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The growth of Atlantic salmon (*Salmo salar* L.) myosatellite cells in culture at two different temperatures

T. W. Matschak and N. C. Stickland

The Royal Veterinary College, Royal College Street, London NW1 0TU (England, UK)
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Abstract. Temperature is known to affect fish growth, and in Atlantic salmon there is an influence on muscle cellularity. Primary muscle cell culture makes it possible to investigate direct effects of temperature on myogenic cells. Salmon myosatellite cells were cultured for the first time in this study. The cells were cultured at either 5 °C or 11 °C. Increased temperature led to an increase in differentiation rate and especially hypertrophic growth ($Q_{10} = 4.0$). No nuclear proliferation was evident in the satellite cell population isolated at either temperature. This may be due to the presence of different subpopulations of myogenic cells at different developmental ages or the presence of indirect factors in vivo.
Key words. Salmon; satellite cells; cell culture; temperature.

Fish muscle constitutes 30–80% of the body mass of the animal[1] and growth is closely linked to muscle growth[2]. In mammalian muscle, growth by hyperplasia, that is recruitment of new muscle fibres, ceases to play a role at around the time of birth[3,4]. In fish, however, both hyperplastic growth and muscle fibre hypertrophy, the increase in size of individual fibres, are important throughout almost the entire life of the animal[5–8].

The growth rate of salmonids is influenced by environmental temperature. Mathers et al.[9] found that fed trout at 10 °C and at 15 °C grow more rapidly than at 5 °C, and this is concomitant with a higher protein retention rate. This is consistent with an increase in protein synthesis rates after acclimatisation to a higher temperature[9,10].

There is evidence from work on pre-hatch salmon that temperature does not affect all growth processes equally. In Atlantic salmon, it has been found that embryos not only develop faster and exhibit a higher growth rate at higher temperatures, but that the muscle cellularity is distinctly influenced by incubation temperature. An increase in temperature results in the fish having fewer but larger muscle fibres by the time of hatching[11,12]. This constitutes a relative increase in growth by hypertrophy as opposed to hyperplasia when compared with fish grown at a lower temperature. In contrast, faster growth in post-hatch fish has been found to be associated with an increase in fibre number[1,8,13,14]. In juvenile salmon also, faster growth is associated with an increase in fibre number[15].

The satellite cell is generally regarded as the source of muscle nuclei for fibre hypertrophy and the addition of new fibres in growth[16,17]. In recent years, cell culture techniques have become available for fish satellite cells[18,19]. The difference in growth mechanism before and after hatching may be due to indirect factors. However, given the potential significance of this cell type in fish muscle growth it is of importance to assess the presence of satellite cells in salmon and to investigate the direct effect of temperature, one of the most important environmental factors in muscle growth of poikilotherms, on these cells. In this study, cell culture was applied to Atlantic salmon satellite cells and the direct effect of temperature on cell volume and nuclear numbers determined.

Materials and methods

Animals. Fish were reared at the DAFS Freshwater Fisheries Laboratory, Pitlochry, Scotland. Juveniles of an average length of 7.2 cm were sent to London and the cells isolated there.

Isolation of myosatellite cells. The method of Koumans et al.[19] was modified. The fish were stunned by a blow to the head and killed by decapitation. In order to sterilise the surface of the fish, they were immersed in 70% ethanol for 30 s, the mucus was scraped off and the fish were sprayed with 70% ethanol. Axial white muscle was excised under sterile conditions and collected in 90% Dulbecco's modified Eagle medium containing non-essential amino acids (DMEM; Gibco BRL, Paisley, United Kingdom) supplemented with 15% Horse serum (HS; Gibco) and PenStrep (50 U/ml penicillin, 100 µg/ml streptomycin; Sigma, Poole, United Kingdom). The medium was buffered using HEPES (Sigma) at a concentration of 10 mM. The tissue was then thoroughly minced, spun down (300 g, 5 min, 4 °C) and washed twice in medium without serum supplement.

