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Larval dietary protein complexity affects regulation of muscle growth and expression of DNA methyltransferases in Senegalese sole

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Abstract

Due to its high protein synthesis and deposition rates, skeletal muscle protein deposition is a major determinant of fish growth. Dietary protein complexity is likely to influence protein utilization and deposition in skeletal muscle, possibly affecting fish myogenesis. In this study, three microdiets were formulated with different degree of hydrolysis of dietary protein as the changing factor: one diet contained a mix of intact protein sources targeting a peptide with molecular weight higher than 20 KDa (Intact); a second diet contained a hydrolysate with polypeptides ranging from 5 to 70 KDa (PartH); and a third diet contained a high level of a protein hydrolysate mostly composed of small peptides (<5 KDa) (HighH). A possible effect on the regulation of muscle growth in Senegalese sole larvae was evaluated through white muscle cellularity and the expression of muscle growth-related genes at 16 and 36 DAH. The PartH diet promoted white muscle growth during the metamorphosis climax (16 DAH), which was reflected on increased body weight. At 36 DAH, different diets induced different expression patterns of genes encoding for the myogenic regulatory factors, which affected muscle growth dynamics, ultimately promoting growth potential in the Intact group. A lower recruitment of small-sized fibres in the PartH and HighH groups led to reduced potential for muscle growth, which resulted on further reduced somatic growth. Accordingly, fish fed the Intact diet grew better up to a late juvenile stage (60 DAH) and were still heavier than others even after 30 days of feeding all groups on the same commercial diet, at 90 DAH. The up-regulation in the transcript levels of genes encoding for *de novo* DNA methyltransferases in the HighH group suggest a potential for nutritional programming in this species.

1- Introduction

Skeletal muscle protein deposition greatly contributes to overall growth in fish juveniles and larvae, when compared to other tissues (Carter, Houlihan, 2001; Houlihan et al., 1995). Due to its high protein synthesis and deposition rates, skeletal muscle is thus a major determinant of fish amino acids (AA) requirements (Houlihan et al., 1995). Fish larvae have a tremendous growth potential (Conceição et al., 2003; Conceição et al., 2011) and its reliance on dietary AA both as fuel for energy production, and as building blocks for growth (Parra, Yúfera, 2001; Parra et al., 1999; Ronnestad et al., 1999; Rønnestad et al., 2003) can be seen as paradoxical considering the poor development of early stages of altricial larvae digestive system (Zambonino Infante et al., 2008). The larvae capacity to digest and absorb dietary protein throughout development are key factors to be considered when formulating microdiets, in order to make the most of its digestive tract capacity to utilize dietary protein and to fully express its maximum growth potential (Conceição et al., 2011; Canada et al., 2017).

Senegalese sole (*Solea senegalensis*) is a fast-growing species that undergoes a complex metamorphosis (Fernández-Díaz et al., 2001). Its digestive system ontogeny follows the general pattern observed in other marine species with altricial development (Fehri-Bedoui et al., 2000; Padrós et al., 2011; Ribeiro et al., 1999a; 1999b; Zambonino Infante et al., 2008). As for most altricial species, it has been assumed that early-stage Senegalese sole larvae have a limited capacity to digest and absorb the native protein sources commonly used in commercial fish microdiets formulations (Engrola et al., 2009; Gamboa-Delgado et al., 2008). Since dietary protein is mainly absorbed as free amino-acids (FAA) and di- or tri-peptides (Ronnestad, Morais, 2008) and early-stage larvae have a poorly developed gut (Zambonino Infante et al., 2008), it has been suggested that the moderate inclusion of pre-digested proteins in microdiets would improve its dietary protein digestibility. In fact, recent results suggest that Senegalese sole pre-metamorphic larvae have a limited capacity to utilize native proteins, whereas larger peptides and intact protein seem more suitable to sole post-larvae and young juveniles anabolic and physiological needs (Canada et al. 2017; Richard et al. 2015)

Several studies reported increased survival and somatic growth in European sea bass (*Dicentrarchus labrax*) (Cahu et al., 2004; 1999; Zambonino Infante et al., 1997), gilthead

sea bream (*Sparus aurata*) (Kolkovski, Tandler, 2000), white seabream (*Diplodus sargus*) (de Vareilles et al., 2012), large yellow croaker (*Pseudosciaena crocea*) (Liu et al., 2006), Asian sea bass (*Lates calcarifer*) (Srichanun et al., 2014) and Atlantic halibut (*Hippoglossus hippoglossus*) (Kvåle et al., 2009; Kvåle et al., 2002) larvae fed microdiets including protein hydrolysates. However, very few studies focused on a possible influence of such diets on muscle growth regulation (Katan et al., 2016; Ostaszewska et al., 2008). Muscle development and growth during early life stages is clearly determinant for the larvae ability to swim, feed and survive (Osse et al., 1997) and was further demonstrated to influence long-term somatic growth (Campos et al., 2014; Galloway et al., 1999; Weatherley et al., 1988). Moreover, early nutrition was recently shown to induce changes on the regulation of skeletal muscle development during early life stages having a long-term effect on somatic growth, which suggests the potential for nutritional programming on muscle growth and somatic growth potential (Alami-Durante et al., 2014).

White skeletal muscle constitutes the bulk of the axial locomotor muscle in Senegalese sole larvae, post-larvae and juveniles. White skeletal muscle fibres, also known as fast-twitch fibres, are used for burst swimming movements (Bone, 1978), which is the main type of locomotion displayed by sole to move and feed (Dinis et al., 1999). The white skeletal muscle development in Senegalese sole follows the general pattern observed in other aquaculture species (Campos et al., 2013b; 2013c). Muscle formation (myogenesis) comprises the recruitment of stem cells to a lineage of myogenic progenitor cells (MPCs) that undergo activation, proliferation, cell cycle exit, differentiation, migration and fusion into already formed muscle fibres (Johnston et al., 2011; Valente et al., 2013). MPCs proliferation and differentiation are ruled by the expression of numerous genes and particularly the four myogenic regulatory factors (MRFs): *myod* and *myf5* are involved in the commitment of myoblasts to form the MPCs population; *myogenin* and *mrf4* drive and keep on the myoblast differentiation that will ultimately result in myotube formation and enlargement (Rescan, 2001). On the other hand, myostatin (*mstn*) functions as a negative regulator of myoblast proliferation and differentiation (Thomas et al., 2000). Muscle growth occurs by both hyperplasia (fibre number increase) and hypertrophy (fibre size increase) (Rowlerson, Veggetti, 2001). During fish post-embryonic and larval development, muscle fibre number increases mainly by stratified hyperplasia, which involves the

recruitment of new fibres in discrete germinal zones found in the lateral margins of the myotome (Rowlerson, Veggetti, 2001). In juvenile and adult stages, new myotubes form on the surface of fast muscle fibres, further fusing or adding nuclei to already existing fibres - mosaic hyperplasia (Rowlerson, Veggetti, 2001). The relative contribution of hyperplasia and hypertrophy was shown to influence long-term growth rate, providing an estimate for individual growth potential (Galloway et al., 1999; Weatherley et al., 1988).

There has been a great effort to understand the regulation of muscle growth by intrinsic factors like genotype (Johnston et al., 1999; Valente et al., 2006) and extrinsic factors such as photoperiod (Johnston et al., 2004; Lazado et al., 2014) and temperature (Campos et al., 2013b; Campos et al., 2013c; Galloway et al., 2006; Silva et al., 2011), in order to optimize broodstock management and larval rearing conditions. Nevertheless, the impact of nutritional factors on fish larval muscle development is far from being understood.

In fish larvae, dietary protein sources (Alami-Durante et al., 1997; Ostaszewska et al., 2008), dietary protein level (Saavedra et al., 2016) and AA supplementation (Aguiar et al., 2005) were shown to affect muscle growth regulation and the somatic growth rate of several species. In rainbow trout, different protein:energy ratios delivered to first-feeding fry induced changes in the regulation of muscle growth during the nutritional challenge period, but also and more remarkably after 3 months of feeding all groups on the same commercial diet (Alami-Durante et al., 2014). This result suggests that the activity of white MPCs might be programmed by nutritional factors (Alami-Durante et al., 2014), although the mechanisms possibly underlying such response are not known. It has recently been suggested that an epigenetic mechanism could promote differential gene expression and modulate Senegalese sole muscle growth in response to different thermal conditions; different rearing temperatures during the pelagic phase induced changes in the methylation status of the *myogenin* putative promoter, its mRNA transcript levels and expression of *dnmt1* and *dnmt3b* (DNA methyltransferases), which was suggested to underlie the rearing temperature effect on muscle cellularity during the metamorphosis climax (Campos et al., 2013a). In addition, the effect of rearing temperature on muscle cellularity during the metamorphosis climax influenced subsequent somatic growth, up to a late juvenile stage (Campos et al., 2013b). Increasing evidence indicates that DNA methylation is labile not only to environmental conditions but also to nutritional factors (Anderson et al., 2012).

