

## Effects of different training protocols on Ca<sup>2+</sup> handling and oxidative capacity in skeletal muscle of Atlantic salmon (*Salmo salar* L.)

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### Summary

The modulation of calcium channel density and oxidative capacity in skeletal muscle after different training protocols were studied in 3-year-old Atlantic salmon smolts. The effect of endurance exercise on dihydropyridine (DHP) and ryanodine (Ry) receptor densities as well as on muscle metabolism were determined by immunoblot and histochemical analysis from swimming muscles of fish subjected to nine different training protocols varying in duration and water current velocity.

In general, exercise training caused a significant increase in the density of both DHP and Ry receptors in both muscle types studied. In red muscle, the most notable increase in DHP and Ry receptor expression was observed in muscle sections from fish swimming against intermediate current velocity for a 2-week period (182.3±16.3%, 234.6±30.3%, respectively). In white muscle, the expression of DHP and Ry receptors was most upregulated after a 6-week swimming period also at intermediate water current velocity (270.4±23.9%,

114.4±15.3%, respectively). As with the activity of enzymes involved in muscle energy supply, endurance exercise resulted in a significant increase in succinate dehydrogenase (SDH) activity, but a significant decrease in phosphorylase activity.

We conclude that the expression of both DHP and Ry receptors was upregulated in the swimming muscles of salmon as a consequence of exercise training. This, along with the increased oxidative enzyme activity, provides benefits to the contraction efficiency of fish muscles while swimming. However, it was also observed that optimal oxidative swimming capacity is achieved only with a proper exercise program, since the most relevant changes in DHP and Ry receptor expression, as well as in oxidative capacity, were seen in the group training with the intermediate swimming velocity.

Key words: dihydropyridine receptor, ryanodine receptor, proper exercise training program, oxidative capacity, fish.

### Introduction

Exercise training induces several adaptations in skeletal muscle of fish, such as an increase in fibre size, alterations in enzyme activities, improvement of oxygen supply, changes in energy requirement and modification of morphology (reviewed by Davison, 1997). Although the changes in metabolic systems responsible for the functional characteristics of muscle have been carefully studied, the results have been quite variable depending upon training protocol and fish species used. Furthermore, the studies on the effects of training on the Ca<sup>2+</sup> regulatory system, one of the major functional elements of skeletal muscle, are still lacking. In this paper, we report the effects of training on metabolism of different skeletal muscles and on the expression of receptors involved in muscle excitation–contraction (EC) machinery by using different training protocols.

Fish skeletal muscle fibres are divided into three distinct layers; red, pink and white. Red fibres are active at sustained

swimming velocities, whereas white fibres are recruited when the swimming velocity increases (reviewed by Altringham and Ellerby, 1999). Fibres differ from each other both metabolically and histologically. Red fibres contain many lipid droplets and mitochondria (Nag, 1972; Johnston, 1980). Thus the activities of oxidative enzymes are higher. Furthermore, the capillary density and myoglobin concentration are high. Thus, red fibres rely on aerobic metabolism and use lipids as their main energy source. White fibres, on the other hand, use anaerobic glycolysis as their energy supply and contain very few mitochondria. White fibres have a large cross-sectional area and weak blood supply. Therefore, the oxygen supply of the fibre is inefficient (Johnston, 1980).

Previous studies suggest that the relative percentage of red fibres increases when training is performed with sustainable swimming velocity (Young and Cech, 1993; Davison, 1997). The training also affects the swimming capacity of fish and leads to improved endurance (Houlihan, 1987; Davison, 1997).

In some cases the critical swimming speed ( $U_{crit}$ ) is also increased (Young and Cech, 1993; Holk and Lykkeboe, 1998). According to Pearson et al. (Pearson et al., 1990) and Young and Cech (Young and Cech, 1993), trained fish seem to recover faster after a forced swimming event.

Training increases the blood oxygen-carrying capacity (Davison, 1997; Gallagher et al., 2001) as well as the number of capillaries per fibre (Davie et al., 1986; Davison, 1997; Sängler and Pötscher, 2000). Furthermore, the myoglobin concentration of fibres has been noted to increase (Davison, 1997). Trained fish seem to have higher levels of enzymes associated with aerobic metabolism compared with the control ones (Davie et al., 1986; Urfi and Talesara, 1989; Farrell et al., 1991; Davison et al., 1997). In some studies, however, the increase in enzyme activities was found to be low or the activities unchanged (Johnston, 1980). Glycolytic enzyme activities have also been shown to increase (Johnston and Moon, 1980a). Moreover, enzymes involved in lipid metabolism have become more active, leading to higher use of lipids as a source of energy in trained fish (Davison, 1997). Although the effects of training have been quite widely studied, no studies have been performed on the effects of training on the EC coupling machinery of muscle.

