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Faculdade de Ciências e Tecnologia

2019/2020

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MSc. Marine Biology

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Declaração de autoria de trabalho

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

Universidade do Algarve, 07 de outubro de 2020

ACKNOWLEDGEMENTS

I would like to thank “Instituto de Ciencias Marinas de Andalucía” that welcomed me in the reseach group, allowing my thesis realization. In particular, I’m extremely grateful to my supervisor, Dr. Manuel Yúfera, Dr. Gonzalo Martínez-Rodríguez and Dr. Neda Gilannejad for their unwavering support and guidance. I had a great pleasure to work with them.

Thanks also to “Aquagroup”, that allowed me to join them in a such delicate period. I express my deepest appreciation to my co-supervisor, Dr. Carmen Navarro Guillén, for invaluable insights into aquaculture, helpful advices and patience. I have very appreciated the time we spend together.

I can not forget the fundamental contribution of Enrico, Laura, Giada, my family and the one of my friends.

They make me, always, maintain a state of serenity and happyness, ideal to approach all the life challenges.

ABSTRACT

The greater amberjack, *Seriola dumerili*, a fast growing pelagic fish, was one of the species selected to increase the farmed fish variety in Europe, due to its adaptation to captivity conditions, good flesh quality and high market demand. Inadequate feeding protocol has been suggested as one of the causes behind mortalities during the larval stage. Aiming to advance feeding optimisation during the early stage of this species, this study examines the ontogeny of digestive function using molecular and biochemical approaches. Gene expression of digestive enzyme precursors and the enzyme activity has been determined during the first 51 days post hatching (dph) and the diurnal period of 19 dph larvae, reared in semi-intensive conditions. The expression of pancreatic proteases precursors (*try3*, *ctra* and *ctrb1*) increased from first-feeding, while gastric chitinase (*chia1*), gastric protease (*pga3*) and proton pump (*atp4a2*) after 10 dph. The precursor genes of pancreatic lipases (*cel1*, *cel2* and *cel3*) peaked between 6 and 10 dph, phospholipase A2 (*pla2g1b*) rose only after 25 pdh, while α-amylase (*amy2a*) increased mainly from 20 dph. The trypsin activity was more evident from first-feeding to 25 dph and chymotrypsin activity from this day onwards. Acidic chitinase and pepsin activity appeared, respectively, at 16 and 30. The activity of 7C-like lipase was evident from first-feeding but, similarly to 4C-like lipase significantly increased from 15 dph, while amylase peaked from 6 to 22 dph. Aminopeptidase and alkaline phosphatase activities started at 20 dph indicating the functional maturation of brush border of the enterocytes. The daily pattern analysis showed a food anticipatory strategy in the expression of proteases related genes. Lipases activity was more evident during the morning hours, followed by amylase, and, in the afternoon, by proteases. Results, also, suggested an alternation in the activity of chymotrypsin and trypsin. These ontogenetic patterns are concordant with a carnivorous marine teleost.

Keywords: daily rhythm, digestive genes, enzymatic activity, larvae ontogeny, *Seriola dumerili*

RESUMO

A limitação principal no setor da aquicultura europeia é a baixa diferenciação de espécies em cultivo. Ao longo da última década, os investimentos em investigação no domínio da aquicultura procuraram aumentar a pouca variedade de peixes de cultura, com sucesso. Uma das espécies visadas é a *Seriola dumerili,* um peixe carnívoro, de crescimento rápido e pelágico. Este é selecionado pela sua adaptação às condições de cativeiro, boa qualidade da carne, elevado valor de mercado e procura. Contudo, o potencial industrial desta espécie é ainda limitado pela baixa produção em massa de juvenis de alta qualidade. Uma elevada taxa de mortalidade, sobretudo durante a transição da alimentação exógena-endógena e desmame limitam a sua produção, além das doenças e do canibalismo. Um importante avanço no cultivo larvar foi verificado com a implementação da tecnologia de mesocosmos e com a inclusão da *Artemia* e de rotíferos na dieta. Apesar disso, vários estudos sugerem que estes problemas ainda não estão totalmente resolvidos, devido a uma lacuna de conhecimento dos requerimentos nutricionais e fisiologia digestiva durante o crescimento das larvas. Na verdade, é referida a falta de sincronia entre a composição do alimento e o desenvolvimento do sistema digestivo. Foi avaliada a atividade de apenas algumas enzimas nesta espécie mas a expressão genética dos seus precursores e os padrões enzimáticos e genéticos diários nunca foram tratados na *Seriola* ssp. Com esse objetivo, este estudo pretende obter pela primeira vez um conhecimento mais aprofundado sobre o desenvolvimento da capacidade máxima digestiva em larvas e juvenis precoces do lírio (*Seriola dumerili*). As larvas foram criadas em condições intensivas, num sistema de aquicultura com recirculação de água (RAS). Com abordagens de biologia molecular e bioquimica, foram avaliadas a expressão dos genes de precursores de enzimas digestivas e a atividade das mesmas enzimas. As amostras incluíram larvas desde os primeiros 30 (expressão) e 51 (enzimas) dias pós-eclosão (dpe). Simultaneamente, foram determinados padrões diários de expressão e atividade enzimática em larvas com 19 dpe, com amostragens periódicas das 7:30h às 21:00h. Se realizou a extração do RNA das larvas e, por transcrição reversa, obteve-se cDNA. A seguir, mediante primers específicos e inclusão de marcadores multiplicaram os genes de interesse e, finalmente, foi avaliada a sua expressão. Por outro lado, para a atividade enzimática, as larvas foram homogeneizadas, isolando o extrato de enzimas pancreáticas e o extrato das células da borda em escova. A utilização de kits apropriados com protocolos específicos permitiu determinar a atividade enzimática, através de medições de absorção e fluorescência. A atividade de proteases, lipases e carboidrases e os respetivos padrões de expressão dos precursores foram medidos e comparados, para ajudar a esclarecer os mecanismos de regulação.

A expressão dos precursores de proteases alcalinas (tripsina, quimotripsina) e de lipases ativadas por

sais biliares foi registada antes e próximo da abertura da boca, respeitivamente. Após a transição para alimentação exógena, observou-se expressão dos carregadores de peptídeos e da quitinase ácida, mas só após o dia 20 registou-se expressão do mRNA de protease ácida, amilase e fosfolipase. Em particular, a pepsina e os precursores *atp4ase* mostram uma forte sincronia com o desenvolvimento do estômago.

Uma atividade precoce da 7C-like lipase parece ter um papel na absorção dos nutrientes do saco vitelino antes da abertura da boca. Durante os primeiros dias de alimentação exógena, o metabolismo das proteínas e dos hidratos de carbono parece ser já relevante. Uma vez que o teor de hidratos de carbono na dieta é baixo, o papel da amilase é ainda desconhecido. Calcula-se que participe na digestão de rotíferos. Do 10 ao 22 dpe, o metabolismo dos lípidos ganha importância, especificamente com a 7C-like lipase. Estes resultados podem evidenciar algumas deficiências na assimilação de proteínas durante o estado larvar, embora outros estudos os considerem comuns. Com o início da alimentação com ração, o metabolismo das proteínas volta a ser altamente relevante. Com efeito, a quimotripsina, a aminopeptidase e a fosfatase alcalina começaram subitamente a manifestar a sua atividade. Por estes motivos, o desmame poderá ter influência na produção da atividade enzimática. Contudo, os valores destas três enzimas diminuem rapidamente, na presença da produção de pepsina, que é um dos fatores indicadores da mudança para digestão adulta. Surpreendentemente, a pepsina não coincide com a atividade da quitinase ácida, que aparece no 19 dpe. A sua síntese deveria ser simultânea, dado que estão relacionadas com o funcionamento do estômago. Uma única larva usada como amostra, em vez de um pool, e a resistência da quitinase alcalina em pH baixo podem justificar estes resultados. Ao mesmo tempo, também se coloca a hipótese de uma falta de sincronia entre o desenvolvimento do estômago e a produção de pepsina. As 4C e 7C-like lipases mostram uma diminuição após o dia 30, sugerindo uma mudança para lípidos mais complexos. A análise dos padrões diários mostrou que a expressão génica se mantém mais constante comparada com a atividade. Entre todos os genes, apenas as transcrições de mRNA relativo ás proteases apresentaram um padrão diário inconstante. Com efeito, os carregadores de tripsina, quimotripsina e peptídeos mostram padrões mais elevados durante a manhã. Como relatado sobre outras espécies, considera-se que uma adaptação aos ritmos de alimentação estará relacionada com a programação genética. Contrariamente às proteases, as outras enzimas não mostram variações durante o período de estudo. Na análise enzimática, são verificadas mais flutuações. Por um lado, as larvas parecem reagir à ingestão de alimento com um aumento de atividade de proteases e amilase. Em particular, a quimiotripsina cresce em poucas horas, enquanto a tripsina leva mais tempo, atingindo o pico no final da tarde. Uma elevada atividade de tripsina à noite

poderá explicar os baixos valores obtidos na análise ontogenética. Por outro lado, as larvas concentraram atividade de lipase nas primeiras horas da manhã, imediatamente antes da alimentação. De forma semelhante à expressão das proteases, as lipases parecem estar envolvidas num mecanismo de antecipação, que limita a perda de energias. Este fator, de acordo com os resultados ontogenéticos, mostra uma vez mais a importância do metabolismo dos lípidos nesta fase larvar.

Tal como verificado em outros estudos, muitas vezes os padrões de expressão não correspondem aos padrões enzimáticos, sugerindo um importante papel e impacto dos mecanismos de regulação. A este propósito, é sabido que muitas respostas metabólicas são determinadas por genes induzidos por fatores ambientais, cujos mecanismos são ainda desconhecidos.

Os resultados de este estudo aumentam o conhecimento da ontogenia, fisiologia e metabolismo do trato digestivo em larvas de *Seriola dumerili*, apontando uma direção para mais estudos. Novas noções deverão melhorar a otimização dos protocolos de alimentação e favorecer a sustentabilidade das culturas. Como resultado, o aumento das taxas de sobrevivência e crescimento irá melhorar os rendimentos dos produtores e os objetivos de sustentabilidade.

Palavras-chave: atividade enzimática, expressão génica, ontogenia de larvas, ritmos diários, *Seriola dumerili*

LIST OF ABBREVIATIONS

actb - beta-actin

amy2a - amylase 2 alpha

ANOVA - analysis of variance

atp4a - proton pump 4a

BAL - bile salt-activated lipase

BB – brush border

cDNA - complementary DNA

cel1 - bile salt-activated lipase 1

cel2 - bile salt-activated lipase 2

cel3 - bile salt-activated lipase 3

chia1 - gastric chitinase 1

ctra - chymotrypsinogen a

ctrb1 - chymotrypsinogen b1

DNA - deoxyribonucleic acid

dph - day post hatching

ef1a - elongation factor 1a

ESD - generalized extreme studentized deviate test

g - grams

h – hours

L – litres

ln - natural logarithm

M - molar

min - minutes

mL – millilitres

mM - millimolar

mRNA - messenger RNA

mg - milligrams

nm - nanometers

PCR - quantitative polymerase chain reaction

pga3 - pepsinogen a3

PLA2 - Phospholipase A2

pla2g1b - phospholipase g1 b

qPCR - quantitative polymerase chain reaction

RAS - recirculating aquaculture system

RFU – relative fluorescence units

RGR - relative growth rate

RNA - ribonucleic acid

SD – standard deviation

slc15a1a - peptide transporter 1a

slc15a1b - peptide transporter 1b

try3 - trypsinogen 3

U – activity units

US$ - United States dollar

µM – micromolar

µL - microlitres

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INTRODUCTION

1. World Aquaculture

Aquaculture is one of the fastest-growing sectors for animal food production, that, in 2014 has produced a value of US$ 160.8 billions (FAO, 2016). The evolution and spread of aquaculture industry is shown by the amount of seafood produced, compared to the one obtained through the capture in nature (Dunham et al., 2001). The value has passed from a 31.1% of aquaculture production, through a 42.1% in 2012, to a 44.1% of total production in 2014 (73.8 million of tons), with 35 countries producing more farmed fish than wild-caught, already, in 2014 (FAO, 2016). 102 million of tons are expected in 2021 from aquaculture seafood production, overtaking the fishery catches (FAO, 2016). For this reason, the increasing demand for fish, due to rising population and incomes, urbanization interlinked with the expansion of fish production, improved distribution channels and benefits for human health (omega 3) will be mainly met by the aquaculture industry growth (Dunham et al., 2001). The most relevant kind of seafood that is produced is represented by the finfish, 49.8 million of tons per US$ 99.2 billions. A total of 362 species of finfishes (including hybrids) are reported to be farmed around all over the word, in 2014 (FAO, 2016). However, its annual growth rate is estimated to decline from 5.4% in the previous decade to 3% percent in the projected period (FAO, 2016). The limiting factors are represented by the lack of availability of space and good quality water, investments and fish seeds (AIFP, 2005; Bellona-Aquaweb, 2009; Eurostat, 2010; Munguti et al., 2014). Moreover, competition between producers and the relative high cost of fish meal put an edge to the development (Asche et al., 2008; FAO, 2016). Another big constraint is the consumption of fish forage and oil for finfishes feeding, finite resources, with just two species of carps that are not supplied with them (Tacon and Metian, 2008; Naylor et al., 2009, FAO, 2016). Finally, in the European contest, the little diversity in the current marine finfish production, which regards only six main species (Atlantic salmon *Salmo salar*, European seabass *Dicentrarchus labrax*, gilthead seabream *Sparus aurata*, turbot *Psetta maxima*, meagre *Argyrosomus regius* and sole *Solea sp*.), largely impedes the growth of the industry (Fernández-Palacios et al., 2015).

