Accepted Manuscript

Dietary protein complexity modulates growth, protein utilisation and the expression of protein digestion-related genes in Senegalese sole larvae

Paula Canada, Luís E.C. Conceição, Sara Mira, Rita Teodósio, Jorge M.O. Fernandes, Carmen Barrios, Francisco Millán, Justo Pedroche, Luisa M.P. Valente, Sofia Engrola

PII: S0044-8486(17)30513-6
DOI: doi: 10.1016/j.aquaculture.2017.05.028
Reference: AQUA 632661

To appear in: *aquaculture*

Received date: 18 March 2017
Revised date: 20 May 2017
Accepted date: 21 May 2017

Please cite this article as: Paula Canada, Luís E.C. Conceição, Sara Mira, Rita Teodósio, Jorge M.O. Fernandes, Carmen Barrios, Francisco Millán, Justo Pedroche, Luisa M.P. Valente, Sofia Engrola , Dietary protein complexity modulates growth, protein utilisation and the expression of protein digestion-related genes in Senegalese sole larvae, *aquaculture* (2017), doi: 10.1016/j.aquaculture.2017.05.028

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

Dietary protein complexity modulates growth, protein utilisation and the expression of protein digestion-related genes in Senegalese sole larvae

Paula Canada[a,b,c], Luís E.C. Conceição[d], Sara Mira[c], Rita Teodósio[c], Jorge M.O. Fernandes[e], Carmen Barrios[c], Francisco Millán[f], Justo Pedroche[f], Luisa M.P. Valente[a,b], Sofia Engrola[c*]

[a] CIMAR/CIIMAR – Centro Interdisciplinar de Investigação Marinha e Ambiental, Terminal de Cruzeiros do Porto de Leixões, Avenida General Norton de Matos, S/N, 4450-208 Matosinhos, Portugal

[b] ICBAS – Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Rua Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal

[c] CCMAR, Centro de Ciências do Mar, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

[d] SPAROS Lda, Área Empresarial de Marim, Lote C, 8700-221 Olhão, Portugal

[e] Faculty of Biosciences and Aquaculture, Marine Genomics Research Group, Nord University, 8049, Bodø, Norway

[f] Instituto de la Grasa (CSIC), Universidad Pablo de Olavide - Edificio 46 Ctra. de Utrera km. 1, 41013-Sevilla, Spain

***Corresponding author:**

Sofia Engrola

Postal address: CCMAR, Centro de Ciências do Mar, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

Email address: sengrola@ualg.pt

Telephone number: +351289800900

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Abstract:

Given its complex metamorphosis and digestive system ontogeny, Senegalese sole larvae capacity to digest and utilize dietary protein is likely to change throughout development. In the present study, we hypothesized that the manipulation of dietary protein complexity may affect Senegalese sole larvae capacity to digest, absorb and retain protein during metamorphosis, as well as the mRNA expression of genes encoding for the precursors of proteolytic enzymes of the digestive tract and the enterocyte peptide transporter PepT1, which may have further impact on somatic growth. Three diets were formulated using approximately the same practical ingredients, except for the main protein source. The Intact diet protein content was mostly based on intact plant protein where the target peptide molecular weight (MW) would be > 70 kDa. The PartH diet protein fraction was mostly based on a protein hydrolysate with a high content of 5–70 kDa peptides. The HighH diet protein fraction was mostly based on a protein hydrolysate with a high content of 5 kDa peptides. A growth trial was performed with larvae reared at 19ºC under a co-feeding regime from mouth opening. The transcription of *pga, tryp1c, ialp*, *ampn* and *pepT1* (encoding respectively for pepsinogenA, Trypsinogen1C, Intestinal alkaline phosphatase, Aminopeptidase N and for the enterocyte peptide transporter 1) was quantified by qPCR, during the metamorphosis climax (16 DAH) and after the metamorphosis was completed (28 DAH). An *in vivo* method of controlled tube-feeding was used to assess the effect on the larvae capacity to utilize polypeptides with different MW (1.0 and 7.2 kDa) representing a typical peptide MW of each of the hydrolysates included in the diets. The PartH diet stimulated growth in metamorphosing larvae (16 DAH), whereas the Intact diet stimulated growth after 36 DAH. The Intact diet stimulated the larvae absorption capacity for 1.0 kDa peptides at 16 DAH, which may have contributed for enhanced growth in later stages. The PartH diet stimulated the transcription of *tryp1c* and *pept1* at 28 DAH, which seemed to reflect on increased post-larvae capacity to retain dietary 7.2 kDa polypeptides. That may indicate a possible strategy to optimize the digestion and utilization of the PartH dietary protein, though it did not reflect into increased growth. The Intact diet promoted the transcription of *pepsinogenA*, which may reflect a reduced gastrointestinal transit time, which could have enhanced the dietary nutrients assimilation, ultimately improving growth. The present results suggest

ACCEPTED MANUSCRIPT

that, whereas pre-metamorphic sole larvae utilize better dietary protein with a moderate degree of hydrolysis, post-metamorphic sole make a greater use of intact protein.

Keywords: protein hydrolysate; proteolytic enzymes; PepT1; metabolism; growth; fish larvae

Highlights:

Proteolytic capacity is a limiting factor for protein digestion in sole early stage larvae.

Sole larvae adjust the way they utilize protein in response to dietary formulation.

Pre-metamorphic sole larvae grow better upon moderately hydrolysed dietary protein.

Dietary intact protein seems to be suitable to sole post-larvae and young juveniles.

Dietary protein complexity should match the developing larvae proteolytic capacity.

Microdiets protein for Senegalese sole should be adapted to each developmental stage.

List of abbreviations

AA	Amino acids
BBM	Brush border membrane
DAH	Days after hatching
FAA	Free amino acids
FPH	Fish protein hydrolysate
MW	Molecular weight
RGR	Relative growth rate

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

1. Introduction:

Senegalese sole (*Solea senegalensis*) is a fast-growing species that undergoes a complex metamorphosis that strongly affects its digestive physiology (Conceição et al., 2007a; Engrola et al., 2010; Fernández-Díaz et al., 2001). As most altricial species, Senegalese sole larvae start exogenous feeding at an early stage of development (2 days after hatching, DAH) before the digestive system is fully developed. Pre-metamorphic larvae do not possess a functional stomach, which indicates a strong dependence on pancreatic enzymes for protein digestion (Ribeiro et al., 1999a). During metamorphosis the spatial organization of the digestive system changes dramatically, concomitantly with an increase of the absorption area, as well as a change on proteolytic activity (Engrola et al., 2009b; Ribeiro et al., 1999a, 1999b). After metamorphosis is completed, settled postlarvae undergo a process of enzymatic maturation of the intestine, characterized by a decrease in cytosolic activity (leucine-alanine peptidase) and an increase in the activity of brush border membrane (BBM) enzymes (aminopeptidase N and alkaline phosphatase) (Ribeiro et al., 1999b). The gastric glands come to be developed between 30 and 40DAH, progressively covering the stomach epithelium (Ribeiro et al., 1999; Yúfera, Darías, 2007). Therefore, Senegalese sole larvae capacity to digest and utilize dietary protein is likely to change remarkably throughout development. Still, Senegalese sole post-larvae, juveniles and even adults never develop a true acidic digestion (Yúfera and Darías, 2007), contrarily to most pleuronectiformes and other altricial fish species with a stomach.

For most altricial species, including Senegalese sole, it has been generally assumed that early-stage larvae have a limited capacity to digest and absorb the native protein sources commonly used in commercial fish feed formulations (Engrola et al., 2009b; Gamboa-Delgado et al., 2008). Since dietary protein is mainly absorbed as free amino-acids (FAA) and di- or tri-peptides (Rønnestad, Morais, 2008), pre-digested proteins have long been introduced in larvae feed formulations in order to ease the dietary protein digestion, with the expectation of promoting absorption and further protein synthesis (Cahu et al., 1999, 2004; Cai et al., 2015; Gisbert et al., 2012; Kolkovksi, Tandler, 2000; Kotzamanis et al., 2007; Kvåle et al., 2002, 2009; Srichanun et al., 2014; Zambonino Infante et al., 1997). In fact, it has been shown that highly hydrolysed (<1.4 kDa) and partially-hydrolysed (10-75 kDa) proteins are absorbed 3.0 and 2.2 times (respectively) faster than intact protein (>65 kDa) within the first 2 h after tube-feeding

ACCEPTED MANUSCRIPT

pre-metamorphic Atlantic halibut (*Hippoglossus hippoglossus*) larvae (Tonheim et al., 2005). However, a trend for a higher oxidation and reduced protein retention for increasing degree of hydrolysis of the tube-fed protein was also found (Tonheim et al., 2005).

Accordingly, while moderate inclusions of hydrolysed protein promoted larval growth and survival, high inclusion levels seem to have detrimental effects on larval performance of European sea bass (*Dicentrarchus labrax*) (Cahu et al., 2004; 1999; Zambonino Infante et al., 1997), gilthead sea bream (*Sparus aurata*) (de Vareilles Sommières, 2013; Kolkovksi, Tandler, 2000), white seabream (*Diplodus sargus*) (de Vareilles et al., 2012), large yellow croaker (*Pseudosciaena crocea*) (Liu et al., 2006), Asian sea bass (*Lates calcarifer*) (Srichanun et al., 2014) and Atlantic halibut (Kvåle et al., 2009, 2002). The lower larval performance has been attributed to a saturation of the peptide transport system in the intestinal BBM due to overloading of short peptides and/or to impaired utilization of the fast absorbed FAA and di or tri-peptides and further decreased protein accretion.

Moderate dietary inclusion levels of protein hydrolysates were also shown to induce gut maturation, by increasing the activity of BBM enzymes in relation to cytosolic protein digestion, setting on the adult mode of protein digestion in European seabass (Cahu et al., 2004; Cahu et al., 1999; Kotzamanis et al., 2007; Zambonino Infante et al., 1997), Atlantic cod (Kvåle et al., 2009), Asian sea bass (Srichanun et al., 2014), but not in the pleuronectid Atlantic halibut (Kvåle et al., 2009). This suggests that the modulation of the digestive enzymes as a response to dietary protein complexity is probably species-specific and mostly dependent on the ontogeny of the digestive system and diet formulation. The expression patterns of genes encoding for digestive enzymes has been proposed as a marker for assessing fish larval development and nutritional condition (Lazo et al., 2011). This marker was used for evaluating the effect of including protein hydrolysates in microdiets for larvae on the modulation of the digestive system in European sea bass (Cahu et al., 2004), Asian sea bass (Srichanun et al., 2014) and large yellow croaker (Cai et al., 2015).

PepsinogenA (pga) encodes for pepsinogen which is synthetized and stored by gastric gland oxynticopentic cells (Lazo et al., 2011). Most of the studied fish have several pepsinogen isoforms which are activated into pepsins with distinct protein structures and enzymatic properties (Zhao et al., 2011). When activated, pepsins hydrolyse

ACCEPTED MANUSCRIPT

proteins into polypeptides and some free amino acids, by cleaving peptide bonds involving aromatic amino-acids and acidic amino-acids. Senegalese sole was suggested to have one single pepsin isoform (Sáenz de Rodrigáñez et al., 2005). *Tryp1C* encodes for one anionic trypsinogen isoform highly expressed in both Senegalese sole juveniles intestine and larvae, displaying the highest expression ratios among *ssetryp1* variants and when compared with other variants (*ssetryp2*, *ssetryp3* and *ssetrypY*) during larval development, its expression throughout larval development being fairly constant after 9DAH (Manchado et al., 2008). Trypsinogens are synthesized in the pancreas as a proenzyme that is further activated by enterokinase and converted into its active form in the intestinal lumen. *Ialp* and *ampn* encode for the intestinal BBM enzymes intestinal alkaline phosphatase and aminopeptidase N which are commonly used as indicators of the maturation of the digestive system in marine fish larvae. *Pept1* encodes for a membrane transporter responsible for the selective transport of di and tri-peptides from the intestinal lumen into the enterocytes (Daniel, 2004). The larvae capacity to absorb and retain dietary protein with different complexities (molecular weight, MW) was assessed during metamorphosis by controlled tube-feeding of representative radiolabelled polypeptides combined with the use of metabolic chambers (Rust et al., 1993; Rønnestad et al., 2001; Conceição et al., 2007b; Richard et al., 2015).

