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Comparative ontogenetic development of two marine teleosts, gilthead seabream and European sea bass: New insights into nutrition and immunity

Héctor Cordero [a], Laura T. Guzmán-Villanueva [b], Elena Chaves-Pozo [c], Marta Arizcun [c], Felipe Ascencio-Valle [b], Alberto Cuesta [a], María A. Esteban [a, *]

[a] Fish Innate Immune System Group, Department of Cell Biology and Histology, Faculty of Biology, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, 30100 Murcia, Spain
[b] Centro de Investigaciones Biológicas del Noroeste (CIBNOR), Av. Politécnico Nacional 195, Col. Playa Palo de Santa Rita, La Paz, B.C.S., 23090, México
[c] Centro Oceanográfico de Murcia, Instituto Español de Oceanografía (IEO), Carretera de la Azohía s/n, Puerto de Mazarrón, 30860, Spain

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ABSTRACT

Gilthead seabream and European sea bass are two of the most commonly farmed fish species. Larval development is critical to ensure high survival rates and thus avoid unacceptable economic losses, while nutrition and immunity are also important factors. For this reason this paper evaluates the ontogenetic development of seabream and sea bass digestive and immune systems from eggs to 73 days post-fertilisation (dpf) by assessing the expression levels of some nutrition-relevant (*tryp*, *amya*, *alp and pept1*) and immune-relevant (*il1b*, *il6*, *il8*, *tnfa*, *cox2*, *casp1*, *tf*, *nccrp1*, *ighm* and *ight*) genes. The results point to similar ontogenetic development trends for both species as regard nutrition and differences in some immunity related genes.

1. Introduction

Gilthead seabream (*Sparus aurata* L.) and European sea bass (*Dicentrarchus labrax* L.) are the most commonly farmed fish species in Europe (FAO, 2014), where a very successful productive industry depends on combining improved knowledge of their biology and improved farm management. However, obtaining larvae from eggs and subsequent larval development is one of the key steps in the productive cycle, as it is for the culture of other fish species of potential interest for the aquaculture industry. Successful marine larviculture requires not only an effective feeding schedule based on the nutritional requirements and digestive capabilities of developing larvae, in which an effective and mature immune system is of prime importance (Conceição et al., 2010; Parker et al., 2012). Suitable production of larva requires large facilities, high maintenance costs and intensive labour to produce the desired amount of life foods constantly. Hence, new advances in our knowledge of the larval digestive system and its interaction with live food in each larvae period constitute a major challenge.

In teleost fish, the emergence of primary and secondary lymphomyeloid organs, head-kidney, spleen and thymus, takes place at different larval stages. The wide variability between species in this respect can be explained by the differing lengths of the embryonic period and larval development, but also by the fact that the development of larvae is strongly affected by culture conditions such as temperature and/or salinity (Falk-Petersen, 2005). In both gilthead seabream and European sea bass, the histological development of the lymphoid organs has been studied previously (Abelli et al., 1996; Josefsson and Tatner, 1993). However, little is known about the timing of the appearance of different immune response gene markers. Furthermore, autologous adaptive immunocompetence acquisition in fish larvae usually follows the first appearance of lymphoid organs (Lam et al., 2004; Nakanishi, 1986) but is not always correlated with the same (Mulero et al., 2008). In addition, as occurs in other vertebrates, some proteins in fish are maternally transferred from the female to an immunologically naïve offspring

* Corresponding author.
E-mail address: aesteban@um.es (M.A. Esteban).

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(Grindstaff et al., 2003; Zhang et al., 2013). Moreover, the maternal transfer of mRNAs to the oocytes after spawning should not be ruled out as gene expression in larvae will determine the timing of immune system development in offspring and the subsequent effective immune response of these offspring (Huttenhuis et al., 2006; Magnadottir et al., 2005).

Improved knowledge of the development of the digestive capability and immunity in fish larvae could help reduce the high mortality rates in hatcheries and overcome a recurring production bottleneck in the aquaculture industry. Thus, the aim of this work was to know the pattern of expression of some genes that code relevant molecules for (i) nutrition, such as the enzyme responsible for hydrolysing proteins (*tryp*), alpha bonds for polysaccharides (*amya*) and phosphate groups (*alp*), and for carrying oligopeptides in the digestive tube (*pept1*); for (ii) different innate immune responses, such as inflammation (interleukin (il) 1 beta (*il1b*), *il6*, *il8*, tumor necrosis factor alpha (*tnfa*) and ciclooxygenase-2: *cox2*), apoptosis (caspase 1: *casp1*), antimicrobial peptides (transferring: *tf*) and cytotoxicity (non-specific cytotoxic cell receptor protein 1: *nccrp1)* and for (iii) adptative immune responses such as different populations of B lymphocyte markers (immunoglobulin M, *ighm*, and immunoglobulin T, *ight*) during the ontogenetic development of two of the most relevant commercial species in the Mediterranean area, the gilthead seabream and the European sea bass.

