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

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ABSTRACT

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

1. Introduction

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

Senegalese sole (*Solea senegalensis*) is a fast-growing species that undergoes a complex metamorphosis (Fernández-Díaz et al., 2001). Its digestive system ontogeny follows the general pattern observed in other marine species with altricial development (Fehri-Bedoui et al., 2000; Padrós et al., 2011; Ribeiro et al., 1999a, 1999b; Zambonino Infante

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et al., 2008). As for most altricial species, it has been assumed that early-stage Senegalese sole larvae have a limited capacity to digest and absorb the native protein sources commonly used in commercial fish microdiets formulations (Engrola et al., 2009; Gamboa-Delgado et al., 2008). Since dietary protein is mainly absorbed as free amino-acids (FAA) and di- or tri-peptides (Ronnestad and Morais, 2008) and early-stage larvae have a poorly developed gut (Zambonino Infante et al., 2008), it has been suggested that the moderate inclusion of pre-digested proteins in microdiets would improve its dietary protein digestibility. In fact, recent results suggest that Senegalese sole pre-metamorphic larvae have a limited capacity to utilize native proteins, whereas larger peptides and intact protein seem more suitable to sole post-larvae and young juveniles anabolic and physiological needs (Canada et al., 2017; Richard et al., 2015)

Several studies reported increased survival and somatic growth in European sea bass (*Dicentrarchus labrax*) (Cahu et al., 1999, 2004; Zambonino Infante et al., 1997), gilthead sea bream (*Sparus aurata*) (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 (*Hippoglossus hippoglossus*) (Kvåle et al., 2009; Kvåle et al., 2002) larvae fed microdiets including protein hydrolysates. However, very few studies focused on a possible influence of such diets on muscle growth regulation (Katan et al., 2016; Ostaszewska et al., 2008). Muscle development and growth during early life stages is clearly determinant for the larvae ability to swim, feed and survive (Osse et al., 1997) and was further demonstrated to influence long-term somatic growth (Campos et al., 2014; Galloway et al., 1999; Weatherley et al., 1988). Moreover, early nutrition was recently shown to induce changes on the regulation of skeletal muscle development during early life stages having a long-term effect on somatic growth, which suggests the potential for nutritional programming on muscle growth and somatic growth potential (Alami-Durante et al., 2014).

White skeletal muscle constitutes the bulk of the axial locomotor muscle in Senegalese sole larvae, post-larvae and juveniles. White skeletal muscle fibres, also known as fast-twitch fibres, are used for burst swimming movements (Bone, 1978), which is the main type of locomotion displayed by sole to move and feed (Dinis et al., 1999). The white skeletal muscle development in Senegalese sole follows the general pattern observed in other aquaculture species (Campos et al., 2013b, 2013c). Muscle formation (myogenesis) comprises the recruitment of stem cells to a lineage of myogenic progenitor cells (MPCs) that undergo activation, proliferation, cell cycle exit, differentiation, migration and fusion into already formed muscle fibres (Johnston et al., 2011; Valente et al., 2013). MPCs proliferation and differentiation are ruled by the expression of numerous genes and particularly the four myogenic regulatory factors (MRFs): *myod* and *myf5* are involved in the commitment of myoblasts to form the MPCs population; *myogenin* and *mrf4* drive and keep on the myoblast differentiation that will ultimately result in myotube formation and enlargement (Rescan, 2001). On the other hand, myostatin (*mstn*) functions as a negative regulator of myoblast proliferation and differentiation (Thomas et al., 2000). Muscle growth occurs by both hyperplasia (fibre number increase) and hypertrophy (fibre size increase) (Rowlerson and Veggetti, 2001). During fish post-embryonic and larval development, muscle fibre number increases mainly by stratified hyperplasia, which involves the recruitment of new fibres in discrete germinal zones found in the lateral margins of the myotome (Rowlerson and Veggetti, 2001). In juvenile and adult stages, new myotubes form on the surface of fast muscle fibres, further fusing or adding nuclei to already existing fibres - mosaic hyperplasia (Rowlerson and Veggetti, 2001). The relative contribution of hyperplasia and hypertrophy was shown to influence long-term growth rate, providing an estimate for individual growth potential (Galloway et al., 1999; Weatherley et al., 1988).

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

In fish larvae, dietary protein sources (Alami-Durante et al., 1997; Ostaszewska et al., 2008), dietary protein level (Saavedra et al., 2016) and AA supplementation (Aguiar et al., 2005) were shown to affect muscle growth regulation and the somatic growth rate of several species. In rainbow trout, different protein:energy ratios delivered to first-feeding fry induced changes in the regulation of muscle growth during the nutritional challenge period, but also and more remarkably after 3 months of feeding all groups on the same commercial diet (Alami-Durante et al., 2014). This result suggests that the activity of white MPCs might be programmed by nutritional factors (Alami-Durante et al., 2014), although the mechanisms possibly underlying such response are not known. It has recently been suggested that an epigenetic mechanism could promote differential gene expression and modulate Senegalese sole muscle growth in response to different thermal conditions; different rearing temperatures during the pelagic phase induced changes in the methylation status of the *myogenin* putative promoter, its mRNA transcript levels and expression of *dnmt1* and *dnmt3b* (DNA methyltransferases), which was suggested to underlie the rearing temperature effect on muscle cellularity during the metamorphosis climax (Campos et al., 2013a). In addition, the effect of rearing temperature on muscle cellularity during the metamorphosis climax influenced subsequent somatic growth, up to a late juvenile stage (Campos et al., 2013b). Increasing evidence indicates that DNA methylation is labile not only to environmental conditions but also to nutritional factors (Anderson et al., 2012). However, studies on epigenetic modifications in response to environmental or nutritional cues are a recent trend in fish. Very few studies have been published concerning nutritional programming on muscle growth (Alami-Durante et al., 2014; Fontagné-Dicharry et al., 2017) and no studies have established a relationship between nutritional status and the epigenetic regulation of myogenesis, through possible changes in DNA methylation status. Campos et al. (2013a, 2013b) results on the influence of temperature on the regulation of sole myogenesis suggest the pelagic phase as a critical time window prone to epigenetic modifications with long-lasting effects on the regulation of myogenesis and subsequent influence on the potential for growth. Therefore, in the present study, we hypothesized that changes in dietary protein complexity would affect the regulation of muscle growth during the metamorphosis climax and up to an early juvenile stage in Senegalese sole, having an impact on long-term somatic growth. The effect on white muscle growth dynamics was analysed at the metamorphosis climax (16 DAH, stage 3), which has been previously recognized as a very relevant time-window in Senegalese sole ontogeny, in which changes in myogenesis induced by external factors were shown to strongly affect long-term somatic growth (Campos et al., 2013b). Muscle cellularity was also analysed in newly-weaned fish (36 DAH), since variable growth rates and size dispersion just after weaning are currently a major constraint for a more successful juveniles' production. After being fed with three experimental diets until 60 DAH, all groups were fed with the same commercial diet until 90 DAH to evaluate the enduring effect on somatic growth. The expression pattern of DNA methyltransferases was analysed in order to understand whether an epigenetic event could possibly underlie the response of muscle growth regulation and somatic growth to dietary protein complexity.

2. Material and methods

2.1. Experimental diets

Three microdiets (Intact, PartH and HighH) were formulated and