Universitat Autònoma de Barcelona

Advances in the Larval Rearing of Meagre (Argyrosomus regius): Diet, weaning protocols, and ontogeny of the digestive and innate immune systems

CINDY CAMPOVERDE VERA

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Ph.D. Thesis

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DOCTORAL THESIS

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The thesis was performed under the direction of Dra. Alicia Estévez and Dr. Karl B. Andree at Institut de Recerca I Tecnologia Agroalimentàries (IRTA) Sant Carles de la Rapita as part of the Aquaculture doctoral program at the Sciences Faculty, Department of Animal Biology, Vegetal Biology and Ecology (2014-2017).

"Advances in the larval rearing of meagre (*Argyrosomus regius*): Diet, weaning protocols and ontogeny of the digestive and innate immune systems".

This dissertation is submitted by **Cindy Campoverde Vera**, doctoral degree candidate at University Autonóma of Barcelona.

Tesis realizada bajo la dirección de Dra. Alicia Estévez y Dr. Karl B. Andree de I´Institut de Recerca i Tecnología Agroalimentàries (IRTA) Sant Carles de la Rápita, Programa de doctorado de Acuicultura, Facultad de Ciencias, Departamento de Biología Animal, Biología Vegetal y Ecología (2014-2017).

"Avances en el cultivo larvario de corvina *(Argyrosomus regius):* Dieta, protocolos de destete y ontogenia de los sistemas digestivo e inmune innato".

Memoria presentada por Cindy Campoverde Vera, para optar al grado de Doctor por la Universidad Autónoma de Barcelona.

Thesis author	Thesis director	Thesis codirector	Thesis tutor
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Dedication

For my mother

&

My son

They have been with me every step of the way with endless love and support.

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Llega la hora de escribir este apartado y tratar de plasmar en una simple hoja lo que debería ser un capítulo en la tesis. Sin embargo, al finalizar un arduo trabajo lleno de vicisitudes y satisfacciones como el desarrollo de una tesis doctoral, es inevitable que me aborde un sentimiento de egocentrismo que me conlleva a concentrar la mayor parte del mérito en lo que he logrado, en un análisis integral y objetivo te muestra inmediatamente que la magnitud de ese aporte hubiese sido imposible sin la participación de personas e instituciones que han hecho posible que esta etapa llegue a un satisfactorio término. Por lo tanto, es para mí un verdadero placer utilizar este espacio para ser justo y consecuente con ellas, expresándoles mi profundo agradecimiento.

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Contents

Summary

Aquaculture production in Europe is dominated by a small group of species, such as salmon, trout, carp, seabass, seabream that limits the number of aquaculture products available on the market, and to some extent, the geographic regions where aquaculture can be done profitably. The market price of these species is often close to or below the minimum cost of production, which has consequent negative influences on the growth of aquaculture in the EU. A sustainable system based on the incorporation of new species could, to a certain extent, reduce the problem by providing a diversified market with increased geographic dispersal of production sites that can reduce transport costs form "farm to table". Thus, in order to increase aquaculture products in the market new species have been selected taking in account characteristics such as rapid growth, ease of cultivation, adaptability to large volumes under intensive rearing, large body size to facilitate automated post-harvest processing and development of value-added consumer products. The purpose of this thesis is to study a species considered promising for Mediterranean aquaculture belonging to the family Sciaenidae and commonly known as meagre (*Argyrosomus regius*). This species is characterized by its high growth rate (1 kg year^{-1}) and good feed conversion rate. However, as other new species, meagre has some bottlenecks present during its early stages of development that need to be solved. Thus, the general objectives of this thesis aims to evaluate the following aspects: (1) morphology and functional development of the digestive system, based on histological and enzymatic activity analyses, (2) study the effect of different strategies for early weaning onto artificial feed, reduce the use of *Artemia* to 50% of the amount used in standard production protocols, and evaluate the effects of these diets on development, digestion, growth, survival, and deformations. Two experiments were carried out using different feeding protocols designed for an early incorporation of artificial feed, then evaluate its effects on morphology and functional development using enzymatic markers that reflect the maturity of the digestive tract. Certain problems inherent to the species, in particular, their cannibalistic behavior during the post-flexion phase had effects on survival rate, although, early weaning had no major influence on the presence of skeletal deformities. In this sense, we proceeded to the (3) study of fatty acid requirements during larval development (DHA -22:6n-3- and DHA/EPA ratio) in order to examine the effects of different live prey enrichments on larval growth and survival, and evaluate their ability to elongate and desaturate fatty acids from their precursors. Two experiments were designed during larval culture using high, medium and low content of DHA demonstrating that the fatty acids requirements (DHA) are species-specific. The use of hemp oil (rich in 18:3n-6 and 18:4n-3) served to demonstrate the inability of meagre larva to elongate and desaturate fatty acids even when the precursors are offered in the live food.

