Ana Rita Gomes Viegas

The effect of L-glutamine supplementation on the digestive physiology of Senegalese sole larvae, *Solea senegalensis* (Kaup, 1858)

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Declaração de autoria de trabalho:

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Ana Rita Gomes Viegas

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Quem faz aquilo que gosta arrisca-se a ser feliz.

Manuel Clemente

Abstract

Senegalese sole (*Solea senegalensis*) is an altricial species, which undergo dramatic transformations before metamorphosing into juvenile. Therefore, larvae must capture and process exogenous food even though the digestive tract may not be fully developed. The inadequate digestion/absorption of exogenous nutrients resulting from late maturation of the enterocytes at the intestinal epithelium has hampered a successful juvenile production of this promising flatfish species.

Glutamine is a conditionally essential FAA which is involved in immune function and intestinal health, and has been widely used in aquaculture.

A 22-day feeding trial was conducted to evaluate the effect of dietary L-glutamine supplementation on the growth performance, survival, metamorphosis, and digestive physiology during the early life stage of Senegalese sole. Two experimental diets were established with different levels of L-glutamine enrichments – 0.0 g l^{-1} (CTL) and 0.5 g l^{-1} (GLN). Experimental treatments were randomly assigned to triplicate groups stocked at a density of 0.26 individuals cm^{-2}, reduced to 0.14 individuals cm^{-2} at 19 DAH, in each tank. The results showed that growth performance and enterocyte height of fish fed L-glutamine-supplemented diet were significantly increased compared to the control group ($p<0.05$). Survival and metamorphosis were not significantly different between dietary groups ($p>0.05$). Significant improvements were observed on specific activity of pepsin-like and amylase from CTL group, whereas BB aminopeptidase was highlighted in GLN group ($p<0.05$). Total activity of pepsin-like, amylase and acid phosphatase from CTL group was greater ($p<0.05$) than GLN group, whereas BB aminopeptidase total activity and IMI assayed through this enzyme showed better results in GLN group. Specific and total activity of trypsin, aminopeptidase, and intestinal and BB alkaline phosphatase did not differ between groups. Also, acid phosphatase specific activity was not affected by dietary L-glutamine. In conclusion, dietary L-glutamine supplementation could improve the growth performance and enterocyte height at the intestinal epithelium of sole post-larvae. Further studies are needed to clarify the effect of L-glutamine supplementation on enzymatic activity.

KEYWORDS: L-glutamine; digestive tract; enterocytes; digestive enzymes; post-larvae; *Solea senegalensis.*

Resumo

Com o contínuo aumento da população, um dos maiores desafios da atualidade baseia-se na procura de novas formas de satisfazer as necessidades alimentares, através de uma dieta saudável, rica em proteínas.

Com a estabilização da produção pesqueira, resultante da sobre-exploração de grande parte dos recursos haliêuticos, e perante o progressivo aumento do consumo global de peixe, a aquacultura surge como uma alternativa viável para a produção de proteína de elevada qualidade nutricional, a um preço relativamente baixo.

A produção de linguado do Senegal (*Solea senegalensis*) tem sido fomentada para a diversificação da aquacultura na Europa. Além de possuir um elevado valor nutritivo, esta espécie de elevado valor comercial apresenta um vasto leque de caraterísticas produtivas vantajosas, que tornam a sua produção economicamente competitiva.

Devido aos avanços tecnológicos e científicos alcançados nas últimas décadas, entre os quais sobre a biologia reprodutiva, comportamento, requisitos nutricionais e fisiologia digestiva, o linguado senegalês tem vindo a exibir sinais de expansão na sua produção a nível industrial.

Consequentemente, a produção de linguado senegalês tem vindo a apresentar uma elevada demanda de forma a atender as necessidades do mercado, que não são completamente satisfeitas pelas capturas pesqueiras. Posto isto, há uma necessidade urgente de aumentar a quantidade de larvas e juvenis de linguado senegalês de elevada qualidade, que passa por solucionar as complicações ainda existentes na fase larvar.

A fase de transição larva-juvenil representa ainda um período crítico na produção de linguado marcado pela elevada taxa de mortalidade, especialmente aquando a metamorfose ou a passagem do alimento vivo para alimento inerte (desmame). Durante o desenvolvimento larvar, as elevadas exigências nutricionais, impostas pelo rápido crescimento e maturação dos tecidos, podem, por vezes, ser limitadas pela reduzida eficiência digestiva. A reduzida capacidade digestiva, que o linguado senegalês apresenta durante o desenvolvimento larvar, pode ser atribuída à maturação tardia do intestino, que ocorre por volta dos 25 dias após eclosão (DAE); três semanas após o início da alimentação exógena. Desta forma, há o interesse em promover um desenvolvimento mais rápido do trato digestivo nas larvas de linguado, na medida em que o epitélio intestinal, ao estar diretamente relacionado com a absorção e digestão dos nutrientes, poderá proporcionar melhores taxas de sobrevivência e assegurar um melhor desempenho produtivo.

Vários estudos têm demonstrado o potencial da glutamina na fisiologia digestiva de diversos organismos aquáticos. Além de impedir a atrofia da mucosa intestinal, este aminoácido é capaz de promover a maturação intestinal através do aumento das vilosidades intestinais, bem como das células epiteliais (enterócitos) nelas presentes.

Devido à versatilidade da glutamina e à miríade de efeitos benéficos associados, a sua utilização tem sido foco de investigação na área da nutrição e visa aumentar a produção comercial de organismos aquáticos. Na última década, a utilização da glutamina como suplemento dietético tem vindo a ser testada nas fases iniciais do desenvolvimento de algumas espécies, como a Tilápia do Nilo (*Oreochromis niloticus*) e linguado (*Cynoglossus semilaevis*).

No âmbito desta necessidade de promover uma rápida maturação do trato digestivo do linguado senegalês, o presente estudo, integrado no projeto DIVERSIAQUAII, teve como principal objetivo avaliar o efeito da suplementação dietética com L-glutamina ao nível da fisiologia digestiva de pós-larvas de *S. senegalensis*. Para tal, foram estabelecidos dois grupos experimentais: Grupo controlo (CTL), no qual as pós-larvas de linguado senegalês foram alimentadas com metanáuplios de *Artemia* sp. enriquecidos com *Red Pepper* (BERNAQUA), uma emulsão comercial (1,0 ± 0,23 mg L-glutamina/g de peso seco de *Artemia* sp.); e o Grupo L-glutamina (GLN), no qual as pós-larvas de linguado senegalês foram alimentadas com metanáuplios de *Artemia* sp. enriquecidos com L-glutamina (em pó) (2,2 ± 0,06 mg L-glutamina/g de peso seco de *Artemia* sp.). Um total de 13 140 larvas (peso seco inicial de 0,37 ± 0,05 mg) de linguado senegalês foram aleatoriamente distribuídas por 6 tanques retangulares (n=3, por cada tratamento experimental). A alimentação foi fornecida *ad libitum* cinco vezes ao dia, desde os 12 até aos 33 DAE. Durante o período experimental foram realizadas cinco amostragens, de forma a avaliar os seguintes parâmetros: comprimento total, peso seco, estado metamórfico, altura dos enterócitos, atividade específica e total das enzimas digestivas e das localizadas na borda em escova dos enterócitos. Com base nos dados de alguns destes parâmetros, foram também determinadas a taxa de crescimento relativa e o índice de maturação intestinal. A taxa de sobrevivência foi obtida com base nos registos de mortalidade registados diariamente.

Relativamente ao comprimento total e peso seco, as pós-larvas de linguado senegalês alimentadas com *Artemia* sp. enriquecida com L-glutamina apresentaram valores superiores aos do grupo CTL. Da mesma forma, a taxa de crescimento relativa do grupo GLN foi significativamente maior a partir dos 26 DAE, comparativamente ao grupo CTL. No entanto, tanto a sobrevivência como os estados metamórficos não diferiram entre tratamentos

experimentais. A suplementação com L-glutamina na dieta das pós-larvas de linguado senegalês promoveu melhorias na altura dos enterócitos do epitélio intestinal.

Em relação aos dados obtidos através da análise enzimática, a atividade específica da pepsina e da amilase do grupo CTL destacaram-se positivamente, em comparação com o grupo GLN; enquanto a atividade específica da aminopeptidase, avaliada ao nível da borda em escova dos enterócitos, foi potenciada pela suplementação de L-glutamina na dieta.

A atividade total da pepsina, amilase e fosfatase ácida, exibidas pelo grupo CTL, foi superior à das pós-larvas de linguado senegalês alimentadas com uma dieta suplementada com L-glutamina. A atividade total da aminopeptidase analisada ao nível da borda em escova dos enterócitos, bem como o índice de maturação intestinal calculado a partir desta enzima, foram promovidos pelo uso de L-glutamina como suplemento dietético no protocolo alimentar das pós-larvas de linguado. Tanto a atividade específica como a atividade total da tripsina, aminopeptidase e fosfatase alcalina (intestinal e da borda em escova dos enterócitos) não diferiram entre tratamentos experimentais. Da mesma forma, não foram observados efeitos da suplementação de L-glutamina na dieta ao nível da atividade específica da fosfatase ácida.

Os resultados obtidos mostram, pela primeira vez, que em pós-larvas de linguado senegalês, o uso de L-glutamina como suplemento dietético pode promover melhorias ao nível do desempenho de crescimento. Além disso, com base nos dados histológicos, os resultados obtidos nesta tese suportam a possibilidade de a suplementação com L-glutamina acelerar a maturação do intestino durante o desenvolvimento larvar de *S. senegalensis.* Porém, é ainda necessário desenvolver outros estudos de forma a clarificar o efeito da suplementação com L-glutamina na atividade enzimática das pós-larvas de linguado senegalês.

Palavras-chave: L-glutamina; trato digestivo; enterócitos; enzimas digestivas; pós-larvas; *Solea senegalensis.*

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LIST OF UNITS AND ABBREVIATIONS

AA	Amino acid
BSA	Bovine serum albumin
BB	Brush border
BBM	Brush border membrane
CTL	Control
DAE	Dias após eclosão
DAH	Days after hatching
DW	Dry weight
FAO	Food and Agriculture Organization of the United Nations
FAA	Free amino acid
GLN	Glutamine
IMI	Intestinal maturation index
pNPP	*para*-nitrophenylphosphate
RGR	Relative growth rate
S	Survival rate
TL	Total length
TCA	Trichloroacetic acid

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1. INTRODUCTION

1.1. General introduction

One of the great challenges at a global level is to search for new ways to meet food needs, through a healthy diet rich in proteins, for an increasing human population expected to exceed 9 billion by 2050 (UN estimates) (Béné *et al.*, 2015). In the past, the growing demand for fish was handled by putting pressure on fish stocks and threatening the sustainability of marine and continental fishing. In this sense, the aquaculture emerges as a potential solution to complement fisheries in the production of aquatic food, and consequently help to mitigate the decline in wild caught stock (FAO, 2016).

Currently aquaculture continues to grow faster than other major food production sectors, and is expected that by 2030 60% of all seafood produced for human consumption will come from aquaculture, 20% higher than in 2016 (FAO, 2018; Global Aquaculture Alliance, 2019).

Due to its high nutritive value and advantageous productive characteristics, Senegalese sole (*Solea senegalensis*, Kaup, 1858) is considered a promising flatfish species for marine aquaculture industry. This species is one of the most important commercial flatfish in Southern European aquaculture, being consistently high-priced in the last few years (FEAP, 2016; Morais *et al.*, 2014). For several decades, the culture of *S. senegalensis* has been possible and commercially attempted, but its intensive production has been slow to take off. Despite research efforts, a successful juvenile production of this species under intensive culture has been often hampered due to its high heterogeneity in growth rates, large size dispersion and difficulties in weaning periods (the transition from live feeds to an inert diet) (Morais *et al.*, 2014). During the early life stage, marked by the complex metamorphosis, the rapid growth displayed by larvae, linked to its intense tissue maturation and physiological functions, can lead to a high demand for nutrients to fulfil their energetic and nutritional requirements. However, the availability of nutrients at first feeding might be limited by the low digestive capacity (Cahu *et al.*, 2003; Holt *et al.*, 2011). The inadequate digestion/absorption of exogenous nutrients by sole larvae could be attributed to the later maturation of the enterocytes at the intestinal epithelium, which occurs between 21 to 27 days after hatching (DAH). Indeed, only after 31 DAH *S. senegalensis* achieve a morphologically complete digestive tract capable of ingesting, digesting and absorbing nutrients, far beyond the onset of exogenous feeding (Dinis *et al.*, 2000; Ribeiro *et al.*, 1999a). In this sense, there has been a great interest in promoting a faster development of the digestive tract in the early stages of Senegalese sole.

Glutamine is traditionally considered as a nonessential amino acid, which becomes conditionally essential during periods of rapid growth of tissues, when its consumption exceeds its provision by body synthesis (DeBerardinis and Cheng, 2010; Lacey and Wilmore, 1990). This free amino acid (FAA) is an important molecule for the immune system and intestinal health (Kim and Kim, 2017), since prevents atrophy of the intestinal mucosal barrier, and it also promotes intestinal growth by increasing villi length (Bartell and Batal, 2007; Murakami *et al.*, 2007).

Due to glutamine's versatility and myriad of beneficial effects, interest in its dietary manipulation to enhance commercial production has risen substantially in young terrestrial animals such as broiler (Bartell and Batal, 2007; Lopes, 2008; Murakami *et al.*, 2007; Sakamoto, 2009; Soltan, 2009; Yi *et al.*, 2005) and weaning piglet (Wang *et al.*, 2008; Wu *et al.*, 1996; Zou *et al.*, 2006). Also, this research has been targeted aquatic animals, although explored during the early life stages only in few species as Nile tilapia (Graciano *et al.*, 2014; Junior, 2014; Silva, 2008; Vesco *et al.*, 2001) and half-smooth tongue sole (Liu *et al.*, 2015).

Can glutamine promote a rapid maturation of the digestive tract of Senegalese sole larvae and, thus promote progresses on the growth performance?

1.2. *Solea senegalensis*

Among the teleost fishes, Senegalese sole is a flatfish from the Soleidae family that inhabits sandy or muddy bottoms throughout the Atlantic and Mediterranean, from the Golf of Biscay to Senegal coasts, and feeds mainly on benthonic invertebrates (Whitehead *et al.*, 1986).

S. senegalensis is a gonochoric species, with females maturing at age 3+, when the total length is around of 32 cm. The spawning season of this species occurs naturally during Spring, between March-June, and in September a second shorter period may occur. In captivity this species has a natural spawning, stopping the egg emission below 16 ºC (Dinis *et al.*, 1999).

Senegalese sole is a valuable flatfish species in the wider European market, which have a tremendous commercial opportunity for diversifying marine aquaculture due to several advantageous productive characteristics, such as the natural spawning of wild-caught broodstocks held in captivity and mass production of offspring, and the rapid development of eggs and larvae (Dinis *et al.*, 1999). In this way, sole has attracted considerable interest during the last 30 years, both at the research and commercial level (Dinis *et al.*, 1999; Garcia and Garcia, 2006). Nevertheless, production of Senegalese sole in southern Europe has failed to reach successful commercial development until recently (FAO Fisheries and Aquaculture Information and Statistics Service, 2011; Flos *et al.*, 2001; Imsland *et al.*, 2003). This can be mostly explained by multiple bottlenecks which compromise the intensive culture of this flatfish species such as: resistance to pathogen infection; outbreaks of infectious disease; incidence of skeletal deformities and pigmentation abnormalities; poor growth rate at high stocking densities; and difficulties in weaning (Morais *et al.*, 2014; Zarza *et al.*, 2003). Consequently, poor fingerlings quality and high mortality rates during weaning periods have led to juvenile scarcity for on-growing purposes (Dinis *et al.*, 1999).

Over the last years, significant progress on larval rearing has been achieved, and are nowadays fairly standardized, with post-larvae being routinely produced and great records on growth and survival rates (Conceição *et al.*, 2007; Engrola *et al.*, 2007; Fernández-Díaz *et al.*, 2001; Morais *et al.*, 2014).

In the last decades, great advances have been achieved in the state of the knowledge in areas as diverse as reproductive biology, behaviour, larval nutritional requirements, physiology, modulation of the immune system in response to environmental parameters, and stress of *S. senegalensis*. In addition, technological improvements have been attained in sole production. Both advances in the state of *S. senegalensis* knowledge and technological developments triggered the expansion of sole production at industrial level: total production of farmed sole

has risen from 68 to 1457 tons in the period 2007–2015 in Europe (FEAP, 2016). This will inevitably lead to an increase in the demand for high-quality juvenile production to attend market needs, which are not completely met by fisheries catches (Dinis *et al.*, 1999; Gavaia *et al.*, 2002; Soares *et al.*, 2002). Given this prospective scenery, there is an urgent need to increase Senegalese sole larvae and juvenile quantity at a high quality, which begins by solving the bottlenecks that still exist in the early larval stage.

1.2.1. Ontogenetic development of Senegalese sole larvae

As the most of marine species common in aquaculture production, *S. senegalensis* is poorly developed at hatching, thus called "altricial" larvae, which must undergo dramatic transformation before metamorphosing into the juvenile (Balon, 2006; Jomori, 2001; Tesser, 2002).

Flatfish species, where *S. senegalensis* is included, are extremely variable in the patterns of metamorphosis that they exhibit. Between and sometimes even within species, size at metamorphosis, as well as duration of metamorphosis and its synchrony with settlement can vary. Moreover, the order of ontogenetic events may also differ (Geffen *et al.*, 2007). According to Padrós *et al.* (2011), Senegalese sole larvae follows developmental patterns similar to other flatfish species and small marine pelagic fish larvae. But specific developmental characteristics in *S. senegalensis* can suffer a wide variation in the timing probably derived from specie's particular environment and behaviour.

Among a wide range of flatfish species, Senegalese sole present one of the most familiar and impressive metamorphosis, characterized by strong anatomical transformations affecting behaviour, feeding and digestive physiology (Fernández-Díaz *et al.*, 2001). Major morphological changes in Senegalese sole occur as the pelagic larvae with bilateral symmetry change to highly asymmetrical juvenile throughout its extremely accentuated metamorphosis (Gavaia *et al.*, 2002), which occurs along the second to third week after hatching and achieves its climax around 14 to 17 DAH (Dinis *et al.*, 1999; Fernández-Díaz *et al.*, 2001; Mai *et al.*, 2009). Around 12 DAH, the left eye migrates towards the right side, and the body suffers a 90° rotation, which is accompanied by the internal organs rearrangement, including the reorganization of the buccal and gill cavity, with migration of the anus towards the pelvic fin (Dinis *et al.*, 1999; Fernández-Díaz *et al.*, 2001; Ribeiro *et al.*, 1999a). According to the same authors, this change, which marks the beginning of metamorphosis, occurs at 12 DAH, but were already detected at 9 DAH by Fehri-Bedoui *et al.* (2000). At the end of metamorphosis, around

18-20 DAH, a benthonic post-larva settles on the blind side (Dinis *et al.*, 1999; Ribeiro *et al.*, 1999b).