In mammals, the EC coupling of skeletal muscles is initiated when t-tubules are depolarised leading to conformational change in the dihydropyridine receptors (DHPRs). In skeletal muscle, DHPRs are directly linked to the ryanodine receptor (RyR) 1, which is found in sarcoplasmic reticulum (SR). As a consequence of DHPR conformational change, RyR1 opens and  $Ca^{2+}$  ions flow to the cytoplasm, initiating contraction. In cardiac muscle, EC coupling is mediated through the entry of calcium ions into the cell cytoplasm, which then triggers calcium-induced calcium release (CICR) from SR via RyR (Lamb, 2000; Fill and Copello, 2002). The mechanism of EC coupling in fish skeletal muscle is still indefinite. It has been proposed that the release of  $Ca^{2+}$  ions from SR could occur both through direct mechanical coupling between DHPR and RyR, and through CICR (O'Brien et al., 1995; Fill and Copello, 2002).

In previous studies with mammals it has been noted that the amount of DHPR is correlated to the contraction force and velocity of muscles (Golden et al., 2003; Mänttari and Järvilehto, 2005). Furthermore, the expression of both receptors increases as a consequence of endurance training (Saborido et al., 1995; Ørtenblad et al., 2000). Since the receptors are an essential part of muscle function and their amount directly affects the power capacity of muscles, we report here the effects of training on the swimming muscles of fish. Moreover, since considerable variability in the effects of training on the metabolism of fish skeletal muscle has been reported, a more consistent way to assess the effects of training on the contractile properties of muscle is used.

### Materials and methods

This study was carried out in spring 2005 (18.4.–15.6.) in the Game and Fishery Research Institute at Taivalkoski,

Finland (65°34' N, 28°15' E). Smolts of Atlantic salmon (*Salmo salar* L.), 3 years of age, were anaesthetised with 100 mg l<sup>-1</sup> ethyl 3-aminobenzoate methanesulfonate salt (Sigma, USA), and then weighed and measured. Fish were divided into distinct groups according to their lengths and reared in a rotation current aquarium system with water coming from the nearby River Iijoki. Natural water temperature and photoperiod were maintained. Fish were fed with commercial fish pellets (Bio-Optimal C80, Bio-Mar, Vantaa, Finland) three times per day (0.73 g per fish). For training groups the amount of food was 1.5 times higher.

After a 2-week adaptation period, fish were divided into nine different training groups ( $N=6$  in each group). During the exercising period, fish were swimming against one of the three different water flow velocities (1, 1.5 or 2  $BL s^{-1}$ ;  $BL$ =body length) 6 hours per day, 5 days per week [modified from Jørgensen and Jobling (Jørgensen and Jobling, 1993)]. The exercising period varied between the groups (2, 4 or 6 weeks). Each training protocol was performed with three different groups (total  $N=162$ ). Control fish swam in a tank against a minimum current velocity used in regular rearing tanks, i.e. 0.5  $BL s^{-1}$  ( $N=54$ ). All the water flow velocities were measured from the area of the tank that the fish preferred.

### Condition factor

At the end of the training period smolts were killed by decapitation. Total length from nose to the end of tail and total mass of the smolts were measured in order to calculate the Fulton's condition factor ( $CF$ )= $(\text{mass} \times \text{length}^{-3}) \times 100$ , where mass is in g and  $\text{length}^{-3}$  is in cm.

### Muscle cross sections

After the measurements, fish were frozen with liquid nitrogen and stored in  $-80^{\circ}C$  until preparation. Blocks of muscles were taken precisely between the adipose fin and tail and 14  $\mu\text{m}$  cross sections were cut with a cryostat microtome at  $-20^{\circ}C$ . To evaluate the density of DHPR and RyR, the cross sections were incubated with 20 nmol l<sup>-1</sup> high affinity (–)-enantiomer of dihydropyridine, labelled with orange fluorophore, and with 0.5  $\mu\text{mol l}^{-1}$  high affinity (–)-enantiomer of ryanodine, labelled with green fluorophore (Molecular Probes, Leiden, Netherlands) for 90 min and processed as described by Mänttari et al. (Mänttari et al., 2001). The control samples were preincubated for 10 min in 10  $\mu\text{mol l}^{-1}$  nifedipine, a DHPR blocker, and 50  $\mu\text{mol l}^{-1}$  dandrolene, a RyR blocker, prior to addition of labelling solution. The images of the sections were obtained using a confocal laser scanning microscope (LSM-5 Pascal, Zeiss, Jena, Germany) with excitation at 543 nm for DHPR and 488 nm for RyR.

Two additional sets of cross sections were simultaneously processed for succinic dehydrogenase (SDH) activity (Nachlas et al., 1957) and for phosphorylase activity, using a modified published method (Dubowitz and Pearse, 1960). To investigate the phosphorylase activity, the sections were first fixed in cold acetone for 2 min, incubated for 1 h at 37°C in substrate solution (0.25 g glucose 1-phosphate, 25 mg AMP, 5 mg

glycogen, 0.45 g NaF, 2.25 g polyvinylpyrrolidone and few drops of insulin in 25 ml of acetate buffer pH 5.9) and briefly dried. The sections were washed with 40% ethanol, dissolved in absolute ethanol for 20 min and dyed in 10% Gmans iodine solution for 5 min. All the sections were scanned using a confocal microscope and the intensity of staining was measured with LSM 5 PASCAL software 3.2 (Zeiss).