Assessing to what extent dietary protein complexity modulates growth, protein utilisation and the expression of protein digestion-related genes in Senegalese sole larvae is paramount to optimize current commercial microdiets, so as to promote growth and a more successful early weaning (Engrola et al., 2013). Even if Senegalese sole larvae qualitative amino-acid (AA) requirements have been well established (Aragão et al., 2004a; Conceição et al., 2007a), information on the larvae capacity to digest proteins with different MWs is still scarce (Engrola et al., 2013; Richard et al., 2015). In the present study, we hypothesize that the manipulation of dietary protein complexity may affect the development of the larvae capacity to digest, absorb and retain protein during metamorphosis, as well as the mRNA expression of *pga, tryp1c, ialp*, *ampn* and *pepT1*, which may have a further impact on somatic growth.

2. Material and Methods

2.1. Husbandry and experimental set-up

ACCEPTED MANUSCRIPT

CCMAR facilities and their staff are certified to house and conduct experiments with live animals ('group-1' license by the 'Direção Geral de Veterinária', Ministry of Agriculture, Rural Development and Fisheries of Portugal). Experiments were performed following the European Directive 2010/63/EU of European Parliament and of the Council of European Union on the protection of animals used for scientific purposes.

Senegalese sole eggs were incubated in an upwelling incubator at 19±0.5ºC and hatching was completed within 24 h. Newly hatched larvae were evenly distributed over 9 white cylindro-conical tanks (100 L) in a semi-closed recirculation system with a density of 60 larvae L^{-1} (6000 larvae/tank). The system was equipped with a mechanical filter, a submerged and a trickling biological filter, a protein skimmer and UV lamps. Larvae were reared in green water conditions until 16DAH, provided by adding frozen *Nannochloropsis* sp. (*Nannochloropsis* 18% FP 472/180908, Acuicultura Y Nutrición de Galicia SL, Spain) to rearing tanks every morning. Abiotic parameters and mortality were daily monitored. Dissolved O_2 in water was maintained at 86.6±7.2% of saturation, temperature at 18.4±0.6ºC and salinity at 37.6±2.3‰. A 10/14 h light/dark photoperiod cycle was adopted and a light intensity of 1000 lux was provided by overhead fluorescent tubes. At 16DAH, during the metamorphosis climax, the larvae were transferred to flat-bottom tanks (30×70×10cm; 21 L), each tank stocking 860 individuals (corresponding to a 4095ind/m^2 density). The system for the benthic rearing was equipped with a mechanical filter, a submerged and a trickling biological filter, a protein skimmer and UV sterilizer. Abiotic parameters were measured and mortality was recorded every morning. Dead larvae were removed and the rearing units were carefully cleaned with minimal disturbance. Dissolved O_2 in water was maintained at 96.6±7.2% of saturation, temperature at 19.6±0.5ºC and salinity at 35.4±0.7 ‰. A 10/14 h light/dark photoperiod cycle was maintained and the light intensity was 400lux at water surface.

The dietary treatments (Intact, PartH and HighH) were randomly assigned to tanks (n = 3 tanks per treatment). From mouth opening (2DAH) until 5DAH larvae were fed rotifers (*Brachionus* sp.) enriched with Easy DHA Selco (INVE, Belgium), at an initial density of 5 rots·mL^{-1} together with the respective inert diet (200-400 μm). *Artemia* AF nauplii (na) (ARTEMIA AF - 480, INVE, Belgium) were introduced at 4DAH and prey density was gradually increased from 4 to 5 na·mL^{-1}, becoming the only prey offered

ACCEPTED MANUSCRIPT

after 5DAH. *Artemia* EG meta-nauplii (M24) (EG SEP-ART Cysts, INVE, Belgium) enriched with Easy DHA Selco were introduced at 12DAH, gradually increasing from 12 to 14 M24·mL^{-1} until 19DAH. Enriched frozen *Artemia* metanauplii were offered to settled larvae (between 16 and 35DAH). Live prey was gradually reduced and substituted by inert diets (SPAROS Lda., Portugal) until complete weaning at 36DAH, according to Engrola et al. (2009a), with the inert diet constituting 50% of the feed supplied (dry matter basis) from 10 to 17DAH, 60% from 17 to 30DAH and more than 80% from 30 to 35DAH. After 36DAH larvae were exclusively fed with the respective inert diet (Intact, PartH and HighH) and considered weaned.

Live prey was delivered 3 times a day (3 h interval) during the pelagic phase and 4 times a day (2.5 h interval) during the benthic phase. First live feed meal was offered 1 h after the lights were on (11.00h) during the pelagic phase and 30 min (09.30h) after during the benthic phase. Inert diet was delivered semi-continuously with automatic feeders (cycles of 2 h of feeding followed by 1 h 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 a large excess of uneaten food (Engrola et al., 2005, 2009a). The amount of inert diet supplied increased from 0.14 mg/larva/day at 2DAH, to 0.25 mg/larvae/day at 16DAH, to 3.89mg/larva/day at 37DAH, and 9.62 mg/larva/day at 60DAH.

2.2. Experimental diets: manufacturing and quality analysis

Three microdiets (Intact, PartH and HighH) were formulated and processed by SPAROS Lda. (Olhão, Portugal) to be isonitrogenous, and isoenergetic but including protein fractions of different complexity, using approximately the same practical ingredients (Table 1). The Intact diet contained a mix of intact plant protein sources – Plant protein Mix 12 (84% Crude Protein, 3.6% Crude Fat, SPAROS, Portugal), targeting a peptide molecular weight > 20 kDa. The PartH diet contained a hydrolysate of the same mixture of plant proteins used in Intact diet - IdG Hydrolysate. This protein hydrolysate was produced at Instituto de la Grasa (CSIC, Sevilla,Spain) according to Villanueva et al. (1999) from pea protein concentrate and wheat gluten, using Alcalase as a food grade proteolytic enzyme. Partially hydrolysed proteins with a high rate of 5 – 20 kDa peptides were targeted with the goal of achieving a compromise between the need to improve plant protein digestibility and to avoid a high leaching rates from formulated diets. The HighH diet contained a high level of a commercial fish protein

ACCEPTED MANUSCRIPT

hydrolysate – CPSP90® (Sopropêche, France) with a predominance of small polypeptides, oligopeptides and di and tri-peptides (<5 kDa). All three diets contained a minimum of 43% marine ingredients, including high levels fish and krill protein hydrolysates, which altogether should make the diets highly attractable and palatable for sole larvae. Moreover, the plant protein Mix used was based on protein concentrates, and thereby the presence of anti-nutritional factors in any of the three diets was highly unlikely.

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

As Senegalese sole post-larvae and juveniles have a peculiar (passive) bottom feeding behaviour, with microdiets remaining 1 min or more in the tank bottom before being eaten (Conceição et al., 2007a; Dinis et al., 2000), feed samples (n = 4 treatment) were submersed in rearing water for 1 min, in order to allow nutrient leaching and to simulate the situation in the rearing tanks. After this period the rearing water was removed and the feed samples were frozen at -80ºC followed by freeze-drying to remove the water. Feed samples were grounded, pooled and analyzed for dry matter (105°C for 24 h), lipid content by petroleum ether extraction using a Soxtherm Multistat/SX PC (Gerhardt, Königswinter, Germany; 150°C), gross energy in an adiabatic bomb calorimeter (Werke C2000; IKA, Staufen, Germany) and crude protein by automatic flash combustion (Leco FP-528, Leco, St. Joseph, USA; N × 6.25). The diets composition after leaching is presented on Table 1.

The amino-acid composition was determined by ultra-high-performance liquid chromatography in a Waters Reversed-Phase Amino Acid Analysis System, using norvaline as an internal standard. In order to do so, samples for total amino-acids and taurine quantification were previously hydrolysed at 6 M HCl at 116ºC, over 22 h. Then all the samples were pre-column derivatized with Waters AccQ Fluor Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) using the AccQ Tag method (Waters, USA). The resultant peaks were analysed with EMPOWER software (Waters,

ACCEPTED MANUSCRIPT

USA). The diet amino acid profiles after rearing-water 1 min immersion are presented on Table 2.

2.3. Sampling and experimental design

2.3.1 Growth and larvae performance

At mouth opening (MO) (2DAH) one pool of 20 individuals was collected from each tank for dry weight (DW) evaluation. Thereafter, individual fish were randomly sampled for dry weight determination at key growth stages: 9DAH (PM: pre-metamorphosis), 16DAH (MC: metamorphosis climax), 28DAH (BSLF: metamorphosis completed) (n=10 per replicate), at 36DAH (weaned post-larvae) (n=20 per replicate) and 60DAH (n=20 per replicate). The larvae and post-larvae were frozen at -80ºC and freeze-dried for dry weight determination to 0.001 mg precision. Growth was expressed as relative growth rate (RGR, % day^{-1}) and was determined during the pelagic phase from mouth opening (2-16DAH), during the benthic phase (16-36DAH and 36-60DAH) and during the whole trial (2-60DAH). RGR was calculated as RGR (% day-1) = $(e^{g}-1)\times100$, where $g = [(\ln_{\text{final weight}} - \ln_{\text{initial weight}})/\text{time}]$ (Ricker, 1958). Survival was evaluated for the benthic phase as the percentage of remaining larvae counted at the end of the trial, relative to the initial larvae number in each flat bottom tank. The results are reported as percentage of survival, relative to the initial larvae number in each tank.

2.3.2 Protein metabolism trials

The effect of the dietary formulations on the digestion, absorption and metabolic utilisation capacities of larvae throughout metamorphosis was assessed through the metabolism of ^{14}C labelled model peptides with different MWs, 1.0 kDa and 7.2 kDa (Richard et al., 2015), at 9DAH (pre-metamorphosis), at 16DAH (metamorphosis climax) and 28DAH (metamorphosis completed).

The determination was performed using the *in vivo* method of controlled tube-feeding described by Rust et al. (1993) and modified by Rønnestad et al. (2001). On the evening prior to protein metabolism trial, Senegalese sole larvae were transferred to the experimental laboratory in order to acclimatise before the experiment was conducted. On the next morning, each treatment larvae were allowed to feed on *Artemia* sp. metanauplii for 30 min. Eight larvae (with guts filled with *Artemia*) from each dietary treatment were anaesthetised with 150-330 µM of MS-222 (depending on larvae age)

ACCEPTED MANUSCRIPT

and tube-fed with two doses of 13.8 ηL of the test ^{14}C-labelled model peptide through a 0.19 mm diameter plastic capillary inserted on a nanoliter injector (World Precision Instruments, USA) firmly attached to a micromanipulator. After capillary withdrawal, each larva was gently rinsed for spillage in two successive wells filled with clean seawater and transferred into incubation chambers filled with 7.5 mL of seawater. Each incubation chamber was air-supplied and connected to a trap chamber containing 5.0 mL of 0.5 M KOH in order to collect $^{14}CO_2$ produced by larvae fed ^{14}C-labelled peptide. At the end of the incubation period (24 h) larvae were removed from the incubation chambers, rinsed with clean water and dissolved in 500 µL of Solvable (PerkinElmer, USA) at 50°C for 12 h for radioactivity counting (disintegrations per minute, dpm). The incubation vials were resealed and 1.0 mL of 1.0 M HCL was gradually injected into the incubation vial, resulting in a progressive decrease of pH to force the $^{14}CO_2$ remaining in the seawater vial to diffuse to the CO_2 trap.

