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1 **Training protocols differently affect AMPK–PGC-1 α signaling**
2 **pathway and redox state in trout muscle.**

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11 **Abbreviations**

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

15 **Abstract**

16 Beneficial effects of physical exercise training are in part related to enhancement of muscle
17 mitochondrial performance. The effects of two different trainings were investigated on
18 transcripts and proteins of the AMPK–PGC-1 α signaling pathway, the mitochondrial
19 functioning (citrate synthase (CS), oxidative phosphorylation complexes, uncoupling proteins
20 (UCP)) and the antioxidant defenses (superoxide dismutase (SOD), glutathione peroxidase
21 (GPx), catalase) in rainbow trout red and white skeletal muscles. One group of trouts swam
22 for 10 days at a moderate intensity (approximately 57% U_{crit} or 2.0 body lengths/s, 23.5
23 h/day) and another group at a high intensity (approximately 90% U_{crit} or 3.2 body lengths/s, 2
24 h/day). In the red muscle, the increase of *Cs* mRNA levels was significantly correlated with
25 the transcripts of *Ampka1*, *Ampka2*, *Pgc-1 α* , the oxidative phosphorylation complexes,
26 *Ucp2 α* , *Ucp2 β* , *Sod1*, *Sod2* and *Gpx1*. After 10 days of training, high intensity training (HIT)
27 stimulates more the transcription of genes involved in this aerobic pathway than moderate
28 intensity training (MIT) in the skeletal muscles, and mainly in the red oxidative muscle.
29 However, no changes in CS, cytochrome c oxidase (COX) and antioxidant defenses activities
30 and in oxidative stress marker (isoprostane plasmatic levels) were observed. The
31 transcriptomic responses are fiber- and training-type dependent when proteins were not yet
32 expressed after 10 days of training. As in mammals, our results suggest that HIT could
33 promote benefit effects in fish.

34 **Keywords**

35 Moderate intensity training
36 High intensity training
37 Skeletal muscle
38 Mitochondrial biogenesis
39 Antioxidant defenses

40 **Introduction**

41 Physical exercise is recognized to improve human health but the involved cellular and
42 molecular mechanisms remain to be fully elucidated (Neufer *et al.*, 2015). The stimulation of
43 the mitochondrial biogenesis and antioxidant defenses induced by physical activity in skeletal
44 muscle are largely considered as contributor to improve overall health (Gabriel and Zierath,
45 2017). These cellular responses and their adaptations are highly dependent on the parameters
46 of the physical exercise protocols such as intensity, duration and frequency (Bishop *et al.*,
47 2019; MacInnis *et al.*, 2019). High intensity interval training (HIIT), characterized by short
48 high intensity fractions interspersed with regular recovery time intervals (Cochran *et al.*,
49 2014), could be even more beneficial for health than moderate intensity continuous training
50 (MICT). In mammals, the mitochondrial biogenesis and the oxidative phosphorylation
51 (OXPHOS) capacities should be more stimulated after HIIT compared to MICT (MacInnis *et*
52 *al.*, 2017).

53 Among the signaling pathways activated in the skeletal muscle during a physical
54 exercise, the AMP-activated protein kinase (AMPK)–peroxisome proliferator-activated
55 receptor- γ (PPAR- γ) coactivator-1 α (PGC-1 α) is the main one stimulating the mitochondrial
56 biogenesis and the oxidative capacities in vertebrates, including fish (Correia *et al.*, 2015;
57 Magnoni *et al.*, 2014; Morash *et al.*, 2014). The AMPK is activated by a cellular stress such
58 as exercise which decreases ATP concentration and then increases AMP:ATP ratio (Winder
59 and Hardie, 1996). This kinase, which stimulates PGC-1 α , is considered as a key molecule in
60 the oxidative metabolism upregulating the OXPHOS. PGC-1 α may interact with transcription
61 factors which lead to the transcription of specific genes such as those encoding antioxidant
62 enzymes and mitochondrial respiratory chain proteins (Bratic and Larsson, 2013; Hawley *et*
63 *al.*, 2014). AMPK and PGC-1 α are both redox sensitive, meaning that this signaling pathway
64 can be influenced by the reactive oxygen species (ROS) (Trewin *et al.*, 2018).
65 The effects of different exercise modalities on the AMPK–PGC-1 α signaling pathway and
66 associated compounds have been studied in human and rodents. The results related to the
67 regulation of this pathway are variable and sometimes contradictory, in part due to the
68 exercise parameters, mainly intensity (Bartlett *et al.*, 2012; Egan *et al.*, 2010; MacInnis *et al.*,
69 2019). Godin *et al.* (2010) suggested these results may be in part explained by differences in
70 fiber type recruitment. The investigations in mammals are often performed on whole skeletal
71 muscle containing mixed type fibers (oxidative type I fibers and glycolytic type IIb fibers)
72 when metabolic responses to physical exercise can also be influenced by the specific fiber
73 type recruitment. Concerning mitochondrial function, MICT and HIIT stimulated for example