The fragmented tissue was then subjected to a digestion in 90% DMEM containing 0.2% collagenase (Sigma)

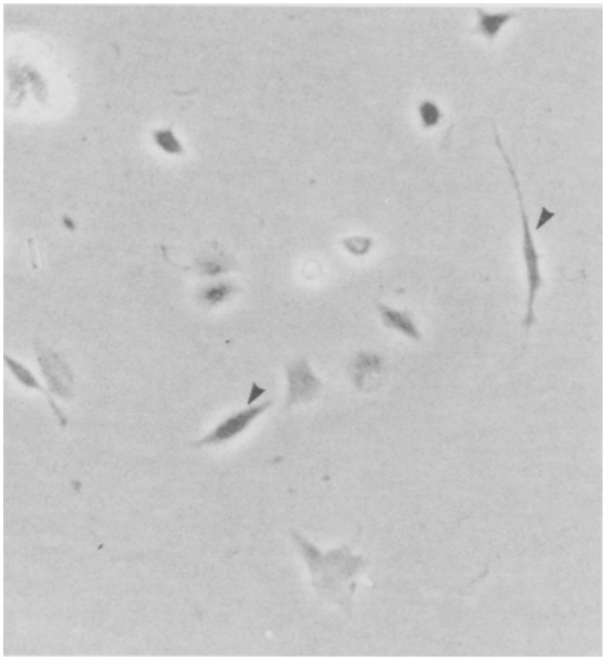


Figure 1. Atlantic salmon satellite cell culture after 24 h at 11 °C. Cells showing the typical spindle shaped morphology of a satellite cell are shown (arrowhead). Phase contrast microscopy; magnification = 200 ×.

for 90 min at 11 °C under gentle agitation (5 ml of solution per g tissue excised). The resulting suspension was washed once in 90% DMEM, triturated five times through a 10 ml pipette and washed again (300 g, 5 min, 4 °C).

The resulting pellet was resuspended in a 0.1% trypsin (Sigma, Poole, United Kingdom) solution (5 ml per g of tissue excised) and incubated for 30 min at 11 °C with gentle agitation. The suspension was centrifuged for 1 min at 300 g, the supernatant aspirated and collected in two volumes of ice-cold medium containing serum (complete medium). The pellet was subjected to another trypsin digestion step (2.5 ml of solution per g of excised tissue) and finally diluted in 2 volumes of complete medium.

All the resulting cell suspensions were spun down (300 g, 15 min, 4 °C). The pellet was resuspended in ice-cold complete medium, triturated and filtered through firstly 40 µm and then 20 µm Nytex filters. The cells were then washed once more and plated at a concentration of 2×10^6 cells/ml.

The myosatellite cells were purified by adhesion to laminin coated culture flasks as described by Koumans et al.[11]. The combined cells from 10 fish were left to adhere for 20 min at 11 °C without disturbance. 8×10^6 cells were plated per 25 cm^2 culture flask. The supernatant containing non-adherent cells was then aspirated and complete medium added.

Cell culture. After isolation and purification, the cells were incubated at either 5 °C or 11 °C in cooled incubators (LMS, Sevenoaks, United Kingdom). The culture medium (90% DMEM supplemented with 15% HS) was changed every ten days and no excessive vacuolisation indicating undernourishment was observed. The growth of the cells was monitored visually every two days.

Analysis. Pilot studies had shown that the cells develop and differentiate much faster at the higher temperature. The cultures incubated at 11 °C were fixed in absolute methanol for 5 min and stained with Leishman's stain (Merck, Lutterworth, United Kingdom) after 20 days of culture when most cells appeared to have fused as judged by visual inspection. The cultures incubated at 5 °C were fixed and stained in the same way after 48 days. After this period of time the 5 °C cultures showed the same appearance in visual microscopic inspection as the 11 °C cultures at 20 days. Briefly, the substratum was covered with the stain, after 1 min an equal amount of phosphate buffer (pH 6.8; Gurr; Merck, Lutterworth, United Kingdom) was added and the cells were incubated for 15 min at room temperature. The stain was then washed off with more phosphate buffer until the cells appeared just pink. Phosphate buffered saline (PBS) containing 0.1% sodium azide was added and the cells were photographed.