Due its complex metamorphosis, the early life stage in *S. senegalensis* is considered a critical period, when the larvae need to fulfill the development of organ systems and further maturation of the physiological functions, mainly at the digestive tract level (Conceição *et al.*, 2010b; Mazurais *et al.*, 2011).

1.2.2. Early ontogeny of digestive system in Senegalese sole

Although basic mechanisms of organ and system development are similar in teleosts, there exist considerable interspecific differences regarding the relative timing of their differentiation, development and functionality during early ontogeny (Treviño *et al.*, 2011).

Digestive system ontogeny in teleosts is commonly divided into three phases (Bisbal and Bengtson, 1995; Buddington, 1985): 1) the lecithotrophic phase, when larvae fully rely on endogenous yolk nutrients; 2) exogenous feeding phase with limited proteolytic capacity due to an immature digestive system; 3) exogenous feeding phase with full capacity to digest dietary protein due to the achieve of a developed and functional stomach.

As described for several marine fish species like, Atlantic cod (*Gadus morhua*) (Kjorsvik *et al.*, 1991; Morrison, 1993), Atlantic halibut (*Hippoglossus hippoglossus*) (Kjorsvik and Reiersen, 1992), gilthead sea bream (*Sparus aurata*) (Sarasquetel *et al.*, 1995), European sea bass (*Dicentrarchus labrax*) (Vu, 1983), red seabream (*Pagrus major*) (Tanaka, 1973), Japanese flounder (*Paralichthys olivaceus*) (Tanaka *et al.*, 1996), Amazonian pimelodid catfish (*Pseudoplatystoma punctifer*) (Gisbert *et al.*, 2014), and palm ruff (*Seriolella violacea*) (Alveal *et al.*, 2018), at hatching (0 DAH) Senegalese sole (Ribeiro *et al.*, 1999a) exhibit a digestive tract as a short and straight tube lying dorsally to the yolk sac, closed at both the mouth and anus, and histologically undifferentiated along its length. At this time, the rudimentary digestive tract present an epithelium formed by a monostratified layer (Fehri-Bedoui *et al.*, 2000; Ribeiro *et al.*, 1999b; Sarasquetel *et al.*, 1996).

At the first DAH, the digestive tract of *S. senegalensis* is differentiated into two section: the prevalvular and postvalvular, which correspond to the anterior (squamous epithelium) and posterior (columnar epithelium) intestine, respectively (Ribeiro *et al.*, 1999a; Zambonino-Infante *et al.*, 2008). However, a late gut differentiation (around 4 DAH) was reported by Fehri-Bedoui *et al.* (2000), as observed in other species as redbanded seabream (*Pagrus auriga*) (Sánchez-Amaya *et al.*, 2007).

Around 2–4 DAH, after mouth opening, the gut of *S. senegalensis* becomes divided by a third portion by a muscular sphincters, also known as pyloric sphincter (Fehri-Bedoui *et al.*, 2000; Ribeiro *et al.*, 1999a). According to Tanaka (1973), this fact is intimately related with the position of the anus.

During development, the digestive tract of *S. senegalensis* suffers some changes concerning the spatial organization in the abdominal cavity resulting from its metamorphosis (Conceição *et al.*, 2007). During this period, the annex glands (liver, pancreas, and gall bladder) starts to differentiate, becoming functional by the 3rd DAH, and well differentiated only at 12–13 DAH (Dinis *et al.*, 2000; Fehri-Bedoui *et al.*, 2000; Ribeiro *et al.*, 1999a, 1999b).

At the end of the lecithotrophic larval stage (about 1–4 DAH), *S. senegalensis* displayed a digestive tract segmented into histologically and functionally distinct regions: bucco-pharynx, fore-, mid- and hindgut (Fehri-Bedoui *et al.*, 2000; Ribeiro *et al.*, 1999a).

The transition of endogenous to exogenous feeding in Senegalese sole, which occurs around 2 DAH, involves the shift to a coiled digestive tract, followed by the complete reabsorption of yolk reserves at 3 DAH; as the larvae becomes ready to receive and digest the first feeding (Dinis *et al.*, 1999; Ribeiro *et al.*, 1999b). As the development of the digestive tract proceeds, around 12 to 20 DAH, Senegalese sole larvae increases its intestinal surface by an elongation of the digestive tract, thereby increasing the gut passage rates and effective digestive time (Ribeiro *et al.*, 1999a).

After some days of exogenous feeding, the nutrients resulting from digestion will be differentially absorbed along the histologically and functionally distinct intestinal regions (Govoni *et al.*, 1986; Tanaka, 1973). The foregut differentiates further into the oesophagus, pyloric caeca and anlage stomach, whereas the midgut and hindgut form the anterior and posterior intestine, respectively (Govoni *et al.*, 1986). The fore- and midgut is most active in digestion and lipid absorption (Chen *et al.*, 2006; Deplano *et al.*, 1991a; Diaz *et al.*, 2002; Sarasquetel *et al.*, 1996), while the hindgut is where the absorption of macromolecules (e.g. protein) by pinocytosis takes place at the enterocytes (epithelial cells) (Chen *et al.*, 2006; Deplano *et al.*, 1991b; Elbal *et al.*, 2004; Gisbert *et al.*, 2004; Govoni *et al.*, 1986; Sarasquete *et al.*, 1995; Segner *et al.*, 1994).

Towards the end of the larval stage, the digestive tract can be radially characterized throughout its length into four distinct layers: mucosa (innermost layer), submucosa, tunica muscularis and tunica serosa (Holt *et al.*, 2011). Mucosa, as the most variable layer in terms of

structure and function, gives several digestive functions to the digestive tract (e.g. protection of the inner layers, absorption, osmoregulation and metabolism of nutrients) (Holt *et al.*, 2011).

Along the mucosal surface two types of tissue can be identified: 1) lamina propria and 2) epithelium, which present different cells (epithelial cells) – goblet (mucous) cells, entero-endocrine (hormones) cells and enterocytes (absorption cells) (Boleli *et al.*, 2002; Junqueira and Carneiro, 2004). Enterocytes, which correspond to the most abundant epithelial cell type in the intestine, are involved in the nutrient absorption, intracellular digestion, and osmoregulation (Holt *et al.*, 2011).

In settled sole post-larvae, the process of intestine maturation, which is characterized by the maturation of enterocytes and the acquisition of an adult mode of digestion, is achieved between 21 and 27 DAH (Ribeiro *et al.*, 1999a).

The last major morphological change of the digestive tract, which can act as an indicator of the beginning of the juvenile stage, occurs when tubular gastric glands develop and their number increases progressively, partially or completely covering the stomach epithelium (Elbal *et al.*, 2004; Ortiz-Delgado *et al.*, 2003; Ribeiro *et al.*, 1999b). In Senegalese sole in spite of the presence of gastric glands around 27 DAH (Ribeiro *et al.*, 1999b), the acidification process has not been detected in either juveniles or adults (Yúfera and Darías, 2007).

In *S. senegalensis* the first digestive glands appears along the mucosa of fundic and pyloric stomach regions by 27 DAH (Ribeiro *et al.*, 1999b). Nevertheless, gastric glands were detected earlier, by the 18th day, in a posterior study carried out by Fehri-Bedoui *et al.* (2000), and only between 30 to 45 DAH in later researches performed by Vieira (2000) (between 35 and 45 DAH), Yúfera and Darías (2007) (around 30-40 DAH) and Sarasquete *et al.* (2001) (35 to 40 DAH). However, developed gastric glands are detected since very earlier, at 8-9 DAH, in Siberian sturgeon (*Acipenser baerii*) (Gisbert *et al.*, 1998), whereas in European bass, gastric glands appear 3–4 weeks after hatching in the cardiac region of the stomach (García-Hernández *et al.*, 2001; Vu, 1983; Zambonino-Infante *et al.*, 2008), between 35 and 45 DAH in gilthead sea bream (Domeneghini *et al.*, 1998), and only at 90 DAH in Atlantic halibut (Luizi *et al.*, 1999). Nonetheless, the development of gastric glands is related to metamorphosis in these species (Buddington and Christofferson, 1985; Gisbert *et al.*, 1998; Sarasquete *et al.*, 1995; Vieira, 2000), and is not necessarily accompanied by the onset of stomach activity since morphology does not always mean functionality (Holt *et al.*, 2011).

One of the most important events which determine the end of the transformation from larvae to juvenile stage in teleosts is the achieve of a completely functional digestive tract

characterized by the adult-like mode of digestion (Yúfera and Darias, 2007; Zambonino-Infante *et al.*, 1997; Zambonino-Infante and Cahu, 2001). In a study focused on the histological and histochemical development of the digestive system of *S. senegalensis* larvae, Ribeiro *et al.* (1999a) observed that after 31 DAH sole larvae are able to ingest, digest and absorb complex nutrients after achieve a morphologically complete digestive tract, equipped with digestive enzymes (Dinis *et al.*, 2000; Holt *et al.*, 2011; Rønnestad *et al.*, 2013; Zambonino-Infante and Cahu, 2001).

1.2.3. The onset of digestive enzymes in Senegalese sole

Digestion processes are well known in growing vertebrates including fish.

The digestive enzymes which are involved in the nutrients digestion include: the enzymes produced by gastric glands at the stomach (pepsin), pancreatic enzymes (e.g. trypsin, amylase, lipase, and chymotrypsin) and intestinal enzymes (aminopeptidase and alkaline phosphatase) (Zambonino-Infante and Cahu, 2001). Although not being digestive enzymes, cytosolic (leucine-alanine peptidase and acid phosphatase) and BBM (e.g. aminopeptidase, alkaline phosphatase and maltase) enzymes have an important role in the absorption/transport of nutrients and cytosolic digestion by the intestinal cells (Ribeiro, 2003; Zambonino-Infante and Cahu, 2001). These enzymes, which are often complementary, develops independently during ontogenesis, and their activity can be adapted to diet composition according to genetically programmed enzymes pattern (Cahu and Zambonino-Infante, 2001) with variation related to fish species and temperature (Kolkovski, 2001).

Due to the post-natal digestive features, it was considered that young larvae can not handle dietary components in a manner exactly analogous to that of juveniles (Buchet *et al.*, 1997; Cahu and Zambonino-Infante, 1995; Peres *et al.*, 1996).

In marine fish larvae, the maturation of the digestive tract which results from the achievement of an adult-like mode of digestion with total digestive capacity, compromises three main stages: 1) acquisition of pancreatic secretions; 2) the onset of the enzymatic activity of the brush border membrane (BBM) at the intestinal epithelium; 3) appearance of pepsin activity concomitant with the development of gastric glands (Gisbert *et al.*, 2009; Rønnestad *et al.*, 2007, 2013; Zambonino-Infante and Cahu, 2001).

At the onset of exogenous feeding, Senegalese sole larvae, as an altricial species, present a digestive tract with no functional stomach or well-differentiated gastric glands (Zambonino-Infante and Cahu, 2001). For this reason, during the first stages, the digestion relies mainly on

pancreatic enzymes and cytosolic intestinal enzymes in an alkaline-neutral pH environment (Ribeiro *et al.*, 1999a; Rønnestad *et al.*, 2007).

The pancreatic enzymes, which are produced by the exocrine pancreas, have a limited capacity for macromolecules digestion that are absorbed by the pinocytotic activity of the hindgut enterocytes for their intracellular digestion (Jobling, 1995; Zambonino-Infante and Cahu, 2001). While trypsin is a proteolytic enzyme responsible for the peptides and proteins hydrolysis (Zambonino-Infante and Cahu, 2001), the substates hydrolysed by amylase is the starch (Jobling, 1995).

Trypsin is considered an important enzyme responsible for activation of other pancreatic enzymes, with the exception of amylase, which is released in its active form (Lazo *et al.*, 2007; Wallig and Sullivan, 2013).

In marine fish larvae, all main pancreatic enzymes (trypsin and amylase) exhibit similar pattern in specific activities during the first three weeks of life: an increase followed by a sharp decline (Ribeiro *et al.*, 1999a).

In *S. senegalensis,* trypsin is present at the moment of mouth opening (Zambonino-Infante and Cahu, 2001) and it specific activity increases from 3 DAH during 4 to 6 days, followed by a decrease to a constant level by the day 10 of age (Martínez *et al.*, 1999; Ribeiro *et al.*, 1999a). The increasing trypsin secretion and activity in the intestinal lumen in the first weeks of development characterizing the normal maturation process of pancreas (Martínez *et al.*, 1999; Ribeiro *et al.*, 1999a). The date of trypsin activity decline depends on the species, ranging from 5 DAH as in barramundi (Ma *et al.*, 2001) to three weeks after hatching as shown in sea bass (Zambonino-Infante and Cahu, 1994a) and red drum (Buchet *et al.*, 2000).

Amylase specific activity in Senegalese sole follows a similar pattern of the sea bass (Zambonino-Infante and Cahu, 1994a) and walleye pollock (*Theragra chalcogramma*) (Oozeki and Bailey, 1995): an increase after first feeding, followed by a decrease to a rather constant level (Ribeiro *et al.*, 1999a). Specific activity of amylase is very high during young larval phase and decreases as the larvae develops. This can be related to the natural predisposition of young larvae to use carbohydrates (Krogdahl and Sundby, 1999), reflecting the different nutritional requirements at different periods of life (Buchet *et al.*, 2000; Ribeiro *et al.*, 1999b; Zambonino-Infante and Cahu, 2001).

After the digestion by the pancreatic enzymes, dietary components are digested by the intestinal enzymes (Rønnestad *et al.*, 2013).

The activity of intestinal peptidases such as the aminopeptidase, which carry out the hydrolysis of peptides resulting from protein digestion by pancreatic proteases, can be detected in both larvae and yolk-sac larvae (Rønnestad *et al.*, 2013). In turbot (Cousin *et al.*, 1987), the activity of aminopeptidase can be found at 1 DAH, but only at the start of feeding in trout and between different species of whitefish hybrids (*Coregonus* sp.) (Lauff and Hofer, 1984), and later, on day 21 after hatching, in milkfish (*Chanos chanos*) (Ferraris *et al.*, 1987). In Senegalese sole, the activity of peptidases has also been detected in juveniles (Ribeiro *et al.*, 2002).

At the mucosal layer of the intestine, facing the lumen, the cytosolic and BBM enzymes are located at the cytoplasm and BBM of the enterocytes, respectively (Zambonino-Infante and Cahu, 2001).

During the first days of larval development, the enzymes located at the cytosol of the enterocytes, facing the gut lumen, are responsible to complete the breakdown of diet components (e.g. peptides) into absorbable monomers (amino acids), allowing their absorption and transport by the columnar cell (enterocyte) (Zambonino-Infante and Cahu, 2001; Zambonino-Infante *et al.*, 2008). The activity of cytosolic enzymes, which are stimulated by incorporating protein hydrolysate in the diet (Zambonino-Infante and Cahu, 2007), are high around first feeding and tend to decrease as the fish larvae develops; concurrent with rising levels of alkaline phosphatase at the BBM (Cahu and Zambonino-Infante, 2001; Kolkovski, 2001; Ribeiro *et al.*, 1999b).

In a study carried out by Martínez *et al.* (1999) in *S. senegalensis*, the activity of alkaline phosphatase at the BBM decreased from day 5 to day 20, and sharply increase from 21 DAH to 27 DAH, when the gastric glands starts to develop (Ribeiro *et al.*, 1999b).

A decrease in cytosolic enzymes concurrent to the increase in activity of a BBM enzyme as the microvillus membrane of enterocytes becomes functional, characterize the progress of the enterocytes maturation, and consequently the maturation of intestine (Zambonino-Infante and Cahu, 2001). Thus, intestinal maturation is often assessed by the alkaline phosphatase/leucine-alanine peptidase and aminopeptidase/leucine-alanine peptidase ratios (Zambonino-Infante and Cahu, 1994a, 1994b). Also, these intestinal maturation indices can be considered as for evaluating the nutritional condition of fish, which switch from a primary or early to an adult mode of digestion (Zambonino-Infante and Cahu, 2001). Once attained the adult-like mode of digestion, Senegalese sole starts to digest small peptides by aminopeptidase and alkaline phosphatase located at the BBM of enterocytes (Cara *et al.*, 2003; Zambonino-Infante and Cahu, 2001).

As the fish larvae achieve the acid digestion, the pinocytotic activity of protein carried out by acid phosphatases stored in lysosomes tends to decrease, and mainly disappear with the transition to the juvenile stage (Cara *et al.*, 2003; Tanaka, 1973).

In *S. senegalensis,* after 3 days of exogenous feeding, the presence of several dense vesicles containing protein in the enterocyte's cytoplasm of the posterior intestine, can reflects the pinocytic absorption of proteins (Ribeiro *et al.*, 1999b). According to Stroband *et al.* (1979), the presence of protein within these vesicles can be related with the strong activity of acid phosphatase, since the activity of this enzyme is related with pinocytic activity and intracellular digestion.

Toward the end of the larval period, the activity of acid proteases (pepsin) released by the gastric glands becomes increasingly important in protein digestion, although accounting only for nearly 10% of total protease activity (Lauf and Hoffer, 1984; Lazo *et al.*, 2007; Martínez *et al.*, 1999; Zambonino-Infante and Cahu, 2007).

In a study performed by Ribeiro *et al.* (1999b) in *S. senegalensis*, no pepsin-like activity was quantified during the first month of life, whereas Zambonino-Infante and Cahu (1994b) reported pepsin specific activity during the sea bass larval stage, although 3 times higher in larvae fed with compound diet than in larvae fed a live food. The absence of this acidic protease in the digestive tract of teleosts during early life stages is thought to be compensated by micropinocytosis and intracellular digestion of proteins in the posterior intestine by acid phosphatases and some aminopeptidases (Cara *et al.*, 2003).

Enzymatic specific activity tends to decrease along the larval development, not because the enzymatic activity decreases but because there is an increase in tissue proteins. On the other hand, the total activity of digestive enzymes increases with age due to an increase in the larval size (Zambonino-Infante and Cahu, 2001).

As the larva becomes juvenile, besides the increase in the surface for nutrient absorption, the plasma membrane of the microvilli, displayed at the surface of each absorptive enterocyte, embeds various enzymes that can help to complete the final stages of nutrient digestion (Fox, 2011).

To summarize, acid phosphatase is an indicator of protein pinocytosis, leucine-alanine peptidase and aminopeptidase of complete protein hydrolysis at an intestinal level, and alkaline phosphatase is an indicator of amino acids absorption (Cara *et al.*, 2003).

The nutrient absorption takes place through the apical region (villus) of the epithelium of each region, where the enterocytes are continuously replaced by the proliferation, a process called epithelium turnover (Blok *et al.*, 2002).