Scintillation cocktail (Ultima Gold XR, Perkin Elmer, USA) was added to all samples and disintegrations per minute (DPM) were counted in a TriCarb 2910TR Low activity liquid scintillation analyser (PerkinElmer, USA). Protein utilization was determined based on the digested/absorbed fraction (A, %), evacuated fraction (E, %), retained fraction (R, %), catabolised fraction (C, %) and total retention (TR, %) calculated as:

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

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

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

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

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

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).

2.3.3 Gene expression

2.3.3.1 RNA extraction and cDNA synthesis

Six pools of 20 whole larvae per dietary treatment (2 pools per tank) were sampled at 16DAH (metamorphosis climax) and at 28DAH (metamorphosis completed), snap-

ACCEPTED MANUSCRIPT

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 2 mL 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 provided in the High Pure RNA Isolation Kit (Roche), by adding 100 µL of 10% DNase I in DNase I Incubation Buffer, mixing and incubating for 15 min at 25ºC. The RNA samples were further purified according to the manufacturer instructions provided with the kit. Purified RNA samples were again quantified using the Nanodrop spectrophotometer. cDNA was synthesized from 1 µg of purified RNA (per pool), using with the M-MLV Reverse Transcriptase Kit (Invitrogen).

2.3.3.2 Quantitave real-time PCR (qPCR)

The relative expression of genes encoding for precursors of the enzymes involved in luminal protein digestion PepsinogenA (*pga)* and Trypsinogen1C (*tryp1c*), for BBM enzymes Intestinal alkaline phosphatase (*iALP*) and Aminopeptidase N (*ampN*) and for the enterocyte membrane peptide transporter 1 (*pept1*) 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).

ACCEPTED MANUSCRIPT

2.4 Data analysis

Statistical analyses followed previously reported methods (Zar, 2010) and IBM SPSS Statistics 22 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 homogeneity of variance (using a Levene's test). All percentage data were arcsin transformed prior to analysis. The overall influence of dietary formulation on survival and growth parameters was tested by one-way ANOVA or a Kruskall-Wallis 1-way ANOVA on ranks, whenever transformed data did not meet normality and homoscedasticity requirements. Pairwise means were compared with Tukey's post-hoc tests or Games-Howell post-hoc tests whenever transformed data did not meet homoscedasticity requirements. The influence of dietary formulation on the larvae capacity to utilize protein was tested by two-way ANOVA, using peptide size and diet as independent factors, followed by Fisher's LSD pairwise comparisons. Whenever there was a significant interaction between the two main effects, this analysis was followed by separate one-way ANOVAs and Tukey's post-hoc (HSD) tests to examine the effect of a diet within a particular age/developmental stage. The differences between groups detected in the relative expression of target genes were tested by a two-way ANOVA using age/developmental stage and diet as independent factors, followed by Fisher's LSD pairwise comparisons. Separate one-way ANOVAS followed by Tukey's post-hoc (HSD) tests to examine the effect of a diet within a particular age/developmental stage were performed in some cases (*tryp1C*, *pepsin* and *pept1*), as there was a significant interaction between the two mains effects. Significance levels were set at p<0.05.

3. Results

3.1 Larval performance

The total substitution of dietary native plant proteins for partially hydrolysed plant protein had a positive effect on larval growth until the metamorphosis climax (1.2 fold higher, when compared to both the Intact and HighH diets) (Fig. 1), with significant differences being detected on larvae dry weight at 16DAH (p=0.002). After the metamorphosis was completed, and the inert diet became the predominant dietary source, the Intact diet fed larvae clearly performed better than those fed the other diets (Fig. 1), with significant differences being detected on dry weight at 36DAH and more

ACCEPTED MANUSCRIPT

remarkably at 60DAH (Fig 1). At end of the experiment, the larvae fed the Intact diet averaged a dry weight 2.17-fold higher than those fed the PartH diet and 2.27-fold higher than those fed the HighH diet. Accordingly, significant differences were detected on relative growth rate (RGR) from 16 to 36DAH (p=0.043), on RGR from 36 to 60DAH (p=0.002) and on overall RGR, from 2 to 60DAH (p=0.000) (Fig. 1). Survival during the benthic phase was significantly affected by diets (p=0.011), reduced in the PartH group (64.7±5.9%), respectively by 15% and 17%, when compared to the Intact (76.2±1.8%) and the HighH (78.0±2.99%) groups.

3.2 Protein metabolism

Senegalese sole larvae presented a higher capacity to absorb 1.0 kDa peptide than 7.2 kDa peptide from 9 to 28DAH (p<0.001) (Fig. 2). The average peptide absorption varied between 60.5 – 80.7% for 1.0 kDa and between 28.0 – 31.6% for 7.2 kDa peptides, with a ratio between 1.0 kDa and 7.2 kDa absorption rates varying between 2.2-fold at 9 and 16DAH and 2.8-fold at 28DAH.

The capacity to absorb either 1.0 kDa or 7.2 kDa peptide was not significantly affected by dietary protein complexity in pre-metamorphic larvae, at 9DAH (p=0.231) (Fig. 2). During the metamorphosis climax, at 16DAH, there was a significant effect of the dietary treatment on the larvae absorption capacity (p=0.001) (Fig. 2). There was also a significant interaction between diet and peptide size as main effects (p=0.000) (Fig.2), with 1.0 kDa peptides being better absorbed by larvae fed the Intact diet than those fed either the PartH or HighH diets (p=0.001), while the 7.2 kDa peptide was equally absorbed among the three groups (p=0.798). After the metamorphosis was completed, at 28DAH, the larvae capacity to absorb either 1.0 kDa or 7.2 kDa peptide was not significantly affected by dietary treatment (p=0.549) (Fig. 2).

The molecular size of the studied peptide fractions did not affect their retention or catabolism throughout the metamorphosis (p>0.05) (Fig. 3). Average retention efficiency calculated as a percentage of the absorbed protein varied between 65.3 – 76.4% for 1.0 kDa and between 65.2 – 74.0% for 7.2 kDa peptides.

Protein retention efficiency and catabolism calculated as a percentage of the absorbed protein were not affected neither by dietary protein complexity (p=0.070) nor the model peptide molecular size (p=0.982) in pre-metamorphic sole (Fig. 3). Similarly no significant effects were found during the metamorphosis climax, at 16DAH, neither

ACCEPTED MANUSCRIPT

from diet ($p=0.346$) nor peptide size ($p=0.496$). After metamorphosis was completed, at 28DAH, although no significant effect was detected neither from diet nor peptide size, there was a significant interaction between these factors as main effects ($p=0.020$) (Fig. 3), with 7.2 kDa peptides being better retained by larvae fed the PartH diet than by those fed either the HighH diet ($p=0.018$), while the 1.0 kDa peptide was equally absorbed amongst the three groups ($p=0.568$).

Total retention calculated as a percentage of the tube-fed protein amount was affected by the model peptide molecular size, with the 1.0 kDa peptide being better retained than the 7.2 kDa model peptide from 9 to 28DAH ($p<0.001$) (Fig. 4). The average peptide total retention varied between 42.2 – 54.1% for 1.0 kDa and between 18.9 – 24.1% for 7.2 kDa peptides, with a ratio between 1.0 kDa and 7.2 kDa total retention rates varying between 2.2-fold at 9 and 16DAH, and 2.8-fold at 28DAH.

The total retention either for 1.0 kDa or 7.2 kDa peptides was not significantly affected by dietary protein complexity in pre-metamorphic larvae, at 9DAH ($p=0.129$). During the metamorphosis climax, at 16DAH, there was an effect of the dietary treatment on the larvae total retention capacity ($p=0.045$). There was also an interaction between diet and peptide size as main effects ($p=0.044$) (Fig. 4), with 1.0 kDa peptides being better retained by larvae fed the Intact diet than those fed either the PartH or HighH diets ($p=0.023$), while the 7.2 kDa peptide was equally retained among the three groups ($p=0.734$). After the metamorphosis was completed (at 28DAH), sole total retention capacity either for 1.0 kDa or 7.2 kDa peptides was not significantly affected by dietary treatment ($p=0.972$) (Fig. 4).

3.3 Expression of protein digestion-related genes

Transcript levels of all the genes encoding for precursors of enzymes involved in luminal protein digestion PepsinogenA (*pga)* and Trypsinogen1C (*tryp1c*), for the BBM enzymes intestinal alkaline phosphatase (*iALP*) and Aminopeptidase N (*ampN*) and for the enterocyte membrane peptide transporter 1 (*pept1*) were higher in post-metamorphic larvae (28DAH) than in metamorphosing larvae (16DAH) (developmental stage effect in two-way ANOVAs, $p<0.001$). During the metamorphosis climax, there was no dietary effect on the relative expression of the studied target genes (Fig. 5; Fig. 6; $p>0.05$). However, at 28DAH, the expression of *tryp1C* ($p=0.012$) and *pept1* ($p=0.046$) was upregulated in the PartH diet fed larvae, with its transcript levels being increased by 2.1-fold (Fig. 5) and 1.4-fold, respectively, when compared to the HighH group (Fig. 6).

ACCEPTED MANUSCRIPT

No differences were found between the Intact and the other groups. At this stage (28DAH), the transcription of *pga* was upregulated (p=0.004) in larvae fed the Intact diet, by 2.0 and 4.1-fold, when compared to PartH and HighH diets, respectively (Fig. 5).

4. Discussion

Given its complex metamorphosis and digestive system ontogeny (Conceição et al., 2007a; Engrola et al., 2009a; Fernández-Díaz et al., 2001; Morais et al., 2004; Ribeiro et al., 1999a, 1999b), it was expected that Senegalese sole larvae capacity to digest and utilize dietary protein would change throughout development. Therefore, the present study aimed to test three microdiets with substantially different peptide MW profiles, including one diet mostly based on native intact protein sources (Intact), one diet mostly based on polypeptides ranging from 5 to 70 kDa (PartH) and one diet mostly based on small polypeptides, oligopeptides and di and tri-peptides (<5 KDa) (HighH). The three microdiets were formulated upon approximately the same practical ingredients (Table 1), with the aim of having the degree of hydrolysis of dietary protein as the main changing factor. The AA profile among the three diets presented only minor changes (see Table 2), and possibly due to the high inclusion level of fish protein hydrolysate in the HighH diet. However, it is believed that this small variation in dietary AA profile did not affect the main results and conclusions of this study, as all three diets seem to exceed in at least 6% (Histidine), and over 18% for most of the indispensable AA requirements (tryptophan was not measured in this study), according to the known requirements for sole juveniles (Silva et al., 2009).

Since protein requirements for sole juveniles are already quite high – 60% for maximum protein accretion (Rema et al., 2008), it is believed that all diets met Sole larvae AA requirements. Furthermore, when analysing the growth results there is no indication that diet PartH would have AA deficiencies (Tables 1 and 2).

This study shows that dietary formulations based on different protein MW profiles differentially affect Senegalese sole somatic growth throughout development. The diet including a protein source partially hydrolysed (target peptide range from 5 to 70 KDa, PartH) promoted growth in metamorphosing larvae in which the digestive system is still very immature and proteolysis relies on pancreatic endoproteases (such as trypsin and chymotrypsin). The diet based on intact plant protein sources (Intact) promoted growth

ACCEPTED MANUSCRIPT

in later stages of development (Fig. 1), when post-larvae already had a fully developed and functional digestive tract, having reached an adult mode of protein digestion (Engrola et al., 2009b; Ribeiro et al., 1999b).

In agreement, previous results suggested that pre-metamorphic halibut larvae had a low capacity to digest and absorb intact protein (Tonheim et al., 2005). According to Tonheim et al. (2005) the absorption efficiency within 20 h past tube-feeding in larvae delivered an intact ^{14}C-labelled salmon serum protein averaged 36% and was increased by 1.75 fold, up to 63%, in larvae tube-fed hydrolysed ^{14}C-labelled salmon serum proteins. Furthermore, the incorporation of a partially hydrolysed ^{14}C-labelled salmon serum protein (15-250 KDa) into body proteins within 10 h past tube-feeding was higher than that of a highly hydrolysed ^{14}C-labelled salmon serum protein (<25 KDa) (Tonheim et al., 2005).

