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Improving growth potential in Senegalese sole (*Solea senegalensis*) through dietary protein: An integrated approach using muscle cellularity, tracer studies and gene expression

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Summary

Fish larvae present tremendous growth potential, displaying high growth rates that are sustained by high protein deposition rates. Besides being the building block for protein synthesis, it is well established that amino acids (AA) are a major energy source during the larval stage of most marine teleost species. Thus, to fully express such a huge growth potential and maximize feed efficiency, high quality dietary protein must be included in larval diets to ensure optimal feed utilization. Dietary protein quality is generally defined by criteria like digestibility, indispensable amino acids (IAA) bioavailability and amino acids (AA) profile. Dietary AA imbalances will result in AA lost for energy production which, considering larvae high growth rates, may have a profound impact on performance. Therefore, regardless the type of diet formulation (based on semi-purified or practical ingredients), it is mandatory to ensure the supply of dietary protein with a balanced AA profile in order to meet the species IAA requirements. It is also essential to adapt the dietary protein complexity to the fish maturing digestive capacity. This is particularly important to optimize protein digestion, absorption and retention in altricial larvae with a marked metamorphosis such as Senegalese sole.

Fish larvae display an allometric growth, with head and visceral tissues initially growing faster than white muscle, and with this tissue growing much faster than any other tissues in later larval stages. In any case, the efficiency of protein retention and synthesis in skeletal muscle is a major determinant of larvae dietary AA requirements throughout development. During the embryonic and larval stages fish muscle development may be modulated by extrinsic factors. The possible effects are usually irreversible due to the rapid pace of ontogenetic change, having potential consequences for larval survival, as well as long-lasting effects on somatic growth. Variations in dietary protein during early larval stages may induce changes in the myogenic processes having long-term irreversible effects on muscle cellularity and impact on growth potential.

Epigenetic modifications, such DNA methylation are labile in response to nutritional cues. DNA methylation is ruled by the activity of DNA methyltransferases (*dnmt1, dnmt3a* and *dnmt3b*) and is involved in the regulation of gene expression, by repressing transcription. It is possible to change the dietary protein absorption and body retention by manipulating the formulation of dietary protein. This may modify the post-prandial availability of methyl-donors, such as methionine, with possible consequences on the DNA methylation and regulation of gene expression in different tissues.

In the present thesis, it was hypothesized that manipulating dietary protein quality and complexity would improve the larvae capacity to digest and retain protein, promoting protein accretion in skeletal muscle and consequently somatic growth.

In **Chapter 2**, dietary protein quality was manipulated according to the ideal protein concept, *i.e.* by adjusting the dietary AA profile to the larval body AA profile, in an attempt to meet Senegalese sole ideal IAA profile. This diet formulation strategy was tested in Senegalese sole larvae from 2 to 51 DAH and had a positive short-term effect on larvae capacity to retain protein (**Chapter 2**), but was not translated into higher somatic growth.

In **Chapter 3**, dietary protein quality was changed by increasing the IAA/DAA ratio, to improve the larvae capacity to retain dietary protein by promoting protein accretion in skeletal muscle. The regulation of muscle growth was evaluated during the metamorphosis climax and at a late juvenile stage, through muscle cellularity and the expression of related genes (*myf5*, *myod2*, *myogenin*, *mrf4*, *myhc* and *mstn1*). Changes in dietary protein exerted a strong influence on sole larvae muscle development, by inducing changes in the myogenic processes, delaying growth in larvae fed the IAA supplemented diet. The expression pattern of DNA methyltransferases was analysed in order to understand whether dietary protein AA profile could induce an epigenetic effect with consequent influence on the potential for growth. Although *dntm3b* expression was reduced the in the larvae fed the IAA/DAA corrected diet, possibly due to a lower protein intake, no correlation was established between the altered expression of *dnmt3b* and the regulation of myogenesis.

In **Chapters 4** and **5**, dietary protein complexity was modified to improve its digestibility, by including protein hydrolysates with different molecular weights in microdiets to be delivered from mouth-opening.

In **Chapter 4**, it was inquired whether the manipulation of dietary protein complexity would affect the mRNA expression of genes encoding for the precursors of proteolytic enzymes and the peptide transporter PepT1 in Senegalse sole metamorphosing and post-metamorphic larvae. A possible effect on the larvae capacity to digest, absorb and retain protein was also evaluated, as well as the impact on somatic growth. Pre-metamorphic sole larvae were shown to utilize better dietary protein with a moderate degree of hydrolysis, while post-metamorphic sole presented a higher feed efficiency when fed the intact protein. Different dietary formulations modulated the patterns of expression of the genes encoding for digestive enzymes precursors and the enterocyte membrane peptide transporter 1 (*pept1*) in post-metamorphic larvae. Sole seems able to adjust protein utilization according to diet formulation, through the adaptation of digestive functions that are regulated at the transcriptional level.

In **Chapter 5**, it was inquired whether dietary changes would affect the regulation of muscle growth during the metamorphosis climax and early juvenile stage. Dietary protein with moderate degree of hydrolysis promoted white muscle growth during the metamorphosis climax. At 36DAH, diets induced changes in the expression patterns of genes encoding for the

myogenic regulatory factors, which affected muscle growth dynamics, ultimately promoting higher growth in the fish fed intact protein. In fish fed the diets including protein hydrolysates, a lower recruitment of small-sized fibers led to reduced growth potential. Sole post-larvae fed a diet mostly based on highly hydrolysed protein displayed higher transcript levels of *dnmt3a* and *dnmt3b*, which was attributed to increased dietary methionine content.

In synthesis, either manipulating the quality or the complexity of dietary protein had impact on the larvae capacity to utilize protein and direct it for growth. Sole larvae seems to be able to adapt its digestive functions and metabolic capacity to dietary protein. Moreover, increasing the dietary IAA/DAA ratio and changing the degree of hydrolysis of dietary protein affected the expression pattern of muscle growth related genes in different ways, with consequences on muscle cellularity and potential for growth.

In conclusion, optimal protein quality seems to change during larval development, what has important consequences for practical larval microdiets formulation. The inclusion of a moderately hydrolysed protein comes up as a promising way to improve growth in early larval stages. However, larger peptides and intact protein seem to be more suitable to sole post-larvae and young juveniles. Therefore, these thesis results suggest that dietary protein fraction formulation of microdiets for Senegalese sole shall be adapted to each developmental stage.

Resumo

Durante a fase larvar, os peixes possuem um enorme potencial de crescimento. Regra geral, apresentam taxas de crescimento elevadas, asseguradas por taxas de deposição proteica igualmente altas. Os aminoácidos são uma importante fonte de energia durante a fase larvar da maioria dos peixes teleósteos, para além de serem os elementos essenciais da síntese proteica. Torna-se assim fundamental providenciar através da dieta uma quantidade suficiente de proteína e de elevada qualidade, por forma a maximizar a conversão alimentar e a expressão do potencial de crescimento que carateriza este período do desenvolvimento larvar. A qualidade da proteína é geralmente definida com base na sua digestibilidade, na biodisponibilidade dos aminoácidos indispensáveis e no perfil de aminoácidos. Quaisquer desequilíbrios no perfil de aminoácidos resultam tendencialmente num aumento do catabolismo, o que poderá comprometer o crescimento somático de forma muito significativa. Por isso, independentemente do tipo de formulação (seja baseada em ingredientes semi-purificados ou práticos) é necessário assegurar o fornecimento de proteína com um perfil de aminoácidos equilibrado e adequado às necessidades da espécie em causa. É igualmente crucial procurar adaptar a complexidade da proteína fornecida ao estádio de maturação do sistema digestivo, de forma a otimizar a digestão, a absorção e a retenção proteica. Este aspeto é particularmente crítico na formulação da componente proteica para espécies altriciais e com uma metamorfose complexa, como é o exemplo do linguado senegalês.

As larvas de peixes apresentam um crescimento alométrico, sendo que em estádios mais precoces, a cabeça e os tecidos viscerais crescem mais rapidamente do que o músculo branco, enquanto que em estádios larvares mais tardios este último cresce muito mais rapidamente do que quaisquer outros tecidos. Assim, a eficiência da retenção e síntese proteica no tecido muscular é um fator determinante das necessidades proteicas durante o desenvolvimento larvar. Nos estádios embrionários e larvares, a plasticidade do tecido muscular em resposta a fatores extrínsecos é normalmente irreversível devido ao ritmo acelerado das alterações ontogénicas. Por isso, quaisquer alterações induzidas no desenvolvimento do músculo durante a fase larvar poderão ter possíveis consequências na sobrevivência, mas também na celularidade do músculo e no potencial de crescimento a longo-prazo.

Modificações epigenéticas, como a metilação do DNA, podem variar em resposta a fatores nutricionais, nomeadamente a disponibilidade de moléculas dadoras de grupos metilo (p. ex. metionina, cisteína, homocisteína). A metilação do DNA é um processo envolvido na regulação da expressão génica e está dependente da atividade das DNA metiltransferases (*dnmt 1, dnmt3a* e *dnmt3b*). A manipulação da componente proteica da dieta poderá resultar em alterações na absorção e retenção proteica, o que poderá levar a variações na

disponibilidade de dadores de grupos metilo em diferentes tecidos (p. ex. músculo). Estas variações eventuais na disponibilidade de dadores de grupos metilo poderão resultar em mudanças no estado de metilação do DNA e consequentemente na regulação da expressão génica.

Nesta tese foi testada a hipótese de a manipulação da qualidade e da complexidade da proteína na dieta resultarem num aumento da capacidade das larvas de linguado para digerirem e reterem proteína, promovendo a deposição proteica no tecido muscular e, por último, o crescimento somático.

No **Capítulo 2**, a qualidade da proteína na dieta foi manipulada de acordo com o conceito da proteína ideal, isto é, aproximando o perfil de aminoácidos da dieta ao perfil de aminoácidos das larvas para o linguado Senegalês. Esta formulação utilizada num ensaio de crescimento dos 2 aos 51 dias após a eclosão e teve um impacto positivo na capacidade de retenção proteica das larvas que, sendo transitório, não se traduziu num aumento do crescimento somático.

No **Capítulo 3**, a qualidade da proteína na dieta foi manipulada através do aumento do rácio entre aminoácidos indispensáveis e aminoácidos dispensáveis (AAI/AAD), com o intuito de melhorar a capacidade de retenção proteica das larvas. Procurou-se com isto promover a deposição proteica no tecido muscular. Durante o clímax da metamorfose e aos 51 dias após a eclosão, foi estudada a celularidade no músculo branco e a expressão de genes envolvidos na miogénese (*myf5*, *myod2*, *myogenin*, *mrf4*, *myhc* e *mstn1*), para avaliar a possível ocorrência de um efeito nutricional na regulação do crescimento muscular. Estas alterações na componente proteica da dieta influenciaram o desenvolvimento muscular de forma significativa, atrasando o crescimento das larvas alimentadas com a dieta em que o rácio AAI/AAD foi aumentado. Também, neste grupo, houve uma redução na expressão das *dnmt3b*, o que foi atribuído a uma eventual alteração na ingestão de proteína. No entanto, não foi estabelecida nenhuma correlação entre a expressão das *dnmt3b* e a regulação da miogénese.

Nos **Capítulos 4** e **5**, a complexidade da proteína na dieta foi manipulada através da inclusão de hidrolisados proteicos com diferentes pesos moleculares, com o intuito de melhorar a digestibilidade da proteína em microdietas introduzidas à abertura de boca.

No **Capítulo 4**, investigou-se o efeito da complexidade da proteína na transcrição de genes que codificam alguns precursores das enzimas proteolíticas e de um gene que codifica um transportador da membrana intestinal específico para péptidos (PepT1). Foi igualmente avaliado o efeito nutricional na capacidade das larvas para digerirem, absorverem e reterem proteína ao longo da metamorfose, bem como no crescimento somático. Enquanto a dieta

baseada em proteína com um grau moderado de hidrólise promoveu o crescimento até à metamorfose, a dieta baseada em proteína intacta promoveu o crescimento em estádios mais tardios do desenvolvimento (a partir dos 36 dias após a eclosão). Formulações diferentes induziram padrões de expressão distintos nos genes que codificam os precursores das enzimas proteolíticas, bem como no gene que codifica o transportador transmembranar específico para péptidos (PepT1). Os resultados apresentados no **capítulo 4** sugerem que as larvas de linguado são capazes de modificar o metabolismo proteico em resposta à formulação da dieta, adaptando o funcionamento do sistema digestivo. Esta adaptação parece ser regulada ao nível da transcrição génica.

No **Capítulo 5** investigou-se o efeito das mesmas dietas testadas no capítulo 4 na regulação do crescimento muscular. A dieta à base de proteína com um grau moderado de hidrólise promoveu o crescimento muscular durante o clímax da metamorfose, o que se traduziu num aumento do crescimento somático. Aos 36 dias após a eclosão, as dietas testadas induziram alterações na expressão dos genes que codificam os fatores reguladores da miogénese, afetando o crescimento muscular e promovendo, por último, o crescimento das larvas alimentadas à base de proteína mais complexa. Os linguados alimentados à base de hidrolisados proteicos, apresentaram sinais de um menor recrutamento de fibras pequenas, o que se poderá ter traduzido numa redução do potencial de crescimento muscular, e consequentemente num menor crescimento somático. Observou-se igualmente um aumento da transcrição das *dnmt3a* e *dnmt3b* nos linguados alimentados à base de proteína com um elevado grau de hidrólise, possivelmente devido a um maior nível de metionina na dieta.

Em síntese, a manipulação da qualidade ou da complexidade da proteína na dieta afeta o desenvolvimento da capacidade das larvas de linguado para utilizarem a proteína com consequências no crescimento. De facto, as larvas parecem adaptar as funções digestivas e capacidade metabólica à composição proteica da dieta. Para além disso, quer a correção do perfil de AA da dieta, através do aumento do rácio AAD/AAI, quer a complexidade da proteína alteraram o padrão de expressão dos genes que codificam fatores reguladores da miogénese; este efeito, refletiu-se por sua vez em alterações a nível da celularidade do músculo, com consequências para o potencial de crescimento.

Em conclusão, a qualidade ótima da proteína a incorporar em dietas para linguado parece variar ao longo do desenvolvimento e tal poderá ter consequências futuras na formulação de microdietas para larvas. A inclusão de proteína com um moderado grau de hidrólise parece ser uma solução promissora para melhorar o crescimento durante os primeiros estádios larvares. No entanto, formas mais complexas parecem ser mais adequadas para estádios larvares mais tardios e para juvenis. Assim, os resultados presentes sugerem que a

formulação da fração proteica das microdietas para larvas de linguado deverá ser alterada de acordo com o estádio de desenvolvimento.

List of abbreviations

AA	Amino acids
ampn	gene encoding for Aminopeptidase N
BBM	Brush border membrane
CAA	Crystalline amino acids
DAA	Dispensable amino acids
DAH	Days after hatching
Dnmt	DNA (cytosine-5)-methyltransferase
DPFF	Days post-first feeding
FAA	Free amino acids
FPH	Fish protein hydrolysate
IAA	Indispensable amino acids
ialp	gene encoding for Intestinal alkaline phosphatase
MPC	Myogenic progenitor cells
Mstn	Myostatin
Myf5	Myogenic factor 5
MyHC	Myosin heavy chain
Myod	Myoblast determination protein
Myog	Myogenin
MRF	Myogenic regulatory factor
Mrf4	Myogenic factor 6
pga	gene encoding for pepsinogenA
pept1	gene encoding for the enterocyte membrane peptide transporter 1
RGR	Relative growth rate
SAM	S-adenosylmethionine
tryp1c	gene encoding for Trypsinogen1C

Chapter 1

General Introduction

1 General introduction

1.1 General aspects of Senegalese sole biology and production

Senegalese sole (*Solea senegalensis* Kaup, 1858) (order Pleuronectiformes, family Soleidae) is a benthonic flatfish that inhabits sandy or muddy bottoms down to 100m, along the coasts and estuaries of North Africa and western Mediterranean up to the Gulf of Biscay (Haedrich, 1986; Quéro, 1984; FAO, 2014). Juvenile and adults feed essentially on invertebrates living in the sediment, such as polychaetes, bivalves and small crustaceans (Cabral, 2000). It is a gonochoric species, the females reaching sexual maturity at approximately 3 years old and 32 cm (Andrade, 1990; Dinis, 1986). Senegalese sole is a batch spawner, spawning from March to July, along the Portuguese coast (Dinis, 1986).

In captivity, eggs are usually obtained from natural spawns of wild broodstock kept in captivity (Dinis et al., 1999; Morais et al., 2016). Depending on the water temperature, the pelagic eggs hatch within 36 to 48h (Dinis and Reis, 1995). The newly hatched larvae are bilaterally symmetric and pelagic (Bedoui, 1995; Dinis et al., 1999; Lagardére, 1979). At 2 days after hatching (DAH) both the mouth and anus are open and larvae start exogenous feeding (Ribeiro et al., 1999a). The digestive tract is differentiated into buccal–pharyngeal cavity, esophagus, an incipient stomach, and anterior and posterior intestine (Ribeiro et al., 1999a). The pancreas and liver are already differentiated at this stage (Ribeiro et al., 1999a).

During the second and third weeks of its life cycle, Senegalese sole larvae undergo a marked metamorphosis that strongly affects its behaviour, feeding and digestive physiology (Conceição et al., 2007b). As in most flatfish, sole metamorphosis is characterized by a 90º body rotation, and the migration of the left eye towards the right side (Fernández-Díaz et al., 2001). The metamorphosis sub-stages are defined in reference to the eye translocation status, from stage 0 (corresponding to pre-metamorphosis) to stage 4 (corresponding to late metamorphosis), and its timing seems to depend on body length rather than on larvae age (Fernández-Díaz et al., 2001). Following a complex anatomical transformation, the larvae settle on the bottom and become benthonic, lying on the blind side of the body. The body rotation usually starts at 4.5-5.0mm/9-15DAH, depending on water temperature and feeding regime (Dinis et al., 1999; Fernández-Díaz et al., 2001; Parra, Yúfera, 2001) and settlement is completed by 7.0-9.5mm/17-20DAH (Bedoui, 1995; Dinis, 1992; Dinis et al., 1999; Fernández-Díaz et al., 2001). During metamorphosis, there is a rearrangement of the internal organs and digestive tract, with migration of the anus towards the pelvic fin. Only around 30DAH the digestive system completes its maturation (Ribeiro et al., 1999a; 1999b).

Senegalese sole larvae and early post-larvae are quite robust and its cultivation is easy, when compared to other marine species. For that reason, culture protocols were established by 1999 (Dinis et al., 1999) and are nowadays fairly standardized, with post-larvae being routinely produced with good growth and at high survival rates (Morais et al., 2016). Due to its wide mouth - >350µm (Parra and Yúfera, 2001), sole can be fed directly with small Artemia *nauplii* from mouth opening onwards, but most of the studies use a mixture of Artemia and rotifers during the first days of rearing (Dinis et al., 1999). After settling, post-larvae are transferred to flat-bottom tanks and are fed live or frozen enriched Artemia. Until recently sole were usually sudden weaned on to an inert diet at approximately 35-40DAH. Historically, some problems have hampered a more successful juvenile production, such as difficulties in weaning, variable growth rates and malformations, which may be related to suboptimal rearing or nutritional conditions during early life stages (Morais et al., 2016). More recently, successful sudden weaning can be achieved at 25-30DAH, and the only significant remaining problem is a large size dispersion (L. Conceição, pers Comm.).

Live feed nutritional composition is considered as sub-optimal for Senegalese sole larvae on what concerns protein quality (Aragão et al., 2004a, 2004b) and that may be the cause of the variable growth rates and malformations. Using a microdiet to supplement live feed in a co-feeding regime can be a possible solution to overcome the inadequacy of the commonly cultivated zooplantonic species to sole nutritional needs. In fact, Engrola et al. (2009) suggested that an early delivery of an inert microdiet in a co-feeding regime would allow breaking down the current difficulties at weaning, by promoting digestive tract maturation and long-term growth. In order to introduce successful variations in sole larval rearing using inert feeds, a suitable microdiet must be developed to meet these larvae nutritional needs.

The basis for formulating a commercial microdiet to be introduced from an early stage (first feeding) to Senegalese sole have been set mostly in the last 15 years (reviewed by Conceição et al., 2007b and Morais et al., 2016). A fair amount of studies have been conducted on sole digestive physiology and have set the basis for a comprehensive morphological and functional characterization of its gastrointestinal ontogeny (Conceição et al., 2007b). Concerning dietary protein, the studies carried out by Aragão et al. (2004a, 2004b) have established the basis for an estimate of sole larvae and post-larvae amino acids (AA) requirements which seem to change throughout ontogenesis. The larvae capacity to regulate dietary protein and AA metabolism has also been extensively studied (Aragão et al., 2004c; Morais et al., 2004b; Pinto et al., 2009; Rønnestad and Conceição, 2012; Rønnestad et al., 2000).

Recent projects have largely contributed to the development of commercial microdiets for several flatfish species that showed to be suitable for a successful weaning of Senegalese

sole post-larvae (MICALA project, 2010-2012 13380/QREN, SOLEAWIN project 2014-2015 310305/FEP/71). Still, there is room for improving the current commercial microdiets and anticipate sudden weaning or to eventually supplement or even definitely replace live feeds.

Senegalese sole is mostly reared in Portugal, Spain and more recently in France and Italy (FEAP, 2016). This species cultivation was initially based on the on-growing of wild juveniles trapped in salt marshes as an added value product in semi-intensive polyculture systems for growing sea bream and sea bass (Ferreira et al., 2010; Yúfera and Arias, 2010). However, nowadays the trend is for its cultivation in intensive growing systems, using commercial feeds and maintaining a highly controlled environment, namely through recirculating water systems (RAS) (Morais et al., 2016). Hopefully, this technological development will result in increased production as it is suggested by the recent production trend: total production of farmed sole has risen from 68 to 1457 tonnes in the period 2007-2015 in Europe (FEAP, 2016). This will inevitably lead to an increase in the demand for high quality juvenile production. This prospective scenery makes it urgent to increase Senegalese sole larvae and juvenile quality, which may be pursued by improving early larvae nutrition.

1.2 Larval growth potential, the role of protein synthesis

The maximum potential for growth is genetically determined and can only be reached under suitable environmental conditions with appropriate nutrient intake. Growth is the net result of retained nutrients utilization, once undigested nutrients and metabolic wastes are processed and excreted, and the maintenance (heat losses and basal metabolism) and voluntary activity have been met (NRC, 2011).

Fish growth is mainly driven by protein deposition (Dumas et al., 2007; Shearer, 1994) and maximal protein deposition and associated carcass lean growth rate determine the nutrient requirements for growth (Schinckel and de Lange, 1996). Due to its high protein synthesis and deposition rates, skeletal muscle protein deposition greatly contributes to overall growth in fish and fish larvae when compared to other tissues (Houlihan et al., 1995a).

Protein deposition or accretion is the net result of the dynamic balance between protein synthesis and protein degradation, in a process called protein turnover (Millward et al., 1975). Protein turnover is the continuous renewal and cycling of tissue proteins through the transfer of amino acids (AA) between the free amino-acid (FAA) and protein pools (Fig.1).

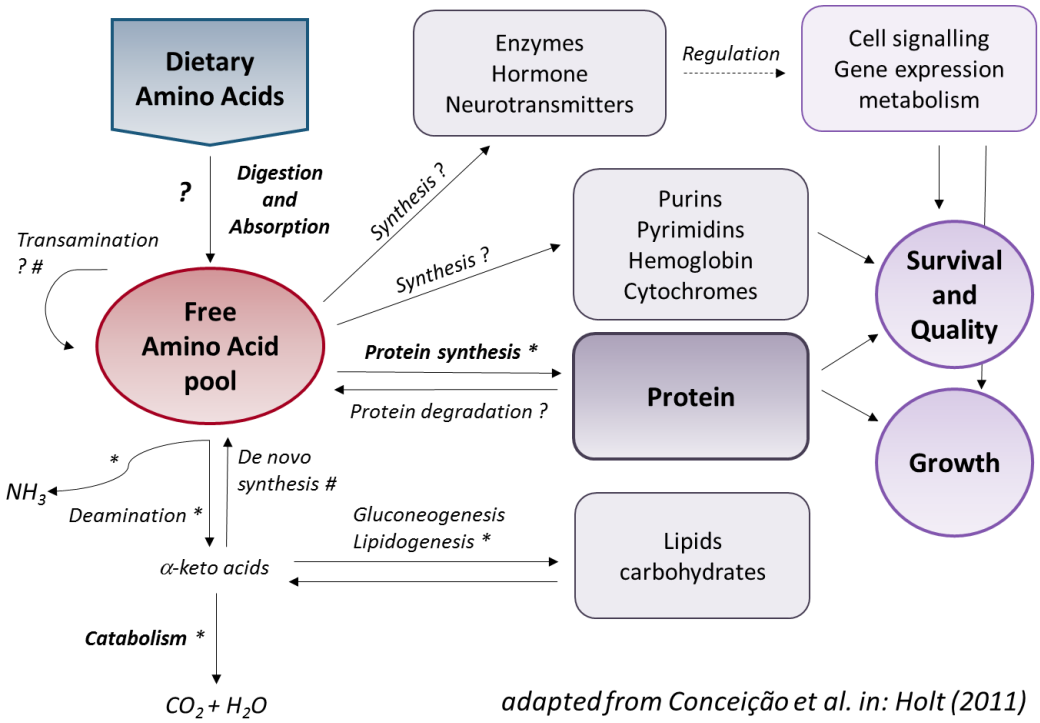

Figure 1: Main routes of amino acid (AA) metabolism in fish larvae and how they may affect larval fish performance. Current knowledge of effects of dietary AA in these routes is given as follows: * = affected by dietary AA composition; ? = unknown if affected by dietary AA composition; # = unknown if relevant in meeting dispensable AA requirements (adapted from Conceição et al., 2011)

Protein turnover can be divided into an obligatory component associated with the maintenance of cell functions, and a variable component related to growth (Reeds, 1989). Protein deposition and growth may result from an increase in the rate of protein synthesis and/or by a decrease in the rate of protein degradation (Millward, 1989; Millward et al., 1975) and it depends exclusively upon dietary AA (Conceição et al., 2011). Dietary AA are mostly absorbed as FAA or as small peptides that are further broken down into FAA (Rønnestad, Morais, 2008). The absorbed AA enter the FAA pool, also called metabolic pool (Cowey and Walton, 1989; Kaushik and Seiliez, 2010), in which the concentrations of FAA are kept within narrow limits (Houlihan et al., 1995a). From this pool, FAA can be used for protein synthesis, or can be either catabolised for energy production, transaminated into other AA, used in gluconeogenesis or lipogenesis, or used in the synthesis of other nitrogen-containing molecules such as purines, pyrimidines or hormones (Fig.1). The utilization of the FAA pools depends on the organisms or tissues metabolic demands and also on the efficiency with which other nutrients are being utilized to meet those demands (Cowey and Walton, 1989; Kaushik

and Seiliez, 2010). The efficiency of AA utilization depends, thus, on the rates of protein synthesis and protein turnover. Higher protein synthesis rates tend to reduce AA catabolism, as the absorbed AA will leave the FAA pool faster, which results in a higher AA utilization efficiency. However, protein synthesis is one of the most energy-demanding cellular processes, which may lead to an increased use of AA for energy production. That can be compromising if other nutrients are not available to sustain energy production. Increased protein turnover usually decreases AA utilization efficiency, as it allows for a higher amount of AA to be available for inevitable catabolism (Conceição et al., 2011).

The dietary nutrient composition may affect the protein deposition efficiency, e.g. low non protein energy content or increased AA catabolism due to dietary AA excess or imbalance will result in increased energy losses and decreased protein deposition efficiency (NRC, 2011). Besides diet composition, many other factors affect the utilization of AA, such as the chemical form of the supplied AA, the protein or AA source, the feed ingredient matrix and a number of biological factors, including species and life stage (NRC, 2011).

Fish larvae have tremendous growth potential, displaying relative growth rates that may exceed 70% a day (Conceição et al., 2003a; Kamler, 1992; Otterlei et al., 1999) and are necessarily sustained by high protein deposition rates. In addition, it is well established that AA are a major energy source during the larval stage of most marine teleost species (Conceição et al., 1993; Finn et al., 2002; Parra et al., 1999; Rønnestad et al., 1999, 2003; Rønnestad and Fyhn, 1993). Thus, fish larvae AA requirements are expected to be higher than those of juveniles or adult fish, usually falling in the range of 50 to 60% (NRC, 2011)**.**

Protein synthesis in fish larvae seems to follow the general trends observed in adult fish and mammals, increasing with growth rate, dietary protein level, temperature and ration size (Conceição et al. 2011). However, whereas in adult fish protein degradation increases concomitantly with protein synthesis, in fish larvae this does not seem to happen (Houlihan et al., 1992): fast-growing larvae just seem to be more efficient in depositing protein than slower growing larvae. In fact, it has been proposed that fish larvae may decrease the rate of protein turnover or either reduce the protein synthesis costs as a response to a strong selective pressure to increase efficiency of protein deposition (Conceição et al., 1997 ; Kiørboe, 1989; Kiørboe et al., 1987).

Fractional rates of protein synthesis (%.day^{-1}), protein turnover and protein degradation tend to decrease with body size and age in fish larvae (Fauconneau et al., 1986a; Houlihan et al., 1995c), as well as in juvenile and adult fish (Houlihan et al., 1986; 1995a; 1995b). This overall decrease in protein synthesis may be explained by the larvae allometric growth, i.e. the increasing relative contribution of white muscle tissue compared to other tissues that are

known to have a higher metabolic activity, such as liver and the digestive tract (Dabrowski, 1986). Therefore, the efficiency of protein retention and synthesis in skeletal muscle is probably a major determinant of larvae dietary AA requirements throughout development.

1.3 Feeding fish larvae

1.3.1 Live feeds *vs.* inert feeds

Most altricial larvae are still difficult to feed upon inert feeds from first feeding and usually require live feeds at least as part of their diet during the early life stages (Cañavate and Fernández-Díaz, 1999, Cahu and Zambonino Infante, 2001; Engrola et al., 2009, 2010; Fernández-Díaz et al., 2006; Koven et al., 2001; Kvåle et al., 2009; Löpez-Alvarado, Kanazawa, 1995; Saavedra et al., 2009b; Seiliez et al., 2006 ; Yúfera et al., 2005; Zambonino Infante et al., 1997). Nonetheless, technical difficulties in mass-producing these organisms are still a constraint to their routine use (Conceição et al., 2010a). Furthermore, despite the successful development of enrichment products and protocols, there is still a lack of consistency on nutritional quality of live feed, which further restricts its use to assess larvae nutritional requirements (Conceição et al., 2010a; NRC, 2011). In fact, particularly on what concerns protein quality, live feed nutritional composition is often sub-optimal for several fish species larvae (Aragão et al., 2004b; Conceição et al., 2010a, 2011; Engrola et al., 2007). In particular for Senegalese sole, despite the high digestibility of *Artemia* protein, it is known that both *Artemia* and rotifers AA profiles do not meet the larvae and post-larvae qualitative IAA requirements (Aragão et al., 2004a; 2004b), which makes the use of microdiets to supplement live feed in a co-feeding regime at least advisable (Engrola et al., 2009).

The development of microdiets with a high and constant nutritional value, easy to maintain and with low production costs is, thus, an imperative conquest for the future sustainability of marine fish larvae production (Kolkovski, 2008; Conceição et al., 2010). However, some obstacles keep on hampering further progress in the use of microdiets for the early larval stages, such as: low attractiveness and low ingestion rates; poor digestibility; high leaching losses of soluble molecules such as FAA, peptides, vitamins and minerals; and difficulties in formulating complete and well balanced feeds due to lack of knowledge on larvae nutritional requirements (Conceição et al., 2011; Kvåle et al., 2006). The poor attractiveness of microdiets has been partially overcome by the inclusion of fish protein hydrolysates with a high percentage of small peptides that are known to act as attractant to fish (Aksnes et al., 2006a; 2006b; Kousoulaki et al., 2013; Velez et al., 2007). The moderate inclusion of pre-digested proteins was suggested to enhance the digestibility and the nutritional value of microdiets (Kolkovski, 2008), but this is still a major obstacle in formulations including practical ingredients. Several attempts have been made to overcome leaching losses of water-soluble protein in microdiets. Yúfera

(2003) used cross linked microencapsulated diets by interfacial polymerization of dietary protein using trimesoyl chloride as a linking agent, obtaining a low efficiency in incorporating free-Lys in microparticles and high retention efficiency after rehydration. Using the same microencapsulation method, Nordgreen et al. (2008) obtained a poor stability of dietary protein quality (AA profile). Onal, Langdon (2004, 2005) achieved substantially reduced leaching losses of water-soluble nutrients through lipid-based encapsulation. Nordgreen et al. (2009) used a protein bound heat coagulated diet to study the stability of dietary protein quality in microdiets containing different concentrations of hydrolysed protein, obtaining losses of approximately 100% for FAA and peptides, and up to 80% for water-soluble protein. Kvåle et al. (2006) compared the efficiency of three different microparticulation technologies in retaining different dietary N-forms, achieving the lowest leaching percentages for supplemented free-Ser (approximately 20%) in a cross linked microencapsulated diet. However, the cod larvae preference for this diet was remarkably low, when compared to a heat coagulated and an agglomerated diet. In fact, a trade-off between acceptability, digestibility and low leaching seems to be a common denominator amongst most of the cited studies. However, Yúfera et al. (2005) reported fair growth rates from 7 to 30DAH in Senegalese sole fed exclusively upon a diet microencapsulated by internal gelation using a matrix of Ca-alginate, suggesting that this technique provides an acceptable compromise between the microdiet digestibility and minimal leaching losses.

