Muscle growth mechanisms in response to isoenergetic changes in dietary non-protein energy source at low and high protein levels in juvenile rainbow trout

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Abstract:

This study investigates muscle growth mechanisms in juvenile rainbow trout in response to isoenergetic changes in dietary non-protein energy (NPE) source (F, fat vs. C, carbohydrates) at two levels of digestible protein to digestible energy (DP/DE) ratio. Fish (initial weight 32.4g) were fed four diets having similar DE levels ($\sim 18 \text{ kJ g}^{-1}$) with a high ($H_{P/E} \sim 26 \text{ mg kJ}^{-1}$) vs. low ($L_{P/E} \sim 14 \text{ mg kJ}^{-1}$) DP/DE ratio using F or C as major NPE source (7 week-experiment). The lowering of dietary DP/DE ratio increased myoblast determination protein 1a (*myod1a*) and decreased myostatin 1b (*mstn1b*) and cathepsin D (*ctsd*) muscle mRNA levels. The isoenergetic change in dietary NPE from F to C decreased *myod1a* and proliferative cell nuclear antigen (*pcna*) muscle mRNA levels. An interaction between DP/DE ratio and NPE source was observed in muscle transcript levels of myogenic factor 6 (*mrf4/myf6*), fast myosin heavy chain (*fmhc*) and fast myosin light chain 2 (*fmhc2*). White muscle total cross-sectional area decreased at low dietary DP/DE ratio and also when NPE source changed from F to C, linked i) to a decreased total number of white muscle fibres, indicating that low dietary DP/DE restricted muscle hyperplasia and that dietary carbohydrate were less efficiently used than fat to sustain muscle hyperplasia, and ii) to decreased percentage of large muscle fibres, indicating limited fibre hypertrophy. Not only the DP level or the DP/DE ratio, but also the isoenergetic change in dietary NPE source (fat vs carbohydrates) thus appears as a potent regulator of muscle hyperplasia and hypertrophy.

Keywords:

Fish nutrition; Dietary macronutrient composition; Myogenesis; Hyperplasia; Hypertrophy; Gene expression; Cellularity.

Highlights of the manuscript:

- Isoenergetic changes in dietary macronutrient composition alter the gene expression of positive actors (*myod1a*, *mrf4*, *pcna*, *fmhc*, *fmlc2*) and negative regulators (*ctsd*, *mstn1b*) of muscle growth.
- Isoenergetic changes in dietary macronutrient composition also alter skeletal muscle cellularity (number and size distribution of muscle fibres).
- A high DP/DE ratio combined with fat as NPE gave the highest hyperplasia and hypertrophy and thereby the highest muscle growth.
- Not only the DP level or the DP/DE ratio, but also type of NPE source appeared as a potent regulator of muscle hyperplasia and hypertrophy.

Statement of relevance

Determining the effects of dietary protein level and non-protein energy source on muscle growth mechanisms will help to develop diets maximizing muscle growth.

1. Introduction

The different steps of myogenesis, i.e., commitment of muscle precursors, myoblast proliferation, migration, alignment, and fusion into myotubes are under the control of diverse regulatory factors among which myogenic regulatory factors (MRFs) and growth factors (Buckingham and Rigby, 2014). In higher vertebrates, myoblast determination protein (MyoD), myogenic factor 5 (Myf5) and myogenic factor 6 (Mrf4/Myf6) function as myogenic determination factors. Myogenin (Myog) acts as a differentiation factor, as can Mrf4 and MyoD, controlling the differentiation of myoblasts into skeletal muscle fibres. Myogenic progenitor cells can either activate Myf5 and MyoD and differentiate or proliferate, providing a reserve cell population for muscle growth during development (Buckingham and Rigby, 2014). MRFs also play a role during activation of muscle satellites cells, the stem cells of adult skeletal muscles, for muscle growth and regeneration. In higher vertebrates, these cells are normally mitotically quiescent but are activated and re-enter the cell cycle in response to stress. The daughter cells of the activated satellite cells undergo multiple rounds of division prior to fusion with the existing or new fibres. Activated satellite cells first express either Myf5 or MyoD, and both factors are co-expressed during the proliferative phase (Sabourin and Rudnicki, 2000). The MRFs similarly drive the normal developmental-related myogenesis of fish (Rescan, 2008) and are also expressed during fish muscle regeneration (Funkenstein et al., 2006; Montfort et al., 2016). In rainbow trout (*Oncorhynchus mykiss*), as in other fish species reaching a large adult size, the growth of white skeletal muscle, which constitutes the bulk of the axial locomotor muscle, occurs after hatching by both the recruitment of new muscle fibres (hyperplasia) and the enlargement of the previously formed fibres (hypertrophy) (Stickland, 1983; Weatherley et al., 1980).

81 The understanding of the regulation of the transcription of myogenic genes by specific
82 nutrients in fish is still fragmentary. Nevertheless, changes in dietary macronutrient source
83 (Alami-Durante et al., 2010b; Cai et al., 2018b; Overturf and Gaylor, 2009; Shi et al., 2017;
84 Valente et al., 2016) and composition (Alami-Durante et al., 2014; Campos et al., 2010;
85 Chapalamadugu et al., 2009) have been found to regulate the transcript level of certain MRFs
86 in fish juveniles. Changes in dietary macronutrient source or composition can also alter in fish
87 juveniles the mRNA level of components of myosin, the most abundant protein in muscle
88 tissue (Alami-Durante et al., 2010b, 2014; Campos et al., 2010; Hevroy et al., 2006; Valente
89 et al., 2016) and the transcript level of negative regulators of muscle growth such as
90 lysosomal proteases (Alami-Durante et al., 2010a; Overturf et al., 2016) and myostatin (Cai et
91 al., 2018b; Galt et al., 2014; Overturf et al., 2016). The changes initiated at molecular level on
92 the genes controlling muscle growth mechanisms (hyperplasia or/and hypertrophy) might lead
93 to changes in muscle cellularity (i.e. in the number or/and the size distribution of muscle
94 fibres). In fish juveniles, white muscle cellularity is being altered by changes in dietary
95 protein source and amino acid supplementation (Alami-Durante et al., 2010a, 2010b, 2018;
96 Cai et al., 2018a; Valente et al., 2016) as well as by changes in the dietary protein level.
97 Importantly, changes in dietary protein level were mostly accompanied by concomitant
98 changes in non-protein energy level and/or source (lipids and/or carbohydrates) (Bjornevik et
99 al., 2003; Overturf et al., 2016, Silva et al., 2009). The white muscle cellularity of fish
100 juveniles has also been shown to be impacted by changes in dietary protein/energy ratio
101 during early life, as demonstrated in rainbow trout (Alami-Durante et al., 2014). However,
102 nothing is known on the potential confounding effects of both dietary factors, i.e. the protein
103 level and the digestible non-protein energy (NPE) level and source, on the muscle growth
104 mechanisms in fast-growing juvenile fish.