74 the cytochrome c oxidase complex in the human *vastus lateralis* when no exercise training
75 effects were observed in isolated fibers I and II (MacInnis *et al.*, 2017).
76 In the present study, the rainbow trout was used as a model for several reasons. First, unlike
77 mammals, fish has anatomically separated fast (white glycolytic muscle) and slow twitch
78 fibers (oxidative red muscle). Such particularity is interesting to investigate the fiber type-
79 specific adaptations to training. Second, fish is also easily to train and may globally respond
80 to endurance training in a similar manner as mammals. For example, training exercise can
81 stimulate mitochondrial biogenesis in fish even if mRNA regulation could be distinct from
82 mammals (McClelland, 2012). Third, the rainbow trout is particularly used in exercise
83 physiology providing a good model for optimizing exercise protocols and for better
84 understanding cellular or molecular mechanisms involved in training exercise adaptation
85 (Kieffer, 2010). Finally, the AMPK–PGC-1 α signaling pathway has been identified in
86 rainbow trout and previously explored in conditions of moderate intensity training by Morash
87 *et al.* (2014).

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

93 **Materials and methods**

94 Fish and experimental conditions

95 The experimental design was made in accordance with the animal experimental
96 authorization n°472802, respecting the three “R” (replace, reduce, refine).
97 Juvenile female rainbow trouts (*Oncorhynchus mykiss*) were obtained from a local fish farm
98 (EARL pisciculture de Lescoat, Lesneven, France). At their arrival at the laboratory, fishes
99 were acclimated for two weeks before experimentation in 400 L polyethylene tanks filled
100 with continuously renewed and aerated water at 15°C with a natural photoperiod (10 h:14 h
101 light:dark). All the trouts were tagged in subcutaneous between the head and the dorsal fin
102 (Glass tag 2 x 12 mm, Biolog-id, Bernay, France). Individual measurements of body mass
103 (BM = 113 \pm 2.2 g) and body length (BL = 20.6 \pm 0.2 cm) were realized.

104 Before starting the training, the critical swimming speed (U_{crit}) was determined to obtain
105 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 $15 \pm 0.5^\circ\text{C}$. U_{crit} was calculated for each animal
110 using Brett's equation: $U_{\text{crit}} = V_{n-1} + V_i \times \frac{t_n}{T_i}$ (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 $3.41 \pm$
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 $15 \pm 0.5^\circ\text{C}$ 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, Hoerd, 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 OD₂₆₀/OD₂₈₀ 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 Mg²⁺) 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

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

180 Enzyme activities and oxidative stress marker

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

183 *Citrate synthase activity*

184 50 mg of frozen red and white muscles were homogenized with a Polytron homogenizer
185 in an extraction buffer (100 mM TRIS HCl, pH 8.1, 4°C). Citrate synthase (CS) activity was
186 assessed at 412 nm on the supernatant using 1 mM DTNB (5,5-dithio-bis-2-nitrobenzoic
187 acid), 12.3 mM acetyl CoA and 100 mM oxaloacetate (Srere, 1969). Measurements were
188 expressed in μmol DTNB reduced/min/g wet tissue.

189 *Cytochrome c oxidase activity*

190 Frozen red and white muscles were homogenized with a Polytron in an extraction buffer
191 (100 mM TRIS, 2 mM EDTA and 2 mM DTE, pH 7.4, 4°C). The homogenate was
192 centrifuged at 12,000 g for 20 min at 4°C. Cytochrome c oxidase (COX) activity was
193 determined on the supernatant at 550 nm using 2 mM reduced cytochrome c and 330 mM
194 sodium phosphate buffer (Smith and Conrad, 1956). COX activity was expressed in nmol
195 cytochrome c oxidized/min/g wet tissue.

196 *Antioxidant enzyme activities*

197 200 mg of frozen red and white muscles were homogenized in an extraction buffer (75
198 mM TRIS and 5 mM EDTA, pH 7.4, 4°C) with a Polytron homogenizer. After a

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

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

206 **CAT activity** was determined at 240 nm through its capacity to transform hydrogen
207 peroxide (H₂O₂) into water and oxygen (Beers and Sizer, 1952). The adding of 200 mM H₂O₂
208 in curves initiated the reaction. CAT activity was expressed in nmol H₂O₂/min/g wet tissue.

209 **GPx activity** was measured at 340 nm with an indirect method adapted from Ross *et al.*
210 (2001) by Farhat *et al.* (2015). Briefly, the activity was determined from the decrease of
211 NADPH induced by a coupled reaction with glutathione reductase. GPx activity was
212 expressed in nmol NADPH oxidized/min/g wet tissue.