The introduction of a microdiet in a co-feeding regime since first-feeding was shown to promote growth and proposed to ease an earlier weaning in Senegalese sole (Engrola et al., 2009). Recent research has led to the development of a commercial microdiet for several flatfish species, produced by cold-extrusion (microaglomeration) and microencapsulation by a proprietary process (Sparos Lda., Olhão, Portugal) that guarantees a good stability of the water-soluble nutrients and was showed to be suitable for a successful weaning of Senegalese sole post-larvae.

Further improvements in inert microdiet technology and formulation will likely lead to a progressive substitution of live feeds and allow for a better understanding of fish larvae nutritional requirements, namely on what concerns protein quality and complexity.

1.3.2 Formulating Protein for fish larvae

1.3.2.1 Protein quality and indispensable amino acids requirements

In order to meet an organism's AA requirements, not only the protein 'quantity' matters. In fact, it is the protein 'quality' rather than the 'quantity' that more directly affects dietary protein utilization. Thus, the protein 'quality' should be considered as a touchstone in the quest of

optimizing dietary AA utilization, to maximize protein accretion and to make the most of the fish larvae growth potential. The protein 'quality' is not easy to define; it should not be seen as a 'single' figure (Bender, 1982) and it is an evolving concept. It was long suggested to be inferred from indispensable amino acids (IAA) profiles (in particular, the quantity and availability of the most limiting IAAs), the *in vivo* and *in vitro* bioavailability of protein and IAA, as well as the efficiency of protein utilization measured as biological evaluation for the target species (through growth and nutrient metabolism studies) (Bender, 1982; Pike, 1991). The Food and Agriculture Association and the World Health Organization (FAO/WHO) have recently stated that the evaluation of protein quality for human nutrition shall be based on the relative digestible content of the IAA and the AA requirement patterns (Lee et al., 2016; Wolfe et al., 2016). This highlights the importance of focusing on dietary protein digestibility, IAA bioavailability and AA requirements when evaluating dietary protein quality, and may well be transposed to the context of dietary formulation for animal nutrition, including fish larvae.

Indispensable amino acids (IAA) refers to all the AA that an organism cannot synthesize on its own (or cannot synthetize in sufficient amounts) and must be provided through the diet, in the form of protein or mixtures of AA. In contrast, the so called dispensable amino acids (DAA) can be synthesized de novo from α-keto acids in the TCA-cycle or through transamination (Cowey and Walton, 1989). It is long known that fish require the same IAA as most other animals (Ketola, 1982; NRC, 2011; Wilson, 1989). It is also known that fish larvae have a tight control of AA metabolism, being able to spare IAA for growth purposes, preferably using dispensable AA for energy production (Rønnestad and Conceição, 2005). Hence, the dietary IAA:DAA ratios may have an impact on larval growth and nitrogen metabolism, similarly to what has been shown in fish juveniles (Alami-Durante et al., 2010; Green et al., 2002; Peres and Oliva-Teles, 2006). Furthermore, any imbalance between AA supply and AA utilization for protein synthesis will always result in increased AA lost for energy production, even though protein turnover may compensate for AA imbalances in postprandrial metabolism (Conceição et al., 2011). Therefore, regardless the type of formulation (based on semi-purified or practical ingredients), it is mandatory to assure the supply of dietary protein in the right quantity and with a balanced AA profile in order to meet the species IAA requirements.

Formulating cost-effective feeds meeting fish AA requirements can represent a challenge, and different approaches have been followed in order to generate reliable estimates of AA requirements for different species, to be taken as reference in commercial feed formulations. The AA requirements of juvenile and adult fish are traditionally determined by dose-response studies using weight gain, protein or IAA deposition as response parameters or by direct or indirect AA oxidation studies (NRC, 2011). However, these methods are difficult to apply to

larvae due to the difficulties in using formulated inert microdiets or in manipulating the protein profile of live feed (see section 1.3.1).

Larval IAA composition remains stable regardless the dietary protein content (Fiogbé and Kestemont, 1995). Whole-body IAA profiles is hence used as an indicator of larvae requirements and is considered a valuable mean to reach a rough determination of larval IAA requirements (Conceição et al., 2011; NRC, 2011). The whole-body IAA profiles at different developmental stages should be taken as reference in species that undergo a marked metamorphosis, such as Senegalese sole, on the account of the ontogenic physiological and morphological changes that may affect the whole-body IAA profile (Aragão et al., 2004a). The use of whole body IAA profile as a method to estimate IAA requirements may be improved if differences in the bioavailability of individual IAA are taken into account (Conceição et al. 2003a; 2003b; 2007b). However the studies evaluating the bioavailability of individual IAA in fish larvae are scarce (Conceição et al., 2003b; Saavedra et al., 2007) and it is largely unknown whether these change with fish species, ontogeny or even diet composition (Conceição et al., 2011). The comparison between the larvae whole-body IAA profile and that of larval feed (live or inert) may be used for detecting possible dietary imbalances (Conceição et al., 2011).

The whole-body AA profile can also be used to define the ideal dietary IAA profile for a given species, i.e., the qualitative AA requirements for growth (Conceição et al., 2003a). The ideal dietary AA profile can be determined as the contribution of each IAA to the larvae whole body IAA profile (or the A/E ratios, i.e. IAA/total IAA × 1000 (Arai, 1981) It is a concept that has been at the basis of many estimates for the “ideal protein” AA profile published for several species (Aragão et al., 2007; Conceição et al., 2003a; Furuya et al., 2004; Green, Hardy, 2004; 2008; Peres, Oliva-Teles, 2009; Rollin et al., 2003). The “ideal protein” as concept has been defined as the AA profile that meets exactly the requirement of the animal with no excess or deficit (Emmert and Baker, 1997; Wang and Fulller, 1989). According to this concept, all dietary IAA should be balanced to be equally limiting.

In the context of feed formulation for fish, three basic approaches exist for meeting digestible IAA requirements: increasing the total protein level; using a combination of different protein sources with different AA profiles; or using crystalline amino acids (CAA) as a supplement (NRC, 2011). These last two approaches allow for the correction of dietary AA profile without increasing the total protein level which could lead to altered AA metabolism, decreased efficiency of dietary protein utilization, and water quality deterioration.

The correction of dietary AA profiles using moderate levels of CAA is a common practice in the formulation of practical aquafeeds (Espe et al., 2007; Silva et al., 2009), for being practical and relatively inexpensive for same IAA (i.e., Lys and Met). Yet, several studies showed that fish

may not use CAA as efficiently as protein bound-AA due to fast and unsynchronized absorption, leading to a great proportion of CAA being lost to catabolism and to reduced somatic growth (Dabrowski et al., 2003; 2010; Peres, Oliva-Teles, 2005; Schumacher et al., 1997; Yamada et al., 1981a, 1981b). This problem may be further complicated, particularly in microdiets, as it may also depend on the dietary ingredient matrix and life stage (Dabrowski et al., 2003), dietary pH (Murai et al., 1981), water stability of the diet and feeding behavior. Still, the supplementation of microdiets with encapsulated CAA has shown positive results in some 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. Such results suggest that this approach is a possible solution for meeting IAA requirements in fish larvae, provided that an effective technique to reduce the solubility and absorption rates of the CAA is available.

1.3.2.2 Protein complexity and digestibility

Proteins are complex molecules varying in size, structure, solubility, and, as a result, digestibility. Since protein are absorbed as FAA or and di or tri-peptides (Rønnestad and Morais, 2008), the digestibility of proteins depends on the extent to which they can be hydrolysed into FAA. Thus, protein digestibility affects directly the way different dietary protein sources allow meeting an organism's AA requirements (NRC, 2011).

Given the wide range of protein sources, the digestibility of dietary protein is considerably variable amongst the ingredients available to be included in fish feed formulation (NRC, 2011). The digestibility of dietary protein is the sum of the digestibility of all the proteins comprising the feed ingredients (NRC, 2011). Hence, processing feed ingredients to partially hydrolyze or remove proteins that are difficult to digest improves its digestibility. The evaluation of dietary protein digestibility, either determined *in vitro* (using an extract or a mixture of proteolytic enzymes available in fish gut) or *in vivo,* directly (estimated from the nitrogen content of ingested feed and the excreted nitrogen, through gills, urine and feces) or indirectly (using an inert tracer, such as chromic oxide or yttrium oxide), is usually a first step for screening new and alternative ingredients to be included in inert diets for fish (NRC, 2011). However, these traditional methods for evaluating bioavailability are either not accurate enough or are too difficult to apply to marine fish larvae, mostly due to their small size, the poor acceptance of microdiets and resulting low or variable ingestion, difficulties in determining feed intake and technological problems associated with the microdiets production process, such as high losses of inert or radioactive labels (Conceição et al., 2007a; 2010b).

The digestibility of a given protein source obviously depends also on the organism's digestive capacity, which in altricial fish is species-specific and stage-specific, corresponding to the

development/ maturation of a functional digestive system (Conceição et al., 2011; NRC, 2011; Zambonino Infante and Cahu, 2001). At the onset of exogenous feeding, altricial larvae have a rudimentary digestive tract with no functional stomach or well-differentiated gastric glands (Zambonino Infante and Cahu, 2001). Due to the lack of hydrochloric acid and pepsin-secreting cells, the preparatory gastric denaturation of ingested proteins is absent in these larvae. Yet, Cahu and Zambonino Infante (1997) and Zambonino Infante and Cahu (2001) suggested that the lack of a stomach does not hinder enzymatic protein digestion in fish larvae, since pancreatic (trypsin, chymotrypsin) and intestinal (Leu-Ala peptidase, alkaline phosphatase and aminopeptidase) enzymes are highly active and seem to allow achieving good growth and survival throughout the metamorphosis. Still, altricial larvae have difficulties in digesting diets based on complex proteins such as fish meal. As compared to denatured proteins, complex or native proteins present a smaller surface area for alkaline enzymatic attack, which may reduce the efficiency of pancreatic and intestinal hydrolysis (Conceição et al., 2011). That is further accentuated due to the short transit time in early stage larvae (Govoni et al., 1986; Rønnestad et al., 2007a). However, a very high level of peptidase activity was detected in early developmental stages of several species, (Zambonino Infante and Cahu, 2001), including Senegalese sole reared upon a co-feeding regime with inert microdiet (Engrola et al., 2009). Moreover, membrane transporters that are specific for oligopeptides, such as PepT1, have been detected in zebrafish (*Danio rerio*) (Verri et al., 2003) and in Atlantic cod (Amberg et al., 2008; Rønnestad et al., 2007b), even before the onset of exogenous feeding. In fact, Artemia, which is a highly digestible protein source for most marine larvae and also for Senegalese sole (Morais et al., 2004b) has a high content of low MW peptides (Rønnestad and Conceição, 2012). These conditions suggest that, despite the limiting proteolytic capacity, early larvae have the capacity for efficiently digest and absorb dietary protein, provided that protein complexity is adapted to its proteolytic capacity. Thus, the provision of protein in the form of hydrolysates could be an effective vehicle to satisfy the AA requirements of early stage altricial larvae. In fact, Tonheim et al. (2005) showed that highly hydrolysed (<1.4KDa) and partially-hydrolysed (10-75 KDa) proteins were absorbed 3 and 2.2 times as fast, respectively, as intact protein (>65KDa) within the first 2 hours after tube-feeding pre-metamorphic Atlantic halibut larvae. Accordingly, moderate inclusions of hydrolysed protein promoted larval growth and survival in several fish species (Carvalho et al., 2004; Kolkovksi and Tandler, 2000; Kvåle et al., 2009; Kvåle et al., 2002; Srichanun et al., 2014; Zambonino Infante et al., 1997).

Throughout development, as gut matures, the enterocytes that line the digestive tract begin to produce a large complement of other enzymes, which increases the efficiency of proteolysis (Zambonino Infante and Cahu, 2001). On the other hand, older larvae have a slower gastrointestinal transit, which contributes for a more efficient digestion and absorption (Govoni

et al., 1986; Rønnestad et al., 2007a; Werner and Blaxter, 1980). The maturation of the digestive system and increased digestive capacity should allow for the inclusion of more complex protein and thus a greater variety of dietary protein sources. However, information on the maximum complexity that each larval stage is able to properly digest remains scarce for most marine species. That is an issue of paramount importance because the degree of hydrolysis is linked with protein water solubility (Carvalho et al., 2004), meaning that the more hydrolysed a protein is (e.g. based on FAA or small peptides) the more leachable will be. As already mentioned (in section 1.3.1), leaching losses of water soluble nutrients was until recently one of the major obstacles in formulating microdiets for fish larvae and in particular for post-settled Senegalese sole that, being a bottom feeder, approximately takes one minute to reach a pellet and feed (Conceição et al., 2007b; Dinis et al., 2000). On the other hand, a high inclusion of extensively hydrolysed protein (mostly based on oligo, di or tri-peptides) was suggested to lead to over loading and saturation of the peptide transport system in the intestinal brush border membrane (BBM) or to impaired utilization of the fast absorbed FAA and di or tri-peptides, further compromising growth (Zambonino Infante et al., 1997; Cahu et al., 1999, 2004; Kolkovksi, Tandler, 2000; Carvalho et al., 2004;; Kvåle et al., 2002, 2009; Liu et al., 2006; Srichanun et al., 2014). Thus, establishing the optimum dietary protein complexity to match the maturing digestive capacity, in order to optimize digestion, absorption and retention of dietary AA at each developmental stage would be a way to improve microdiets protein utilization in altricial larvae that would certainly improve protein accretion and somatic growth.

Senegalese sole, as most altricial species, has a poor capacity to digest complex protein in early developmental stages (Engrola et al., 2009, 2010; Gamboa-Delgado et al., 2008). However, Richard et al. (2015) recent work with Senegalese sole, have shown that, whereas 1.0KDa oligopeptides are highly digestible and its retention efficiency is constant throughout development, the digestibility and body retention of larger polypeptides (6.8KDa) are poor in pre-metamorphic larvae – 12DAH, but improve throughout development (from 12 to 34DAH). These results support the idea that more complex protein may be incorporated into the microdiets, following the maturation of the digestive system and subsequent increasing proteolytic capacity. The impact of dietary protein complexity on the efficiency of protein utilization has been recently studied at short-term, in a tracer study using the tube-feeding technique combined with the use of metabolic chambers (Richard et al., 2015). Future experiments may further explore the concept through long-term responses.

1.4 Improving the utilization of dietary protein: tracers studies and the regulation of the digestive capacity

The assessment of fish larval nutritional requirements has been hampered due to the organism's small size, the fact that most species do not grow well on inert microdiets and difficulties to determine feed intake and diet digestibility (Conceição et al., 2007a). To overcome these difficulties, tracer methodologies have been intensively used in recent larval nutrition studies (e.g., Morais et al., 2006, 2007; Morais and Conceição, 2008; Saavedra et al., 2008a; 2008b; Engrola et al., 2009; 2010; Pinto et al., 2010a; 2010b; Hamre et al., 2011; Navarro-Guillén et al., 2014; Richard et al., 2015; Rocha et al., 2016)

Tracer methodologies are used to quantify *in vivo* feed intake, digestion, absorption and utilization of nutrients in fish larvae, settled on the principle that the tracer mimics the target nutrient (tracee), reflecting the metabolic fate of the actual dietary nutrient. Thus a suitable tracer should be chemically identical to the molecule of interest (the tracee), but carrying some distinctive characteristic that enables its accurate quantification (Wolfe, 1992). Isotopic tracers have one or more of the naturally occurring atoms in the tracee molecule replaced in a specific position(s) by an isotope of an atom with a less common abundance. Both stable (e.g., containing ^{13}C or ^{15}N) and radioactive tracer molecules (e.g., containing ^{14}C, ^{35}S or ^{3}H) are available. However, besides being expensive and time-consuming, the stable isotopes low detection sensitivity limits their utilization in larvae. Thus, despite the handling and environmental risks associated with the use of radioactive tracers, its detection sensitivity makes them much more suitable to assess nutrients metabolic utilization even in very small larvae (Conceição et al. 2007a).

Amongst the several techniques using tracers that have been developed and applied to fish larvae nutrition research (reviewed in Conceição et al. 2007a; 2010), the tube-feeding technique combined with the use of metabolic chambers is the most commonly used method to assess digestibility, absorption and metabolic fate of labelled nutrients. The tube-feeding technique was firstly developed for fish larvae by Rust et al. (1993) and further modified and combined with the use of metabolic chambers by Rønnestad et al. (2001). Briefly, it consists of tube-feeding larvae with ^{14}C-labelled nutrients using a capillary fitted in a microinjector and then transfer them to single metabolic chambers containing seawater. At the end of the incubation period, through the manipulation of the seawater pH, it is possible to promote the diffusion of the CO_2 out of the seawater into a CO_2 trap and therefore distinguish between the unabsorbed and the catabolised nutrients. Since it allows delivering the studied nutrients directly into the larval gut, this method overcomes the difficulties of measuring feed intake and the constraints associated with the microdiets production process, such as high losses of water

soluble low molecular weight nutrients and the difficult labelling with inert, stable or radioactive labels (Conceição et al., 2007a). Due to the handling stress imposed to the larvae when using this technique, it should be kept in mind that the rates of nutrient digestion, absorption and utilization may be altered (Conceição et al., 2007a; 2010b). So the results of these trials do not necessarily represent the digestive and metabolic performance of an undisturbed larvae feeding *ad libitum* in a culture system. Still, when these methods are used under standardized conditions, they can serve as tools to assess and compare the performance between treatments and to study ontogenetic changes (Conceição et al., 2007a; 2010b).

The growing knowledge brought up by gene cloning and from the genome and transcriptome sequencing programs for different aquaculture produced fish species, allows for a more comprehensive understanding on how nutritional factors influence gene expression and regulate the digestive capacity (Conceição et al., 2010b).The ontogeny of the digestive system is genetically programmed, the expression of digestive enzymes following a spatio-temporal pattern of gene expression which is probably species-specific (Lazo et al., 2011). Therefore the expression of genes encoding for digestive enzymes and enzymes precursors could be used as markers for fish development (Lazo et al., 2011), enabling a better understanding of the molecular events underlying the ontogeny of enzymatic activity and digestive capacity.

The nutritional condition of fish larvae may be reflected in the expression patterns of genes encoding for digestive enzymes throughout development, as it has been suggested in recent research (Cai et al., 2015; Sánchez-Amaya et al., 2009; Srichanun et al., 2014; Vagner et al. 2007; Wang et al., 2006). The expression of genes encoding for intermediary gluconeogenic enzymes was shown to change in response to dietary lipids, but the enzymatic activity remained unchanged (Ducasse-Cabanot et al., 2007). These findings reinforce the idea that unraveling the molecular mechanism underlying the digestive system ontogeny and digestion would expand the knowledge of larval physiology and provide a further insight into some physiological and biological responses to dietary changes. Besides understanding the nutritional regulation of genes encoding for digestive enzymes, the cloning/sequencing and expression of genes related with the utilization of dietary nutrients, such as membrane transporters, may also contribute for a better perception of the animal's physiological adaptation to different dietary formulations.

1.5 Somatic growth potential and muscle growth: muscle cellularity and the regulation of myogenesis

The skeletal muscle characteristics and the way muscle tissue grows are major factors influencing overall growth capacity in fish, as in mammals and poultry (Chang, 2007; Johnston, 1999; 2001). Growth and muscle function are mainly related to fiber-type composition, that

determines fiber contractile speed (slow vs. fast) and metabolism (oxidative vs. glycolytic) (Johnston, 2001). Red fibers, also named slow-twitch fibers, have slow contraction speeds, high mitochondrial and myoglobin content, a rich blood supply and an aerobic metabolism. These fibers are used in sustained swimming movements (Bone, 1978). White fibers, also known as fast-twitch fibers, have fast contraction, large diameters, low mitochondrial and myoglobin content, a poor blood supply and a preferential anaerobic metabolism (Bone, 1978). These fibers are used for burst swimming movements. Pink fibers have intermediary characteristics and operate at intermediate swimming speed (Scapolo and Rowlerson, 1987). In fish, red and white muscle fibers are arranged in discrete anatomical regions (Bone, 1978; Mascarello et al., 1995; Moutou et al., 2005; Silva et al., 2008; Campos et al. 2013) largely located in the hypaxial and epaxial muscles along the length of the body and comprise 40% to 60% of body mass (Reviewed by Valente et al., 2013). Pink fibers are located between the red and the white fibers (Scapolo and Rowlerson, 1987).

The process whereby the skeletal muscle lineage is determined and develops into mature muscle fibers is called myogenesis and it seems to be a conserved process amongst fish, mammals and poultry (Rehfeldt et al., 2011; Valente et al., 2013). The specification of stem cells to a myogenic lineage (myogenic progenitor cells – MPCs) involves its activation, proliferation, cell cycle exit, differentiation, migration and fusion (Johnston, 2006; Valente et al., 2013) (Fig.2). 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, Myf-5, myogenin* and *MRF-4*) (reviewed by Rescan (2001)). *MyoD* (myoblast determination factor) and *Myf-5*, also called primary MRFs, are required for the commitment of myoblasts to form the MPC population. The MPCs are then activated by hepatocyte growth factor/scatter factor and committed to final differentiation. Active MPCs undergo a proliferation phase, being controlled by positive (e.g. peptide growth factor) and negative (e.g. *myostatin*) signaling pathways. Following proliferation, the MPC progeny enter the differentiation program that will lately result in myotube formation and enlargement, involving the expression of the so-called secondary MRFs (*myogenin*, *MRF-4*) and also the *MEF2* gene family members.

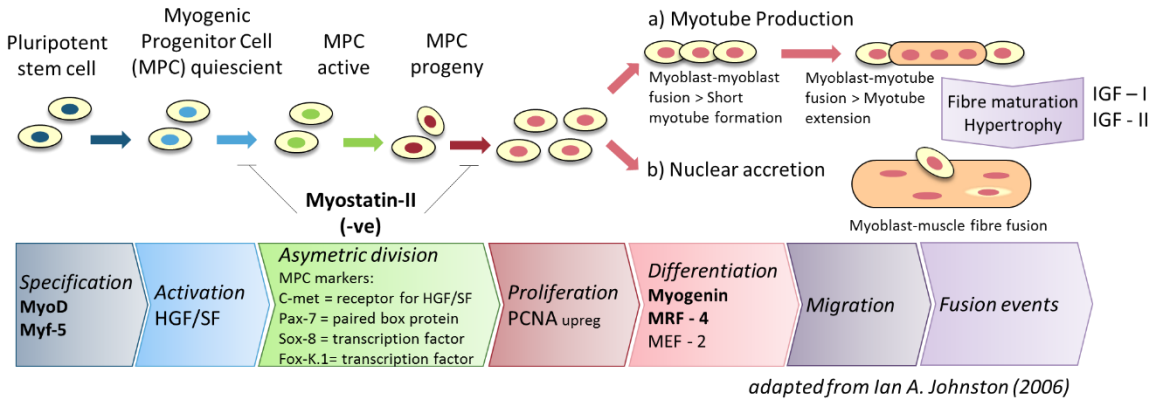

Figure 2: Schematic description of the main events of myogenesis in teleost skeletal muscle (adapted from Johnston (2006).

The MPC progeny can migrate through the muscle and either fuse in myoblast-myoblast or myoblast-myotube fusion events (Fig.2A) or fuse with mature muscle fibers (myoblast-muscle fiber fusion) in order to add nuclei to the growing muscle fibers (Fig.2B) (reviewed by Johnston (2006)). Once formed, the myotubes mature into muscle fibers (Fig.2), and produce muscle-specific contractile proteins, such as myosin heavy chain (MyHC) and myosin light chain (MyLC) (reviewed by Goldspink et al. 2001). The regulation of fiber size is thought to be controlled by signaling pathways involving insulin-like growth factors I (IGF-1) and II (IGF-II) (Johnston, 2006).

In teleosts, myogenesis occurs in three distinct phases (reviewed by Valente et al., 2013). During the embryonic phase, the primary myotome is formed in the embryo: medial paraxial mesoderm cells differentiate into a superficial layer of slow (or red) muscle fibers (or adaxial cells); subsequently, the deep fast (or white) muscle fibers differentiate from posterior somite cells. Anterior cells of the somite then migrate to the lateral surface of the primary myotome and contribute muscle progenitor cells for primary myotome expansion/growth (reviewed in Stellabotte and Devoto, 2007). Stratified hyperplasia is the main mechanism responsible for the increase of muscle fibers number after hatching, and involves the recruitment of new fibers in discrete germinal zones found in the lateral margins of the myotome (Rowlerson and Veggetti, 2001). This phase is crucial to provide larvae the capacity for cruise swimming and search for exogenous food, but according to Valente et al (2013) there is no clear relation between the start of stratified hyperplasia and the onset of exogenous feeding. In Senegalese sole, and according to Campos et al. (2012), stratified hyperplasia starts during pre-metamorphosis stage (8DAH, 0.41 cm) and lasts up to 30DAH in larvae measuring approximately 1.3 cm. In a third and last phase called mosaic hyperplasia, new myotubes form

on the surface of existing muscle fibers, further fusing to form new muscle fibers or adding nuclei to the larger already existing fibers, to keep size of nuclear domains constant during hypertrophic growth (Rowlerson, Veggetti, 2001). Mosaic hyperplasia is the main mechanism for expanding fiber number in the juvenile and adult stages of most species. The duration of this phase can largely vary among fish species (Valente et al., 2013). In Senegalese sole this phase was reported to start by 22DAH, in larvae with 0.85 cm (Campos et al., 2012), but no study has still reported its end point. From a certain stage onwards, growth exclusively consists of fiber enlargement (hypertrophy). Thus, throughout the lifecycle of fish, both hyperplasia (formation of new fibers, Fig.2) and hypertrophy (increase in size of existing fibers, Fig.2B) contribute to muscle growth. The relative contribution of hyperplasia and hypertrophy in fish was shown to be related to the 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. Muscle cellularity studies focus on the number and size distribution of the different muscle fibers within a body or muscle section. This classical approach has been extensively used to characterize skeletal muscle phenotype and the way it grows, namely by determining the relative contribution of hyperplasia to muscle growth under different rearing conditions (Alami-Durante et al., 2010; 1997; Campos et al., 2013a; 2013b; Galloway et al., 1999; Johnston et al., 1999; 2004; López-Albors et al., 2003; Silva et al., 2009; 2011; Valente et al., 1999).

The process of myogenesis (both hyperplasia and hypertrophy) seem to be controlled either by intrinsic - genotype (Johnston et al., 1999; Valente et al., 2006) - and extrinsic factors, such as photoperiod (Johnston et al., 2004; Lazado et al., 2014), temperature (Campos et al., 2013b; 2013c; Galloway et al., 2006; Silva et al., 2011) and nutrition (Aguiar et al., 2005; Alami-Durante et al., 1997; Valente et al. (2016)). In fish, most of the studies on myogenesis focus on the effects of either environmental conditions or fasting and malnutrition (Valente et al., 2013), whereas the influence of dietary macronutrients on muscle development and growth is still rudimentary. Myoblast differentiation and the regulation of myotube maturation and hypertrophy seem to be controlled by signaling pathways involving insulin-like growth factor (IGFI and IGFII), so the mTOR-IGF axis was suggested as a likely molecular link between nutrition and skeletal muscle development (Seiliez et al., 2008). Recently, Yoon and Chen (2013) showed that mTOR is a serine/threonine protein kinase that functions as a master regulator of cell growth, proliferation and various types of cellular differentiation, including skeletal myogenesis. IGFII transcription, which is required for myoblast differentiation, is controlled by mTOR pathway that depends on the availability of AA (Yoon, Chen, 2013). Thus, it is likely that the AA composition of fish larval diets exerts an influence on muscle development. During the embryonic and larval stages, fish muscle plasticity in response to extrinsic factors is usually irreversible due to the rapid pace of ontogenetic change, having

potential consequences for larval survival as well as long-lasting effects on fast-twitch muscle cellularity (Rehfeldt et al., 2011). Thus, variations in dietary protein during early larval stages may induce changes in the myogenic processes having long-term irreversible effects on somatic growth. The selection of best diets during these early stages might hence be a promising tool to conditioning larvae further growth.

In Senegalese sole, the effect of different incubation and rearing temperatures on muscle cellularity and the expression of genes regulating myogenesis was evaluated throughout ontogenetic development (Campos et al., 2013b; 2013c). These studies allowed identifying critical developmental time windows in which environmental variations induced changes in the regulation of myogenesis, exerting a long-term effect on muscle cellularity and somatic growth. Campos et al. (2013b; 2013c) provided a reference for the time windows prone to environmental variations and the target genes to be possibly used as markers of myogenesis to be used in the optimization of rearing conditions for Senegalese sole.

1.6 Dietary protein and Epigenetics

Epigenetics studies heritable traits (or “phenotypes”) that result from potentially reversible molecular modifications in DNA and chromatin, though not involving alterations in the underlying DNA sequence (Berger et al., 2009). Such modifications occur throughout the life course and can be heavily influenced by external factors (Anderson et al. 2012). Thus, external effects on the epigenome may alter gene expression, potentially giving rise to phenotypic disparity. Epigenetic modifications include chromatin remodeling, histone tail modifications non-coding RNA and microRNA gene regulation and DNA methylation (Anderson et al. 2012).

DNA methylation is one of the best studied mechanisms of epigenetic modification of DNA (Berger et al., 2009). It is a biological process that results in the addition of methyl groups to DNA and it contributes to the epigenetic network that controls the gene expression, by silencing the expression of germline-specific genes and repetitive elements (Zhang et al. 2015). In mammals, DNA methylation is an essential regulator of embryogenesis, as dynamic changes in the methylation patterns prepare embryos for further differentiation (Reik et al., 2001). Two main waves of genome-wide epigenetic reprogramming occur in mammals, affecting pre-implantation development and primordial germ cell formation (Reik et al., 2001). In fish, there is also a dynamic change in methylation of the embryonic genome: the embryonic genome is globally demethylated after fertilisation, but re-methylation increases rapidly and is re-established during the gastrula stage (Mhanni, McGowan, 2004).

Increasing evidence indicates that DNA methylation is labile in response to nutritional cues (Anderson et al., 2012; Zhang, 2015). DNA methylation relies on the one-carbon metabolism pathway, which is dependent upon the activity of several enzymes in the presence of dietary methyl donors, such as folate, choline, betaine, vitamins B2, B6 and B12 and methionine (Fig.3) (Anderson et al., 2012; Zhang, 2015). In the one-carbon cycle, methionine is converted into S-adenosylmethionine (SAM), the universal cellular methyl donor (Selhub, 1999). DNA cytosine methyltransferases (dnmts) covalently attach SAM methyl groups to the 5′-position of cytosine (in CpG dinucleotides), thus methylating DNA and repressing transcription. There are three DNA cytosine methyltransferases with different functions. DNMT3A and DNMT3B are responsible for addition of methyl group de novo (Okano et al., 1999), whereas DNMT1 is responsible for maintenance of DNA methylation patterns (Detich et al., 2001).

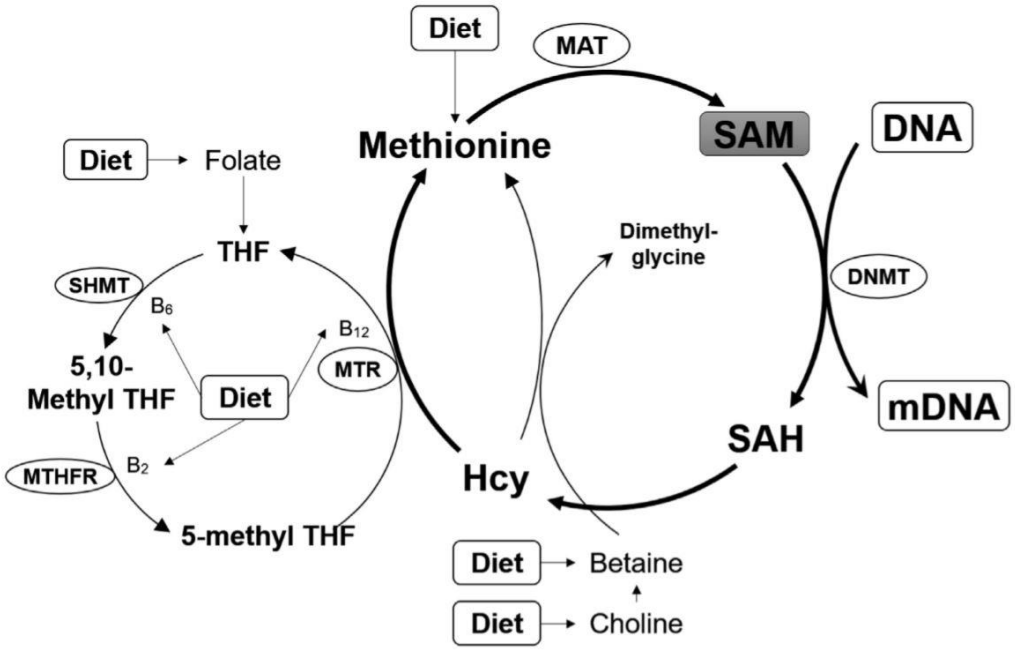

Fig. 3. Possible ways that nutrition influences patterns of DNA methylation (Revised from McKay and Mathers, 2011, adapted from Zhang, 2015). MAT = methionine adenosyltransferase; SAM = S-adenosyl methionine; SHMT = serine hydroxymethyltransferase; THF = tetrahydrofolate; DNMT = DNA methyltransferase; MTR = 5-methyltetragydrofolate-homocysteine methyltransferase; MTHFR = methylentetrahydrofolate reductase; Hcy = homocysteine; SAH = S-adenosylhomocysteine; mDNA = methylated DNA.