The immune system is another aspect of great importance during larval rearing, since the success of larval culture is based not only in nutrition, but also on the defense mechanisms that the larva possess against potential pathogens present in the water. The larva depends on a suite of important protective molecules of the innate immune system that deal with potential pathogens until the adaptive or specific system is completely mature. Most of

the mortality afflicting industrial aquaculture occurs during the critical stages larval growth, thus limiting the production of juveniles. In this sense, to evaluate the time in which the larva is more susceptible to external factors and its ability to cope with an immune response, we have studied (4) the ontogeny of the innate immune system during larval development, focused on the organogenesis of the major lymphoid organs (thymus, kidney, and spleen), and lymphoid tissues associated with mucosal gut and gills. For this purpose, larval samples were taken in several developmental periods including the critical change in development (metamorphosis) where rapid changes in growth require tissue modification and increased bioenergetic consumption. For this objective we have used histological techniques and specific stains to detect the main changes in composition and structures in these organs and tissues. *A. regius* shows similar patterns to most teleosts during organogenesis, but the time of appearance of these structures during their growth is species-specific. In addition, and to complete this study of the development of the immune system (5) the expression of certain genes related to the non-specific immune system was analyzed in order to identify possible markers of immunity during growth. Particularly, we wanted to determine the presence during early developmental periods of significant immune gene transcripts as well changes in expression level in larvae and juvenile tissues, and study whether the relative expression of these genes may be correlated to observed morphological changes seen by histology. The results indicated that the genes under study for this work can be detected during the early stages of development and we found indications that their expression profiles may be influenced by significant dietary changes made during the larval and early juvenile culture.

Resumen

La producción acuícola en Europa está dominada por un pequeño grupo de especies, como salmón, trucha, carpa, lubina, dorada, que a su vez limita el número de productos acuícolas disponibles en el mercado. Estas especies han experimentado algunos problemas en relación al precio de mercado que menudo se encuentra cerca o por debajo del coste mínimo de producción, como consecuencia de ello el crecimiento de la acuicultura en la UE se ha visto limitado, no alcanzando las cantidades previstas a la vez que se han ido incrementando las importaciones desde otros países. Sin embargo, un sistema sostenible basado en la incorporación de nuevas especies podría mermar en cierto modo gran parte del problema. Actualmente, el nivel de concienciación en la industria de la acuicultura de investigar la biología de nuevas especies contribuye a fortalecer la sostenibilidad económica, lo que trae como consecuencia un mayor interés por buscar nuevas especies con ciertas características como: rápido crecimiento, facilidad de cultivo, adaptabilidad a grandes volúmenes de cultivo y alta productividad, que ofrezcan excelentes oportunidades para el procesamiento y desarrollo de valor añadido. El propósito de este trabajo, es el estudio de una especies considerada prometedora para la acuicultura mediterránea, perteneciente a la familia Sciaenidae y conocida comúnmente como corvina (*Argyrosomus regius*). Esta especie se caracteriza por su alta tasa de crecimiento (1 kg año^{-1}) y su buen índice de conversión entre otras. Sin embargo, como toda especie nueva cuenta con algunos cuello de botellas presentes durante sus primeras etapas de desarrollo que necesitan ser resueltos. Así los objetivos generales de esta tesis van dirigidos a estudiar los siguientes aspectos: (1) morfología y desarrollo funcional del sistema digestivo de la larva, basado en el análisis histológico y actividad enzimática durante su fase de crecimiento, (2) estudio del efecto de diferentes estrategias de alimentación temprana de alimento artificial y reducción del uso de nauplios de *Artemia* (destete temprano) a un 50% de la cantidad utilizada en protocolos estándar de producción, evaluando los efectos en el desarrollo, particularmente en crecimiento, supervivencia, digestión y deformaciones. Dos experimentos fueron diseñados con diferentes protocolos de alimentación orientados especialmente en la incorporación temprana de alimento artificial, evaluando su efecto en la morfología y desarrollo funcional, mediante marcadores enzimáticos que reflejan la madurez del tracto digestivo. Sin embargo ciertos problemas inherentes a la especie particularmente, su conducta caníbal durante la fase de post-flexión marcaron efectos sobre la supervivencia (%), no obstante el destete temprano no tuvo mayor influencia en el desarrollo de deformaciones a nivel de estructuras esqueléticas. En este sentido se procedió al (3) estudio de los requerimientos nutricionales durante el desarrollo larvario, especialmente en la composición de ácidos grasos, con el intento de examinar los efectos sobre el crecimiento y supervivencia mediante diferentes composición de ácidos grasos en larvas, específicamente contenido de DHA y relación DHA/EPA, así como también, evaluar su capacidad de poder elongar y desaturar ácidos grasos de sus precursores usando una emulsión con bajo contenido de DHA (aceite Hemp). Dos experimentos fueron diseñados durante el cultivo larvario, utilizando varios contenidos de DHA (alto, medio, bajo) demostrado que los requerimientos en cuantos ácidos grados (DHA) son