1.1 New horizons in aquaculture production

As an answer to aquaculture limitations, the recirculation aquatic systems, RAS, have been developed at industrial scale. It is mainly spread in the European continent, justified as a sustainable development technique for aquaculture production (Eurostat, 2010). RAS system is a technology for intensive fish farming, that preserves space and water, aiming to an increase the production while

reducing the nutrient discharged (MAFF, 2007). It offers a promising solution to water use conflicts, water quality, and waste disposal Biofilters, through bacteria nitrification, and solid removals recycle 90-99% of water, removing waste products and uneaten feeds (Heinen et. al, 1996; Badiola et al., 2012). Since the water is reused, the total consumption is about 20% compared to the flow-through water systems, resulting, also, very useful in areas where water and space are expensive (Helfrich and Libey, 1991). At the same time, this technology could be implemented in latitudes with unfavourable climates, that do not lead to a profitable production, or close to the big cities, reducing hauling distances and transport costs (Helfrich and Libey, 1991). It makes possible the control over environmental and water quality parameters, avoiding loss and optimizing the production, preventing diseases and parasites (Helfrich and Libey, 1991). However, due to its complexity, it is expensive and hard to handle and it requires skilled technical assistance and constant supervision to check the parameters (Schneider et al., 2006; Lekang, 2007). For this reason, its potential was recognized just in the '90s, passing from 300 tonnes in 1986 to more than 23,000 in 2009 in Europe (Martins et al., 2010).

In order to promote and improve new fish species spread farming in Europe, the DIVERSIFY project (2013-2018) aimed to fund the biological information exploration of finfish species («Diversify-eu - News» s.d.), such as reproduction, nutrition, husbandry and pathogens, of 6 different species. The greater amberjack *Seriola dumerili*, was identified as a valuable candidate because of both biological and market suitability point of view. First of all, its flesh quality, and the simplicity of its transformation attracted the market, while the remarkable profit encouraged the production (Muraccioli et al., 2000; Nakada, 2002). In addition, the great adaptability to captivity conditions, even under intensive conditions (RAS) and the rapid growth grade under cultured conditions, faster than other regularly farmed species as *Dicentrarchus labrax* and *Sparus aurata* (Garcia and Diaz, 1995; Divanach, 2004; Jerez et al., 2006), revealed an important potential and opportunity for the aquaculture sector. In fact, *S. dumerili* is able to reach 6 kg in just 2.5 years (Mazzola et al., 2000).

2. Seriola dumerili

Seriola dumerili (Risso, 1810), known as greater amberjack, belongs to the Carangidae (Liu, 2001) family. It is a marine fish species with circumglobal distribution, living in temperate water but adapting to a wide temperature range (from 15 °C to 27 °C) (Thompson et al., 1999; Nakada, 2000; Liu 2001). It attends pelagic and epibenthic coastal habitats, presenting gregarious juveniles (Mazzola, et al., 2000). Greater amberjacks have opportunistic predatory feeding behaviour with diet subjected to ontogenetic variations. Zooplankton and nekton species represent the main food items until they reach 20 cm length. After this period, juveniles move closer to coastal areas, where they switch the diet to fishes and

cephalopods (FAO, 2019**).**

2.1 *Seriola* spp. and limitations in aquaculture

Japan was the first country involved in *Seriola* spp. production, cultivating *Seriola quinqueradiata* since 1960. Nowadays, this species still dominates the *Seriola* spp. production, with 160,477 tonnes in 2011 (Lovatelli et al., 2013). From 1990, *Seriola* spp. production has rapidly increased and spread all over the world (Daniel et al., 2013). At the moment, several species are farmed, like yellowtail (*S. quinqueradiata*) in Japan and South Korea, yellowtail kingfish (*S. lalandi*) in Japan and Australia, longfin yellowtail (*S. rivoliana*) in the United States, and Pacific yellowtail (*S. mazatlana*) in North and Central America (Sicuro and Luzzana, 2016). In particular, greater amberjack has a long farming history, that started in 1978 in Japan, where it is still considered important, producing 38,770 tonnes in 2013 (Miwa et al., 2011; Matsunari et al., 2013a). *S. dumerili* farming further developed in the Mediterranean region in the second half of the 1980's, where major producers have been Spain, Italy, Malta, Croatia and Turkey. Recently, Taiwan, Korea, China and Saudi Arabia started the cultivation. (Sicuro and Luzzana, 2016).

Seriola spp. need a very particular diet, because requires a higher amount of crude proteins (47/50%) compared to other Mediterranean species (Jover et al., 1999; Takakuwa et al., 2006). Rotifers, *Artemia*, assisted by mesocosm technology, compose the larval rearing feeding. However, industrial potential of this species is still limited by mass production of high quality juveniles (Hamasaki et al., 2009; Yamamoto et al., 2013). For the recent development of this cultivation, specific microdiets has not been developed yet and there are just few studies about the requirement of docosahexaenoic acid (Matsunari et al., 2013a) and taurine (Matsunari et al., 2005, 2013b), during the first 30 days after hatching. Early weaning, an early transition between live feed and artificial diet, seems to be a good solution to reduce the mass mortality event before the 30 days. However, this technique, apparently, increased the aggressiveness of the larvae and led to cannibalism episodes (Miki et al., 2011). At the same time, some cultivations were heavily affected by viruses, bacteria and parasites infection, as anisakid nematodes, also dangerous for humans (Mushiake, 2006; Yoshinaga et al., 2006; FAO, 2020). Despite the research applied on impacts of biotic and abiotic parameters in *Seriola* spp. culture, a bottleneck is still present in the production during the hatchery production of seed (FAO, 2020). According to Roo et al. (2014) just 0.5% of larvae survive under intensive condition and 2.5% under semi-intensive. Several studies assess that feeding is the most limiting factor, claiming about a lack of timing between the food composition and the development of the digestive system of the larvae (Zaiss et al., 2006; Darias et al., 2007a; Kozarić et al. 2008; Shahriari et al., 2014).

Adjustments are supposed to lead to improved survival and growth rates (Chen et al., 2007).

3. Early digestive tract development of fish larvae

Fish larvae face remarkable morphological and physiological changes during their growth. The digestive system has the role for reducing the complexity of the molecules ingested with the help of digestive enzymes, and of absorbing the derived nutrients through the digestive epithelium. Fishes are divided in three categories, according to their stomach development. Agastric fishes do not develop a stomach in their entire life. Precocial ones present a stomach already existing at the onset of their first feeding, while altricial are subjected to a metamorphosis, developing a functional stomach during the larval development (Rønnestad et al., 2013). The complexity of the last group of fishes make them the most studied in research, because their larval rearing is more difficult, compared with the other categories. Also, *S. dumerili* is an altricial fish and the digestive tract expands from a short and strait tube, closed on the anus and mouth ends in the yolk-sac (Rønnestad et al., 2013). The stomach formation is considered the most variable factor in digestive system, fluctuating between few days and several months, even inside the same family (Rønnestad et al., 2013). Its first function is just as a reservoir of food (Rønnestad et al., 2013). The stomach transition stages have relevant importance, because they are used to identify the steps in growth (Tanaka, 1973). Finally, it acquires a digestive function, through gastric glands formation, that are responsible for H^+ secretion. This factor leads to pH decrease in lumen and pepsinogen production. A remarkable inter-specific variability is observed in the progress and extent of acidification, as studied in *S. aurata, Pagrus pagrus, Morone saxatilis and Paralichthys olivaceus* (Rush et al., 1993; Rønnestad et al., 2000; Yúfera et al., 2004; Darias et al., 2005).

The intestine development of fish larvae is gradual, resulting in segmented, histologically differentiated and looped gut in juveniles (Rønnestad et al., 2013). At the onset of the exogenous feeding, the gut can be grouped in three sections, foregut, midgut and hindgut, each one divided from the other through a muscular sphincter. The absorption capacity of the gut increases with growth, elongating the mucosa, folding and increasing the thickness of the epithelium, producing enterocytes and larger microvilli (Rønnestad et al., 2013). In this way, the residence time of food in the gut is increased as the mixing with digestive secretions (Kamisaka and Rønnestad 2011).

Liver (presenting also a reserve function), gallbladder, pancreas and relatively outlets are already differentiated at the yolk-sac stages. In fact, Langerhans cells and hepatocytes can be observed from the first feeding (Tanaka, 1969; Sarasquete et al., 1993; Guyot et al., 1998; Micale et al., 2008). In particular, pancreatic enzymes production and activity, as trypsin, have a remarkable importance in the

digestive process, attracting the attention of many studies in relation to fish diet (Rønnestad et al., 2013).

From an overall point of view, the ontogenetic development of the digestive system is divided in several phases (Buddington, 1985; Boulhic and Gabaudan, 1992; Bisbal and Bengtson, 1995). The first one (endotrophic or lecitotrophic stage) goes from hatching to the completion of the endogenous feeding, where the larvae nutrition is based on the yolk sac and oil globules. The second phase, not always present, is the mixed endogenous-exogenous feeding. That is, the opening of the mouth and the start of feeding occurred before the complete reabsorption of yolk reserves. The third phase (exotrophic stage) starts when the yolk sac is completely reabsorbed and the nutrition depends exclusively from the exogenous feeding. The commencement of feeding is the most critical period, because larvae are very sensible to starvation, in case of inappropriate or insufficient feeding (Kamler, 1992; Watanabe and Kiron, 1994, Yúfera and Darias 2007). During the first days of feeding up to the maturation of the gut and the development of a well-formed brush border in enterocytes, the larvae depend, primarily, on pinocytosis, intracellular digestion and absorption, for processing complex polypeptides (Govoni et al., 1986). With the gastric glands formation, the digestion capacity attains still higher hydrolysing capacity, similar to that in the juveniles and adults (Watanabe, 1982). For this reason, larvae prefer a feeding easy to digest, like rotifers. In the third phase, the larvae meet the metamorphosis, developing, gradually, the digestive systems and becoming able to accept inert pellet feeding (Bisbal and Bengtson, 1995; Gordon and Hecht, 2002). Timing, quantity and quality of exogenous feeding are fundamental for the larvae survival. A delay, or an inappropriate food supply after yolk absorption may lead in abnormal growth, slow development, digestive tract degeneration or loss ability of food digestion/ingestion (Kamler ,1992; Chen et al., 2007).

3.1 Digestive capacity in fish larvae

Enzymes from pancreas are fundamental in the protein digestion of fish first life stages, missing a functional stomach and its acid protease, pepsin (Rønnestad et al., 2013). Their activities vary between species, but they are usually produced from the onset of the first feeding (Rønnestad et al., 2013). On this purpose, the most important enzyme in marine fish larvae is trypsin, a serine protease that hydrolyses proteins (Govoni et al., 1986). Its activity, in fast growing species, has a relation with rearing success (Rønnestad et al., 2013). Trypsin activity is, conventionally, divided in four phases correlated to the ontogenetic development. Initially, during the yolk-sac stage and the endogenous feeding, the activity increases (Rønnestad et al., 2013). A first decrement appears in the middle stage, when larvae face the “critical period” of transition, correlated with low growth and high mortality rates,

following unclear mechanisms (Rønnestad et al., 2013). Then, a correct food supply leads to an activity recovery and, as a consequence, to fast growth again (Rønnestad et al., 2013). Finally, after the metamorphosis stage, it decreases, due to the stomach formation and pepsin secretion. Chymotrypsin is another enzyme produced by pancreas with a complementary activity to trypsin (Stryer, 1988). It is a serine alkaline protease, contributing to protein digestion which activity is present from hatching and increases during the larval stage (Rønnestad et al., 2013). Pepsin, an enzyme correlated to stomach formation during metamorphosis, is slowly developed and it is able to work just after a pH luminal acidification (Yúfera et al., 2004; Darias et al., 2007a; Yúfera et al., 2012). Pepsin is able to digest complex proteins, like collagen, enriching the diet of the fish (Gildberg, 2004). In the gut, in addition to pancreatic enzymes, there are enzymes associated with he enterocytes that also contribute to the final digestion of proteins and peptides. The brushborder membrane enzymes are located in the apical cell membrane facing the gut lumen. Between these enzymes are aminopeptidases, that hydrolyse small peptides that are the result of cleaving by pancreatic proteases (Rønnestad et al., 2013). The resulting small peptides and free amino-acids are easily absorbed by the enterocytes in the midgut (Rønnestad et al., 2013). Finally, there is speculation about exogenous enzymes role, from live food preys, that may support to some extent tryptic activity in some marine species (Rønnestad et al., 2013).

In fish larvae, phospholipids represent the most important lipid, from the nutrition point of view, probably because of the troubles for fish larvae to synthesize them (Coutteau et al., 1997). In fact, they provide energy, regulate proteins, compose membranes and they, also, act as messengers (Coutteau et al., 1997; Tocher et al., 2008). In this context, the main phospholipase (phospholipase A_2 - PLA2) is produced by the pancreas and is present in all the types of cells B (Murakami and Kudo, 2002). Lipids digestion activity is also present as gastric lipases, that hydrolyses triacylglycerols in free fatty acids and monoacylglycerols, for an easier digestion (Xiong et al., 2011). In the intestine, dietary lipids are absorbed by enterocytes, through the emulsioning activity mediated by phospholipids and bile salts, in some species available from the first feeding (Diaz and Connes, 1997; Diaz et al., 1997a,b). Lipases act from the first feeding and their activity, in several species, remain stable or drop during the larval stage (Murray et al., 2003, 2006; Perez-Casanova et al., 2004, 2006; Darias et al., 2007b). At the same time, from the first exogenous feeding, in some species gut, a neutral lipase activity (bile salt-activated lipase - BAL) is noticed. BAL may also be involved in phospholipids digestion, being observed in fish larvae species, without PLA2 activity in high phospholipids diet (Geurden et al., 1995; Fontagné et al., 1998; Cahu et al., 2003; Sæle et al., 2011). As the proteases, lipolytic enzymatic contribution from live preys may be important since rotifers have a very high lipase activity, compared to fishes. In the intestine,

these enzymes may collaborate with pinocytosis, with the same mechanism of protein absorption, digesting intact phospholipids in the posterior part of the gut (Farber et al., 2001; Hoehne-Reitan et al., 2001).