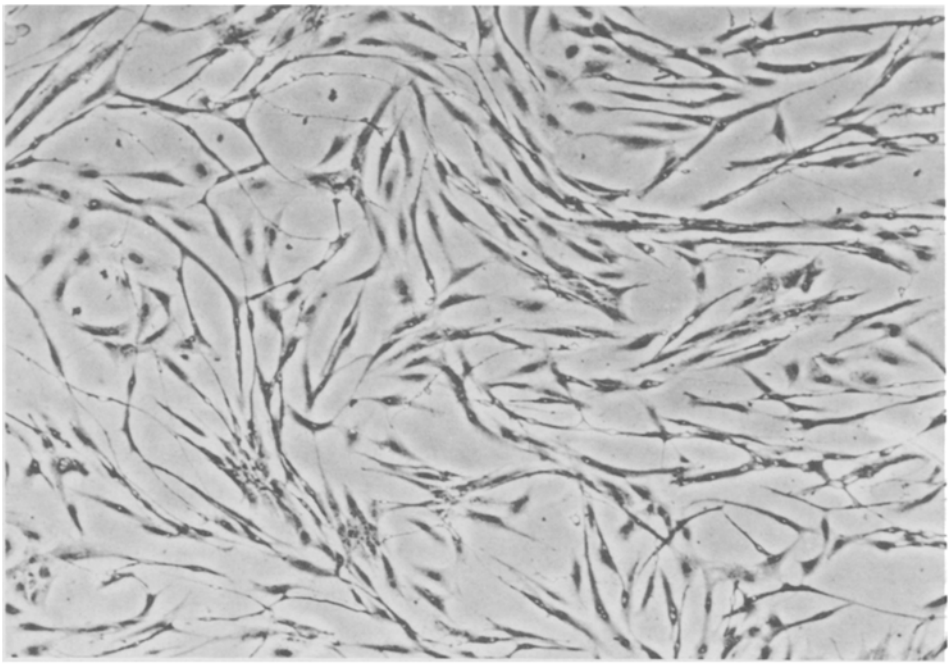
Ten randomly chosen fields of vision from the fixed and stained cultures, each corresponding to an area of 0.13 mm^2, for each temperature were used to determine nuclear numbers and cell size with a Seescan image analyser. For a comparison of the two temperature treatments at 20 days unstained cultures were used. The original number of adherent cells was determined also from unstained cultures.

Antibody staining. To verify that the cultured cells produced muscle specific protein, the cells were stained using an anti-myosin heavy chain mouse monoclonal antibody (83b6; a kind gift from Dr. G. K. Dhoot). The cells were fixed for 5 min in −20 °C cold 50% acetone/50% ethanol. Incubation took place in PBS containing 0.5% bovine serum albumin at a dilution of 1:200. Horseradish peroxidase conjugated rabbit anti-mouse immunoglobulin (Dako Ltd. High Wycombe, United Kingdom) was used as the secondary antibody. Chromogen, H_2O_2, and substrate mixture (AEC) from a DakoPAP kit (Dako Ltd.) were applied to visualise the bound antibody.