*SDS-PAGE and western blotting*

White muscle samples were taken caudally behind the adipose fin from exactly the same point on each fish, homogenised in 6 vol of homogenization buffer (62.5 mmol l<sup>-1</sup> Tris-HCl, pH 6.8) and denatured at 70°C for 7 min. SDS-PAGE (Laemmli, 1970) was performed using a 7.5% separating gel and a 3.5% stacking gel. Each sample contained 24 µg of protein [determined by the Bradford method (Bradford, 1976)]. The proteins were electrophoretically separated at 150 V for 40 min. The separated proteins were electroblotted to nitrocellulose membrane according to the method of Towbin et al. (Towbin et al., 1979). Membranes were incubated for 2 h in primary antibody (L-type  $Ca^{2+}$  CP α1S; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; monoclonal anti-ryanodine receptor clone 34 C; Sigma-Aldrich Inc., St Louis, MO, USA) and with secondary antibody (blotting grade affinity purified goat anti-mouse IgG H+L alkaline phosphatase conjugate, Bio-Rad, Hercules, CA, USA) also for 2 h. Antibody detection was performed with bromo-4-chloro-3-indolyl phosphate mono-(–toluidinium) salt/nitro blue tetrazolium (BCIP/NBT) substrate for 12 min. The dilution for the primary antibody was 1:250 for dihydropyridine receptors and 1:4000 for ryanodine receptors. The optical densities of the detected bands were analysed with FluorS MultiImager program (Bio-Rad).

All the experiments were performed in accordance with the Animal Ethics Committee of the University of Oulu (licence no. 083/04).

*Statistics*

Data are presented as mean ± s.e.m. and analyzed for protein expression levels and oxidative capacity in salmon swimming muscles with R2.2.1 for Windows software. Differences between control and trained groups were evaluated by analysis of independent samples *t*-test. The differences between changes in training groups were evaluated by two-way ANOVA using training velocity and duration of training as the major factors.

**Results**

*Condition of fish*

In order to evaluate the change in the condition of fish after training, Fulton’s CFs were calculated. The results are presented in Table 1. The most notable change (0.077) was seen between the group training at a velocity of 1 BL s<sup>-1</sup> for 4 weeks and group training at velocity of 2 BL s<sup>-1</sup> for 6 weeks. The groups differed statistically from each other (*P*=0.002).

Table 1. Condition factors of salmon smolts subjected to different training protocols of swimming velocity and duration of training

Velocity (BL s <sup>-1</sup> )	Duration (weeks)	Condition factor
1	2	0.932±0.054
1.5	2	0.919±0.059
2	2	0.920±0.059
Control (0.5)	2	0.955±0.068
1	4	0.896±0.055
1.5	4	0.914±0.067
2	4	0.923±0.062
Control (0.5)	4	0.907±0.058
1	6	0.915±0.075
1.5	6	0.915±0.086
2	6	0.973±0.046
Control (0.5)	6	0.923±0.059

Values are means ± s.d. (N=18 in each group).

*Expression of DHPR and RyR*

To determine the density of DHP and Ry receptors in muscle samples, both fluorescent labelling and western blotting methods were used. Generally, for both types of muscle investigated the densities of DHPR and RyR were significantly higher in the exercising groups compared with the control ones. The results from fluorescent labelling and western blotting are presented in Figs 1 and 2, respectively. In red muscle, the highest DHPR expression was found in fish swimming against the intermediate current velocity of 1.5 BL s<sup>-1</sup> for 2 weeks. RyR expression was also high in the group training for 2 weeks at intermediate velocity. The trained groups differed significantly from each other, both as a result of swimming velocity and duration of training (for DHPR *F*=4.37, *P*=0.0143, with swimming velocity as the major factor, and *F*=18.60, *P*=5.62×10<sup>-8</sup> with duration of training as the major factor; for RyR *F*=6.19, *P*=0.0026, with swimming velocity as the major factor and *F*=7.17, *P*=0.0011, with duration of training as the major factor). The most notable changes were seen in the group swimming at a velocity of 1.5 BL s<sup>-1</sup> for 2 weeks. For red muscle western blot analysis was not performed since the antibodies did not recognize the receptors.