The role of carbohydrases in the digestion process during the fish early stages has received less attention than proteases and lipases, partly, due to the low carbohydrate content of the planktonic preys (Dhont and Van Stappen, 2003; Hamre, 2006). The ability to digest carbohydrates depends on fish feeding habits. During larval rearing, carbohydrates are present in the feed in many forms. The activity of α-amylase, an enzyme synthesized by the pancreas that digests complex carbohydrates, has been determined in many larval species (Ma et al., 2005; Suzer et al., 2007; Gisbert et al., 2009). The majority of fish larvae presents a high activity of α-amylase after the first feeding and a gradual decrease during maturation (Martínez et al., 1999; Ribeiro et al., 1999). In other cases, the activity peak is reached in the middle of larval stage, disappearing during the transition to juveniles (Álvarez-González et al., 2008; Gisbert et al., 2009). On the other hand, sometimes, a second peak in the end of the larval stage (Babaei et al., 2011; Sanz et al., 2011), or a continuous increase of the activity (Zouiten et al., 2008) are recorded. These differences, probably, just reflect the different feeding habits and the feeding protocols (Yúfera et al., 2018). In fact, carnivorous habits tend to reduce amylase activity when the stomach is becoming functional, while herbivorous and omnivorous species seem to exhibit an increase in activity as they approach the juvenile stage. Another enzyme, maltase, presents an initial activity increment after the first feeding and almost a constant level afterwards in *Dentex dentex* (Gisbert et al., 2009). Furthermore, mRNA expression of chitinase, involved in the crustacean exoskeleton assimilation, was detected in the gastric glands of some species, like *S. dumerili* (Kurokawa et al., 2004; Wu et al., 2011).

3.2 Digestive genes expression and regulation

Many studies about digestive enzyme activities have been published. However, regarding gene expression, published works are limited to just few species (Hansen et al., 2013; Sahlmann et al., 2015; Moguel-Hernández et al., 2016, Yúfera et al., 2018). Synthesis of mRNA codifying the digestive enzyme precursors is the previous step to synthetize the functional protein and its study is crucial to shed light on how the assimilation of macronutrient is programmed, activated and modulated, through the regulated secretion of enzymes (Yúfera et al., 2018). However, impediments have limited the research. Firstly, the elevated cost of this molecular analysis, even though new methods are making this process more accessible. Secondly, the long process to obtain transcript sequences and, thirdly, the complications in results interpretation, due to the possible existence of several isoforms that may codify

for other proteins (Yúfera et al., 2018). Low expression of genes encoding for trypsin and BAL precursors was noticed in *Salmo salar,* between 7-27 days post hatching (dph), while pepsin and α-amylase precursors expression levels appeared later, at 17 and 46 dph, respectively (Sahlmann et al., 2015). Equally to the enzymatic analysis, literature provided variability in results according to larvae feeding habits. Investigations on *Labrus bergylta* and *Gadus morhua s*howed high levels of amylase precursor expression on the onset of the first feeding (Kortner et al., 2011; Hansen et al., 2013), while in *Pelteobagrus fulvidraco* pepsinogen expression was detected since 1 dph (Gao et al., 2013). Ontogenetic profiles of activity and gene expression of proteases, lipases and carbohydrases present several types of results in literature. Generally, time patterns of increase and decrease and peaks of maximum values for both, activity and gene expression, are coincident (Srichanun et al., 2013; Sahlmann et al., 2015). However, this study suggests, that a delay or, in some cases, an anticipation between the detection of the molecular expression and of corresponding activity is also normal. In fact, the factors involved in the regulation may be synthesised with a different time sequence (Yúfera et al., 2018). On the other hand, some studies highlighted that gene expression for the main digestive enzymes seem to be just genetically programmed (Péres et al., 1998; Gilannejad et al., 2019). At the same time, other authors claim that genetic expression or enzymatic activity or both, in diverse larval stages, as in European seabass, are influenced by dietary proteins content (Cahu et al., 2004, Wang et al., 2006). In European seabass and gilthead seabream larvae, diet is also able to regulate lipase activity, but it does not affect the gene expression (Morais et al., 2004b; Mata-Sotres et al,. 2014). The variety of these results suggest a species-specific component and a probable age-dependent effect in the transcriptional or post-transcriptional regulation of the enzyme function (Yúfera et al., 2018).

3.3 Related literature in *Seriola* spp.

A study focused on the histological and biochemical development of the digestive system in *S. dumerili* larvae has been recently published by Pérez et al. (2020). During endogenous feeding, the digestive tract is reported to be limited to a closed straight tube located dorsally to the yolk sac. The liver develops early, with hepatic cells that appear at 2-3 dph. They are initially located behind the yolk sac under the anterior intestine and they later surround the anterior part of the intestine. The pancreas presents an undifferentiated tissue at 2 dph, but at 4-5 dph it starts to be differentiated in endocrine and exocrine regions. The first differentiation events of the digestive system occurs with the mouth and the anus opening, at 3-4 dph. During this period, the digestive tract starts to be divided in distinct regions. At 6 dph, the esophageal mucosa folds leave space for a following stomach development, in proximity of cardiac and pyloric sphincters. From 8-10 to 30 dph supranuclear vacuoles are visible in the larval

hindgut, while first taste buds appear along the buccopharyngeal epithelium at 12 dph. From 15 dph, goblet cells start to materialize in the esophagus, increasing their number over time. At the same time, the first pharyngeal teeth appeared at the posterior part of the buccopharynx area. The stomach functionality acquisition is demonstrated by the first gastric glands appearance at 17-20 dph, that are located at the pyloric portion. This event is the one that register the highest interest in publications, presenting variations due to different rearing temperatures and feeding protocols. In fact, in Cruces et al. (2016) and Pérez et al. (2020) studies it is reported between day 17 and 20 dph, while the one from Wu et al. (2011) at 13 dph. Finally, in the midgut area, supranuclear bodies develop between 20-25 dph while first goblet cells appear 23-25 dph.

The mRNA expression of digestive enzymes precursors is a topic still untreated by literature on this species. No papers have been published about their patterns. Besides, according to what was previously told, more interest was noticed by enzymes activity. However, in most of the publications, the attention was put to variations of the enzyme activity in response to changes in environmental factors, like the water temperature or dissolved oxygen concentration (Kofuji et al., 2005; Miegel et al., 2010; Bowyer et al., 2014). In other studies, the correlation between diets and digestive enzymes activity is evaluated (Ma et al., 2014; Navarro-Guillén, 2019). Investigations more focused on ontogenetic development of digestive tract and enzymes are presented by Chen et al. (2006), Wu et al. (2011) and Pérez et al. (2020).

4. Objectives

The **main objective** of this study is to attain a deep knowledge of the development of the full digestive capacity in larvae and early juvenile of the greater amberjack *S. dumerili*. The final aim is to have a good understanding of this development in order to improve feeding procedures during this early stages.

Specific objectives:

1- To assess the appearance and sequential pattern of the different digestive enzymes at molecular level by examining the gene expression of key digestive enzymes precursors.

2- To assess the appearance and sequential pattern of the activity of key digestive enzymes.

3- To examine the diurnal pattern of larvae digestive functionality from the molecular and biochemical perspectives.

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Molecular and enzymatic analysis of feed digestion in *Seriola dumerili* larvae

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ABSTRACT

The greater amberjack, *Seriola dumerili*, a fast growing pelagic fish, was one of the species selected to increase the farmed fish variety in Europe, due to its adaptation to captivity conditions, good flesh quality and high market demand. Inadequate feeding protocol has been suggested as one of the causes behind mortalities during the larval stage. Aiming to advance feeding optimisation during the early stage of this species, this study examines the ontogeny of digestive function using molecular and biochemical approaches. Gene expression of digestive enzyme precursors and the enzyme activity has been determined during the first 51 days post hatching (dph) and the diurnal period of 19 dph larvae, reared in semi-intensive conditions. The expression of pancreatic proteases precursors (*try3*, *ctra* and *ctrb1*) increased from first-feeding, while gastric chitinase (*chia1*), gastric protease (*pga3*) and proton pump (*atp4a2*) after 10 dph. The precursor genes of pancreatic lipases (*cel1, cel2* and *cel3*) peaked between 6 and 10 dph, phospholipase A2 (*pla2g1b*) rose only after 25 pdh, while α-amylase (*amy2a*) increased mainly from 20 dph. The trypsin activity was more evident from first-feeding to 25 dph and chymotrypsin activity from this day onwards. Acidic chitinase and pepsin activity appeared, respectively, at 16 and 30. The activity of 7C-like lipase was evident from first-feeding but, similarly to 4C-like lipase significantly increased from 15 dph, while amylase peaked from 6 to 22 dph. Aminopeptidase and alkaline phosphatase started at 20 dph indicating the functional maturation of brush border of the enterocytes. The daily pattern analysis showed a food anticipatory strategy in the expression proteases related genes. Lipases activity was more evident during the morning hours, followed by amylase, and, in the afternoon, by proteases. Results, also, suggested an alternation in the activity of chymotrypsin and trypsin. These ontogenetic pattern are concordant with a carnivorous marine teleost.

Keywords: daily rhythm, enzymatic activity, digestive genes, larvae ontogeny, *Seriola dumerili*

1. INTRODUCTION

The greater amberjack *Seriola dumerili* (Risso, 1810) is a pelagic teleost inhabiting temperate and warm marine waters around the world (Thompson et al., 1999). This species is gathering an increasing interest for the aquaculture industry worldwide (Sicuro and Luzzana, 2016), being also considered a primary candidate for diversification of marine aquaculture in the Mediterranean region. This species has a high tolerance to handling in captivity and farming conditions, exhibits faster growth than most commonly farmed Mediterranean gilthead seabream *Sparus aurata* and European sea bass *Dicentrarchus labrax* and has an excellent flesh quality (Muraccioli et al., 2000; Nakada, 2002; Jerez et al., 2006). As in other fish species that are currently being commercially farmed, a crucial step in the way to industrialisation is to develop suitable spawning and larval rearing methodologies to obtain robust and healthy juveniles. Larval rearing techniques of greater amberjack has progressed mainly during the last 15 years using different methodologies from mesocosms to intensive systems and adjusting feeding with live prey and weaning protocols (Papandroulakis et al., 2005; Papadakis et al., 2008; Hamasaki et al., 2009; Matsunari et al., 2013; Yamamoto et al., 2013; Navarro-Guillén et al., 2019). Nevertheless, there are still some problems to solve during this stage to prevent mortalities and size dispersion due to pathologies, malnutrition and cannibalism (Mushiake, 2006; Yoshinaga et al., 2006; Miki et al., 2011; FAO, 2020).

To provide adequate feeding is one of the principal conditions for success in fish larval rearing (Yúfera and Darias, 2007; Hamre et al., 2013; Rønnestad et al., 2013). A good ingestion and an efficient digestion of nutrients are necessary to meet the high demand of matter and energy able to support such high growth during the larval stage, particularly in fast growing species as greater amberjack with daily growth rates up to 11% (Mazzola et al., 2000; Navarro-Guillén et al., 2019). Consequently, good growth and survival requires an appropriate prey-food sequence matching the predatory capacities of developing larvae (Polo et al., 1992; Yúfera, 2011) and their nutritive requirements (Hamre et al., 2013) as well as a suitable weaning onto inert feeds (Engrola et al., 2009; Parma et al., 2013). Furthermore, an efficient digestion of ingested food (live prey and inert feed) is a key factor along this developmental period toward the juvenile stage.

Digestive function changes progressively during the larval stage from first feeding for improving the hydrolysing capacity up to attain the definitive digestion mode of juvenile and adults. These changes are linked to ontogenetic anatomical modifications in the digestive system (Yúfera at al., 2011). These anatomical and physiological changes appear progressively during the first weeks of larval growth supported by an adequate feeding. Food dependence is still more exigent in a fast

developing species in which the changes may occur faster. Digestion is a complex process with a tight regulation depending on external and internal signals. The enzymatic precursors are synthetized from their corresponding mRNA transcripts that on turn are synthetized at due time, depending on the genetic developmental programme and the mentioned signals; these enzyme precursors are then released and activated in presence of food (Yúfera et al., 2018). Therefore, there are two main factors affecting the activity level of the different digestive enzymes during the larval stage, the age and feeding conditions. There is an abundant literature referring the studies on the ontogeny of the digestive enzyme activities in marine fish (see reviews by Lazo et al., 2011; Gisbert et al., 2013; Rønnestad et al., 2013; Yúfera et al., 2018). The information on the ontogeny of the gene expression of RNAm codifying digestive enzymes, although less abundant and more fragmentary, is also important (Murray et al., 2003, 2006; Darias et al., 2005, 2006; Sæle et al., 2010; Kortner et al., 2011a,b; Galaviz et al., 2012, 2015; Hansen et al., 2013; Srichanun et al., 2013; Murashita et al., 2013, 2014; Mazurais et al., 2015; Mata-Sotres et al., 2016a; Moguel-Hernández et al., 2016; Gilannejad et al., 2019, 2020; Khoa et al., 2019, 2021). However, the digestive response during feeding has been less studied in fish larvae. In most of species the ingestion and digestion are related to light/dark or presence/absence of food cycles but detailed daily sampling is necessary to determine these daily patterns what is a more laborious task (Tillner et al., 2013; Navarro-Guillén et al., 2015, 2017; Mata-Sotres et al., 2016b; Zeytin et al., 2016). These studies indicated that molecular expression and activity level vary along the daily cycle and not necessarily progressing in parallel (Mata-Sotres et al., 2016b; Yúfera et al., 2018).

Ontogeny of the digestive capacity has been studied in yellowtail kingfish *Seriola lalandi* (Chen et al., 2006; Ma et al., 2014). There are also two recent studies on this subject on greater amberjack, one focusing the weaning period (Navarro-Guillén et al., 2019) and other describing the ontogenetic pattern during the first month after hatching (Pérez et al., 2020), both of them examining only changes in the enzymatic activities. In the present study, we have determined the ontogenetic pattern of both the digestive enzyme activity and the gene expression of enzyme precursors during the first weeks of life. In addition, we have determined their patterns during the diurnal period in order to get a first insight on the daily cycle of the digestive functionally.