The reduced availability of methyl donors should result in low SAM synthesis and global DNA hypomethylation, and vice versa. Information about the effects of methy-donors on DNA

methylation is available from several studies with rodent models (Pogribny et al., 2008, 2009; Waterland, 2006, 2008).

As the formulation of dietary protein may lead to changes in the dietary protein absorption and body retention (as discussed in sections 1.2 and 1.3.1), it may subsequently lead to changes in the availability of methyl-donors, such methionine, with possible consequences on the DNA methylation and regulation of gene expression in different tissues. In rodents, maternal low protein was shown to increase the expression of IGFII in the liver of offspring, via altered DNA methylation and *dnmt1* and *dntm3a* expression (Gong et al., 2014). In pigs, maternal dietary protein restriction and excess affected the *dnmt3a* and *dnmt3b* transcription, global methylation, and the expression of condensin I subunit genes in the offspring's liver. It has simultaneously affected the *dnmt3a* transcription and the expression of condensin I subunit genes, in the offspring's skeletal muscle (Altmann et al., 2012). It was not reported whether the amount of methyl-donors is reduced in these specific studies, but it is commonly accepted that protein restriction or under-nutrition correlates with reduced methyl-donor availability.

Studies on the epigenetic modulation of DNA methylation by nutrition are a recent trend in fish and up to now no published literature is available. However, 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 fast-twitch muscle cellularity of Senegalese sole during metamorphosis climax (Campos et al., 2013a), and influenced subsequent somatic growth in later stages (Campos et al., 2013b). Campos et al. (2013a; 2013b) results suggest the pelagic phase in sole as a critical developmental time window prone to epigenetic modifications with long-lasting effects on the regulation of myogenesis and subsequent influence on the potential for growth.

1.7 Objectives

This thesis aims to contribute for establishing optimal dietary protein quality for Senegalese sole larvae, using practical microdiets, in an attempt to overcome some of the current problems in this species larviculture, such as the difficult early adaptation to inert diets and highly variable growth rates. Different diet formulation strategies were employed in an attempt to improve Senegalese sole larvae capacity to utilize and deposit protein throughout metamorphosis, so to further maximize growth potential.

In **Chapter 2**, dietary protein quality was manipulated according to the ideal protein concept, i.e. by adjusting the dietary AA profile to the larval body AA profile, in an attempt to meet Senegalese sole ideal IAA profile. This first study aimed to improve the larvae capacity to digest and retain dietary protein and thus promote somatic growth up to a juvenile stage. The larvae capacity to absorb and retain dietary protein was assessed throughout the metamorphosis as it seems to be a critical developmental window particularly susceptible to nutritional conditions in Senegalese sole.

In **Chapter 3**, dietary protein quality was manipulated by increasing the IAA/DAA ratio, as to improve the larvae capacity to retain dietary protein in order to promote protein accretion in skeletal muscle and consequently muscle growth. In this chapter, the expression pattern of DNA methyltransferases was analysed in order to understand whether dietary protein AA profile could induce an epigenetic effect with consequent influence on the potential for growth.

In **Chapters 4** and **5**, dietary protein complexity was manipulated to improve its digestibility, by including protein hydrolysates with different molecular weights. In **Chapter 4**, it was inquired whether the manipulation of dietary protein complexity might affect the mRNA expression of genes encoding for the precursors of proteolytic enzymes and the peptide transporter PepT1 in Senegalse sole larvae. A possible effect on the larvae capacity to digest, absorb and retain protein was also evaluated, as well as the impact on somatic growth. The larvae capacity to digest the dietary protein was indirectly addressed through the larvae capacity to digest, absorb and retain polypeptides with different MW (1.0 and 7.2KDa) representing a typical peptide MW of each of the hydrolysates included in the diets.

In **Chapter 5**, it was inquired whether changes in dietary protein complexity may affect the regulation of muscle growth during the metamorphosis climax and up to an early juvenile stage. The concept that nutrition may modulate an epigenetic event that will regulate muscle growth was analyzed through the expression pattern of DNA methyltransferases.

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Dietary indispensable amino acids profile affects protein utilisation and growth of Senegalese sole larvae

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Abstract

In diet formulation for fish it is critical to assure that all the indispensable amino acids (IAA) are available in the right quantities and ratios. This will allow to minimize dietary AA imbalances that will result in unavoidable AA losses for energy dissipation rather than for protein synthesis and growth. The supplementation with crystalline amino acids (CAA) is a possible solution to correct the dietary amino acid (AA) profile that has shown positive results for larvae of some fish species. This study tested the effect of supplementing a practical microdiet with encapsulated CAA as to balance the dietary IAA profile and to improve the capacity of Senegalese sole larvae to utilize AA and maximize growth potential. Larvae were reared at 19ºC under a co-feeding regime from mouth opening. Two microdiets were formulated and processed as to have as much as possible the same ingredients and proximate composition. The control diet (CTRL) formulation was based on commonly used protein sources. A balanced diet (BAL) was formulated as to meet the ideal IAA profile defined for Senegalese sole: the dietary AA profile was corrected by replacing 4% of encapsulated protein hydrolysate by CAA. The *in vivo* method of controlled tube-feeding was used to assess the effect on the larvae capacity to utilize protein, during key developmental stages. Growth was monitored until 51DAH. The supplementation of microdiets with CAA in order to balance the dietary AA had a positive short-term effect on the Senegalese sole larvae capacity to retain protein. However that did not translate into increased growth. On the contrary, larvae fed a more imbalanced (CTRL group) diet attained a better performance. Further studies are needed to ascertain whether this was due to an effect on the voluntary feed intake as a compensatory response to the dietary IAA imbalance in the CTRL diet or due to the higher content of tryptophan in the BAL diet.

Key words: indispensable amino acids; balanced diet; metabolism; growth; fish larvae

2.1 Introduction:

Fish larvae have a high requirement for amino acids (AA) for protein deposition (Conceição et al. 2011, Rønnestad 2003) which is the primary determinant of fish growth. An efficient protein synthesis requires that all indispensable amino acids (IAA) are present at an optimum ratio at the same time (Carter and Houlihan 2001), meaning that AA imbalances will result in AA

losses. Still, the use of some dietary AA for processes other than growth losses is unavoidable, like for energy production, transamination into other AA, lipogenesis or gluconeogenesis, synthesis of other nitrogen-containing molecules such enzymes and hormones or purines and pyrimidines (Conceição et al. 2011). Considering the high growth potential of fish larvae, AA losses due to dietary deficiencies have a larger negative impact in larvae than in juveniles (Conceição et al. 2003). Therefore the dietary protein quantity and quality supplied to early stage larvae is of paramount importance to the optimization of larval growth.

Senegalese sole (*Solea senegalensis*, Kaup 1858) is a promising flatfish species for intensive aquaculture in Southern Europe countries because of its high market value, good growth and robustness (Morais et al. 2014). A number of obstacles during larval cultivation have hampered the industry from reaching expected production levels. A major constraint is the variable success of weaning, with high mortality rates, leading to juvenile scarcity for on-growing purposes (Morais et al. 2014) Therefore and despite the significant progress from using different weaning strategies (Engrola et al. 2005, 2007), this species cultivation at a commercial scale has been so far dependent on live feed during early life stages. An early delivery of an inert microdiet in a co-feeding regime was suggested by Engrola et al. (2009a) as a possible solution to overcome the current difficulties at weaning. Regardless of several attempts made to formulate microdiets for Senegalese sole larvae, the results concerning early larval performance (e.g., survival, growth and metamorphosis completion) are still far behind those obtained with live feeds (Fernández-Díaz et al. 2006, Gamboa-Delgado et al. 2008, Yúfera et al. 2005). Therefore, in order to optimize Senegalese sole larvae growth and to promote a more successful weaning, current commercial microdiets may be improved (Engrola, unpublished results), namely on what concerns AA composition and bioavailability.

The formulation of an inert microdiet to be delivered at mouth opening implies a deep knowledge of larval nutritional physiology and a high degree of technology. It is known that Senegalese sole is a fast growing species that undergoes a complex metamorphosis that strongly affects its behaviour, feeding and digestive physiology (Conceição et al. 2007, Fernández-Díaz et al. 2001).At mouth opening, larvae do not possess a functional stomach, which indicates a strong dependence on pancreatic enzymes for protein digestion (Engrola et al. 2009a, Ribeiro et al. 1999a). During the metamorphosis the spatial organization of the digestive system in the abdominal cavity changes dramatically, which is sided by an increase of the volume available for digestion and area of absorption, as well as a change on proteolytic activity (Engrola et al. 2009a, Ribeiro et al. 1999a). Only long after the metamorphosis is completed, gastric glands are fully developed and the stomach becomes functional. This remarkable transformation during the second and third weeks of Senegalese sole life is sided

by likely changes in the IAA requirements (Aragão et al. 2004b) and live feed used in Senegalese sole standard rearing protocols (both rotifers and *Artemia* nauplii) does not meet such requirements (Aragão et al. 2004a, 2004b).

Therefore, in the present study, an inert was introduced at an early developmental stage in an attempt to meet Senegalese sole larvae qualitative AA requirements. The ideal dietary AA profile based on whole body IAA profile (Wilson and Cowey 1985) was used as a reference to formulate the protein fraction composition. The ideal dietary AA profile can be defined as the contribution of each IAA to the larvae whole body IAA profile (or the A/E ratios, i.e. IAA/total IAA × 1000, (Arai 1981) and it is a concept that has been at the basis of many estimates for the "ideal protein" AA profile published for several species (Aragão et al. 2007, Conceição et al. 2003, Furuya et al. 2004, Green and Hardy 2004, 2008, Peres and Oliva-Teles 2009, Rollin et al. 2003). According to Aragão et al. (2004b), the ingredients commonly used as native protein sources on the formulation of commercial feeds do not provide a balanced AA profile for Senegalese sole larvae, thus, in this study, crystalline amino acids (CAA) were used to correct the IAA profile in the inert diet.

The supplementation of inert microdiets with crystalline amino acids in order to balance the dietary AA profile is a possible solution for cost-effective fish-feed formulations, that has been slowly adopted by aquaculture industry possibly due to a highly variable success, namely on what concerns the efficiency with which fish can use CAA when compared to intact protein, and subsequent impact on growth (reviewed in (TNRC 2011)). However, the supplementation with CAA as to balance dietary AA profile has shown positive results for some altricial larvae, such as white bream *Diplodus sargus* (Saavedra et al. 2009a, 2009b) 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 shown to improve the retention of a ^{14}C-labelled protein hydrolysate in an *in-vivo* tube-feeding trial, suggesting a positive impact of a balanced dietary AA profile on nitrogen utilisation and growth (Aragão et al. 2004c). However, no feeding trial was ever conducted to test the effect of correcting the dietary AA profile with CAA on the larvae capacity to utilize protein and direct it for growth.

The metamorphosis period is a time window particularly susceptible to nutritional conditions in this Senegalese sole (Pinto et al. 2010, Villalta et al. 2008 , Yúfera et al. 2005). Different diets induced changes in the capacity of metamorphosing larvae to digest and utilize protein, which reflected on somatic growth (Engrola et al. 2010, 2009b). Thus, in the present study, it was hypothesized that supplementing microdiets with CAA in order to meet a balanced dietary AA profile would improve the larvae capacity to digest and retain protein during metamorphosis,

having a positive long-term effect on somatic growth. A growth trial was performed, together with a tracer study using the tube-feeding technique (Rust et al. 1993) modified by Rønnestad et al. (2001) at key developmental stages. Using this combination of methods, the present study assessed to what extent the digestibility and metabolic utilisation (AA retention and AA catabolism) of polypeptides with different MW (1.0 KDa, 6.8KDa) can explain differences in growth performance.

2.2 Material and Methods

2.2.1. Husbandry and experimental set-up

The experiment was done at the research facilities of the CCMAR/University of Algarve, Portugal Fertilized eggs were provided by Sea8, Safiestela, Póvoa do Varzim, Portugal.

Eggs were incubated in an upwelling incubator at 19ºC and hatching was completed within the next day. Newly hatched larvae were evenly placed in each of 6 white cylindro-conical tanks (100L) in a semi-closed recirculation system with a density of 100 larvae L−1 (10,000 larvae/tank). The system was equipped with a mechanical filter, a submerged and a trickling biological filter, a protein skimmer and UV sterilizer. Larvae were reared in green water conditions until 25 days after-hatching (DAH) (SL=8.22mm; DW=1.46mg), 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 monitored daily. Dissolved oxygen in water was maintained at 90.9±7.9% of saturation, temperature at 18.9±0.4ºC and salinity at 36.8±0.1‰. A 10/14h light/dark photoperiod cycle was adopted and a light intensity of 1000lux was provided by overhead fluorescent tubes. After settling (25DAH) larvae were transferred to flat-bottom tanks (30×70×10cm; 21L), each tank stocking 635 individuals (corresponding to a 3024ind/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 oxygen in water was maintained at 92.3±4.4% of saturation, temperature at 21.5±0.9ºC and salinity at 39.4±3.1 ‰. A 10/14h light/dark photoperiod cycle was adopted and the light intensity was 400lux at water surface.

2.2.2 Feeding protocol

The two treatments (inert microdiets formulated as to have different IAA profiles) were randomly assigned to tanks (n = 3 replicates 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} and the respective inert diet (200-400μm). 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 at 5DAH. Artemia EG meta-nauplii (M24) (EG SEP-ART Cysts, INVE, Belgium) enriched with Easy DHA Selco were introduced at 12DAH, increasing from 12 to 14 M24·mL−1 until 19DAH. Enriched frozen *Artemia metanauplii* were offered to settled larvae (between 19 and 25DAH). Live prey was gradually reduced and substituted by inert diet until complete weaning at 38DAH, according to Engrola et al. (2009b), with the inert diet constituting 60% of the feed supplied (dry matter basis) from 8 to 20 DAH, 75% from 20 to 26DAH and more than 75% from 27 to 38DAH. After 39DAH, larvae were exclusively fed with inert diet and considered weaned.

Feeding was always done close to satiation 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). Live prey was delivered 3 times a day (3h interval) during the pelagic phase and 4 times a day (2h30 interval) during the benthic phase. Inert diet was delivered semi-continuously with automatic feeders (cycles of 2 h of feeding followed by 1h break). First live feed meal was offered 1hour after the lights were on (11.00h) during the pelagic phase and 30 min (9.30h) after during the benthic phase. The amount of microdiet supplied daily to each tank was the same daily and for both treatment groups. The amount of microdiet supplied increased from 0.22 mg/larva/day at 2 DAH, to 0.79 mg/larvae/day at 19 DAH, to 9.39mg/larva/day at 39 DAH, and 16.19 mg/larva/day at 51 DAH.

2.2.3 Feed manufacturing and quality analysis

The compositions of both diets and processing details are provided in Table 1. The diets were formulated and processed by SPAROS Lda. (Olhão, Portugal) to be isonitrogenous (crude protein 60% dry matter), isolipidic and isoenergetic and formulated with the same practical ingredients. Only part of the protein fraction was manipulated to result in different IAA balances (Table 1). The CTRL diet was formulated to have a protein content based on native protein and a fish protein hydrolysate – CPSP90® (Sopropêche, France) (Table 1), what would have resulted in an imbalanced dietary AA profile according to (Aragão et al. 2004b). A second diet (BAL) consisted in replacing 4% of the encapsulated CPSP90® fraction in diet CTRL with encapsulated crystalline-FAA, in an attempt to correct the dietary IAA profile according to the ideal dietary AA profile as suggested by Aragão et al. (2004b).

Table 1: Composition and proximate analyses of the experimental diets

	Diets	
	CTRL	BAL
Ingredients (% dry matter)		
Whey Protein isolate [a]	10	10
FPH - non encapsulated[b]	10	10
Squid meal[c]	8.5	8.5
FPH encapsulated[b]	20	16
Fish Gelatine[d]	14	14
Autolysed yeast[e]	1.7	1.7
Krill Meal[f]	9.0	9.0
Fish oil[g]	4.5	4.5
DHA-rich oil [h]	2.0	2.0
Copepod oil [i]	5.5	5.5
PVO40.01 Premix[j]	5.0	5.0
Vit C[k]	3.0	3.0
Vit E[l]	1.0	1.0
Taurine (Sigma)[m]	1.5	1.5
Chitosan[n]	3.3	3.3
Mono Ca Phosphate[o]	1.0	1.0
L-Arginine HCl[p]		0.25
DL-Methionine[p]		0.75
L-Lysine HCl[p]		0.25
L-Histidine HCl[p]		0.15
L-Leucine[p]		1.00
L-Isoleucine[p]		0.30
L-Phenylalanine[p]		1.30
Proximate analyses (% dry matter)		
Crude protein	60.9	60.1
Crude fat	14.6	13.6
Gross Energy (MJ kg^{-1})	17.5	17.6

[a]Isolate Crystal Whey. Weider. USA;
[b]Fish protein hydrolysate - CPSP 90®. Sopropêche. France;
[c]Super prime without guts. Sopropêche, France;
[d]Fish edible gelatine. Lapi Gelatine. Italy;
[e]Hylisis. ICC. Brazil;
[f]Qrill. Aker Biomarine. Norway;
[g]Marine oil omega 3: Henry Lamotte Oils GmbH. Germany;
[h]DHA 70% Algatrium, Brudy Technology SL., Spain;
[i]Phosphonorse, K/S Tromsø Fiskeindustri A/S & Co., Norway;
[j]PVO40.01 Premix for marine fish, PREMIX Lda, Portugal. Vitamins (IU or mg/kg diet): sodium menadione bisulphate, 10 mg; retinyl acetate, 8000 IU; DL-cholecalciferol, 1700 IU; thiamin, 8 mg; riboflavin, 20 mg; pyridoxine, 10 mg; cyanocobal-amin, 0.02 mg; nicotinic acid, 30 mg; folic acid, 6 mg; inositol, 300 mg; biotin, 0.7 mg; calcium panthotenate, 70 mg; betaine, 400 mg. Minerals (mg/kg diet): cobalt carbonate, 0.1 mg; copper sulphate, 5 mg; ferric sulphate, 60 mg; potassium iodide, 1.5 mg; manganese oxide, 20 mg; sodium selenite, 0.25 mg; zinc oxide, 30 mg; sodium chloride, 80 mg; excipient: wheat middlings.;
[k]Ascorbil monophosphate, PREMIX Lda, Portugal;
[l]α-Tocopherol, PREMIX Lda, Portugal;
[m]Taurine T0625 Sigma-Aldrich Corporation, Germany;
[n]Chitosan 448869, Sigma-Aldrich Corporation, Germany;
[o] Mono-calcium phosphate, Fosfitalia, Italy;
[p] Crystalline AA, Ajinomoto Eurolysine SAS, France

Water soluble ingredients, such as fish protein hydrolysate (CPSP 90®, Sopropêche, France), crystalline-AA and vitamin and mineral premix (Pre-Mix PVO040®, Premixportugal, Portugal) were encapsulated, using internal chitosan microparticles made from a suspension with a proportion of 2:12:3 (chitosan: CPSP 90® /crystalline-AA: premix) (Santos et al. 2012). For this purpose, a chitosan (Sigma, USA) solution was prepared at 2% (w/v) in 1% (w/w) acetic acid (Carl Roth, Germany). After grinding below 50µm and complete dissolution of chitosan, 12% (w/v) of CPSP 90® Sopropêche, France) or crystalline-AA and 1% (w/v) premix (Pre-Mix PVO040®, Premixportugal, Portugal) were added. The suspension was atomized in a laboratory scale spray-dryer (Lab-Plant SD-04, United Kingdom) using the following conditions: inlet temperature of 160 ± 1 °C, outlet temperature of 120 ± 4 °C, solution flow of 5 ± 0.5 mL/min, maximum blower level (100 units).

All dietary ingredients, including the microparticles produced by spray-drying, were initially mixed according to each target formulation in a double-helix 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 60 ºC, 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, microdiets usually remaining at least for one minute in the tank bottom before being eaten (Conceição et al. 2007, Dinis et al. 2000), it seems to be more adequate to analyse and interpret the present results in the light of the AA contents attained after 1 min leaching than using those obtained in the microdiet itself. Feed samples (n = 4 treatment) were hence submersed in rearing water for 1 minute, in order to allow nutrient leaching and to simulate the situation as in the rearing tanks. After this period the rearing water was removed and the feed samples were frozen at -80ºC and freeze-dried to remove the water. Feed samples were ground, pooled and analyzed for dry matter (105 °C for 24 h), crude protein by automatic flash combustion (Leco FP-528, Leco, St. Joseph, USA; N × 6.25), lipid content by petroleum ether extraction using a Soxtherm Multistat/SX PC (Gerhardt, Königswinter, Germany; 150 °C), and gross energy in an adiabatic bomb calorimeter (Werke C2000; IKA, Staufen, Germany). The diet composition after 1 min immersion in rearing-water (39 ‰) is presented on table 1.

The amino-acid composition was determined by ultra-high-performance liquid chromatography (UPLC) 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, USA). Tryptophan was determined by HPLC with fluorescence detection (extinction 280 nm, emission 356 nm), after alkaline hydrolysis with barium hydroxide octahydrate for 20 h at 110°C (Commission Directive 2000). The dietary amino acids profiles are presented in Table 2.

Table 2: Determined amino acid content (% dry matter) of the experimental diets after 1 min leaching in seawater.

	Diets	
	CTRL	BAL
Indispensable amino-acids (IAA)		
Arginine	4.63	4.77
Histidine	0.94	1.03
Lysine	5.48	4.94
Threonine	2.48	2.50
Isoleucine	2.55	2.55
Leucine	4.08	4.34
Valine	2.71	2.63
Methionine+Cysteine	1.36	1.85
Phenylalanine+Tyrosine	3.44.	4.34.
Tryptophane	0.48	0.59
IAA sum	**28.13**	**29.53**
Dispensable amino-acids (DAA)		
Aspartic acid + Asparagine	5.94	5.46
Glutamic acid + Glutamine	8.90	8.06
Alanine	4.24	4.14
Glycine	6.21	6.65
Proline	4.12	4.17
Serine	2.53	2.62
Taurine	0.93	0.85
IAA/DAA ratio	0.86	0.92

2.2.4 Sampling and experimental design

2.2.4.1 Growth and larvae performance

From each replicate tank, 1 pool of 20 individuals was collected for dry weight (DW) evaluation at mouth opening (2DAH). Thereafter, individual fish were randomly sampled for dry weight and standard length at 13DAH (pre-metamorphosis), 19DAH (metamorphosis climax), 25DAH (metamorphosis completed) (n=10 per replicate tank), at 38DAH (weaned post-larvae) and 51DAH (n=20 per replicate tank). Sole were individually photographed and measured

(standard length), using Axio Vision L.E. 4.8.2.0 (Carl Zeiss Micro Imaging GmbH) and kept frozen at -80ºC for dry weight determination to 0.001mg precision after freeze-drying. Growth was expressed as relative growth rate (RGR, % day^{-1}) and was determined during the pelagic phase from mouth opening (2-25DAH), during the benthic phase (25-51DAH) and during the whole trial (2-51DAH). RGR was calculated as RGR (% day-1) = $(e^{g}-1)\times 100$, where g = [(ln $_{final\ weight}$ - ln $_{initial\ weight}$)/time] (Ricker 1958). The coefficient of variation (CV/ %) calculated as CV = (treatment standard deviation/treatment mean) $\times 100$ was used to determine the inter-individual weight variation within each group (in a total of 30 fish per group on 13, 25 and 51DAH and 60 fish per group on 38 and 51DAH). Survival during the benthic phase was evaluated by counting the remaining larvae in the rearing tanks at the end of the experiment (51 DAH). The results are presented as percentage of survival, relative to the initial larvae number in each tank.

2.2.4.2 Protein metabolism trials

The effect of dietary amino acid profile on the digestion, absorption and metabolic utilisation capacities of larvae throughout the metamorphosis was assessed through the metabolism of ^{14}C labelled model peptides with different molecular weights, 1.0KDa and 6.8KDa (Richard et al. 2015) , at 13DAH (pre-metamorphosis), at 19DAH (metamorphosis climax) and at 25DAH (metamorphosis completed).

This 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 upon *Artemia* sp. *metanauplii* for 30 min. Then 8 larvae (with guts well filled with Artemia) from each dietary treatment were anaesthetised with 150-330µM of MS-222 (depending on larvae age) and tube-fed with two doses of 13.8 ηL of the test ^{14}C labelled model peptide (193ηg of 1.0KDa peptide; 635ηg of 6.8KDa peptide) through a 0.19mm diameter plastic capillary inserted on a nanoliter injector (World Precision Instruments, Sarasota, USA) firmly attached to a micromanipulator (Richard et al. 2015). 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 5.0mL 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 radio activity counting (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.

All samples were then added scintillation cocktail (Ultima Gold XR, Perkin Elmer, USA) 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, %), retained fraction (R, %), catabolised fraction (C, %) calculated as:

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

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

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

, 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).

2.2.5 Data analysis

In order to compare the experimental diets, the A/E ratios (Arai 1981) were calculated, on a weight basis, for the two diets (CTRL and BAL) at two stages: when the inert diet became the predominant (25DAH) and exclusive (51DAH) source of food. A dietary deficiency for a given IAA is assumed to occur when the relative difference between the A/E ratios of Senegalese sole post-larvae and juveniles and the experimental diets A/E ratios, calculated as ($_{diet}$A/E ratio – $_{fish}$A/E ratio) × ($_{fish}$A/E ratio)$^{-1}$ ×100, is negative (Fig.1). For the purpose of the discussion, an IAA was considered potentially limiting in the diet whenever relative difference between the $_{fish}$A/E ratio and $_{diet}$A/E ratio for that given IAA was lower than -10%. The first-limiting AA for a given diet is considered to be the IAA with the lowest relative difference between its contribution to the diet and to the larval A/E ratio (Conceição et al. 2003, Conceição et al. 1998b). The first-limiting AA will set the limit for AA utilisation above which protein synthesis cannot proceed. Therefore, the absolute value of this relative difference is considered to be an estimate of the unavoidable AA loss (percent of total AA absorbed) for a given diet (Conceição et al. 2003, Conceição et al. 1998b). Cysteine and tyrosine were included with the IAA, since they can only be synthesized from methionine and phenylalanine, respectively.

Statistical analyses followed previously reported methods (Zar 2010) and IBM SPSS Statistics 19 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 AA profile on survival and growth parameters was tested by one-way ANOVA or Wilcoxon-Mann-Whitney test whenever data did not meet normality and homoscedasticity requirements. The influence of dietary AA profile on the larvae capacity to utilize protein was tested by two-way ANOVA using peptide size and diet as independent factors. A one-way ANOVA was also performed to test the influence of developmental stage on the larvae capacity to utilize different sized peptides. Significance levels were set at $P<0.05$.

2.3 Results

2.3.1 Diets

The amino acid (AA) profile of the experimental inert diets (Fig.1) showed that a 4% replacement of the encapsulated fish protein hydrolysate (CPSP90®, Sopropêche, France) fraction by a crystalline-AA (CAA) mixture resulted in a more balanced diet (BAL) for Senegalese sole larvae. When compared to the CTRL diet, the BAL diet formulation was effective in reducing the estimated unavoidable losses of the microdiet supplied AA from 37% and 31% to 24 and 26%, respectively, for 25 and 51DAH fish. This occurred by increasing the A/E ratio of Methionine+Cysteine (Met+Cys), which appeared as the CTRL diet first-limiting AA in both ages (Fig. 1). In the BAL diet, Val and His appear as the first-limiting AA, respectively for 25DAH larvae and 51DAH (Fig. 1). The BAL formulation has also reduced the relative difference between the microdiet and fish A/E ratios for Histidine (His), Leucine (Leu) and Phenylalanine+Tyrosine (Phe+Tyr), preventing Leu and Phe+Tyr of becoming potentially limiting AA, respectively for 25DAH and 51DAH fish. The BAL diet formulation has also reduced the excess of Arginine (Arg), Threonine (Thr) and most effectively of Lysine (Lys) for 51DAH fish. Tryptophane (Trp) also appears to be increased in the BAL diet, when compared to the CTRL diet (Fig.1). While the CTRL diet seems to be slightly deficient in Trp for 25DAH larvae, in the BAL diet the Trp A/E ratio in the diet is in slight excess for 25 and 51DAH fish (Fig.1).

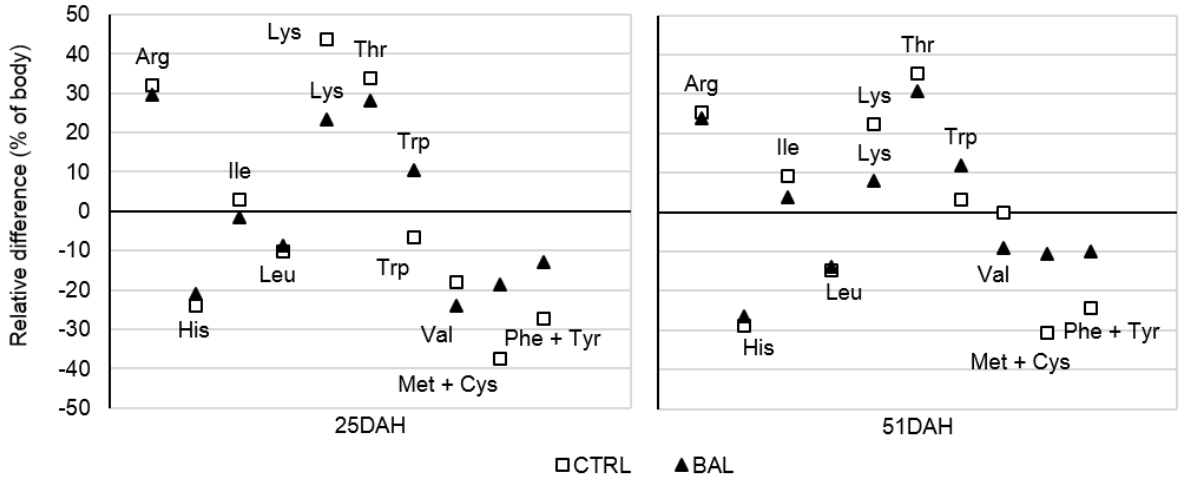

Fig. 1 Relative difference between the fish whole body A/E ratios and the experimental diets A/E ratios, calculated as ($_{diet}$A/E ratio – $_{fish}$A/E ratio) × ($_{fish}$A/E ratio)$^{-1}$ ×100, where $_{diet}$A/E ratio and $_{fish}$A/E ratio are the dietary and whole body A/E ratios for a given IAA, during the period when the inert diet became the predominant (25DAH) or exclusive (51DAH) source of food

2.3.2 Growth

The dietary protein composition had a significant effect on larval growth, with significant differences being detected on sole standard length at 38DAH ($p=0.045$) and more remarkably at 51DAH ($p<0.001$) (Table 3), as well as on dry weight at 51DAH ($p<0.001$) (Fig. 2). At end of the experiment (at 51DAH), the larvae fed CTRL diet averaged a dry weight 1.66-fold higher than those fed the BAL diet ($P<0.001$). In general, larval growth was highly variable in all treatments, with the coefficient of variation for dry weight increasing with age (table 3). The coefficient of variation was not influenced by diet ($p > 0.05$).

Sole relative growth rate averaged 23.9%/day during the pelagic phase and 6.6 %/day during the benthic phase (Fig.2). Sole survival during the benthic phase, from 19 to 51DAH, was not significantly affected by diets (table 3, $P=0.246$), averaging 63.0%.

Table 3: Senegalese sole standard length (SL/mm) and coefficient of variation (CV/%) during the pelagic (13 and 19DAH) and the benthic phase (25 to 51DAH); values are means ± s.d. (N=30 at 13, 19 and 25DAH; N=60 at 38 and 51DAH). Survival during the benthic phase (% of initial number of larvae in each tank); values are means ± s.d. (N=3/treatment). Different superscript letters at each developmental stage indicate significant differences ($P<0.05$; 1 way-ANOVA was used to test differences between groups in RGR, survival and SL at 25DAH; Wilcoxon-Mann-Whitney was used to test differences between groups in SL at 13, 19, 25, 38 and 51DAH).

