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RESEARCH PAPER

How do exercise training variables stimulate processes related to mitochondrial biogenesis in slow and fast trout muscle fibres?

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Abstract

Exercise is known to be a powerful way to improve health through the stimulation of mitochondrial biogenesis in skeletal muscle, which undergoes cellular and molecular adaptations. One of the current challenges in human is to define the optimal training stimulus to improve muscle performance. Fish are relevant models for exercise training physiology studies mainly because of their distinct slow and fast muscle fibres. Using rainbow trout, we investigated the effects of six different training protocols defined by manipulating specific training variables (such as exercise intensity, volume, duration and frequency), on mRNAs and some proteins related to four sub-systems (AMP-activated protein kinase–peroxisome proliferator-activated receptor γ coactivator-1 α signalling pathway, mitochondrial function, antioxidant defences and lactate dehydrogenase (LDH) metabolism) in both red and white muscles (RM and WM, respectively). In both muscles, high-intensity exercise stimulated more mRNA types and enzymatic activities related to mitochondrial biogenesis than moderate-intensity exercise. For volume, duration and frequency variables, we demonstrated fibre type-specific responses. Indeed, for high-intensity interval training, RM transcript levels are increased by a low training volume, but WM transcript responses are stimulated by a high training volume. Moreover, transcripts and enzymatic activities related to mitochondria and LDH show that WM tends to develop aerobic metabolism with a high training volume. For transcript stimulation, WM requires a greater duration and frequency of exercise than RM, whereas protein adaptations are efficient with a long training duration and a high frequency in both muscles.

KEYWORDS

exercise training, mitochondria, skeletal muscle

1 | INTRODUCTION

The practice of regular physical exercise is recommended to reduce the risk of many chronic illnesses such as cardiovascular diseases

(Karjalainen et al., 2015). Exercise training promotes numerous health benefit effects that include metabolic changes such as increases in maximal oxygen uptake ($\dot{V}_{O_{2max}}$), insulin sensitivity and oxidation of fatty acids (Bamman et al., 2014). It has also been largely demonstrated

that endurance training can improve muscle aerobic capacity by stimulating mitochondrial biogenesis (i.e. the generation of new mitochondrial components that enhance mitochondrial function and content) (Gabriel & Zierath, 2017). Studies are still needed, however, to (i) define the best training exercise protocols for stimulating mitochondrial biogenesis, and (ii) identify the precise cellular and molecular events involved and their temporal changes (MacInnis & Gibala, 2017; Trewin et al., 2018).

Currently, the challenge is to optimise training protocols to stimulate mitochondrial biogenesis adaptations in skeletal muscle. Interval training often consists of short periods of high intensity exercise interspersed by periods of recovery and represents a time efficient exercise type leading to many mitochondrial adaptations similar to those of moderate endurance training (Richards et al., 2011). However, no consensus has been reached on the most relevant training variable to stimulate mitochondrial biogenesis among relative work intensity, duration and frequency of exercise (MacInnis & Gibala, 2017). Indeed, although some human studies have shown that exercise intensity is the most important variable determining the stimulation of citrate synthase activity (used as marker of mitochondrial biogenesis) (MacInnis et al., 2019), but other studies underline exercise training volume, which is the product of exercise intensity, duration and frequency (Bishop et al., 2019). For ethical and methodological reasons (cohorts in parallel, biopsy size limitation), such experimentation is difficult to conduct in human. New models are therefore needed to take such investigations further.

Fish are a relevant model to explore mitochondrial adaptations to different modes of exercise training. Compared with mammals, fish offer the advantage of having clearly separated slow and fast fibres (McClelland, 2012; Pengam et al., 2020). This is a relevant criterion for exercise physiology studies because red oxidative and white glycolytic fibres are recruited differently depending on the type and the duration of exercise (Hvas & Oppedal, 2017). Energetic and electromyographic studies in salmonids, including rainbow trout, have defined the swimming velocities where red and white fibres are preferentially recruited (Altringham & Ellerby, 1999; Burgetz et al., 1998).

Several cellular pathways involved in mitochondrial biogenesis have been proposed to be responsible for mediating the adaptive responses to exercise training (Ji et al., 2016). Among these, the AMP-activated protein kinase (AMPK)-peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α) signalling pathway is the main pathway that increases oxidative capacities in vertebrates, including fish (Lin et al., 2005). The depletion of ATP induced by a cellular stress such as exercise increases the AMP:ATP ratio, which activates AMPK (Jäger et al., 2007). This kinase upregulates PGC-1 α , which in turn stimulates mitochondrial and antioxidant genes (Olesen et al., 2010). This signalling pathway is regulated in part by reactive oxygen species (ROS) because some proteins, including AMPK and PGC-1 α , are known to be redox sensitive (Trewin et al., 2018).

In a previous study performed in rainbow trout, we demonstrated that, as in mammals, a high intensity-low volume training is more

New Findings

• What is the central question of this study?

Exercise is known to promote mitochondrial biogenesis in skeletal muscle, but what are the most relevant training protocols to stimulate it?

• What is the main finding and its importance?

As in mammals, training in rainbow trout affects slow and fast muscle fibres differently. Exercise intensity, relative to volume, duration and frequency, is the most relevant training variable to stimulate the processes related to mitochondrial biogenesis in both red and white muscles. This study offers new insights into muscle fibre type-specific transcription and expression of genes involved in mitochondrial adaptations following training.

efficient than a moderate intensity-high volume training at stimulating the transcripts related to the AMPK-PGC-1 α signalling pathway, mitochondrial functioning and antioxidant defences (Pengam et al., 2020). These responses were also greater in red muscle (RM) than in white muscle (WM). However, the question remains as to whether these fibre type-specific responses were more closely related to exercise intensity or to training volume.

The present study aimed to investigate the influence of exercise variables (intensity, volume, training duration and frequency) on the AMPK-PGC-1 α signalling pathway in RM and WM.

For this purpose, four swimming protocols were developed for rainbow trout. Transcripts and proteins involved in the AMPK-PGC-1 α signalling pathway, mitochondrial compounds, antioxidant defences and lactate dehydrogenase metabolism were explored in RM and WM and the data compared to a previous study (Pengam et al., 2020).

2 | METHODS

2.1 | Ethical approval

The experiment was designed in accordance with animal experimental authorisations no. 472802 and no. 16046. The three 'Rs' (replace, reduce and refine) were respected during the stabling and experiments. Juvenile female rainbow trout (*Oncorhynchus mykiss*) were acquired from a local fish farm (EARL Pisciculture de Lescoat, Lesneven, France) and were killed by cerebral concussion at the end of the experiment. The present study complied with principles and standards for reporting experiments in *Experimental Physiology* (Grundy, 2015).

TABLE 1 Training protocols of the experimental groups

Experimental group	<i>n</i>	Training type	Volume (AU)	Intensity (% U_{crit})	Duration (days)	Frequency (session/day)
HIIT1, 10 ^a	12	HIIT	1747	90	10	1 × 2 h
HIIT1, 20	10		3493		20	1 × 2 h
HIIT2, 10	10		3493		10	2 × 2 h
HIIT2, 20	10		6987		20	2 × 2 h
MICT, 10 ^a	12	MICT	13,395	57	10	—
MICT, 20	10		25,850		20	—

^aExperimental groups used in a previous study (Pengam et al., 2020). HIIT, high-intensity interval training; MICT, moderate-intensity continuous training; U_{crit} , critical swimming speed; *n*: number of fish per group.

2.2 | Animals and experimental procedures

2.2.1 | Animals

On their arrival in the laboratory, the trout were acclimated for 2 weeks 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). The experimental work was performed in winter. All trout were tagged subcutaneously between the head and the dorsal fin (Glass tag 2 × 12 mm, Biolog-id, Bernay, France). The body mass (134.4 ± 25.3 g; *n* = 96) and body length (BL; 21.9 ± 1.5 cm; *n* = 96) were measured for each animal.

The present study included six experimental training groups (Table 1), two of which come from a previous study (Pengam et al., 2020). The training protocols of these six groups differed according to the following exercise variables: intensity, volume, duration and frequency. Training volume was calculated according to the formula given below (also used in mammals, Granata et al., 2018):

$$\text{Training volume (AU)} = \text{training duration (days)} \\ \times \text{exercise intensity (\% } U_{crit}) \times \text{exercise timing (hours/day)}$$

Fish were randomly divided among three polyethylene tanks of 450 l with aerated tap water maintained at $15 \pm 0.5^\circ\text{C}$ and half renewed every day. One of these tanks contained untrained fish presenting only spontaneous movements, which were used as controls (*n* = 32). The other two circular tanks (diameter: 128 cm; height: 75 cm) were used for the experimental training groups: moderate intensity continuous training (MICT, *n* = 22) or high intensity interval training (HIIT, *n* = 42). In order to obtain uniform water flow, a smaller circular tank (diameter: 50 cm; height: 47 cm) was placed within each circular tank at the centre.

2.2.2 | Training protocols

Before starting the training, the critical swimming speed (U_{crit}) was determined to work out the water velocity corresponding to the training intensity protocols. For each experiment, the U_{crit} of eight fish was measured in a Brett-type swimming tunnel according to Brett

(1964) and Farrell (2008). The mean U_{crit} (3.26 ± 0.52 BL/s) was not significantly different between experimental groups. Before the beginning of the U_{crit} test, each fish was placed in the swimming tunnel for 1 h with a water velocity of 0.05 m/s to acclimate. Then, water velocity was increased by 0.10 m/s every 10 min until exhaustion, in water maintained at $15 \pm 0.5^\circ\text{C}$. U_{crit} was calculated for each animal using Brett's equation: $U_{crit} = V_{n-1} + V_i \times \frac{t_n}{T_i}$, where V_{n-1} is the velocity of the last completed swimming speed (m/s); V_i is the velocity increment (0.1 m/s); t_n is the duration of the last step (min) and T_i is the duration of each step (10 min).

Fish of the MICT groups swam at approximately 57% U_{crit} for 23.5 h per day for 10 or 20 consecutive days. Water flow was stopped once per day for 30 min during which the fish were fed *ad libitum*. For HIIT regimes, fish were exercised at approximately 57% U_{crit} for 10 min to acclimate them to the water flow and were then exposed to a swimming speed of approximately 90% U_{crit} . Trout swam 5 days a week once daily for 2 h (HIIT1) or twice daily for 2 h (HIIT2), with a 2-h rest period. These training treatments were continued for 10 or 20 days (Table 1).