2. MATERIALS AND METHODS

2.1 Larval rearing and sampling

Greater amberjack larval rearing was performed in the facilities of Futuna Blue España S.L. (Puerto de Santa María, Cádiz, Spain). Larvae from three successive spawning were reared in three 20

m^3 tanks in a RAS system. Photoperiod was 14 h light: 10 h dark, water salinity was maintained around 35 gL^{-1} and temperature 24.5 ºC. Larvae were fed according to the following protocol: rotifers (*Brachionus plicatilis*) from 3 to 15 days post hatching (dph), *Artemia* nauplii from 10 to 30 dph, copepods nauplii and copepodites (*Acartia* sp + *Tigriopus* sp) from 5 to 30 dph and commercial feed from 22 dph onwards (Fig. 1). After 30 dph, larvae were exclusively fed on inert diet and considered weaned.

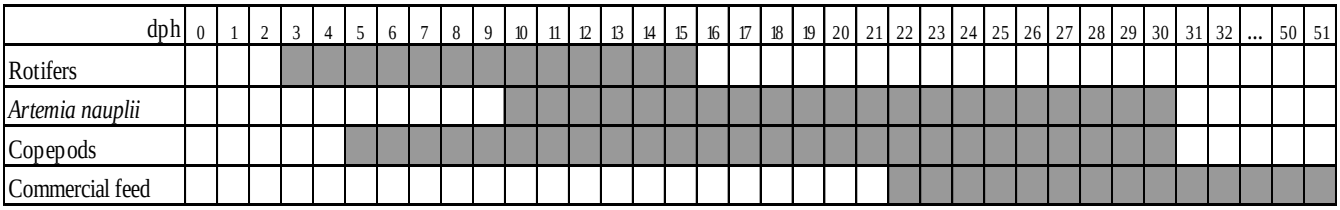

Fig. 1 Schematic drawing of experimental feeding regime of greater amberjack larvae during the experimental period (0 to 51 dph).

Larvae were collected periodically from 1 dph to 30 dph for gene expression analysis, and from 1 dph to 51 dph for enzyme activity analyses. Larvae were always sampled at the same time (11:00 h) to avoid differences between sampling points due to feeding condition. From the pool of larvae sampled each day from the three tanks, 6 individual larvae were used for gene analyses and 5 (1 - 47 dph) and 3 (51 dph) individual larvae for enzymatic activity analyses.

After sampling, larvae were slaughtered with an overdose of tricaine methanesulfonate (MS-222), rinsed in distilled water and immediately preserved in RNA*later* (Thermo Fisher Scientific) or frozen and then freeze-dried for the posterior analysis of gene expression levels or digestive enzymes activity, respectively. Previous to enzymatic analysis, the same larvae were used for the determination of individual dry weight. Individual growth was also evaluated through the relative growth rate (RGR, % day^{-1}) following the formula in Ricker (1958):

$$RGR - (e^{g} - 1) * 100, \text{ where } g - [(In_{\text{final weight}} - In_{\text{initial weight}}) / \text{time}]$$

To evaluate the diurnal pattern of gene expression levels and digestive enzyme activities, at 19 dph additional samples were collected along the daytime and processed as described above. Sampling times were set at 7:30, 9:30, 13:30, 17:00 and 21:00 h.

2.2 Quantification of gene expression levels (qPCR)

For the gene expression analyses, RNA was extracted from the whole larvae using

NucleoSpin® RNA XS kit (Macherey-Nagel) for individuals with a mass inferior to 5 mg, and NucleoSpin® RNA kit (Macherey-Nagel) for heavier ones (from 30 dph). Samples were incubated in a solution composed by a lysis buffer (RA1) and a reducing agent. A rotor stator homogenizer shredded the tissues. Ethanol was added to the homogenized lysate, then a filtration bound the RNA. A membrane desalting buffer (MDB) desalted the silica membrane of the filter, while rDNase RNAse free mixture digested the gDNA from the column. RA2 and RA3 buffers, respectively, inactivated rDNAse and washed the silica membrane. Finally, RNase free water eluted highly pure RNA from the column. RNA was stored, until the utilization, at -80°C.

The extraction process effectiveness was determined in two ways. The first one consisted in measuring the RNA concentration with a Qubit® 2.0 Fluorometer (Invitrogen, life Technologies) using Qubit® RNA BR Assay Kit (Molecular Probes®, Life technologies). The second control method implied the Bioanalyzer 2100 and the RNA 6000 Nano kit (Agilent Technologies, LifeSciences). Agilent 2100 Bioanalyzer checked the RNA quality, giving us the RNA integrity number, a coefficient that ranges from 1 (low quality) and 10 (high quality). Samples resulting with a lower coefficient than 7.5 or 15 ng/µL concentrations were discarded. RNA was reverse transcribed, in order to obtain cDNA. Nuclease-free water, reverse transcriptase and a reaction mix, from the qScript™ cDNA synthesis kit (Quanta BioSciences) were added to each RNA. The kit included a thermal cycle procedure: 5 minutes at 22 °C, 30 minutes at 42 °C and 5 minutes at 85 °C. To quantify the gene expression primers, functioning as the DNA amplification starting point, had to be set. Digestive genes were taken from a previous RNAseq study on the same *S. dumerili* stock using liver, kidney, pituitary gland and brain tissues. BLAST software utilisation obtained the whole predicted cDNA sequences per each gene, through digital alignment with GenBank database. Primers for trypsinogen (*try*), chymotrypsinogen (*ctrb*), pepsinogen (*pga*), proton pump (*atp4a*), amylase (*amy2a*), bile salt-activate lipase (*cel*), chitinase (*chia*) and peptide transporter 1 (*slc15a1*) were designed from poly-A cDNA sequences using the software Primer3 v.0.4.0 (available in http://bioinfo.ut.ee/primer3-0.4.0/), and they were synthesized by IDT® (Integrated DNA Technologies). Assay linearity and amplification efficiency of each gene were assessed by iQSYBR® Green Supermix (Bio-Rad kit, in order to check the primers effectiveness. They were tested in a temperature gradient between 55 and 65 °C. PCR setting was: 95 °C, 10 min; [95 °C, 15 s; 60 °C, 30 s]×40 cycles; melting curve 60–95 °C, Δ 0.5 °C/5 s. Efficiency and R^2 consisted in the parameters used to check the primers replication efficiency. The accepted values ranged between 90 % and 110 % for the efficiency, while for R^2 had to be greater than 0.950 (Table 1). Relative gene expressions were quantified in a CFX 96 system (Bio-Rad) under the control of Bio-Rad

CFX Maestro 1.1 software.

Table 1. Oligonucleotides used for qPCR quantification, their amplicon length, position in the sequence, primers concentration in the reaction, amplification efficiency and R^2 of the calibration curve.

GENE	sequence	amplicon lenght	position		primers concentration (nM)	efficiency (%)	R^2
sdQchia1-F1	CAAAGCCCACCACCACAC	127	1208	1629	200	105.9	0.998
sdQchia1-R1	CCCTGGAAGCACTGGAAATA		1334		200		
sdQcel1-F1	ATGCCACAAATGCTCAGTG	141	1500	1766	200	90.4	0.998
sdQcel1-R1	TTTCACCCACAAGGTTTATTG		1640		200		
sdQcel2-F1	CACCAACCCTACCTGAGCAT	146	1560	1835	200	94.5	0.995
sdQcel2-R1	CAGTTCTCCGTCTCGTCCTC		1705		200		
sdQcel3-F1	AGCCTTTCGTCACACCACT	118	1443	1823	200	105	0.999
sdQcel3-R1	ACACGCAGTCCTCCTTTGTT		1560		200		
sdQpla2g1b-F1	CACCTGTCTGAATAACAACAACG	141	328	480	200	95.6	1
sdQpla2g1b-R1	CATCCCTCGGCTCACTTG		468		200		
sdQctrb1-F1	TCTCCAACGAGCAGTGTAAGAG	116	573	937	200	95.9	0.999
sdQctrb1-R1	ACAGACCAGAGGACCACCAG		688		200		
sdQctra-F1	GGCAACAAGATCAGCAACCT	128	615	911	200	97	0.999
sdQctra-R1	CAGGACACAATTCCAACCAG		742		200		
sdQamy2a-F1	GTGGTAATCGTGGTTTCATCG	122	1439	1722	200	96.3	0.998
asQamy2a-R1	CCTGTTTCCTTCCTTCTGTCC		1560		200		
sdQslc15a1a-F1	CGGCTGGTTGTTTACTGTTG	109	1959	2408	200	98.3	0.996
sdQslc15a1a-R1	CAGGAGAGAGGCAAAGAGGA		2067		200		
sdQslc15a1b-F1	TGATGTTGTCGTATGCTCCAG	122	2700	2874	200	100	0.997
sdQslc15a1b-R1	AATGAAACCACTGCTGATGC		2821		200		
sdQpga3-F1	GGCTCAGTACATCGGTCTGG	130	1101	1297	200	105.6	0.996
sdQpga3-R1	GTCCCATTTGCACCCTTCT		1230		200		
sdQactb-F	CAGTGGTTGGCGCATACTTA	145	1435	1738	200	92	0.999
sdQactb-R	GAAGAGGTCACGATTGGGTTT		1560		200		
sdQef1a-F	CCCTGGATCACCTTCTCTGA	142	1533	1808	200	93.6	0.999
sdQef1a-R	TAAGAGGCACCGTCATGTGA		1674		200		
sdQgh-F	AGGCGAAGAGTTGCTGAGAC	105	575	885	200	97	0.998
sdQgh-R	GGAGAGAGCCGACATTTAGC		679		200		
sdQatp4a-F2	TGGTGGTTTGTTCCTCTTCC	90	2886	3433	200	90.9	0.999
sdQatp4a-R2	CCAACTTCCTGGGTATCTTCTG		2975		200		
sdQtry-F3	CCACGGAGGACGACAAGAT	80	63	882	200	99.6	0.987
sdQtry-R3	GCCAGAGTTCAGAGACACCTG		142		200		

Two internal genes, *actb* and *ef1a*, were used as references, due to their transcriptional stability. In these genes, the ontogenic M coefficient was 0.34 while the daily was 0.44. These values are included in the acceptable range ($0<M<0.5$) from BioRad CFX Manager. Reactions for qPCR were carried, containing a mix of forward and reverse primers, replicated per each gene, iQ SYBR® Green supermix and cDNA. As the previous passage, PCR protocol was: 95 °C, 10 min; [95 °C, 15 s; 60 °C, 30 s]×40 cycles; melting curve 60–95 °C, Δ 0.5 °C/5 s. All the samples were run in triplicate.

2.3 Digestive enzyme activity analyses

To prepare the enzyme extracts, every single fish was mechanically homogenized using an Ultra-Turrax® Homogenizer T-18 (IKA®-Werke). Extracts were prepared following the protocol described by Gisbert et al. (2018), consisting in two steps; the first one to extract gastric and pancreatic enzymes, and a second step for brush border (BB) membranes purification. Briefly, larvae were homogenized in 50 mM mannitol - 2 mM Tris (pH 7) and centrifuged at 9000 g, 10 min, 4 °C. The supernatant was recovered and used for the analysis of gastric and pancreatic enzymes (pepsin, acidic chitinase, trypsin, chymotrypsin, amylase and lipases). 10 mM $CaCl_2$ was added to the remaining pellet of cell debris and a second centrifugation was performed at 16000g, 40 min, 4 °C. The supernatant was discarded and the pellet (containing BB) was dissolved in 50 mM mannitol - 2 mM Tris (pH 7). This fraction was used for the analysis of BB enzyme activities (alkaline phosphatase and aminopeptidase-N). All samples were kept in ice during the process described above in order to avoid enzymes denaturation and/or damage. Enzyme extracts were kept at −20 °C until analysis.

Pepsin activity was determined by the method of Anson (1938): 15 µL of extracts were mixed with 1 mL of 2% acid-denatured bovine hemoglobin (Sigma) diluted in 0.2 M HCl-glycine buffer (pH 2). After incubation at room temperature for 20 min, the reaction was stopped by adding 0.5 mL of 20% trichloroacetic acid (TCA, VWR), cooled to 4 °C for 15 min and then centrifuged at 11000 g for 15 min. The absorbance of the resulting supernatant was measured at 280 nm. Blanks were constructed by adding the enzyme extracts to the reaction mixture just after the TCA.

Acidic chitinase activity was assayed through a modification of German et al. (2015) protocol. The fluorogenic substrate 4-methylumbelliferyl-N-acetyl--d-glucosaminide (69585, Sigma-Aldrich) was dissolved in HCl-glycine buffer (pH 3), to a final concentration of 200 µM. For analysis, 90 µL of substrate and 15 µL of fish homogenate were added to the microplate. Fluorescence was measured at 365 nm (excitation) and 450 nm (emission).

For pancreatic protease activities, the fluorogenic substrates Boc-Gln-Ala-Arg-7-methylcoumarin hydrochloride (BOC, B4153-Sigma-Aldrich) and N-SuccinylAla-Ala-Pro-Phe-7-amido-4-methylcoumarin (S9761, Sigma-Aldrich) were used for trypsin and chymotrypsin analyses, respectively. Substrates were diluted in dimethyl sulfoxide (DMSO), to a final concentration of 20 µM. For analysis, 5 µL of these substrates, 190 µL of 50 mM Tris +10 mM $CaCl_2$ buffer (pH 8.5) and 15 µL of the fish homogenate were added to the microplate (Rotllant et al., 2008). Fluorescence was measured at 355 nm (excitation) and 460 nm (emission).