		Diets	
		CTRL	BAL
Pelagic phase			
13DAH	SL (mm)	5.8±0.4	5.8±0.3
	CV (%)	23.8±8.9	18.6±5.0
19DAH	SL (mm)	6.7±0.8	6.5±0.5
	CV (%)	24.2±6.4	16.6±5.7
Benthic phase			
25DAH	SL (mm)	8.4±1.1	8.1±0.8
	CV (%)	29.3±14.2	24.1±6.3
38DAH	SL (mm)	10.5±1.7^{a}	9.9±1.4^{b}
	CV (%)	50.2±10.7	39.7±14.5
51DAH	SL (mm)	15.3±2.7^{a}	13.1±2.1^{b}
	CV (%)	54.6±10.8	44.1±9.5
Survival 19-51 DAH (%)		57.2±11.3	68.7±9.6

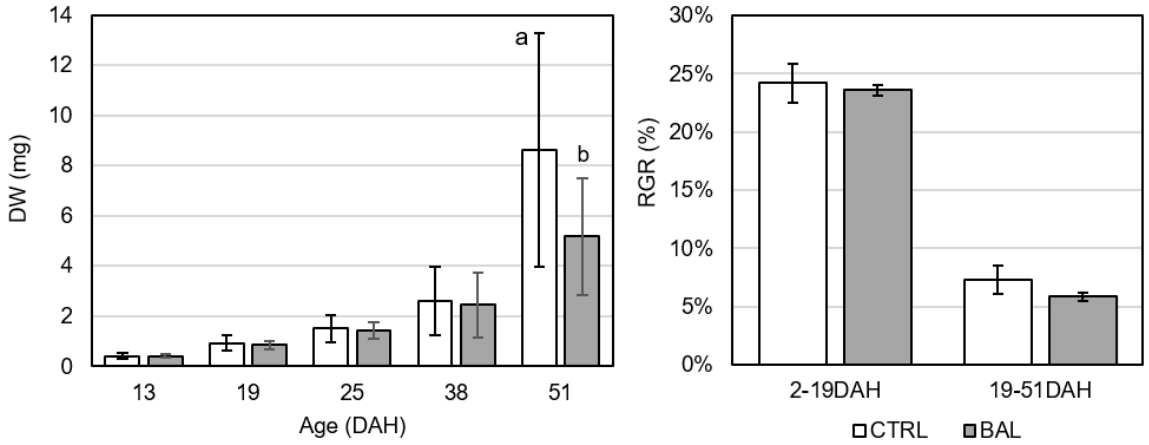

Fig. 2 Sole dry weight (DW/mg); values are means ± s.d. (N=30 at 13, 19 and 25DAH; N=60 at 38 and 51DAH). Relative growth rate (RGR) during the pelagic (2-19DAH) and the benthic phase (19-51DAH); values are means ± s.d. (N=3/treatment). Different superscript letters at each developmental stage indicate significant differences ($P<0.05$; 1 way-ANOVA was used to test differences between groups in RGR and DW at 25DAH; Wilcoxon-Mann-Whitney was used to test differences between groups in DW at 13, 19, 25, 38 and 51DAH)

2.3.3 Protein metabolism

The larvae capacity to digest and absorb either 1KDa or 6.8KDa peptide was not significantly affected ($p>0.05$) by dietary protein quality throughout metamorphosis (fig.3). Larvae presented a higher capacity to absorb 1.0KDa peptide than 6.8KDa peptide, from 13 to 25DAH ($p<0.001$) (fig.3). The average peptide absorption varied between 80.7 – 85.8% for 1.0KDa and between 38.3 – 47.1% for 6.8KDa peptides, with a ratio between 1.0KDa and 6.8KDa absorption rates varying between 1.8-fold at 13DAH, 2.1-fold at 19DAH and 1.9-fold at 25DAH. Absorption capacity did not change significantly between stages, throughout the metamorphosis, either for 1.0KDa or 6.8KDa ($P=0.373$), although there was a tendency to decrease on the 6.8KDa absorption rate during the metamorphosis (19DAH), 1.2-fold lower than before (13DAH) and 1.1-fold lower than after the metamorphosis was completed (25DAH) (fig.3)). Moreover, at 19DAH there was a significant interaction between diet and peptide size, with 1KDa peptide being better absorbed in BAL group, while the 6.8 KDa peptide may be better (or equally) absorbed in CTRL group.

Protein retention efficiency and catabolism of 13DAH sole were not affected ($P=0.747$) by dietary AA profile (fig.4). The peptide molecular size did not affect the retention or catabolism efficiency in pre-metamorphosis larvae ($P=0.286$) (fig.4). Average retention efficiency of 13DAH larvae were, 80.2 ± 0.9% for 1.0KDa peptide and 75.1 ± 1.2% for 6.8KDa peptide.

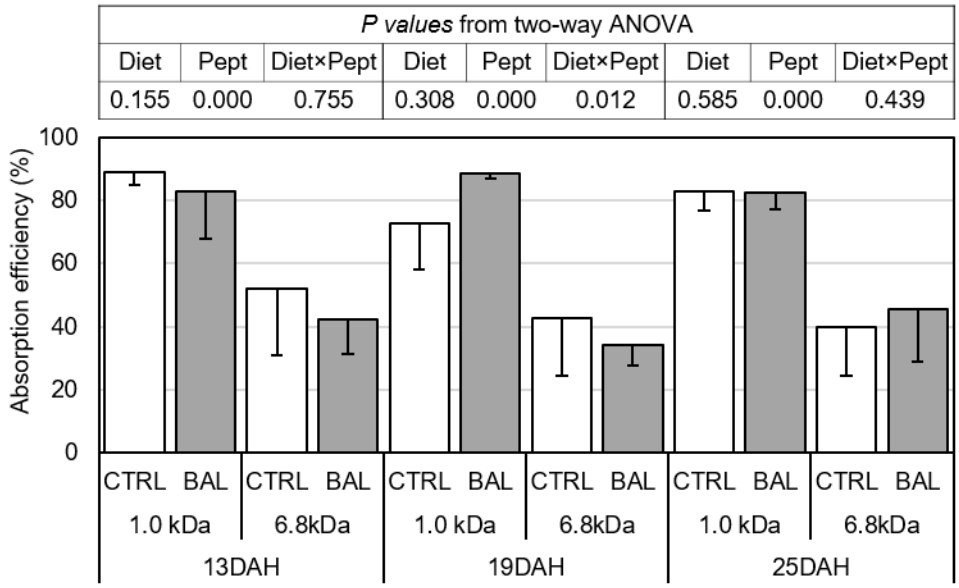

Fig. 3 1.0KDa and 6.8KDa peptides absorption (sum of the % of radiolabel in the body and in the metabolic trap in relation to the total tracer fed) in sole larvae at 13 (pre-metamorphosis), 19 (metamorphosis climax) and 25DAH (post-metamorphosis). Values are means ± SD, n = 6-8. Comparisons between groups fed different diets and tube-fed different molecular sized peptides were made for each developmental stage using 2-way ANOVA (values given above graph)

The BAL diet formulation had a positive impact on the larvae capacity to retain either 1KDa or 6.8KDa peptides during the metamorphosis climax (P=0.039) (fig.4). Larvae fed CTRL diet presented a lower retention (70.9 ± 10.6%) and higher catabolism (29.1 ± 10.6%) for 1.0KDa peptides when compared with larvae fed BAL diet. In 19DAH larvae fed the BAL diet, the retention of 1.0KDa peptides was higher by 1.18-fold, while catabolism was decreased by 0.56-fold, when compared to the CTRL group. In the BAL group, the retention of 6.8KDa peptides was 1.06-fold higher, while catabolism was 0.81-fold lower, than that of the CTRL group. The molecular size of the studied peptide fractions did not affect their retention or catabolism during the metamorphosis climax (P=0.695) (fig.4). Average retention efficiency of 13DAH larvae were, 77.4 ± 9.1% for ^{14}C labelled 1.0KDa peptide and 78.7 ± 3.2% for ^{14}C labelled 6.8KDa peptide.

At 25 DAH, there was no longer an effect of the diet on larvae protein retention (P=0.605) (fig.4). However, when comparing the larvae capacity to metabolize smaller or larger peptides,

although 1.0KDa peptides were still better absorbed than 6.8KDa peptides (P<0.001), the later became better retained and less catabolized (P=0.032) (fig.4).

When comparing metabolism between different developmental stages, in general, there is a slight increase of larvae capacity to retain 6.8KDa peptides from the pre-metamorphosis stage to the post-metamorphosis (P=0.043) (fig.4).

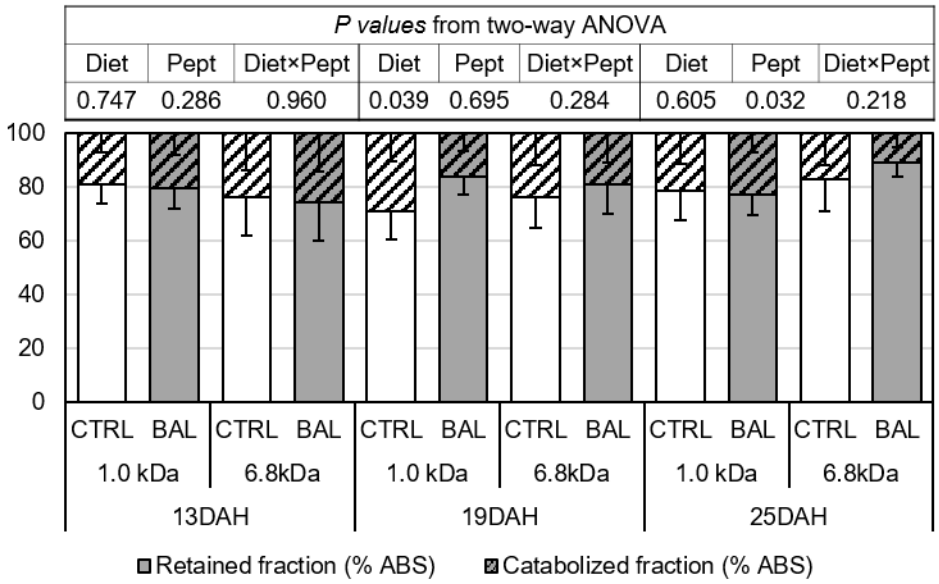

Fig. 4 1.0KDa and 6.8KDa peptides retained fraction (% of radiolabel in the body in relation to absorbed label; empty bars, white = CTRL, grey = BAL), and catabolized fraction (% of radiolabel in the metabolic trap in relation to absorbed label; dashed bars, white = CTRL, grey = BAL) in sole larvae at 13 (pre-metamorphosis), 19 (metamorphosis climax) and 25DAH (post-metamorphosis). Values are means ± SD, n = 6-8. Comparisons between groups fed different diets and tube-fed different molecular sized peptides were made for each developmental stage using 2-way ANOVA (values given above graph)

2.4 Discussion

The partial replacement (4%) of the encapsulated fish protein hydrolysate by a mixture of encapsulated crystalline-AA (CAA) did result in a more balanced diet (BAL), when compared to a diet without any attempt of correcting IAA putative deficiencies (CTRL diet). When compared to the CTRL diet, the BAL diet formulation has reduced the unavoidable losses of the microdiet supplied AA from 37% and 31% to 24 and 26%, respectively, for 25 and 51DAH fish, as well as the deficiency of His, Leu, Met+Cys and Phe+Tyr and the excess of Arg, Lys

and Thr. However, His, Met+Cys and Phe+Tyr remained limiting in the BAL diet, particularly during the feeding periods where the inert feed became the predominant or exclusive source of food. On the other hand, although the correction of the dietary IAA profile did decrease the excess of Arg and Thr, it was not sufficient to balance the IAA dietary profile in relation to the larvae A/E ratio for these two IAA and prevent them from being irreversibly lost to catabolism. In short, the BAL diet was better balanced, even if not fully balanced, in IAA for Senegalese sole larvae, when considering the ideal dietary IAA profile defined by Aragão et al. (2004b). The positive impact of the BAL diet on retention of both peptides assayed during metamorphosis climax supports that this diet was in fact more balanced in IAA than the CTRL diet. Moreover, this is in accordance with previous data from Aragão et al. (2004c) who showed a higher retention of AA in *Artemia* fed Senegalese sole postlarvae, after Leu-Gly and Phe-Ala dipeptides supplementation, in order to balance the dietary AA profile. It is also consistent to what has been suggested by Saavedra et al. (2009b) for *Diplodus sargus* larvae that showed a reduced nitrogen excretion when reared upon AA balanced diets, in a co-feeding regime.

The observed differences between diet formulation and IAA profiles measured after diet 1min immersion in seawater were most likely due to leaching losses of some of the IAA, as these are highly soluble molecules. In fact, one major difficulty on devising microdiets for fish larvae is the small size of the feed particles compared with pellet used for juveniles. Larval microdiets present a very high surface/volume ratio meaning that the diffusion distance from its core is very short. So, while larvae do not possess the digestive machinery developed enough to utilize complex protein, a more soluble nitrogen form such as CAA, that would allow to finely tune dietary protein quality, are often easily lost by leaching (Kvale et al. 2007, Kvale et al. 2006, Nordgreen et al. 2008, Yúfera et al. 2002).

Throughout development there was a shift on the larvae retention capacity: until the metamorphosis was completed there was no difference in the retention of different sized peptides and just then the 6.8KDa peptide became more efficiently retained than the 1.0KDa, later in development in both groups. That is accordance to Tonheim et al. (2005) findings on Atlantic halibut (*Hippoglossus hippoglossus*) larvae that suggest that, when compared to larger peptides, small peptides would be were very rapidly absorbed, which would lead to impaired retention, increasing their use for energy production. According to Tonheim et al. (2005), when compared to larvae tube-fed a partially hydrolysed ^{14}C-labelled salmon serum protein (15-250 KDa), those tube-fed a highly hydrolysed ^{14}C-labelled salmon serum protein (<25 KDa) displayed an absorption rate measured in the first 4h past tube-feeding increased by 1.36-fold and the catabolism after 10h past tube-feeding significantly increased. The fact that, in the present study, only older larvae retain 6.8KDa peptides more efficiently than 1KDa peptides could be explained by an improved digestive capacity in older larvae. However, no such

differences were observed in the present study for absorption efficiency (Fig. 3). An alternative explanation may be the lower growth rate and metabolism of older larvae (Conceição et al. 1998a), making them less efficient in using fast-absorbed lower molecular weight peptides, due to transient AA imbalances. The 6.8KDa polypeptide fraction could also be better adapted to the AA anabolic and physiological need of young sole juveniles rather than younger larval stages as it was suggested by Richard et al. (2015). These authors have also obtained an increasing trend of 6.8KDa peptide retention throughout the ontogeny. Another possible explanation would be a gradual decrease in the sole larvae reliance on AA for energy production, leading to an apparent sparing of AA for growth purposes.

Concerning the development of larvae capacity to digest and absorb different sized peptides, as protein sources, throughout metamorphosis, it was expected that smaller peptides would be more easily absorbed than larger peptides. Since the sole digestive system at such young stages is still very immature, not having any pepsin activity, it relies proteolysis on pancreatic endoproteases (such as trypsin and chymotrypsin) activity, which may not be as efficient to access the cleavage sites of partially folded 6.8KDa peptides as those of 1.0KDa peptides. However it was a little surprising that there was no major increase on the larvae capacity to absorb larger molecules throughout development, though similar results have also been observed in other studies with same sized model peptides (Engrola et al. 2013, Richard et al. 2015). Perhaps an increase would be noticed at later stages, as it was observed in 34DAH sole larvae by Richard et al. (2015). The 20% decrease on the 6.8KDa absorption rate during the metamorphosis climax (19DAH) is comparable to (Engrola et al. 2009b) previous results, according to which Senegalese sole reared upon a co-feeding regime from an early stage displayed a reduced capacity to digest *Artemia* during the metamorphosis climax. This is in line with the decrease on the activity of alkaline proteases during the metamorphosis climax described in this species by Ribeiro et al. (1999b), and comparable to that of other flatfish species (Bolasina et al. 2006).

The positive effect of a dietary AA profile correction on the larvae capacity to utilize small-sized peptides was observed only during the metamorphosis climax, and was not enough to allow the larvae fed a more balanced diet (BAL) to attain a better a performance. In fact, replacing 4% of the encapsulated CPSP90® fraction by a crystalline-AA mixture, in order to balance the dietary IAA profile, did not improve Senegalese sole larvae growth. On the contrary, larvae fed the CTRL diet attained a faster growth from an early stage, what has become accentuated throughout time towards the end of the experiment.

This could eventually be explained by the impaired utilization of the fast absorbed crystalline (free) AA compared to the protein-bound AA which needs the action of digestion prior to

absorption, which would have led to a decrease in protein accretion. Rønnestad et al. (2000) have shown that free AA are absorbed much faster than intact protein in Senegalese sole post-larvae (23DAH), at 21ºC, and (Rønnestad and Conceição 2012) proposed that even a highly digestible protein – the one from *Artemia* – takes more than 2 hours to be fully digested by Senegalese sole metamorphosing larvae, at 21ºC. However, this is not a likely explanation as the BAL diet was enriched with free AA at expense of a protein hydrolysate (rich in free AA and di- and tri-peptides) which are equally fast absorbed by fish larvae (Rønnestad and Conceição 2012).

Given the positive effect of the BAL diet formulation on the larvae capacity to retain protein, it is likely that the differences found in growth are due to a possible effect on the voluntary feed intake. It was not possible to quantify the voluntary feed intake on the experimental microdiets, as no viable technique has been so far developed to measure feed intake on microdiets in fish larvae. However, the daily visual observation allowed to notice a clear excess of remaining feed in the tanks fed the crystalline AA-supplemented diet (BAL).

The larvae fed a more imbalanced diet (CTRL treatment) may have increased the voluntary feed intake as to compensate for AA deficiencies. This hypothesis may be contrasting to what has been reported for mammals (Bellinger et al. 2005, Gietzen and Aja 2012, Hao et al. 2005) and to what was reported for rainbow trout juveniles (*Oncorhynchus mykiss)* that showed a reduced feed intake when fed Lys devoid diets (Yamamoto et al. 2001). Furthermore, no mechanism is known to explain an increased feed intake as to compensate for inferior quality of the dietary IAA composition in fish. However, a study from (Dabrowski et al. 2007) reported an increased feed intake in midas juveniles (*Amphilophus citrinellum*) feeding on a FAA based diet and two IAA devoid diets when compared to a control group fed a protein based diet. On a second experiment, using different feeding strategies combining complementary IAA-imbalanced diets, the groups fed the imbalanced diets showed a significant increase of the voluntary feed intake when compared to a control group fed a balanced FAA-based diet. Similarly, in the present study the CTRL group may have increased voluntary feed intake as to compensate for the dietary AA imbalance, overcoming the lower retention efficiency on small peptides observed during the metamorphosis climax. Ultimately, that would have led to a higher growth rate in larvae fed the CTRL diet.

Another possible explanation for a possibly higher feed intake in larvae fed the CTRL diet is a higher percentage of CPSP, a commercial fish protein hydrolysate with a high percentage of small peptides (<5.0KDa) that are known to act as attractant to fish (Aksnes et al. 2006a, Aksnes et al. 2006b, Kousoulaki et al. 2013, Velez et al. 2007), and namely to Senegalese sole (Barroso et al. 2013). However IAA, such as the ones replacing the CPSP in the BAL diet,

are also well known as attractants (Goh and Tamura 1980, Hara 2006, Knutsen 1992, Kolkovski et al. 1997, Kousoulaki et al. 2013, Mackie et al. 1980, Yacoob and Browman 2007). So this CPSP effect is not a likely explanation.

Ultimately, a higher level Trp of in the BAL diet might have contributed to a reduced voluntary feed intake in these groups. The supplementation with encapsulated crystalline Trp was shown to be quite effective: while this essential amino-acid appears as limiting in the CTRL diet, it appears in a slight excess the BAL diet. The Trp is precursor of serotonin (5-hydroxiTryptamine, 5-HT) which participates as a messenger in the central nervous system (CNS) and peripherally in gastrointestinal and vascular systems. The oral administration of 5-HT reduced feed intake in the european sea bass (*Dicentrarchus labrax)* (Rubio et al. 2006). In juvenile groupers (*Epinephelus coioides*) there was is reduction of growth with increased levels of dietary Trp supplementation, possibly due to depressed appetite and feed consumption (Hseu et al. 2003). Therefore, the supplementary Trp in the BAL diet could have led a reduced voluntary feed intake and a subsequent decrease in somatic growth in larvae fed the BAL diet.

In conclusion, the present results suggest that the replacement of the encapsulated CPSP fraction by a an encapsulated crystalline-AA mixture, in order to better balance the dietary IAA profile brings a positive short-term effect on the development of the Senegalese sole larvae capacity to retain small-sized peptides. However, that did not reflect on the somatic growth in the present study. On the contrary, larvae fed a more imbalanced (CTRL group) diet attained a better performance. Further studies are needed to ascertain whether this is due to an effect on the voluntary feed intake as a compensatory response to the dietary IAA imbalance or due to the higher content of tryptophan in the BAL diet.

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Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical approval: Experiments were performed by trained scientists and 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

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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 utilisation, which requires that all the indispensable amino acids (IAA) 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 utilise 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.

Keywords: dietary protein, amino acids profile, muscle growth, gene expression, Senegalese sole

3.1 Introduction

In most teleost larvae (Alami-Durante, 1990; Alami-Durante et al., 2006; Campos et al., 2013c; Khemis et al., 2013; Osse, 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 fibers (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 program that will lately result in myotubes 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 fiber number) and hypertrophy (increase of fiber size) from hatching to until approximately 40% of maximum fish length (Rowlerson, Veggetti, 2001; Weatherley et al., 1988). During post-embryonic and larval development, muscle fiber number increases mainly by stratified hyperplasia, a phase of myogenesis that involves the recruitment of new fibers in discrete germinal zones found in the lateral margins of the myotome (Rowlerson, Veggetti, 2001). In juvenile and adult stages, in a second phase called mosaic hyperplasia, new myotubes form on the surface of fast muscle fibers, further fusing or adding nuclei to already existing fibers, to keep size of nuclear domains constant during hypertrophic growth (Rowlerson, 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 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 acids (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 utilisation and growth (Aragão et al., 2004b).

In the present study, it was hypothesized 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 analyzed in order to understand if growth differences could be associated with an epigenetic event.

3.2 Material and Methods

3.2.1 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 replacing 8% of the encapsulated fish protein hydrolysate fraction of the CTRL diet with encapsulated crystalline AA in order to ensure a higher IAA supply (Table 1). Water soluble ingredients, such as fish protein hydrolysate, crystalline-AA and vitamin and mineral premix were encapsulated, using internal chitosan microparticles made from a suspension with a proportion of 2:12:3 (chitosan: fish protein hydrolysate /crystalline-AA: premix) (Santos et al., 2012). For this purpose, a chitosan (Sigma, USA) solution was prepared at 2% (w/v) in 1% (w/w) acetic acid (Carl Roth, Germany). After complete dissolution of chitosan, 12% (w/v) of fish protein hydrolysate (CPSP 90®, Sopropêche, France) or crystalline-AA and 1% (w/v) vitamin and trace minerals pre-mixture (Pre-Mix PVO040®, Premixportugal, Portugal) were added. The suspension was atomized in a laboratory scale spray-dryer (Lab-Plant SD-04, United Kingdom) using the following conditions: inlet temperature of 160 ± 1 °C, outlet temperature of 120 ± 4 °C, solution flow of 5 ± 0.5 mL/min, maximum blower level (100 units).

All dietary ingredients, including the microparticles produced by spray-drying, were initially mixed according to each target formulation in a double-helix 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 60 ºC, being subsequently crumbled (Neuero Farm, Germany) and sieved to desired size ranges.

Senegalese sole has a passive bottom feeding behaviour, so that microdiets commonly remain for one min or more on the bottom of the tank before being eaten. Feed samples (*n* = 4 per treatment) were hence submersed in rearing water for 1 minute, in order to allow nutrient leaching and simulate a similar nutritional situation observed in the rearing tanks. Most leaching of AA in microdiets occurs over the first minute (Yúfera et al., 2002). After this period the rearing water was removed and the feed samples were frozen at -80ºC and freeze-dried before further analysis. Feed samples were ground, pooled and analyzed for dry matter (105 °C for 24 h), crude protein by automatic flash combustion (Leco FP-528, Leco, St. Joseph, USA; N × 6.25), lipid content by petroleum ether extraction using a Soxtherm Multistat/SX PC (Gerhardt, Königswinter, Germany; 150 °C), and gross energy in an adiabatic bomb calorimeter (Werke C2000; IKA, Staufen, Germany). Diet composition after immersion in the rearing-water for one min is presented on Table 1.

The dietary amino acid composition was determined by ultra-high-performance liquid chromatography (UPLC) in a Waters Reversed-Phase Amino Acid Analysis System, using norvaline as an internal standard. Samples were hydrolysed at 6 M HCl at 116ºC, over 22 h, and then 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, USA). Tryptophan was determined by HPLC with fluorescence detection (extinction 280 nm, emission 356 nm), after alkaline hydrolysis with barium hydroxide octahydrate for 20 h at 110°C (Commision Directive, 2000). Diet AA profiles after immersion in the rearing-water for one min are presented on Table 2.

3.2.2 Husbandry and experimental set-up

Experiments were performed by trained scientists and following the European Directive 2010/63/EU of the European Parliament and the Council of the European Union on the protection of animals used for scientific purposes.

Table 1: Ingredients and proximate composition of the experimental diets after one min leaching in seawater.

	Diets	
	CTRL	SUP
Ingredients (% dry matter)		
Whey Protein isolate [a]	10	10
FPH - non encapsulated[b]	10	10
Squid meal[c]	8.5	8.5
FPH encapsulated[b]	20	11.2
Fish Gelatine[d]	14	13
Autolysed yeast[e]	1.7	1.7
Krill Meal[f]	9	9
Fish oil[g]	4.5	5.5
DHA-rich oil [h]	2	2
Copepod oil [i]	5.5	5.5
Premix[j]	5	5
Vit C[k]	3	3
Vit E[l]	1	1
Taurine (Sigma)[m]	1.5	1.5
Chitosan[n]	3.3	3.3
Mono Ca Phosphate[o]	1	1
L-Arginine HCl[p]		1
DL-Methionine[p]		0.75
L-Lysine HCl[p]		2
L-Tryptophan[p]		0.5
L-Histidine HCl[p]		0.6
L-Leucine[p]		1
L-Isoleucine[p]		0.5
L-Phenylalanine[p]		2.5
Proximate analyses (% dry matter)		
Crude protein	60.93	59.82
Crude fat	14.61	13.90
Gross Energy (MJ kg^{-1})	17.46	17.53

[a]Isolate Crystal Whey. Weider. USA;
[b]Fish protein hydrolysate - CPSP 90®. Sopropêche. France;
[c]Super prime without guts. Sopropêche, France;
[d]Fish edible gelatine. Lapi Gelatine. Italy;
[e]Hylisis. ICC. Brazil;
[f]Qrill. Aker Biomarine. Norway;
[g]Marine oil omega 3: Henry Lamotte Oils GmbH. Germany;
[h]DHA 70% Algatrium, Brudy Technology SL., Spain;
[i]Phosphonorse, K/S Tromsø Fiskeindustri A/S & Co., Norway;
[j]PVO40.01 Premix for marine fish, PREMIX Lda, Portugal;
[k]Ascorbil monophosphate, PREMIX Lda, Portugal;
[l]α-Tocopherol, PREMIX Lda, Portugal;
[m]Taurine T0625 Sigma-Aldrich Corporation, Germany;
[n]Chitosan 448869, Sigma-Aldrich Corporation, Germany;
[o] Mono-calcium phosphate, Fosfitalia, Italy;
[p] Crystalline AA, Ajinomoto Eurolysine SAS, France

Table 2: Determined amino acid content (% dry matter) of the experimental diets after one min leaching in seawater.

	Diets		% Deviation
	CTRL	SUP	(CTRL *vs* SUP)
Indispensable amino-acids (IAA)			
Arginine	4.63	4.82	4.2
Histidine	0.94	1.11	18.2
Lysine	5.48	6.34	15.8
Threonine	2.48	2.82	13.8
Isoleucine	2.55	2.81	10.4
Leucine	4.08	4.67	14.6
Valine	2.71	2.60	-3.8
Methionine	1.26	1.47	16.6
Phenylalanine	1.97	3.56	81.0
Cysteine	0.10	0.10	-1.9
Tyrosine	1.47	1.28	-12.9
Tryptophane	0.48	0.65	35.7
IAA sum	**28.13**	**32.24**	**14.6**
Dispensable amino-acids (DAA)			
Aspartic acid + Asparagine	5.94	5.11	-13.9
Glutamic acid + Glutamine	8.90	8.31	-6.6
Alanine	4.24	3.78	-10.7
Glycine	6.21	5.56	-10.4
Proline	4.12	4.06	-1.56
Serine	2.53	2.40	-5.12
Taurine	0.93	0.94	1.5
IAA/DAA ratio	**0.86**	**1.07**	**24.8**

% Deviation calculated as
(SUP_{AAi} content - $CTRL_{AAi}$ content)/ $CTRL_{AAi}$content ×100

Senegalese sole (*Solea senegalensis*) eggs were incubated in an upwelling incubator at 19±0.5ºC and hatching was completed within the next day (24h). Newly hatched larvae were evenly distributed by 6 white cylindro-conical tanks (100L) in a semi-closed recirculation system with a density of 100 larvae L^{-1} (10^4 larvae/tank). The system was equipped with a mechanical filter, a submerged and a trickling biological filter, a protein skimmer and UV sterilizer. Larvae were reared in green water conditions until 25 days after-hatching (DAH), provided by adding frozen *Nannochloropsis* sp. (Nannochloropsis 18% FP 472/180908, Acuicultura Y Nutrición de Galicia SL) to the rearing tanks every morning. Abiotic parameters and mortality were daily monitored. Dissolved oxygen in water was maintained at 90.8±8.5% of saturation, temperature at 18.9±0.5ºC and salinity at 36.8±0.1‰. A 10/14h light/dark photoperiod cycle was adopted and a light intensity of 1000lux at water surface was provided by overhead fluorescent tubes. After settling (25 DAH) larvae were transferred to flat-bottom tanks (30×70×10cm; 21L), each tank stocking 635 individuals (corresponding to a density of

3024 ind/m^2). 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 oxygen was maintained at 93.1±4.5% of saturation, temperature at 21.5±0.9ºC and salinity at 39.4±3.1‰. A 10/14h light/dark photoperiod cycle was maintained and the light intensity was 400lux at water surface, following CCMAR´s standard Senegalese sole rearing conditions.

The two dietary treatments (CTRL and SUP) were randomly assigned to tanks (n = 3 tanks per treatment). From mouth opening (2 DAH) until 5 DAH 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 (200-400μm). *Artemia* AF *nauplii* (na) (ARTEMIA AF - 480, INVE, Belgium) were introduced at 4 DAH and prey density was gradually increased from 4 to 5 na·mL^{-1}, becoming the only prey offered after 5 DAH. *Artemia* EG *metanauplii* (M24) (EG SEP-ART Cysts, INVE, Belgium) enriched with Easy DHA Selco were introduced at 12 DAH, gradually increasing from 12 to 14 M24·mL−1 until 19DAH. Enriched frozen *Artemia metanauplii* were offered to settled larvae (between 19 and 25 DAH). Live prey was gradually reduced and substituted by inert diet (200-400μm) until complete weaning at 38 DAH, according to Engrola et al. (2009). After 39 DAH larvae were exclusively fed with the respective inert diet (CTRL and SUP; 400-600μm) and considered weaned.

During the pelagic phase, live prey was delivered 1h after the lights were on, at 11.00h, then at 14.00h and at 17.00h. During the benthic phase, frozen *Artemia* was delivered 30 min after the lights were switched on, at 9.30 and then at 12.00h, 14.30h and 17.00h. Inert diet was delivered semi-continuously with automatic feeders (cycles of 2 h of feeding followed by 1h 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 excess of uneaten food (Engrola et al., 2005).

3.2.3 Larvae performance

The sampling points were selected at key stages throughout Senegalese sole larval development, with emphasis on the metamorphosis time-window as defined by Fernández-Díaz et al. (2001): at mouth-opening (MO); pre-metamorphosis - stage 1 (Pre-Met); metamorphosis climax - stage 3 (Met); metamorphosis completed - stage 4 (Post-Met); and weaned post-larvae with a fully developed digestive system (Weaned). A final sampling point at a later juvenile stage during the benthic phase was also selected at 51 DAH for both treatments (Juvenile).

At MO, one pool of 20 individuals was collected from each tank for dry weight evaluation. Then after, individual fish (*n*=10-20 per replicate) were randomly sampled for dry weight and standard length determination at the selected sampling points. The fish were killed by over-anaesthesia (MS-222, 400 $mg.L^{-1}$) and individually photographed and measured (standard length), using Axio Vision L.E. 4.8.2.0 **(**Carl Zeiss MicroImaging GmbH**),** frozen at -80ºC and freeze-dried for dry weight determination to 0.001mg precision. Growth was expressed as relative growth rate (RGR, % day^{-1}) and was determined during the pelagic phase from mouth opening (2-25 DAH), during the benthic phase (25-51 DAH) and during the whole trial (2-51 DAH). 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 during the benthic phase was evaluated by counting the remaining fish in the rearing tanks at the end of the experiment (51 DAH).

3.2.4 Protein metabolism trials

The *in vivo* method of controlled tube-feeding described by Rust et al. (1993) and modified by Rønnestad et al. (2001a) was used to assess the effect of dietary IAA levels on the larvae capacity retain protein throughout the metamorphosis time-window, at the following stages: Pre-Met, Met and Post-Met. Selected ^{14}C labelled model peptides of 1.0KDa and 6.8KDa (Richard et al., 2015) were offered to the larvae to determine the capacity of sole to utilize different size nitrogen forms (including catabolism and retention). One day prior to protein metabolism trial, Senegalese sole larvae from each dietary treatment were transferred to the experimental laboratory to acclimatise. Larvae were fasted for 12h and freely allowed to swim in white trays previously prepared with clean seawater and aeration. Larvae were then fed *Artemia* sp. *metanauplii* for 30 min. Eight larvae with *Artemia*-filled guts from each dietary treatment were anaesthetised with 150-330µM of MS-222 (depending on larvae age) and tube-fed each tracer peptide. A 0.19-mm diameter plastic capillary inserted on a nanoliter injector (World Precision Instruments, Sarasota, USA) firmly attached to a micromanipulator was used. The injection volume used was 13.8 nL. After capillary withdrawal, larvae were gently rinsed for spillage through two successive wells filled with clean seawater and transferred into incubation chambers filled with 7.5 mL of seawater. An airflow connection was provided between each incubation chamber and a CO_2 trap (5 mL, KOH 0.5 M) used to collect all $^{14}CO_2$ produced by labelled peptide larval catabolism. At the end of the incubation period (24 h) larvae were rinsed with clean water and sampled. Larvae bodies were solubilised with Solvable (Perkin-Elmer, USA) and samples incubated at 50ºC for 24h. The incubation vials were resealed and 1 mL of 1.0 M HCL was gradually injected into the incubation vial, to force the catabolised $^{14}CO_2$ remaining in the seawater vial to diffuse to the CO_2 trap. Thus, ^{14}C released through AA catabolism could be accurately estimated. All samples were added scintillation cocktail (Ultima Gold XR, Perkin Elmer, USA) and disintegrations per minute (DPM) were

counted in a TriCarb 2910TR Low activity liquid scintillation analyser (PerkinElmer, USA). All counts were corrected for quench and lumex.

