Assessment of muscle biochemical and histochemical criteria for flesh quality in salmonids

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ASSESSMENT OF MUSCLE BIOCHEMICAL AND HISTOCHEMICAL CRITERIA FOR FLESH QUALITY IN SALMONIDS.

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Abstract
Fish flesh is composed of muscle, conjonctive and adipose tissues. Muscle and conjonctive tissues are supposed to play a significant role in the textural properties of the flesh. Muscle experience large qualitative and quantitative changes during development including during late development in large commercial size fish. This paper try to related these changes with flesh quality variability.
Skeletal muscle could be divided in a superficial red muscle and a deep white muscle. The contractile and metabolic characteristics of these two muscles have been extensively studied in a lot of fish species. We analysed further the variabilities of some of these characteristics within each muscle. Different criteria which described the physical, biochemical and metabolic characteristics of muscle have been developed. The validity of these criteria for the assessment of variability of flesh quality was tested in rainbow trout and brown trout.
The size of fibres was the most important criteria that was analysed in fish. The diameter of fibre is homogeneous in red muscle and heterogeneous in white muscle which is the main tissue of the flesh. Methods to analysed automatically the size of fibres in fish have been developed using image analysis. The direct relationship between mean size of fibres in white muscle and texture parameters have been tested in pan-size rainbow trout and in large size brown trout. The size of fibres explained a small part of the variability of texture in small size rainbow trout but seemed not to explain neither water-holding capacity nor texture in large size brown trout.
The contractile characteristics of muscle were assessed by the analysis of one contractile protein: myosin. Different myosin subunits isoforms were present in red and white muscle. Within the white muscle a variability related to body size was also observed in these isoforms for large size brown trout. The consequences of such changes on quality are not known.
The metabolic characteristics of muscle were also studied but the analysis of glycogen content and distribution. The glycogen content was more important in red muscle than in white muscle. A specific distribution of glycogen between fibre was observed in white muscle: the glycogen content being higher in small diameter fibre than the large one. In experimentally stressed fish the glycogen content of large fibres was more affected than that of the small fibres.

Keywords muscle, biochemistry, histochemistry, image analysis, glycogen, myosin, flesh quality, salmonids

Introduction

Fish flesh is composed of muscle, conjonctive and adipose tissues. Both the relative proportion and the characteristics of these tissues are changing during development of fish. Recent works on muscle growth have demonstrated that large qualitative and quantitative changes occur within muscle during development including late development up to large commercial size fish (Stickland 1983, Kiessling et al 1991). It is not known however how these changes affect quality of the flesh. Lipid content and composition in adipose tissues and in muscle are especially important for flavour of the flesh (Josephson and Lindsay 1986). Furthermore muscle and conjonctive tissues are supposed to play a significant role in the textural properties of the flesh (Dunajski 1979, Haard 1992, Borressen 1992). Further analysis of the characteristics of these different tissues are thus required and this work deal more specifically with muscle characteristics.

Skeletal muscle is composed of a red superficial muscle, a white deep muscle and an intermediate pink muscle (Johnston 1982). The differences in the contractile and metabolic characteristics of these muscles have been extensively studied in a lot of fish species (see for instance Rowlerson et al 1985). Schematically, red muscle is composed of slow fibres which demonstrated a low contractile activity associated with an active oxidative metabolism and white muscle is composed of fast fibres which have a high contractile activity associated with a low glycolytic metabolism. Water holding capacities and mechanical behaviour of the flesh are related to fibre types in mammals and this is probably true in fish although very few data are available on that subject (Hatae et al 1990). It could be mentioned however, that red muscle constitute only a minor part of the flesh and flesh quality is certainly more dependent of white muscle characteristics.
Furthermore, within each of these muscle, variabilities of metabolic and contractile characteristics due to non homogeneous composition of fibres could be suspected (Rowlerson et al 1985, Kiessling et al 1991, Martinez et al 1993, Koomans et al 1993). The consequences for flesh quality are not known.
The aims of this work were to analysed the physical, biochemical and metabolic characteristics of each muscle using different criteria which have been developed or adapted for that purpose. The validity of these criteria for the assessment of variability of flesh quality was tested in rainbow trout and brown trout.

Material and Methods

Fish and treatments

Rainbow trout came from our freshwater experimental facilities (SEDI Le Drennec FRANCE). Fish were reared in normal conditions for production except for the following specific experiment. Pan size rainbow trout were reared for 5 months in tanks normally supplied with water but without any flow rate (> 0.25 L/s Control group) or with a large flow rate (2.5 - 3.0 L/s Exercice group.
Rainbow Trout were anaesthetized using monophenyl ether ethylen glycol (0.4 p. thousand) and then they were killed by a sharp blow on the head.
Brown trout came from our sea water experimental facilities (SEMII Camaret sur mer). Brown trout were sampled from a genetic breeding program for individual growth selection (PROSPER, Chevassus 1991). Brown trout were anaesthetized by immersion in chilling water and CO2 diffusion, then they were blooded by section of branchial arches.

Tissues sampling and treatments.

A transverse section of muscle was taken in the posterior part of dorsal muscle between adipose fin and anal fin for histology analysis. These samples were normally frozen in isopentane solution cooled up to solidification (-80°C) by liquid nitrogen. Then they were stored at -80°C. For determination of fibre size, muscle samples were alternatively treated as follow. Samples were fixed for 3-4 hours in 2 % paraformaldehyde solution in phosphate buffer (NaH2PO4 5mM, Na2HPO4 10 mM, pH 7.4), then rinsed twice (30 min) with phosphate buffer, immersed successively in 10 %, 15 % and 20 % sucrose solutions respectively for 1h, 2 h and overnight and frozen as described above in cold isopentane. The whole procedure was realized at 0-2 °C.
Red superficial muscle was finely dissected (within 2-3 min) and a sample of dorsal white muscle was taken and both were frozen in liquid nitrogen and stored at -20°C until biochemical analysis.

