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Cloning and molecular ontogeny of digestive enzymes in fed and food-deprived developing gilthead seabream (*Sparus aurata*) larvae

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ABSTRACT

We have determined the expression pattern of key pancreatic enzymes precursors (*trypsinogen*, *try*; *chymotrypsinogen*, *ctrb*; *phospholipase A*$_2$, *pla2*; *bile salt-activated lipase*, *cel*; and *α-amylase*, *amy2a*) during the larval stage of gilthead seabream (*Sparus aurata*) up to 60 days after hatching (dph). Previously, complete sequences of *try*, *cel*, and *amy2a* were cloned and phylogenetically analyzed. One new isoform was found for *cel* transcript (*cel1b*). Expression of all enzyme precursors was detected before the mouth opening. Expression of *try* and *ctrb* increased during the first days of development and then maintained high values with some fluctuations during the whole larval stage. The prolipases *pla2* and *cel1b* increased from first-feeding with irregular fluctuation until the end of the experiment. Contrarily, *cel1a* maintained low expression values during most of the larval stage increasing at the end of the period. Nevertheless, *cel1a* expression was negligible as compared with *cel1b*. The expression of *amy2a* sharply increased during the first week followed by a gradual decrease. In addition, a food-deprivation experiment was performed to find the differences in relation to presence/absence of gut content after the opening of the mouth. The food-deprived larvae died at 10 dph. The expression levels of all digestive enzymes increased up to 7 dph, declining sharply afterwards. This expression pattern up to 7 dph was the same observed in fed larvae, confirming the genetic programming during the early development. Main digestive enzymes in gilthead seabream larvae exhibited the same expression profiles than other marine fish with carnivorous preferences in their juvenile stages.

1. Introduction

Fish larvae are considered a transitional stage in which ontogeny and growth present substantial changes in structure, size, body shape and physiology of the individual. The basic mechanisms of organs development, including the digestive tract, are similar in all the teleost, although there are considerable differences regarding the relative timing in ontogeny (Zambonino-Infante et al., 2008). The digestive capacity during the early development of the digestive system is provided by the pancreatic enzymes (proteases, lipases and glucosidases) in conjunction with alkaline proteolytic enzymes secreted by the intestine, prior to acid digestion (Lazo et al., 2011; Rønnestad et al., 2013).

Numerous studies have been conducted to understand the general patterns of digestive activities during early ontogeny in many fish species (Lazo et al., 2000; Zambonino-Infante et al., 2008; Gisbert et al., 2009; Yang et al., 2010; Srichanun et al., 2013; Suzer et al., 2013; Murashita et al., 2014). These studies showed that digestive enzyme activities are good indicators of the digestive capacity of fish and directly reflect both the development of the digestive tract and the nutritional status of the fish (Rønnestad et al., 2013). Moreover, the levels of secretion of main pancreatic enzymes (trypsin, lipase, and amylase) are commonly used as indicators of digestive system function and maturation (Ribeiro et al., 1999; Cahu et al., 2004; Murray et al., 2006; Zambonino-Infante and Cahu, 2007; Zambonino-Infante et al., 2008).

In contrast, the expression patterns during the ontogeny of the mRNA transcripts encoding the digestive enzyme precursors are comparatively scarce (Kortner et al., 2011; Hansen, 2012; Srichanun et al., 2013; Murashita et al., 2014). Therefore, although the molecular expression patterns of digestive enzymes can be used as markers for fish larval development (Lazo et al., 2011), the molecular mechanisms for the regulation of digestion are not well understood. It seems that gene expression of some digestive enzymes is genetically programmed during the first days of feeding, but a potential effect of feeding level and diet composition has also been reported (Gamboa-Delgado et al., 2011; Hachero-Cruzado et al., 2014).

The gilthead seabream (*Sparus aurata*) is a marine teleost of primary interest for the Mediterranean aquaculture. This species has been profusely studied since the 80s and there is a good knowledge of its

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physiology during the larval development to juvenile (Yúfera et al., 2011). Histological (Sarasquete et al., 1995) and functional (Moyano et al., 1996) development of the digestive tract during early ontogeny has been previously described. Gilthead seabream larvae have a relatively long development which last during the first two months of life, after which the appearance of gastric glands marks the transition to juvenile (Elbal et al., 2004). While the enzymatic activities of some key digestive enzymes were already studied during the first month of life (Moyano et al., 1996), the profiles of the corresponding mRNA transcripts remain unexplored.

In this study, we have determined the expression patterns of the main pancreatic enzyme precursors during the whole larval stage of gilthead seabream. We have analyzed the profiles of *trypsinogen* (*try*), *chymotrypsinogen* (*ctrb*), *phospholipase* A_2 (*pla2*), *bile salt-activated lipase* (isoforms *cel1a* and *cel1b*) and *α-amylase* (*amy2a*) transcripts. Previously, the full-length cDNAs of *trypsinogen*, *bile salt-activate lipase* and *α-amylase* were cloned.

2. Materials and methods

2.1. Rearing conditions

Gilthead seabream fertilized eggs were supplied in 2012 by the Servicio Central de Investigación de Cultivos Marinos (SCI-CM) at the Faculty of Marine and Environmental Sciences (University of Cádiz, Puerto Real, Cádiz, Spain; Operational Code REGA ES11028000312) and transferred to ICMAN facilities. Upon arrival the eggs were incubated under the same environmental condition than the hatched larvae. Larvae were reared in 3 circular 250 L tanks under constant temperature (19 °C), salinity (34 g L^{-1}) and photoperiod (12 h light:12 h darkness), and fed *ad libitum* with rotifers (*Brachionus rotundiformis* Bs-strain and *Brachionus plicatilis* S-1-strain; Yúfera, 1982) from day 4 post-hatching (dph) till 24 dph, supplied at a density of 10 rotifers/mL and enriched with the microalgae *Nannocholopsis gaditana*, and subsequently with *Artemia sp*. nauplii and metanauplii from 18 dph until the end of experiment at 60 dph (Polo et al., 1992).

2.2. Experimental design

To determine the expression patterns of digestive enzymes during the larval development of gilthead seabream, 9 larvae (3 individuals per tank) were taken at 14 different sampling times (1, 3, 4, 5, 7, 10, 15, 18, 20, 25, 30, 34, 40 and 60 dph). All samples were taken at 3 pm to avoid variations caused by the daily feeding rhythms. At this time of the day (in the middle of the light period) the larvae are actively feeding and show increasing gut content at all tested ages (Mata-Sotres et al., 2015). To determine the effect of food deprivation on gene expression, larvae were reared in the absence of food in a parallel experiment. Samples were taken at 3, 5, 7 and 9 dph. In addition, 10–30 extra larvae were taken for each sampling time for growth determination. Larvae dry weight was measured in a micro-balance (Mettler Toledo, XP2U), and the total length was measured by a light microscope (Wild Heerbrugg, M5). Specific growth rate (d^{-1}) was calculated as the slope of the exponential regression fitted to dry weight data points vs. larval age during the exponential growth phase.

All sampled larvae were anesthetized and killed with an overdose of ethyl-4-amino-benzoate and immediately stored in RNA*later* (Ambion, LifeTechnologies), an RNA stabilizing solution for molecular analyses. In all molecular biology protocols involving commercial kits cited here and elsewhere in this study, the manufacturer's instructions were followed, except where noted. All experimental procedures complied with the Guidelines of the European Union Council (2010/63/EU) for the use and experimentation of laboratory animals and were reviewed and approved by the Spanish National Research Council (CSIC) bioethical committee.

2.3. Cloning of trypsinogen, bile-salt activated lipase and α-amylase

cDNAs for trypsinogen (GenBank acc. no. AY835386), bile-salt activated lipase (GenBank acc. no. DQ073423), and α-amylase (GenBank acc. no. AY741554) from red porgy *Pagrus pagrus* were used as ^{32}P radio labeled probes (Darias et al., 2005, 2006) for screening a gilthead sea bream gastrointestinal tract cDNA library constructed in lambda ZAP (Stratagene, Agilent Technologies; discontinued) as previously described in Balmaceda-Aguilera et al. (2012). *In vivo* excision of 3 single positives of the screening was performed for each using *Escherichia coli* XL-1-Blue MRF′ and SOLR strains (Stratagene, Agilent Technologies Life Sciences). Excised pBluescript SK(−) containing the specific clones were double digested by *Eco*RI and *Xho*I (Takara), and the inserts were separated from the vector in a 1% agarose gel in 1× TBE (130 mM Tris–HCl, 45 mM boric acid, 2.5 mM EDTA Na_2 in water, pH ~9.0), stained with GelRed™ (Biotium), and visualized with the ChemiDoc™ XRS + System using Image Lab™ Software (Bio Rad). Two of the clones for each enzyme precursor were fully sequenced in both strands in the Central Services for Science and Technology (SCCYT) from the University of Cadiz (Cádiz, Spain), using an ABI PRISM® 3100 Genetic Analyzer and the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Life Technologies). Blast analyses (http://blast.ncbi.nlm.nih.gov/Blast.cgi) confirmed identities with *trypsinogen*, *bile-salt activated lipase* and *α-amylase* from other fish species, although the 2 last lacked the 5′ ends including a good part of the open reading frames.

