Looking for markers of muscle growth and flesh quality in fish

Abstract
Substantial progress in aquaculture has been made in the last ten years. Research on flesh quality and especially on muscle growth regulation is, however, still insufficient. In vitro and in vivo studies were performed in order to evaluate AKT, AKTP, MAPK, MAPKP, and c-Met as indicators of growth and muscle quality and to determine the best conditions in terms of feeding and water circulation for sustainable and efficient fish aquaculture. Expression analyses on the level of protein and/or mRNA in myocyte cultures promote the in vitro studies as an useful tool to identify the molecules that may indicate the stage of the muscle development and presence of satellite cells, in an attempt to determine those conditions that result in better muscle growth and flesh quality.

Key Words: rainbow trout (Oncorhynchus mykiss), gilthead sea bream (Sparus aurata), expression analysis, myocyte culture, myogenesis

Zusammenfassung
Titel der Arbeit: Suche nach Markern von Muskelwachstum und Fleischqualität beim Fisch

Schlüsselwörter: Regenbogenforelle (Oncorhynchus mykiss), Goldbrasse (Sparus aurata), Expressionsanalysen, Myozyten-Kultivierung, Myogenese

Introduction
A great deal of progress in aquaculture has been made in the last ten years and knowledge of growth protocols, nutrition requirements, diet formulation etc., for many fish species has increased significantly. The substitution of vegetable products for fish meal and fish oil has also been studied, and we are now much closer to finding the key to producing fish food with less dependency on fish compounds. Research on flesh quality and especially on muscle growth regulation is, however, still insufficient. Fish myogenesis is controlled by molecules similar to those described in other vertebrates; MRFs (MyoD, myogenin, mrf4, myf5), MEFs, and myostatin are described in several species (JOHNSTON, 2006). The continuous growth of many cultured species offers an interesting model for the study of myogenesis regulation.

Our objectives are to study the molecules in fish muscle that may be indicators of growth and muscle quality and that will be useful for the selection of species families and to determine the best conditions for sustainable and efficient fish aquaculture.
Material and methods

*In vitro* studies were done with rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*) juveniles from Truchas del Segre and Cupimar, respectively, maintained in the fish facilities of the Faculty of Biology (University of Barcelona). Trout and gilthead sea bream myocytes were isolated following CASTILLO et al. (2002), and MONTSERRAT et al. (2007). Myocyte culture was used to investigate the appearance of regulatory molecules throughout the *in vitro* development. The molecules studied were AKT, AKTP, MAPK, MAPKP, and c-Met. Western blot analyses were performed as described (CASTILLO et al., 2006). PCNA was studied by microscope observation of muscle cells stained with the antibody, and MyoD2 was analyzed by real time PCR based on the sequence described by TAN and DU (2002). Flow cytometry and a specific antibody were used to analyze the presence of the marker CD34.

*In vivo* studies. Trout juveniles were fed a control diet with fish meal or a diet containing plant protein instead of fish meal for 3 months. Trout juveniles were maintained in regular tanks or in tanks with water circulation to provoke forced swimming activity for 3 weeks. Dorsal muscle was taken and kept frozen until the preparation of samples for Western blot analysis.

Results and Discussion

*In vitro studies*

In fish myocytes we found the presence of MAPK and AKT at different stages of the *in vitro* development (Figure 1). The MAPK and its phosphorylated form present the highest levels at early stages, in myogenic precursor cells and myocytes. Later its expression decreased, and its response to IGFs was much lower. Considering the role of MAPK (CASTILLO et al., 2004) in proliferation, it makes sense to find these molecules highly expressed at the beginning of the culture and reduced once differentiated.

On the other hand, AKT and AKTP levels were present during the different stages of myocyte development, and they seemed to be even more active in the differentiated myotubes. These results agree with the AKT pathway’s involvement in differentiation and in several metabolic processes (CASTILLO et al., 2006), which are maximized at the myotube stage.

