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Ontogeny changes and weaning effects in gene expression patterns of digestive enzymes and regulatory digestive factors in spotted rose snapper (*Lutjanus guttatus*) larvae

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Abstract The study of digestive physiology is an important issue in species that have been introduced in aquaculture like the spotted rose snapper (*Lutjanus guttatus*). The aims of this study were to describe the expression of digestive enzymes (trypsinogen, chymotrypsinogen, α-amylase, lipoprotein lipase, phospholipase A and pepsinogen) and their relation with orexigenic (neuropeptide Y, NPY) and anorexigenic (cholecystokinin, CCK) factors during the larval development and to evaluate the effect of weaning in their expression. The results showed that the transcripts of all the assayed digestive enzymes, with the exception of pepsinogen, and NPY and CCK were already present in *L. guttatus* from the hatching stage. The expression of all the enzymes was low during the yolk-sac stage (0–2 days after hatching, DAH), whereas after the onset of exogenous feeding at 2 DAH, their expression increased and fluctuated throughout larval development, which followed a similar pattern as in other marine fish species and reflected changes in different types of food items and the progressive maturation of the digestive system. On the other hand, weaning of *L. guttatus* larvae from live prey onto a microdiet between 25 and 35 DAH significantly affected the relative expression of most pancreatic digestive enzymes during the first weaning days, whereas chymotrypsinogen 2 and lipoprotein lipase remained stable during this period. At the end of co-feeding, larvae showed similar levels of gene expression regardless of the diet (live prey vs. microdiet), which indicated that larvae of *L. guttatus* were able to adapt their digestive capacities to the microdiet. In contrast, feeding *L. guttatus* larvae with live feed or microdiet did not affect the expression of CCK and NPY. The relevance of these findings with regard to current larval rearing procedures of *L. guttatus* is discussed.

Keywords Spotted rose snapper · *Lutjanus guttatus* · Digestive function · Enzyme expression · Regulatory digestive factors · Weaning

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Introduction

Understanding the functions and limitations in processing capacities of the digestive system during early

life stages continues to be a key area for aquaculture-related research on fish larvae (Rønnestad et al. 2013). In this context, the efficiency of food utilization depends on the physiological capacities in fish to digest and transform ingested nutrients. The analysis of digestive enzyme activities is an easy and reliable biochemical method that can provide insight into the digestive physiology of fish larvae (Rønnestad et al. 2013), their nutritional condition (Bolasina et al. 2006; Cara et al. 2007) and assist in defining their nutritional requirements (Twining et al. 1983). In addition, a comprehensive analysis of the ontogenic changes during the early life stages of fish is essential for the proper design of feeding strategies and formulation of compound diets (Gisbert et al. 2013).

Studies have identified two critical phases during marine fish larval rearing that might be considered as bottlenecks, both of which are related to larval feeding and digestive capacities (García-Gasca et al. 2006; García-Ortega 2009; Qu et al. 2012). The first one occurs during the onset of exogenous feeding, when larvae must successfully start to seek and capture food, whereas the second one occurs during weaning, which in aquaculture is the transition from live food to compound diets (Pérez-Casanova et al. 2006; Muguet et al. 2011 among others). It has been suggested that these critical stages are associated with a low digestive capacity present in the larvae at these periods in comparison with later stages of development. Although the basic mechanisms of organ development are similar in all teleosts, there are considerable differences in the relative timing of ontogeny. The time required for the development of organs and their associated physiological functions are affected by the ontogeny of each species and biotic and abiotic factors such as temperature, water quality, food availability and composition during the early stages of life (Zambonino-Infante and Cahu 2001; Álvarez-González et al. 2006; García-Gasca et al. 2006; Pérez-Casanova et al. 2006).

In this context, many studies have described the morphological and functional development of the digestive system by means of histological and biochemical approaches in order to improve larval rearing and feeding protocols (Lemieux et al. 2003; Gisbert et al. 2004, 2009; García-Gasca et al. 2006; Pérez-Casanova et al. 2006; Sveinsdóttir et al. 2006; Kortner et al. 2011a). Recently, those approaches have been complemented by molecular tools to provide data on the expression patterns of genes involved in the development and functionality of the digestive system during early ontogeny (Galaviz et al. 2012; Srichanun et al. 2013; Mata-Sotres et al. 2016). Knowledge of gene expression levels and pattern of production of digestive enzyme precursors constitute a valuable tool that complements the information on the nutritional condition of an organism obtained through enzymatic indicators. Expanding the knowledge on the molecular mechanisms underlying the ontogenetic development of the digestive system and its regulation would aid in finding solutions to nutritional problems by providing clues as to which molecular pathways may be disrupted and/or at what stage particular pathways are ontogenetically mature (Gisbert et al. 2013).

The spotted rose snapper, *Lutjanus guttatus* (Steindachner 1869) is a tropical marine fish commercially important in the Mexican Pacific coast and a species with a great potential for aquaculture. In recent years, several studies have provided insight into some aspects of its culture, including reproduction, larval rearing and juvenile nutrition (Ibarra-Castro and Duncan 2007; Boza-Abarca et al. 2008; García-Ortega 2009; Abdo de la Parra et al. 2010; Ibarra-Castro and Álvarez-Lajonchére 2011; Álvarez-Lajonchére et al. 2012). Two recent studies have provided data on the ontogeny of the digestive system of this species by means of assaying the activity of the main pancreatic digestive enzymes (Moguel-Hernández et al. 2014) and gene expression patterns of alkaline and acid proteases (Galaviz et al. 2012), whereas little is known about the expression profile of the enzymes associated with proximal component digestion (trypsinogen, chymotrypsinogen, α-amylase, and phospholipase A and pepsinogen) and nutrient regulation (lipoprotein lipase), as well as neuronal (neuropeptide Y, NPY) and hormonal (cholecystokinin, CCK) key regulatory factors that stimulate (orexigenic) or inhibit (anorexigenic) food intake during larval ontogeny. This information may be of use for providing insight into the regulation of digestive enzymes and understanding the digestive and nutritional physiology of fish larvae during larval ontogeny.

The aim of this study was to describe the gene expression of several digestive enzymes (trypsinogen, chymotrypsinogen, α-amylase, phospholipase A and pepsinogen), as well as that of lipoprotein lipase involved in the regulation of circulating triglycerides, and their relation with orexigenic (NPY) and

anorexigenic (CCK) factors during the larval development of the spotted rose snapper.

Materials and methods

Larval fish rearing and sample collection

Fertilized eggs (fertilization rate = 95 %) were obtained from a single, spontaneous spawning of spotted rose snapper broodstock (20 males and 10 females of 1.5–3 kg) kept in captivity in Centro de Investigación en Alimentación y Desarrollo in Mazatlán, Sinaloa (México) and shipped to the Unidad Piloto de Maricultivos at CICIMAR-IPN in La Paz, Baja California Sur (Mexico). At their arrival, eggs were incubated in two cylindrical conical tanks filled with 120 L of mechanically filtered, UV-sterilized seawater at 26 °C, with moderate aeration from the bottom and continuous water flow (500 mL min^{-1}) until they hatched (hatching rate = 97.5 %). At 1 day after hatching (DAH), larvae were transferred from incubation tanks to 16 120-L fiberglass tanks (density of 100 larvae L^{-1}) connected to a recirculation system where they were reared until the early juvenile stage (35 DAH). During this period, water temperature was 26.7 ± 0.2 °C (mean ± standard deviation, SD), salinity was 36.0 ± 0.6 g L^{-1}, and dissolved oxygen was 4.0 ± 0.4 mg L^{-1} (~50 % saturation), and the photoperiod was 13L:11D (light/dark). Light intensity averaged 1200 lux at the water surface. Microalgae, *Nannochloropsis oculata*, were added to rearing tanks at a density of 300,000 cells mL^{-1} from 2 to 18 DAH as green water.