específicos a nivel de especie, además de evidenciar la incapacidad de la larva por elongar y desaturar cuando sus precursores son ofrecidos en el alimento vivo.

El sistema inmune es otros de los aspectos de gran importancia durante la cría larvaria ya que por lo general el éxito de un cultivo larvario se basa no solo en la nutrición sino también en los mecanismos de defensa con que cuenta la larva frente a innumerable agentes patógenos presentes en el medio de cultivo. La larva durante su desarrollo depende de una serie de importantes moléculas de protección que hacen frente a potenciales patógenos hasta que su sistema adaptativo o específico está completamente maduro. Particularmente, la mayor parte de la mortalidad durante un cultivo peces se presenta durante las etapas más críticas de crecimiento, limitando la producción de juveniles. En este sentido, para evaluar el tiempo en que la larva es más susceptible a factores externos, y su capacidad de afrontar una respuesta inmune, (4) se estudió la ontogenia del sistema inmune innato durante el desarrollo larvario, enfocado en la organogénesis de los principales órganos linfoides tales como el timo, el riñón y el bazo, además de tejidos linfoides asociados a las mucosas del intestino y branquias. Para este propósito se tomaron muestras de larvas en los períodos más importantes del crecimiento, considerando también las fases críticas de cambio en el desarrollo (metamorfosis), para este fin, se utilizaron técnicas histológicas mediante tinciones específicas para detectar los principales cambios a nivel de composición y estructuras en estos órganos y tejidos. *A. regius*, presenta patrones similares a la mayoría de teleósteos durante la organogénesis, pero el tiempo de aparición de estas estructuras durante su crecimiento es específico. Además y para completar este estudio del desarrollo del sistema inmune (5) se analizó la expresión de ciertos genes relacionados con el sistema inmune inespecífico con el propósito de identificar posibles marcadores de inmunidad durante el crecimiento, particularmente su presencia durante períodos tempranos de desarrollo así como cambios a nivel de expresión en larvas y tejidos de juveniles, además, evaluar si la expresión relativa de estos genes puede estar correlacionada con cambios morfológicos observados por la histología. Los resultados mostraron que estos genes pueden detectarse durante la etapa temprana de desarrollo y sus perfiles de expresión pueden estar influenciados por la dieta durante el cultivo.

Report on paper impact factors

Dra. Alicia Estevéz and Dr. Karl B Andree, as the supervisor of the Doctoral Thesis titled "**Advances in the larval rearing of meagre (*Argyrosomus regius*): Diet, weaning protocols and ontogeny of the digestive and innate immune systems.**" performed by Cindy Campoverde Vera. In light of the aforementioned certifies the Impact Factor in each of the scientific papers that are part of this thesis. In addition, it is declared that none of the papers have been included in any other Doctoral Thesis apart from the present.

