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1 **Larval dietary protein complexity affects regulation of muscle growth and expression**
2 **of DNA methyltransferases in Senegalese sole**
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30

31 **Abstract**

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

53

54 ## 1- Introduction

55 Skeletal muscle protein deposition greatly contributes to overall growth in fish juveniles and
56 larvae, when compared to other tissues (Carter, Houlihan, 2001; Houlihan et al., 1995). Due
57 to its high protein synthesis and deposition rates, skeletal muscle is thus a major determinant
58 of fish amino acids (AA) requirements (Houlihan et al., 1995). Fish larvae have a tremendous
59 growth potential (Conceição et al., 2003; Conceição et al., 2011) and its reliance on dietary
60 AA both as fuel for energy production, and as building blocks for growth (Parra, Yúfera,
61 2001; Parra et al., 1999; Ronnestad et al., 1999; Rønnestad et al., 2003) can be seen as
62 paradoxical considering the poor development of early stages of altricial larvae digestive
63 system (Zambonino Infante et al., 2008). The larvae capacity to digest and absorb dietary
64 protein throughout development are key factors to be considered when formulating
65 microdiets, in order to make the most of its digestive tract capacity to utilize dietary protein
66 and to fully express its maximum growth potential (Conceição et al., 2011; Canada et al.,
67 2017).
68 Senegalese sole (*Solea senegalensis*) is a fast-growing species that undergoes a complex
69 metamorphosis (Fernández-Díaz et al., 2001). Its digestive system ontogeny follows the
70 general pattern observed in other marine species with altricial development (Fehri-Bedoui et
71 al., 2000; Padrós et al., 2011; Ribeiro et al., 1999a; 1999b; Zambonino Infante et al., 2008).
72 As for most altricial species, it has been assumed that early-stage Senegalese sole larvae have
73 a limited capacity to digest and absorb the native protein sources commonly used in
74 commercial fish microdiets formulations (Engrola et al., 2009; Gamboa-Delgado et al.,
75 2008). Since dietary protein is mainly absorbed as free amino-acids (FAA) and di- or tri-
76 peptides (Ronnestad, Morais, 2008) and early-stage larvae have a poorly developed gut
77 (Zambonino Infante et al., 2008), it has been suggested that the moderate inclusion of pre-
78 digested proteins in microdiets would improve its dietary protein digestibility. In fact, recent
79 results suggest that Senegalese sole pre-metamorphic larvae have a limited capacity to utilize
80 native proteins, whereas larger peptides and intact protein seem more suitable to sole post-
81 larvae and young juveniles anabolic and physiological needs (Canada et al. 2017; Richard et
82 al. 2015)
83 Several studies reported increased survival and somatic growth in European sea bass
84 (*Dicentrarchus labrax*) (Cahu et al., 2004; 1999; Zambonino Infante et al., 1997), gilthead

85 sea bream (*Sparus aurata*) (Kolkovksi, Tandler, 2000), white seabream (*Diplodus sargus*)
86 (de Vareilles et al., 2012), large yellow croaker (*Pseudosciaena crocea*) (Liu et al., 2006),
87 Asian sea bass (*Lates calcarifer*) (Srichanun et al., 2014) and Atlantic halibut (*Hippoglossus*
88 *hippoglossus*) (Kvåle et al., 2009; Kvåle et al., 2002) larvae fed microdiets including protein
89 hydrolysates. However, very few studies focused on a possible influence of such diets on
90 muscle growth regulation (Katan et al., 2016; Ostaszewska et al., 2008). Muscle development
91 and growth during early life stages is clearly determinant for the larvae ability to swim, feed
92 and survive (Osse et al., 1997) and was further demonstrated to influence long-term somatic
93 growth (Campos et al., 2014; Galloway et al., 1999; Weatherley et al., 1988). Moreover, early
94 nutrition was recently shown to induce changes on the regulation of skeletal muscle
95 development during early life stages having a long-term effect on somatic growth, which
96 suggests the potential for nutritional programming on muscle growth and somatic growth
97 potential (Alami-Durante et al., 2014).
98 White skeletal muscle constitutes the bulk of the axial locomotor muscle in Senegalese sole
99 larvae, post-larvae and juveniles. White skeletal muscle fibres, also known as fast-twitch
100 fibres, are used for burst swimming movements (Bone, 1978), which is the main type of
101 locomotion displayed by sole to move and feed (Dinis et al., 1999). The white skeletal muscle
102 development in Senegalese sole follows the general pattern observed in other aquaculture
103 species (Campos et al., 2013b;, 2013c). Muscle formation (myogenesis) comprises the
104 recruitment of stem cells to a lineage of myogenic progenitor cells (MPCs) that undergo
105 activation, proliferation, cell cycle exit, differentiation, migration and fusion into already
106 formed muscle fibres (Johnston et al., 2011; Valente et al., 2013). MPCs proliferation and
107 differentiation are ruled by the expression of numerous genes and particularly the four
108 myogenic regulatory factors (MRFs): *myod* and *myf5* are involved in the commitment of
109 myoblasts to form the MPCs population; *myogenin* and *mrf4* drive and keep on the myoblast
110 differentiation that will ultimately result in myotube formation and enlargement (Rescan,
111 2001). On the other hand, myostatin (*mstn*) functions as a negative regulator of myoblast
112 proliferation and differentiation (Thomas et al., 2000). Muscle growth occurs by both
113 hyperplasia (fibre number increase) and hypertrophy (fibre size increase) (Rowlerson,
114 Veggetti, 2001). During fish post-embryonic and larval development, muscle fibre number
115 increases mainly by stratified hyperplasia, which involves the recruitment of new fibres in

