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# Endurance exercise decreases protein synthesis and ER-mitochondria contacts in mice skeletal muscle

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Regulation of protein synthesis during exercise

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## Abstract

Exercise is important to maintain skeletal muscle mass through stimulation of protein synthesis, which is a major ATP-consuming process for cells. However, muscle cells have to face high-energy demand during contraction. The present study aimed to investigate protein synthesis regulation during aerobic exercise in mouse hindlimb muscles. Male C57Bl/6J mice ran at 12 m/min for 45 min or at 12 m/min for the first 25 min followed by a progressive increase in velocity up to 20m/min for the last 20 min. Animals were injected I.P. with 40 nmol/g of body weight of puromycin and sacrificed by cervical

dislocation immediately after exercise cessation. Analysis of gastrocnemius, plantaris, quadriceps, soleus and tibialis anterior muscles revealed a decrease in protein translation assessed by puromycin incorporation, without significant differences among muscles or running intensities. The reduction of protein synthesis was associated with a marked inhibition of mTORC1-dependent phosphorylation of 4EBP1, a mechanism consistent with reduced translation initiation. A slight activation of AMPK consecutive to the running session was measured but did not correlate with mTORC1 inhibition. More importantly, exercise resulted in a strong upregulation of REDD1 protein and gene expressions, while transcriptional regulation of other recognized exercise-induced genes (IL6, KLF15, and Rcan1) did not change. Consistently with the recently discovered role of REDD1 on mitochondria-associated membranes, we observed a decrease in mitochondria-ER interaction following exercise. Collectively, these data raise questions concerning the role of MAM disruption in the regulation of muscle proteostasis during exercise and, more generally, in cell adaptation to metabolic stress.

#### **Key words**

AMPK, MAMs, protein synthesis, REDD1, running exercise

#### **New & Noteworthy**

How muscles regulate protein synthesis to cope with the energy demand during contraction is poorly documented. Moreover, it is unknown whether protein translation is differentially affected among mice hindlimb muscles under different physiological exercise modalities. We showed here that 45 min of running decreases puromycin incorporation similarly in five different mice muscles. This decrease was associated with a strong increase in REDD1 protein expression and a significant disruption of the mitochondria and sarcoplasmic reticulum interaction.

## Introduction

Regular physical activity exerts beneficial effects on health, which are largely related to muscle adaptations (17). For example, endurance training improves metabolic profile as illustrated by a reduced whole body adiposity and a greater insulin sensitivity (8, 17). At the muscle level, metabolic adaptations to repeated endurance exercises involve a transition in muscle typology from glycolytic to more oxidative fibers and an alteration of mitochondrial function and/or network (17, 27). Endurance exercise also prevents from skeletal muscle atrophy induced by disuse or various pathologies (41). The maintenance or the increase in muscle mass relies on protein balance and particularly on protein synthesis. While studies have often focused on resistance exercise, a stimulus known for promoting muscle protein accretion (28), knowledge about the regulation of protein synthesis with endurance exercise remains more limited. Considering that protein synthesis is an energy demanding process (34, 45), one may suppose that it will be reduced proportionately to the increased metabolic disturbance occurring during exercise. However, it is unclear, whether muscle typology or exercise intensity influence protein synthesis in response to an endurance exercise bout.

The mammalian target of rapamycin complex 1 (mTORC1) is a central node regulating translation of capped mRNA into polypeptides through activation of initiation and elongation factors (14). For instance, phosphorylation of eIF4-binding protein 1 (4EBP1) by mTORC1 allows eIF4E-eIF4G interaction, which is a crucial event for mRNAs recruitment to ribosomes (34). Most of the studies (e.g. (24, 37)) report the activation of mTORC1 as a proxy for protein synthesis, partly due to methodological difficulties to directly measure mRNA translation. Among the negative regulators of mTORC1, AMP activated protein kinase (AMPK) and Regulated in Development and DNA Damage 1 (REDD1) have been proposed to inhibit protein synthesis during exercise (13, 31). AMPK activation depends on the cell energy status, notably the AMP:ATP ratio, and promotes both ATP generation and inhibition of biosynthetic pathways (23). During muscle contractions, AMPK activation depends upon exercise intensity (29, 37, 48). While the exact mechanisms remain to be uncovered, REDD1 protein expression is also enhanced by energetic stress and thus aerobic exercise (7, 19, 24). REDD1 reduces protein

synthesis and alters mitochondrial function in skeletal muscle (6, 7). The effect of REDD1 on mitochondrial activity might originate from its role on endoplasmic reticulum (ER)-mitochondria contact sites (called hereafter MAMs for mitochondria-associated membranes). MAMs are a key hub linking mitochondrial respiration, mTORC1 signaling and protein synthesis (4, 7). Recently, our team showed that REDD1 reduced MAMs formation in muscle cells, which was associated with a decrease in protein synthesis (7). However, it is still unknown whether exercise influences MAMs dynamic. Hence, our study aims to determine how exercise intensity and/or muscle typology alter protein translation and its regulation in mice. We hypothesize that protein synthesis will be inversely correlated to exercise intensity and that the decrease in protein synthesis will be associated with reduced mTORC1 activity and MAM formation.

## **Materials and methods**

### *Animals*

Experiments were carried out on 5-month old male C57Bl/6J mice from our own stock. Animals (3-4 per cage) were housed in a 12-h/12-h light/dark cycle at 22°C with food and water ad libitum. Experiments were conducted in the morning ( $\approx$ 10 am) with mice having free access to food until the beginning of the exercise bout. Food was removed in the control group while the other groups started their running exercise. These experiments were performed according to the European directives (86/609/CEE) and approved by the national ethics committee (referral file APAFIS#9706-2,017,042,516,166,776 v3).