In white muscle, the expression pattern following the training was not that conclusive. The highest change in the density of both receptor molecules were found in muscles of fish exercising with intermediate swimming velocities. The group training at 1 BL s<sup>-1</sup> for 2 weeks, however, was an exception, since the DHPR density analysed by western blotting was deviant. The increase in percentage of receptor expression differed significantly between the training groups and it seemed that most significant change occurred after 6 weeks of training (for RyR *F*=26.43, *P*=1.31×10<sup>-10</sup>, duration of training as the major factor; for DHPR analyzed by western blotting *F*=24.70, *P*=4.79×10<sup>-10</sup> and for DHPR

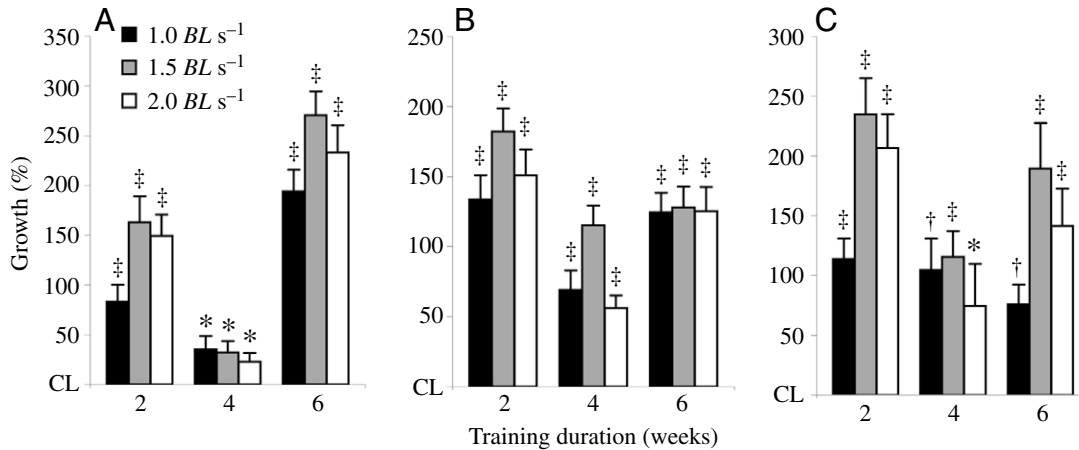


Fig. 1. The alteration of the staining intensities of dihydropyridine (DHP; A,B) and ryanodine (Ry; C) receptors in white (A) and red (B,C) muscle sections of salmon. Sections were incubated in 20 nmol l<sup>-1</sup> high affinity (-)-enantiomer of dihydropyridine labelled with orange fluorophore and in 0.5 μmol l<sup>-1</sup> high affinity (-)-enantiomer of ryanodine labelled with green fluorophore. Exercise protocol: three different swimming velocities, 1, 1.5 and 2 BL s<sup>-1</sup>, and three different training periods, 2, 4 and 6 weeks. Difference between control and trained group significant at \**P*<0.05, †*P*<0.01 or ‡*P*<0.001. CL, control level.

analyzed by fluorescent labelling  $F=75.82$ ,  $P=2.20 \times 10^{-16}$ . The analysis of change in white muscle RyR fluorescence could not be performed reliably since the fluorescence was not detectable.

*Enzyme activities*

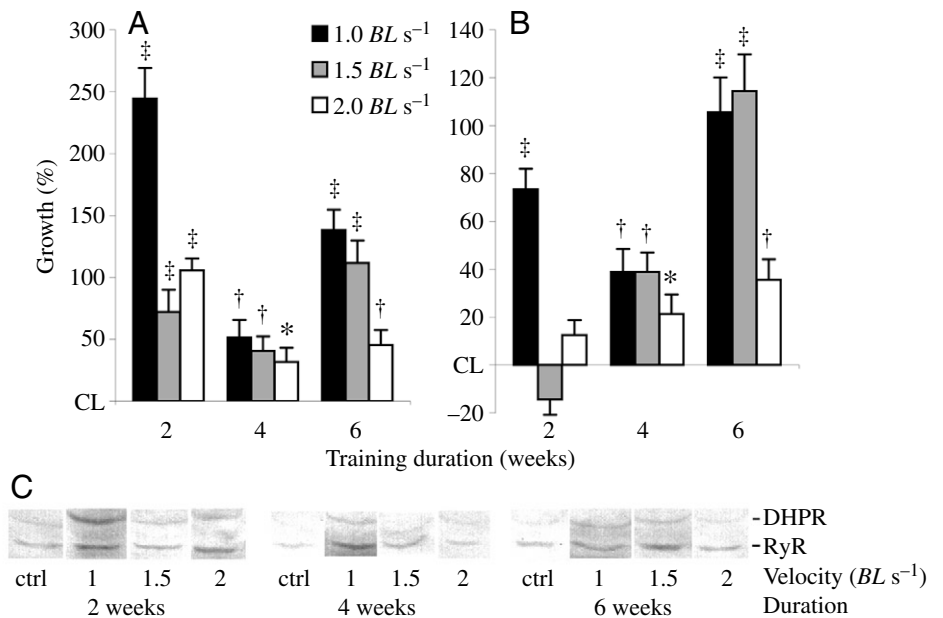
The activity of enzymes involved in muscle energy supply was determined from cross sections incubated in substrate solutions. The results are presented in Fig. 3. Generally, training resulted in significant increase in the activity of SDH, but a decrease in phosphorylase activity. In red muscle, the highest change in SDH activity was found in the group training with intermediate swimming velocity for 4 weeks. The change in phosphorylase activity was higher in the group training for

longer periods ( $F=11.74$ ,  $P=1.78 \times 10^{-5}$ , duration of training as the major factor), though the group training with intermediate swimming velocity for 6 weeks was an exception.