2.2.3 | Tissue sampling

To avoid potential effects of acute exercise on the results, samples were collected 65 h after the last training session (Leek et al., 2001; MacInnis et al., 2017). RM and WM were rapidly excised along the last 5–10 cm in front of the caudal fin and more precisely at the lateral line level for RM. Plasma samples were collected from 5% EDTA–blood by centrifugation at 3000 g for 5 min at room temperature. Muscles and plasma were immediately frozen in liquid nitrogen and stored at -80°C until further analyses.

2.3 | RNA extraction, reverse transcription and real-time RT-PCR

Total RNA was isolated from 30 mg RM and WM using the NucleoSpin® RNA Set for NucleoZOL (Macherey Nagel, Hoerd, France) and stored at -80°C as previously described in Pengam et al. (2020). RNA concentrations were measured with a SimpliNano™

TABLE 2 Primer sequences used for Real-Time RT-PCR analysis

Target gene	Abbreviation	Primer sequences (5' to 3')	Database	Accession number
18S ribosomal RNA	18S rRNA ¹	(F) CGGAGGTTCTGAAGACGATCA (R) TCGCTAGTTGGCATCGTTTAT	GenBank	AF308735.1
Adenosine monophosphate kinase α 1	<i>Ampkα1</i>	(F) GCCACCATCAAAGAGATCCG (R) TCAAACCTCTCACACACCTCC	GenBank	XM_021590586
Adenosine monophosphate kinase α 2	<i>Ampkα2</i>	(F) CGCTCAAGAGGGCTACCATT (R) CAGACTCGGTGCTCTCAAAC	GenBank	XM_021604240
Peroxisome proliferator-activated receptor- γ coactivator-1 α	<i>Pgc-1α</i> ²	(F) CAACCACCTTGCCACTTCCT (R) GGTTCCAGAGATCTCCACAC	GenBank	FJ710605.1
Citrate synthase	<i>Cs</i> ¹	(F) AACCAACCTCACTCATCACCA (R) GCAGCAGAAGCAGCCCATAA	GenBank	XM_021566131
Superoxide dismutase 1	<i>Sod1</i> ³	(F) AGGCTGTTTGCCTGCTCAA (R) CCAATCAGCTTCACAGGACCAT	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) TTAAGAAGCGCGTCTTGATG	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) TTGTACCCGTTCTGTAGCAG (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) GCCGTTCTCTTTAGGTGTAA	GenBank	NP_008293
Cytochrome c oxidase 4	<i>Cox4</i> ⁴	(F) TACGTGGGGACATGGTGTT (R) CCCAGGAGCCCTTCTCCTTC	Sigenae	tcav0004c.i.22_3.1.s.om.8
Adenosine triphosphate synthase 6	<i>Atp synthase 6</i>	(F) CTTGACCAATTTATGAGCCC (R) TCGGTTGATGAACACCCTTG	GenBank	NP_008295
Lactate dehydrogenase A	<i>Ldh-a</i>	(F) AGGTCATGGACCTGCAGCAT (R) GGCTGTACTTGACGATCTGG	GenBank	XM_021564046.1
Lactate dehydrogenase B	<i>Ldh-b</i>	(F) GGTCCAGAGGAACGTCAACA (R) TGCCGATGACACGGTGTGTTG	GenBank	XM_021577545.1

The hybridisation temperature was 60°C for all primers. Primers were designed by ourselves except those with a subscript number. (F): Forward, (R): Reverse. References: ¹Magnoni et al. (2013); ²Eya et al. (2015); ³Gunnarsson et al. (2009); ⁴Kolditz et al. (2008).

(Biochrom Spectrophotometers/Fisher Scientific, Illkirch, France) spectrophotometer and their purity was assessed using OD₂₆₀/OD₂₈₀ ratios. Their integrity was also checked by electrophoresis on a 1.5% agarose gel with ethidium bromide.

Each sample of RNA was reverse transcribed with the qScript™ cDNA synthesis kit (Quanta BioSciences, VWR, Fontenay-sous-Bois, France) containing a reaction mix and reverse transcriptase. Complementary DNA was diluted 10-fold for PCR experiments and stored at -20°C.

Real-time RT-PCR was realised with a 7500 Fast Real-Time PCR system (Applied Biosystems/Thermo Fisher Scientific, Illkirch, France) as previously described in Pengam et al. (2020). Briefly, target genes were amplified and quantified by SYBR® Green incorporation (EurobioGreen® Mix qPCR 2x Lo-Rox; Eurobio Ingen, Courtaboeuf,

France) with specific primers (presented in Table 2). The cycling conditions consisted in a denaturing step at 95°C for 2 min, followed by 40–50 cycles of amplification (denaturation: 95°C for 5 s; annealing/extension: 60°C for 30 s). At the end of amplification, a melting curve programme was carried out. 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 normalised using 18S ribosomal RNA (rRNA) 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 target gene mRNA/18S rRNA and mRNA levels of each

experimental group were then expressed as fold change with respect to the untrained group, which was set at 1.

2.4 | Enzyme activities and oxidative stress marker

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

2.4.1 | Citrate synthase activity

For each tissue type, 50 mg of frozen muscle were homogenised with a Polytron homogeniser in an extraction buffer (100 mM Tris-HCl, pH 8.1, 4°C). Citrate synthase (CS) activity was determined as previously described in Pengam et al. (2020).

2.4.2 | Lactate dehydrogenase activity

Tissue samples, 70 mg of frozen RM and 150 mg of frozen WM, were homogenised with a Polytron in an extraction buffer (100 mM Tris, 2 mM EDTA and 2 mM dithioerythritol, pH 7.4, 4°C). The homogenate was centrifuged at 12,000 *g* for 20 min at 4°C. Lactate dehydrogenase (LDH) activity was determined on the resulting supernatant at 340 nm using 40 mM sodium pyruvate and 40 mM NADH. Activity was calculated based on oxidation of NADH and expressed in μmol oxidised NADH/min/mg protein.

2.4.3 | Antioxidant enzyme activities

For each tissue type, 200 mg of frozen muscles were homogenised in an extraction buffer (75 mM Tris and 5 mM EDTA, pH 7.4, 4°C) with a Polytron homogeniser. After 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 as previously described in Pengam et al. (2020). Catalase activity was not detectable in WM.

2.4.4 | Plasmatic oxidative stress marker

Total plasmatic 8-isoprostane (free and esterified in lipids) concentration was measured using an enzyme-linked immunosorbent assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's protocol. All samples were hydrolysed using 15% KOH and incubated for 60 min at 40°C and then neutralised with potassium phosphate buffer. A further purification step was then 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, plasmatic 8-isoprostane concentration was measured at 410 nm and expressed in pg/ml.

2.5 | Statistical analysis

All values are given as means with standard deviation (SD). Statistics were performed using Statistica v. 13 software (StatSoft, Paris, France). Normality of population distributions was tested using the Shapiro-Wilk test. The appropriate tests were then used to detect significant differences: one-way ANOVA or Kruskal-Wallis tests followed by a *post hoc* test (HSD) or a Mann-Whitney test, respectively. The significance threshold was set at $P < 0.05$.

Correlations were calculated and a principal component analysis (PCA) were performed using R software and the *FactoMineR* package. Correlation matrix coefficients were obtained with a Pearson test using the R *corrplot* package.

3 | RESULTS

3.1 | mRNA correlations and principal component analysis

Based on the mRNA data from the six training protocols, numerous mRNA levels of each subsystem (AMPK-PGC-1 α signalling pathway, mitochondrial functioning, antioxidant defences and LDH metabolism) were found to be correlated with one another in both RM and WM (Figure 1, left). The first two dimensions of the PCA accounted for more than 64% of the total variance in both muscles (Figure 1, right). Some differences were observed between RM and WM. Correlations were more numerous and higher between AMPK-PGC-1 α signalling pathway mRNAs and both mitochondrial functioning and antioxidant defence subsystems in RM than in WM. In WM, the correlations between mitochondrial functioning, antioxidant defences and LDH metabolism were more numerous and higher than in RM. In contrast to RM, *Pgc-1 α* and *Cs* were not correlated in WM.

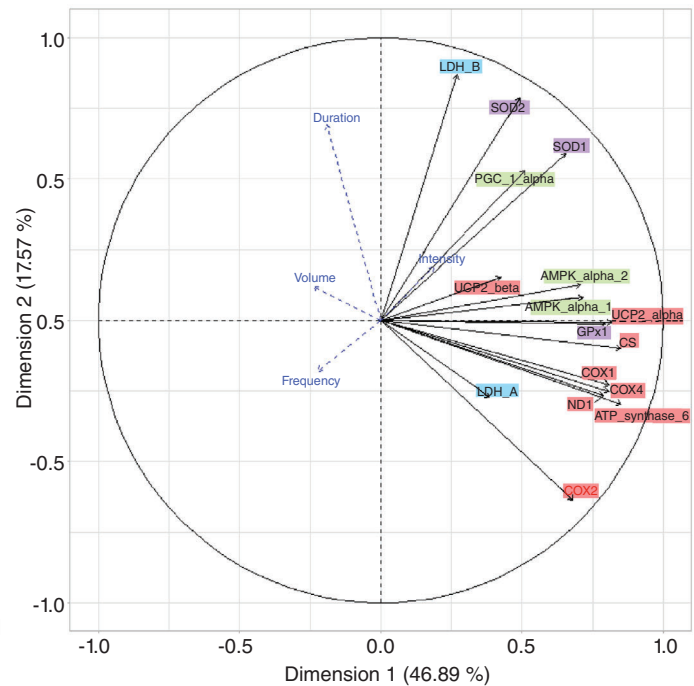
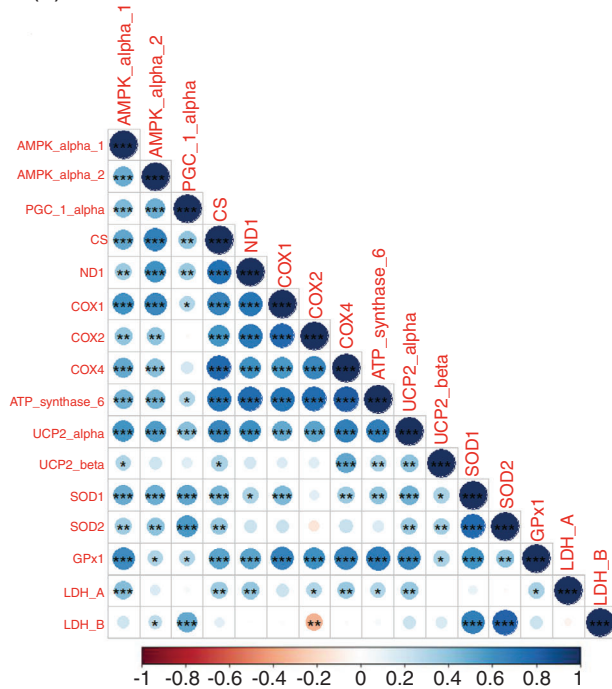
3.2 | Influence of training variables on mRNA levels, enzymatic activities and 8-isoprostane

To analyse the effects of exercise intensity and training volume, we present these results on the same figures (Figures 2–4). MICT (57% U_{crit}) and HIIT (90% U_{crit}) protocols were each pooled for statistical analysis of the exercise intensity effects.