### *Exercise protocol*

Running sessions were performed on a motor-driven treadmill (Exer-6M Treadmill; Columbus Instruments). First, all mice were acclimatized to treadmill for 10-15 min at 12 m/min on three consecutive days. The last acclimatization session took place 48h before the exercise protocol. Then,

mild intensity exercise (M; n=8) consisted of a 45 min bout of running at a velocity of 12 m/min, which represents about 50% of maximal aerobic velocity in our conditions (7). Heavy intensity exercise (H; n=8) started by 25 min running at 12 m/min followed by increments of 2 m/min every 5 min. Exercise was stopped after a total duration of 45 min with the last 5-min performed at a speed of 20 m/min (approximately 75-80% of maximal aerobic velocity; (7)). We covered the front end of the treadmill to reduce the potential stress associated with exercise on treadmill and attract the mice to run forward. Animals from M and H groups were sacrificed immediately after the 45 min running bout by cervical dislocation. Mice from control group (C; n=8) were maintained in their cage. All animals were injected intraperitoneously (I.P.) with 40 nmol/g of body weight of puromycin (8 µl/g of a solution at 5 mM in PBS) 20 min before cervical dislocation and tissue harvest. Exercise was interrupted (<15s) for the M and H groups to allow puromycin injection. Tibialis anterior (TA), gastrocnemius (GAS), soleus (SOL), plantaris (PLA) and quadriceps (QUA) muscles were removed in the same order consistently, within 10 min following exercise cessation, and immediately snap-frozen in liquid nitrogen.

### *Immunoblotting*

Frozen muscles were powdered and homogenized in lysis buffer containing 50 mM Tris-HCl, 100 mM NaCl, 5% glycerol, 1 mM EDTA, 1 mM EGTA, 50 mM β-glycerophosphate, 50 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1% Triton X-100, and protease inhibitor cocktail with Fastprep-24 (MP Biomedical, Santa Ana, CA). Total proteins (50 µg) were loaded on a 4–15% acrylamide precast gel (Bio-Rad, Hercules, CA) before SDS-PAGE and transferred onto a nitrocellulose membrane with the Trans-Blot® Turbo™ Transfer System (Bio-Rad). Membranes were blocked in 5% nonfat dry milk in Tris-buffered saline (TBS) for 1 h and then incubated overnight at 4°C with primary antibody at 1:1000 except for puromycin (1:2000) and REDD1 (1:500) in TBS 0.1% Tween and 5% of nonfat dry milk or BSA according to the manufacturer's recommendations. Antibodies 4EBP1 (#9452), 4EBP1 ser<sup>65</sup> (#9451), Acetyl-CoA Carboxylase (ACC; #3676), ACC ser<sup>79</sup> (#11818), AMPK (#5832), AMPK thr<sup>172</sup> (#2535), ERK1/2 (#4696), ERK1/2 Thr202/Tyr204 (#4370), p38 (#9212), p38 thr<sup>180</sup>/tyr<sup>182</sup> (#9211), S6 (#2317), and S6 Ser240/244

(#5364) were purchased from Cell Signaling Technology (Danvers, MA), puromycin antibody (MABE34) from Merck (Darmstadt, Germany), and REDD1 antibody (#10638-1-AP) from ProteinTech (Chicago, IL). Protein bands were visualized using peroxidase-conjugated secondary antibody and enhanced chemiluminescent (ECL) reagent with the Chemidoc™ Touch Imaging System and Image Lab™ Touch Software (Bio-Rad). Puromycin and REDD1 contents were normalized using stain-Free technology to loading controls on the membrane (Fig. 1). Phosphorylated protein 4EBP1, ACC, AMPK, ERK1/2, p38 and rpS6 were normalized to total protein content.

#### *Duo-link proximity ligation in situ assay*

*In situ* duo-link proximity ligation assay was performed on paraffin-embedded sections of skeletal muscle to monitor MAM number as previously described (7). Soleus and plantaris muscles from C and M conditions (n=4/group) were fixed in 4% paraformaldehyde on ice for 4-5h. Longitudinal sections were stained for nucleus with haematoxylin and immunolabelled with rabbit anti-inositol trisphosphate receptor (IP3R; Santa Cruz Biotechnology, Inc., Dallas, TX; #sc-28614) and mouse anti-voltage-dependent anion channel (VDAC; Abcam, Cambridge, UK; #ab14734) primary antibodies. Then, samples were processed for proximity ligation assay according to the manufacturer protocol (Olink Bioscience, Uppsala, Sweden). Secondary anti-mouse and anti-rabbit IgG conjugated antibodies were added to complementary oligonucleotide extensions (probe MINUS and PLUS). If the distance between the IP3R and VDAC proteins was below 40 nm, the secondary antibodies conjugated oligonucleotides hybridized with a connector oligonucleotide allowing the formation of a circular DNA template. After ligation and amplification steps, this DNA template was detected following hybridization using an HRP coupled-oligonucleotide probe. Then, an immunohistochemistry HRP enzymatic reaction generated spots, each one resulting from a single endogenous IP3R/VDAC interaction. Finally, these spots were quantified with a 20x magnification lens using a bright-field microscopy device (10 fields per muscle).

## RNA isolation and RT-qPCR

Total RNA was extracted from muscle samples using TRIzol Reagent (ThermoFisher Scientific, Courtaboeuf, France). RNA concentration was determined by spectrophotometry (Biodrop, Cambridge, UK) and purity was checked by the OD 260nm/OD 280nm ratio (>1.9). RNA quality was checked by electrophoresis on 1% agarose gel. Reverse transcription was performed with 2 µg of total RNA using the High-Capacity cDNA Reverse Transcription kit (4368813, Applied Biosystems, Courtaboeuf, France), and according to the manufacturer's instructions. Quantitative PCR (qPCR) analysis was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems) with 10 µl of Powerup SYBR Green Master Mix (A25742, Applied Biosystems), 10 nM of both forward and reverse primers (Table 1), and 5 µl of diluted cDNA (final volume of 15 µl). All PCR assays were performed in duplicate using the following cycling conditions: 50°C for 2 min, then 95°C for 2 min followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. The relative mRNA levels were normalized with the arithmetic mean of *α-tubulin* (*α-tub*) and *acidic ribosomal phosphoprotein P0* (*Arp*), as these housekeeping genes remained unaffected by the experimental conditions. Results were expressed using the comparative cycle threshold (Ct) method to generate  $\Delta\Delta C_t$  values with template dilutions ranging from  $10^1$  to  $10^6$  copies. The PCR overall efficiency (E) was calculated from the slopes of the standard curves according to the equation  $E = [10^{(-1/\text{slope})}] - 1$  and this value was higher than 95% for all assays. The relative abundance of each sample was then normalized according to the equation: Relative Quantity (RQ) =  $2^{-\Delta\Delta C_t}$  (39). All the experiments were conducted according to the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (9).