In the present study, while the PartH diet promoted growth during the pelagic phase, those larvae fed the Intact diet were then probably not able to digest the dietary protein efficiently, due to a limited proteolytic capacity to digest complex protein, as previously observed (Engrola et al., 2013; Richard et al., 2015).

Being mostly based on low MW peptides, the HighH diet may have caused an overloading of di- and tri-peptides and subsequently led to a saturation of the peptide transport system in the intestinal BBM, as previous suggested by Kotzamanis et al. (2007), de Vareilles et al. (2012); de Vareilles Sommières (2013), respectively, for European sea bass, white seabream and gilthead seabream early stage larvae. Alternatively, the rapid hydrolysis of small peptides may have produced an excess of amino acids that, being absorbed at different rates (Conceição et al., 2011), with different efficiencies (Conceição et al., 2002; Rønnestad et al., 2001; Saavedra et al., 2008a, 2008b), depending on different transport systems (Rønnestad, Morais, 2008), may have led to transitory AA imbalances and subsequent decreased protein accretion and reduced growth. According to Kotzamanis et al. (2007), 15 days post hatching (dph) European sea bass larvae still relying proteolysis on pancreatic endoproteases and cytosolic peptidases, grew better upon a diet with a 10% inclusion of a commercial fish protein hydrolysate (FPH) than upon a diet with a 19% FPH inclusion; in 22dph larvae, a 10% inclusion of a less extensively hydrolysed FPH (with a major part of 0.5-2.5 kDa peptides) promoted growth better than a 10% inclusion of a more extensively hydrolysed sardine protein (with a major part 0.2-0.5 kDa peptides). According to de

ACCEPTED MANUSCRIPT

Vareilles et al. (2012) white seabream larvae grew better from 2 to 15DAH upon a diet with a 15% inclusion of a FPH mostly based on 0.5-30 kDa peptides than a upon a diet with a similar inclusion of a FPH mostly based on <0.5 kDa peptides; the latter group displaying altered muscle protein turnover pointing to enhanced catabolism, as revealed in proteomic analysis. In gilthead seabream larvae, a dietary 15% inclusion of FPH (with a major part 0.5-30 kDa peptides) promoted growth at 28DAH better than a dietary 30% inclusion of the same FPH (de Vareilles Sommières, 2013). When compared to a microdiet with a 10% inclusion of a commercial FPH mostly based on <5 kDa peptides, a microdiet with a 20% inclusion of the same hydrolysate delivered from first-feeding increased nitrogen excretion in 20DAH gilthead sea bream larvae and reduced the larvae capacity to retain dietary protein at 30DAH (de Vareilles Sommières, 2013). In Atlantic halibut pre-metamorphic larvae, Tonheim et al. (2005) suggest that, when compared to larger peptides, small peptides would be rapidly absorbed, which would lead to impaired retention, thus increasing their use for energy production. When comparing larvae tube-fed a partially hydrolysed ^{14}C-labelled salmon serum protein (15-250 kDa) with larvae tube-fed a highly hydrolysed ^{14}C-labelled salmon serum protein (<25 kDa), the latter displayed not only a higher absorption rate (1.36-fold increased) but also higher levels of retained FAA measured in the first 2h past tube- feeding and increased catabolism after 10h past tube-feeding (Tonheim et al., 2005). This possible explanation for reduced growth at 16DAH in the HighH group is supported by the decreasing tendency in the retention of both model-peptides in pre-metamorphic larvae fed the HighH diet, either calculated as a percentage of absorbed protein (Fig. 3) or a percentage of tube-fed protein (Fig. 4).

Furthermore, there was no major effects of the diet on pre-metamorphic (9 DAH) larvae capacity to absorb or retain different sized model peptides (Fig. 2 and 3), except for a tendency for increased absorption of 7.2 kDa peptides in the PartH fed larvae (Fig. 2). The larvae capacity to absorb the different sized ^{14}C -labelled model peptides (1.0 kDa and 7.2 kDa) was used as an indirect way to access the larvae capacity to digest the experimental diets, as these diets are based on different protein sources with different MW profiles, the PartH including IdGH which is mostly based on polypeptides with MW>5.0 kDa (77%) and the HighH including FPH which is mostly based on small peptides with MW <5 kDa (87%) (Table 1). The tendency for increased absorption of 7.2 kDa peptides in those larvae fed the PartH suggests a better digestibility of this diet

ACCEPTED MANUSCRIPT

at this pre-metamorphic stage, which may have contributed for increased growth towards the metamorphosis climax (16DAH) (Fig. 1).

Since sole metamorphosing larvae digestive system is still far from being fully developed, (Ribeiro et al., 1999b), the larvae fed the Intact diet were probably not able to utilize intact dietary protein efficiently. Thus it is possible that these larvae make a better use of the small peptides available in the Intact diet supplied by the 13% inclusion of FPH which is mostly composed (87%) of peptides with a MW<5.0 kDa, so as to compensate for their lower capacity to digest complex protein, as it is suggested by a higher absorption of the 1.0 kDa peptides in the Intact group (Fig.2) Another possible explanation for a higher absorption of low MW peptides in this group would be an effect of the Intact diet formulation on gut transit time. In mammals, hydrolysed protein formulas have accelerated gastrointestinal transit of milk, when compared to intact protein formulas (Mihatsch et al., 2001; Staelens et al., 2008). Fast gut evacuation was long shown to have a negative effect on nutrient assimilation in fish larvae (Govoni et al., 1986). If in the present study, the diets including hydrolysates (PartH and HighH) have accelerated gut transit; a slower gastrointestinal transit of the Intact diet would have led to a more efficient proteolysis in the Intact diet and a more effective absorption of dietary small peptides. No differences were detected on the retention of different sized model peptides, calculated as a percentage of the absorbed protein (Fig. 3). However, there was a transitory positive effect of the Intact diet formulation on the absorption and total retention of small-sized peptides during the metamorphosis climax, which may have contributed for enhanced growth at later stages (from 36DAH onwards) (Fig. 1, 2 and 4). The metamorphosis period seems to be a time window particularly susceptible to nutritional conditions in Senegalese sole (Engrola, et al., 2009b; Parra, Yúfera, 2001; Pinto et al. 2010, Villalta et al. 2008, Yúfera et al. 2005). Different feeding regimes induced changes in the capacity of metamorphosing larvae to digest and utilize protein, which reflected on further somatic growth (Engrola et al. 2010, 2009b).

The expression patterns of genes encoding for proteolytic enzymes and the enterocyte membrane peptide transporter 1 (*pept1*) were used as markers for assessing the effect of including protein hydrolysates in microdiets for larvae on the modulation of the digestive system of Senegalese sole. Since the ontogeny of the digestive system is genetically programmed, the expression of digestive enzymes following a spatio-

ACCEPTED MANUSCRIPT

temporal pattern of gene expression, the expression of genes encoding for digestive enzymes and enzymes precursors has been proposed as a marker for fish larvae development (Lazo et al., 2011). However, since gene expression does not always translate into protein synthesis and actual enzyme activity, it can be discussed whether *per se* it should be used as a marker of the digestive system functionality. The nutritional condition of fish larvae could be reflected in the gene expression patterns of some digestive enzymes during ontogenesis, which may underlie the ontogeny of enzymatic activity and digestive capacity. In fact, dietary protein amount and nature have significantly affected both the transcription and activity of proteolytic enzymes, such as trypsin (Cahu et al., 2004; Cai et al., 2015; Wang et al., 2006), pepsin (Wang et al., 2006) and aminopeptidase N (Cai et al., 2015; Srichanun et al., 2014) in several species. Furthermore, dietary induced changes in enzymes precursors mRNA transcription give insight into the potential for nutritional programming at a certain time window of larval development (Rocha et al., 2016; Vagner et al., 2009).

In the present study, no differences were detected in the mRNA levels of the studied genes encoding for the enzymes precursors (pepsinogen (*pga)*, trypsinogen (*tryp1c*), alkaline phosphatase (*ialp*) and aminopeptidase N (*ampn*)) nor for the enterocyte membrane peptide transporter 1 (*pept1*) during the metamorphosis climax (16DAH) (Fig.5 and 6). In European seabass, a 19% inclusion of FPH promoted gut maturation in 20 dph larvae, by increasing aminopeptidase N activity and alkaline phosphatase/ Leu-Ala peptidase and aminopeptidase N/Leu-Ala peptidase activity ratios (Cahu et al., 1999). However, regarding the present results on the larvae protein metabolic capacities, no such effect was observed when measured as the expression of genes encoding for BBM or cytosolic enzymes. Regarding the dietary effect found on the capacity to absorb 1.0 kDa (Fig. 2), higher transcript levels of *pept1* in the Intact group would be expected. PepT1 is a low-affinity/high capacity H^+ dependent co-transporter (Verri et al., 2003) responsible for the selective transport of di and tri-peptides from the intestinal lumen into the enterocytes (Daniel, 2004). In fish juveniles, *pept1* expression responds to drastic changes in feed availability (Hakim et al., 2009; Terova et al., 2009) and to dietary protein sources, including di and tri-peptides peptides and certain FAA (Bakke et al., 2010; Frøystad-Saugen et al., 2009; Kwasek et al., 2012; Ostaszewska et al., 2010; 2010b; Terova et al., 2013), having been suggested as a useful marker of protein quality and absorption efficiency (Terova et al., 2013). In late stage large yellow croaker

ACCEPTED MANUSCRIPT

larvae, *pept1* transcript levels were affected by the dietary moderate inclusion of different size-fractioned fish hydrolysates (Cai et al., 2015). However, in early stage Atlantic cod larvae *pept1* mRNA expression did not change in response to type of live feed (Amberg et al., 2008).

After metamorphosis was completed, at 28DAH, the transcript levels of the genes encoding for digestive enzymes precursors (*pga, tryp1c*, *ialp* and *ampn*) and the peptide transporter 1 (*pept1*) were generally increased in comparison to those measured during the metamorphosis climax (16DAH). (Figs. 5 and 6). That is in line with the known development of Senegalese sole digestive system (Conceição et al., 2007a). After the metamorphosis is completed, there is an increase on the pancreatic enzymes activity (including trypsin) and the onset of the intestine enzymatic maturation process, while the elongated and folded intestine provides increased absorption area. The onset of intestinal maturation in fish is characterized by the decrease in cytosolic activity and a concomitant increase in the activity of BBM enzymes (aminopeptidase N and alkaline phosphatase) (Zambonino Infante, Cahu, 2001).

While in the present study the *pga* expression was practically undetectable during the metamorphosis climax, there was an already noticeable expression at 28DAH (Fig. 4). Ribeiro et al. (1999a) reported Senegalese sole gastric glands formation and fully development at 27DAH, and Fehri-Bedoui et al. (2000) observed gastric glands at 18DAH. It is not surprising that the expression of the gene encoding for the enzymes precursor *pga* starts earlier. Gastric gland formation precedes the pepsinogen expression in some fish (Darias et al., 2007; Huang et al., 1998; Miwa et al., 1992; Murray et al., 2006), but in winter flounder (*Pleuronectes americanus*), Douglas et al. (1999) detected *pepsinogenIIA* expression as early as 13dph, before the gastric glands were formed.

At 28DAH, dietary formulations induced different patterns of expression in the genes encoding for digestive enzymes precursors and the enterocyte membrane peptide transporter 1 (*pept1*). While the transcription of *pga* was promoted in the Intact diet fed post-larvae, the transcription of the studied trypsinogen isoform (*tryp1c*) and *pept1* (Fig. 5 and 6) was promoted in post-larvae fed the PartH diet. The transcript levels of those genes encoding for intestinal alkaline phosphatase (*ialp*) and aminopeptidase N (*ampn*) also tended to be higher in PartH fed post-larvae, although not significantly (Fig. 6).