116 discrete germinal zones found in the lateral margins of the myotome (Rowlerson, Veggetti,
117 2001). In juvenile and adult stages, new myotubes form on the surface of fast muscle fibres,
118 further fusing or adding nuclei to already existing fibres - mosaic hyperplasia (Rowlerson,
119 Veggetti, 2001). The relative contribution of hyperplasia and hypertrophy was shown to
120 influence long-term growth rate, providing an estimate for individual growth potential
121 (Galloway et al., 1999; Weatherley et al., 1988).
122 There has been a great effort to understand the regulation of muscle growth by intrinsic
123 factors like genotype (Johnston et al., 1999; Valente et al., 2006) and extrinsic factors such
124 as photoperiod (Johnston et al., 2004; Lazado et al., 2014) and temperature (Campos et al.,
125 2013b; Campos et al., 2013c; Galloway et al., 2006; Silva et al., 2011), in order to optimize
126 broodstock management and larval rearing conditions. Nevertheless, the impact of nutritional
127 factors on fish larval muscle development is far from being understood.
128 In fish larvae, dietary protein sources (Alami-Durante et al., 1997; Ostaszewska et al., 2008),
129 dietary protein level (Saavedra et al., 2016) and AA supplementation (Aguiar et al., 2005)
130 were shown to affect muscle growth regulation and the somatic growth rate of several
131 species. In rainbow trout, different protein:energy ratios delivered to first-feeding fry induced
132 changes in the regulation of muscle growth during the nutritional challenge period, but also
133 and more remarkably after 3 months of feeding all groups on the same commercial diet
134 (Alami-Durante et al., 2014). This result suggests that the activity of white MPCs might be
135 programmed by nutritional factors (Alami-Durante et al., 2014), although the mechanisms
136 possibly underlying such response are not known. It has recently been suggested that an
137 epigenetic mechanism could promote differential gene expression and modulate Senegalese
138 sole muscle growth in response to different thermal conditions; different rearing temperatures
139 during the pelagic phase induced changes in the methylation status of the *myogenin* putative
140 promoter, its mRNA transcript levels and expression of *dnmt1* and *dnmt3b* (DNA
141 methyltransferases), which was suggested to underlie the rearing temperature effect on
142 muscle cellularity during the metamorphosis climax (Campos et al., 2013a). In addition, the
143 effect of rearing temperature on muscle cellularity during the metamorphosis climax
144 influenced subsequent somatic growth, up to a late juvenile stage (Campos et al., 2013b).
145 Increasing evidence indicates that DNA methylation is labile not only to environmental
146 conditions but also to nutritional factors (Anderson et al., 2012). However, studies on

147 epigenetic modifications in response to environmental or nutritional cues are a recent trend
148 in fish. Very few studies have been published concerning nutritional programming on muscle
149 growth (Alami-Durante et al., 2014; Fontagné-Dicharry et al., 2017) and no studies have
150 established a relationship between nutritional status and the epigenetic regulation of
151 myogenesis, through possible changes in DNA methylation status. Campos et al. (2013a,
152 2013b) results on the influence of temperature on the regulation of sole myogenesis suggest
153 the pelagic phase as a critical time window prone to epigenetic modifications with long-
154 lasting effects on the regulation of myogenesis and subsequent influence on the potential for
155 growth. Therefore, in the present study, we hypothesized that changes in dietary protein
156 complexity would affect the regulation of muscle growth during the metamorphosis climax
157 and up to an early juvenile stage in Senegalese sole, having an impact on long-term somatic
158 growth. The effect on white muscle growth dynamics was analysed at the metamorphosis
159 climax (16DAH, stage 3), which has been previously recognized as a very relevant time-
160 window in Senegalese sole ontogeny, in which changes in myogenesis induced by external
161 factors were shown to strongly affect long-term somatic growth (Campos et al., 2013b).
162 Muscle cellularity was also analysed in newly-weaned fish (36DAH), since variable growth
163 rates and size dispersion just after weaning are currently a major constraint for a more
164 successful juveniles' production. After being upon three experimental diets until 60 DAH,
165 all groups were fed upon the same commercial diet until 90 DAH to evaluate the enduring
166 effect on somatic growth. The expression pattern of DNA methyltransferases was analyzed
167 in order to understand whether an epigenetic event could possibly underlie the response of
168 muscle growth regulation and somatic growth to dietary protein complexity.
169

170 **2. Material and Methods**

171 *2.1 Experimental diets*

172 Three microdiets (Intact, PartH and HighH) were formulated and processed by SPAROS Lda.
173 (Olhão, Portugal) to be isoenergetic and high protein diets, using approximately the same
174 ingredients, but including protein fractions of different complexity, as previously described
175 by Canada et al. (2017) (Table 1). The Intact diet contained a mix of intact plant protein
176 sources – Plant protein Mix 12 (84% CP, 3.6% CF, SPAROS Lda., Portugal), targeting a
177 peptide molecular weight (MW) higher than 20KDa. The PartH diet contained a hydrolysate

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

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

201 The microdiets were grounded, pooled and analysed for dry matter (105 °C for 24 h) and ash
202 content by combustion in a muffle furnace (Nabertherm L9/11/B170, Bremen, Germany; 550
203 °C for 6 h). As sole have a passive bottom feeding behaviour, with microdiets remaining one
204 min or more in the tank bottom before being eaten (Conceição et al., 2007; Dinis et al., 2000),
205 microdiet samples (n = 4/treatment) were submersed in rearing water for 1 min, in order to
206 allow nutrient leaching and simulate the situation observed in the rearing tanks. After this
207 period the rearing water was removed and the feed samples were frozen at -80ºC and freeze-
208 dried before analysed. These samples were then grounded, pooled and analyzed for dry