3.2.1 | Exercise intensity

Overall, we observed that 90% U_{crit} tended to increase more transcript types in both muscles compared with 57% U_{crit} (Figures 2 and 3). In RM, 90% U_{crit} significantly increased *Ampk α 1*, *Pgc-1 α* , *Ucp2 α* and *Ldh-a* mRNA contents (Figure 2) and tended to increase SOD activity ($P = 0.10$; Figure 4a). In WM, 90% U_{crit} stimulated more *Pgc-1 α* , *Cs* ($P = 0.06$), *Nd1*, *Ucp2 α* and *Ldh-b* gene transcriptions compared with

(a) Red muscle



(b) White muscle

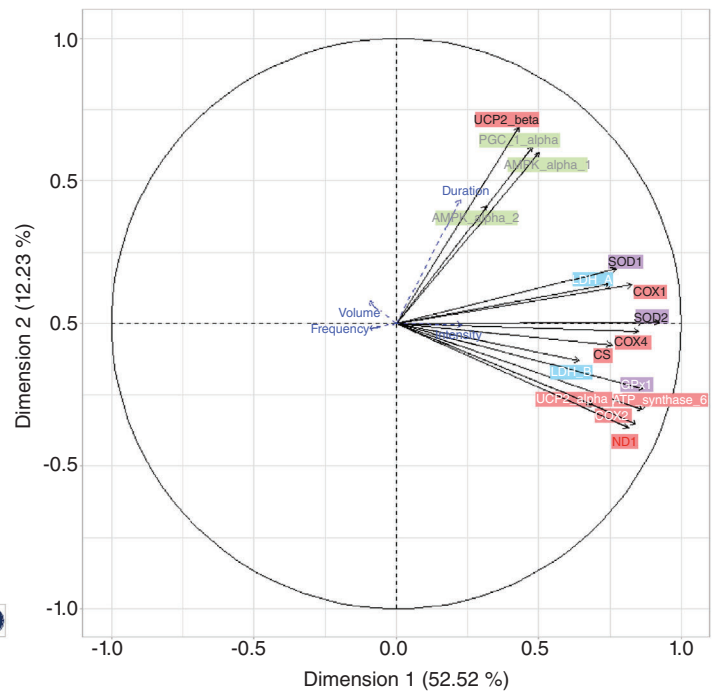
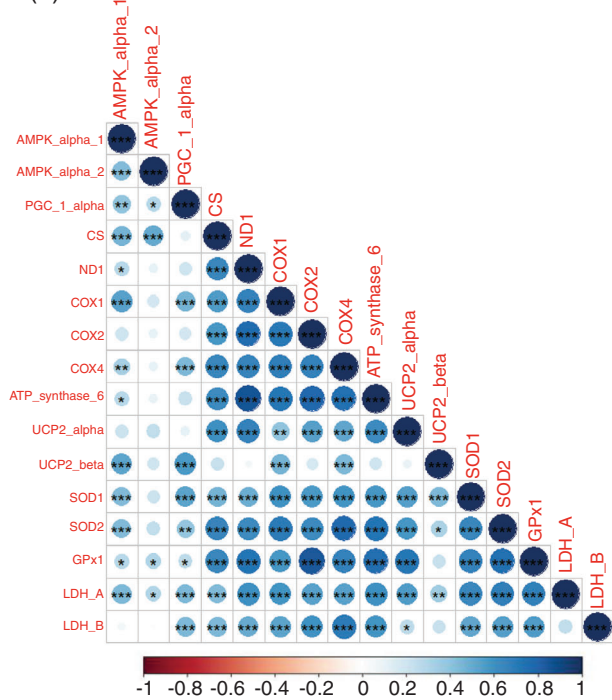


FIGURE 1 Correlations (left) and correlation circles of the first two dimensions of a multiple factor analysis (principal component analysis, PCA) (right) performed using mRNA levels of the AMPK-PGC-1 α signalling pathway (green), mitochondrial functioning (red), antioxidant defences (purple) and lactate dehydrogenase subunits (blue) in red ($n = 62$) (a) and white ($n = 63$) (b) muscles of the six experimental groups (Table 1). For the correlations, the colour scale indicates the degree of correlation; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. For the PCA, exercise intensity, volume, duration and frequency are projected as supplementary variables (in blue, dotted lines)

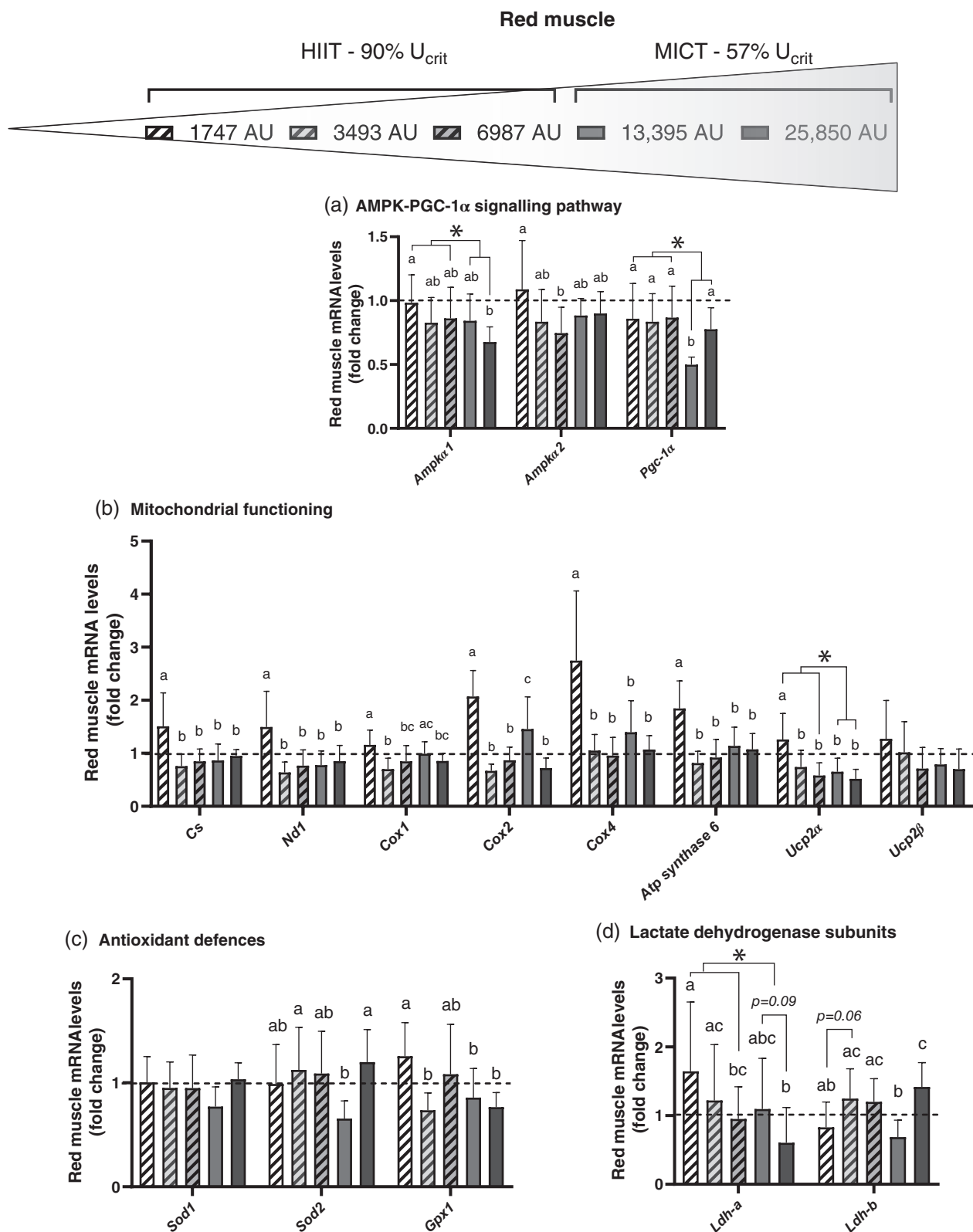


FIGURE 2 Effects of exercise intensity (57% U_{crit} , $n = 22$ and 90% U_{crit} , $n = 40$) and training volume (1747 AU, $n = 11$; 3493 AU, $n = 19$; 6987 AU, $n = 10$; 13,395 AU, $n = 12$ and 25,850 AU, $n = 10$) on mRNA levels of the AMPK-PGC-1 α signalling pathway (a), mitochondrial functioning (b), antioxidant defences (c) and lactate dehydrogenase subunits (d) in red muscle. Hatched bars represent high exercise intensity groups (HIIT, 90% U_{crit}) and non-hatched bars represent moderate exercise intensity groups (MICT, 57% U_{crit}). Results of each experimental group are expressed as fold change compared with the respective untrained group, which was set at 1. Results are shown as means with SD. Different letters indicate significant differences ($P < 0.05$) between experimental groups. *Significant differences between high and moderate exercise intensity groups

