

**SHORT COMMUNICATION**

# Fueling the engine: induction of AMP-activated protein kinase in trout skeletal muscle by swimming

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**ABSTRACT**

AMP-activated protein kinase (AMPK) is well known to be induced by exercise and to mediate important metabolic changes in the skeletal muscle of mammals. Despite the physiological importance of exercise as a modulator of energy use by locomotory muscle, the regulation of this enzyme by swimming has not been investigated in fish. We found that sustained swimming (40 days at 0.75 body lengths  $s^{-1}$ ) increased AMPK activity in red and white trout skeletal muscle (3.9- and 2.2-fold, respectively) as well as the expression of AMPK target genes involved in energy use: lipoprotein lipase and citrate synthase in red and white muscle and CPT1 $\beta$ 1b and PGC-1 $\alpha$  in red muscle. Furthermore, electrical pulse stimulation of cultured trout myotubes increased AMPK activity and glucose uptake (1.9- and 1.2-fold, respectively) in an AMPK-dependent manner. These results suggest that AMPK may play an important mediatory role in the metabolic adaptation to swimming in fish skeletal muscle.

**KEY WORDS:** AMPK, Skeletal muscle, Swimming, Fish

**INTRODUCTION**

AMP-activated protein kinase (AMPK) is a phylogenetically conserved enzyme that acts as a ‘metabolic master switch’ mediating the cellular adaptation to environmental or nutritional stress factors. This fuel-sensing enzyme is activated by phosphorylation when a cellular stress increases the AMP/ATP ratio, such as by exercise in skeletal muscle (Jørgensen et al., 2006). AMPK activation leads to a concomitant inhibition of energy-consuming biosynthetic pathways not acutely required for survival and the activation of metabolic pathways that regenerate the ATP.

It is well known that lipids, carbohydrates and proteins constitute possible sources of metabolic energy that can be mobilized and utilized in animals. In fish, the contractile skeletal muscle requires larger amounts of oxidative substrates during swimming than while at rest and, depending on the duration and intensity of the activity, lipids and carbohydrates can be the main source of energy. Given that exercise-induced activation of AMPK in skeletal muscle increases glucose and lipid metabolism in mammals (Hardie and Sakamoto, 2006), we hypothesized that AMPK could play a similar

role in fish. In this regard, we have recently shown that AMPK is present in trout skeletal muscle and that AMPK can be pharmacologically activated in cultured trout skeletal muscle cells, resulting in an increase in glucose uptake and utilization (Magnoni et al., 2012). Despite the importance of AMPK in the regulation of energy use in fish skeletal muscle, as in mammals, the regulation of the activity of this enzyme by swimming-induced contractile activity has not been described in fish to date. Elucidating whether AMPK activity in the fish skeletal muscle is activated by swimming is important for our understanding of how energy balance is maintained during swimming in fish. Therefore, the present study was undertaken to understand the effects of sustained swimming on the activation of AMPK in locomotory muscles of fish and the changes that may be associated with the use of energy by this tissue by looking specifically into the transcriptional regulation of key genes related to the provision and use of energy in red and white muscle. To further characterize these observed metabolic effects, we investigated changes in AMPK activity and glucose uptake in trout myotubes in response to electrical stimulation.

