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**Title page**

**Original Article**

***Authors*:** Victor A. Andrade-Souza[1], Thaysa Ghiarone[1], Andre Sansonio[1], Kleiton Augusto Santos Silva[1,2], Fabiano Tomazini[1], Lucyana Arcoverde[1], Jackson Fyfe[3], Enrico Perri[4], Nicholas Saner[4], Jujiao Kuang[4], Romulo Bertuzzi[5], Carol Gois Leandro[1], David J. Bishop[4,6]* and Adriano E. Lima-Silva[1,7]

***Title of Article*:** Exercise twice-a-day potentiates skeletal muscle signalling responses associated with mitochondrial biogenesis in humans, which are independent of lowered muscle glycogen content

***Author's Institutions:***
[1]Department of Physical Education and Sports Science, Academic Center of Vitoria, Federal University of Pernambuco, Vitoria de Santo Antao, Pernambuco, Brazil
[2]Department of Medicine, University of Missouri School of Medicine, Columbia, Missouri, USA
[3]School of Exercise and Nutrition Sciences, Faculty of Health, Deakin University, Burwood, Victoria, Australia
[4]Institute for Health and Sport, Victoria University, Melbourne, Victoria, Australia
[5]Endurance Performance Research Group, School of Physical Education and Sport, University of São Paulo, Sao Paulo, Brazil
[6]School of Medical and Health Sciences, Edith Cowan University, Joondalup, Western Australia, Australia
[7]Human Performance Research Group, Academic Department of Physical Education, Technological Federal University of Parana, Curitiba, Parana, Brazil

***Correspondence and requests for materials should be addressed to*:**
David John Bishop
Institute for Sport and Health, Victoria University. PO Box 14428, Melbourne VIC 8001, Australia. Phone: +61 3 9919 9471. E-mail address: David.Bishop@vu.edu.au

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## Abstract

Endurance exercise begun with reduced muscle glycogen stores seems to potentiate skeletal muscle protein abundance and gene expression. However, it is unknown whether this greater signalling responses is due to low muscle glycogen *per se* or to performing two exercise sessions in close proximity - as a first exercise session is necessary to reduce the muscle glycogen stores. In the present study, we manipulated the recovery duration between a first muscle glycogen-depleting exercise and a second exercise session, such that the second exercise session started with reduced muscle glycogen in both approaches but was performed either two or 15 h after the first exercise session (so-called "twice-a-day" and "once-daily" approaches, respectively). We found that exercise twice-a-day increased the nuclear abundance of transcription factor EB (TFEB) and nuclear factor of activated T cells (NFAT) and potentiated the transcription of peroxisome proliferator-activated receptor-□ coactivator 1 alpha (PGC-1α), peroxisome proliferator-activated receptor alpha (PPARα) and peroxisome proliferator-activated receptor beta/delta (PPARβ/δ) genes, in comparison with the once-daily exercise. These results suggest that the elevated molecular signalling reported with previous "train-low" approaches can be attributed to performing two exercise sessions in close proximity rather than the reduced muscle glycogen content *per se*. The twice-a-day approach might be an effective strategy to induce adaptations related to mitochondrial biogenesis and fat oxidation.

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Endurance exercise is a powerful stimulus affecting cytoplasmic and nuclear proteins, and genes encoding mitochondrial proteins, with a subsequent increase in mitochondrial biogenesis (i.e., the generation of new mitochondrial components leading to increased mitochondrial content and respiratory function)[1-8]. While these responses are affected by the nature of the exercise (e.g., the exercise intensity[2, 9]), there is evidence substrate availability is also a potent modulator of this response[10-13]. It has been hypothesised that initiating endurance exercise with low muscle glycogen stores (the so-called "train-low" approach) results in a greater increase in the transcription of genes associated with mitochondrial biogenesis[14-17]. If performing exercise with reduced muscle glycogen modifies the transcriptional response, it could be hypothesised that there will also be concomitant changes in the content of nuclear proteins that regulate gene transcription. However, while this information could provide important mechanistic insights, the response of nuclear proteins to the train-low approach in humans has not been assessed.

Although the train-low strategy seems to be a potent approach to potentiate skeletal muscle signalling responses related to mitochondrial biogenesis[14, 18-20], there are also contrasting findings showing no effects[17, 21-24] and a consensus is yet to be reached. Furthermore, much of the evidence supporting the train-low approach is based on performing a first exercise session to reduce muscle glycogen stores, which is followed by a second exercise session one to three hours later – the so-called "twice-a-day" approach[18-22, 24, 25]. Although the second exercise session will start with reduced muscle glycogen stores, it is challenging to determine if any changes are due to performing the second exercise session with low muscle glycogen or performing the second exercise session soon after the first. It is well known that the transcriptional response of many

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genes peak 3 to 6 hours post exercise and return to basal levels within 8 to 12 h[5, 13, 26-29]. Thus, it is possible that reported increases in gene expression with the twice-a-day approach can be attributed to performing the second exercise session close to the first, when there is an already increased expression of genes associated with mitochondrial biogenesis.

Therefore, the aim of this study was to investigate whether previous reports of greater exercise-induced signalling with the train-low approach can be attributed to low muscle glycogen *per se* or to the cumulative effect of performing two exercise sessions in close proximity. In the present study, we manipulated the recovery duration between a first muscle glycogen-depleting exercise and a second exercise session (i.e., a "once-daily" *vs*. a "twice-a-day" approaches). In the once-daily condition, muscle glycogen content was reduced via evening exercise (prolonged exercise) followed by a carbohydrate (CHO)-restricted period, and a second exercise (i.e., high-intensity interval exercise; HIIE) on the next day (i.e., 15 h between exercises sessions). In the twice-a-day condition, the same exercises were used but with a short recovery period between exercise sessions (i.e., 2 h between exercise sessions). A HIIE session undertaken without a prior muscle glycogen-depleting exercise served as a control.

We demonstrated for the first time that performing two exercise sessions in close succession increases the abundance of transcription factor EB (TFEB) and nuclear factor of activated T cells (NFAT) proteins in the nucleus. We also showed that exercise twice-a-day potentiates the transcription of peroxisome proliferator-activated receptor-coactivator 1 alpha (PGC-1α), peroxisome proliferator-activated receptor alpha (PPARα) and peroxisome proliferator-activated receptor beta/delta (PPARβ/δ) - genes

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that have been associated with mitochondrial biogenesis and fat metabolism. These data suggest that the elevated molecular signalling when starting an exercise session with reduced muscle glycogen content can be attributed to performing two exercise sessions in close succession rather than the reduced muscle glycogen content *per se*. Further research is required to investigate if the twice-a-day approach is a more effective training strategy to induce adaptations related to mitochondrial biogenesis and fat oxidation.

## Results

**Muscle glycogen concentration.** To examine whether the second exercise session started with similarly low levels of muscle glycogen in the twice-a-day and once-daily approaches, we analysed the muscle glycogen content via enzymatic analysis with fluorometric assay detection[30]. Prior to the HIIE, muscle glycogen concentration was similarly lower in both the twice-a-day and once-daily conditions, compared to the control condition (Fig. 1). Muscle glycogen concentration was below 300 $mmol \cdot kg^{-1} \cdot$dry mass for both the twice-a-day and once-daily conditions. This value has been suggested as an "upper limit threshold", above which muscle glycogen will not modulate the activation of acute and chronic skeletal muscle adaptations with the "train-low" approach[31]. Muscle glycogen concentration decreased similarly after the HIIE in all three conditions ($P < 0.05$) and remained lower 3 h post HIIE in both the twice-a-day and once-daily conditions, compared to the control condition. Post HIIE muscle glycogen concentration remained above 100 $mmol \cdot kg^{-1} \cdot$dry mass. This value has been hypothesised as a "lower limit threshold", below which muscle glycogen will not continue modulating genes related to mitochondrial biogenesis[31]. These findings suggest that the HIIE in both twice-a-day and once-daily approaches were performed

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with muscle glycogen levels compatible with the "train-low" approach. In addition, the HIIE was initiated with similarly low levels of muscle glycogen in both the once-daily and twice-a-day conditions. Therefore, any differences in cytosolic and nuclear protein abundance and gene expression responses between these two exercising approaches can be attributed to the different recovery duration between the two exercise, rather than the starting muscle glycogen concentration.

(PLEASE INSERT FIGURE 1 HERE)

**Cytosolic proteins relative abundance pre and post HIIE.** Given that the muscle glycogen concentration was similar between both the twice-a-day and once-daily conditions, we next investigated whether the two exercising approaches had a similar effect on exercise-induced changes in cytosolic protein relative abundance. Representative blots are presented in Fig. 2a. Cytosolic p53 protein relative abundance increased immediately post HIIE in all three conditions (Fig. 2c), with no differences between conditions. Cytosolic PGC-1α, phosphorylated p53 (p-p53$^{Ser15}$), PHF20 protein (PHF20), TFEB, p38 mitogen-activated protein kinase (p38MAPK), phosphorylated 5' adenosine monophosphate-activated protein kinase (p-AMPK$^{Thr172}$) and NFAT relative abundance were unaffected by the exercise approach or time (Fig. 2b, 2d-i). Collectively, these data reveal that neither "train-low" approach influenced exercise-induced changes in the cytosolic proteins assessed in the present study.

(PLEASE INSERT FIGURE 2 HERE)

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**Nuclear protein relative abundance pre and post HIIE.** Given that cytosolic proteins can rapidly translocate to the nucleus, and the exercise-induced changes are often not observed in the cytoplasm[4, 32], we next investigated whether either "train-low" approach altered exercise-induced changes in nuclear protein relative abundance. Representative blots are presented in Fig. 3a. Nuclear PGC-1α, p53, and p-p53$^{Ser15}$ relative abundance increased post HIIE (Fig. 3b-d), with no clear differences between the three conditions. The relative abundance of nuclear PHF20, p38MAPK, and p-AMPK$^{Thr172}$ was unaffected by either "train-low" approach or time (Fig. 3e-g). However, nuclear TFEB relative abundance was greater in the twice-a-day compared to the once-daily condition both pre and post HIIE (Fig. 3h). Moreover, nuclear NFAT relative abundance was also greater in the twice-a-day compared to both the once-daily and control condition post HIIE (Fig. 3i). Because TFEB and NFAT can act as transcription factors for several genes involved in mitochondrial biogenesis[32-35], potential downstream pathways were further explored.

