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Abbreviations

COX, cytochrome c oxidase; CS, citrate synthase; HIT, high intensity training; MIT, moderate intensity training; OXPHOS, oxidative phosphorylation; PGC-1α, peroxisome proliferator-activated receptor-γ (PPAR-γ) coactivator-1α; UCP, uncoupling protein.

Abstract

Beneficial effects of physical exercise training are in part related to enhancement of muscle mitochondrial performance. The effects of two different trainings were investigated on transcripts and proteins of the AMPK–PGC-1α signaling pathway, the mitochondrial functioning (citrate synthase (CS), oxidative phosphorylation complexes, uncoupling proteins (UCP)) and the antioxidant defenses (superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase) in rainbow trout red and white skeletal muscles. One group of trouts swam for 10 days at a moderate intensity (approximately 57% U_{crit} or 2.0 body lengths/s, 23.5 h/day) and another group at a high intensity (approximately 90% U_{crit} or 3.2 body lengths/s, 2 h/day). In the red muscle, the increase of *Cs* mRNA levels was significantly correlated with the transcripts of *Ampkα1*, *Ampkα2*, *Pgc-1α*, the oxidative phosphorylation complexes, *Ucp2α*, *Ucp2β*, *Sod1*, *Sod2* and *Gpx1*. After 10 days of training, high intensity training (HIT) stimulates more the transcription of genes involved in this aerobic pathway than moderate intensity training (MIT) in the skeletal muscles, and mainly in the red oxidative muscle. However, no changes in CS, cytochrome c oxidase (COX) and antioxidant defenses activities and in oxidative stress marker (isoprostane plasmatic levels) were observed. The transcriptomic responses are fiber- and training-type dependent when proteins were not yet expressed after 10 days of training. As in mammals, our results suggest that HIT could promote benefit effects in fish.

Keywords

Moderate intensity training

High intensity training

Skeletal muscle

Mitochondrial biogenesis

Antioxidant defenses

Introduction

Physical exercise is recognized to improve human health but the involved cellular and molecular mechanisms remain to be fully elucidated (Neufer *et al.*, 2015). The stimulation of the mitochondrial biogenesis and antioxidant defenses induced by physical activity in skeletal muscle are largely considered as contributor to improve overall health (Gabriel and Zierath, 2017). These cellular responses and their adaptations are highly dependent on the parameters of the physical exercise protocols such as intensity, duration and frequency (Bishop *et al.*, 2019; MacInnis *et al.*, 2019). High intensity interval training (HIIT), characterized by short high intensity fractions interspersed with regular recovery time intervals (Cochran *et al.*, 2014), could be even more beneficial for health than moderate intensity continuous training (MICT). In mammals, the mitochondrial biogenesis and the oxidative phosphorylation (OXPHOS) capacities should be more stimulated after HIIT compared to MICT (MacInnis *et al.*, 2017).

Among the signaling pathways activated in the skeletal muscle during a physical exercise, the AMP-activated protein kinase (AMPK)–peroxisome proliferator-activated receptor-γ (PPAR-γ) coactivator-1α (PGC-1α) is the main one stimulating the mitochondrial biogenesis and the oxidative capacities in vertebrates, including fish (Correia *et al.*, 2015; Magnoni *et al.*, 2014; Morash *et al.*, 2014). The AMPK is activated by a cellular stress such as exercise which decreases ATP concentration and then increases AMP:ATP ratio (Winder and Hardie, 1996). This kinase, which stimulates PGC-1α, is considered as a key molecule in the oxidative metabolism upregulating the OXPHOS. PGC-1α may interact with transcription factors which lead to the transcription of specific genes such as those encoding antioxidant enzymes and mitochondrial respiratory chain proteins (Bratic and Larsson, 2013; Hawley *et al.*, 2014). AMPK and PGC-1α are both redox sensitive, meaning that this signaling pathway can be influenced by the reactive oxygen species (ROS) (Trewin *et al.*, 2018).

The effects of different exercise modalities on the AMPK–PGC-1α signaling pathway and associated compounds have been studied in human and rodents. The results related to the regulation of this pathway are variable and sometimes contradictory, in part due to the exercise parameters, mainly intensity (Bartlett *et al.*, 2012; Egan *et al.*, 2010; MacInnis *et al.*, 2019). Godin *et al.* (2010) suggested these results may be in part explained by differences in fiber type recruitment. The investigations in mammals are often performed on whole skeletal muscle containing mixed type fibers (oxidative type I fibers and glycolytic type IIb fibers) when metabolic responses to physical exercise can also be influenced by the specific fiber type recruitment. Concerning mitochondrial function, MICT and HIIT stimulated for example

the cytochrome c oxidase complex in the human *vastus lateralis* when no exercise training effects were observed in isolated fibers I and II (MacInnis *et al.*, 2017).

In the present study, the rainbow trout was used as a model for several reasons. First, unlike mammals, fish has anatomically separated fast (white glycolytic muscle) and slow twitch fibers (oxidative red muscle). Such particularity is interesting to investigate the fiber type-specific adaptations to training. Second, fish is also easily to train and may globally respond to endurance training in a similar manner as mammals. For example, training exercise can stimulate mitochondrial biogenesis in fish even if mRNA regulation could be distinct from mammals (McClelland, 2012). Third, the rainbow trout is particularly used in exercise physiology providing a good model for optimizing exercise protocols and for better understanding cellular or molecular mechanisms involved in training exercise adaptation (Kieffer, 2010). Finally, the AMPK–PGC-1α signaling pathway has been identified in rainbow trout and previously explored in conditions of moderate intensity training by Morash *et al*. (2014).

The present study aimed to compare a moderate intensity training (MIT) and a high intensity training (HIT) (10 days for both) on the transcriptional and enzymatic activity responses involved in AMPK–PGC-1α signaling pathway, mitochondrial activity and antioxidant systems. We hypothesized that these two training intensities could differently affect red and white muscles in rainbow trout.

Materials and methods

Fish and experimental conditions

The experimental design was made in accordance with the animal experimental authorization n°472802, respecting the three "R" (replace, reduce, refine).

Juvenile female rainbow trouts (*Oncorhynchus mykiss*) were obtained from a local fish farm (EARL pisciculture de Lescoat, Lesneven, France). At their arrival at the laboratory, fishes were acclimated for two weeks before experimentation in 400 L polyethylene tanks filled with continuously renewed and aerated water at 15°C with a natural photoperiod (10 h:14 h light:dark). All the trouts were tagged in subcutaneous between the head and the dorsal fin (Glass tag 2 x 12 mm, Biolog-id, Bernay, France). Individual measurements of body mass (BM = 113 ± 2.2 g) and body length (BL = 20.6 ± 0.2 cm) were realized.