Two different studies are presented in this report: a first study dedicated to describing the changes in the digestive enzymes (trypsinogen, chymotrypsinogen, α-amylase, lipoprotein lipase, phospholipase A and pepsinogen) and their relation with orexigenic (NPY) and anorexigenic (CCK) factors during the larval development of *L. guttatus* (study 1), and a second one focused on evaluating the effects of weaning on the expression of the above-mentioned genes (study 2). The larval rearing protocol used in the study 1 was the following (Fig. 1): spotted rose snapper larvae were fed copepod, *Pseudodiaptomus euryhalinus*, nauplii from 2 to 18 DAH (density: two nauplii mL^{-1}), rotifers *Brachionus rotundiformis* enriched with *N. oculata* from 2 to 7 DAH (density: 3 rotifers mL^{-1})

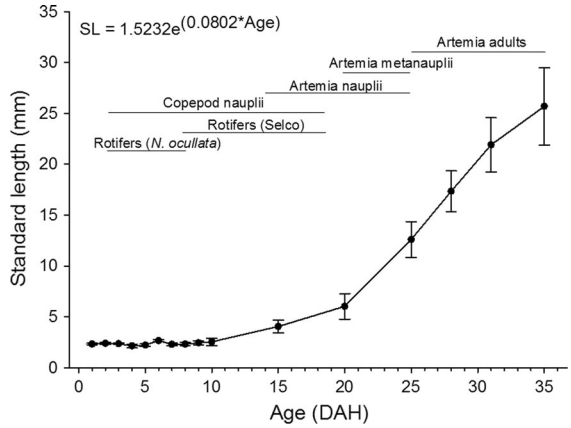

Fig. 1 Changes in standard length (mm, SL) and growth regression equation and rearing protocol in spotted rose snapper, *Lutjanus guttatus* larvae. Each *point* represents the mean ($n = 15$), while the *vertical bars* show the standard deviation

and with S. presso Selco™ (INVE Aquaculture, Thailand) from 8 to 18 DAH (density: 3 rotifers mL^{-1}), *Artemia* sp. metanauplii (*Artemia* cysts, Vitellus, Bernaqua, Belgium) enriched with S.presso Selco™ from 14 to 24 DAH (density: 0.5 nauplii mL^{-1}), 10-day-old *Artemia* metanauplii from 22 to 24 DAH (density: 3 metanauplii mL^{-1}), and 20-day-old *Artemia* adults from 25 to 35 DAH (density: 3 adults mL^{-1}). Live preys were enriched according to instructions provided by the manufacturer of the commercial enriching products. Larvae were sampled directly from the rearing tanks using a 100 μm sieve. Samples were taken at hatching, 1, 2, 3, 5, 10, 15, 20 ($n = 30$ larvae), 25, 28, 31 and 35 ($n = 10$ larvae) DAH. Fish were sampled early in the morning before feeding to avoid possible effects to the expression of digestive enzyme genes of the larvae due to the presence of prey in the larval digestive tract. After sampling, larvae were killed with an overdose of anesthetic (tricaine methanesulfonate, Sigma-Aldrich, St. Louis, USA), washed in distilled water and frozen at −80 °C in RNAlater® (Sigma-Aldrich) until they were processed. In addition, 15 anesthetized larvae from each sampling point were photographed under a binocular microscope (SZ-CTV, Olympus, Japan) and measured for standard length (SL) with Image Pro Plus v4.5 (Media Cybernetics, MD, USA) digital image analyzer software.

For the weaning experiment (study 2), larvae were kept under the same conditions as in the first study until 23 DAH when 508 larvae were removed from the

general rearing tank and split into 4 tanks (120 L) at a density of 1.1 larvae L^{-1} (127 larvae per tank). Tanks were divided into two groups, the first group continued to be fed with *Artemia* adults (3 adults mL^{-1}) up to 35 DAH (Group LF), whereas the second group (Group MD) was progressively weaned onto the inert microdiet Otohime B2 (Red Mariculture Inc., USA) (particle size: 360–650 μm; proximate composition: 51 % protein, 11 % fat, 3 % fiber, 15 % ash, 2.3 % calcium, 1.5 % phosphorus) between 25 and 30 DAH. Weaning was conducted by co-feeding; by decreasing the proportion of live food and increasing the amount of inert diet over a period of 5 days (proportion inert diet/*Artemia*: 25 DAH, 15/85; 26 DAH, 30/70; 27 DAH, 45/55; 28 DAH, 60/40; 29 DAH, 80/20). At 30 DAH, only the inert diet was offered to fish from group MD. The amount of diet per day was estimated at 20 % stocked fish biomass. In both groups (MD and LF), feed was divided into four portions, which were provided throughout the day at regular times. At 25, 28, 31 and 35 DAH, 10 larvae per tank were sampled early in the morning before feed was administered in rearing tanks (first meal of the day). Each sampled larva was weighed on an analytical balance (TE64, Sartorius, Germany) and measured using an electronic digital caliper (12777-830, Traceable, USA) for SL. Fish samples were stored in RNAlater® and kept at −80 °C until further analysis as previously described for the study 1.

RNA extraction, cDNA synthesis and qPCR assays

Each sample of pooled larvae was homogenized in TRIzol® Reagent (Invitrogen, USA) then subjected to DNase treatment with RQ1 RNase-Free DNase kit (Promega, Madison, USA). RNA concentration and quality were determined by spectrophotometry ($A_{260/280}$) and a denaturing electrophoresis in TAE agarose gel (1.5 %) (Masek et al. 2005). For each sample 2 μg of RNA was reverse transcribed into cDNA using SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen™, Life Technologies, Warrington, UK) primed with Oligo $(dT)_{12–18}$ and random hexamers.

To obtain the specific sequences of α-amylase (*amy*), lipoprotein lipase (*lpl*), phospholipase A (*pla2*), chymotrypsinogen 1 (*ctr1*), chymotrypsinogen 2 (*ctr2*), cholecystokinin (*cck*), neuropeptide Y (*npy*) and elongation factor 1α (*ef1a*) genes, an alignment of marine teleost homologs for each of these gene sequences obtained from GenBank were made using BioEdit Sequence Alignment Editor ver 7.0.5.2 (Hall 1999). Consensus primers designed from conserved regions identified in these alignments were used for amplification of *L. guttatus*-specific gene sequences, which after sequencing, were used for designing primers for the *L. guttatus*-specific qPCR assays (Table 1).