However, studies on epigenetic modifications in response to environmental or nutritional cues are a recent trend in fish. Very few studies have been published concerning nutritional programming on muscle growth (Alami-Durante et al., 2014; Fontagné-Dicharry et al., 2017) and no studies have established a relationship between nutritional status and the epigenetic regulation of myogenesis, through possible changes in DNA methylation status. Campos et al. (2013a, 2013b) results on the influence of temperature on the regulation of sole myogenesis suggest the pelagic phase as a critical time window prone to epigenetic modifications with long-lasting effects on the regulation of myogenesis and subsequent influence on the potential for growth. Therefore, in the present study, we hypothesized that changes in dietary protein complexity would affect the regulation of muscle growth during the metamorphosis climax and up to an early juvenile stage in Senegalese sole, having an impact on long-term somatic growth. The effect on white muscle growth dynamics was analysed at the metamorphosis climax (16DAH, stage 3), which has been previously recognized as a very relevant time-window in Senegalese sole ontogeny, in which changes in myogenesis induced by external factors were shown to strongly affect long-term somatic growth (Campos et al., 2013b). Muscle cellularity was also analysed in newly-weaned fish (36DAH), since variable growth rates and size dispersion just after weaning are currently a major constraint for a more successful juveniles' production. After being fed with three experimental diets until 60 DAH, all groups were fed with the same commercial diet until 90 DAH to evaluate the enduring effect on somatic growth. The expression pattern of DNA methyltransferases was analyzed in order to understand whether an epigenetic event could possibly underlie the response of muscle growth regulation and somatic growth to dietary protein complexity.

2. Material and Methods

2.1 Experimental diets

Three microdiets (Intact, PartH and HighH) were formulated and processed by SPAROS Lda. (Olhão, Portugal) to be isoenergetic and high protein diets, using approximately the same ingredients, but including protein fractions of different complexity, as previously described by Canada et al. (2017) (Table 1). The Intact diet contained a mix of intact plant protein sources – Plant protein Mix 12 (84% CP, 3.6% CF, SPAROS Lda., Portugal),

targeting a peptide molecular weight (MW) higher than 20KDa. The PartH diet contained a hydrolysate of the same plant protein mixture used in Intact diet - IdG Hydrolysate. This hydrolysate was produced at Instituto de la Grasa (CSIC, Sevilla, Spain) according to Villanueva et al. (1999), using pea protein concentrate and wheat gluten as sources and Alcalase as a food grade proteolytic enzyme. Partially hydrolysed proteins with a high proportion of 5 – 20 KDa peptides were targeted with the goal of achieving a compromise between the need to improve plant protein digestibility and to avoid high leaching rates from formulated diets. The HighH diet contained a high level of a commercial fish protein hydrolysate with a predominance of small polypeptides, oligopeptides and di and tri-peptides (<5 KDa). All three diets contained a minimum of 43% marine ingredients, including high levels fish and krill protein hydrolysates, which all together should make the diets highly palatable for sole larvae. Moreover, the plant protein Mix used was based on protein concentrates, and thereby the presence of anti-nutritional factors in any of the three diets is highly unlikely. All diets were formulated to meet Senegalese sole post-larvae requirements for crude fat, long chain polyunsaturated fatty acids (LC-PUFA) content and in particular DHA content, according to Pinto et al. (2016). Moreover, the inclusion level of Algatrium (rich in PUFA) and Phosphonorse (rich in marine phospholipids) was the exactly same in the three diets. Fish oil inclusion was very similar between diets (Table 1).

All dietary ingredients were initially mixed according to each target formulation in a mixer, being thereafter ground twice in a micropulverizer hammer mill (SH1, Hosokawa-Alpine, Germany). Diets were then humidified and agglomerated through low-shear extrusion (Dominioni Group, Italy). Upon extrusion, diets were dried in a convection oven (OP 750-UF, LTE Scientifics, United Kingdom) for 4 h at 60 °C, being subsequently crumbled (Neuero Farm, Germany) and sieved to desired size ranges.

The microdiets were grounded, pooled and analysed for dry matter (105 °C for 24 h) and ash content by combustion in a muffle furnace (Nabertherm L9/11/B170, Bremen, Germany; 550 °C for 6 h). As sole have a passive bottom feeding behaviour, with microdiets remaining one min or more in the tank bottom before being eaten (Conceição et al., 2007; Dinis et al., 2000), microdiet samples ($n = 4/\text{treatment}$) were submersed in rearing water for 1 min, in order to allow nutrient leaching and simulate the situation observed in the rearing tanks. After this period the rearing water was removed and the feed

samples were frozen at -80°C and freeze-dried before analysed. These samples were then grounded, pooled and analyzed for dry matter (105°C for 24 h), crude protein by automatic flash combustion (Leco FP-528, Leco, St. Joseph, USA; $\text{N} \times 6.25$) and gross energy in an adiabatic bomb calorimeter (Werke C2000; IKA, Staufen, Germany). The amino-acid composition of the microdiet samples after leaching for 1 min was determined by ultra-high-performance liquid chromatography (UPLC) in a Waters Reversed-Phase Amino Acid Analysis System, using norvaline as an internal standard. In order to do so, samples for total amino-acids and taurine quantification were previously hydrolysed at 6 M HCl at 116°C , over 22 h. Then all the samples were pre-column derivatized with Waters AccQ Fluor Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) using the AccQ Tag method (Waters, USA). The resultant peaks were analysed with EMPOWER software (Waters, USA). The diet composition before (dry matter and ash content) and after leaching (crude protein, crude fat and gross energy) is presented on Table 1. The dietary AA profile is presented in Table 2, like previously published elsewhere (Canada et al., 2017).

2.2 Husbandry and experimental set-up

This experiment was performed by trained scientists and following the European Directive 2010/63/EU of European Parliament and of the Council of European Union on the protection of animals used for scientific purposes. CCMAR facilities and their staff are certified to house and conduct experiments with live animals ('group-1' license by the 'Direção Geral de Veterinária', Ministry of Agriculture, Rural Development and Fisheries of Portugal). Following the 3R's policy, the number of animals was reduced by sharing the same trial and the same microdiets between two studies with different purposes: i) a first study focused on the effect of dietary protein complexity on larval protein utilization and the expression of protein digestion-related genes in relation to somatic growth (Canada et al., 2017); and ii) the present study focused on the effect of dietary protein complexity on the regulation of fish muscle growth dynamics (muscle cellularity and muscle growth related genes) and its impact on long-term somatic growth.

Like previously described in Canada et al. (2017), Senegalese sole eggs were incubated in an upwelling incubator at $19 \pm 0.5^{\circ}\text{C}$ and hatching was completed within the next day (24h). Newly hatched larvae were evenly distributed over 9 white cylindro-conical tanks (100L) in

a semi-closed recirculation system with a density of 60 larvae L⁻¹ (6000 larvae/tank). The system was equipped with a mechanical filter, a submerged and a trickling biological filter, a protein skimmer and UV sterilizer. Sole was reared according to previously established rearing protocols for Senegalese sole larvae (Engrola et al. 2005). Larvae were reared in green water conditions until 16 days after hatching (DAH), provided by adding frozen *Nannochloropsis* sp. (*Nannochloropsis* 18% FP 472/180908, Acuicultura Y Nutrición de Galicia SL) to rearing tanks every morning. Abiotic parameters and mortality were daily monitored. Dissolved oxygen in water was maintained at 86.6±7.2% of saturation, temperature at 18.4±0.6°C and salinity at 37.6±2.3‰. While larvae were fed live prey, a light intensity of 1000 lux was provided by overhead fluorescent tubes under a 10h/14h light/dark photoperiod cycle. At 16DAH, during the metamorphosis climax, the larvae were transferred to flat-bottom tanks (30×70×10cm; 21L), each tank stocking 860 individuals (corresponding to a 4095ind/m² density). The system for the benthic rearing was equipped with a mechanical filter, a submerged and a trickling biological filter, a protein skimmer and UV sterilizer. Abiotic parameters were monitored and mortality was recorded every morning. Dead larvae were removed and the rearing units were carefully cleaned with minimal disturbance. Dissolved oxygen in water was maintained at 96.6±7.2% of saturation, temperature at 19.6±0.5°C and salinity at 35.4±0.7‰. During the benthic phase post-larvae were fed both frozen artemia and inert diet. At this stage, sole feeds on the bottom of the tank, displaying a calm behavior and spending most of the time settled on the bottom, swimming short distances at a time. So light intensity was reduced to 400lux to minimize stress and the photoperiod cycle was maintained at 10h/14h light/dark. The photoperiod was decided based on live prey feeding schedule during the pelagic phase, and was maintained during the benthic phase.

