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SHORT COMMUNICATION

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Effect of fasting and refeeding on in vitro muscle cell proliferation in rainbow trout (*Oncorhynchus mykiss*)

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Abstract The effects of short-term fasting and refeeding were studied on satellite cells extracted from white epaxial muscle of juvenile rainbow trout (1–3 g body weight). In vitro changes in the proliferation of satellite cells were analyzed using bromodeoxyuridine (BrdU) incorporation over a 24-h period. Proliferation in fed control fish was characterized by an initial basal proliferation rate of 5–10% BrdU-labeled nuclei·day^{-1}, followed by an exponential increase at a rate of +18–20%·day^{-1}, up to a maximum of 60–70% BrdU-labeled nuclei·day^{-1}. Characteristics of satellite cells extracted from starved fish, namely extraction yield, morphology, and proliferation, were different from those of fed fish. Fasting (8–10 days) completely suppressed initial proliferation of satellite cells in vitro over a period of 4 days. After this delay, proliferation resumed and changes in proliferation rates over time were similar to those of the control group. In fish fed for 4 days after an 8-day fast, the initial proliferation rate and the changes in proliferation rates over time were completely restored. These findings demonstrate that satellite cells express different behavior depending on feeding status, which could be due to the presence of different satellite cell populations.

Key words Fish · Growth · Muscle · Satellite cells · Fasting · Oncorhynchus mykiss (Teleostei)

Introduction

Postlarval muscle growth in fish results in an increase in both the size and number of fibers (Koumans et al. 1993b). The recruitment of new fibers (Weatherley and Gill 1980) is the main process contributing to red and white muscle growth in juveniles. The presence of different generations of fibers generates a classical mosaic aspect in muscle sections (Weatherley and Gill 1980).

The muscle hyperplasic process during postlarval growth seems to be slightly different to what is observed during embryogenesis or larval development (Johnston 1996). In juveniles, new fibers are assumed to be the result of recruitment, division, and fusion of satellite cells adjacent to existing fibers (Alfei et al. 1994; Akster et al. 1995). These satellite stem cells may have the same origin as those observed at the periphery of somites during early stages (Stoiber and Sänger 1996). The mosaic pattern in muscles is not randomly distributed, as the satellite cells are located at the apex of existing fibers.

It has been demonstrated by direct analysis of the number of fibers, or hypothesized through the measurement of small fiber percentages, that many factors affect the hyperplasic growth of muscle (Fauconneau et al. 1997) . The recruitment of new fibers decreases in both red and white muscle tissues with ageing. This is associated with a decrease in the number of satellite cells (Alfei et al. 1989; Koumans et al. 1993a) but also with a decrease in the proliferation rate of muscle cells measured both in vivo (Alfei et al. 1989, 1994) and in vitro (Koumans et al. 1991, 1993a).

It is known that fish are well adapted to relatively long periods of fasting and demonstrate extensive capacities for rapid recovery. The mechanisms for such recovery have been searched for extensively at the level of total fish growth and a number of circulating hormones are thought to be involved. However, with the exception of a few studies carried out on rainbow trout white muscle (Weatherley and Gill 1981; Kiessling et al. 1990; Fauconneau et al. 1994), little attention has been paid to muscle growth, and particularly to either white muscle fiber hypertrophy and hyperplasia or governing growth factors.

The aim of this work was to characterize the proliferation of myosatellite cells in vitro and to determine the effects of fasting and refeeding on in vitro proliferation of muscle cells in rainbow trout fingerlings.

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Materials and methods

Fish and treatments

In experiment 1, two groups of fish (1.8 g mean body weight) were constituted. One group (n=200) was starved for 8 days and the control group (n=100) was fed ad libitum over the same period of time. At the end of this first period, 15–20 fish from each group were sampled for muscle cell extraction. Fish from fasted groups were refed ad libitum for 8 days and compared with regularly fed fish. At the end of this second period, 20 fish were sampled from the refed group, muscle cell extraction was performed, and proliferation was analyzed.

In experiment 2, three groups of 40 fish (2.5 g mean body weight) were constituted from a group of regularly fed fish and subjected to three different feeding schedules. One group was fed ad libitum for 4 days then starved for 10 days, another group fasted during 10 days and was then refed ad libitum for 4 days, and the control group was regularly fed ad libitum during the 14 days of the experiment. Fish were weighed individually at the end of the experiment and 20 fish from each group were sampled for muscle cell extraction.