Quantitative real-time polymerase chain reaction (qPCR)

Real-time qPCR was performed using an ABI PRISM 7300 (Applied Biosystems, Alcobendas, Spain) to analyze gen expression of α-amylase (*amy*), lipoprotein lipase (*lpl*), phospholipase A (*pla2*), trypsinogen (*trp*), chymotrypsinogen 1 (*ctr1*), chymotrypsinogen 2 (*ctr2*), pepsinogen (*pep*), cholecystokinin (*cck*), neuropeptide Y (*npy*), elongation factor 1α (*ef1a*) and 18S rDNA (*18S*). The two last genes were used as reference genes. Quantitative PCR analyses for each gene were performed in triplicate (pooled larvae) in a total volume of 20 μL containing 2 μL cDNA, 1 μL primers (20 μmol L^{-1}), 10 μL SYBR® Green (Life Technologies, Warrington, UK) and 7 μL of sterile water. A negative control was included (NTC-no template control) for each set of reactions on each 96-well plate. All reactions were performed using the following conditions: 1 cycle at 95 °C for 10 min; 40 cycles at 95 °C for 15 s, 62 °C for 30 s. A final melt curve stage completed the program, enabling confirmation of the amplification of a single product in each reaction. The absence of primer-dimer formation in the NTC was also confirmed. A standard curve was obtained by amplification of a dilution series of cDNA for calculation of the efficiency (%E) for each assay. For data analysis, the RT-qPCR results were imported into the software qBase + (Biogazelle, Zwijnaarde, Belgium), where relative expression units (REU) were calculated employing target and run-specific amplification efficiencies and using the geometric mean of 2 reference genes (*EF1* and *18S; M* = 0.5, CV = 0.17). Data on gene expression were expressed as mean ± standard error of the mean (SEM) calculated from 3 replicates.

Statistics

Gene expression data were analyzed using one-way ANOVA, Kruskal–Wallis test for compared digestive

Table 1 Primer sequences for qPCR and amplicon size for spotted rose snapper *Lutjanus guttatus*

Gene/NCBI accession	Direction	Sequence	Size (bp)	% E	Source
α-Amylase (*amy*)	Sense	CACTCTTCATGTGGAAGCTGGTTC	110	94	(1)
KJ908179	Antisense	CCATAGTTCTCAATGTTGCCACTGC			
Lipoprotein lipase *(lpl)*	Sense	AAGCTGGTGTCTGCCCTCTACG	190	93	(1)
KJ908180	Antisense	AGCCACATGTGCTCCCAGACTG			
Phospholipase A2 *(pla2)*	Sense	CGACTACGGCTGCTACTGCGG	130	98	(1)
KJ908181	Antisense	TCGTCACAGCTGTAGTCGTAGAAC			
Trypsinogen *(trp)*	Sense	ATGAGCTCCACTGCTGACAGAAAC	156	100	(2)
HM754476	Antisense	AGAGTCACCCTGGCAAGAGTCCTT			
Chymotrypsinogen 1*(ctr1)* KJ908182	Sense	CTCACTGCCGCGTGTCTCCAAG	105	89	(1)
	Antisense	GCCCTGGAAATGGACTTGACCTG			
Chymotrypsinogen 2 *(ctr2)* KJ908183	Sense	GGTGTCCCTGCAGGATTACACC	170	89	(1)
	Antisense	CTTCATGACCTGGATGTCCTCAGC			
Pepsinogen *(pep)*	Sense	TGTCTTCGACAACATGATCAAGCA	184	90	(2)
HM754478	Antisense	GCTGTCCATCTTGATCTGCCAGTA			
Cholecystokinin (*cck*)	Sense	TCTCCTCGAGGCGGACACCCAC	130	99	(1)
KJ908184	Antisense	CTGCGCACAGAACCTTTCCTG			
Neuropeptide Y (*npy*) KJ908185	Sense	GTACTACTCAGCCCTGAGACAC	105	96	(1)
	Antisense	CCTTCAACAGCAGCTCTGAGAC			
Elongation factor 1-alpha (*ef1-alpha*) KJ908186	Sense	GCATGGTCGTCACCTTCGCTCC	150	94	(1)
	Antisense	CCACGTATCCACGACGGATTTCC			
18S rDNA *(18S)*	Sense	CTGAACTGGGGCCATGATTAAGAG	165	98	(2)
HM754479	Antisense	GGTATCTGATCGTCGTCGAACCTC			

Sources: (1) current study, (2) Galaviz et al. (2012)

enzymes and regulatory digestion factors between sampling days. For the second experiment, a two-way ANOVA was performed to compare the expression of digestive enzymes and regulatory digestion factors, including larvae fed Artemia and larvae fed with commercial diet during weaning, having as variables the types of diet and time of sampling. Also a post hoc test Newman-Keuls were made to identify interactions between treatments. All statistics were conducted using Statistica 8 for Windows (Statistica® 8, USA), with a significance level of $P < 0.05$.

Results

Changes in gene expression of digestive enzymes and regulatory factors during larval ontogeny

Growth of spotted rose snapper from hatching up to 35 DAH was exponential (Fig. 1), reaching a maximum SL at the end of the study of 25.7 ± 3.8 mm (mean ± SD). No significant growth ($P > 0.05$) was observed during the first 10 days of development. The yolk sac was totally depleted between 2 and 3 DAH.

Transcripts of all the assayed digestive enzymes (with the exception of pepsinogen), and digestive regulatory factors were found in *L. guttatus* from hatching. The expression of all the enzymes was low during the yolk-sac stage (0–2 DAH), and after the onset of exogenous feeding at 2 DAH, their expression increased and fluctuated throughout larval development. The expression of *amy* significantly increased from hatching and 1 DAH to 5 DAH ($P = 0.0001$), reaching a maximal relative expression (8.82 ± 0.69 REUs) and from that point, a progressive decrease in expression until the end of the larval period ($P < 0.05$; Fig. 2a).

The mRNA of *lpl* and *pla2* were detected at relatively low levels in yolk–sac larvae aged 0–1 DAH (0.35 ± 0.02 and 0.008 ± 0.001 REU, respectively). The expression levels of *lpl* increased at 5 DAH with a