Before starting the training, the critical swimming speed (U_{crit}) was determined to obtain the water velocity corresponding to the training intensity protocols. The U_{crit} of 8 fishes was

106 measured in a Brett-type swimming tunnel according to Brett (1964) and Farrell (2008).
107 Before the experimentation, each fish was placed into the swimming tunnel 1 h with a water
108 velocity at 0.05 m/s to acclimate. Then, water velocity was increased by 0.10 m/s every 10
109 min until exhaustion, in water maintained at 15 ± 0.5°C. U_{crit} was calculated for each animal
110 using Brett's equation: $U_{crit} = V_{n-1} + V_i \times \frac{t_n}{T_i}$ (in which V_{n-1} is the velocity of the last
111 completed swimming speed (m/s); V_i is the velocity increment (0.1 m/s); t_n is the duration of
112 the last step (min) and T_i is the duration of each step (10 min)). The mean of U_{crit} was 3.41 ±
113 0.16 BL/s (n=8).
114 For the training experiment, 36 healthy fishes from the same animal batch were
115 randomly transferred and divided into three polyethylene tanks of 450 L with aerated tap
116 water maintained at 15 ± 0.5°C and renewed in half every day. One of these tanks kept fishes
117 presenting only spontaneous movements and were used as control, "Untrained" (n=12). The
118 other two circular tanks (diameter: 128 cm; height: 75 cm) kept experimental training groups:
119 "MIT" (n=12) and "HIT" (n=12). In order to get a uniform water flow, a little circular tank
120 (diameter: 50 cm; height: 47 cm) was added in the center of each tank. The two training
121 protocols lasted 10 days during which trouts were observed every day.
122 The MIT program exposed trouts to a water flow of 0.41 m/s corresponding to a swimming
123 speed of 57% U_{crit} (2.0 BL/s) during 23.5 h per day. Water flow was stopped one time per day
124 during 30 min when fishes were fed to satiation.
125 For HIT program, trouts started to swim at 0.41 m/s (57% U_{crit}) for 10 min to acclimate to the
126 water flow and then, were exposed to a water flow of 0.65 m/s corresponding to a swimming
127 speed of 90% U_{crit} (3.2 BL/s) for 2 h/day.
128 Trained animals were killed by cerebral commotion 65 h after the 10 days training
129 programs to avoid potential acute effects of exercise. BL and BM were recorded. At the
130 beginning and at the end of the experiment, condition factor (CF) was calculated for each fish
131 using the following equation: $CF = \frac{BM \times 100}{BL^3}$ where BM is expressed in g and BL in cm
132 (Fulton, 1902). Red and white muscles were rapidly excised along the last 5 to 10 cm before
133 caudal fin and more precisely at the lateral line level for red muscle. Plasma samples were
134 collected from 5% EDTA-blood by centrifugation at 3,000 g for 5 min at room temperature.
135 All these samples were immediately frozen in liquid nitrogen and stored at -80°C for further
136 analyses.

137 RNA extraction

138 Total RNA was isolated from red and white muscles using the NucleoSpin® RNA Set
139 for NucleoZOL (Macherey Nagel, Hoerdt, France). Briefly, 30 mg of tissue previously
140 ground in liquid nitrogen and stored at -80°C were homogenized for 2 x 15 s with an
141 Ultraturrax in 500 µL of NucleoZOL. After adding 200 µL of DNase/RNase-free water and
142 incubating 15 min at room temperature, samples were centrifuged (12,000 g, 15 min, 4°C).
143 500 µL of the supernatant were collected and the same volume of MX buffer was added. This
144 solution was transferred into NucleoSpin® columns. RNA was fixed at the silica membrane of
145 the columns by centrifugation (8,000 g, 1 min, room temperature). The silica membranes
146 were then washed and dried with the RA3 buffer containing ethanol. RNA was eluted in 40
147 µL of DNase/RNase-free water and stored at -80°C until use. RNA concentrations were
148 measured with a SimpliNano™ spectrophotometer and their purity was assessed using
149 OD_{260}/OD_{280} ratios. Their integrity was also checked by an electrophoresis on a 1.5% agarose
150 gel with ethidium bromide.

151 mRNA quantification by Real-Time Reverse Transcriptase-PCR (RT-PCR)

152 1,000 ng of each sample RNA was reverse transcribed with the qScript™ cDNA
153 synthesis kit (Quanta BioSciences, VWR, France) containing a reaction mix (dNTPs,
154 oligo(dt) and random primers, enzyme specific buffer and Mg^{2+}) and the Reverse
155 Transcriptase. Complementary DNA (cDNA) was diluted 10-fold for PCR experiments and
156 stored at -20°C. Real-Time RT-PCR was realized with a 7500 Fast Real-Time PCR system
157 (Applied Biosystems, Thermo Fisher Scientific, France). Target genes were amplified and
158 quantified by SYBR®green incorporation (EurobioGreen® Mix qPCR 2x Lo-Rox ; Eurobio
159 Ingen, Courtaboeuf, France) with specific primers presented in **Table 1**. The cycling
160 conditions consisted in a denaturing step at 95°C for 2 min, followed by 40 to 50 cycles of
161 amplification (denaturation: 95°C for 5 s; annealing/extension: 60°C for 30 s). Finally, a
162 melting curve program was carried out from 60°C to 95°C with a heating rate of 0.1°C per s,
163 showing a single product with a specific melting temperature for each gene and sample
164 evaluated.
165 Standard curves were established to determine and compare the transcription level of the
166 different target genes in the three experimental groups. To obtain these curves, all genes were
167 first amplified from a pool of RT products prepared with samples from control and
168 experimental groups. PCR products were separated on a 2% agarose gel with ethidium

bromide and purified using the Nucleospin gel and PCR Clean-Up® kit (Macherey Nagel, Hoerdt, France). These products were then quantified using a SimpliNano™ spectrophotometer before proceeding to a serial dilution from 10 pg/µL to 0.001 fg/µL. A seven-point standard curve was used to determine the PCR efficiency of each primer pair (between 90% and 100%) and the transcription level of the different genes in all samples. Each gene was amplified in a single run from triplicates for standard points and duplicates for sample points. Quantification was normalized using 18S ribosomal RNA (rRNA) considered as a reference gene. This choice was validated by the absence of significant differences in 18S rRNA levels between experimental groups for each muscle (red and white) ($p>0.05$). All mRNA levels were first calculated with the ratio: $\frac{target\ gene\ \text{mRNA}}{18S\ \text{rRNA}}$ and then expressed as fold change with the untrained group which was set to 1.

Enzyme activities and oxidative stress marker

All measurements were performed at 15°C and determined using a plate reader (SAFAS Xenius, Monaco). All samples were measured in duplicate.

Citrate synthase activity

50 mg of frozen red and white muscles were homogenized with a Polytron homogenizer in an extraction buffer (100 mM TRIS HCl, pH 8.1, 4°C). Citrate synthase (CS) activity was assessed at 412 nm on the supernatant using 1 mM DTNB (5,5-dithio-bis-2-nitrobenzoic acid), 12.3 mM acetyl CoA and 100 mM oxaloacetate (Srere, 1969). Measurements were expressed in µmol DTNB reduced/min/g wet tissue.

Cytochrome c oxidase activity

Frozen red and white muscles were homogenized with a Polytron in an extraction buffer (100 mM TRIS, 2 mM EDTA and 2 mM DTE, pH 7.4, 4°C). The homogenate was centrifuged at 12,000 g for 20 min at 4°C. Cytochrome c oxidase (COX) activity was determined on the supernatant at 550 nm using 2 mM reduced cytochrome c and 330 mM sodium phosphate buffer (Smith and Conrad, 1956). COX activity was expressed in nmol cytochrome c oxidized/min/g wet tissue.