Lipase activities were determined using 4-methylumbelliferyl butyrate (19362, Sigma-Aldrich)

and 4-methylumbelliferyl heptanoate (M2514, Sigma-Aldrich). Substrates were dissolved in phosphate buffer (pH 7) to a final concentration of 0.4 mM, modified method from Rotllant et al. (2008). 15 µL of the fish homogenate was added to the microplate and mixed with 250 µL of substrate for the analysis. Fluorescence was measured at 355 nm (excitation) and 460 nm (emission).

Ultra Amylase Assay Kit (E33651, Molecular Probes), was used for amylase analysis. The substrate contained in this kit is a starch derivative labeled with a fluorophore dye. Substrate was diluted in 3-(N-morpholino) propane sulfonic acid (MOPS; pH 6.9) and substrate solvent (sodium acetate; pH 4.0), to a final concentration of 200 µg/mL. For analysis, 50 µL of the substrate solution and 15 µL of the fish extract were added to the microplate. Fluorescence was measured at 485 nm (excitation) and 538 nm (emission).

For alkaline phosphatase analysis the substrate used was 4-Methylumbelliferyl phosphate disodium salt, (MUP, M8168 Sigma-Aldrich). A 1 mmol/L stock solution of MUP was prepared by dissolving the substrate in borate buffer (pH 8). 15 µL of the enzymatic extract was added to the microplate and mixed with 100 µL of substrate for the analysis (modified from Fernley and Walker, 1965). Fluorescence was measured at 360 nm (excitation) and 440 nm (emission).

For aminopeptidase-N activity, the fluorogenic substrate Nα-Benzoyl-L-arginine-7-amido-4-methylcoumarin hydrochloride (B7260 Sigma-Aldrich) was diluted in dimethyl sulfoxide (DMSO), to a final concentration of 20 µM. For analysis, 5 µL of this substrate, 190 µL of 50 mM Tris buffer (pH 8.5) and 15 µL of the fish homogenate were added to the microplate (Rotllant et al., 2008). Fluorescence was measured at 355 nm (excitation) and 460 nm (emission).

Pepsin activity was indicated in activity unites (U) per mg of fish dry weight. One activity unit was defined as the amount of enzyme that is required to hydrolyse haemoglobin to give 1 µg of tyrosine in 1 min at pH 2. For that, the molar extinction coefficient of tyrosine in HCl-glycine buffer (pH 2) was calculated. For the remaining enzymes analysed, for which fluorogenic substrates were used, the liberation of the fluorophore was kinetically followed, and activities were expressed as relative fluorescence units (RFU) per mg of fish dry weight.

2.4 Statistics

Outliers were excluded through the use of GraphPad software and, in the specific, Grubb test with ESD method (https://www.graphpad.com/quickcalcs/Grubbs1.cfm), with alpha set as 0.05. Statistical analysis was performed with GNU PSPP 1.2.0 software. Significant differences in gene expression levels and enzyme activities during ontogeny and the diurnal cycle were analysed by one-way ANOVA after assessing equality of variances by a Levene's test. Post hoc multiple comparisons were carried out

using Tukey's test ($P > 0.05$). Results are given as means and standard deviations (SD).

3. RESULTS

Greater amberjack larvae showed exponential growth during the experiment. At the end of the experiment (51 dph) larvae reached a final dry weight of 0.372 ± 0.025 g (Fig. 2). Average RGR, considering all the experimental period, was 20.61% day^{-1}. When analyzed by age intervals, average RGR for the period of development with a moderate growth performance (1-30 dph) was 20.53% day^{-1}, followed by RGR of 29.36, 5.87 and 34.33% day^{-1} for the intervals 30-39, 39-47 and 47-51 dph, respectively.

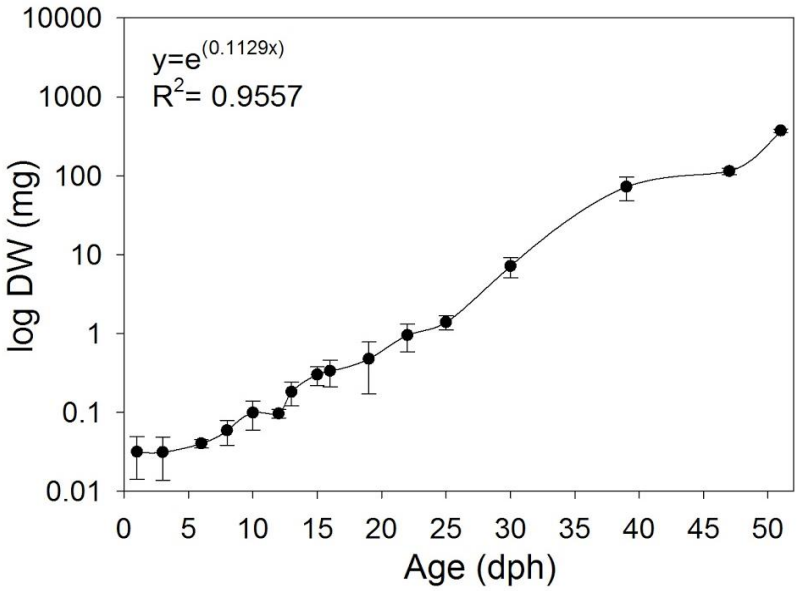

Fig. 2. Growth of *Seriola dumerili* from 1 to 51 dph measured as dry weight (mg $larva^{-1}$). Values are represented as means ± SD. Different letters represent significant differences in dry weight between ages ($p < 0.05$).

3.1 Ontogeny of the digestive function in greater amberjack larvae

3.1.1 Ontogeny of the molecular expression of digestive enzymes precursors

With respect to genes related to stomach development and functionality, expression of *pga3* and *atp4a* only started at 19 dph, with a progressive increase in expression levels up to the end of the experiment (Fig. 3). On the other hand, an increasing trend was observed for *chia1* expression pattern from 10 dph onwards until the end of the experiment, with a slight and non-significant decrease between 20 and 22 dph (Fig. 3).

Concerning alkaline digestion, expression of proteases precursors, trypsinogen and chymotrypsinogen, were detected from early stages of development. The expression pattern of *try*

started at 3 dph with a significant increment up to 13 dph. It was observed a gradual decrease from 13 to 22 dph, afterwards the expression increased to reach the maximum level at 25 dph, maintaining similar values until the end of the experiment (Fig. 3). Regarding chymotrypsinogen transcripts, *ctra* and *ctrb1*, both followed similar ontogenetic expression patterns. Expression pattern increased from 3 dph, reaching the maximum levels at 8 dph. Similar to trypsinogen expression, a decrease was observed until 22 dph, followed by a marked increase at 25 dph, which specifically for *ctra* was maintained until the end of the experiment (Fig. 3). Peptide transporters expression patterns, *slc15a1a* and *slc15a1b*, were similar and characterized by low expression levels during the first days. Preceded by oscillations between 10 and 20 dph, both transcripts reached maximum expression levels at 22 dph, followed by a decreasing pattern between 22 and 30 dph (Fig. 3).

The three genes coding for carboxyl ester lipases (*cel1*, *cel2* and *cel3*) were expressed from early stages of development (3 dph), reaching the maximum levels of expression between 6 and 10 dph, followed by a decreasing expression pattern until 30 dph (Fig. 4). By contrast, bile salt activated lipase precursor, *pla2g1b*, showed a delayed expression pattern, being noticed only at 30 dph (Fig. 4).

Low expression levels were recorded for the amylase precursor, *amy2a*, up to 19 dph. A notable peak of expression was observed at 25 dph, followed by a significant decrease at 30 dph (Fig. 4).

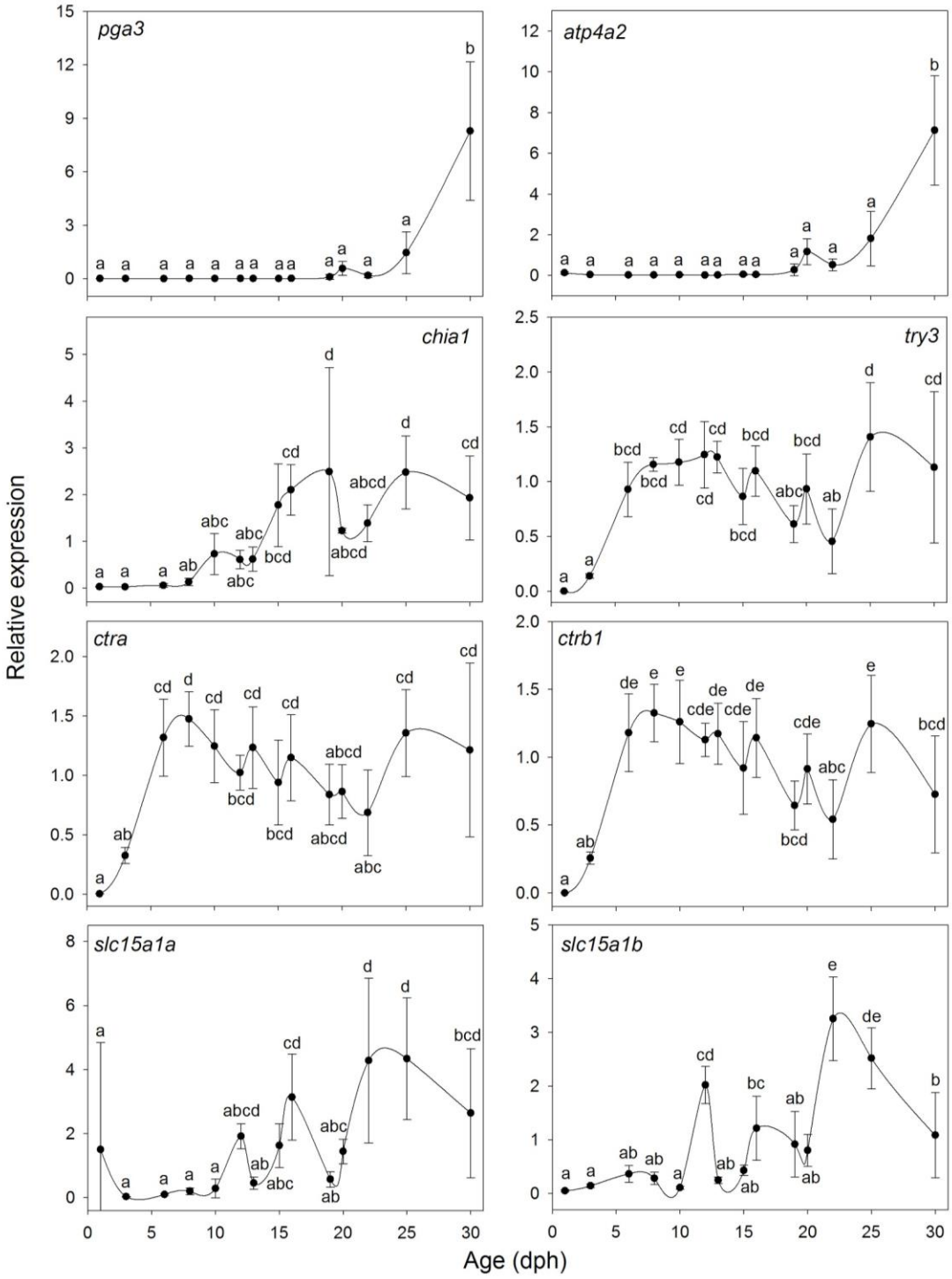

Fig. 3. Gastric protease (*pga3*), proton pump (*atp4a2*) and chitinase (*chia1*), and pancreatic proteases (*try3*, *ctra* and *ctrb1*) and peptide transporters (*slc15a1a* and *slc15a1b*) precursors relative expression during *Seriola dumerili* larvae development. Values are represented as means ± SD (n = 6). Different letters represent significant differences in relative expression between ages ($p < 0.05$).

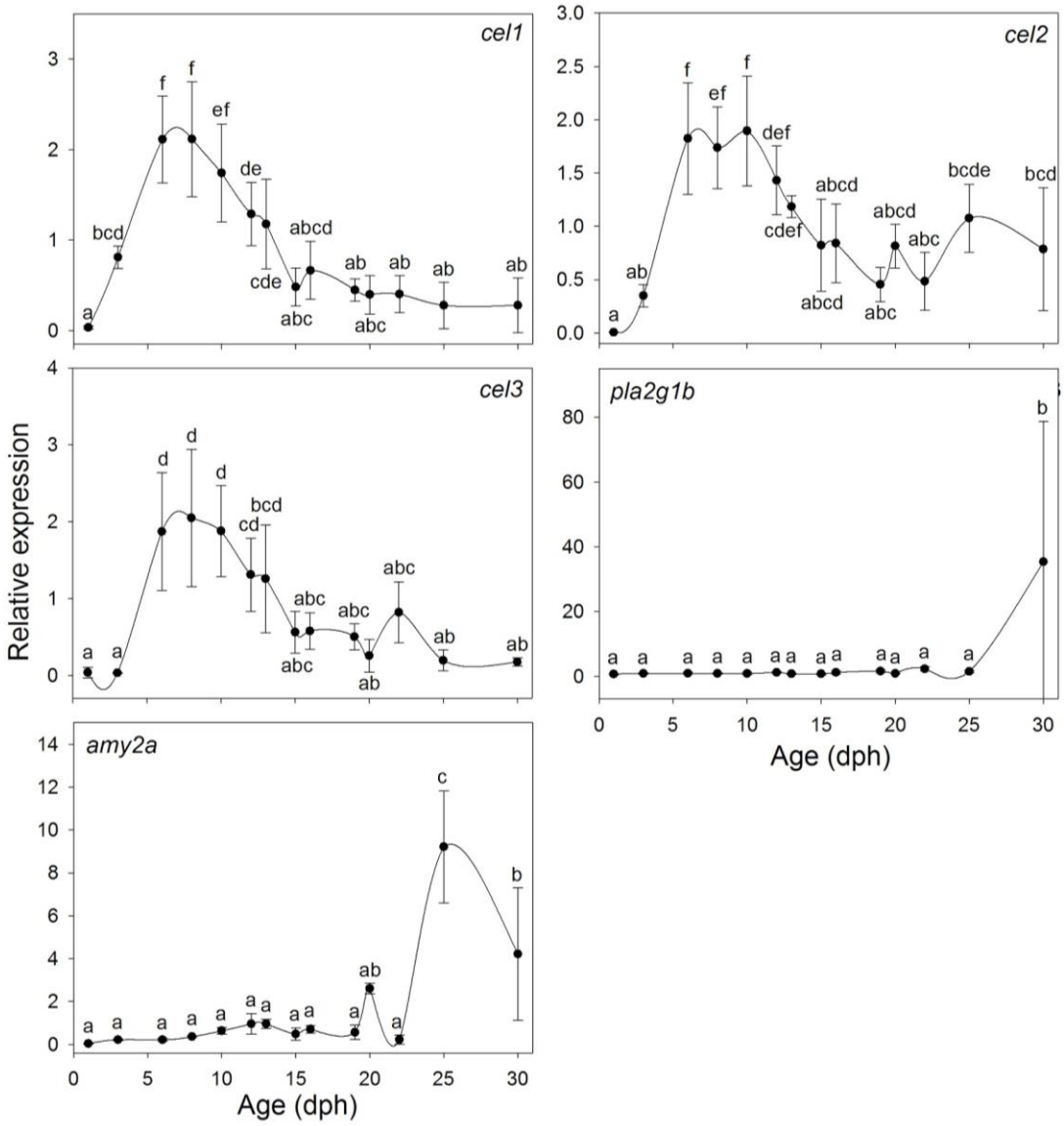

Fig. 4. Carboxyl ester lipases (*cel1*, *cel2* and *cel3*), bile salt activated lipase (*pla2g1b*) and amylase (*amy2a*) precursors relative expression during *Seriola dumerili* larvae development. Values are represented as means ± SD (n = 6). Different letters represent significant differences in relative expression between ages ($p < 0.05$).