Biochemical treatment and analysis.

Myofibrillar proteins were extracted from muscle samples using successive mixing (Polytron 10 000 rpm) and centrifugation (2000 g, 15 min) in 5 to 10 volumes of low ionic strength buffer at 0-2°C. Three buffers were successively used (A/ KCL 80mM, Tris-HCl 10 mM, EDTA 4 mM, ß-mercaptoethanol 5 mM, sodium azide 1 mM pH 7.0; B/ KCL 50 mM, EDTA 1 mM, ß-mercaptoethanol 5 mM, sodium azide 1mM pH 6.4; B/ KCL 50 mM, ß-mercaptoethanol 5 mM, sodium azide 1 mM pH 6.4). The final residue was resuspended in buffer C. A sample of the suspension was taken and diluted in 1N Sodium Hydroxide solution for protein analysis using Lowry et al. (1951) method. Another sample was taken and diluted 3 to 4 times in 2% SDS solution (Tris HCl 62.5 mM, ß-mercaptoethanol 5 %, glycerol 2 %, bromophenol 20 mg/l pH 6.8) for electrophoresis.

Myofibrillar protein were separated using PAGE procedure (Laemmli 1970). For myosin light chain separation, 10 to 20 µg protein were separated on 13 % acrylamide slab gels (60mm x 80mm x 1mm). The migration was realized at 100-120V (60 mA) and lasted 1 to 2 hours. For myosin heavy chain, 0.5 to 1 µg protein were separated on 4 % acrylamide slab gels (1 mm) containing 37.5 % glycerol. The migration was realized at 180-200V (20 mA) and lasted 4 to 5 hours. Absorbencies due to Light Chain bands on the 13 % acrylamide gels were analyzed after numerization of the gel with a CCD camera followed by automatic searching and quantification of bands on grey level image using E1d software (Trubuil et al. 1993).

Glycogen, glucose/glucose6P and lactate were measured according to Fernandez et al. (1992). Glycogen was extracted from muscle samples (200 mg) by mixing in 10 volumes of PCA 0.5M. Aliquotes of the suspension were taken and use for glycogen analysis. The remain was centrifuged (4000 g, 20 min) and the supernatant was used for lactate analysis.
An aliquote of muscle suspension was neutralized (KOH 30%) and submitted to an amylo-glucosidase reaction (3 hours , 37 °C, pH 4.8). Glucose and glucose 6P were analysed after hexokinase and glucose 6P dehydrogenase reaction (3 hours, 37°C, pH 7.5) by the production of NADH (O.D. at 340 nm). An aliquote of the suspension was analysed directly for glucose and glucose 6P content using the same procedure. The result was subtracted from the first determination to give the glycogen content.
Lactate was measured on the previous supernatant after lactate dehydrogenase reaction using the production of NADH (O.D. at 340 nm).

Muscle section preparation and treatments.

Sections (15 to 20 µm) of freeze muscle sample were realized at -24°C, laid on glass. The section were immersed in 1 % azorubine acid solution (molybdic acid 0.5%, acetic acid 1%) during 1.5 h for non specific coloration of fibres. Then section were normally dried and included in resin solution.

For actomyosin ATPase determination the procedure of Guth and Samaha 1970 was used with small modifications on 10 µm muscle sections. The sections were preincubated at different acid and basic pH and for different times. The optimum acid conditions for differentiating red and white muscle fibres ATPase activity was pH 4.3 during 3 min. The preincubation in basic solution even during short times (few minutes) desactivated both red and white fibres ATPase activities. Then ATPase was revealed using normal procedure with ATP incubation and ammonium sulphur inorganic phosphate revelation.
Succinic dehydrogenase activity was revealed on muscle section using procedure of Nachlas et al. 1957.
Glycogen was revealed on the section using the PAS reaction (Van del Laarse et al. 1992).

Tissue section analysis

Section were visualized on a microscope at x 16 to x 25 magnification and photographs (24 x 36 slides) representative of the section were taken.

For fibres size determination, two different procedures were used. The first procedure is a semi-automatic analysis. The slides were projected on a digitalizer tablet connected to a PC computer. The principal points of the profile of fibres were pointed using a mobile cursor and the computer calculated the area of fibres.
The second procedure is an automatic analysis. The slides laid on a light tablet were numerized using a monochrome CCD camera (Cohu CCD 512) connected to a numerization card (Matrox PIP 1024) in a PC computer. A specific software was realized for automatic analysis of fibres size using classical image analysis routine of the software Visilog (Noesis France) (Fig 1). After acquisition of the image (512 x 512 frame), the format of the image was change from a rectangular to an hexagonal grid to take into account the polygonal shape of the fibres. Then, it was transformed in a binary image using a two levels threshold (the intensity of azorubine tainting of small fibres is generally higher than that of large fibre) which separated clearly fibres and extracellular network. The centre of fibres were search using a fast erosion routine. A step of manual correction of the centre (suppression or addition) was proposed. The number of 1 pixel erosions necessary to reach the centre of the fibre was affected as intensity level to each centre. This image was analysed as a labelled image were each object is composed of the set of centres affected by the same intensity level (the same erosion level). The number of centre of each object correspond to the

euler number of the object. The analysis of the labelled image produced directly a distribution of fibre size. A calibration procedure and a transformation to relative distribution of fibre size was then performed. The fibres were classified according to the number of 1 pixel erosions necessary to reach their centres.