(PLEASE INSERT FIGURE 3 HERE)

**Mitochondrial-related gene expression pre, post, and 3 h post HIIE.** Since we observed a greater relative abundance of nuclear TFEB and NFAT with the twice-a-day approach, we further compared the expression of several related genes. Pre HIIE, total PGC-1α mRNA content was ~9-fold higher in the twice-a-day compared to both the once-daily and control conditions (Fig. 4a). Three hours post HIIE, total PGC-1α mRNA content increased in all three conditions compared with their respective pre-values; however, total PGC-1α mRNA content remained ~10-fold higher in the twice-a-day compared to both control and once-daily conditions. Similarly, PGC-1α isoform 4

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mRNA content was ~24-fold higher at pre HIIE in the twice-a-day compared to both the once-daily and control conditions (Fig. 4c). At 3 h post HIIE, the PGC-1α isoform 4 mRNA content increased in all three conditions compared with their respective pre-values; however, the PGC-1α isoform 4 mRNA content remained ~10-fold higher in the twice-a-day compared to the control and once-daily conditions. Additionally, PGC-1α isoform 1 mRNA content was higher in the twice-a-day compared to the once-daily when all time points where considered (two-way ANOVA, main effect of condition, $p < 0.05$, Fig. 4b). There was, however, no effect of condition for p53, TFEB, chromodomain-helicase-DNA-binding protein 4 (CHCHD4), p21, mitochondrial transcription factor A (Tfam), NADH dehydrogenase subunit β (NDUFβ; mitochondrial complex I), succinate dehydrogenase subunit β (SDHβ; mitochondrial complex II), cytochrome c (mitochondrial complex III), and cytochrome c oxidase subunit IV (COX IV; mitochondrial complex IV) mRNA content (Fig. 4d-l). The mRNA content of representative subunits of mitochondrial complexes II, III, and IV increased 3 h post HIIE to a similar extent for all three conditions. Thus, the transcription of PGC-1α (and its isoforms) are potentiated by performing two exercise sessions in close succession, although exercise-induced increases in mitochondrial complex genes were not affected by the different "train-low" approaches.

(PLEASE INSERT FIGURE 4 HERE)

**Fat transport and lipolysis related genes pre, post, and 3 h post HIIE.** Since the twice-a-day approach increased the nuclear abundance of TFEB, which could affect genes related to fat metabolism[32, 35], we also assessed changes in a selection of genes associated with fat transport and lipolysis. The content of carnitine palmitoyltransferase

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I subunit A (CPT1A) mRNA was higher in both the twice-a-day and once-daily conditions compared with the control condition at 3 h post HIIE; however, there was no difference between the two "train-low" approaches (Fig. 5a). There was an increase in mitochondrial uncoupling protein 3 (UCP3) mRNA content 3 h post exercise in all three conditions. The UCP3 mRNA content was, however, significantly higher only in the twice-a-day compared to the control condition at 3 h post HIIE (Fig. 5b). Pre HIIE, the PPARα mRNA content was ~11-fold higher in the twice-a-day compared to both the once-daily and control conditions (Fig. 5c). Three hours post HIIE, the PPARα mRNA content increased ~7- and 9-fold in the once-daily and control conditions, respectively, compared with their respective pre-values; however, the PPARα mRNA content remained ~16-fold higher in the twice-a-day compared to the once-daily and control conditions. The PPARβ/δ mRNA content was higher in the twice-a-day than in the control condition post HIIE, and higher than both the once-daily and control conditions at 3 h post HIIE (Fig. 5d). The PPARβ/δ mRNA content was higher at 3 h post HIIE compared to pre- and post HIIE only for the twice-a-day approach. The citrate synthase (CS) mRNA content was higher 3 h post HIIE compared to post HIIE for all three conditions. However, the peroxisome proliferator-activated receptor gamma (PPARγ), β-hydroxyacyl-CoA dehydrogenase (β-HAD), and fatty acid translocase cluster of differentiation 36 (CD-36) mRNA content were unaffected by "train-low" approach or time (Fig. 5e-h). Thus, performing two exercise sessions in close succession potentiates the transcription of PPARα, PPARβ/δ and UCP3, while exercise-induced changes in CPT1A mRNA content seemed to be influenced by low muscle glycogen stores *per se* as it was similarly increased by HIIE in both the twice-a-day and the once-daily conditions.

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(PLEASE INSERT FIGURE 5 HERE)

**Glycolysis related genes pre, post and 3 h post HIIE.** As the train-low strategy has been reported to affect CHO pathways[4], we also assessed exercise-induced changes in genes associated with CHO metabolism. The mRNA content of phosphofructokinase (PFK) and glucose transporter 4 (GLUT4) was unaffected by exercising approach or time. While pyruvate dehydrogenase kinase isoenzyme 4 (PDK4) mRNA content increased 3 h post HIIE, there was no difference for the three different exercising approaches (Fig. 6a-c). These findings indicate that exercise-induced changes in genes related to CHO metabolism were not influenced by muscle glycogen stores or the proximity of the two exercise sessions in the present study.

(PLEASE INSERT FIGURE 6 HERE)

**Physiological responses pre, post, and 3 h post HIIE.** The twice-a-day approach was associated with a higher heart rate, ventilation, and oxygen uptake, and a lower plasma glucose concentration during HIIE than both the once-daily and the control condition (Fig. 7a-c and Table 1). Plasma glucose was also lower during HIIE in the once-daily compared to the control condition (Table 1). In addition, the respiratory exchange ratio was lower and the serum free fatty acid concentration higher than the control only for the twice-a-day approach (Fig. 7d and Table 1). Serum glycerol was higher and plasma lactate lower post HIIE in both the twice-a-day and once-daily conditions compared with the control condition (Table 1). Plasma epinephrine and norepinephrine concentration was not influenced by the exercising approach undertaken. Together,

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these findings suggest a higher systemic physiological stress and a higher fat oxidation rate with the twice-a-day compared with the once-daily and control conditions.

(PLEASE INSERT FIGURE 7 HERE)

(PLEASE INSERT TABLE 1 HERE)

## Discussion

There is continued debate about whether beginning exercise with low muscle glycogen stores potentiates the exercise-induced increase in genes associated with mitochondrial biogenesis and metabolism[10, 11, 31, 36-39]. Some of this controversy may relate to the observation that much of the evidence supporting the train-low approach is based on performing the experimental exercise session a few hours after a glycogen-lowering exercise session[18-22, 25]. Thus, it is difficult to determine if any observed effects are due to performing the second exercise session with low muscle glycogen stores and/or performing the second exercise session close to the first. We aimed to resolve this controversy by performing the same exercise session with similar starting muscle glycogen stores, but either 2 or 15 hours following the previous glycogen-lowering exercise session. In contrast to previous suggestions, our results indicate that the greater exercise-induced nuclear protein abundance (TFEB and NFAT) and transcription of genes involved in mitochondrial biogenesis (PGC-1α, PPARα, PPARβ/δ) with the so-called “train-low” approach can be attributed to performing two exercise sessions in close proximity and is not due to low muscle glycogen *per se*.

Despite the different recovery periods between exercise sessions for the two train-low approaches, muscle glycogen prior to the HIIE was reduced to a similar extent

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in both the twice-a-day and once-daily condition compared with the control condition (45 and 42% from control, respectively, Fig. 1). This level of muscle glycogen concentration is consistent with previous studies that have utilised either the twice-a-day[19, 20] or once-daily[17] approach. The similar low muscle glycogen levels when commencing the HIIE in both "train-low" conditions allowed us to investigate whether any differences for exercise-induced gene or protein expression could be attributed to performing HIIE with reduced muscle glycogen stores or to performing HIIE close to a previous exercise session.

The train-low approach has been associated with a greater exercise-induced activation of genes associated with mitochondrial biogenesis and fat metabolism[14, 17, 19, 20]. However, to our knowledge, no study has investigated the effect of different train-low strategies on exercise-induced changes in the nuclear abundance of proteins associated with the transcriptional activation of mitochondrial biogenesis and fat metabolism. Consistent with previous research[14, 17, 40, 41], immediately post HIIE there was greater PGC-1α, p-p53, and p53 relative protein abundance in the nucleus (Fig. 3b-d). However, there were no significant differences between either of the train-low approaches and the control condition. Similarly, neither train-low approach affected the nuclear abundance of p-AMPK or p-p38 MAPK (Fig. 3f,g). This indicates that lowering muscle glycogen levels did not affect the nuclear abundance of these proteins, at rest or following HIIE. We subsequently investigated the nuclear abundance of other proteins that may contribute to previous reports of greater exercise-induced increases in gene expression with different train-low approaches.

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Despite evidence from animal studies[32, 34, 35, 42, 43], to our knowledge this is the first study to report an exercise-induced increase in the nuclear protein abundance of TFEB and NFAT in human skeletal muscle. Another novel finding of the present study was that the nuclear abundance of both NFAT and TFEB was significantly greater with the twice-a-day approach compared to both the once-daily and control conditions (Fig. 3h,i). Furthermore, the exercise-induced increase in the nuclear abundance of NFAT was only significant in the twice-a-day condition. As activated calcineurin dephosphorylates both NFAT[34, 42] and TFEB[32, 35, 43], leading to their translocation to the nucleus[32-35, 42, 43], this suggests there might be greater calcineurin activation when a second exercise session is performed soon after a prior exercise session. Unfortunately, we did not have sufficient muscle sample to test this hypothesis and further research is required. Nonetheless, as activated calcineurin has been implicated in the expression of many genes[33], we also investigated the effect of our two different train-low approaches on the expression of selected metabolic and mitochondrial genes.

In the twice-a-day condition, we observed significantly greater exercise-induced increases in genes that have been reported to be regulated by calcineurin[33] (e.g., PGC-1α, PPARα, PPARβ/δ; Fig. 4 and 5). This effect was not observed in the once-daily condition; this suggests that the greater exercise-induced increase in these genes can be attributed to performing HIIE soon after the prior exercise session, rather than beginning HIIE with lowered muscle glycogen levels. There were not greater exercise-induced increases in other investigated genes that have previously been reported to be activated by calcineurin (e.g., PPARγ, CS, CD-36, and GLUT4; Fig. 4, 5 and 6). In the case of PPARγ, others have also reported no effect of exercise on PPARγ gene expression[44] and this probably explains why we observed no effect of any condition on

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the exercise-induced expression of this gene. Regarding CS, CD-36, and GLUT4, others have reported that the expression of these genes is not increased until more than 6 h post exercise[44-46] and it may be that our biopsy timing (3 h post HIIE) did not allow us to detect significant differences in the expression of these genes with exercise or between conditions.

Consistent with previous research, we observed a robust increase in PGC-1α mRNA content following HIIE[2, 14, 17]. However, although it has been suggested that commencing exercise with low muscle glycogen can amplify the increase in genes associated with mitochondrial biogenesis[14, 16, 17], there were no differences in post-HIIE PGC-1α mRNA content between the once-daily and control conditions. However, in the present study, the total PGC-1α and PGC-1α4 mRNA content were higher (~10-fold) in the twice-a-day approach, compared to the once-daily and control 3 h post HIIE (Fig. 4a-c). Additionally, PPARα and PPARβ/δ mRNA content was also higher in the twice-a-day compared to the once-daily and control 3 h post HIIE (~16- to 2-fold, respectively; Fig. 5c-d). Together, PGC-1α, PPARα and PPARβ/δ regulate the expression of genes related to fat metabolism[33]. Recent findings have also indicated that PPARβ/δ is a potent protector of PGC-1α against degradation, and can regulate mitochondrial respiratory chain proteins, which suggests PPARβ/δ also plays an essential role in exercise-induced mitochondrial biogenesis[47]. Therefore, our findings indicate the twice-a-day approach may be a more effective strategy to increase both mitochondrial biogenesis and fat metabolism. This is consistent with studies that have reported greater increases in CS activity[20, 25] and whole-body fat oxidation during sub-maximal exercise[18, 20] when training twice-a-day compared to once-daily training.