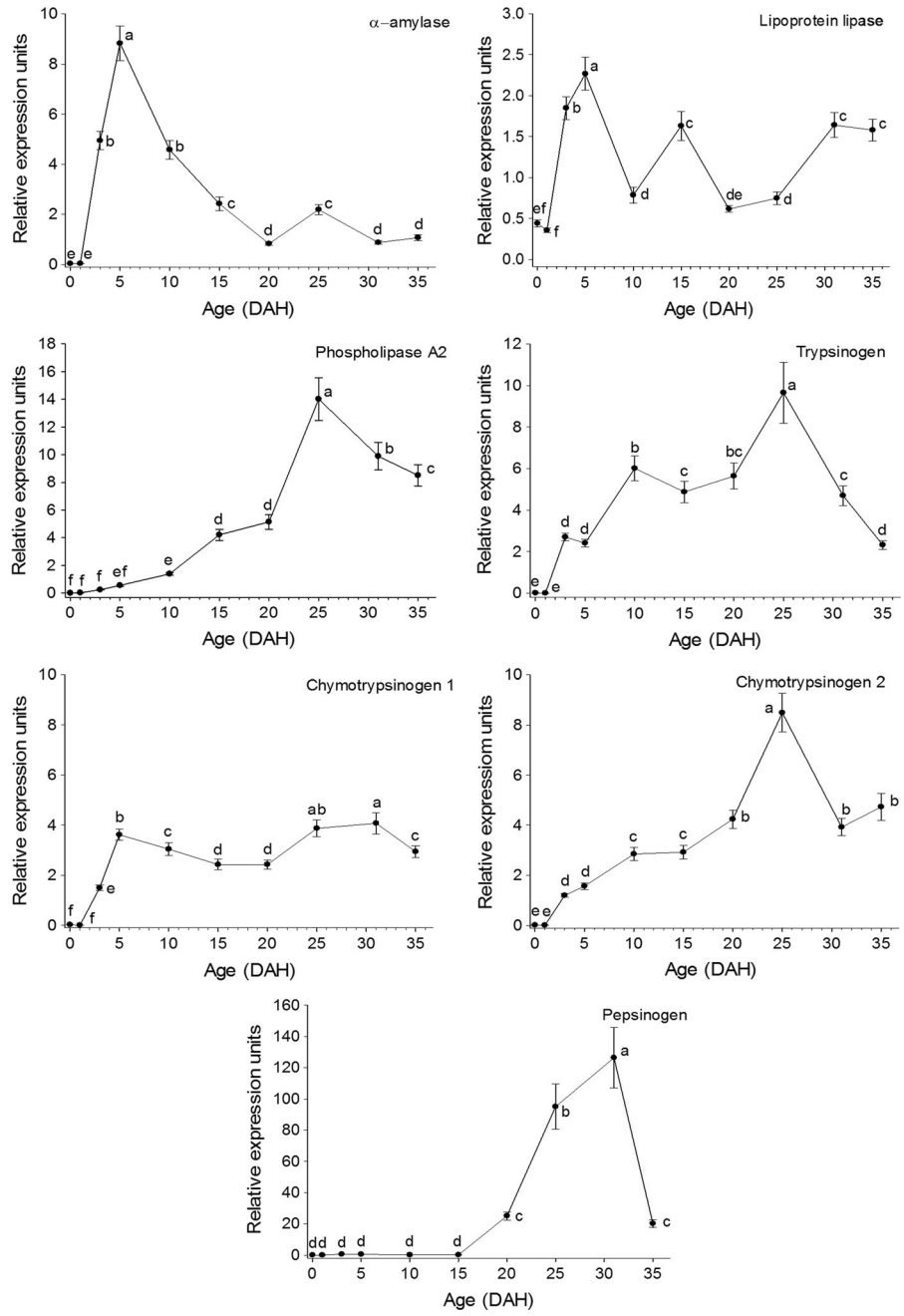

Fig. 2 Relative gene expression of digestive enzymes during larval development of the spotted rose snapper, *Lutjanus guttatus* (mean ± SE, $n = 3$ pooled larvae). *Different lower case letters* indicate significant differences ($P \leq 0.05$)

peak in expression of 2.26 ± 0.2 REU ($P < 0.05$), which coincided with the complete transition to exogenous feeding on copepods and rotifers enriched with *N. oculata*. At 15 DAH, another peak in expression of *lpl* was detected (1.63 ± 0.17 REU) and coincided with the start of *Artemia* metanauplii delivery in larval rearing tanks, *lpl* relative expression values decreased between 20 and 25 DAH, and recovered similarly to those observed at 15 DAH between 30 and 35 DAH ($P < 0.001$; Fig. 2b). On the other hand, *pla2* expression progressively increased from hatching to 20 DAH (5.144 ± 0.52 REU) and then abruptly increased further at 25 DAH (14.01 ± 1.55 REU; $P = 0.0001$) after which a decrease in the expression was observed until the end of the experiment (Fig. 2c, $P < 0.001$).

The alkaline proteases, *trp*, *ctr1* and *ctr2,* were detected during all the stages of development. The relative expression of *trp* was very low between 0 and 1 DAH ($0.01 \pm 3.29 \times 10^{-3}$ and $7.15 \times 10^{-4} \pm 2.65 \times 10^{-4}$ REUs, respectively). It progressively increased until 25 DAH (9.65 ± 1.46 REU), and after this peak in activity it sharply decreased in expression by approximately 80 % at 35 DAH (2.32 ± 0.21 REU) (Fig. 2d; $P < 0.01$). Two different isoforms of chymotrypsinogen (1 and 2) were found, showing different ontogenic expression patterns. The expression of *ctr1* was low during the yolk-sac stage (0 DAH = $8.01 \times 10^{-3} \pm 9.43 \times 10^{-4}$; 2 DAH = 0.02 ± 0.004 REU), and it increased until 5 DAH (3.61 ± 0.21 REU). After 5 DAH, *ctr1* expression slightly decreased until 20 DAH (2.43 ± 0.18 REU) and increased once again between 25 and 31 DAH (4.07 ± 0.42 REU) ($P < 0.05$). Between 31 and 35 DAH, the expression of *ctr1* decreased around 27 % (Fig. 2e; $P < 0.001$). The expression of *ctr2* progressively increased from 1 DAH ($6.34 \times 10^{-3} \pm 5.78 \times 10^{-4}$ REU) until 25 DAH (8.49 ± 0.76 REU) when it peaked ($P < 0.05$). After 25 DAH, *ctr2* expression decreased at 31 DAH and remained constant until the end of the study at 35 DAH (4.73 ± 0.53 REU; Fig. 2f). The expression of *pep* was very low during the first 15 DAH ($7.25 \times 10^{-3} \pm 1.68 \times 10^{-3}$ REU, 0 DAH; 0.52 ± 0.06 REU, 3 DAH; 0.11 ± 0.01 REU, 15 DAH). It started to increase after 20 DAH (25.05 ± 2.61 REU) peaking at 30 DAH (126.34 ± 19.29 REU), after then, *pep* expression levels sharply decreased ca. 85 % at 35 DAH (20.16 ± 2.58 REU) (Fig. 2g; $P < 0.001$).

The ontogenic patterns of mRNA expression of the two appetite regulatory factors considered in this study, *cck* and *npy*, were similar regardless of their different putative functions. Thus, the expression of *cck* was around 1.01 ± 0.11 REU during the first 3 DAH. It progressively increased until 10 DAH, reaching a maximum value in relative gene expression (2.07 ± 0.27 REU) ($P < 0.05$). After then, *cck* expression decreased until 20 DAH (0.66 ± 0.06 REU) and remained relatively constant until 35 DAH (0.66 ± 0.08 REU) ($P > 0.05$; Fig. 3a). On the other hand, the *npy* expression was low at the beginning of the development, but it increased significantly between 10 and 15 DAH (3.47 ± 0.23 and 3.56 ± 0.40 REU, respectively) ($P < 0.05$), followed by a significant decrease in the expression from 20 to 35 DAH ($P < 0.05$; Fig. 3).

Effects of weaning on gene expression of digestive enzymes and regulatory factors

There were no significant differences ($P > 0.05$) in body weight (g) or standard length (mm) between *L. guttatus* larvae fed with *Artemia* or weaned into a microdiet during different sampling days (Fig. 4). However, a significant growth was observed during the studied period. Larvae at 28 DAH were significantly larger ($P < 0.05$) than larvae at 25 DAH, but significantly smaller ($P < 0.05$) than larvae at 31 and 35 DAH. Survival rates were 62.6 ± 2.8 % in LF larvae and 54.3 ± 4.4 % in MD larvae, although no significant difference was observed ($P > 0.05$).