Protein utilization was determined based on the digested/absorbed fraction (A, %), retained fraction (R, %), catabolised fraction (C, %) calculated as:

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

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

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

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).

3.2.5 Fast-twitch muscle cellularity

Standard histological and morphometric techniques (Silva et al., 2009; Valente et al., 1999) were used to analyse fast-twitch muscle cellularity during the metamorphosis climax and at the juvenile stage 51 DAH. Three fish per tank were collected, killed by over-anaesthesia (MS-222, 400 mgl^{-1}) and measured. Fish were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, Missouri, USA) for 24h, washed with 1×PBS and stored in 70º ethanol, at 4ºC until further processing. Juvenile fish (51 DAH) were decalcified in 5% nitric acid and 5% sodium sulphate. All samples were dehydrated in a graded ethanol series (diluted from Etanol 99.5%, AGA, Prior Velho), cleared in xylol (Prolabo, VWR International LLC, Radnor, PA, USA) and finally included in paraffin (Merck, KGaA, Darmstadt). Fish were sectioned (7 µm) transversely to the body axis, mounted on coated slides with 3-aminopropyltriethoxysilane (APES) (Sigma-Aldrich, St. Louis, Missouri, USA) and double stained with haematoxylin (Haematoxylin Gill II, Merck, KGaA, Darmstadt, Germany) and eosin (Eosin Y, VWR, Geldenaakseosan, Leuven) before placing a cover slip.

Morphometric variables were measured in transversal body sections of individual fish, at a perianal location. In both larvae and juveniles, the total number of fast-twitch fibers (N) were counted and the total cross-sectional area [CSA (mm^2)], the total cross section muscle area [Muscle CSA (mm^2)] and the fiber cross-sectional area (μm^2) were measured. Fiber diameter (µm) was estimated from the fiber cross-sectional area (μm^2) data assuming that muscle fibers cross-section is round shaped. The mean fiber diameter and percentage of small fibers (<5 µm) were estimated from a minimum of 500 cross-sectioned fast-twitch fibers representative of the whole cross sectional muscle area. The fiber density (total number/mm^2) was calculated by dividing the total number of fast-twitch fibers (N) counted by the total cross section muscle area [Muscle CSA (mm^2)]. Muscle fiber outlines were traced using a 400× magnification using

an Olympus BX51 microscope (Olympus Europa GmbH, Hamburg, Germany) with the Cell^B Basic imaging software.

3.2.6 Gene expression

3.2.6.1 RNA extraction and cDNA synthesis

Nine pools of 20 whole larvae at the metamorphosis climax and 10 whole fish at the juvenile stage - 51 DAH (3 pools per tank) were sampled per dietary treatment, snap-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 2mL 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, purified using the High Pure RNA Isolation Kit (Roche) and again quantified using the Nanodrop spectrophotometer. cDNA was synthesized from 1µg of purified RNA (per pool), using with the M-MLV Reverse Transcriptase Kit (Invitrogen).

3.2.6.2 Quantitave real-time PCR (qPCR)

The relative expression of the myogenic regulatory factors (*myf5, myod2, mrf4, myog), myhc, mstn1* as well as genes encoding for the proteins responsible for *de novo* DNA methylation and DNA methylation maintenance (*dnmt1, dnmt3a,* and *dnmt3b*) 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).

Table 3: Primers used in qPCR

Gene	Fwd sequence (5′→3′)	Rev sequence (5′→3′)	Accession no (GenBank)	Size (bp)	Annealing temp. (ºC)	E (%)
myf5	GAGCAGGTGGAGAACTACTACG	CCAACCATGCCGTCAGAG	FJ515910	89	60	103
mrf4	GAGAGGAGGAGGCTCAAGAAG	CAGGTCCTGTAATCTCTCAATG	EU934042	137	58	96
myog	GTCACAGGAACAGAGGACAAAG	TGGTCACTGTCTTCCTTTTGC	EU934044	118	60	94
myod2	ACAGCCACCAGCCCAAAC	GTGAAATCCATCATGCCATC	FJ009108	194	60	111
myhc	GAAAAATCTGACAGAGGAAATGG	CCTTGGTGAGAGTGTTGACTTTG	FJ515911	143	60	96
mstn1	GGGAGATGACAACAGGGATG	TGGATCCGGTTCAGTGGC	EU934043	91	60	108
dnmt1	GATCCCAGTGAGGAGTACGG	AAGAAGGTCCTCATAAGTAGCGTC	KC129104	117	62	103
dnmt3a	AACTGCTGTAGGTGTTTCTGTGTG	CGCCGCAGTAACCCGTAG	KC129105	134	60	101
dnmt3b	ATCAAGCGATGTGGCGAGC	CGATGCGGTGAAAGTCAGTCC	KC129106	91	60	96
rps4	GTGAAGAAGCTCCTTGTCGGCACCA	AGGGGGTCGGGGTAGCGGATG	AB291557	101	60	95
ubq	AGCTGGCCCAGAAATATAACTGCGACA	ACTTCTTCTTGCGGCAGTTGACAGCAC	AB291588	135	60	93

For each gene, its GenBank accession numbers, amplicon size (bp), Annealing temperatures (ºC) and qPCR amplification efficiencies (E) are indicated.

3.2.7 Data analysis

Statistical analyses followed previously reported methods (Zar, 2010) and IBM SPSS Statistics 19 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 for homogeneity of variance using a Levene's test. Data were log transformed when required and percentages were arcsin transformed prior to analysis.

The influence of diet on the larvae capacity to utilize protein was tested by two-way ANOVA using peptide size and diet as independent factors. The differences between groups detected in growth and muscle growth parameters as well as in the relative expression of target genes were tested by a one-way ANOVA. A Pearson's coefficient correlation was used to compare the relative expression of genes regulating muscle growth *versus* muscle growth parameters, using the mean value of each triplicate tank (N =6). Significant levels were set at $P<0.05$.

To compare the distribution of muscle fiber size, a nonparametric method was used to fit smoothed probability density functions (PDFs) using the statistical program for the analysis of muscle fiber populations (Johnston et al., 1999b). Bootstrapping was used to distinguish random variation in diameter distribution from treatment differences. A Kruskall-Wallis test was used to test the null hypothesis that PDFs of muscle fiber diameter in the two treatments were identical.

3.3 Results

3.3.1 Diets

The amino acids (AA) contents of the experimental inert diets showed that an 8% replacement of the encapsulated fish protein hydrolysate fraction by a crystalline-AA mixture was effective in increasing most IAA levels in the SUP diet resulting in a 14.6% increase in the sum of the indispensable amino acids (IAA) and a 24.8% increase in the IAA/DAA ratio (Table 2). The most significant differences between diets (CTRL and SUP) were found in phenylalanine (Phe) and tryptophan (Trp) contents, which were increased respectively by 81 and 36% in the SUP diet, when compared to the CTRL diet. Moreover, valine (Val) and tyrosine (Tyr) levels were reduced respectively by 4 and 13% in the SUP diet, when compared to the CTRL diet (Table 2), as they were not supplemented in crystalline form (Table 1).

3.3.2 Protein metabolism

Protein retention efficiency and catabolism of Pre-Met larvae were not significantly affected (P=0.637) by dietary IAA levels (Fig.1). The peptide molecular size did not affect the retention or catabolism efficiency in Pre-Met larvae (P=0.592) (Fig.1). Average retention efficiency of Pre-Met larvae was 77.5±3.4%.

Neither the dietary IAA level (P=0.076) nor the different sized peptides (P=0.485) had a significant impact on larvae retention efficiency during the metamorphosis climax (Met) (Fig.1). However, at this stage, there was a significant interaction between diet and peptide size, with the 1.0KDa peptide being better retained in SUP group and the 6.8KDa peptide being similarly retained by both groups. Average retention efficiency of Met larvae was 77.3±6.5%.

Further on the metamorphosis process, at the Post-Met stage, there was also no effect of the diet on larvae metabolic efficiency either for 1.0KDa or 6.8KDa peptides (P=0.83) (Fig.1). However, when comparing the larvae capacity to metabolize smaller or larger peptides, the 6.8KDa peptide was better retained and less catabolized than 1.0KDa (P=0.008) (Fig.1): average retention efficiency of Post-Met larvae was 76.5±3.0% for 1.0KDa peptides and 86.0±4.7% for 6.8KDa peptides.

When comparing larvae metabolism between different developmental stages, regardless of the diets, larvae capacity to retain 6.8KDa peptides increased throughout metamorphosis; significant differences were observed between the Pre-Met and Post-Met stages (P=0.023).

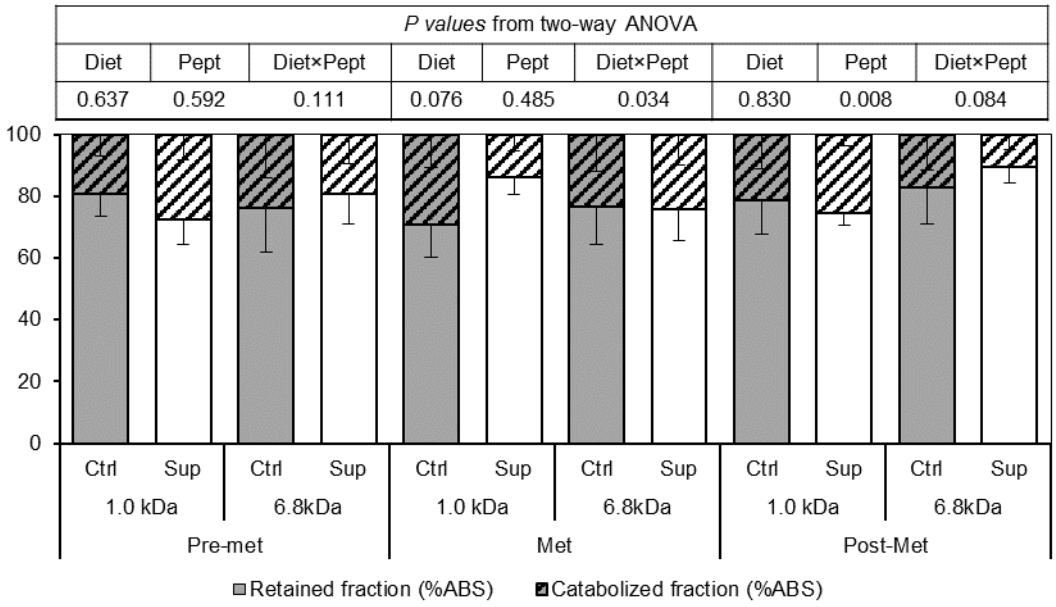

Fig. 1. 1.0KDa and 6.8KDa peptide retention (% of radiolabel in the body in relation to absorbed label), and catabolism (% of radiolabel in the metabolic trap in relation to absorbed label) after 24 h of incubation, in Senegalese sole larvae during the metamorphosis time-window: at pre-metamorphosis – stage 1 (Pre-Met), metamorphosis climax – stage 3 (Met) and post-metamorphosis – stage 4 (Post-Met). Values are means ± s.d., n = 8. Comparisons between groups fed different diets and tube-fed different molecular sized peptides were made using 2-way ANOVA.

3.3.3 Larval performance

In the present trial, both experimental groups (CTRL and SUP) achieved the selected key developmental stages simultaneously, with no significant inter-individual variation: mouth-opening occurred at 2 DAH; Pre-Met (stage 1) occurred at 13 DAH; Met (stage 3) occurred at 19 DAH; Post-Met (stage 4) occurred at 25 DAH; and all post-larvae were weaned at 38 DAH. The last sampling of juvenile fish was intentionally carried out at the same age, 51 DAH, in both treatments (CTRL and SUP), when all fish had already acquired a benthic behaviour.

The supplementation with encapsulated crystalline-IAA affected larval growth throughout the trial, with significant differences being found in the overall RGR (P=0.04) (Table 4; Fig.2). Dry weight was similar between dietary treatments during the pelagic phase, but in the benthic phase fish fed the CTRL diet performed better than those fed the SUP diet. This response to diet was remarkably accentuated with time and throughout the benthic phase (Fig. 2). At the

end of the trial, the fish fed the CTRL diet were 2.1-fold heavier than those fed the SUP diet. Survival was not significantly affected by dietary formulation (Table 4).

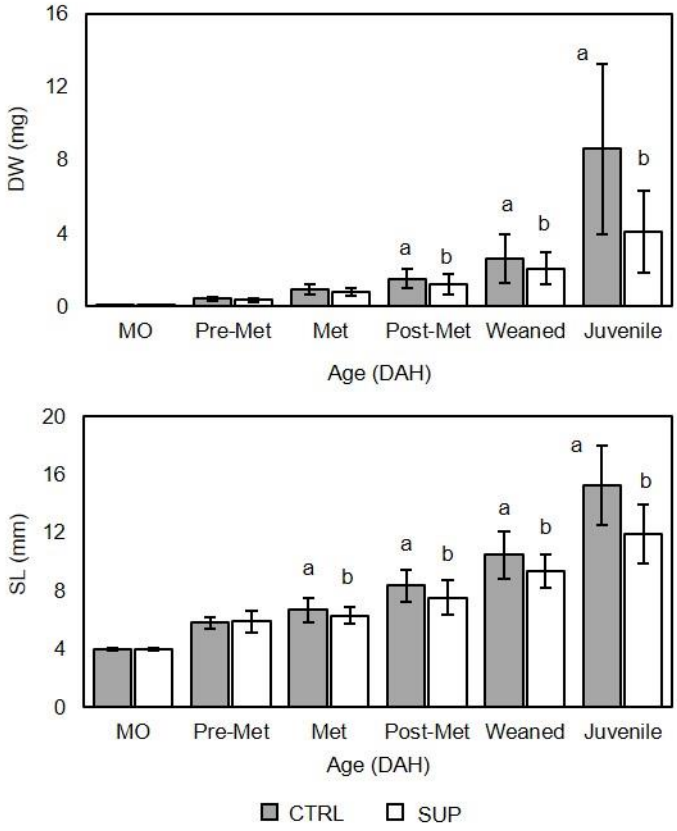

Fig. 2. Sole dry weight (DW) and standard length (SL) during the pelagic phase (at mouth opening (MO), pre-metamorphosis – stage 1 (Pre-Met) and metamorphosis climax – stage 3 (Met)) and the benthic phase (at post-metamorphosis –stage 4 (Post-Met), weaned post-larvae (Weaned) and a late juvenile stage, at 51 DAH (Juvenile)). Values are means ± s.d. of treatments replicates (*n*=30 during the pelagic phase; *n*=60 during the benthic phase). Different superscript letters at each developmental stage indicate significant differences (P<0.05, 1 way-ANOVA) between dietary treatments.

3.3.4 Dietary effect on fast-twitch skeletal muscle growth

Although the dietary IAA level significantly affected larvae body length during the metamorphosis climax (P=0.045), muscle cross-sectional area was similar between groups at this stage. Neither the total number of fibers (N) nor the mean fiber diameter were significantly affected by the dietary treatment at this stage (P>0.05, Table 4), and these parameters did not correlate with fish length (P>0.05). There was also no significant correlation between fish

length and the percentage of small fibers (P>0.05) which was also not affected by dietary treatment (P>0.05, Table 4). Moreover, fiber size distribution remained similar among groups (Fig.3). Significant differences between diets at the Met stage were only detected for fiber density (P=0.021) (Table 4).

Table 4: Standard length (mm), relative growth rate (RGR, %) and white muscle growth morphometric variables measured at a peri-anal location (*N*=9/treatment) at metamorphosis climax – stage 3 (Met) and a juvenile stage, 51 DAH (Juvenile). Relative growth rate (RGR, %) and survival (%) estimated for each tank (*N*=3/treatment). Values are means ± s.d. Comparisons between groups fed with different diets were made using one-way ANOVA. Dissimilar superscript letters indicate a significant difference (P<0.05) between groups.

		Diets	
		CTRL	SUP
Pelagic phase			
Met	SL (mm)	6.7±0.8^{a}	6.3±0.6^{b}
	CSA (mm²)	0.70±0.10	0.64±0.07
	Muscle CSA (mm²)	0.11±0.02	0.09±0.01
	Total number of fibers N	1381±176	1444±185
	Fiber density (total number/mm²)	13227±1805^{b}	15845±2397^{a}
	mean fiber diameter (µm)	8.27±0.99	8.04±0.53
	% small fibers (<5 µm)	15.65±8.91	13.22±4.39
	RGR 2-19 DAH (%.day^{-1})	24.2±1.7	23.4±0.6
Benthic phase			
Juvenile	SL (mm)	15.3±2.7^{a}	11.9±2.0^{b}
	CSA (mm²)	2.56±0.51^{a}	1.78±0.51^{b}
	Muscle CSA (mm²)	0.53±0.13^{a}	0.34±0.13^{b}
	Total number of fibers N	3118±552^{a}	2481±536^{b}
	Fiber density (total number/mm²)	6071±1197^{b}	7695±1494^{a}
	mean fiber diameter (µm)	11.16±1.25^{a}	9.96±0.94^{b}
	% small fibers (<5 µm)	4.06±3.08	5.40±3.72
	RGR 19-51 DAH (%.day^{-1})	7.27±1.21	5.06±1.12
	Survival rate (19-51 DAH) (%)	57.2±11.3	49.4±15.1
Overall growth			
	RGR 2-51 DAH (%.day^{-1})	12.9±0.3^{a}	11.0±1.0^{b}

Between the Met and the Juvenile stages there was a significant enlargement of muscle fibers and muscle CSA, reflected on the shift of PDFs distribution towards the right-hand of graphic (Fig. 3). The total number of fibers increased by 2.3-fold in the CTRL group while it increased by 1.8-fold in the SUP group during the 19-51 DAH period. By the end of the trial, at 51 DAH, muscle CSA was 1.6-fold larger in the CTRL than in the SUP group (P=0.008) (Table 4). This CSA increase was paralleled by a significantly higher total number of fibers (P=0.024) and larger mean fiber diameter (P=0.035) in the CTRL group, compared to the SUP group (Table 4). Fiber density was significantly higher in the SUP fish than in the CTRL (P=0.022), but both

the percentage of small fibers (Table 4) and fiber size distribution (Fig.3) remained similar among dietary treatments at 51DAH. Moreover, no significant correlation could be observed between fish length and any of the muscle cellularity parameters at the juvenile stage (P>0.05).

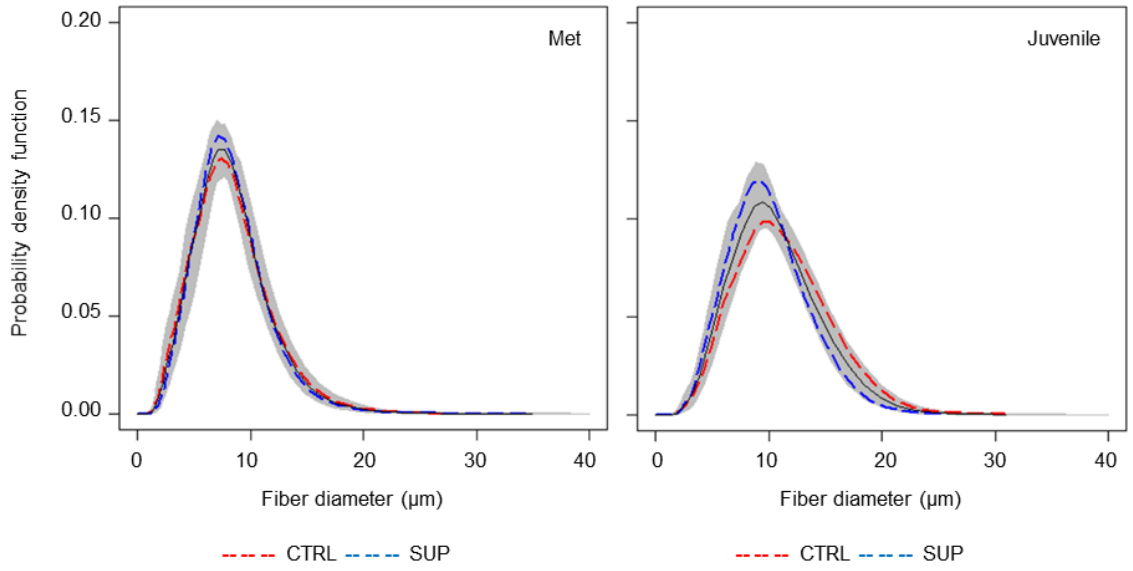

Fig. 3. Probability density functions (PDFs) distributions of fast muscle fibers at the metamorphosis climax – stage 3 (Met) and late juvenile stage, 51 DAH (Juvenile). The dashed lines show the mean PDF for each group and the solid line central to the shaded area is the average PDF for combined groups (CTRL and SUP; *n*=9/treatment). The shaded area shows 1000 bootstrap estimates from combined populations of fiber diameter.

3.3.5 Expression of growth-related genes and DNA methyltransferases

During the metamorphosis climax (Met), *myog* was significantly upregulated in the larvae fed the CTRL diet compared to those fed the SUP diet (P=0.010) (Fig 4). At this stage, *myog* expression was positively correlated with both the CSA (R=0.895, P<0.05) and the percentage of fibers < 5µm (R=0.873, P<0.05) (Table 5). Also at this stage, the expression of *myf5*, *myod2* and *mrf4* was similar between dietary treatments (Fig 4). In 51 DAH juveniles, *mrf4* transcript levels were 1.4-fold higher in the SUP group, compared to the CTRL group (P=0.008) (Fig 4). No significant differences were found in the transcript levels of the other myogenic factors at 51 DAH.

The expression of an important structural gene in muscle, *myhc*, during the metamorphosis climax (Met) was 1.8-fold higher in the CTRL group, compared to the SUP group (P=0.002) (Fig 4), being positively correlated with standard length (R=0.953, P<0.01), CSA (R=0.840,

P<0.05) and muscle CSA (R=0.905, P<0.05) (Table 5). However, in 51 DAH juveniles no significant difference was detected on the *myhc* transcript levels (Fig 4).

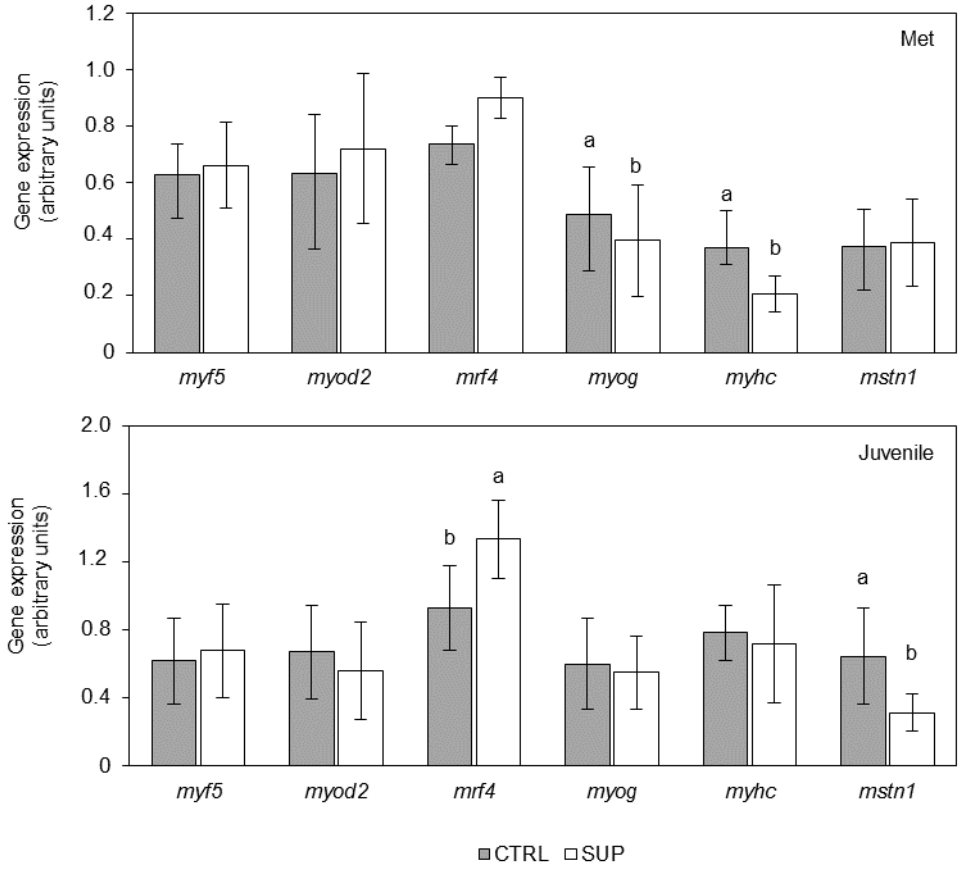

Fig. 4. Expression of genes encoding for myogenic regulatory factors *myf5*, *mrf4*, *myod2*, *myog*, *mstn1*and *myhc* at the metamorphosis climax – stage 3 (Met) (whole body pools of 20 individuals) and late juvenile stage, 51 DAH (Juvenile) (whole body pools of 10 individuals). mRNA expression was normalized to transcript levels of *ubq* and *rps4*. Values are presented means ± s.d., n = 9. Different superscript letters indicate significant differences (P<0.05, 1-way ANOVA) between the dietary treatments at each developmental stage.

No effect was found in the *mstn1* mRNA levels during the metamorphosis climax (Met). Interestingly, in 51 DAH juveniles, *mstn1* showed a 2.1-fold upregulation (P=0.002) in the CTRL group compared with the SUP group (Fig 4). A positive correlation was also observed between *mstn1* expression and relative growth rate during the benthic period (P=0.028; r=0.860) (Table 5).

Table 5: correlations (Pearson's coefficient) between gene expression and muscle growth parameters in Senegalese sole larvae, during the metamorphosis climax –stage 3 (Met) and at a late juvenile stage, 51 DAH (Juvenile)

Genes	SL	CSA	MuscleCSA	Number of fibers	Density	Avg fiber diameter	% of small fibers	RGR (19-51DAH)	RGR (2-51DAH)
Met									
myf5	NS	NS	NS	NS	NS	NS	NS	NS	NS
mrf4	NS	NS	NS	NS	NS	NS	NS	NS	NS
myog	NS	P=0.016, r=0.895	NS	NS	NS	NS	P=0.023, r=0.873	NS	P=0.021, r=0.880
myod2	NS	NS	NS	NS	NS	NS	NS	NS	NS
myhc	P=0.003, r=0.953	P=0.037, r=0.840	P=0.013, r=0.905	NS	P=0.029, r= -0.858	NS	P=0.042, r=0.829	P=0.031, r=0.854	P=0.046, r=0.854
mstn1	NS	NS	NS	NS	NS	NS	NS	NS	NS
dnmt1	NS	NS	NS	NS	NS	NS	P=0.019, r=0.886	NS	NS
dnmt3b	P=0.022, r=0.875	P=0.016, r=0.896	P=0.013, r=0.907	NS	P=0.031, r= -0.853	NS	NS	NS	NS
Juvenile									
myf5	NS	NS	NS	NS	NS	NS	NS	NS	NS
mrf4	P=0.002. r=-0.960	NS	NS	NS	NS	NS	NS	NS	NS
myog	NS	NS	NS	NS	NS	NS	NS	NS	NS
myod2	NS	NS	NS	NS	NS	NS	NS	NS	NS
myhc	NS	NS	NS	NS	NS	NS	NS	NS	NS
mstn1	NS	NS	NS	NS	NS	NS	NS	P=0.028, r=0.860	NS
dnmt1	P=0.004, r=0.946	NS	NS	NS	NS	NS	NS	P=0.019, r=0.886	NS
dnmt3a	NS	NS	P=0.049, r=0.813	NS	NS	NS	NS	NS	NS

Statistical significance was set at P<0.05 (*N*=6)

There was no effect of dietary IAA level on the expression of the DNA methyltransferases *dnmt1* and *dnmt3a*, neither during the metamorphosis climax (Met) nor at 51 DAH. However *dnmt3b* transcript levels were significantly higher in the CTRL group, when compared to the SUP group, at both the metamorphosis climax (1.6-fold, P<0.001) and at 51 DAH (1.4-fold, P=0.045).

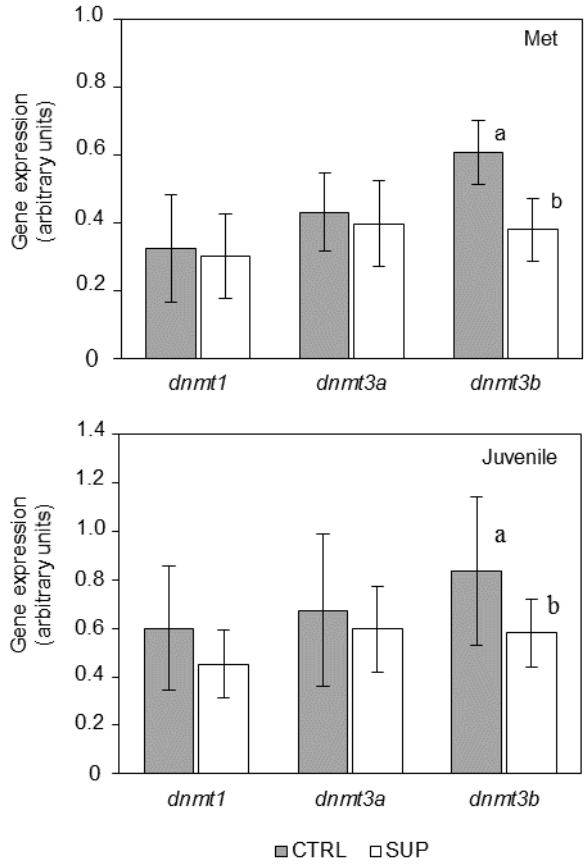

Fig. 5. Expression of genes related to DNA methylation (*dnmt1, dnmt3a* and *dnmt3b*) at the metamorphosis climax – stage 3 (Met) (whole body pools of 20) and late juvenile stage, 51 DAH (Juvenile) (whole body pools of 10). mRNA expression was normalized to those of *ubq* and *rps4*. Values are presented means ± s.d., n = 9. Dissimilar superscript letters indicate significant differences (P<0.05, 1-way ANOVA) between the dietary treatments at each developmental stage.

3.4 Discussion

3.4.1 Effect of IAA supplementation on protein utilization and somatic growth

Replacing 8% of the encapsulated fish protein hydrolysate fraction by a crystalline-AA mixture in the SUP diet was an effective way of increasing the sum of IAA, the IAA/DAA ratio and the

level of most IAA (Table 2). However, this supplementation was not sufficient to fully correct the dietary AA profile. Considering Senegalese sole larvae IAA requirements suggested by Aragão et al. (2004a) as reference Met and His remained possibly limiting in the SUP diet. This was probably due to leaching losses as these supplemented IAA are highly soluble molecules. A major challenge on devising the protein fraction for larvae microdiets is the small size of the feed particles, in which the high surface/volume ratio reduces the diffusion distance from the core to the surface. As a consequence, soluble protein forms such as FAA that would allow to finely tune dietary AA profile are easily lost by leaching (Kvale et al., 2007; Kvale et al., 2006; Nordgreen et al., 2008; Yúfera et al., 2002).

Dietary IAA supplementation did not improve larvae capacity to utilize small peptides, as no significant differences were found between larvae fed the CTRL diet and those fed the SUP diet. A positive impact of the SUP diet would have been expected in accordance with previous data from Aragão et al. (2004b) that have shown a higher retention of [^{14}C]-protein hydrolysate in *Artemia* fed Senegalese sole post-larvae (36-40 DAH), after Leu-Gly and Phe-Ala dipeptides supplementation in an *in vivo* tube-feeding trial. However, in the present study, it is likely that IAA dietary supplementation was not sufficiently effective to have an effect on the larvae metabolic capacity and did not promote growth, similarly to what was reported in white seabream (*Diplodus sargus*) larvae fed microencapsulated diets supplemented with crystalline-AA (Lys and Trp) (Saavedra et al., 2009b). On the contrary, larvae fed the non-supplemented diet (CTRL group) grew faster since an early stage. These results might be explained by the impaired utilization of the fast absorbed crystalline (free) AA compared to the protein-bound AA which needs the action of digestion prior to absorption. That would have led to a decrease in protein accretion. Rønnestad et al. (2000) showed that free AA are absorbed much faster than intact protein, and Rønnestad and Conceição (2012) proposed that even a highly digestible protein – the one from *Artemia* – takes more than 2 hours to be fully digested. Furthermore, the absorption of individual AA depends on different transport systems (Ronnestad, Morais, 2008) and seems to proceed at different rates (Conceição et al., 2011; Dabrowski, 1983) and with different efficiencies, depending on the species and developmental stage (Conceição et al., 2002; Rønnestad et al., 2001b; Saavedra et al., 2008a; 2008b). As a consequence, different absorption rates between individual AA may lead to transitory AA imbalances in the cellular FAA pool where the protein synthesis occurs, leading to increased AA catabolism. If that was the case, a significant part of the supplemented crystalline IAA could have been lost to catabolism, thus resulting in less IAA available for protein synthesis and compromising long-term growth.