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In the twice-a-day condition we also observed a greater circulating FFA at pre, post, and 3-h post HIIE (Table 1), and a lower RER throughout the HIIE (Fig. 7), which is indicative of greater fat oxidation. The increase in circulating FFA might activate calcineurin[52], resulting in the regulation of skeletal muscle metabolism via coordinated changes in gene expression[33]. Activated calcineurin will lead to a translocation of both NFAT and TFEB to the nucleus[32-35, 42, 43]. Translocated NFAT will promote the expression of PPARα mRNA, while both PPARβ/δ and PPARα mRNA will be overexpressed under the influence of TFEB. This is in accordance with our findings of increased nuclear protein relative abundance of TFEB and NFAT (Fig.3 h and i), with a consequent larger increase in PPARα and PPARβ/δ gene expression only in the twice-a-day approach (Fig.5 c and d). Thus, our results indicate that the twice-a-day training might be more effective to induce adaptions in fat metabolism.

It has been suggested that the greater exercise-induced upregulation of cell signalling pathways with the train-low approach may be due to enhanced activation of AMPK and p38 MAPK[19, 2119, 2119, 2119, 21], but contradictory results have been reported[17, 19, 21, 53]. For example, Cochran et al.[21] observed larger increases in p-p38 MAPK but not p-AMPK, while Yeo et al.[19] reported larger increases in p-AMPK but without alterations in p-p38 MAPK, with the train-low approach. On the other hand, Gejl et al.[53] and Psilander et al.[17] found no effect of their train-low approaches on both p-AMPK and p-p38 MAPK protein content, which is consistent with our findings (Fig. 2g-i). There are no obvious explanations for these contradictory results, although muscle glycogen content before the second exercise session did differ between studies (~170 $mmol \cdot kg^{-1} \cdot$dry mass[17], ~250 $mmol \cdot kg^{-1} \cdot$dry mass[19], ~300 $mmol \cdot kg^{-1} \cdot$dry mass[21], ~400 $mmol \cdot kg^{-1} \cdot$dry mass[53], ~250 $mmol \cdot kg^{-1} \cdot$dry mass in the present study). The “train-low” protocols

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used in each study also differed and consisted of either a prolonged exercise followed by a moderate-intensity interval exercise 14h later[17], a prolonged exercise followed by a HIIE two hours later[19], two HIIE performed three hours apart[21], or a HIIE followed by a prolonged exercise seven hours later[53]. However, activation of AMPK and p38 MAPK does not seem to be associated with muscle glycogen levels or a particular train-low regime.

In contrast to our results, two studies have reported greater exercise-induced increases in genes associated with mitochondrial biogenesis (e.g., PGC-1α) with the once-daily approach (~5-fold) compared to a control condition (~2- to 4-fold;[14, 17]). In the present study, PGC-1α increased similarly between the once-daily and the control condition (~5-fold). However, one notable difference is that pre-exercise muscle glycogen levels were lower in these previous two studies (105 and 170 $mmol \cdot kg^{-1} \cdot$dry mass, respectively), compared with the current study (~250 $mmol \cdot kg^{-1} \cdot$dry mass). Nonetheless, this appears unlikely to explain the contrasting findings as all three studies had pre-exercise muscle glycogen levels between 100 and 300 $mmol \cdot kg^{-1} \cdot$dry mass, which has been hypothesised to be a critical level to enhance exercise-induced molecular signalling[31].

In summary, our findings indicate that the greater exercise-induced signalling with the so-called “train-low” approach can mostly be attributed to the performance of two exercise sessions in close succession rather than exercising with a reduced muscle glycogen content. We presented evidence that performing two exercise sessions separated by a short recovery period increases the nuclear abundance of TFEB and NFAT and potentiates the transcription of PGC-1α, PPARα and PPARβ/δ. Although we

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have identified novel molecular mechanism by which the twice-a-day approach might be a more effective strategy to induce adaptations related to mitochondrial biogenesis and fat oxidation, further research is required to determine if training using the twice-a-day approach results in greater changes in mitochondrial content and function and fat oxidation.

# Methods

## Participants

Eight healthy, physically-active men, who were accustomed to cycling [age: 30.8 ± 3.5 years, body mass: 78.7 ± 9.9 kg, height: 1.76 ± 0.07 m, body fat: 13.6 ± 5.1%, maximal oxygen uptake ($\dot{V}O_{2max}$): 37.1 ± 6.4 $mL \cdot kg^{-1} \cdot min^{-1}$, maximal aerobic power (MAP): 229.6 ± 38.2 W, and first (LT1) and second (LT2) lactate thresholds: 78.1 ± 20.9 and 170.0 ± 37.7 W, respectively] participated in this study. Participants were informed about the procedures, risks, and benefits associated with the protocol, before they signed a consent form agreeing to participate in this study, which was approved by the Research Ethics Committee of Federal University of Pernambuco. The study was conducted according to the principles presented in the Declaration of Helsinki.

## Study overview

Each participant completed three experimental trials in a randomized, crossover design. An overview of the experimental design is shown in Fig. 8. Briefly, in the once-daily approach participants performed the muscle glycogen-depleting exercise in the

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evening before (2000 – 2200 h) followed by an overnight fast. On the experimental day morning (0800 h), participants ate a low-CHO breakfast (CHO: 42.7 ± 5.0 kcal, 7%; fat: 365.9 ± 43.1 kcal, 60%; protein: 201.3 ± 23.7 kcal, 33%) and performed the HIIE session (1300 h). In the twice-a-day approach, participants ate a low-CHO breakfast on the morning of the experimental day (0800 h), and then performed a muscle glycogen-depleting exercise (0900-1100 h), followed by a 2-h rest period and a HIIE (1300 h). During the control, participants consumed the same low-CHO breakfast as in both experimental trials (0800 h), and then performed the same HIIE session (i.e., 1300 h). Water was provided ad libitum throughout the experiments. Skeletal muscle biopsies from the *vastus lateralis* and venous blood samples were taken before, immediately after, and 3 h after completion of the HIIE sessions. Each experimental trial was separated by approximately two weeks to washout any residual effect of fatigue or damage caused by exercise and the multiple muscle biopsies.

**Exercise protocols**

*Preliminary test*

One week prior to the commencement of this study, participants performed a graded exercise test to volitional fatigue on a cycloergometer (Ergo-Fit 167, Pirmasens, Germany). The test commenced at 50 W, and thereafter intensity was increased by 25 W every 4 min, with a 1-min break between stages, until volitional exhaustion[54]. The test was interrupted when the participant could no longer maintain the required cadence (70 rpm). Strong verbal encouragement was provided to each participant.

$\dot{V}O_2$ was measured breath-by-breath throughout the test using an automatic analyzer (Cortex, Metalyzer 3B®, Saxony, Germany). Before each test, the gas analyzer

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was calibrated using ambient air and a cylinder of known gas concentration (12% $O_2$ and 5% $CO_2$). The volume was calibrated using a 3-L syringe (Quinton Instruments, Washington, US). Capillary ear lobe blood samples were taken at rest and immediately after each 4-min stage of the test for determination of plasma lactate concentration. The LT1 was visually identified by two experienced investigators as the first increase in plasma lactate concentration above resting level. The LT2 was calculated by the modified Dmax method[55]. This was determined by the point on the polynomial regression curve that yields the maximal perpendicular distance to the straight line connecting the LT1 and the final stage of the test. $\dot{V}O_{2max}$ was defined as the highest 30-s average $VO_2$ during the test and MAP was determined as the highest workload reached. If a participant did not complete the final 4-min stage, then the MAP was determined using the following equation:

$$MAP = PO_{last} + (t/240 \cdot 25) \quad (1)$$

where $PO_{last}$ is the power output in watts of the last completed stage performed by the participant, $t$ is the time (in seconds) sustained during the last incomplete stage, and 25 corresponds to the increments in power (Watts) at each stage.

*Muscle glycogen-depleting exercise*

To reduce muscle glycogen stores, participants cycled for 100 min at a power output corresponding to 50% of the difference between LT1 and LT2 (124 ± 27 W, 54 ± 5% of MAP). Then, after an 8-min rest, participants performed six 1-min exercise bouts at 125% MAP (287 ± 46 W) interspersed with 1-min rest periods[56]. This protocol has been shown effective for reducing the muscle glycogen content[57, 58].

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*High-intensity interval exercise*

The HIIE sessions were preceded by a 5-min warm-up at 90% LT1. Participants completed ten 2-min intervals at an intensity of 20% of the difference between the LT2 and MAP (182 ± 38 W, 79 ± 5% of MAP). Each 2-min bout was interspersed with a 1-min passive recovery period[54]. Participants were required to maintain a pedal frequency of 70-80 rpm during each 2-min bout. The $\dot{V}O_2$, carbon dioxide production, RER, and $\dot{V}E$ were measured breath-by-breath throughout each HIIE session using the same gas analyzer described for the graded exercise test. Data were then converted to 30-s intervals for further analysis.

**Diet and exercise control before starting experimental manipulation**

Participants were asked to register all foods and beverages consumed during the 48-h preceding the start of the first experimental trial (Fig. 8). Dinner of two days before, and breakfast, lunch and dinner of one day before the experimental trial were replicated during the subsequent experimental trials (Fig. 8). They were given verbal and written instructions on how to repeat this before the subsequent experimental approach. Checklists were used to check any deviations from the menu. Participants were also instructed to avoid any strenuous exercise as well as alcohol and caffeine consumption for the 24 h prior to each experimental trial.

**Blood collection and analysis**

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Blood samples were collected from an antecubital vein and separated into three different tubes. Two millilitres of blood were collected in tubes containing sodium fluoride and EDTA (Hemogard™ Fluoride/EDTA, BD Vacutainer®, USA). Blood was centrifuged at 4,000 rev.min$^{-1}$ for 10 min at 4º C with the resulting plasma transferred to 2-mL tubes and immediately analysed for plasma glucose and lactate concentrations. Plasma glucose and lactate concentrations were analysed with a commercially available enzymatic kit (Glucose Liquiform and Enzymatic Lactate, respectively, Labtest, Lagoa Santa, Minas Gerais, Brazil). A further 8 mL of blood was collected in tubes containing Clot activator and gel for serum separation (SST II Plus, BD Vacutainer®, USA), and another 3 mL of blood was collected in tubes containing sodium heparin (Sodium Heparin$^{N}$ Plus, BD Vacutainer®, USA). Both were then centrifuged at 4,000 rev.min$^{-1}$ for 10 min at 4º C, and the resulting serum/plasma frozen and stored in liquid nitrogen for later analyses of concentration of FFA and glycerol concentrations, and plasma catecholamine. Serum FFA and glycerol concentrations were determined by an enzymatic colorimetric method (EFFA-100 and EGLY-200, BioAssay, Hayward, California, USA). Plasma catecholamine concentrations were determined by using ion-pairing reverse phase liquid chromatography coupled with electrochemical detection[56].