Antioxidant enzyme activities

200 mg of frozen red and white muscles were homogenized in an extraction buffer (75 mM TRIS and 5 mM EDTA, pH 7.4, 4°C) with a Polytron homogenizer. After a

centrifugation at 12,000 g for 10 min at 4°C, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities were determined on the resulting supernatant:

SOD activity was assessed at 480 nm using an indirect method that inhibits the adrenaline to adenochrome reaction with the xanthine/hypoxanthine reaction as a superoxide anion producer (Misra and Fridovich, 1972). One unit (U) of SOD activity corresponds to the amount of sample needed to cause 50% inhibition relative to the control without tissue. SOD activity was expressed in U/mg wet tissue.

CAT activity was determined at 240 nm through its capacity to transform hydrogen peroxide (H_2O_2) into water and oxygen (Beers and Sizer, 1952). The adding of 200 mM H_2O_2 in curves initiated the reaction. CAT activity was expressed in nmol H_2O_2/min/g wet tissue.

GPx activity was measured at 340 nm with an indirect method adapted from Ross *et al.* (2001) by Farhat *et al.* (2015). Briefly, the activity was determined from the decrease of NADPH induced by a coupled reaction with glutathione reductase. GPx activity was expressed in nmol NADPH oxidized/min/g wet tissue.

Oxidative stress marker

Total plasmatic 8-isoprostane (free and esterified in lipids) were measured in duplicate using an Elisa kit (Cayman Chemical, Ann Arbor, Michigan, USA) according to the manufacturer's protocol. All samples were hydrolyzed using 15% KOH and incubated 60 min at 40°C and then neutralized with potassium phosphate buffer. Another step of purification was necessary with ethanol. Finally, samples were extracted using ethyl acetate containing 1% methanol and SPE Cartridges (C-18) (Cayman Chemical). After 18 h of incubation, 8-isoprostane plasmatic concentration was measured at 410 nm and expressed in pg/mL.

Statistical analysis

All results were expressed as mean ± standard error of mean (SEM). Statistics were performed using Statistica v. 12 software (StatSoft, France). Normality and homogeneity of population were respectively tested using Shapiro-Wilk and Levene tests. Adapted tests were then performed: one-way ANOVA or Kruskall-Wallis tests were followed respectively by a post-hoc test (HSD) or a Mann-Whitney test. Significance threshold was set at $p<0.05$. A Pearson test was used to analyze the correlations between mRNA variables, the threshold for significance was set at $p<0.05$.

Results

Condition factor

The condition factor (CF), commonly used in fish farm to follow fish health, was measured for each animal (including the untrained group) at the beginning and at the end of the experiment. Trainings (MIT and HIT) had no effects on the CF (**Table 2**).

Influence of two training protocols (MIT and HIT) on muscle transcripts

AMPK–PGC-1α signaling pathway

In the red muscle, HIT induced a ~30% increase in the *Ampkα2* mRNA content compared to untrained and MIT groups ($p<0.05$). No changes were observed on the *Ampkα1* mRNA levels between groups. In the MIT group, the *Pgc-1α* mRNA content was significantly reduced by 2-fold ($p<0.05$) compared to untrained and HIT groups in red and white muscles. In the white muscle, both MIT and HIT decreased the *Ampkα1* mRNA levels whereas the *Ampkα2* mRNA quantity was unchanged (**Figure 1, A and B**).

Mitochondrial functioning

HIT up-regulated the *Cs* mRNA levels compared to untrained and MIT groups in both types of trout skeletal muscle. In the red muscle, the mRNA levels of *Nd1*, *Cox1*, *Cox2*, *Cox4* and *Atp synthase 6* were also increased by HIT compared to the untrained group. The MIT group showed a 65% increase ($p<0.05$) in the *Cox2* mRNA content in the red muscle and a decrease in *Nd1* and *Cox1* mRNA levels in the white muscle compared to the untrained group. In addition, the *Nd1*, *Cox2*, *Cox4* and *Atp synthase 6* mRNA levels were up-regulated by HIT in the red muscle when compared to the MIT group. In the white muscle, a decrease in the *Nd1* mRNA levels by MIT was shown compared to untrained and HIT groups whereas no modifications were observed for the *Cox2*, *Cox4* and *Atp synthase 6* gene transcriptions. In the red muscle, HIT raised the *Ucp2α* and *Ucp2β* mRNA levels compared to the MIT group. In the white muscle, both types of training did not modify these two genes contents (**Figure 2, A and B**).

Antioxidant defenses

In the red muscle, the *Sod1* and *Sod2* mRNA levels in HIT group were ~20% higher ($p<0.05$) than in the MIT group. In the same muscle, HIT induced a ~50% increase ($p<0.05$) in the *Gpx1* mRNA content compared with untrained and MIT groups (**Figure 3, A**).

However, no training effects on the antioxidant enzymes gene transcription were observed in the white muscle (**Figure 3, B**).

Correlations analysis in skeletal muscles

In the red muscle, the mRNA level of *Cs* was significantly correlated (moderate correlation: $0.4 \leq R < 0.7$ or strong correlation: $0.7 \leq R < 1$) with transcripts of compounds related to the AMPK–PGC-1α signaling pathway (*Ampkα1*, *Ampkα2* and *Pgc-1α*), the mitochondrial functioning (*Nd1*, *Cox*, *Atp synthase 6* and *Ucp2α*) and antioxidant defenses (*Sod1*, *Sod2* and *Gpx1*) (**Table 3**). In the white muscle, *Cs* transcripts were significantly correlated with the mRNA of *Ampkα2*, mitochondrial functioning compounds (except *Cox2* and *Ucp2β*) and all target antioxidant systems (**Table 3**).

Enzymatic activities (citrate synthase, cytochrome c oxidase and antioxidant systems)

No significant effects were observed on the enzymatic CS, COX, SOD, CAT and GPx activities with training in both skeletal muscles (**Table 4**).

Oxidative stress marker

Neither MIT nor HIT modified the plasmatic concentration of 8-isoprostane (**Table 5**) considered as an oxidative stress marker (the arachidonic acid peroxidation product).

Discussion

The present paper is the first to concurrently study the effects of moderate and high intensity trainings on the signaling pathway cascade AMPK–PGC-1α, OXPHOS complexes and antioxidant defenses in red and white muscles of trout. The three main findings of this study are: 1) HIT stimulated more than MIT the signaling pathway cascade involving AMPK–PGC-1α, OXPHOS complexes and antioxidant enzymatic systems in trout muscles; 2) these training adaptations were largely more activated in the red oxidative muscle than in the white glycolytic muscle; 3) as in mammals, HIT could have benefit effects in fish by stimulating this signaling pathway cascade and antioxidant defenses.

As in mammals, AMPK serves as a cellular energy sensor in fish. Activation of AMPK has been reported in numerous environmental and endogenous conditions such as exposure to hypoxia or anoxia, changed in nutritional states and increased in activity (Craig *et al.*, 2018; Fuentes *et al.*, 2013; Magnoni *et al.*, 2014). AMPKα isoforms are extremely conserved across

vertebrates, including fish (Craig *et al.*, 2018). Among them, we studied the mRNA encoding for AMPKα1 and AMPKα2 in the rainbow trout skeletal muscle. Our data showed that the responses of AMPKα isoforms were fibers- and training-type depending. In fish, the role of each skeletal muscle fiber is exclusive of a swimming type. Aerobic and sustained swimming recruits preferentially the oxidative skeletal muscle (red muscle) whereas the glycolytic skeletal muscle (white muscle) is mainly involved in anaerobic and burst swimming (Palstra *et al.*, 2013).