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

222 *2.2 Husbandry and experimental set-up*

223 This experiment was performed by trained scientists and following the European Directive
224 2010/63/EU of European Parliament and of the Council of European Union on the protection
225 of animals used for scientific purposes. CCMAR facilities and their staff are certified to house
226 and conduct experiments with live animals ('group-1' license by the 'Direção Geral de
227 Veterinaria', Ministry of Agriculture, Rural Development and Fisheries of Portugal).
228 Following the 3R´s policy, the number of animals was reduced by sharing the same trial and
229 the same microdiets between two studies with different purposes: i) a first study focused on
230 the effect of dietary protein complexity on larval protein utilization and the expression of
231 protein digestion-related genes in relation to somatic growth (Canada et al., 2017); and ii) the
232 present study focused on the effect of dietary protein complexity on the regulation of fish
233 muscle growth dynamics (muscle cellularity and muscle growth related genes) and its impact
234 on long-term somatic growth.

235 Like previously described in Canada et al. (2017), Senegalese sole eggs were incubated in an
236 upwelling incubator at 19±0.5ºC and hatching was completed within the next day (24h).
237 Newly hatched larvae were evenly distributed over 9 white cylindro-conical tanks (100L) in
238 a semi-closed recirculation system with a density of 60 larvae L^{-1} (6000 larvae/tank). The

239 system was equipped with a mechanical filter, a submerged and a trickling biological filter,
240 a protein skimmer and UV sterilizer. Sole was reared according to previously established
241 rearing protocols for Senegalese sole larvae (Engrola et al. 2005). Larvae were reared in
242 green water conditions until 16 days after hatching (DAH), provided by adding frozen
243 *Nannochloropsis* sp. (Nannochloropsis 18% FP 472/180908, Acuicultura Y Nutrición de
244 Galicia SL) to rearing tanks every morning. Abiotic parameters and mortality were daily
245 monitored. Dissolved oxygen in water was maintained at 86.6±7.2% of saturation,
246 temperature at 18.4±0.6ºC and salinity at 37.6±2.3‰. While larvae were fed live prey, a light
247 intensity of 1000 lux was provided by overhead fluorescent tubes under a 10h/14h light/dark
248 photoperiod cycle. At 16DAH, during the metamorphosis climax, the larvae were transferred
249 to flat-bottom tanks (30×70×10cm; 21L), each tank stocking 860 individuals (corresponding
250 to a 4095ind/m^2 density). The system for the benthic rearing was equipped with a mechanical
251 filter, a submerged and a trickling biological filter, a protein skimmer and UV sterilizer.
252 Abiotic parameters were monitored and mortality was recorded every morning. Dead larvae
253 were removed and the rearing units were carefully cleaned with minimal disturbance.
254 Dissolved oxygen in water was maintained at 96.6±7.2% of saturation, temperature at
255 19.6±0.5ºC and salinity at 35.4±0.7‰. During the benthic phase post-larvae were fed both
256 frozen artemia and inert diet. At this stage, sole feeds on the bottom of the tank, displaying a
257 calm behavior and spending most of the time settled on the bottom, swimming short distances
258 at a time. So light intensity was reduced to 400lux to minimize stress and the photoperiod
259 cycle was maintained at 10h/14h light/dark. The photoperiod was decided based on live prey
260 feeding schedule during the pelagic phase, and was maintained during the benthic phase.

261 The dietary treatments (Intact, PartH and HighH) were randomly assigned to tanks (n = 3
262 tanks per treatment). From mouth opening (2DAH) until 5DAH larvae were fed rotifers
263 (*Brachionus* sp.) enriched with Easy DHA Selco (INVE, Belgium), at an initial density of 5
264 rots·mL^{-1} together with the respective inert diet (200-400µm). *Artemia* AF nauplii (na)
265 (INVE, Belgium) were introduced at 4DAH and prey density was gradually increased from
266 4 to 5 na·mL−1, becoming the only prey offered after 5DAH. *Artemia* EG metanauplii (M24)
267 (INVE, Belgium) enriched with Easy DHA Selco were introduced at 12DAH, gradually
268 increasing from 12 to 14 M24·mL^{-1} until 19DAH. Enriched frozen *Artemia* metanauplii were
269 offered to settled larvae (between 16 and 35DAH). Live prey was gradually reduced and

270 replaced by inert diet until complete weaning at 36DAH, according to Engrola et al. (2009),
271 with the inert diet constituting 60 % of the feed supplied (dry matter basis) from 6 to 16DAH,
272 more than 60 % from 17 to 30DAH and more than 80 % from 30 to 36 DAH.

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

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

284 *2.3 Somatic growth*

285 At mouth opening (MO) (2DAH), one pool of 20 individuals was collected from each tank
286 for dry weight (DW) evaluation. Thereafter, individual fish were randomly sampled for dry
287 weight determination at 16DAH (MC: metamorphosis climax), 36DAH (weaned early
288 juvenile) (*n*=20 per replicate), 60DAH, at a late juvenile stage, and after being fed exclusively
289 upon the inert diets for 24days (*n*=20 per replicate), and at 90DAH after being fed exclusively
290 upon a single commercial diet for 30 days (*n*=20 per replicate) The larvae and early juveniles
291 were frozen at -80ºC and freeze-dried for dry weight determination to 0.001mg precision.
292 Growth was expressed as relative growth rate (RGR, % day^{-1}) and was determined during
293 the pelagic phase from mouth opening to metamorphosis climax (2-16DAH), during the
294 benthic phase (16-36DAH, 36-60DAH and 60-90DAH) and during the whole trial (2-
295 60DAH). RGR was calculated as RGR (% day-1) = $(e^{g}-1)\times100$, where g = $[(\ln_{\text{final weight}} - \ln$
296 $_{\text{initial weight}})/\text{time}]$ (Ricker, 1958).