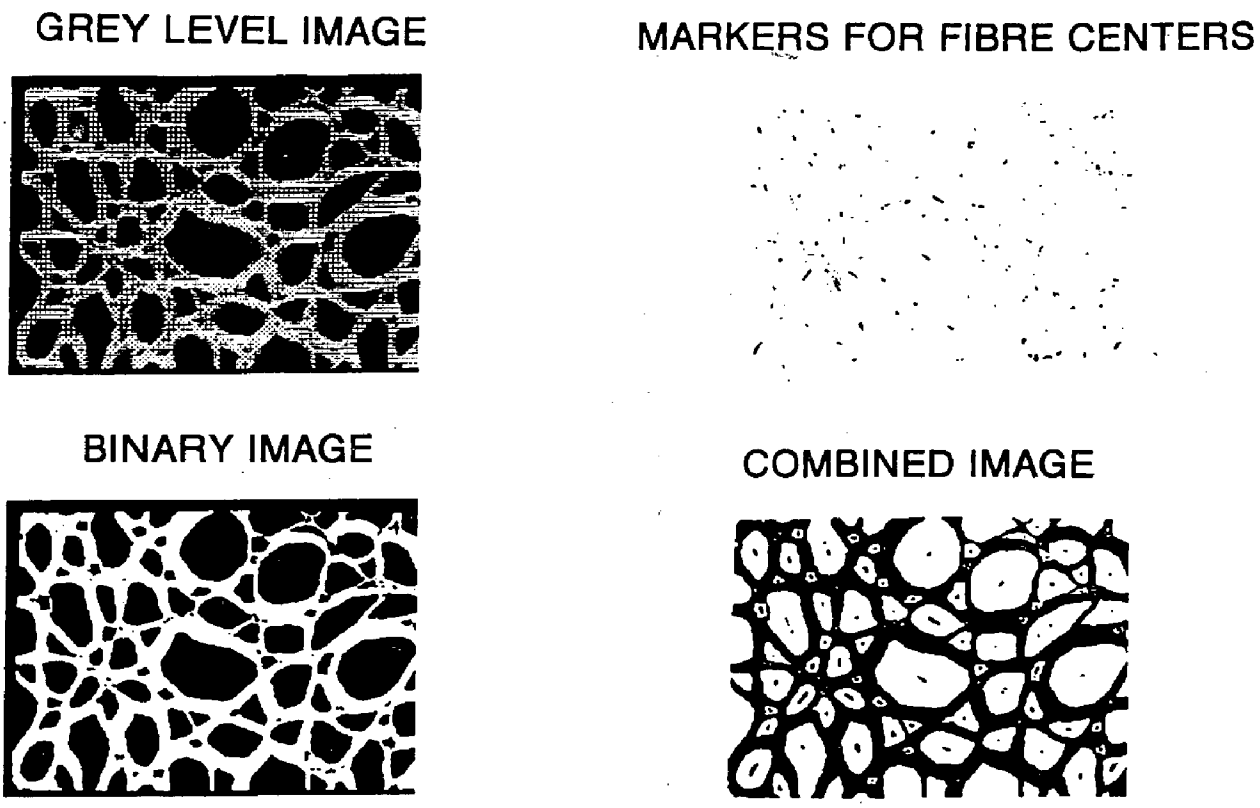

Figure 1: Schematic representation of different steps of automatic fibre size analysis using image analysis.

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For glycogen analysis a semi automatic procedure was used. The slide of the PAS glycogen section was projected on a opaque board and pattern of the network surrounding fibres was copied manually on a transparent using a felt-tip pen. The network image was numerized using the system already described. Then the PAS glycogen slide was also numerized after manual matching of the two images. The network image was transformed as a binary image, then after an inversion to obtained the image of fibre as objects it was transformed to a labelled image. Each fibre was thus identified and different quantitative shape and area parameters could be calculated. Different criteria of the intensity related to PAS coloration could also be analyzed in the area of each fibre using the PAS glycogen image. The area and the mean intensity of PAS coloration within each fibre was only retained.

Calculation and Statistical analysis.

The percentage of small fibres (< 25 µm) and the mean diameter of large fibres (> 25 µm) were calculated from the distribution of fibres. Multivariate analysis were performed using STATGRAPHICS software. Multidimensionnal analysis (Principal components analysis) were realized using SPAD software.

Results and Discussion

Size of fibres

It is well known that the distribution of fibre size are completely different in red and white muscle (Johnston 1982) and that was confirmed in our analysis (Figure 2). Furthermore, the diameter of fibre is homogeneous in red muscle and heterogeneous in white muscle which is the main tissue of the flesh. Such heterogeneity in white muscle has been related to the specific growth process of muscle in fish by recruitment of new fibres (Stickland 1983, Kiessling et al 1991). Consequently, the variability in muscle characteristics is related both to the presence and importance of small diameter fibres and to the more classical increase in the size of fibres. The two parameters used to described the distribution of fibre diameters is thus justified in term of growth process. Is is so also for the variability of quality of fish flesh. That was analysed in pan-size rainbow trout and large size brown trout.

The variability of fibre size parameters in white muscle was analysed in 40 pan-size rainbow trout (250-300g) and tentatively related to chemical composition, colour and texture of raw and cooked flesh. The variability in the characteristics of the flesh was related to different exercice (see Fauconneau et al 1993 for more details). It was demonstrated in salmon that a moderate exercice modified the distribution of fibre size with an increase in the number of small diameter fibres and the appearance of very large diameter fibres (Totland et al 1987). None of these effects were observed in our experiment where fish were submitted to intense exercice. The chemical and physical characteristics of the raw and cooked flesh were clearly separated (Figure 3). However none of the parameters of muscle fibres size were related to these characteristics. Part of the variability of percentage of small diameter fibres and mean diameter of large fibres seems to be related to characteristics of cooked flesh.