**Muscle tissue samples and analysis**

Nine separate incisions (three per trial) were made into the vastus lateralis under local anaesthesia (2% Xylestesin®), and a muscle sample taken using Bergström needle[59] adapted for manual suction[60]. Samples were taken approximately 1 cm apart from a previous biopsy site. Samples [mean: 118 ± 48 mg; range: 49 to 248 mg] were immediately snap-frozen in liquid nitrogen, and then stored at in -80ºC until subsequent analyses. Muscle samples were taken at rest (pre HIIE), immediately after HIIE (post

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HIIE) and 3 h after HIIE (3 h post HIIE). Biopsies were subsequently analysed for muscle glycogen content, as well as gene and protein expression (described subsequently).

**Muscle glycogen concentration**

Approximately 2 to 3 mg of freeze-dried muscle tissue was powdered and dissected free of all visible non-muscle tissue. Powdered muscle tissue was then extracted with 250 µL of 2 M HCl, incubated at 95°C for 2 h (agitated gently every 20 min), and then neutralized with 750 µL of 0.66 M NaOH. Glycogen concentration was subsequently assayed in triplicate via enzymatic analysis with fluorometric detection[61] and the mean value reported as millimoles per kilogram dry weight.

**Western blotting**

*Muscle homogenate preparations and protein assays*

Approximately 20 mg of frozen muscle tissue was homogenized using a TissueLyser II (Qiagen, Valencia, CA) in a 1:20 dilution of ice-cold RIPA buffer (pH 7.4) containing: 0.15 M NaCl, 1% Triton-X100, 0.5% sodium deoxycholate, 0.05 M Tris, 0.1% SDS, 0.1 M EDTA, 1% protease/phosphatase inhibitor cocktail (100X, Cell Signalling Technology [CST], #5872, St. Louis, MI). Homogenates were rotated end-over-end for 60 min at 4°C. Protein content of muscle homogenate was measured in triplicate using a Bradford assay (Bio-Rad protein assay dye reagent concentrate, Bio-Rad Laboratories, Hercules, CA) against bovine serum albumin standards (BSA, A9647, Sigma-Aldrich). Nuclear and crude cytosolic fractions were prepared from 40 to 60 mg of wet muscle using a commercially-available nuclear extraction kit (NE-PER, Pierce, USA). Muscle samples were homogenized in CER-I buffer containing a

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protease/phosphatase inhibitor cocktail (CST #5872). Following centrifugation, the supernatant was taken and pellets containing nuclei were washed five times in PBS to remove cytosolic contamination, before nuclear proteins were extracted by centrifugation in high-salt NER buffer supplemented with the same inhibitors cocktail following manufacturers' instruction. Sufficient muscle was available to prepare subcellular fractions from eight participants.

*Immunoblotting*

RIPA-buffered homogenate was diluted in 4X Laemmli buffer (0. 25 M Tris, 8% SDS, 40% glycerol, 0.04% bromophenol blue, 20% 2-mercaptoethanol) and equal amounts of total protein (10 to 20 µg) were loaded on Criterion™ 4-20% TGX Stain-Free™ Precast Gels (Bio-Rad). All samples for a participant were loaded in adjacent lanes on the same gel. Four to six different dilutions of a mixed-homogenate internal standard were also loaded on each gel and a calibration curve plotted of density against protein content. From the subsequent linear regression equation protein abundance was calculated from the measured band intensity for each sample on the gel[62]. Gel electrophoresis ran for 90 min at 80-150 V. Proteins were turbo-transferred to a 0.2 µm PVDF membrane at 25 V for 10 min. Membranes were blocked for 60 min at room temperature in 5% non-fat dry milk (NFDM) diluted in Tris-buffered saline with 0.1% Tween-20 (TBST). Membranes were then washed in TBST and incubated overnight at 4°C – with the appropriate primary antibody: monoclonal anti-PGC-1α (CST #2178), polyclonal anti-p-ACC$^{Ser79}$ (CST #3361), polyclonal anti-AMPK (CST #2532), polyclonal anti-p-AMPK$^{Thr172}$ (CST #2531), polyclonal anti-p38 MAPK (CST #9212), polyclonal anti-p-p38 MAPK$^{Thr180/Tyr182}$ (CST #9211), PHF20 (CST #3934), p53 (CST

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#2527), NFAT2 (CST #8032) and Phospho-(Ser) 14-3-3 Binding Motif Antibody anti-TFEB (CST #9601) diluted (1:1,000) in 5% BSA and 0.02% sodium azide in TBST. Following TBST washes the membranes were incubated in the relevant secondary antibody: Goat anti-rabbit IgG (Perkin Elmer/ NEF812001EA), diluted (1:10,000) in 5% NFDM in TBST, for 60 min at room temperature. After further washes, membranes were incubated in chemiluminescent solution (1.25 mM 294 luminol, 0.2 mM ρ-coumaric acid, 100 mM Tris pH 8.5, 0.009% $H_2O_2$) for 2 min and images were taken with a ChemiDoc Imaging System fitted (Bio-Rad). Densitometry was performed with Image Lab 5.0 software (Bio-Rad). Images are typically displayed with at least five bandwidths above and below the band of interest.

**Real-Time quantitative PCR**

*RNA extraction*

Total RNA from approximately 10 to 15 mg of frozen muscle was homogenized in 800 µL of TRIzol reagent (Thermo Fisher Scientific, Waltham, USA) using a TissueLyser II (Qiagen, Valencia, CA)[63]. The concentration and purity of each sample was assessed using a NanoDrop One/One$^c$ (Thermo Fisher Scientific). As representative, RNA integrity of a subset of samples was measured using a Bio-Rad Experion microfluidic gel electrophoresis system (7007104, Experion RNA StdSens Analysis kit) and determined from the RNA quality indicator. RNA was stored at −80°C until reverse-transcription was performed.

*Reverse transcription*

1 µg RNA, in a total reaction volume of 20 µL, was reverse-transcribed to cDNA using a Thermocycler (S1000, Bio-Rad) and Bio-Rad iScript RT Supermix (170-

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8840) per the manufacturer's instructions. Priming was performed at 25°C for 5 min and reverse transcription for 30 min at 42°C. All samples, including RT-negative controls, were performed during the same run. cDNA was stored at −20°C until subsequent analysis.

*qPCR*

Relative mRNA expression was measured by qPCR (QuantStudio 7 Flex, Applied Biosystems, Foster City, CA) using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Primers were designed using Primer-BLAST[64] to include all splice variants, and were purchased from Sigma-Aldrich (see Supplementary Table 1 for primer details). All reactions were performed in duplicate on 384-well MicroAmp optical plates (4309849, Applied Biosystems) using an epMotion M5073 automated pipetting system (Eppendorf AG). Total reaction volume of 5 µL contained 2 µL of diluted cDNA template, 2.5 µL of mastermix, and 0.3 µM or 0.9 µM primers. All assays ran for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The stability of six potential reference genes were determined by *BestKeeper*[65] and *NormFinder*[66] software, and the three most stably expressed genes were TBP, 18S, and ACTB (Supplementary Table 1). There were no main effects of training approach, time, nor interaction effects, for the Ct values of the three most stable housekeeping genes ($P > 0.05$). Expression of each target gene was normalized to the geometric mean of expression of the three reference genes[67], and using the $2^{-\Delta\Delta Ct}$ method (where Ct is the quantification cycle)[68].

**Statistical analysis**

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Statistical analysis was performed using the GraphPad Prism software version 6.01. All data were checked for normality with the Kolomogorov-Smirnov test. To compare the responses before, immediately after, and 3 h after each HIIE session, data were analysed with two-way repeated measures ANOVA (exercising approach *vs*. time). A Bonferroni's post-hoc test was used to locate the differences. All values are expressed as means ± SD. Significance was accepted when $p < 0.05$.

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**Author contributions**

V. A. A-S., R. B., D. J. B., and A. E. L-S. designed the experiments. V. A. A-S., T. G., A. S., K. A. S. S., F. T., L. A., and A. E. L-S. performed the experiments. V. A. A-S., T. G., A. S., K. A. S. S., F. T., L. A., and A. E. L-S. conducted the exercise analyses at Department of Physical Education and Sports Science, Academic Center of Vitoria, Federal University of Pernambuco and V. A. A-S., J. F., E. P., N. S., and J. K. conducted qPCR and western blot analyses at Institute for Health and Sport, Victoria University. V. A. A-S analyzed data and discussed analyses and results with J. F., J. K., D. J. B., and A. E. L-S. T.G., K. A. S. S., R. B., and C. G. L. supported data analyses.

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V. A. A-S., K. A. S. S., J. K., D. J. B., and A. E. L-S made the figures. All authors wrote and approved the manuscript.

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## Figure legends

**Figure 1 | Muscle glycogen concentration pre, post, and 3 h post the high-intensity interval exercise (HIIE).** Data are presented as mean ± standard deviation. n = 8. * significantly lower than control at the same time point ($P < 0.05$); # significantly lower than pre HIIE for the same condition ($P < 0.05$). Two-way analysis of variance (ANOVA) with Bonferroni post hoc test.

**Figure 2 | Cytosolic protein relative abundance pre and post the high-intensity interval exercise (HIIE). (a)** Representative immunoblots corresponding to total and phosphorylated protein relative abundance measured in the cytosolic fraction, pre and post the HIIE in the control, twice-a-day, and once-daily approaches; **(b)** cytosolic peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-1α); **(c)** cytosolic p53 (p53); **(d)** cytosolic phosphorylated p53 (p-p53$^{Ser15}$); **(e)** cytosolic PHF20 (PHF20); **(f)** cytosolic phosphorylated 5' adenosine monophosphate-activated protein kinase (p-AMPK$^{Thr172}$); **(g)** cytosolic phosphorylated p38 mitogen-activated protein kinase (p-p38MAPK); **(h)** cytosolic transcription elongation factor EB (TFEB); **(i)** cytosolic nuclear factor of activated T cells (NFAT). n = 8 for all proteins. Data are presented as fold changes from control pre (mean ± standard deviation). # significantly higher than pre HIIE for the same condition (P < 0.05). Two-way analysis of variance (ANOVA) with Bonferroni post hoc test.

**Figure 3 | Nuclear protein relative abundance pre and post the high-intensity interval exercise (HIIE). (a)** Representative immunoblots corresponding to total and phosphorylated protein relative abundance measured in the nuclear fraction, pre and post the HIIE in the control, twice-a-day, and once-daily approaches; **(b)** nuclear peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-1α); **(c)** nuclear p53 (p53); **(d)** nuclear phosphorylated p53 (p-p53$^{Ser15}$); **(e)** nuclear PHF20 (PHF20); **(f)**

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nuclear phosphorylated AMPKThr172 (p-AMPK$^{Thr172}$); **(g)** nuclear phosphorylated p38MAPK (p-p38MAPK); **(h)** nuclear transcription elongation factor EB (TFEB); **(i)** nuclear factor of activated T cells (NFAT). n = 8 for all proteins. Data are presented as fold changes from control pre (mean ± standard deviation). * significantly higher than the once-daily and control condition at the same time point (P < 0.05); † significantly higher than the once-daily condition at the same time point (P < 0.05). # significantly higher than pre HIIE for the same condition (P < 0.05). Two-way analysis of variance (ANOVA) with Bonferroni post hoc test.