In order to obtain the 5′-ends of *cel* and *amy2a*, the 5′-RACE protocol from the FirstChoice® RLM RACE Kit (Ambion, Life Technologies) was followed. In brief, cDNA was synthesized from 1 μg of total RNA from gastrointestinal tract with random decamers from the kit. Two rounds of PCR were run with two nested reverse primers (Table 1), designed from the 5′ ends, allowing an overlap of at least 150 bp between the RACE fragments and the previously obtained library clones. 5′-RACE Outer and Inner primers from the kit were used as forward oligonucleotides. PCR reactions were performed with 1 U of the proof reading VELOCITY DNA polymerase (Bioline) with the first strand cDNA (corresponding to 25 ng of input total RNA), manufacturer's PCR buffer (1× final concentration), 0.5 μM each forward and reverse primers, 0.2 mM dNTPs mixture, and 1.5 mM $MgCl_2$ in a total volume of 20 μL. The samples were cycled at 98 °C for 5 min; 98 °C for 30 s, 65 to 55 °C in touchdown for 30 s, 72 °C for 1 min, during 35 cycles; 72 °C for 10 min, in a Mastercycler®proS (Eppendorf). The PCR products were run in a 1% agarose gel and visualized as described above. Products were purified from gel using the NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel). Fragments were cloned in pJET1.2/blunt Cloning Vector, using the CloneJET PCR Cloning Kit (Fermentas, Life Sciences), and sequenced using pJET1.2 forward and reverse sequencing primers (Bioarray, Elche, Spain). eBiox (v1.5.1) software was used for fragment assembly. Translation of the sequences to the open reading frame (ORF) was performed with eBiox (v1.5.1) software. Sequences were analyzed for identity at nucleotide and amino acid levels using BLAST/NCBI. Conserved domains were retrieved with CDD/NCBI (Marchler-Bauer et al., 2015) and InterPro/EMBL-EBI (Mitchell et al., 2015).

2.4. Phylogenetic analyses

The evolutionary history for trypsinogen, bile salt-activated lipase and α-amylase from *S. aurata* were inferred by the neighbor-joining method using complete protein sequences available in GenBank for each gene. The robustness of the trees was tested with bootstrapping tests (1000 replicates) and the evolutionary distances were computed using the Poisson correction method. The phylogenetic trees were constructed with MEGA 6.0 software (Tamura et al., 2013).

Table 1
Oligonucleotides used for 5′RACE (A) and qPCR (B). Primer sequences are indicated, as well as amplicon sizes (bp), reaction efficiencies (E) and Pearson's coefficients of determination (R^2) for q-PCR reactions.

Gene	Fwd sequence (5′–3′)	Rev sequence (5′–3′)	Size (bp)	E	R^2
(A)					
Amy-R1	–	TAGTCTTTCTCCAGGGCGAG	–	–	–
Amy-R2	–	TTGGCACTGTGCTTGAACTC	–	–	–
Cel-R1	–	CTAACACTAGCTCCGCCTGC	–	–	–
Cel-R2	–	AAGCGCAGGATCAGTCATCT	–	–	–
(B)					
try	TGAACATCCCCATCCTGTCT	GTAGCCCCAGGACACAACAC	172	1.00	1.00
cel1a	TTTGGAGGAGACCCTGACA	GCTCTCTTAAACAGTCCTTTGTTAA	107	0.92	0.96
cel1b	TTTGGAGGAGACCCTGACA	GCTCTCTTAAACAGTCCTTTGTTGT	107	1.00	0.99
amy2a	AACCACGACAACCAGAGAGG	GCCCATCCAGTCATTCTGAT	186	1.00	0.99
ctrb	ATCCAACGGCTTTCATTTCTG	GCCATAGCCCTTATTGTGCTC	124	1.00	0.99
pla2	CCAGACCATCTTCACCATCC	CACCCAATCCACAGGAGTTC	114	0.97	0.99
actb	TCCTGCGGAATCCATGAGA	GACGTCGCACTTCATGATGCT	108	1.00	0.99

2.5. Quantification of gene expression levels (qPCR)

Samples, preserved in RNA*later* (Ambion), were individually processed for total RNA extraction using the NucleoSpin® RNA XS (larvae from 1 to 19 dph) or the NucleoSpin® RNA II (larvae from 20 dph to 60 dph) kits (Macherey-Nagel), including the optional on column RNase free DNase digestion step. An Ultra-Turrax® T8 (IKA®-Werke) was used to homogenize the larval tissue before the extraction. RNA quality was checked in a Bioanalyzer 2100 and with the RNA 6000 Nano kit (Agilent Technologies). RNA quantity was measured spectrophotometrically at 260 nm with the BioPhotometer Plus (Eppendorf). Only RNA samples with a RNA integrity number (RIN) higher than 8.0 were used for expression quantification.

Total RNA (50 ng) from each larval sample was reverse-transcribed in a 20 μL reaction using the qScript™ cDNA synthesis kit (Quanta BioSciences) in a Mastercycler®proS (Eppendorf). The reverse transcription program consisted in 5 min at 22 °C, 30 min at 42 °C, 5 min at 85 °C and finally kept at 4 °C. Optimization of qPCR conditions was previously made on primer annealing temperature (ranging from 50 to 60 °C), different primer concentrations (100, 200 and 400 nM) and template concentration (calibration curve with five 1:10 dilution series in triplicate from 10 ng to 1 pg of input RNA). The assay linearity and amplification efficiency for each pair of primers are shown in Table 1. qPCR reactions were performed in duplicate with 1 ng of cDNA (estimated from the input of total RNA), forward and reverse primers for the digestive enzyme precursors (200 nM each, Table 1) and PerfeCTa™ SYBR® Green FastMix™ (Quanta BioSciences). PCR conditions were: an initial denaturation and polymerase activation step during 10 min at 95 °C; 40 cycles of denaturing for 15 s at 95 °C, annealing and extension for 45 s at 60 °C; and a final melting curve from 60 °C to 95 °C for 20 min to check for primer–dimer artefacts. Relative gene quantification was calculated by the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001) using automated threshold and walking baseline for determining the C_T values. The cDNA from an individual from the 18 dph was used as calibrator or control in triplicate for all the larval time points, developmental stages, experimental conditions and genes studied, including the reference gene, and being repeated in all the analyzed plates. β-actin was used as the internal reference gene (acc. no. X89920) for each sample in duplicate. Reactions were conducted in 10 μL, in white twin.tec real-time PCR plates 96 (Eppendorf) covered with adhesive Masterclear real-time Film (Eppendorf). All the analyses were performed analyzing individually 9 larvae in each age and treatment. GenBank accession numbers for the studied genes are: EU163287 for *try*, AY550953 for *ctrb*, JX975714 for *cel1a*, KR779817 for *cel1b*, AM972037 for *pla2* and KR779816 for *amy2a*.

2.6. Data analysis

In order to identify significant differences in gene expression and growth, one-way ANOVA analyses were performed followed by post-hoc comparisons by the Tukey test. Statistical significance was accepted at $p < 0.05$. Statistical analysis was performed using the software STATISTICA 8.0™ (StatSoft, Inc. USA). Results are given as means ± standard error of the means.

3. Results

3.1. Cloning of trypsinogen, bile salt-activated lipase and α-amylase cDNA sequences

The full-length cDNA sequences obtained for gilthead seabream were 859 bp for *try* (GenBank acc. no. EU163287), 1781 bp for *cel* (GenBank acc. no. KR779817) and 1615 bp for *amy2a* (GenBank acc. no. KR779816). In addition, each nucleotide sequence comprised an ORF of 726 bp, 1689 bp and 1539 bp, respectively. The encoding predicted amino acid sequences were 241 for *try*, 562 for *cel1b* and 512 for *amy2a* (Figs. 1, 2 and 3 in Supplementary Material).

Cloned *try* from *S. aurata* had an identity at nucleotide level of 99 and 96% with other *trypsinogens* from *S. aurata* (GenBank acc. no. AY550954 and AY550948, respectively) and 97% with *S. aurata trypsinogen II* precursor (GenBank acc. no. DQ443543). With other sparids, the identity was 96% with red seabream *Pagrus major* (GenBank acc. no. AB678427) and 95% with white seabream *Diplodus sargus* (GenBank acc. no. EU163288). Whereas at amino acid level the identities were 99 and 96% with trypsinogen-like from *S. aurata* (GenBank acc. no. AAT45259, and ABE68639 and AAT45253, respectively), 94% with trypsinogen from *P. major* (GenBank acc. no. BAL14139), 93% with *D. sargus* (GenBank acc. no. ABX89622), 86 and 85% with *Pundamilia nyererei* (NCBI Ref. Seq. nos. XP_005736222 and XP_005736224, respectively), and 86% with Japanese flounder *Paralichthys olivaceus* trypsinogen 1 (GenBank acc. no. BAA82362) and 85% with trypsinogen 2 (GenBank acc. no. ABS32239) (Fig. 1).