Another possibility is that the differences found in growth are due to a possible effect on the voluntary feed intake. In the present study, it was not possible to quantify the larva voluntary feed intake on the experimental diets since no viable technique has been so far developed to measure consistently feed intake over time in marine fish larvae. However, there was an apparent clear excess of remaining feed in the tanks fed the SUP diet. It can be hypothesised that larvae fed the CTRL diet may have increased their voluntary feed intake to compensate for IAA deficiencies, as previously reported for midas (*Amphilophus citrinellum*) (Dabrowski et al., 2007) and rainbow trout juveniles (*Oncorhynchus mykiss*) (Alami-Durante et al., 2010). Ultimately, a higher level of tryptophan (Trp) in the SUP diet might also have reduced voluntary feed intake in this group (Table 2). Trp is the precursor of serotonin (5-hydroxitryptamine, 5-HT) which participates as a messenger in the central nervous system and peripherally in gastrointestinal and vascular systems. Higher dietary Trp levels were shown to induce variations on brain 5-HT content, probably leading to depressed appetite and feed consumption and ultimately to reduced growth in juvenile groupers (*Epinephelus coioides*) (Hseu et al., 2003). In the present study, a higher voluntary feed intake in the CTRL group would have led to a positive net nitrogen and energy balance throughout the experiment, leading to a higher growth rate. Further studies involving innovative approaches able to determine feed intake in fish larvae are required to validate such hypothesis.

3.4.2 Effect on the regulation of muscle growth

Dietary supplementation with encapsulated crystalline-IAA affected larval growth throughout the trial, and significant differences on fish length started being noticed during the metamorphosis climax (Fig 2, Table 4). At this stage (Met), fish fed the SUP diet were significantly smaller than their control counterparts (P=0.045, Table 4) but muscle cross-sectional area was similar between groups, mostly due to the higher number of fast-twitch fibers in the SUP group. Moreover, the expression pattern of key genes regulating myogenesis was affected by the dietary treatments in Met larvae, with the expression of *myog* and *myhc* transcript levels being significantly reduced in the SUP group (Fig. 4). *Myogenin* is an indicator of myogenic cell recruitment for stratified hyperplasia, the second phase of myogenesis occurring at this developmental stage in Senegalese sole, as previously described by Campos et al (2013b; 2013c). Although the down-regulation of *myogenin* in the SUP fed larvae did not translate into changes on total number of fibers between dietary treatments during the Met stage, it might partially explain the reduced total number of fibers, smaller cross-sectional muscle area and reduced somatic growth rate in later stages (51 DAH juveniles). In fact, in Met larvae, *myogenin* expression was positively correlated with the percentage of small-sized muscle fibers (Table 5) that is known to be a good indicator of further muscle growth potential

(Valente et al., 1999). Thus, the up-regulation of *myogenin* in the CTRL group in response to dietary IAA levels at the Met stage may have anticipated a greater increase of the total number of fibers from the Met to the juvenile stage that would ultimately have sustained a higher growth rate. In pike perch (*Sander lucioperca*) larvae (Ostaszewska et al., 2008), different types of feed and dietary formulations led to different growth rates and altered muscle growth dynamics. Increased proliferative capacity of MPCs and a higher contribution of hyperplasia was reported in fast-growing groups. A similar response was reported for pacu larvae (*Piaractus mesopotamicus*) (Leitão et al., 2011) subjected to different types of feed, dietary formulations and feeding regimes, including starvation.

In the present study, the group fed the SUP diet showed no signs of reduced contribution of hyperplasia to muscle growth, as indicated by a similar percentage of small fibers between diet groups, but it displayed some signs of delayed muscle growth at the juvenile stage. Differences in juvenile muscle cellularity were not significantly correlated with fish length. Instead, they probably reflect a lower feed intake and subsequent lower AA availability for protein accretion in the SUP group that ultimately resulted in decreased fiber size and reduced fish length. The downregulation of *myogenin* in the SUP group (Fig. 4) is consistent with previous results reported in common carp (*Cyprinus carpio*) fingerlings subjected to a restrictive diet (Kamaszewski et al., 2014) and in rainbow trout (*Oncorhynchus mykiss*) juveniles subjected to starvation (Johansen, Overturf, 2006). Moreover, *myogenin* expression was clearly responsive to AA availability in myocyte cells isolated from gilthead seabream (*Sparus aurata*) (Velez et al., 2014) and to refeeding in a primary culture of Atlantic salmon (*Salmo salar*) myocytes (Bower, Johnston, 2010). These results suggest that *myogenin* expression is responsive to a possibly lower availability of AA for protein synthesis in the SUP group.

Unlike the observed up-regulation of *myogenin* in the CTRL group (significant at the Met stage)*, mrf4* exhibited an opposite tendency, being significantly less expressed in the CTRL group at the juvenile stage (Fig.4). Secondary MRF's (those involved in the inducing and maintaining the muscle differentiation) showed different patterns of expression in response to starvation and refeeding in rainbow trout juveniles (Johansen, Overturf, 2006) and in response to refeeding in Atlantic salmon isolated myocytes (Bower et al., 2008). Senegalese sole post-larvae (35-51 DAH) exposed to different rearing temperatures during the pelagic phase also showed a tendency to increase the expression of *mrf4* in a slow-growing group, during a compensatory growth phase at 83 DAH, long after the exposure to the environmental challenge (Campos et al., 2013b).

At the juvenile stage (51 DAH), *mstn1* was up-regulated in the CTRL group (Fig.4). Similarly, Campos et al. (2013b) reported an up-regulation of *mstn1* in the fast-growing S. sole groups

at a juvenile stage (83 DAH). In other fish species, the relation between *myostatin* mRNA levels and muscle growth is surprising: depression of growth induced by environmental conditions does not correlate with an up-regulation of *myostatin* as expected (Rescan, 2005). In fact, different *myostatin* paralogues have been reported in salmonids and exhibit distinct expression patterns in muscle and non-muscle tissues (Rescan et al., 2001; Roberts, Goetz, 2001; Valente et al., 2006). In Senegalese sole, high transcript levels of *mstn1* were found in juveniles liver (Campos et al., 2010). As gene expression was analyzed in the whole fish and not only in the muscle, it is possible that a higher expression of *mstn1* might be associated with other physiological mechanisms and not only with skeletal muscle growth regulation, as previously suggested by Campos et al. (2013b).

The down-regulation of *myhc* in SUP fed Met larvae (Fig.4) did not translate into significant changes on fiber size between dietary treatments (Table 4; Fig. 3), but might be partially related with the slightly higher total number of fibers and higher fiber density observed in the SUP group at the Met stage. In fact, at this stage, *myhc* expression was negatively correlated with fiber density and positively correlated with muscle CSA (Table 5). Thus, the down-regulation of *myhc* in Met larvae may explain, at least in part, the further reduced size of fast fibers and total cross-sectional muscle area during the juvenile stage (51 DAH). *Myhc* was suggested as an index to monitor “specific growth rate” under variable nutritional conditions in rainbow trout (Overturf, Hardy, 2001) and was shown to be correlated with muscle protein accretion in Atlantic salmon juveniles (Hevrøy et al., 2006). Similarly, in the present study, the reduced expression of *myhc* in the SUP group at the Met stage (Fig.4) may be related with a lower availability of AA to promote protein synthesis in the muscle.

3.4.3 Epigenetic effect

DNA methylation relies on the one-carbon metabolism pathway, which is dependent upon the activity of several enzymes in the presence of dietary methyl donors, such as folate, choline, betaine and methionine (Anderson et al., 2012). In the one-carbon cycle, methionine is converted into S-adenosylmethionine (SAM), the universal cellular methyl donor (Selhub, 1999). DNA cytosine methyltransferases (*dnmt*s) covalently attach SAM methyl groups to the 5′-position of cytosine (in CpG dinucleotides), thus methylating DNA and repressing transcription. In the present study, the reduced expression of *dnmt3b* and a decreasing tendency in the expression of *dnmt1* and *dnmt3a* in the SUP group during the metamorphosis climax (Met) and at the juvenile stage (Fig. 5) could be associated with a possible lower protein intake. Further studies are needed to ascertain whether this was due to a lower availability of methyl group donors, as a consequence of lower feed intake, and whether it would possibly lead to DNA hypomethylation in skeletal muscle. Previous studies in Senegalese sole showed

that rearing temperature during the pelagic phase induced changes in the expression of *dnmt1* and *dnmt3b* DNA methyltransferases during metamorphosis, eventually mediating an epigenetic regulation of muscle growth, through altered expression of *myogenin* (Campos et al., 2013a; 2013b) . Therefore, the pelagic phase and in particular the metamorphosis climax could be a susceptible time window for nutritional programming in Senegalese sole.

3.5 Conclusions

The present results suggest that supplementing microdiets with crystalline-AA in order to correct IAA dietary deficiencies does not bring a clear effect on the Senegalese sole larvae capacity to retain different-sized peptides and does not improve long-term somatic growth. In fact, fish fed a non-supplemented diet performed better. This 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*), as well as the expression of *dnmt3b*. As this gene encodes for a DNA methyltransferase essential for *de novo* methylation, an epigenetic effect at the transcriptional regulation level is suggested as a possible explanation for the differences found in growth as a response to a nutritional cue.

3.6 Acknowledgements

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

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 plant protein hydrolysate with a high incorporation of 5–70 kDa peptides. The HighH diet protein fraction was mostly based on a commercial fish protein hydrolysate with a high incorporation 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 membrane 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 *pga* (*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.

Key words: protein hydrolysate; proteolytic enzymes; PepT1; metabolism; growth; fish larvae

4.1 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 d 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., 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 (Ronnestad, 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., 2004; Cahu et al., 1999; Cai et al., 2015; Gisbert et al., 2012; Kolkovksi, Tandler, 2000; Kotzamanis et al., 2007; Kvåle et al., 2009; Kvåle et al., 2002; 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 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, 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 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., 2007), 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.

4.2. Material and Methods

4.2.1. 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±0.5ºC 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 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 (9.30h) 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.

4.2.2. 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 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.

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.

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

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

4.2.3. Sampling and experimental design

4.2.3.1 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.

4.2.3.2 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) 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).

4.2.3.3 Gene expression

4.2.3.3.1 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-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).

4.2.3.3.2 Quantitave real-time PCR (qPCR)

The relative expression of genes encoding for precursors of the enzymes involved in luminal protein digestion Pepsin (*pga)* and Trypsin (*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™ 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).

4.2.4 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 $p<0.05$.

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

4.3 Results

4.3.1 Larval performance

The total substitution of dietary native plant proteins for partially hydrolysed plant protein had a positive effect (1.2 fold higher, when compared to both the Intact and HighH diets) on larval growth until the metamorphosis climax (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 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.

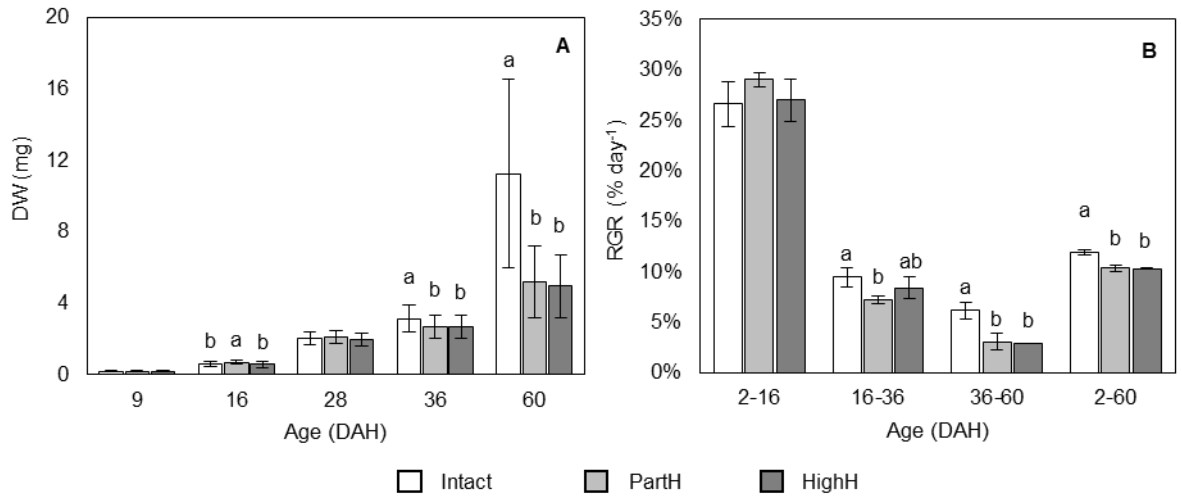

Fig. 1 A. Sole dry weight (DW/mg); values are means ± s.d. (*n*=30 at 9, 16 and 28 d after hatching (DAH); *n*=60 at 36 and 60 DAH). **B.** Relative growth rate (%/day, RGR) during the pelagic phase (2-16 DAH), the benthic phase (16-36 DAH and 36-60 DAH) and the whole trial (2-60 DAH); values are means ± 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)

4.3.2 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).

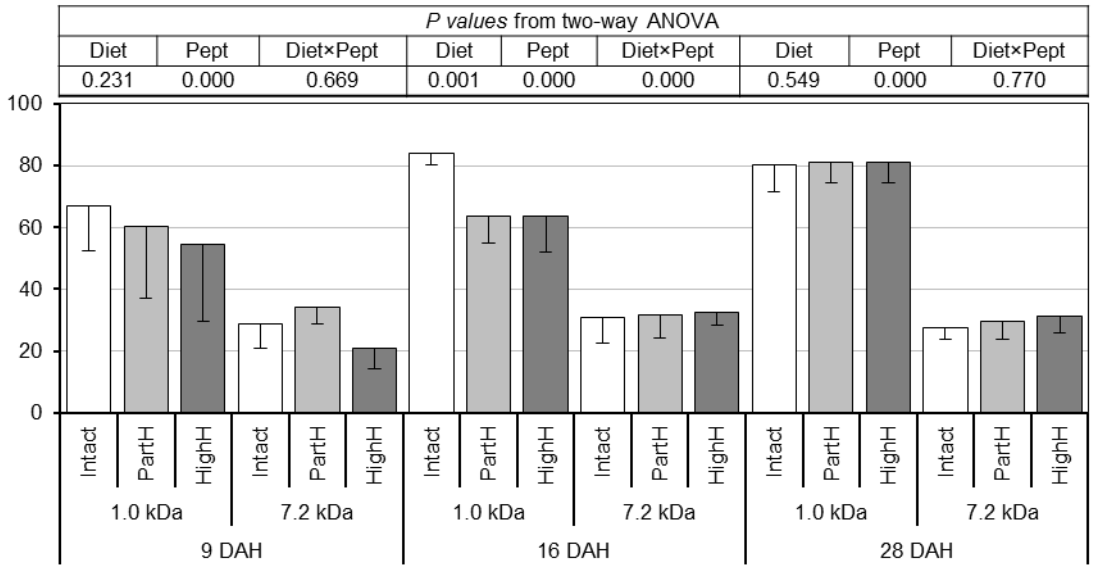

Fig. 2 1.0 kDa and 7.2 kDa peptides absorbed fraction (sum of the % of radiolabel in the body and in the metabolic trap in relation to the total tracer fed; empty bars, white = Intact, light grey = PartH, dark grey = HighH) in sole larvae at 9 (pre-metamorphosis), 16 (metamorphosis climax) and 28 DAH (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.

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 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).

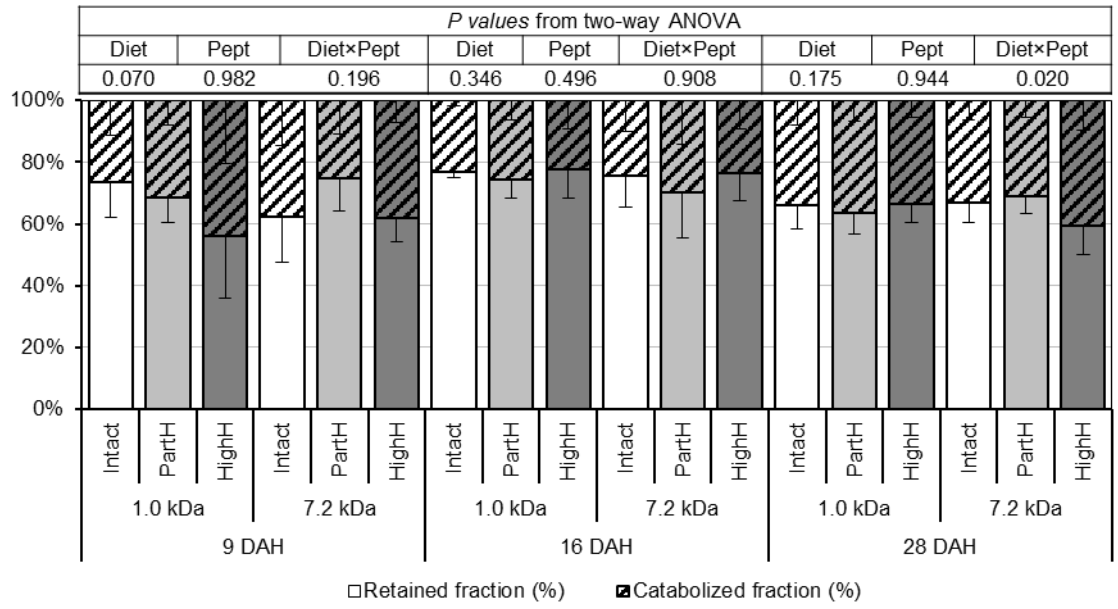

Fig. 3 1.0 kDa and 7.2 kDa peptides retained fraction (% of radiolabel in the body in relation to absorbed label; empty bars, white = Intact, light grey = PartH, dark grey = HighH), and catabolized fraction (% of radiolabel in the metabolic trap in relation to absorbed label; dashed bars, white = Intact, 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.

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, in 28DAH sole, the total retention capacity either 1.0 kDa or 7.2 kDa peptide was not significantly affected by dietary treatment ($p=0.972$) (Fig. 4).

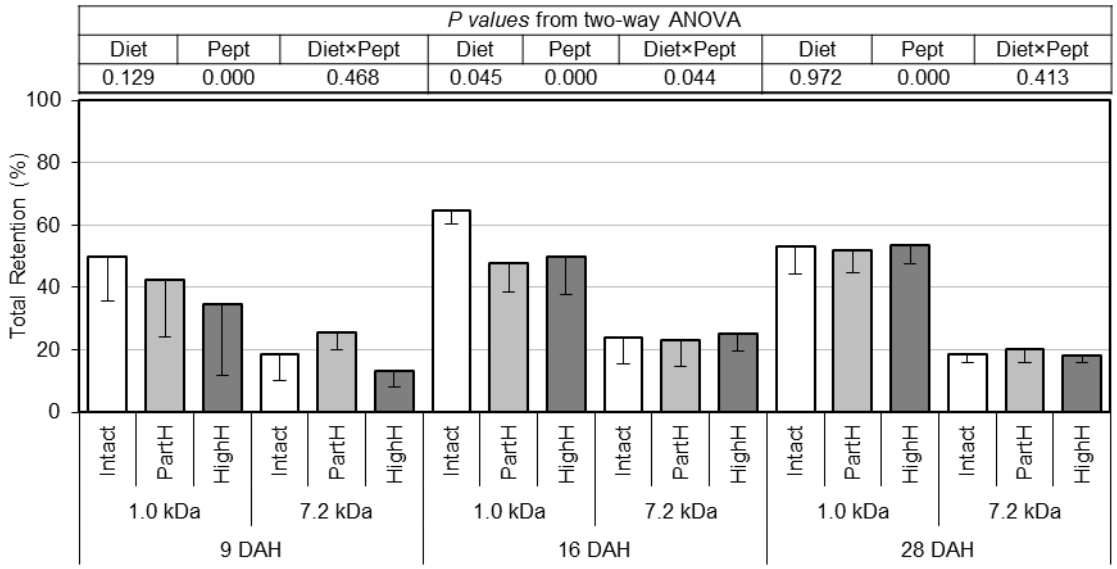

Fig. 4 1.0 kDa and 7.2 kDa peptides total retention (% of radiolabel in the body in relation to tube-fed protein label) in sole larvae at 9 (pre-metamorphosis), 16 (metamorphosis climax) and 28DAH (post-metamorphosis). Values are mean ± s.d., *n*=6-13. Comparisons between groups fed different diets and 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.

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 Pepsin (*pga)* and Trypsin (*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). 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).

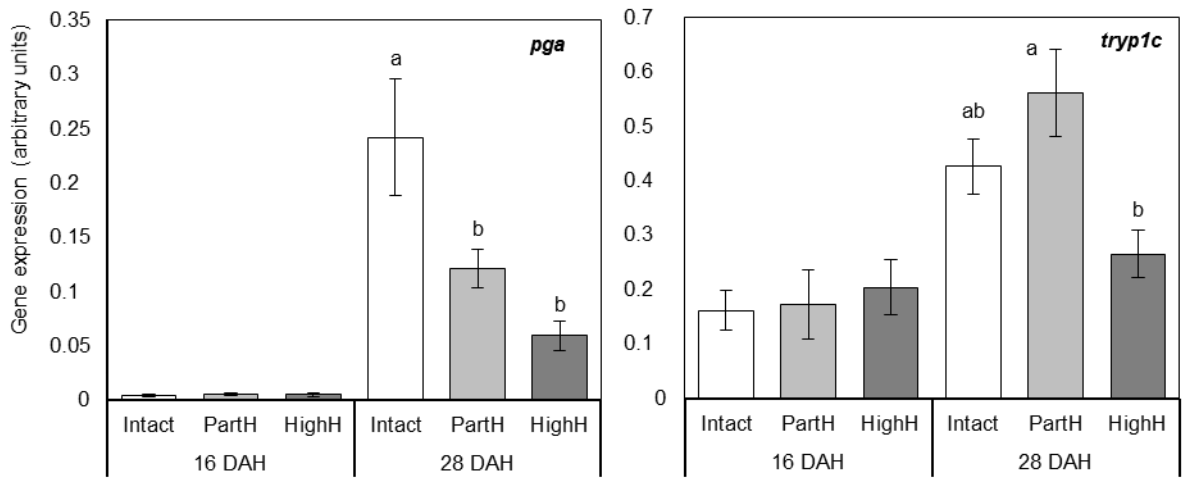

Fig.5 Expression of genes encoding for precursors of pepsin (*pga*) and trypsin (*tryp1c*) involved in luminal protein digestion at 16 (metamorphosis climax) (whole body pools of 20 individuals) and 28 DAH (post-metamorphosis) (whole body pools of 20 individuals). mRNA expression was normalized to transcript 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.

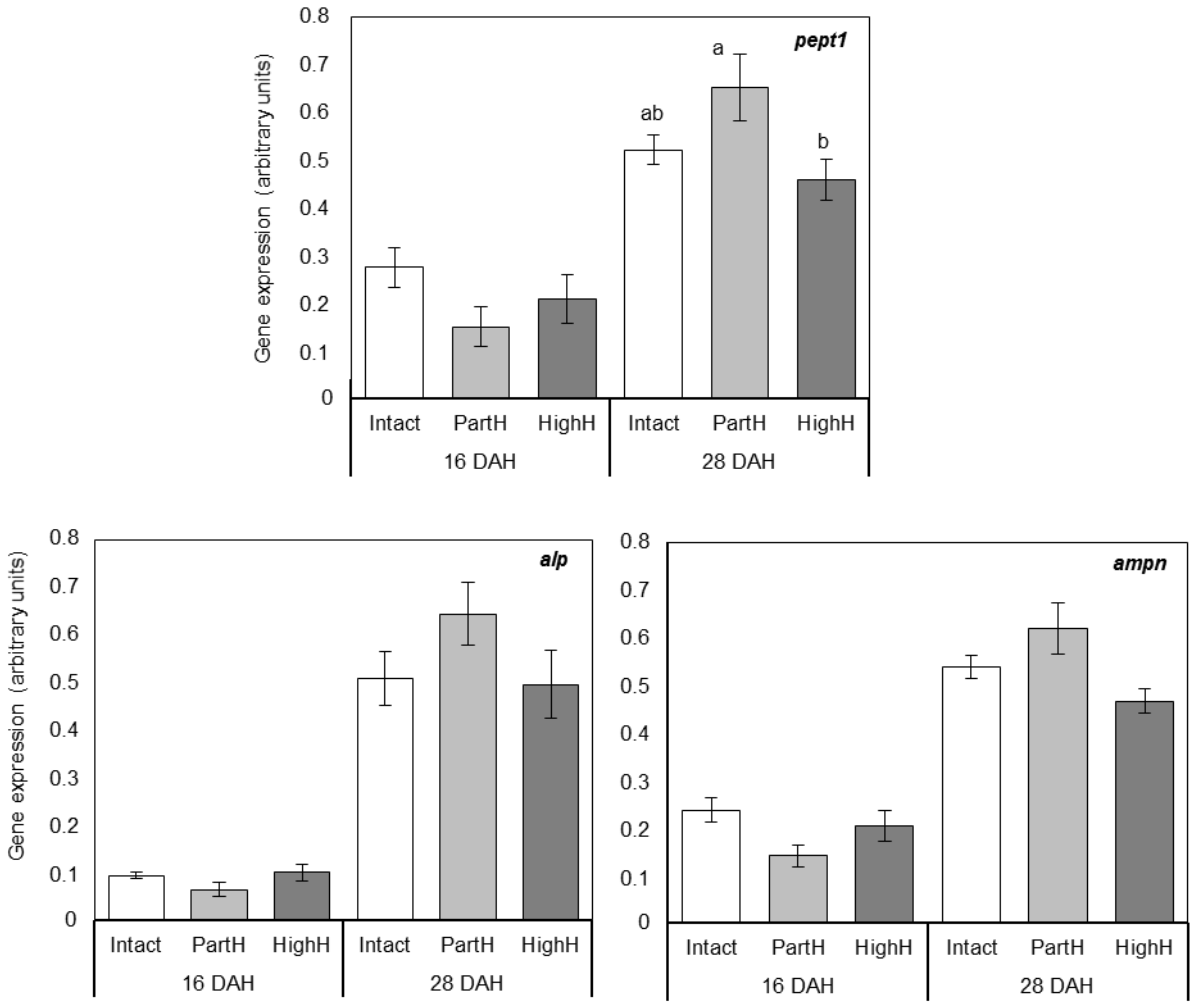

Fig.6 Expression of genes encoding for the enterocyte membrane peptide transporter 1 (*pept1*) and for the BBM enzymes intestinal alkaline-phosphatase (*alp*) and aminopeptidase N (a*mpN*), at 16 (metamorphosis climax) (whole body pools of 20 individuals) and 28 DAH (post-metamorphosis) (whole body pools 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.

4.4. 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, in the present study, we aimed to test three microdiets with substantially different peptide MW profiles, including one diet mostly

based on native intact plant 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. However, being practical formulations, other factors have also changed. Initially it was planned to use the same a mix of plant protein sources to produce both the hydrolysates to be included in the PartH and HighH diets. However, the hydrolysate produced with this plant protein mixture, hydrolysed so to have a dominance of low molecular weight peptides (<5 KDa), was extremely hygroscopic, which made it impossible to use in microdiets for sole larvae. Thus, this plant protein based hydrolysate was replaced by a commercial fish protein hydrolysate – CPSP90® (Sopropêche, France) with a high content (87%) of small polypeptides, oligopeptides and di and tri-peptides (<5 KDa) to be included in the HighH diet. This replacement is believed not to have affected attractability or palatability of the three microdiets for sole larvae, since all three diets contained a minimum of 43% marine ingredients, including high levels fish and krill protein hydrolysates (Table 1). 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.

In this study, dietary formulations based on different protein MW profiles have differentially affected the 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 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). Thus, 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).

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 (Ronnestad, 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 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, although 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 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 be able to utilize dietary intact 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).

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., 2010a; 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 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 the transcription of the studied trypsinogen isoform (*tryp1c*) and *pept1* (Fig. 5 and 6) was promoted in 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 the effect of Intact formulation on gut transit time. When compared to 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 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.

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

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Dietary protein complexity affects growth and the expression pattern of muscle growth related genes in Senegalese sole (*Solea senegalensis*) larvae

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Dietary protein complexity affects growth and the expression pattern of muscle growth related genes in Senegalese sole (*Solea senegalensis*) larvae

Abstract

The inclusion of pre-digested protein in microdiets for fish larvae may affect the genetic regulation of muscle growth, and possibly underlie epigenetic events. 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 higher than 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). 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 somatic growth. At 36 DAH, 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 fibers in the PartH and HighH groups led to reduced potential for muscle growth, which resulted on further reduced somatic growth. The up-regulation in the transcript levels of genes encoding for *de novo* DNA methyltransferases in the HighH group is likely due to increased methionine levels in this diet, which may result in a general increase in DNA methylation.

Key words: protein hydrolysate; MRF; muscle growth, gene expression, Senegalese sole

5.1 Introduction:

Protein deposition is the main determinant of fish growth (Carter, Houlihan, 2001; Houlihan et al., 1995) and fish larvae, more than juvenile and adult fish, have a particularly high requirement for amino acids (AA) (Conceição et al., 2011; Rønnestad et al., 2003). Fish larvae have a tremendous growth potential (Conceição et al., 2003; et al., 2011) and its reliance on dietary AA both as fuel for energy production, and as building blocks for growth (Parra, Yúfera, 2001; Parra et al., 1999; Rønnestad et al., 1999, 2003; Rønnestad, Fyhn, 1993;) can be seen as paradoxical considering the development of the 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 for fish larvae, in

order to make the most of its digestive tract capacity to utilize dietary protein and support high growth rates, so as to fully express its maximum growth potential (Conceição et al., 2011).

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 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 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 (Ronnestad, 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 for fish larvae would improve its dietary protein digestibility. Previous studies reported increased survival and somatic growth in European sea bass (*Dicentrarchus labrax*) (Cahu et al., 2004; 1999; Zambonino Infante et al., 1997), gilthead sea bream (*Sparus aurata*) (Kolkovksi, Tandler, 2000), white seabream (*Diplodus sargus*) (de Vareilles et al., 2012), large yellow croaker (*Pseudosciaena crócea*) (Liu et al., 2006), Asian sea bass (*Lates calcarifer*) (Srichanun et al., 2014) and Atlantic halibut (*Hippoglossus hippoglossus*) (Kvåle et al., 2009, 2002) larvae fed microdiets including protein hydrolysates. However, very few studies focused on a possible influence on muscle growth regulation (Katan et al., 2016; Ostaszewska et al., 2008). Muscle development and growth during early life stages is clearly determinant of 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).

As in most teleost larvae, Senegalese sole white skeletal muscle constitutes the bulk of the axial locomotor muscle and its development 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 (MPC) that undergo activation, proliferation, cell cycle exit, differentiation, migration and fusion into already formed muscle fibers (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 MPC population; *myogenin* and *mrf4* drive and keep on the myoblast

differentiation that will ultimately result in myotube formation and enlargement (reviewed by 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, Veggetti, 2001). During post-embryonic and larval development, muscle fibre number increases mainly by stratified hyperplasia, which involves the recruitment of new fibers in discrete germinal zones found in the lateral margins of the myotome (Rowlerson, Veggetti, 2001). In juvenile and adult stages, new myotubes form on the surface of fast muscle fibers, further fusing or adding nuclei to already existing fibers - mosaic hyperplasia (Rowlerson, 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 (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), 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. According to Alami-Durante et al. (2014), different protein:energy ratios delivered to first-feeding rainbow trout 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. This result suggests that the activity of white MPCs might be programmed by nutritional factors, 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). To our best knowledge, in fish, no relationship has been established between nutritional status and the epigenetic regulation of myogenesis.

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 a late early juvenile stage in Senegalese sole. The expression pattern of DNA methyltransferases was analyzed in order to understand whether there could be an epigenetic event possibly underlying the response of muscle growth regulation and somatic growth to different dietary formulations.

5.2. Material and Methods

5.2.1. Experimental diets

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 ingredients (Table 1). The Intact diet contained a mix of intact plant protein sources – Plant protein Mix 12 (84% CP, 3.6% CF, SPAROS, Portugal), targeting a peptide molecular weight (MW) higher than 20KDa. The PartH diet contained a hydrolysate of the same plant protein mixture used in INTACT diet - IdG Hydrolysate. This hydrolysate was produced at Instituto de la Grasa (CSIC, Sevilla, Spain) according to (Villanueva et al., 1999), using pea protein concentrate and wheat gluten as sources and 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 hydrolysate with a predominance of small polypeptides, oligopeptides and di and tri-peptides (<5 KDa). All three diets contain a minimum of 43% marine ingredients, including high levels fish and krill protein hydrolysates, which all together should make the diets highly 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 is 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 60 ºC, being subsequently crumbled (Neuero Farm, Germany) and sieved to desired size ranges.

The microdiets were grounded, pooled and analyzed for dry matter (105 °C for 24 h) and ash content by combustion in a muffle furnace (Nabertherm L9/11/B170, Bremen, Germany; 550 °C for 6 h).

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 (DM,%)	93.5	90.7	91.7
Ash (% DM)	8.6	10.9	10.1
Crude protein (% DW) after leaching for 1 min	64.7	61.1	65.8
Gross Energy (Kj/g) after leaching for 1 min	20.1	20.1	20.2

[a] Proprietary SPAROS product for marine fish: 93% CP, 1.3% CF.
[b] Proprietary SPAROS product for marine fish: 84% CP, 3.6% CF.
[c] Proprietary SPAROS protein hydrolysate, resulting from hydrolysis of Plant protein Mix; peptide molecular weight profile: >70 KDa (12%); 20-70 KDa (28%); 5-20 KDa (27%); <5 KDa (23%)
[d] CPSP 90, Sopropêche, France; peptide molecular weight profile:>20 KDa (1%); 20-10 KDa (4%); 10-5 KDa (8%); 5-1 KDa (48%); 1-0.5KDa (18%); <0.5KDa (21%)
[e] ICC, Brazil
[f] Aquativ, France
[g] DHA-rich oil
[h] Marine phospholipids and marine oils, Tromsø Fiskeindustri A/S, Norway
[i] Marine oil omega 3: Henry Lamotte Oils GmbH, Germany
[j] Proprietary SPAROS premixes / products for marine fish.