Weaning *L. guttatus* between 25 and 35 DAH, significantly affected the relative expression of all assayed digestive enzymes over time ($P < 0.05$; Table 2), with the exception of *ctr2*, which did not show significant differences in gene expression between the two experimental groups. On the first day of weaning, no significant differences were observed between the expressions of digestive enzymes between the LF and

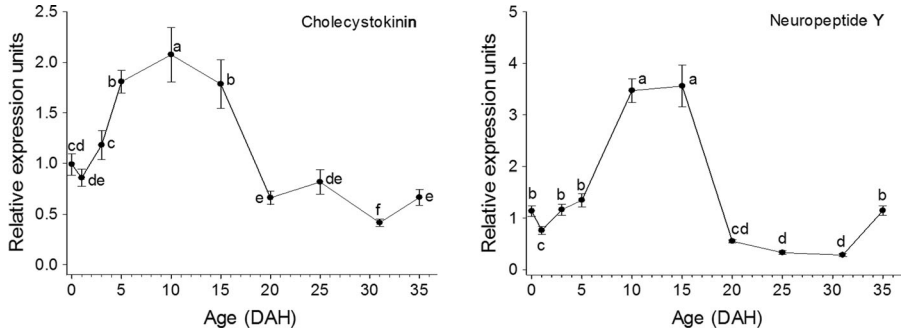

Fig. 3 Relative gene expression of cholecystokinin and neuropeptide Y during larval development of the spotted rose snapper, *Lutjanus guttatus* (mean ± SE, $n = 3$ pools of larvae). Different letters indicate significant differences ($P \leq 0.05$)

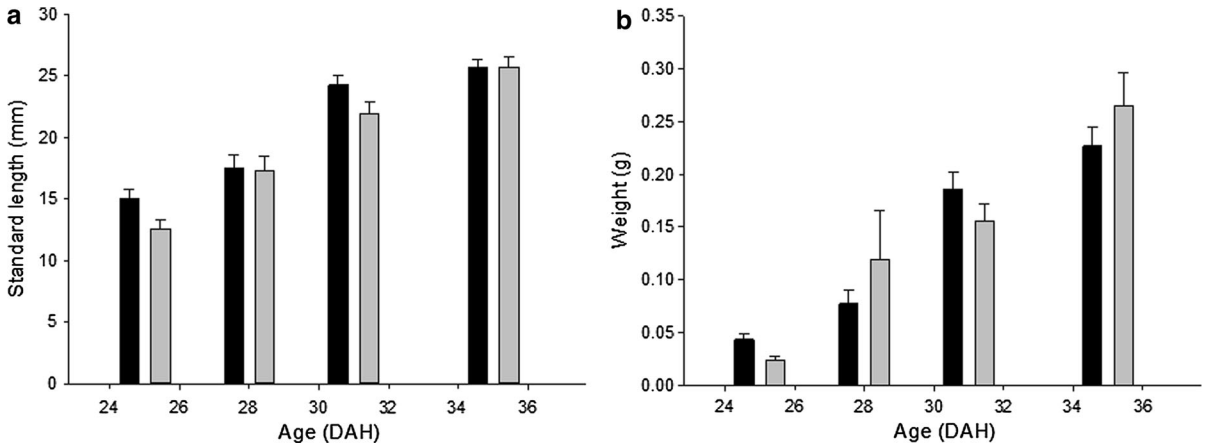

Fig. 4 Standard length (**a**) and body weight (**b**) of spotted rose snapper, *Lutjanus guttatus* larvae during weaning. The *black bars* represent the larvae fed with microparticulate diet (MD) and the *gray bars* those fed with *Artemia* metanauplii (LF). Data are shown as mean ± SE calculated from 20 specimens from each experimental group

Table 2 Relative expression of digestive enzymes of larval spotted rose snapper *Lutjanus guttatus* comparing larvae fed with *Artemia* (LF) and inert diet (MD) (mean ± SE, $n = 2$ pools of larvae)

Enzymes	25 DAH		28 DAH		31 DAH		35 DAH	
	LF	MD	LF	MD	LF	MD	LF	MD
α-amy	1.43 ± 0.19	1.32 ± 0.15	0.83 ± 0.03^{b}	2.29 ± 0.15^{a}	0.48 ± 0.02^{b}	1.11 ± 0.01^{a}	0.61 ± 0.01	0.85 ± 0.01
lpl	0.58 ± 0.07	0.76 ± 0.31	1.28 ± 0.16	1.63 ± 0.15	1.58 ± 0.09^{a}	0.91 ± 0.21^{b}	1.41 ± 0.01^{a}	0.59 ± 0.01^{b}
pla2	0.68 ± 0.17	1.19 ± 0.04	1.36 ± 0.53^{b}	2.21 ± 0.12^{a}	0.95 ± 0.28^{b}	2.51 ± 0.24^{a}	0.56 ± 0.01	0.35 ± 0.05
trp	1.03 ± 0.28	2.11 ± 0.50	1.87 ± 0.24^{b}	3.62 ± 0.56^{a}	0.69 ± 0.06	1.16 ± 0.31	0.34 ± 0.03	0.27 ± 0.00
ctr1	1.01 ± 0.13	1.04 ± 0.13	1.06 ± 0.15^{b}	1.94 ± 0.22^{a}	0.82 ± 0.11^{b}	1.68 ± 0.36^{a}	0.68 ± 0.00	0.51 ± 0.01
ctr2	1.24 ± 0.18	1.61 ± 0.34	1.72 ± 0.14	2.06 ± 0.53	0.76 ± 0.11	1.27 ± 0.14	0.76 ± 0.01	0.21 ± 0.00
pep	0.73 ± 0.14	0.72 ± 0.30	0.81 ± 0.14^{b}	2.79 ± 0.12^{a}	1.81 ± 0.51	1.71 ± 0.67	0.21 ± 0.01^{b}	1.57 ± 0.01^{a}

Different lower case letters indicate significant differences between the types of food in each enzyme expression ($P < 0.05$)

α-amy amylase, *lpl* lipoprotein lipase, *pla2* phospholipase 2, *trp* trypsinogen, *ctr1* chymotrypsinogen 1, *ctr2* chymotrypsinogen 2, *pep* pepsinogen

MD groups. However, between 28 and 31 DAH (4 and 7 days after the onset of weaning), the relative expressions of *amy*, *pla2*, *trp* and *ctr1* in fish from the MD group were significantly higher than in larvae fed only with *Artemia* metanauplii (LF group) ($P < 0.05$). The relative expression of lipoprotein lipase was significantly higher in fish from the LF group when compared with the fish from the MD group (1.58 ± 0.09 vs. 0.91 ± 0.21 REU at 31 DAH and 1.41 ± 0.01 vs. 0.59 ± 0.01 REU at 35 DAH). The relative expression of pepsinogen was quite variable during the weaning period, whereas the REUs of pepsinogen were higher in fish from the MD group than in those from the LF group at 28 and 35 DAH ($P < 0.05$), but there were no significant differences in REUs among both groups at 25 and 31 DAH ($P > 0.05$). In regard to *cck* and *npy*, no significant differences ($P > 0.05$) were found in their relative expression when comparing the two experimental groups (Table 3).