Bile salt-activated lipase (*cel1b*) from *S. aurata* had an identity at nucleotide level of 97% with another *cel* from *S. aurata* (GenBank acc. no. JX975714), 87% with bicolor damselfish *Stegastes partitus cel*-like (NCBI Ref. Seq. no. XM_008288338), 84% with Pacific bluefin tuna *Thunnus orientalis cel1* (GenBank acc. no. AB859992) and *S. partitus cel*-like (NCBI Ref. Seq. no. XM_008288337), and 80% with *cel*-like from zebra mbuna *Maylandia zebra* (NCBI Ref. Seq. no. XM_004538437), Burton's mouthbrooder *Haplochromis burtoni* (NCBI Ref. Seq. no. XM_005941359), Japanese puffer *Takifugu rubripes* (NCBI Ref.

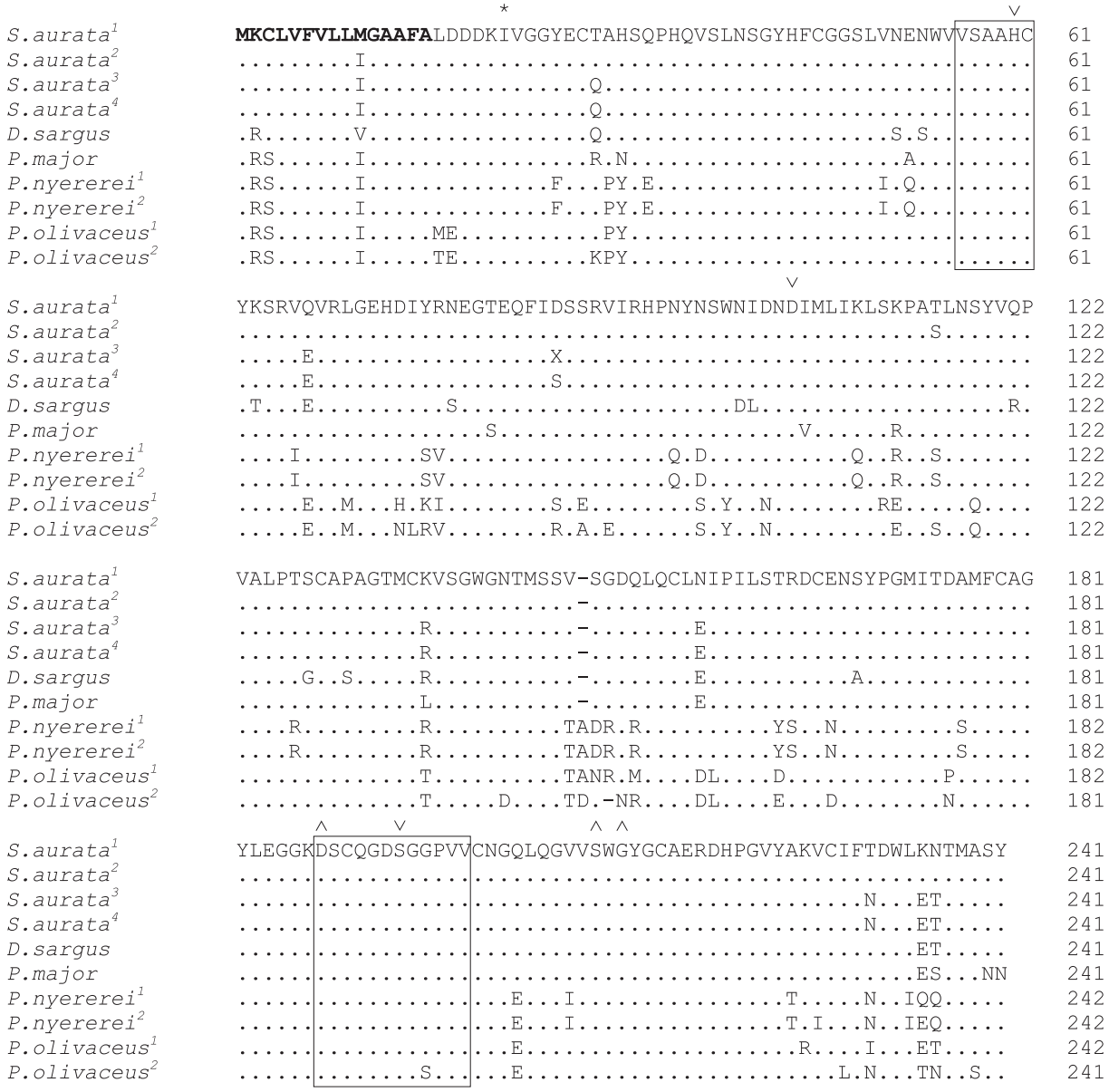

Fig. 1. Alignments of trypsinogen amino acid sequences from *S. aurata* (GenBank acc. nos. [1]ABX89621, [2]AAT45259, [3]AAT45253, and [4]ABE68639), *Diplodus sargus* (GenBank acc. no. ABX89622), *Pagrus major* (GenBank acc. no. BAL14139), *Pundamilia nyererei* (NCBI Reference Sequences [1]XP_005736222 and [2]XP_005736224), and *Paralichthys olivaceus* (GenBank acc. nos. [1]BAA82362 and [2]ABS32239). Dots indicate identity and hyphens represent gaps. The conserved amino acid for the cleavage site is indicated with an asterisk (*), whereas the amino acids for the active and substrate binding sites, from the trypsin-like serine proteases superfamily, are indicated with down (˅) and up (˄) arrowheads, respectively. The signal peptide (1–15) is shown in bold, and the histidine active site [LIVM]-[ST]-A-[STAG]-H-C and the serine active site [DNSTAGC]-[GSTAPIMVQH]-x(2)-G-[DE]-S-G-[GS]-[SAPHV]-[LIVMFYWH]-[LIVMFYSTANQH] for the trypsin family of serine proteases are boxed.

Seq. no. XM_003978375) and *P. nyererei* (NCBI Ref. Seq. no. XM_005725601). Whereas at amino acid level the identities were 96% with Cel from *S. aurata* (GenBank acc. no. AGV13286), 86% with *S. partitus* Cel-like (NCBI Ref. Seq. no. XP_008286560), 83% with *T. orientalis* Cel1 (GenBank acc. no. BAO01447), 82% with *S. partitus* Cel-like (NCBI Ref. Seq. no. XP_008286559), and 80% with *T. rubripes* (NCBI Ref. Seq. no. XP_003978424) (Fig. 2).

Reported *α-amylase* sequence (*amy2a*) from *S. aurata* had the highest identity at nucleotide level with thicklip grey mullet *Chelon labrosus amy2a* with a 96% (GenBank acc. no. KF684941), 95% with *D. sargus amy2a* (GenBank acc. no. EU163286) and *P. major amy2a* (GenBank acc. no. AB678421), and 90% with *T. orientalis amy2a.1* (GenBank acc no. AB678419). Whereas at amino acid level the identities were 96% with *C. labrosus* Amy2a (GenBank acc. no. AIC81809), 94% with *P. major* Amy2a (BAL14133), 93% with *D. sargus* Amy2a (GenBank acc. no. ABX89620), 92% with *E. coioides* (GenBank acc. no. ACJ26844), and 91% with *T. orientalis* Amy2a.1 and Amy2a.2 (GenBank acc. nos. BAL14131 and BAL14132, respectively) (Fig. 3).

Trypsinogen showed the conserved sites for cleavage domain (Ile^{21}), for the active domain (His^{60}, Asp^{104} and Ser^{195}), and the substrate binding domain (Asp^{189}, Ser^{210}, Gly^{212}) from trypsin-like serine protease superfamily (Fig. 1). Bile-salt activated lipase showed the conserved sites for the substrate binding pocket domain (Gly^{123}–Gly^{124}–Gly^{125}, Glu^{210}–Ser^{211}–Ala^{212}, Ala^{215}, Arg^{362}, Ser^{366}–Tyr^{367}, Gly^{404}, Ala^{456}, Leu^{459}) and the catalytic triad domain (Ser^{211}, Asp^{336}, His^{455}) from the esterase-lipase superfamily and the carboxylesterase family (Fig. 2).

