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PII: S0044-8486(16)30119-3
DOI: doi: 10.1016/j.aquaculture.2016.03.010
Reference: AQUA 632047

To appear in: *Aquaculture*

Received date: 15 December 2015
Revised date: 3 March 2016
Accepted date: 4 March 2016

Please cite this article as: Canada, Paula, Engrola, Sofia, Mira, Sara, Teodósio, Rita, Fernandes, Jorge M.O., Sousa, Vera, Barriga-Negra, Lúcia, Conceição, Luís E.C., Valente, Luisa M.P., The supplementation of a microdiet with crystalline indispensable amino-acids affects muscle growth and the expression pattern of related genes in Senegalese sole (*Solea senegalensis*) larvae, *Aquaculture* (2016), doi: 10.1016/j.aquaculture.2016.03.010

The supplementation of a microdiet with crystalline indispensable amino-acids affects muscle growth and the expression pattern of related genes in Senegalese sole (*Solea senegalensis*) larvae

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Abstract

The full expression of growth potential in fish larvae largely depends on an efficient protein utilisation, which requires that all the indispensable amino acids (IAA) are provided at an optimum ratio. The effect of supplementing a practical microdiet with encapsulated crystalline-AA to correct possible IAA deficiencies was evaluated in Senegalese sole larvae. Two isonitrogenous and isoenergetic microdiets were formulated and processed to have approximately the same ingredients and proximate composition. The control diet (CTRL) was based on protein sources commonly used in the aquafeed industry. In the supplemented diet (SUP) 8% of an encapsulated fish protein hydrolysate was replaced by crystalline-AA in order to increase the dietary IAA levels. The microdiets were delivered from mouth-opening upon a co-feeding regime until 51 days after hatching (DAH). The larvae capacity to utilise protein was evaluated using an *in vivo* method of controlled tube-feeding during relevant stages throughout development: pre-metamorphosis (13 DAH); metamorphosis climax (19 DAH) and metamorphosis completion (25 DAH). Somatic growth was monitored during the whole trial. A possible effect on the regulation of muscle growth was evaluated through muscle cellularity and the expression of related genes (*myf5*, *myod2*, *myogenin*, *mrf4*, *myhc* and *mstn1*) at metamorphosis climax (19 DAH) and at a juvenile stage (51 DAH). The SUP diet had a negative impact on larvae somatic growth after the metamorphosis, even though it had no effect on the development of Senegalese sole larvae capacity to retain protein. Instead, changes in somatic growth may reflect alterations on muscle growth regulation, since muscle cellularity suggested delayed muscle development in the SUP group at 51 DAH. Transcript levels of key genes regulating myogenesis changed between groups, during the metamorphosis climax and at the 51 DAH. The group fed the SUP diet had lower *dnmt3b* mRNA levels compared to the CTRL group. Further studies are needed to ascertain whether this would possibly lead to an overall DNA hypomethylation in skeletal muscle.

Keywords: dietary protein, amino acids profile, muscle growth, gene expression, Senegalese sole

1. Introduction

In most teleost larvae (Alami-Durante, 1990; Alami-Durante et al., 2006; Campos et al., 2013c; Khemis et al., 2013; Osse, Van den Boogaart, 1995) white skeletal muscle constitutes the bulk of the axial locomotor muscle, sustaining larvae burst swimming performance (Beamish, 1978) and their ability to capture prey while living in the water column. Therefore, white muscle growth during early life stages has a clear impact on the larvae capacity to swim, feed and survive (Osse et al., 1997). Moreover, in farmed species, there has been a great effort over the years to provide the best conditions for successful development of embryos and small larvae, as early environmental conditions can strongly affect muscle growth during early stages and influence the subsequent growth potential at later life stages (Campos et al., 2014; Galloway et al., 1999; Weatherley et al., 1988).

Muscle formation (myogenesis) is a complex process common to all vertebrates that involves the specification of stem cells to a myogenic lineage of myogenic progenitor cells – MPC- which then undergo activation, proliferation, cell cycle exit, differentiation, migration and fusion into muscle fibers (Johnston, 2006; Valente et al., 2013). Proliferation and differentiation of the MPCs are dependent on the programmed expression of four muscle-specific basic helix-loop-helix transcription factors, called myogenic regulatory factors (MRFs): *myod* (myoblast determination factor) and *myf5* are required for the commitment of myoblasts to form the MPC population; *myog* and *mrf4* induce and maintain the muscle differentiation program that will lately result in myotubes formation and enlargement (reviewed by Rescan (2001)). Myostatin is a negative regulator of muscle growth that inhibits myoblast proliferation (Thomas et al., 2000). *Myhc* (myosin heavy chain) encodes for myosin, which is a major structural protein of skeletal muscle and was shown to be correlated with muscle protein accretion in Atlantic salmon juveniles (Hevrøy et al., 2006). Fish muscle growth occurs both by hyperplasia (increase of fiber number) and hypertrophy (increase of fiber size) from hatching to until approximately 40% of maximum fish length (Rowlerson, Veggetti, 2001; Weatherley et al., 1988). During post-embryonic and larval development, muscle fiber number increases mainly by stratified hyperplasia, a phase of myogenesis that involves the recruitment of new fibers in discrete germinal zones found in the lateral margins of the myotome (Rowlerson, Veggetti, 2001). In juvenile and adult stages, in a second phase called mosaic hyperplasia, new myotubes form on the surface of fast

muscle fibers, further fusing or adding nuclei to already existing fibers, to keep size of nuclear domains constant during hypertrophic growth (Rowlerson, Veggetti, 2001). The relative contribution of hyperplasia and hypertrophy in fish was shown to be related to growth rate and final size attained by each species (Galloway et al., 1999; Weatherley et al., 1988), thus giving an estimate of individual growth potential.

In spite of the increased efforts to understand the regulation of myogenesis by intrinsic factors like genotype (Johnston et al., 1999a; Valente et al., 2006) and extrinsic factors such as photoperiod (Giannetto et al., 2013; Johnston et al., 2004; Lazado et al., 2014) and temperature (Campos et al., 2013b; 2013c; Galloway et al., 2006; Silva et al., 2011), studies evaluating the impact of nutritional factors on fish larvae muscle development are is still scarce. Different nutritional conditions, such as dietary protein sources (Alami-Durante et al., 1997; Ostaszewska et al., 2008) and lysine supplementation (Aguiar et al., 2005) were shown to affect muscle growth regulation and the somatic growth rate in fish larvae. More recently, Alami-Durante et al. (2014) suggested that in rainbow trout the activity of white MPCs might be early programmed by early nutrition. According to these authors, diets with different protein:energy ratios delivered to first-feeding rainbow trout larvae induced changes in white muscle cellularity in parallel with changes in the expression of muscle-growth related genes during the nutritional challenge period (from first-feeding to 75 days of feeding, but also and more remarkably after 3 months of feeding all groups on the same commercial diet. However, the mechanisms by which this early nutritional cue might have printed long-term changes in the expression of muscle growth related genes are not known. Campos et al. (2013a) have recently 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 in the expression of *dnmt1* and *dnmt3b* DNA methyltransferases, which catalyse the methylation of CpG dinucleotides, silencing gene expression. These changes resulted in alterations in the white muscle cellularity of Senegalese sole during metamorphosis climax (Campos et al., 2013a), and influenced subsequent somatic growth in later stages (Campos et al., 2013b). Increasing evidence indicates that DNA methylation is labile, not only to environmental conditions but also to nutritional factors, such as the availability of dietary methyl donors (reviewed by

Anderson et al. (2012)). However, to our best knowledge, the relationship between nutritional status and the epigenetic regulation of myogenesis has never been established in fish.

Fish larvae have high protein requirements and high obligatory amino acid (AA) losses for energy production (Conceição et al., 2011), and therefore dietary indispensable amino acids (IAA) levels may be a limiting factor. Moreover, ingredients commonly used as native protein sources on the formulation of commercial feeds may not meet the Senegalese sole larvae nutritional requirements on what concerns IAA (Aragão et al., 2004a). The supplementation of experimental inert microdiets with crystalline AA is a possible solution to increase dietary IAA levels. Such a strategy has shown positive results in other fish species, such as white bream *Diplodus sargus* (Saavedra et al., 2009a) and gilthead seabream *Sparus aurata* (Aragão et al., 2007), by improving survival, growth and/or larval quality. In Senegalese sole post-larvae, the supplementation with potential limiting IAA was also shown to improve the retention of a ^{14}C-labelled protein hydrolysate in an *in-vivo* tube-feeding trial, suggesting a positive impact on nitrogen utilisation and growth (Aragão et al., 2004b).