In a situation of imbalance in the cell renewal process toward an increase of proliferation, the growth in the number of epithelial cells lead to an increase in both height and density of the villi. This will enable the development of the intestinal mucosa, and, thereby, a greater number of enzymes to be produced, helping in the extracellular digestion (Maiorka *et al.*, 2002).

In this sense, the maturation of the intestine is an event of great importance for larvae, since good growth and survival is achieved with early maturation of enterocytes. This maturational process is genetically programmed but can be disrupted when larvae are fed diets that do not meet their specific needs (Zambonino-Infante and Cahu, 1994a, 2001; 2007). Diets containing only native protein or high protein hydrolysate levels (Zambonino-Infante *et al.*, 1997) may induce a delay in the decrease in cytosolic activities, and consequently in the increase (or onset) in BBM enzyme activities (Zambonino-Infante *et al.*, 2008). While diets containing a moderate amount of protein hydrolysate may induce an earlier maturation of enterocytes, which can resulting in survival and growth performance improvements (Zambonino-Infante and Cahu, 1999).

Several studies suggested that earlier stimulation of the digestive tract through different substrates can promote the development of the intestinal epithelium, improving digestion and absorption processes (Noy and Pinchasov, 1993). Among the nutrients known to promote proliferation of fish enterocytes that are involved in nutrient absorption, is glutamine (Jiang *et al.*, 2009; Pohlenz *et al.*, 2012a).

1.3. Glutamine

Glutamine is an amino acid that exists in two forms: D-glutamine and L-glutamine, an optically active form of glutamine with L-configuration, which is the form found in foods and supplements (National Center for Biotechnology Information).

Glutamine is a neutral glucogenic amino acid, which presents a structure composed by two amino groups, namely the α-amino and the easily-hydrolysable side-chain amide group. These features enable the role played by glutamine as a non-toxic nitrogen vehicle (Darmaun and Humbert, 2000; Hawthorne, 1984; Roth, 2008).

Glutamine metabolism is largely controlled by two intracellular enzymes: glutamine synthetase (GS) and glutaminase (GLS). GS is responsible for triggering the reaction that synthesizes glutamine from ammonium ion (NH_4^+) and glutamate through ATP consumption,

whereas GLS is responsible for glutamine hydrolysis, which converts it into glutamate and NH_4^+ again (Damian and Pitts 1970; Neu *et al.*, 1996; Newsholme *et al.*, 2003a).

Glutamine comprise a large proportion of the body pool of amino acids, both in the free form and incorporated into protein, and its turnover rate exceed that of other amino acids (Wu *et al.*, 2011).

Glutamine is classified as a non-essential (dispensable) amino acid due to the ability of most cells to produce it (Labow *et al.*, 2001; Lacey and Wilmore, 1990). However, glutamine becomes conditionally essential under hyper-catabolic states, such as malnutrition, infection/inflammation and during periods of fast growth, stress or critically illness, when its levels are insufficient to meet the increased body's demands (Lacey and Wilmore, 1990; Lobley *et al.*, 2001; Wu *et al.*, 2011). In such deficiency conditions, glutamine reserves, particularly in the skeletal muscle, are depleted, as a result of the concomitant increase in GLS expression and inhibition of the GS action (Labow *et al.*, 2001; Lobley *et al.*, 2001; Smith, 1990).

It is now known that a large number of body tissues and cells use glutamine at high rates and that glutamine utilization is essential for their function. These tissues and cells include skeletal muscle, kidney, intestine, liver, specific neurons in the central nervous system, cells of the immune system, and pancreatic ß-cells (Curi, 2000; Young and Ajami, 2001). Among these cells and tissues, the liver, since contains both GS and GLS enzymes, is the only one which is capable to switch from net glutamine consumption to net production, depending on physiological and nutritional conditions (Meynial-Denis, 2016).

Glutamine is part of functional amino acids (Wu, 2009). Within the body glutamine plays many roles, including as a provider of carbon for energy, and it not only participates as an important mediator in numerous metabolic pathways but also acts as regulator of certain key physiologic processes including glycogen synthesis, gluconeogenesis and lipolysis (Barbosa *et al.*, 2006; Fu *et al.*, 2005; Rhoads *et al.*, 2006). Therefore, this amino acid plays an important role in maximizing efficency of food utilization, enhancing protein accretion, reducing adiposity, and improving health (Suenaga *et al.*, 2008). In addition, glutamine is able to up-regulate antioxidant genes expression (Wang *et al.*, 2008), prevent oxidant-induced apoptosis (Brasse-Lagnel *et al.*, 2009), and modulate intestinal oxidative status, being precursor of glutathione, the most abundant antioxidant in the intestine (Cheng *et al.*, 2011; Wu *et al.*, 2004).

Glutamine appears to be the most versatile amino acid (Krebs, 1980), essential for many important homeostatic functions and for the optimal functioning of a number of tissues in the body, namely at the level of the intestine (Li *et al.*, 2007; Souba, 1993; Newsholme *et al.*, 2003).

1.4. The important role of glutamine on the intestine

Glutamine is the body's main source of FAA's in fish plasma and muscle (Rowbottom *et al.*, 1996), which is obtained endogenously through the intestinal lumen by protein digestion (Carrascosa *et al.*, 1984). Nevertheless, can be attained through exogenous proteins (i.e., diet). In this case, immediately after the ingestion of the diet, glutamine absorption occurs at the intestinal lumen, through the microvilli presented at the enterocytes. The higher the glutamine concentration in the lumen, the greater its transport through the nitrogen transport system (Souba *et al.*, 1990).

The intestine, as a major tissue of glutamine consumption, are capable of metabolizing large amounts of this amino acid supplied by both the bloodstream and/or diet, to convert it into other amino acids (Burrin *et al.*, 2000; Windmueller and Spaeth, 1980). Indeed, glutamine is the major fuel for rapidly proliferating cells, such as the cells of immune system, intestinal epithelium (enterocytes) and intestinal mucosa, which place huge demands on the body for availability energy and nutrients (Lobley *et al.*, 2001; Wu *et al.*, 2011).

The essential role of glutamine for cell proliferation can be linked to its utilization as a precursor for amino acids, nucleotides, and glutathione. Moreover, during cell proliferation, this FAA serves as an anaplerotic substrate to allow replenishment of Krebs cycle. Indeed, during proliferation, a large part of glutamine is uptaken in the cell mitochondria where it is converted into NH_4^+ and glutamate by GLS. Then, glutamate is converted through a second deamination into α-ketoglutarate, a Krebs cycle intermediate essential for the cell cycle progression during proliferation (Labrou, 2019; Newsholme *et al.*, 1985; Rhoads *et al.*, 1997).

Glutamine is quantitatively the most important donor of nitrogen required for the pyrimidine and purine synthesis, which are crucial for the normal development, maturation and repair of the intestinal mucosa (Newsholme *et al.*, 2003a). Moreover, as a precursor of N-acetyl glucosamine and N-acetyl galactosamine for intestinal mucin synthesis, this amino acid plays a critical role in maintaining intestinal mucosal integrity and function, being extensively catabolized by enterocytes (Curi *et al.*, 2005).

Due to the major role of the intestines in the immune system, glutamine benefit overall immune health by supporting not only the intestinal cells, but also the immune cells (Calder and Yaqoob, 1999; Chassaing *et al.*, 2014).

Over the last few decades, the beneficial effects of dietary glutamine supplementation in improving nutrition status have already been extensively reported in mammals, revealing its unique and complex physiological functions, especially in aspects such as growth promoting

and digestive structure protection (Bartell and Batal, 2007; Souba *et al.*, 1990; Wu *et al.*, 2011). In weaning piglets (Wang *et al.*, 2008) and artificially reared rats (Potsic *et al.*, 2002), dietary glutamine supplementation has a positive effect by preventing intestinal dysfunction and atrophy, and maintaining the intestinal epithelial integrity, respectively. Yi *et al.* (2005) also pointed out that dietary glutamine supplementation affected the growth performance and small intestinal morphology of hatchling broilers, by improving weight gain, feed efficiency and livability.

In recent years, few studies were carried out to assess the role of glutamine in aquatic species and its correlation with physiological functions of the digestive tract. A previous study in juveniles Jian carp (*Cyprinus carpio* var. Jian) demonstrated that the inclusion of 1.2–2.0% glutamine in the diet can improve intestinal weight, fold height and digestive enzyme activity, which resulted in progress on the growth performance (Yan and Qui-Zhou, 2006). Dietary glutamine can also increase microvillus height and enterocyte in the intestine of hybrid striped bass (*Morone chrysops* × *M. saxatilis*) (Cheng *et al.*, 2012). Also, glutamine together with arginine have been found to improve immune responses as well as the intestinal development and feed efficiency of juvenile red drum (*Sciaenops ocellatus*) (Cheng *et al.*, 2011). In channel catfish (*Ictalurus punctatus*), Pohlenz *et al.* (2012a) showed that efficient utilization of free dietary glutamine by intestinal mucosal cells, resulted in enhancement of the enteric microstructure along with increased migration rates of enterocytes and modified plasma amino acids profiles. In addition, Yu *et al.* (2016) pointed out that suitable levels of glutamine in diets can improve intestinal function, villus height and density in the intestine, and significantly increase trypsin and lipase activity in sea cucumber (*Apostichopus japonicus*), as well as it antioxidant capacity by improving the activity of catalase. Recently, Qu *et al.* (2019) showed that diets containing glutamine can improve growth performance and increase lipase and trypsin activity, mucosal thickness and the number of lymphocytes in juvenile grass carp (*Ctenopharyngodon idella*). Similarly, Liu *et al.* (2015) reported that dietary glutamine supplementation could improve survival and growth performance by maximizing the activities of digestive enzymes in half-smooth tongue sole post-larvae (*Cynoglossus semilaevis*). These results provide support for the use of glutamine in improving intestinal structure and function in aquatic animals. Nevertheless, concerning the physiological function, as growth promoting and digestive structure protection, dietary glutamine supplementation still almost scarcely reported in marine fish larvae.

Compared with juvenile and adult individuals, fish larval phase is a critical stage when larvae need to fulfill the development of organ systems and further maturation of tissues and physiological functions (Mazurais *et al.*, 2011). The immature digestive system of larvae and its inadequate digestion/absorption of exogenous nutrients could be a main cause for high mortality rates during weaning periods in marine larvicultura (Kjørsvik *et al.*, 2011; Liu *et al.*, 2015).

The major challenge to the digestive tract is the high demands for glutamine during the early stages, when the intestinal mucosa suffers a reduction (villous atrophy), resulting from increased proteolysis of the skeletal muscle and subsequent translocation of glutamine to other organs. Nevertheless, in those critical situations, the addition of this amino acid with trophic action on the intestinal mucosa can be beneficial (Smith and Wilmore, 1990), as it improves the physiological processes of digestion and absorption of nutrients in the diet (Zavarize *et al.*, 2010); showing progresses on the productive performance.

2. THESIS AIMS AND HYPOTHESES

Senegalese sole, one of the most important commercial flatfish in Southern European aquaculture, is consistently high-priced in recent years due to its high nutritive value and advantageous productive characteristics. However, Senegalese sole production has been often hampered by variable growth rate and size dispersion during metamorphosis, and mainly by the high mortality at weaning, linked to the later maturation of the digestive tract. In this sense, this thesis, integrated in the DIVERSIAQUAII project, was conducted to test the hypothesis that supplementation with dietary L-glutamine in *Solea senegalensis* larvae may promote an earlier maturation of digestive tract. It should provide insights to understand of how growth performance, survival rate, enterocyte maturation and enzyme activities are affected by the dietary L-glutamine supplementation. It is expected that results should lead to improve juveniles production in quantity at a high quality.

Specific objectives:

- Define an adequate dose of L-glutamine to enrich live feed;
- Evaluate the growth performance, survival rate and metamorphosis progress during the larval period;
- Analyze the digestive physiology through the development of the enterocytes and enzyme activities of the intestinal epithelium.

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3. MATERIALS AND METHODS

3.1. Biological material and larval rearing

S. senegalensis eggs were obtained by natural spawning from a broodstock adapted to captivity at the aquaculture research station (EPPO) of IPMA – Instituto Português do Mar e da Atmosfera, Portugal (37°02′ N, 7°49′ W).

After hatching (day 0) larvae were reared in 1500 L circular tank, where were kept under controlled light and temperature conditions until 11 DAH.

After mouth opening (2 DAH) sole larvae were fed rotifers – *Brachionus* sp. – at a starting density of 5 prey ml^{-1}, which was gradually increased until 7 DAH. A second type of live food, *Artemia* sp. nauplii, was introduced at 3 DAH and its proportion in the diet was gradually increased, becoming the only prey offered to larvae from 7 DAH onwards. *Artemia* sp. metanauplii (EG strain INVE, Aquaculture) were provided to the larvae after 12 DAH, at a starting density of 8 prey ml^{-1}. Between the 13 and 15 DAH live *Artemia* sp. metanauplii were gradually changed to frozen *Artemia* sp. metanauplii, allowing an easier catch to the benthic larvae. From 16 DAH onwards sole larvae were fed frozen *Artemia* sp. metanauplii exclusively (**Figure 1**).

During the experimental trial, fish larvae were fed manually and daily *ad libitum* in five meals – 10:00, 12:00, 14:00, 16:00 and 17:00 –, based on predicted maximum growth. Daily adjustments were made based on visual inspection to avoid large excess of uneaten preys.

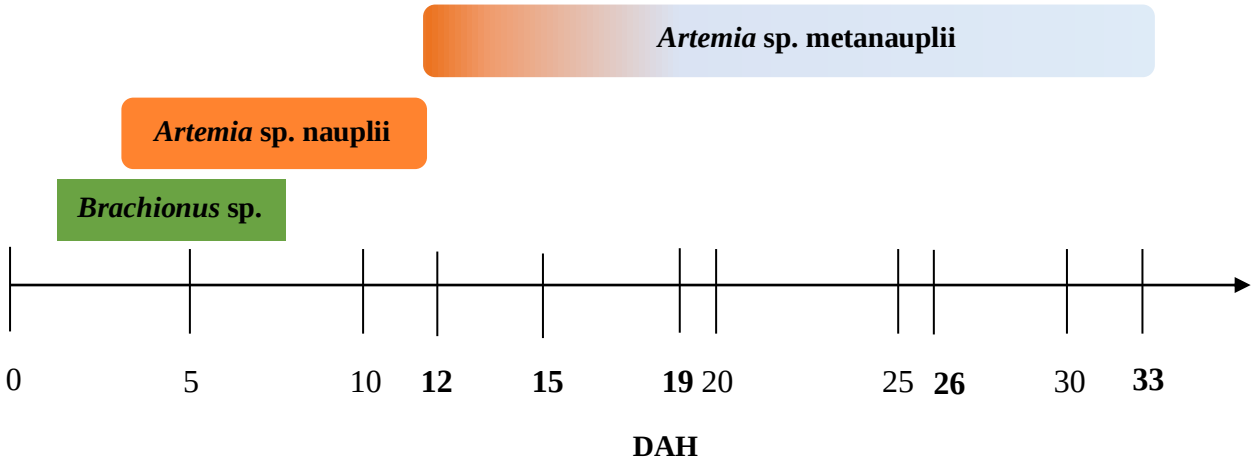

Figure 1 – Schematic plan of the feeding regime for both experimental groups. The feeding regime includes *Brachionus* sp. (green), *Artemia* sp. nauplii and metanauplii, in live (orange) and frozen (light blue) form. Sampling procedure was carried out at five different ages: 12, 15, 19, 26 and 33 DAH (bolt).

At 12 DAH, sole larvae (13 140 individuals) were transferred and randomly distributed by the six flat-bottom tanks (120 cm long, 70 cm width and 15 cm water column) in a seawater circulating system (**Figure 2A** and **B**), at a density of 0.26 individuals cm^{-2}.

Experimental period started from 12 DAH onwards until 33 DAH. At 19 DAH density was reduced to 0.14 individuals cm^{-2}, since its around 18 to 20 DAH that the benthic lifestyle is acquired (Ribeiro *et al.*, 1999b).

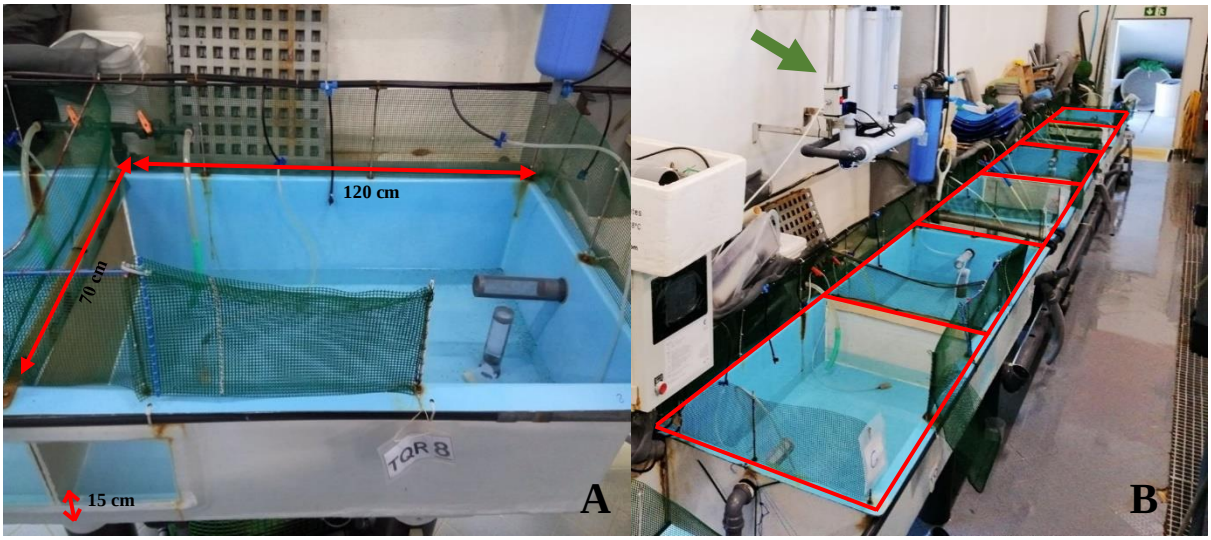

Figure 2 – Schematic of experimental assay composed by six blue flat-bottom fiberglass tanks (120 cm side, 70 cm depth and 15 cm water column) equipped with outlet filters – surface and depth filter –, airstones, surface skimmers and tubing (**A**). Each tank connected to a seawater circulating system composed by a mechanical filter backwashing, a tank flushing, and a UV sterilizer (indicated by the green arrow) (**B**).

During the whole experiment, tanks were routinely cleaned in the morning before feeding, dead larvae were removed and counted for survival rate determination, and water temperature, dissolved oxygen and salinity were monitored. Water temperature was ranged between 18.4 to 23 ºC, dissolved oxygen remained at 6.8 ± 0.72 mg l^{-1} and salinity ranged from 35 to 37 g l^{-1}. Fish larvae were reared under natural photoperiod corresponding to ca. 12 hours lightness to 12 hours darkness cycle.