297 *2.4 White muscle cellularity*

298 Standard histological and morphometric techniques (Silva et al., 2009b; Valente et al., 1999)
299 were used to analyse fast-twitch muscle cellularity at two developmental stages: 16DAH
300 (metamorphosis climax – stage 3) and 36DAH (weaned post-larvae). Three fish per tank
301 were collected, killed by over-anaesthesia (MS-222, Sigma-Aldrick, USA; 400 mgL^{-1}) and
302 measured for standard length (SL, mm). Fish were then fixed in 4% paraformaldehyde in
303 phosphate-buffered saline (PBS) (Sigma-Aldrich, USA) for 24h, washed with 1×PBS and
304 stored in 70º ethanol, at 4ºC until further processing. Paraffin (Merck, Germany) embedded
305 fish were sectioned (7 μm) transversely to the body axis, using a microtome (RM2245, Leica,
306 Germany), mounted on coated slides with 3-aminopropyltriethoxysilane (APES) (Sigma-
307 Aldrich, USA) to improve section adhesion and double stained with haematoxylin
308 (Haematoxylin Gill II, Merck, Germany) and eosin (Eosin Y, VWR, Belgium) before placing
309 a cover slip.
310 Morphometric variables were measured in transversal body sections of individual fish, at a
311 peri-anal location. In larvae and early juveniles (16 and 36DAH), the total number of fast-
312 twitched fibres (N) were counted, and total cross-sectional area [CSA (mm^2)], total cross
313 section muscle area [Muscle CSA (mm^2)] and fast-twitch fibre cross-sectional area (μm^2)
314 were measured. Fibre diameter (μm) was estimated from the fibre cross-sectional area (μm^2)
315 data assuming that muscle fibres cross-section is round shaped. The mean fibre cross-
316 sectional diameter and percentage of small fibres (with a diameter <3 μm at 16DAH and <5
317 μm at 36DAH) were estimated from a minimum of 600 fibres which cross-sectional area was
318 measured over the total cross section muscle area. The percentage of small fibres is an
319 indicator of the growth potential and the chosen fibre size class to define the population of
320 small fibres should correspond to the left-tail of the fibre size class distribution (Ian Johnston,
321 University of St Andrews, pers. Comm). The fibre density (total number/mm^2) was calculated
322 by dividing the total number of fast-twitched fibres (N) counted by the total cross section
323 muscle area [Muscle CSA (mm^2)]. Muscle fibre outlines were traced using a 400×
324 magnification. This study was performed using an Olympus BX51 microscope (Olympus
325 Europa GmbH, Germany) with the Cell^B Basic imaging software. The relative contribution
326 of hypertrophy and hyperplasia to the increase of muscle cross-sectional area was estimated
327 as follows: Δ Muscle CSA (μm^2) = $N_{m\times}\Delta A$ (μm^2) + $A_{m\times}\Delta N$, where Δ was calculated between

328 two sampling times (t and t + 1), N_m and A_m refer to the mean total number of fibres and
329 fibre area at t, respectively (Silva et al., 2009b; Valente et al., 1999).

330 *2.5 Gene expression*

331 *2.5.1 RNA extraction and cDNA synthesis*

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

346 *2.5.2 Quantitave real-time PCR (qPCR)*

347 The relative expression of the MRFS (*myf5, myod2, mrf4, myog), mstn1* as well as genes
348 encoding for the proteins responsible for *de novo* DNA methylation (*dnmt3a,* and *dnmt3b*)
349 and methylation maintenance (*dnmt1*) were quantified using real-time PCR. Specific primers
350 for qPCR were used (see Table 3 for primer sequences, GenBank accession numbers,
351 amplicon sizes, annealing temperatures (ºC) and qPCR amplification efficiencies).
352 Quantification of gene expression was performed by qPCR with Sso Fast Evagreen supermix
353 (Bio-Rad) on a CFX96^{TM} Real-Time PCR Detection System (Bio-Rad). Samples were
354 denatured for 30s at 95°C and then subjected to 40 cycles of amplification with the following
355 thermocycling parameters: denaturation for 5s at 95°C and annealing/extension for 10s (see
356 Table 3 for annealing temperatures (ºC)). Specificity of the qPCR reaction and the presence

357 of primer dimers were checked by examining the melting curves with a dissociation protocol
358 from 65 to 95 °C (in 0.5ºC increment, for 5 s). Five-point standard curves of a 5-fold dilution
359 series (1:5–1:3125) of pooled RNA were used for PCR efficiency calculation. Minus reverse
360 transcriptase controls were checked for every gene. All samples were run in triplicate. CT
361 values were determined using the baseline subtracted curve fit method using the CFX
362 Manager Software with a fluorescence threshold automatically set. Profiling of mRNA
363 transcription levels (qPCR) were used to quantify gene expression, using data normalised
364 against the geometric average of transcript levels of two reference genes (*ubq* and *rps4*)
365 obtained from GeNorm (Vandesompele et al., 2002), as previously reported (Fernandes et
366 al., 2008). Reference genes were selected based on previous works on Senegalese sole larvae.
367 Three genes (*ubq*, *rps4* and *ef1a1*) were tested and two (*ubq*, *rps4*) were selected based on
368 the stability of its expression in all samples.