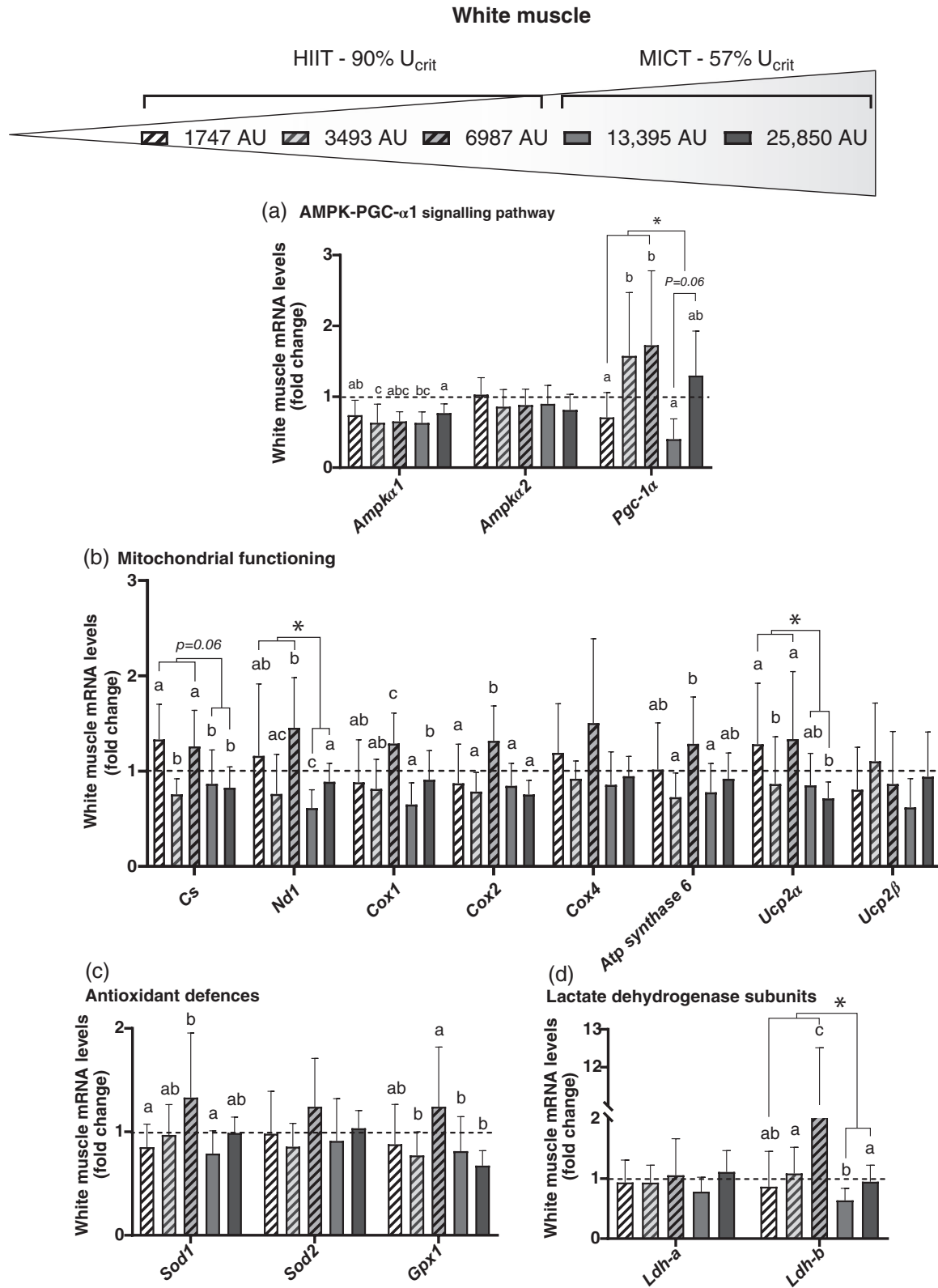


FIGURE 3 Effects of exercise intensity (57% U_{crit} , $n = 21$ and 90% U_{crit} , $n = 42$) and training volume (1747 AU, $n = 12$; 3493 AU, $n = 20$; 6987 AU, $n = 10$; 13,395 AU, $n = 11$ and 25,850 AU, $n = 10$) on the studied mRNA levels in white muscle. Data analysis and presentation details are the same as those given in Figure 2 caption

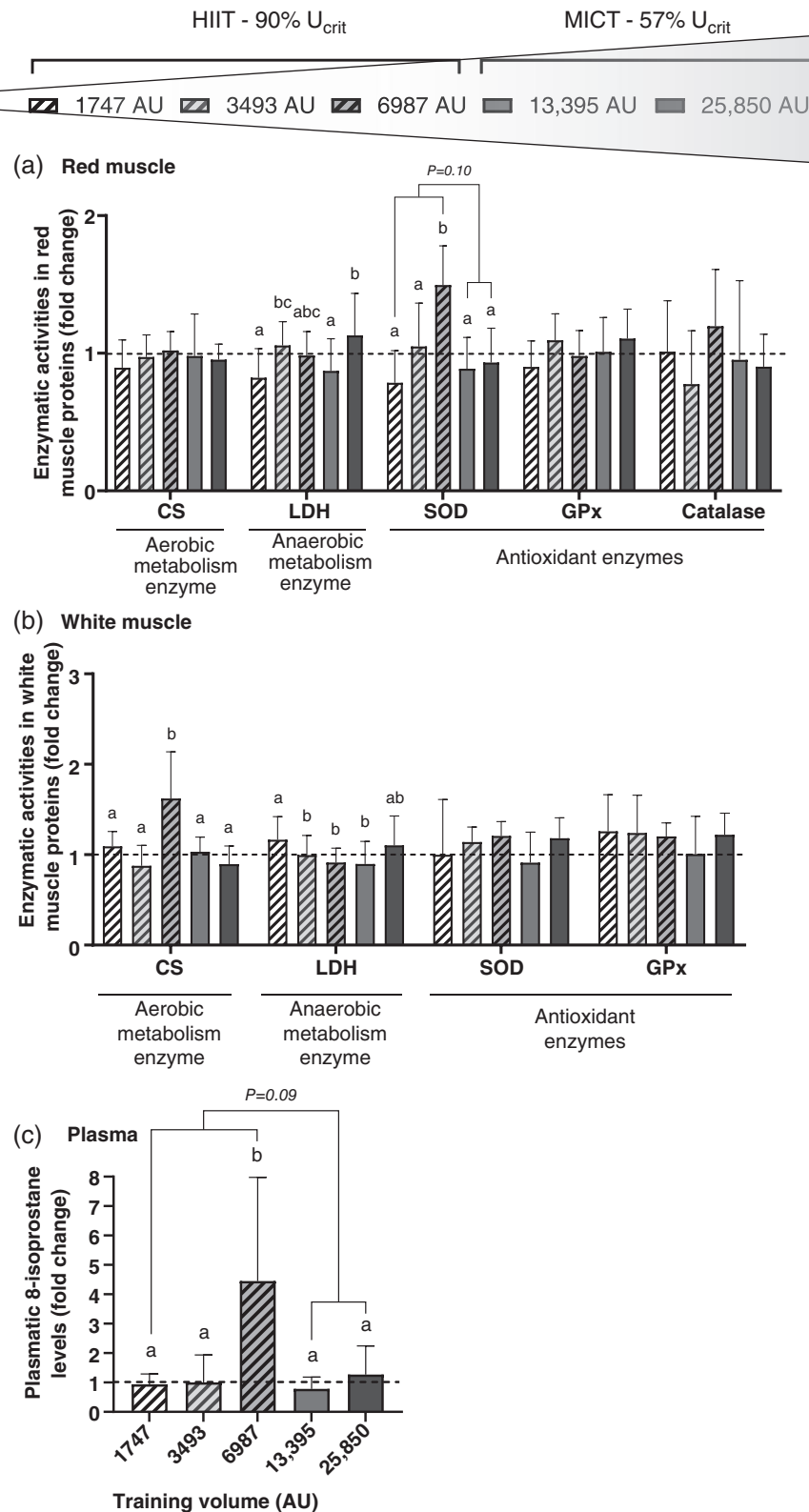


FIGURE 4 (a, b) Effects of exercise intensity (57% U_{crit} , $n = 22$ and 90% U_{crit} , $n = 42$) and training volume (1747 AU, $n = 11$; 3493 AU, $n = 19$; 6987 AU, $n = 10$; 13,395 AU, $n = 12$ and 25,850 AU, $n = 10$) on citrate synthase (CS), lactate dehydrogenase (LDH), superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase activities in red (a) and white (b) muscles. (c) Effects of exercise intensity (57% U_{crit} , $n = 22$ and 90% U_{crit} , $n = 38$) and training volume (1747 AU, $n = 11$; 3493 AU, $n = 18$; 6987 AU, $n = 9$; 13,395 AU, $n = 12$ and 25,850 AU, $n = 10$) on plasmatic 8-isoprostane levels. Data analysis and presentation details are the same as those given in Figure 2 caption

57% U_{crit} (Figure 3). Plasmatic 8-isoprostane concentration tended to increase at 90% U_{crit} ($P = 0.09$; Figure 4c).

3.2.2 | Training volume

For the analysis of training volume effects, we pooled the results from the protocols with the same training volume (HIIT1, 20 days and HIIT2, 10 days).

Overall, in RM, a low training volume upregulated mRNA levels (Figure 2). Indeed, the lowest training volume (1747 AU) stimulated *Ampk α* ($\alpha 1$ and $\alpha 2$), mitochondrial compounds (*Cs*, *Nd1*, *Cox 1*, 2 and 4, *Atp synthase 6* and *Ucp2 α*), *Gpx1* and *Ldh-a* mRNAs. The highest MICT training (25,850 AU) only increased *Pgc-1 α* and *Sod2* mRNA levels compared with the lowest MICT volume (13,395 AU). Concerning the enzyme activities, SOD increased significantly at the highest HIIT volume (6987 AU) compared with the other protocols. LDH activity was greater at the highest MICT volume compared with the lowest HIIT and MICT volumes (Figure 4a).

Overall, in WM, an intermediate training volume (6987 AU) stimulated the most mRNA levels (Figure 3). Indeed, the highest HIIT volume (6987 AU) largely stimulated more gene transcription (*Pgc-1 α* , mitochondrial compounds, *Sod1*, *Gpx1* and *Ldh-b*) than the other training protocols. Among the MICT protocols, the highest volume stimulated *Ampk α 1*, *Pgc-1 α* ($P = 0.06$), *Nd1*, *Cox1* and *Ldh-b* mRNA levels. CS activity was upregulated by the highest HIIT volume and LDH activity by the lowest HIIT volume (Figure 4b).

The highest volume of HIIT largely increased plasmatic 8-isoprostane concentration while MICT had no effect (Figure 4c).

3.2.3 | Training duration

For each training duration (10 and 20 days), HIIT and MICT results were pooled.