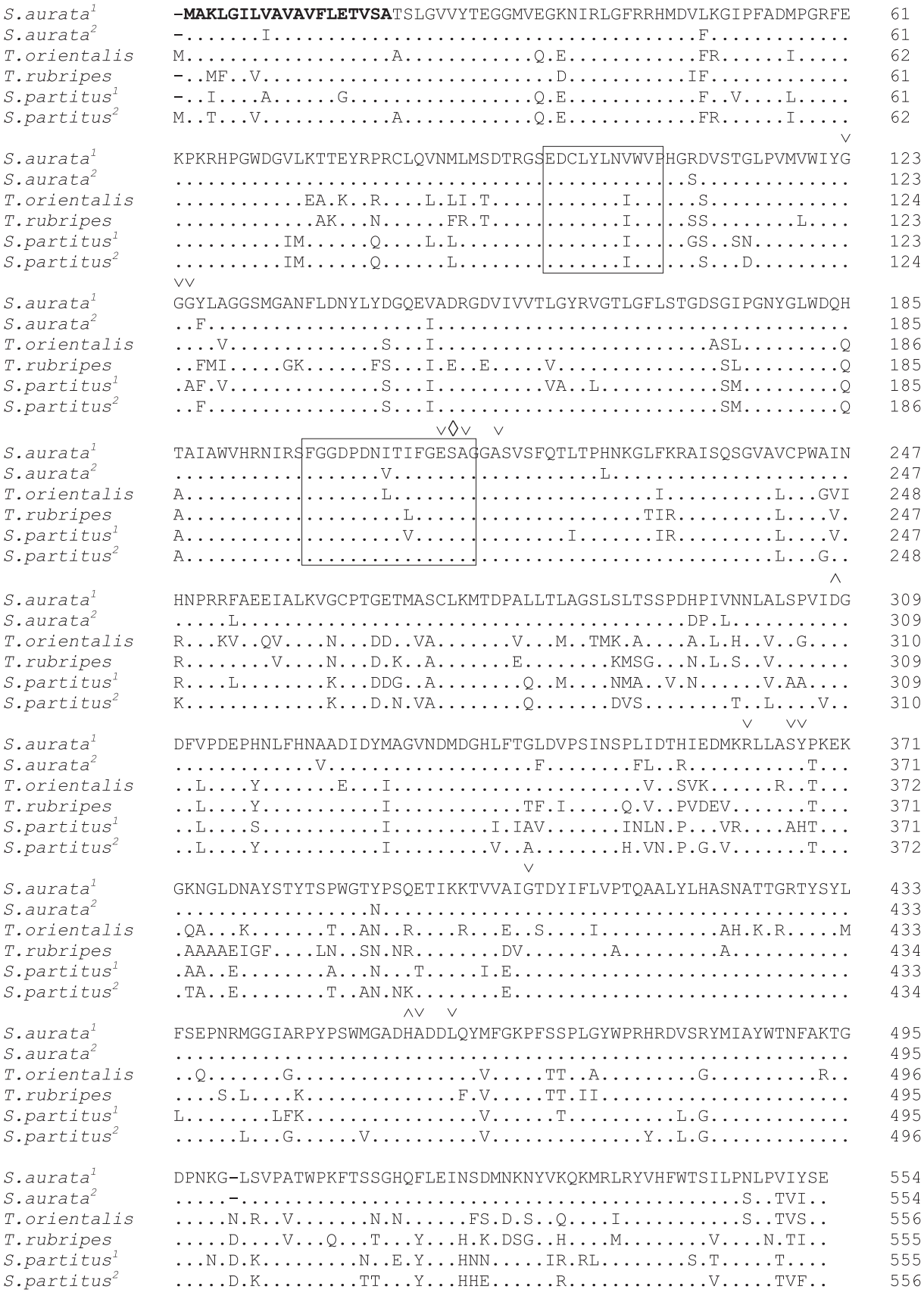

Fig. 2. Alignments of bile-salt activated lipase amino acid sequences from *S. aurata* (GenBank acc. no. [1]ALB35088; and [2]AGV13286), *Thunnus orientalis* (GenBank acc. no. BAO01447), *Takifugu rubripes* (NCBI Reference Sequence XP_003978424), and *Stegastes partitus* (NCBI Reference Sequences [1]XP_008286559 and [2]XP_008286560). Dots indicate identity and hyphens represent gaps. The amino acids from the conserved sites for the substrate binding pocket domain and the catalytic triad domain, from the esterase-lipase superfamily and carboxylesterase family, are indicated with down (˅) and up (˄) arrowheads (a rhombus – ◊ – when the amino acid is involved in both sites), respectively. The signal peptide (1–19) is shown in bold, and the conserved signature 2 [EDA]-[DG]-C-L-[YTF]-[LIVT]-[DNS]-[LIV]-[LIVFYW]-x-[PQR] and the serine active site F-[GR]-G-x(4)-[LIVM]-x-[LIV]-x-G-x-[STAG]-G for carboxylesterases type-B are boxed.

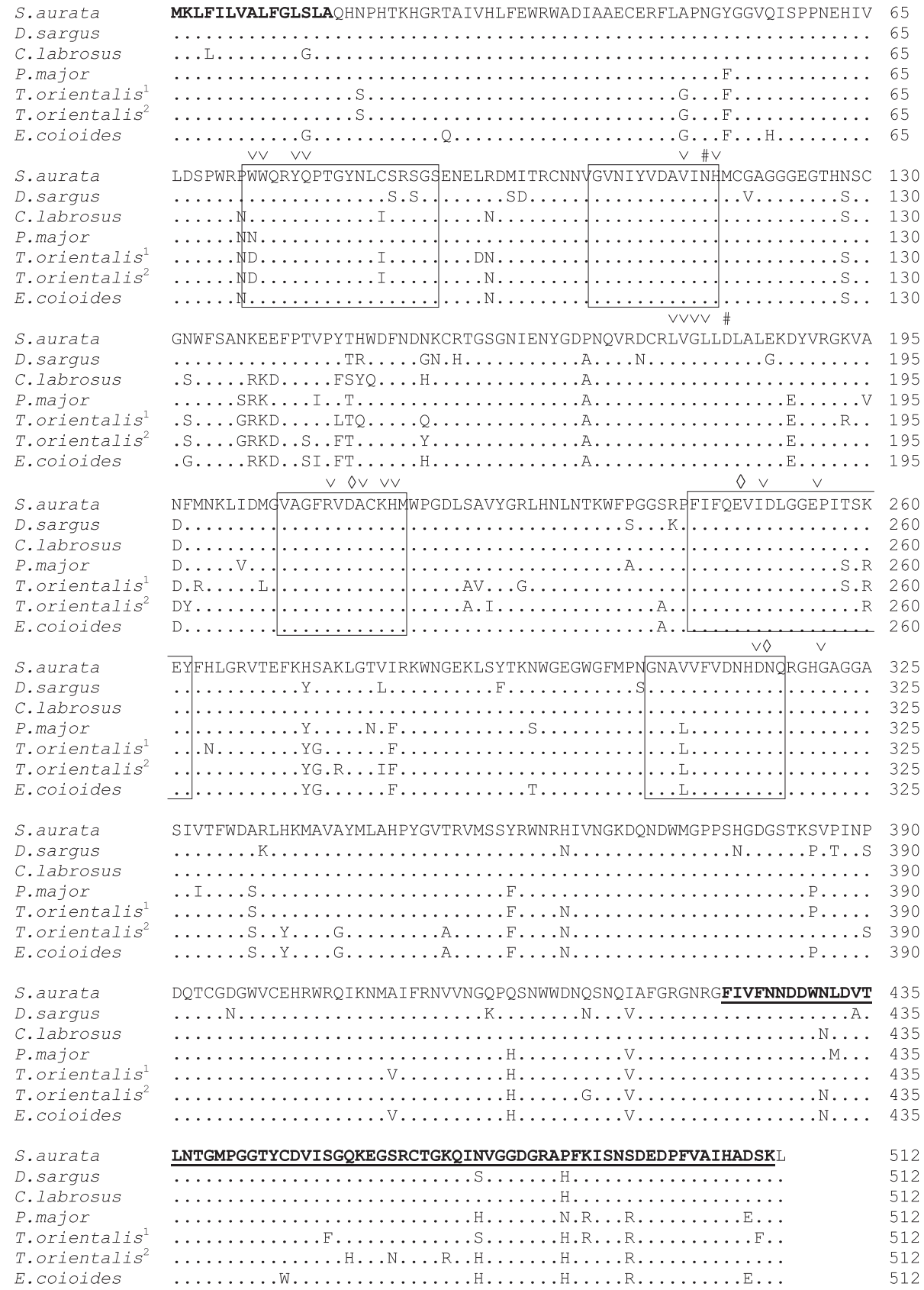

Fig. 3. Alignments of α-amylase amino acid sequences from *S. aurata* (GenBank acc. no. ALB35087), *Diplodus sargus* (GenBank acc. no. ABX89620), *Chelon labrosus* (GenBank acc. no. AIC81809), *Pagrus major* (GenBank acc. no. BAL14133), *Thunnus orientalis* (GenBank acc. nos. [1]BAL14131 – Amy2a.1 – and [2]BAL14132—Amy2a.2), and *Epinephelus coioides* (GenBank acc. no. ACJ26844). Dots indicate identity. The conserved amino acids for the calcium binding and for the active sites, from the glycoside hydrolase family 13 and alpha amylase family, are indicated with number signs (#) and down arrowheads or rhombi (˅, ◊), respectively. Whereas for the catalytic site the amino acids are indicated with a rhombus (◊) and they are coincident with those of the active site. The signal peptide (1–15) is shown in bold, the C-terminal beta-sheet domain (421–511) in bold and underlined and the 5 element fingerprint that provides a signature for alpha amylase family is boxed.