The variability of fibre size parameter was also analysed in 70 large size brown trout. At that stage the recruitment of small fibres is stopped (Stickland 1983) although we have observed that it seems to reappeared in the anterior part of dorsal muscle (Kiessling et al 1991). In our experiment samples were realized in the posterior part of muscle, thus we increase the threshold for the calculation of small diameter fibres (25 to 35 µm). In that experiment no relationship with chemical and physical characteristics: colour, water-holding capacity and texture of fish flesh were observed (see Bauvineau et al. in that issue).

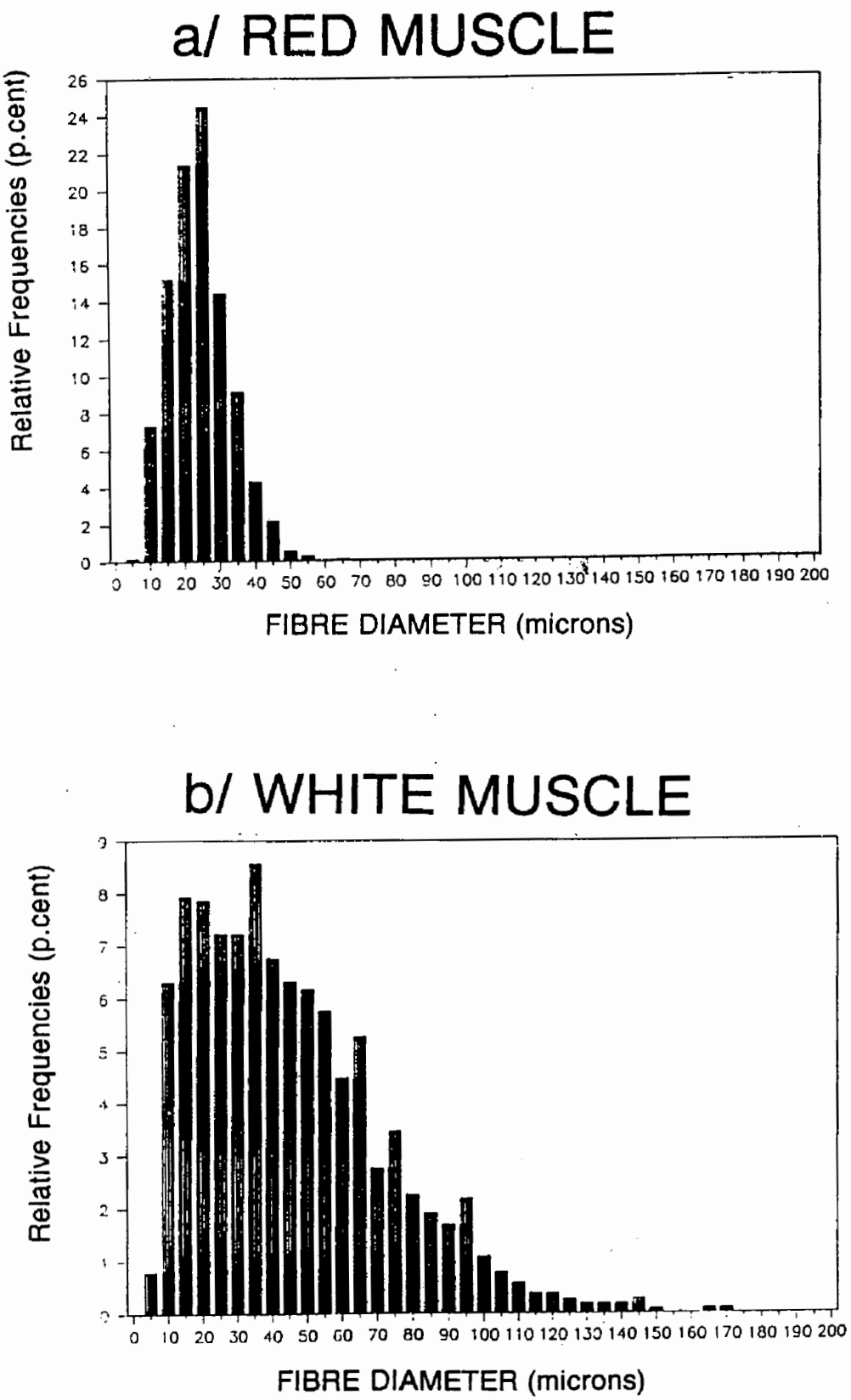


Figure 2: Distribution of fibre diameter in white and red muscle of rainbow trout (500-600g BW).