The observed increase in the transcript levels of *pga* in the Intact fed post-larvae was possibly due to the effect of Intact formulation on gut transit time. When compared to

ACCEPTED MANUSCRIPT

hydrolysed protein formulas, intact protein formulas slow down the gastric emptying in human pre-term infants (Mihatsch et al., 2001; Staelens et al., 2008). A slower transit time in the Intact fed post-larvae may have promoted the up-regulation of *pga*, due to a possibly prolonged presence of protein and peptides in the stomach and stomach distension which are known to stimulate myenteric reflexes to release acetylcholine which stimulates the gastric gland oxynticopentic cells to synthetize and store pepsinogen (Webb, Rønnestad, 2011). Senegalese sole was suggested to have one single pepsin isoform, with a pH functional optimum between 2 and 2.5, its activity becoming residual at pH over 4.0 (Sáenz de Rodrigáñez et al., 2005). Yúfera and Darías (2007) showed that Senegalese sole gastric pH remains above 6.0 in adult fish and above 7.0 in larvae, regardless the gut content. Thus it is generally accepted that this species would not have a very efficient gastric protein digestion, due to the lack of a true acidic environment in its stomach. Therefore, it is unlikely that the upregulation of *pga* transcription would translate into increased pepsin activity and enhanced proteolysis in the stomach of Senegalese sole post-larvae fed the Intact diet.

Nevertheless, a higher *pga* expression may suggest a more developed digestive system, since the onset of a functional stomach with functional gastric glands marks the passage from a larval to an adult mode of protein digestion in most gastric altricial fish species (Lazo et al., 2011). The present results warrant further research on a possible dietary effect on gut transit time. It has been suggested that the proton pump H^+/K^+-ATPase expression would change in response to dietary formulation in rainbow trout (*Oncorhynchus mykiss*) (Sugiura et al., 2006). Thus it would also be interesting to further investigate for a possible effect on gastric pH and pepsin activity at stomach actual pH values, even though previous results point for a non-effective enzymatic proteolysis in Senegalese sole stomach (Yúfera, Darías, 2007).

The increased expression of *tryp1c* and *pept1* and the observed tendency for increased transcript levels of *ialp* and *ampn* suggest that the digestion and utilization of dietary protein with a lower MW (5 to 70 kDa) were optimized in the PartH fed post-larvae, through improved pancreatic and intestinal protein digestion.

The significantly lower transcript levels of *trypC1* in those post-larvae fed the HighH diet suggest that the dietary inclusion of a more hydrolysed protein (FPH) seems to inhibit pancreatic proteolytic capacity, similarly to what has been reported for sea bass

ACCEPTED MANUSCRIPT

larvae fed high inclusion levels of highly hydrolysed fish protein (Cahu et al., 2004; 1999).

Terova et al. (2013) suggested PepT1 as a useful marker of protein quality and absorption efficiency, thus an up-regulation of *pepT1* (Fig. 6) in the PartH group would indicate that this diet was suitable for sole post-larvae, compared to the other two diets, but the ParthH diet did not promote absorption capacity or growth at this stage or later on (Fig.1 and 2). However, the *tryp1C* and *pept1* expression patterns seemed to reflect on post-larvae capacity to utilize dietary low MW polypeptides, since the PartH diet has also promoted the 7.2 kDa model-peptides retention calculated as a percentage of the absorbed protein at 28DAH (Fig. 3). It is thus probable that the increased expression of *tryp1C* and *pept1* underlie a better utilization of low MW polypeptides, mostly present in the PartH diet. However, the increased retention of 7.2 kDa peptides in PartH fed post-larvae was not reflected on somatic growth (Fig. 1 and 3).

On the contrary, the Intact diet clearly promoted growth during the benthic phase, particularly towards the end of the trial (Fig. 1). It is indeed very likely that Senegalese sole becomes able to digest and utilize complex protein from a certain ontogeny stage. In Atlantic halibut, Tonheim et al. (2005) showed that the larvae capacity to absorb intact protein significantly increased from 25 to 31 dpff and while 25 dpff larvae displayed a limited absorption capacity for increasing tube-fed protein amount, the absorption efficiency for increasing protein amount was significantly raised at 31dpff. In Senegalese sole, the larvae capacity to retain 6.8 kDa polypeptides increased throughout development (Canada et al., 2016; Engrola et al., 2013; Richard et al., 2015), which suggests that young sole juveniles AA anabolic and physiological needs may be better adapted to larger peptides rather than younger larval stages. The present results suggest that from a certain point of Senegalese sole development, the pre-hydrolysis of dietary protein is no longer beneficial and microdiets should include mostly intact protein.

5. Conclusion

The present results suggest that the proteolytic capacity is, in fact, a limiting factor for dietary protein digestion in early larval stages (metamorphosing larvae) and the inclusion of partially hydrolysed diets may promote Senegalese sole early larval growth (up to 1.2 fold higher). However, there is a shift on the effect of dietary protein

ACCEPTED MANUSCRIPT

complexity throughout development, as the intact protein based microdiet ended up promoting growth later at the juveniles stage (Fig .7). Taking in account the present findings, we suggest that the dietary protein fraction formulation of microdiets for Senegalese sole, and possibly other species, should be adapted to each developmental stage (e.g pelagic vs. benthic).

Acknowledgements

This work was funded by Project EPISOLE (FCT) [PTDC/MAR/110547/2009], through project CCMAR/Multi/04326/2013 (Portugal) from FCT (Portugal), and by project MICALA — I&DT Co-Promoção No. 13380 (Portugal, supported by POAlgarve 21, QREN and European Union). 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, (CCMAR) and Hernâni Gerós for revising the manuscript.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

References

Amberg, J.J., Myr, C., Kamisaka, Y., Jordal, A.E., Rust, M.B., Hardy, R.W., Koedijk, R., Rønnestad, I., 2008. Expression of the oligopeptide transporter, PepT1, in larval Atlantic cod (*Gadus morhua*). Comparative biochemistry and physiology. Part B, Biochemistry & molecular biology. 150, 177-182.

Aragão, C., Conceição, L.E.C., Fyhn, H.-J., Teresa Dinis, M., 2004a. Estimated amino acid requirements during early ontogeny in fish with different life styles: gilthead seabream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*). Aquaculture. 242, 589-605.

Bakke, S., Jordal, A.E., Gomez-Requeni, P., Verri, T., Kousoulaki, K., Aksnes, A., Rønnestad, I., 2010. Dietary protein hydrolysates and free amino acids affect the spatial expression of peptide transporter PepT1 in the digestive tract of Atlantic cod (*Gadus morhua*). Comparative biochemistry and physiology. Part B, Biochemistry & molecular biology. 156, 48-55.

Cahu, C., Rønnestad, I., Grangier, V., Zambonino Infante, J.L., 2004. Expression and activities of pancreatic enzymes in developing sea bass larvae (*Dicentrarchus labrax*) in relation to intact and hydrolyzed dietary protein; involvement of cholecystokinin. Aquaculture. 238, 295-308.

Cahu, C.L., Zambonino Infante, J. L. , Quazuguel, P., Le Gall, M.M., 1999. Protein hydrolysate vs. fish meal in compound diets for 10-day old sea bass *Dicentrarchus labrax* larvae. Aquaculture. 171, 109-119.

Cai, Z., Li, W., Mai, K., Xu, W., Zhang, Y., Ai, Q., 2015. Effects of dietary size-fractionated fish hydrolysates on growth, activities of digestive enzymes and aminotransferases and expression of some protein metabolism related genes in large yellow croaker (*Larimichthys crocea*) larvae. Aquaculture. 440, 40-47.

Canada, P., Engrola, S., Richard, N., Lopes, A.F., Pinto, W., Valente, L.M., Conceicao, L.E., 2016. Dietary indispensable amino acids profile affects protein utilization and growth of Senegalese sole larvae. Fish physiology and biochemistry. 1-16

Conceição, L.E.C., Rønnestad, I., Tonheim, S.K., 2002. Metabolic budgets for lysine and glutamate in unfed herring (*Gluea harengus*) larvae. Aquaculture. 206, 305-312.

Conceição, L.E.C., Ribeiro, L., Engrola, S., Aragão, C., Morais, S., Lacuisse, M., Soares, F., Dinis, M.T., 2007a. Nutritional physiology during development of Senegalese sole (*Solea senegalensis*). Aquaculture. 268, 64-81.

Conceição, L.E.C., Morais, S., Rønnestad, I., 2007b. Tracers in fish larvae nutrition: A review of methods and applications. Aquaculture. 267, 62-75.

Conceição, L.E.C., Aragão, C., Rønnestad, I., 2011. Proteins. in: (ed.)., J.H. (Ed.), Larval Fish Nutrition. John Wiley & Sons, Inc. UK, West Sussex, UK, pp. 83-116.

Daniel, H., 2004. Molecular and integrative physiology of intestinal peptide transport. Annu Rev Physiol. 66, 361-384.

Darias, M.J., Murray, H.M., Gallant, J.W., Douglas, S.E., Yúfera, M., Martínez-Rodríguez, G., 2007. Ontogeny of pepsinogen and gastric proton pump expression in red porgy (*Pagrus pagrus*): Determination of stomach functionality. Aquaculture. 270, 369-378.

de Vareilles, M., Richard, N., Gavaia, P.J., Silva, T.S., Cordeiro, O., Guerreiro, I., Yúfera, M., Batista, I., Pires, C., Pousão-Ferreira, P., Rodrigues, P.M., Rønnestad, I., Fladmark, K.E., Conceição, L.E.C., 2012. Impact of dietary protein hydrolysates on skeleton quality and proteome in *Diplodus sargus* larvae. Journal of Applied Ichthyology. 28, 477-487.

de Vareilles Sommières, M.D.M.S.d.L., 2013. Dietary Nitrogen Utilisation and Protein Expression in Fish Faculdade de Ciências e Tecnologias. Universidade do Algarve, Faro, Portugal, pp. 186.

Dinis, M.T., Ribeiro, L., Conceição, L.E.C., Aragão, C., 2000. Larvae digestion and new weaning experiments in *Solea senegalensis*. . in: Méditerranéennes, C.O. (Ed.), Recent advances in Mediterranean aquaculture finfish species diversification. CIHEAM, Zaragoza, pp. 193-204

ACCEPTED MANUSCRIPT

Douglas, S.E., Gawlicka, A., Mandla, S., Gallant, J.W., 1999. Ontogeny of the stomach in winter flounder: characterization and expression of the pepsinogen and proton pump genes and determination of pepsin activity. Journal of fish biology. 55, 897-915.

Engrola, S., Conceicao, L.E., Gavaia, P., Cancela, M.L., Dinis, M.T., 2005. Effects of pre-weaning feeding frequency on growth, survival and deformation of senegalese sole, *Solea senegalensis* (Kaup, 1858). The Israeli Journal of Aquaculture - Bamidgeh. 57, 10-18.

Engrola, S., Mai, M., Dinis, M.T., Conceição, L.E.C., 2009a. Co-feeding of inert diet from mouth-opening does not impair protein utilization by Senegalese sole (*Solea senegalensis*) larvae. Aquaculture. 287, 185-190.

Engrola, S., Figueira, L., Conceição, L.E.C., Gavaia, P., Ribeiro, L., Dinis, M.T., 2009b. Co-feeding in Senegalese sole larvae with inert diet from mouth opening promotes growth at weaning. Aquaculture. 288, 264-272.

Engrola, S., Dinis, M.T., Conceição, L.E.C., 2010. Senegalese sole larvae growth and protein utilization is depressed when co-fed high levels of inert diet and Artemia since first feeding. Aquaculture Nutrition. 16, 457-465.