369 *2.6 Data analysis*

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

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

382 To compare the distribution of muscle fibre size, a nonparametric method was used to fit
383 smoothed probability density functions (PDFs) using the statistical program for the analysis
384 of muscle fibre populations (Johnston et al., 2004). Bootstrapping was used to distinguish
385 random variation in diameter distribution from treatment differences. A Kruskal-Wallis test

386 with 1000 bootstrap replicates was used to test the null hypothesis that PDFs of muscle fibre
387 diameter in the three treatments were identical.

388 **3. Results**

389 *3.1 Dietary effect on white skeletal muscle growth and somatic growth*

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

397 Although the smoothed probability density functions (PDFs) of muscle fibre populations did
398 not differ significantly between groups at 16DAH, the percentage of fibres with diameter
399 within the range 7-11 μm was higher in PartH and HighH groups, when compared to the
400 Intact group (P=0.001) and the percentage of fibres with diameter within the range 11-15 μm
401 was higher in the PartH group than in the Intact group (P=0.002) (Table 4). The smoothed
402 PDFs of muscle fibre populations indicated a high percentage of small-sized muscle fibres in
403 all dietary treatments, as all curves skewed to the left-hand tail of the distribution (Fig. 1).
404 There was a significant correlation between the percentage of very small fibres (<3μm) and
405 the relative growth rate (RGR) in the subsequent period, from 16 to 36DAH (Table 5). At
406 36DAH, fish fed the Intact diet were already heavier than those fed the other diets and grew
407 better until 60DAH (Table 4).

408 Between 16 and 36DAH there was a significant enlargement of muscle fibres in all groups
409 (Table 4), reflected on the shift of PDFs distribution towards the right-hand of the plot (Fig.
410 1). The average fibre size increased from 16 to 36DAH by 1.8, 1.6 and 1.7-fold respectively
411 in the Intact, PartH and HighH groups, being similar among groups at 36DAH (Table 4).
412 This was paralleled by a 2.3, 2.3 and 2.0-fold increase of total number of fibres in the Intact,
413 PartH and HighH groups, respectively, which reflected in a muscle CSA increase of 6.2-fold,
414 4.8-fold and 4.4-fold in the Intact, PartH and HighH groups, respectively (Table 4).

415 Moreover, there was significant contribution of the total number of fibres to the muscle cross-
416 sectional area, as a positive correlation between these two parameters was detected at this
417 stage (P=0.031) (Table 5). Although PDFs distributions at 36DAH did not differ significantly
418 among treatments (Fig. 1), the percentage of small fibres (diameter <5µm) was higher in the
419 group fed the Intact diet, when compared to those fed the PartH and the HighH diets
420 (P=0.039) (Table 4). Moreover, there was a positive correlation between the percentage of
421 small fibres (diameter <5µm) and the relative growth rate in early juvenile fish (Table 5).
422 The relative contribution of hypertrophy to white muscle growth in the 16-36DAH period
423 was higher in the HighH group (63.4±7.0%) than in the PartH group (52.6±6.4%) (P=0.026),
424 but not significantly different from that in the Intact group (55.6.±8.2%). No further
425 differences were found in the white muscle cellularity variables at 36DAH (Table 4).

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

430 *3.2 Expression of growth-related genes and DNA methyltransferases*

431 The relative expression of the genes encoding for the myogenic regulatory factors did not
432 differ significantly between groups fed different diets during the metamorphosis climax (Fig.
433 2A). However, at 36DAH, the expression of m*yf5* (P=0.027) and *myod2* (P=0.029) was
434 significantly upregulated in the group fed the HighH diet, in which the transcript levels were
435 increased, respectively, by 2.24 and 2.05-fold, when compared to the PartH group, no
436 differences being found in pair-wise comparison between the HighH and Intact groups
437 (Fig.2B). At this stage, the transcript levels of *myog* (P=0.004) were also upregulated in the
438 group fed the HighH diet, increased by 1.51 and 1.77-fold when compared to the groups fed
439 Intact and PartH diets, respectively (Fig.2B). No significant differences were found in the
440 transcript levels of *mrf4*. At this stage, fibre density was negatively correlated with the
441 expression of *myod2* and *myog* (Table 5). No dietary effect was found in the *mstn1* mRNA
442 levels during the metamorphosis or at 36DAH (Fig.2B). However, fibre density was
443 negatively correlated with the expression of *mstn1* (Table 5).

444 There was no effect of dietary protein complexity on the DNA methyltransferase *dnmt1*
445 transcript levels in 36DAH Senegalese sole (Fig.3). However *dnmt3a* (P=0.042) and *dnmt3b*
446 (P=0.041) transcript levels were significantly higher in fish fed the HighH diet, when
447 compared to those fed either the Intact or PartH diets (Fig.3). Moreover, the expression of
448 *dnmt3a* was negatively correlated with fibre density and accordingly, it was positively
449 correlated with the fibre diameter (Table 5). The expression of *dnmt3b* was negatively
450 correlated with the total number of fibres (Table 5).
451

452 **4. Discussion**

453 *4. 1 Effect of dietary protein complexity on larval performance*

454 The three microdiets (Intact, PartH and HighH) tested in this study were formulated with the
455 aim of having the degree of hydrolysis of dietary protein as the main changing factor, using
456 approximately the same practical ingredients. Nevertheless, the proportions of marine and
457 plant ingredients included in each diet might have also induced some other differences
458 between diets. Since all diets included high levels fish and krill protein hydrolysates, the
459 attractability or palatability to sole larvae were not likely to have changed between the diets,
460 which was confirmed by the visual observation of fish behaviour towards the diets. All diets
461 exceeded the indispensable amino acid (IAA) requirements for sole juveniles (Silva et al.,
462 2009a) and were balanced according to the ideal protein concept (Arai, 1981) taking as
463 reference the Senegalese sole post-larvae whole-body AA profile (Aragão et al., 2004). Thus,
464 the effects of dietary formulation variations on the regulation of muscle growth and larval
465 performance were most likely due to dietary protein complexity and not to any AA
466 imbalances.