The dietary treatments (Intact, PartH and HighH) were randomly assigned to tanks ($n = 3$ tanks per treatment). From mouth opening (2DAH) until 5DAH larvae were fed rotifers (*Brachionus* sp.) enriched with Easy DHA Selco (INVE, Belgium), at an initial density of 5 rots·mL⁻¹ together with the respective inert diet (200-400µm). *Artemia* AF nauplii (na) (INVE, Belgium) were introduced at 4DAH and prey density was gradually increased from 4 to 5 na·mL⁻¹, becoming the only prey offered after 5DAH. *Artemia* EG metanauplii (M24) (INVE, Belgium) enriched with Easy DHA Selco were introduced at 12DAH,

gradually increasing from 12 to 14 M24·mL⁻¹ until 19DAH. Enriched frozen *Artemia metanauplii* were offered to settled larvae (between 16 and 35DAH). Live prey was gradually reduced and replaced by inert diet until complete weaning at 36DAH, according to Engrola et al. (2009), with the inert diet constituting 60 % of the feed supplied (dry matter basis) from 6 to 16DAH, more than 60 % from 17 to 30DAH and more than 80 % from 30 to 36 DAH.

Live prey was delivered 3 times a day (3h interval) during the pelagic phase and 4 times a day (2h30 interval) during the benthic phase. The first live feed meal was offered 1hour after the lights were on (11.00h) during the pelagic phase and 30 min (9.30h) after during the benthic phase (Engrola et al., 2005). Inert diet was delivered semi-continuously with automatic feeders (cycles of 2 h of feeding followed by 1h break). After 36DAH fish were exclusively fed upon the respective inert diet (Intact, PartH and HighH) and considered weaned. After 60 DAH all groups were fed upon the same commercial diet until 90 DAH (Gemma Diamond, Skretting).

The amount of feed distributed to each tank was based on predicted maximum growth and daily adjustments were done based on visual inspection to avoid excess of uneaten food (Engrola et al., 2005).

2.3 Somatic growth

At mouth opening (MO) (2DAH), one pool of 20 individuals was collected from each tank for dry weight (DW) evaluation. Thereafter, individual fish were randomly sampled for dry weight determination at 16DAH (MC: metamorphosis climax), 36DAH (weaned early juvenile) ($n=20$ per replicate), 60DAH, at a late juvenile stage, and after being fed exclusively upon the inert diets for 24days ($n=20$ per replicate), and at 90DAH after being fed exclusively upon a single commercial diet for 30 days ($n=20$ per replicate) The larvae and early juveniles were frozen at -80°C and freeze-dried for dry weight determination to 0.001mg precision. Growth was expressed as relative growth rate (RGR, % day⁻¹) and was determined during the pelagic phase from mouth opening to metamorphosis climax (2-16DAH), during the benthic phase (16-36DAH, 36-60DAH and 60-90DAH) and during the whole trial (2-60DAH). RGR was calculated as $RGR (\% \text{ day}^{-1}) = (e^g - 1) \times 100$, where $g = [(\ln \text{ final weight} - \ln \text{ initial weight}) / \text{time}]$ (Ricker, 1958).

2.4 White muscle cellularity

Standard histological and morphometric techniques (Silva et al., 2009b; Valente et al., 1999) were used to analyse fast-twitch muscle cellularity at two developmental stages: 16DAH (metamorphosis climax – stage 3) and 36DAH (weaned post-larvae). Three fish per tank were collected, killed by over-anaesthesia (MS-222, Sigma-Aldrich, USA; 400 mgL⁻¹) and measured for standard length (SL, mm). Fish were then fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (Sigma-Aldrich, USA) for 24h, washed with 1×PBS and stored in 70° ethanol, at 4°C until further processing. Paraffin (Merck, Germany) embedded fish were sectioned (7 µm) transversely to the body axis, using a microtome (RM2245, Leica, Germany), mounted on coated slides with 3-aminopropyltriethoxysilane (APES) (Sigma-Aldrich, USA) to improve section adhesion and double stained with haematoxylin (Haematoxylin Gill II, Merck, Germany) and eosin (Eosin Y, VWR, Belgium) before placing a cover slip.

Morphometric variables were measured in transversal body sections of individual fish, at a peri-anal location. In larvae and early juveniles (16 and 36DAH), the total number of fast-twitched fibres (N) were counted, and total cross-sectional area [CSA (mm²)], total cross section muscle area [Muscle CSA (mm²)] and fast-twitch fibre cross-sectional area (µm²) were measured. Fibre diameter (µm) was estimated from the fibre cross-sectional area (µm²) data assuming that muscle fibres cross-section is round shaped. The mean fibre cross-sectional diameter and percentage of small fibres (with a diameter <3 µm at 16DAH and <5 µm at 36DAH) were estimated from a minimum of 600 fibres which cross-sectional area was measured over the total cross section muscle area. The percentage of small fibres is an indicator of the growth potential and the chosen fibre size class to define the population of small fibres should correspond to the left-tail of the fibre size class distribution (Ian Johnston, University of St Andrews, pers. Comm). The fibre density (total number/mm²) was calculated by dividing the total number of fast-twitched fibres (N) counted by the total cross section muscle area [Muscle CSA (mm²)]. Muscle fibre outlines were traced using a 400× magnification. This study was performed using an Olympus BX51 microscope (Olympus Europa GmbH, Germany) with the Cell[^]B Basic imaging software. The relative contribution of hypertrophy and hyperplasia to the increase of muscle cross-sectional area was estimated as follows: $\Delta \text{Muscle CSA } (\mu\text{m}^2) = N_{m \times} \Delta A$

$(\mu\text{m}^2) + A_{m \times} \Delta N$, where Δ was calculated between two sampling times (t and $t + 1$), N_m and A_m refer to the mean total number of fibres and fibre area at t , respectively (Silva et al., 2009b; Valente et al., 1999).

2.5 Gene expression

2.5.1 RNA extraction and cDNA synthesis

Six pools of 20 individuals per dietary treatment (2 pools per tank) were sampled at 16DAH (metamorphosis climax – stage 3) and 36DAH (weaned post-larvae), snap-frozen in liquid nitrogen and kept at -80°C until further analysis. Each pool was grinded using pre-chilled pestle and mortar by adding liquid nitrogen, and then transferred to a 2mL sterile centrifuge tube. Total RNA was extracted according to the Tri reagent method (Sigma-Aldrich, USA). Assessment of RNA quality was performed by agarose gel electrophoresis. RNA samples were then quantified with a Nanodrop spectrophotometer (Nanodrop Technologies). In order to remove any traces of genomic DNA contamination, total RNA samples were treated with DNaseI provided in the High Pure RNA Isolation Kit (Roche), by adding 100 μL of 10% DNase I in DNase I Incubation Buffer, mixing and incubating for 15 min at 25°C . The RNA samples were further purified according to the manufacturer instructions provided with the kit. Purified RNA samples were again quantified using the Nanodrop spectrophotometer. cDNA was synthesized from 1 μg of purified RNA (per pool), using with the M-MLV Reverse Transcriptase Kit (Invitrogen, USA).