## Statistics

Data are presented as mean + SEM. A two-ways analysis of variance (ANOVA) was performed to determine the respective effects of exercise intensity and muscle type on the dependent variables (protein and gene expressions) after confirming data normality using the skewness and kurtosis coefficients. Differences between groups were identified using the Bonferroni post-test for multiple



comparisons when ANOVA was significant. Mann-Whitney U test was used to analyze data differences from duo-link proximity ligation assay (n=4/group) and gene expressions on SOL muscles (n=3/group). The critical alpha level was set at 5%.

## Results

Puromycin administration has been shown to be a reliable method to measure protein synthesis (18). Analysis of puromycin incorporation revealed a significant negative effect of exercise on protein translation (Fig. 1A). Contrary to our expectations, the 17 to 32% decrease in protein synthesis was not significantly different between mild (M) and heavy (H) exercise modalities and/or among the five studied muscles. mTORC1-dependent phosphorylation of 4EBP1 on ser<sup>65</sup>, an event required to promote cap-dependent translation initiation, displayed a marked decrease during exercise (from -47 to -80%) with no difference between M and H groups (Fig. 1B). A significant, albeit weak, correlation between puromycin content and 4EBP1 phosphorylation was observed on the mean data from the five muscles ( $r^2=0.42$ ;  $p=0.033$ ). Phosphorylation of S6 ribosomal protein (rpS6) on ser<sup>240/244</sup>, a downstream target of mTORC1/S6 kinase signaling (32), was noted in three muscles (GAS, PLA and TA). Consistently with 4EBP1 phosphorylation changes, a significant decrease in rpS6 ser<sup>240/244</sup> phosphorylation during exercise (40-50% decrease except for TA in M modality, -19%) was observed, with no differences between M and H modalities (Fig. 1C).

Given the lack of differences in protein synthesis between the five muscles, we checked whether running exercise exerted differential strain on these muscles. Therefore, we measured p38 mitogen-activated protein kinase (MAPK) activation as it senses signals arising from mechanical strains and Ca<sup>2+</sup><sub>intra</sub> fluxes during aerobic exercise (27). Phosphorylation on thr<sup>180</sup>/tyr<sup>182</sup> was positively altered by exercise with no difference between M and H modalities (Fig. 2A). A main effect of muscle type and a positive interaction between intensity and muscle type were observed, SOL muscle displaying greater

responses to exercise (+1790 and +1970% in M and H condition vs. C, respectively) compared to other muscles ( $\approx$  +50% in GAS; +160% in PLA, and QUA vs. C) in agreement with the higher level of neural activation recorded for the SOL during running (38).

ERK1/2 MAPK activation was assessed in three muscles (GAS, SOL, and TA). Along with a p38 activation, we recorded a main positive effect of exercise, with muscles from H group exhibiting significant greater values compared to C group muscles (Fig 2B). In addition, exercise-induced ERK1/2 activation was higher in the SOL (+830 and +1300% in M and H vs. C groups, respectively) compared to GAS (+130%) and TA (+70%) muscles.

Then, we investigated known regulators of mTORC1 and protein synthesis. Running had a main positive effect on AMPK phosphorylation (Fig. 3A). Post-hoc analysis revealed that the H modality elicited greater AMPK thr<sup>172</sup> protein content compared to control (+15% in SOL; +30% in QUA; +74% in GAS; +166% in PLA and +210% in TA). There were no differences between muscles and no muscle x intensity interaction. Values from the M condition were not different from the two other groups (C and H).

We also analyzed the phosphorylation pattern of ACC, a recognized substrate of AMPK (47). AMPK-mediated phosphorylation of ACC was altered by exercise with M and H modalities displaying greater values compared to C group (Fig. 3B). Muscle type had a main effect on ACC phosphorylation while there was no interaction between muscle and intensity. Indeed, post-hoc analysis revealed that exercise-induced ACC phosphorylation tended to be greater in TA vs. GAS and SOL ( $p=0.013$  and  $p=0.014$  respectively; with corrected  $\alpha$  risk set to 0.005).

Exercise strongly induced REDD1 mRNA and protein expression (Fig. 4A and B). REDD1 protein content was significantly greater in M (+110 to +500% depending on muscle type) and H conditions (+170 to +880% depending on muscle type) vs. C condition ( $p=0.0002$  and  $p<0.0001$ , respectively). We observed a main effect of muscle type on REDD1 protein expression with SOL tending to have lower values compared to GAS and PLA ( $p=0.019$  and  $p=0.022$ , respectively with Bonferroni corrected  $\alpha$  risk set to

0.005). Statistical analysis also showed a significant interaction between intensity and muscle type on REDD1 protein content. *Redd1* gene expression was enhanced by exercise, with no difference between M (+330 to +775% depending on muscles type) and H modalities (+250 to +1120% depending on muscle type). As for protein, we observed a main effect of muscle type and a significant interaction between muscle type and intensity on REDD1 mRNA level. Indeed, TA and PLA muscles exhibited greater increase in REDD1 mRNA (+650 to 1120%) compared to QUA muscle (+330 and +250% in M and H modalities, respectively, compared to C). All mRNA expression values from SOL were analyzed separately due to the lack of data for the H intensity. M condition induced a 170% increase in REDD1 mRNA of SOL muscle. There was no significant correlation between REDD1 protein and mRNA (not shown).

Gordon et al. recently showed that the increase in REDD1 mRNA expression following running exercise was partially dependent on glucocorticoid receptor (19). Kruppel-like factor 15 (KLF15) mRNA expression shares common features with REDD1 since it is induced by glucocorticoids (40) as well as endurance exercise (21). In addition, both REDD1 and KLF15 suppress mTORC1 activity in muscle (40). However, in contrast to REDD1, there were no exercise or muscle type effects on KLF15 expression (Fig. 5A).

Therefore, we wanted to determine whether expressions of other previously identified exercise-responsive genes were induced in our conditions (i.e. immediately after exercise). We focused on calcium-dependent genes since it may mirror muscle recruitment. Indeed, activation of motoneurons during muscle contractions results in enhanced intracellular calcium ( $\text{Ca}^{2+}_{\text{intra}}$ ) flux. Increased  $\text{Ca}^{2+}_{\text{intra}}$  then activates  $\text{Ca}^{2+}$ -dependent enzymes such as the phosphatase calcineurin. We measured mRNA expression of *regulator of calcineurin 1* (*Rcan1*, a.k.a *MCIP1*) and *interleukin-6* (*IL-6*), two genes with expressions related to calcineurin activity (1–3). Exercise had a main significant effect on *Rcan1* mRNA level (Fig. 5B). However, there was no effect of muscle type or muscle x intensity interaction. Post-hoc analysis revealed significantly greater values for M vs. H modality. As for *Rcan1*, we observed a main

effect of exercise intensity on IL-6 mRNA expression with no influence of muscle type or significant muscle x intensity interaction (Fig. 5C). In contrast to Rcan1 data, H group displayed greater IL6 gene expression compared to M group.