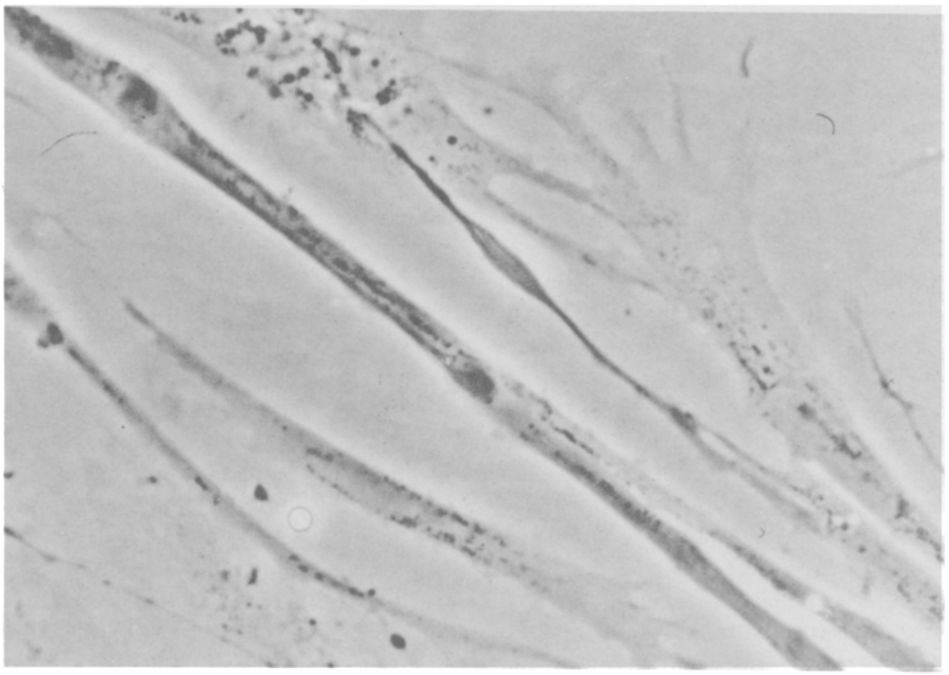
Statistics. Nuclear numbers and cell sizes were compared using a Student t-test.

Results

Morphology and immunocytochemistry. Cells exhibiting the spindle shaped morphology of satellite cells[18,19] were first detected after 24 h of culture (fig. 1). Fusion of cells was first observed after 5 days of culture for 11 °C and 9 days of culture at 5 °C. At least 50% of the cells had fused after 13 days at the higher temperature and after



A

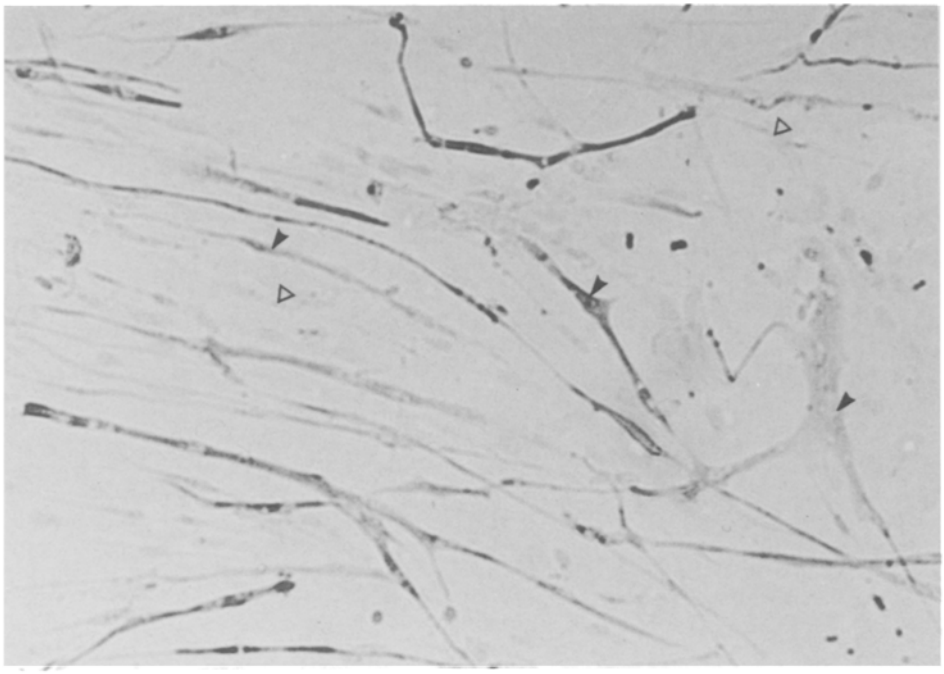


B

Figure 2. Atlantic salmon satellite cell culture.
A) Appearance of cultures incubated at 5 °C for 48 days after isolation. Almost all of the cells had undergone fusion. Phase contrast microscopy; magnification = 80 ×.
B) Myotubes formed from the isolated satellite cells cultured for 20 days at 11 °C. Note the myotube containing several nuclei. Phase contrast microscopy; magnification = 400 ×.