Therefore, in order to improve our knowledge on the regulation of fish muscle growth by macronutrients, we analyse here the effects of feeding isoenergetic diets (in terms of DE) differing in digestible protein (DP, high or low) and in the main source of non-protein energy (NPE, fat vs. carbohydrates) on the muscle growth mechanisms of rainbow trout juveniles by using molecular (expression of myogenic and muscle growth-related genes) and cellular (number and size distribution of muscle fibres) approaches.

2. Materials and methods

2.1. Growth experiment and sampling procedures

The experiment was conducted with a certificate for experimenting on live animals (N°B6410006) and complied with the EU Directive for the protection of animals used for scientific purposes (N°2010/63/EU) and the French Decree for animal experimentation (N°2013-118). Twelve groups of 35 juvenile rainbow trout (*Oncorhynchus mykiss*, initial mean weight of 32.4g) were reared in an INRA experimental fish farm (Donzacq, France; agreement N°A40-228-1). The experimental setup consisted of 12 independent circular tanks (150 L) in a flow-through system (flow rate 0.4 L sec⁻¹) supplied with natural spring water (temperature of 17±1°C) under natural light regimen (February-April). The fish were fed in triplicate (3 tanks per diet) twice a day to visual satiety with one of the four test diets formulated to be isoenergetic and to contain either a high (H_{P/E} diets) or a low (L_{P/E} diets) digestible protein to digestible energy (DP/DE) ratio. At each DP/DE ratio, a contrast was created in the non-protein energy source (NPE) by substituting fat by an iso-energetic amount of gelatinized maize starch (on DE basis). Cellulose was added as an inert filler at the high fat diets. This resulted in four diets highly contrasting in macronutrient composition: H_{P/E}F,

H_{P/E}C, L_{P/E}F, L_{P/E}C (Table 1). The four diets had a similar digestible energy content (~ 18 kJ g⁻¹) and contrast in DP/DE ratio between H_{P/E} diets (~26 mg kJ⁻¹) and L_{P/E} diets (~14 mg kJ⁻¹) (measured digestible nutrient values; Saravanan et al., 2012). In total, the feeding trial lasted for 7 weeks. During the first 6 weeks (growth period) we assessed feed intake, growth and nutrient utilization, and then fish were allowed to recover for 1 week (recovery period) before sampling for histological and molecular analyses. During the growth period, mortality was monitored daily and fish were group weighed every 2 weeks to calculate intermediate growth and feed intake. A random sample of 36 h feed deprived fish were euthanized (overdose of anesthesia, 2-phenoxy-ethanol) and stored at -20°C for subsequent analyses of whole body composition, at the beginning (35 fish) and end (8 fish per tank i.e. 24 per dietary treatment) of the growth period. At the end of the 6 weeks, all fish were counted and weighed to calculate the final body weight of fish. The fish were then continued to be fed their respective diets for a period of 1 week (recovery period) prior to sampling (7 h post-feeding; 3 fish per tank i.e. 9 fish per dietary treatment) for muscle histological and molecular analyses. These fish were euthanized (overdose of anesthesia, 2-phenoxy-ethanol), weighed and measured, and a transverse section of 1 cm width was cut at their vent level, fixed in Serra's liquid, dehydrated and preserved in butanol until further histological analysis. For analysis of expression of muscle growth-related genes, pieces of dorsal white muscle from the same 9 fish per treatment were frozen in liquid nitrogen and stored at -80°C until molecular analysis.

2.2. Chemical analyses

Whole fish from each tank were ground, pooled and fresh moisture content was determined. Fish were subsequently freeze-dried before further analyses. The nutrient composition of fish and diets were analysed according to the following procedures. Feed and

whole body samples were analyzed for dry matter (105°C for 24 h), protein (Kjeldahl; N x 6.25) after acid digestion, fat content of feed (Folch et al., 1957) using dichloromethane instead of chloroform and fat content of fish by petroleum ether extraction (Soxhlet; 40-60°C) and gross energy content by adiabatic bomb calorimeter (IKA-Werke C5000). Ash contents were determined by combustion in muffle furnace (550°C for 12 h). Starch content was determined as glucose, using the amyloglucosidase/ hexokinase/glucose-6-phosphate dehydrogenase method after ethanol (40%) extraction and starch decomposition in dimethylsulfoxide/ HCl (ISO, 2005).

2.3. *Quantitative histological analyses*

The muscle samples kept in butanol were embedded in paraffin and cut transversely into sections (10µm thick) that were stained with hematoxylin and orange G (Gabe, 1968). Muscle cellularity was examined after 7 weeks of feeding on one section per fish, located at the vent level. The total cross-sectional areas of white muscle (TCSAWM) and the individual cross-sectional areas of white muscle fibres were quantified with ImagePro software. Three muscle areas were selected per fish (Fig. 1) in order to quantify stratified hyperplasia, mosaic hyperplasia, maximum hypertrophy and to measure a number of muscle fibres higher than 1000 per fish, as previously assessed for rainbow trout juveniles (Alami-Durante et al., 2018). The individual equivalent diameter of white fibres (diameter of a circle of equal area to that of the muscle fibre; hereafter referred to as “fibre diameter”, d) was then determined. The total number of white muscle fibres (TNWF) was estimated from the total cross-sectional area of white muscle and the mean cross-sectional area of measured white muscle fibres.

2.4. *RNA extraction and quantitative RT-PCR*

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181 Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA)
182 according to the manufacturer's instructions, and stored in Nuclease-free water at -20°C.
183 Samples were subjected to electrophoresis on 1% agarose gels to confirm the integrity of the
184 28S and 18S rRNA bands, and RNA purity was assessed by the 260/280 nm absorbance ratio.
185 cDNA was generated from 1 µg of total RNA using 200 U SuperScript™ III Reverse
186 Transcriptase (Invitrogen) and 500 ng of random primers (Promega) in a total volume of
187 20 µL. RNA samples were heated at 25°C for 10 min and then at 55°C for 1 h to generate
188 cDNA, followed by heat inactivation at 70°C for 15 min.