2.5.2 Quantitative real-time PCR (qPCR)

The relative expression of the MRFS (*myf5*, *myod2*, *mrf4*, *myog*), *mstn1* as well as genes encoding for the proteins responsible for *de novo* DNA methylation (*dnmt3a*, and *dnmt3b*) and methylation maintenance (*dnmt1*) were quantified using real-time PCR. Specific primers for qPCR were used (see Table 3 for primer sequences, GenBank accession numbers, amplicon sizes, annealing temperatures ($^\circ\text{C}$) and qPCR amplification efficiencies). Quantification of gene expression was performed by qPCR with Sso Fast Evagreen supermix (Bio-Rad) on a CFX96TM Real-Time PCR Detection System (Bio-Rad). Samples were denatured for 30s at 95°C and then subjected to 40 cycles of amplification

with the following thermocycling parameters: denaturation for 5s at 95°C and annealing/extension for 10s (see Table 3 for annealing temperatures (°C)). Specificity of the qPCR reaction and the presence of primer dimers were checked by examining the melting curves with a dissociation protocol from 65 to 95 °C (in 0.5°C increment, for 5 s). Five-point standard curves of a 5-fold dilution series (1:5–1:3125) of pooled RNA were used for PCR efficiency calculation. Minus reverse transcriptase controls were checked for every gene. All samples were run in triplicate. CT values were determined using the baseline subtracted curve fit method using the CFX Manager Software with a fluorescence threshold automatically set. Profiling of mRNA transcription levels (qPCR) were used to quantify gene expression, using data normalised against the geometric average of transcript levels of two reference genes (*ubq* and *rps4*) obtained from GeNorm (Vandesompele et al., 2002), as previously reported (Fernandes et al., 2008). Reference genes were selected based on previous works on Senegalese sole larvae. Three genes (*ubq*, *rps4* and *efla1*) were tested and two (*ubq*, *rps4*) were selected based on the stability of its expression in all samples.

2.6 Data analysis

Statistical analyses followed previously reported methods (Zar, 2010) and IBM SPSS Statistics 19 was the software used for all the statistical analysis performed. All data were tested for normality using a Kolmogorov-Smirnov (whenever $n > 30$) or Shapiro-Wilk (whenever $n < 30$) test and for homogeneity of variance using a Levene's test. Data were log transformed when required and percentages were arcsin transformed prior to analysis.

Comparisons between groups fed different diets were made using one-way ANOVA followed by a Tukey post-hoc test, or a Kruskal-Wallis one-way ANOVA on ranks followed a Games-Howell post-hoc test, whenever data did not meet equal variance requirements, either for growth, muscle growth parameters or relative expression of target genes. A Pearson's correlation coefficient was used to compare the relative expression of genes regulating muscle growth vs muscle growth parameters, using the mean value of each triplicate tank ($n = 6$).

To compare the distribution of muscle fibre size, a nonparametric method was used to fit smoothed probability density functions (PDFs) using the statistical program for the analysis of muscle fibre populations (Johnston et al., 2004). Bootstrapping was used to distinguish

random variation in diameter distribution from treatment differences. A Kruskal-Wallis test with 1000 bootstrap replicates was used to test the null hypothesis that PDFs of muscle fibre diameter in the three treatments were identical.

3. Results

3.1 Dietary effect on white skeletal muscle growth and somatic growth

The total number of fast-twitch muscle fibres and fibre density were not significantly different between groups at 16DAH, but the average fibre diameter ($P=0.023$) and muscle cross-sectional area ($P=0.046$) were significantly higher in the PartH group than in the Intact group at 16DAH (Table 4). Accordingly, 16DAH larvae fed the PartH diet were heavier when compared to those fed either the Intact or the HighH diets (Table 4). At this stage, there was a strong correlation between fibre diameter and Muscle CSA and a negative correlation between fibre density and Muscle CSA (Table 5).

Although the smoothed probability density functions (PDFs) of muscle fibre populations did not differ significantly between groups at 16DAH, the percentage of fibres with diameter within the range 7-11 μm was higher in PartH and HighH groups, when compared to the Intact group ($P=0.001$) and the percentage of fibres with diameter within the range 11-15 μm was higher in the PartH group than in the Intact group ($P=0.002$) (Table 4). The smoothed PDFs of muscle fibre populations indicated a high percentage of small-sized muscle fibres in all dietary treatments, as all curves skewed to the left-hand tail of the distribution (Fig. 1). There was a significant correlation between the percentage of very small fibres ($<3\mu\text{m}$) and the relative growth rate (RGR) in the subsequent period, from 16 to 36DAH (Table 5). At 36DAH, fish fed the Intact diet were already heavier than those fed the other diets and grew better until 60DAH (Table 4).

Between 16 and 36DAH there was a significant enlargement of muscle fibres in all groups (Table 4), reflected on the shift of PDFs distribution towards the right-hand of the plot (Fig. 1). The average fibre size increased from 16 to 36DAH by 1.8, 1.6 and 1.7-fold respectively in the Intact, PartH and HighH groups, being similar among groups at 36DAH (Table 4). This was paralleled by a 2.3, 2.3 and 2.0-fold increase of total number of fibres in the Intact, PartH and HighH groups, respectively, which reflected in a muscle CSA increase of

6.2-fold, 4.8-fold and 4.4-fold in the Intact, PartH and HighH groups, respectively (Table 4). Moreover, there was significant contribution of the total number of fibres to the muscle cross-sectional area, as a positive correlation between these two parameters was detected at this stage ($P=0.031$) (Table 5). Although PDFs distributions at 36DAH did not differ significantly among treatments (Fig. 1), the percentage of small fibres (diameter $<5\mu\text{m}$) was higher in the group fed the Intact diet, when compared to those fed the PartH and the HighH diets ($P=0.039$) (Table 4). Moreover, there was a positive correlation between the percentage of small fibres (diameter $<5\mu\text{m}$) and the relative growth rate in early juvenile fish (Table 5). The relative contribution of hypertrophy to white muscle growth in the 16-36DAH period was higher in the HighH group ($63.4\pm 7.0\%$) than in the PartH group ($52.6\pm 6.4\%$) ($P=0.026$), but not significantly different from that in the Intact group ($55.6\pm 8.2\%$). No further differences were found in the white muscle cellularity variables at 36DAH (Table 4).

From 60 to 90DAH, after being fed upon the same commercial diet for 30 days, all the groups grew at similar relative growth rates (Table 4). At 90DAH fish fed the Intact diet were still heavier (DW = 79.8 ± 27.3 mg) than those fed the PartH (DW = 39.2 ± 21.4 mg) and HighH (DW = 42.2 ± 21.3 mg), respectively by 2.0 and 1.9-fold ($P<0.001$).

3.2 Expression of growth-related genes and DNA methyltransferases

The relative expression of the genes encoding for the myogenic regulatory factors did not differ significantly between groups fed different diets during the metamorphosis climax (Fig. 2A). However, at 36DAH, the expression of *myf5* ($P=0.027$) and *myod2* ($P=0.029$) was significantly upregulated in the group fed the HighH diet, in which the transcript levels were increased, respectively, by 2.24 and 2.05-fold, when compared to the PartH group, no differences being found in pair-wise comparison between the HighH and Intact groups (Fig.2B). At this stage, the transcript levels of *myog* ($P=0.004$) were also upregulated in the group fed the HighH diet, increased by 1.51 and 1.77-fold when compared to the groups fed Intact and PartH diets, respectively (Fig.2B). No significant differences were found in the transcript levels of *mrf4*. At this stage, fibre density was negatively correlated with the expression of *myod2* and *myog* (Table 5). No dietary effect was found in the *mstn1* mRNA

levels during the metamorphosis or at 36DAH (Fig.2B). However, fibre density was negatively correlated with the expression of *mstn1* (Table 5).

There was no effect of dietary protein complexity on the DNA methyltransferase *dnmt1* transcript levels in 36DAH Senegalese sole (Fig.3). However *dnmt3a* (P=0.042) and *dnmt3b* (P=0.041) transcript levels were significantly higher in fish fed the HighH diet, when compared to those fed either the Intact or PartH diets (Fig.3). Moreover, the expression of *dnmt3a* was negatively correlated with fibre density and accordingly, it was positively correlated with the fibre diameter (Table 5). The expression of *dnmt3b* was negatively correlated with the total number of fibres (Table 5).