Our data showed that both trainings decreased the *Ampkα1* mRNA levels in the white muscle (**Figure 1, B**) whereas only HIT increased the *Ampkα2* mRNA level in the red muscle (**Figure 1, A**). Our results were difficult to compare to others because Morash *et al.* (2014) quantified total *Ampk* mRNA and not separated isoforms in the trout muscle. They showed a 10-fold rise in the red muscle after one week of MIT with a return to the control level after 2 and 4 weeks of training. So, it seems that the *Ampk* mRNA could be regulated during the first days of training in skeletal muscle.

In mammal skeletal muscles, AMPK regulates the expression of PGC-1α. This transcriptional co-factor could be considered as the key player of adaptations induced by endurance exercise training and it has an important role in the regulation of genes involved in mitochondrial biogenesis and antioxidant defenses (Olesen *et al.*, 2010). Several studies in mammals focused on the effects of acute exercise but less on the effects of chronic exercise. Among them, Hyatt *et al.* (2016) showed that 10 days of MIT increased the *Pgc-1α* mRNA content in the rat *plantaris* muscle (mainly composed of type II fibers) with no changes in the *soleus* muscle (mainly composed of type I fibers), that suggested a *Pgc-1α* transcriptomic response fiber-type dependent. In the present study, compared to untrained and HIT groups, the *Pgc-1α* transcripts levels were surprisingly down-regulated after 10 days of MIT in both red and white skeletal muscles (**Figure 1, A and B**). In the literature, a decrease in PGC-1α expression was often related to reduce mitochondrial performance in mammals (Mortensen *et al.*, 2007) or in fish (Gilbert *et al.*, 2014). Here, in the red muscle, it was not the case because the mRNA of the OXPHOS complexes are, for most of them, unchanged or increased after 10 days of MIT. Differently, in the white muscle, MIT decreased *Ampkα1*, *Pgc-1α* and some electron transport chain (ETC) complexes mRNA too (see below).

HIT had no effect on the *Pgc-1α* mRNA levels compared to the untrained group but numerous transcripts of the mitochondrial functioning were also upregulated mainly in the red muscle (see below). Thus, we can suppose that during both training conditions (MIT and HIT) the regulation of the *Pgc-1α* gene has already been occurred at least in the red muscle. This

regulation could happened earlier in comparison to the overexpression of *Pgc-1α* mRNA reported in the red muscle after 30 or 40 days of sustained swimming (Magnoni *et al.*, 2013, 2014). However, our results were closed to those reported in zebrafish when one week of moderate interval training (twice daily for 3 h separated by 2 h rest period) increased the *Pgc-1α* mRNA quantity in the skeletal muscle with a return to the control value after 8 weeks of training (LeMoine *et al.*, 2010).

Because it is known that PGC-1α stimulates mitochondrial gene and antioxidant defenses during oxidative stress periods, such as physical exercise (Gilbert *et al.*, 2014), we studied the training effects on mitochondria activity. The first main global result was that the mRNAs of genes involved in mitochondrial functioning were more regulated in the red muscle than in the white muscle. Secondly, we showed that HIT upregulated more target genes (*Cs*, OXPHOS complexes and *Ucp*) than MIT in the oxidative fibers. So, as in mammals, oxidative metabolism genes regulation should predominate in type I fibers (Wang and Sahlin, 2012).

Citrate synthase (CS), enzyme involved in the Krebs cycle, is considered as a mitochondrial biogenesis marker. Compared to untrained and MIT groups, HIT upregulated the *Cs* mRNA levels in the both muscles (**Figure 2, A and B**) but neither MIT nor HIT had effect on the CS enzymatic activity (**Table 4**). In fish, studies focused on the training adaptations of *Cs* mRNA or CS activity separately but not simultaneously. Among them, Magnoni *et al.* (2013, 2014) showed an increase in *Cs* mRNA levels in trout muscles after 30 days of MIT but they did not determined *Cs* mRNA levels in the early stages of training. In other studies, it was reported that CS activity increased after 30 days of MIT in trout (Morash *et al.*, 2014) or zebrafish muscles (McClelland *et al.*, 2006). So, we suggest that the translation of *Cs* mRNA may not have occurred in our study maybe because of short duration and/or intensity training. In mammals, it is often reported that mRNA precede (Perry *et al.*, 2010) or coincide (Daussin *et al.*, 2012) with changes in mitochondrial enzyme activity and protein content. In vertebrate, including fish, HIT appears to be an effective training to improve the mitochondrial biogenesis in the oxidative muscle even if the time course of responses in fish could be different from mammals (Bexfield *et al.*, 2009; MacInnis *et al.*, 2017).

Mitochondrial DNA encodes for 13 proteins in the OXPHOS (including ND1, COX1, COX2 and ATP synthase 6) whereas the remaining mitochondrial proteins (including COX4) are encoded by nuclear genes (Smeitink *et al.*, 2001). In our study, HIT stimulated analyzed OXPHOS transcripts (*Nd1*, *Cox (1, 2 and 4)*, *Atp synthase 6*) in the red muscle, confirming that HIT stimulated mitochondrial functioning transcripts (**Figure 2, A**). Differently, there

were no changes in the white muscle (**Figure 2, B**). MIT only stimulated *Cox2* mRNA in the oxidative red muscle. Our training protocols did not significantly modify the COX activity but HIT tended to increase in white muscle when compared to MIT (p= 0.07; **Table 4**). McClelland *et al.* (2006) showed that COX activity increased in zebrafish but after 30 days of sustained swimming. Few studies focused on the OXPHOS complexes mRNA in the present training conditions in mammals and at our knowledge, none in fish. Our results confirmed a fiber-type molecular response depending on training-type.

The UCPs are present in the inner mitochondrial membrane and principally known for its thermogenesis function. By dissipating the proton electrochemical gradient, UCP2 would have also a common role as redox sensors by reducing mitochondrial ROS production because of a more important electron transport through the mitochondrial ETC (Ji and Zhang, 2014). In fish, the effects of different environmental stressors (temperature, hypoxia, nutrient) have been studied on UCP2 (Bermejo-Nogales *et al.*, 2014) but not the training effects at our knowledge. In the present paper, two UCP2 isoform genes (*Ucp2α* and *Ucp2β*) have been studied which have 93% of similitude in their sequences in rainbow trout (Coulibaly *et al.*, 2006). In the red muscle, HIT increased the transcription of the gene encoding for UCP2α compared to untrained and MIT groups. This training stimulated *Ucp2β* mRNA compared to the other groups (**Figure 2, A**). Neither MIT nor HIT affected the *Ucp2α* and *Ucp2β* mRNA levels in the white muscle (**Figure 2, B**). HIT would enhance the transcription of *Ucp2α* in the red muscle. This mild uncoupling of mitochondrial OXPHOS may represent a process of defense against oxidative stress in mitochondria by reducing the potential electron leak associated to physical exercise.