213 *Oxidative stress marker*

214 Total plasmatic 8-isoprostane (free and esterified in lipids) were measured in duplicate
215 using an Elisa kit (Cayman Chemical, Ann Arbor, Michigan, USA) according to the
216 manufacturer's protocol. All samples were hydrolyzed using 15% KOH and incubated 60 min
217 at 40°C and then neutralized with potassium phosphate buffer. Another step of purification
218 was necessary with ethanol. Finally, samples were extracted using ethyl acetate containing
219 1% methanol and SPE Cartridges (C-18) (Cayman Chemical). After 18 h of incubation, 8-
220 isoprostane plasmatic concentration was measured at 410 nm and expressed in pg/mL.

221 Statistical analysis

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

229 **Results**

230 Condition factor

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

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

235 *AMPK–PGC-1 α signaling pathway*

236 In the red muscle, HIT induced a ~30% increase in the *Ampka2* mRNA content
237 compared to untrained and MIT groups ($p < 0.05$). No changes were observed on the *Ampka1*
238 mRNA levels between groups. In the MIT group, the *Pgc-1 α* mRNA content was significantly
239 reduced by 2-fold ($p < 0.05$) compared to untrained and HIT groups in red and white muscles.
240 In the white muscle, both MIT and HIT decreased the *Ampka1* mRNA levels whereas the
241 *Ampka2* mRNA quantity was unchanged (**Figure 1, A and B**).

242 *Mitochondrial functioning*

243 HIT up-regulated the *Cs* mRNA levels compared to untrained and MIT groups in both
244 types of trout skeletal muscle. In the red muscle, the mRNA levels of *Nd1*, *Cox1*, *Cox2*, *Cox4*
245 and *Atp synthase 6* were also increased by HIT compared to the untrained group. The MIT
246 group showed a 65% increase ($p < 0.05$) in the *Cox2* mRNA content in the red muscle and a
247 decrease in *Nd1* and *Cox1* mRNA levels in the white muscle compared to the untrained group.
248 In addition, the *Nd1*, *Cox2*, *Cox4* and *Atp synthase 6* mRNA levels were up-regulated by HIT
249 in the red muscle when compared to the MIT group. In the white muscle, a decrease in the
250 *Nd1* mRNA levels by MIT was shown compared to untrained and HIT groups whereas no
251 modifications were observed for the *Cox2*, *Cox4* and *Atp synthase 6* gene transcriptions. In
252 the red muscle, HIT raised the *Ucp2 α* and *Ucp2 β* mRNA levels compared to the MIT group.
253 In the white muscle, both types of training did not modify these two genes contents (**Figure 2,**
254 **A and B**).

255 *Antioxidant defenses*

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

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

261

262 Correlations analysis in skeletal muscles

263 In the red muscle, the mRNA level of *Cs* was significantly correlated (moderate
264 correlation: $0.4 \leq R < 0.7$ or strong correlation: $0.7 \leq R < 1$) with transcripts of compounds
265 related to the AMPK–PGC-1 α signaling pathway (*Ampka1*, *Ampka2* and *Pgc-1 α*), the
266 mitochondrial functioning (*Nd1*, *Cox*, *Atp synthase 6* and *Ucp2 α*) and antioxidant defenses
267 (*Sod1*, *Sod2* and *Gpx1*) (**Table 3**). In the white muscle, *Cs* transcripts were significantly
268 correlated with the mRNA of *Ampka2*, mitochondrial functioning compounds (except *Cox2*
269 and *Ucp2 β*) and all target antioxidant systems (**Table 3**).

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

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

273 *Oxidative stress marker*

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

276 **Discussion**

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

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

289 vertebrates, including fish (Craig *et al.*, 2018). Among them, we studied the mRNA encoding
290 for AMPK α 1 and AMPK α 2 in the rainbow trout skeletal muscle. Our data showed that the
291 responses of AMPK α isoforms were fibers- and training-type depending. In fish, the role of
292 each skeletal muscle fiber is exclusive of a swimming type. Aerobic and sustained swimming
293 recruits preferentially the oxidative skeletal muscle (red muscle) whereas the glycolytic
294 skeletal muscle (white muscle) is mainly involved in anaerobic and burst swimming (Palstra
295 *et al.*, 2013).

296 Our data showed that both trainings decreased the *Ampka1* mRNA levels in the white muscle
297 (**Figure 1, B**) whereas only HIT increased the *Ampka2* mRNA level in the red muscle (**Figure**
298 **1, A**). Our results were difficult to compare to others because Morash *et al.* (2014) quantified
299 total *Ampk* mRNA and not separated isoforms in the trout muscle. They showed a 10-fold rise
300 in the red muscle after one week of MIT with a return to the control level after 2 and 4 weeks
301 of training. So, it seems that the *Ampk* mRNA could be regulated during the first days of
302 training in skeletal muscle.