3.1.2 Ontogeny of digestive enzyme activities

Regarding acidic enzymes, pepsin activity was recorded for the first time at 39 dph. The activity pattern trended to increase between 39 and 47 dph, followed by a decrease at 51 dph (Fig. 5). On the other hand, acidic chitinase activity was recorded for the first time at 19 dph. Activity statistically increased with development reaching the maximum at 30 dph and thereafter maintained similar values until 51 dph (Fig. 6).

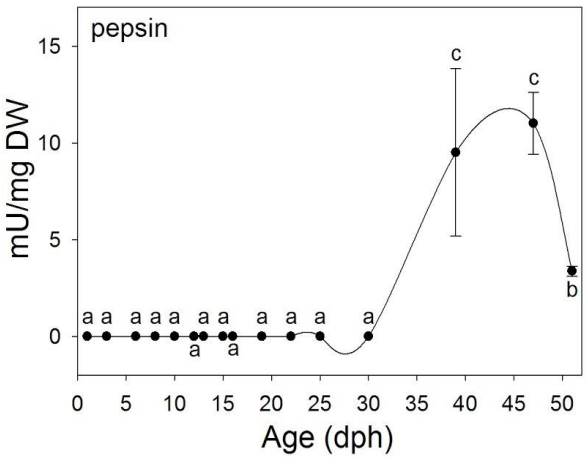

Fig. 5. Pepsin activity (mU mg larva DW^{-1}) during *Seriola dumerili* larvae development. Values are represented as means ± SD (n = 5 from 1 to 47 dph; n = 4 at 39 dph, n = 3 at 51 dph). Different letters represent significant differences in activity between ages ($p < 0.05$).

Regarding alkaline digestion, trypsin presented low activity levels between 1 and 6 dph, with a marked increase at 8 dph, achieving the highest activity value at 10 dph. A statistical decline was observed in trypsin activity from 12 dph onwards, reaching between 19 and 51 dph values similar to those recorded at first days post-hatching. Chymotrypsin activity started to be detected at 30 dph, followed by a marked decrease from 39 dph onwards. Trypsin : chymotrypsin ratio (T:C) was 1.78 ± 0.27, 33.61 ± 11.01, 26.27 ± 13.62 and 75.41 ± 60.57 for 30, 39, 47 and 51 dph, respectively (Fig. 6).

The activity of lipase toward 4 and 7 carbon substrates showed slightly different patterns along ontogeny. Overall, higher values of activity were measured toward heptanoate, while much lower activity was measured toward butyrate. Thus, 7C-like lipase showed activity from 1 dph, with a progressive increment along development, reaching the maximum activity value at 25 dph, followed by a gradual decrease. On the other hand, 4C-like lipase activity started at 15 dph, being coincident with 7C-like lipase in a maximum peak at 25 dph. A marked decrease in activity was observed from 30 dph onwards (Fig. 6).

Amylase activity reached the highest activity value at 8 dph, maintaining the activity levels until 22 dph, followed by low levels of activity between 25 and 51 dph (Fig. 6).

Concerning brush border enzyme activities, on average, aminopeptidase activity was low during the period under study. Activity was recorded for the first time at 22 dph, however, a statistical decline was observed at 25 dph, followed by basal levels of activity until 51 dph. By contrast, alkaline phosphatase activity started at 15 dph, showing two peaks of higher activity, the first one between 22 and 25 dph and the second one at 51 dph (Fig. 6).

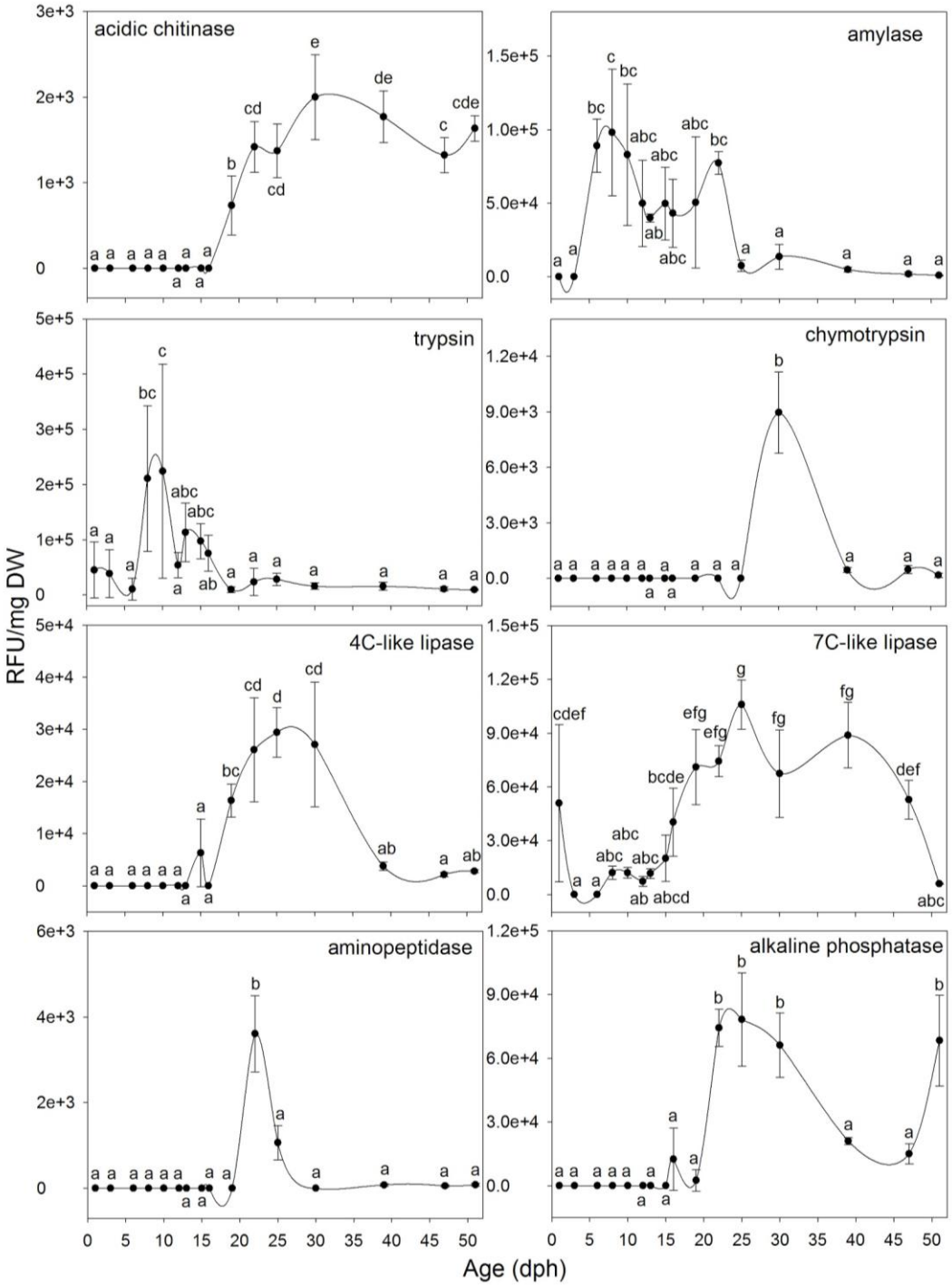

Fig. 6. Acidic chitinase, amylase, trypsin, chymotrypsin, lipases toward 4 and 7 carbon substrates, aminopeptidase and alkaline phosphatase activities during *Seriola dumerili* larvae development. Values are represented as means ± SD (n = 5 from 1 to 47 dph; n = 3 at 51 dph). Different letters represent significant differences in activity between ages ($p < 0.05$).

3.2 Diurnal pattern of digestive function

3.2.1 Diurnal pattern of the molecular expression of digestive enzymes precursor

Genes related to acidic digestion (*pga3*, *atp4a* and *chia1*) did not reveal a correlation with feeding time and showed constant expression levels during the analyzed period of time (Fig. 7). Regarding genes involved in alkaline digestion, some mRNA transcripts reached the highest expression level before feeding time (7:30h), as observed for *try*, *ctra*, *ctrb1*, *slc15a1a*, *slc15a1b* (Fig. 7). On the other hand, *cel1*, *cel3*, *pla2g1b* and *amy2a* expression levels were maintained constant in all sampling points (Fig. 8).

3.2.2 Diurnal pattern of digestive enzyme activities

The enzymes detected at the diurnal pattern analysis are shown in Fig. 9. Both pancreatic proteases showed late increasing patterns of activity, with peaks of activity starting at 13:30 and 21:00h for chymotrypsin and trypsin, respectively. After increasing, chymotrypsin activity levels were maintained until the end of the day. Both lipases showed the highest activity values at 7:30h, followed by a gradual decrease in activity during the day. This decline was smoother for the lipase toward butyrate. Amylase activity increased at 09:30h and activity levels were maintained for the rest of the day. Alkaline phosphatase was the only enzyme showing constant levels of activity along the period of the day under study.

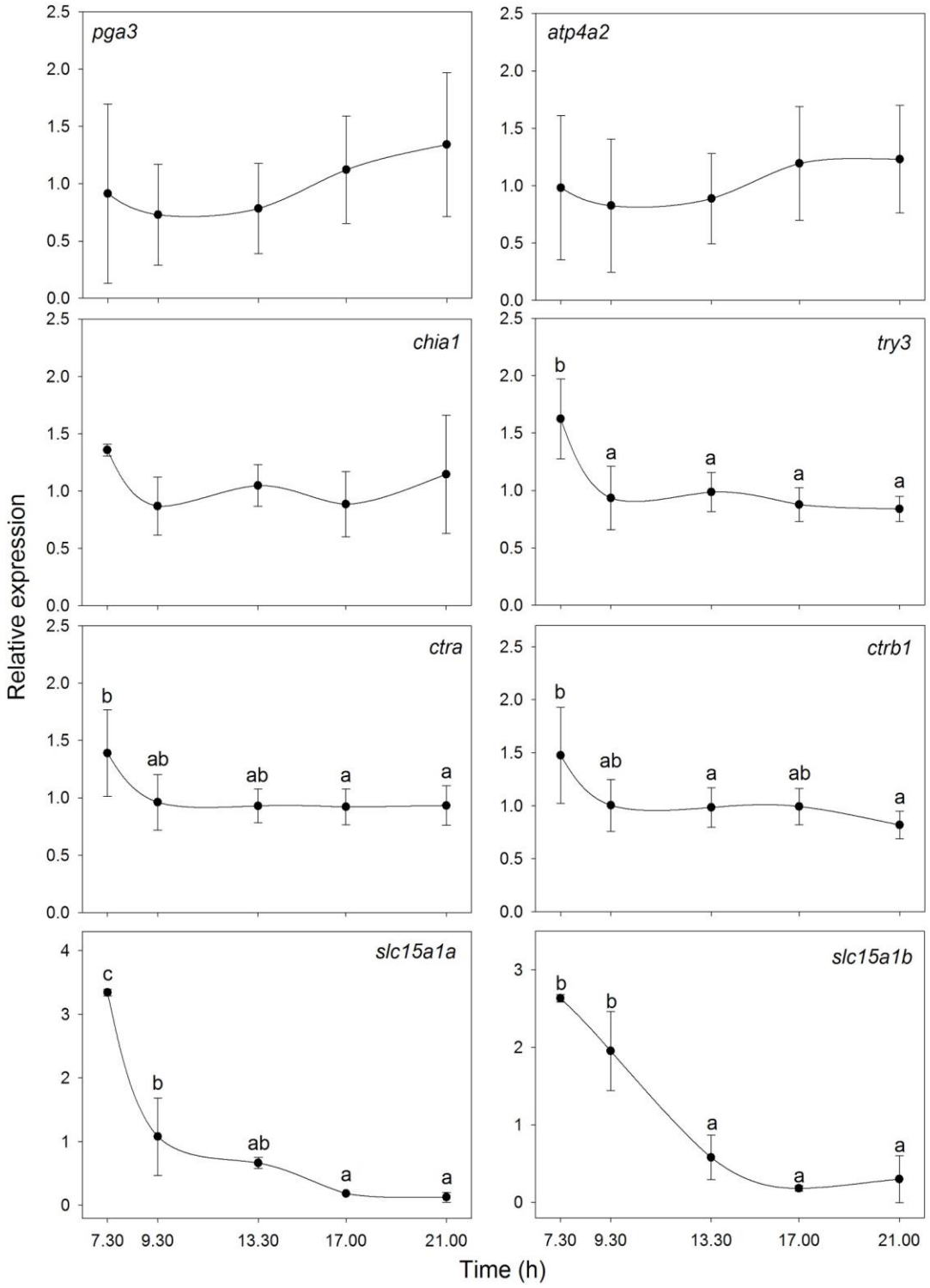

Fig. 7. Diurnal pattern of gastric protease (*pga3*), proton pump (*atp4a2*) and chitinase (*chia1*), and pancreatic proteases (*try3*, *ctra* and *ctrb1*) and peptide transporters (*slc15a1a* and *slc15a1b*) precursors relative expression of *Seriola dumerili* larvae at 19 dph. Values are represented as means ± SD (n = 6). Different letters represent significant differences in relative expression between sampling points ($p < 0.05$). Absence of letters indicates no statistical differences in relative expression between sampling points ($p > 0.05$).