189 The primers used for myogenic regulatory factors [myogenic factor 5 (*myf5*), myoblast
190 determination protein 1a (*myod1a*), myogenin (*myog*), myogenic factor 6 (*mrf4/myf6*),] and
191 those used for genes involved in cell proliferation [proliferative cell nuclear antigen (*pcna*)],
192 muscle structure [fast myosin heavy chain (*fmhc*), fast myosin light chain 2 (*fmlc2*)],
193 proteolytic pathways [cathepsin D (*ctsd*), calpain 2 (*capn2*)], growth regulation [myostatin 1a
194 (*mstn1a*), myostatin 1b (*mstn1b*)] were previously published, like those for 18S RNA (Alami-
195 Durante et al., 2010a, 2014; Chauvigné et al., 2003; Donato et al., 2008; Johansen and
196 Overturf, 2006; Kamangar et al., 2006; Moghadam et al., 2007; Salem et al., 2006). All
197 primers were synthesised by Eurogentec (Belgium) and amplicons confirmed by sequencing
198 (Eurofins Genomics, Ebersberg, Germany).

199 For real-time RT-PCR assays, the Roche Lightcycler 480 system was used (Roche
200 Diagnostics, Neuilly-sur-Seine, France). The assays were performed using a reaction mix of 6
201 µL per sample, each of which contained 2 µL of diluted (dilution=50) cDNA template, 0.12
202 µL of each primer (10 µmol L⁻¹), 3 µL Light Cyclers 480 SYBR® Green I Master mix and
203 0.76 µL DNase/RNase-free water (5 Prime GmbH, Hamburg, Germany). The PCR protocol
204 was initiated at 95°C for 10 min for initial denaturation of the cDNA and hot-start DNA

polymerase activation and continued with 40 cycles of a two-step amplification program (15 s at 95°C; 30 s at 60°C). Melting curves were systematically monitored (temperature gradient at 0.5°C 10 s⁻¹ from 55 to 94°C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. Each real-time RT-PCR run included replicate samples (duplicate of reverse transcription and PCR amplification) and negative controls (reverse transcriptase- and cDNA template-free samples). Standard curves, consisting of five serial dilutions in triplicate of a pool of cDNAs, were obtained for each cDNA template by plotting CT values against the log₁₀ of the different dilutions. Real-time PCR efficiency (E) was calculated from standard curves according to Pfaffl (2001). *18S* mRNA abundance was similar for the four conditions and was used for the normalisation of qPCR data. The 18S-adjusted data were analysed using the $\Delta\Delta CT$ method (Pfaffl, 2001) with the H_{P/E}F-fed juveniles as a control.

2.5. Statistical analysis

Statistical analyses were performed with R 3.2.3 Software (R Development Core Team, Vienna, Austria, www.R-project.org) and SigmaStat 3 computing program (SPSS, Chicago, IL, USA). Statistical analyses were performed either on means of triplicates (body growth, feed intake, whole body composition) or on individuals (muscle cellularity, muscle gene expressions) as previously assessed (Alami-Durante et al., 2010b). Differences between dietary groups were analyzed using two-way ANOVA to test the effect of dietary DP/DE ratio and NPE source and their interaction. The Newman-Keuls multiple range test was used to compare means when a significant difference ($P < 0.05$) was found. These analyses were performed on data previously ln-transformed (weight, length, total cross-sectional area of white muscle, white muscle fibre diameters), arcsin-transformed (percentage of white muscle

fibres in a diameter class), square root-transformed (total number of white muscle fibres), or ranked (mRNA relative expressions) as Levene tests revealed a non-homogeneous variance for many raw data. Pearson product moment correlation coefficients were calculated to assess links between trout individual body growth, muscle cellularity and gene expression data. Correlations were considered significant when $P < 0.05$.

3. Results

3.1. Trout growth performance

The trout's feeding response to these four diets, in terms of intake, nutrient retentions, metabolism and body growth has been described previously (Saravanan et al., 2012, Figueiredo-Silva et al., 2013). In short, trout growth performance was affected by dietary DP/DE ratio and NPE source (Table 2). At high DP/DE ratio, final body weight, thermal growth coefficient (TGC) and protein gain (PG) were not significantly different between groups fed diet $H_{P/E}F$ and $H_{P/E}C$. At low DP/DE intake, final body weight, TGC and PG were lower in trout fed carbohydrate ($L_{P/E}C$) relative to fat ($L_{P/E}F$) as main NPE source. Trout lipid gain (LG) was higher in L- than C-fed trout. The body growth of trout fed the $L_{P/E}F$ diet did not differ significantly from that of fish fed diet $H_{P/E}C$. The feed intake of $L_{P/E}F$ -fed trout did not differ from that of $H_{P/E}F$ - and $H_{P/E}C$ -fed trout. The lowest growth was found in fish fed diet $L_{P/E}C$ which had the lowest feed intake. The protein retention efficiency of $L_{P/E}F$ -fed trout was higher than that of the trout fed with the three other diets.

254 3.2. Transcription of genes involved in myogenesis, cell proliferation, muscle structure and
255 function, proteolysis and growth regulation

256
257 Dietary DP/DE ratio and NPE source affected the gene expression of the myogenic
258 regulatory factor *myod1a* without interaction ($H_{P/E} < L_{P/E}$ and $F > C$) while an interaction
259 between both dietary factors was seen for the gene expression of myogenic regulatory factor
260 *mrf4* (highest in $L_{P/E}F$), with higher gene expression of *mrf4* in $L_{P/E}F$ -fed trout than in trout
261 fed the 3 other diets (Table 3). The transcript levels of the myogenic regulatory factors *myf5*
262 and *myog* were not affected by changes in dietary composition. The muscle transcript level of
263 *pcna*, involved in cell proliferation, was not affected by changes in DP/DE but decreased
264 when NPE was carbohydrates ($F > C$). The relative mRNA abundance of the two fast myosin
265 chains studied (*fmhc* and *fmlc2*) was affected by DP/DE and NPE interaction, and down
266 regulated in $L_{P/E}C$ -fed juveniles. The muscle transcript level of *ctsd*, a lysosomal protease,
267 was decreased when dietary DP/DE was lowered ($H_{P/E} > L_{P/E}$). The gene expression of *capn2*, a
268 non-lysosomal protease, was unaffected by dietary changes. The two growth factor studied
269 (*mstn1a* and *mstn1b*) respond differently to isoenergetic changes in dietary macronutrient
270 composition: the transcript level of *mstn1a* remained stable while that of *mstn1b* decreased
271 when dietary DP/DE was lowered ($H_{P/E} > L_{P/E}$).

