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

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ABSTRACT

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

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

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

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

1. Introduction

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

Muscle formation (myogenesis) is a complex process common to all vertebrates that involves the specification of stem cells to a myogenic lineage of myogenic progenitor cells — MPC — which then undergo activation, proliferation, cell cycle exit, differentiation, migration and fusion into muscle fibres (Johnston, 2006; Valente et al., 2013). Proliferation and differentiation of the MPCs are dependent on the programmed expression of four muscle-specific basic helix–loop-helix transcription factors, called myogenic regulatory factors (MRFs): *myod* (myoblast determination factor) and *myf5* are required for the commitment of myoblasts to form the MPC population; *myog* and *mrf4* induce and maintain the muscle differentiation programme that will lately result in myotube formation and enlargement (reviewed by Rescan, 2001). Myostatin is a negative regulator of muscle growth that inhibits myoblast proliferation (Thomas et al., 2000). *Myhc* (myosin heavy chain) encodes for myosin, which is a major structural protein of skeletal muscle and was shown to be correlated with muscle protein accretion in Atlantic salmon juveniles (Hevrøy et al., 2006). Fish muscle growth occurs both by hyperplasia (increase of fibre number) and hypertrophy (increase of fibre size) from hatching to until approximately 40% of maximum fish length (Rowlerson and Veggetti, 2001; Weatherley et al., 1988). During post-embryonic and larval development, muscle fibre number increases mainly by stratified hyperplasia, a phase of myogenesis that 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, in a second phase called mosaic hyperplasia, new myotubes form on the surface of fast muscle fibres, further fusing or adding nuclei to already existing fibres, to keep size of nuclear domains constant during hypertrophic growth (Rowlerson and Veggetti, 2001). The relative contribution of hyperplasia and hypertrophy in fish was shown to be related to growth rate and final size attained by each species (Galloway et al., 1999; Weatherley et al., 1988), thus giving an estimate of individual growth potential.

In spite of the increased efforts to understand the regulation of myogenesis by intrinsic factors like genotype (Johnston et al., 1999a; Valente et al., 2006) and extrinsic factors such as photoperiod (Giannetto et al., 2013; Johnston et al., 2004; Lazado et al., 2014) and temperature (Campos et al., 2013b, 2013c; Galloway et al., 2006; Silva et al., 2011), studies evaluating the impact of nutritional factors on fish larvae muscle development are is still scarce. Different nutritional conditions, such as dietary protein sources (Alami-Durante et al., 1997; Ostaszewska et al., 2008) and lysine supplementation (Aguiar et al., 2005) were shown to affect muscle growth regulation and the somatic growth rate in fish larvae. More recently, Alami-Durante et al. (2014) suggested that in rainbow trout the activity of white MPCs might be early programmed by early nutrition. According to these authors, diets with different protein:energy ratios delivered to first-feeding rainbow trout larvae induced changes in white muscle cellularity in parallel with changes in the expression of muscle-growth related genes during the nutritional challenge period (from first-feeding to 75 days of feeding, but also and more remarkably after 3 months of feeding all groups on the same commercial diet. However, the mechanisms by which this early nutritional cue might have printed long-term changes in the expression of muscle growth related genes are not known. Campos et al. (2013a) have recently suggested that an epigenetic mechanism could promote differential gene expression and modulate Senegalese sole muscle growth in response to different thermal conditions. Different rearing temperatures during the pelagic phase induced changes in the methylation status of the *myogenin* putative promoter, its mRNA transcript levels and in the expression of *dnmt1* and *dnmt3b* DNA methyltransferases, which catalyse the methylation of CpG dinucleotides, silencing gene expression. These changes resulted in alterations in the white muscle cellularity of Senegalese sole during metamorphosis climax (Campos et al., 2013a), and influenced subsequent somatic growth in later stages (Campos et al., 2013b). Increasing evidence indicates that DNA methylation is labile, not only to environmental conditions but also to nutritional factors, such as the availability of dietary methyl donors (reviewed by Anderson et al., 2012). However, to our best knowledge, the relationship between nutritional status and the epigenetic regulation of myogenesis has never been established in fish.

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

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

2. Material and methods

2.1. Experimental diets

Two diets (CTRL and SUP) were formulated and processed by SPAROS Lda. (Olhão, Portugal) to be isonitrogenous, isolipidic and isoenergetic, using the same practical ingredients. The CTRL diet was formulated to have a protein content based on native protein and a fish protein hydrolysate (Table 1). A second diet (SUP) consisted in