Discussion

Changes in gene expression of digestive enzymes and regulatory factors during larval ontogeny

Under current experimental conditions, *L. guttatus* larvae showed good growth performance and development, comparable to those described in previous studies (Galaviz et al. 2012; Moguel-Hernández et al. 2014).

Table 3 Relative expression of cholecystokinin (*cck*) and neuropeptide Y (*npy*) of larval spotted rose snapper *Lutjanus guttatus* comparing larvae fed with *Artemia* metanauplii (LF) and microdiet (MD) (mean ± SE, $n = 2$ pools of larvae)

	25 DAH		28 DAH		31 DAH		35 DAH	
	LF	MD	LF	MD	LF	MD	LF	MD
cck	1.51 ± 0.37	1.31 ± 0.37	0.95 ± 0.18	1.36 ± 0.32	0.76 ± 0.17	0.41 ± 0.05	0.93 ± 0.01	1.56 ± 0.08
npy	0.54 ± 0.01	0.58 ± 0.32	1.54 ± 0.78	1.43 ± 0.27	0.55 ± 0.06	0.93 ± 0.02	1.97 ± 0.04	2.05 ± 0.04

Therefore, the expression patterns found in the present study here can be related and compared to the above-mentioned studies.

The ontogeny of the digestive system of marine fish larvae has been studied during the last two decades, providing a valuable tool to better understand the digestive physiology of larvae and has been used to establish feeding protocols to optimize mass larval rearing (Lazo et al. 2011). Although there exists some conservation among different fish species regarding the ontogeny of the onset of digestive enzyme synthesis, the intensity and timing of occurrence of certain enzymes is a very plastic phenomenon and can be influenced by changes in the rearing conditions, the composition and type of diet, or be due to the growth and development of new organs and tissues (see review in Rønnestad et al. 2013), as well as be related to the geographic region of origin of the species (tropical vs. temperate species) (Cahu et al. 2004).

Transcripts of all the assayed digestive enzymes (with the exception of pepsinogen) and digestive regulatory factors were found in *L. guttatus* from hatching. Similar results have been reported in different species (Zambonino-Infante et al. 2008; Srichanun et al. 2013; Murashita et al. 2014; Galaviz et al. 2015; Mata-Sotres et al. 2016) supporting the hypothesis that the digestive capacity of the larvae during the first days of development is genetically pre-programmed in order to prepare the larva to maximize its digestive capacities at the onset of the exogenous feeding when endogenous reserves contained in the yolk become limiting and the larva needs to acquire nutrients and energy from the diet. In the case of *L. guttatus* has been confirmed with the detection of enzymatic activity before mouth opening (Moguel-Hernández et al. 2014).

In *L. guttatus*, *amy* expression was high during the first 5 days of development (2.56 ± 0.13 mm), but decreased during the rest of larval development. This expression pattern is consistent with the results reported by Moguel-Hernández et al. (2014) regarding α-amylase enzyme activity, where larvae of 2.21 ± 0.13 mm had a higher specific activity than older fish, which had lower levels of enzyme activity, indicating a good transcriptional regulation of α-amylase synthesis during early ontogeny. This pattern in expression and activity also has been observed in other marine fish species like European sea bass *Dicentrarchus labrax* (Péres et al. 1998; Cahu and Zambonino-Infante 2001), Californian halibut *Paralichthys californicus* (Álvarez-González et al. 2006), common dentex *Dentex dentex* (Gisbert et al. 2009), ballan wrasse *Labrus bergylta* (Hansen et al. 2013), totoaba *Totoaba macdonaldi* (Galaviz et al. 2015) and gilthead sea bream (Mata-Sotres et al. 2016). Cahu and Zambonino-Infante (2001) mentioned that the fluctuations in α-amylase activity during development are a good example of the influence of age on the activity of pancreatic enzymes, as the decrease in α-amylase-specific activity during larval development seems to be more genetically programmed rather than nutritionally induced, which would be in agreement with the carnivorous feeding habits of *L. guttatus*. The increase in *amy* expression in 25 DAH in *L. guttatus* might be attributed to changes in the diet, from rotifers to *Artemia* nauplii. A similar result was obtained by Ma et al. (2005) in *Pseudosciaena crocea and* Galaviz et al. (2015) in *T. macdonaldi*, which were attributed to the high levels of glycogen in live prey, as α-amylase can be stimulated in fish larvae by glycolytic polymers like glycogen and starch.

Lipids are the main source of energy for developing fish larvae, and the n-3 highly unsaturated fatty acids (HUFA) have been identified as essential dietary components for marine fish, since they cannot synthesize them *de novo* (Wold et al. 2007). Lipids are assembled in lipoprotein particles such as chylomicrons and very low-density lipoproteins (VLDL) in the enterocytes, which distribute the lipids to the body

(Rønnestad et al. 2013). The enzymes of lipoprotein metabolism, including lipoprotein lipase (LPL) and hepatic lipase have been found in both, marine and freshwater species (Tocher 2003). Lipoprotein lipase is a ubiquitous glycosylated enzyme, which is involved in the regulation of circulating triacylglycerides (Albalat et al. 2007). Once LPL is synthesized, the enzyme is secreted and transferred to the luminal surface of capillary endothelial cells where it hydrolyzes triglycerides circulating in the form of chylomicrons and very low-density lipoproteins into free fatty acids (FFA) and 2-monoacylglycerols. The resulting FFA can be utilized by different tissues, such as adipose tissue or by other peripheral tissues, such as muscle and heart (Albalat et al. 2007; Han et al. 2011). LPL has been identified at the molecular level in various species, including red sea bream (*Pagrus major*) (Oku et al. 2002), gilthead sea bream (Saera-Vila et al. 2005), and European sea bass (Ibañez et al. 2008) among others. Lipoprotein lipase expression in *L. guttatus* was detected from hatching with variations in a "saw-tooth" profile during larval development. These variations coincided with changes in food sources and changes in triacylglycerol levels: (1) from the nutrients contained in the yolk sac to live prey, (2) from rotifers enriched with *N. oculata* to rotifers enriched with Spresso Selco^{TM}; and (3) to *Artemia* nauplii and metanauplii. Albalat et al. (2007) reported that in juvenile *S. aurata*, the *lpl* mRNA values in the adipose tissue were regulated by the concentration of insulin, whereas in rats it has also been correlated to the temporal accumulation of triacylglycerols in the liver (Ramírez et al. 1983). Regarding phospholipids, marine phospholipids are carriers of HUFAs and therefore good lipid sources for starter feeds for marine fish larvae. Marine fish larvae possess a high capacity to utilize phospholipids, and micro diets containing more marine phospholipids than marine triacylglycerols have resulted in better growth performance in larvae (Gisbert et al. 2005; Wold et al. 2007). The expression profile of *pla2* in *L. guttatus* showed a continuous increase throughout development until 25 DAH, followed by a decrease in the expression, which is consistent with the high nutritional requirements of fish larvae on phospholipids during early ontogeny (Cahu et al. 2009). The decrease in the expression after 25 DAH could be related to maturation in the digestive system of larvae at this age, as the expression levels (current study) and activity (Moguel-Hernández et al. 2014) of digestive proteases seemed to indicate. However, it has also been observed in other species that *pla2* expression is determined in part by the type of lipids present in the food (Cahu et al. 2003; Gisbert et al. 2005; Hansen et al. 2013); thus, in the case of the *L. guttatus*, it should not be discarded that the lipid profile of enriched live prey may have had some impact on *pla2* expression. However, further research regarding the lipid profile in the diet and its effect on *lpl* and *pla2* activities and gene expression is required.