467 Several studies evaluated the inclusion of pre-digested proteins in microdiets for fish larvae
468 (Cai et al., 2015; de Vareilles et al., 2012; Gisbert et al., 2012; Kotzamanis et al., 2007; Kvåle
469 et al., 2009; Kvåle et al., 2002; Skalli et al., 2014; Srichanun et al., 2014), but to our
470 knowledge very few focused on the dietary effects of hydrolysates on the regulation of
471 muscle growth (Katan et al., 2016; Ostaszewska et al., 2008). In the present study, the tested
472 microdiets were mostly based on protein sources with substantially different peptide MW
473 profiles to be delivered to Senegalese sole from a very early developmental stage, i.e. from

474 mouth-opening. On the contrary, in the previous studies the protein hydrolysates included in
475 microdiets were mostly composed of free amino-acids and di- or tri-peptides (<0.2KDa) or
476 oligopeptides (0.5-2.5 KDa) (Cahu et al., 2004; 1999; Cai et al., 2015; de Vareilles et al.,
477 2012; Gisbert et al., 2012; Kotzamanis et al., 2007; Skalli et al., 2014; Srichanun et al., 2014;
478 Zambonino Infante et al., 1997).

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

493 *4.2 Effect of dietary protein complexity on the regulation of muscle growth*

494 Different dietary formulations based on different protein MW profiles induced differences in
495 the white muscle cellularity that were reflected on somatic growth during the metamorphosis
496 climax. Although the smoothed probability density functions (PDFs) of muscle fibre
497 populations did not differ significantly among treatments at 16DAH, significant differences
498 were detected in the percentage of medium to large-sized muscle fibres. The percentage of
499 fibres with diameter within the range 7-11 μm was lower in the Intact group, when compared
500 to the PartH and HighH groups, and the percentage of fibres with diameter within the range
501 11-15 μm was lower in the Intact group than in the PartH group, which together with a
502 reduced average fibre diameter and similar fibre number, suggests a delayed muscle growth
503 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 <3μm), 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 <3μm) 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

535 compared to that fed the PartH diet. The transcription of *myog* was also significantly. higher
536 in the group fed the HighH diet, when compared to both the Intact and PartH groups Since
537 *myog* encodes a highly conserved myogenic regulatory factor that is involved in terminal
538 muscle differentiation (Rescan, 2001), its upregulation might have sustained the
539 differentiation of myoblasts to further fuse into already formed muscle fibres, promoting
540 hypertrophy rather than hyperplasia in the HighH group, being upstream supported by the
541 upregulated *myf5* and *myod2* transcription. This is further corroborated by the negative
542 correlation observed between fibre density and the transcript levels of *myod2* and *myog*
543 (Table 5), and confirmed by the relative contribution of hypertrophy towards white muscle
544 growth in the 16-36DAH period, which was significantly higher in the HighH group (63%)
545 than in the PartH group (52%). Also in Senegalese sole, differences in the transcript levels
546 of both *myod2* and *myog* between groups reared at different water temperatures were
547 associated with changes in fibre diameter and not in the total fibre number during the
548 metamorphosis climax (Campos et al., 2013b), suggesting that the upregulation of these
549 MRFs seem to promote fibre hypertrophy without affecting total fibre number. A higher
550 contribution of hypertrophy to muscle growth and the reduced recruitment of small fibres
551 (evidenced by a low percentage of fibres < 5 μm at 36DAH) are predictive of a lower growth
552 potential in the HighH group. The relation between hypertrophy-sustained muscle growth
553 and further reduced somatic growth has been proposed in common carp (Alami-Durante et
554 al., 1997), Atlantic cod (Galloway et al., 1999), pike perch (Ostaszewska et al., 2008) and
555 pacu (Leitão et al., 2011) larvae subjected to different types of feed, dietary formulations and
556 feeding regimes.
557 A significant downregulation of *myf5*, *myod2* and *myog* transcription in the PartH group, and
558 lower percentage of small fibres compared to the Intact group, suggests a reduced recruitment
559 capacity of new fibres in fish fed the PartH diet. This is further supported by a negative
560 correlation between the *myod2* and *myog* transcript levels and fibre density. Decreased
561 recruitment of new fibres would have led to reduced growth potential in PartH fed fish. Also
562 in Senegalese sole, a significant downregulation of most MRFs in earlier stages was reported
563 in a slow-growing group exposed to a low rearing temperature during the pelagic phase
564 (Campos et al., 2013b). Pike perch larvae fed a formulated diet including a casein hydrolysate
565 (Ostaszewska et al., 2008) and Atlantic cod larvae reared upon a fish hydrolysate