Cell extraction

The protocol used is similar to that developed for carp by Koumans et al. (1990), with some adaptations for rainbow trout. All the experiments were carried out at 15°C. Fish were sampled, weighed, killed by a sharp blow to the head, and immersed for 30 s in 70% ethanol. The skin was removed, the red superficial muscle was scrapped off, and the dorsal white muscle was carefully dissected. Samples were immersed in cold (0°C) basal medium 5 ml/g muscle: Dulbeco's modified Eagle medium (DMEM), 9 mM $NaHCO_3$, 20 mM HEPES, pH 7.4, Posm 300 mosmol/kg supplemented with 15% horse serum. An antibiotic cocktail was added to the basal medium (penicillin 100 U/ml, streptomycin 100 µg/ml, Fungizone 0.25 µg/ml, gentamycine 75 µg/ml).

Muscle samples were cut into small pieces, centrifuged (300 *g*, 5 min), and the pellet was washed twice with the basal medium without serum. The muscle tissue was then digested with collagenase (Type Ia Sigma 0.2%solution in DMEM) for 1 h at 18°C, then centrifuged (300 g, 5 min), and the pellet was washed with basal medium and resuspended in basal medium 5 ml/g/muscle. The cells were then submitted to mechanical dissociation (five triturations through a 1.4×100 mm needle) and centrifuged (300 *g*, 20 min).

The cells were then submitted twice to trypsin digestion (1:250 trypsin, 0.1% solution in DMEM; Sigma) for 20 min at 18°C, then centrifuged (300 *g*, 5 min) and the two supernatants were pooled, diluted (1:1 v/v) in cold basal medium supplemented with 15% horse serum, and centrifuged (300 g, 20 min, 4°C). The supernatant was resuspended in 20 ml basal medium and the suspended cells were then submitted to mechanical dissociation (ten triturations through a 1.4×100 mm needle) and centrifuged (300 *g*, 20 min). The suspension was filtered successively on 100-µm and 50-µm nylon screen and centrifuged (300 *g*, 20 min, 4°C). The cells were resuspended in the basal medium supplemented with 10% fetal calf serum to reach a final concentration of 1.5×10^6 cells·ml^{-1}.

The extraction yield of satellite cells from starved fish ($4.0\cdot10^6$ cells·g^{-1}) was slightly higher than that of fed fish ($3.7\cdot10^6$ cells·g^{-1}) in experiment 1. The extraction yields of satellites cells in starved ($2.9\cdot10^6$ cells·g^{-1}) and starved/refed ($2.9\cdot10^6$ cells·g^{-1}) groups were also slightly higher than those observed in the fed controls ($2.4\cdot10^6$ cells·g^{-1}).

Cell cultures

The crude cell suspension was enriched in satellite cells by making use of their high affinity for laminin. To this end, plastic culture plates or glass coverslips (1.1 cm^2) were treated successively with poly-L-lysine 16 µg·cm^{-2} (MW more than 300, 000; solution 100 µg·ml^{-1} in distilled water, 2.5 h at 15°C; Sigma) and laminin 2 µg·cm^{-2} (L2020; 20 µg·ml^{-1} in DMEM, 24 h at 18°C; Sigma).

The cells were seeded on laminin-treated plates at a concentration of 100,000 to 200,000 cells·cm^{-2} and left for 25 min at 18°C. The supernatant was then removed, the cells were washed twice very gently with DMEM, then cultured at 18°C in the basal medium supplemented with 10% fetal calf serum. The medium was renewed every day for the proliferation studies.

Proliferation analysis in vitro: bromodeoxyuridine incorporation

For proliferation studies, a solution of bromodeoxyuridine (BrdU; 10 µmol·l^{-1} in culture medium; Boehringer) was added to the wells. Incorporation of BrdU from the culture medium was then measured over a 24-h period. At the end of this period, the glass coverslips were washed in DMEM then immersed in cold (–20°C) 70% ethanol, 50 mM glycine solution and stored until analysis. The glass coverslips were washed in PBS 10% (phosphate buffer with 0.2% BSA and 0.2% saponine), then incubated with a first monoclonal antibody (anti-BrdU clone BMC 9318; Boehringer), washed in PBS 10% pH 7.4, incubated with a second antibody (anti-mouse coupled with FITC 480 nm/535 nm), and washed in PBS 10%. Finally, the glass coverslips were incubated in a Hoechst 33258 (molecular probe) dye solution for the detection (355 nm/450 nm) of total cell nuclei.

Measurements and calculations

In vitro-labeled fluorescent nuclei counts were obtained from two representative areas (2×0.0625 mm^2) of the total glass coverslip culture (more than 100 nuclei in each area). The total number of nuclei was assessed using Hoechst revelation and the number of proliferative nuclei was assessed using BrdU incorporation. A proliferative index was calculated based on the mean proliferative nuclei to total nuclei ratio in the two selected areas and expressed as a percentage per day.