22 days at 5 °C with virtually all cells having fused after 20 days and 48 days, at 11 °C and 5 °C respectively (fig. 2A). The formed myotubes showed the typical morphology described in the literature (fig. 2B).

In addition to the morphological criteria, the ability of the cells to synthesise muscle protein, in this case myosin heavy chain, was confirmed by immunocytochemistry. The reactivity of the antibody was tested on frozen sections of juvenile salmon. 83b6 was found to bind to red and white muscle fibres but not to any other tissue in the fish (results not shown). In the cultures the antibody did bind to the majority but not to all myotubes (fig. 3A). Cross-striated cells could be detected after the antibody staining (fig. 3B).



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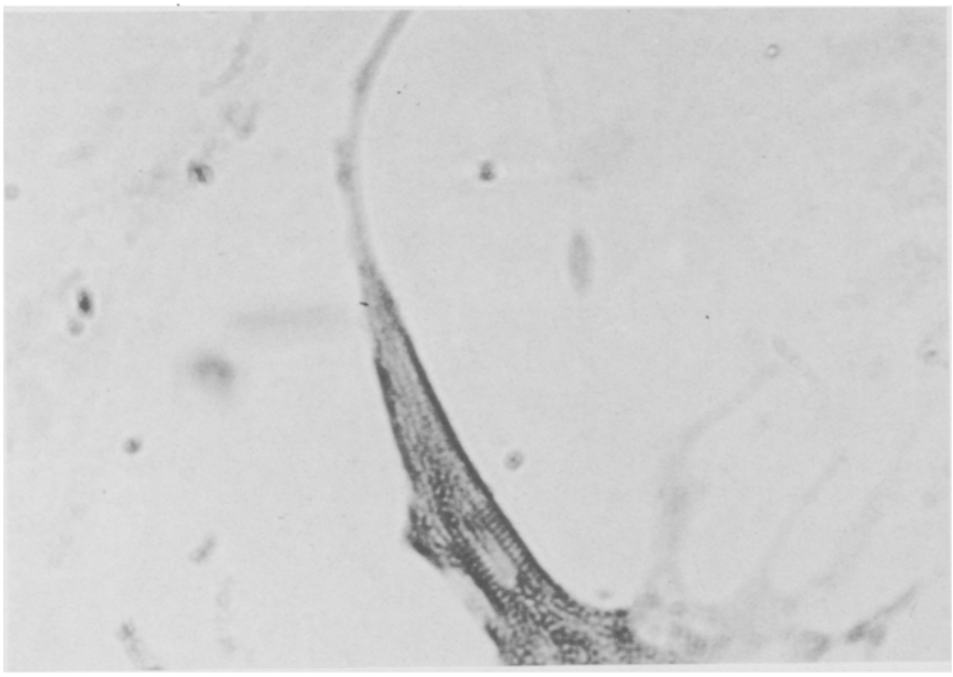