α-Amylase showed the conserved sites for the active domain (Trp^{73}–Trp^{74}, Tyr^{77}–Gln^{78}, Val^{113}, Asn^{115}–His^{116}, Leu^{177}–Val^{178}–Gly^{179}–Leu^{180}, Arg^{210}, Asp^{212}–Ala^{213}, Lys^{215}–His^{216}, Glu^{248}, Ile^{250}, Glu^{255}, His^{314}–Asp^{315}, His^{320}), the calcium binding domain (Asn^{115}, Asp^{182}), and the catalytic domain (Asp^{212}, Glu^{248}, Asp^{315}), from the alpha amylase catalytic domain family superfamily (Fig. 3).

3.2. Phylogenetic analyses

The respective phylogenetic trees have been reconstructed in order to examine the evolutionary histories of gilthead seabream proteins from trypsinogen (Try), bile-salt activated lipase (Cel) and α-amylase (Amy2a). Gilthead seabream trypsinogen clustered with other teleosteans trypsinogen type 1, and was grouped within the family Sparidae, including *D. sargus* and *P. major* (Fig. 4).

Bile salt-activated lipases were separated in two main clusters. The first one included Cel proteins from teleost species, while the second cluster included the tetrapods and the only teleost in this group, zebrafish *Danio rerio*. The *S. aurata* Cel proteins were clustered with the corresponding proteins of *S. partitus*. Notably most of the species showed at least two isoforms (Fig. 5).

α-Amylase sequences were mainly clustered in two groups, one of them containing Amy2a from teleosts, and the second from tetrapods. *S. aurata* Amy2a was located in the same cluster than other sparid (*D. sargus*) (Fig. 6).

3.3. Larval growth

Gilthead seabream larvae exhibited a good growth during the experiment (Fig. 7). The larvae grew exponentially up to 50 dph at a rate of

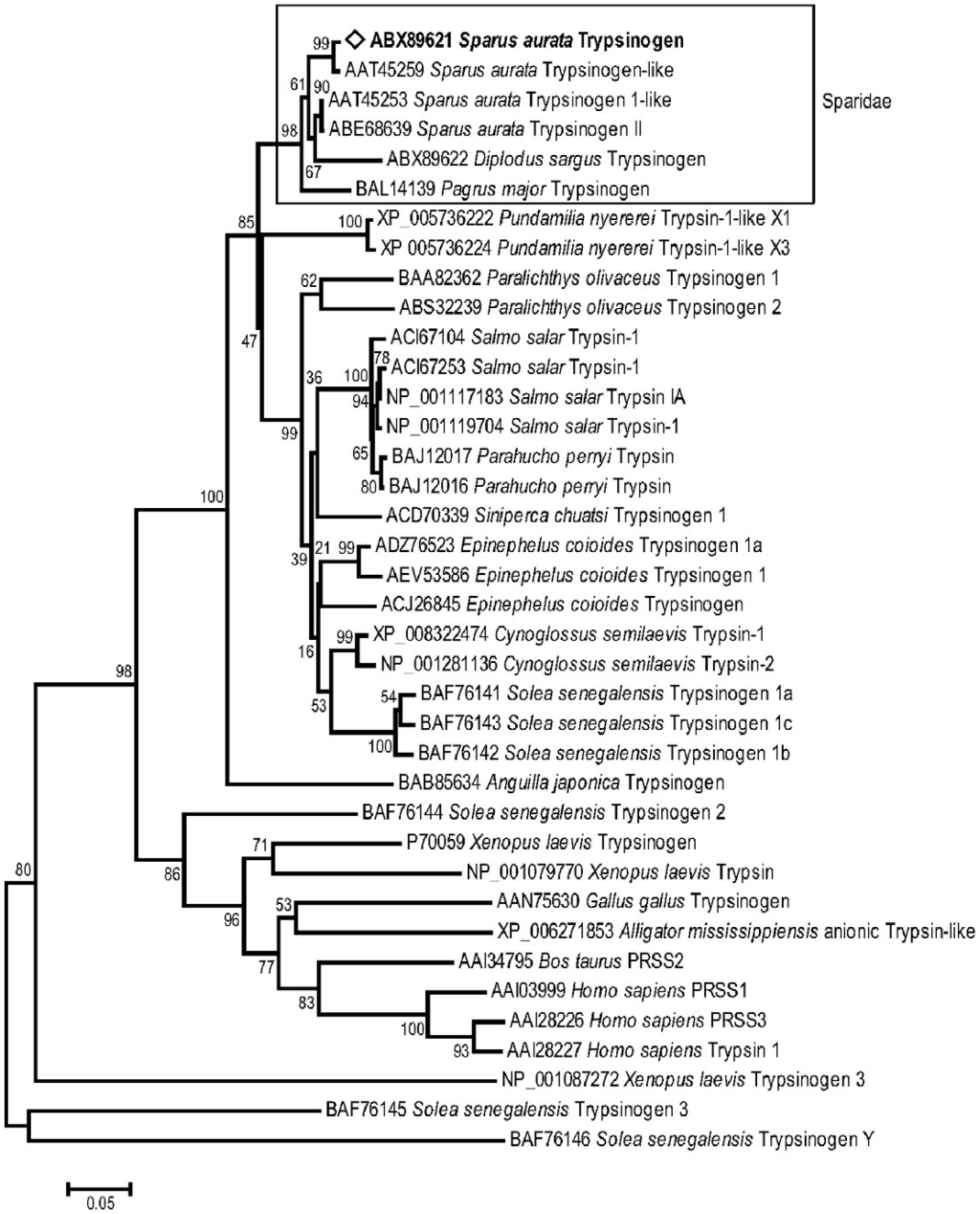

Fig. 4. Evolutionary relationship of 38 taxa for trypsinogen (Try). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches. GenBank or NCBI Reference Sequence accession numbers appear to the left of each taxon.