272
273 3.3. Recruitment and growth dynamics of skeletal white muscle fibres

274
275 After 7 weeks of feeding, the total cross-sectional area of white muscle and the total
276 number of white fibres were higher in $H_{P/E}$ - than in $L_{P/E}$ -fed trout and, for each DP/DE ratio,
277 higher in trout fed fat instead of carbohydrate as major NPE source (Table 4). The shape of
278 the diameter distribution of white muscle fibres was different in the trout fed the four diets

(Figure 2). Seven weeks of feeding with the control diet (H_{P/E}F) led to a bimodal distribution of white fibre diameters, with a first mode at 10µm and a second mode at 27.5µm. Modes at 10µm and 27.5µm were also present in the H_{P/E}C-fed trout, despite a different shape of diameter distribution. The distribution of fibre diameters of L_{P/E}F- fed trout presented two closed modes (7.5 µm and 17.5µm), and that of L_{P/E}C- fed trout only a mode at 15µm. Trout fed with a high vs. low DP/DE ratio had a higher mean and median diameter of white muscle fibres; they also had a higher percentage of muscle fibres with a diameter higher than 40µm, and a higher maximal diameter of white muscle fibres (Table 4). Trout fed fat as main NPE source had a higher percentage of muscle fibres with a diameter higher than 60µm and a higher maximal diameter of white muscle fibres. Dietary DP/DE ratio and NPE source interact to regulate the percentage of muscle fibres with a diameter lower than 20µm. Feeding the L_{P/E}F- and L_{P/E}C-diets led to the highest percentages of small white muscle fibres (43-45%) and feeding the H_{P/E}C-diet to the lowest percentage of small white muscle fibres (28%), that of the H_{P/E}F-fed trout being intermediate (36%).

3.4. Correlations between individual trout body growth, muscle cellularity and muscle transcript levels

As shown in Table 5, trout body weight was positively correlated to the gene expression of *fmlc2* (P=0.011), *mstn1a* (P=0.008) and *mstn1b* (P<0.0001), and not correlated to that of *fmhc* (P=0.063). Trout fork length was also positively correlated to *fmlc2* (P=0.019), *mstn1a* (P=0.023) and *mstn1b* (P=0.0001) transcript levels, and close to positively correlated (P=0.053) to *fmhc* transcript level. The total cross-sectional area of white muscle was positively correlated to *mstn1a* (P=0.008) and *mstn1b* (P<0.0001) transcript levels, and close to positively correlated (P=0.054) to *fmlc2* transcript level. The total number of white muscle

fibre was positively correlated with *mstn1b* transcript level ($P=0.037$). The percentage of white muscle fibres with a small diameter ($<20\mu\text{m}$) was positively correlated with the transcript level of *mrf4* ($P=0.048$), and negatively correlated with the transcript level of *mstn1b* ($P=0.004$). The linear relationships existing between these three actors of muscle growth are reported on figure 3. The number of white muscle fibres with a large diameter ($>80\mu\text{m}$) and the maximal diameter of white muscle fibre were both positively correlated with the mRNA level of *mstn1b* ($P<0.0001$ and $P=0.002$, respectively). The gene expressions of *myf5*, *myod1a*, *myog*, *pcna*, *ctsd* and *capn2* were not correlated to trout body characteristics and muscle cellularity parameters (data not shown).

4. Discussion

The present study investigates macronutrient-induced changes in muscle growth mechanisms of rainbow trout. For this, we used juvenile trout fed isoenergetic diets with an experimentally high contrast in the level of DP and in DP/DE ratio (below or above the optimal DP/DE ratio of 17- 19 mg kJ^{-1} (Dias et al., 1999) or 21 mg kJ^{-1} (NRC, 2011) for rainbow trout), while fat was exchanged by an isoenergetic amount of carbohydrates (effect NPE source) at each DP/DE ratio. Trout's feeding response to these four diets, in terms of intake, nutrient retentions, metabolism and body growth has been analyzed in details elsewhere (Saravanan et al., 2012, Figueiredo-Silva et al., 2013). Overall, food intake and whole body growth were reduced by feeding diets with low vs high DP/DE and by replacing fat by starch as NPE source. The efficiency of protein retention for growth (body protein gain per unit DP intake) was higher at low than at high DP level but only when fat was used as

major NPE source. In contrast, the source of NPE had no effect on protein sparing at DP levels above the fish DP requirement. Knowing that protein growth largely reflects muscle growth, the observed divergent protein growth responses form a unique experimental starting point for further studies on the effects of macro nutrients on mechanisms determining muscle growth.

Overall, our study confirms that the expression of certain MRFs and genes coding for muscle structural proteins can be manipulated in fish juveniles by changes in macronutrient supply (Alami-Durante et al., 2014; Campos et al., 2010; Chapalamadugu et al., 2009). Yet, the previous studies do not allow to determine whether the observed transcriptional changes resulted from dietary changes in digestible protein (DP) or in digestible energy (DE) or in non-protein energy (NPE) supply. These confounding effects are further examined in the present study.

4.1. Effect of dietary protein level

Feeding the juvenile trout with the L_{P/E} diets, containing a protein and amino acid level below established requirements (NRC, 2011), enhanced the muscle mRNA level of *myod1a*, a salmonid paralog of *myod1* gene expressed in proliferating and differentiating cells (Bower and Johnston, 2010). The upregulation of *myod1a* transcript level in L_{P/E}-fed trout might have been triggered by an amino acid deficiency, as also seen in our previous study in trout juveniles following a dietary methionine deficiency (Alami-Durante et al., 2018). Similarly, other essential amino acids, such as lysine and histidine have been shown to affect *myod* gene expression in juveniles of other fish species (Cai et al., 2018b; Michelato et al., 2017). The question whether it is the overall low amino acid supply or a specific amino acid deficiency

which caused the upregulation of *myod1a* transcript level in L_{P/E}-fed trout deserves further investigation.