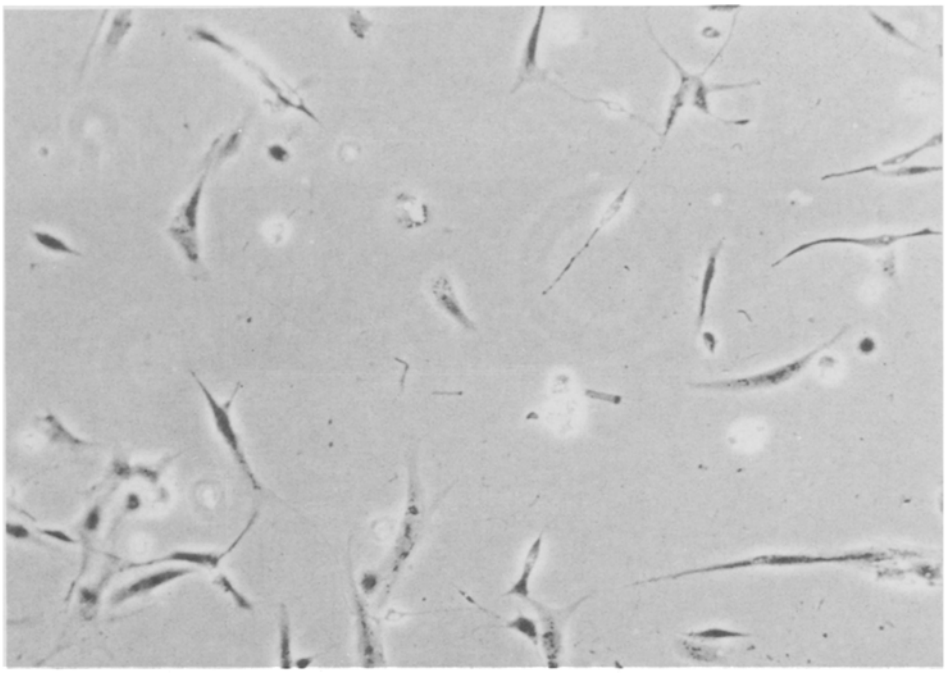
B

Figure 3. Binding of mouse monoclonal antibody specific for myosin heavy chain (83b6) to cultured Atlantic salmon satellite cells.
A) Cells cultured at 5 °C for 50 days. Bound antibody is shown as a dark stain. Some myotubes with bound antibody are emphasised with filled arrowheads; some antibody-negative cells are highlighted with open arrowheads. Magnification = 200 ×.
B) Strongly stained myotube from the above culture. Note the cross-striations visible within the cell. Magnification = 600 ×.

Nuclear numbers and cell size. The results are summarised in the table. The number of cells that attached to the laminin coated substratum after 20 min at 11 °C was 2.6×10^5 cells per g of tissue. This represents about 5% of the total number of cells (30×10^6) plated. After 20 days of culture there was no statistically significant difference in nuclear numbers between the two temperature treatments ($p = 0.855$). However, the development and growth in cell size had proceeded at a much faster speed at the higher temperature. This is reflected in a highly significant difference in the area occupied by the cells ($p < 0.001$) and is also clearly visible (fig. 4A, C). The cultures incubated at 5 °C were grown on to 48 days, by which time the appearance of the culture was

Table. Percentage of substratum occupied by cells and nuclear numbers per mm^2. Standard errors are given. nd = not determined.

Temp., Age	0 d	5 °C, 20 d		11 °C, 20 d	5 °C, 48 d		11 °C, 20 d
Area (%)	0.3 ± 0.03	6.4 ± 0.8		29.4 ± 0.8	40.2 ± 4.8		38.6 ± 4.1
p			< 0.001			0.797	
Number/mm^2	156 ± 6	118 ± 6		117 ± 5	165 ± 10		167 ± 9
p			0.855			0.857	