303 In mammal skeletal muscles, AMPK regulates the expression of PGC-1 α . This
304 transcriptional co-factor could be considered as the key player of adaptations induced by
305 endurance exercise training and it has an important role in the regulation of genes involved in
306 mitochondrial biogenesis and antioxidant defenses (Olesen *et al.*, 2010). Several studies in
307 mammals focused on the effects of acute exercise but less on the effects of chronic exercise.
308 Among them, Hyatt *et al.* (2016) showed that 10 days of MIT increased the *Pgc-1 α* mRNA
309 content in the rat *plantaris* muscle (mainly composed of type II fibers) with no changes in the
310 *soleus* muscle (mainly composed of type I fibers), that suggested a *Pgc-1 α* transcriptomic
311 response fiber-type dependent. In the present study, compared to untrained and HIT groups,
312 the *Pgc-1 α* transcripts levels were surprisingly down-regulated after 10 days of MIT in both
313 red and white skeletal muscles (**Figure 1, A and B**). In the literature, a decrease in PGC-1 α
314 expression was often related to reduce mitochondrial performance in mammals (Mortensen *et*
315 *al.*, 2007) or in fish (Gilbert *et al.*, 2014). Here, in the red muscle, it was not the case because
316 the mRNA of the OXPHOS complexes are, for most of them, unchanged or increased after 10
317 days of MIT. Differently, in the white muscle, MIT decreased *Ampka1*, *Pgc-1 α* and some
318 electron transport chain (ETC) complexes mRNA too (see below).

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

323 regulation could happen earlier in comparison to the overexpression of *Pgc-1 α* mRNA
324 reported in the red muscle after 30 or 40 days of sustained swimming (Magnoni *et al.*, 2013,
325 2014). However, our results were closed to those reported in zebrafish when one week of
326 moderate interval training (twice daily for 3 h separated by 2 h rest period) increased the *Pgc-*
327 *1 α* mRNA quantity in the skeletal muscle with a return to the control value after 8 weeks of
328 training (LeMoine *et al.*, 2010).

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

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

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

357 were no changes in the white muscle (**Figure 2, B**). MIT only stimulated *Cox2* mRNA in the
358 oxidative red muscle. Our training protocols did not significantly modify the COX activity but
359 HIT tended to increase in white muscle when compared to MIT ($p=0.07$; **Table 4**).
360 McClelland *et al.* (2006) showed that COX activity increased in zebrafish but after 30 days of
361 sustained swimming. Few studies focused on the OXPHOS complexes mRNA in the present
362 training conditions in mammals and at our knowledge, none in fish. Our results confirmed a
363 fiber-type molecular response depending on training-type.
364 The UCPs are present in the inner mitochondrial membrane and principally known for its
365 thermogenesis function. By dissipating the proton electrochemical gradient, UCP2 would
366 have also a common role as redox sensors by reducing mitochondrial ROS production
367 because of a more important electron transport through the mitochondrial ETC (Ji and Zhang,
368 2014). In fish, the effects of different environmental stressors (temperature, hypoxia, nutrient)
369 have been studied on UCP2 (Bermejo-Nogales *et al.*, 2014) but not the training effects at our
370 knowledge. In the present paper, two UCP2 isoform genes (*Ucp2 α* and *Ucp2 β*) have been
371 studied which have 93% of similitude in their sequences in rainbow trout (Coulibaly *et al.*,
372 2006). In the red muscle, HIT increased the transcription of the gene encoding for UCP2 α
373 compared to untrained and MIT groups. This training stimulated *Ucp2 β* mRNA compared to
374 the other groups (**Figure 2, A**). Neither MIT nor HIT affected the *Ucp2 α* and *Ucp2 β* mRNA
375 levels in the white muscle (**Figure 2, B**). HIT would enhance the transcription of *Ucp2 α* in
376 the red muscle. This mild uncoupling of mitochondrial OXPHOS may represent a process of
377 defense against oxidative stress in mitochondria by reducing the potential electron leak
378 associated to physical exercise.

379 During physical exercise, ROS are generated from different sources (mitochondrion,
380 NADPH oxidase and xanthine oxidase) by contracting skeletal muscle (He *et al.*, 2016). Even
381 if mitochondrion is often cited as a main source (Davies *et al.*, 1982; Gredilla *et al.*, 2004;
382 Powers and Jackson, 2008), the primary site remains unclear (Powers *et al.*, 2011). It is
383 known that ROS are necessary for numerous cellular adaptive mechanisms (Ljubcic *et al.*,
384 2010). Single bouts of acute exercise may enhance ROS overproduction and cause potential
385 oxidative damage. But, regular aerobic exercise, through ROS acting as signaling agents, can
386 improve muscle oxidative balance by upregulation of endogenous antioxidant defenses
387 systems (Steinbacher and Eckl, 2015). Among the main enzymatic antioxidants, SOD reduce
388 the superoxide anion to hydrogen peroxide through two isoforms: SOD1 (Cu/Zn-SOD) and
389 SOD2 (Mn-SOD) respectively present in the cytoplasm and in mitochondria. GPx and CAT
390 mainly transform the hydrogen peroxide to water (Birben *et al.*, 2012).