Another consequence of feeding the L_{P/E} diets was the downregulation of the gene expression of *mstn*, a secreted growth factor known to negatively regulate muscle growth in mammals by decreasing satellite cell proliferation and differentiation and by attenuating myonuclear addition and muscle protein synthesis (McCroskery et al., 2003; McPherron et al., 1997; Taylor et al., 2001; Welle et al., 2006; Yang et al., 2007). In fish, endogenous expression of *mstn* paralogs is rarely related to muscle growth rate, and *mstn* functions as a general inhibitor of cell proliferation and growth (Gabillard et al., 2013). It was previously shown that rainbow trout juveniles fed for 5 weeks with a high-fat (40%P:25%L) instead of a low-fat (40%P:10%L) diet had lower muscle *mstn1a* and *mstn1b* mRNA levels, suggesting that lipids downregulate *mstn* expression (Galt et al., 2014). But, as 15% of the fish oil from the high-fat diet was replaced by 15% wheat flour in the low-fat diet (Galt et al., 2014), not only the dietary lipid but also the dietary energy content and protein/energy ratio might have modified *mstn1a* and *mstn1b* transcription. A longer study also in trout juveniles showed enhanced muscle *mstn1a* mRNA in response to a 50% P:15% L-diet relative to 62% P:6%L- and 43%P:20%L- diets, without difference in *mstn1b* expression (Overturf et al., 2016). The three diets used in that study also differed in energy content, complicating interpretation of results. Based on observations in starved trout (Johansen and Overturf, 2006), the latter study suggested that the *mstn1a* muscle mRNA in trout fed the 62%P:6%L and 43%P:20%L diets were decreased to prevent excess muscle tissue wastage in these fish (Overturf et al., 2016). Our results, which allow to specify the effects of isoenergetic changes in macronutrient supply, showed that *mstn1b* muscle gene expression is decreased when dietary protein and DP/DE ratio are decreased, whatever the NPE source. The higher and the lower muscle *mstn1b* mRNA levels were thus occurring in juveniles having the higher (H_{P/E}) and lower

(L_{P/E}) total cross-sectional area of white muscle (TCSAWM), respectively. This result may seem at first sight surprising knowing that *mstn* functions to control tissue mass. Yet, at the end of the feeding trial, the TCSAWM, as well as the total number of white fibres, the number of large fibres and the maximum white fibre diameter, integer the initial muscle status (number and size of fibres at the start of the trial) and the hyperplastic and hypertrophic processes that occurred during the 7-week trial. These processes might have been regulated by a level of *mstn* different from that actually seen at the end of the trial, and the transcription of muscle *mstn* possibly decreased over time in slow-growing trout to partly counteract diet-induced restrictions in muscle growth. This hypothesis is supported by findings in tilapia showing that *mstn* mRNA level may rise initially in the early stages of adverse nutritional conditions but consistently decreased when these conditions persisted (Rodgers et al., 2003). We believe that the action of the *mstn* present in muscle at the end of the trial is detectable at the level of fibres having a diameter below 20µm, as this diameter class represents recent myogenic events (new recruitment and early growth of myotubes). The percentage of white muscle fibres in this diameter class was negatively correlated to *mstn1b* mRNA level (P=0.0043), supporting the negative regulatory function of *mstn* in muscle growth. The lower muscle *ctsd* transcription in L_{P/E}-fed trout might similarly be a cellular response to decrease lysosomal proteolysis and thus limit atrophy. *Capn2* gene expression was not affected, implying a lesser sensitivity of non-lysosomal proteases to changes in dietary macronutrient composition, in line with previous findings (Overturf et al., 2016).

At the cellular level, feeding the L_{P/E} diets highly decreased white muscle growth (TCSAWM ~ 30% lower compared to H_{P/E} diets), linked to alterations in both hyperplasia, as indicated by the smaller number of white muscle fibres, and to fibre hypertrophy, as indicated by the smaller number of large (>40 µm) fibres compared to H_{P/E}-fed trout. This was not observed in a study (Silva et al., 2009) with blackspot sea bream juveniles fed diets with

similar variations in protein contents (24-64%, vs 27-51% in the present study). In sea bream, the low protein diet indeed decreased white fibre number but affected neither the growth of white muscle (TCSA) neither the mean diameter of white muscle fibres. These discrepancies might be due to differences in dietary macronutrient balance and/or DE content (variable in the study by Silva et al., 2009 vs. constant in present conditions).

4.2. Effect of NPE source

Feeding the juvenile trout with C- instead of F-diets decreased the muscle transcript level of *myod1a* with potential consequences on proliferation and differentiation of muscle cells, as suggested by its *in vitro* expression pattern (Bower and Johnston, 2010). A down regulation of the gene expression of another *myod* paralog (*myod1b*) has similarly been observed in juvenile rainbow trout fed a high amount of carbohydrates (Chapalamadugu et al., 2009). Feeding the juvenile trout with C- instead of F-diets also decreased the muscle mRNA level of *pcna*, which functions as a cofactor of DNA polymerase- δ necessary for cell proliferation (Bravo et al., 1987). This suggests that carbohydrates were less efficiently used than fat for cell proliferation, with potential effects not only on hyperplasia but also on hypertrophy as myonuclear accretion is necessary for maintenance of nucleocytoplasmic ratio during fibre hypertrophy. Cellular analyses confirmed that muscle hyperplasia was decreased by the isoenergetic change in the main NPE source from F to C, as revealed by a 19% lower number of muscle fibres in C-fed trout, indicating that carbohydrates were not as efficient as fat to sustain white muscle hyperplasia and this irrespective of the protein level. Carbohydrates were also less efficient to sustain fibre hypertrophy, as revealed by the lower percentage of fibres with a large diameter ($> 60\mu\text{m}$) and the lower maximal fibre diameter in C-fed trout. This does not imply that carbohydrates should be excluded from trout diets as

their absence is detrimental to muscle growth; it indeed depressed the muscle protein/DNA ratio and exacerbated muscle protein degradation (Peragon et al., 1999) and also downregulated *myod1b*, *myf5* and *myog* gene expressions (Chapalamadugu et al., 2009). A balanced level of dietary carbohydrate thus appeared not only necessary to increase protein and lipid retention by preventing the catabolism of these nutrients for energy needs, reduce nitrogen excretion and provide metabolites for biological synthesis (Kamalam et al., 2017) but also to favor white muscle hyperplasia and hypertrophy.