During the early days of larval development, *L. guttatus*, like other marine fish larvae, have no functional stomach and therefore, no secretion of hydrochloric acid and pepsin. Thus, the protein hydrolysis starts with an alkaline intestinal digestion, which is mainly provided by trypsin and chymotrypsin, and other alkaline proteases (e.g., carboxpeptidases) that are synthesized and secreted by the pancreas into the intestinal lumen. Synthesis and secretion rate of pancreatic enzymes during the first days of development seems to be related to feed intake, and later in development also to diet composition (Hjelmeland et al. 1984; Zambonino-Infante and Cahu 1994; Rønnestad et al. 2013). Thus, starvation, reduced feed intake, or an unbalanced diet may result in a decrease in secretion and, consequently, in the activity of trypsin and chymotrypsin (Hoehne-Reitan and Kjørsvik 2004). Therefore, evaluation of the expression of these enzymes can serve as a proxy for nutritional status in fish larvae, as well as for evaluating different feeding protocols and diets as reported by Ueberschär (1993). In *L. guttatus*, *trp* expression was found from hatching with a continuous increase throughout larval development with a peak in expression at 25 DAH, which may be indicative of the complete maturation of the exocrine pancreas (Galaviz et al. 2012). When expression values of *trp* were compared with the enzymatic activity of this alkaline protease (Moguel-Hernández et al. 2014), the maximum levels of enzyme activity were detected 5 days later than the corresponding peak in gene expression, which confirmed the translational regulation of synthesis of this protease by dietary protein content (Cahu et al. 2004). A similar delay in the translation of trypsin was reported by Srichanun et al. (2013) in *Lates calcarifer* larvae.

In the case of chymotrypsinogen, two isoforms were found and analyzed in this study. The expression of *chym1* was relatively constant throughout

development in *L. guttatus*; however, *chym2* presented a similar pattern of expression as *trp* with a peak at 25 DAH, which matched the pattern of enzyme activity previously reported for this species reared under similar conditions (Moguel-Hernández et al. 2014). A similar trend of these two alkaline proteases (trypsin and chymotrypsin) was also observed in the early stages of Dover sole *Solea solea* larval development (Parma et al. 2013) and in gilthead sea bream (Mata-Sotres et al. 2016), and it may be related to their specific properties and activity. In this sense, among the two endopeptidases analyzed in this study, trypsin, which has specific affinity for peptide bonds involving lysine or arginine, seemed to have a predominant role in protein digestion in comparison with chymotrypsin, which has more affinity for specific bonds involving tyrosine and phenylalanine (Gisbert et al. 2013). Such differences in activity between these two endopeptidases may reflect differences in the protein composition and amino acid profile of live feed (Conceição et al. 2003).

The appearance of pepsin signals the onset of acid digestion in the stomach after the appearance of the gastric glands, and this feature has been considered as a major change toward an adult-type of digestion (Govoni et al. 1986). In our study, *pep* expression was initially detected at 20 DAH (6.3 mm), whereas in a previous study in *L. guttatus* larvae, Galaviz et al. (2012) reported the expression of pepsinogen from 18 DAH (6.6 mm), which indicated that the development of the stomach in *L. guttatus* is a well-conserved process that generally occurs within the same range of body sizes independently of the larval age, regardless of the differences in rearing conditions and larval growth. Srichanun et al. (2013) also reported the expression of pepsinogen in Asian sea bass *L. calcarifer* larvae from 18 DAH and in Pacific bluefin tuna *Thunnus orientalis*, expression of pepsinogen was reported to increase from 19 DAH (Murashita et al. 2014).

Digestion is controlled in a hierarchical manner beginning with hormones released into the blood circulation which, upon finding their cognate receptors, trigger various processes in the digestive system from contraction of smooth muscles in the intestine, to HCl secretion in the stomach and pancreatic enzymes into the anterior intestine. In addition, the internal feedback mechanisms that regulate appetite and food intake are believed to operate in fish quite similarly as in higher vertebrates, in spite of the paucity of information on larval stages (Gomes et al. 2015; Bonacic et al. 2016). These mechanisms are based on signals generated by the ingested food and the energy balance of the organism, and travel from peripheral tissues to relevant centers of homeostatic control in the central nervous system (Volkoff 2006). In this study, we decided to not focus on the complex interrelationships existing between the gut and brain that control feeding behavior and digestion, and we centered our efforts in getting basic knowledge on two of such effectors, *cck* and *npy* together with the main digestive enzymes to understand how the levels of expression may be altered during larval development in *L. guttatus*. The number of signaling molecules known to modulate one or more gastrointestinal characteristics continues to increase and includes hormones, neurotransmitters, and neuromodulators (Rønnestad et al. 2013). A major regulatory hormone is CCK, upon binding its receptors in the gut CCK induces the release of bile salts and pancreatic enzymes during the regulation of satiety (anorexigenic factor); the peristaltic movements, also stimulates release of gallbladder contents and trypsin secretion in the intestine and also inhibits gastric emptying (Tillner et al. 2013). It is produced in specific cells scattered in the mucosa of the proximal intestine and is secreted into the plasma in response to the presence of nutrients in the lumen (Cahu et al. 2004). Like in cod *Gadus morhua* (Kortner et al. 2011b) and red drum *Sciaenops ocellatus* (Webb et al. 2010) larvae, the expression of this hormone in spotted rose snapper was detected from hatching until the end of the study. However, the levels of *cck* mRNA displayed a moderate but consistent decrease after 15 DAH. Similar results were reported by Kortner et al. (2011b) in *G. morhua*, and they assumed that *cck* is principally expressed in the brain during early larval stages. So the observed decrease in *cck* expression during the experimental period might be due to the decreased proportion of brain tissue with respect to the whole larval body (RNA was obtained from whole larvae). Neuropeptide Y (an orexigenic factor) had the same pattern of expression as *cck* in *L. guttatus* and also is primarily expressed in the brain (Volkoff et al. 2005; Kortner et al. 2011b). The similar pattern in the expression of these two regulatory factors suggests that both were synthesized in parallel to regulate food ingestion and satiety. In the present study, the REU levels of *npy*

were almost double than those found for *cck* and this may reflect that the need for stimulation of food ingestion surpasses the need for controlling satiety (rather than the inverse), which may be related to the voracious appetite reported for most fish larvae during early life stages (Pittman et al. 2013). Moreover, larvae in the present study were sampled in the morning prior to feeding and assuming that no feeding occurred during the night before, the high REU levels of *npy* could reflect a mild starvation condition of the larvae. Further research is needed in order to further characterize daily rhythms of *cck* and *npy*, and the relationship between feed frequency and their expression.