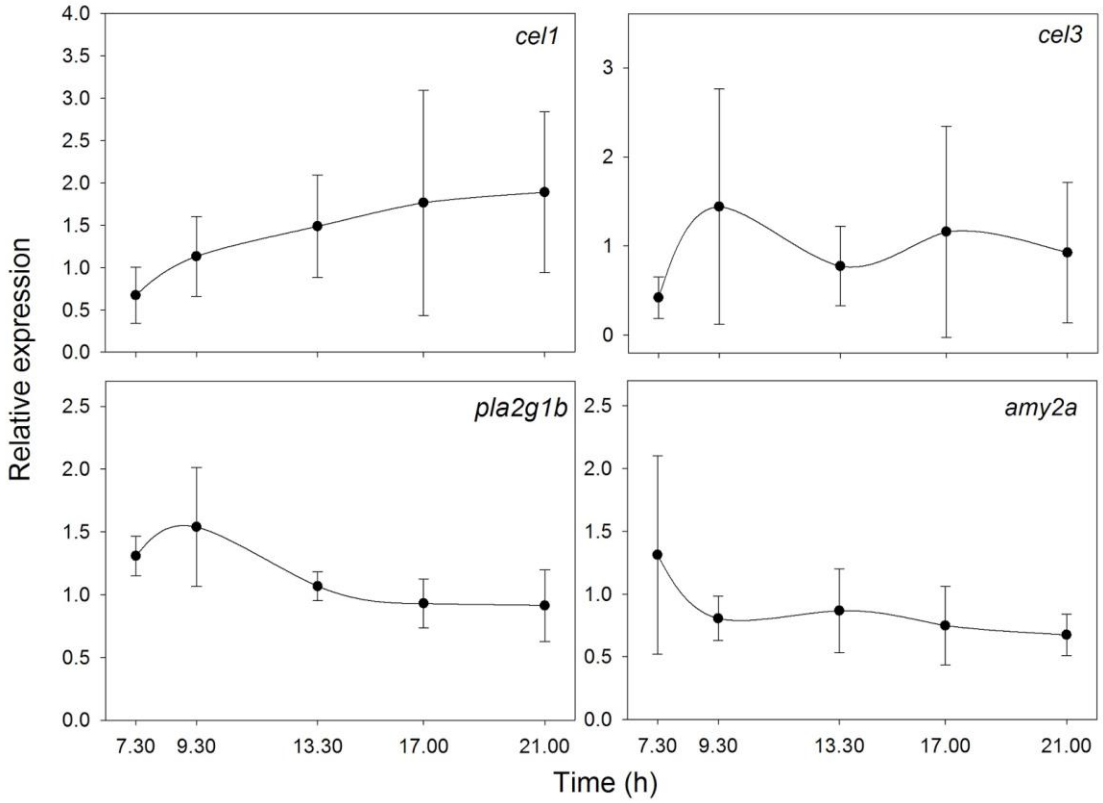

Fig. 8. Diurnal pattern of carboxyl ester lipases (*cel1*, *cel2* and *cel3*), bile salt activated lipase (*pla2g1b*) and amylase (*amy2a*) precursors relative expression of *Seriola dumerili* larvae at 19 dph. Values are represented as means ± SD (n = 6). Different letters represent significant differences in relative expression between ages ($p < 0.05$). Absence of letters indicates no statistical differences in relative expression between sampling points ($p > 0.05$).

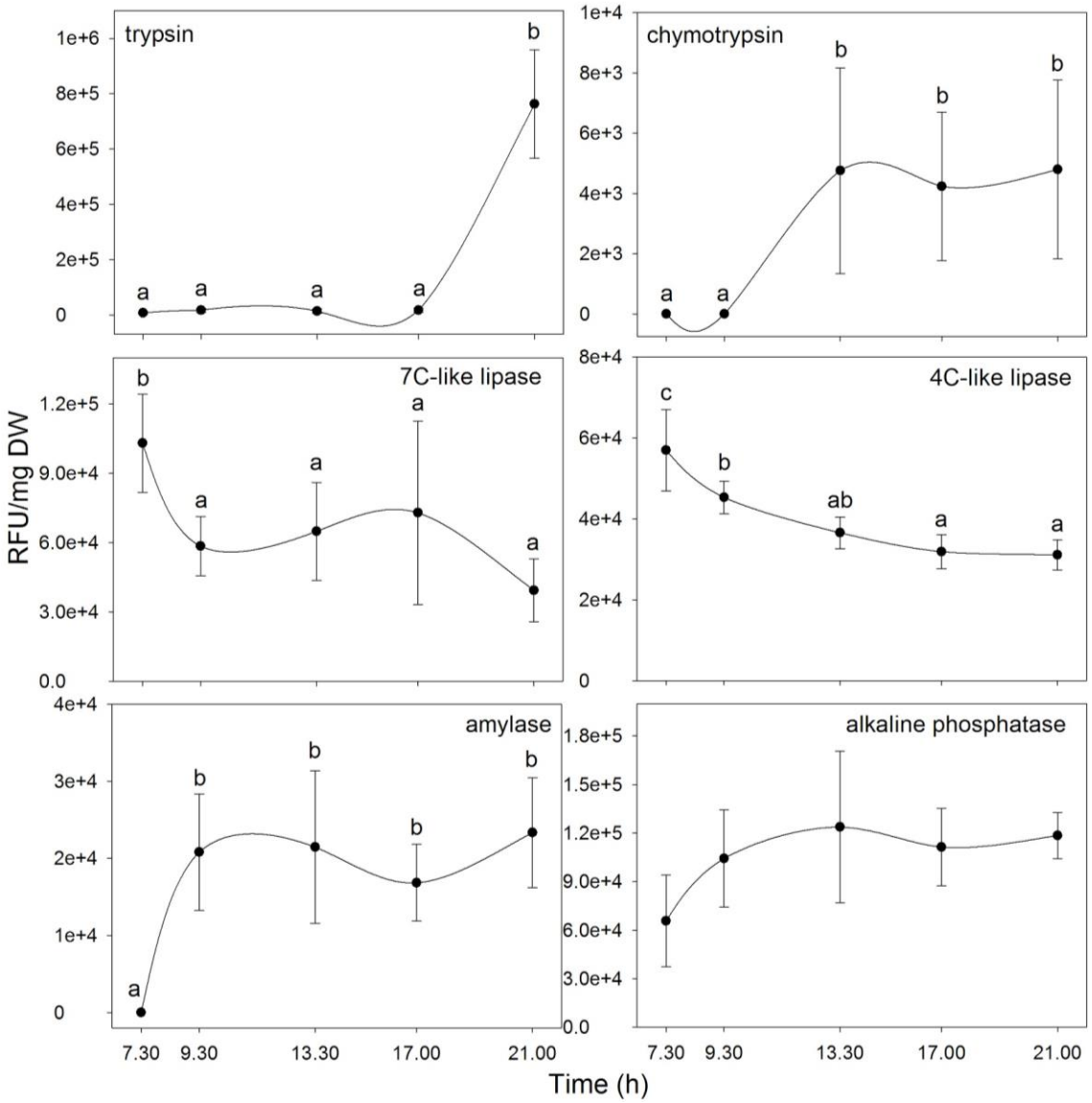

Fig. 9. Diurnal pattern of trypsin, chymotrypsin, lipases toward 4 and 7 carbon substrates, amylase and alkaline phosphatase activities of *Seriola dumerili* larvae at 19 dph. Values are represented as means ± SD (n - 5). Different letters represent significant differences in activity between sampling points ($p < 0.05$). Absence of letters indicates no statistical differences in activity between sampling points ($p > 0.05$).

4. DISCUSSION

A good understanding of the digestive capacity and of the mechanisms driving the digestive functionality is essential to provide appropriate food and define effective feeding protocols during the larval development of a fish species. In the present study, the appearance and functionality profiles of key digestive enzymes have been examined in detail at molecular and activity level in order to complete the previous knowledge on greater amberjack. In addition, the expression of two peptide transporter isoforms that could be representative of the protein hydrolysis and the posterior amino acids and peptides absorption have also been determined. Growth is basically protein deposition and to

obtain the required amino acids from the ingested nutrients is a paramount task in fast growing fish larvae. Intending to advance still more in the understanding of the digestive function of this species, the changes of these enzymes during the diurnal period have been examined in 19 dph larvae. A constant exponential growth was obtained during the experimental period and it may be assumed that the observed patterns are representative of the digestive function of greater amberjack in rearing conditions.

4.1 Ontogeny of digestive function

In general, ontogenetic pattern of the digestive functionally is quite similar for most of studied teleosts, and the main differences were observed in relation to the feeding habits that will be attained at the juvenile stage, from herbivorous to strict carnivorous and/or presence or absence of stomach, that may affect the appearance sequence of different enzymes (Yúfera et al., 2018). During the larval stage, in the absence of gastric glands, the bulk enzymatic contribution for nutrient digestion is secreted by the pancreas and intestinal epithelium. The end of the larval mode of digestion starts with the appearance of gastric glands and acidic enzymatic activity, in a process that leads to the completion of the stomach and full acidic digestion capacity, therefore, to the adult mode of digestion. In the case of large pelagic and piscivorous teleost as seriola or tuna species, the developmental process occurs relatively fast and the first gastric glands in the incipient stomach appear generally along the second and third week after hatching at 23-25 °C of water temperature (Kaji et al., 1996, 1999; Chen et al., 2006; Yúfera et al., 2014; Teles et al., 2017; Pérez et al., 2020). Such event that may occurs even before of the notochord flexion is determinant for the protein hydrolysing efficiency because thereafter the acidic digestion appears progressively allowing to digest larger prey than before.

Trypsin is the most important enzyme linked to fish early digestive capacity (Rønnestad et al., 2013), with early expression and activity patterns, making larvae capable of digesting proteins from mouth opening. Fast growing species are characterized by a trend to decrease it trypsin activity early, due to a precocious stomach development and the consequent transition to adult digestion. This has been observed for yellowtail amberjack, Pacific bluefin tuna (*Thunnus thynnus*) and meagre (*Argyrosomus regius*) (Miyashita et al., 1998; Chen et al., 2006; Solovyev et al., 2016), and is also in agreement with results in the present study for greater amberjack, with a clear decline in trypsin activity from 10 dph onwards, while data from longfin yellowtail (*Seriola rivoliana*) revealed a sharp increase of trypsin after first feeding (Teles et al., 2019). On the contrary, in commonly marine farmed species with slower growth rates, as red seabream *(Pagrus major*), Southern flounder (*Paralichthys lethostigma*), gilthead seabream and Senegalese sole (*Solea senegalensis*), a longer prevalence of

trypsin activity and a later switch to adult digestion is highlighted (Faulk and Holt, 2009; Navarro-Guillén et al., 2015; Mata-Sotres et al., 2016b; Khoa et al., 2019), suggesting a slightly slower larvae digestive development. This quick decline of trypsin activity might lead to think in an early appearance of pepsin activity in greater amberjack larvae as described for other fast-growing fish species; 10 dph for longfin yellowtail (Teles et al., 2019) and 15 dph for yellowtail amberjack (Ma et al., 2014). However, in the present study pepsin was detected for the first time at 39 dph. Previous studies on greater amberjack digestive development are contradictory towards this issue, while Navarro-Guillén et al. (2019) detected pepsin only at 61 dph, Pérez et al. (2020) detected pepsin activity in a pool of 11 to 15 dph larvae, although a significant increase was only observed in pooled larvae from 20 to 30 dph. Variations may be explained mainly by methodological differences, since Pérez et al. (2020) analysed pooled larvae of different close ages and in Navarro-Guillén et al. (2019) and in the present study enzyme activities were measured in individual larvae. Differences in rearing conditions, such as temperature and feeding protocols, might also explain developmental differences. Chymotrypsin also contributes to protein digestion in the early stages of larval development, as described for sharpsnout seabream (*Diplodus puntazzo*), red drum (*Sciaenops ocellatus*), meagre, and longfin yellowtail (Applebaum et al., 2001; Aktulun et al., 2008; Solovyev et al., 2016; Teles et al., 2019). On the contrary, in red seabream larvae chymotrypsin activity peaked at 25 dph (Khoa et al., 2019). In the present study chymotrypsin activity showed a late, sharp increment at weaning (30 dph) followed by an abrupt decline. Conceição et al. (2003) reported that differences in trypsin and chymotrypsin activity patterns may reflect dietary differences in protein composition. This factor might justify the early chymotrypsin detection in longfin yellowtail larvae from Teles et al. (2019), where inert feed was included in the feeding plan earlier than in the present study, which might stimulate early chymotrypsin secretion. A higher trypsin: chymotrypsin ratio (T:C) is correlated with an elevated absorption rate of essential amino acids for protein synthesis, promoting growth potential, as reported by Rungruangsak-Torrissen et al. (2000, 2006) and Rønnestad et al. (2013). In agreement with this, in the present study, ages tending to a higher T:C ratio were linked to intervals with higher RGR. Trypsin has been described to be the key protease under conditions favouring growth, while chymotrypsin plays a major role when growth is limited or depressed (Rungruangsak-Torrissen et al., 2006). Concordant with this, the high RGR of greater amberjack larvae might justify the predominantly low chymotrypsin activity during the period of study. However, this disparity in results confirm that still a gap of knowledge exists about chymotrypsin significance in the larval digestive process, and that could also be related to the time of the day of sampling, as it is explained below.