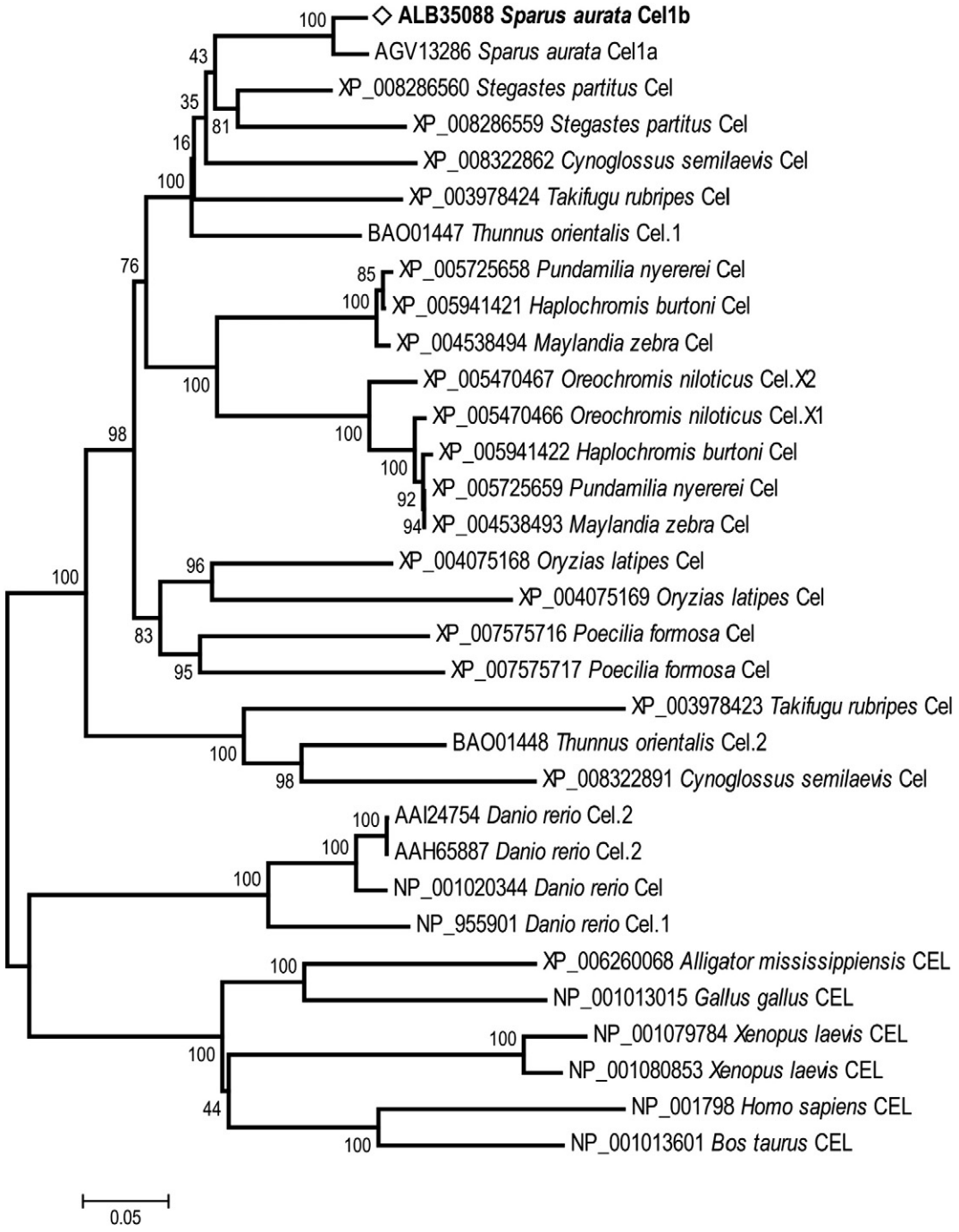

Fig. 5. Evolutionary relationship of 32 taxa for bile salt-activated lipase (Cel). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches. GenBank or NCBI Reference Sequence accession number appears to the left of each taxon.

0.076 d^{-1} on a dry weight basis. At the end of the experiment (60 dph) the larvae attained a final dry weight of 2.30 ± 0.28 mg and total length of 16.72 ± 0.07 mm.

3.4. Ontogeny of the molecular expression of digestive enzymes precursors

Expression of proteases was detected as early as hatching. The expression pattern of *try* increased from hatching up to the onset of feeding (4 dph) and thereafter maintained similar values with some fluctuations until the end of the experiment, although a significant increase (1.50 ± 0.06) was observed later in the development at 40 dph (Fig. 8A). Expression of *ctrb* sharply increased from hatching to 7 dph and after a short decline maintained similar values up to the end of the experimental period (Fig. 8B).

pla2 was the only enzyme which pattern of expression had a tendency to decrease before mouth opening (4 dph) showing a reduction in the expression level from hatching (0.65 ± 0.19) to the minimum expression level at 5 dph (0.22 ± 0.03). Relatively low values were maintained up to 18 dph and afterwards the expression increased to reach the maximum level ($p < 0.05$) at 60 dph (2.48 ± 0.42) (Fig. 9A). The expression of *cel1a* exhibited a continuous increase from hatching

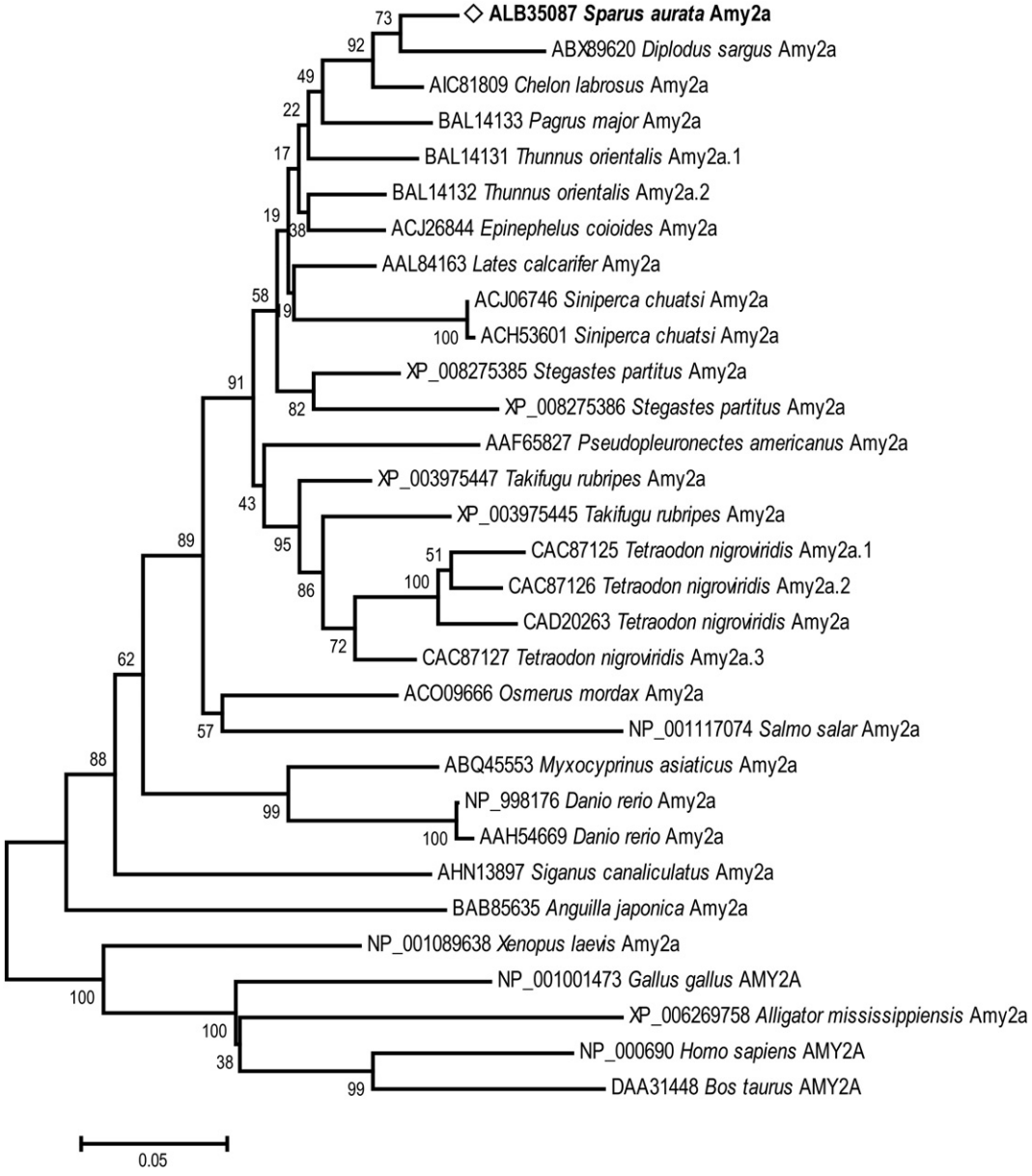

Fig. 6. Evolutionary relationship of 31 taxa for α-amylase (Amy2a). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches. GenBank or NCBI Reference Sequence accession number appears to the left of each taxon.

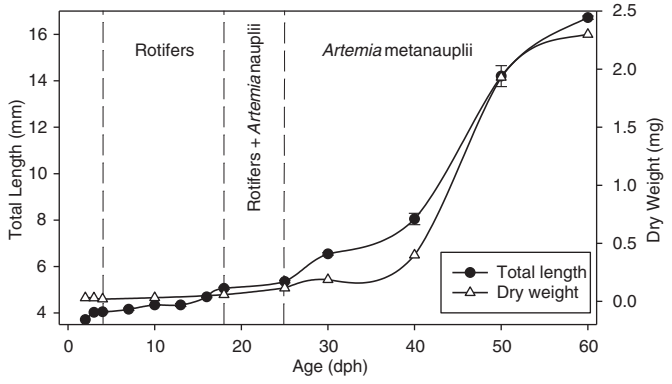

Fig. 7. Growth pattern in total length and dry weight of gilthead seabream during larvae development. Feeding regime is indicated between dashed lines. Values are represented as means ± SE (n = 10).

(0.15 ± 0.01) to 15 dph, dropping afterwards to low values until 34 dph. After this age, the expression increased to the end of the experimental period (2.62 ± 0.41) (Fig. 9B). On the other hand, *cel1b* expression increased from hatching to 10 dph (0.83 ± 0.03) and after a significant decline on 18 dph continued increasing slowly up to 60 dph (1.25 ± 0.09) (Fig. 9C). The determination of baseline C_T values from cel isoforms showed a specific higher expression level for *cel1b* ($C_T = 20.44 \pm 0.14$) than *cel1a* ($C_T = 27.02 \pm 0.09$), almost 100 times higher.

The expression profile of *amy2a* exhibited a sharp increase from hatching ($0.03 \pm 4.56\ 10^{-3}$) to 5 dph (5.01 ± 0.25), when the maximum expression was reached ($p < 0.05$). From this age onwards the expression decreased until 60 dph (0.25 ± 0.04) (Fig. 10).