Engrola, S., Richard, N., Lopes, A.F., Pinto, W., Conceição, L.E.C., 2013. Linking weaning success to larval digestive capacity using radiolabelled peptide fractions. Commun Agric Appl Biol Sci. . 78, 115-118.

Fehri-Bedoui, R., Dinis, M.T., Maamouri, F., 2000. Étude histologique du développement de l'appareil digestif et de la vessie gazeuse de la sole, *Solea senegalensis* (Soleidae). Cybium. 24, 343-358.

Fernandes, J.M., Mommens, M., Hagen, O., Babiak, I., Solberg, C., 2008. Selection of suitable reference genes for real-time PCR studies of Atlantic halibut development. Comparative biochemistry and physiology. Part B, Biochemistry & molecular biology. 150, 23-32.

Fernández-Díaz, C., Yúfera, M., Cañavate, J.P., Moyano, F.J., Alarcón, F.J., Díaz, M., 2001. Growth and physiological changes during metamorphosis of Senegal sole reared in the laboratory. Journal of fish biology. 58, 1086-1097.

Frøystad-Saugen, M.K., Lilleeng, E., Bakke-McKellep, A.M., Vekterud, K., Valen, E.C., Hemre, G.I., Krogdahl, Å., 2009. Distal intestinal gene expression in Atlantic salmon (*Salmo salar* L.) fed genetically modified maize. Aquaculture Nutrition. 15, 104-115.

Gamboa-Delgado, J., Cañavate, J.P., Zerolo, R., Le Vay, L., 2008. Natural carbon stable isotope ratios as indicators of the relative contribution of live and inert diets to growth in larval Senegalese sole (*Solea senegalensis*). Aquaculture. 280, 190-197.

Gisbert, E., Skalli, A., Fernández, I., Kotzamanis, Y., Zambonino-Infante, J.L., Fabregat, R., 2012. Protein hydrolysates from yeast and pig blood as alternative raw materials in microdiets for gilthead sea bream (*Sparus aurata*) larvae. Aquaculture. 338-341, 96-104.

Govoni, J.J., Boehlert, G.W., Watanabe, Y., 1986. The physiology of digestion in fish larvae. Environmental Biology of Fishes. 16, 59-77.

Hakim, Y., Harpaz, S., Uni, Z., 2009. Expression of brush border enzymes and transporters in the intestine of European sea bass (*Dicentrarchus labrax*) following food deprivation. Aquaculture. 290, 110-115.

Huang, L., Schreiber, A.M., Soffientino, B., Bengston, D.A., Specker, J., L., 1998. Metamorphosis of summer flounder (*Paralichthys dentatus*): Thyroid status and the timing of gastric gland formation. Journal of Experimental Zoology. 280, 413-420.

Kolkovksi, S., Tandler, A., 2000. The use of squid protein hydrolysate as a protein source in microdiets for gilthead seabream *Sparus aurata* larvae Aquaculture Nutrition. 6, 11-15.

Kotzamanis, Y.P., Gisbert, E., Gatesoupe, F.J., Zambonino Infante, J., Cahu, C., 2007. Effects of different dietary levels of fish protein hydrolysates on growth, digestive enzymes, gut microbiota, and resistance to *Vibrio anguillarum* in European sea bass (*Dicentrarchus labrax*) larvae. Comparative biochemistry and physiology. Part A, Molecular & integrative physiology. 147, 205-214.

ACCEPTED MANUSCRIPT

Kvåle, A., Harboe, T., Mangor-Jensen, A., Hamre, K., 2009. Effects of protein hydrolysate in weaning diets for Atlantic cod (*Gadus morhua* L.) and Atlantic halibut (*Hippoglossus hippoglossus* L.). Aquaculture Nutrition. 15, 218-227.

Kvåle, A., Harboe, T., Espe, M., Naess, T., Hamre, K., 2002. Effect of predigested protein on growth and survival of Atlantic halibut larvae (*Hippoglossus hippoglossus* L.). Aquaculture Research. 33, 311-321.

Kwasek, K., Terova, G., Wojno, M., Dabrowski, K., Wick, M., 2012. The effect of dietary dipeptide lysine–glycine on growth, muscle proteins, and intestine PepT1 gene expression in juvenile yellow perch. Reviews in Fish Biology and Fisheries. 22, 797-812.

Lazo, J.P., Darias, M.J., Gisbert, E., 2011. Ontogeny of the digestive tract. in: Holt, G.J. (Ed.), Larval Fish Nutrition. John Wiley & Sons, Inc UK, West Sussex, UK, pp. 5-46.

Liu, F., Mai, K.-s., Ai, Q.-h., Duan, Q.-y., Xu, W., Tan, B.-p., Zhang, W.-b., Ma, H.-m., Liufu, Z.-g., 2006. Effects of dietary fish protein hydrolysate levels on growth, survival and body composition of larvae in *Pseudosciaena crocea*. Journal of Fisheries of China. 30, 502-508.

Manchado, M., Infante, C., Asensio, E., Crespo, A., Zuasti, E., Canavate, J.P., 2008. Molecular characterization and gene expression of six trypsinogens in the flatfish Senegalese sole (*Solea senegalensis* Kaup) during larval development and in tissues. Comparative biochemistry and physiology. Part B, Biochemistry & molecular biology. 149, 334-344.

Mihatsch, W.A., Högel, J., Pohlandt, F., 2001. Hydrolysed protein accelerates the gastrointestinal transport of formula in preterm infants. Acta Paediatrica. 90, 196-198.

Miwa, S., Yamano, K., Inui, Y., 1992. Thyroid hormone stimulates gastric development in flounder larvae during metamorphosis. Journal of Experimental Zoology. 261, 424-430.

Morais, S., Lacuisse, M., Conceição, L.E.C., Dinis, M.T., Rønnestad, I., 2004. Ontogeny of the digestive capacity of Senegalese sole (*Solea senegalensis*), with respect to digestion, absorption and metabolism of amino acids from Artemia. Marine Biology. 145.

Murray, H.M., Gallant, J.W., Johnson, S.C., Douglas, S.E., 2006. Cloning and expression analysis of three digestive enzymes from Atlantic halibut (*Hippoglossus hippoglossus*) during early development: Predicting gastrointestinal functionality. Aquaculture. 252, 394-408.

Ostaszewska, T., Dabrowski, K., Kamaszewski, M., Grochowski, P., Verri, T., Rzepkowska, M., Wolnicki, J., 2010. The effect of plant protein-based diet supplemented with dipeptide or free amino acids on digestive tract morphology and PepT1 and PepT2 expressions in common carp (*Cyprinus carpio* L.). Comparative biochemistry and physiology. Part A, Molecular & integrative physiology. 157, 158-169.

Parra, G., Yúfera, M., 2001. Comparative energetics during early development of two marine fish species, *Solea senegalensis* (Kaup) and *Sparus aurata* (L.). Journal of Experimental Biology. 204, 2175-2183.

Rema, P., Conceição, L.E.C., Evers, F., Castro-Cunha, M., Dinis, M.T., Dias, J., 2008. Optimal dietary protein levels in juvenile Senegalese sole (*Solea senegalensis*). Aquaculture Nutrition. 14, 263-269.

Ribeiro, L., Zambonino-Infante, J.L., Cahu, C., Dinis, M.T., 1999a. Development of digestive enzymes in larvae of *Solea senegalensis*, Kaup 1858. Aquaculture. 179, 465-473.

Ribeiro, L., Sarasquete, C., Dinis, M.T., 1999b. Histological and histochemical development of the digestive system of *Solea senegalensis* (Kaup, 1858) larvae. Aquaculture. 171, 293-308.

Richard, N., Engrola, S., Palma, P., Simes, D.C., Conceição, L.E.C., 2015. Assessment of protein digestive capacity and metabolic utilisation during ontogeny of Senegalese sole larvae: A tracer study using in vivo produced radiolabelled polypeptide fractions. Aquaculture. 441, 35-44.

Ricker, W.E., 1958. Handbook of computations for biological statistics of fish populations. Bull. Fish. Res. Board Can. 119, 1-300.

ACCEPTED MANUSCRIPT

Rocha, F., Dias, J., Geurden, I., Dinis, M.T., Panserat, S., Engrola, S., 2016. High-glucose feeding of gilthead seabream (Sparus aurata) larvae: Effects on molecular and metabolic pathways. Aquaculture. 451, 241-253.

Rønnestad, I., Morais, S., 2008. Digestion. in: Finn, R.N., Kapoor, B.G. (Eds.), Fish Larval Physiology. Science Publishers, Enfield, NH, pp. 201-262.

Rønnestad, I., Rojas-Garcıa, C.R., Tonheim, S.K., Conceição, L.E.C., 2001. In vivo studies of digestion and nutrient assimilation in marine fish larvae. Aquaculture. 201.

Rust, M., Hardy, R.W., Stickney, R.R., 1993. A new method for force-feeding larval fish. Aquaculture. 116, 341-352.

Saavedra, M., Conceicao, L.E., Pousao-Ferreira, P., Dinis, M.T., 2008a. Metabolism of tryptophan, methionine and arginine in *Diplodus sargus* larvae fed rotifers: effect of amino acid supplementation. Amino acids. 35, 59-64.

Saavedra, M., Conceição, L.E.C., Helland, S., Pousão-Ferreira, P., Dinis, M.T., 2008b. Effect of lysine and tyrosine supplementation in the amino acid metabolism of *Diplodus sargus* larvae fed rotifers. Aquaculture. 284, 180-184.

Sáenz de Rodrigáñez, M., Alarcón, F.J., Martínez, M.I., Ruiz, F., Díaz, M., Moyano, F.J., 2005. Caracterización de las proteasas digestivas del lenguado senegalés *Solea senegalensis* Kaup, 1858. Bol. Inst. Esp. Oceanogr. 21 95-104.

Silva, J.M.G., Espe, M., Conceição, L.E.C., Dias, J., Valente, L.M.P., 2009. Senegalese sole juveniles (*Solea senegalensis* Kaup, 1858) grow equally well on diets devoid of fish meal provided the dietary amino acids are balanced. Aquaculture. 296, 309-317.

Srichanun, M., Tantikitti, C., Kortner, T.M., Krogdahl, Å., Chotikachinda, R., 2014. Effects of different protein hydrolysate products and levels on growth, survival rate and digestive capacity in Asian seabass (*Lates calcarifer* Bloch) larvae. Aquaculture. 428-429, 195-202.

Staelens, S., Van den Driessche, M., Barclay, D., Carrie-Faessler, A.L., Haschke, F., Verbeke, K., Vandebroek, H., Allegaert, K., Van Overmeire, B., Van Damme, M., Veereman-Wauters, G., 2008. Gastric emptying in healthy newborns fed an intact protein formula, a partially and an extensively hydrolysed formula. Clin Nutr. 27, 264-268.

Sugiura, S.H., Roy, P.K., Ferraris, R.P., 2006. Dietary acidification enhances phosphorus digestibility but decreases H+/K+-ATPase expression in rainbow trout. The Journal of experimental biology. 209, 3719-3728.

Terova, G., Corà, S., Verri, T., Rimoldi, S., Bernardini, G., Saroglia, M., 2009. Impact of feed availability on PepT1 mRNA expression levels in sea bass (*Dicentrarchus labrax*). Aquaculture. 294, 288-299.

Terova, G., Robaina, L., Izquierdo, M., Cattaneo, A., Molinari, S., Bernardini, G., Saroglia, M., 2013. PepT1 mRNA expression levels in sea bream (*Sparus aurata)* fed different plant protein sources. Springerplus. 2, 17.

Tonheim, S.K., Espe, M., Hamre, K., Rønnestad, I., 2005. Pre-hydrolysis improves utilisation of dietary protein in the larval teleost Atlantic halibut (*Hippoglossus hippoglossus* L.). Journal of Experimental Marine Biology and Ecology. 321, 19-34.