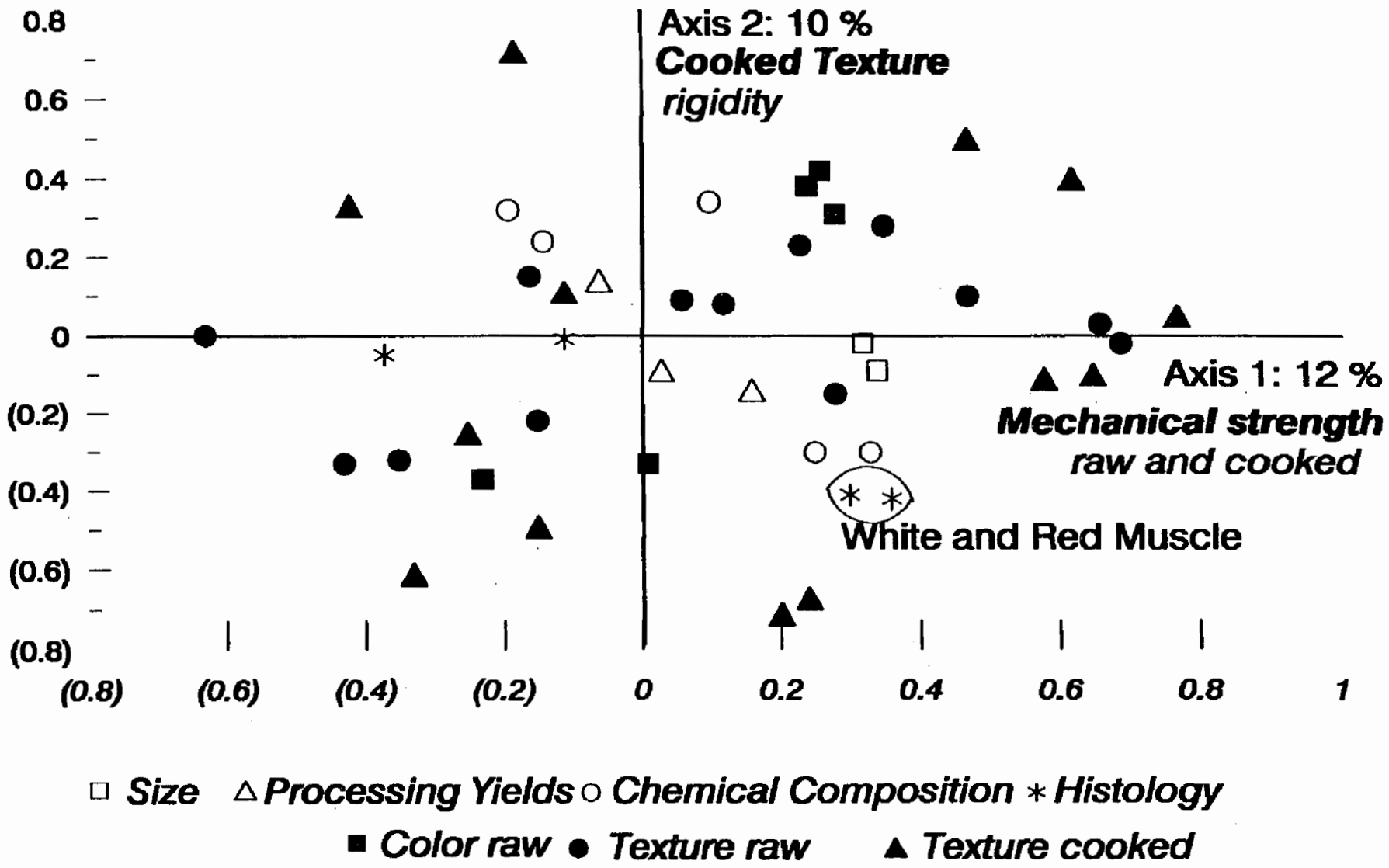


Figure 3: Principal components analysis of 53 parameters related to morphology, chemical composition, mean fibre sizes in white and red muscle, mean cell sizes in dorsal and adipose tissues, color and texture of raw and cooked flesh. Analysis on 40 pan-size (250-300 g) rainbow trout.

Biochemical fibres type.

The contractile characteristics of muscle were assessed by the analysis of one contractile protein: myosin in rainbow trout and brown trout. Different myosin subunits isoforms were found in red and white muscle (Table 1). This was observed for myosin heavy chain of rainbow trout and brown trout. It has already been found in Arctic charr and Atlantic salmon (Martinez et al 1991, Martinez et al 1993). The same was observed for myosin light chain as it has already been found in rainbow trout and Atlantic salmon (Martinez et al. 1993, Fauconneau et al. 1994). These different isoforms are expressed in different fibre types which composed the two muscles: slow twitch oxidative fibre type in red muscle and fast twitch glycolytic fibre type in white muscle (Johnston 1982).

Table 1 Isoforms of myosin subunits found in brown trout (2.7 kg)

	RED MUSCLE	WHITE MUSCLE
Myosin Heavy Chain		
MHCs	100 %	
MHCf	trace	100 %
Myosin Ligth Chain		
LC1s, LC2s	63	5
(n=32)	(8)	(2)
LC1f/LC3f, LC2f	37	95
(n=32)	(8)	(2)
LC3f/LC1f	1.8	1.5
	(0.3)	(0.7)

It is however more interesting to analysed the variability within on muscle of these characteristics. We found in red muscle of large size brown trout and rainbow trout the presence of light chains specific from fast myosin (LC_1fast, LC_2fast and LC_3fast) in relatively large proportion. It could be explained partially by contaminations or remains of red muscle by intermediate muscle. However such light chains were not observed in smaller fish where red muscle has been dissected in the same conditions (Fauconneau et al 1994). The presence of fast fibres within red muscle has been observed also by Rowlerson et al. (1985) in relatively large rainbow trout. Thus it seems that red muscle is not so homogeneous.

The relative proportions of light chains was also analysed in white muscle. The myosin molecule is composed of two heavy chains and four light chains. One light chain, the alkali LC_2 is always present in duplicate in myosin molecule, the other light chains LC_1 and LC_3 are present in variable proportion but in a stoechiometric ratio with LC_2. It is known that during differentiation of fast fibres LC_1 is first expressed then as fibres matured, LC_3 became more and more important (Martinez et al 1993). The ratio LC_3/LC_1 thus reflect the state of differentiation of fast fibres. During the last stages of rearing of brown trout (1.5 to 5 kg), it appeared that the ratio of LC_3/LC_1 increase with size (Figure 5). Thus muscle is more mature at 5 kg than at 1 kg. The consequences of such changes on quality are not known but changes due to body size have already been observed in brown trout (Bauvineau et al 1993).

Histochemical fibre types

The classical determination of fibre types using actomyosin ATPase and SDH only confirm that metabolic and contractile activities of white and red muscle are different (Johnston 1982, Rowlerson et. 1985). It could be added also that glycogen content of red muscle is higher than that of white muscle. This is directly a consequence of higher metabolic activity in red than in white muscle (Johnston 1982, Fauconneau et al 1984). Furthermore, the analysis of distribution of glycogen in white muscle is especially interesting.