3.5. Molecular expression in non-fed larvae

Under food deprivation gilthead seabream larvae died at 10 dph. *try*, *ctrb*, *cel1a*, *cel1b* and *amy2a* showed an increase in their expression up to a maximum on 7 dph (*try*: 0.35 ± 0.05, *ctrb*: 2.00 ± 0.19, *cel1a*: 0.94 ± 0.18, *cel1b*: 0.83 ± 0.06, and *amy2a*: 7.40 ± 0.60), decreasing afterwards

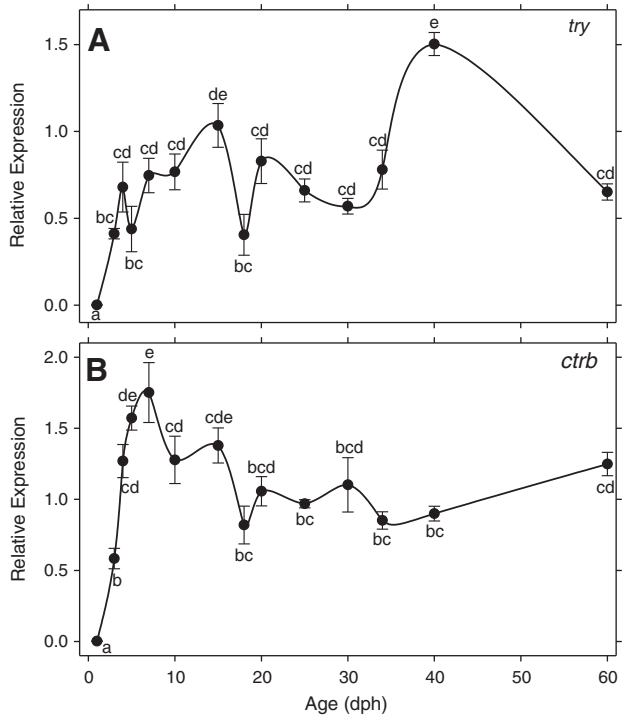

Fig. 8. Proteases (A) *try* and (B) *ctrb* relative expression during larvae development (n = 9). Different letters represent different statistically significant values between ages ($p < 0.05$).

(Fig. 11). For *pla2*, a steady decreasing pattern was observed during the experimental period, starting with the maximum expression level at hatching (0.70 ± 0.11).

4. Discussion

A better understanding of the digestive physiology will contribute to increase the performance of the rearing protocol for fish larvae (Rønnestad et al., 2013). In this work, we have characterized a complete set of genes for pancreatic digestive enzymes during the ontogeny in gilthead seabream larvae. Firstly, we cloned the full-length cDNA transcripts of some of these genes (*try*, *cel1b*, and *amy2a*) in which the complete sequences were not yet available at the time of the experiment. These genes had a high identity at nucleotide and amino acid levels to their homologous from other teleosts present in GenBank, as well as with others sequences already published for this species. Moreover, motifs analyses for each of the sequences demonstrated the presence of conserved domains. Taking together, all these features assure the pertaining of each one of the cloned cDNAs to their respective gene families. In addition, the structural properties of the three digestive enzymes cloned in the present study demonstrate that the corresponding mRNAs are translated into the corresponding functional proteins.

Given at the time of the experiment *pla2* cDNA sequence was not available for *S. aurata* in GenBank, a nucleotide Blast search was made using *P. major pla2* (acc. no.: AB050633) on *S. aurata* (taxiid: 8175) expressed sequence tags (est) database, obtaining a 85% identity with clone AM972037. Posterior phylogenetic analysis (data not shown) revealed this sequence clustered together with both intestinal and pancreatic phospholipase A2 proteins, but being different to int-Pla2 from *S. aurata*. Then we assumed this to be the pancreatic form of *pla2*, although the cloning of the full-length cDNA will be necessary to confirm this aspect, as well as to characterize the morphological localization of the mRNA and/or the protein.

Phylogenetic analyses showed that trypsinogen is closer to other trypsinogens previously cloned from *S. aurata* and other sparids, being

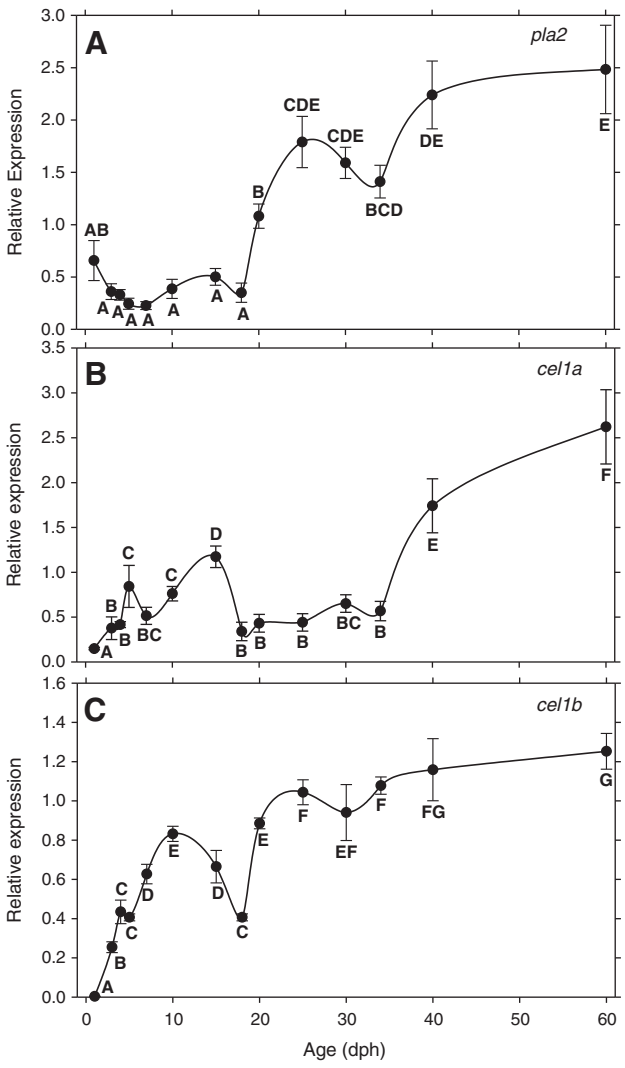

Fig. 9. Relative expression of gilthead seabream lipases during larvae development (n = 9): (A) *phospholipase A_2* (*pla2*) and *bile salt-activated lipase* isoforms: (B) *cel1a* and (C) *cel1b*. Different letters represent different statistically significant values between ages ($p < 0.05$).

all them similar to trypsinogens 1 from other teleosts, and belonging to trypsinogen type I or anionic trypsinogens. Bile-salt activated lipase was clustered to a similar, but not identical, previously reported Cel protein in *S. aurata* (GenBank acc. no. AGV13286; Benedito-Palos et al.,

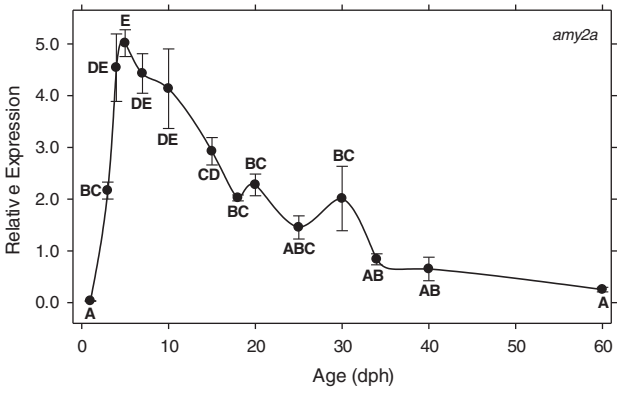

Fig. 10. Relative expression of *amy2a* during larvae development (n = 9). Different letters represent different statistically significant values between ages ($p < 0.05$).

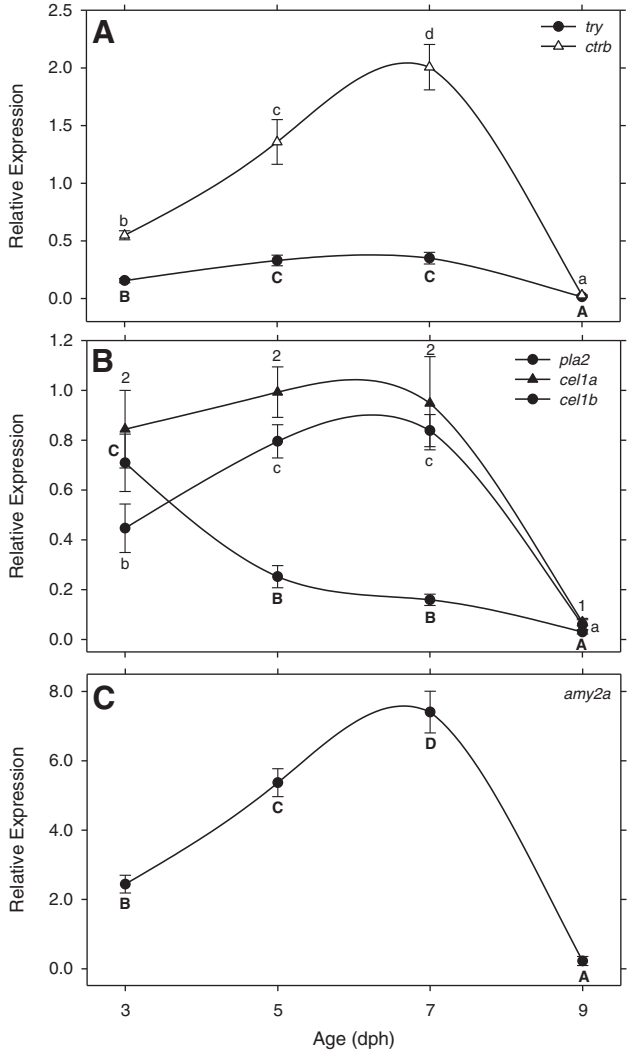

Fig. 11. Relative expression of digestive enzymes in unfed larvae from 3 to 9 dph (n = 9): (A) proteases (*try* and *ctrb*), (B) lipases (*pla2*, *cel1a* and *cel1b*), and (C) carbohydrases (*amy2a*). Different letters represent different statistically significant values between ages ($p < 0.05$).