Since REDD1 can control MAM dynamic (7), we assessed MAM number via the interaction between two proteins that localize on the outer mitochondrial membrane (VDAC) and the sarcoplasmic reticulum membrane (IP3R). M condition induced a 30 and 44% decrease of VDAC/IP3R interaction in PLA and SOL muscles, respectively (Fig. 6). In addition, basal ER-mitochondria contacts tended to be higher in SOL vs. PLA muscle (+40%;  $p=0.083$ ).

## **Discussion**

In the present study, we measured puromycin incorporation, activation, and expression of relevant protein synthesis regulators immediately after an endurance exercise bout. We found a 20-30% inhibition of protein translation with no significant differences either between mild and heavy intensities or between the five muscles studied. These results are consistent with the similar reduction of mTORC1 activity regardless of muscle type or running intensity. The decrease in 4EBP1 phosphorylation with exercise suggests a slower initiation of protein translation, which is in agreement with the impeded polysome formation previously observed by Williamson et al. in mice muscle following a 30 min running bout (46). Interestingly, these observations were concomitant with a decrease in muscle mitochondria-ER interaction. We further investigated the putative mechanisms involved in this regulation.

### **Regulation of protein synthesis**

mTORC1 is considered a master regulator of protein synthesis in muscle cells following anabolic stimuli (28). While many studies reported similar variations between mTORC1 activity and protein synthesis

(6, 7, 13, 18, 30, 36, 46), Philp et al. observed an increase in myofibrillar and mitochondrial protein synthesis despite a reduction in mTORC1 activity 30 min after an endurance exercise bout (33). In addition, rapamycin administration, which inhibits mTORC1 activity, modestly reduced myofibrillar protein synthesis suggesting a marginal role of mTORC1 in endurance exercise-induced regulation of protein synthesis. However, one may hypothesize that the effect of rapamycin was modest because mTORC1 activity was already low. Moreover, it is important to note that our data may differ from Philip's due to a different muscle collection time (immediately vs. 30 min post exercise, respectively) as well as a different timing of protein synthesis tracer injection (during vs. after exercise, respectively). Although the precise role of mTORC1 in the protein synthesis response to endurance exercise remains to be elucidated, our data suggest that alteration of mTORC1 activity (but not in ERK1/2 or p38 activities) could be responsible for the reduced protein anabolism during exercise.

AMPK and REDD1 are two negative regulators of mTORC1. AMPK acts through direct phosphorylation on Raptor (20) or tumor suppressor complex 2 (TSC2) (25), whereas REDD1 has been proposed to inhibit Akt activation (11) or to release TSC2 from its association with inhibitory 14-3-3 proteins (12). In the present study, phosphorylation of AMPK and its downstream target ACC were not only slightly altered by exercise but also did not correlate with puromycin uptake or 4EBP1 phosphorylation. Similar to our study, others had previously reported an uncoupling between AMPK phosphorylation and protein synthesis or 4EBP1 phosphorylation (24, 37). In addition, genetic deactivation of AMPK does not alter inhibition of neither protein synthesis nor 4EBP1 phosphorylation in response to ex-vivo contraction (36). Altogether, it appears that AMPK is not the main nor the earliest repressor of protein synthesis during muscle contraction. Indeed, our results suggest that REDD1 could transiently inhibit muscle protein translation, even for mild intensity exercise. In addition, we previously showed that REDD1 deletion resulted in muscle AMPK overactivation following fasting, hypoxia and exercise (7). Therefore, AMPK might be preferentially activated in conditions when muscle cells are in high energy demand.

The robust increase in both REDD1 protein (3 to 10-fold) and mRNA (3 to 12-fold) observed right after an exercise session reinforces the idea that REDD1 is important for muscle cell adaptation to contractions. *Redd1* gene induction could originate from several signals activated by exercise, among which glucocorticoids release (19), cellular hypoxia (15), adrenaline secretion (49), oxidative stress (22, 26), and potentially heat stress (16, 43). In addition, exercise activates MEK pathway in skeletal muscle (44, 46), as attested here by the increase in ERK1/2 phosphorylation, and MEK stabilizes and protects REDD1 from proteasomal degradation in adipocytes and HEK cells (35). Thus, the rapid increase in REDD1 protein level during running could also be related to MEK signaling. Overall, our data provide evidences that REDD1 (mRNA and protein expressions) is a valuable biomarker to assess muscle stress during exercise.

We have previously demonstrated that REDD1 i) localizes in the crude mitochondrial fraction after endurance exercise, ii) interacts with some MAM components including glucose regulated protein 75 (GRP75; also called mortalin/PBP74/mtHSP70), and iii) disrupts MAM (7). In turn, the reduced interaction between mitochondria and ER may decrease ATP availability for ER-dependent protein translation, which represents a major energy consuming process (34, 45). Indeed, we have showed that REDD1 silencing led to an increase in MAM interaction and protein synthesis in muscle cells (7). Accordingly, in our study, M exercise led to a clear decrease in ER-mitochondria interaction based on a proximity ligation assay performed on muscles. Furthermore, basal MAM formation tended to be greater in the SOL, a muscle with high protein turnover (10). These results support the hypothesis that protein synthesis in muscle cells could be tightly regulated by the connection between these two organelles, and that REDD1 might control this mechanism.