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

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

410 To explore the relationships among the components analyzed, correlations between
411 different mRNA levels of parameters were assessed. This analysis demonstrates that, mainly
412 in the red muscle, the *Cs* mRNA is positively correlate to almost all the studied transcripts
413 related to AMPK–PGC-1 α signaling pathway, OXPHOS and antioxidant enzymatic defenses.
414 The significant positive correlation was moderate or strong for *Ampka1*, *Ampka2* and *Pgc-1 α*
415 ($0.64 < R < 0.71$) and OXPHOS components ($0.40 < R < 0.81$). The antioxidant systems
416 presented strong relationships in the red muscle ($0.74 < R < 0.82$) (**Table 3**). These results
417 suggested a coordinate regulation of the mitochondrial biogenesis with the AMPK–PGC-1 α
418 signaling pathway and the activation of antioxidant defenses including UCP2 α as observed in
419 mammals (Gouspillou *et al.*, 2014). In the mouse skeletal muscle, overexpression of PGC-1 α
420 induced a stimulation of the mitochondrial biogenesis involving a rise of the expression of
421 genes encoding for OXPHOS complexes such as *Cox* (Leick *et al.*, 2008). Based on our
422 results and those of LeMoine *et al.* (2010), PGC-1 α seems sensitive to the exercise stimulus in
423 fish. That confirms, as in mammals, its involvement in the regulation of mitochondrial
424 biogenesis at least in conditions of exercise (Magnoni *et al.*, 2014).

425 To a lesser extent, in the white muscle, *Cs* transcripts were moderately correlated with the
426 *Ampka2* mRNA ($R=0.50$), mitochondrial functioning compounds (except *Cox2* and *Ucp2 β*)
427 and antioxidant defenses (except *Sod1*) (**Table 3**). MIT and HIT had few effects on transcripts
428 in white muscle in comparison to red muscle. Red muscle fibers are recruited during slow or
429 moderate intensity movements when both red and white fibers are recruited during high
430 intensity movements (Jayne and Lauder, 1994). In salmonids, studies showed that aerobic
431 metabolism predominates in swimming up to 70 to 90% of their U_{crit} and that the recruitment
432 of white fibers in rainbow trout initiated at swimming speeds around 80% U_{crit} (Hvas and
433 Oppedal, 2017; Webb, 1971). Therefore in our study, an intensity of 90% U_{crit} was chosen for
434 HIT in order to stimulate not only red muscle (as supposed during MIT) but potentially also
435 white muscle. Our data show mRNA adaptations of the studied signaling pathway,
436 mitochondrial compounds and antioxidant defenses mainly in the red muscle after HIT.
437 During HIT, when fishes swam at 90% U_{crit} , the red oxidative muscle recruitment should be
438 even greater than during MIT and the white glycolytic muscle should be also mobilized but in
439 a lesser extent. We can hypothesize than a longer period of high intensity exercise (> 2 h/day)
440 could have been more efficient in stimulating mitochondrial biogenesis in white muscle.

441 Our study has some methodological limitations related to the choice of some protocol
442 parameters. Ten days of training might be considered as too short in comparison to the
443 durations of training used in previous studies. In fish, the CS activity is increased only after
444 30 days of sustained swimming whereas mRNAs are stimulated during the early stages
445 between 1 and 2 weeks (LeMoine *et al.*, 2010; McClelland *et al.*, 2006; Morash *et al.*, 2014).
446 In a perspective to explore the temporal changes of adaptations of the AMPK–PGC-1 α
447 signaling pathway and antioxidant systems to training, we chose to first explore their early
448 changes. In the present paper, we clearly showed after 10 days of training the stimulation of
449 mRNA levels mainly with the HIT protocol, but the duration was probably not long enough to
450 observe concomitant protein changes. One of our perspectives remains to investigate a longer
451 training duration.

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

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

463 **Conclusion**

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

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662

663 **Figure captions**

664

665 **Figure 1.** Effects of MIT (moderate intensity training) and HIT (high intensity training) on the
666 *Ampka1*, *Ampka2* and *Pgc-1 α* mRNA levels in red (**A**) and white (**B**) muscles of the rainbow trout.
667 Results were expressed as fold change with the untrained group, which was set to 1. Results were
668 shown as means \pm SEM. Similar letters indicate no significant differences ($p>0.05$) between groups.