In the present study, it was hypothesized that increasing dietary IAA levels by supplementing microdiets with crystalline amino acids would impact on the larvae capacity to retain protein throughout metamorphosis and up to a juvenile stage. A growth trial was performed in conjunction with metabolic, muscle cellularity and gene expression studies. The expression pattern of DNA methyltransferases was analyzed in order to understand if growth differences could be associated with an epigenetic event.

2. Material and Methods

2.1 Experimental diets

Two diets (CTRL and SUP) were formulated and processed by SPAROS Lda. (Olhão, Portugal) to be isonitrogenous, isolipidic and isoenergetic, using the same practical ingredients. The CTRL diet was formulated to have a protein content based on native protein and a fish protein hydrolysate (Table 1). A second diet (SUP) consisted in replacing 8% of the encapsulated fish protein hydrolysate fraction of the CTRL diet with encapsulated crystalline AA in order to ensure a higher IAA supply (Table 1). Water soluble ingredients, such as fish protein hydrolysate, crystalline-AA and vitamin and mineral premix were encapsulated, using internal chitosan microparticles made from a suspension with a proportion of 2:12:3 (chitosan: fish protein hydrolysate /crystalline-AA: premix) (Santos et al., 2012). For this purpose, a chitosan (Sigma, USA) solution was prepared at 2% (w/v) in 1% (w/w) acetic acid (Carl Roth, Germany). After complete dissolution of chitosan, 12% (w/v) of fish protein hydrolysate (CPSP 90®, Sopropêche, France) or crystalline-AA and 1% (w/v) vitamin and trace minerals pre-mixture (Pre-Mix PVO040®, Premixportugal, Portugal) were added. The suspension was atomized in a laboratory scale spray-dryer (Lab-Plant SD-04, United Kingdom) using the following conditions: inlet temperature of 160 ± 1 °C, outlet temperature of 120 ± 4 °C, solution flow of 5 ± 0.5 mL/min, maximum blower level (100 units).

All dietary ingredients, including the microparticles produced by spray-drying, were initially mixed according to each target formulation in a double-helix 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 ºC, being subsequently crumbled (Neuero Farm, Germany) and sieved to desired size ranges.

Senegalese sole has a passive bottom feeding behaviour, so that microdiets commonly remain for one min or more on the bottom of the tank before being eaten. Feed samples (n = 4 per treatment) were hence submersed in rearing water for 1 minute, in order to allow nutrient leaching and simulate a similar nutritional situation observed in the rearing tanks. Most leaching of AA in microdiets occurs over the first minute (Yúfera et al., 2002). After this period the rearing water was removed and the feed samples were frozen at -80ºC and freeze-dried before further analysis. Feed samples were ground,

pooled and analyzed for dry matter (105 °C for 24 h), crude protein by automatic flash combustion (Leco FP-528, Leco, St. Joseph, USA; N × 6.25), lipid content by petroleum ether extraction using a Soxtherm Multistat/SX PC (Gerhardt, Königswinter, Germany; 150 °C), and gross energy in an adiabatic bomb calorimeter (Werke C2000; IKA, Staufen, Germany). Diet composition after immersion in the rearing-water for one min is presented on Table 1.

The dietary amino acid composition was determined by ultra-high-performance liquid chromatography (UPLC) in a Waters Reversed-Phase Amino Acid Analysis System, using norvaline as an internal standard. Samples were hydrolysed at 6 M HCl at 116ºC, over 22 h, and then 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). Tryptophan was determined by HPLC with fluorescence detection (extinction 280 nm, emission 356 nm), after alkaline hydrolysis with barium hydroxide octahydrate for 20 h at 110°C (Commision Directive, 2000). Diet AA profiles after immersion in the rearing-water for one min are presented on Table 2.

2.2 Husbandry and experimental set-up

Experiments were performed by trained scientists and following the European Directive 2010/63/EU of the European Parliament and the Council of the European Union on the protection of animals used for scientific purposes.

Senegalese sole (*Solea senegalensis*) eggs were incubated in an upwelling incubator at 19±0.5ºC and hatching was completed within the next day (24h). Newly hatched larvae were evenly distributed by 6 white cylindro-conical tanks (100L) in a semi-closed recirculation system with a density of 100 larvae L^{-1} (10^4 larvae/tank). The system was equipped with a mechanical filter, a submerged and a trickling biological filter, a protein skimmer and UV sterilizer. Larvae were reared in green water conditions until 25 days after-hatching (DAH), provided by adding frozen *Nannochloropsis* sp. (Nannochloropsis 18% FP 472/180908, Acuicultura Y Nutrición de Galicia SL) to the rearing tanks every morning. Abiotic parameters and mortality were daily monitored. Dissolved oxygen in water was maintained at 90.8±8.5% of saturation, temperature at 18.9±0.5ºC and salinity at 36.8±0.1‰. A 10/14h light/dark photoperiod cycle was adopted and a light intensity of 1000lux at water surface was provided by overhead

fluorescent tubes. After settling (25 DAH) larvae were transferred to flat-bottom tanks (30×70×10cm; 21L), each tank stocking 635 individuals (corresponding to a density of 3024 ind/m^2). 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 measured and mortality was recorded every morning. Dead larvae were removed and the rearing units were carefully cleaned with minimal disturbance. Dissolved oxygen was maintained at 93.1±4.5% of saturation, temperature at 21.5±0.9ºC and salinity at 39.4±3.1‰. A 10/14h light/dark photoperiod cycle was maintained and the light intensity was 400lux at water surface, following CCMAR´s standard Senegalese sole rearing conditions.

The two dietary treatments (CTRL and SUP) were randomly assigned to tanks (n = 3 tanks per treatment). From mouth opening (2 DAH) until 5 DAH 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) (ARTEMIA AF - 480, INVE, Belgium) were introduced at 4 DAH and prey density was gradually increased from 4 to 5 na·mL^{-1}, becoming the only prey offered after 5 DAH. *Artemia* EG *metanauplii* (M24) (EG SEP-ART Cysts, INVE, Belgium) enriched with Easy DHA Selco were introduced at 12 DAH, gradually increasing from 12 to 14 M24·mL−1 until 19DAH. Enriched frozen *Artemia metanauplii* were offered to settled larvae (between 19 and 25 DAH). Live prey was gradually reduced and substituted by inert diet (200-400μm) until complete weaning at 38 DAH, according to Engrola et al. (2009). After 39 DAH larvae were exclusively fed with the respective inert diet (CTRL and SUP; 400-600μm) and considered weaned.

During the pelagic phase, live prey was delivered 1h after the lights were on, at 11.00h, then at 14.00h and at 17.00h. During the benthic phase, frozen *Artemia* was delivered 30 min after the lights were switched on, at 9.30 and then at 12.00h, 14.30h and 17.00h. Inert diet was delivered semi-continuously with automatic feeders (cycles of 2 h of feeding followed by 1h break). 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 Larvae performance

The sampling points were selected at key stages throughout Senegalese sole larval development, with emphasis on the metamorphosis time-window as defined by Fernández-Díaz et al. (2001): at mouth-opening (MO); pre-metamorphosis - stage 1 (Pre-Met); metamorphosis climax - stage 3 (Met); metamorphosis completed - stage 4 (Post-Met); and weaned post-larvae with a fully developed digestive system (Weaned). A final sampling point at a later juvenile stage during the benthic phase was also selected at 51 DAH for both treatments (Juvenile).

At MO, one pool of 20 individuals was collected from each tank for dry weight evaluation. Then after, individual fish (n=10-20 per replicate) were randomly sampled for dry weight and standard length determination at the selected sampling points. The fish were killed by over-anaesthesia (MS-222, 400 $mg.L^{-1}$) and individually photographed and measured (standard length), using Axio Vision L.E. 4.8.2.0 **(**Carl Zeiss MicroImaging GmbH**),** frozen at -80ºC 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 (2-25 DAH), during the benthic phase (25-51 DAH) and during the whole trial (2-51 DAH). RGR was calculated as RGR (% day-1) = $(e^{g}-1)\times100$, where g = [(ln $_{\text{final weight}}$ - ln $_{\text{initial weight}}$)/time] (Ricker, 1958). Survival during the benthic phase was evaluated by counting the remaining fish in the rearing tanks at the end of the experiment (51 DAH).