As sole have a peculiar (passive) bottom feeding behaviour, with microdiets remaining one min or more in the tank bottom before being eaten (Conceição et al., 2007; Dinis et al., 2000), microdiet samples (*n* = 4/treatment) were submersed in rearing water for 1 min, in order to allow nutrient leaching and to simulate the situation as 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. The microdiet samples after leaching for 1 min were grounded, pooled and analyzed for dry matter (105 °C for 24 h), crude protein by automatic flash combustion (Leco FP-528, Leco, St. Joseph, USA; N × 6.25) and gross energy in an adiabatic bomb calorimeter (Werke C2000; IKA, Staufen, Germany). The amino-acid composition of the

microdiet samples after leaching for 1 min was determined by ultra-high-performance liquid chromatography (UPLC) in a Waters Reversed-Phase Amino Acid Analysis System, using norvaline as an internal standard (Table 2). 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, USA). The diet composition before (dry matter and ash content) and after leaching (crude protein and gross energy) is presented on Table 1.

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

5.2.2. Husbandry and experimental set-up

Experiments were performed by trained scientists and 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. CCMAR facilities and their staff are certified to house and conduct experiments with live animals ('group-1' license by the 'Direção Geral de Veterinaria', Ministry of Agriculture, Rural Development and Fisheries of Portugal).

Senegalese sole eggs were incubated in an upwelling incubator at 19±0.5ºC and hatching was completed within the next day (24h). Newly hatched larvae were evenly distributed over 9 white cylindro-conical tanks (100L) 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 sterilizer. Larvae were reared in green water conditions until 16 days after-hatching (DAH), provided by adding frozen *Nannochloropsis* sp. (Nannochloropsis 18% FP 472/180908, Acuicultura Y Nutrición de Galicia SL) to rearing tanks every morning. Abiotic parameters and mortality were daily monitored. Dissolved oxygen 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 10/14h light/dark photoperiod cycle was adopted and a light intensity of 1000lux was provided by overhead fluorescent tubes. At 16DAH, during the metamorphosis climax, the larvae were transferred to flat-bottom tanks (30×70×10cm; 21L), 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 monitored and mortality was recorded every morning. Dead larvae were removed and the rearing units were carefully cleaned with minimal disturbance. Dissolved oxygen 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 10/14h 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 (200-400μm). *Artemia* AF nauplii (na) (INVE, Belgium) were introduced at 4DAH and prey density was gradually increased from 4 to 5 na·mL−1, becoming the only prey offered after 5DAH. *Artemia* EG metanauplii (M24) (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 diet until complete

weaning at 36DAH, according to Engrola et al. (2009a). After 36DAH fish were exclusively fed with the respective inert diet (Intact, PartH and HighH) and considered weaned.

Live prey was delivered 3 times a day (3h interval) during the pelagic phase and 4 times a day (2h30 interval) during the benthic phase. The first live feed meal was offered 1hour after the lights were on (11.00h) during the pelagic phase and 30 min (9.30h) after during the benthic phase. Inert diet was delivered semi-continuously with automatic feeders (cycles of 2 h of feeding followed by 1h 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).

5.2.3. Somatic growth and survival

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 9DAH (PM: pre-metamorphosis), 16DAH (MC: metamorphosis climax), 28DAH (BSLF: metamorphosis completed) (*n*=10 per replicate), at 36DAH (weaned early juvenile) (*n*=20 per replicate) and 59DAH (*n*=20 per replicate). The larvae and early juveniles were frozen at -80ºC and freeze-dried for dry weight determination to 0.001mg 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 (16-60DAH) and during the whole trial (2-60DAH). RGR was calculated as RGR (% day-1) = $(e^{g}-1)\times 100$, 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.

5.2.4. Fast-twitch muscle cellularity

Standard histological and morphometric techniques (Silva et al., 2009; Valente et al., 1999a) were used to analyse fast-twitch muscle cellularity at two developmental stages: 16DAH (metamorphosis climax – stage 3) and 36DAH (weaned post-larvae). Three fish per tank were collected, killed by over-anaesthesia (MS-222, Sigma-Aldrick, USA; 400 mgL^{-1}) and measured as previously described. Fish were then were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (Sigma-Aldrich, USA) for 24h, washed with 1×PBS and stored in 70º ethanol, at 4ºC until further processing. Paraffin (Merck, Germany) embedded fish were sectioned (7 µm) transversely to the body axis, using a microtome (RM2245, Leica, Germany), mounted on coated slides with 3-aminopropyltriethoxysilane (APES) (Sigma-Aldrich, USA) to improve section adhesion and double stained with haematoxylin (Haematoxylin Gill II, Merck, Germany) and eosin (Eosin Y, VWR, Belgium) before placing a cover slip.

Morphometric variables were measured in transversal body sections of individual fish, at a peri-anal location. In larvae and early juveniles (16 and 36DAH), the total number of fast-twitched fibers (N) were counted, and total cross-sectional area [CSA (mm^2)], total cross section muscle area [Muscle CSA (mm^2)] and fibre cross-sectional area (μm^2) were measured. Fibre diameter (µm) was estimated from the fibre cross-sectional area (μm^2) data to assuming that muscle fibers cross-section is round shaped. The mean fibre cross-sectional diameter and percentage of small fibers (<5 µm) were estimated from a minimum of 500 fibers which cross-sectional area was measured over the total cross section muscle area. The fibre density (total number/mm²) was calculated by dividing the total number of fast-twitched fibers (N) counted by the total cross section muscle area [Muscle CSA (mm^2)]. Muscle fibre outlines were traced using a 400× magnification. This study was performed using an Olympus BX51 microscope (Olympus Europa GmbH, Germany) with the Cell^B Basic imaging software. The relative contribution of hypertrophy and hyperplasia to the increase of muscle cross-sectional area was estimated as follows: Δ Muscle CSA (μm^2) = $N_{m\times}\Delta A$ (μm^2) + $A_{m\times}\Delta N$, where Δ was calculated between two sampling times (t and t + 1), N_m and A_m refer to the mean total number of fibers and fiber area at t, respectively (Valente et al., 1999b).

5.2.5 Gene expression

5.2.5.1 RNA extraction and cDNA synthesis

Six pools of 20 individuals per dietary treatment (2 pools per tank) were sampled at 16DAH (metamorphosis climax) and at 36DAH, snap-frozen in liquid nitrogen and kept at −80 °C until further analysis. Each pool was grinded using pre-chilled pestle and mortar by adding liquid nitrogen, and then transferred to a 2mL sterile centrifuge tube. Total RNA was extracted according to the Tri reagent method (Sigma-Aldrich, USA). 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 1µg of purified RNA (per pool), using with the M-MLV Reverse Transcriptase Kit (Invitrogen, USA).

5.2.5.2 Quantitave real-time PCR (qPCR)

The relative expression of the MRFS (*myf5, myod2, mrf4, myog), mstn1* as well as genes encoding for the proteins responsible for *de novo* DNA methylation (*dnmt3a,* and *dnmt3b*) and methylation maintenance (*dnmt1*) 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). Samples were denatured for 30s at 95°C and then subjected to 40 cycles of amplification with the following thermocycling parameters: denaturation for 5s at 95°C and annealing/extension for 10s (see Table 3 for annealing temperatures (ºC)). 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 (in 0.5ºC increment, for 5 s). 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).

5.2.6 Data analysis

Statistical analyses followed previously reported methods (Zar, 2010) and IBM SPSS Statistics 19 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 for homogeneity of variance using a Levene's test. Data were log transformed when required and percentages were arcsin transformed prior to analysis.

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

To compare the distribution of muscle fibre size, a nonparametric method was used to fit smoothed probability density functions (PDFs) using the statistical program for the analysis of muscle fibre populations (Johnston et al., 1999). Bootstrapping was used to distinguish random variation in diameter distribution from treatment differences. A Kruskal-Wallis test with 1000 bootstrap replicates was used to test the null hypothesis that PDFs of muscle fibre diameter in the three treatments were identical.

Table 3: Primers used in qPCR

Gene	Fwd sequence (5′→3′)	Rev sequence (5′→3′)	Accession no (GenBank)	Size (bp)	Annealing temp. (ºC)	E (%)
myf5	GAGCAGGTGGAGAACTACTACG	CCAACCATGCCGTCAGAG	FJ515910	89	60	103
mrf4	GAGAGGAGGAGGCTCAAGAAG	CAGGTCCTGTAATCTCTCAATG	EU934042	137	58	96
myog	GTCACAGGAACAGAGGACAAAG	TGGTCACTGTCTTCCTTTTGC	EU934044	118	60	94
myod2	ACAGCCACCAGCCCAAAC	GTGAAATCCATCATGCCATC	FJ009108	194	60	111
mstn1	GGGAGATGACAACAGGGATG	TGGATCCGGTTCAGTGGC	EU934043	91	60	108
dnmt1	GATCCCAGTGAGGAGTACGG	AAGAAGGTCCTCATAAGTAGCGTC	KC129104	117	62	103
dnmt3a	AACTGCTGTAGGTGTTTCTGTGTG	CGCCGCAGTAACCCGTAG	KC129105	134	60	101
dnmt3b	ATCAAGCGATGTGGCGAGC	CGATGCGGTGAAAGTCAGTCC	KC129106	91	60	96
rps4	GTGAAGAAGCTCCTTGTCGGCACCA	AGGGGGTCGGGGTAGCGGATG	AB291557	101	60	95
ubq	AGCTGGCCCAGAAATATAACTGCGACA	ACTTCTTCTTGCGGCAGTTGACAGCAC	AB291588	135	60	93

For each gene, its GenBank accession numbers, amplicon size (bp), Annealing temperatures (ºC) and qPCR amplification efficiencies (E) are indicated.

5.3 Results

5.3.1 Larval performance

The total replacement of dietary native plant proteins for partially hydrolyzed plant protein had a positive effect on larval growth until metamorphosis climax (Fig.1), with significant differences being detected on dry weight at 16DAH (P=0.002). After metamorphosis, and when the inert feed became the predominant dietary source, the fish fed the Intact diet clearly performed better than those fed the other diets (Fig. 1), displaying a higher dry weight from 36DAH onwards (Fig 1). At end of the experiment, the fish fed the Intact diet averaged a dry weight 2.17-fold higher than fish fed the PartH diet and 2.27-fold higher than that fed the HighH diet. Accordingly, significant differences among treatments were detected on RGR from 16 to 36DAH (P=0.047) and on overall RGR, from 2 to 60DAH (P=0.000) (Table 4, Fig.1), with fish fed the Intact diet growing faster. Survival rate was determined only during the benthic phase (16-60 DAH) and was significantly affected by diets (P=0.011), being reduced in the PARTH group by 11.5 and 13.3%, when compared respectively with the Intact and the HighH groups (Table 4).

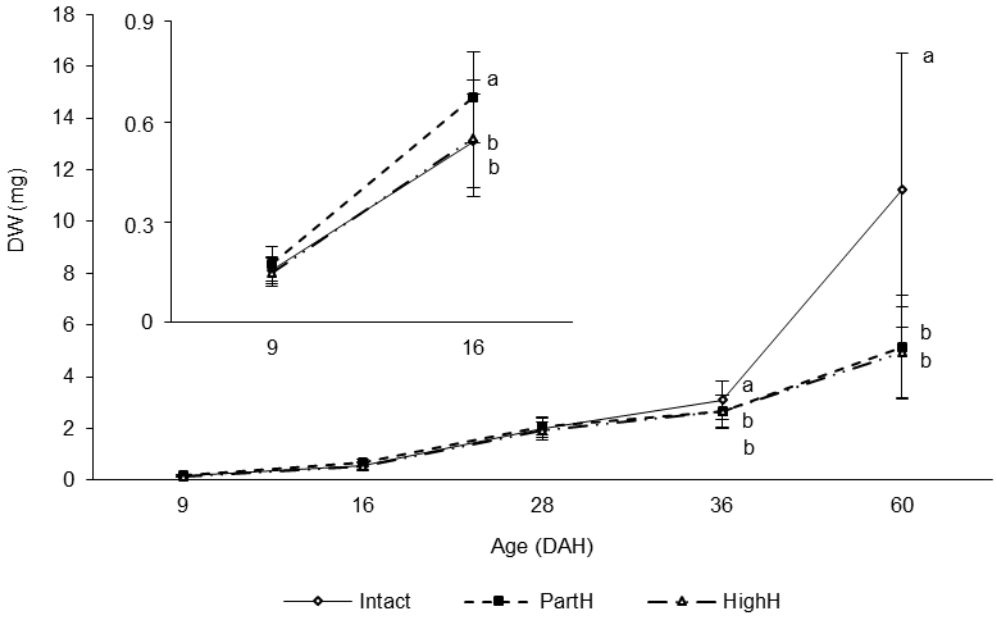

Figure 1: Sole dry weight during the pelagic (13 to 16DAH) and the benthic phase (16 to 60 DAH). Values are means ± S.D. of treatments replicates. Comparisons between groups fed with different diets were made using one-way ANOVA followed by Tukey-Kramer post-hoc tests. Dissimilar superscript letters indicate a significant difference (P<0.05) between treatments.

5.3.2 Dietary effect on white skeletal muscle growth

The total number of fast-twitch muscle fibers and fiber density were not significantly different between groups at 16DAH, but the average fibre diameter (P=0.023) and muscle cross-sectional area (P=0.046) were significantly higher in the PartH group than in the Intact group at 16DAH (Table 4). Moreover, there was a strong correlation between fiber diameter and Muscle CSA at this stage and a negative correlation between fiber density and Muscle CSA (Table 5). Altough the smoothed probability density functions (PDFs) of muscle fibre populations did not differ significantly between groups at 16DAH, the percentage of fibers with diameter within the range 7-11 μm was higher in PartH and HighH groups, when compared to the Intact group (P=0.001) and the percentage of fibers with diameter within the range 11-15 μm was higher in the PartH group than in the Intact group (P=0.002) (Table 4). The smoothed PDFs of muscle fiber populations indicated a high percentage of small-sized muscle fibers in all dietary treatments, as all curves skewed to the left-hand tail of the distribution (Fig. 2). There was a significant correlation between the percentage of very small fibers (<3μm) and the relative growth rate (RGR) in the subsequent period, from 16 to 36DAH (Table 5).

Between the metamorphosis climax and 36DAH there was a significant enlargement of muscle fibers in all groups (Table 4), reflected on the shift of PDFs distribution towards the right-hand of the plot (Fig. 2). The average fiber size increased from 16 to 36DAH by 1.8, 1.6 and 1.7-fold respectively in the Intact, PartH and HighH groups, being similar among groups at 36DAH (Table 4). This was paralleled by a 2.3, 2.3 and 2.0-fold increase of total number of fibers in the Intact, PartH and HighH groups, respectively, which reflected in a muscle CSA increase of 6.2-fold, 4.8-fold and 4.4-fold in the Intact, PartH and HighH groups, respectively (Table 4). Moreover, there was significant contribution of the total number of fibers to the muscle cross-sectional area, as a positive correlation was detected at this stage (P=0.039) (Table 5). Although PDFs distributions at 36DAH did not differ significantly among treatments (Fig. 2), the percentage of small fibers (diameter <5μm) was higher in the group fed the Intact diet, when compared to those fed the PartH and the HighH diets (P=0.039) (Table 4). Moreover, there was a positive correlation between the percentage of small fibers (diameter <5μm) and the relative growth rate in early juvenile fish (Table 5). The relative contribution of hypertrophy to white muscle growth in the 16-36DAH period was higher in the HighH group (63.4±7.0%) than in the PartH group (52.64±6.4%) (P=0.026), but not significantly different from that in the Intact group (55.6.±8.2%). No further differences were found in the white muscle cellularity variables at 36DAH (Table 4).

Table 4: Standard length (mm) and white muscle cellularity parameters measured at a peri-anal location. (N=9/treatment). Relative growth rate (RGR, (% day^{-1}) and survival (%) estimated for each tank (N=3/treatment). Values are means ± s.d. Comparisons between groups fed different diets were made using one-way ANOVA followed by a Tukey post-hoc test, or a Kruskal-Wallis one-way ANOVA followed a Games-Howell post-hoc test, whenever data did not meet equal variance requirements. Dissimilar superscript letters indicate a significant difference (P<0.05) between groups.

	Diets		
	Intact	PartH	HighH
16 DAH			
RGR (2-16DAH) (% day^{-1})	26.6±2.2	29.0±0.7	26.9±2.1
SL (mm) group	5.6±0.2	5.8±0.3	5.9±0.3
Muscle CSA (mm^2)	$0.07±0.01^{b}$	$0.08±0.01^{a}$	$0.08±0.03^{ab}$
Total number of fibers N	1252±144	1329±180	1323±199
Fiber density (total number/mm^2)	19493±4172	15755±1549	16558±4265
mean fiber diameter (D, µm)	$5.75±0.64^{b}$	$6.61±0.41^{a}$	$6.35±0.86^{ab}$
Percentage of fibers with			
D<3µm	21.1±8.9	14.4±3.2	17.0±6.5
3<D<7µm	53.1±4.2	48.7±4.4	49.5±6.2
7<D<11µm	$16.6±4.1^{b}$	$23.9±4.1^{a}$	$21.7±4.1^{a}$
11<D<15µm	$6.1±1.4^{b}$	$8.9±1.9^{a}$	$7.9±3.0^{ab}$
D>15µm	2.6±1.3	3.3±1.0	2.7±3.0
36 DAH			
RGR (16-36DAH) (% day^{-1})	$4.4±0.4^{a}$	$3.3±0.2^{b}$	$3.9±0.5^{ab}$
SL (mm) group	11.9±0.9	11.3±0.8	10.9±0.5
Muscle CSA (mm^2)	0.40±0.06	0.41±0.12	0.38±0.07
Total number of fibers N	3015±458	3074±582	2617±373
Fiber density (total number/mm^2)	7538±1155	7831±1266	7002±723
mean fiber diameter (µm)	10.15±0.38	10.70±0.72	10.96±0.78
Percentage of fibers with			
D<5µm	$10.7±2.4^{a}$	$8.2±2.0^{b}$	$8.0±1.6^{b}$
5<D<10µm	44.8±4.8	45.2±3.2	42.3±4.6
10<D<15µm	28.7±2.4	28.5±3.3	30.4±1.4
15<D<20µm	8.4±2.6	9.2±1.8	9.6±2.4
D>20µm	5.1±2.1	6.2±2.8	6.6±2.3
Overall growth & Survival			
RGR (2-60DAH) (% day^{-1})	$11.9±0.3^{a}$	$10.3±0.3^{b}$	$10.3±0.3^{b}$
Survival (16-60DAH) (%)	$76.2±1.8^{a}$	$64.7±5.9^{b}$	$78.0±2.99^{a}$

Table 5: Correlations (Pearson's coefficient) between white muscle cellularity parameters and somatic growth or gene expression data in Senegalese sole larvae, at 16 DAH (metamorphosis climax) and at 36 DAH.

	Total nr of fibers	Fiber density	Fiber diameter	% of small fibers
16DAH				
Muscle CSA	NS	P=0.002, r=-0.867	P=0.000, r=0.946	NS
RGR (2-16DAH)	NS	NS	NS	NS
RGR(16-36DAH)	NS	NS	NS	P=0.029, r=0.719
myf5	NS	NS	NS	NS
myod2	NS	NS	NS	NS
myog	NS	NS	NS	NS
mrf4	NS	NS	NS	NS
mstn1	NS	NS	NS	NS
36DAH				
Muscle CSA	P=0.031, r=0.712	NS	NS	NS
RGR(16-36DAH)	NS	NS	NS	NS
RGR(36-60DAH)	NS	NS	NS	P=0.032, r=0.712
myf5	NS	NS	NS	NS
myod2	NS	P=0.013, r=-0.783	NS	NS
myog	NS	P=0.044, r=-0.679	NS	NS
mrf4	NS	NS	NS	NS
mstn1	NS	P=0.046, r=-0.674	NS	NS
dnmt1	NS	NS	NS	NS
dnmt3a	NS	P=0.025, r=-0.731	P=0.038, r=0.695	P=0.037, r=0.696
dnmt3b	P=0.013, r=-0.783	NS	NS	NS

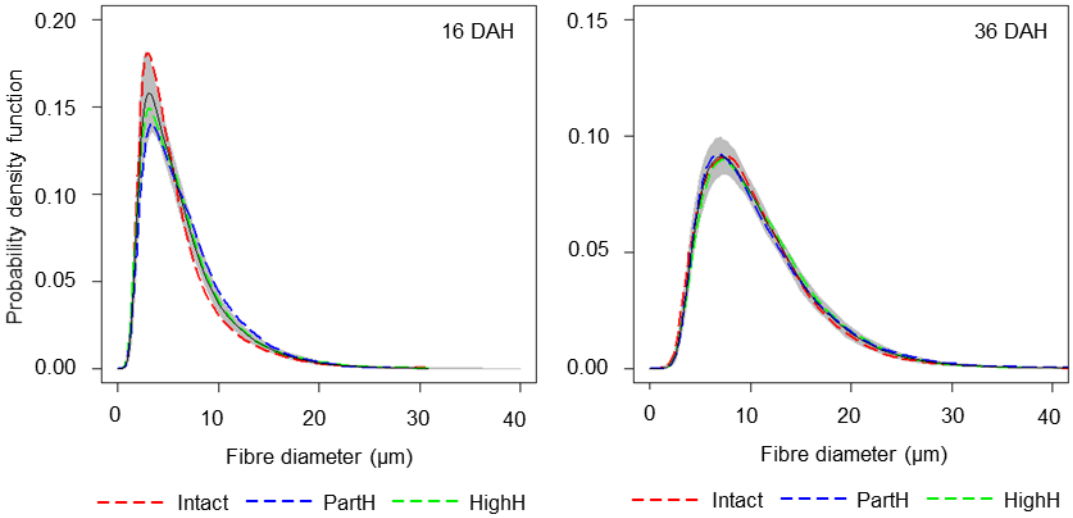

Figure 2: Probability density functions (PDFs) distributions of fast muscle fibers at 16 and 36DAH. The dashed lines show the mean PDF for each group and the solid line central to the shaded area is the average PDF for combined groups (Intact, PartH and HighH; n=9/treatment). The shaded area shows 1000 bootstrap estimates from combined populations of fibre diameter.

5.3.3 Expression of growth-related genes and DNA methyltransferases

The relative expression of the genes encoding for the myogenic regulatory factors did not differ significantly between groups fed different diets during the metamorphosis climax (Fig. 3A). However, at 36DAH, the expression of m*yf5* (P=0.027) and *myod2* (P=0.029) was significantly upregulated in the group fed the HighH diet, in which the transcript levels were increased, respectively, by 2.24 and 2.05-fold, when compared to the PartH group, no differences being found in pair-wise comparison between the HighH and Intact groups (Fig.3B). At this stage, the transcript levels of *myog* (P=0.004) were also upregulated in the group fed the HighH diet, increased by 1.51 and 1.77-fold when compared to the groups fed Intact and PartH diets, respectively (Fig.3B). No significant differences were found in the transcript levels of *mrf4*. At this stage, fiber density was negatively correlated with the expression of *myod2* and *myog* (Table 5). No dietary effect was found in the *mstn1* mRNA levels during the metamorphosis or at 36DAH (Fig.3B). However, fiber density was negatively correlated with the expression of *mstn1* (Table 5).

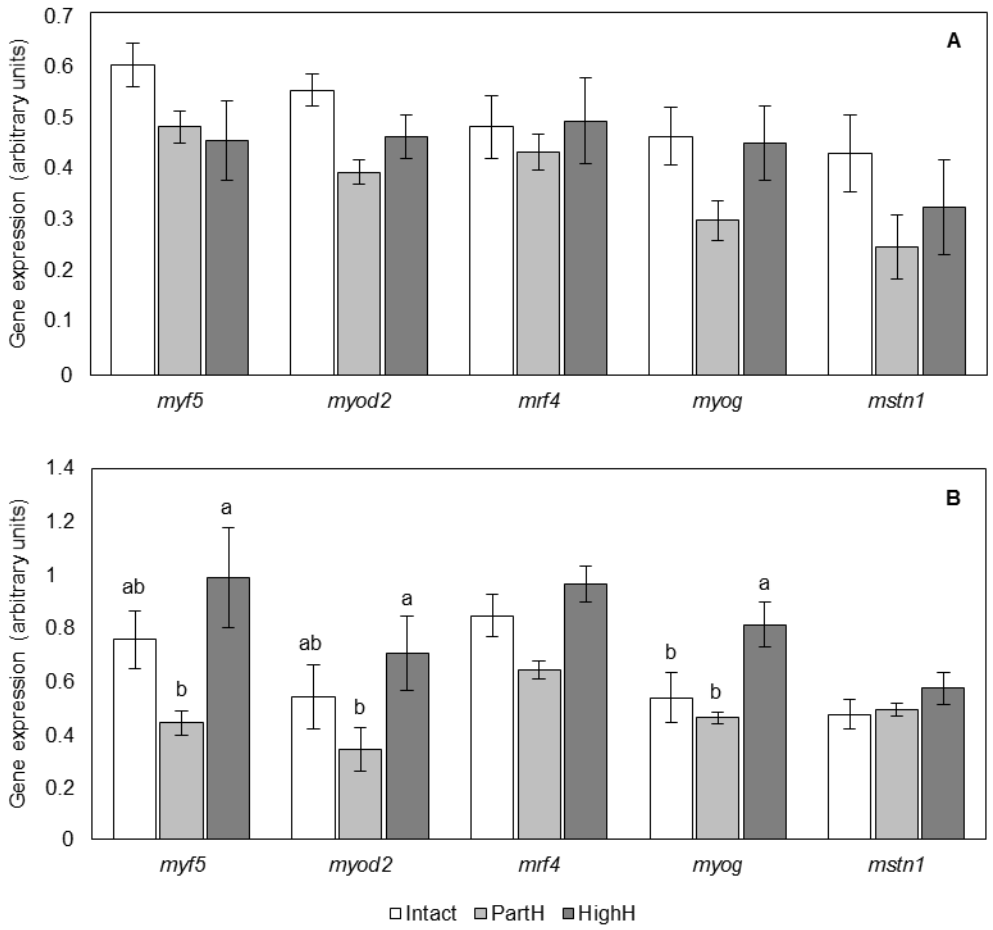

Figure 3: Expression of genes encoding for myogenic regulatory factors *myf5*, *mrf4*, *myod2*, *myog* and *mstn1* at 16DAH (A) and 36DAH (B) (whole body pools of 20 individuals). mRNA expression was normalized to transcript levels of *ubq* and *rps4*. Values are presented means ± s.e.m., n = 6. Different superscript letters indicate significant differences (P<0.05, 1-way ANOVA) between the dietary treatments at each developmental stage.

There was no effect of dietary protein complexity on the DNA methyltransferase *dnmt1* transcript levels in 36DAH Senegalese sole (Fig.4). However *dnmt3a* (P=0.042) and *dnmt3b* (P=0.041) transcript levels were significantly higher in fish fed the HighH diet, when compared to those fed either the Intact or PartH diets (Fig.4). Moreover, the expression of *dnmt3a* was negatively correlated with fiber density and accordingly, it was positively correlated with both the fibre diameter and percentage of small fibers (Table 5). The expression of *dnmt3b* was negatively correlated with the total number of fibers (Table 5).

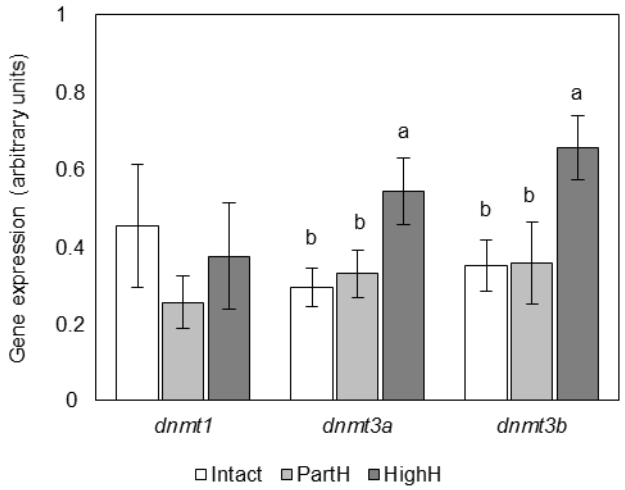

Figure 4: Expression of genes related to DNA methylation (*dnmt1, dnmt3a* and *dnmt3b*) at 16DAH and 36DAH (whole body pools of 20 individuals). mRNA expression was normalized to that of *ubq* and *rps4*. Values are presented means ± s.e.m., n = 6. Dissimilar superscript letters indicate significant differences (P<0.05, 1-way ANOVA) between the dietary treatments at each developmental stage.

5.4 Discussion

5.*4.1 Effect of dietary protein complexity on larval performance*

The three microdiets (Intact, PartH and HighH) used in this study were formulated with the aim of having the degree of hydrolysis of dietary protein as the main changing factor, using approximately the same practical ingredients (Table 1). Nevertheless, the proportions of marine and plant ingredients included in each diet might have also induced some other differences between diets. Since all diets included high levels fish and krill protein hydrolysates (Table 1), the attractability or palatability to sole larvae were not likely to change between the diets, which was confirmed by the visual observation of fish behaviour towards the diets. Since all diets exceeded the indispensable amino acid (IAA) requirements for sole juveniles (Silva et al., 2009), the minor variations observed in the dietary AA profiles (Table 2) did not affect the main results and conclusions of this study.

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

Senegalese sole from a very early developmental stage, i.e. at mouth-opening. On the contrary, in the previous studies the protein hydrolysates included in microdiets were mostly composed of free amino-acids (FAA) and di- or tri-peptides (<0.2KDa) or oligopeptides (0.5-2.5KDa).

Given the Senegalese sole complex metamorphosis and digestive system ontogeny (Conceição et al., 2007; Engrola et al., 2009b; Fernández-Díaz et al., 2001; Morais et al., 2014), it was expected that the larvae capacity to digest and utilize dietary protein would change throughout development. Indeed in the present study, different dietary formulations based on different protein MW profiles had different effects on Senegalese sole somatic growth throughout development (Fig.1). The diet including a partially hydrolyzed plant protein mixture (target peptide range from 5 to 70 KDa) (PartH) promoted growth in metamorphosing larvae (Fig.1), having been considered as more digestible to pre-metamorphic larvae (results presented and discussed in Chapter 2). The diet based on intact plant protein sources (Intact) promoted the absorption and total retention of small-sized peptides during the metamorphosis climax, due to a possibly induced a slower gastrointestinal transit (results presented and discussed in Chapter 2), what would have contributed for enhanced growth in later stages, from 36DAH onwards (Fig.1). These results are in line with Richard et al. (2015) previous outcome, according to which, larger peptides are better suited to sole young juvenile AA anabolic and physiological needs than to early stage larvae.

5.4.2 Effect of dietary protein complexity on the regulation of muscle growth

Different dietary formulations based on different protein MW profiles induced differences in the muscle cellularity that were reflected on somatic growth during the metamorphosis climax. Although the smoothed probability density functions (PDFs) of muscle fibre populations did not differ significantly among treatments at 16DAH (Fig. 2), significant differences were detected in the percentage of large-sized muscle fibers (Table 4). The percentage of fibers with diameter within the range 7-11 µm was lower in the Intact group, when compared to the PartH and HighH groups and the percentage of fibers with diameter within the range 11-15 µm was lower in the Intact group than in the PartH group, which together with a reduced average fibre diameter and similar fibre number, suggests a delayed muscle growth in the Intact fed larvae. Accordingly, the PartH fed larvae had a significantly larger average fibre diameter, compared to the Intact group (Table 4), which reflected on a higher muscle cross-sectional area (as suggested by the positive correlation between average fibre diameter and muscle- cross-sectional area, Table 5) and probably contributed to increased dry weight during the metamorphosis climax (Fig.1) Also in Senegalese sole metamorphosing larvae exposed to different rearing temperatures during the pelagic phase, muscle growth of fast-growing fish

was related to increased fast-twitch fibre size and not the fibre number (Campos et al., 2013b). Campos et al. (2013b) a reported a significant increase in the transcript levels of both primary MRFs and *myog* at a pre-metamorphic stage, and *myod2* and *myog* during the metamorphosis climax, in the fastest-growing group . In pacu larvae (*Piaractus mesopotamicus*) reared upon different types of feed, dietary formulations and feeding regimes, the fastest-growing group displayed a higher frequency of small fibers and trend for higher *myod* expression (Leitão et al., 2011). Increased proliferative capacity of MPCs and a higher contribution of hyperplasia was also reported in pike perch (*Sander lucioperca*) larvae fast-growing groups, as a response to type of feed and dietary formulation (Ostaszewska et al., 2008). In the present study, the relative expression of the genes encoding for myogenic regulatory factors was similar among dietary treatments, during the metamorphosis climax, and could not be related to muscle cellularity parameters. Yet and despite the possibly delayed muscle growth in the Intact diet fed larvae during the pelagic phase, this group displayed a tendency for a higher percentage of small fibers (diameter <3µm), representing a 1.24 and 1.47-fold increase, when compared with the HighH and PartH groups (Table 4). This increase in the percentage of small fibers suggests a possible gain in the proliferative capacity of the larvae reared upon the Intact diet. This could have promoted subsequent growth in this group, a hypothesis that is supported by the significant correlations between the percentage of very small fibers (diameter <3µm) during the metamorphosis climax and relative growth rate in the 16-36DAH period. In fact, when compared to the groups fed the PartH and HighH diets, the Intact group grew significantly better from 36DAH onwards (Fig.1).