4. Discussion

4.1 Effect of dietary protein complexity on larval performance

The three microdiets (Intact, PartH and HighH) tested in this study were formulated with the aim of having the degree of hydrolysis of dietary protein as the main changing factor, using approximately the same practical ingredients. Nevertheless, the proportions of marine and plant ingredients included in each diet might have also induced some other differences between diets. Since all diets included high levels fish and krill protein hydrolysates, the attractability or palatability to sole larvae were not likely to have changed between the diets, which was confirmed by the visual observation of fish behaviour towards the diets. All diets exceeded the indispensable amino acid (IAA) requirements for sole juveniles (Silva et al., 2009a) and were balanced according to the ideal protein concept (Arai, 1981) taking as reference the Senegalese sole post-larvae whole-body AA profile (Aragão et al., 2004). Thus, the effects of dietary formulation variations on the regulation of muscle growth and larval performance were most likely due to dietary protein complexity and not to any AA imbalances.

Several studies evaluated the inclusion of pre-digested proteins in microdiets for fish larvae (Cai et al., 2015; de Vareilles et al., 2012; Gisbert et al., 2012; Kotzamanis et al., 2007; Kvåle et al., 2009; Kvåle et al., 2002; Skalli et al., 2014; Srichanun et al., 2014), but to our knowledge very few focused on the dietary effects of hydrolysates on the regulation of muscle growth (Katan et al., 2016; Ostaszewska et al., 2008). In the present study, the

tested microdiets were mostly based on protein sources with substantially different peptide MW profiles to be delivered to Senegalese sole from a very early developmental stage, i.e. from mouth-opening. On the contrary, in the previous studies the protein hydrolysates included in microdiets were mostly composed of free amino-acids and di- or tri-peptides (<0.2KDa) or oligopeptides (0.5-2.5 KDa) (Cahu et al., 2004; 1999; Cai et al., 2015; de Vareilles et al., 2012; Gisbert et al., 2012; Kotzamanis et al., 2007; Skalli et al., 2014; Srichanun et al., 2014; Zambonino Infante et al., 1997).

Given the Senegalese sole complex metamorphosis and digestive system ontogeny (Conceição et al., 2007; Fernández-Díaz et al., 2001; Morais et al., 2016), it was expected that the larvae capacity to digest and utilize dietary protein would change throughout development. Indeed, according to has been previously reported by Canada et al. (2017), different dietary formulations based on different protein MW profiles had different effects on Senegalese sole somatic growth throughout development. The diet including a partially hydrolyzed plant protein mixture (target peptide range from 5 to 70 KDa) (PartH) was likely more digestible to pre-metamorphic larvae (Canada et al., 2017) promoting growth towards the metamorphosis climax. However, during the metamorphosis climax, the diet based on intact plant protein sources (Intact) promoted the absorption and total retention of small-sized peptides (Canada et al., 2017), what would have contributed for enhanced growth in later stages from 36DAH onwards. These results are in agreement with Richard et al. (2015) that suggested that larger peptides are better suited to sole young juvenile AA anabolic and physiological needs than to early stage larvae.

4.2 Effect of dietary protein complexity on the regulation of muscle growth

Different dietary formulations based on different protein MW profiles induced differences in the white muscle cellularity that were reflected on somatic growth during the metamorphosis climax. Although the smoothed probability density functions (PDFs) of muscle fibre populations did not differ significantly among treatments at 16DAH, significant differences were detected in the percentage of medium to large-sized muscle fibres. The percentage of fibres with diameter within the range 7-11 μm was lower in the Intact group, when compared to the PartH and HighH groups, and the percentage of fibres with diameter within the range 11-15 μm was lower in the Intact group than in the PartH

group, which together with a reduced average fibre diameter and similar fibre number, suggests a delayed muscle growth in the Intact fed larvae. Accordingly, the PartH fed larvae had a significantly larger average fibre diameter, compared to the Intact group, which was reflected on a higher muscle cross-sectional area (as suggested by the positive correlation between average fibre diameter and muscle- cross-sectional area) and probably contributed to increased dry weight during the metamorphosis climax. Also in Senegalese sole metamorphosing larvae exposed to different rearing temperatures during the pelagic phase, muscle growth of fast-growing fish was related to increased fast-twitch fibre size and not the fibre number (Campos et al., 2013b). Campos et al. (2013b) reported a significant increase in the transcript levels of both primary MRFs and *myog* at a pre-metamorphic stage, and *myod2* and *myog* during the metamorphosis climax, in the fastest-growing group. In pacu larvae (*Piaractus mesopotamicus*) reared upon different types of feed, dietary formulations and feeding regimes, the fastest-growing group displayed a higher frequency of small fibres and a trend for higher *myod* expression (Leitão et al., 2011). Increased proliferative capacity of MPCs and a higher contribution of hyperplasia was also reported in pike perch (*Sander lucioperca*) larvae fast-growing groups, as a response to type of feed and dietary formulation (Ostaszewska et al., 2008). In the present study, the relative expression of the genes encoding for myogenic regulatory factors was similar among dietary treatments, during the metamorphosis climax, and could not be related to muscle cellularity parameters. Yet and despite the possibly delayed muscle growth in the larvae fed the Intact diet during the pelagic phase, this group displayed a tendency for a higher percentage of small fibres (diameter $<3\mu\text{m}$), representing a 1.24 and 1.47-fold higher proportion of those fibres, when compared with the HighH and PartH groups, respectively. This higher percentage of small fibres suggests a possible gain in the proliferative capacity of the larvae reared upon the Intact diet, what would have promoted subsequent growth in this group. This hypothesis is supported by the significant correlations between the percentage of very small fibres (diameter $<3\mu\text{m}$) during the metamorphosis climax and relative growth rate in the following period (16-36DAH). In fact, when compared to the groups fed the PartH and HighH diets, the Intact group grew significantly better from 36DAH onwards.

At 36DAH, and contrarily to what was observed during the metamorphosis climax, different dietary formulations induced different expression patterns of genes encoding for myogenic regulatory factors. At this stage, the transcription of the genes encoding for the primary MRFs (*myf5* and *myod2*) was significantly upregulated in the group fed the HighH diet, when compared to that fed the PartH diet. The transcription of *myog* was also significantly higher in the group fed the HighH diet, when compared to both the Intact and PartH groups. Since *myog* encodes a highly conserved myogenic regulatory factor that is involved in terminal muscle differentiation (Rescan, 2001), its upregulation might have sustained the differentiation of myoblasts to further fuse into already formed muscle fibres, promoting hypertrophy rather than hyperplasia in the HighH group, being upstream supported by the upregulated *myf5* and *myod2* transcription. This is further corroborated by the negative correlation observed between fibre density and the transcript levels of *myod2* and *myog* (Table 5), and confirmed by the relative contribution of hypertrophy towards white muscle growth in the 16-36DAH period, which was significantly higher in the HighH group (63%) than in the PartH group (52%). Also in Senegalese sole, differences in the transcript levels of both *myod2* and *myog* between groups reared at different water temperatures were associated with changes in fibre diameter and not in the total fibre number during the metamorphosis climax (Campos et al., 2013b), suggesting that the upregulation of these MRFs seem to promote fibre hypertrophy without affecting total fibre number. A higher contribution of hypertrophy to muscle growth and the reduced recruitment of small fibres (evidenced by a low percentage of fibres < 5 µm at 36DAH) are predictive of a lower growth potential in the HighH group. The relation between hypertrophy-sustained muscle growth and further reduced somatic growth has been proposed in common carp (Alami-Durante et al., 1997), Atlantic cod (Galloway et al., 1999), pike perch (Ostaszewska et al., 2008) and pacu (Leitão et al., 2011) larvae subjected to different types of feed, dietary formulations and feeding regimes.

A significant downregulation of *myf5*, *myod2* and *myog* transcription in the PartH group, and lower percentage of small fibres compared to the Intact group, suggests a reduced recruitment capacity of new fibres in fish fed the PartH diet. This is further supported by a negative correlation between the *myod2* and *myog* transcript levels and fibre density. Decreased recruitment of new fibres would have led to reduced growth potential in PartH

fed fish. Also in Senegalese sole, a significant downregulation of most MRFs in earlier stages was reported in a slow-growing group exposed to a low rearing temperature during the pelagic phase (Campos et al., 2013b). Pike perch larvae fed a formulated diet including a casein hydrolysate (Ostaszewska et al., 2008) and Atlantic cod larvae reared upon a fish hydrolysate supplemented diet (Katan et al., 2016) have displayed reduced somatic growth, but as a result of delayed muscle growth, including reduced muscle cross-sectional area. The present study results suggest a lower proliferative capacity in the PartH fed fish, but not at the point of reducing muscle cross-sectional area.