In RM, 10 days of training stimulated some of the mitochondrial functioning mRNAs (including *Nd1*, $P = 0.055$; *Cox2*, *Atp synthase 6* and *Ucp2 α*) and *Ldh-a* mRNA whereas 20 days led to greater upregulation of *Pgc-1 α* , *Sod1*, *Sod2* and *Ldh-b* mRNAs (Figure 5). In RM, 20 days of training stimulated LDH and SOD activities and tended to increase GPx activity ($P = 0.07$) with no change in WM (Figure 6a,b). In WM, 20 days of training increased numerous transcripts (including *Ampk α 1*, *Pgc-1 α* , *Cox1*, *Ucp2 β* , *Sod1*, *Sod2* and *Ldh-b*) (Figure 7). No significant training duration effects were shown for plasmatic 8-isoprostane levels (Figure 6c).

3.2.4 | HIIT frequency

In RM, *Ampk α 2*, *Cs*, *Cox 1*, 2 and 4, *Ucp2 α* , *Ucp2 β* and *Sod2* mRNA contents were upregulated by one session of HIIT (HIIT1) (Figure 8), whereas in WM, two sessions of HIIT (HIIT2) increased mRNA levels (*Nd1*, *Cox2*, *Atp synthase 6*, *Gpx1* and *Ldh-b*), except *Ampk α 1* and

Ucp2 β mRNAs, which were more stimulated by HIIT1 (Figure 9). HIIT2 stimulated CS and SOD activities more in both muscles, CAT activity in RM ($P = 0.08$) (Figure 10a, b) and also plasmatic 8-isoprostane (Figure 10c).

4 | DISCUSSION

This study explored the effects of six different training protocols, created by manipulating exercise variables (intensity, volume, duration and/or frequency), on molecular mechanisms related to mitochondrial biogenesis in slow and fast muscle fibres of rainbow trout. The primary goal of the present study was to determine the most relevant training variable to stimulate these events. Our main findings were: (i) WM tended to develop aerobic metabolism with a high training volume, confirmed by the mitochondria and LDH responses; (ii) gene transcriptions and enzymatic activities in slow and fast fibres and plasmatic 8-isoprostane concentrations were more stimulated by HIIT than by MICT; (iii) the lowest HIIT volume stimulated mRNA responses in RM whereas the highest HIIT volume stimulated them in WM; (iv) a longer training duration (20 days vs. 10 days) and a higher frequency (2×2 h/day vs. 1×2 h/day) promoted protein expression in trout muscles.

The present study focuses on the effects of training and not of acute exercise on skeletal muscle. So, the time point for muscle sampling after the last exercise is important to define. Recent studies sampled skeletal muscle 48–72 h after the last training session to avoid the acute effects of exercise on the muscle responses (MacInnis et al., 2017; Miyamoto-Mikami et al., 2018). In our study, we chose an intermediary time point of 65 h for tissue sampling with the risk of losing some early mRNA expression but to be sure to explore training effects.

It is a challenge to define a training protocol for humans that improves overall health and is adapted to today's rhythm of life (Gibala et al., 2012). While it is agreed that exercise is a powerful stimulus to improve the processes beneficial to health, such as mitochondrial biogenesis, the optimal training stimulus is still debated (Bishop et al., 2014). The dose responses of exercise variables (intensity, volume, duration and frequency) that maximise molecular and cellular responses are not well known (MacInnis & Gibala, 2017). Several studies demonstrated that high intensity training represents a very time-efficient mode of training to promote mitochondrial biogenesis (Gibala et al., 2012; Gillen et al., 2016; MacInnis et al., 2019), but further study is still needed to gain a better understanding of the cellular process involved in training-induced responses. Fish make relevant experimental animal models for exercise physiology studies because they can be easily prompted to swim against a water flow (Li et al., 2017). Fish have separated slow and fast muscle fibres (McClelland, 2012), which are differently recruited according to the training type as in mammals (Godin et al., 2010; Jayne & Lauder, 1994). Recent studies in fish have shown similarities with mammals in training-activated signalling pathways such as AMPK–PGC-1 α , one of the main pathways involved in cellular energetic state and mitochondrial biogenesis (Magnoni et al., 2014; Morash et al.,

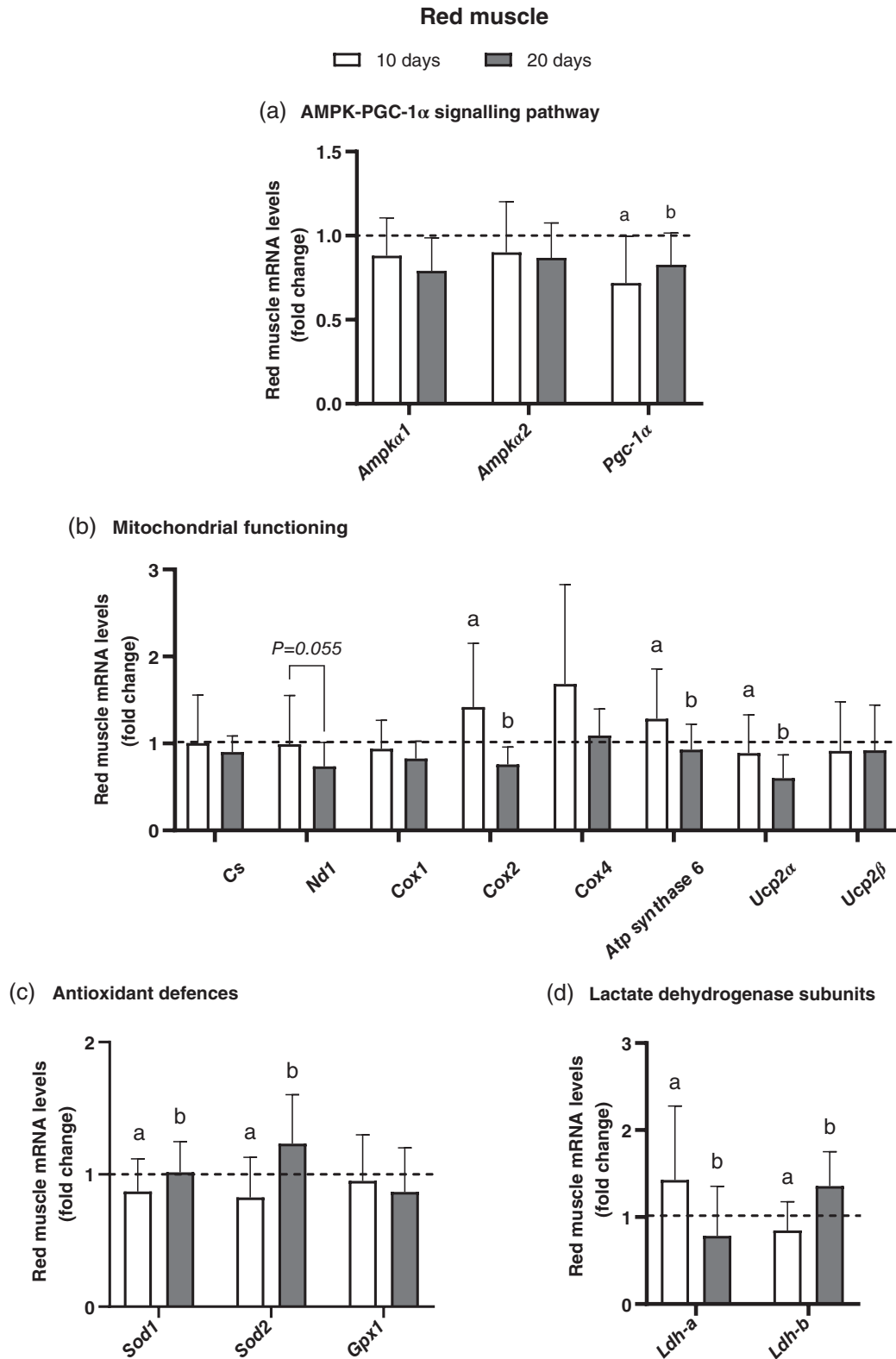


FIGURE 5 Effects of training duration (10 days, $n = 33$ and 20 days, $n = 29$) on mRNA levels of the AMPK-PGC-1 α signalling pathway (a), mitochondrial functioning (b), antioxidant defences (c) and lactate dehydrogenase subunits (d) in red muscle. For each training duration (10 and 20 days), HIIT and MICT results were pooled. Results of each experimental group are expressed in fold change compared with the respective untrained group, which was set at 1. Results are shown as means with SD. Different letters indicate significant differences ($P < 0.05$) between experimental groups

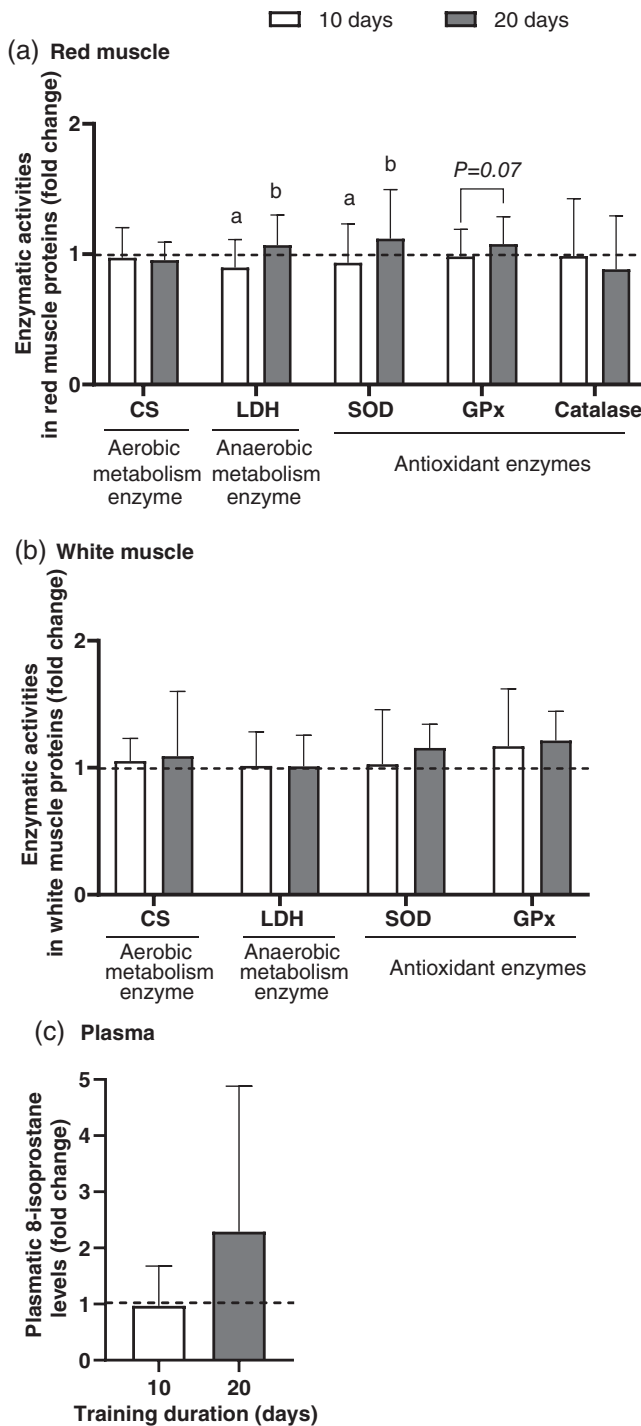


FIGURE 6 (a, b) Effects of training duration (10 days, $n = 34$ and 20 days, $n = 30$) on citrate synthase (CS), lactate dehydrogenase (LDH), superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase activities in red (a) and white (b) muscles. (c) Effects of training duration (10 days, $n = 32$ and 20 days, $n = 28$) on plasmatic 8-isoprostane levels. Data analysis and presentation details are the same as those given in Figure 5 caption