The size (diameter) of myosatellite cells was measured for 24 nuclei per treatment (8 nuclei per well in triplicate), 2 days after seeding. Comparisons between treatments at each culture stage were performed using Student's t-test.

Results

Experiment 1

In the first part of the experiment, the growth rate of fed fish was 3.2%·day^{-1} and no growth was observed in starved fish. In the second part of the experiment, a significant growth rate of 5.5%·day^{-1} was observed in refed fish during the 1st week, which decreased to 2.8%·day^{-1} during the 2nd week. These growth rates, however, were similar to those of fed fish.

Satellite cells in starved fish were small (4±0.5 µm, mean ± standard deviation) and had a spherical or triangular shape compared with that of fed fish, which were significantly (P<0.001) larger (5±0.6 µm) and spindle shaped (Fig. 1).

The initial proliferation rate of cells in fed fish was 12%·day^{-1}. The stimulated cell proliferation rate in fed fish increased (+16%·day^{-1}) up to a plateau value of 60%·day^{-1}, reached after 5 days (Fig. 2a).

During the first 2–3 days of culture, there was no proliferation of cells in the starved group (Figs. 1, 2a). Pro-

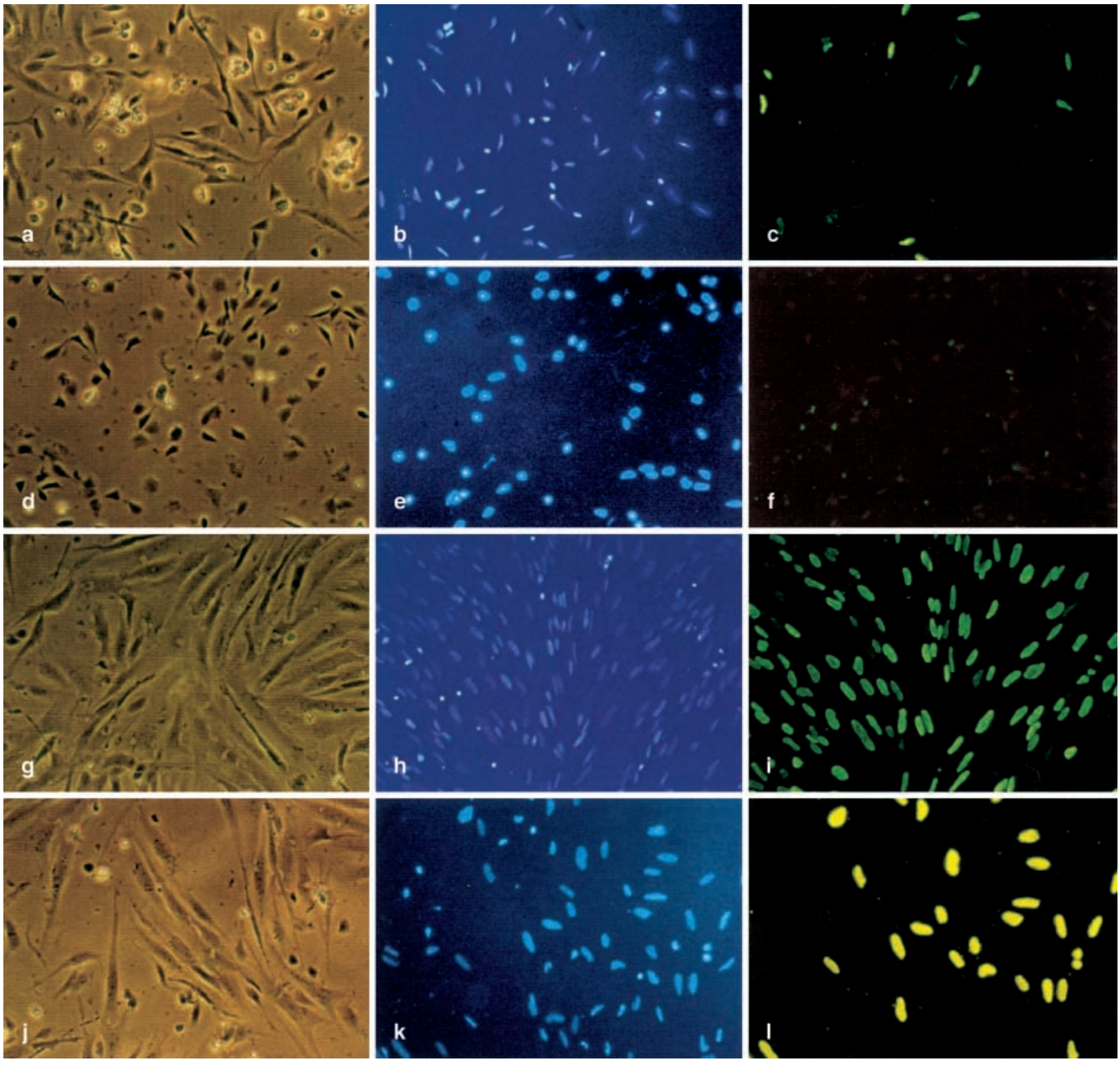

Fig. 1a–l Appearance (**a,d,g,j**) of Hoechst-positive nuclei (**b,e,h,k**), and BrdU-positive nuclei (**c,f,i,l**) of satellite cells in vitro after 2 days (**a–f**) and 8 days (**g–l**) of culture. Satellite cells were extracted from regularly fed (**a–c, g–i**) and 10-day starved (**d–f, j–l**) juvenile rainbow trout (1.8 g body weight)

liferation rate subsequently increased in a manner (+18%·day^{-1}) similar to that of control fed fish and reached a maximum of 55%·day^{-1} after 8 days.