During physical exercise, ROS are generated from different sources (mitochondrion, NADPH oxidase and xanthine oxidase) by contracting skeletal muscle (He *et al.*, 2016). Even if mitochondrion is often cited as a main source (Davies *et al.*, 1982; Gredilla *et al.*, 2004; Powers and Jackson, 2008), the primary site remains unclear (Powers *et al.*, 2011). It is known that ROS are necessary for numerous cellular adaptive mechanisms (Ljubicic *et al.*, 2010). Single bouts of acute exercise may enhance ROS overproduction and cause potential oxidative damage. But, regular aerobic exercise, through ROS acting as signaling agents, can improve muscle oxidative balance by upregulation of endogenous antioxidant defenses systems (Steinbacher and Eckl, 2015). Among the main enzymatic antioxidants, SOD reduce the superoxide anion to hydrogen peroxide though two isoforms: SOD1 (Cu/Zn-SOD) and SOD2 (Mn-SOD) respectively present in the cytoplasm and in mitochondria. GPx and CAT mainly transform the hydrogen peroxide to water (Birben *et al.*, 2012).

MIT did not modify the mRNA levels of the antioxidant enzymes (*Sod1*, *Sod2* and *Gpx1*; **Figure 3, A and B**) and the enzymatic activities (SOD, CAT and GPx; **Table 4**) in both muscles. 8-isoprostanes, as radical attack targets are recognized to be a relevant marker of the oxidative stress (Montuschi *et al.*, 2004). Its plasmatic concentration was unaffected by MIT (**Table 5**) suggesting that no ROS deleterious effects and overproduction occurred after 10 days of MIT in rainbow trout.

Differently from MIT conditions, HIT stimulated the antioxidant defenses mRNA levels in the oxidative muscle only (**Figure 3, A**). The antioxidant enzymes activities remained unchanged in both muscles (**Table 4**). As for CS and COX results, translation of antioxidant enzymes mRNA may not occurred maybe because of short duration and/or intensity training. In rat, 12 weeks of HIT permitted to stimulate the enzymatic activities of SOD and GPx only in the *soleus* muscle (Criswell *et al.*, 1993). Thereby, HIT seemed also to induce fiber type-specific antioxidant transcript responses mainly activated in the oxidative muscle. Otherwise, the absence of oxidative stress, supported by no change in 8-isoprostane plasmatic levels (**Table 5**) was conform to the mitohormesis theory. This theory explains that ROS generation associated with repeated exercise can result in antioxidant adaptations to facilitate protection in subsequent oxidative insult (Bo *et al.*, 2013). The absence of oxidative stress at the systemic level induced by the training protocols is coherent with the calculated condition factors reflecting a good health of the fishes (**Table 2**).

To explore the relationships among the components analyzed, correlations between different mRNA levels of parameters were assessed. This analysis demonstrates that, mainly in the red muscle, the *Cs* mRNA is positively correlate to almost all the studied transcripts related to AMPK–PGC-1α signaling pathway, OXPHOS and antioxidant enzymatic defenses. The significant positive correlation was moderate or strong for *Ampkα1*, *Ampkα2* and *Pgc-1α* ($0.64 < R < 0.71$) and OXPHOS components ($0.40 < R < 0.81$). The antioxidant systems presented strong relationships in the red muscle ($0.74 < R < 0.82$) (**Table 3**). These results suggested a coordinate regulation of the mitochondrial biogenesis with the AMPK–PGC-1α signaling pathway and the activation of antioxidant defenses including UCP2α as observed in mammals (Gouspillou *et al.*, 2014). In the mouse skeletal muscle, overexpression of PGC-1α induced a stimulation of the mitochondrial biogenesis involving a rise of the expression of genes encoding for OXPHOS complexes such as *Cox* (Leick *et al.*, 2008). Based on our results and those of LeMoine *et al.* (2010), PGC-1α seems sensitive to the exercise stimulus in fish. That confirms, as in mammals, its involvement in the regulation of mitochondrial biogenesis at least in conditions of exercise (Magnoni *et al.*, 2014).

To a lesser extent, in the white muscle, *Cs* transcripts were moderately correlated with the *Ampkα2* mRNA (R=0.50), mitochondrial functioning compounds (except *Cox2* and *Ucp2β*) and antioxidant defenses (except *Sod1*) (**Table 3**). MIT and HIT had few effects on transcripts in white muscle in comparison to red muscle. Red muscle fibers are recruited during slow or moderate intensity movements when both red and white fibers are recruited during high intensity movements (Jayne and Lauder, 1994). In salmonids, studies showed that aerobic metabolism predominates in swimming up to 70 to 90% of their U_{crit} and that the recruitment of white fibers in rainbow trout initiated at swimming speeds around 80% U_{crit} (Hvas and Oppedal, 2017; Webb, 1971). Therefore in our study, an intensity of 90% U_{crit} was chosen for HIT in order to stimulate not only red muscle (as supposed during MIT) but potentially also white muscle. Our data show mRNA adaptations of the studied signaling pathway, mitochondrial compounds and antioxidant defenses mainly in the red muscle after HIT. During HIT, when fishes swam at 90% U_{crit}, the red oxidative muscle recruitment should be even greater than during MIT and the white glycolytic muscle should be also mobilized but in a lesser extent. We can hypothesize than a longer period of high intensity exercise (> 2 h/day) could have been more efficient in stimulating mitochondrial biogenesis in white muscle.

Our study has some methodological limitations related to the choice of some protocol parameters. Ten days of training might be considered as too short in comparison to the durations of training used in previous studies. In fish, the CS activity is increased only after 30 days of sustained swimming whereas mRNAs are stimulated during the early stages between 1 and 2 weeks (LeMoine *et al.*, 2010; McClelland *et al.*, 2006; Morash *et al.*, 2014). In a perspective to explore the temporal changes of adaptations of the AMPK–PGC-1α signaling pathway and antioxidant systems to training, we chose to first explore their early changes. In the present paper, we clearly showed after 10 days of training the stimulation of mRNA levels mainly with the HIT protocol, but the duration was probably not long enough to observe concomitant protein changes. One of our perspectives remains to investigate a longer training duration.

The timing of sampling after training (65 h) might be long but was decided on the basis of different elements. First, LeMoine *et al.* (2010) compared training and acute exercise mRNA responses and concluded that 24 h of resting period were not sufficient to avoid the last exercise training session effects. Second, the present study using fish as model was performed in a perspective of human health. For mammals including human, the muscle biopsy is currently performed 48 or 72 h after the final training session to avoid potential acute exercise

effects (Ghiarone *et al.*, 2019; MacInnis *et al.*, 2017). Third, the time of recovery is often reported to be slower in fish in comparison to mammals in part due to difference in body temperature (Milligan *et al.*, 2000). So, for the above reasons and to be consistent with these studies in mammals, a resting period of 65 h was chosen to avoid any acute exercise effects with the risk of losing some early mRNA expression levels.