At 36DAH, the transcription of *myf5*, *myod2* in the Intact group was probably high enough to keep up with the increase of the MPC population and further fibre recruitment. In fact, fish fed the Intact diet had a significantly higher percentage of small fibres, which has probably contributed for a higher growth potential, as further suggested by a significant correlation between the percentage of small fibres at 36DAH (diameter $<5\mu\text{m}$) and relative growth rate in the 36-60DAH period. Indeed, the percentage of small-sized muscle fibres is long known to be a good indicator of further muscle growth potential both in juveniles and larvae (Alami-Durante et al., 1997; Galloway et al., 1999; Silva et al., 2009b). At 90 DAH, after being fed upon a common commercial diet for 30 days, fish fed the Intact diet were still heavier than those fed the PartH and HighH diets, which reinforces the impact of muscle growth dynamics on long-term somatic growth in response to dietary protein complexity. However, it cannot be excluded that 30 days may not be enough to evaluate whether this impact on long-term somatic growth is reversible or not.

In spite of the negative correlation between *mstn1* transcription and fibre density detected at 36DAH, different diets did not induce significant changes in the *mstn1* mRNA levels during the metamorphosis or at 36DAH. Since *mstn1* encodes for myostatin, which is a negative regulator of MPCs activation and proliferation, a negative correlation between *mstn1* transcription and density or fibre recruitment and muscle growth would be expected. However, the relation between *myostatin* mRNA levels and muscle growth is surprising: depression of growth induced by environmental conditions does not correlate with an up-regulation of *myostatin* as expected (Rescan, 2005). While Atlantic cod larvae fed a fish hydrolysate supplemented diet displayed reduced growth and delayed muscle growth along with upregulated *mstn1* transcription, Senegalese sole fast-growing groups displayed an up-

regulation of *mstn1* in late juvenile stages (Campos et al., 2013b; Canada et al., 2016). Moreover, *mstn1* transcription did not respond to environmental changes in Senegalese sole metamorphosing larvae and post-larvae (Campos et al., 2013b). Similarly, the present results show no clear dietary effect on the expression of *mstn1* at 16 or 36DAH. Moreover, in the present study, gene expression was analyzed in the whole fish and not only in the muscle, so it is also possible that the expression of *mstn1* might be associated with other physiological mechanisms and not only with skeletal muscle growth regulation (Campos et al., 2010).

4.3 Epigenetic effect

Late post-larvae fed the HighH diet had higher *dnmt3a* and *dnmt3b* mRNA levels, when compared to those fed the other diets, which may be associated with the dietary methionine availability in the HighH diet. *Dnmt3a* and *dnmt3b* are *de novo* DNA cytosine methyltransferases which covalently attach S-adenosylmethionine (SAM) methyl groups to the 5'-position of cytosine (in CpG dinucleotides), thus methylating DNA and repressing transcription during embryogenesis and cell differentiation (Turek-Plewa, Jagodzinski, 2005). DNA methylation relies on the availability of SAM, the universal cellular methyl donor (Selhub, 1999). SAM generates from methionine in a ATP-dependent reaction in the one-carbon cycle which depends on the activity of several enzymes and the presence of dietary methyl donors, such as folate, choline, betaine and methionine (Anderson et al., 2012). The HighH diet had a higher methionine content, increased by 28% and 88% when compared with the Intact and the PartH diets, respectively. A higher availability of methionine may underlie the increased transcription of *dnmt3a* and *dnmt3b* in the HighH group, which may be associated with a general increase in DNA methylation. However, further studies would be needed to ascertain whether there were actually changes in the availability of methyl group donors in the tissues of the fish fed the different diets, and whether the increased expression of genes encoding for DNA methyltransferases would possibly lead to DNA hypermethylation. In fact, although theoretically the supplementation of methionine would increase the DNA methylation of genes and down-regulate gene expression (Waterland, 2006), in practice the effect of a methionine-supplemented diet on the DNA methylation is not always as expected (Zhang, 2017). The effect of methionine

supplementation on the regulation of one C-cycle, namely on the production of either S-adenosylmethionine (SAM) or S-adenosylhomocysteine (SAH) is not consistent, neither is its effect on the expression of the DNMTs or in the methylation patterns, which seems to be tissue-specific (revised by Zhang, 2017). In fact, not always the up-regulation of DNMTs lead to global or region-specific hypermethylation, which in turn not always leads to gene expression silencing (revised by Zhang, 2017).

In the present study, the correlations detected between the expression of *dnmt3a*, fibre density and the fibre diameter, and between the expression of *dnmt3b* and the total number of fibres might suggest an epigenetic event possibly underlying the changes in muscle growth in response to dietary protein complexity. However, given the present state of knowledge about the mechanisms governing global and region-specific DNA methylation specifically in the context of nutri-epigenomics (see Zhang, 2017), such discussion would require further research on the methylation status of genes or genes promoters regulating myogenesis.

5. Conclusions

Dietary protein complexity did affect the regulation of myogenesis throughout Senegalese sole development, by delaying muscle growth until the metamorphosis climax (16DAH) in larvae fed the Intact diet, and by either reducing the recruitment of small fibres in both the PartH and HighH groups or also by promoting hypertrophy sustained muscle growth in the HighH group in an early juvenile stage, at 36DAH. The dietary protein complexity induced changes in the regulation of myogenesis that have ultimately reduced the potential for muscle growth in those groups fed microdiets mostly based on hydrolysed protein sources. The subsequent impact on somatic growth remained until a late juvenile stage (60DAH) and even after 30 days of feeding all groups on the same commercial diet, at 90DAH.

The expression of *dnmt3a* and *dnmt3b* was up-regulated in the HighH group, possibly due to a higher content of methionine in the HighH diet. As these genes encode for a DNA methyltransferases essential for *de novo* methylation, its expression up-regulation may be associated with a general DNA hypermethylation. Future studies should be carried on possible ways to modulate the metabolic pathways in the long-term through epigenetic modifications.

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Conflict of interest:

The authors declare that they have no conflict of interest.

Authorship:

P.C. performed the experiment, the statistical analysis and wrote the manuscript. R.T, P.C. and S.M. performed gene expression analysis. P.C. and V.S performed histological analysis. J.P and M.Y manufactured the IdG Hydrolysate and revised the manuscript. L.V., L.C, S.E. and J.F conceived the study, supervised the research and contributed to writing of the manuscript.

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Figure captions:

Fig. 1: Probability density functions (PDFs) distributions of fast muscle fibres at 16 (metamorphosis climax) and 36DAH (weaned post-larvae). The dashed lines show the mean PDF for each group and the solid line central to the shaded area is the average PDF for combined groups (Intact n 11, PartH n 9 and HighH n 11, at 16DAH; Intact n 7, PartH n 8 and HighH n 8, at 36DAH). The shaded area shows 1000 bootstrap estimates from combined populations of fibre diameter.

Fig. 2: Expression of genes encoding for myogenic regulatory factors *myf5*, *mrf4*, *myod2*, *myog* and *mstn1* at 16DAH (A) and 36DAH (B) (whole body pools of 20 individuals). mRNA expression was normalized to transcript levels of *ubq* and *rps4*. Values are presented means \pm s.e.m., n = 6. Different superscript letters indicate significant differences (P<0.05, 1-way ANOVA) between the dietary treatments at each developmental stage.

Fig. 3: Expression of genes related to DNA methylation (*dnmt1*, *dnmt3a* and *dnmt3b*) at 36DAH (whole body pools of 20 individuals). mRNA expression was normalized to that of *ubq* and *rps4*. Values are presented means \pm s.e.m., n = 6. Dissimilar superscript letters indicate significant differences (P<0.05, 1-way ANOVA) between the dietary treatments at each developmental stage.