At 36DAH, and contrarily to what was observedduring the metamorphosis climax , different dietary formulations induced different expression patterns of genes encoding for myogenic regulatory factors. At this stage, the transcription of the genes encoding for the primary MRFs (*myf5* and *myod2*) was significantly upregulated in the group fed the HighH diet, when compared to that fed the PartH diet (Fig.3). The transcription of *myog* was also significantly higher in the group fed the HighH diet, when compared to both the Intact and PartH groups (Fig.3). Since *myog* encodes a highly conserved myogenic regulatory factor that is involved in terminal muscle differentiation (Rescan, 2005), its upregulation might have sustained the differentiation of myoblasts to further fuse into already formed muscle fibers, promoting hypertrophy rather than hyperplasia in the HighH group, being upstream supported by the upregulated *myf5* and *myod2* transcription (Fig.3). This is further corroborated by the negative correlation observed between fiber density and the transcript levels of *myod2* and *myog* (Table 5), and confirmed by the relative contribution of hypertrophy towards white muscle growth in the 16-36DAH period, which was significantly higher in the HighH group (63%) than in the PartH group (52%). A higher contribution of hypertrophy to muscle growth and the reduced

recruitment of small fibers (evidenced by a low percentage of fibers < 5 µm, Table 4) are predictive of a lower growth potential in the HighH group. The relation between hypertrophy-sustained muscle growth and further reduced somatic growth has been proposed in common carp (Alami-Durante et al., 1997), Atlantic cod (Galloway et al., 1999), pike perch (Ostaszewska et al., 2008) and pacu (Leitão et al., 2011) larvae subjected to different types of feed, dietary formulations and feeding regimes.

A significant downregulation of *myf5*, *myod2* and *myog* transcription in the PartH group, and lower percentage of small fibers compared to the Intact group, suggests a reduced recruitment capacity of new fibers (Table 4).This is further supported by a negative correlation between the *myod2* and *myog* transcript levels and fibre density (Table 5). Decreased recruitment of new fibers would have led to reduced growth potential in PartH fed fish. Also in Senegalese sole, a significant downregulation of most MRFs in earlier stages was reported in a slow-growing group exposed to a low rearing temperature during the pelagic phase (Campos et al., 2013b). Pike perch larvae fed a formulated diet including a casein hydrolysate (Ostaszewska et al., 2008) and Atlantic cod larvae reared upon a fish hydrolysate supplemented diet (Katan et al., 2016) have displayed reduced somatic growth, but as a result of delayed muscle growth, including reduced muscle cross-sectional area. The present study results suggest a delay in the muscle growth of the PartH fed fish, but not at the point of reducing muscle cross-sectional area; neither the hypertrophic muscle growth in the HighHgroup reflected in reduced muscle cross-sectional area.

At 36DAH, the transcription of *myf5*, *myod2* in the Intact group was probably high enough to keep up with the increase of the MPC population and further fibre recruitment. In fact, fish fed the Intact diet had a significantly higher percentage of small fibers (Table 4), which has probably contributed for a higher growth potential, as further suggested by a significant correlation between the percentage of small fibers (diameter <5µm) and relative growth rate in the 36-60DAH period (Table 5). Indeed, the percentage of small-sized muscle fibers is long known to be a good indicator of further muscle growth potential both in juveniles and larvae (Alami-Durante et al., 1997; Galloway et al., 1999; Valente et al., 1999b).

In spite of the negative correlation between *mstn1* transcription and fiber density detected at 36DAH (Table 5), different diets did not induce significant changes in the *mstn1* mRNA levels during the metamorphosis or at 36DAH (Fig.3). Since *mstn1* encodes for myostatin, which is a negative regulator of MPC activation and proliferation, a negative correlation between *mstn1* transcription and density or fibre recruitment and muscle growth would be expected. However, the relation between *myostatin* mRNA levels and muscle growth is surprising: depression of growth induced by environmental conditions does not correlate with an up-regulation of *myostatin* as expected (Rescan, 2005). While Atlantic cod larvae fed a fish hydrolysate

supplemented diet displayed reduced growth and delayed muscle growth along with upregulated *mstn1* transcription, Senegalese sole fast-growing groups displayed an up-regulation of *mstn1* in late juvenile stages (Campos et al., 2013b; Canada et al., 2016). Moreover, m*stn1* transcription did not respond to environmental changes in Senegalese sole metamorphosing larvae and post-larvae (Campos et al., 2013b). It is thus not surprising that no clear dietary effect was found on the expression of *mstn1* at 16 or 36DAH. In the present study, gene expression was analyzed in the whole fish and not only in the muscle, so it is also possible that the expression of *mstn1* might be associated with other physiological mechanisms and not only with skeletal muscle growth regulation (Campos et al., 2010).

5.4.3 Epigenetic effect

Late post-larvae fed the HighH diet had higher *dnmt3a* and *dnmt3b* mRNA levels, when compared to those fed the other diets, which may be associated with the dietary methionine contents. *Dnmt3a* and *dnmt3b* are *de novo* DNA cytosine methyltransferases which covalently attach S-adenosylmethionine (SAM) methyl groups to the 5′-position of cytosine (in CpG dinucleotides), thus methylating DNA and repressing transcription during embryogenesis and cell differentiation (Turek-Plewa, Jagodzinski, 2005). DNA methylation relies on the availability of SAM, the universal cellular methyl donor (Selhub, 1999). SAM generates from methionine in a ATP-dependent reaction in the one-carbon cycle which depends on the activity of several enzymes and the presence of dietary methyl donors, such as folate, choline, betaine and methionine (Anderson et al., 2012). The HighH diet had a higher level of methionine, increased by 28% and 88%, when compared with the Intact and the PartH diets, respectively (Table 2). A higher availability of methionine may underlie the increased transcription of *dnmt3a* and *dnmt3b* in the HighH group, which may be associated with a general increase in DNA methylation.

5.5 Conclusion

Dietary protein complexity did affect the regulation of myogenesis throughout Senegalese sole development, by delaying muscle growth until the metamorphosis climax (16 DAH) in larvae fed the Intact diet, and by either reducing the recruitment of small fibers in both the PartH and HighH or also by promoting hypertrophy sustained muscle growth in the HighH group in an early juvenile stage, at 36DAH. The dietary protein complexity induced changes in the regulation of myogenesis that have ultimately reduced the potential for muscle growth in those groups fed microdiets mostly based on hydrolysed protein sources. The expression of *dnmt3a* and *dnmt3b* was up-regulated in the group fed the HighH diet, possibly due to a higher dietary content of methionine. As these genes encode for a DNA methyltransferases essential for *de*

novo methylation, its expression up-regulation may be associated with a general DNA hypermethylation.

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6. General discussion

6.1 Feed formulating strategies affect protein utilization and somatic growth

Different diet formulation strategies were employed in the framework of this PhD thesis to improve Senegalese sole larvae capacity to utilize and deposit protein throughout metamorphosis and maximize growth potential: meeting the ideal indispensable amino acids (IAA) profile by adjusting the dietary amino acids (AA) profile to the larval body AA profile (**Chapter 2**); meeting the IAA requirements, by increasing the content of IAA (**Chapter 3**); and decreasing the complexity (molecular weight) of dietary protein, as to make it more digestible for the fish (**Chapters 4 and 5**). All these experiments were run in similar conditions (environmental conditions, feeding regime and even sampling points). So the comparison between results among the three experiments seems tangible and pertinent considering the goal of this PhD thesis, even considering the multiple factors involved when attempting to compare across studies (broodstock origin, parental nutrition, batch quality, eggs and hatchlings quality, among others).

Generally, it was clearly demonstrated that different formulating strategies had different effects on the development of the larvae capacity to utilize and retain protein for growth purposes.

Manipulating dietary protein quality, by adjusting the dietary AA profile to the larval body AA profile, in an attempt to meet Senegalese sole ideal IAA profile, had a positive short-term effect on the Senegalese sole larvae capacity to retain protein, but did not translate into increased growth (**Chapter 2**). Manipulating dietary protein quality by increasing the IAA/DAA ratio had no effect on the development of Senegalese sole larvae capacity to retain protein and has decreased somatic growth in post-metamorphic fish (**Chapter 3**). In **Chapters 4 and 5**, when comparing a diet mostly based on native protein sources (Intact), a diet mostly based on low MW peptides (<5KDa, HighH), and a diet mostly based on polypeptides ranging from 5 to 70 KDa (PartH), the later promoted growth (DW, up to 1.2 fold higher) in metamorphosing larvae, which digestive system is still very immature, relying proteolysis on pancreatic endoproteases (such as trypsin and chymotrypsin).

Adjusting the dietary AA profile to the larval body AA profile, through a 4% supplementation with crystalline amino acids (CAA), appears as a promising way to meet the ideal AA profile and improve the retention of dietary protein even for a short time (**Chapter 2**). Moreover, a diet formulation mostly based on polypeptides (PartH diet) arises as the most promising way to promote growth of early stage larvae, by providing a protein form better matching the early larvae digestive capacity (**Chapter 4**). These results corroborate the general premise that the proteolytic capacity is a limiting factor for dietary protein digestion in early larval stages

(metamorphosing larvae), which is also supported by generally lower transcript levels of genes encoding for enzyme-precursors involved in protein digestion at 16 days after hatching (DAH) compared to those observed in post-larvae (**Chapter 4**). Furthermore, the present data highlight the importance of dietary protein digestibility during early larval stages and the relevance of assessing the ideal protein complexity matching the digestive system maturation status at each developmental stage as previously suggested by Richard et al (2015). According to what had been proposed by Richard et al. (2015) as a short-term effect, the present thesis results suggest that pre-metamorphic sole larvae utilize better dietary protein with a moderate degree of hydrolysis, i.e. in the 5-70 KDa MW range, which reflected into increased somatic growth during the metamorphosis climax.

In later stages of development, diets supplemented with CAA, at 4 % (**Chapter 2**) or 8% (**Chapter 3**), have delayed growth when compared to a CTRL diet mostly based on native protein and a commercial fish protein hydrolysate (FPH). Both diets mostly based on hydrolysed proteins (PartH and HighH) have also delayed growth in post-larvae and juveniles, as compared to the Intact diet (**Chapters 4 and 5**). Though both CTRL (**Chapters 2 and 3**) and Intact diets (**Chapters 4 and 5**) generally gave better growth results in late developmental stages, a high inclusion of FPH (30%) in the CTRL diet may explain a lower growth in post-weaned fish, when compared to the Intact diet (FPH 13%). Lower protein accretion, possibly caused by transitory AA imbalances in the free amino acids (FAA) pool, as result of an excess of amino acids arriving at the intestinal epithelium, appears as a common denominator for the observed reduced growth in both diets supplemented with crystalline amino-acids (**Chapters 2 and 3**) and in the diet mostly based on oligo, di and tri-peptides (HighH diets, **Chapters 4 and 5**). This highlights the importance of post-prandial AA imbalances, as a cause for decreased protein synthesis and growth, particularly in early stage larvae (Conceição et al., 2011).

Concerning the effect of manipulating dietary protein quality on protein utilization and growth, according to the ideal protein concept (**Chapter 2**) or by increasing the IAA/DAA ratio (**Chapter 3**), the thesis outcome may agree with the some of the concerns regarding the supplementation of CAA in fish feeds. Several studies showed that fish may not utilize CAA as efficiently as protein bound-AA, due to fast and unsynchronized absorption, leading to a great proportion of CAA being lost to catabolism and subsequently having a detrimental effect on somatic growth (Dabrowski et al., 2003, 2010; Peres, Oliva-Teles, 2005; Schumacher et al., 1997; Yamada et al., 1981a; 1981b). FAA seem to be transported across the brush-border membrane (BBM) by saturable carrier-mediated transporters and apparent diffusion (Bakke-McKellep et al., 2000; Storelli et al., 1989; Vilella et al., 1990). The fact that different amino acids are 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 (Ronnestad, Morais, 2008) is an additional factor affecting the post-prandial FAA pool balance. In fact, the BAL diet formulation allowed to partially balance the dietary AA profile considering the ideal protein concept (Arai, 1981), and promoted the metamorphosing larvae capacity to retain oligopeptides (**Chapter 2**), according to what had been previously suggested by for sole post-larvae (Aragão et al., 2004). However, a higher supplementation with CAA, in order to increase the IAA/DAA ratio, has not shown any benefits, possibly due to AA excess and loss to catabolism and even through excretion, though that could not be confirmed by the tube ^{14}C labelled model-peptides analysis (**Chapter 3**). Perhaps a supplementation with di and tri-peptides, as previously suggested by Zambonino-Infante et al. (1997), would be more effective in promoting the retention of dietary AA, as it would make use not only of the FAA transport system, but also of the peptide transport system. In fact, Aragão et al. (2004) have used di-peptides (Leu-Gly and Phe-Ala) in order to balance the dietary AA profile and improved retention of dietary AA in *Artemia* fed Senegalese sole post-larvae. Nevertheless, probably more than the molecular form, individual IAA bioavailability, considering each IAA absorption rate and efficiency, should be taken in consideration when trying to formulate the ideal dietary AA profile, as it has been suggested by Conceição et al. (2011). The IAA bioavailability has been estimated for gilthead seabream (Conceição et al., 2003) and sharpsnout seabream (Saavedra et al., 2007), but it is largely unknown whether each IAA bioavailability changes with species, ontogeny or even diet composition (Conceição et al., 2011). For that reason, it could have been uncertain to take those data on IAA bioavailability as reference for the present studies. Estimating the bioavailability of each IAA for Senegalese sole at different developmental stages would be useful, as it would allow formulating a dietary AA profile corrected for individual IAA bioavailability. That would certainly enable fine tuning the dietary AA profile and get closer to the ideal AA profile for Senegalese sole. Anyhow, correcting the dietary AA profile in an effective way will always require an effective technique to reduce the leaching losses of the dietary CAA. In fact, in the study presented in **Chapter 2**, leaching losses prevented reaching the intended dietary AA profile in the BAL diet and actually adjusting it to the larval body AA profile. That was only fairly accomplished, but was enough to promote the retention of dietary oligopeptides. Recent improvements in inert microdiet technology to avoid leaching, e.g., integrating advanced binding techniques with microencapsulation of water soluble nutrients (Conceição L., pers. Comm.), will allow for better tuning of the dietary AA profile in microdiets for Senegalese sole larvae in a near future. As it has been discussed in **Chapters 2 and 3**, given its magnitude, the differences found in growth between the CTRL diet and the BAL diet (**Chapter 2**) and the CTRL diet and the SUP diet (**Chapter 3**) were most

likely due to induced changes in voluntary feed intake. This assumption brings up other possible effects of manipulating the dietary AA profile. In fact, Dabrowski et al. (2007) has previously reported that midas (*Amphilophus citrinellum*) juveniles fed imbalanced diets displayed a significant increase of the voluntary feed intake when compared to a control group fed a balanced FAA-based diet. These authors have actually suggested that combining complementary IAA-imbalanced diets would be a useful strategy to meet the IAA requirements and promote protein accretion. A small dietary excess of Tryptophan (Trp) either in the BAL diet or in the SUP diet may have contributed for a reduced feed intake. Trp is a precursor of serotonin (5-hydroxiTryptamine, 5-HT) and its administration to fish has been suggested to have an anorectic effect (Hseu et al., 2003; Rubio et al., 2006). This hypothesis brings up the need to be cautious and consider the roles of each IAA in several physiological functions (NRC, 2011; Wu, 2009), when manipulating the dietary IAA. In fact, the supplementation of certain IAA may be useful and beneficial by promoting some important functions, such as metamophorsis (Pinto et al., 2010a), but a dietary excess of certain IAA may be toxic (see NRC, 2011) or induce adverse or undesirable effects.

Concerning the effect of dietary protein complexity on protein utilization and growth (**Chapters 4** and **5**), an eventual saturation of peptide transport systems in the intestinal BBM caused by an overloading of di- and tri-peptides is a possible explanation for the detrimental effect of the HighH diet on Senegalese sole larvae growth. This is in accordance with previous results for other species larvae fed microdiets with high inclusions of highly hydrolyzed proteins (Cahu et al., 2004; de Vareilles et al., 2012; de Vareilles Sommières, 2013; Kolkovksi, Tandler, 2000; Kvåle et al., 2009; 2002; Liu et al., 2006; Srichanun et al., 2014; Zambonino Infante et al., 1997). The results presented in **Chapter 4** suggest that a limited proteolytic capacity affects early stage larvae capacity to digest dietary native protein hypothesis, which agrees has it has been previously proposed by Engrola et al., (2010; 2009b) and Gamboa-Delgado et al. (2008). On the other hand, a limited absorptive capacity is probably another factor affecting the utilization of dietary protein and keeping up a post-prandial FAA equilibrium, which may be disrupted if there is a dietary excess of CAA or a high inclusion of extensively hydrolysed proteins. The Intact diet formulation had a transitory positive effect on the absorption and retention of small-sized peptides during the metamorphosis climax, which seemed to reflect on enhanced growth at later stages (**Chapter 4**). This suggests the absorptive capacity as possible indicator for the larvae performance in later developmental stages.

The results on metabolism provided new insights into optimizing sole larvae diet formulation, as larvae seem to adjust the way they utilize dietary protein to the dietary formulation (**Chapter**

4). Moreover, the oligopeptides come up as an important dietary protein source during the metamorphosis climax (**Chapters 2 and 4**).

The PartH diet, rich in 5-70 KDa peptides, seemed to stimulate the pre-metamorphic larvae capacity to absorb 7KDa polypeptides. The Intact diet stimulated the absorption and retention of 1KDa oligopeptides in metamorphosing larvae, in a presumptive attempt to make a better use of the oligopeptides present in the Intact diet as to compensate for their low capacity to digest complex protein. The PartH diet stimulated the retention of 7KDa polypeptides in post-metamorphic larvae, in an apparent attempt to optimize the utilization of the predominant peptides in this diet, which was also suggested by the increased expression of *tryp1C*, encoding for a trypsinogen isoform, and *pept1*, encoding for a membrane transporter responsible for the selective transport of di and tri-peptides from the intestinal lumen to the enterocytes (Daniel, 2004). These results discussed in **Chapter 4** suggest that sole larvae adjust the digestive machinery and metabolic capacity to dietary protein complexity.

The fact that the Intact diet promoted the absorption and retention of oligopeptides (**Chapter 4**) and the BAL diet promoted the retention of oligopeptides (**Chapter 2**) in metamorphosing larvae highlights the importance of oligopeptides as a dietary protein source at this challenging stage. The different dietary formulations affected the larvae metabolic capacity most clearly during the metamorphosis climax. These findings confirm that in Senegalese sole this time window is particularly susceptible to nutritional conditions as suggested by previous studies (Engrola et al., 2009a, 2010; ; Pinto et al., 2010b; Villalta et al., 2008 and Yúfera et al., 2005).

The results discussed in **Chapter 4** highlight the gut transit time as a factor probably affecting the digestion and utilization of dietary protein in sole larvae, though it has not been analysed in the framework of this PhD. The gut transit time has been pinpointed as a critical factor on dietary protein utilization by fish larvae (Conceição et al. 2011), particularly on slowly digested and absorbed proteins (Tonheim et al., 2005). Conversely, as discussed in **Chapter 4**, Intact diet formulation was suggested to slow down sole larvae gut transit time, promoting a better absorption of dietary oligopeptides in metamorphosing larvae. In post-metamorphic larvae, a possibly slower gut transit time was proposed to stimulate the expression of *pga*, encoding for pepsinogen A, the only pepsinogen isoform present in sole's stomach. According to Govoni et al. (1986), a slower gut transit time may enhance nutrient assimilation and possibly contribute for increased growth in later stages. The present results suggest gut transit time as a future target of research to improving dietary protein utilization.

Regarding the effect of manipulating dietary protein complexity throughout development, this thesis suggests that while polypeptides within the 5-70 KDa range seem to be easily digestible to pre-metamorphic larvae (**Chapter 4**), oligopeptides are a valuable dietary protein source for

metamorphosing larvae (**Chapter 2, 3 and 4**), and larger peptides and intact protein may be more suitable as dietary protein to sole post-larvae and young juveniles (**Chapter 4**). This last observation is further supported by the larvae increasing capacity to retain 6.8 kDa polypeptides throughout development (**Chapter 2**), confirming recent results from Richard et al. (2015). Thus, from a certain point of Senegalese sole development, a high dietary inclusion of pre-hydrolysed is no longer beneficial, and microdiets should include mostly intact protein.

In conclusion, when formulating dietary protein for sole several factors should be considered: the degree of maturation of the digestive system; the development of the proteolytic capacity; the metabolic capacity that seems to change throughout development; and lastly the feed transit time that is known to increase with age and may be prone to change with formulation.

6.2 Formulation strategies affect early muscle growth and somatic growth potential

As mentioned in section 1.5, the skeletal muscle characteristics and the way muscle tissue grows are major factors influencing overall growth capacity in fish (Johnston, 1999;, 2001; Valente et al., 2013). Myoblast differentiation and the regulation of myotube maturation and hypertrophy seem to be controlled by signalling pathways involving insulin-like growth factors (IGFI and IGFII) and IGFII transcription is controlled by mTOR pathway that depends on the availability of AA (Yoon, Chen, 2013). Thus, in this thesis, it was hypothesized that the changes in dietary protein would exert an influence on sole larvae muscle development, by inducing changes in the myogenic processes that would possibly have long-term irreversible effects on somatic growth.

The present thesis results showed that increasing the dietary content of IAA through an 8% supplementation with crystalline-AA led to changes on the regulation of muscle growth that were associated with changes in the expression patterns of some muscle growth markers (*myogenin* and *mrf4*, *myhc* and *mstn1*) (**Chapter 3**). Although that was mainly attributed to possible changes in feed intake that were induced by the SUP diet formulation and subsequent reduced protein availability for accretion and growth, the modifications observed in myogenesis regulation provided valuable insights into the effect of protein on the regulation of muscle growth and its long-term consequences. In general, when compared to the CTRL diet, the SUP diet formulation seemed to delay muscle growth, which was mainly revealed by muscle cellularity at two key developmental stages (during the metamorphosis climax and at a late juvenile stage) (**Chapter 3**). The expression of *myogenin* and *myhc* changed in response to dietary protein, being significantly reduced in metamorphosing larvae fed the SUP diet. This down-regulation of *myogenin* in the SUP fed larvae did not translate into changes in the total number of fibers in the muscle cross-section. Since *myogenin* is an indicator of myogenic cell

recruitment for stratified hyperplasia in Senegalese sole (Campos et al., 2013b; 2013c), its down-regulation in the SUP fed larvae during the metamorphosis climax was suggested to partially explain the reduced total number of fibers, smaller cross-sectional muscle area and reduced somatic growth rate in juveniles. A positive correlation was observed between *myogenin* expression and the percentage of small-sized muscle fibers in metamorphosing larvae, evidencing its major role in promoting muscle growth potential at later stages. Thus, the up-regulation of *myogenin* in the CTRL group in response to dietary protein during the metamorphosis climax may have supported a greater increase of the total number of fibers from the metamorphosis climax to the juvenile stage that would ultimately have sustained a higher growth rate. The reduced expression of *myhc* in the SUP group during the metamorphosis climax may to be related with a lower availability of AA for protein synthesis in the muscle, partially explaining the further reduced size of fast fibers and total cross-sectional muscle area during the juvenile stage. The *mrf4* was significantly more expressed in SUP fed juveniles than in CTRL, which is in general accordance with previous results from Campos et al. (2013b) in a slow-growing group during a compensatory growth phase at 83DAH. Surprisingly, the *mstn1*, a myostatin isoform that functions as a negative regulator of myoblast proliferation and differentiation (Thomas et al., 2000; Valente et al., 2006), was upregulated in the CTRL fed juveniles, that displayed an increased growth rate. The fact that *mstn1* transcription was analysed in the whole fish and the existence of several *myostatin* paralogues exhibit distinct expression patterns in muscle and non-muscle tissues are possible explanations for such result.

In **Chapter 5**, different dietary formulations based on different protein MW profiles induced differences in muscle cellularity during the metamorphosis climax (16DAH), with the PartH diet (mostly based on 5 to 70 KDa peptides) promoting fast-twitch muscle growth, which was reflected on increased somatic growth. At 36DAH, 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. At this stage, the transcription of *myf5* and *myod2* was significantly upregulated in the fish fed the HighH diet compared to fish the PartH diet. The transcription of *myogenin* was also significantly higher in the fish fed the HighH diet than infish fed either the Intact or PartH diets. *Myogenin* encodes a highly conserved myogenic regulatory factor that is involved in terminal muscle differentiation (Rescan, 2005). Hence, *myogenin* upregulation might have sustained the differentiation of myoblasts to further fuse into already formed muscle fibers, promoting hypertrophy rather than hyperplasia in the HighH group, being upstream supported by the upregulation of *myf5* and *myod2* transcription. A higher contribution of hypertrophy to muscle growth and the reduced recruitment of small fibers were suggested to be predictive of a lower growth potential in the

HighH group. On the other hand, a significant downregulation of *myf5*, *myod2* and *myogenin* transcription in the PartH group, and lower percentage of small fibers compared to the Intact group, suggests a reduced recruitment capacity of new fibers. Decreased recruitment of new fibers would have led to reduced growth potential in PartH fed fish . The transcription of *myf5* and *myod2* in the Intact group was probably high enough to keep up with the increase of the MPC population and further fiber recruitment. Accordingly, fish fed the Intact diet had a higher percentage of small fibers, which has probably contributed for a higher growth potential.

In **Chapters 3** and **5**, myogenin appears as the most responsive MRF to dietary protein manipulation. *In vitro* studies have previously shown that *myogenin* expression was clearly responsive to AA availability in gilthead seabream myocyte cells (*Sparus aurata*) (Velez et al., 2014) and to refeeding a primary culture of Atlantic salmon (*Salmo salar*) myocytes (Bower, Johnston, 2010). However, the effect of any environmental or nutritional cue, such as dietary protein, on the regulation of muscle growth should always be evaluated by a more holistic approach, preferentially using an *in vivo* study that combines both the phenotype (e.g. muscle cellularity) and the genotype information (e.g the expression of MRFs or, if possible other factors involved in myogenesis) (see section 1.5). Myogenesis is a very complex process and any induced changes cannot be interpreted in a simple and straightforward way (e.g. upon the expression of a single MRF or a single morphometric parameter). That comes out of an overall look into the results discussed in **Chapters 3** and **5**. While a muscle growth delay was observed in the SUP (**Chapter 3**) and in the PartH groups (**Chapter 5**) and associated with reduced *myogenin* expression and decreased myogenic cell recruitment capacity, in the HighH group (**Chapter 5**) the reduced growth potential seemed to be caused by a preferential hypertrophic growth. On the other hand, a steady expression of *myf5* and *myod2* was proposed to keep up the recruitment of small-sized fibers in the Intact fed fish (**Chapter 5)**. This was further confirmed by the muscle cellularity analysis that evidenced a higher percentage of small fibers that was able to support long-term growth in those fish fed the Intact diet. The present results support the percentage of small fibers as a good indicator of somatic growth potential in fish larvae, in agreement what has been long suggested by Alami-Durante et al., (1997), Galloway et al. (1999) and Valente et al. (1999).

6.3 Formulation strategies may induce epigenetic effects

DNA methylation is one of the best studied mechanisms of epigenetic modification of DNA (Berger et al., 2009). It is a biological process that results from the addition of methyl groups to DNA and contributes to the epigenetic network that controls gene expression (Zhang, 2015). DNA methylation relies on the one-carbon metabolism pathway, which is dependent upon the activity of several enzymes in the presence of dietary methyl donors, such as folate, choline,

betaine, vitamins B2, B6 and B12 and methionine (Anderson et al., 2012; Zhang, 2015). In the one-carbon cycle, methionine is converted into S-adenosylmethionine (SAM), the universal cellular methyl donor (Selhub, 1999). DNA cytosine methyltransferases (dnmts) covalently attach SAM methyl groups to the 5′-position of cytosine (in CpG dinucleotides), thus methylating DNA and repressing transcription. The reduced availability of methyl donors should result in low SAM synthesis and global DNA hypomethylation, and *vice versa*, as it has been suggested in mammals (Pogribny et al., 2008; 2009; Waterland, 2006; 2008).

In the present thesis it was hypothesized that manipulating the formulation of dietary protein might lead to changes in the dietary protein absorption and body retention, and subsequently modify the post-prandial availability of methyl-donors, such methionine, with possible consequences on the DNA methylation and regulation of gene expression in different tissues.

In **Chapter 3**, a higher expression of *dnmt3b* and an increasing tendency in the expression of *dnmt1* and *dnmt3a* in fish fed the CTRL diet, during the metamorphosis climax and at the juvenile stage, was suggested to be associated with a higher availability of methyl group donors. This could be a consequence of a higher protein intake, due to a possibly stimulating effect of the CTRL diet formulation on voluntary feed intake. In pigs, maternal dietary protein restriction and excess affected both the *dnmt3a* transcription and the expression of condensin I subunit genes, in the offspring's skeletal muscle (Altmann et al., 2012). It has also affected the *dnmt3a* and *dnmt3b* transcription, the global methylation, and the expression of condensin I subunit genes in the offspring's liver. Altmann et al. (2012) did not report whether the amount of methyl-donors was actually reduced, but it is commonly accepted that protein restriction or under-nutrition correlates with reduced methyl-donor availability. In the present thesis, there were no signs of protein restriction or under-nutrition in the CTRL or SUP groups, as both exhibited normal growth rates, but a lower post-prandial availability of methyl-donors as a result of a lower protein intake might explain the reduced expression of *dnmt*'s in the SUP fed fish.

In **Chapter 5**, the up-regulation in the transcript levels of genes encoding for *de novo* DNA methyltransferases in the HighH group were proposed to be associated with the higher methionine content of this diet, as compared to the Intact or the PartH diets.

In general terms, the results of the present thesis support the hypothesis that nutrition may induce changes in post-prandial availability of methyl-donors that might alter the expression of *dnmt*'s, with putative consequences on DNA methylation.

Further studies are needed to ascertain whether there were actually changes in the availability of methyl group donors in the tissues of the fish fed the CTRL and HighH diets, and whether

the increased expression of genes encoding for DNA methyltransferases would possibly lead to DNA hypermethylation.

Previous studies in Senegalese sole showed that rearing temperature during the pelagic phase induced changes in the expression of *dnmt1* and *dnmt3b* DNA methyltransferases during metamorphosis, eventually mediating an epigenetic regulation of muscle growth, through altered expression of *myogenin* (Campos et al., 2013a; 2013b; 2013c). However, the present thesis did not target establishing a relation between the altered expression of DNA methyltransferases and the regulation of myogenesis, what should be done in the future.

6.4. Conclusions and recommendations

An overall look into the results presented in this thesis highlights the following conclusions and recommendations:

- Manipulating dietary protein quality upon different concepts (according to the ideal protein concept or by increasing the IAA/DAA ratio) has different effects on the development of the larvae capacity to utilize and retain protein for growth purposes. A partial adjustment of the dietary AA profile to the larval body AA profile according to the ideal protein concept had a positive short-term effect on the Senegalese sole larvae capacity to retain protein (**Chapter 2**), but none of the strategies translated into increased growth (**Chapter 2** and **3**). However, post-prandial AA imbalances were pointed as a cause for decreased protein synthesis and growth, thus it necessary to find a more effective way of correcting the dietary AA profile. The correction of the dietary AA profile for individual IAA bioavailability will probably allow reaching for the ideal dietary AA profile for Senegalese sole, but that implies determining the IAA bioavailability in Senegalese sole at different developmental stages. The roles of individual IAA in several physiological functions should also be carefully considered, when manipulating the dietary IAA, to avoid toxic or other negative effects.

- The proteolytic capacity was confirmed as a limiting factor for dietary protein digestion in early larval stages (metamorphosing larvae) and the manipulation of dietary protein complexity should match the developing larvae proteolytic capacity (**Chapter 4**).

- Larvae are able to adjust the way they utilize dietary protein in response to the dietary formulation, by adapting the digestive machinery functioning, what seems to be regulated at transcriptional level (**Chapter 4**).

- The gut transit time is a factor possibly affecting the digestion and utilization of dietary protein in sole larvae; the effects of dietary protein formulation on the gut transit time

should be seen as a future target on improving dietary protein utilization in sole larvae (**Chapter 4**).

- While polypeptides within the 5-70 KDa range seem to be digestible to pre-metamorphic larvae (**Chapter 4**), oligopeptides are a valuable dietary protein source for metamorphosing larvae (**Chapter 2, 3** and **4**), and larger peptides and intact protein may be more suitable to sole post-larvae and young juveniles (**Chapter 2** and **4**). The complexity of dietary protein should increase in parallel to Senegalese sole development **(Chapter 4 and 5**); ultimately this process will require the inclusion of a greater variety of dietary protein sources, and the sequential use of different feeds.

- Changes in dietary protein exerted a strong influence on sole larvae muscle development, by inducing changes in the myogenic processes that were reflected on somatic growth potential (**Chapters 3** and **5**). The dietary induced changes in myogenesis were variable; either delayed muscle growth or preferential hypertrophic growth were both associated with somatic growth impairment; this highlights the plasticity of myogenesis as affected by nutritional factors.

- The percentage of small fibers was confirmed as a good indicator of somatic growth potential in Senegalese sole larvae (**Chapter 5**).

- Dietary induced changes in post-prandial availability of methyl-donors alters the expression of *dnmt*'s; further studies are needed to ascertain whether the altered expression of genes encoding for DNA methyltransferases in response to dietary protein would lead to changes in DNA methylation, and explain differences in growth potential.

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