2014), which demonstrated the existence of a new Cel isoform in this species. Finally, α-amylase clustered, as expected, with other teleosts.

4.1. Expression patterns during the larval growth and development

In our study, *S. aurata* larvae exhibited good growth and development, comparable to those described in previous studies fed on live prey (Polo et al., 1992; Yúfera et al., 1993; Sarasquete et al., 1995; Moyano et al., 1996; Parra and Yúfera, 2000). Therefore, the expression patterns found here can be related to other developmental results reported in those studies.

In marine fish larvae the pancreatic alkaline proteases are the predominant proteolytic enzymes (Zambonino-Infante et al., 2008). Trypsinogen and chymotrypsinogen are pancreatic enzyme precursors for the serine proteases trypsin and chymotrypsin. In the intestine, enterokinase removes the N-terminal activation peptide on trypsinogen and converts it into its active form, trypsin. In turn, the resulting trypsins activate their own proenzymes (Rønnestad et al., 2013).

After the initial increase, *try* and *ctrb* maintained similar values during almost the whole larval stage with some irregular fluctuations. Similar *try* expression patterns were reported for Japanese flounder *P. olivaceus* (Srivastava et al., 2002), winter flounder *Pseudopleuronectes americanus* (Murray et al., 2003), *P. pagrus* (Darias et al., 2006), bullseye puffer fish *Sphoeroides annulatus* (García-Gasca et al., 2006), yellow catfish *Pelteobagrus fulvidraco* (Wang et al., 2006), European seabass *Dicentrarchus labrax* (Darias et al., 2008), spotted rose snapper *Lutjanus guttatus* (Galaviz et al., 2012), as well as during the first month of exogenous feeding in Atlantic salmon *Salmo salar* (Sahlmann et al., 2015). However, in some studies with qPCR, *try* also showed a decreasing trend after the maximum was reached, shortly after first feeding, in some species, such as Atlantic cod *Gadus morhua* (Kortner et al., 2011), Asian seabass *Lates calcarifer* (Srichanun et al., 2013) and *T. orientalis* (Murashita et al., 2014), while *ctrb* may also show a constant trend after first feeding (*Solea senegalensis*, Gamboa-Delgado et al., 2011). These close expression patterns exhibited by *try* and *ctrb* would support the suggested complementary action of trypsin and chymotrypsin during the early larval development before the appearance of the acidic digestion (Rønnestad et al., 2013).

Bile salt-activated is a unique pancreatic lipase which hydrolyzes neutral lipids in teleost while phospholipase A_2 is responsible for hydrolyzing phospholipids in the intestine of marine fishes (Sæle et al., 2010; Hansen et al., 2013). There are many recent descriptions in GenBank about two *cel* isoforms discovered in many fish species in several genome sequencing projects. To confirm these, two different reverse primers were tested to describe their expression patterns. We found that both *cel* isoforms, *cel1a* (Benedito-Palos et al., 2014) and *cel1b* (cloned in this work), are expressed along the larval development. As in other fish species, molecular expressions of these lipases in *S. aurata* were also detected as early as hatching, proving that fish larvae have the capacity to digest dietary lipids before mouth opening (Murray et al., 2003; Sæle et al., 2010; Srichanun et al., 2013; Murashita et al., 2014). Taken as a whole, *cel1b* and *pla2* showed an increasing trend with larval age. A similar expression profile for *cel* was found in *T. orientalis* (Murashita et al., 2014), haddock *Melanogrammus aeglefinus* (Perez-Casanova et al., 2004), *G. morhua* (Kortner et al., 2011) and *L. calcarifer* (Srichanun et al., 2013). Nevertheless, in species as Atlantic halibut *Hippoglossus hippoglossus* and *P. americanus* the expression remained at very low levels during first 60 dph, suggesting an age dependent expression (Murray et al., 2003, 2006). The importance of *cel1a* isoform was negligible as compared to *cel1b* that was almost 100 times more expressed. Nevertheless, *cel1a* expression increased at the end of the larval stage, becoming the main isoform during the juvenile stage in this species as reported by Benedito-Palos et al. (2014). Likewise, an increasing *pla2* expression pattern was also found in *T. orientalis* (Murashita et al., 2014) and *L. calcarifer* (Srichanun et al., 2013). Nonetheless, *pla2* expression was stable from the start of feeding until 62 dph in *G. morhua* (Sæle et al., 2010). Furthermore, the gene expression pattern of pancreatic lipases appears to be species specific (Murashita et al., 2014).

The expression of *amy2a* increased sharply from hatch to the onset of feeding decreasing afterwards until the end of experiment. The same expression profile was reported in *D. labrax* (Darias et al., 2008), *P. pagrus* (Darias et al., 2006), *G. morhua* (Kortner et al., 2011), *L. calcarifer* (Srichanun et al., 2013) and *T. orientalis* (Murashita et al., 2014). Natural diet of fish larvae has very low amount of carbohydrates, therefore the digestive function of amylase is not completely understood (Darias et al., 2006; Hansen et al., 2013). The apparent genetic pre-determination of the process which drives the expression patterns suggest the importance of carbohydrates as nutrients during the first stages of development even in larvae from carnivorous fishes (Lazo et al., 2011; Srichanun et al., 2013; Murashita et al., 2014). However, an increasing trend profile of the α-amylase expression during the ontogeny has also been described in species with omnivorous habits regardless of the offered diet composition (Parma et al., 2013; Kim et al., 2014).

The detection of mRNA expression of all these enzymes before the onset of exogenous feeding has been observed in other fish larvae (Zacarias-Soto et al., 2006; Shan et al., 2008; Zambonino-Infante et al.,

2008; Lazo et al., 2011; Hansen, 2012; Rønnestad et al., 2013; Srichanun et al., 2013; Suzer et al., 2013) and supports the hypothesis that the expression of digestive enzymes is genetically preprogrammed in fish during early developmental stages.

An expression decline or change in the tendency during the development was detected at 18 dph for all tested genes. Whether this fact is consequence of the change of prey or a developmental issue associated to the gut functional maturation is an open question that will require specific research.

4.2. Expression patterns in unfed larvae

A quick and successful onset of the exogenous feeding is crucial for the subsequent development of the larvae. A relatively short non-feeding period may induce deformities as well as permanent feeding problems that seriously affect larval survival (Yúfera et al., 1993; Kailasam et al., 2007; Yúfera and Darias, 2007). In the present work, the larvae maintained unfed after the opening of the mouth died as expected at day 10 dph. This is in agreement with a previous study with this species (Yúfera et al., 1993). Interestingly, the expression of all studied digestion-related genes fell down after 7 dph, the day determined as the moment of irreversible starvation in the mentioned study. That is, after day 7, the larvae still alive are unable to start feeding although the food is supplied. The present results show that this incapacity to predate and ingest is accompanied by a strong limitation to synthesize the digestive enzymes. On the other hand, during the three first days after the mouth opening, the expression patterns of fasted larvae had the same profile than fed larvae, confirming the genetically preprogrammed enzymatic machinery of early fish larvae (Ribeiro et al., 1999; Lazo et al., 2000; Darias et al., 2008; Galaviz et al., 2012; Srichanun et al., 2013). These findings confirm how genes could be used as markers for digestive capacity and nutritional status in fish larvae (Lazo et al., 2011).

In conclusion, this study presents the expression pattern of the main pancreatic enzymes in the gilthead seabream during larval ontogeny, which is similar to other marine fish species with carnivorous preferences. This suggests that these molecular profiles have an important species-specific type of feeding preference component. Detectable expression of the mRNA transcripts codifying the pancreatic enzymes found at hatching confirms the readiness to produce the corresponding enzymes at mouth opening. Moreover, the starvation condition also confirmed the genetically preprogrammed enzymatic machinery during the early development of fish larvae.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpb.2015.09.006.

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