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

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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 19 °C 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 utilisation 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 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.

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

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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 40 DAH, progressively covering the stomach epithelium (Ribeiro et al., 1999b; Yúfera and 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 and 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 and 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 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 and 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 utilisation 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, 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 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 9 DAH (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

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 ± 0.5 °C 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