4.3. Effect of DP/DE ratio-NPE source interaction

Dietary DP/DE ratio and NPE source interacted to regulate the gene expression of *mrf4*, a myogenic regulatory factor with an *in vitro* biphasic expression, playing a role in cell proliferation and differentiation (Bower and Johnston, 2010). In our study, *mrf4* gene expression was the highest in L_{P/E}F-fed trout which had the best protein retention efficiency, retaining 44% of the ingested DP compared to only 32-35% in the other trout. This suggests that *mrf4* might be a relevant molecular marker of protein retention efficiency in trout juveniles, in line with previous results in Senegalese sole juveniles (Campos et al., 2010). The gene expression of *mrf4* was here also positively correlated to the percentage of white muscle fibres with a small diameter (<20µm) suggesting that *mrf4* might also be a relevant molecular marker of early myogenic events.

Dietary DP/DE ratio and NPE source also interacted to regulate the gene expression of myosin chains. The significantly lower transcript level of *fmhc* and *fmlc2* in the L_{P/E}C-fed trout suggests a lower ability to synthesize myosin chains in these fish, which might in part explain the lowest protein growth and body growth of these fish. In Senegalese sole juveniles fed diets differing in energy and macronutrient composition (Campos et al., 2010) the mean

fmlc2 gene expression in white muscle was correlated to the mean body protein retention efficiency and lipid gain, which was not observed here. This discrepancy might be linked to species-specific differences in body composition (leaner in sole than in trout) and metabolism. A lower ability to synthesize myosin chains in L_{P/E}C-trout, as suggested by their lower *fmhc* and *fmlc2* gene expressions, would also have potential negative effects on fibre hypertrophy, as confirmed by cellular phenotyping. The shape of the distribution of white fibre diameters was indeed unimodal in L_{P/E}C-trout and its mode was small, confirming that this diet has prevented the hypertrophic growth of muscle fibres. The growth dynamics of muscle fibres were different in trout fed the diet H_{P/E}F, which allowed the highest muscle growth, as in these fish a population of small white fibres (diameter < 20µm), was clearly separated from a second population of bigger fibres. The population of small fibres most likely represents recruitment and early growth of new muscle fibres, and the other population fibres initially present at the start of the trial that had grown. Of interest, the percentage of small muscle fibres (diameter < 20 µm) was regulated by an interaction between the dietary DP/DE ratio and NPE source. The overall lower proportion of small fibres in trout fed the high versus low (H_{P/E}- vs L_{P/E}) diets most likely resulted from the higher fibre hypertrophy in H_{P/E}-trout, increasing the relative proportion of large fibres (diameter > 40 µm) rather than from reduced hyperplasia (higher number of muscle fibres in H_{P/E}-trout). Instead, the lower percentage of small fibres (diameter < 20µm) following the isoenergetic replacement of F by C in H_{P/E}C- vs. H_{P/E}F-fed trout most likely resulted for its part from decreased hyperplasia, as indicated by a lower number of muscle fibres, and decreased hypertrophy, as shown by the lower percentage of large fibres (diameter > 40 µm) and lower maximal diameter of fibres.

In juvenile Atlantic salmon fed isonitrogenous and isoenergetic diets differing in marine protein sources (Hevroy et al., 2006) and in juvenile spotted wolfish fed a commercial feed at different rearing temperatures (Imsland et al., 2006) *fmhc* expression was linearly

related to individual specific growth rate ($R=0.39$, $P<0.5$ and $R=0.44$, $P<0.001$, respectively), suggesting *fmhc* as a marker of muscle growth. This does not apply to present conditions, as the correlation between *fmhc* gene expression and individual trout weight was not significant. *Fmhc*, which gene expression did not correlate ($r=0.23$; $P=0.18$) with individual muscle area, also does not appear here as a valid molecular marker of white muscle growth, in line with other results in trout juveniles (Alami-Durante et al., 2010b, 2018). As opposed, the correlation between *fmlc2* gene expression and individual trout length ($r=0.39$; $P=0.02$) and weight ($r=0.42$; $P=0.01$) and between *fmlc2* gene expression and individual white muscle total cross-sectional area ($r=0.33$; close to significance, $P=0.05$) indicates that *fmlc2* gene expression might be not only a good molecular marker of individual juvenile growth, as previously seen in gilthead sea bream juveniles fed with commercial diets (Georgiou et al., 2014), but also of individual white muscle growth. Further studies in juvenile fish under different nutritional conditions are needed to specify this finding.

5. Conclusion

Our findings demonstrate that isoenergetic changes in dietary macronutrient composition alter the gene expression of positive actors (*myod1a*, *mrf4*, *pcna*, *fmhc*, *fmlc2*) and negative regulators (*ctsd*, *mstn1b*) of muscle growth and have consequences on skeletal muscle cellularity (number and size distribution of muscle fibres). Not only the dietary protein level or DP/DE ratio, but also the source of dietary NPE appeared as a potent regulator of muscle hyperplasia and hypertrophy.

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Figure captions

Fig.1. Transversal section of a dorsal quadrant of rainbow trout trunk after 7 weeks of feeding showing the position of the 3 areas used to quantify stratified hyperplasia, mosaic hyperplasia and fibre maximal hypertrophy.

WM white muscle, *RM* red muscle, *m* myosept, *SC* spinal cord, *NC* notochord, *vs* vertical septum, *hs* horizontal septum, *s* skin.

Fig.2. Distribution of white skeletal muscle fibre diameters in juvenile trout after 7 weeks of feeding. Values are means (n=9). Variability is reported in Table 4 for clarity.

H_{P/E}F – High P/E ratio with fat as main non-protein energy source; *H_{P/E}C* - High P/E ratio with carbohydrate as main non-protein energy source; *L_{P/E}F* - Low P/E ratio with fat as main non-protein energy source; *L_{P/E}C* - Low P/E ratio with carbohydrate as main non-protein energy source.

Fig.3. Scatter plots showing the relations between the individual gene expression of *mrf4* and percentage of small (d<20µm) white muscle fibres ($y=27.584 + 9.280x$; $R=0.316$; $P=0.033$) and between the individual gene expression of *mstn1b* and percentage of small (d<20µm) white muscle fibres ($y=47.548 - 11.670x$; $R=0.476$; $P=0.004$) in rainbow trout juveniles after 7 weeks of feeding (n=36).