566 supplemented diet (Katan et al., 2016) have displayed reduced somatic growth, but as a result
567 of delayed muscle growth, including reduced muscle cross-sectional area. The present study
568 results suggest a lower proliferative capacity in the PartH fed fish, but not at the point of
569 reducing muscle cross-sectional area.
570 At 36DAH, the transcription of *myf5*, *myod2* in the Intact group was probably high enough
571 to keep up with the increase of the MPC population and further fibre recruitment. In fact, fish
572 fed the Intact diet had a significantly higher percentage of small fibres, which has probably
573 contributed for a higher growth potential, as further suggested by a significant correlation
574 between the percentage of small fibres at 36DAH (diameter <5µm) and relative growth rate
575 in the 36-60DAH period. Indeed, the percentage of small-sized muscle fibres is long known
576 to be a good indicator of further muscle growth potential both in juveniles and larvae (Alami-
577 Durante et al., 1997; Galloway et al., 1999; Silva et al., 2009b). At 90 DAH, after being fed
578 upon a common commercial diet for 30 days, fish fed the Intact diet were still heavier than
579 those fed the PartH and HighH diets, which reinforces the impact of muscle growth dynamics
580 on long-term somatic growth in response to dietary protein complexity. However, it cannot
581 be excluded that 30 days may not be enough to evaluate whether this impact on long-term
582 somatic growth is reversible or not.

583 In spite of the negative correlation between *mstn1* transcription and fibre density detected at
584 36DAH, different diets did not induce significant changes in the *mstn1* mRNA levels during
585 the metamorphosis or at 36DAH. Since *mstn1* encodes for myostatin, which is a negative
586 regulator of MPCs activation and proliferation, a negative correlation between *mstn1*
587 transcription and density or fibre recruitment and muscle growth would be expected.
588 However, the relation between *myostatin* mRNA levels and muscle growth is surprising:
589 depression of growth induced by environmental conditions does not correlate with an up-
590 regulation of *myostatin* as expected (Rescan, 2005). While Atlantic cod larvae fed a fish
591 hydrolysate supplemented diet displayed reduced growth and delayed muscle growth along
592 with upregulated *mstn1* transcription, Senegalese sole fast-growing groups displayed an up-
593 regulation of *mstn1* in late juvenile stages (Campos et al., 2013b; Canada et al., 2016).
594 Moreover, m*stn1* transcription did not respond to environmental changes in Senegalese sole
595 metamorphosing larvae and post-larvae (Campos et al., 2013b). Similarly, the present results
596 show no clear dietary effect on the expression of *mstn1* at 16 or 36DAH. Moreover, in the

597 present study, gene expression was analyzed in the whole fish and not only in the muscle, so
598 it is also possible that the expression of *mstn1* might be associated with other physiological
599 mechanisms and not only with skeletal muscle growth regulation (Campos et al., 2010).

600 *4.3 Epigenetic effect*

601 Late post-larvae fed the HighH diet had higher *dnmt3a* and *dnmt3b* mRNA levels, when
602 compared to those fed the other diets, which may be associated with the dietary methionine
603 availability in the HighH diet. *Dnmt3a* and *dnmt3b* are *de novo* DNA cytosine
604 methyltransferases which covalently attach S-adenosylmethionine (SAM) methyl groups to
605 the 5′-position of cytosine (in CpG dinucleotides), thus methylating DNA and repressing
606 transcription during embryogenesis and cell differentiation (Turek-Plewa, Jagodzinski,
607 2005). DNA methylation relies on the availability of SAM, the universal cellular methyl
608 donor (Selhub, 1999). SAM generates from methionine in a ATP-dependent reaction in the
609 one-carbon cycle which depends on the activity of several enzymes and the presence of
610 dietary methyl donors, such as folate, choline, betaine and methionine (Anderson et al.,
611 2012). The HighH diet had a higher methionine content, increased by 28% and 88% when
612 compared with the Intact and the PartH diets, respectively. A higher availability of
613 methionine may underlie the increased transcription of *dnmt3a* and *dnmt3b* in the HighH
614 group, which may be associated with a general increase in DNA methylation. However,
615 further studies would be needed to ascertain whether there were actually changes in the
616 availability of methyl group donors in the tissues of the fish fed the different diets, and
617 whether the increased expression of genes encoding for DNA methyltransferases would
618 possibly lead to DNA hypermethylation. In fact, although theoretically the supplementation
619 of methionine would increase the DNA methylation of genes and down-regulate gene
620 expression (Waterland, 2006), in practice the effect of a methionine-supplemented diet on
621 the DNA methylation is not always as expected (Zhang, 2017). The effect of methionine
622 supplementation on the regulation of one C-cycle, namely on the production of either S-
623 adenosylmethionine (SAM) or S-adenosylhomocysteine (SAH) is not consistent, neither is
624 its effect on the expression of the DNMTs or in the methylation patterns, which seems to be
625 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 promotors 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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665

666 **Conflict of interest:**

667 The authors declare that they have no conflict of interest.

668

669

670 **Authorship:**

671 P.C. performed the experiment, the statistical analysis and wrote the manuscript. R.T, P.C. 672 and S.M. performed gene expression analysis. P.C. and V.S performed histological analysis. 673 J.P and M.Y manufactured the IdG Hydrolysate and revised the manuscript. L.V., L.C, S.E. 674 and J.F conceived the study, supervised the research and contributed to writing of the 675 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 16DAH). 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 ± 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 ± 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)

	Diets		
	Intact	PartH	HighH
Ingredients (% dry matter)			
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
Proximate analyses			
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.