2014; Pengam et al., 2020; Trewin et al., 2018). Other signalling pathways stimulating mitochondrial biogenesis, such as p38 mitogen-activated protein kinase and the tumour suppressor p53, are also phylogenetically conserved in fish but studied to a lesser extent than in mammals (Magnoni et al., 2013).

In fish, AMPK-PGC-1 α signalling pathway activation can also be related to environmental stress responses such as hypoxia or anoxia (Craig et al., 2018). In order to verify the relationships between the four explored subsystems (AMPK-PGC-1 α signalling pathway, mitochondrial functioning, antioxidant defences and LDH metabolism) in a physical exercise adaptation context, we assessed the correlations between mRNA levels of these groups. Overall, numerous correlations between quantified mRNAs were demonstrated (Figure 1), suggesting that training adaptations involve these coordinated metabolic processes as in mammals. However, the most correlated subsystems were not strictly the same ones in RM and WM. The strongest and most numerous correlations in RM were established between *Ampk α* ($\alpha 1$ and $\alpha 2$) mRNAs and both mitochondrial functioning and antioxidant defence subsystems, whereas in WM they were found between mitochondrial functioning, antioxidant defences and LDH metabolism. These differences could be related to the specific metabolism of each muscle and to the difference in the recruitment of fibres according to exercise type (Kristensen et al., 2015). It is known that WM has few mitochondria and relies predominantly on anaerobic metabolism for energy production during high intensity exercise such as burst swimming. RM fibres, which have a high mitochondria content, are mainly involved in aerobic and sustained swimming (Tudorache et al., 2013). So, as in mammals, the AMPK-PGC-1 α signalling pathway should be related to mitochondria functioning (CS and oxidative phosphorylation (OXPHOS)) in the skeletal muscle but should be less evident in WM than in RM. Indeed, *Pgc-1 α* and *Cs* mRNAs were correlated in RM but not in WM. In contrast to mammals (Olesen et al., 2010), PGC-1 α did not play a direct role in CS regulation in zebrafish mixed muscles mainly composed of WM (LeMoine et al., 2010). It is possible that differences in training protocols might explain the differences in training responses between fish and mammals (McClelland et al., 2006). Moreover, it might be interesting to explore other co-transcription factors such as p53 that stimulate mitochondrial content, at least in mammals (Huertas et al., 2019).

To adjust the exercise intensity of the training protocols, the critical swimming speed (U_{crit}) was determined. This represents the indicative maximum aerobic performance of the fish and can thus be assimilated to maximal oxygen uptake ($\dot{V}O_{2max}$) in mammals (McClelland, 2012). In salmonids, like rainbow trout, aerobic metabolism, which recruits mainly oxidative fibres (RM), predominates in swimming up to 70–90% of the U_{crit} . Recruitment of glycolytic fibres (WM) is known to be initiated at swimming speeds between 80% U_{crit} (for most of the studies, Hvas & Oppedal, 2017; Jones, 1982) and 86% U_{crit} (Beddow & McKinley, 1999). So, we chose an exercise intensity of 90% U_{crit} for HIIT in order to increase the probability to recruit not only oxidative fibres but also glycolytic fibres.

High exercise intensity upregulated *Ampk α 1*, *Pgc-1 α* , *Ucp2 α* and *Ldh*-a mRNAs in RM (Figure 2) and *Pgc-1 α* , *Cs* ($P = 0.06$), *Nd1*, *Ucp2 α* and

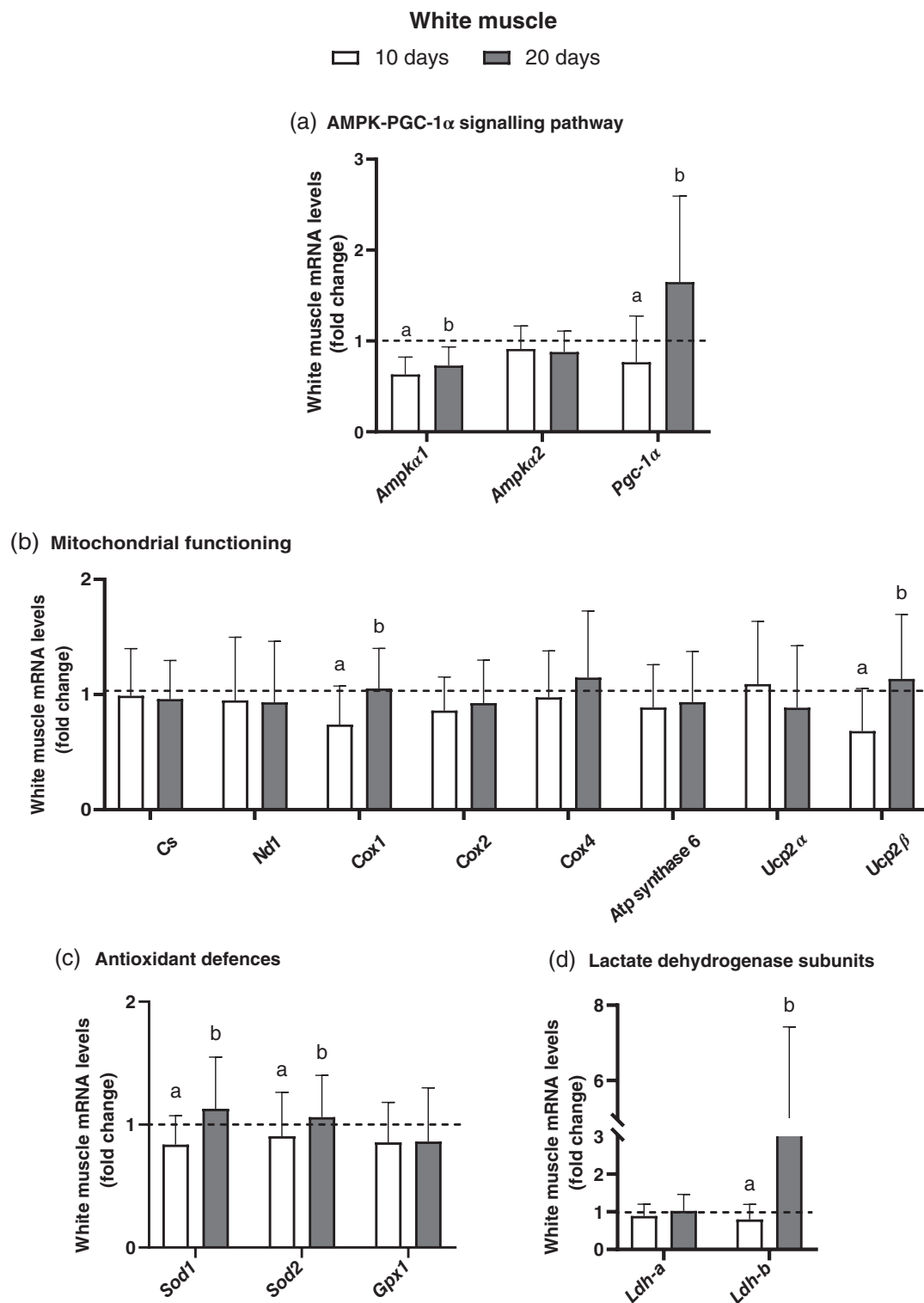


FIGURE 7 Effects of training duration (10 days, $n = 33$ and 20 days, $n = 30$) on studied mRNA levels in white muscle. Data analysis and presentation details are the same as those given in Figure 5 caption

Ldh-b mRNAs in WM (Figure 3). In parallel, RM SOD enzymatic activity ($P = 0.10$) and plasmatic 8-isoprostane levels ($P = 0.09$) (Figure 4a,c) tended to increase. In trout muscles, whatever the exercise protocol, it is clear that high exercise intensity (90% U_{crit}) stimulates more gene transcriptions than moderate exercise intensity (57% U_{crit}), but not

protein expression. PCA also indicated that exercise intensity was the factor most correlated with mRNAs in both muscles (Figure 1). It is known that mitochondrial biogenesis could result from the cumulative effects of transient increases in mRNA transcripts encoding mitochondrial proteins after successive exercise sessions (Perry et al.,