Several authors mention that both *cck* and *npy* expression can be influenced by the composition of food (Volkoff et al. 2005; Webb et al. 2010; Kortner et al. 2011b; Tillner et al. 2013). For instance, CCK is involved in the secretion of trypsin in larval fish; thus, dietary protein content, in combination with protein and peptide chain lengths and intraluminal proteolytic activity may regulate CCK levels (Rønnestad et al. 2013) However, as many digestive tract hormones are also found in the brain, whole-larva levels of CCK and NPY cannot be regarded as representative of just intestinal values; thus, further research is needed in order to characterize the relationships between CCK and NPY in the gut and brain in *L. guttatus* and their role in the regulation of energy metabolism, nutrient partitioning, and the control of feeding behavior.

Effects of weaning on gene expression of digestive enzymes and regulatory factors

Culture of fish from the larval stage requires a good understanding of the development of their digestive system, as it is this stage of development where the fish are at their most vulnerable and significant mortalities can occur due simply to improper nutrition or feeding practices. Furthermore, to formulate the most palatable and digestible diet, it is necessary to know at what age particular nutrients can be properly broken down and absorbed by the digestive system, which depends to a large degree on what types of enzymes are being produced at each stage of development (Gisbert et al. 2013). Following the previous work of Moguel-Hernández et al. (2014), early weaning *L. guttatus* was performed at 25 DAH, when pepsin activity was present.

No differences in weight and length were observed among *L. guttatus* larvae fed with *Artemia* (LF) and weaned onto a microdiet (MD), whereas survival rates only differed by 9 % between the two groups (63 % LF and 54 % MD), which were considered as very promising results in terms of growth performance and survival. Similar results were reported in other marine fish larvae, reflecting the ability of larvae to digest inert diet (Cahu and Zambonino-Infante 2001; Holt et al. 2011; Nguyen et al. 2011; Galaviz et al. 2012). However, further research is needed in order to evaluate new weaning protocols for earlier ages based on microdiets for *L. guttatus* larvae with improved nutritional quality and physical properties.

Regarding the effects of weaning on the gene expression of different digestive enzymes, the lack of differences in gene expression between both experimental groups on the first day of weaning may be attributed to the fact that the time-lapse between diet administration and gene expression was not long enough to induce any marked effect on gene expression. However, differences in gene expression for most of the genes studied, with the exception of *ctr2* and *lpl*, were found between 3 and 6 days after the onset of weaning. Nevertheless, at the end of the experiment, no significant differences were observed between treatments. This is suggestive that the larvae of *L. guttatus* have some adaptability to the diet, and the expression levels of these enzymes are similar to those needed to digest the live feed. Similarly, Engrola et al. (2007) also noted that the profile of digestive enzymes in Senegalese sole *Solea senegalensis* had a period of adaptation to inert diets.

As mentioned above, *amy* expression is genetically programmed, decreasing throughout development due to the carnivorous feeding habits of this species. However, it can be modulated by the diet as observed in this study, where the amylase expression was higher in the larvae fed with the microparticulate diet compared to those fed with *Artemia* metanauplii, and this is likely influenced by differences in the nature and concentrations of carbohydrates, especially starch, in the microdiet with regard to *Artemia* metanauplii as shown by Cahu et al. (2004). Similar results were found in *D. labrax* where the decrease in *amy* expression and activity during larval development were lower in larvae fed with diets incorporating a higher quantity of carbohydrates (Péres et al. 1998; Cahu and Zambonino-Infante 2001). These results

showed that the molecular mechanisms that control the dietary adaptation of this carbohydrase are independently regulated, age-dependent and influenced by the composition and the quantity of the diet. The expression levels of *lpl* were higher at the end of the weaning period (31 and 35 DAH) in the LF group compared to those of the MD group. By contrast, *pla2* expression was higher in the MD group larvae, which indicated that expression levels of *lpl* and *pla2* in *L. guttatus* larvae were regulated by the type of diet and the amount of body and dietary triacylglycerols and phospholipids, respectively. In this sense, the type and amount of fatty acids in the diet has also been shown to influence both the expression and activity of lipolytic enzymes in *D. labrax* (Zambonino-Infante and Cahu 2001), *Seriola quinqueradiata* (Murashita et al. 2008), *G. morhua* (Saele et al. 2010) and *L. bergylta* (Hansen et al. 2013).

Considering the digestive proteases, Péres et al. (1998) reported two patterns of *trp* expression in *D. labrax* larvae fed with live prey and microdiets. They suggested that the nature of the protein modulates the transcription of mRNA of proteases. This was also observed in *L. guttatus* larvae, as expression levels of *trp*, *ctr1* and *pep* were affected by co-feeding during the transition from live prey to the microdiet at 28 DAH, period during which larvae fed with the inert diet showed higher *trp*, *ctr1* and *pep* expression levels than those fed the live prey, which might be due to the nature of dietary proteins (Nguyen et al. 2011), but also related to a higher need of proteases for digesting the microdiet due to the different peptide molecular weight distribution of the dietary protein fraction (Holt et al. 2011; Skalli et al. 2014). This increase in protease activity during weaning has also been observed in species like *S. senegalensis* (Engrola et al. 2007) and *S. ocellatus* (Kolkovski 2001), while in *Rachycentron canadum* (Nguyen et al. 2011) no differences between the larvae fed *Artemia* and those fed inert diet were found and the authors suggested that it was due to ingestion rates rather than the dietary protein profile. However, the effect of dietary components and inclusion levels in the diet on gene expression during weaning of *L. guttatus* larvae remains to be evaluated.

Regarding the effects of weaning on the expression of *cck* and *npy*, we did not find differences among both experimental groups, whereas it should not be excluded that the chosen sampling strategy at the beginning of the day prior to meal delivery might have masked potential differences between experimental groups due to the circadian nature of these peptides. CCK is involved in trypsin production in larval fish, whereas dietary protein content, in combination with protein and peptide chain lengths and intraluminal proteolytic activity, regulates CCK levels (Rønnestad et al. 2013). In the present study, an increase in *trp* levels was not correlated with higher levels of *cck* transcripts in *L. guttatus* weaned onto a microdiet, which seemed to indicate that other gut hormones and neuropeptides, like secretin, bombesin or PYY, among others, might be involved and play a more dominant or potentiating role in digestive physiology in fish larvae than previously expected, as Tillner et al. (2014) reported.

Conclusions

In conclusion, this study presents the expression patterns of pancreatic enzymes and gastric pepsinogen, and *cck* and *npy* during larval development of *Lutjanus guttatus*, which is similar to marine species with carnivorous habits. Overall results indicate that the digestive system in this species was completely functional between 6.0 and 6.3 mm in SL. To our knowledge, this is the first report of the expression of *lpl* during larval development. The changes in the expression of digestive enzyme genes in *L. guttatus* larvae showed that the molecular mechanisms that control the adaptation of pancreatic and gastric enzymes were age-dependent and influenced by the type of diet. Further, as there were few significant differences in growth and gene expression during early weaning of *L. guttatus* larvae, the microparticulate diet used herein presents a viable option for *Artemia* replacement during commercial production of this species.

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