### **Effect of muscle type**

We choose to analyze five hindlimb muscles that are commonly used in exercise physiology studies. Three of these muscles (GAS, QUAD and TA) are rich in type IIB (glycolytic) fibers while one (SOL) contains predominantly type I and IIA (oxidative) fibers. The last one muscle (PLA) displays a mixed

phenotype (5). Previous studies illustrated a different susceptibility of protein synthesis to repeated contractions between glycolytic and oxidative muscles, the latter being less affected by exercise (10, 30, 36). However, these discrepancies with our results could be explained by two factors. Firstly, the level of muscle recruitment in the experimental models used in these studies (electrostimulation) is far greater compared to our conditions of exercise, thus contributing to the high levels of protein synthesis and/or mTORC1 inhibition recorded by others (10, 30, 36). Secondly, experiments on isolated muscles induced exogenous electrostimulation levels that are similar across the tested muscles, while during running session, muscles will be differentially recruited depending on the exercise modalities (e.g. SOL >> TA, (38)). The striking difference in p38 phosphorylation observed in this study between SOL and the four other muscles further confirms that running elicited various mechanical strains. Therefore, even if protein synthesis of glycolytic muscles is more affected by contraction than in oxidative ones (10, 30, 36), the expected stronger effect on protein synthesis could be balanced by a poorer recruitment of these fast twitch muscles during the running session.

#### **Methodological considerations**

We hypothesized that running velocity would impact protein synthesis and TORC1 activity as suggested by others (10, 37). Bylund-Fellenius et al. proposed that protein synthesis is inversely related to the energy state. Assuming that running at 20 m/min elicits greater metabolic changes compared to 12 m/min, it seemed reasonable to expect higher variations of the energy status in H compared to M group. In fact, AMPK activation was significantly increased in H but not in M conditions compared to C. As AMPK negatively regulates TORC1 activity, a greater AMPK phosphorylation should have caused higher TORC1 inhibition in H modality. One may wonder if the duration of running at higher speeds (i.e. 18-20 m/min) during H exercise modality was sufficient to induce higher strenuous alterations in cell homeostasis compared to M modality. We also may have missed a difference in TORC1 activity between M and H intensity since the decrease in 4EBP1 phosphorylation could reach a plateau after

45 min of exercise, regardless of the running velocity. Indeed, 4EBP1 phosphorylation continues to decrease from 10 to 30 min of exercise in human and mouse muscles (37, 46).

One major difference in the measurement of puromycin uptake vs. 4EBP1 phosphorylation is that the former reflects the average protein translation during 20 min starting from puromycin injection whereas the latter is an endpoint marker. This emphasizes that the puromycin incorporation technique is likely less sensitive to acute variations than monitoring 4EBP1 phosphorylation. Moreover, puromycin incorporation is a global marker of protein synthesis, while 4EBP1 phosphorylation controls the translation of a subfamily of messengers depending on TORC1 signaling (TOP mRNAs; (42)). Therefore, these two methods can bring interesting and complementary information on translation dynamic during exercise.

In summary, our findings give new insights into how muscle cells face the high ATP need. Our results show that muscle protein building is impaired even with light intensity exercise. This work also highlights the fast and dynamic changes affecting mTORC1 activity and MAMs in the regulation of muscle proteostasis during running. Importantly, the present results may also apply to other conditions of cell adaptation in particular energetic context. For example, it would be interesting to determine whether such mechanisms are also effective in cancer, neurodegenerative disorders or during ischemia as MAMs number and REDD1 expression are both altered in these conditions.

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## **Disclosures**

None.



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527     **Tables**

528

529     **Table 1.** Real Time PCR Primer-Characteristics for cDNAs Amplification

Gene	GenBank Acc. No.	Forward primer	Reverse primer	Amplicon size (bp)
<i>Arp</i>	NM_007475.5	5'-ACTGGTCTAGGACCCGAGAAG-3'	5'-CTCCACCTTGTCTCCAGTC-3'	124
<i>α-Tub</i>	NM_011653.2	5'-CTGGAACCCACGGTCATC-3'	5'-GTGGCCACGAGCATAGTTATT-3'	114
<i>IL-6</i>	NM_001314054.1	5'-TGGTACTCCAGAAGACCAGAGG-3'	5'-AACGATGATGCACTTGCAGA-3'	128
<i>Klf15</i>	NM_023184.3	5'-TGTGCCCATTGCCGCCAACCTA-3'	5'-GTGCCTTGACAACTCATCTG-3'	265
<i>Rcan1</i>	NM_019466.4	5'-ACCGTGTGGAATTGTCCTTCTC-3'	5'-GGGACCCAGTAATACACATGCA-3'	70
<i>Redd1</i>	NM_029083.2	5'-CCAGAGAAGAGGGCCTTGA-3'	5'-CCATCCAGGTATGAGGAGTCTT-3'	112

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## Figure legends

**Figure 1. Exercise decreases puromycin incorporation, 4EBP1 and rpS6 phosphorylation in mouse hindlimb muscles.** ■ control, ■ mild and □ heavy exercise groups; GAS: gastrocnemius, PLA: plantaris, QUA: quadriceps, SOL: soleus, and TA: Tibialis anterior muscles; LC: loading control. Puromycin content (A) was normalized to loading control (stain-free method; n=6-8/group), whereas 4EBP1 (B; n=5-7/group) and rpS6 phosphorylation (C; n=6/group) were normalized to total 4EBP1 and rpS6 expressions, respectively. Bonferroni post-hoc test revealed significant differences between M and H groups vs. C group for all three variables ( $p<0.0167$ ).

**Figure 2. Running exercise markedly enhanced p38 and ERK1/2 phosphorylation in mouse hindlimb muscles.** ■ control, ■ mild and □ heavy exercise groups; GAS: gastrocnemius, PLA: plantaris, QUA: quadriceps, SOL: soleus, and TA: Tibialis anterior muscles. A) p38 phosphorylation (n=6-8/group except for GAS and SOL of C group and SOL of H group, n=5); \* significantly different from the corresponding control group and † significantly different from the four other muscles for the corresponding intensity according to Bonferroni correction for multiple comparisons ( $p<0.001$ ). B) ERK1/2 phosphorylation (n=5-7/group) in SOL muscle is significantly higher compared to the four other muscles according to Bonferroni correction for multiple comparisons ( $p<0.0167$ ).