**RESULTS AND DISCUSSION**

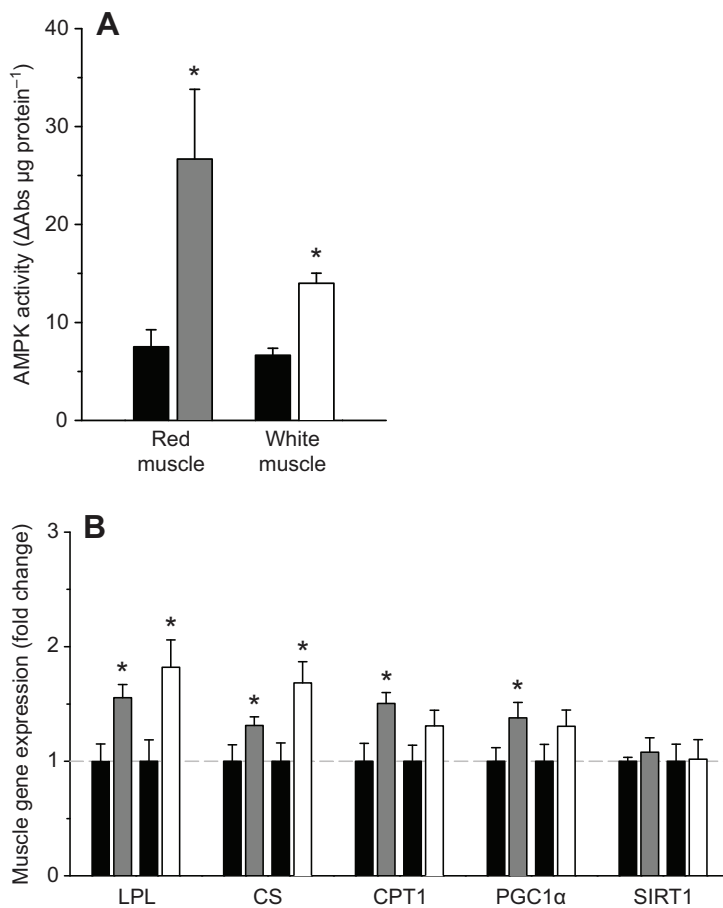
In mammals, it has been extensively documented that AMPK is activated in skeletal muscle during exercise and after electrical pulse stimulation (EPS)-evoked contraction of skeletal muscle cells in culture (Hardie and Sakamoto, 2006; Nedachi et al., 2008). AMPK activation in skeletal muscle has several metabolic consequences, including the transcriptional activation of genes involved in catabolic processes linked to ATP generation, such as lipid oxidation and glycolysis, which may in turn assist replenishment of cellular levels (Hardie and Sakamoto, 2006). In the present study, we investigated whether AMPK can be enzymatically activated in trout skeletal muscle by swimming-induced activity and show that AMPK activity was significantly increased by swimming in red and white muscle (3.9- and 2.2-fold, respectively; Fig. 1A). Basal AMPK activity levels did not differ significantly between red and white muscle in the control resting group. Given the known metabolic effects of AMPK activation in skeletal muscle in mammals (Hardie and Sakamoto, 2006) and fish (Magnoni et al., 2012), we next investigated the expression of AMPK target genes involved in the regulation of glucose and lipid catabolism in skeletal muscle. The mRNA levels of lipoprotein lipase (LPL) were augmented by swimming in red and white skeletal muscle, reinforcing its pivotal role in lipid mobilization and use by these tissues (Fig. 1B). The mRNA levels of carnitine palmitoyltransferase 1 $\beta$ 1b (CPT1 $\beta$ 1b), one of the five known isoforms of CPT1 in trout, which controls the entry of fatty acids into the mitochondria for their oxidation, were also increased by swimming in trout skeletal muscle, but only significantly in red muscle. Similarly, the mRNA levels of citrate synthase (CS), an enzyme controlling one of the flux-determining steps of the tricarboxylic acid cycle, were upregulated by swimming in both red and white muscle of trout.

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**Fig. 1. Effects of sustained swimming on AMPK activity and expression of AMPK target genes in red and white skeletal muscle in trout.** (A) Effects of sustained swimming on AMPK activity in trout skeletal muscle. AMPK activity levels in the control resting group are shown by the black bars and those from red and white muscle from trout subjected to swimming are shown by the shaded and white bars, respectively. AMPK activity in homogenates of red and white muscle was determined as described in the Materials and methods and the results are expressed as the difference ( $\Delta$ Abs) between the absorbance measured in the absence or presence of Compound C ( $10 \mu\text{mol l}^{-1}$ ), normalized to the amount of protein in the muscle extract. Data are means  $\pm$  s.e.m. of 10 fish, each measured in duplicate. (B) Effects of sustained swimming on the expression of AMPK target genes in skeletal muscle. The mRNA expression levels of lipoprotein lipase (LPL), citrate synthase (CS), carnitine palmitoyltransferase 1  $\beta$ 1b (CPT1 $\beta$ 1b), peroxisome proliferator-activated receptor- $\gamma$  coactivator (PGC-1 $\alpha$ ) and NAD<sup>+</sup>-dependent protein deacetylase sirtuin-1 (SIRT1) in red (shaded bars) and white (white bars) muscle of trout subjected to sustained swimming. Total red and white muscle RNA was isolated, reverse transcribed to cDNA and mRNA expression levels were determined by qPCR as described in the Materials and methods. Results are expressed as fold stimulation above the control resting group (black bars), which was set to 1, and are means  $\pm$  s.e.m. of 10 fish, each performed in triplicate. Asterisks indicate significant differences from control (\* $P < 0.05$ ).