Vagner, M., Robin, J.H., Zambonino-Infante, J.L., Tocher, D.R., Person-Le Ruyet, J., 2009. Ontogenic effects of early feeding of sea bass (Dicentrarchus labrax) larvae with a range of dietary n-3 highly unsaturated fatty acid levels on the functioning of polyunsaturated fatty acid desaturation pathways. The British journal of nutrition. 101, 1452-1462.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of mutiple internal control genes. Genome Biology. 3, 0034.0031-0034.0011.

Verri, T., Kottra, G., Romano, A., Tiso, N., Peric, M., Maffia, M., Boll, M., Argenton, F., Daniel, H., Storelli, C., 2003. Molecular and functional characterisation of the zebrafish (*Danio rerio*) PEPT1-type peptide transporter1. FEBS Letters. 549, 115-122.

Villanueva, A., Vioque, J., Sánchez-Vioque, R., Clemente, A., Pedroche, J., Bautista, J., Millán, F., 1999. Peptide characteristics of sunflower protein hydrolysates. Journal of the American Oil Chemists' Society. 76, 1455-1460.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Wang, C., Xie, S., Zhu, X., Lei, W., Yang, Y., Liu, J., 2006. Effects of age and dietary protein level on digestive enzyme activity and gene expression of Pelteobagrus fulvidraco larvae. Aquaculture. 254, 554-562.

Webb, K., Rønnestad, I., 2011. Regulation of digestive processes in: Holt, G.J. (Ed.), Larval Fish Nutrition. John Wiley & Sons, Inc, West Sussex, UK, pp. 265-282.

Yúfera, M., Darías, M.J., 2007. Changes in the gastrointestinal pH from larvae to adult in Senegal sole (*Solea senegalensis*). Aquaculture. 267, 94-99.

Yúfera, M., Fernández-Díaz, C., Pascual, E., 2005. Food microparticles for larval fish prepared by internal gelation. Aquaculture. 248, 253-262.

Zambonino Infante, J.L., Cahu, C.L., Peres, A., 1997. Partial Substitution of Di- and Tripeptides for Native Proteins in Sea Bass Diet Improves *Dicentrarchus labrax* Larval Development. Journal of Nutrition, 308-614.

Zambonino Infante, J.L., Cahu, C.L., 2001. Ontogeny of the gastrointestinal tract of marine fish larvae. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology. 130, 477-487.

Zar, J.H., 2010. Biostatistical analysis. Pearson Prentice Hall.

Zhao, L., M Budge, S., E Ghaly, A., S Brooks, M., 2011. Extraction, Purification and Characterization of Fish Pepsin: A Critical Review. Journal of Food Processing & Technology. 2, 126

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Figure captions:

Fig. 1 A. Sole dry weight (DW/mg); values are means ± s.d. (*n*=30 at 9, 16 and 28 d after hatching (DAH); *n*=60 at 36 and 60 DAH). **B.** Relative growth rate (%/day, RGR) during the pelagic phase (2-16 DAH), the benthic phase (16-36 DAH and 36-60 DAH) and the whole trial (2-60 DAH); values are means ± s.d. (*n*=3 replicates/treatment). Different superscript letters at each developmental age or time period indicate significant differences ($p<0.05$; one way-ANOVA was used to test differences between groups in RGR and DW at 9, 16, 28, 36 DAH; Kruskall-Wallis one-way ANOVA was used to test differences between groups in DW at 60DAH)

Fig. 2 1 kDa and 7.2 kDa peptides absorbed fraction (sum of the % of radiolabel in the body and in the metabolic trap in relation to the total tracer fed; empty bars, white = Intact, light grey = PartH, dark grey = HighH) in sole larvae at 9 (pre-metamorphosis), 16 (metamorphosis climax) and 28 DAH (post-metamorphosis). Values are mean ± s.d., *n*=6-13. Comparisons between groups fed different diets and tube-fed different molecular sized peptides were made for each developmental stage using two-way ANOVA (values given above graph), followed by Fisher's LSD pairwise comparisons.

Fig. 3 1 kDa and 7.2 kDa peptides retained fraction (% of radiolabel in the body in relation to absorbed label; empty bars, white = Intact, light grey = PartH, dark grey = HighH), and catabolized fraction (% of radiolabel in the metabolic trap in relation to absorbed label; dashed bars, white = Intact, light grey = PartH, dark grey = HighH) in sole larvae at 9 (pre-metamorphosis), 16 (metamorphosis climax) and 28DAH (post-metamorphosis). Values are mean ± s.d., *n*=6-13. Comparisons between groups fed different diets and tube-fed different molecular sized peptides were made for each developmental stage using two-way ANOVA (values given above graph), followed by Fisher's LSD pairwise comparisons.

Fig. 4 1 kDa and 7.2 kDa peptides total retention (% of radiolabel in the body in relation to tube-fed protein label) in sole larvae at 9 (pre-metamorphosis), 16 (metamorphosis climax) and 28DAH (post-metamorphosis). Values are mean ± s.d., *n*=6-13. Comparisons between groups fed different diets and tube-fed different molecular sized peptides were made for each developmental stage using two-way ANOVA (values given above graph), followed by Fisher's LSD pairwise comparisons.

ACCEPTED MANUSCRIPT

Fig.5 Expression of genes encoding for precursors of pepsin (*pga*) and trypsin (*tryp1c*) involved in luminal protein digestion at 16 (metamorphosis climax) (whole body pools of 20 individuals) and 28 DAH (post-metamorphosis) (whole body pools of 20 individuals). mRNA expression was normalized to transcript levels of *ubq* and *rps4*. Values are presented mean ± s.e.m., *n*=6. Comparisons between groups fed different diets and different ages/developmental stages were made stage using two-way ANOVA, followed by Fisher's LSD pairwise comparisons. Different superscript letters indicate significant differences ($p<0.05$) between dietary treatments at each developmental stage.

Fig.6 Expression of genes encoding for the enterocyte membrane peptide transporter 1 (*pept1*) and for the BBM enzymes intestinal alkaline-phosphatase (*alp*) and aminopeptidase N (a*mpN*), at 16 (metamorphosis climax) (whole body pools of 20 individuals) and 28 DAH (post-metamorphosis) (whole body pools of 20 individuals). mRNA expression was normalized to transcript levels of *ubq* and *rps4*. Values are presented mean ± s.e.m, *n*=6. Comparisons between groups fed different diets and different ages/developmental stages were made stage using two-way ANOVA, followed by Fisher's LSD pairwise comparisons. Different superscript letters indicate significant differences ($p<0.05$) between dietary treatments at each developmental stage.

Fig.7 Summary of the general effects of dietary protein complexity on the transcription of protein digestion-related genes, protein metabolism and somatic growth in Senegalese sole larvae and post-larvae

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Table 1

Composition and proximate analyses of the experimental diets

	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)			
Crude protein (% DW)	64.7	61.1	65.8
Crude fat (% DW)	14.5	18.9	15.8
Gross Energy (Kj/g)	20.1	20.1	20.25

[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.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

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

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Table 3

Primers used in qPCR

Gene	Fwd sequence (5′→3′)	Rev sequence (5′→3′)	Accession nr (GenBank)	Size (bp)	Annealing temp. (ºC)	E(%)
pga	ACGGCACTGGCAGCATGAATGGAT	ACAGGGACAACATCGTCGGAAGCA	KX832916	181	62	104
tryp1c	TCTGCGCTGGATACCTGGAGGGA	GCAGCTCACCGTTGCACACAACA	AB359191	81	62	100
iALP	GTTGACCAGCAGATGCCAGACAG	CAGAACAGATTTGACCTCATTCCCGATA	KX832910	147	62	97
ampN	CTGGCGTGGGACTTTGTGCGAGAT	CCGTTGATGAGGTTGGAGAAGGAGAAGG	KX832911	89	60	100
pepT1	TCAGGACCATCAGGAGAAGCAGAGG	AACACAATCAGAGCTACCACCATGAGAG	KX832912	195	62	98
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