In white muscle, the most significant increase in SDH activity was seen after 6 weeks of training ( $F=4.75$ ,  $P=0.0099$ , duration of training as the major factor) though increased activity could be seen also after shorter training periods. Training seemed, however, to decrease the phosphorylase activity of white muscle.

There was a significant difference in the enzyme activities between the two muscle types studied. The SDH activity was higher ( $P=8.92 \times 10^{-13}$ ) and that of phosphorylase lower ( $P=2.20 \times 10^{-16}$ ) in red muscle than white muscle. All the measured values are presented in Tables 2 and 3.

Fig. 2. (A) The change in the optical density unit (oDu) values of dihydropyridine receptor (DHPR) measured from white muscle homogenates analysed by western blotting. (B) The change in the optical density unit values of ryanodine receptor (RyR) from white muscle homogenates of salmon. (C) Membranes containing electrophoretically separated DHPR and RyR from white muscle of control and trained salmon. Exercise protocol: three different swimming velocities, 1 (low), 1.5 (intermediate) and 2 BL s<sup>-1</sup> (high) and three different training durations, 2, 4 and 6 weeks. Difference between control (ctrl) and trained group significant at \**P*<0.05, †*P*<0.01 or ‡*P*<0.001. CL, control level.



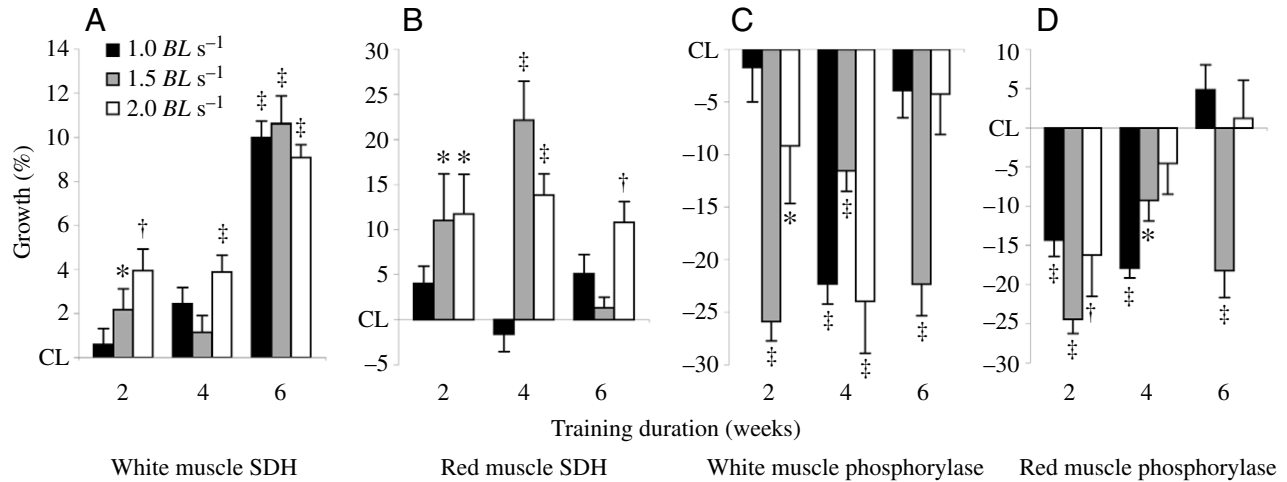


Fig. 3. The alteration of staining intensities of succinate dehydrogenase (SDH; A,B) and phosphorylase (C,D) in red (B,D) and white (A,C) muscles of salmon measured from muscle sections obtained from fish exercised with different training protocols. Exercise protocol: three different swimming velocities, 1, 1.5 and 2  $BL s^{-1}$ , and three different training durations, 2, 4 and 6 weeks. Difference between control and trained group significant at \* $P < 0.05$ , † $P < 0.01$  or ‡ $P < 0.001$ . CL, control level.

### Discussion

Exercise training has been shown to affect fish skeletal muscle metabolism and histology (reviewed by Davison, 1997). The plasticity of EC coupling components is, however, not so well understood. This study demonstrates for the first time the effect of exercise on the expression of DHP and Ry receptors in fish swimming musculature. The main result is the discovery of augmentation of the expression of DHP and Ry receptors in both white and red muscles of Atlantic salmon after intermediate training. Furthermore, as a result of increasing muscle performance, the activity of the aerobic enzyme SDH is increased. The activity of the glycolytic enzyme phosphorylase was, by contrast, reduced.