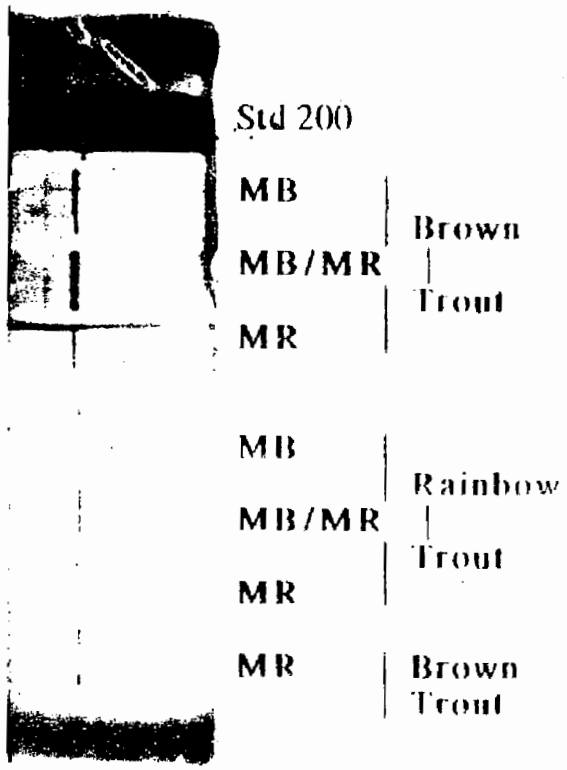


Figure 4: Myosin heavy chain in white and red muscle of pan-size (250-300g BW) rainbow trout and brown trout.

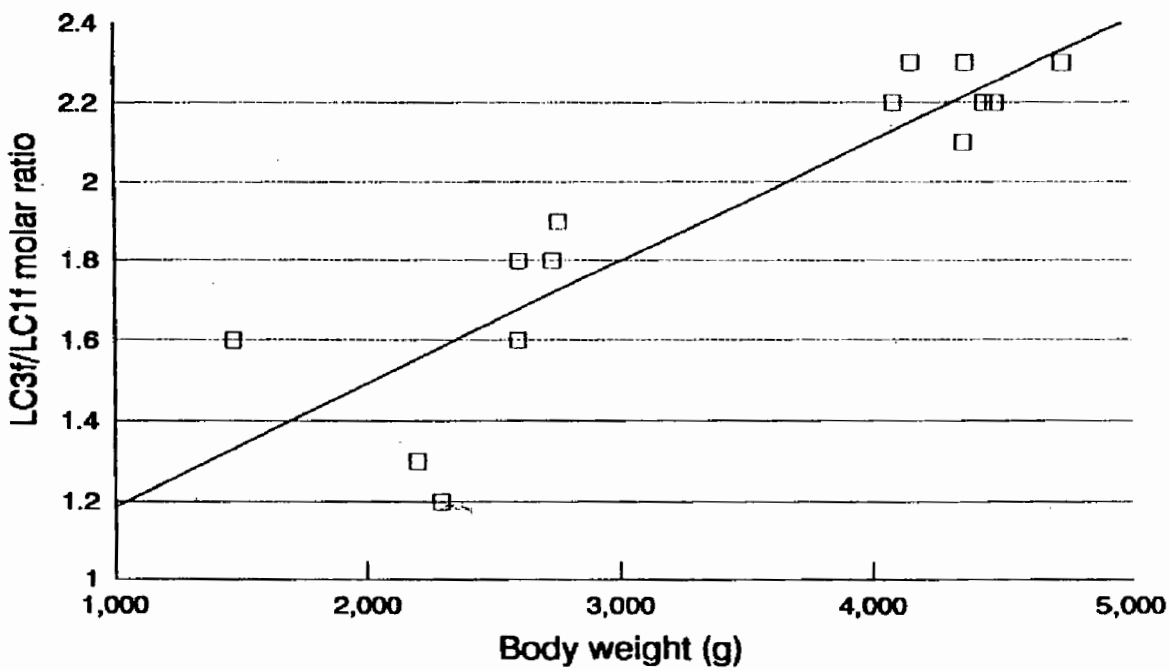


Figure 5: Changes in alkali ligth chain ratio (LC3f/LC1f) used as an index of white muscle differenciation in the flesh of large size brown trout (1.5 to 4.5 kg). Each point is the mean of 10 individuals.

We found in rainbow trout and in brown trout that the glycogen content is higher in small diameter fibres than in the large one (Figure 6). Such difference has been mentioned earlier (Rowlerson et al. 1985, Kiessling et al. 1991), however it has never been quantified. We observed for instance that the glycogen / size relationships distribution seemed to be affected by intrinsic factors such as triploidy in brown trout. It has been observed in rainbow trout that triploidy induces an alteration in muscle protein metabolism (Fauconneau et al. 1990). Such differences in muscle metabolism could explain the slightly lower growth performances of triploid fish.

The consequences for quality of the glycogen distribution and especially changes after slaughtering were analysed. Glycogen constitute the energy store of white muscle *in vivo*. *In vitro* glycogen is the only source of energy and its consumption is directly involve in the post-mortem changes of pH through the production of lactate (Azam et al. 1990). In rainbow trout and brown trout, we found in experimentally stressed fish (injection of adrenalin compared with control fish or fish injected with a beta agonist Suacron R), that the glycogen content of muscle was depleted and that the glycogen content of large fibres was more affected than that of the small fibres (Figure 7). Such heterogeneities in glycogen distribution and in depletion of glycogen were also observed in response to fasting (Table 2). These changes in response to stress and fasting have probably some consequences on the behaviour of the flesh during post-mortem storage and on final quality.