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

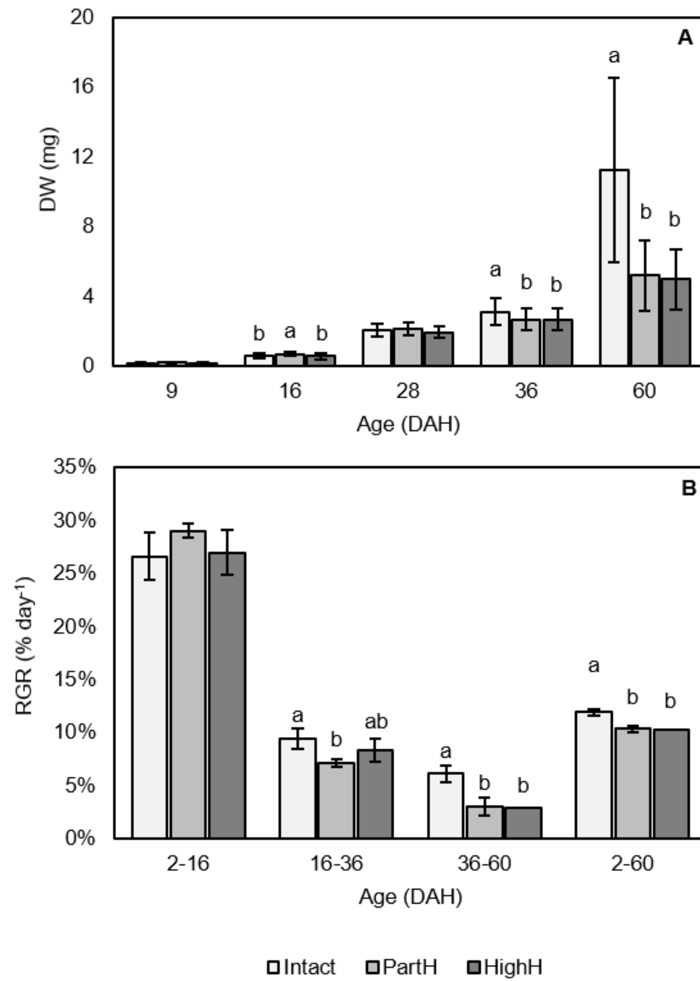

Fig. 1

ACCEPTED MANUSCRIPT

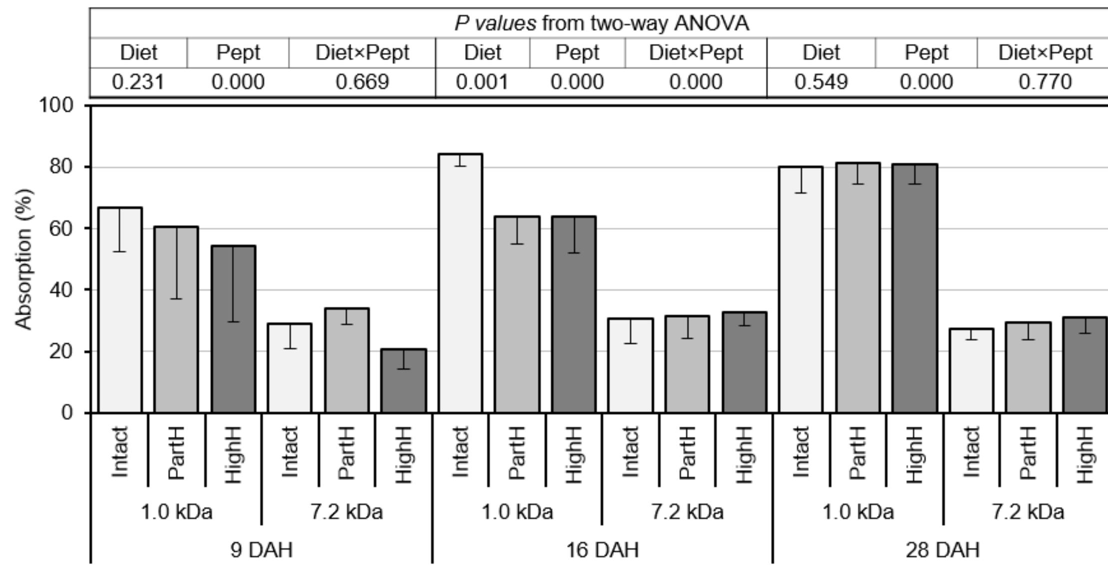

Fig. 2

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

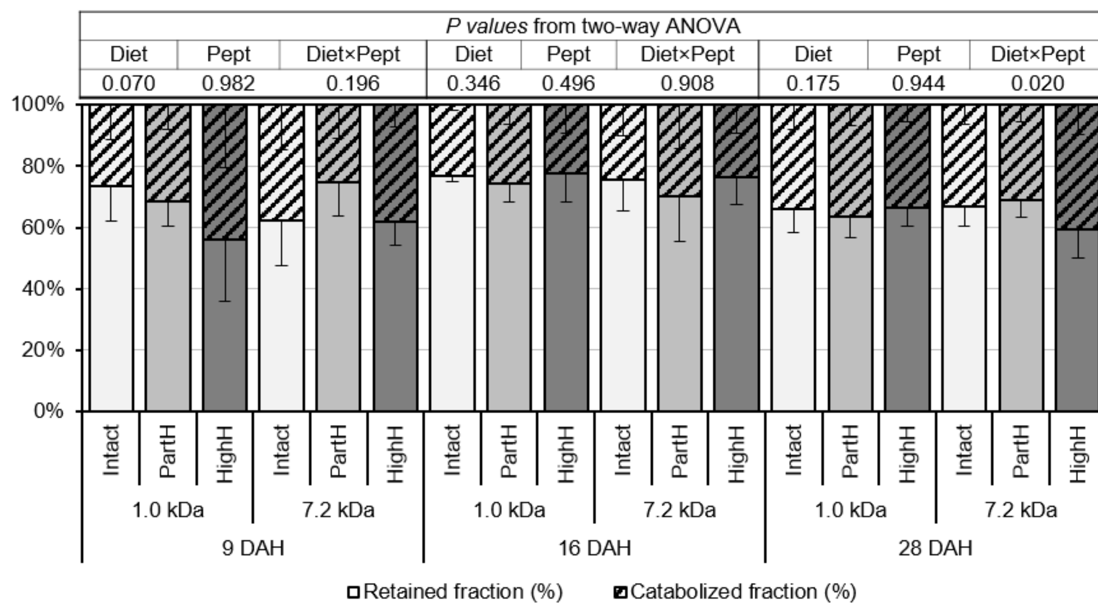

Fig. 3

ACCEPTED MANUSCRIPT

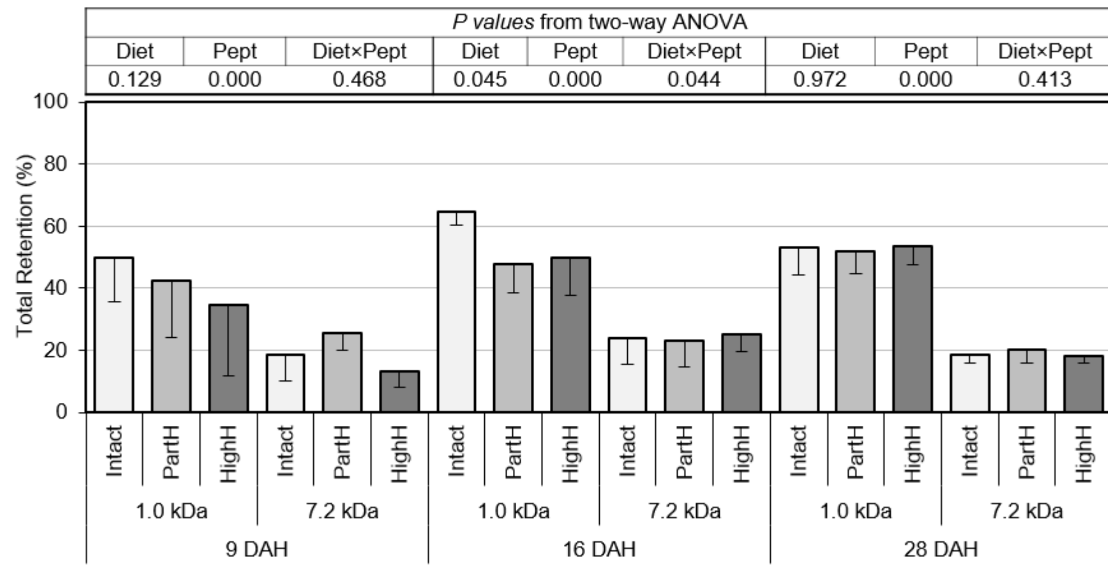

Fig. 4

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

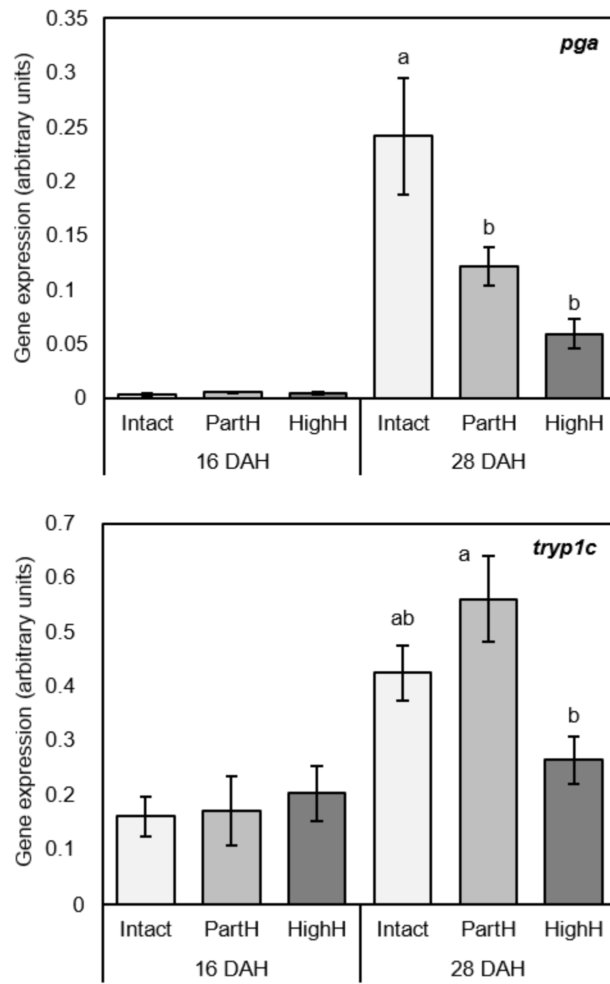

Fig. 5

ACCEPTED MANUSCRIPT

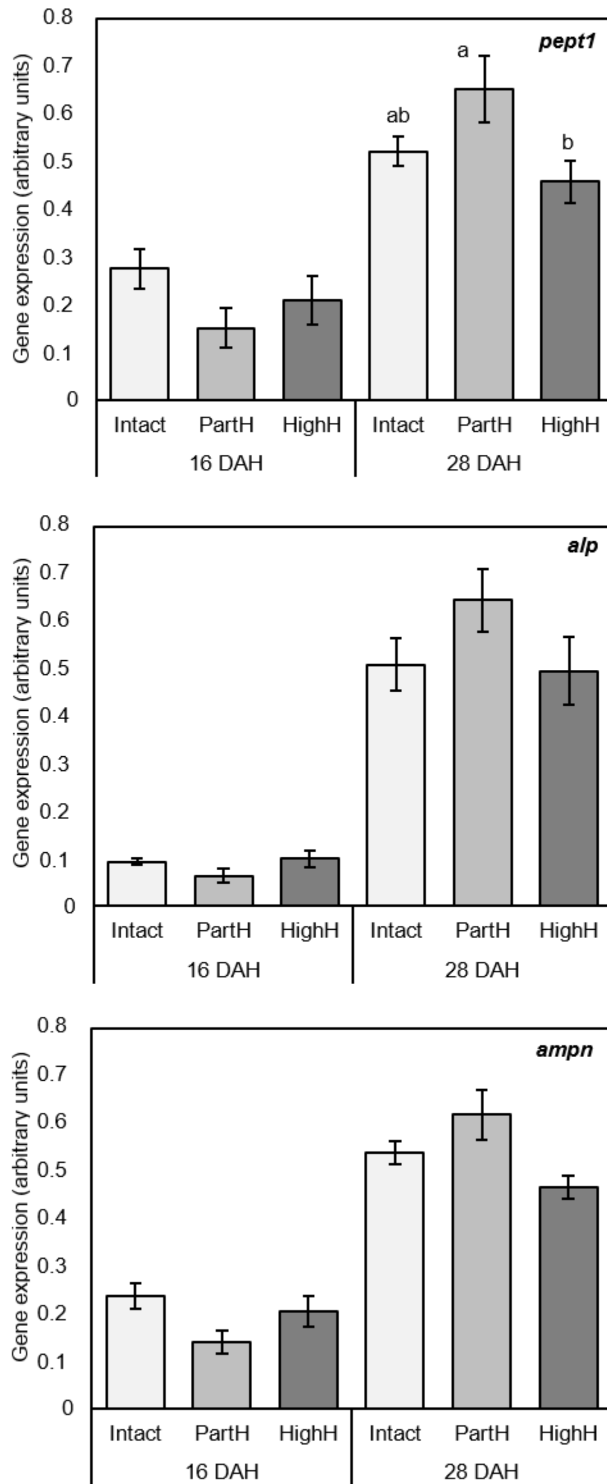

Fig. 6

ACCEPTED MANUSCRIPT

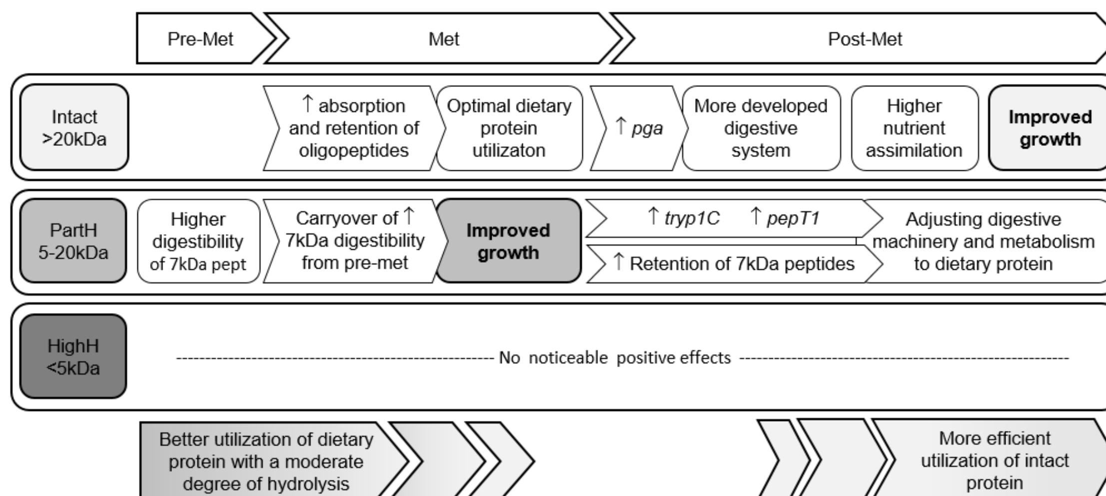

Fig. 7

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Highlights:

- Proteolytic capacity is a limiting factor for protein digestion in sole early stage larvae.
- Sole larvae adjust the way they utilize protein in response to dietary formulation.
- Pre-metamorphic sole larvae grow better upon moderately hydrolysed dietary protein.
- Dietary intact protein seems to be suitable to sole post-larvae and young juveniles.
- Dietary protein complexity should match the developing larvae proteolytic capacity.
- Microdiets protein for Senegalese sole should be adapted to each developmental stage.

ACCEPTED MANUSCRIPT