Until 14 DAH the water renewal was 40%, afterwards the water exchange rate was gradually increased reaching 80% by the end of the experiment, which allowed the self-cleaning of the cultivation unit.

To avoid losses of live prey with outlet flow, outlet was protected with 150 µm mesh filter during the day, which was replaced by 500 µm mesh filter during the night.

3.2. Experimental design

In order to study the effect of L-glutamine supplementation on the growth performance, survival rate, metamorphosis and digestive physiology during the early life stage of *Senegalese sole*, two experimental groups were established: Control group (CTL), where sole larvae were fed *Artemia* sp. metanauplii enriched with commercial emulsion – Red Pepper (BERNAQUA) –, according to the standard enrichment established by EPPO; and L-glutamine group (GLN), where *Artemia* sp. metanauplii offered to sole larvae was supplemented with L-glutamine (powder) after the standard enrichment.

Each experimental group was carried out in triplicate (n=3) and was carried out between 12 and 33 DAH.

3.2.1. Live feed production and enrichment

Brine shrimp *Artemia* sp. cysts originating from Great Salt Lake, USA, were incubated for 24 hours in 100 L cylindro-conical tanks, under standard conditions (Van Stappen, 1996). The newly hatched *Artemia* sp. nauplii (Instar I) were rinsed with clean seawater and passed through a SEP-Art magnetic device designed to remove unhatched cysts and empty shells, and then collected on a 150 µm sieve. For *Artemia* sp. metanauplii (Instar II) enrichment, newly hatched nauplii were stocked (200 nauplii ml^{-1}) in a single cylindro-conical 200 L container with seawater at 28 °C, with strong aeration. After reach their first feeding stage (instar II) (Van Stappen, 1996), *Artemia* sp. metanauplii were enriched with Red Pepper (BERNAQUA) (0.4 g l^{-1}; INVE Technologies; 200 metanauplii ml^{-1}; salinity 35 g l^{-1} and temperature 28 °C) during 12 hours. This procedure – bioencapsulation – was necessary to increase the nutritive value of the *Artemia* sp., to give to the sole larvae an optimal quality feed.

3.2.2. Glutamine enrichment – Experimental group

Following Red Pepper enrichment, part of the *Artemia* sp. metanauplii was enriched for 1 hour longer with a mix previously prepared by combining L-glutamine (powder), obtained from Sparos, Lda., and Red Pepper (emulsion), at a concentration of 0.5 g l^{-1} and 0.08 g l^{-1}, respectively (200 metanauplii ml^{-1}; salinity 35 g l^{-1}; temperature 28 ºC) (**Figures 3A, B** and **C**).

After enrichment, *Artemia* sp. metanauplii were collected on a 150 µm mesh filter and resuspended in clean seawater (at 28 ºC) (**Fig. 3D**).

Artemia sp. metanauplii to be offered to both experimental groups were harvested, washed on 150 µm mesh with sterilized seawater, and disinfected with hydrogen peroxide at

100 ppm. Then, *Artemia* sp. metanauplii were kept at low temperatures (at 4 ºC) to maintain their nutritional value by delaying their metabolic rates.

In the beginning of the experiment, harvested *Artemia* sp. metanauplii were washed in freshwater when still alive, counted, and frozen in a - 20 ºC freezer. From 13 DAH onwards, *Artemia* sp. metanauplii previously enriched and frozen were offered to allow an easier to catch by benthic sole larvae.

Artemia sp. metanauplii from both experimental groups were collected to assess L-glutamine enrichment efficacy. Thus, samples were washed several times with distilled water, and then analysed according to Aragão *et al.* (2004) for total FAA content focusing on the glutamine.

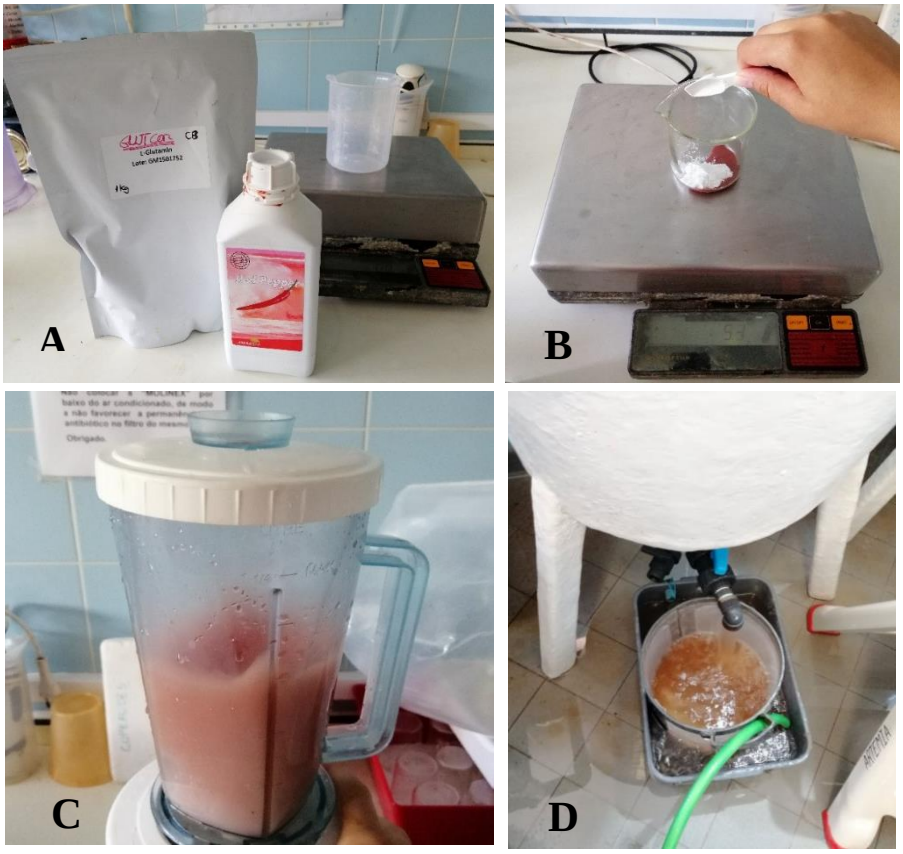

Figure 3 – *Artemia* sp. metanauplii enrichment preparation by combining L-glutamine (powder) and Red Pepper (emulsion) (**A**) at different concentrations (0.5 g L^{-1} and 0.08 g l^{-1}, respectively) using a balance (**B**) to weigh it and a blending (**C**) to mix it. Following the enrichment period (1 hour at 28 ºC; 200 metanauplii ml^{-1}; salinity 35 g l^{-1}), *Artemia* sp. were collected on a 150 µm mesh and resuspended in clean seawater (**D**).

3.3. Sampling

At the beginning of the experimental period (12 DAH), concurrent to the beginning of the metamorphosis, a total of 225 Senegalese sole larvae were sampled for biometric (n=30), histological (n=15) and enzymatic (n=180) analysis. This was performed immediately after fish distribution by the tanks, to evaluate the initial condition of sole larvae.

During the experimental period, Senegalese sole larvae from both experimental groups were sampled regularly. At 15, 19, 26 and 33 DAH pooled samples of larvae were collected for biometric (n=45, per experimental group) and enzymatic analysis (n=180, per each experimental group, except on day 33, where n=120). For histological assays, larvae were sampled at 19 and 33 DAH (n=9, per each experimental group).

In all samplings, larvae were harvested randomly to a mesh sieve. All samplings were performed in the morning before feeding distribution in order to have no influence on the weight, besides minimize the potential effect of exogenous enzymes from undigested prey in the digestive tract of the Senegalese sole larvae (Kolkovski, 2001).

3.4. Analytical methods

3.4.1. Growth performance and survival

Senegalese sole was measured using a micrometric magnifying glass Zeiss Stemi 200-C (**Figure 4A**) with transmitted light. During this process, each larva was photographed using a Cannon PowerShot GS 5.0 MP (**Figure 4B**) incorporated into the micrometric magnifying glass. Length was measured from the tip of the snout to the end of the caudal fin of the individuals – total length (TL) –, as shown in **Figure 4C**. Afterwards, sole larvae were then careful rinsed with distilled water to remove any remaining salt, and immediately frozen in liquid nitrogen, for posterior freeze-dry (at 60 ºC for 48 hours) and consequent determination of dry weight (DW). Senegalese sole larvae were divided in three pools of 5 individuals with similar sizes and weighted in an analytic microbalance with a precision of 1 µg (Sartorius M5P) (**Figure 4D**).

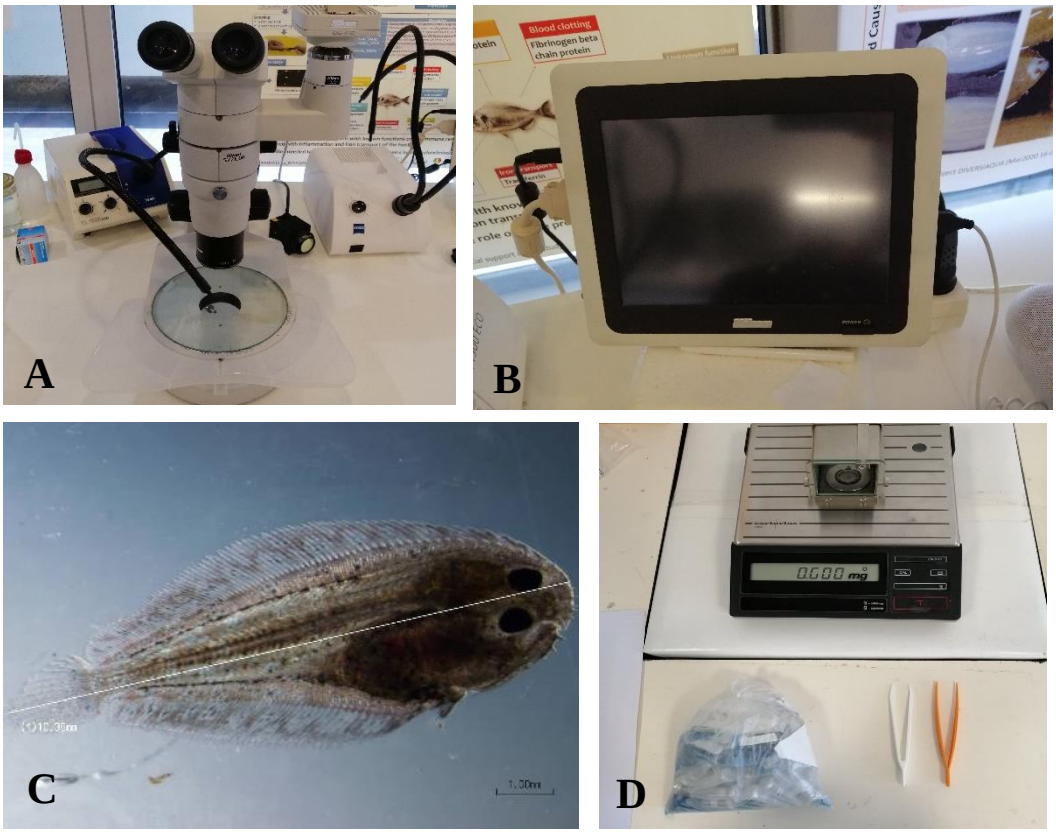

Figure 4 – Performing of the Senegalese sole measurements using a micrometric magnifying glass Zeiss Stemi 200-C with transmitted light (**A**), and a Cannon PowerShot GS 5.0 MP (**B**) to get an image to determine the TL (**C**). Dry sole larvae were weighted in an analytic microbalance with a precision of 1 µg (Sartorius M5P) (**D**).

The data obtained from periodical weight sampling and daily counts of dead individuals, were used to determine the relative growth rate (RGR) and survival rate (S), respectively.

RGR was calculated according to Ricker (1958):

$$\text{RGR (\% day}^{-1}) = \left(e^{\frac{\ln DWf - ln\ DWi}{tf - ti}} - 1 \right) * 100,$$

where DW_f and DW_i represent final and initial DW (in mg), respectively, and t_f and t_i the rearing period (in days), respectively.

Survival rate was calculated as the ratio of fish larvae surviving until the end of the experiment over the number of fish larvae present in the beginning, during the experimental period. Senegalese sole larvae used for analysis were also considered alive.

$$\text{S (\%)} = \frac{\text{Total no. of fish larvae alive until the end}}{\text{Total no. of fish larvae}} * 100$$

3.4.2. Metamorphosis

Metamorphosis was evaluated based on the main external morphological features of the Senegalese sole larvae, applying the metamorphosis degrees described by Padrós *et al.* (2011) – 3a, 3b, 3c, 4c and 5 – (**Appendix I**), together with the left eye migration index described by Fernández-Díaz *et al.* (2001) (**Appendix II**). Metamorphosis was assessed individually based on fish images used for TL determination.

3.4.3. Histology

Senegalese sole sampled for histology were euthanized using 2–phenoxyethanol (4.17 ml l^{-1}) and fixed overnight in 10% neutral buffered formalin. Afterwards, sole larvae were washed three times in running tap water before they were preserved in 70% ethanol, at 4 ºC for posterior analysis.

Following fixation, biological material was dehydrated in a graded series of ethanol (70, 85 and 100%) before being embedded in resin previously prepared according to the Technovit 7100 resin kit (**Appendix III**), allowing the blocks to be polymerized for two days at room temperature. Afterwards, polymerized resin blocks were mounted on wooden blocks (4 cm x 2.8 cm x 0.7 cm) using Historesin Mounting Media (Leica Biosystems Nussloch GmbH) glue. Then, tissue sections of 5–7 µm thickness were prepared using a rotation microtome (Microm Leizt® HM340E) and a circular water-bath (Electrothermal®) set at about 40 ºC to stretch the cuts and improve adherence to the microscopy slide.

After tissue sectioning, slides were stained with Hematoxylin–Eosin (H–E) according to the embedding process presented in **Table 1**.

Table 1- Staining procedure – Hematoxylin-eosin method – displayed as steps of immersion in different reagents and corresponding times, according to Neuromuscular Lab (2015) with modifications.

Steps	Reagent	Time (minutes:seconds)
1	Hematoxylin (Ehrlich)	10
2	Clean water	1
3	Clean water	1
4	Acid water (120mM HCl)	:30
5	Scott's Tap water (pH 7.7)	3
6	Eosin	3
7	Clean water	2
8	Clean water	2
9	Ethanol (95%)	1

Finally, once dried at room temperature, all stained preparations were mounted permanently using DPX for posterior analysis under light microscope equipped with a digital camera (Nikon D5-Fi2, Japan). Larval sections were used to measure the enterocyte height at the intestinal epithelium, through the images which were taken at 4, 10 and 40x magnification and analysed using a digital image analysis software (ImageJ 1.49v, National Institutes of Health, USA; http://imagej.nih.gov/ij).

3.4.4. Enzymatic analysis

Pooled samples of larvae were collected, washed in distilled water to remove any remaining salt, and frozen in liquid nitrogen (- 196 ºC) to prevent protein autolysis. Then, Senegalese sole larvae were stored at - 80 ºC until analysis.

Fish larvae dissection – preparation of extracts

All procedure was carried out under a dissecting stereo microscope on a petri dish maintained on ice (at 0 ºC) (**Figure 5A**) to preserve enzymes activity, as described previously by Ribeiro *et al.* (1999b) for Senegalese sole. Whole body was used for enzymatic analysis in larvae younger than 20 DAH – 12, 15 and 19 DAH – explained by the small size of the individuals. After this date, in older larvae – 26 and 33 DAH –, the head and the caudal part were cut according to the dissection cut lines showed in **Figure 5B**, leaving the abdomen to prepare the crude extract, as described by Guerreiro (2008) (**Figure 5C**). Dissected samples were frozen at - 80 °C pending analysis.

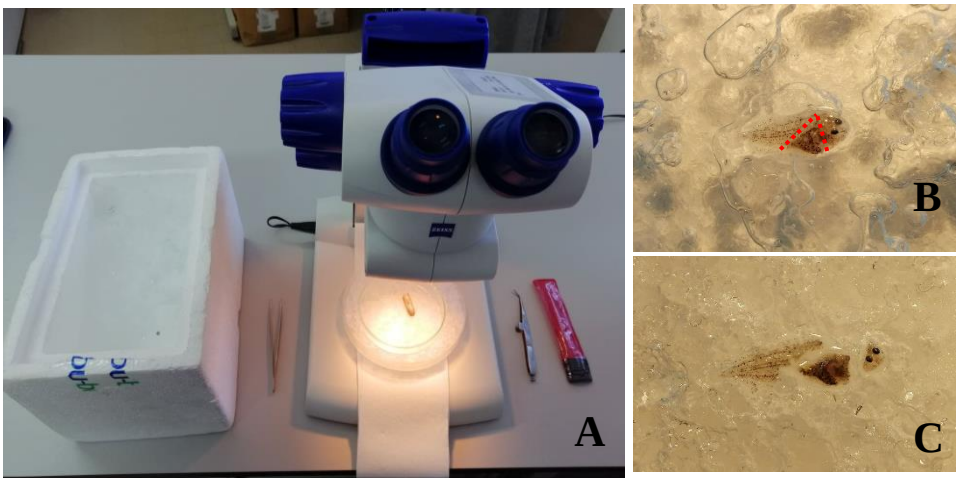

Figure 5 – Steps of Senegalese sole abdominal dissection carried out under a stereo microscope on a petri dish maintained on ice (**A**). Two almost perpendicular clean cuts (dashed red lines) on the ocular side of the sole were performed (**B**), leaving the abdomen to prepare the crude extract (**C**) (Lazo *et al.*, 2007).

Both whole larval body and abdominal cavities obtained by dissection were weighted in a balance [Precisa 100A-300M (1 ± 0.00001 g)] to calculate the homogenization volume.

All the enzymatic assays were performed by testing enzyme activities of tissue homogenates against dissolved substrates whereby reaction products or the disappearance of substrates were measured photometrically.

Pooled samples for each replicate were homogenized for periods of 2 x 30 seconds using an electric tissue homogenizer (Ultra-Turrax) in 30 volumes (v/w) of ice-cold Tris-Manitol and Tris-HCl at pH 7.0. All samples were kept in ice during the process described above to avoid enzymes denaturation and/or damage. From this homogenate 2 ml aliquot was collected for BB purification. Homogenate volume (1 ml) was centrifuged at 3300 x *g* for 15 seconds at 4 ºC and the supernatant extract was collected and stored at - 20 ºC pending enzymatic activity and protein quantification. BB purification was performed as described by Guerreiro *et al.* (2010). Briefly, once the samples have been homogenized as descried above, tissue homogenates were centrifuged at 9000 x *g* for 10 minutes after the addition of a certain volume of $CaCl_2$ significant in membrane stability. The supernatants were transferred to new vials and submitted to a new centrifugation at 26000 x *g* (at 4 ºC) for 40 seconds. The supernatant was discarded and the precipitated resuspended with DL-Dithiothreitol (DDT) (Guerreiro, 2008). After all, the extracts for enzyme assays were stored frozen (- 80 ºC) until enzymatic activity and protein quantification.