2.4 Protein metabolism trials

The *in vivo* method of controlled tube-feeding described by Rust et al. (1993) and modified by Rønnestad et al. (2001a) was used to assess the effect of dietary IAA levels on the larvae capacity retain protein throughout the metamorphosis time-window, at the following stages: Pre-Met, Met and Post-Met. Selected ^{14}C labelled model peptides of 1.0KDa and 6.8KDa (Richard et al., 2015) were offered to the larvae to determine the capacity of sole to utilize different size nitrogen forms (including catabolism and retention). One day prior to protein metabolism trial, Senegalese sole larvae from each dietary treatment were transferred to the experimental laboratory to acclimatise. Larvae were fasted for 12h and freely allowed to swim in white trays previously prepared with clean seawater and aeration. Larvae were then fed *Artemia* sp. *metanauplii* for 30 min. Eight larvae with *Artemia*-filled guts from each dietary treatment were anaesthetised with 150-330µM of MS-222 (depending on larvae age) and tube-fed each tracer peptide.

A 0.19-mm diameter plastic capillary inserted on a nanoliter injector (World Precision Instruments, Sarasota, USA) firmly attached to a micromanipulator was used. The injection volume used was 13.8 nL. After capillary withdrawal, larvae were gently rinsed for spillage through two successive wells filled with clean seawater and transferred into incubation chambers filled with 7.5 mL of seawater. An airflow connection was provided between each incubation chamber and a CO_2 trap (5 mL, KOH 0.5 M) used to collect all $^{14}CO_2$ produced by labelled peptide larval catabolism. At the end of the incubation period (24 h) larvae were rinsed with clean water and sampled. Larvae bodies were solubilised with Solvable (Perkin-Elmer, USA) and samples incubated at 50ºC for 24h. The incubation vials were resealed and 1 mL of 1.0 M HCL was gradually injected into the incubation vial, to force the catabolised $^{14}CO_2$ remaining in the seawater vial to diffuse to the CO_2 trap. Thus, ^{14}C released through AA catabolism could be accurately estimated. All samples were added scintillation cocktail (Ultima Gold XR, Perkin Elmer, USA) and disintegrations per minute (DPM) were counted in a TriCarb 2910TR Low activity liquid scintillation analyser (PerkinElmer, USA). All counts were corrected for quench and lumex.

Protein utilization was determined based on the digested/absorbed fraction (A, %), retained fraction (R, %), catabolised fraction (C, %) calculated as:

$$A\ (\%) = (R_{body} + R_{CO2\ trap}) / (R_{body} + R_{CO2\ trap} + R_{sw}) \times 100$$

$$R\ (\%) = R_{body} / (R_{body} + R_{CO2\ trap}) \times 100$$

$$C\ (\%) = R_{CO2\ trap} / (R_{body} + R_{CO2\ trap}) \times 100$$

where R_{body}, $R_{CO2\ trap}$ and R_{sw} are the total radioactivity contents (DPM) in larva body, CO_2 trap and incubation seawater expressed as the percentage of total tracer fed (i.e., the sum of radioactivity contents (DPM) of the larva body, CO2 trap and incubation seawater).

2.5 Fast-twitch muscle cellularity

Standard histological and morphometric techniques (Silva et al., 2009; Valente et al., 1999) were used to analyse fast-twitch muscle cellularity during the metamorphosis climax and at the juvenile stage 51 DAH. Three fish per tank were collected, killed by over-anaesthesia (MS-222, 400 mgl^{-1}) and measured. Fish were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, Missouri, USA) for 24h, washed with 1×PBS and stored in 70º ethanol, at 4ºC until further processing. Juvenile fish (51 DAH) were decalcified in 5% nitric acid and 5%

sodium sulphate. All samples were dehydrated in a graded ethanol series (diluted from Etanol 99.5%, AGA, Prior Velho), cleared in xylol (Prolabo, VWR International LLC, Radnor, PA, USA) and finally included in paraffin (Merck, KGaA, Darmstadt). Fish were sectioned (7 μm) transversely to the body axis, mounted on coated slides with 3-aminopropyltriethoxysilane (APES) (Sigma-Aldrich, St. Louis, Missouri, USA) and double stained with haematoxylin (Haematoxylin Gill II, Merck, KGaA, Darmstadt, Germany) and eosin (Eosin Y, VWR, Geldenaakseosan, Leuven) before placing a cover slip.

Morphometric variables were measured in transversal body sections of individual fish, at a perianal location. In both larvae and juveniles, the total number of fast-twitch fibers (N) were counted and the total cross-sectional area [CSA (mm^2)], the total cross section muscle area [Muscle CSA (mm^2)] and the fiber cross-sectional area (μm^2) were measured. Fiber diameter (μm) was estimated from the fiber cross-sectional area (μm^2) data assuming that muscle fibers cross-section is round shaped. The mean fiber diameter and percentage of small fibers (<5 μm) were estimated from a minimum of 500 cross-sectioned fast-twitch fibers representative of the whole cross sectional muscle area. The fiber density (total number/mm^2) was calculated by dividing the total number of fast-twitch fibers (N) counted by the total cross section muscle area [Muscle CSA (mm^2)]. Muscle fiber outlines were traced using a 400× magnification using an Olympus BX51 microscope (Olympus Europa GmbH, Hamburg, Germany) with the Cell^B Basic imaging software.

2.6 Gene expression

2.6.1 RNA extraction and cDNA synthesis

Nine pools of 20 whole larvae at the metamorphosis climax and 10 whole fish at the juvenile stage - 51 DAH (3 pools per tank) were sampled per dietary treatment, snap-frozen in liquid nitrogen and kept at −80 °C until further analysis. Each larvae 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). 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, purified using the High Pure RNA Isolation Kit (Roche) and again quantified using the Nanodrop

spectrophotometer. cDNA was synthesized from 1µg of purified RNA (per pool), using with the M-MLV Reverse Transcriptase Kit (Invitrogen).

2.6.2 Quantitave real-time PCR (qPCR)

The relative expression of the myogenic regulatory factors (*myf5, myod2, mrf4, myog), myhc, mstn1* as well as genes encoding for the proteins responsible for *de novo* DNA methylation and DNA methylation maintenance (*dnmt1, dnmt3a,* and *dnmt3b*) 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 (ºC) and qPCR amplification efficiencies). Quantification of gene expression was performed by qPCR with Sso Fast Evagreen supermix (Bio-Rad) on a CFX96^{TM} Real-Time PCR Detection System (Bio-Rad). 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. 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).

2.7 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.

The influence of diet on the larvae capacity to utilize protein was tested by two-way ANOVA using peptide size and diet as independent factors. The differences between groups detected in growth and muscle growth parameters as well as in the relative expression of target genes were tested by a one-way ANOVA. A Pearson's coefficient

correlation was used to compare the relative expression of genes regulating muscle growth *versus* muscle growth parameters, using the mean value of each triplicate tank (N =6). Significant levels were set at $P<0.05$.

To compare the distribution of muscle fiber size, a nonparametric method was used to fit smoothed probability density functions (PDFs) using the statistical program for the analysis of muscle fiber populations (Johnston et al., 1999b). Bootstrapping was used to distinguish random variation in diameter distribution from treatment differences. A Kruskall-Wallis test was used to test the null hypothesis that PDFs of muscle fiber diameter in the two treatments were identical.

3. Results

3.1 Diets

The amino acids (AA) contents of the experimental inert diets showed that an 8% replacement of the encapsulated fish protein hydrolysate fraction by a crystalline-AA mixture was effective in increasing most IAA levels in the SUP diet resulting in a 14.6% increase in the sum of the indispensable amino acids (IAA) and a 24.8% increase in the IAA/DAA ratio (Table 2). The most significant differences between diets (CTRL and SUP) were found in phenylalanine (Phe) and tryptophan (Trp) contents, which were increased respectively by 81 and 36% in the SUP diet, when compared to the CTRL diet. Moreover, valine (Val) and tyrosine (Tyr) levels were reduced respectively by 4 and 13% in the SUP diet, when compared to the CTRL diet (Table 2), as they were not supplemented in crystalline form (Table 1).

3.2 Protein metabolism

Protein retention efficiency and catabolism of Pre-Met larvae were not significantly affected ($P=0.637$) by dietary IAA levels (Fig.1). The peptide molecular size did not affect the retention or catabolism efficiency in Pre-Met larvae ($P=0.592$) (Fig.1). Average retention efficiency of Pre-Met larvae was 77.5±3.4%.

Neither the dietary IAA level ($P=0.076$) nor the different sized peptides ($P=0.485$) had a significant impact on larvae retention efficiency during the metamorphosis climax (Met) (Fig.1). However, at this stage, there was a significant interaction between diet and peptide size, with the 1.0KDa peptide being better retained in SUP group and the 6.8KDa peptide being similarly retained by both groups. Average retention efficiency of Met larvae was 77.3±6.5%.