**Figure 4 | Mitochondrial-related gene expression pre, post, and 3 h post the high-intensity interval exercise (HIIE). (a)** peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-1α) total gene expression; **(b)** PGC-1α isoform 1 gene expression; **(c)** PGC-1α isoform 4 gene expression; **(d)** p53 (p53) gene expression; **(e)** transcription elongation factor EB (TFEB) gene expression; **(f)** Chromodomain-helicase-DNA-binding protein 4 (CHCHD4) gene expression; **(g)** p21 protein (p21) gene expression; **(h)** mitochondrial transcription factor A (Tfam) gene expression; **(i)** NADH dehydrogenase (NDUFβ) (mitochondrial complex I) gene expression; **(j)** succinate dehydrogenase subunit β (SDHβ) (mitochondrial complex II) gene expression; **(k)** cytochrome c (mitochondrial complex III) gene expression; **(l)** cytochrome c oxidase subunit IV (COXIV) (mitochondrial complex III) gene expression. n = 8 for all genes (except Tfam, n = 7). Data are presented as fold changes from control pre (mean ± standard deviation). * significantly higher than the once-daily and control condition at the same time point (P < 0.05); # significantly higher than pre HIIE for the same condition (P < 0.05); □ significantly higher than post HIIE for the same condition (P < 0.05). Two-way analysis of variance (ANOVA) with Bonferroni post hoc test.

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**Figure 5 | Genes related to fat transport and lipolysis pre, post, and 3 h post the high-intensity interval exercise (HIIE). (a)** carnitine palmitoyltransferase I (CPT1) gene expression; **(b)** mitochondrial uncoupling protein 3 (UCP3) gene expression; **(c)** peroxisome proliferator-activated receptor α (PPARα) gene expression; **(d)** peroxisome proliferator-activated receptor β/δ (PPARβ/δ) gene expression; **(e)** peroxisome proliferator-activated receptor γ (PPARγ) gene expression; **(f)** citrate synthase (CS) gene expression; **(g)** β-hydroxyacyl-CoA dehydrogenase (β-HAD) gene expression; **(h)** fatty acid translocase cluster of differentiation 36 (CD-36) gene expression. n = 8 for all genes (except CS and UCP3, where n = 7). Data are presented as fold changes from control pre (mean ± standard deviation). * significantly higher than the once-daily condition at the same time point ($P < 0.05$); † significantly higher than the control condition at the same time point ($P < 0.05$); # significantly higher than pre HIIE for the same condition ($P < 0.05$); □ significantly higher than post HIIE for the same condition ($P < 0.05$). Two-way analysis of variance (ANOVA) with Bonferroni post hoc test.

**Figure 6 | Genes related to carbohydrate metabolism pre, post, and 3 h post the high-intensity interval exercise (HIIE). (a)** phosphofructokinase (PFK) gene expression; **(b)** glucose transporter 4 (GLUT4) gene expression; **(c)** pyruvate dehydrogenase kinase isoenzyme 4 (PDK4) gene expression. n = 8 for all genes. Data presented as fold changes from control pre (mean ± standard deviation). # significantly higher than pre HIIE for the same condition ($P < 0.05$). Two-way analysis of variance (ANOVA) with Bonferroni post hoc test.

**Figure 7 | Physiological and systemic responses during the high-intensity interval exercise (HIIE). (a)** Heart rate (HR); **(b)** Ventilation ($\dot{V}E$); **(c)** Oxygen uptake ($\dot{V}O_2$); **(d)** Respiratory exchange ratio (RER). n = 8 for all variables. Data are presented as

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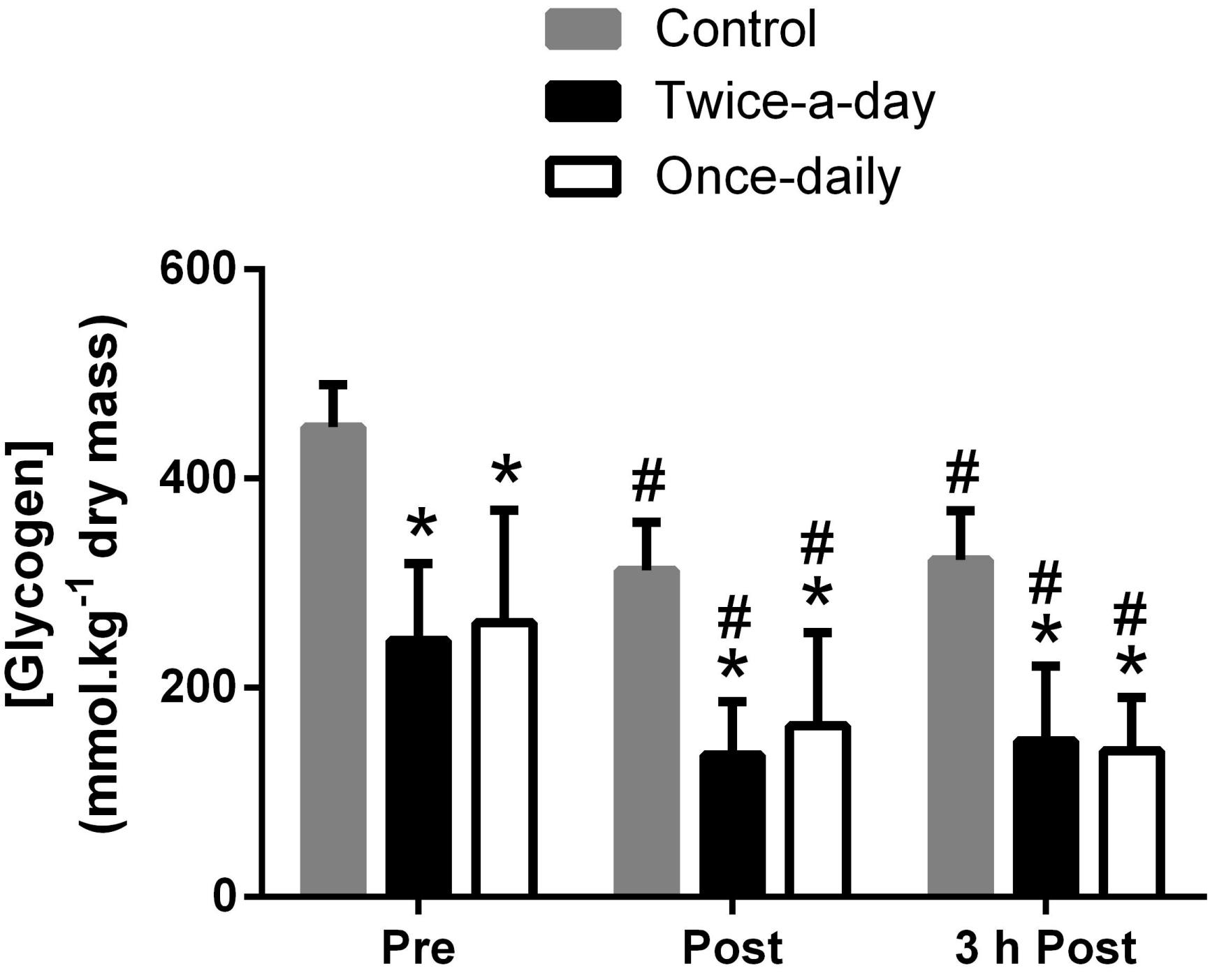
mean ± standard deviation. Time effect has been omitted for clarity. INT: Interval. Two-way analysis of variance (ANOVA) with Bonferroni post hoc test.

**Figure 8 | Experimental design.** D, dinner; B, breakfast; L, lunch; MG-DE, muscle glycogen-depleting exercise; HIIE, high-intensity interval exercise. Open dashed circles indicates that participants replicated their normal diet, while closed circles that participants ate a low-carbohydrate (CHO) breakfast [CHO: 42.7 ± 5.0 kcal (~7%), fat: 365.9 ± 43.1 kcal (~60%), protein: 201.3 ± 23.7 kcal (~33%)]. Black arrows indicate time point.


Control
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Pre
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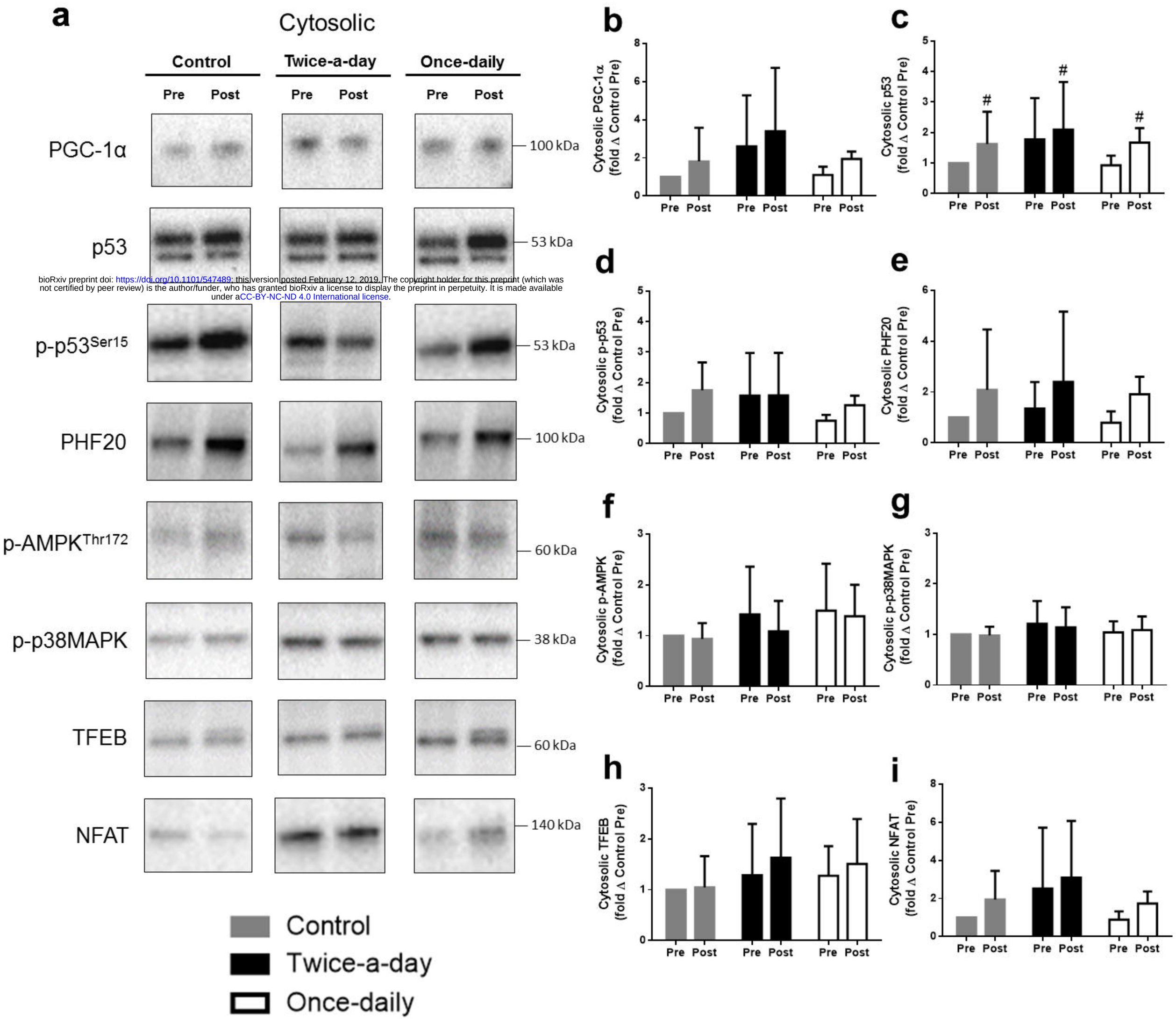