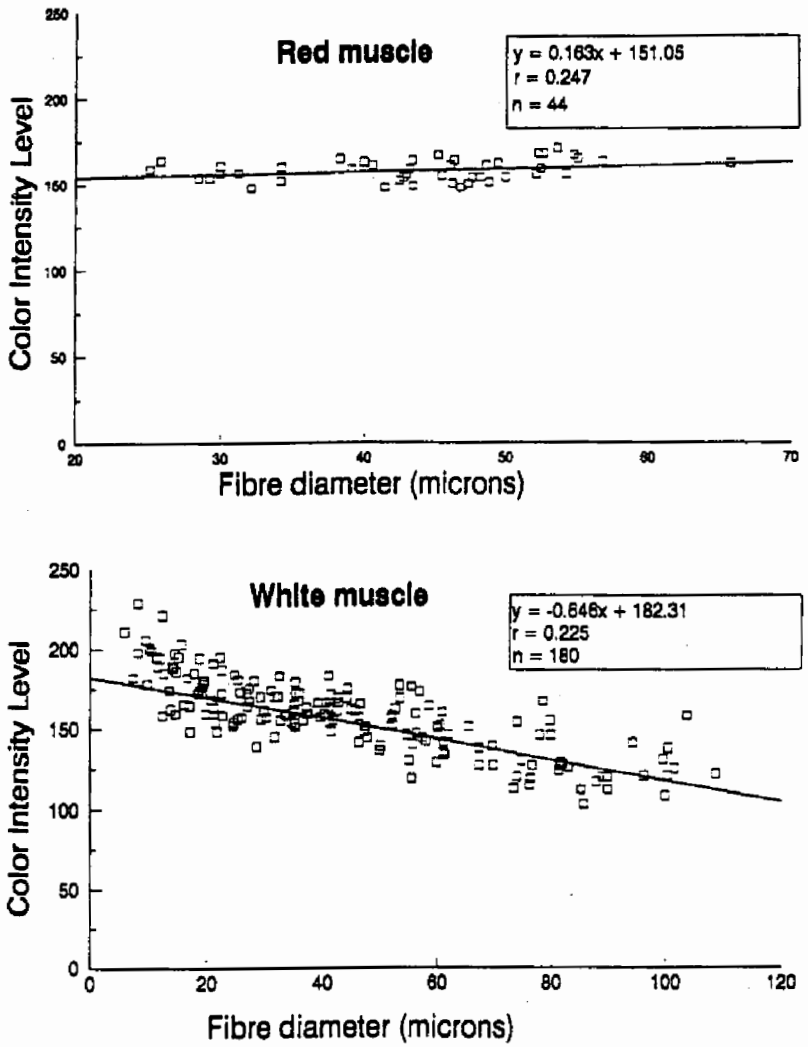
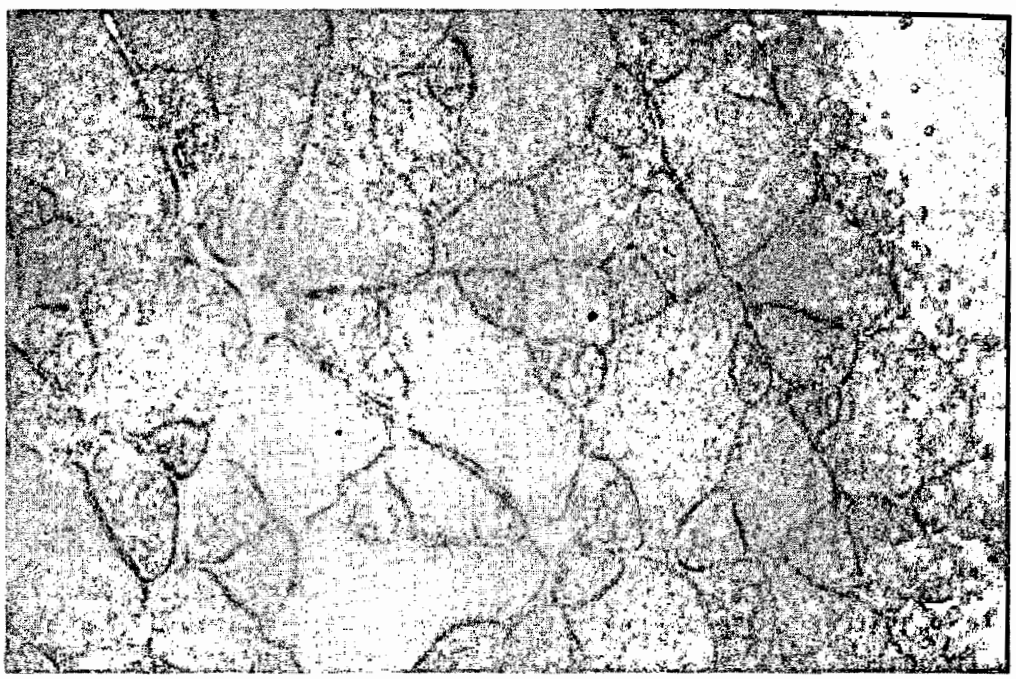


Figure 6: Glycogen (color intensity using PAS reaction) and mean diameter (microns) distribution of red and white muscle fibres of pan-size rainbow trout (200-250g BW).