3.4.4.1. Enzymatic assays

The enzymes assayed were: 1) acid protease (pepsin-like) produced by the gastric glands at the stomach; 2) pancreatic enzymes – trypsin and amylase; 3) intestinal enzymes (aminopeptidase and alkaline phosphatase), and those enzymes presented at the cytosol (acid phosphatase) and BBM (aminopeptidase and alkaline phosphatase) of the enterocytes. Besides these enzymes, protein content was determined.

Pepsin-like – acid protease

Pepsin-like activity quantification was conducted according to the method described by Anson (1938) using 2.5% hemoglobin (pH 2.0 adjusted by HCl) as substrate. In the reaction tube 500 µl of substrate was incubated at 37 ºC for 5 minutes. Afterwards 100 µl of homogenate was added and incubated for 20 minutes. After this period, 500 µl of 5% trichloroacetic acid (TCA) was added to stop the reaction before another incubation period of 5 minutes. In the blank tubes, TCA was added to the substrate prior to the addition of the homogenate.

The precipitates were centrifuged for 10 minutes at 16000 x *g* (at 4 ºC) after a period of 15 minutes (at 4 ºC) and the optical density of the test tubes supernatant was read immediately at 280 nm against the blank. Adequate blanks were run for each assay to exclude interactions from other enzymes in the crude extract and to exclude background reaction due to dissociation of chromogenic substrates.

Trypsin

Trypsin activity was assayed according to the method described by Holm *et al.* (1988) using BAPNA (Nα-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride) as substrate in Tris-$CaCl_2.{}_2H_2O$ buffer, pH 8.2. All processes were performed at 25 ºC. The reaction was read in a spectrophotometer during 2 minutes at 407 nm.

Amylase

Amylase activity was measured using starch as the substrate dissolved in phosphate buffer ($NaH_2PO_4 \cdot {}_2H_2O$) at pH 7.4 (Métais and Bieth, 1968). In a reaction tube 100 µl of starch solution and 50 µl of the homogenate were incubated at 37 ºC for 30 minutes. At the end of this period 20 µl of HCl was added to stop the reaction. Finally, to the reaction tube were added 400 µl of deionized water and 2 ml of iodine solution. Blanks were similarly prepared except that the 50 µl of the homogenate was added only after the reaction was stopped with HCl. The color developed in these tubes was read against the iodine solution at 580 nm. Adequate blanks were run for the same purpose of pepsin-like assay.

Aminopeptidase

The activity of aminopeptidase was determined according to the method described by Maroux *et al.* (1973) using L-leucine *p* – nitroanilide as substrate in DMSO and phosphate buffer at pH 8.0. All processes were performed at 37 ºC. The reaction was read in a spectrophotometer during 4 minutes at intervals of 15 seconds at 405 nm.

Alkaline phosphatase

Alkaline phosphatase activity was determined according to the method described by Bessey *et al.* (1946) using *para*-nitrophenylphosphate (pNPP) as substrate in $NaCO_3$ buffer at pH 9.8. All processes were performed at 37 ºC. The reaction was read in a spectrophotometer during 4 minutes at intervals of 15 seconds at 407 nm.

Acid Phosphatase

According to Terra *et al.* (1979), acid phosphatase activity was determined using pNPP as substrate made in a solution of citrate buffer (citric acid and sodium citrate), pH 4.8. All processes were performed at 37 ºC. The reaction was read in a spectrophotometer during 4 minutes at intervals of 15 seconds at 407 nm.

Protein analysis

Protein quantification of the extracts were made according to the method described by Bradford (1976) using bovine serum albumin (BSA) as standard to measure total soluble protein in enzyme crude extracts. After preparing the calibration straight with water and stock solution (BSA stock and deionized water), a variable amount of homogenate was mixed with the Bradford solution. The reaction was run in triplicate and read in a spectrophotometer for 2 minutes at 595 nm.

Enzyme activity expression

Enzyme activities were calculated as μmoles of substrate hydrolysed per minute (i.e., U). Amylase activity was expressed as the equivalent enzyme activity which was required to hydrolyse 1 mg of starch in 30 minutes. Pepsin-like activity was expressed as 1 U representing 1 mM equivalent of tyrosine liberated per minute per mg of protein.

Enzyme activities were expressed as specific activities, i.e., U/mg protein or mU/mg protein, and as segmental activities, i.e., total activity per larva (U/mg larva or mU/mg larva). For the calculation of these values, the extinction coefficients show in the **Appendix IV** were used.

Analytical assays were carried out in duplicates for each collected sample and all spectrophotometric analysis were performed with a Jenway 6300 UV-visible spectrophotometer using quartz cuvettes.

Intestinal maturation index (IMI)

As Zambonino-Infante *et al.* (1997), several authors have determined the intestinal maturation index (IMI) using leucine-alanine peptidase as the cytosolic enzyme. However, in the present study, acid phosphatase, a lysosomal enzyme present in the cytoplasm of the enterocytes, was used instead of leucine-alanine peptidase, once both these enzymes present an equivalent role. In this sense, the IMI was set as the ratio between BBM enzyme total activity (aminopeptidase and alkaline phosphatase) and cytosolic enzyme total activity (acid phosphatase).

3.5. Statistical analysis

All data of TL, DW, RGR, S, enterocyte height, specific and total activity of enzymes, as well as the two IMIs, were expressed as mean ± standard deviation of treatment replicates.

Assumptions for normality and homogeneity of variance were tested when needed with Kolmogorov-Smirnov test and Levene's test, respectively. The effect of dietary L-glutamine supplementation was assessed by comparing data between the two experimental groups with the parametric Student t-test for TL, DW, RGR, enterocyte height, specific and total activity of enzymes, and IMIs at the different development stages. Larval growth within each experimental group was analyzed through One Way ANOVA along experimental period. When differences were detected, Fisher's Least Significant Difference (LSD) was used as post hoc test.

The statistical analysis of metamorphic stages of Senegalese sole was performed using Chi-square test, by comparing differences between the two experimental groups at the same age.

For all statistical analysis, the significance level was set at $p<0.05$. All analysis were carried out by the statistical software SPSS (version 21.0), and Excel were used to make graphs.

4. RESULTS

***Artemia* sp. enrichment with L-glutamine**

Protocol to supplement *Artemia* sp. with L-glutamine was successfully established at EPPO, since the results obtained showed that glutamine content was two times higher in *Artemia* sp. supplemented with this amino acid (2.2 ± 0.06 mg L-glutamine/g *Artemia* sp. DW) when compared to *Artemia* sp. enriched according to the standard protocol deprived of L-glutamine supplementation (1.0 ± 0.23 mg L-glutamine/g *Artemia* sp. DW).

4.1. Growth performance and survival

The TL of Senegalese sole post-larvae showed a growing trend in both experimental groups – CTL and GLN. During all the trial, Senegalese sole post-larvae from GLN treatment were slightly longer ($p<0.05$), when compared to those from CTL group. Differences between dietary treatments were higher at 26 DAH, followed by 33 DAH and lower at 19 and 15 DAH (**Figure 6**).

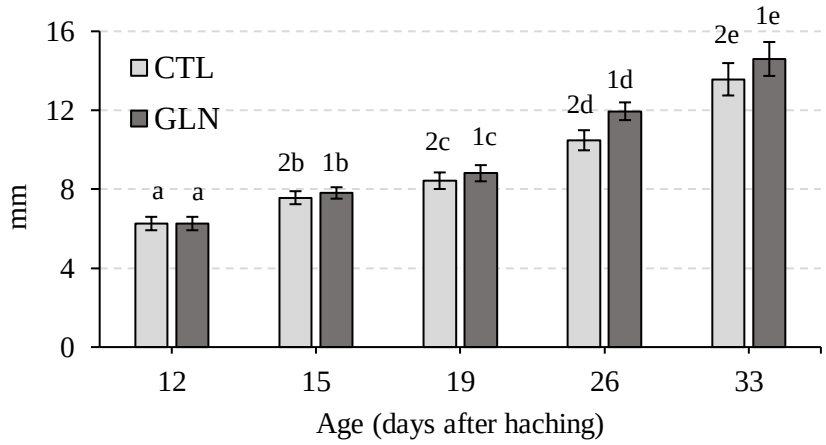

Figure 6 – Total length during larval development of Senegalese sole reared under two distinct feeding regimes: fed on *Artemia* sp. (CTL) and *Artemia* sp. supplemented with L-glutamine (GLN). Mean ± SD, n=3, per each experimental group. Different superscript numbers indicate significant differences ($p<0.05$) between treatments, and different letters indicate significant differences ($p<0.05$) between ages within each treatment.

DW of Senegalese sole exhibited a growing trend over the experiment in both experimental groups – CTL and GLN: a slow increase from 12 to 19 DAH, followed by a rapidly increase from 19 DAH onwards. (**Figure 7**). Similar to TL, DW of Senegalese sole post-larvae from GLN treatment were slightly longer ($p<0.05$), when compared to those from CTL group; except on day 15 after hatching, when no significant differences ($p>0.05$) were observed between treatments. The differences between experimental groups became more evident after 26 DAH, followed by the day 33 (DAH) and lower at 19 DAH.

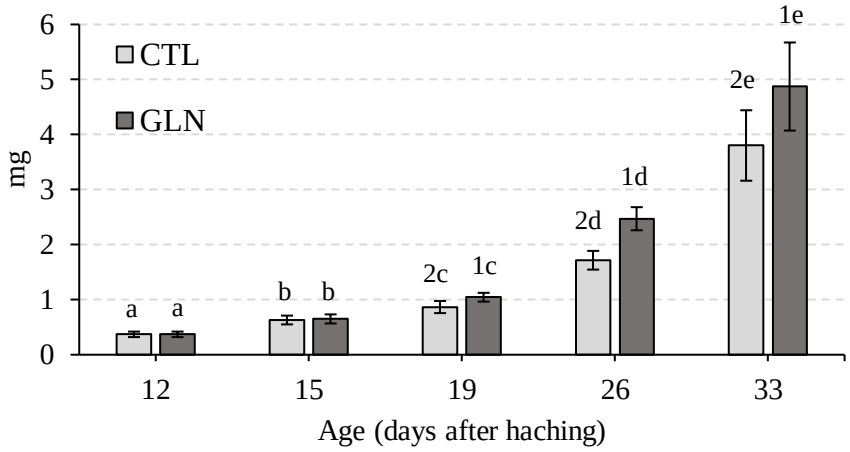

Figure 7 – Dry weight during larval development of Senegalese sole reared under two distinct feeding regimes: fed on *Artemia* sp. (CTL) and *Artemia* sp. supplemented with L-glutamine (GLN). Mean ± SD, n=3, per each experimental group. Different superscript numbers indicate significant differences ($p<0.05$) between treatments, and different letters indicate significant differences ($p<0.05$) between ages within each treatment.

During the experimental period, RGR of Senegalese sole post-larvae exhibited a strong decline from 15 to 19 DAH, more evident in CTL group. Then, RGR showed a slight decrease until 26 DAH in both experimental groups, followed by a slight increase in CTL group, whereas GLN treatment decreased until 33 DAH. Until 19 DAH, there were no significant differences ($p>0.05$) between RGR from experimental groups. However, from 26 DAH onwards, sole post-larvae from GLN group exhibited higher RGR ($p<0.05$) than those from CTL group. Overall, no significant differences were observed in the S ($p>0.05$) between sole post-larvae fed with standard *Artemia* sp. (CTL) and *Artemia* sp. supplemented with L-glutamine (GLN) (**Table 2**).

Table 2 – Relative growth rate (RGR) and survival rate (S) of Senegalese sole post-larvae in both experimental groups – CTL and GLN.

	DAH	Experimental groups	
		CTL	GLN
RGR (% day^{-1})	12		
	15	^{a}19.3 ± 3.65	^{a} 20.79 ± 5.25
	19	^{b}12.86 ± 1.88	^{ab}15.94 ± 1.04
	26	^{2b}11.58 ± 0.34	^{1b}14.51 ± 0.66
	33	^{2b}11.73 ± 0.30	^{1b}13.05 ± 0.43
S (%)	12		
	15		
	19	98.37 ± 0.79	98.81 ± 0.50
	26		
	33	98.86 ± 0.08	99.00 ± 0.47

Data are expressed as mean ± standard deviation (mean ± SD); n=3, per each experimental group.
Different superscript numbers indicate significant differences ($p<0.05$) between treatments, and different letters indicate significant differences ($p<0.05$) between different ages within each treatment.

4.2. Metamorphosis

Results on metamorphosis progress, which were determined using the main external morphological features, are depicted in **Figure 8**. In the beginning of the experiment, at 12 DAH, 56.70% and 43.30% of Senegalese sole from both experimental groups were at the stages 3a and 3b, respectively. At 15 DAH, the majority (95.60%) of sole from CTL was at stage 3c, while a minority (4.40%) was still at the prior stage. Similar relative frequencies were observed in sole age 15 DAH from GLN group, which registered a minority (4.40%) at stage 3b and the majority (93.30%) at the stage 3c, but a small percentage (2.20%) was already at the next stage (4c). After four days, at 19 DAH, Senegalese sole post-larvae from both groups were at the stages 3c and 4c, even though at different proportions: CTL group registered 8.90% of individuals at the stage 3c, and the majority (91.10%) at the stage 4c, whereas GLN treatment exhibited lower (2.20%) and higher (97.80%) percentages of individuals at the stages 3c and 4c, respectively. Metamorphosis success (100% of larvae in stage 5) was achieved in both experimental groups at the same age – 26 DAH. During the studied period, there was no significant differences ($p>0.05$) in metamorphic stages between dietary treatments.

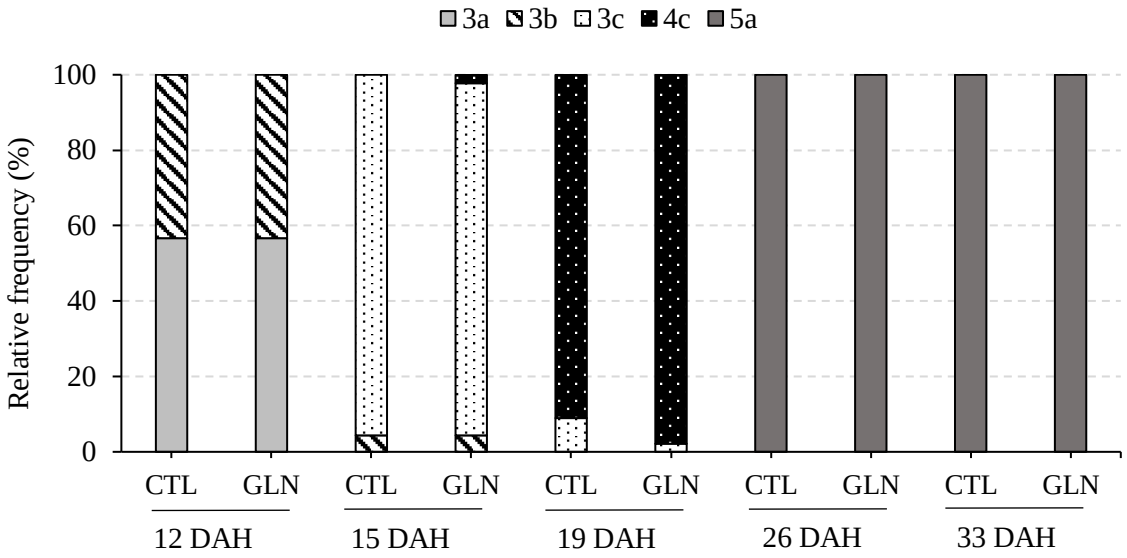

Figure 8 – Relative frequency (%) representation of metamorphic stages during larval development of Senegalese sole reared under two distinct feeding regimes: fed on *Artemia* sp. (CTL) and *Artemia* sp. supplemented with L-glutamine (GLN). Classification of the 3a, 3b, 3c, 4c and 5 stages was carried according to Padrós *et al.* (2011) and Fernández-Díaz *et al.* (2001). n=210, per each experimental group.

4.3. Histology

The enterocyte height measured at the intestinal epithelium of Senegalese sole showed a growing trend along the larval development in both CTL and GLN treatments (**Figure 9**). During all the trial, enterocyte height in Senegalese sole post-larvae from GLN treatment were higher ($p<0.05$), when compared to those from CTL group.

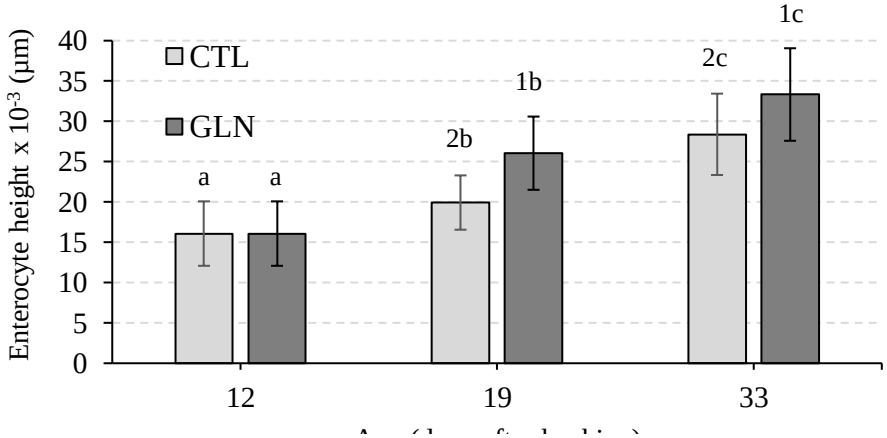

Figure 9 – Enterocyte height at the intestinal epithelium during larval development of Senegalese sole reared under two distinct feeding regimes: fed on *Artemia* sp. (CTL) and *Artemia* sp. supplemented with L-glutamine (GLN). Mean ± SD, n=3, per each experimental group. Different superscript numbers indicate significant differences ($p<0.05$) between treatments, and different letters indicate significant differences ($p<0.05$) between ages within each treatment.

4.4. Enzymatic analysis

Specific activity

Specific activity of pepsin-like assayed along the larval development of Senegalese sole post-larvae differed between experimental groups. CTL group registered an increase from 12 to 15 DAH, followed by a decrease at 19 DAH; then, by the day 26 (DAH), mean values obtained were reached the same level obtained on day 15 (DAH), increasing until the end of the experiment. Senegalese sole post-larvae from GLN group displayed a constant level of pepsin-like specific activity from 12 to 15 DAH, followed by an increase until 26 DAH, registering a decrease by the end of the trial. Significant differences were observed at 19 DAH, when the GLN group showed higher pepsin-like specific activity, and by the end of the studied period, when the CTL group stood out by its higher mean values of specific activity of this acid protease ($p>0.05$) (**Figure 10**).