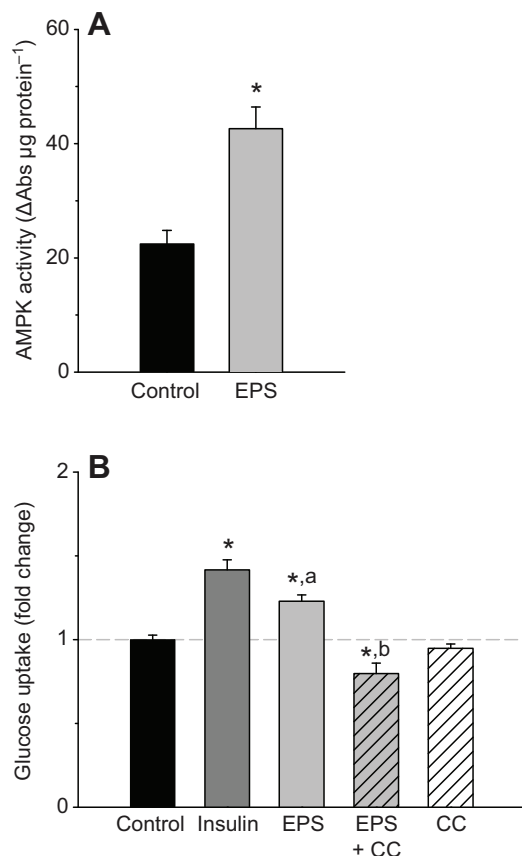
Furthermore, the mRNA levels of peroxisome proliferator-activated receptor- $\gamma$  coactivator (PGC-1 $\alpha$ ), an important transcriptional regulator of genes involved in energy metabolism and mitochondrial biogenesis in mammals, were also upregulated in red muscle of swimming trout. Therefore, the swimming-induced expression of genes involved in the tricarboxylic acid cycle (CS) as well as in lipid catabolism (LPL, CPT1 $\beta$ 1b) stresses the importance of lipids as metabolic fuels of preference in the locomotory muscle of trout. These findings are in accordance with the reported prominent role of lipids fueling the locomotory muscle of trout during sustained swimming (Magnoni and Weber, 2007), and support the notion that contractile activity in skeletal muscle may stimulate fatty acid metabolism through  $\beta$ -oxidation. An induction of the expression of PGC-1 $\alpha$  by swimming in skeletal muscle has also been shown in zebrafish (LeMoine et al., 2010); however, it has been suggested that PGC-1 $\alpha$  may not be a master regulator of mitochondrial biogenesis in fish, as in mammals. Nevertheless, based on the results presented here, we hypothesize that the increase in the mRNA expression levels of CS, LPL, CPT1 $\beta$ 1b and PGC1- $\alpha$  may occur as a result of the induction of AMPK activity by swimming in trout skeletal muscle. This idea is supported by data showing that pharmacological activation of AMPK increases the mRNA expression levels of CS and PGC1- $\alpha$  in trout myotubes (Magnoni et al., 2012) and by the reported correlation between AMPK activity and PGC-1 $\alpha$  content in the skeletal muscle of flounder (Fuentes et al., 2013).

In mammals, another member of the signaling cascade initiated by AMPK activation by exercise is the NAD<sup>+</sup>-dependent protein deacetylase sirtuin-1 (SIRT1), involved in the transcriptional regulation by deacetylation of nuclear proteins including PGC-1 $\alpha$

(Cantó et al., 2009). Our results show that the mRNA levels of SIRT1 did not change in red or white muscle of swimming trout when sampled after 40 days of swimming. Further research is needed to clarify the possible earlier involvement of SIRT1 in the metabolic response of skeletal muscle to swimming in fish.

To provide further evidence on the activation of AMPK by contractile activity in trout skeletal muscle cells, we show here that application of EPS, an *in vitro* model frequently utilized to simulate the effects of exercise in mammalian myotubes (Nedachi et al., 2008), significantly increased AMPK activity (1.9-fold) in trout myotubes (Fig. 2A). In light of the observation that pharmacological activation of AMPK in trout myotubes stimulates glucose uptake (Magnoni et al., 2012), we also show that EPS significantly increased glucose uptake (>1.2-fold) in trout myotubes, as was shown in mammalian myotubes (Nedachi et al., 2008). Interestingly, Compound C, a broadly used AMPK inhibitor, completely blocked the stimulatory effects of EPS on glucose uptake in trout myotubes (Fig. 2B). This observation, coupled with data showing that EPS stimulates AMPK activity (present study) and that pharmacological activation of endogenous AMPK stimulates glucose uptake in trout myotubes (Magnoni et al., 2012), strongly suggests that the stimulatory effects of EPS on glucose uptake in trout myotubes may be mediated by AMPK.

In summary, in the present study we provide data showing, for the first time in fish, that AMPK activity in skeletal muscle is increased by sustained swimming in trout and by the application of EPS on cultured trout myotubes. Our results indicate that AMPK may be an important metabolic sensor in fish skeletal muscle, particularly under conditions of increased energy use such as those elicited by swimming or electrical stimulation of skeletal muscle cells.