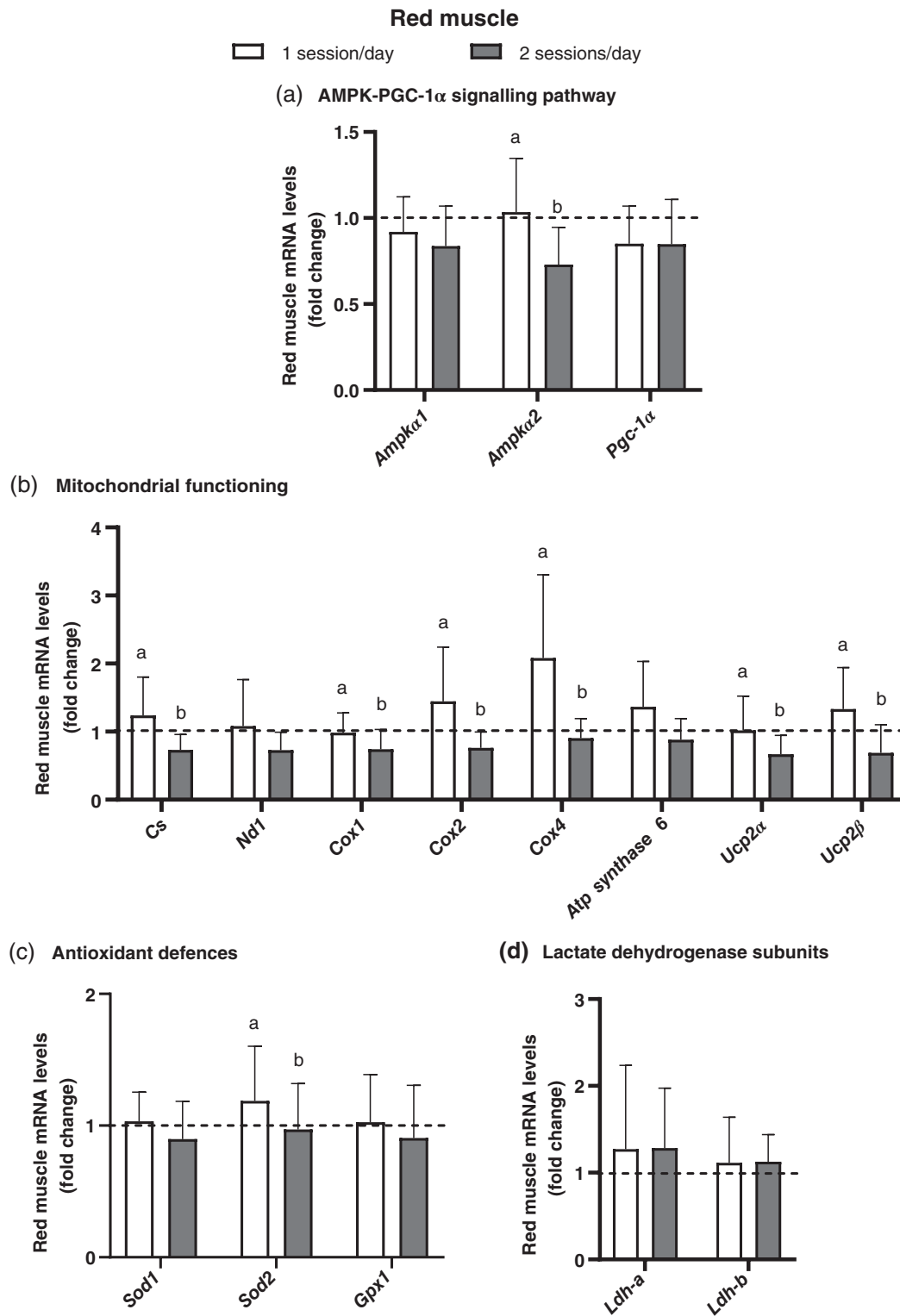


FIGURE 8 Effects of HIIT frequency (1 session/day, $n = 20$ and 2 sessions/day, $n = 20$) on mRNA levels of the AMPK-PGC-1 α signalling pathway (a), mitochondrial functioning (b), antioxidant defences (c) and lactate dehydrogenase subunits (d) in red muscle. Results of each experimental group are expressed as fold change compared with the respective untrained group, which was set at 1. Results are shown as means with SD. Different letters indicate significant differences ($P < 0.05$) between experimental groups

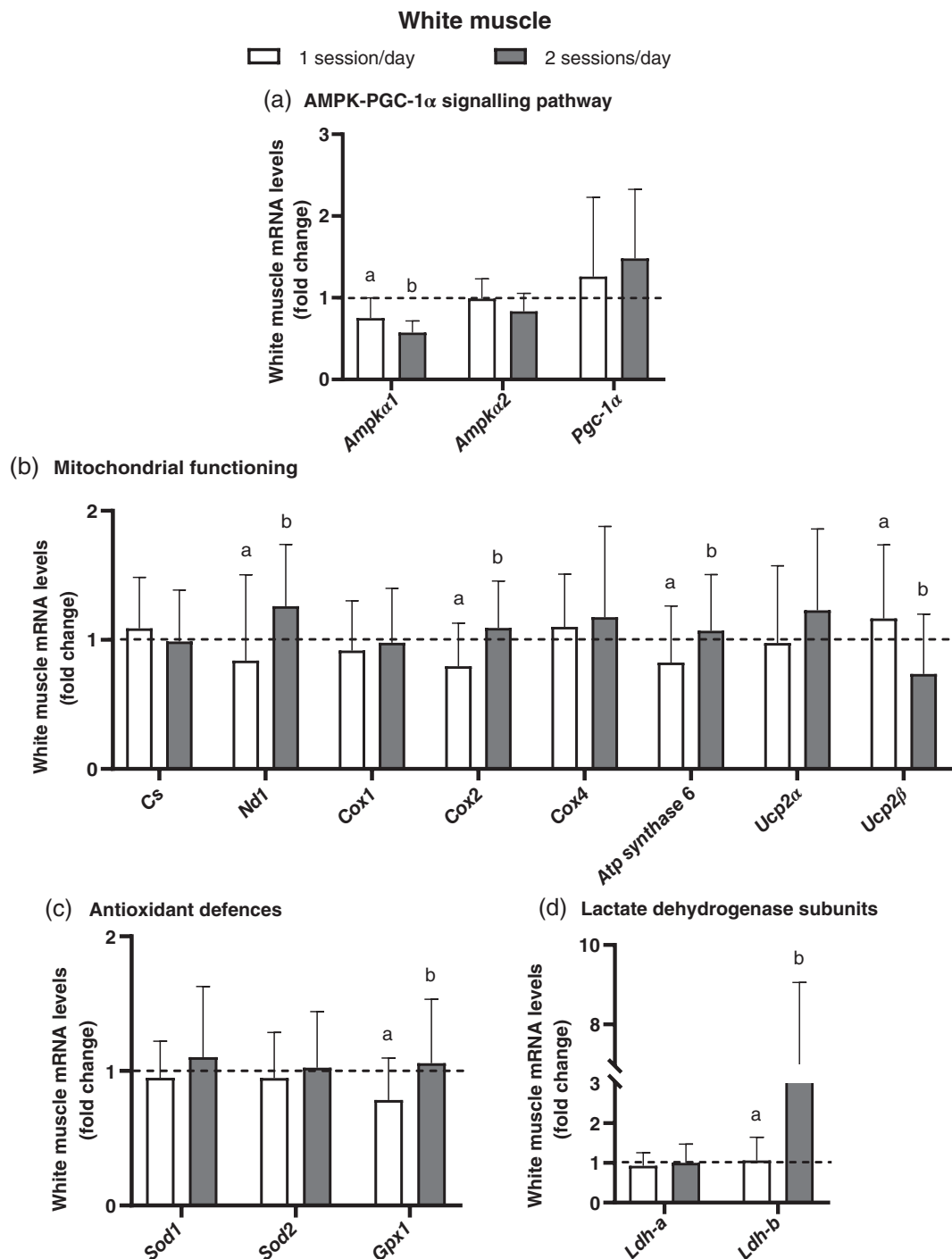


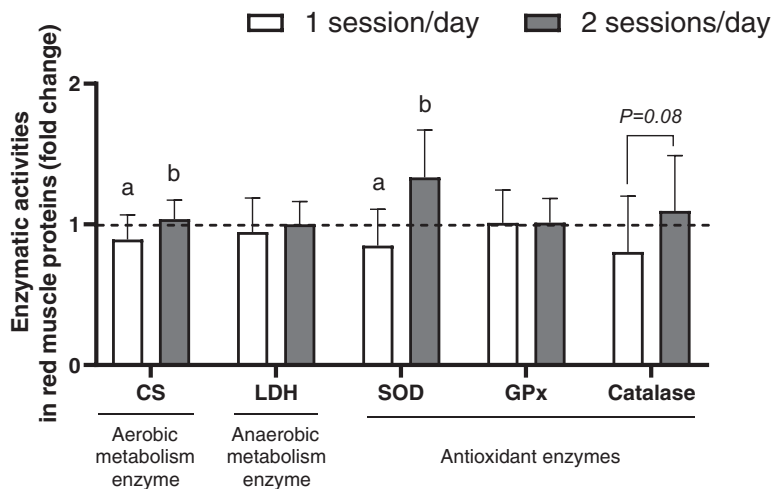
FIGURE 9 Effects of HIIT frequency (1 session/day, $n = 22$ and 2 sessions/day, $n = 20$) on studied mRNA levels in white muscle. Data analysis and presentation details are the same as those given in Figure 8 caption

2010). Our results show that numerous muscle mRNA responses were amplified following HIIT.

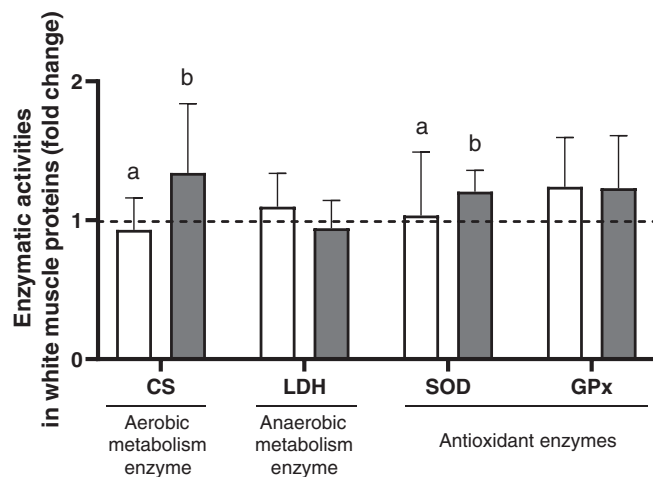
The present data obtained from six training protocols confirm our previous results showing that the signalling pathway cascade involving AMPK-PGC-1 α , OXPHOS complexes and antioxidant enzymatic systems is more stimulated by high exercise intensity than by moderate exercise intensity in trout muscles (Pengam et al., 2020). Only a few studies have compared different exercise intensities in

fish. Among these, Castro et al. (2011) showed that increased exercise intensity improved disease resistance in Atlantic salmon through the stimulation of the immune response, antioxidant defences and cardiovascular function. In mammals, it has been recently shown that high exercise intensity stimulated mitochondrial biogenesis more than moderate exercise intensity through the increase of *Pgc-1 α* mRNA content (MacInnis et al., 2019) and through the stimulation of mitochondrial content and aerobic capacities (Laursen et al., 2007;

(a) Red muscle



(b) White muscle



(c) Plasma

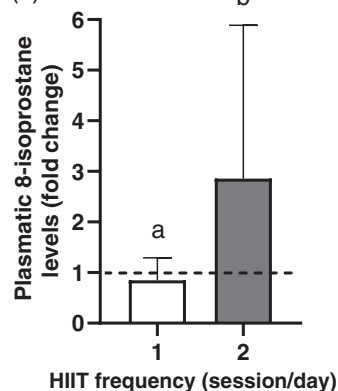


FIGURE 10 (a, b) Effects of HIIT frequency (1 session/day, $n = 22$ and 2 sessions/day, $n = 20$) on citrate synthase (CS), lactate dehydrogenase (LDH), superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase activities in red (a) and white (b) muscles. (c) Effects of HIIT frequency (1 session/day, $n = 20$ and 2 sessions/day, $n = 18$) on plasmatic 8-isoprostane levels. Data analysis and presentation details are the same as those given in Figure 8 caption

MacInnis & Gibala, 2017; MacInnis et al., 2017). In mammals, as in fish, the biochemical mechanisms involved for mitochondrial biogenesis in muscle seem to be mediated by a high intensity of exercise.