Trypsin specific activity displayed a strong increase in both CTL and GLN groups from the beginning of the experiment (12 DAH) until 19 DAH; a four-fold increase was observed during this period. This activity decreased slightly between 19 and 26 DAH in CTL group as well as in GLN treatment, followed by an increase until 33 DAH, displaying almost the same values as at 19 DAH. Along the larval development, Senegalese sole from GLN treatment exhibited lower mean values of trypsin specific activity than those from CTL group, but no significant differences ($p>0.05$) were observed (**Figure 10**).

A high level of amylase specific activity was observed at 12 DAH in both treatments. On day 15 (DAH), the specific activity of this pancreatic enzyme exhibited a sharp decrease in both groups, remaining at the same level until the end of the experiment, two times lower than the first sampling point. Along the rearing period, amylase specific activity was very similar between treatments ($p>0.05$), except at 26 DAH, when Senegalese sole post-larvae from the CTL group exhibited higher values ($p<0.05$) in comparison to the post-larvae fed on *Artemia* sp. supplemented with L-glutamine (**Figure 10**).

The evolution of aminopeptidase activity from both treatments showed a profile marked by some variations along the larval development: a decrease from the beginning of the experiment until 15 DAH, remaining almost constant for four days in CTL group, whereas GLN treatment registered a slight increase; then both groups registered a strong increase at 26 DAH, followed by a slight decrease until the end. No significant differences ($p>0.05$) were observed along the studied period (**Figure 10**).

Specific activity of alkaline phosphatase remained at a constant level until 15 DAH, then showed a strong increase between 15 and 26 DAH, followed by a slight decrease at 33 DAH. During all the rearing period, Senegalese sole post-larvae from CTL and GLN groups presented similar levels of alkaline phosphatase specific activity. This was statistically corroborated since no significant differences ($p>0.05$) were recorded between treatments (**Figure 10**).

Specific activity of acid phosphatase assayed in both treatments remained relatively constant between 12 and 15 DAH, followed by a slight increase until the day 19 (DAH), more evident in GLN group. Then, between 19 and 26 DAH, acid phosphatase specific activity exhibited a strong increase in both groups, followed by a slight increase in CTL group, while GLN registered a decrease until 33 DAH. No significant differences ($p>0.05$) were observed for the specific activity of acid phosphatase between both experimental groups (**Figure 10**).

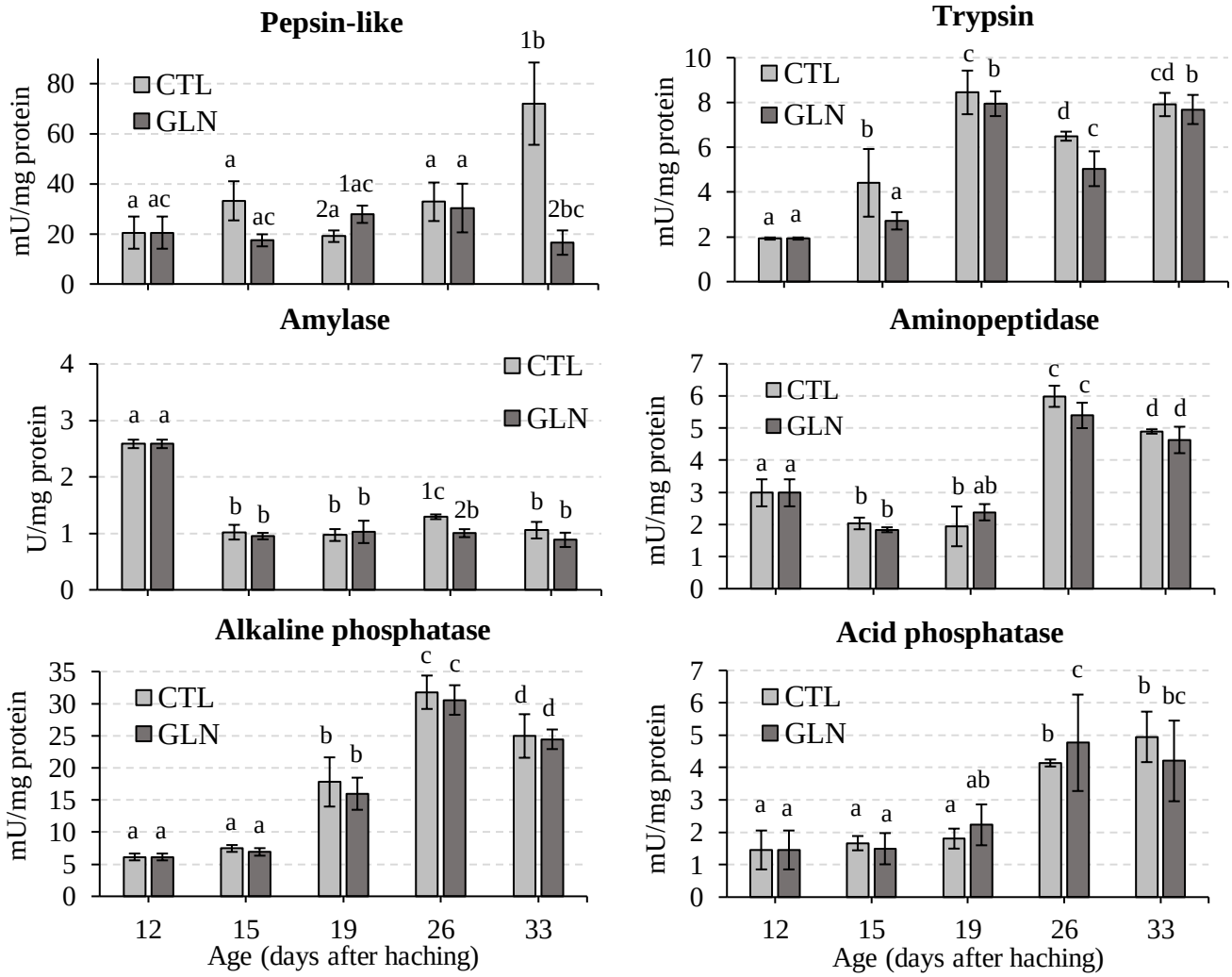

Figure 10 – Specific activity of digestive enzymes during larval development of Senegalese sole reared under two distinct feeding regimes: fed on *Artemia* sp. (CTL) and *Artemia* sp. supplemented with L-glutamine (GLN). Mean ± SD, n=3, per each experimental group. Different superscript numbers indicate significant differences ($p<0.05$) between treatments, and different letters indicate significant differences ($p<0.05$) between different ages within each treatment.

The profile marked by the aminopeptidase specific activity, determined at the BBM of the enterocytes in Senegalese sole post-larvae, differed between the two groups. Regarding to the CTL group, this activity increased suddenly from 12 to 15 DAH, followed by a gradual decrease until the end of the experiment. On the other hand, between 12 and 15 DAH, GLN treatment registered a constant level of this activity, which increased on day 19, followed by a decrease until 26 DAH; at the end of the experiment, aminopeptidase specific activity at the BBM increased again. At 15 DAH, the specific activity of this BBM enzyme was significantly higher ($p<0.05$) in the CTL group, when compared to the other group. However, the opposite was verified on the day 33, when GLN group stood out by the higher values, statistically significant ($p<0.05$). On the other sampling points (19 and 26 DAH) no significant differences ($p>0.05$) were observed (**Figure 11**).

At the enterocyte's BBM of the intestinal epithelium, Senegalese sole post-larvae from both experimental groups exhibited a strong increase in the alkaline phosphatase specific activity from 12 to 15 DAH. Then, the mean values exhibited by GLN group remained constant from 15 DAH onwards, whereas CTL group registered a slight increase at 19 DAH, followed by a decrease at 26 DAH, and peaking at the end. No significant differences ($p>0.05$) were observed between the two groups in whole the experiment (**Figure 11**).

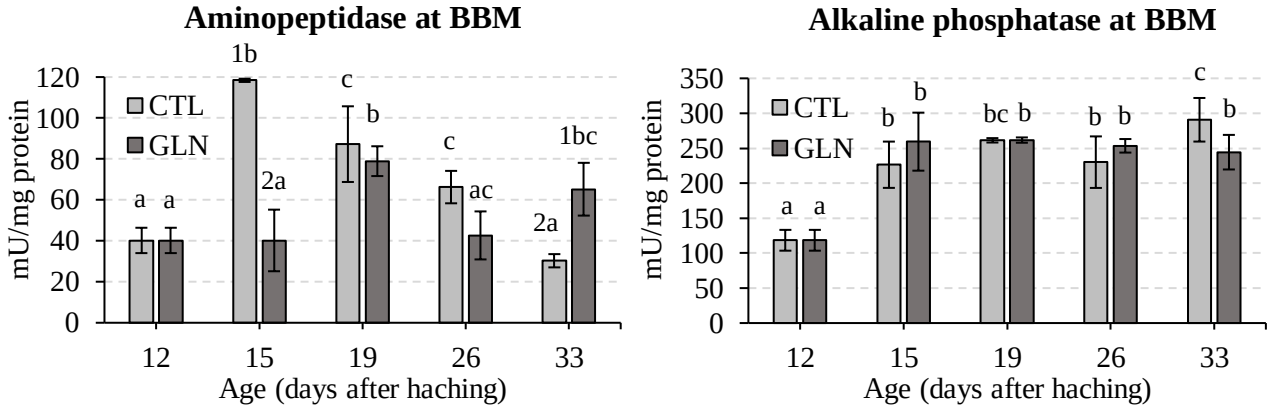

Figure 11 – Specific activity of BBM enzymes during larval development of Senegalese sole reared under two distinct feeding regimes: fed on *Artemia* sp. (CTL) and *Artemia* sp. supplemented with L-glutamine (GLN). Mean ± SD, n=3, per each experimental group. Different superscript numbers indicate significant differences ($p<0.05$) between treatments, and different letters indicate significant differences ($p<0.05$) between different ages within each treatment.

Total activity

Pepsin-like total activity remained relatively constant at a low level from 12 to 26 DAH in both treatments, followed by a strong increase at the end of the experiment, more evident for the CTL group. Along the rearing period, no significant differences ($p>0.05$) were observed for pepsin-like total activity between the two groups, except for the last sampling point, when this activity was significantly higher ($p<0.05$) for the CTL group, compared to the GLN treatment (**Figure 12)**.

Trypsin total activity assayed in both treatments exhibited a growing trend during the period of study, except between 15 to 19 DAH, when CTL group showed a slight decrease, as GLN treatment from 19 to 26 DAH. At the end of the experiment, both groups exhibited a strong increase in the total activity of this pancreatic enzyme. No significant differences ($p>0.05$) were observed along the rearing period (**Figure 12)**.

Amylase total activity showed a profile marked by a gradual decrease from 12 to 19 DAH more evident in CTL group, as observed in the increase until the end of the experiment. At 19 DAH, there was a higher ($p<0.05$) amylase total activity in the GLN treatment, whereas at the day 26, sole post-larvae from CTL group stood out by its higher ($p<0.05$) mean values (**Figure 12)**.

In both treatments, the total activity of aminopeptidase remained at a low and constant level between 12 and 15 DAH, followed by a slight decrease at the day 19, more evident for the CTL group. Then, the profile of this enzyme exhibited a strong increase until the end of the experiment. No significant differences ($p>0.05$) were observed between the two experimental groups during all the rearing period (**Figure 12)**.

Alkaline phosphatase total activity of Senegalese sole exhibited a similar pattern in both treatments: a gradual increase from 12 to 19 DAH, followed by a strong increase until the end of the experiment. Globally, the total activity of this intestinal enzyme was higher in GLN group, compared to the CTL group, but no significant differences ($p>0.05$) were observed (**Figure 12)**.

Concerning the acid phosphatase, in both groups the total activity of this enzyme showed low levels between 12 and 15 DAH, followed by a slight decrease at 19 DAH, more evident in CTL group. Then, from 19 onwards, both treatments showed a strong increase in the total activity of this lysosomal enzyme, reaching mean values 5 times higher than those assayed in the beginning. During all the studied period, there were no significant differences ($p>0.05$) between groups (**Figure 12)**.

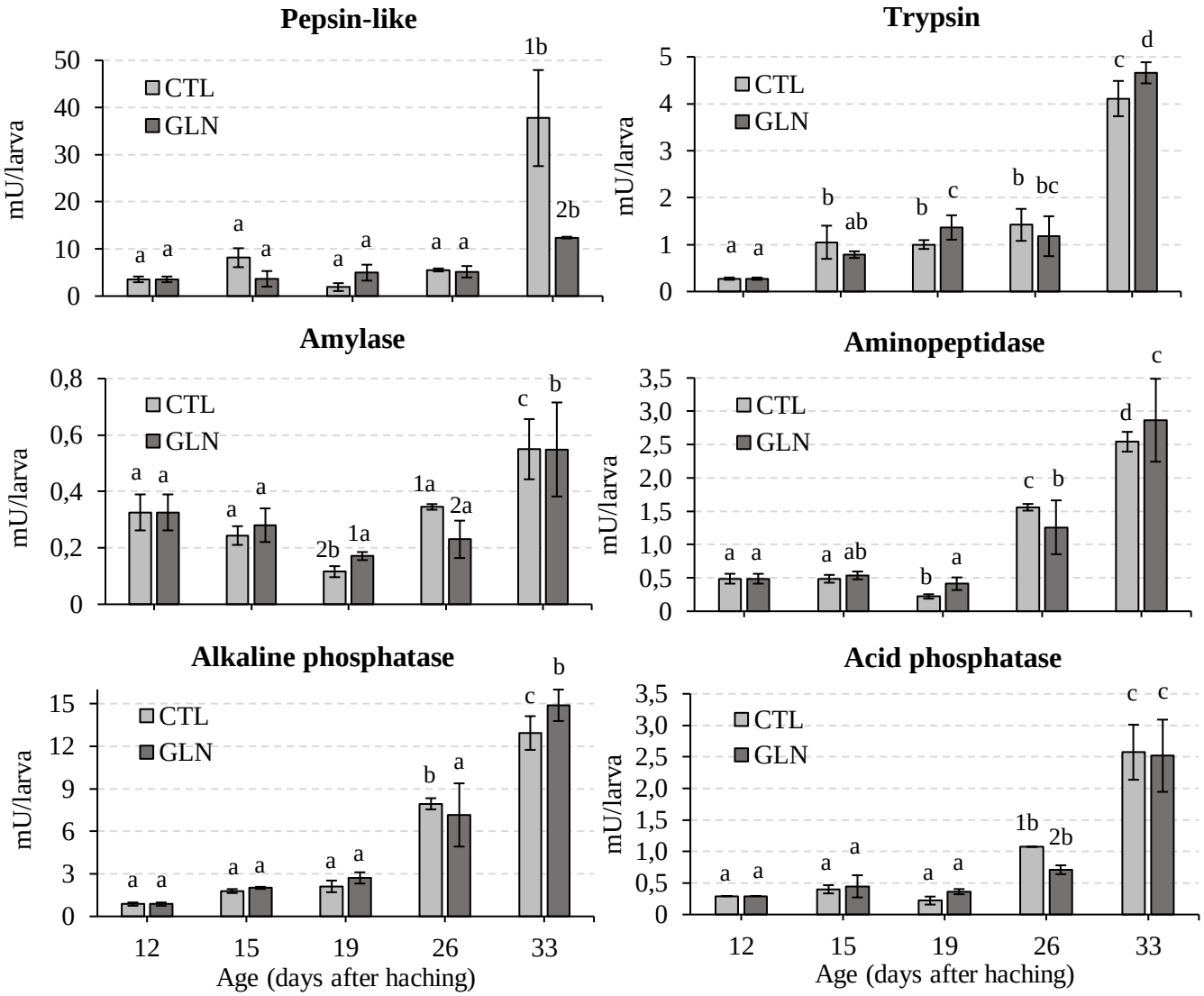

Figure 12 – Total activity of digestive enzymes during larval development of Senegalese sole reared under two distinct feeding regimes: fed on *Artemia* sp. (CTL) and *Artemia* sp. supplemented with L-glutamine (GLN). Mean ± SD, n=3, per each experimental group. Different superscript numbers indicate significant differences ($p<0.05$) between treatments, and different letters indicate significant differences ($p<0.05$) between different ages within each treatment.

Regarding the total activity of the aminopeptidase assayed at the BBM, the pattern exhibited by both groups were completely distinct. CTL group showed a gradual increase along the rearing period, with a slight decrease at 19 DAH. On the other hand, GLN group decrease from 12 to 15 DAH, remaining at the same level until 26 DAH; at the end of the experiment reached a peak, almost 5 times higher than the mean value observed in the prior sampling point. At 15 and 26 DAH, the mean values of the aminopeptidase total activity analyzed at the BBM of Senegalese sole post-larvae was significantly higher ($p<0.05$) in CTL group, when compared to the group which received L-glutamine supplementation in the diet. However, at the end of

the experiment, GLN group was highlighted by it significant higher ($p<0.05$) mean values than CTL group (**Figure 13**).

Alkaline phosphatase total activity, determined at the intestinal BBM, showed a gradual increase along the larval development in both CTL and GLN groups, except between 15 and 19 DAH, when CTL group remained relatively constant, whereas GLN treatment registered a slight decrease. No significant differences ($p>0.05$) were statistically found between the two experimental groups (**Figure 13**).

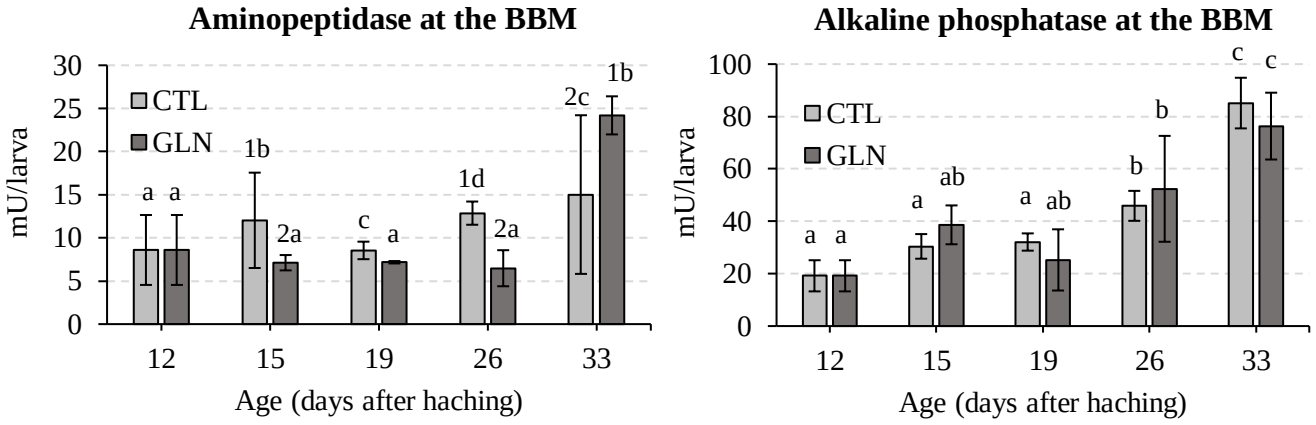

Figure 13 – Total activity of BBM enzymes during larval development of Senegalese sole reared under two distinct feeding regimes: fed on *Artemia* sp. (CTL) and *Artemia* sp. supplemented with L-glutamine (GLN). Mean ± SD, n=3, per each experimental group. Different superscript numbers indicate significant differences ($p<0.05$) between treatments, and different letters indicate significant differences ($p<0.05$) between different ages within each treatment.