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734 Figure 1

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742 Figure 3

743 **Table 1**

744 Formulation and composition of the experimental diets

Diets ¹	H _{P/E} F	H _{P/E} C	L _{P/E} F	L _{P/E} C
<i>Ingredients (%)</i>				
Protein mixture ²	66.0	66.0	35.9	35.9
Oils ³	11.0	1.0	19.1	9.1
Gelatinized maize starch ⁴	5.0	30.0	24.3	49.3
Cellulose ⁵	15.0	0.0	15.0	0.0
Other ⁶	3.0	3.0	5.7	5.7
<i>Analysed nutrient content on DM basis (g kg⁻¹)⁷</i>				
Dry matter (DM; g kg ⁻¹ diet)	938	924	949	947
Crude protein (Nx6.25)	519	511	276	261
Crude fat	152	34	207	143
Total carbohydrates	254	380	444	528
Starch	49	303	246	456
Ash	75	75	73	68
Digestible energy (DE, kJ g ⁻¹)	18.70	18.27	18.74	18.19
DP/DE (mg kJ ⁻¹)	26.5	26.8	14.1	13.7

745

746 ¹H_{P/E}F - High DP/DE ratio diet with fat as main non-protein energy source; H_{P/E}C - High DP/DE ratio
747 diet with carbohydrate as main non-protein energy source; L_{P/E}F - Low DP/DE ratio diet with fat as
748 main non-protein energy source; L_{P/E}C - Low DP/DE ratio diet with carbohydrate as main non-protein
749 energy source

750 ²Protein mixture (% mixture): 50% fishmeal (Sopropêche 56100 Lorient, France), 16.5% soybean
751 protein concentrate (Sopropêche 56100 Lorient, France) , 16.5% pea protein concentrate (Roquette
752 62080 Lestrem, France), 16.5% wheat gluten (Roquette 62080 Lestrem, France) and 0.5% DL
753 methionine (Ajinomoto Eurolysine 75017 Paris, France).

754 ³ Oils: rapeseed oil (Daudruy 59640 Dunkerque, France) in H_{P/E} diets; 5% (% diet) fish oil
755 (Sopropêche 56100 Lorient, France) and the remaining part from rapeseed oil in L_{P/E} diets.

756 ⁴ Gelatinized maize starch: Roquette 62080 Lestrem, France.

757 ⁵ Cellulose: Rettenmeier et Sohne 73494 Rosenberg, Germany

758 ⁶ Other (% diet): 2% Diamol (indigestible marker, Diamol GM, Franz Bertram Hamburg, Germany);

759 1% vitamin and mineral premix (INRA UPAE 78200 Jouy en Josas). For L_{P/E}-diets 0.4% CaCO₃,

760 1.8% Ca(HPO₄)₂, and 0.5% Na₂CO₃ were added.

761 ⁷ See Saravanan et al. (2012) for further details.

762

763 **Table 2**

764 Growth performance of the rainbow trout juveniles fed with the H_{P/E}F-, H_{P/E}C-, L_{P/E}F- and L_{P/E}C-diets for 6 weeks.

	Diets ¹								<i>P-value</i>		
	H _{P/E} F		H _{P/E} C		L _{P/E} F		L _{P/E} C		DP/DE ratio	NPE source	DP/DE x NPE
	Mean	SD	Mean	SD	Mean	SD	Mean	SD			
Initial BW ²	32.4	0.6	32.5	0.7	32.3	0.5	32.4	0.7	0.79	0.79	1.00
Final BW	103.7 ^a	9.0	96.6 ^{ab}	4.0	84.4 ^b	5.0	59.5 ^c	2.4	<0.01	<0.01	<0.05
TGC ³	2.1 ^a	0.2	2.0 ^{ab}	0.1	1.7 ^b	0.1	1.0 ^c	0.1	<0.01	<0.01	<0.01
FI ⁴	16.6 ^a	0.2	14.7 ^b	0.3	15.4 ^{ab}	0.9	11.6 ^c	0.3	<0.01	<0.01	<0.01
PG ⁵	0.15 ^a	0.01	0.15 ^a	0.004	0.11 ^b	0.01	0.06 ^c	0.01	<0.01	<0.01	<0.01
LG ⁶	0.12	0.01	0.07	0.01	0.12	0.02	0.06	0.01	0.92	<0.01	0.50
PRE ⁷	31.6 ^b	2.5	35.4 ^b	1.5	44.0 ^a	2.1	34.1 ^b	4.0	<0.01	0.08	<0.01

765

766 ¹H_{P/E}F – High P/E ratio with fat as main non-protein energy source; H_{P/E}C - High P/E ratio with carbohydrate as main non-protein energy source;

767 L_{P/E}F - Low P/E ratio with fat as main non-protein energy source; L_{P/E}C - Low P/E ratio with carbohydrate as main non-protein energy source.

768 ²BW, body weight (g); ³TGC, thermal growth coefficient; ⁴FI, feed intake (g/kg metabolic BW per day); ⁵PG, protein gain (g/kg metabolic BW

769 per day); ⁶LG, lipid gain (g/kg metabolic BW per day); ⁷PRE, protein retention efficiency (% digestible intake). See Saravanan et al. (2012) and
770 Figueiredo-Silva et al. (2013) for further details.

771 Values are means $\pm$ SD (n=3). Row means with different superscript letters were significantly different and assigned only if interaction effect
772 was significant (P<0.05).

773

774 **Table 3**

775 Relative mRNA abundance of myogenic and muscle growth-related genes in the juvenile rainbow trout fed with the H_{P/E}F-, H_{P/E}C-, L_{P/E}F- and
 776 L_{P/E}C-diets for 7 weeks.