a



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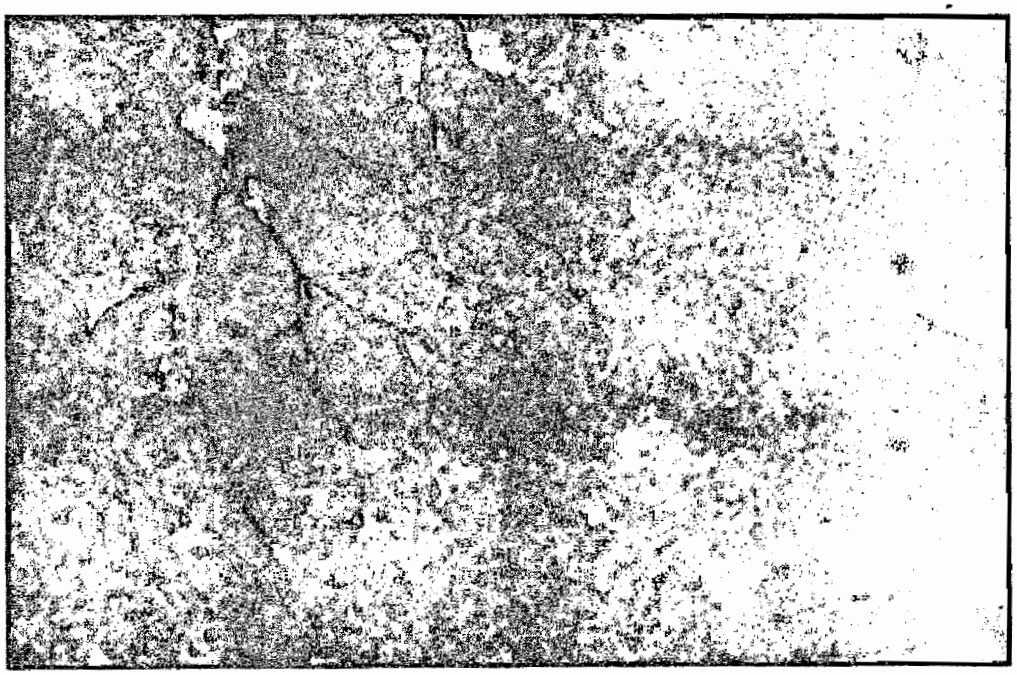

Figure 7: Glycogen content and distribution in white muscle fibres of rainbow trout (1.5 kg BW) injected either non stressed (a/ injection of a beta agonist Suacron R) or stressed (b/ injection of adrenaline).

Table 2 Effect of experimental stress on glycogen (absorbance unit) and white muscle fibre diameter (microns) relation ship. Brown trout (2800 g BW) were either injected with adrenaline or with a beta-agonist (suacron Solvay France)

	CONTROL	ADRENALIN	Beta AGONIST
Small diameter fibres	16.4	11.1	16.2
(relative percentage)	(3.7)	(4.2)	(8.9)
White fibres diameter	77.5	74.4	74.2
(microns)	(8.7)	(8,7)	(4.5)
Glycogen	17,6	15,0	18,0
(µmol/g)	(4.5)	(3.0)	(3.6)
Maximal Intensity	195	194	211
(Absorbance)	(26)	(22)	(17)
Slope of regression	-0.07	0.10	0.07
	0.03	0.05	0.03

Conclusions

The different characteristics measured on muscle demonstrated that there was an heterogeneity in the main muscle of the flesh: white muscle. This criteria gave a basis for a specific typing of fish muscle in fish based on fibre size distribution, biochemical (LC_3f relative distribution) and metabolic (glycogen distribution) characteristics. Such heterogeneity is certainly related to growth process but it has yet to be proved. There should be in the future a growing interest on the study of the relationship between such muscle typing and flesh quality.

Résumé

La chair du poisson est composée de muscle, de tissus conjonctif et adipeux. Le muscle et les tissus conjonctifs sont supposés jouer un rôle significatif dans les propriétés de la texture de la chair. Le muscle subit de grands changements qualitatifs et quantitatifs durant le développement y compris dans ses phases ultimes liées à la production de poissons commerciaux de grande taille. Ce papier tente d'établir un rapport entre ces changements et les variabilités de qualité de chair. Le muscle squelettique peut se diviser en muscle rouge superficiel et muscle blanc profond. les propriétés contractiles et métaboliques de ces deux muscles ont été étudiées chez de nombreuses espèces de poissons. Nous analysons de façon plus approfondie la variabilité de certaines de ces caractéristiques dans chaque muscle. Différents critères décrivant les caractéristiques physique, biochimique et métabolique du muscle ont été développées. La validité de ces critères pour l'estimation de la variation de la qualité de la chair a été testée chez la truite arc-en-ciel et la truite commune. La taille des fibres a été le critère le plus important étudié chez les poissons. Le diamètre des fibres est homogène dans le muscle rouge et hétérogène dans le muscle blanc, qui est le principal tissu de la chair. Des méthodes d'analyse automatique de la taille des fibres chez les poissons ont été développées grâce à l'analyse d'image. Des relations directes entre la taille moyenne des fibres du muscle blanc et des paramètres de texture ont été recherchées chez des truites arc-en-ciel de taille portion et chez la grande truite commune.

La taille des fibres explique une faible part de la variabilité de texture des premières et ne semblent pas expliquer du tout ni la capacité de rétention en eau, ni la texture des secondes.

Les caractéristiques contractiles du muscle ont été appréhendées par l'analyse d'une protéine contractile la myosine. Différentes sous unités isoformes de myosine sont présentes dans les muscles rouge et blanc. A l'intérieur du muscle blanc de la grande truite commune, on a observé une variabilité liée à la taille du corps dans ces isoformes. Les conséquences de tels changements sur la qualité sont inconnues.

Les caractéristiques métaboliques du muscle ont été étudiées par l'analyse des teneurs en glycogène et de sa distribution. La teneur en glycogène est plus importante dans le muscle rouge que dans le muscle blanc.

Dans ce dernier on a constaté une distribution spécifique de la teneur en glycogène, celle-ci étant plus importante dans les fibres de petit diamètre que dans celles de grand diamètre.
Dans le cas de poissons stressés expérimentalement, la teneur en glycogène des grandes fibres est plus affectée que celle des petites fibres.

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