Further on the metamorphosis process, at the Post-Met stage, there was also no effect of the diet on larvae metabolic efficiency either for 1.0KDa or 6.8KDa peptides (P=0.83) (Fig.1). However, when comparing the larvae capacity to metabolize smaller or larger peptides, the 6.8KDa peptide was better retained and less catabolized than 1.0KDa (P=0.008) (Fig.1): average retention efficiency of Post-Met larvae was 76.5±3.0% for 1.0KDa peptides and 86.0±4.7% for 6.8KDa peptides.

When comparing larvae metabolism between different developmental stages, regardless of the diets, larvae capacity to retain 6.8KDa peptides increased throughout metamorphosis; significant differences were observed between the Pre-Met and Post-Met stages (P=0.023).

3.3 Larval performance

In the present trial, both experimental groups (CTRL and SUP) achieved the selected key developmental stages simultaneously, with no significant inter-individual variation: mouth-opening occurred at 2 DAH; Pre-Met (stage 1) occurred at 13 DAH; Met (stage 3) occurred at 19 DAH; Post-Met (stage 4) occurred at 25 DAH; and all post-larvae were weaned at 38 DAH. The last sampling of juvenile fish was intentionally carried out at the same age, 51 DAH, in both treatments (CTRL and SUP), when all fish had already acquired a benthic behaviour.

The supplementation with encapsulated crystalline-IAA affected larval growth throughout the trial, with significant differences being found in the overall RGR (P=0.04) (Table 4; Fig.2). Dry weight was similar between dietary treatments during the pelagic phase, but in the benthic phase fish fed the CTRL diet performed better than those fed the SUP diet. This response to diet was remarkably accentuated with time and throughout the benthic phase (Fig. 2). At the end of the trial, the fish fed the CTRL diet were 2.1-fold heavier than those fed the SUP diet. Survival was not significantly affected by dietary formulation (Table 4).

3.4 Dietary effect on fast-twitch skeletal muscle growth

Although the dietary IAA level significantly affected larvae body length during the metamorphosis climax (P=0.045), muscle cross-sectional area was similar between groups at this stage. Neither the total number of fibers (N) nor the mean fiber diameter were significantly affected by the dietary treatment at this stage (P>0.05, Table 4), and these parameters did not correlate with fish length (P>0.05). There was also no

significant correlation between fish length and the percentage of small fibers (P>0.05) which was also not affected by dietary treatment (P>0.05, Table 4). Moreover, fiber size distribution remained similar among groups (Fig.3). Significant differences between diets at the Met stage were only detected for fiber density (P=0.021) (Table 4).

Between the Met and the Juvenile stages there was a significant enlargement of muscle fibers and muscle CSA, reflected on the shift of PDFs distribution towards the right-hand of graphic (Fig. 3). The total number of fibers increased by 2.3-fold in the CTRL group while it increased by 1.8-fold in the SUP group during the 19-51 DAH period. By the end of the trial, at 51 DAH, muscle CSA was 1.6-fold larger in the CTRL than in the SUP group (P=0.008) (Table 4). This CSA increase was paralleled by a significantly higher total number of fibers (P=0.024) and larger mean fiber diameter (P=0.035) in the CTRL group, compared to the SUP group (Table 4). Fiber density was significantly higher in the SUP fish than in the CTRL (P=0.022), but both the percentage of small fibers (Table 4) and fiber size distribution (Fig.3) remained similar among dietary treatments at 51DAH. Moreover, no significant correlation could be observed between fish length and any of the muscle cellularity parameters at the juvenile stage (P>0.05).

3.5 Expression of growth-related genes and DNA methyltransferases

During the metamorphosis climax (Met), *myog* was significantly upregulated in the larvae fed the CTRL diet compared to those fed the SUP diet (P=0.010) (Fig 4). At this stage, *myog* expression was positively correlated with both the CSA (R=0.895, P<0.05) and the percentage of fibers < 5µm (R=0.873, P<0.05) (Table 5). Also at this stage, the expression of *myf5*, *myod2* and *mrf4* was similar between dietary treatments (Fig 4). In 51 DAH juveniles, *mrf4* transcript levels were 1.4-fold higher in the SUP group, compared to the CTRL group (P=0.008) (Fig 4). No significant differences were found in the transcript levels of the other myogenic factors at 51 DAH.

The expression of an important structural gene in muscle, *myhc*, during the metamorphosis climax (Met) was 1.8-fold higher in the CTRL group, compared to the SUP group (P=0.002) (Fig 4), being positively correlated with standard length (R=0.953, P<0.01), CSA (R=0.840, P<0.05) and muscle CSA (R=0.905, P<0.05) (Table 5). However, in 51 DAH juveniles no significant difference was detected on the *myhc* transcript levels (Fig 4).

No effect was found in the *mstn1* mRNA levels during the metamorphosis climax (Met). Interestingly, in 51 DAH juveniles, *mstn1* showed a 2.1-fold upregulation (P=0.002) in the CTRL group compared with the SUP group (Fig 4). A positive correlation was also observed between *mstn1* expression and relative growth rate during the benthic period (P=0.028; r=0.860) (Table 5).

There was no effect of dietary IAA level on the expression of the DNA methyltransferases *dnmt1* and *dnmt3a*, neither during the metamorphosis climax (Met) nor at 51 DAH. However *dnmt3b* transcript levels were significantly higher in the CTRL group, when compared to the SUP group, at both the metamorphosis climax (1.6-fold, P<0.001) and at 51 DAH (1.4-fold, P=0.045).

4. Discussion

4.1 Effect of IAA supplementation on protein utilization and somatic growth

Replacing 8% of the encapsulated fish protein hydrolysate fraction by a crystalline-AA mixture in the SUP diet was an effective way of increasing the sum of IAA, the IAA/DAA ratio and the level of most IAA (Table 2). However, this supplementation was not sufficient to fully correct the dietary AA profile. Considering Senegalese sole larvae IAA requirements suggested by Aragão et al. (2004a) as reference Met and His remained possibly limiting in the SUP diet. This was probably due to leaching losses as these supplemented IAA are highly soluble molecules. A major challenge on devising the protein fraction for larvae microdiets is the small size of the feed particles, in which the high surface/volume ratio reduces the diffusion distance from the core to the surface. As a consequence, soluble protein forms such as FAA that would allow to finely tune dietary AA profile are easily lost by leaching (Kvale et al., 2007; Kvale et al., 2006; Nordgreen et al., 2008; Yúfera et al., 2002).

Dietary IAA supplementation did not improve larvae capacity to utilize small peptides, as no significant differences were found between larvae fed the CTRL diet and those fed the SUP diet. A positive impact of the SUP diet would have been expected in accordance with previous data from Aragão et al. (2004b) that have shown a higher retention of [^{14}C]-protein hydrolysate in *Artemia* fed Senegalese sole post-larvae (36-40 DAH), after Leu-Gly and Phe-Ala dipeptides supplementation in an *in vivo* tube-feeding trial. However, in the present study, it is likely that IAA dietary supplementation was not sufficiently effective to have an effect on the larvae metabolic capacity and did not

promote growth, similarly to what was reported in white seabream (*Diplodus sargus*) larvae fed microencapsulated diets supplemented with crystalline-AA (Lys and Trp) (Saavedra et al., 2009b). On the contrary, larvae fed the non-supplemented diet (CTRL group) grew faster since an early stage. These results might be explained by the impaired utilization of the fast absorbed crystalline (free) AA compared to the protein-bound AA which needs the action of digestion prior to absorption. That would have led to a decrease in protein accretion. Rønnestad et al. (2000) showed that free AA are absorbed much faster than intact protein, and Rønnestad and Conceição (2012) proposed that even a highly digestible protein – the one from *Artemia* – takes more than 2 hours to be fully digested. Furthermore, the absorption of individual AA depends on different transport systems (Ronnestad, Morais, 2008) and seems to proceed at different rates (Conceição et al., 2011; Dabrowski, 1983) and with different efficiencies, depending on the species and developmental stage (Conceição et al., 2002; Rønnestad et al., 2001b; Saavedra et al., 2008a; 2008b). As a consequence, different absorption rates between individual AA may lead to transitory AA imbalances in the cellular FAA pool where the protein synthesis occurs, leading to increased AA catabolism. If that was the case, a significant part of the supplemented crystalline IAA could have been lost to catabolism, thus resulting in less IAA available for protein synthesis and compromising long-term growth.