Conclusion

This study was the first to compare MIT and HIT on the AMPK–PGC-1α, mitochondrial compounds and antioxidant defenses molecular responses in the trout skeletal muscles. Overall, the transcriptional response to swim training was more activated in the red muscle than in the white muscle. We suggested that the transcripts stimulation could be delayed or less regulated by training in the white muscle in these training conditions. Several mammalian studies showed that HIIT stimulates more than MICT mitochondrial biogenesis and the muscle oxidative capacity (Godin *et al.*, 2010). The present study reported that also in fish HIT was more relevant than MIT to stimulate the signaling pathway cascade involving AMPK–PGC-1α, mitochondrial compounds and antioxidant defenses in the red muscle. The use of fish models may provide further insights for understanding the mechanisms by which exercise training improves health.

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662

Figure captions

Figure 1. Effects of MIT (moderate intensity training) and HIT (high intensity training) on the *Ampkα1*, *Ampkα2* and *Pgc-1α* mRNA levels in red **(A)** and white **(B)** muscles of the rainbow trout. Results were expressed as fold change with the untrained group, which was set to 1. Results were shown as means ± SEM. Similar letters indicate no significant differences ($p>0.05$) between groups.

Figure 2. Effects of MIT (moderate intensity training) and HIT (high intensity training) on *citrate synthase (Cs)*, *NADH dehydrogenase 1 (Nd1)*, *cytochrome c oxidase 1, 2 and 4 (Cox1, 2 and 4)*, *ATP synthase 6* and *uncoupling proteins (Ucp) 2α* and *2β* mRNA levels in red **(A)** and white **(B)** muscles of the rainbow trout. Results were expressed as fold change with the untrained group, which was set to 1. Results were shown as means ± SEM. Similar letters indicate no significant differences ($p>0.05$) between groups.

Figure 3. Effects of MIT (moderate intensity training) and HIT (high intensity training) on the *superoxide dismutase (Sod) 1* and *2* and *glutathione peroxidase 1 (Gpx1)* mRNA levels in red **(A)** and white **(B)** muscles of the rainbow trout. Results were expressed as fold change with the untrained group, which was set to 1. Results were shown as means ± SEM. Similar letters indicate no significant differences ($p>0.05$) between groups.

Tables

Table 1. Primer sequences used for Real-Time RT-PCR analysis. The hybridization temperature was 60°C for all primers. Primers were own designed except those with a subscript number.

Target gene	Abbreviation	Primer sequences (5' to 3')	Data base	Accession number
18S ribosomal RNA	*18S rRNA*[1]	(F) CGGAGGTTCGAAGACGATCA (R) TCGCTAGTTGGCATCGTTTAT	GenBank	AF308735.1
Adenosine monophosphate kinase α1	*Ampkα1*	(F) GCCACCATCAAAGAGATCCG (R) TCAAACTTCTCACACACCTCC	GenBank	XM_021590586
Adenosine monophosphate kinase α2	*Ampkα2*	(F) CGCTCAAGAGGGCTACCATT (R) CAGACTCGGTGCTCTCAAAC	GenBank	XM_021604240
Peroxisome proliferator-activated receptor-γ coactivator 1α	*Pgc-1α*[2]	(F) CAACCACCTTGCCACTTCCT (R) GGTTCCAGAGATCTCCACAC	GenBank	FJ710605.1
Citrate synthase	*Cs*[1]	(F) AACCAACCTCACTCATCACCA (R) GCAGCAGAAGCAGCCCATAA	GenBank	XM_021566131
Superoxide dismutase 1	*Sod1*[3]	(F) AGGCTGTTTGCGTGCTCAA (R) CCAATCAGCTTCACAGGACCAT	GenBank	AF469663.1
Superoxide dismutase 2	*Sod2*[3]	(F) CCACACCATCTTCTGGACAAAC (R) GGAGCCAAAGTCACGGTTGA	Tigr	TC123543
Glutathione peroxidase 1	*Gpx1*[3]	(F) CCTGGGAAATGGCATCAAGT (R) GGGATCATCCATTGGTCCATAT	Tigr	TC94794
Uncoupling protein 2α	*Ucp2α*	(F) ACCAAACCGACGTTACCATGG (R) TTAAGAAGCGCGTCCTTGATG	GenBank	NM_001124654.1
Uncoupling protein 2β	*Ucp2β*	(F) TATTGCCATGGTGACCAAGGA (R) CTACAGAGGAGTGGTGTAGTT	GenBank	NM_001124571.1
NADH dehydrogenase 1	*Nd1*	(F) TTGTACCCGTTCTGTTAGCAG (R) TAGTTTTAGGCCGTCTGCGAT	GenBank	NP_008290
Cytochrome c oxidase 1	*Cox1*	(F) TCAACCAACCACAAAGACATTG (R) CACGTTATAGATTTGGTCATCC	GenBank	NP_008292
Cytochrome c oxidase 2	*Cox2*[2]	(F) GAGGCAATAAAGGCTGTTTGGT (R) GCCGTTCCTTCTTTAGGTGTAA	GenBank	NP_008293
Cytochrome c oxidase 4	*Cox4*[4]	(F) TACGTGGGGGACATGGTGTT (R) CCCAGGAGCCCTTCTCCTTC	Sigenae	tcav0004c.i.22_3.1.s.om.8
Adenosine triphosphate synthase 6	*Atp synthase 6*	(F) CTTCGACCAATTTATGAGCCC (R) TCGGTTGATGAACCACCCTTG	GenBank	NP_008295

(F): Forward, (R): Reverse.

References: [1]Magnoni *et al.* (2013) [2]Eya *et al.* (2015) [3]Gunnarsson *et al.* (2009) [4]Kolditz *et al.* (2008)

Table 2. Condition factors of rainbow trouts.

	Untrained n=12	MIT n=12	HIT n=12
Initial CF*	1.28 ± 0.02	1.27 ± 0.02	1.29 ± 0.04
Final CF**	1.30 ± 0.02	1.31 ± 0.03	1.30 ± 0.04

Results were shown as means ± SEM. No significant differences were observed.

*Initial CF: condition factor measured at the beginning of the experiment.

** Final CF: condition factor measured at the end of the experiment.

CF: condition factor = $\frac{\text{BM x } 100}{\text{BL}^3}$ with body mass (BM) in g and body length (BL) in cm.

MIT: moderate intensity training; HIT: high intensity training.

Table 3. Correlations between *Cs* mRNA vs. *Ampkα1, Ampkα2, Pgc-1α, Nd1, Cox1, Cox2, Cox4, Atp synthase 6, Sod1, Sod2, Gpx1, Ucp2α and Ucp2β* mRNA in red and white muscles.

	Red muscle n=35	**White muscle n=35**
	Cs	
Ampkα1	R=0.66*	R=0.27
Ampkα2	R=0.71*	R=0.50*
Pgc-1α	R=0.64*	R=0.01
Nd1	R=0.79*	R=0.58*
Cox1	R=0.70*	R=0.42*
Cox2	R=0.61*	R=0.25
Cox4	R=0.80*	R=0.49*
Atp synthase 6	R=0.81*	R=0.40*
Ucp2α	R=0.74*	R=0.64*
Ucp2β	R=0.40*	R=0.29
Sod1	R=0.74*	R=0.34*
Sod2	R=0.82*	R=0.59*
Gpx1	R=0.74*	R=0.43*

The threshold for significance was set at $p<0.05$ and significant correlations were represented by *.

Table 4. Effects of MIT and HIT on CS, COX, SOD, CAT and GPx activities in red and white muscles.