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

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

680 **Tables**

681

682 **Table 1.** Primer sequences used for Real-Time RT-PCR analysis. The hybridization temperature was
 683 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	<i>18S rRNA</i> ¹	(F) CGGAGGTTTCGAAGACGATCA (R) TCGCTAGTTGGCATCGTTTAT	GenBank	AF308735.1
Adenosine monophosphate kinase α 1	<i>Ampka1</i>	(F) GCCACCATCAAAGAGATCCG (R) TCAAACCTTCTCACACACCTCC	GenBank	XM_021590586
Adenosine monophosphate kinase α 2	<i>Ampka2</i>	(F) CGCTCAAGAGGGCTACCATT (R) CAGACTCGGTGCTCTCAAAC	GenBank	XM_021604240
Peroxisome proliferator-activated receptor- γ coactivator 1 α	<i>Pgc-1α</i> ²	(F) CAACCACCTTGCCACTTCCT (R) GGTTCAGAGATCTCCACAC	GenBank	FJ710605.1
Citrate synthase	<i>Cs</i> ¹	(F) AACCAACCTCACTCATCACCA (R) GCAGCAGAAGCAGCCATAA	GenBank	XM_021566131
Superoxide dismutase 1	<i>Sod1</i> ³	(F) AGGCTGTTTGCCTGCTCAA (R) CCAATCAGCTTACAGGACCAT	GenBank	AF469663.1
Superoxide dismutase 2	<i>Sod2</i> ³	(F) CCACACCATCTTCTGGACAAAC (R) GGAGCCAAAGTCACGGTTGA	Tigr	TC123543
Glutathione peroxidase 1	<i>Gpx1</i> ³	(F) CCTGGGAAATGGCATCAAGT (R) GGGATCATCCATTGGTCCATAT	Tigr	TC94794
Uncoupling protein 2 α	<i>Ucp2α</i>	(F) ACCAAACCGACGTTACCATGG (R) TTAAGAAGCGCGTCCTTGATG	GenBank	NM_001124654.1
Uncoupling protein 2 β	<i>Ucp2β</i>	(F) TATTGCCATGGTGACCAAGGA (R) CTACAGAGGAGTGGTGTAGTT	GenBank	NM_001124571.1
NADH dehydrogenase 1	<i>Nd1</i>	(F) TTGTACCCGTTCTGTTAGCAG (R) TAGTTTTAGGCCGTCTGCGAT	GenBank	NP_008290
Cytochrome c oxidase 1	<i>Cox1</i>	(F) TCAACCAACCACAAAGACATTG (R) CACGTTATAGATTTGGTCATCC	GenBank	NP_008292
Cytochrome c oxidase 2	<i>Cox2</i> ²	(F) GAGGCAATAAAGGCTGTTTGGT (R) GCCGTTCTTTCTTTAGGTGTAA	GenBank	NP_008293
Cytochrome c oxidase 4	<i>Cox4</i> ⁴	(F) TACGTGGGGGACATGGTGT (R) CCCAGGAGCCCTTCTCCTTC	Sigenae	tcav0004c.i.22_3.1.s.om.8
Adenosine triphosphate synthase 6	<i>Atp synthase 6</i>	(F) CTTTCGACCAATTTATGAGCCC (R) TCGGTTGATGAACCACCCTTG	GenBank	NP_008295

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

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

686

687 **Table 2.** Condition factors of rainbow trouts.

688			
	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

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

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

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

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

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

697 **Table 3.** Correlations between *Cs* mRNA vs. *Ampka1*, *Ampka2*, *Pgc-1a*, *Nd1*, *Cox1*, *Cox2*, *Cox4*, *Atp*
 698 *synthase 6*, *Sod1*, *Sod2*, *Gpx1*, *Ucp2a* and *Ucp2b* mRNA in red and white muscles.

	Red muscle n=35	White muscle n=35
	<i>Cs</i>	
<i>Ampka1</i>	R=0.66*	R=0.27
<i>Ampka2</i>	R=0.71*	R=0.50*
<i>Pgc-1a</i>	R=0.64*	R=0.01
<i>Nd1</i>	R=0.79*	R=0.58*
<i>Cox1</i>	R=0.70*	R=0.42*
<i>Cox2</i>	R=0.61*	R=0.25
<i>Cox4</i>	R=0.80*	R=0.49*
<i>Atp synthase 6</i>	R=0.81*	R=0.40*
<i>Ucp2a</i>	R=0.74*	R=0.64*
<i>Ucp2b</i>	R=0.40*	R=0.29
<i>Sod1</i>	R=0.74*	R=0.34*
<i>Sod2</i>	R=0.82*	R=0.59*
<i>Gpx1</i>	R=0.74*	R=0.43*

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

700

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

	Red muscle			White muscle		
	Untrained n=12	MIT n=12	HIT n=12	Untrained n=12	MIT n=12	HIT n=12
CS ($\mu\text{mol DTNB reduced}/\text{min}/\text{g WT}$)	25.4 \pm 2.1	25.3 \pm 2.4	24.9 \pm 1.6	2.9 \pm 0.2	3.2 \pm 0.2	3.1 \pm 0.2
COX ($\text{nmol cyt. c oxidized}/\text{min}/\text{g WT}$)	40.4 \pm 7.3	41.34 \pm 5.3	44.60 \pm 6.0	6.3 \pm 1.0	5.9 \pm 1.1	8.26 \pm 1.1
SOD ($\text{U}/\text{mg WT}$)	3.9 \pm 0.4	3.7 \pm 0.3	3.2 \pm 0.3	0.31 \pm 0.02	0.33 \pm 0.04	0.30 \pm 0.04
CAT ($\text{nmol H}_2\text{O}_2/\text{min}/\text{g WT}$)	354.8 \pm 29.2	338.0 \pm 50.7	372.3 \pm 42.1	ND	ND	ND
GPx ($\text{nmol NADPH}/\text{min}/\text{g WT}$)	275.1 \pm 22.7	293.0 \pm 18.6	254.7 \pm 17.7	151.3 \pm 17.1	167.6 \pm 21.5	185.0 \pm 13.4