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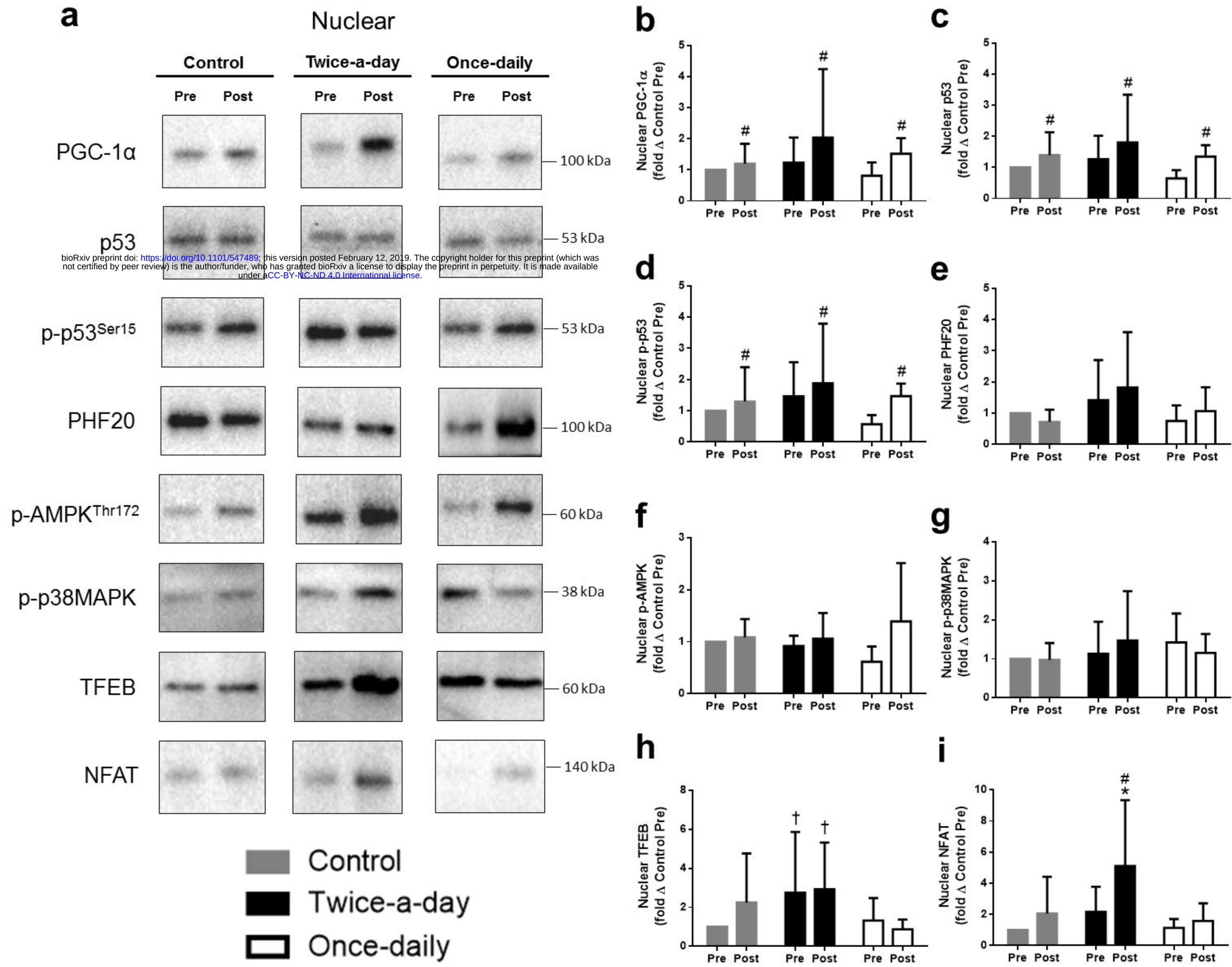


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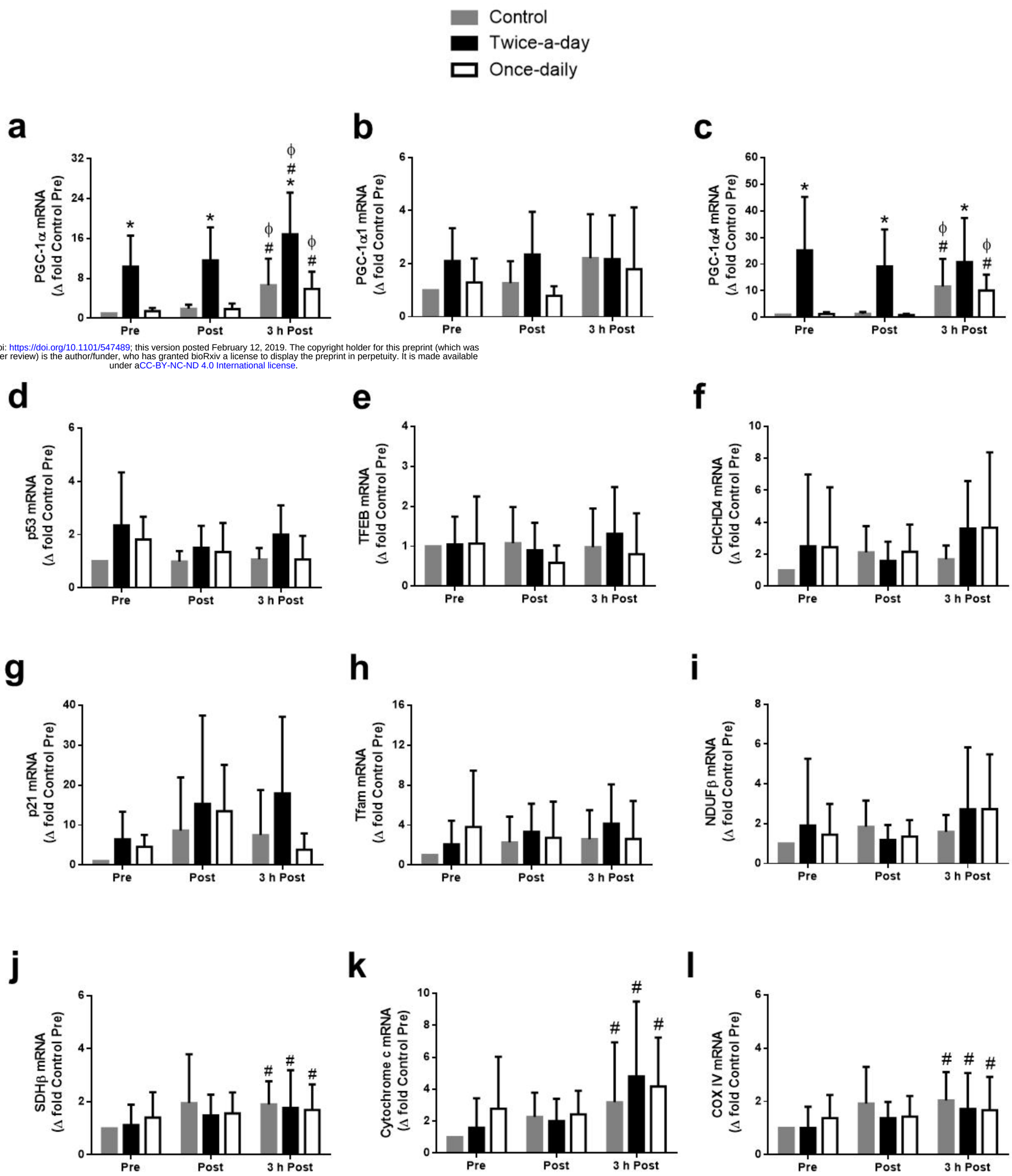


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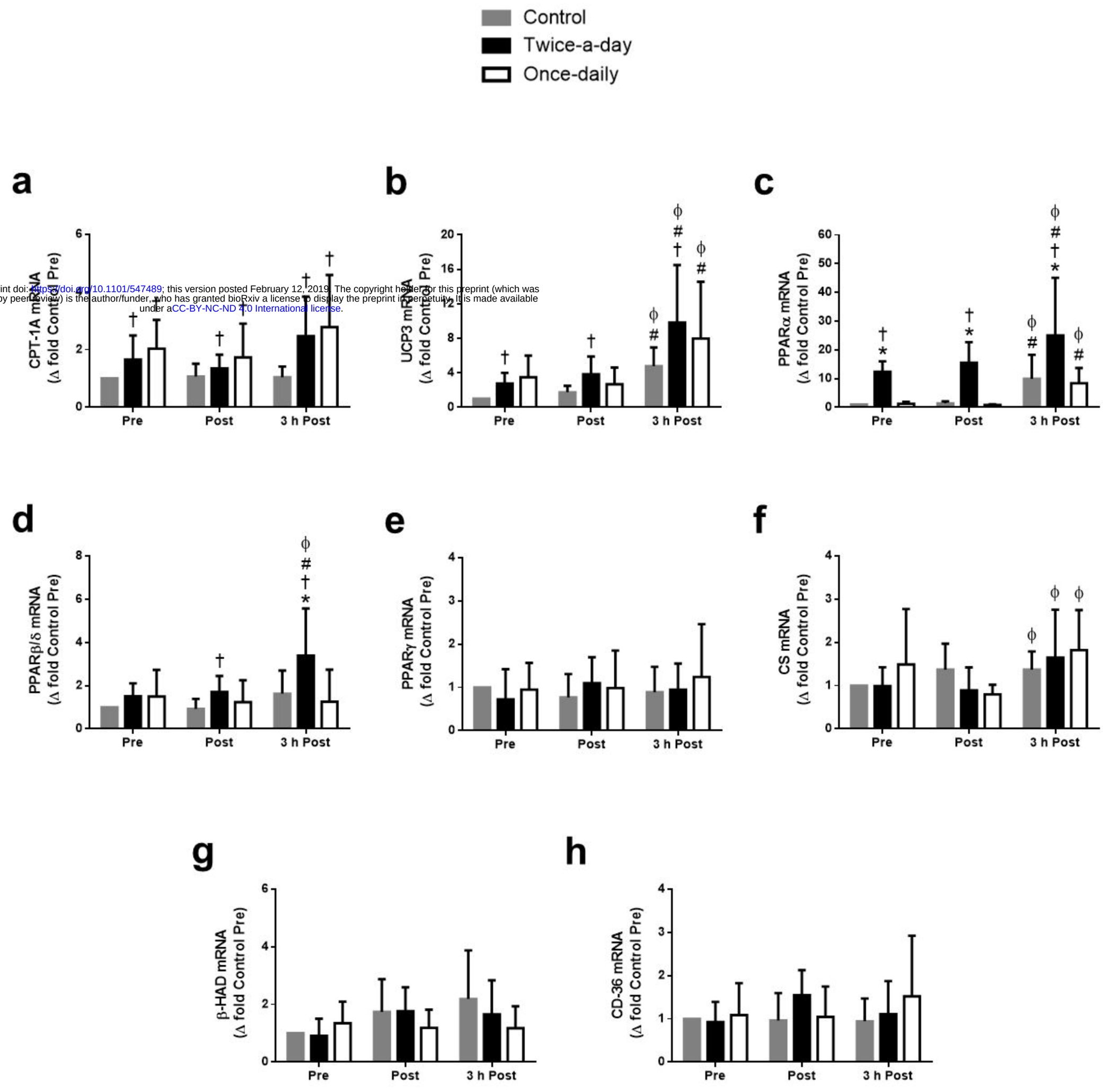