Chronological order:

1. AUTHORS: M.M. Solovyev, C. Campoverde, S. Öztürk, C. Moreira, M. Diaz, F.J. Moyano, A. Estévez, E. Gisbert.

TITLE: **Morphological and functional description of the development of the digestive system in meagre (*Argyrosomus regius*): An integrative approach**

Journal: Aquaculture, 464 (2016) 381–391.
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2. AUTHORS: C. Campoverde, C. Rodriguez, J. Perez, E. Gisbert, A. Estévez

TITLE: **Early weaning in meagre *Argyrosomus regius*: Effects on growth, survival, digestion and skeletal deformities**

Journal: Aquaculture Research, 48 (2017) 5289-5299
DOI: 10.1111/are.13342.

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3. AUTHORS: C. Campoverde, A. Estévez

TITLE: **The effect of live food enrichment with docosahexaenoic acid (22:6n-3) rich emulsions on growth, survival and fatty acid composition of meagre (*Argyrosomus regius*) larvae.**

Journal: Aquaculture 478 (2017) 16-24
DOI: 10.1016/j.aquaculture.2017.05.012.

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4. AUTHORS: C. Campoverde, D.J. Milne, A. Estévez, N. Duncan, C.J. Secombes, K.B. Andree.

TITLE: **Ontogeny and modulation after PAMPs stimulation of β-defensin, hepcidin, and piscidin antimicrobial peptides in meagre (*Argyrosomus regius*).**

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5. AUTHORS: C. Campoverde, D.J. Milne, A. Estévez, E. Gisbert, C.J. Secombes, K.B. Andree.

TITLE: **Ontogeny of the innate immune system meagre (*Argyrosomus regius*) during early rearing.**

Journal: Fish Shellfish Immunology, ***preparing for submission***

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6. AUTHORS: C. Campoverde, F. Carella, A. Estévez, E. Gisbert, K.B. Andree.

TITLE: **Ontogeny of lymphoid organs and mucosa tissues system in meagre (*Argyrosomus regius*) during early rearing.**

Journal: Fish Biology, ***preparing for submission***

IF: 1.519

Presentations in conferences:

- "Nuevos avances en el cultivo larvario de corvina (*Argyrosomus regius*)". III Foro de estudiantes Ecuatorianos en Europa. Octubre, 2015. Berlin, Alemania. (Oral Presentation).

- "Progress in understandig the ontogeny of the immune system in meagre (*Argyrosomus regius*)". Campoverde C, Milne D, Andree K, Gisbert E, Estévez A, Secombes C. European aquaculture society, Rotterdam, 2015. (Poster).

- "Early weaning in meagre *Argyrosomus regius*: Effects on growth, survival, digestion and skeletal deformation". Campoverde C, Estévez A. Rodríguez C, Pérez J, Gisbet E. International Conference & Exposition. September, 2016. Edinburg, Scotland. (Poster).

- "Early weaning in meagre Argyrosomus regius: Effects on growth, survival, digestion and skeletal deformation". Campoverde C, Estévez A. Rodríguez C, Pérez J, Gisbet E. III Jornada científica del departamento de Biología Animal y Biología animal y Ecología. Barcelona, España. Junio 2017. (Oral Presentation).

- "Gene expression analysis of antimicrobial peptides during larval rearing and grow-out of meagre (*Argyrosomus regius*)". World Aquaculture Society meeting celebrated in Cape Town (South-Africa). June 2017.

- "Ontogeny of limphoid organs and mucosal immune tissues in meagre (*Argyrosomus regius*)". Campoverde C, Carella F, Bellot O, Estévez A, Gisbert E, Andree K. International Conference European Aquaculture Society. September 2017. Dubrovnik, Croatia. Septiembre, 2017. (Poster).