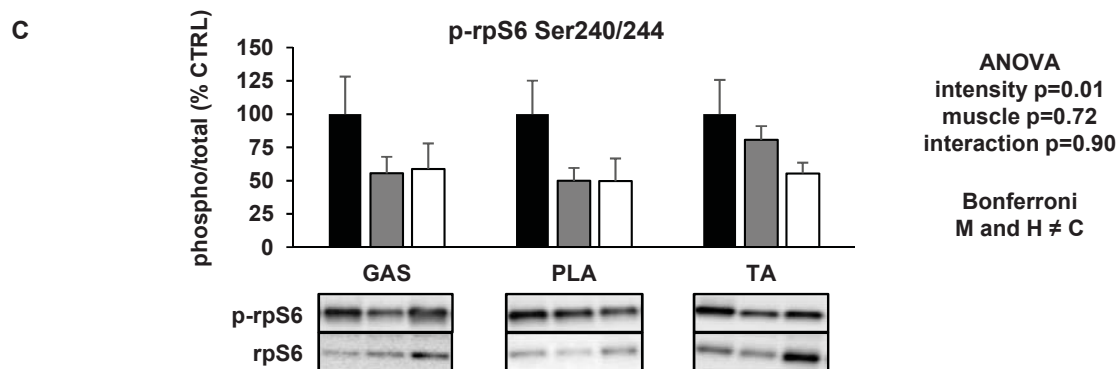
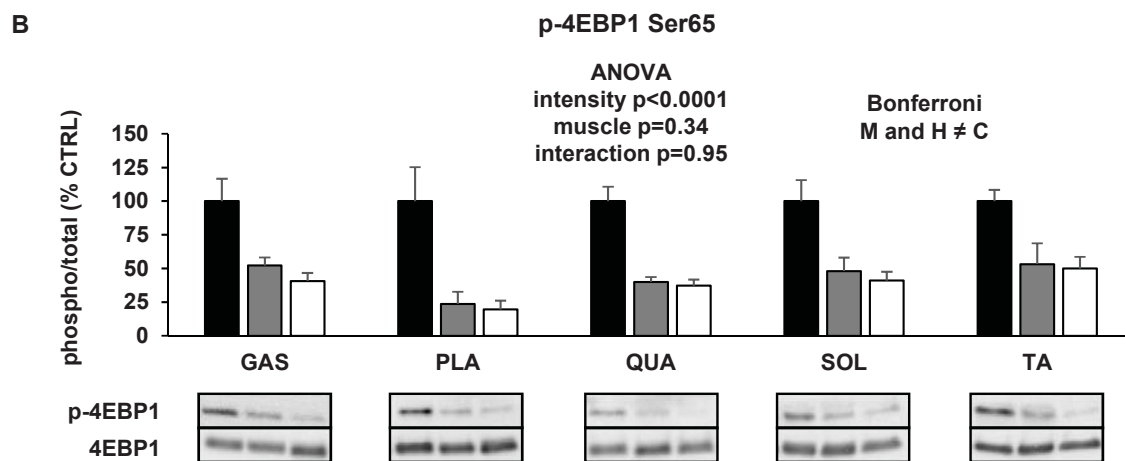
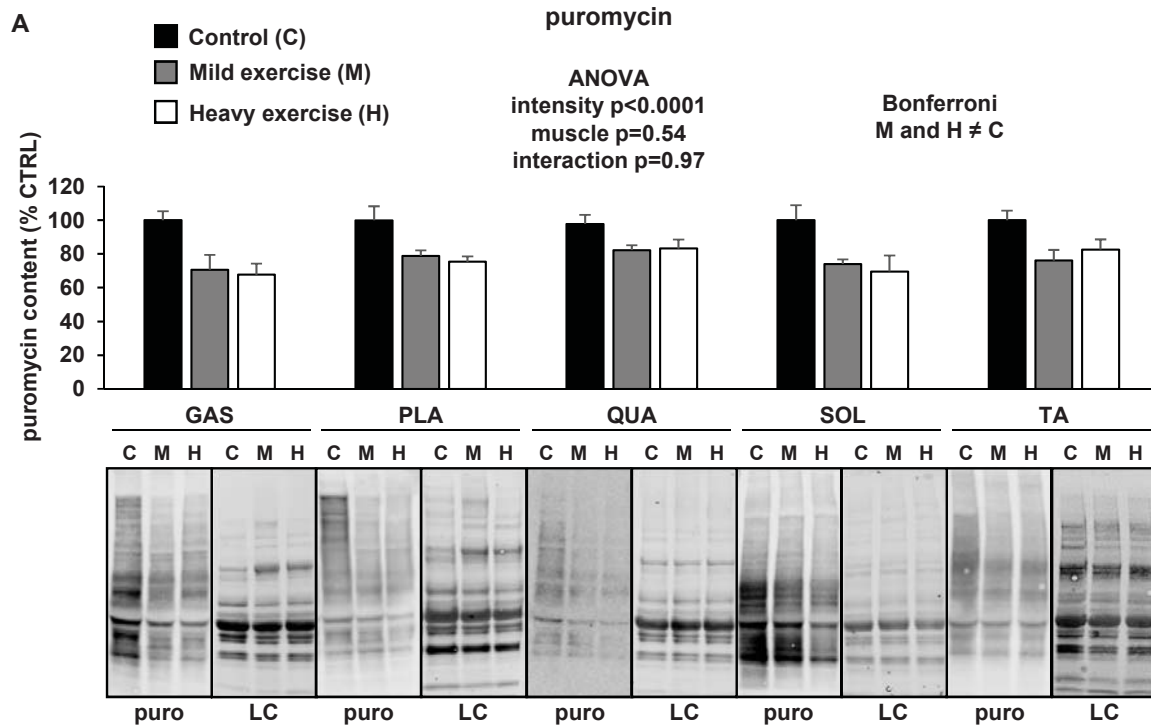
**Figure 3. Heavy intensity exercise activates AMPK in mouse hindlimb muscles.** ■ control, ■ mild and □ heavy exercise groups; GAS: gastrocnemius, PLA: plantaris, QUA: quadriceps, SOL: soleus, and TA: Tibialis anterior muscles. A) AMPK phosphorylation (n=4-6/group) is significantly increased in H vs. C group according to Bonferroni correction for multiple comparisons ( $p<0.0167$ ). B) ACC phosphorylation (n=5-8/group) is greater in H and M groups compared to C group according to Bonferroni correction for multiple comparisons ( $p<0.0167$ ).

**Figure 4. REDD1 protein and mRNA are strongly induced by endurance exercise in mouse hindlimb muscles.** ■ control (C), ■ mild (M) , and □ heavy (H) exercise groups; GAS: gastrocnemius, PLA: plantaris, QUA: quadriceps, SOL: soleus, and TA: Tibialis anterior muscles; A) REDD1 protein expression was normalized to loading control (LC) using the stain-free technology (n=6-8/group). \* and \$ significantly different from the corresponding C group and the corresponding M group, respectively, according to Bonferroni correction for multiple comparisons ( $p < 0.0167$ ). B) REDD1 mRNA levels were analyzed in GAS, PLA, QUA, and TA muscles (n=5-7/group) by a two-way ANOVA followed by multiple comparisons with the Bonferroni post-hoc test. \* significantly different from the corresponding C group ( $p < 0.0167$ ). A Mann-Whitney test was used to analyze SOL values (n=3); \*  $p < 0.05$  vs. C group.