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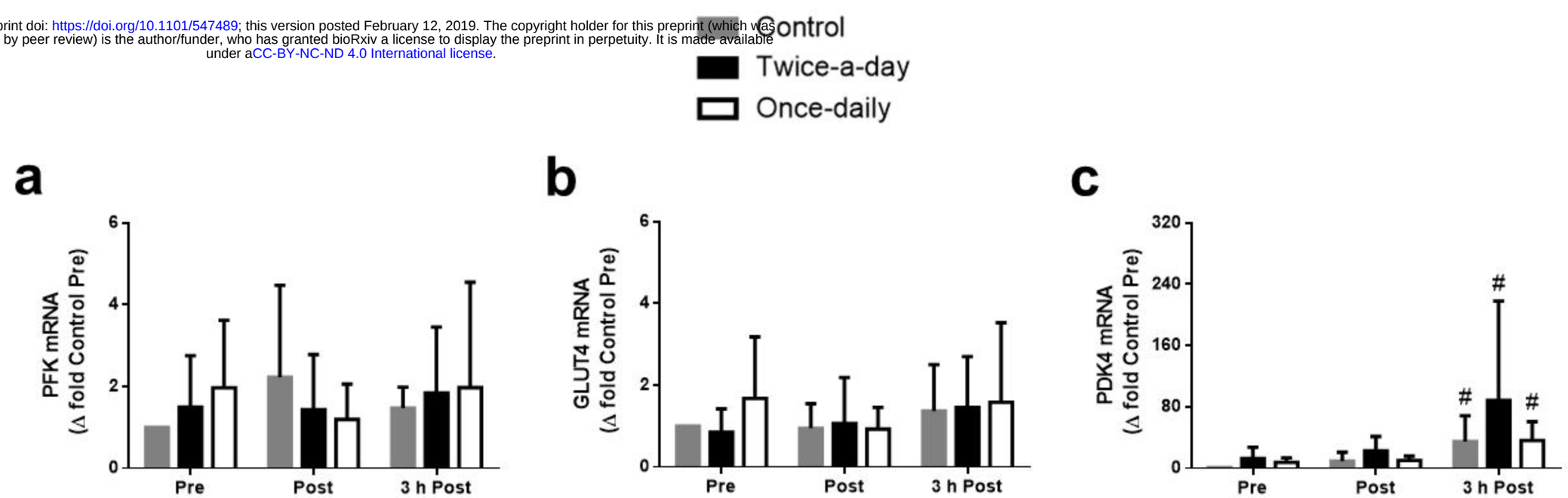

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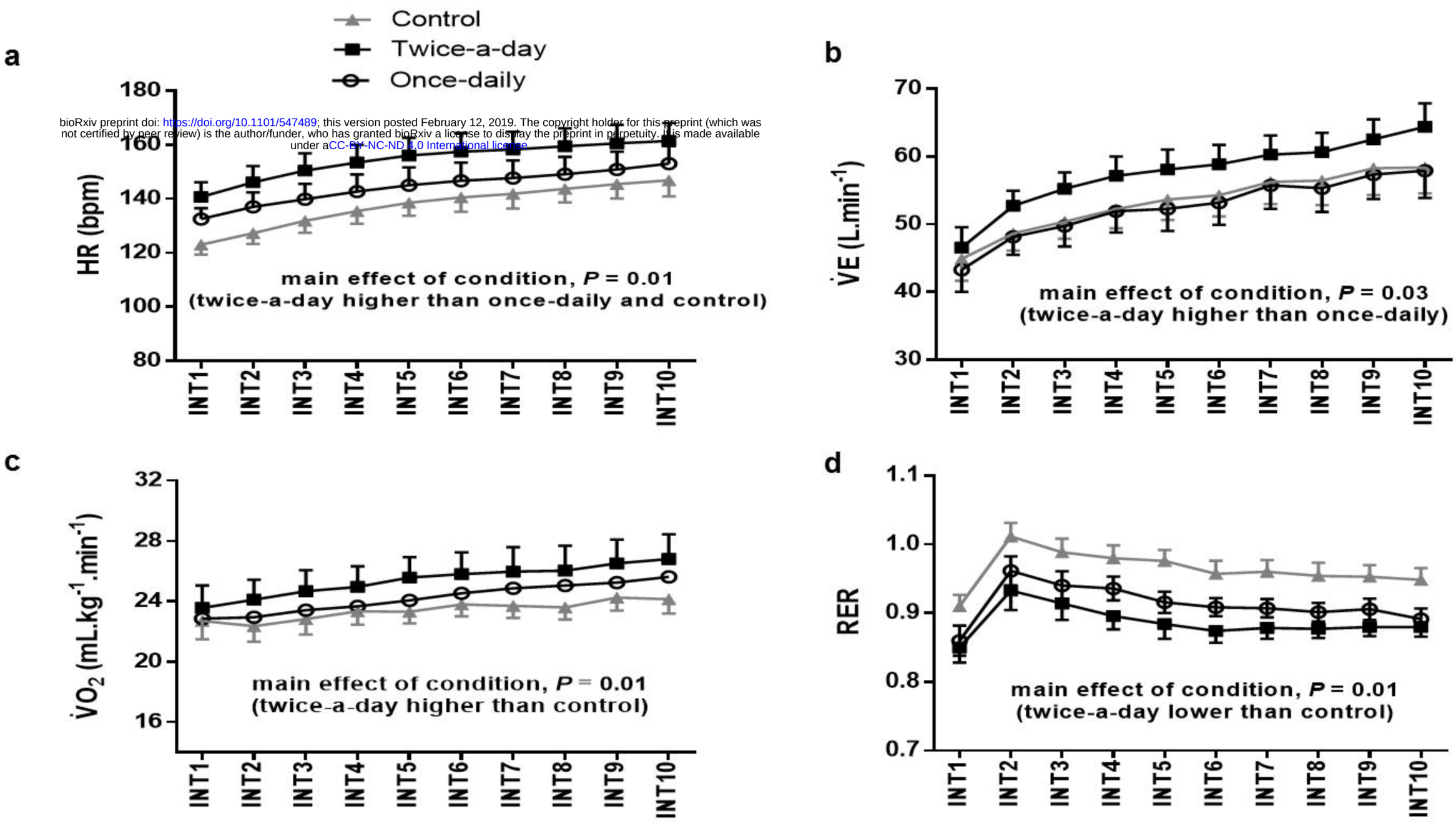

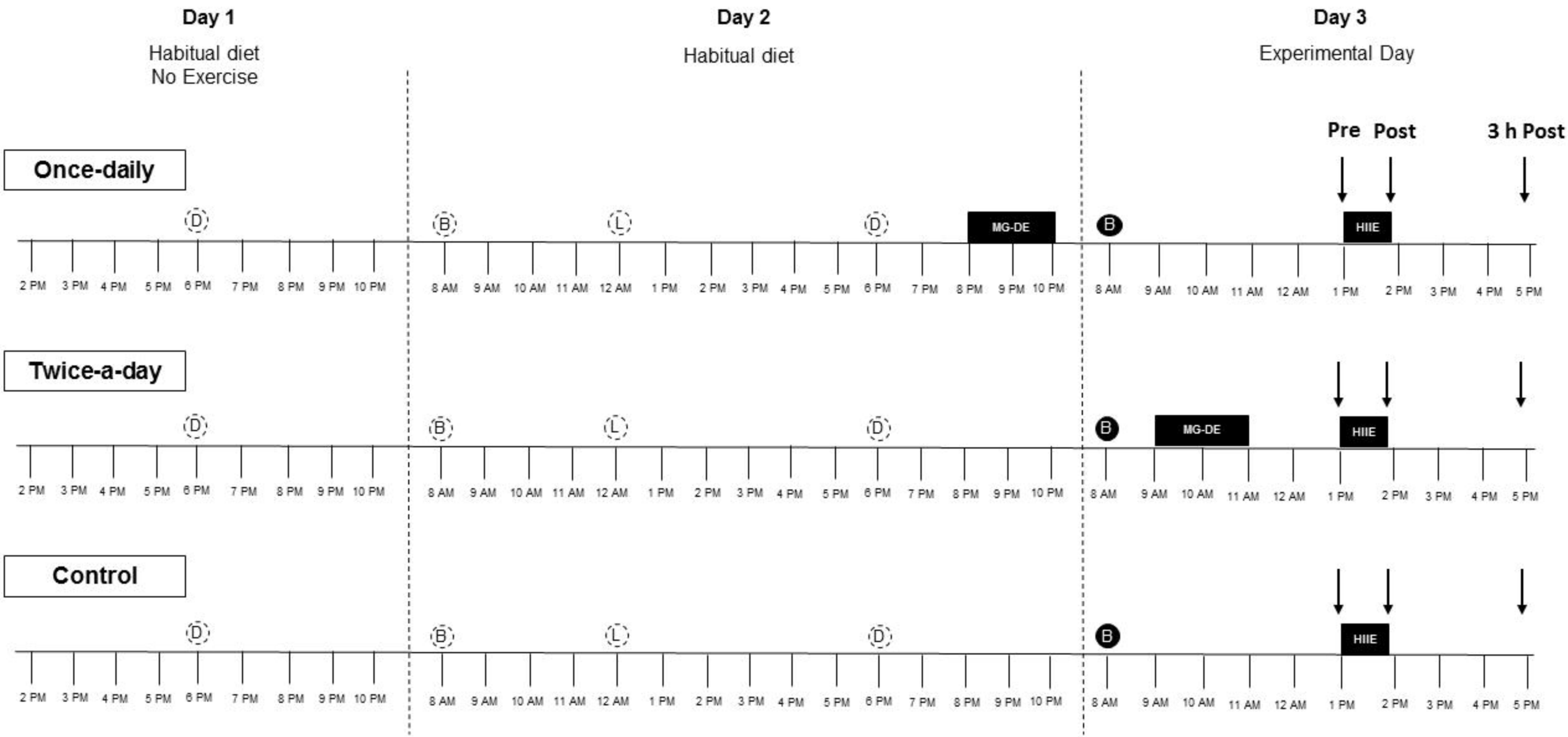
Day 1
Habitual diet
No Exercise
Day 2
Habitual diet
Day 3
Experimental Day
Pre
Post
3 h Post
Once-daily
Twice-a-day
Control
D
B
L
MG-DE
HIIE
2 PM 3 PM 4 PM 5 PM 6 PM 7 PM 8 PM 9 PM 10 PM
8 AM 9 AM 10 AM 11 AM 12 AM 1 PM 2 PM 3 PM 4 PM 5 PM 6 PM 7 PM 8 PM 9 PM 10 PM
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**Table 1 | Plasma lactate, glucose, epinephrine and norepinephrine concentrations, and serum free fat acid (FFA) and glycerol concentrations at pre, post and 3 h post the high-intensity interval exercise (HIIE).**