Intestinal maturation index (IMI)

Intestinal maturation index (IMI) based on aminopeptidase assayed at the BBM of the enterocytes showed distinct patterns of variation between the two experimental groups. CTL group showed an increase until 19 DAH, followed by a strong decrease until the end. On the other hand, for GLN treatment, this index remained constant until 19 DAH, then fell down at 26 DAH, and registered a slight increase at 33 DAH. In general, for the same age, the IMI exhibited by CTL group was higher than the GLN treatment, but the difference was not significant ($p>0.05$), except on day 33, when GLN treatment was higher ($p<0.05$) than the CTL group.

IMI based on BB alkaline phosphatase exhibited similar patterns between the two experimental groups. CTL group increased from 12 to 19 DAH, then fell down until the end of the experiment, whereas GLN group showed an increase until 15 DAH, followed by a progressive decrease until 33 DAH. No significant differences ($p>0.05$) were founded between treatments during all the studied period.

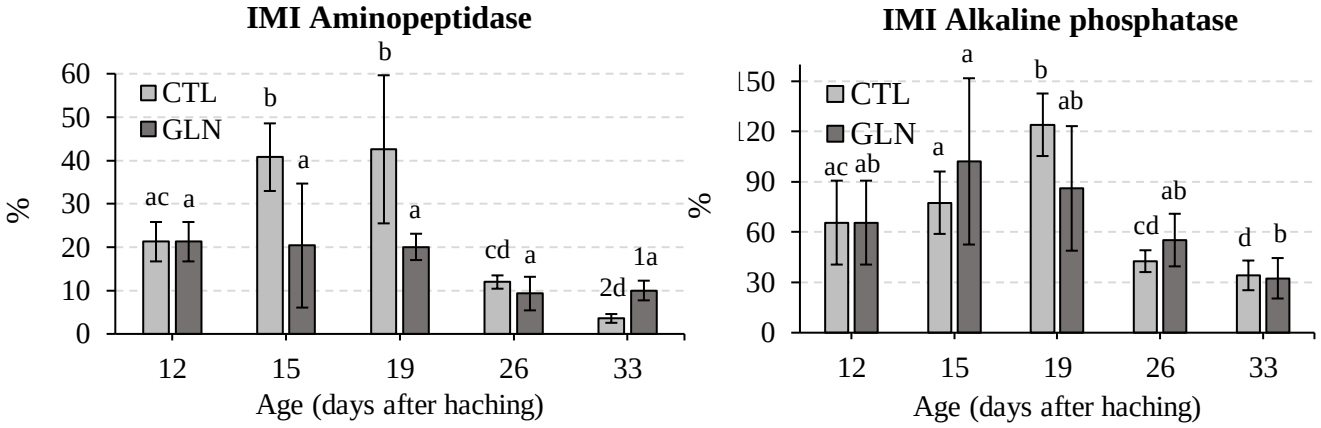

Figure 14 – Intestinal maturation indices (%) during larval development of Senegalese sole reared under two distinct feeding regimes: fed on *Artemia* sp. (CTL) and *Artemia* sp. supplemented with L-glutamine (GLN). Mean ± SD, n=3, per each experimental group. Different superscript numbers indicate significant differences ($p<0.05$) between treatments, and different letters indicate significant differences ($p<0.05$) between different ages within each treatment.

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5. DISCUSSION

The variable growth rates and large size dispersion during metamorphosis, as well as difficulties in weaning periods which results in high mortalities rates of *S. senegalensis*, have been a focal point for aquaculture research over the last years (Morais *et al.*, 2014). These difficulties founded in the Senegalese sole production can be related to the later maturation of the digestive tract, which occurs around 31 DAH, far beyond the onset of exogenous feeding (Ribeiro *et al.*, 1999a).

Several researches have shown a beneficial role of glutamine on the digestive tract. Indeed, glutamine represents a key energetic substrate to cells with high turnover, such as enterocytes (Wu *et al.*, 2011), and provide nitrogen required for the pyrimidine and purine synthesis, which are crucial for the normal development, maturation and repair of the intestinal mucosa (Newsholme *et al.*, 2003a). Due to its regulatory function on the growth performance of animals by improving physiological processes of digestion and absorption of nutrients in the diet (Zavarize *et al.*, 2010), glutamine has been widely evaluated in the recent years as a novel feed additive in aquaculture. Therefore, in the present study, the effects of dietary L-glutamine supplementation on the digestive physiology were assessed experimentally, aiming its application as feed supplement to improve digestive capacity of farmed Senegalese sole during the early life stage and, thereby, improve juveniles production in quantity and quality. This was evaluated by enriching live feed – *Artemia* sp. metanauplii – with L-glutamine, before being offered to *S. senegalensis* post-larvae. To our knowledge, this was the first time the effects of dietary L-glutamine supplementation were assessed in Senegalese sole. Thus, no direct comparison was made between the experimental results and similar research.

Table 3 – Influence of dietary glutamine supplementation on the growth performance parameters in several fish species. The increasing arrow (↑) indicates the optimal supplementation level, whereas the decreasing arrow (↓) indicates the negative effect triggered by glutamine supplementation. Missing values means no differences between treatments. G, Growth expressed as relative growth rate, specific growth rate, final body weight or percent weight gain; S, Survival; E, enterocyte height, Amy, amylase specific activity; Try, trypsin specific activity; Alk, alkaline phosphatase specific activity; BBAlk, alkaline phosphatase specific activity at the BBM.

Fish species	Dietary glutamine supplementation	Stage development	Performance	Reference
Cyprinus carpio var. (Jian carp)	0, 0.4, 0.8, 1.2, 1.6 and 2.0 %	Juvenile	↑G at 2.0%	[1]Yan and Qui-Zhou (2006)
Sciaenops ocellatus (red drum)	0, 1.0 and 2.0 %	Juvenile	↑G at 2.0%, ↓S at 2.0%, ↑E at 2.0%	Cheng *et al.* (2011)
Acipenser schrenckii × *Huso dauricus* (hybrid sturgeon)	0, 0.30, 0.60, 0.90, 1.20 and 1.50 %	Juvenile	↑G at 1.50%, ↑Amy at 1.20%	Qiyou *et al.* (2011)
Morone chrysops × *M. saxatilis* (hybrid striped bass)	0, 1.0 and 2.0 %	Juvenile	↑G and S at 1.0%	Cheng *et al.* (2012)
Cynoglossus semilaevis (half-smooth tongue sole)	0, 0.5, 1.0 and 2.0 %	Post-larvae	↑G and S at 0.5%, ↑Try at 0.5%, ↑Amy at 2.0%, ↑Alk at 0.5%, ↓Alk at 1.0%, ↑BBAlk at 2.0% ↑Leucine-aminopeptidase at 2.0%	Liu *et al.* (2015)
Ctenopharyngodon idella (grass carp)	0, 3, 6, 9 and 12 g/Kg	Juvenile	↑G at 6 g/Kg, ↑S at 3 and 6 g/Kg ↓S at 9 and 12 g/Kg, ↑Try at 9 g/Kg	Qu *et al.* (2019)
Oreochromis niloticus (Nile tilapia)	0, 1.0, 2.0, 3.0 %	Fingerling Juvenile	↑G at 3 % ↑G at 1 %	[1]Silva (2008)
	0, 1.0, 2.0, 3.0 %	Juvenile	↑G at 3 %	[1]Silva *et al.* (2010b)
	0, 0.5, 1.0, 1.5 and 2.0%	larvae		Vesco *et al.* (2001)
	0, 0.75, 1.5, 2.25, 3.0%	Fingerlings		Junior (2014)
	0, 1.0, 2.0, 3.0, 4.0%	Adults		Quadros (2010)
	0, 5.0, 10.0, 15.0, 20.0 g/Kg [2](Aminogut®)	Post-larvae	↑S at 20 g/Kg	Graciano *et al.* (2014)
Oreochromis niloticus × *O.aureus* (hybrid tilapia)	0, 0.2, 0.4, 0.6, 0.8 and 2.0%	Juvenile		Yang *et al.* (2008)
Hoplias lacerdae (Giant trahira)	0, 2.0, 4.0, 6.0, 8.0, 10.0 g/Kg	Juvenile		Ramos (2014)
Sparus aurata (gilthead sea bream)	0, 0.5, 1.0, 2.0%	Juvenile		Coutinho *et al.* (2016)
Scophthalmus maximus L. (turbot)	0, 1.0 and 2.0%	Juvenile		Zhang *et al.* (2017)
Ictalurus punctatus (channel catfish)	0, 5.0, 10.0, 15.0, 20.0, 30.0 g/Kg	Juvenile	↑E at 20 g/Kg	Pohlenz *et al.* (2012a)

[1] dietary L-glutamine and L-glutamate supplementation

[2] commercial product composed of glutamine and glutamic acid

During the experimental period, Senegalese sole post-larvae fed on *Artemia* sp. deprived of L-glutamine enrichment exhibited similar values of growth performance compared to other studies carried out with the same species (Dinis *et al.*, 1999), but higher than those reported by Ribeiro *et al.* (1999a) and Fernández *et al.* (2009). This allowed to validate the rearing conditions applied in this experiment.

In the present trial, growth performance of *S. senegalensis* post-larvae was significantly enhanced by dietary L-glutamine supplementation, which was consistent with the findings in Jian carp (Yan and Qui-Zhou, 2006), red drum (Cheng *et al.*, 2011), hybrid sturgeon (Qiyou *et al.*, 2011), hybrid striped bass (Cheng *et al.*, 2012), half-smooth tongue sole (Liu *et al.*, 2015) and grass carp (Qu *et al.*, 2019) (**Table 3**). Positive effects of dietary glutamine on the growth performance were also reported in fingerlings (Silva, 2008) and juveniles (Silva, 2008; Silva *et al.*, 2010b) of Nile tilapia fed diets supplemented with L-glutamine and L-glutamate (**Table 3**). These are in agreement with other studies which consistently report beneficial effects of glutamine supplementation on the growth performance for different animal models, including swine (Kitt *et al.*, 2002; Lackeyram *et al.*, 2001; Teixeira *et al.*, 2014; Wu *et al.*, 1996; Zou *et al.*, 2006), poultry (Bartell and Batal, 2007; Soltan, 2009; Yi *et al.*, 2001), broiler chickens (Sakamoto, 2009; Souza, 2005), and sea cucumbers (Yu *et al.*, 2016).

The beneficial effects of glutamine on the growth performance can be triggered by its capacity to mediate numerous metabolic pathways. This amino acid acts as a nitrogen source to promote the synthesis of non-essential amino acids, leading to protein deposition, which results in weight gain (Newsholme *et al.*, 2003b). Also, glutamine can promote animal growth (Newsholme *et al.*, 2003b) by its capacity to act as a stimulus for muscle synthesis (Forti, 2003) and reduce catabolism in skeletal muscle.

Contrary to the present study, some authors did not observe any effect of dietary glutamine supplementation on the growth performance, when tested in larvae (Vesco *et al.*, 2001), fingerlings (Junior, 2014), juveniles (Silva, 2008) and adults (Quadros, 2010) of Nile tilapia, as well as during it sex reversal in post-larval phase (Graciano *et al.*, 2014) (**Table 3**). These results support those showed by Yang *et al.* (2008) in hybrid tilapia, as Ramos (2014) in Giant trahira, Coutinho *et al.* (2016) in gilthead sea bream, and Zhang *et al.* (2017) in turbot (**Table 3**). Concerning terrestrial vertebrates, Maiorka *et al.* (2000), Fisher da Silva (2001) and Murakami *et al.* (2007) also did not found positive effects on growth performance of broiler chickens fed supplemented diets with glutamine (1.0%), as Chamorro *et al.* (2010) in weaned rabbits, House *et al.* (1994) in piglets and Plaizier *et al.* (2001) in dairy cows.

The absence of substantial differences in the growth performance of animals fed diets supplemented with glutamine might be explained by a wide range of factors. In rapidly growing species such as red drum, limited supply of dietary glutamine would not be able to support the high metabolic activity of enterocytes or the proliferation, maturation and/or physiological functions of intestinal lymphocytes (Cheng *et al.*, 2011) as is the case in humans (Le Becquer *et al.*, 2001), pigs (Wu *et al.*, 1996) and rats (Mandir and Goodlad, 1999). Also, digestive tract anatomy can influence dietary glutamine effects, inasmuch as the different sizes of intestine between species can lead to different enteric metabolism and more so as it related do glutamine utilization (Cheng *et al.*, 2011). Even within species, the utilization of glutamine and its metabolites can also be affected by a range of factors, such as age, developmental stage, environment, and pathological state (Lobley *et al.*, 2001). Diseases or pathologies (illness, inflammation) related to the presence of an infectious agents can channeled glutamine toward the proliferation of lymphocytes, instead of enterocytes at the intestinal epithelium. This may lead to the absence of glutamine effects on the growth performance, as the digestion/absorption of nutrients become limited by the poor functional capacity of epithelial cells. The absence of dietary glutamine effect on growth performance might also be explained be the needs for this amino acid for maintaining normal survival, as reported by Zhang *et al.* (2017) in a study performed in juvenile turbot.

The absence of beneficial effects of dietary glutamine supplementation on growth performance can also be explained by the presence of other dietary amino acids, which might have either competitive or inhibitory effects capable of weak the possible inducive effect of glutamine, as reported by Coutinho *et al.* (2016) in a study performed in gilthead sea bream juveniles.

These apparently conflicting results are not exclusive to fish (Lobley *et al.*, 2001) and seem to reveal species specific physiological differences that still need to be understood (Pohlenz *et al.*, 2012a).

Survival rate of fish depends on biotic (e.g. stocking density) as well as abiotic (e.g. water system and water chemistry) factors (Jenning and Philipp, 1994).

Along the rearing period of Senegalese sole, there was a control of the water system as well as the chemical parameters of water. Regarding to the biotic factors, both predation and competition factors were absent during the rearing period. Live food was offered *ad libitum*, therefore the probability of zooplankton being a decisive factor in the survival rate was reduced. Also, the stocking density of sole post-larvae was reduced at 19 DAH to ensure that this

parameter did not affect the survival. Thus, in the current study, the live feed enrichment was the key parameter which could most influence the survival rate of Senegalese sole post-larvae.

In the present study, survival rate of *S. senegalensis* post-larvae was high in both experimental groups, reflecting the good quality of sole. Furthermore, diet supplemented with L-glutamine did not promote any improvement on the survival rate of *S. senegalensis* post-larvae. Although much higher, this result was consistent with the study of Cheng *et al.* (2011), in which no significant differences were observed in survival (ranged between 89–92%) of juvenile red drum fed 1.0 and 2.0% of dietary glutamine (**Table 3**). These results were also similar to those of Qu *et al.* (2019) reported in juvenile grass carp (97.33% to 99.3% of survival rate), and those demonstrated by Cheng *et al.* (2012) and Qiyou *et al.* (2011) in juvenile hybrid striped bass and hybrid sturgeon, respectively, although survival rate were higher in these two studies (**Table 3**), compared to the values obtained for *S. senegalensis* in the present work. However, these findings are inconsistent with other studies in half-smooth tongue sole (Liu *et al.*, 2015) and Nile tilapia post-larvae during its sex reversal (Graciano *et al.*, 2014), although lower values were obtained in both species when compared with the data from the current study (**Table 3**).

The onset and the duration of sole metamorphosis are highly influenced by feed availability and type (Fernández-Díaz *et al.*, 2001). However, in the present study, dietary L-glutamine supplementation did not influence the duration of metamorphosis of *S. senegalensis* post-larvae, which was completed by 19–20 DAH, as reported in other studies by Dinis and Reis (1995), Ribeiro *et al.* (1999a) and Dinis *et al.* (1999).

Previously, intestinal morphology and digestive enzymes have been found to play vital roles in the digestive processes of the digestive tract. Both these factors are extremely important in fish growth, and are commonly recognized as indicators of intestinal function (Cahu *et al.*, 1998; Cara *et al.*, 2003; Refstie *et al.*, 2006).

Our results showed that *S. senegalensis* post-larvae fed diets containing L-glutamine exhibited significantly higher enterocyte height when compared to sole post-larvae from control group. Similar results were reported for red drum (Cheng *et al.*, 2011) and channel catfish (Pohlenz *et al.*, 2012a). These are in agreement with other studies performed in other vertebrates, as swine (Wu *et al.*, 1996) and poultry (Bartell and Batal, 2007; Lopes, 2008; Murakami *et al.*, 2007; Sakamoto, 2009; Soltan, 2009; Yi *et al.*, 2005).

Although not for the enterocyte height, other studies have demonstrated that dietary glutamine supplementation can improve development and function of the intestine in aquatic

animals (Cheng *et al.*, 2012; Liu *et al.*, 2006; Qiyou *et al.*, 2011; Qu *et al.*, 2019; Souba *et al.*, 1990; Yan and Qui-Zhou, 2006; Yu *et al.*, 2016). Contrasting results from those obtained in the present work were reported by Yi *et al.* (2001) and Souza (2005) in broiler chicken.

Considering the histological results of the enterocytes at the intestinal epithelium, the present experiment supports the notion of an efficient L-glutamine utilization by *S. senegalensis* post-larvae. This evidence the glutamine's role as metabolic fuel and growth factor for enterocytes (Souba *et al.*, 1985). The increase in enterocyte height and its surface area results in a better absorption of available nutrients in the intestine (Caspary, 1992). Thus, in the present study, the increase in enterocyte height might indicate that sole fed diet supplemented with L-glutamine might have had greater nutrient absorption and utilization, likely improving growth.

Digestive enzymes play a key role in nutrients digestion, which activities can directly reflect the digestive capacity, and, thereby, affect growth and survival of fish larvae (Abi-Ayad and Kestemont, 1994; Cahu *et al.*, 1998). According to Galgani *et al.* (1988), and Kumulu and Jones (2007), the enzyme activities are affected by dietary intake. Hence, knowledge on digestive enzyme activities is a key tool in nutrition studies since can be used as indicators of nutrition condition for fish larvae (Cheng *et al.*, 2012; Kjørsvik *et al.*, 2011; Liu *et al.*, 2015; Zhao *et al.*, 2015).