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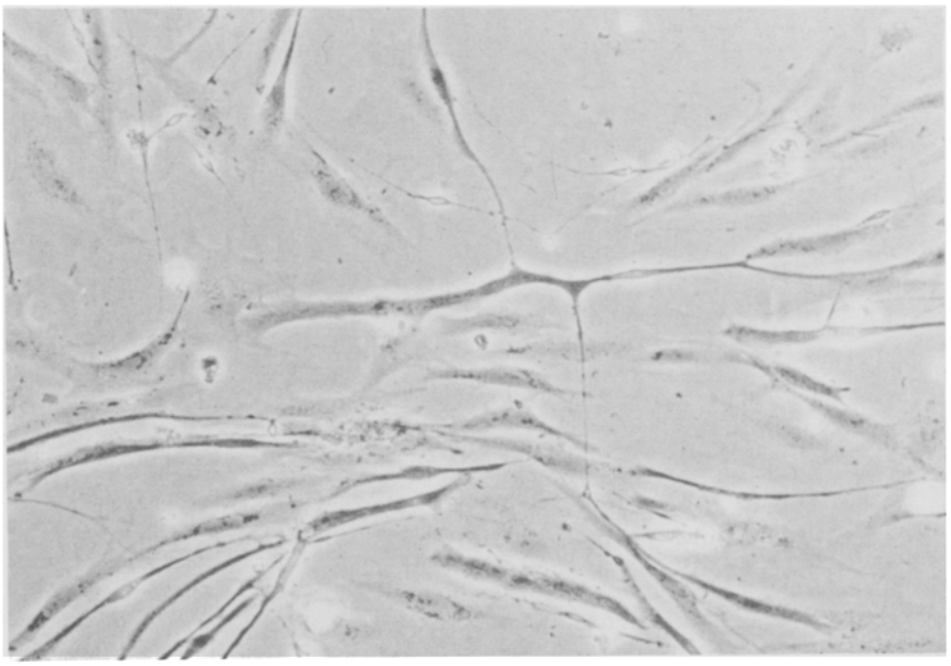
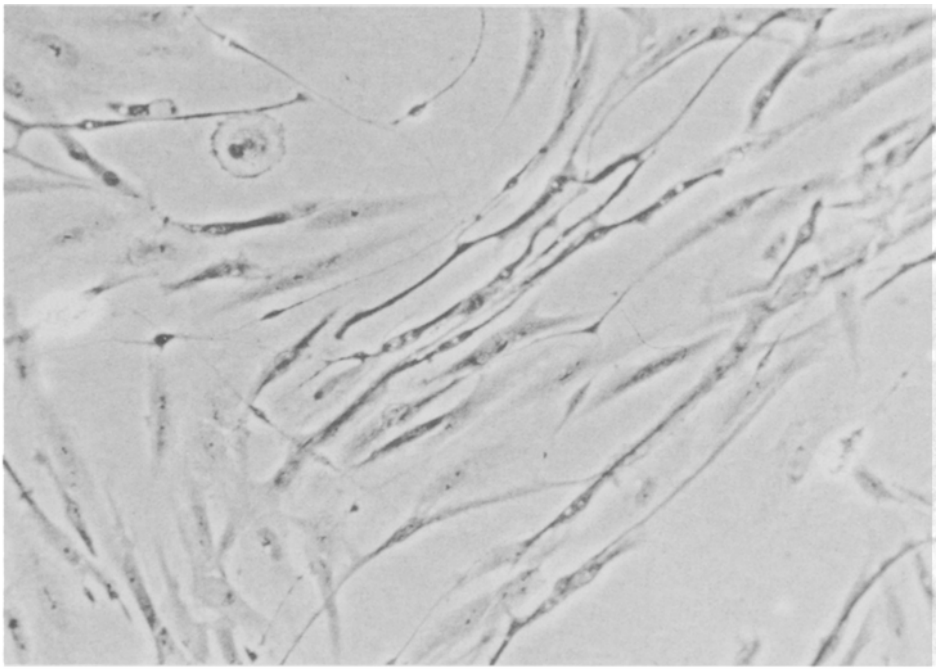


B

Figure 4. Parts A, B. (for legend see part C on following page).



C

Figure 4. Satellite cell culture from Atlantic salmon. Phase contrast microscopy was used.
A) Cells cultured at 5 °C for 20 days.
B) Cells cultured at 5 °C for 48 days.
C) Cells cultured at 11 °C for 20 days.
Note that *B*) and *C*) are virtually indistinguishable in appearance and that virtually all cells have fused in these cultures. Magnification = 200×.

identical to the one incubated at the higher temperature for 20 days (fig. 4C). Image analysis revealed that there was still no statistically significant difference in nuclear numbers between the two temperatures at this stage ($p = 0.857$). The nuclear numbers determined in this case are higher than above. This is due to the fact that unstained cultures were used for comparing the cultures at day 20 whereas stained cells were analyzed when day 20 at 11 °C was compared with day 48 at 5 °C. This led to an underestimate of the actual number present in the former case. Interestingly, the cell sizes at 5 °C had increased to an extent that there was no significant difference between the two temperature treatments any more ($p = 0.797$; 5 °C at 48 days and 11 °C at 20 days).