Another possibility is that the differences found in growth are due to a possible effect on the voluntary feed intake. In the present study, it was not possible to quantify the larva voluntary feed intake on the experimental diets since no viable technique has been so far developed to measure consistently feed intake over time in marine fish larvae. However, there was an apparent clear excess of remaining feed in the tanks fed the SUP diet. It can be hypothesised that larvae fed the CTRL diet may have increased their voluntary feed intake to compensate for IAA deficiencies, as previously reported for midas (*Amphilophus citrinellum*) (Dabrowski et al., 2007) and rainbow trout juveniles (*Oncorhynchus mykiss*) (Alami-Durante et al., 2010). Ultimately, a higher level of tryptophan (Trp) in the SUP diet might also have reduced voluntary feed intake in this group (Table 2). Trp is the precursor of serotonin (5-hydroxitryptamine, 5-HT) which participates as a messenger in the central nervous system and peripherally in gastrointestinal and vascular systems. Higher dietary Trp levels were shown to induce variations on brain 5-HT content, probably leading to depressed appetite and feed

consumption and ultimately to reduced growth in juvenile groupers (*Epinephelus coioides*) (Hseu et al., 2003). In the present study, a higher voluntary feed intake in the CTRL group would have led to a positive net nitrogen and energy balance throughout the experiment, leading to a higher growth rate. Further studies involving innovative approaches able to determine feed intake in fish larvae are required to validate such hypothesis.

4.2 Effect on the regulation of muscle growth

Dietary supplementation with encapsulated crystalline-IAA affected larval growth throughout the trial, and significant differences on fish length started being noticed during the metamorphosis climax (Fig 2, Table 4). At this stage (Met), fish fed the SUP diet were significantly smaller than their control counterparts (P=0.045, Table 4) but muscle cross-sectional area was similar between groups, mostly due to the higher number of fast-twitch fibers in the SUP group. Moreover, the expression pattern of key genes regulating myogenesis was affected by the dietary treatments in Met larvae, with the expression of *myog* and *myhc* transcript levels being significantly reduced in the SUP group (Fig. 4). *Myogenin* is an indicator of myogenic cell recruitment for stratified hyperplasia, the second phase of myogenesis occurring at this developmental stage in Senegalese sole, as previously described by Campos et al (2013b; 2013c). Although the down-regulation of *myogenin* in the SUP fed larvae did not translate into changes on total number of fibers between dietary treatments during the Met stage, it might partially explain the reduced total number of fibers, smaller cross-sectional muscle area and reduced somatic growth rate in later stages (51 DAH juveniles). In fact, in Met larvae, *myogenin* expression was positively correlated with the percentage of small-sized muscle fibers (Table 5) that is known to be a good indicator of further muscle growth potential (Valente et al., 1999). Thus, the up-regulation of *myogenin* in the CTRL group in response to dietary IAA levels at the Met stage may have anticipated a greater increase of the total number of fibers from the Met to the juvenile stage that would ultimately have sustained a higher growth rate. In pike perch (*Sander lucioperca*) larvae (Ostaszewska et al., 2008), different types of feed and dietary formulations led to different growth rates and altered muscle growth dynamics. Increased proliferative capacity of MPCs and a higher contribution of hyperplasia was reported in fast-growing groups. A similar response was reported for pacu larvae (*Piaractus mesopotamicus*)

(Leitão et al., 2011) subjected to different types of feed, dietary formulations and feeding regimes, including starvation.

In the present study, the group fed the SUP diet showed no signs of reduced contribution of hyperplasia to muscle growth, as indicated by a similar percentage of small fibers between diet groups, but it displayed some signs of delayed muscle growth at the juvenile stage. Differences in juvenile muscle cellularity were not significantly correlated with fish length. Instead, they probably reflect a lower feed intake and subsequent lower AA availability for protein accretion in the SUP group that ultimately resulted in decreased fiber size and reduced fish length. The downregulation of *myogenin* in the SUP group (Fig. 4) is consistent with previous results reported in common carp (*Cyprinus carpio*) fingerlings subjected to a restrictive diet (Kamaszewski et al., 2014) and in rainbow trout (*Oncorhynchus mykiss*) juveniles subjected to starvation (Johansen, Overturf, 2006). Moreover, *myogenin* expression was clearly responsive to AA availability in myocyte cells isolated from gilthead seabream (*Sparus aurata*) (Velez et al., 2014) and to refeeding in a primary culture of Atlantic salmon (*Salmo salar*) myocytes (Bower, Johnston, 2010). These results suggest that *myogenin* expression is responsive to a possibly lower availability of AA for protein synthesis in the SUP group.

Unlike the observed up-regulation of *myogenin* in the CTRL group (significant at the Met stage), *mrf4* exhibited an opposite tendency, being significantly less expressed in the CTRL group at the juvenile stage (Fig.4). Secondary MRF's (those involved in the inducing and maintaining the muscle differentiation) showed different patterns of expression in response to starvation and refeeding in rainbow trout juveniles (Johansen, Overturf, 2006) and in response to refeeding in Atlantic salmon isolated myocytes (Bower et al., 2008). Senegalese sole post-larvae (35-51 DAH) exposed to different rearing temperatures during the pelagic phase also showed a tendency to increase the expression of *mrf4* in a slow-growing group, during a compensatory growth phase at 83 DAH, long after the exposure to the environmental challenge (Campos et al., 2013b).

At the juvenile stage (51 DAH), *mstn1* was up-regulated in the CTRL group (Fig.4). Similarly, Campos et al. (2013b) reported an up-regulation of *mstn1* in the fast-growing S. sole groups at a juvenile stage (83 DAH). In other fish species, 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). In fact, different *myostatin* paralogues have been reported in salmonids and exhibit distinct expression patterns in muscle and non-muscle tissues (Rescan et al., 2001; Roberts, Goetz, 2001; Valente et al., 2006). In Senegalese sole, high transcript levels of *mstn1* were found in juveniles liver (Campos et al., 2010). As gene expression was analyzed in the whole fish and not only in the muscle, it is possible that a higher expression of *mstn1* might be associated with other physiological mechanisms and not only with skeletal muscle growth regulation, as previously suggested by Campos et al. (2013b).

The down-regulation of *myhc* in SUP fed Met larvae (Fig.4) did not translate into significant changes on fiber size between dietary treatments (Table 4; Fig. 3), but might be partially related with the slightly higher total number of fibers and higher fiber density observed in the SUP group at the Met stage. In fact, at this stage, *myhc* expression was negatively correlated with fiber density and positively correlated with muscle CSA (Table 5). Thus, the down-regulation of *myhc* in Met larvae may explain, at least in part, the further reduced size of fast fibers and total cross-sectional muscle area during the juvenile stage (51 DAH). *Myhc* was suggested as an index to monitor "specific growth rate" under variable nutritional conditions in rainbow trout (Overturf, Hardy, 2001) and was shown to be correlated with muscle protein accretion in Atlantic salmon juveniles (Hevrøy et al., 2006). Similarly, in the present study, the reduced expression of *myhc* in the SUP group at the Met stage (Fig.4) may be related with a lower availability of AA to promote protein synthesis in the muscle.

4.3 Epigenetic effect

DNA methylation relies on the one-carbon metabolism pathway, which is dependent upon the activity of several enzymes in the presence of dietary methyl donors, such as folate, choline, betaine and methionine (Anderson et al., 2012). In the one-carbon cycle, methionine is converted into S-adenosylmethionine (SAM), the universal cellular methyl donor (Selhub, 1999). DNA cytosine methyltransferases (*dnmt*s) covalently attach SAM methyl groups to the 5′-position of cytosine (in CpG dinucleotides), thus methylating DNA and repressing transcription. In the present study, the reduced expression of *dnmt3b* and a decreasing tendency in the expression of *dnmt1* and *dnmt3a* in the SUP group during the metamorphosis climax (Met) and at the juvenile stage (Fig. 5) could be associated with a possible lower protein intake. Further studies are needed to ascertain whether this was due to a lower availability of methyl group donors, as a

consequence of lower feed intake, and whether it would possibly lead to DNA hypomethylation in skeletal muscle. Previous studies in Senegalese sole showed that rearing temperature during the pelagic phase induced changes in the expression of *dnmt1* and *dnmt3b* DNA methyltransferases during metamorphosis, eventually mediating an epigenetic regulation of muscle growth, through altered expression of *myogenin* (Campos et al., 2013a; 2013b) . Therefore, the pelagic phase and in particular the metamorphosis climax could be a susceptible time window for nutritional programming in Senegalese sole.

5. Conclusions

The present results suggest that supplementing microdiets with crystalline-AA in order to correct IAA dietary deficiencies does not bring a clear effect on the Senegalese sole larvae capacity to retain different-sized peptides and does not improve long-term somatic growth. In fact, fish fed a non-supplemented diet performed better. This led to changes on the regulation of muscle growth associated with changes in expression patterns of muscle growth markers during the trial (secondary MRFs *myogenin* and *mrf4*, *myhc* and *mstn1*), as well as the expression of *dnmt3b*. As this gene encodes for a DNA methyltransferase essential for *de novo* methylation, an epigenetic effect at the transcriptional regulation level is suggested as a possible explanation for the differences found in growth as a response to a nutritional cue.