**Figure 5. No transcriptional induction of Kruppel-like factor 15 (KLF15), regulator of calcineurin 1 (Rcan1) and interleukin-6 (IL-6) by endurance exercise in mouse hindlimb muscles.** ■ control, ■ mild and □ heavy exercise groups; GAS: gastrocnemius, PLA: plantaris, QUA: quadriceps, SOL: soleus, and TA: Tibialis anterior muscles. GAS, PLA, QUA and TA mRNA expressions (n=5-7/group) were analyzed by a two-way ANOVA followed by multiple comparisons with the Bonferroni post-hoc test. Bonferroni post-hoc test revealed significant differences between M vs. H group for Rcan1 and IL6 mRNA expressions ( $p < 0.0167$ ). A Mann-Whitney test was used to analyze SOL mRNA expressions (n=3).

**Figure 6. Mild intensity exercise reduces ER-mitochondria contacts in PLA and SOL muscles.** ■ control (C), and ■ mild (M) exercise groups; PLA: plantaris and SOL: soleus muscles. \* $p < 0.05$  compared to control group following a Mann-Whitney test (n=4/group).

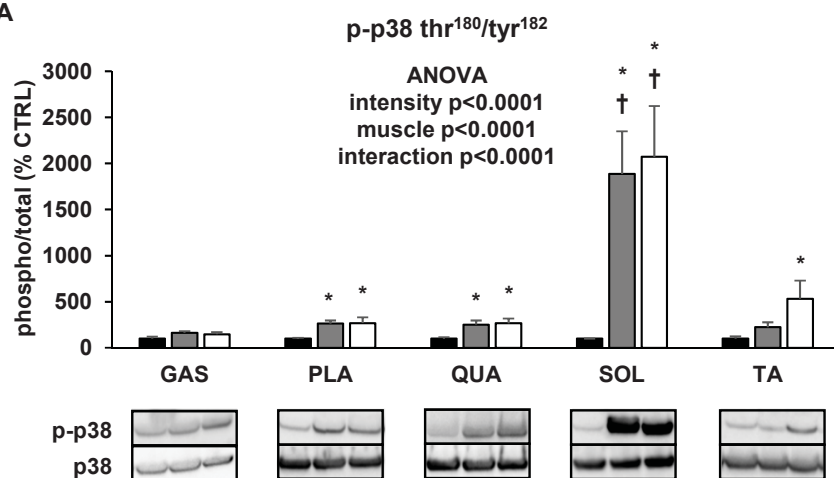
**Figure 1**





**Figure 2**

**A**



**B**

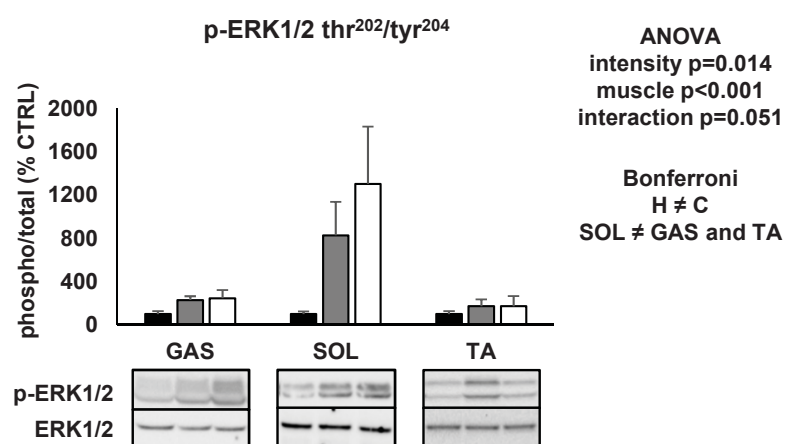


Figure 3

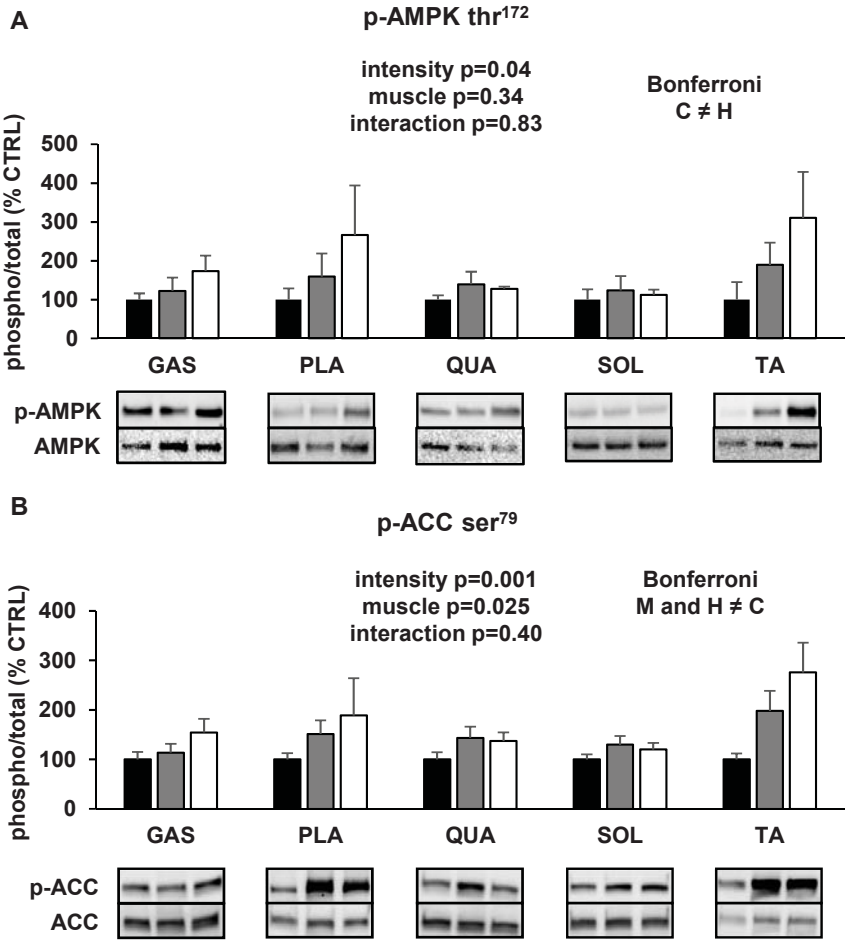


Figure 4

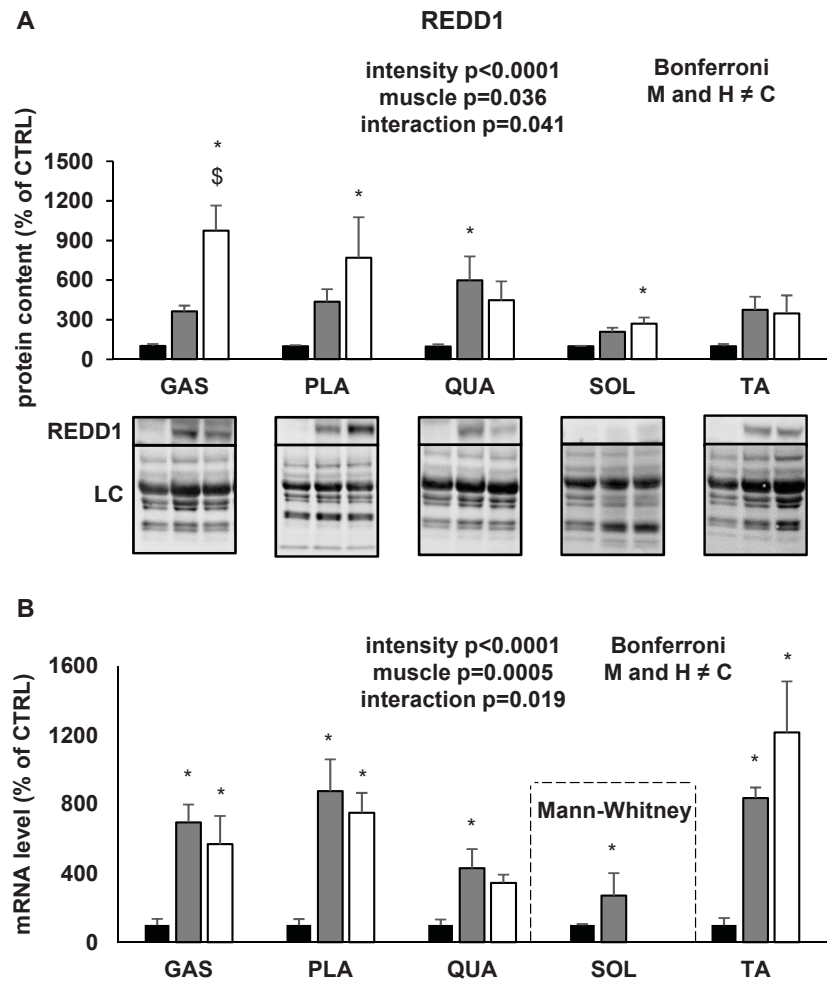


Figure 5

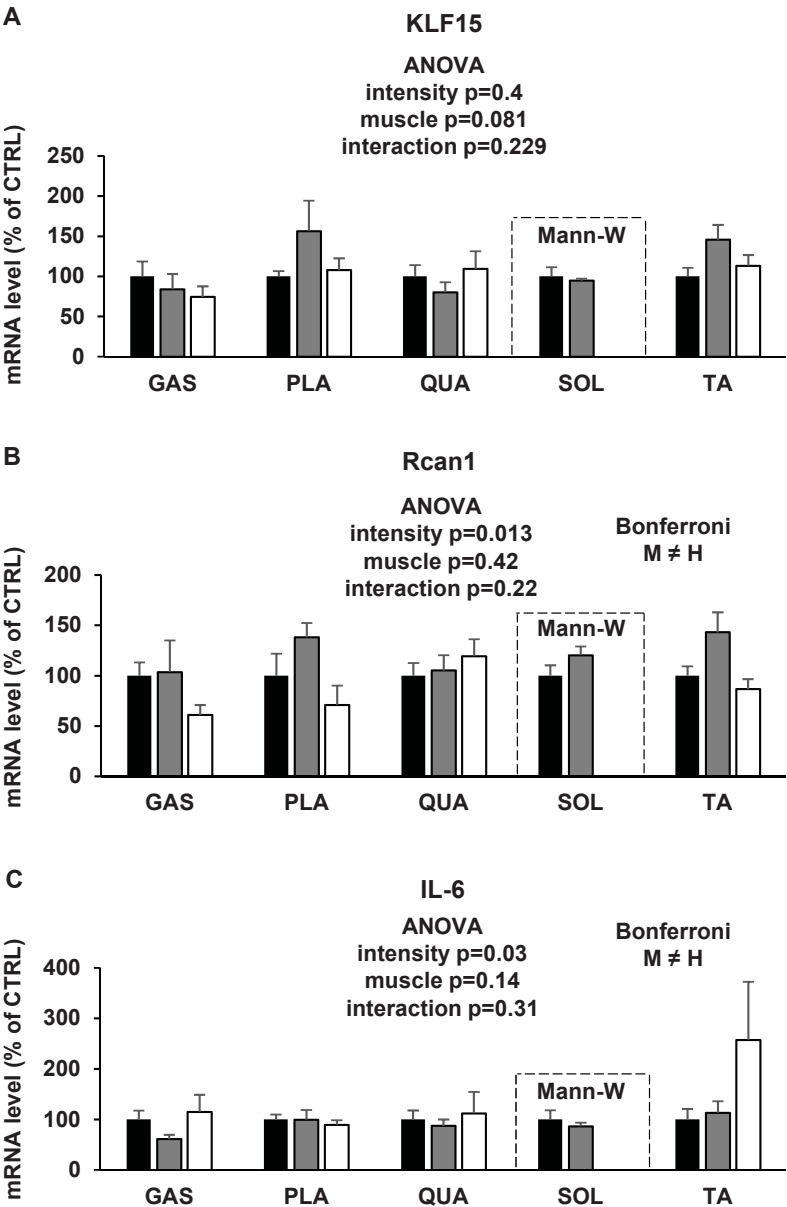


Figure 6