	Red muscle			**White muscle**		
	Untrained n=12	MIT n=12	HIT n=12	Untrained n=12	MIT n=12	HIT n=12
CS (µmol DTNB reduced/min/g WT)	25.4 ± 2.1	25.3 ± 2.4	24.9 ± 1.6	2.9 ± 0.2	3.2 ± 0.2	3.1 ± 0.2
COX (nmol cyt. c oxidized/min/g WT)	40.4 ± 7.3	41.34 ± 5.3	44.60 ± 6.0	6.3 ± 1.0	5.9 ± 1.1	8.26 ± 1.1
SOD (U/mg WT)	3.9 ± 0.4	3.7 ± 0.3	3.2 ± 0.3	0.31 ± 0.02	0.33 ± 0.04	0.30 ± 0.04
CAT (nmol H_2O_2/min/g WT)	354.8 ± 29.2	338.0 ± 50.7	372.3 ± 42.1	ND	ND	ND
GPx (nmol NADPH/min/g WT)	275.1 ± 22.7	293.0 ± 18.6	254.7 ± 17.7	151.3 ± 17.1	167.6 ± 21.5	185.0 ± 13.4

Results were shown as means ± SEM. No significant differences were observed between groups. MIT: moderate intensity training; HIT: high intensity training; CS: citrate synthase; COX: cytochrome c oxidase; SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; WT: wet tissue; ND: not detected.

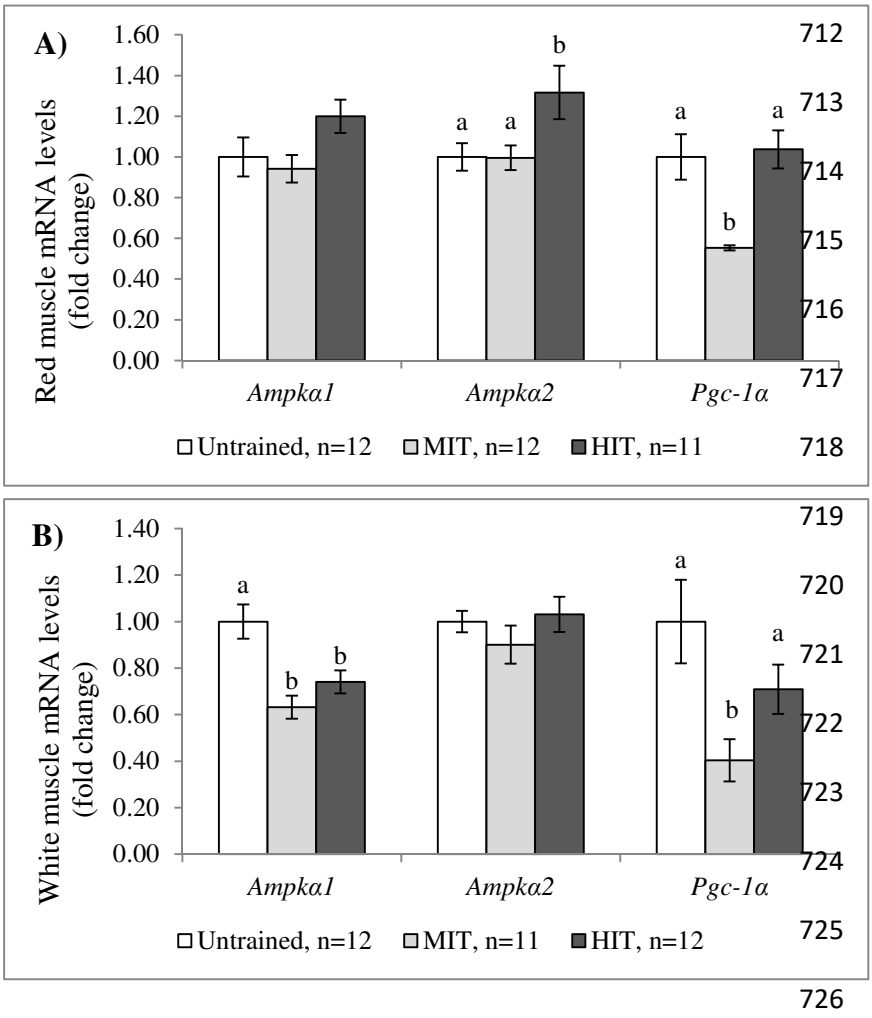
706 **Table 5.** Effects of MIT and HIT on plasmatic 8-isoprostane concentrations.

	Untrained n=12	MIT n=12	HIT n=11
8-isoprostane (pg/mL)	165 ± 24	129 ± 20	154 ± 19

707 Results were shown as means ± SEM. No significant differences were observed between groups.
708 MIT: moderate intensity training; HIT: high intensity training.

Figures

Figure 1.