703 Results were shown as means \pm SEM. No significant differences were observed between groups. MIT: moderate
 704 intensity training; HIT: high intensity training; CS: citrate synthase; COX: cytochrome c oxidase; SOD:
 705 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

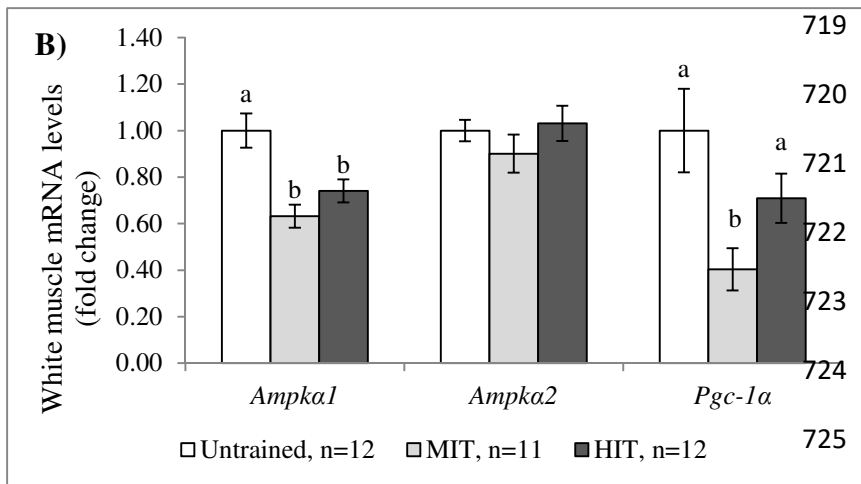
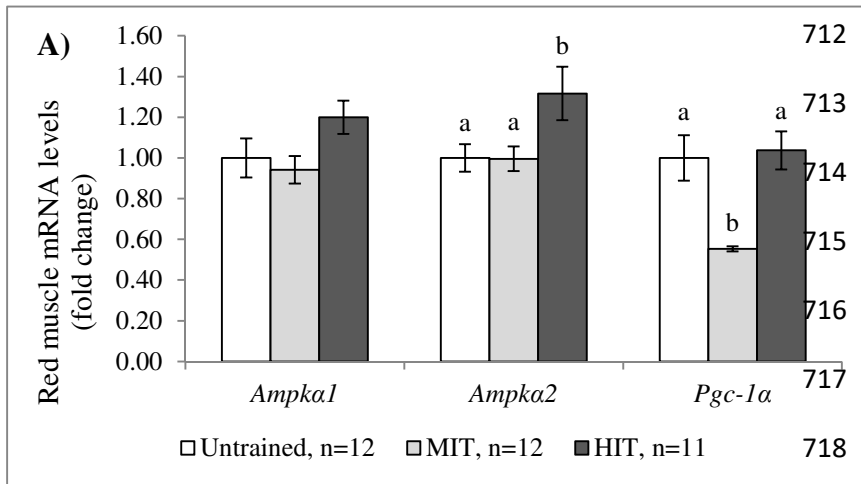
707 Results were shown as means ± SEM. No significant differences were observed between groups.