Acknowledgements

This work was funded by Project EPISOLE (FCT) [PTDC/MAR/110547/2009] from FCT (Portugal). P. Canada was supported by FCT grant SFRH/BD/82149/2011. Sofia Engrola was supported by FCT investigator grant IF/00482/2014/CP1217/CT0005 funded by the European Social Fund, the Operational Programme Human Potential and the Foundation for Science and Technology of Portugal (FCT). The authors acknowledge the collaboration of Wilson Pinto and André Santos (SPAROS Lda), the Aquagroup team, Ana Filipa Lopes and Sara Ferreira in particular (CCMar), Cláudia Figueiredo-Silva and the Evonik Industries AG (Essen, Germany).

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Figure captions:

Fig. 1. 1.0KDa and 6.8KDa peptide retention (% of radiolabel in the body in relation to absorbed label), and catabolism (% of radiolabel in the metabolic trap in relation to absorbed label) after 24 h of incubation, in Senegalese sole larvae during the metamorphosis time-window: at pre-metamorphosis – stage 1 (Pre-Met), metamorphosis climax – stage 3 (Met) and post-metamorphosis – stage 4 (Post-Met). Values are means ± s.d., n = 8. Comparisons between groups fed different diets and tube-fed different molecular sized peptides were made using 2-way ANOVA.

Fig. 2. Sole dry weight (DW) and standard length (SL) during the pelagic phase (at mouth opening (MO), pre-metamorphosis – stage 1 (Pre-Met) and metamorphosis climax – stage 3 (Met)) and the benthic phase (at post-metamorphosis –stage 4 (Post-Met), weaned post-larvae (Weaned) and a late juvenile stage, at 51 DAH (Juvenile)). Values are means ± s.d. of treatments replicates (n=30 during the pelagic phase; n=60 during the benthic phase). Different superscript letters at each developmental stage indicate significant differences ($P<0.05$, 1 way-ANOVA) between dietary treatments.

Fig. 3. Probability density functions (PDFs) distributions of fast muscle fibers at the metamorphosis climax – stage 3 (Met) and late juvenile stage, 51 DAH (Juvenile). 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 (CTRL and SUP; n=9/treatment). The shaded area shows 1000 bootstrap estimates from combined populations of fiber diameter.

Fig. 4. Expression of genes encoding for myogenic regulatory factors *myf5*, *mrf4*, *myod2*, *myog*, *mstn1*and *myhc* at the metamorphosis climax – stage 3 (Met) (whole body pools of 20 individuals) and late juvenile stage, 51 DAH (Juvenile) (whole body pools of 10 individuals). mRNA expression was normalized to transcript levels of *ubq* and *rps4*. Values are presented means ± s.d., n = 9. Different superscript letters indicate significant differences ($P<0.05$, 1-way ANOVA) between the dietary treatments at each developmental stage.

Fig. 5. Expression of genes related to DNA methylation (*dnmt1*, *dnmt3a* and *dnmt3b*) at the metamorphosis climax – stage 3 (Met) (whole body pools of 20) and late juvenile stage, 51 DAH (Juvenile) (whole body pools of 10). mRNA expression was normalized to those of *ubq* and *rps4*. Values are presented means ± s.d., n = 9. Dissimilar superscript letters indicate significant differences ($P<0.05$, 1-way ANOVA) between the dietary treatments at each developmental stage.

Table 1

Ingredients and proximate composition of the experimental diets after one min leaching in seawater.

	Diets	
	CTRL	SUP
Ingredients (% dry matter)		
Whey Protein isolate [a]	10	10
FPH - non encapsulated[b]	10	10
Squid meal[c]	8.5	8.5
FPH encapsulated[b]	20	11.2
Fish Gelatine[d]	14	13
Autolysed yeast[e]	1.7	1.7
Krill Meal[f]	9	9
Fish oil[g]	4.5	5.5
DHA-rich oil [h]	2	2
Copepod oil [i]	5.5	5.5
Premix[j]	5	5
Vit C[k]	3	3
Vit E[l]	1	1
Taurine (Sigma)[m]	1.5	1.5
Chitosan[n]	3.3	3.3
Mono Ca Phosphate[o]	1	1
L-Arginine HCl[p]		1
DL-Methionine[p]		0.75
L-Lysine HCl[p]		2
L-Tryptophan[p]		0.5
L-Histidine HCl[p]		0.6
L-Leucine[p]		1
L-Isoleucine[p]		0.5
L-Phenylalanine[p]		2.5
Proximate analyses (% dry matter)		
Crude protein	60.93	59.82
Crude fat	14.61	13.90
Gross Energy (MJ kg^{-1})	17.46	17.53

[a]Isolate Crystal Whey. Weider. USA;
[b]Fish protein hydrolysate - CPSP 90®. Sopropêche. France;
[c]Super prime without guts. Sopropêche, France;
[d]Fish edible gelatine. Lapi Gelatine. Italy;
[e]Hylisis. ICC. Brazil;
[f]Qrill. Aker Biomarine. Norway;
[g]Marine oil omega 3: Henry Lamotte Oils GmbH. Germany;
[h]DHA 70% Algatrium, Brudy Technology SL., Spain;
[i]Phosphonorse, K/S Tromsø Fiskeindustri A/S & Co., Norway;
[j]PVO40.01 Premix for marine fish, PREMIX Lda, Portugal;
[k]Ascorbil monophosphate, PREMIX Lda, Portugal;
[l]α-Tocopherol, PREMIX Lda, Portugal;
[m]Taurine T0625 Sigma-Aldrich Corporation, Germany;
[n]Chitosan 448869, Sigma-Aldrich Corporation, Germany;
[o] Mono-calcium phosphate, Fosfitalia, Italy;
[p] Crystalline AA, Ajinomoto Eurolysine SAS, France

Table 2

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

	Diets		% Deviation (CTRL *vs* SUP)
	CTRL	SUP	
Indispensable amino-acids (IAA)			
Arginine	4.63	4.82	4.2
Histidine	0.94	1.11	18.2
Lysine	5.48	6.34	15.8
Threonine	2.48	2.82	13.8
Isoleucine	2.55	2.81	10.4
Leucine	4.08	4.67	14.6
Valine	2.71	2.60	-3.8
Methionine	1.26	1.47	16.6
Phenylalanine	1.97	3.56	81.0
Cysteine	0.10	0.10	-1.9
Tyrosine	1.47	1.28	-12.9
Tryptophane	0.48	0.65	35.7
IAA sum	**28.13**	**32.24**	**14.6**
Dispensable amino-acids (DAA)			
Aspartic acid + Asparagine	5.94	5.11	-13.9
Glutamic acid + Glutamine	8.90	8.31	-6.6
Alanine	4.24	3.78	-10.7
Glycine	6.21	5.56	-10.4
Proline	4.12	4.06	-1.56
Serine	2.53	2.40	-5.12
Taurine	0.93	0.94	1.5
IAA/DAA ratio	**0.86**	**1.07**	**24.8**

% Deviation calculated as (SUP_{AAi} content - $CTRL_{AAi}$ content)/ $CTRL_{AAi}$content ×100

Table 3

Primers used in qPCR

Gene	Fwd sequence (5′→3′)	Rev sequence (5′→3′)	Accession no (GenBank)	Size (bp)	Annealing temp. (ºC)	E (%)
myf5	GAGCAGGTGGAGAACTACTACG	CCAACCATGCCGTCAGAG	FJ515910	89	60	103
mrf4	GAGAGGAGGAGGCTCAAGAAG	CAGGTCCTGTAATCTCTCAATG	EU934042	137	58	96
myog	GTCACAGGAACAGAGGACAAAG	TGGTCACTGTCTTCCTTTTGC	EU934044	118	60	94
myod2	ACAGCCACCAGCCCAAAC	GTGAAATCCATCATGCCATC	FJ009108	194	60	111
myhc	GAAAAATCTGACAGAGGAAATGG	CCTTGGTGAGAGTGTTGACTTTG	FJ515911	143	60	96
mstn1	GGGAGATGACAACAGGGATG	TGGATCCGGTTCAGTGGC	EU934043	91	60	108
dnmt1	GATCCCAGTGAGGAGTACGG	AAGAAGGTCCTCATAAGTAGCGTC	KC129104	117	62	103
dnmt3a	AACTGCTGTAGGTGTTTCTGTGTG	CGCCGCAGTAACCCGTAG	KC129105	134	60	101
dnmt3b	ATCAAGCGATGTGGCGAGC	CGATGCGGTGAAAGTCAGTCC	KC129106	91	60	96
rps4	GTGAAGAAGCTCCTTGTCGGCACCA	AGGGGGTCGGGGTAGCGGATG	AB291557	101	60	95
ubq	AGCTGGCCCAGAAATATAACTGCGACA	ACTTCTTCTTGCGGCAGTTGACAGCAC	AB291588	135	60	93

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

Table 4

Standard length (mm), relative growth rate (RGR, %) and white muscle growth morphometric variables measured at a peri-anal location (*N*=9/treatment) at metamorphosis climax – stage 3 (Met) and a juvenile stage, 51 DAH (Juvenile). Relative growth rate (RGR, %) and survival (%) estimated for each tank (*N*=3/treatment). Values are means ± s.d. Comparisons between groups fed with different diets were made using one-way ANOVA. Dissimilar superscript letters indicate a significant difference ($P<0.05$) between groups.