Abbreviations

ALA	linolenic acid	GI	gastrointestinal tract
AP	alkaline phosphatase	NTC	no template control
ARA	arachidonic acid	DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid	DW	dry weight
gDNA	genomic DNA	Elovl	elongase
RNA	ribonucleic acid	EPA	eicosapentaenoic acid
rRNA	ribosomal RNA	FA	fatty acid
mRNA	messenger RNA	Fad	fatty acyl desaturase
tRNA	transfer RNA	GALT	gut associated lymphoid tissue
cDNA	complementary DNA	IECs	intestinal epithelial cells
dsDNA	double stranded DNA	LC-PUFA	long chain polyunsaturated fatty acid
ssDNA	single stranded DNA	MALT	mucosa associated lymphoid tissues
ssRNA	single stranded RNA	MUFA	monounsaturated fatty acid
dsRNA	double stranded RNA	NFkB	nuclear factor kB
PCR	polymerase chain reaction	PAMP	pathogen-associated molecular pattern
qPCR	quantitative real-time PCR	poly (I:C)	polyinosinic-polycytidylic acid
RT	reverse transcription	PRRs	Pattern-recognition receptors
Ct	cycle threshold	PUFA	polyunsaturated fatty acid
dph	days post hatch	RACE	rapid amplification of cDNA ends
wph	weeks post hatch	SGR	specific growth rate
IgM	immunoglobulin M	TFA	total fatty acid
AMP	antimicrobial peptide	UTR	untranslated region
LPS	lipopolysaccharide	β-glucan	beta-glucans
TLR	toll-like receptor	MMC	melanomacrophage centers
EST	expressed sequence tag		

CHAPTER 1

General Introduction

Cindy Campoverde

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1. Aquaculture status global

Aquaculture, the cultivation and breeding of aquatic species (fish, crustaceans, molluscs and algae) represents an important source of food for the world's human population, contributing significantly to welfare and food security. One of the great challenges at a global level is the need to feed an increasing human population that will reach 9 billion people by 2050 [1]. In the past, the growing demand for fish was handled by putting pressure on natural resources and threatening the sustainability of marine and continental fishing, but also there has been a push towards aquaculture development. Fisheries captures have not increased significantly since the early 1990´s with levels (90-95 million tonnes) likely indicating that oceans are being exploited close to their maximum capacity (Figure 1). While the production of aquatic animals from aquaculture accounted for 73.8 million tonnes by 2014. It is expected that with the stagnation of wild captures and the increase of the world population, aquaculture will continue growing as the main source of high-quality protein (plant and animal) supply, rich in essential oils of aquatic origin. In recent decades, aquaculture production has increased the supply of fish for human consumption contributing 39% of fish for human consumption in 2004 vs. 7% in 1974. Following this approach, with the population growth, the apparent consumption of fish per capita worldwide recorded an average of 9.9 kg (1960) with potential increases to approximately 20 kg for 2014-2015.

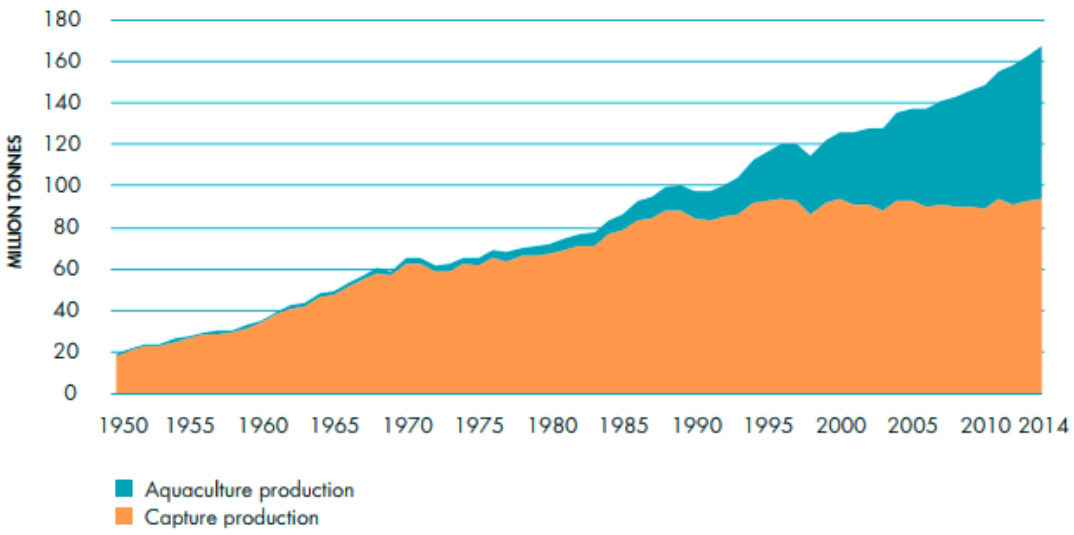

Fig. 1. Global aquaculture production.