It has been reported that the inclusion of glutamine in diets can induce changes in specific enzyme activities involved in digestive processes. In the present study, different digestive enzyme activities were analyzed during the experimental study at five different ages of Senegalese sole to assess the effect of dietary L-glutamine supplementation on digestive physiology.

Regarding pepsin-like specific activity, this enzyme was enhanced by dietary L-glutamine only at 19 DAH, however this pattern changed at the end of the experimental period, and CTL group exhibited higher pepsin activity. The presence of pepsin-like activity in sole before 20 DAH was controversial some years ago once Martínez *et al.* (1999) reported activity of pepsin-like since 12 DAH sole, whereas Ribeiro *et al.* (1999a, 1999b) reported the appearance of gastric glands around 27 DAH, and pepsin-like activity was not quantified at 30 DAH for the same species. In a recent study, Mamani (2016) mentioned that the presence of some acid proteases activity in *Anisotremus scapularis* larvae fully homogenized could be explained by the activity of lysosomal proteases of enterocytes at the posterior intestine, which are involved in intracellular protein digestion, and also by the activity of other acid proteases

(e.g. lysosomal cathepsins) present in the body of larvae (Lazo *et al.*, 2007; Walford and Lam, 1993). Thus, in the present study, the presence of pepsin-like activity until 19 DAH sole are not linked to the stomach formation, since no gastric glands were observed on the histological section used for intestinal epithelium measurements, suggesting that its detection could be attributed to cathepsins activity instead of pepsin-like as both react to the same substrate. Nevertheless, at the end of the experiment, gastric glands were present in the stomach of sole from GLN treatment, but its development can not be attributed to the L-glutamine supplementation in the diet, since exhibited lower mean values of pepsin-like activity than did sole from CTL group.

Trypsin specific activity showed a decrease from 19 to 26 DAH more evident in sole fed diet supplemented with L-glutamine, which increased at the end of the experimental period. This result are in agreement with the data reported for the above-mentioned digestive enzyme. In the absence of a gastric digestion, trypsin is responsible for proteins digestion. Contrasting results were reported by Qu *et al.* (2019), who found significantly highest trypsin activity in juvenile grass carp fed diet supplemented with glutamine (9 and 12 g glutamine/Kg diet). In a study performed in half-smooth tongue sole post-larvae (Liu *et al.*, 2015), fish fed the diet supplemented with glutamine displayed higher activities of trypsin in intestinal segments than did control group (**Table 3**).

Concerning the other pancreatic enzyme, amylase, the present study showed that sole fed diet deprived of L-glutamine had highest mean values, although only statistically significant at 26 DAH. Similar results were obtained in grass carp fed a glutamine-containing diet at 3, 6, 9 and 12 g/Kg, which had worst results than did control group (Qu *et al.*, 2019). Contrary to these results, Liu *et al.* (2015) reported a significantly higher activity of amylase in half-smooth tongue sole post-larvae fed diet supplemented with glutamine than those fed the control diet, as well as shown by Qiyou *et al.* (2011) in hybrid sturgeon (**Table 3**).

The reason why amylase specific activity was higher in CTL group than in sole fed L-glutamine-supplemented diet could be attributed to the filling degree of the gut and the nutritional condition of the fish. According to Vonk and Western (1984) and Munilla-Móran *et al.* (1990), higher levels of amylase are detected when fish are satiated. Thus, sole from CTL could be more satiated, compared to those fed L-glutamine-supplemented diet. Indeed, during the experimental period, tanks from CTL treatment presented more food waste than those belonging to the GLN treatment.

Aminopeptidase specific activity, which carry out the hydrolysis of peptides, was not influenced by L-glutamine supplementation in the diet. The same was observed for specific activity of alkaline phosphatase, which is involved in the nutrient absorption at the intestinal epithelium. But there are no similar studies to allow a direct comparison of both these intestinal enzymes.

Like the most enzymes above-mentioned, specific activity of acid phosphatase, which is an indicator of protein pinocytosis (Cara *et al.*, 2003), did not differ between CTL and GLN treatments. But no studies are available to compare this result.

Digestion and absorption of nutrients depend on the activity of the digestive enzymes, in particular those located in the BBM of the intestine, such as aminopeptidase and alkaline phosphatase, which are responsible for the final stages of breaking down and assimilation of feed ingredients (Silva *et al.*, 2010a).

Previous study has demonstrated that uptake of glutamine can promote the maturation of larval intestinal development (Liu *et al.*, 2015). Our results showed that Senegalese sole fed diet containing L-glutamine exhibit significantly higher BB aminopeptidase than did control sole. The increasing levels of aminopeptidase specific activity at the BBM demonstrated that dietary L-glutamine could promote the maturation of enterocytes in the early life stage of Senegalese sole. This result agree with those of Liu *et al.* (2015), who reported that dietary glutamine supplementation significantly increase leucine-aminopeptidase specific activity in BBM of half-smooth tongue sole (**Table 3**).

On the other hand, the alkaline phosphatase specific activity assayed at the BBM of sole fed diet supplemented with L-glutamine did not differ from those fed control diet. However, previous studies have been reported positive effects of dietary glutamine on the BB alkaline phosphatase (Liu *et al.*, 2015), highlighted this enzyme to support the maturation of larval intestinal development (Cahu *et al.*, 1998; Segner *et al.*, 1989)**.**

In general, the specific activity of digestive enzymes assayed in sole fed dietary L-glutamine exhibited lower values than did CTL group. The reason why this happened could be attributed to the increase in tissue proteins in sole fed L-glutamine-supplemented diet (Zambonino Infante and Cahu, 2001), therefore resulting in lower specific activity.

The total activity of digestive enzymes increased with age and post-larvae size in both experimental groups. However, higher values were most exhibited by sole post-larvae from CTL treatment.

Regarding the IMI assessed based on alkaline phosphatase and aminopeptidase BB activity, both showed a decrease over larval development whether in CTL group or GLN group. These results were unexpected since IMIs tend to increase over development progress, accompanied by rising BB.

The reason why dietary glutamine positively influenced activities of digestive enzymes could attribute to its benefit effects on fish intestinal morphology.

The intestine is a major tissue of glutamine utilization that can convert glutamine into other amino acids; in turn, glutamine can provide energy to intestinal epithelial cells such as enterocytes for rapid division (Windmueller and Spaeth, 1980).

Although glutamine crosses the BBM of the enterocyte, part of this amino acid acquired through the live feed and supplementation is consumed by the intestinal cells themselves (Adibi, 2003). Thus, it is possible that the additional L-glutamine may be funneled towards other physiological purposes instead of being used to fulfil fish energy demand. For instance, it was already reported that dietary glutamine supplementation increased enterocytes migration in channel catfish (Pohlenz *et al.*, 2012a); enhanced the enteric microstructure in Jian carp (Yan and Qui-Zhou, 2006), red drum (Cheng *et al.*, 2011), hybrid sturgeon (Zhu *et al.*, 2011), and channel catfish (Pohlenz *et al.*, 2012a); improved antioxidant defense system in hybrid sturgeon (Qiyou *et al.*, 2011; Zhu *et al.*, 2011); enhanced the innate immune system of red drum (Cheng *et al.*, 2011), hybrid sturgeon (Zhu *et al.*, 2011), and hybrid striped bass (Cheng *et al.*, 2012); and improved both innate and adaptative immune responses in channel catfish (Pohlenz *et al.*, 2012b, 2012c).

As seen with other amino acids, some levels of glutamine supplementation could cause adverse effects in several animal species, including fish (Garlick, 2001). However, in the present study, no adverse effects were seen in the growth performance of Senegalese sole post-larvae fed on *Artemia* sp. supplemented with L-glutamine (2.2 ± 0.06 mg L-glutamine/g *Artemia* sp. DW). This allow to consider this dose an adequate supplementation to the sole post-larvae.

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6. CONCLUSIONS AND FUTURE PERSPECTIVES

In conclusion, the results of the present study confirm that dietary L-glutamine supplementation (0.5 g l^{-1}) in the diet of Senegalese sole significantly improved growth performance and enterocyte height during larval development. This allow to reinforce the hypothesis of beneficial action of L-glutamine in the digestive tract, with consequent benefit in digestion and absorption of nutrients during the phase preceding the weaning, which is considered critical in the development of *S. senegalensis*. In addition, during the early stage of Senegalese sole, dietary L-glutamine supplementation can improve the digestive capacity by enhancing the activity of some digestive enzymes and an enzyme located at the BBM of the enterocytes. Although the results from the evaluation of dietary L-glutamine supplementation are very promising, there is still potential for further improvements regarding digestive capacity during the early life stage of Senegalese sole, since the effect of this amino acid on the most enzymes is still unclear. It seems that follow-up trials will be needed to identify the optimal concentrations of L-glutamine as supplement in the diet. Furthermore, future research should test earlier introduction and over a longer period. This would allow to test the effect of L-glutamine supplementation on the weaning of *S. senegalensis*, and answer to the remaining questions: can L-glutamine lead to a better adaptation to inert diet, by enhancing the maturation of the digestive tract of *S. senegalensis*? Can the inert diet be potentially introduced earlier?

There is a close relationship between the process of intestine maturation, which implies the maturation of enterocytes and the acquisition of an adult mode of digestion, and the digestion/absorption of exogenous nutrients, which are important processes when introducing the inert diet. Therefore, the practicability and efficacy of dietary L-glutamine supplementation for Senegalese sole may provide an experimental basis to use this amino acid in fish production in aquaculture, contributing to the successful juvenile production of this promising flatfish species, by promoting an earlier maturation of digestive tract.

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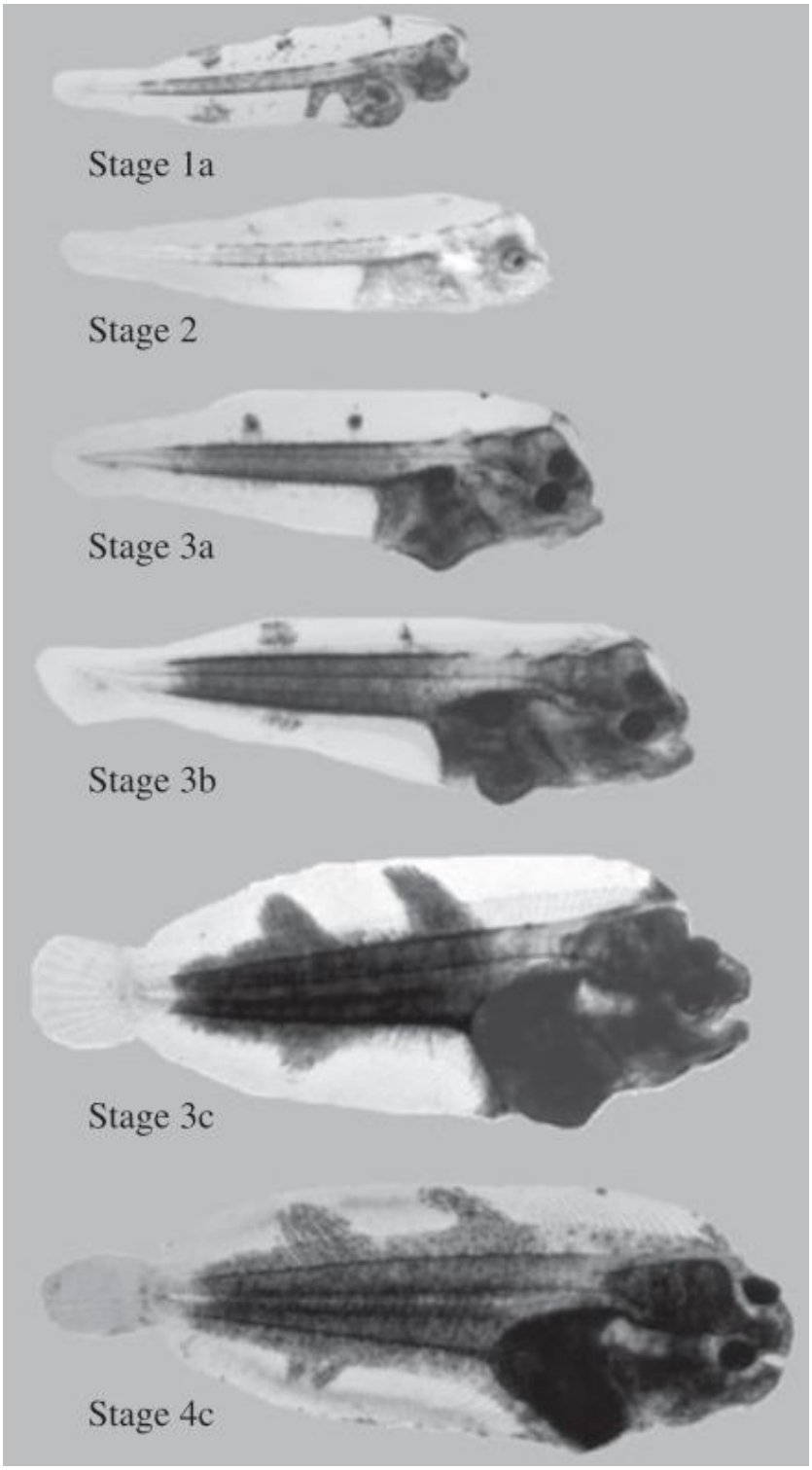
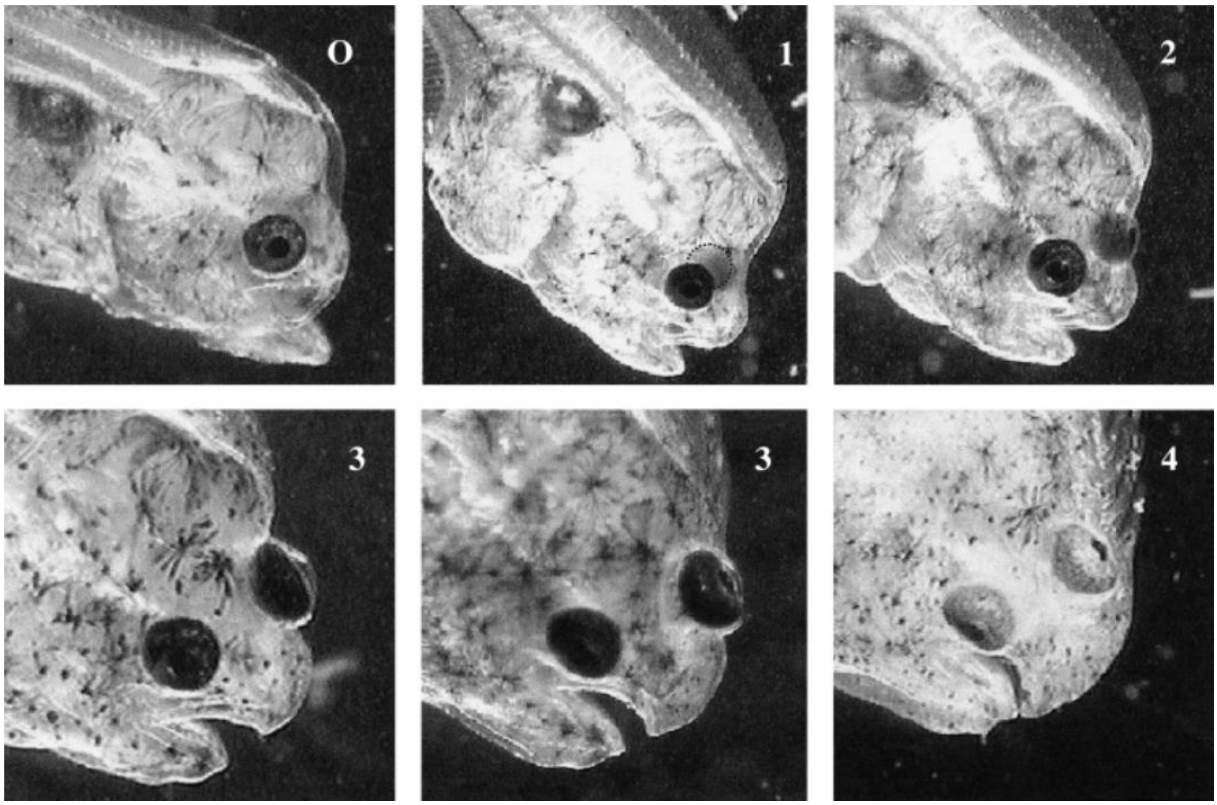
8. APPENDICES

Appendix I – Morphological characteristics at different developmental stages and substages of *Solea senegalensis* larvae (Padrós *et al*., 2011).



Appendix II – Eye position in the different stages of metamorphosis for *Solea senegalensis* (Fernández-Díaz *et al.*, 2001).



Appendix III – Histology procedures (Resin) (Electron Microscopy Sciences)

1. **Tissue processing**

1st step – Dehydration

In order to remove the water from the tissue which is present – either bound to the tissue, or free in the tissue, samples were dehydrated in a graded series of ethanol (85 and 100%) by immersing tissue in ethanol solutions for 2 hours each concentration. A series of increasing concentrations is used to ensure that the water in the tissue is gradually replaced by the alcohol and to avoid excessive distortion of the tissue. This step was carried out at room temperature.

2nd step – Pre–infiltration, Infiltration and Polymerization

Preparation of working solutions

During the inclusion process two solutions – A and B – were prepared according to the step-by-step instructions' Kit Technovit 7100 commercially acquired.

Solution A – Infiltration solution

1g of Technovit® 7100 hardener I (1 bag) was dissolved in 100 ml of Technovit® basic solution and mixed with a magnetic stirrer in the hood until completely dissolved. The flask was wrapped with aluminum foil and stored at 4 °C.

Solution B – Polymerization solution

Polymerization solution was prepared by mixing 15 ml of mix infiltration medium (unused) with 1 ml of Technovit® 7100 hardener II using a glass rod, for approximately 3–5 minutes of mix. This solution was used for inclusion itself and it must be made only when it to be used.

a. Pre–infiltration

After 4 hours of dehydration and having the solution A prepared, the alcohol was removed from the samples which was placed in a 1:1 (100% alcohol: infiltration solution) for 2 hours.

b. Infiltration

After 2 hours, the pre–infiltration solution was removed from the vials, and the samples were soaked in infiltration solution for 20 hours.

c. Polymerization

Using a standard pipetting aids and a disposable container, the histoform embedding cavities were filled halfway with polymerization solution (disposable pipette) and positioned the prepared sole larvae therein before the form being filled (only the cavity, not the entire recess). In order to avoid the delay of process, the forms were covered with a clear plastic and therefore the polymerization occurred completely after 48 hours at room temperature.

Appendix IV – Enzyme activities

Table 1 - All the enzymatic activities are reported in worth units (U), where one U was defined as the amount of enzyme that catalyzes the formation of 1 μg of substrate per minute, using different molar extinction coefficients depending on the substrate used for each enzyme.

Enzyme	**Molar extinction coefficient (**ϵmolar)
Acid protease (pepsin-like)	0.0082
Trypsin	0.0082
Aminopeptidase	8200
Alkaline phosphatase	18300
Acid phosphatase	18300