In fish skeletal muscle the exact mechanism of EC coupling is still unknown. However, it is clear that both DHP and Ry receptors are crucial for triggering  $Ca^{2+}$  release from SR (Fill and Copello, 2002). Moreover, in mammalian muscles it has been shown that the expression of receptors correlates positively with both the contraction force and velocity (Golden et al., 2003; Mänttari and Järvilehto, 2005). Therefore, the effect of exercise training on the protein expression of both receptors was studied. Since the muscle contraction activity increases as a result of increased swimming activity, the coupling process must occur effectively, i.e. continuously and with high velocity. Because of the increased power capacity obtained with the high level of receptors in muscles, the expression of both receptors is up-regulated after training.

Table 2. Expression of dihydropyridine and ryanodine receptors and enzyme activities in red muscle of salmon

Velocity ( $BL s^{-1}$ )	Duration (weeks)	DHPR (intensity)	RyR (intensity)	SDH (1/intensity) $\times 1000$	Phosphorylase (1/intensity) $\times 1000$
1	2	63.6 $\pm$ 4.7‡	36.5 $\pm$ 3.0‡	8.7 $\pm$ 0.16	9.5 $\pm$ 0.23‡
1.5	2	76.8 $\pm$ 4.4‡	57.1 $\pm$ 5.2‡	9.3 $\pm$ 0.43*	8.4 $\pm$ 0.20‡
2	2	68.2 $\pm$ 5.1‡	52.4 $\pm$ 4.8‡	9.3 $\pm$ 0.37*	9.3 $\pm$ 0.58†
Control (0.5)	2	27.2 $\pm$ 2.9	17.1 $\pm$ 3.0	8.4 $\pm$ 0.22	11.3 $\pm$ 0.42
1	4	57.4 $\pm$ 4.7‡	52.7 $\pm$ 6.9†	7.5 $\pm$ 0.15	9.8 $\pm$ 0.15‡
1.5	4	73.1 $\pm$ 4.8‡	55.6 $\pm$ 5.5‡	9.4 $\pm$ 0.33‡	10.8 $\pm$ 0.32*
2	4	53.0 $\pm$ 3.1‡	44.9 $\pm$ 9.1*	8.7 $\pm$ 0.18‡	11.4 $\pm$ 0.47
Control (0.5)	4	34.0 $\pm$ 2.5	25.8 $\pm$ 3.4	7.8 $\pm$ 0.24	12.2 $\pm$ 0.58
1	6	52.1 $\pm$ 3.2‡	27.6 $\pm$ 2.7†	7.2 $\pm$ 0.15	12.8 $\pm$ 0.39
1.5	6	53.1 $\pm$ 3.5‡	45.5 $\pm$ 6.0‡	6.9 $\pm$ 0.08	10.0 $\pm$ 0.41‡
2	6	52.5 $\pm$ 4.0‡	37.9 $\pm$ 4.9‡	7.6 $\pm$ 0.16†	12.3 $\pm$ 0.60
Control (0.5)	6	23.2 $\pm$ 3.2	15.7 $\pm$ 2.8	6.9 $\pm$ 0.23	12.4 $\pm$ 0.38

DHPR, dihydropyridine receptor; RyR, ryanodine receptor.

Values are means  $\pm$  s.e.m. ( $N=18$  in each group). Significant difference between control and trained group: \* $P < 0.05$ , † $P < 0.01$  or ‡ $P < 0.001$ .

Table 3. Expression of dihydropyridine and ryanodine receptors and enzyme activities in white muscle of salmon

Velocity ( $BL\ s^{-1}$ )	Duration (weeks)	DHPR (intensity)	DHPR (oDu)	RyR (oDu)	SDH (1/intensity) $\times 1000$	Phosphorylase (1/intensity) $\times 1000$
1	2	13.3 $\pm$ 1.2 <sup>‡</sup>	0.034 $\pm$ 0.0025 <sup>‡</sup>	0.043 $\pm$ 0.0021 <sup>‡</sup>	6.3 $\pm$ 0.05	14.5 $\pm$ 0.48
1.5	2	19.0 $\pm$ 1.9 <sup>‡</sup>	0.017 $\pm$ 0.0018 <sup>‡</sup>	0.021 $\pm$ 0.0016	6.4 $\pm$ 0.06*	10.9 $\pm$ 0.27 <sup>‡</sup>
2	2	18.0 $\pm$ 1.5 <sup>‡</sup>	0.021 $\pm$ 0.0010 <sup>‡</sup>	0.028 $\pm$ 0.0015	6.5 $\pm$ 0.05 <sup>†</sup>	13.4 $\pm$ 0.81*
Control (0.5)	2	7.2 $\pm$ 1.1	0.010 $\pm$ 0.0000	0.025 $\pm$ 0.0022	6.2 $\pm$ 0.05	14.9 $\pm$ 0.40
1	4	18.4 $\pm$ 1.8*	0.016 $\pm$ 0.0015 <sup>†</sup>	0.022 $\pm$ 0.0015 <sup>†</sup>	6.5 $\pm$ 0.05 *	13.7 $\pm$ 0.33 <sup>‡</sup>
1.5	4	18.0 $\pm$ 1.6*	0.014 $\pm$ 0.0012 <sup>†</sup>	0.022 $\pm$ 0.0013 <sup>†</sup>	6.4 $\pm$ 0.05	15.6 $\pm$ 0.34 <sup>‡</sup>
2	4	16.7 $\pm$ 1.2*	0.014 $\pm$ 0.0012*	0.019 $\pm$ 0.0013*	6.6 $\pm$ 0.05 <sup>‡</sup>	13.5 $\pm$ 0.88 <sup>‡</sup>
Control (0.5)	4	13.6 $\pm$ 0.8	0.010 $\pm$ 0.0008	0.016 $\pm$ 0.0016	6.4 $\pm$ 0.05	17.8 $\pm$ 0.42
1	6	15.9 $\pm$ 1.2 <sup>‡</sup>	0.020 $\pm$ 0.0014 <sup>‡</sup>	0.026 $\pm$ 0.0018 <sup>‡</sup>	6.8 $\pm$ 0.05 <sup>‡</sup>	16.3 $\pm$ 0.45
1.5	6	20.0 $\pm$ 1.3 <sup>‡</sup>	0.018 $\pm$ 0.0015 <sup>‡</sup>	0.027 $\pm$ 0.0019 <sup>‡</sup>	6.8 $\pm$ 0.08 <sup>‡</sup>	13.2 $\pm$ 0.51 <sup>‡</sup>
2	6	18.0 $\pm$ 1.5 <sup>‡</sup>	0.012 $\pm$ 0.0010 <sup>†</sup>	0.017 $\pm$ 0.0011 <sup>†</sup>	6.8 $\pm$ 0.04 <sup>‡</sup>	16.2 $\pm$ 0.65
Control (0.5)	6	5.4 $\pm$ 0.6	0.008 $\pm$ 0.0007	0.013 $\pm$ 0.0012	6.2 $\pm$ 0.04	17.3 $\pm$ 0.62