		Diets	
		CTRL	SUP
Pelagic phase			
Met	SL (mm)	6.7±0.8^{a}	6.3±0.6^{b}
	CSA (mm²)	0.70±0.10	0.64±0.07
	Muscle CSA (mm²)	0.11±0.02	0.09±0.01
	Total number of fibers N	1381±176	1444±185
	Fiber density (total number/mm²)	13227±1805^{b}	15845±2397^{a}
	mean fiber diameter (µm)	8.27±0.99	8.04±0.53
	% small fibers (<5 µm)	15.65±8.91	13.22±4.39
	RGR 2-19 DAH (%.day^{-1})	24.2±1.7	23.4±0.6
Benthic phase			
Juvenile	SL (mm)	15.3±2.7^{a}	11.9±2.0^{b}
	CSA (mm²)	2.56±0.51^{a}	1.78±0.51^{b}
	Muscle CSA (mm²)	0.53±0.13^{a}	0.34±0.13^{b}
	Total number of fibers N	3118±552^{a}	2481±536^{b}
	Fiber density (total number/mm²)	6071±1197^{b}	7695±1494^{a}
	mean fiber diameter (µm)	11.16±1.25^{a}	9.96±0.94^{b}
	% small fibers (<5 µm)	4.06±3.08	5.40±3.72
	RGR 19-51 DAH (%.day^{-1})	7.27±1.21	5.06±1.12
	Survival rate (19-51 DAH) (%)	57.2±11.3	49.4±15.1
Overall growth			
	RGR 2-51 DAH (%.day^{-1})	12.9±0.3^{a}	11.0±1.0^{b}

Table 5

Correlations (Pearson's coefficient) between gene expression and muscle growth parameters in Senegalese sole larvae, during the metamorphosis climax –stage 3 (Met) and at a late juvenile stage, 51 DAH (Juvenile)

Genes	SL	CSA	MuscleCSA	Number of fibers	Density	Avg fiber diameter	% of small fibers	RGR (19-51DAH)	RGR (2-51DAH)
Met									
myf5	NS	NS	NS	NS	NS	NS	NS	NS	NS
mrf4	NS	NS	NS	NS	NS	NS	NS	NS	NS
myog	NS	P=0.016, r=0.895	NS	NS	NS	NS	P=0.023, r=0.873	NS	P=0.021, r=0.880
myod2	NS	NS	NS	NS	NS	NS	NS	NS	NS
myhc	P=0.003, r=0.953	P=0.037, r=0.840	P=0.013, r=0.905	NS	P=0.029, r= -0.858	NS	P=0.042, r=0.829	P=0.031, r=0.854	P=0.046, r=0.854
mstn1	NS	NS	NS	NS	NS	NS	NS	NS	NS
dnmt1	NS	NS	NS	NS	NS	NS	P=0.019, r=0.886	NS	NS
dnmt3b	P=0.022, r=0.875	P=0.016, r=0.896	P=0.013, r=0.907	NS	P=0.031, r= -0.853	NS	NS	NS	NS
Juvenile									
myf5	NS	NS	NS	NS	NS	NS	NS	NS	NS
mrf4	P=0.002. r=-0.960	NS	NS	NS	NS	NS	NS	NS	NS
myog	NS	NS	NS	NS	NS	NS	NS	NS	NS
myod2	NS	NS	NS	NS	NS	NS	NS	NS	NS
myhc	NS	NS	NS	NS	NS	NS	NS	NS	NS
mstn1	NS	NS	NS	NS	NS	NS	NS	P=0.028, r=0.860	NS
dnmt1	P=0.004, r=0.946	NS	NS	NS	NS	NS	NS	P=0.019, r=0.886	NS
dnmt3a	NS	NS	P=0.049, r=0.813	NS	NS	NS	NS	NS	NS

Statistical significance was set at P<0.05 (*N*=6)