Regarding lipases activity, high 7C-like lipase activity levels were recorded at hatching, decreasing at 3 dph and rising again from 13 dph onwards, in concomitance to a decrease in overall alkaline proteases activity. In agreement to what described for other species (Rønnestad et al., 2013), lipases are suggested to be implied in yolk sac lipids absorption, that are consumed before mouth opening, reported at day 3 dph in greater amberjack (Pérez et al., 2020). The role of lipases during larvae-juvenile transition is still debated. On one hand, a low proteolytic level might be correlated with high lipases activity and excessive dietary lipids, which according to Ma et al. (2014), may suppress amino acids absorption and metabolism, compromising growth efficiency. On the other hand, fast-growing larvae and early juveniles require a higher dietary lipid to satisfy the energy demand derived from larvae fast metabolism (Jover et al., 1999; Navarro-Guillén et al., 2019). In this study, 7C-like lipase seems to be the most active lipase during greater amberjack larval stage. In accordance with Navarro-Guillén et al. (2019), a decline in lipases activity was observed from 30 dph onwards. Similarly, Pérez et al. (2020) described a tendency to lower lipase activity from 11-15 dph in greater amberjack larvae. Results for other fast-growing species showed similar patterns, as observed for longfin yellowtail and yellowtail amberjack (Chen et al., 2006; Teles et al., 2018).

Acidic chitinase activity results did not match with those for pepsin in terms of stomach functionality. This might be due to several reasons, as methodological reasons, since pepsin activity was measured by spectrophotometry and acidic chitinase through fluorometry (more sensitive). An unsynchronised development of gastric glands might also explain this mismatch between results, as described for meagre and Senegalese sole (Ribeiro et al., 1999; Solovyev et al., 2016). Since the whole larva was used for enzymatic analysis, the possible resistance of intestinal alkaline chitinase to acidic pH might also justify the earlier detection of chitinase activity at low pH. Amylase results are in line with those described by Pérez et al. (2020) for greater amberjack, but also with those described for gilthead seabream (Moyano et al., 1996), large yellow croaker (*Pseudosciaena crocea*) (Ma et al., 2005) and Atlantic bluefin tuna (Mazurais et al 2015), characterized by an activity decline from 10 dph, interpreted as digestive developmental progress. A slower and less evident decrement in comparison to the previous mentioned species has been observed in meagre and yellowtail amberjack (Chen et al., 2006; Suzer et al., 2013; Solovyev et al., 2016), while a second peak of activity at the end of larval stage has been described for red drum (Lazo et al., 2000). On the other hand, a continuous increase of amylase activity during the development has been described for thicklip grey mullet (*Chelon labrosus*) and Senegalese sole (Navarro-Guillén et al., 2015; Gilannejad et al., 2020).

Brush border enzymes are considered as indicators of a transition from a primary to an adult

mode of digestion, their activity increases concomitantly with a decrease in cytosolic enzymes (Cara et al., 2003; Rønnestad et al., 2013). Similar enzymatic patterns confirm the correlation between alkaline phosphatase and aminopeptidase, however, not much is known about their specific function in fish larvae. As observed for greater amberjack larvae in the present study, in which aminopeptidase-n and alkaline phosphatase activities started at 20 dph, a late appearance of both enzymatic activities along development has been described for Senegalese sole, meagre and California halibut (*Paralichthys californicus*) (Ribeiro et al., 1999; Suzer et al., 2013; Álvarez-González et al., 2006). On the other hand, activity since the first days after hatching has been recorded in other species like blackspot sea bream (*Pagellus bogaraveo*), white seabream (*Diplodus sargus*), yellowtail amberjack and longfin yellowtail (Cara et al., 2003; Chen et al., 2006; Ribeiro et al., 2008; Teles et al., 2019). Results lead to think that these two enzymes present species-specific patterns. As suggested by Solovyev et al. (2016), high levels during the first days after hatching might be correlated with the fast development of the intestinal mucosa. On the contrary, constant values might indicate a slower increase in the absorption surface of the intestinal epithelium with larval development (Ribeiro et al., 1999).

The ontogenetic pattern of mRNA transcript expression of the examined proenzymes was not linked to that observed for the corresponding activities. In general, the transcript expressions were detected earlier in development than the activity of the corresponding enzymes. The molecular expression and the biochemical activity reflect the produced and used molecules, respectively, and not necessarily may exhibit similar patterns because they depend on different regulation mechanisms. In addition, the analysed activity of a given enzyme may correspond to different isoforms analysed at molecular level (Yúfera et al., 2018). This discordance between molecular expression and biochemical activity has been therefore described in previous studies (Sánchez-Paz et al., 2003; Wang et al., 2006; Mata-Sotres et al., 2016b). Transcripts encoding for the same proenzymes showed close similar ontogenetic patterns, as observed for *ctra* and *ctrb1*, *slc15a1a* and *slc15a1b*, and *cel1*, *cel2* and *cel3*.

The importance of intestinal protein digestion in the larval stage of greater amberjack was also evidenced by the gene expression results. The expression of trypsinogen and chymotrypsinogen was detected from early stages, maintaining high expression levels during the whole period of study. Similar patterns have been observed for gilthead seabream and red seabream (Mata-Sotres et al., 2016a; Khoa et al., 2019). On the contrary, thicklip grey mullet showed delayed expression patterns for both proenzymes, with expression starting around 50 dph (Gilannejad et al., 2020). Analogous conclusions can be obtained from the peptide transporters *slc15a1a* and *slc15a1b* expression levels obtained in the present study, trending to increase with age. Similar ontogenetic expression patterns have been

recorded in Atlantic cod (*Gadus morhua*), rainbow trout (*Oncorhynchus mykiss*), Japanese eel (*Anguilla japonica*) and Nile tilapia (*Oreochromis niloticus*) (Amberg et al., 2008; Ostaszewska et al., 2010; Ahn et al., 2013; Huang et al., 2015). Previous functional and biophysical studies demonstrated that both carriers interact with trypsin, suggesting a role in clustering proteolytic activity to the site of peptide transport (Beale et al., 2015). This is also supported by the similarity in the expression patterns between *try3* and *slc*-group transcripts observed in the present study. Regarding acidic protein digestion and stomach functionality, *pga3* and *atp4a* showed a highly expression synchronism. If compared with previous studies on the same and other species, a wide variation in the ontogenetic pepsinogen expression pattern can be observed. Wu et al. (2011) reported pepsinogen expression in greater amberjack larvae at 13 dph in the primordial stomach and at 20 dph in the fully developed gastric glands, while in the present study it was recorded at 20 dph using the whole larvae. Persian sturgeon (*Acipenser persicus*) larvae revealed earlier pepsinogen expression (10 dph), while for giant grouper (*Epinephelus lanceolatus*), red seabream, thicklip grey mullet and Southern flounder pepsinogen expression was detected for the first time between 20 and 40 dph (Anderson et al., 2016; Faulk and Holt, 2009; Gilannejad, 2019, 2020; Khoa et al., 2019).

mRNA transcripts of bile salt activated lipases (*cel1*, *cel2* and *cel3*) showed similar expression patterns. As expected due to the importance of lipid metabolism in fast-growing species, early expression patterns were recorded, with a peak of higher expression levels between 10 and 15 dph in all of them. Patterns are in line with those described for other fast-growing species as Pacific bluefin tuna (Murashita et al., 2014), while when compared to species with slower growth rates, such as thicklip grey mullet and Persian sturgeon, the later showed delayed expression patterns (Gilannejad et al., 2019, 2020). Contrarily to what described for *cel* group transcripts, thicklip grey mullet, gilthead seabream and the Pacific bluefin tuna showed earlier *pla2g1b* expression in relation to the results observed in the present study for greater amberjack larvae (Murashita et al., 2014; Mata-Sotres et al., 2016a; Gilanneyad et al., 2020). Heterogeneous results from other species as Atlantic cod and Japanese eel may indicate species-specific ontogenetic patterns (Sæle et al., 2010; Murashita et al., 2013).

Literature about ontogenetic expression of acidic chitinase precursor in fish larvae is scarce. Since it is related to stomach development, an expression pattern similar to that for pepsinogen would be expected. However, in the present study, *chia1* expression was recorded in younger larvae than *pga3* expression (16 and 25 dph, respectively). Wu et al. (2011) reported *chi* expression in the primordial stomach of greater amberjack at 13 dph, concomitantly with pepsinogen expression. In other fish species, *chia1* expression appeared at 27 dph for Japanese flounder (*Paralichthys olivaceus*), at 40 dph

for spotted halibut (*Verasper variegatus*), and between 25 and 38 dph in seven-band grouper (*Epinephelus septemfasciatus*) (Wu et al., 2011; Khoa et al., 2021). The expression of amylase mRNA transcript (*amy2a*) in greater amberjack larvae was really low until 20 dph, reaching maximum expression levels at 25 dph. Similar but earlier ontogenetic patterns have been described for other fast-growing species, as Atlantic bluefin tuna (Mazurais et al., 2015). It is interesting to notice that in gilthead seabream, European sea bass and red porgy (*Pagrus pagrus*), no fast-growing species, *amy2a* expression started earlier in development (Darias et al., 2006, 2008; Mata-Sotres et al., 2016a). Results reveal the wide heterogeneity and variability between species, probably as results of adaptations to different metabolic requirements, feeding habits and environmental conditions.

4.2 Diurnal functional patterns

There is very few and fragmentary information on daily patterns of digestion in fish larvae. Overall, the enzyme activity and the expression of proenzyme transcripts are related to the amount of gut content and its filling and emptying cycles, although this relation may differ for the different enzymes and it is not clear that exist for all of them (Fujii et al., 2007; Tillner et al., 2014; Navarro-Guillén et al., 2015, 2017; Mata-Sotres et al., 2016b). Gut content has not been measured in the present study but it can be assumed to be similar to other visual feeder larvae, that is, increasing the gut content during the light period and declining afterwards (Shoji et al., 2001; Mata-Sotres et al., 2016a). In this study a complete daily cycle was not examined because the samples could only be obtained in the period from 07:30 to 21:00 hours due to logistical restrictions. The start of the dark period and presumably the cease of ingestion occurred by 21:00 hours.

In relation to molecular expression, a statistically significant decreasing trend was found in the expression of those genes related to alkaline digestion of proteins and peptides transport. In *try3* and *ctr* the decrease occurred during the first morning hours, while the expression of the peptides transporters declined during more time up to the afternoon. Contrarily, the rest of genes related to lipids and carbohydrates digestion did not show significant changes during the diurnal period. The same occurred with genes related to gastric digestion (*pga3*, *chia1* and *atp4a2*). As such, this partial view of the daily cycle would indicate that protein related genes have been restocked during the night as na anticipatory strategy to be used at the start of feeding in the morning, while the transcripts of other enzyme precursors apparently are maintained at constant levels but their expression may increase or decrease during the dark period. These patterns are similar to that found in gilthead seabream larvae (Mata-Sotres et al., 2016b). In that study, the minimum expression values were generally observed at the beginning of the night and the highest at the transition from dark to light period, although with some

differences among the different enzymes.

The trypsin activity has been reported to increase in parallel to gut content in several studies (Mata et al., 2016b; Gilannejad et al., 2021). In this study, both alkaline proteases increased during the diurnal period, first the chymotrypsin by the start of afternoon, and then the trypsin at the end of the light period. This interesting pattern has also been found in 10g early Senegal sole juveniles (Gilannejad et al., 2016), although in this nocturnal feeder species the chymotrypsin activity predominated during the night-time and the trypsin activity during the light period. The amylase activity increased in the morning after the supply of food, possibly as a strategy to obtain more energy to support the high demand derived from the feeding, digestion and growth processes. Contrarily, activity of both lipases (4C- and 7C-like lipase) decreased progressively during the diurnal period. Lipase activity decreased during the daytime in 30 dph seabream larvae but not at other ages during the development (Mata-Sotres et al., 2016b).

We choose a priori the age of 19 dph for the diurnal sampling searching for an intermediate point during the development. Unfortunately, this age falls just in a transitional moment in the digestion capacities (the change from trypsin to chymotrypsin; before the increase of pepsin and alkaline phosphatase) and in which the activity was minimal for most of the enzymes. Therefore, these diurnal patterns may be poor representatives for some enzymes, as for alkaline phosphatase. In any case, we found that activity level varies along the daily cycle and that a single morning sample may not represent properly the capacity at a given age. Obviously, whole daily cycle sampling performed at different ages during the development would provide a complete figure of the digestive function in greater amberjack developing larvae.

5. CONCLUSIONS

The detailed expression and activity ontogenetic profiles of digestive enzymes reported in this study contributed to advancing in the knowledge of the digestive function in developing larvae of greater amberjack under rearing conditions. The described patterns are in agreement with what is expected for a carnivorous and piscivorous fish species. Although there are some discrepant features that could be due to species-specific characteristics or to the particular experimental conditions and feeding performed in this study. A wider analysis on daily and nocturnal patterns at different ages would shed light more specifically to regulation mechanisms and the potential changes in the utilisation of different macronutrients during the developmental course to juvenile.

Acknowledgments

This research was funded by the project RTI208-096134-B-I00 from the Spanish Ministry of Science, Innovation and Universities (MCIU), the State Research Agency (AEI) and the European Regional Development Fund (FEDER/ERDF) granted to MY. PG was supported by a Erasmus fellowship (Eramsus+). CNG acknowledges UID/Multi/04326/2019 financed by the FCT (Portugal).

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