**Fig. 2. Effects of electrical pulse stimulation (EPS) on AMPK activity and AMPK-mediated glucose uptake in trout myotubes in culture.** (A) Effects of EPS on AMPK activity. (B) Effects of EPS on glucose uptake. Cells were electrically stimulated and AMPK activity or glucose uptake was subsequently determined as described in the Materials and methods. AMPK activity is expressed as the difference ( $\Delta$ Abs) between the absorbance measured in the absence or presence of Compound C (CC;  $10 \mu\text{mol l}^{-1}$ ), normalized to the amount of protein in the cell extract. Glucose uptake is expressed as fold stimulation above the control group, which was set to 1. Data are means  $\pm$  s.e.m. of five independent experiments, each performed in triplicate. The effects of EPS on glucose uptake were tested in the absence or presence of CC ( $40 \mu\text{mol l}^{-1}$ ). Insulin was used as a positive control ( $1 \mu\text{mol l}^{-1}$ ) during the last 30 min of incubation. Asterisks indicate significant differences from control, and different letters indicate significant differences between myotubes exposed to EPS in the absence or presence of CC ( $*P < 0.05$ ).

Furthermore, our results suggest that the cellular and molecular mechanisms involved in the response of skeletal muscle to contractile stimuli have been well conserved between fish and mammals.

## MATERIALS AND METHODS

### Exercise induction experiments

Rainbow trout [*Oncorhynchus mykiss* (Walbaum 1792);  $n=20$ , 1.8 kg body mass, 50 cm length] were acquired from a Danish supplier (Frederiksværk Aleexport, Frederiksværk, Denmark) and transferred to a 6000 l swim-flume at Leiden University (The Netherlands). The animals were randomly assigned to a treatment group that swam at a speed of 0.75 body lengths  $\text{s}^{-1}$  for 40 days (swimmers,  $n=10$ ) or to a control resting group ( $n=10$ ) that was subjected to a minimal flow. Holding and swimming conditions are described in detail in Palstra et al. (Palstra et al., 2013). Red and white muscle tissue samples were dissected, frozen in liquid  $\text{N}_2$  and stored at  $-80^\circ\text{C}$  for analysis. These experiments were approved by the Animal Experimental Committee (DEC) of Leiden University under number 08107.

### Isolation and culture of trout muscle cells

Trout muscle cells were obtained from brown trout [*Salmo trutta* (Linnaeus 1758)] weighing 8–14 g that were supplied by the Piscifactoria de Baga (Generalitat de Catalunya, Spain) and maintained in the facilities at the University of Barcelona in a closed-water flow circuit under controlled conditions as described in Magnoni et al. (Magnoni et al., 2012). Trout muscle cell isolation and culture was performed as described previously (Magnoni et al., 2012). Trout muscle cells were cultured until they spontaneously differentiated into myotubes after 8–10 days. Media, enzymes and reagents used during isolation and culture were purchased from Sigma-Aldrich (Tres Cantos, Spain). The experimental protocols used here have been reviewed and approved by the Ethics and Animal Welfare Committee of the University of Barcelona, Spain.

### EPS of trout myotubes

Cell culture plates containing differentiated trout myotubes were placed between the connection cards of two electrical stimulators (ESCC), that were attached to a transparent plastic support (Marotta et al., 2004). The cards were connected by standard electrical cables to a custom-made stimulator (Universitat Politècnica de Catalunya, Barcelona, Spain) that generates variable electrical pulses (i.e. pulse duration, 10  $\mu\text{s}$ –10 s; pulse frequency, 0.01–1000 Hz; and voltage, 10 mV–150 V). Before immersion in the culture medium, platinum wire electrodes were sterilized with 70% ethanol and subsequently dried under ultraviolet light. The ESCC system was placed inside a water-jacketed cell incubator set at  $18^\circ\text{C}$  during the experiments (Nuair, Plymouth, MN, USA). Myotubes were stimulated by applying four series of EPS of 2 ms at 30 V and 2 Hz for 60 min, with intervals of 60 min without the generation of EPS between the series (three to four wells per plate in five independent experiments). A group of three to four wells in each plate did not receive EPS during the experiments and served as controls. Immediately after the last EPS, myotubes were used for the determination of AMPK activity or glucose uptake. The viability of electrically stimulated myotubes was assessed by measuring lactate dehydrogenase (LDH) activity released into the culture medium using a commercial kit (Spinreact, SantEsteve de Bas, Girona, Spain), following the manufacturer's instructions. No statistically significant differences in the levels of LDH activity in the medium were found between myotubes subjected to EPS and the controls ( $28.04 \pm 1.70$  and  $31.53 \pm 2.03 \text{ U l}^{-1}$ , respectively).