In 2014, fish harvested from aquaculture amounted to 73.8 million tonnes, with an estimated first-sale value of US$160.2 billion, consisting of 49.8 million tonnes of finfish (US$99.2 billion) [2].

World aquaculture production of fish accounted for 44.1 percent of total production (including for non-food uses) from capture fisheries and aquaculture in 2014, up from 42.1 percent in 2012 and 31.1 percent in 2004 (Figure 2). This data showed a general trend of an increasing share of aquaculture production in total fish production. Therefore an increase in aquaculture will be the main driver of change in the fisheries sector in the near future.

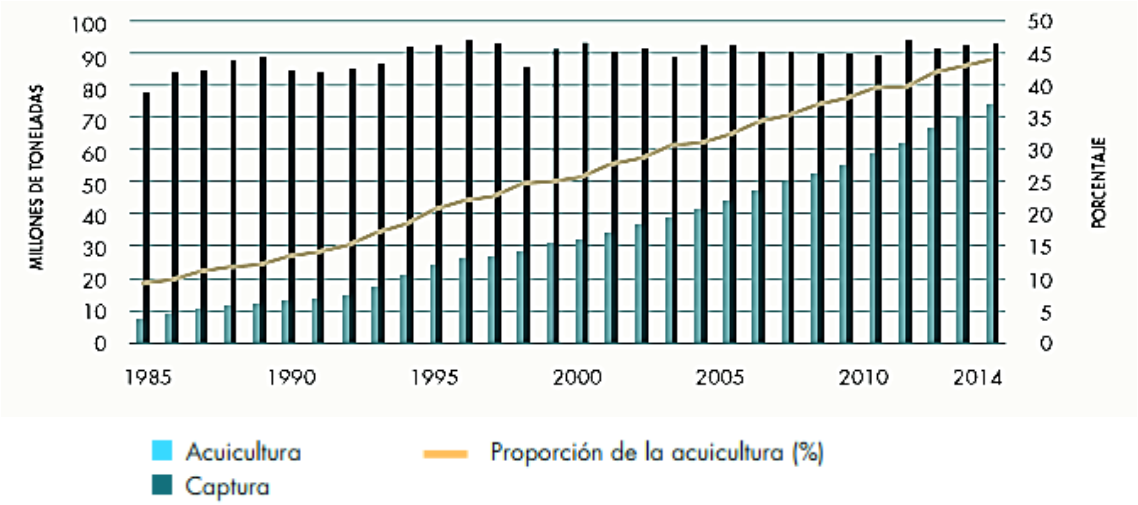

Fig. 2. Proportion of aquaculture of aquatic animals in total production. (FAO 2016).

1.1 Marine Mediterranean Aquaculture

In the Mediterranean region, aquaculture has expanded rapidly over the past two decades with an annual growth rate that increased from 4% in 1980 to 13% in 2000 with a tendency towards diversification of cultured species. Although, Mediterranean aquaculture production was based mainly on molluscs during the 1990s, today the proportion of farms producing fish continues to increase. Aquaculture production in the Mediterranean has increased almost 3 times from the first records in 1994 to those from 2003.

Table. I Aquaculture in the Mediterranean Production by species (FAO 2006).

Mejillón mediterráneo *(Mytilus galloprovincialis)*	147,920 t.
Dorada *(Sparus aurata)*	74,078 t.
Lubina *(Dicentrarchus labrax)*	43,804 t.
Lisa *(Mugil cephalus)*	42,546 t.
Almeja japonesa *(Ruditapes philippinarum)*	25,000 t.
Otras especies de lubina	20,982 t.
Ostra japonesa *(Crassostrea gigas)*	8,608 t.
Otros peces marinos	4,894 t.
Trucha *(Salmonidos)*	1,194 t.
Corvina ocelada *(Sciaenops ocellatus)*	438 t.