Experiment 2

The mean final body weight of fed fish, 4.1±0.4 g, was significantly higher than that of starved fish (3.1±0.2 g). This corresponds to a specific growth rate of 3.4%·day^{-1} in fed fish, whereas no growth was observed in starved fish. The body weight of starved and refed fish (2.6±0.2 g) tended to be higher than that of starved fish, although this difference was not significant.

The aspect of muscle cells after seeding and adhesion was different in fasted and regularly fed fish (Fig. 1). The initial cell proliferation rate of the fasted/refed group (20%·day^{-1}) was significantly higher than that of the fed control group (4%·day^{-1}). The stimulated proliferation rate of cells from fed fish increased (25%·day^{-1}) up to a plateau value of 60%·day^{-1}, which was reached after 6–7 days. The same changes were observed in the cells from the fasted/refed group (Fig. 2b).

During the first 4–5 days of culture, no cell proliferation was observed for the starved group (Fig. 1). The proliferation rate subsequently increased in a manner

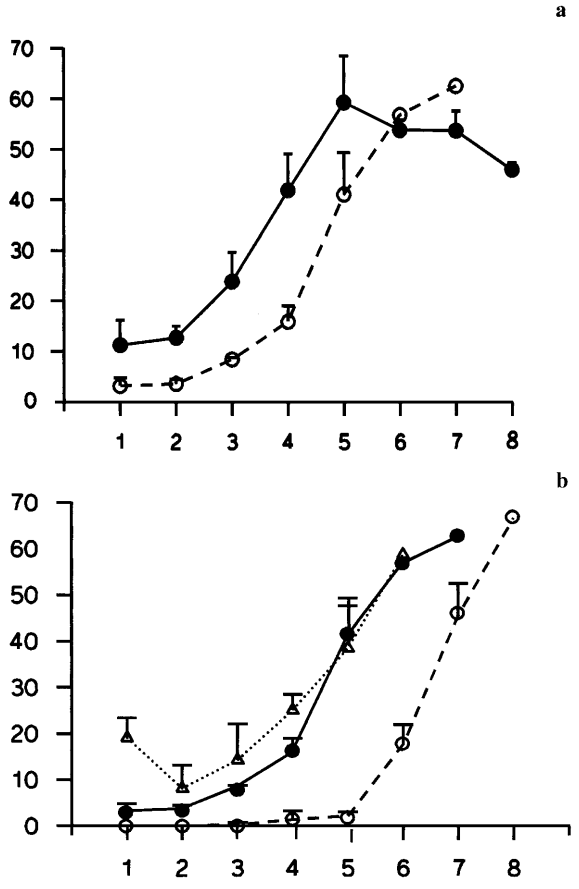

Fig. 2a, b Changes in proliferation rate over time (percentage BrdU-positive nuclei or Hoechst-positive nuclei per 24 h) in satellite cells extracted from: **a** regularly fed (*closed circle, continuous line*) and 10-day starved (*open circle, dashed line*) juvenile rainbow trout (1.8 g body weight); and **b** regularly fed (*closed circle, continuous line*), 8-day starved (*open circle, dashed line*) and 8-day starved and 4-day refed (*triangle, dotted line*) juvenile rainbow trout (2.5 g body weight)

similar (20–25%·day^{-1}) to that of fed controls and reached a maximum after 8 days.