Table 1

Composition and proximate analyses of the experimental diets (modified after Canada et al., 2017)

	<i>Diets</i>		
	Intact	PartH	HighH
<i>Ingredients (% dry matter)</i>			
Marine protein Mix ^a	15	15	15
Plant protein Mix 12 ^b	41.5	0	14.6
IdG Hydrolysate (IdGH) ^c	0	40.5	5
Fish protein hydrolysate (FPH) ^d	13	13	36
Autolysed yeast Hilyses ^e	1	1	1
Krill hydrolysate HC6 ^f	5	5	5
Algatrium ^g	2.5	2.5	2.5
Phosphonorse ^h	4	4	4
Fish oil ⁱ	6	7	5
Vit & Min Premix ^j	8	8	8
AA mix ^j	4.0	4.0	3.9
<i>Proximate analyses</i>			
Dry matter (DM,%)	93.5	90.7	91.7
Ash (% DM)	8.6	10.9	10.1
Crude protein (% DW) ^{after leaching for 1 min}	64.7	61.1	65.8
Crude fat (% DW) ^{after leaching for 1 min}	14.5	18.9	15.8
Gross Energy (Kj/g) ^{after leaching for 1 min}	20.1	20.1	20.2

^a Proprietary SPAROS product for marine fish: 93% CP, 1.3% CF.

^b Proprietary SPAROS product for marine fish: 84% CP, 3.6% CF.

^c Proprietary SPAROS protein hydrolysate, resulting from hydrolysis of Plant protein Mix; peptide molecular weight profile: >70 KDa (12%); 20-70 KDa (28%); 5-20 KDa (27%); <5 KDa (23%)

^d CPSP 90, Sopropêche, France; peptide molecular weight profile:>20 KDa (1%); 20-10 KDa (4%); 10-5 KDa (8%); 5-1 KDa (48%); 1-0.5KDa (18%); <0.5KDa (21%)

^e ICC, Brazil

^f Aquativ, France

^g DHA-rich oil

^h Marine phospholipids and marine oils, Tromsø Fiskeindustri A/S, Norway

ⁱ Marine oil omega 3: Henry Lamotte Oils GmbH, Germany

^j Proprietary SPAROS premixes / products for marine fish: L-Lysine HCl 30% L-Tryptophan 8%, Taurine 38% Betaine 25%

Table 2

Determined amino acid content (% dry matter) of the experimental diets after 1 min leaching in seawater.

	<i>Diets</i>		
	Intact	PartH	HighH
<i>Indispensable amino-acids (IAA)</i>			
Arg	5.37	4.24	5.85
His	1.15	0.97	1.18
Lys	5.25	4.62	5.83
Thr	2.27	2.12	2.39
Ile	2.37	2.10	2.24
Leu	4.12	3.68	4.03
Val	2.50	2.10	2.50
Met	1.67	1.14	2.13
Phe	2.77	2.42	2.52
Cys	0.10	0.08	0.12
Tyr	1.34	1.18	1.24
IAA sum	28.93	24.63	30.04
<i>Dispensable amino-acids (DAA)</i>			
Aspartic acid + Asparagine	5.20	4.79	5.33
Glutamic acid + Glutamine	7.90	6.65	6.21
Alanine	3.94	3.39	4.62
Glycine	5.98	4.89	6.54
Proline	4.06	3.37	3.73
Serine	2.44	2.16	2.44
Taurine	1.12	1.14	0.97

Table 3

Primers used in qPCR

Gene	Fwd sequence (5'→3')	Rev sequence (5'→3')	Accession nr (GenBank)	Size (bp)	Annealing temp. (°C)	E(%)
<i>myf5</i>	GAGCAGGTGGAGAACTACTACG	CCAACCATGCCGTCAGAG	FJ515910	89	60	103
<i>mrf4</i>	GAGAGGAGGAGGCTCAAGAAG	CAGGTCCTGTAATCTCTCAATG	EU934042	137	58	96
<i>myog</i>	GTCACAGGAACAGAGGACAAAG	TGGTCACTGTCTTCTTTTGC	EU934044	118	60	94
<i>myod2</i>	ACAGCCACCAGCCCAAAC	GTGAAATCCATCATGCCATC	FJ009108	194	60	111
<i>mstn1</i>	GGGAGATGACAACAGGGATG	TGGATCCGGTTCAGTGCC	EU934043	91	60	108
<i>dnmt1</i>	GATCCCAAGTGGAGTACGG	AAGAAGTCCTCATAAGTAGCGTC	KC129104	117	62	103
<i>dnmt3a</i>	AACTGCTGTAGGTGTTTCTGTGTG	CGCCGCAGTAACCCGTAG	KC129105	134	60	101
<i>dnmt3b</i>	ATCAAGCGATGTGGCGAGC	CGATGCGGTGAAAGTCAGTCC	KC129106	91	60	96
<i>rps4</i>	GTGAAGAAGCTCCTTGTCGGCACCA	AGGGGGTCGGGGTAGCGGATG	AB291557	101	60	95
<i>ubq</i>	AGCTGGCCCAGAAATATAACTGCGACA	ACTTCTTCTGCGGCAGTTGACAGCAC	AB291588	135	60	93

For each gene, its GenBank accession numbers, amplicon size (bp), Annealing temperatures (°C) and qPCR amplification efficiencies (E) are indicated

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Table 4

Growth performance and muscle cellularity parameters at key developmental stages: 16DAH (metamorphosis climax), 36DAH (weaned post-larvae) and juveniles.

	<i>Diets</i>		
	Intact	PartH	HighH
<i>16 DAH</i>			
RGR (2-16DAH) (% day ⁻¹)	26.6±2.2	29.0±0.7	26.9±2.1
DW (mg)	0.54±0.14 ^b	0.67±0.14 ^a	0.55±0.17 ^b
SL (mm)	5.6±0.2	5.8±0.3	5.9±0.3
Muscle CSA (mm ²)	0.07±0.01 ^b	0.08±0.01 ^a	0.08±0.03 ^{ab}
Total number of fibres N	1252±144	1329±180	1323±199
Fibre density (total number/mm ²)	19493±4172	15755±1549	16558±4265
Fibre diameter (D, µm)	5.75±0.64 ^b	6.61±0.41 ^a	6.35±0.86 ^{ab}
Percentage of fibres with			
D<3 µm	21.1±8.9	14.4±3.2	17.0±6.5
3<D<7 µm	53.1±4.2	48.7±4.4	49.5±6.2
7<D<11 µm	16.6±4.1 ^b	23.9±4.1 ^a	21.7±4.1 ^a
11<D<15 µm	6.1±1.4 ^b	8.9±1.9 ^a	7.9±3.0 ^{ab}
D>15 µm	2.6±1.3	3.3±1.0	2.7±1.3
<i>36 DAH</i>			
RGR (16-36DAH) (% day ⁻¹)	9.46±1.0 ^a	7.1±0.4 ^b	8.3±1.0 ^{ab}
DW (mg)	3.09±0.76 ^a	2.66±0.64 ^b	2.65±0.64 ^b
SL (mm)	11.9±0.9	11.3±0.8	10.9±0.5
Muscle CSA (mm ²)	0.40±0.06	0.41±0.12	0.38±0.07
Total number of fibres N	3015±458	3074±582	2617±373
Fibre density (total number/mm ²)	7538±1155	7831±1266	7002±723
Fibre diameter (µm)	10.15±0.38	10.70±0.72	10.96±0.78
Relative contribution of hypertrophy (%)	55.6±8.21 ^{ab}	52.6±6.42 ^b	63.4±7.02 ^a
Percentage of fibres with			
D<5 µm	10.7±2.4 ^a	8.2±2.0 ^b	8.0±1.6 ^b
5<D<10 µm	44.8±4.8	45.2±3.2	42.3±4.6
10<D<15 µm	28.7±2.4	28.5±3.3	30.4±1.4
15<D<20 µm	8.4±2.6	9.2±1.8	9.6±2.4
D>20 µm	5.1±2.1	6.2±2.8	6.6±2.3
<i>Growth after 36 DAH</i>			
RGR (36-60DAH) (% day ⁻¹)	6.1±0.8 ^a	3.0±0.8 ^b	2.9±0.0 ^b
RGR (60-90DAH) (% day ⁻¹)	6.7±0.8	6.9±0.9	7.4±0.5

Relative growth rate (RGR) calculated for each tank (n=3) as $RGR (\% \text{ day}^{-1}) = (e^g - 1) \times 100$, where $g = [(\ln \text{ final weight} - \ln \text{ initial weight}) / \text{time}]$. Dry weight (DW) at 16DAH (n=30) and 36 DAH (n=60). Standard length (SL) measured from the tip of the snout to the posterior end of the midlateral portion of the hypural plate, and white muscle cellularity parameters measured at a peri-anal location in individuals sampled for histological analysis (n=11 for 16DAH, except for PartH diet (n=9); n=8 for 36DAH, except for Intact diet (n=7)).