	Diets		
	Intact	PartH	HighH
Indispensable amino-acids (IAA)			
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**
Dispensable amino-acids (DAA)			
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. (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
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

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

	Diets		
	Intact	PartH	HighH
16 DAH			
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
36 DAH			
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
Growth after 36 DAH			
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 (% day-1) = (eg-1)×100, where g = [(ln final weight - ln initial weight)/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
myf5	NS	NS	NS	NS
myod2	NS	NS	NS	NS
myog	NS	NS	NS	NS
mrf4	NS	NS	NS	NS
mstn1	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
myf5	NS	NS	NS	NS
myod2	NS	P=0.013, r=-0.783	NS	NS
myog	NS	P=0.044, r=-0.679	NS	NS
mrf4	NS	NS	NS	NS
mstn1	NS	P=0.046, r=-0.674	NS	NS
dnmt1	NS	NS	NS	NS
dnmt3a	NS	P=0.025, r=-0.731	P=0.038, r=0.694	NS
dnmt3b	P=0.013, r=-0.783	NS	NS	NS

Fig.1

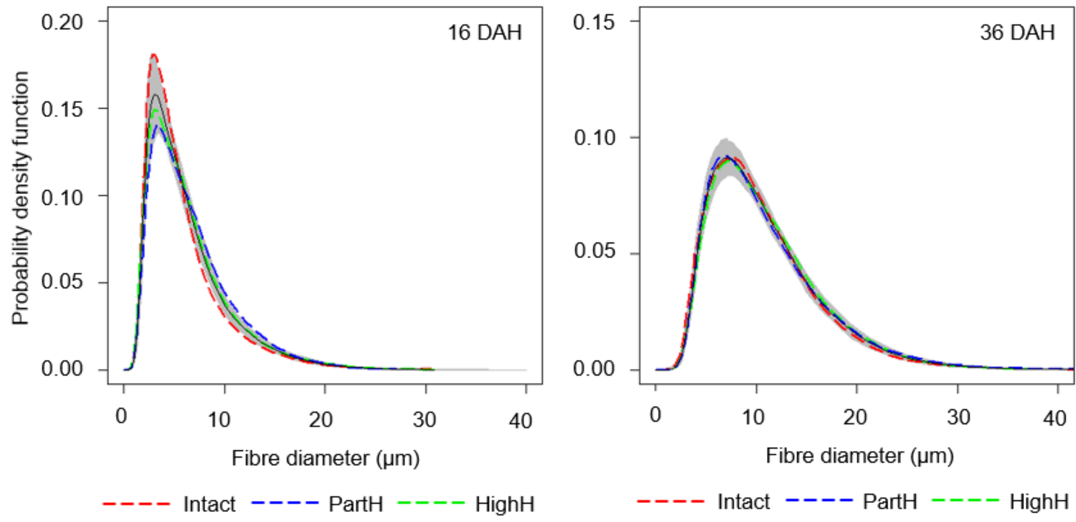

Fig.2

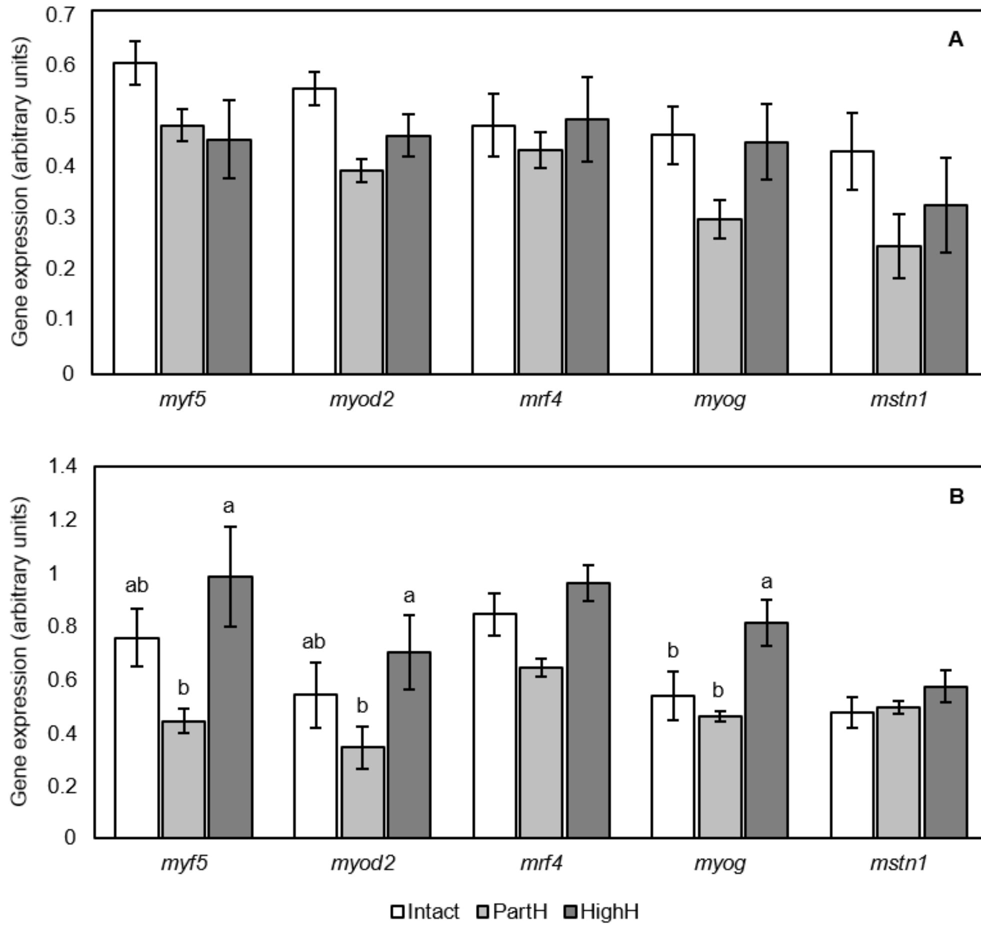

Fig.3