Dihydropyridine receptor (DHPR) and ryanodine receptor (RyR) optical density unit (oDu) values were detected on membranes containing electrophoretically separated DHP and Ry receptors, treated with specific antibodies against the receptors.

Values are means  $\pm$  s.e.m. ( $N=18$  in each group). Significant difference between control and trained group: \* $P<0.05$ , <sup>†</sup> $P<0.01$  or <sup>‡</sup> $P<0.001$ .

Similar results have been previously described in mammals (Saborido et al., 1995; Ørtenblad et al., 2000). Furthermore, in our previous study with salmon it was revealed that the expression of DHPR and the activity of oxidative enzymes increased during downstream migration associated with increased swimming activity (Mänttari et al., 2005).

Alteration in the expression of DHP as well as Ry receptors was seen in both red and white muscles at all three swimming velocities investigated. In some previous studies it has been shown that the recruitment order of swimming muscles is red>pink>white as a function of increasing swimming velocity (reviewed by Altringham and Ellerby, 1999). It has been noted, however, that white muscle is also active at sustainable swimming speeds (Johnston and Moon, 1980a; Johnston and Moon, 1980b; Wilson and Egginton, 1994; Day and Butler, 1996). This may explain the improved performance of white muscle, at lower swimming velocities, observed at molecular level in this study. Furthermore, in previous investigations it has also been noted that white muscle characteristics change as a result of training with sustainable swimming velocities (Davie et al., 1986; Gruber and Dickson, 1997; Sängner and Pötscher, 2000). The change in white muscle characteristics could, also, be partly the result of global changes in gene expression induced by alteration in red muscle activity.

The Fulton's condition factor was calculated in order to evaluate the effect of the overall condition of fish on muscle characteristics. Though the groups differed statistically from each other, the changes between groups were minor (highest difference was 0.077). Moreover, there could not be seen any trends in CFs between groups. Therefore, we concluded that the alterations in muscle characteristics were due to training, not due to the change in the overall condition of fish.

In the present investigation the antibodies used to analyze the expression of receptors did not recognise the receptors in

red muscles. One reason for this could be a lower expression of receptors in red muscle. However, it has been previously shown that the muscle types differ from each other by the isoform of RyR (Franck et al., 1998; Morrissette et al., 2000). In white muscle there are two types of RyRs;  $\alpha$ -RyR-fast and  $\beta$ -RyR (O'Brien et al., 1993; Morrissette et al., 2000). In the red muscle, on the other hand, only  $\alpha$ -RyR-slow is expressed (Franck et al., 1998; Morrissette et al., 2000). For DHPR there are no comparisons made between muscle types and the investigations are limited to analysis of the sequence of DHPR in white muscle (Grabner et al., 1991). In mammals, it has been shown that skeletal muscles contain multiple DHPR isoforms (Fill and Copello, 2002). The differential expression of isoforms due to training could, thus, explain the slight difference in results of expression of DHP receptors analysed by western blotting and fluorescence labelling. In the future it would be interesting to study the differences of receptor isoforms between muscle types. This way the effect of training on different receptor isoforms could also be analysed.