### Determination of AMPK activity

For the determination of AMPK activity in skeletal muscle, red and white muscle samples from swimming ( $n=10$ ) and resting ( $n=10$ ) trout were homogenized in 5 volumes of ice-cold homogenization buffer containing 50  $\text{mmol l}^{-1}$  Tris-HCl, pH 8.0, 150  $\text{mmol l}^{-1}$  NaCl, 1% Nonidet P-40 (v/v), 0.5% sodium deoxycholate, 0.1% SDS, 10  $\mu\text{l ml}^{-1}$  protease inhibitor cocktail and 10  $\mu\text{l ml}^{-1}$  phosphatase inhibitor cocktail (Sigma-Aldrich). The homogenate was left for 45 min on ice, centrifuged at 14,000 g for 10 min and the supernatant containing the proteins was used for the assays. AMPK activity was determined using a commercial kit (CycLex Co. Ltd, Nagano, Japan), according to a previously published protocol (Magnoni et al., 2012). Specific AMPK activity was calculated as the difference between the absorbance measured in the absence or presence of 6-[4-(2-piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine (Compound C;  $10 \mu\text{mol l}^{-1}$ ).

For the determination of AMPK activity in trout myotubes, cells were washed and scraped from the plates with PBS and lysates were obtained using ice-cold RIPA buffer in the presence of protease and phosphatase inhibitors as described in Magnoni et al. (Magnoni et al., 2012). AMPK activity in lysates from a total of five independent experiments (each performed in triplicate) was determined and calculated as indicated above and as described in detail in Magnoni et al. (Magnoni et al., 2012).

### RNA extraction, cDNA synthesis and quantitative real-time PCR

Total RNA was extracted from red and white muscle samples from swimming ( $n=10$ ) and resting ( $n=10$ ) trout using Trizol reagent (Invitrogen, Barcelona, Spain), and reverse-transcribed to cDNA using SuperScript III Transcriptase (Invitrogen), oligo(dT) primer and random hexamer primers

(Promega, Barcelona, Spain), according to the manufacturer's protocols. cDNA was diluted 1:25 for target mRNA and 1:1000 for 18S, used as the reference gene. Quantitative real-time PCR reactions were carried out in a MyiQ Detection System (Bio-Rad, Madrid, Spain), according to previously published procedures (Magnoni et al., 2012). All the samples were run in triplicate and fluorescence was measured at the end of every extension step. Fluorescence readings were used to estimate the values for the threshold cycles ( $C_t$ ). Values for each sample were expressed as fold change, calculated relative to control group and normalized for each gene against those obtained for 18S. Expression of 18S was not affected by swimming in red (controls,  $C_t=17.95\pm 0.46$ ; swimmers,  $C_t=18.26\pm 0.59$ ) or in white muscle (controls,  $C_t=18.21\pm 0.52$ ; swimmers,  $C_t=18.10\pm 0.58$ ). For all primer pairs, a dilution curve obtained from a serially diluted cDNA was used to ensure that PCR efficiency was higher than 90%. Primer sequences, amplicon sizes and GenBank accession numbers of the target genes are presented in supplementary material Table S1.

#### Determination of glucose uptake in trout myotubes

Determination of 2-deoxyglucose uptake in trout myotubes was performed as described previously (Magnoni et al., 2012). Recombinant human insulin (Sigma-Aldrich) was added to myotubes not receiving EPS as a positive control, at a concentration of  $1\ \mu\text{mol l}^{-1}$  for 30 min prior to the glucose uptake assays. In cells incubated with Compound C, the inhibitor was added to a final concentration of  $40\ \mu\text{mol l}^{-1}$  before application of EPS. Protein content in the lysates was measured using the Bio-Rad Protein Assay kit (Bio-Rad). Glucose uptake was measured in triplicate, normalized to total protein and expressed as fold induction with respect to non-stimulated cells.

#### Statistical analysis

Analysis of differences between groups was performed using the Mann-Whitney rank sum test or unpaired Student's *t*-test and were considered statistically significant when  $P<0.05$ . Values are given as means  $\pm$  s.e.m.

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#### Competing interests

The authors declare no competing financial interests.

#### Author contributions

L.J.M., A.P.P. and J.V.P. designed the study; L.J.M. and A.P.P. conducted the experiments; L.J.M. drafted the manuscript and J.V.P. edited the manuscript.

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#### Supplementary material

Supplementary material available online at <http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.099192/-/DC1>

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