727 **Figure 2.**

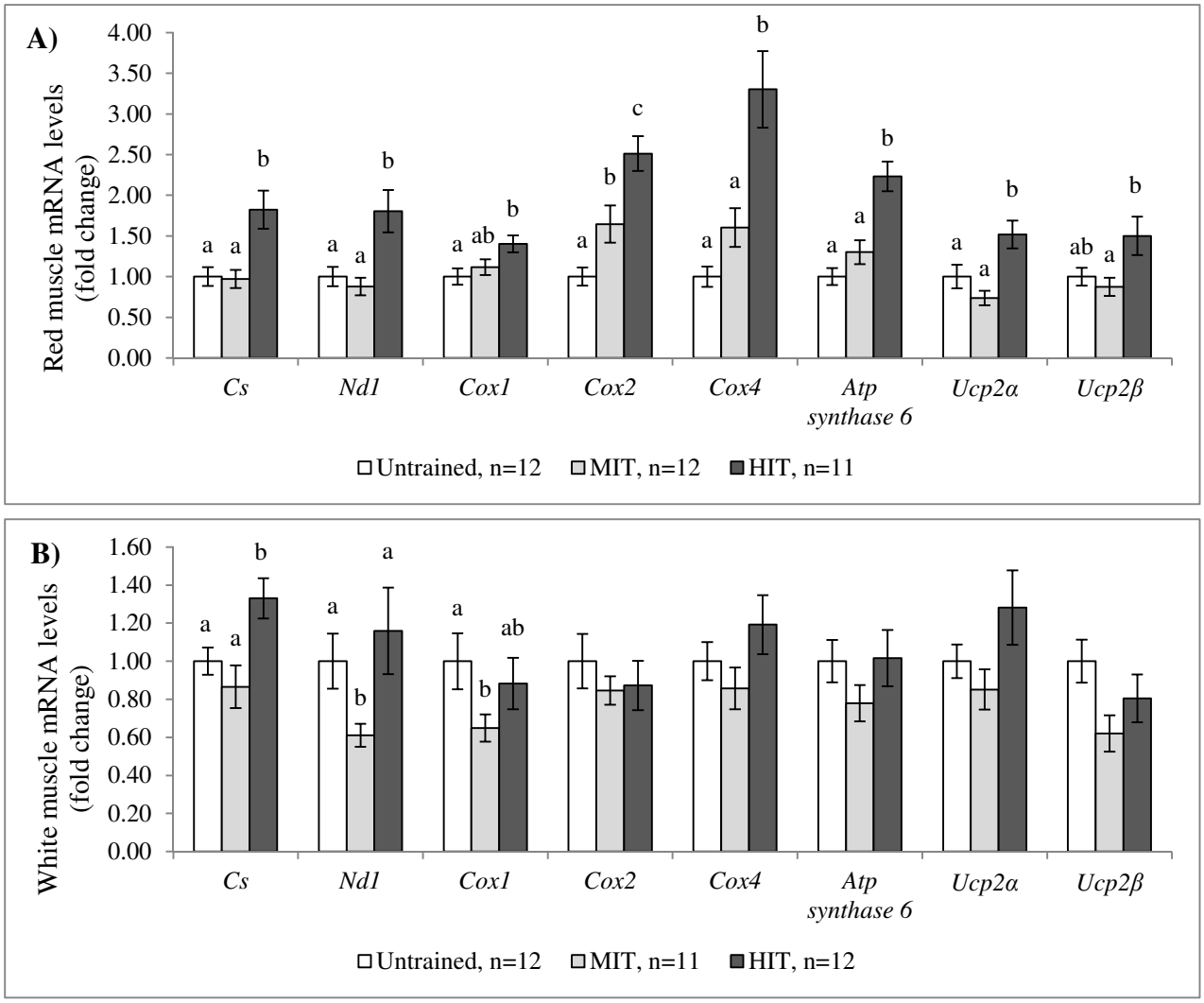
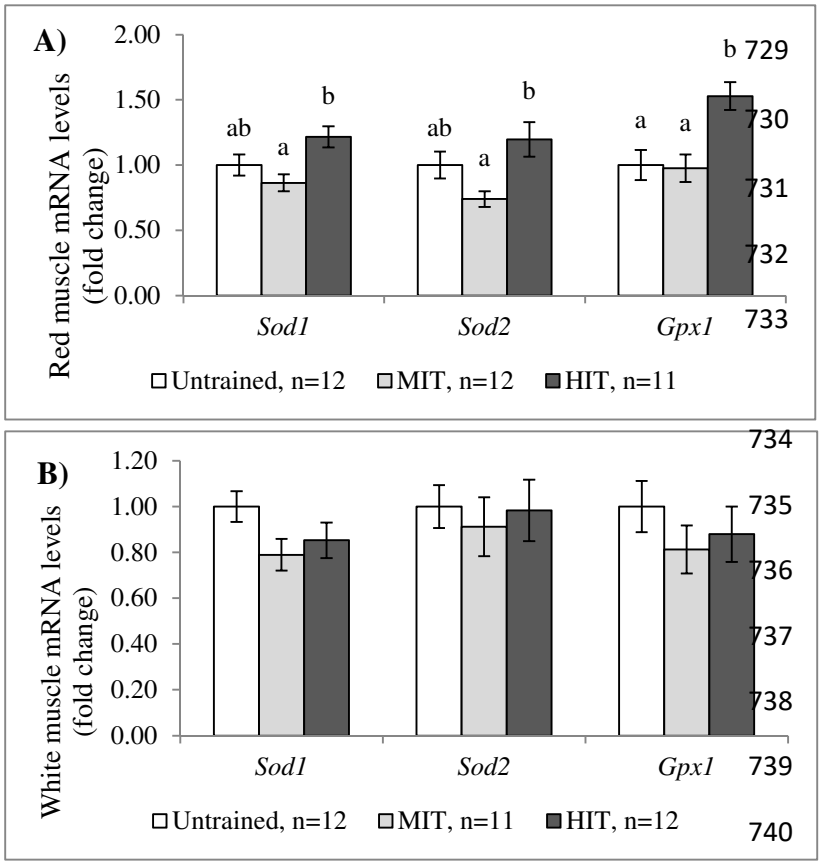
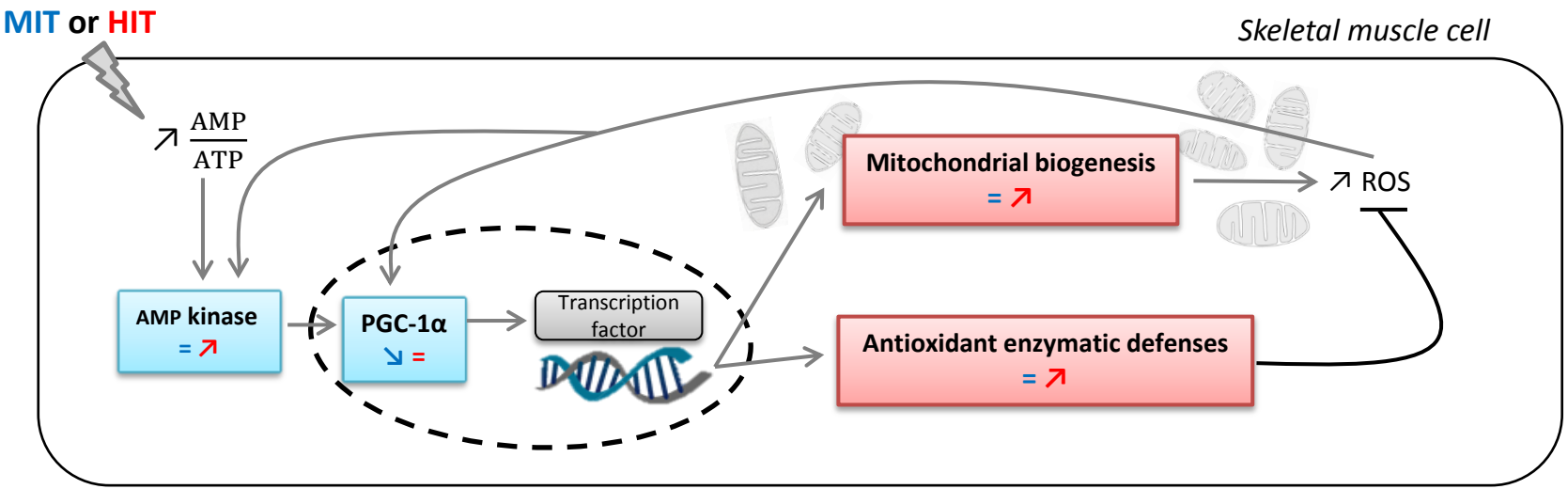

A)
Red muscle mRNA levels (fold change)
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
a a b
a a b
a ab b
a b c
a a b
a a b
a a b
ab a b
Cs
Nd1
Cox1
Cox2
Cox4
Atp synthase 6
Ucp2α
Ucp2β
Untrained, n=12
MIT, n=12
HIT, n=11
B)
White muscle mRNA levels (fold change)
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
a a b
a b a
a b ab
Cs
Nd1
Cox1
Cox2
Cox4
Atp synthase 6
Ucp2α
Ucp2β
Untrained, n=12
MIT, n=11
HIT, n=12

Figure 3.


A)
Red muscle mRNA levels (fold change)
2.00
1.50
1.00
0.50
0.00
ab
a
b
ab
a
b
a
a
b
Sod1
Sod2
Gpx1
Untrained, n=12
MIT, n=12
HIT, n=11
B)
White muscle mRNA levels (fold change)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Sod1
Sod2
Gpx1
Untrained, n=12
MIT, n=11
HIT, n=12



Transcript responses after 10 days of MIT (Moderate Intensity Training) and HIT (High Intensity Training) in rainbow trout red muscle