Rainbow trout and gilthead sea bream are different in terms of phylogeny, and biological cycle, and the cell culture is done at different salinity and temperature (MONTSERRAT et al., 2007). Furthermore, the time required to reach different muscle stages is longer in sea bream than in trout. However, it is interesting to note that in both species MAPK and AKT activation follow a similar pattern, suggesting a good evolutionary conservation of these pathways and their regulative role in muscle growth.
c-Met is the receptor of the hepatic growth factor and is considered a good marker of the satellite cells (WOZNIAK et al., 2003). Our studies on rainbow trout and gilthead sea bream showed that in a myocyte culture, c-Met presents the highest expression during the first and especially the middle stages of the culture. However once the cells reached the myocyte stage the levels decreased rapidly, and in the stage of myotube c-met was almost non detectable (Figure 2). Myocytes exposed to IGF-I (10-100nM) for 24 h presented higher levels of c-Met than control cells.

Figure 1.
STIMULATION of MAPK and AKT PHOSPHORYLATION by IGF-I (100 nM) in gilthead sea bream muscle cells at different stages of development. Cells were incubated with peptides for 30 minutes, lysed, and 30 µg of protein were loaded in each lane and subjected to a 10% SDS-PAGE under reducing conditions. Results of three independent experiments are shown as a percentage of stimulation over basal and normalized for the content of total MAPK or Akt. Different letters indicate significant differences (P<0.05).

Figure 2.
c-Met EXPRESSION IN RAINBOW TROUT MYOCYTE CULTURE at different stages of cell development. Cells were lysed and 40µg of protein were loaded in each lane and were subjected to 12% SDS-PAGE under reducing conditions. Primary antibody against c-Met (final dilution 1:500) and secondary antibody anti-rabbit (1:10000) were used. A densitometry analysis is shown above. Results are expressed as arbitrary units of densitometry and mean ± SE. Significant differences (p<0.05) are represented by an asterisk in the figure.
In rainbow trout, the profile observed for c-Met coincides with that observed for CD34, a molecule that labels those cells that have not yet been determined to myoblasts (TAMAKI et al., 2002). Similarly, in gilthead sea bream, PCNA analyses, that label the proliferative nuclei on cultured cells, showed the highest levels in the myocyte stage, decreasing progressively from that moment. Little information is available on the presence of these molecules in fish (BRODEUR et al., 2003; JOHNSTON, 2006), but these preliminary results suggest a function similar to that observed in mammals.

Finally, studies on MyoD2 in gilthead sea bream myocytes showed the highest expression during the first stages of development in satellite cells and early myocytes, but this expression decreased with the progress of the culture. Such a pattern of MyoD2 agrees with that observed in gilthead sea bream during myogenesis (TAN and DU, 2002), which would indicate that this myogenic factor plays a primary role in lineage determination.

In vivo studies
Trout fed with an experimental diet with a high level of plant protein showed less growth than in the control fish fed with a diet without substitutes. The control group presented a significantly higher c-Met level in white muscle, which might suggest that best growth is accompanied by a large number of myogenic precursors and higher proliferation. The same tendency was observed in gilthead sea bream.

Juveniles of rainbow trout subjected to forced swimming presented higher values of total AKT compared to control fish, but MAPK did not show variation between groups (Figure 3). This response would agree with the higher level of physical activity in the fish, which would then need a greater supply of metabolites.

![Figure 3. AKT EXPRESSION IN RAINBOW TROUT SUBJECTED TO FORCED SWIMMING.](image)

Myocyte culture provides a useful tool with which to identify the molecules that may indicate the stage of the muscle development and presence of satellite cells, in an attempt to determine those conditions that result in better muscle growth and flesh quality. Thus the combination of certain molecules (MAPK, c-Met, MyoD…) can suggest a muscle with a strong potency for proliferation and growth. Further studies will be necessary to verify these hypotheses.
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