| | Pre HIIE | Post HIIE | 3 h post HIIE |
|---|---|---|---|
| ***Plasma*** | | | |
| **[Lactate] (mmol·L$^{-1}$)** | | | |
| *Control* | 1.2 ± 0.7 | 6.2 ± 2.0 □ | 1.3 ± 0.4 |
| *Once-daily* | 0.9 ± 0.3 | 3.9 ± 1.0 □† | 1.2 ± 0.4 |
| *Twice-a-day* | 1.4 ± 0.5 | 4.0 ± 1.6 □† | 2.0 ± 0.7 |
| **[Glucose] (mmol·L$^{-1}$)** | | | |
| *Control* | 5.2 ± 0.8 | 5.5 ± 0.9 | 5.3 ± 0.8 |
| *Once-daily* | 5.2 ± 0.5 | 5.0 ± 0.8 † | 4.8 ± 0.7 †# |
| *Twice-a-day* | 4.4 ± 0.7 *† | 4.8 ± 1.1 □† | 4.3 ± 1.0 *† |
| **[Epinephrine] (pg·mL$^{-1}$)** | | | |
| *Control* | 32.2 ± 18.4 | 39.3 ± 22.7 | 36.7 ± 17.8 |
| *Once-daily* | 56.7 ± 25.4 | 54.2 ± 25.7 | 35.8 ± 34.8 |
| *Twice-a-day* | 43.1 ± 13.6 | 43.7 ± 19.0 | 36.7 ± 10.4 |
| **[Norepinephrine] (pg·mL$^{-1}$)** | | | |
| *Control* | 68.0 ± 34.6 | 231.1 ± 141.8 □ | 120.4 ± 90.3 |
| *Once-daily* | 70.7 ± 33.8 | 350.6 ± 139.8 □ | 105.5 ± 77.5 |
| *Twice-a-day* | 76.7 ± 49.1 | 287.5 ± 147.6 □ | 113.8 ± 44.6 |
| ***Serum*** | | | |
| **[FFA] (µM)** | | | |
| *Control* | 194.9 ± 118.2 | 118.6 ± 72.6 | 130.3 ± 72.1 |
| *Once-daily* | 155.5 ± 79.9 | 236.5 ± 151.4 | 158.0 ± 62.8 |
| *Twice-a-day* | 209.9 ± 125.5 † | 260.6 ± 151.3 † | 230.4 ± 98.2 † |
| **[Glycerol] (mmol·L$^{-1}$)** | | | |
| *Control* | 0.105 ± 0.021 | 0.098 ± 0.025 | 0.098 ± 0.037 |
| *Once-daily* | 0.090 ± 0.037 | 0.141 ± 0.022 † | 0.100 ± 0.039 |
| *Twice-a-day* | 0.106 ± 0.031 | 0.169 ± 0.046 † | 0.102 ± 0.023 |

Data are presented as mean ± standard deviation. n = 8 for all variables. * significantly different from the once-daily at the same time point ($P < 0.05$); † significantly different from the control condition at the same time point ($P < 0.05$); # significantly different from pre HIIE for the same condition ($P < 0.05$); □ significantly different from pre and post HIIE for the same condition, except for glucose that was significantly different only from post HIIE ($P < 0.05$). Two-way analysis of variance (ANOVA) with Bonferroni post hoc test.

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**Supplementary Table 1 | Details of PCR primers used for RT-qPCR**

| Gene | Primer Efficience (%) | Forward Sequence | Reverse Sequence |
|---|---|---|---|
| PGC-1α | 103.6 | 5´-CAGCCTCTTTGCCCAGATCTT-3´ | 5´-TCACTGCACCACTTGAGTCCAC-3´ |
| PGC-1α1 | 102.9 | 5´-ATGGAGTGACATCGAGTGTGCT-3´ | 5´-GAGTCCACCCAGAAAGCTGT-3´ |
| PGC-1α4[69] | 111.9 | 5´-TCACACCAAACCCACAGAGA-3´ | 5´-TCACACCAAACCCACAGAGA-3´ |
| COX IV | 103.6 | 5´-GAGCAATTTCCACCTCTGC-3´ | 5´-CAGGAGGCCTTCTCCTTCTC-3´ |
| CPT1 | 111.0 | 5´-ACAGTCGGTGAGGCCTCTTA-3´ | 5´-CCACCAGTCGCTCACGTAAT-3´ |
| NDUF | 109.0 | 5´-TCAGATTGCTGTCAGACATGG-3´ | 5´-TGGTGTCCCTTCTATCTTCCA-3´ |
| SDH | 105.0 | 5´-AAATGTGGCCCCATGGTATTG-3´ | 5´-AGAGCCACAGATGCCTTCTCTG-3´ |
| Cytochrome C | 98.8 | 5´-GGGCCAAATCTCCATGGTCT-3´ | 5´-TCTCCCCAGATGATGCCTTT-3´ |
| GLUT4 | 103.6 | 5´-CTTCATCATTGGCATGGGTTT-3´ | 5´-AGGACCGCAAATAGAAGGAAGA-3´ |
| β-HAD | 80.6 | 5´-TGGACAAGTTTGCTGCTGAACAT-3´ | 5´-TTTCATGACAGGCACTGGGT-3´ |
| CD-36 | 119.0 | 5´-TTGATTGAAAAATCCTTCTTAGCCA-3´ | 5´-TGGTTTCTACAAGCTCTGGTTCTT-3´ |
| PFK | 97.6 | 5´-AAGACATCAAGAATCTGGTGGTTA-3´ | 5´-TCCAAAAGTGCCATCACTGC-3´ |
| CS | 113.5 | 5´-TGGGGTGCTGCTCCAGTATT-3´ | 5´-CCAGTACACCCAATGCTCGT-3´ |
| PPARα | 92.7 | 5´-GGCAGAAGAGCCGTCTCTACTTA-3´ | 5´-TTTGCATGGTTCTGGGTACTGA-3´ |
| PPAR□ | 109.0 | 5´-CTTGTGAAGGATGCAAGGGTT -3´ | 5´-GAGACATCCCCACTGCAAGG -3´ |
| UCP3 | 89.5 | 5´-CCACAGCCTTCTACAAGGGATTTA-3´ | 5´-ACGAACATCACCACGTTCCA-3´ |
| Tfam | 109.3 | 5´-CCGAGGTGGTTTTCATCTGT-3´ | 5´-GCATCTGGGTTCTGAGCTTT-3´ |
| PDK4 | 99.7 | 5´-GCAGCTACTGGACTTTGGTT-3´ | 5´-GCGAGTCTCACAGGCAATTC-3´ |
| p53 | 101.8 | 5´-GTTCCGAGAGCTGAATGAGG-3´ | 5´-TTATGGCGGGAGGTAGACTG-3´ |
| PPARβ/δ | 103.7 | 5´-CATCATTCTGTGTGGAGACCG-3´ | 5´-AGAGGTACTGGGCATCAGGG-3´ |
| TFEB | 102.0 | 5´-CAGATGCCCAACACGCTACC-3´ | 5´-GCATCTGTGAGCTCTCGCTT-3´ |
| CHCHD4 | 107.0 | 5´-GCTTGGCTGTTCCTTGTTATTC -3´ | 5´-GTTTCCTCTCTTGCTGCTACTC -3´ |
| p21 | 99.8 | 5´-GCAGACCAGCATGACAGATTT -3´ | 5´-GATGTAGAGCGGGCCTTTGA -3´ |
| GAPDH | 106.0 | 5´-AATCCCATCACCATCTTCCA-3´ | 5´-TGGACTCCACGACGTACTCA-3´ |
| B2M | 98.0 | 5´-TGCTGTCTCCATGTTTGATGTATCT-3´ | 5´-TCTCTGCTCCCCACCTCTAAGT-3´ |
| TBP | 99.0 | 5´-CAGTGACCCAGCAGCATCACT-3´ | 5´-AGGCCAAGCCCTGAGCGTAA-3´ |
| Cyclophilin | 100.0 | 5´-GTCAACCCCACCGTGTTCTTC-3´ | 5´-TTTCTGCTGTCTTTGGGACCTTG-3´ |
| 18S | 99.0 | 5´-CTTAGAGGGACAAGTGGCG-3´ | 5´-GGACATCTAAGGGCATCACA-3´ |
| ACTB | 107.0 | 5´-GAGCACAGAGCCTCGCCTTT-3´ | 5´-TCATCATCCATGGTGAGCTGGC-3´ |

PGC- 1α, peroxisome proliferator-activated receptor-□ coactivator 1α; COX IV, cytochrome c oxidase subunit IV; CPT1, carnitine palmitoyltransferase 1; NDUF, NADH:ubiquinone oxidoreductase; SDH, succinate dehydrogenase; GLUT4, Glucose transporter type 4; β-HAD, 3-hydroxyacyl-CoA dehydrogenase; CD36, fatty acid translocase cluster of differentiation 36; PFK, phosphofructokinase; CS, citrate synthase; PPARα, peroxisome proliferator-activated receptor alpha; PPAR□, peroxisome proliferator-activated receptor delta; UCP3, uncoupling protein 3; Tfam, mitochondrial transcription factor A; PDK4, pyruvate dehydrogenase kinase 4; p53, p53 protein; PPARβ/δ, peroxisome proliferator-activated receptor beta/delta; TEFB, transcription elongation factor; CHCHD4, coiled-coil-helix-coiled-coil-helix domain containing 4; p21, p21 protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; B2M, β-2-microglobulin; TBP, TATA-box binding protein; 18S, 18S ribosomal RNA; ACTB, actin beta.