The enhancement of DHPR and RyR expression in swimming muscles was accompanied by an increase in SDH activity and a reduction in phosphorylase activity. In mammals, training has been shown to increase the oxidative capacity of skeletal muscle (Bell et al., 2000; Serrano et al., 2000; Allen et al., 2001). After exercise, the enzymes involved in the citric acid cycle are more active and the number as well as the size of mitochondria increases. The activity of glycolytic enzymes, e.g. phosphorylase, increases only when the training regime includes a significant amount of sprint exercise (Exner et al., 1973). In fish, the effects of training have been quite versatile. In some cases the oxidative enzyme activities and the number and the size of mitochondria have been increased (Davison, 1997). On the other hand, there are also investigations where the values were unchanged (Johnston, 1980). In general, if the

endurance training occurs at lower swimming velocities, the oxidative capacity of swimming muscles seems to increase. However, in some investigations, glycolytic enzyme activities have been observed to increase (Johnston and Moon, 1980a). According to the data obtained from the present study, intermediate sustained swimming enhances the activity of oxidative enzymes, but at the same time decreases the activity of glycolytic enzymes. We also detected a relationship between enzyme activities and the training protocol used, i.e. the optimal oxidative capacity of swimming muscles is achieved only with a suitable exercise program.

In red muscle, the increase of SDH activity was not as remarkable as in white muscle. It has been shown that red muscle cells contain about twenty times more mitochondria and thus the SDH activity is about seven times higher compared to white muscles (Johnston, 1980). Since the onset level of red muscle oxidative capacity is high, the change in red muscle SDH activity after exercising is not so remarkable. The increase in DHPR and RyR expression, however, was more significant in red muscles. This could be explained by the fact that training occurred with quite low, sustainable swimming velocity when red muscles are active continuously.

In general, some variability in the parameters measured, both with respect to duration and velocity of training, was observed in the present study. Training induced changes in red muscle characteristics (DHPR, RyR and phosphorylase) already after 2 weeks. However, the SDH activity showed the highest increase in the group training for 4 weeks. The slower response in SDH activity could be explained by the high initial level of oxidative enzymes in red muscle (Johnston, 1980), which enhances the aerobic capacity for muscle in the beginning of the training. The most relevant changes in red muscle in all the measured parameters were seen in the groups training with intermediate swimming velocities. The effect of training on the parameters investigated seemed to decline when the training period was prolonged. According to previous investigations (Beddow and McKinley, 1999), red muscle is active all the time in all the swimming velocities used in the present study. Training could, thus, be too intense for the optimal contractile properties of red muscle when both the swimming velocity and the duration increased. In white muscle, on the other hand, the SDH activity enhanced when training velocity and duration increased. White muscle is probably more efficiently recruited when the activity of red muscle declines. A similar trend with respect to duration of training was seen also in other parameters measured. The most relevant increase in the expression of DHP and Ry receptors was, however, observed even at slower swimming velocities. It is concluded, therefore, that also for white muscle the optimal contractile properties are achieved with intermediate swimming velocity. Taken together, more intense training seems to be too strenuous for white, and even more so for red muscles of fish, weakening the swimming capacity of both muscle types.

The parrs turned into smolts during the experiment because

of the northerly location of the rearing station and the time of the year. This is also seen in the slight alteration of the parameters in the muscles of fish swimming at the lowest velocity. However, since the control fish were from the same age group as the trained ones, the different training groups were comparable. Despite the smoltification, training increased the swimming capacity of fish in general and induced a change toward oxidative metabolism.

The major purpose of this study was to find out what kind of training regimen enhances both Ca<sup>2+</sup> handling and oxidative capacity most in swimming muscles of salmon. It is well known that reared fish do not survive in nature as well as the wild ones (Juttila et al., 2003; Kallio-Nyberg et al., 2004; Jokikokko et al., 2006). In spring, salmon smoltify and start migration of hundreds of kilometres. This kind of event requires high oxidative capacity, especially in red muscles. It has been noted that wild fish have superior swimming capacity compared with the reared ones (McDonald et al., 1998). Thus the condition of fish swimming musculature probably has an effect on fish survival rates, which will be studied in our ongoing research project.

As a conclusion, the endurance training has a significant impact on fish skeletal muscle characteristics associated with both muscle contraction efficiency and oxidative capacity. First, the density of receptors involved in the muscle EC coupling mechanism increases. Second, the oxidative metabolism of swimming muscles increases. Moreover, a relationship between training protocol used, and both indicators of improved muscle performance studied, was detected. It was also observed that optimal oxidative swimming capacity is achieved only with a proper exercise program.

#### **List of abbreviations**

AMP	adenosine mono phosphate
BCIP/NTB	bromo-4-chloro-3-indolyl phosphate mono- (-toluidinium) salt/nitro blue tetrazolium
Bl	body length
CICR	calcium-induced calcium release
DHP	dihydropyridine
DHPR	dihydropyridine receptor
EC	excitation-contraction
oDu	optical density unit
Ry	ryanodine
RyR	ryanodine receptor
SDH	succinate dehydrogenase
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SR	sarcoplasmic reticulum
$U_{crit}$	critical swimming speed

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