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

709 **Figures**

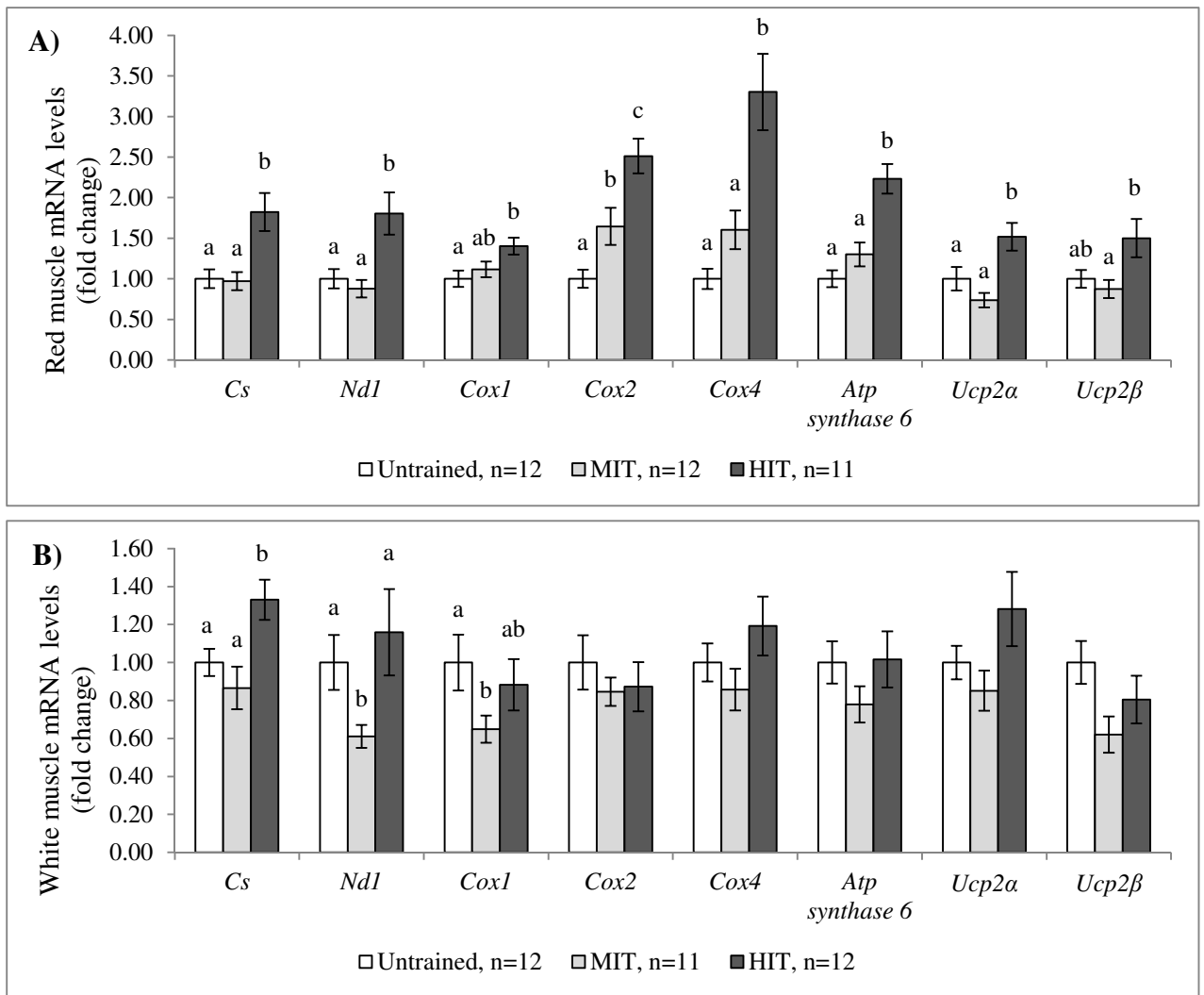
710

711 **Figure 1.**

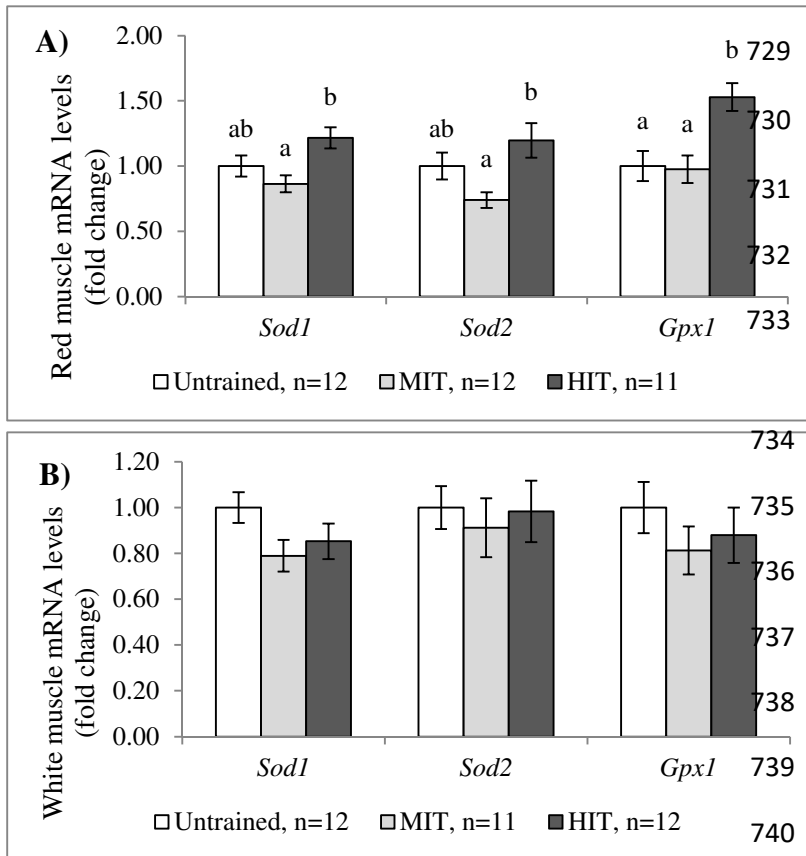


726

727 **Figure 2.**



728 **Figure 3.**



MIT or HIT

Skeletal muscle cell



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