^{a,b} Mean values within a row with unlike superscript letters were significantly different (one-way ANOVA followed by a Tukey post-hoc test (α 5%), or a Kruskal-Wallis one-way ANOVA followed a Games-Howell post-hoc test (α 5%), whenever data did not meet equal variance requirements.

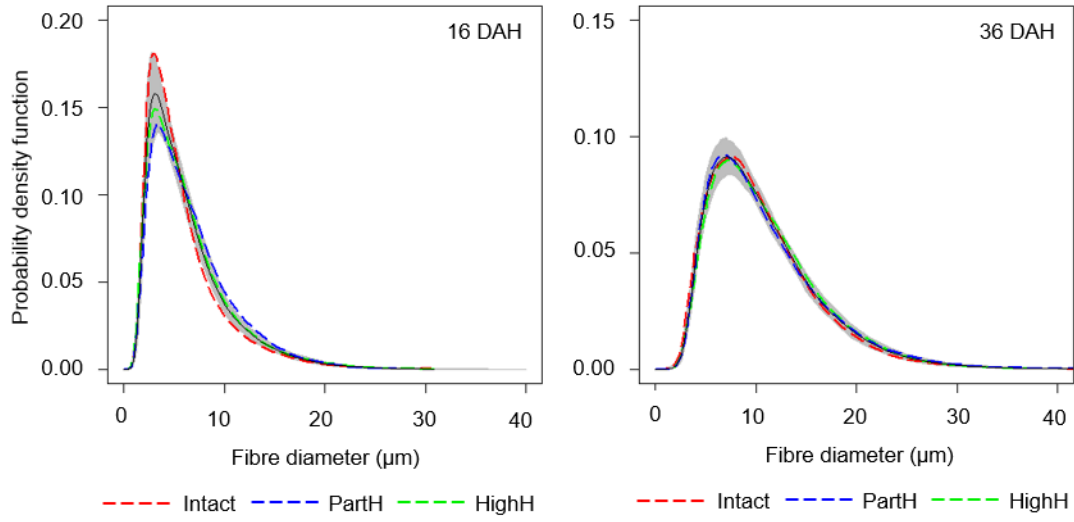
Table 5

Correlations (Pearson's coefficient, n=9) between white muscle cellularity parameters and somatic growth or gene expression data in Senegalese sole larvae, at 16 DAH (metamorphosis climax) and at 36 DAH (weaned post-larvae).

	Total nr of fibres	Fibre density	Fibre diameter	% of small fibres
16DAH				
Muscle CSA	P=0.004, r=0.850	P=0.002, r=-0.867	P=0.007, r=0.817	NS
RGR (2-16DAH)	NS	NS	NS	NS
RGR(16-36DAH)	NS	NS	NS	P=0.035, r=0.702
<i>myf5</i>	NS	NS	NS	NS
<i>myod2</i>	NS	NS	NS	NS
<i>myog</i>	NS	NS	NS	NS
<i>mrf4</i>	NS	NS	NS	NS
<i>mstn1</i>	NS	NS	NS	NS
36DAH				
Muscle CSA	P=0.031, r=0.713	NS	NS	NS
RGR(16-36DAH)	NS	NS	NS	NS
RGR(36-60DAH)	NS	NS	NS	P=0.032, r=0.712
<i>myf5</i>	NS	NS	NS	NS
<i>myod2</i>	NS	P=0.013, r=-0.783	NS	NS
<i>myog</i>	NS	P=0.044, r=-0.679	NS	NS
<i>mrf4</i>	NS	NS	NS	NS
<i>mstn1</i>	NS	P=0.046, r=-0.674	NS	NS
<i>dnmt1</i>	NS	NS	NS	NS
<i>dnmt3a</i>	NS	P=0.025, r=-0.731	P=0.038, r=0.694	NS
<i>dnmt3b</i>	P=0.013, r=-0.783	NS	NS	NS

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Fig.1



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Fig.2

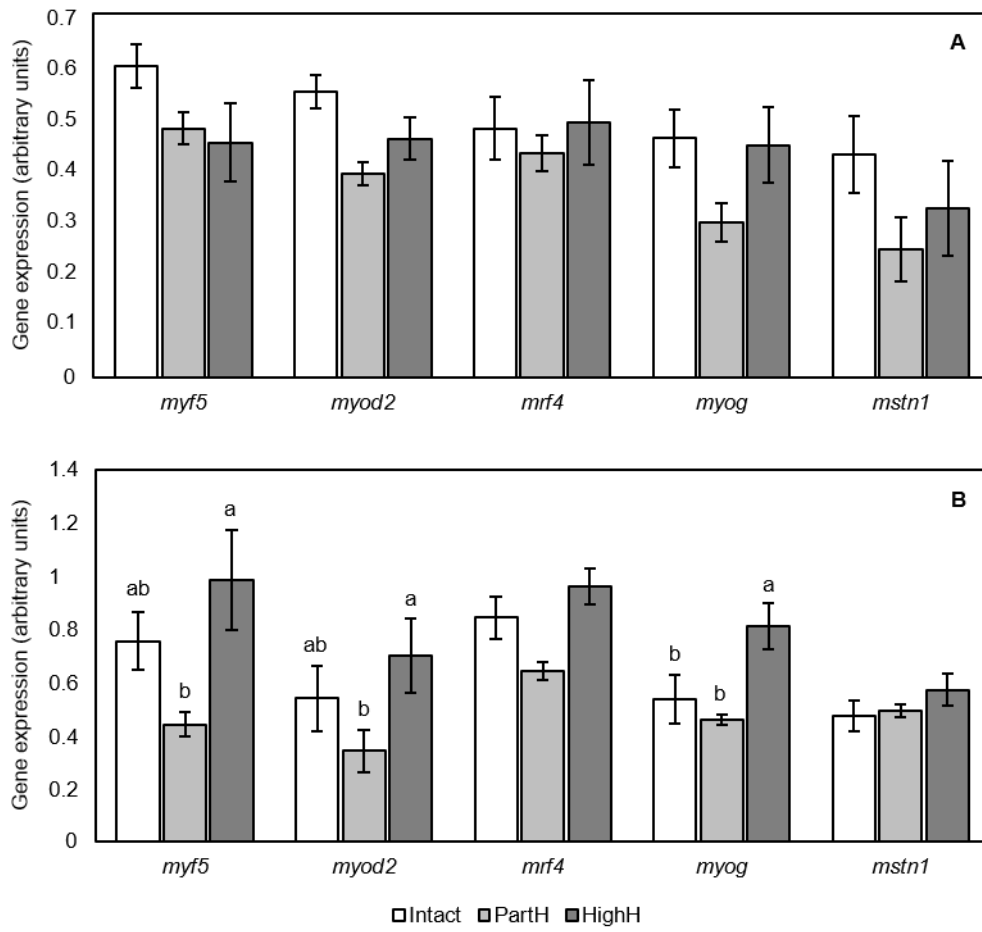
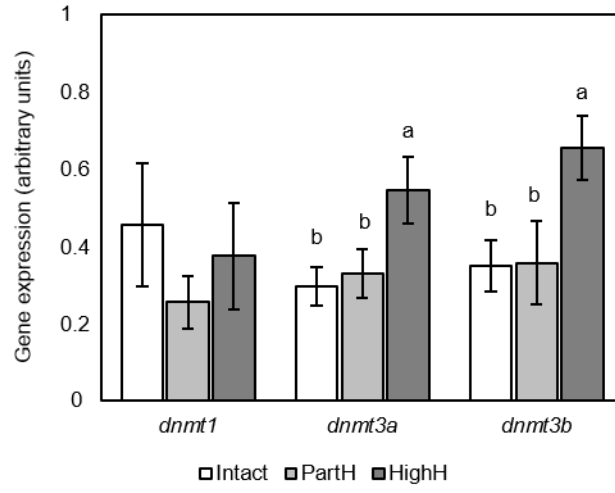


Fig.3



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Highlights:

Moderately hydrolysed protein promotes muscle growth of pre-metamorphic sole larvae.

Dietary intact protein increased growth potential in sole post-larvae and juveniles.

Changes in dietary protein complexity affected myogenesis in sole larvae.

Changes in dietary protein complexity affected the expression of *de novo* DNMTs.

ACCEPTED MANUSCRIPT