If minimal exercise intensity is likely required to improve mitochondrial biogenesis (Bishop et al., 2019), training volume may also be a key determinant of training-induced changes in mitochondrial content (Granata et al., 2018). Five training volumes

differing in their intensity, duration and frequency were tested here, consisting more precisely of three HIIT volumes ($<10,000$ AU) and two MICT volumes ($>10,000$ AU). For volumes $<10,000$ AU, the lowest volume induced the most mRNA responses in RM whereas the highest volume had the greatest influence in WM. For volumes $>10,000$ AU, the highest volume stimulated the most mRNA responses in both muscles.

In RM, considering <10,000 AU, the lowest volume (1747 AU) stimulated *Ampk α 2* mRNA and OXPHOS transcripts (*Nd1*; *Cox 1*, 2 and 4; and *Atp synthase 6*) (Figure 2a,b). *Cs* mRNA was increased by the lowest training volume but its enzymatic activity was not (Figures 2b and 3a). So, aerobic metabolism seems to be initiated at the transcriptional level, with a low training volume in RM, but is not yet reflected at the protein level. Inversely, WM needs the highest volume (6987 AU) to stimulate *Pgc-1 α* , *Cs* and OXPHOS mRNAs (*Nd1*, *Cox1*, *Cox2* and *Atp synthase 6*) (Figure 3a,b). In contrast to RM, CS activity increased in WM with high training volume (Figure 4b). In zebrafish, McClelland et al. (2006) also showed that a high volume of HIIT stimulated mRNA and CS activity in mixed muscles mainly composed of WM. Considering only volumes >10,000 AU, the highest volume (25,850 AU) increased *Pgc-1 α* in both muscles more than the lowest volume (13,395 AU) (Figures 2a and 3a) and increased *Nd1* and *Cox1* mRNAs in WM (Figures 3b). As shown in human (Fiorenza et al., 2018), high intensity matched with high training volume seems to be required to stimulate mitochondrial biogenesis in fish skeletal muscle.

The development of a more aerobic phenotype in RM and WM with low and high HIIT volumes, respectively, and in both muscles with a high MICT volume is confirmed by the modifications of LDH metabolism. LDH is a tetrameric enzyme composed of the subunits A and/or B, which have distinct functions: the LDH-A isoenzyme transforms pyruvate to lactate in tissues dependent on anaerobic glycolysis and LDH-B permits lactate oxidation in tissues dependent on aerobic metabolism, such as cardiac muscle (Lim et al., 1975; Wieser et al., 1987). In RM, high HIIT and MICT volumes concurrently increased *Ldh-b* mRNA content and LDH activity (transformation pyruvate into lactate), while *Ldh-a* mRNA content decreased (Figures 2d and 4a). It is known that lactate is continuously produced under aerobic conditions, particularly when rates of glycogenolysis and glycolysis are high, as during HIIT (Horii et al., 2017). Thus, in our conditions of high HIIT and MICT volumes, the lactate production was elevated in RM and lactate was more oxidised, supposed by the *Ldh-a* and *Ldh-b* mRNA levels. In WM, *Ldh-b* mRNA increased with high HIIT and MICT volumes (Figure 3d) and LDH activity decreased with high HIIT volume (Figure 4b) suggesting a lower anaerobic capacity. Our results are coherent with those of He et al. (2013), which showed that a training of 4 weeks at moderate intensity tended to increase LDH activity in juvenile common carp RM whereas it tended to decrease it in WM. Together, the overall results detailed above indicate a fibre-dependent volume response of LDH metabolism and suggest that WM tends to develop an aerobic phenotype with a high training volume.

During physical exercise, ROS production is necessary for numerous cellular adaptive events, but can lead to oxidative stress at high concentrations (He et al., 2016; Ljubicic et al., 2010). Among the antioxidant processes involved in the adaptations to exercise, cytosolic and/or mitochondrial antioxidant enzymes such as SOD, GPx and CAT are frequently reported, but also uncoupling proteins (UCPs), which are encased in the inner mitochondrial membrane. By dissipating the proton electrochemical gradient, UCPs would have an indirect antioxidant role by reducing mitochondrial ROS production because of more rapid electron transport throughout the mitochondrial electron transport chain (Ji & Zhang, 2014). In RM the highest HIIT volume

stimulated SOD activity (Figure 4a) while in WM it increased *Sod1*, *Gpx1* and *Ucp2 α* mRNA levels (Figure 3b,c) with no change in enzymatic activities (Figure 4b). The highest level of oxidative stress (quantified by plasmatic 8-isoprostane) was observed with the highest HIIT volume (Figure 4c). In fish, mitochondrial content is ~10-fold greater in RM than in WM (Leary et al., 2003). As mitochondria are often considered to be the main source of ROS production (Powers & Jackson, 2008; Westermann, 2010), they are probably produced to a lesser extent in WM. We can cautiously suppose that the high ROS production during cumulative sessions of exercise induced antioxidant adaptations to prevent deleterious effects of oxidative stress (Steinbacher & Eckl, 2015). Our findings suggest that high HIIT volume increases antioxidant defences in fish muscles.

The training volume also depends on duration and/or frequency of exercise. In RM, when training duration increased, mitochondrial functioning mRNAs (*Nd1*, $P = 0.055$; *Cox2*, *Atp synthase 6* and *Ucp2 α*) and *Ldh-a* mRNAs were down-regulated whereas *Pgc-1 α* and *Ldh-b* mRNAs (Figure 5a, b, d) and LDH activity (Figure 6a) were stimulated. In WM, the longer training duration (20 days) initiated aerobic adaptations through the stimulation of *Ampk α 1*, *Pgc-1 α* , *Cox1*, *Ucp2 β* and *Ldh-b* mRNAs (Figure 7a, b, d). So, aerobic metabolism was initiated in both muscles but earlier in RM than in WM. Davie et al. (1986) also reported that increased training duration promoted aerobic capacities, particularly in the trout WM, through the shift of isoform from LDH-A to LDH-B.

After 20 days of training, antioxidant defences were stimulated, as confirmed by the rise of *Sod1* and *Sod2* mRNAs in both muscles (Figures 5c and 6c) and the stimulation of SOD and GPx ($P = 0.07$) activities in RM (Figure 6a). Supported by the duration effect on plasmatic 8-isoprostane level (Figure 6c), we cautiously hypothesise that antioxidant defences could become efficient after more than 10 days of training.

While one session of HIIT (HIIT1) was sufficient to stimulate most of the mRNA levels in RM (Figure 8), two sessions of HIIT (HIIT2) were required for transcriptional adaptations in WM (Figure 9). Mitochondrial biogenesis seemed to be promoted by HIIT2 through CS activity stimulation in both muscles (Figures 10a, b). After HIIT2, the activation of oxidative processes was also confirmed by the rise of plasmatic 8-isoprostane concentration (Figure 10c), the concomitant increase in SOD activity in both muscles and the upward trend of CAT activity in RM ($P = 0.08$) after HIIT2 (Figure 10a, b). In mammals, more numerous and repeated HIIT sessions influence mitochondrial content and cardiometabolic health-related adaptations (increase of $\dot{V}_{O_{2max}}$ and decrease of total body fat) (Ghiarone et al., 2019; Stavrinou et al., 2018). Based on these few data, it would seem that, as in mammals, fish aerobic adaptations including mitochondrial biogenesis could be more stimulated when exercise frequency increases.

5 | CONCLUSION

The present paper investigates the effects of six training protocols on the AMPK-PGC-1 α signalling pathway, mitochondrial compounds, redox state and LDH metabolism molecular mechanisms in trout

oxidative and glycolytic muscles. Exercise intensity appears to be the most relevant training variable to stimulate the studied signalling pathway and its related compounds in both muscles. For a given exercise intensity, the training volume seems to play an important role in the cascade of responses involved in aerobic metabolism with fibre type-specific responses. Indeed, glycolytic fibres need a greater training volume than oxidative fibres for molecular adaptations. Moreover, adaptations seem to occur earlier in RM than in WM. It is important to note that the total muscular mass of rainbow trout is composed of ~4% RM and ~96% WM (Stickland, 1983). Considering the total muscle mass, therefore, just a few WM adaptations could have an important impact on the fish's physiology. In WM, training that combines high-intensity and high-volume could initiate a possible metabolic shift from anaerobic to aerobic metabolism. Data collected on fish skeletal muscles are coherent with those obtained in mammals, which confirms that rainbow trout is a relevant model to study physical exercise physiology. This study provides new insights regarding mitochondrial adaptations induced by different training stimuli and could contribute to the improvement of exercise programme design. In a future study, it would be interesting to explore training protocols with the same exercise volume but combined with different exercise intensities.

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COMPETING INTERESTS

The authors have no conflicts of interest to declare.


AUTHOR CONTRIBUTIONS

M.P., C.M., B.S. and A.A. designed the experiment. M.P., B.S. and A.A. collected the fish samples (muscles and plasma) after training. M.P., A.G. and M.I. completed all the biochemical and molecular analyses. M.P., C.M., B.S. and A.A. wrote the initial draft of the manuscript and all authors contributed, reviewed and approved the final version of the submitted manuscript. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

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