Discussion

Salmon satellite cells were cultured for the first time in this study. Myosatellite cells are small, spindle-shaped cells found under the basal lamina of muscle fibres and are generally held responsible for the formation of new muscle fibres as well as for supplying nuclei to existing fibres undergoing growth (mammals[16]; fish[17]). Their presence has been confirmed in many fish species (e.g. trout[18]; eel[20]; carp[21]).

Satellite cells have been previously cultured from carp[13,19] and from trout[18]. In this study, the cultured cells were identified as satellite cells on the basis of the following criteria: 1) The morphology of the cells was in accordance with that found for carp and for trout. 2) The vast majority of the cells isolated proceeded to fuse and formed myotubes. 3) Myofibrillar protein was demonstrated in a substantial number of these myotubes formed (see fig. 3A). This further demonstrates the involvement of myosatellite cells in the development of fish muscle. Interestingly, a yield of cells similar to that found in the carp[13] was achieved in the salmon.

This is the first study in which an attempt was made to analyse the direct influence of temperature on myosatellite cells. The analysis concentrated on two factors, namely nuclear proliferation and increase in cell size. Temperature influences the growth rate of fish muscle in salmonids[9], and muscle growth is associated with an increase in nuclear numbers[22,23]. However, in this study it was found that no proliferation took place in the myosatellite cell population isolated. This is consistent with the findings of Koumans et al.[19] who found only a very small proportion of proliferating cells in the carp, as measured by BrdU incorporation 17 h after isolation, and no change in the number of muscle nuclei. Powell et al.[18] found no obvious proliferation in the trout.

In contrast, it has been found that in older carp a higher percentage of myosatellite cells show proliferation[24]. Koumans et al.[24] speculate that this is due to two subpopulations of satellite cells being present in fish. One subpopulation, which exhibits a postmitotic nature, would be responsible for hyperplasia and is therefore found at stages growing mainly by this mechanism. The other subpopulation would be involved in growth by muscle fibre hypertrophy. In salmon growth occurs mainly by hyperplasia in fish longer than 6.5 cm[15] and the satellite cells isolated in this study may therefore

represent the above mentioned postmitotic subpopulation.

However, an alternative explanation may be the presence of indirect factors, such as hormones or growth factors, leading to proliferation in vivo. This could also explain the findings in the carp[19]. The presence of at least some proliferation at isolation is shown by BrdU incorporation. These cells seem to rapidly lose their proliferative capacity, however, and undergo differentiation. With only 10% of the cells in the carp showing BrdU incorporation a change in nuclear numbers would thus not become apparent.

Temperature influenced the speed of differentiation in the cultures. Fusion occurred earlier and proceeded much faster at the higher temperature. However, more interestingly, cell size was very dramatically affected. At day 20 the cells raised at 11 °C were 4.6 times larger than the cells incubated at 5 °C. It has to be borne in mind, however, that after 20 days less than 50% of the lower temperature cells had fused whereas most of the higher temperature cells had undergone fusion. The large difference found may therefore represent a developmental difference, rather than a difference in the hypertrophic growth of myofibres. When cultures containing mostly differentiated myotubes are compared, i.e. after 20 days for 11 °C and after 48 days for 5 °C, a Q_{10} of 4.0 can be calculated. This is not dissimilar to the Q_{10} of 5.75 found for total protein growth in fed rainbow trout[9] and may suggest a direct temperature control of hypertrophic growth. The result clearly shows that a surprisingly high increase in hypertrophic growth of myotubes can be effected directly by temperature.

In summary this study has shown that, in the salmon, there is an absence of proliferative activity in at the least a subset of myosatellite cells. This may be due to the absence of indirect factors necessary for proliferation. Temperature had a very pronounced direct effect on fusion, differentiation and hypertrophic growth of satellite cells and myotubes in culture.

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