Fig 1

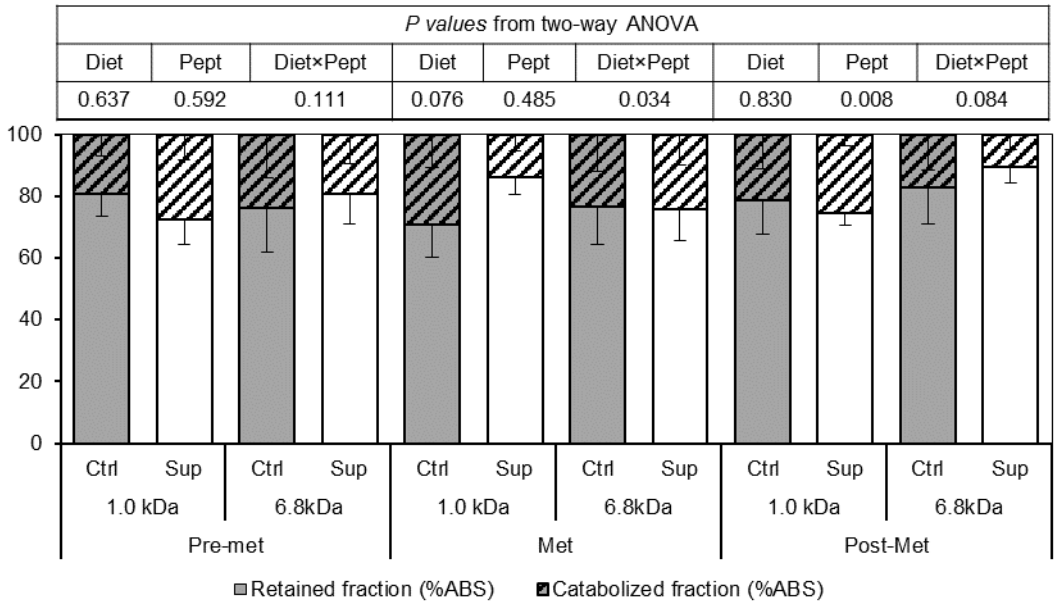


Fig 2

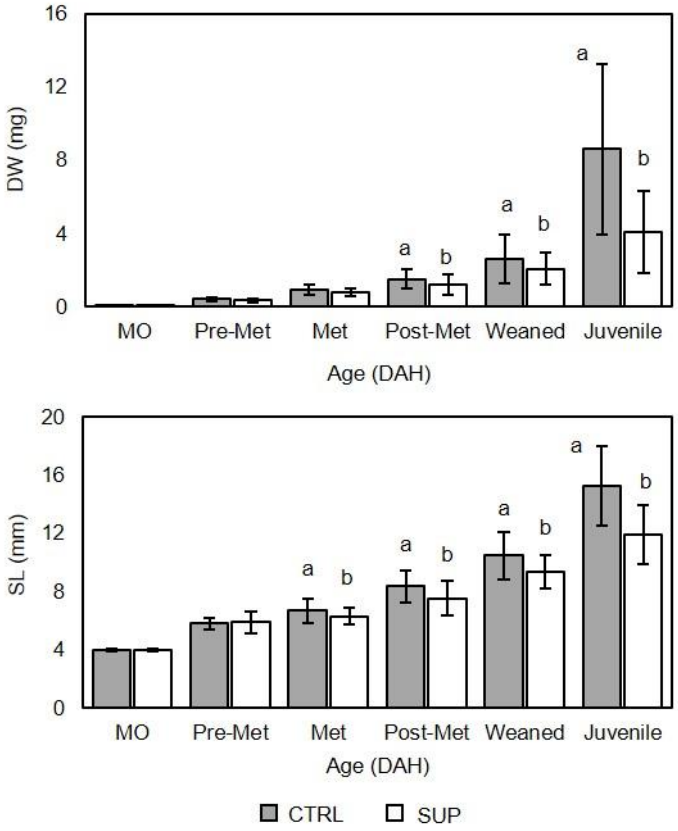


Fig 3

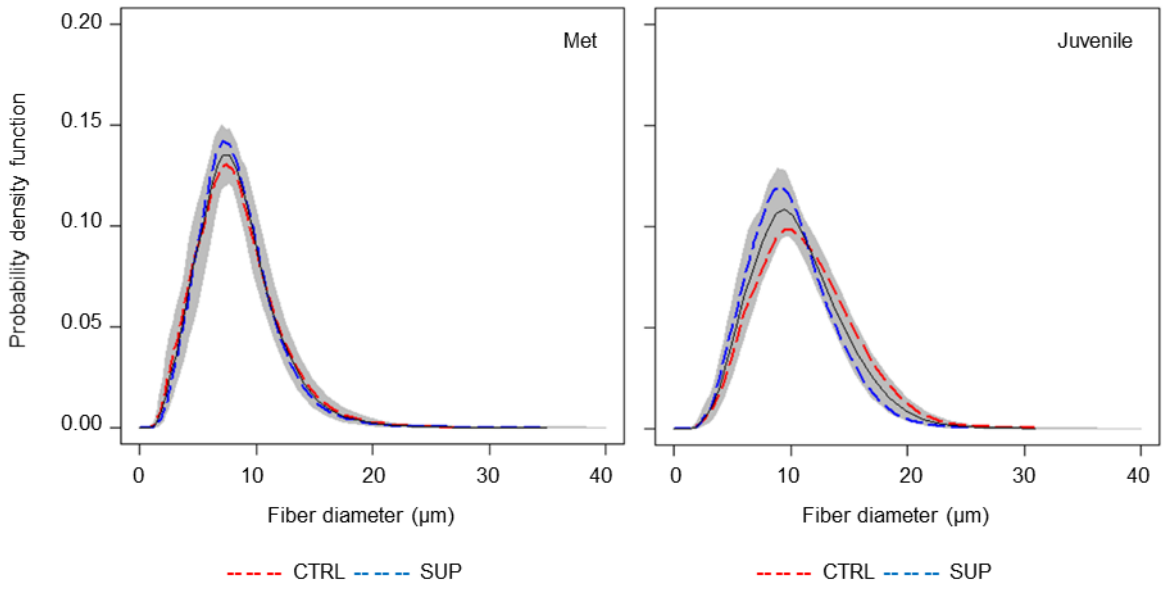


Fig 4

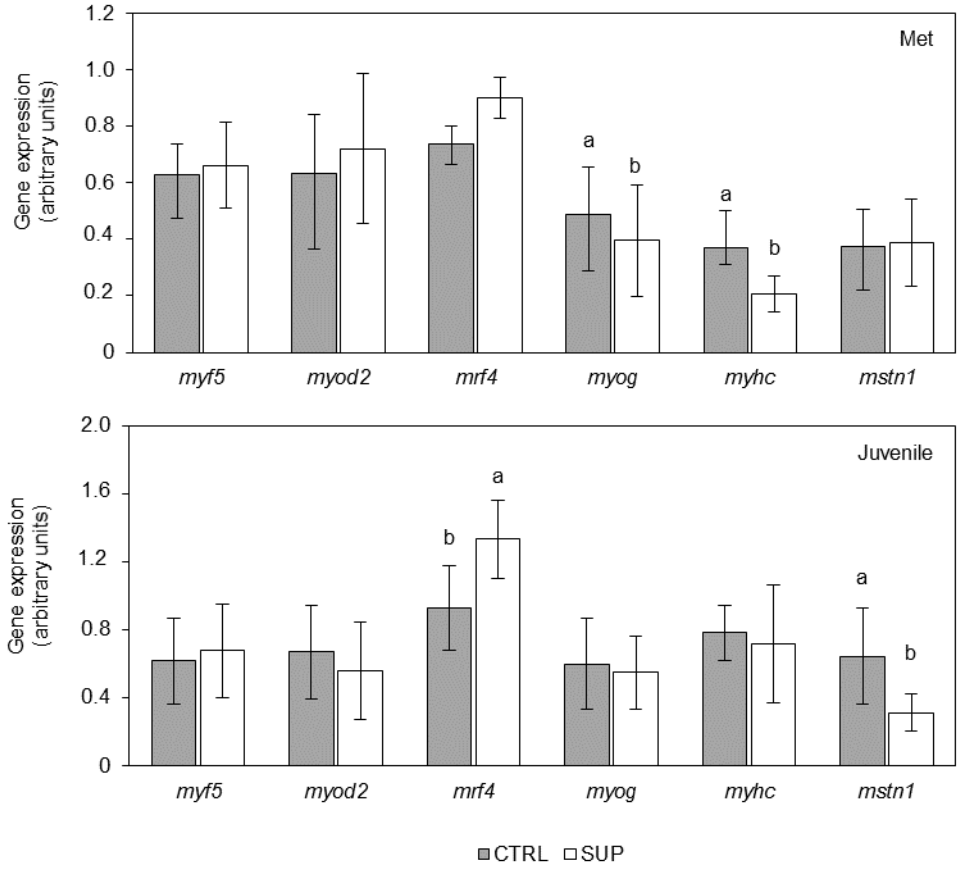
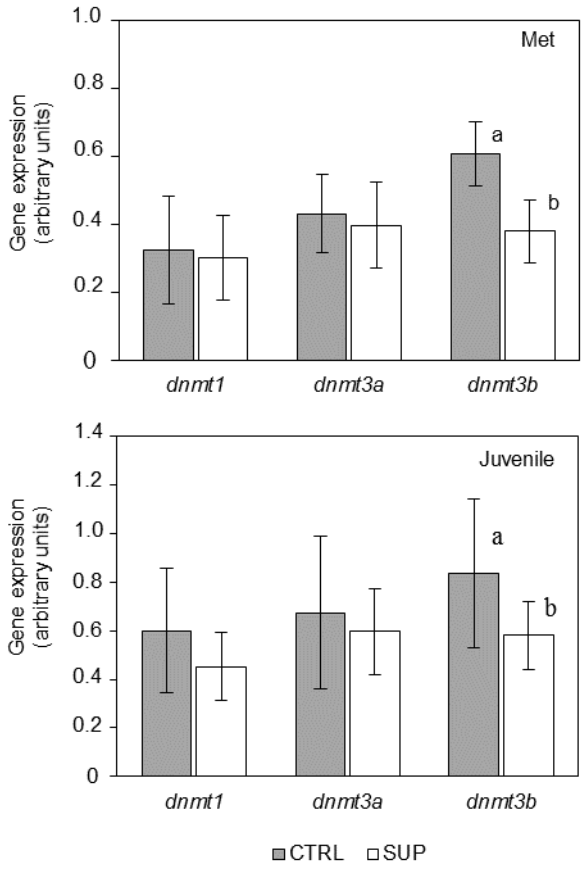


Fig 5



Statement of relevance

In farmed fish species, there has been a great effort over the years to provide the best conditions for successful development of embryos and small larvae, as early environmental conditions can strongly affect muscle growth during early stages and influence the subsequent growth potential at later life stages. In spite of increased efforts to understand the regulation of myogenesis by intrinsic factors like genotype and extrinsic factors such as photoperiod and temperature, studies evaluating the impact of nutritional factors on fish larvae muscle development are still very scarce.

This work will raise interest to the discussion on whether a nutritional cue during an early developmental stage can impact on the regulation of muscle growth and on further growth potential in a metamorphosing farmed fish species, such as Senegalese sole. Supplementing microdiets with crystalline-AA in order to correct dietary IAA did not improve Senegalese sole larvae somatic growth and led to changes on the regulation of muscle growth associated with changes in expression patterns of muscle growth markers during the trial (secondary MRFs *myogenin* and *mrf4*, *myhc* and *mstn1*). Dietary IAA affected the expression of a DNA methyltransferase essential for *de novo* methylation, *dnmt3b,* suggesting that an epigenetic effect at the transcriptional regulation level may explain differences found in somatic growth as a response to a nutritional cue.

This work may contribute to lay down basis for future studies on nutritional programming of muscle growth in fish larvae of important farmed species.

Highlights

Early life nutrition impacts growth potential of a metamorphosing farmed fish species

Dietary amino-acids profile affects the regulation of muscle growth in sole larvae

Transcript levels of key genes regulating myogenesis changed with dietary IAA profiles

Dietary IAA affected the expression of *dnmt3b* suggesting an epigenetic effect underling growth

Results lay down basis for future nutritional programming studies on fish larvae growth