	Diets ¹								<i>P-value</i>		
	H _{P/E} F		H _{P/E} C		L _{P/E} F		L _{P/E} C		DP/DE ratio	NPE source	DP/DE x NPE
	Mean	SE	Mean	SE	Mean	SE	Mean	SE			
<i>myf5</i>	1.08	0.15	1.79	0.23	1.35	0.23	1.22	0.23	0.480	0.192	0.114
<i>myod1a</i>	1.03	0.08	0.88	0.05	1.37	0.14	0.94	0.06	0.033	0.004	0.229
<i>myog</i>	1.02	0.07	1.21	0.06	1.15	0.06	1.08	0.06	0.828	0.365	0.064
<i>myf4</i>	1.01 ^b	0.06	1.04 ^b	0.08	1.55 ^a	0.10	1.09 ^b	0.09	0.006	0.040	0.020
<i>pcna</i>	1.02	0.07	0.84	0.03	0.98	0.04	0.91	0.05	0.323	0.022	0.574
<i>fmhc</i>	1.00 ^a	0.04	1.08 ^a	0.04	1.07 ^a	0.05	0.88 ^b	0.04	0.079	0.201	0.005
<i>fmlc2</i>	1.01 ^a	0.05	1.05 ^a	0.05	0.99 ^a	0.06	0.81 ^b	0.03	0.017	0.172	0.039
<i>capn2</i>	1.00	0.04	1.14	0.10	0.98	0.05	1.03	0.08	0.441	0.270	0.665
<i>ctsd</i>	1.03	0.09	1.23	0.07	0.89	0.04	0.96	0.06	0.006	0.063	0.355
<i>mstn1a</i>	1.14	0.23	0.75	0.12	0.81	0.15	0.77	0.16	0.416	0.182	0.452

<i>mstn1b</i>	1.03	0.08	0.92	0.14	0.65	0.08	0.51	0.07	<0.001	0.100	0.881
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778 ¹H_{P/E}F – High P/E ratio with fat as main non-protein energy source; H_{P/E}C - High P/E ratio with carbohydrate as main non-protein energy source;

779 L_{P/E}F - Low P/E ratio with fat as main non-protein energy source; L_{P/E}C - Low P/E ratio with carbohydrate as main non-protein energy source.

780 *myf5*, myogenic factor 5; *myod1a*, myogenic differentiation 1a; *myog*, myogenin; *mrf4*, myogenic factor 6; *pcna*, proliferating cell nuclear

781 antigen; *fmhc*, fast myosin heavy chain; *fmlc2*, fast myosin light chain 2; *capn2*, calpain 2; *ctsd*, cathepsin D; *mstn1a*, myostatin 1a; *mstn1b*,

782 myostatin1b.

783 Data are normalized to 18S mRNA and expressed as fold-changes of mRNA abundance compared with the control group H_{P/E}F.

784 Values are means ± SE (n=9). Row means with different superscript letters were significantly different and assigned only if interaction effect was

785 significant (P<0.05).

786

787 **Table 4**

788 Cellularity of the dorsal skeletal white muscle of the juvenile rainbow trout fed with the H_{P/E}F-, H_{P/E}C-, L_{P/E}F- and L_{P/E}C-diets for 7 weeks.

	Diets ¹								<i>P-value</i>		
	H _{P/E} F		H _{P/E} C		L _{P/E} F		L _{P/E} C		DP/DE ratio	NPE source	DP/DE x NPE
	Mean	SE	Mean	SE	Mean	SE	Mean	SE			
Dorsal white muscle											
TCSA (mm ²)	79.2	2.2	66.6	1.8	55.7	2.6	41.2	2.1	<0.001	<0.001	0.105
TNWF	33659	1862	27359	926	28445	673	23102	1635	0.002	<0.001	0.964
White muscle fibres											
d mean (µm)	32.1	1.3	32.8	1.1	27.0	0.8	25.5	0.8	<0.001	0.640	0.240
d median (µm)	28.0	1.1	29.9	1.0	22.8	0.7	22.0	0.9	<0.001	0.642	0.147
d ≤20µm (%)	36.2 ^b	1.2	28.4 ^c	2.1	43.1 ^a	1.5	44.8 ^a	2.6	<0.001	0.110	0.023
20<d≤40µm (%)	35.0	1.2	40.3	1.5	35.9	1.0	37.1	1.7	0.443	0.032	0.167
40<d≤60µm (%)	19.0	1.2	22.4	1.1	14.9	1.2	14.6	1.1	<0.001	0.189	0.130
60<d≤80µm (%)	7.5	0.8	6.9	0.6	5.1	0.6	3.3	0.4	<0.001	0.045	0.194
d>80µm (%)	2.3	0.3	2.1	0.3	1.0	0.2	0.2	0.1	<0.001	0.007	0.076

d max (μm)	117.4	5.9	104.6	5.8	97.2	3.3	85.8	2.0	<0.001	0.008	0.946
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790 ¹H_{P/E}F – High P/E ratio with fat as main non-protein energy source; H_{P/E}C - High P/E ratio with carbohydrate as main non-protein energy source;

791 L_{P/E}F - Low P/E ratio with fat as main non-protein energy source; L_{P/E}C - Low P/E ratio with carbohydrate as main non-protein energy source.

792 TCSEA, total cross sectional area of white muscle; TNWF, total number of white muscle fibres; d, diameter of white muscle fibres.

793 Values are means ± SE (n=9). Row means with different superscript letters were significantly different and assigned only if interaction effect was

794 significant (P<0.05).

795

796 **Table 5.**

797 Correlations (Pearson's coefficients) between individual body growth, muscle cellularity and muscle gene expressions (n=35) after 7 weeks of
798 feeding.

	<i>mrf4</i>	<i>fmhc</i>	<i>fmlc2</i>	<i>mstn1a</i>	<i>mstn1b</i>
L _F	-0.095	0.330#	0.394*	0.383*	0.602***
BW	-0.139	0.317	0.423*	0.440**	0.654***
TCSAWM	-0.212	0.232	0.328#	0.438**	0.654***
TNWF	0.012	0.261	0.270	0.279	0.354*
WF, dmean	-0.372*	0.034	0.134	0.145	0.435**
WF, d≤20μm	0.336*	-0.166	-0.220	-0.103	-0.470**
WF, d>80μm	-0.114	0.204	0.283	0.289	0.645***
WF, dmax	-0.091	0.095	0.242	0.263	0.516**

799

800 L_F, fish fork length; BW, fish body weight; TCSAWM, total cross sectional area of white muscle; TNWF, total number of white muscle fibres; d,
801 diameter of white muscle fibres; *mrf4*, myogenic factor 6; *fmhc*, fast myosin heavy chain; *fmlc2*, fast myosin light chain 2; *mstn1a*, myostatin 1a;
802 *mstn1b*, myostatin1b. ***P<0.001; **P<0.01; *P<0.05; #P=0.05.