Discussion

Cultures of muscle satellite cells have been developed for different fish species: salmon (Powell et al. 1989; Matschak and Stickland 1995), carp (Koumans et al. 1990), zebrafish (Sepich et al. 1994), catfish (Cook et al. 1995), and rainbow trout (Greenlee et al. 1995; Rescan et al. 1995). In vitro, these satellite cells usually only differentiate into small myotubes (two to four nuclei; Koumans et al. 1990; Greenlee et al. 1995), although, under the conditions of the present study, differentiation into very large myotubes (containing more than 15–20 nuclei) has been seen to occur within 6–8 days (Rescan et al. 1994, 1995; Fauconneau and Paboeuf 1998). This indicates the existence of different populations of satellite cells (Koumans et al. 1991, 1993a, 1993b; Fauconneau and Paboeuf 2000) and their sensitivity to isolation processes and culture conditions. The existence of these different populations could also explain the differences in cell extraction yields between fed and starved fish (mean difference of 10±5%, n=5), as observed in the two experiments presented here as well as in three other experiments realized under the same conditions and using the same procedure for cell isolation (B. Fauconneau and G. Paboeuf, unpublished results).

Proliferation of satellite cells was only observed in cells extracted from rainbow trout and cultured on basal lamina components: laminin or fibronectin (Greenlee et al. 1995; Rescan et al. 1995). Different means have been used to analyze the proliferation of muscle cells in vivo (Alfei et al. 1989, 1993, 1994; Akster et al. 1995). In vitro, however, this proliferation has only been quantified based on the increase in cell numbers (Greenlee et al. 1995). In the present study, an accurate measurement of satellite cell proliferation in vitro is described, which is based on the incorporation of BrdU (Zölzer et al. 1994) into nuclei labeled with Hoechst dye over a 24-h period. Under the culture conditions used here, satellite cells exhibited an initial basal proliferation rate of 5–10%·day^{-1}, which could be related to "active" satellite cells already engaged in vivo in a myogenic process (Alfei et al. 1989; Fauconneau and Paboeuf 2000). A stimulated proliferation rate (an increase by +18–20%·day^{-1} up to 60–70%·day^{-1}) was subsequently observed for "quiescent" cells cultured with the growth factors supplied by fetal calf serum (Fauconneau and Paboeuf 2000) up to a confluent state observed after 5–7 days of culture. These two phases were greatly affected by feeding status.

The characteristics of satellite cells extracted from starved fish differed extensively in terms of their size, morphology, and initial proliferation rate to those satellite cells extracted from fed fish. This suggests that the active cell population observed in continuously fed fish completely disappeared in starved fish, in which only quiescent cells could be observed. Complementary studies on instantaneous in vivo proliferation rates analyzed using the expression of a cell division marker (proliferative cell nuclear antigen associated with the expression of a cyclin; Zölzer et al. 1994) also suggest that the proliferation of muscle cells is completely suppressed in situ in starved fish (B. Fauconneau and G. Paboeuf, unpublished results). Proliferation was resumed in starved and refed fish and this demonstrates the adaptation of fish muscle cells to changes in feeding status. This adaptation would certainly contribute both to hyperplasic and hypertrophic growth processes.

The timing for the initiation of this stimulated proliferation was very dependent on feeding status (4-day delay in fasted fish). This could be directly related to differences in the environment of satellite cells in vivo. In physiological as well as in experimental fasting, both hypertrophic and hyperplasic components of muscle growth are stopped (Weatherley et al. 1979; Kiessling et al. 1990; Fauconneau et al. 1994). Fish starvation also

resulted in various physiological adaptations: a decrease in metabolic rate and an increase in the catabolism of energy (lipid) stores. This is thought to be associated with a decrease in the concentration of circulating anabolic hormones and a decrease in the expression of growth factors in muscle. Thus, the cellular, metabolic, endocrine, and paracrine environment of satellite cells are affected by fasting, and this probably induces a "resistant" state in the quiescent cells, which requires a few days of stimulation by fetal calf serum to be compensated. The duration of this stimulation period is probably related to the expression of receptors for the different growth factors present in the fetal calf serum.

The results of the present study clearly demonstrate, for the first time, that differences in satellite cell characteristics are related to feeding status in fish and that these differences are attributable to the existence of different satellite cell populations in growing fish. Further investigations are required to determine whether or not these different populations contribute to hyperplasic and hypertrophic growth processes in muscle (Koumans et al. 1993a, 1993b; Stoiber and Sänger 1996).

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