2. Material and methods

2.1. Experimental design

Broodstocks of gilthead seabream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) were bred and kept at the *Instituto Español de Oceanografía* (*IEO*, Mazarrón, Murcia), where the fry was bred and maintained. Natural seawater (38‰ salinity) was heated to 17 ± 1 °C and filtered through mechanical and biological substrates. The temperature increased naturally and reached 26 °C by the end of the experiment. Gilthead seabream larvae were bred using the "green water" technique in a 5000-l round tank with an initial density of about 60 eggs/l. During the experiment, the light intensity was 1000 lux at the water surface, and the photoperiod was 16:8 (L:D). Water renewal was limited to 2% daily during the first 20 days of culture and was achieved by the addition of 70 ml/m^3 of a microalgae concentrated solution (Phytobloom, Necton) containing 80% *Nanochloropsis oculata*. Subsequently, continuous water renewal (30%/h) and light aeration were provided in the tank. Larvae were successively fed with enriched (Selco, Inve Animal Health) rotifers from 6 to 24 days post-fertilisation (dpf), *Artemia nauplii* (Inve Animal Health) from 20 to 35 dpf, enriched Instar II *Artemia* from 31 to 58 dpf and a commercial dry pellet diet (Skretting) from 54 dpf onward. The European sea bass larvae were kept in dark during the first 40 days after hatching. The light intensity of 1000 lux at the water surface and the 16:8 (L:D) photoperiod were maintained. The specimens were subsequently fed with enriched Instar II *Artemia* and a commercial dry pellet diet (Skretting) from 54 dpf onwards. Three pools of eggs and larvae at different time points post fertilisation (0, 3, 6, 10, 13, 17, 24, 31, 45, 59 and 73 dpf) were sampled and stored at −80 °C in TRIzol® reagent (Life Technologies) for latter RNA isolation. The experiments described comply with the Guidelines of the European Union Council (2010/63/EU), the Bioethical Committee of the University of Murcia (Spain) and the *Instituto Español de Oceanografía* (Spain) for the use of laboratory animals.

2.2. Analysis of gene expression by real-time PCR (qPCR)

Total RNA was extracted from pooled eggs or larvae using TRIzol® reagent according to the manufacturer's instructions. Total RNA (1 μg) was treated with DNase I to remove genomic DNA and the first strand of was cDNA synthesized by reverse transcription using SuperScript III Reverse Transcriptase (Life Technologies) with an oligo-dT_{18} primer (Life Technologies). To check integrity, 1 μl of each RNA sample was run in 2% agarose gel.

Real-time PCR was performed as described elsewhere (Cordero et al., 2015). Four putative endogen genes were evaluated following the method described by Pfaffl et al. (2004). The stability of the Ct-values for each candidate in both species is represented in Supplementary Figure S1. Based on another specific study for European sea bass larval stages (Mitter et al., 2009) and an analysis of the four endogen gene transcriptions by the BestKeeper® software, the gene expression of each target gene was corrected by the most suitable reference gene (*ef1a*) according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Gene names follow the guidelines of the Zebrafish Nomenclature Committee (ZNC). The primers used were designed using the OligoPerfect™ Designer Tool (Thermo Fisher Scientific) and are shown in Table 1. Before the experiments, the specificity of each primer pair was studied using positive and negative samples. Amplified products from positive samples were run in 2% agarose gels and sequenced. After these verifications, all amplifications were performed in duplicate cDNAs and repeated once to confirm the results. Negative controls with no template were always included in the reactions.

2.3. Statistical analysis

The results are expressed as mean ± standard error mean (SEM). Data were statistically analysed by one-way analysis of variance (ANOVA) to determine differences between groups followed by a post hoc Tukey test. Normality of the data was previously assessed using a Shapiro-Wilk test and homogeneity of variance was also verified using the Levene test. Statistical analyses were conducted using SPSS for Windows V19. Differences were considered statistically significant when $P < 0.05$ and represented with different letters for each stage.

3. Results

3.1. Digestive and transport component transcription increased during the first days

The transcript profile of the genes that code for several digestive enzymes (*tryp*, *amya* and *alp*) and a transport protein located in the digestive tube (*pept1*) (Fig. 1) was analysed. The expression of *tryp*, was expressed in eggs of both species, and higher values were found from 6 dpf onward, with comparable levels of expression in both species. On the other hand, *amya* was undetected in eggs, and later gene expression levels increased from 3 to 17 dpf in both species. The expression of *tryp*, *alp* and *pept1* showed very similar profiles in both species, the only exception being the undetectable *pept1* in European sea bass eggs.

3.2. Innate immune-related genes were expressed differently in both species during development

The following transcription of genes involved in inflammation, phagocytosis, apoptosis and cytotoxicity innate immune responses were studied (Fig. 2): interleukin (il) 1 beta (*il1b*), *il6*, *il8*, tumor necrosis factor alpha (*tnfa*), ciclooxygenase-2 (*cox2*), caspase 1 (*casp1*), transferrin (*tf*) and non-specific cytotoxic cell receptor protein 1 (*nccrp1*). The mRNA transcripts of *il1b*, *tnfa* were not detected in eggs in either of the species analysed, while *il6* transcripts were not detected in gilthead seabream eggs (Fig. 2). Two of