Mediterranean aquaculture is mainly represented by molluscs and fish, with very few crops of other groups (crustaceans or macroalgae). However, Mediterranean marine fish aquaculture concentrates on a small group of species such as Seabream (*Sparus auratus*) and Seabass (*Dicentrarchus labrax*) covering 100% of the total marine production (table 1). In recent years, the production of these species has saturated the market, with a deep decrease in final product price and the sector has been facing a crisis scenario. According to FAO statistics, in 2012 only 5% of total production of Mediterranean marine fish was derived from new marine fish species. The sector, especially of certain Mediterranean species such as seabream and seabass have reached their maturity and have forced production regions look for alternatives of new species covering other market niches such as larger size fish that facilitate their preparation and can be processed into portions (fillets, loins) [3]. The low concentration of lipids and their balanced distribution in muscle gives some species a high dietetic value and a positive image for consumers.

1.2 Global Production

Meagre (*Argyrosomus regius*, corvina in Spanish) is a new species that has been considered for large scale aquaculture production very recently. The production of this species was almost non-existent in 2000, but now meagre production has reached 4871 tons in Europe and it is being considered a highly-prized species in some European regions, although, given its limited wild capture and the very recent onset of their production through aquaculture, it is little known in the market. The main countries that catch this species are Ghana, Mauritania, Egypt and France, whereas aquaculture is confined to Corsica, Italy and Spain. The aquaculture production reported for meagre in 2010 and 2011 was more than 14,000 tonnes (Figure 3). This production was reduced both in 2012 (31%) and in 2013 (55%), as compared with 2014 when there was a slight increase of 11,770 tonnes (FAO 2016).

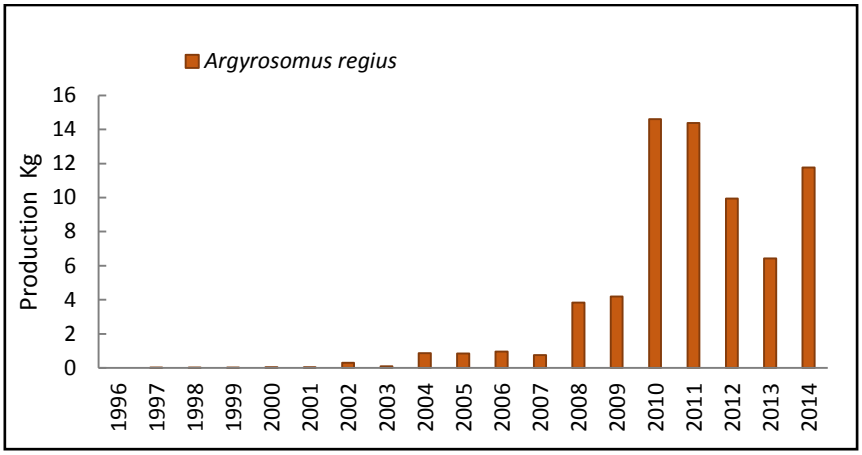

Fig. 3. Global aquaculture production of meagre (*Argyrososmus regius*).

1.3 Meagre (*Argyrosomus regius*).

Meagre, *Argyrsomus regius* is a member of the family Sciaenidae, including 270 species that inhabit saltwater, freshwater and brackish waters [4]. It is a carnivorous species, with an external morphology elongated and slender, almost fusiform, and slightly compressed body. Its distribution ranges from the Mediterranean Sea and the Black Sea, as well as occurring along the Atlantic coast of Europe where it lives in coastal waters, although it can be found near the bottom of the continental shelf or near the surface, at depths of 15-200 m [5]. This species was chosen for the diversification of aquaculture in the Mediterranean region due to its high potential. Its culture started in Europe at the end of the 90`s in France and Italy, followed by Spain (2004), Greece and Turkey (2007), and later on in Egypt [6]. This species shows certain characteristics that determine its aquaculture suitability such as its large size, good processing yield, low fat content, excellent taste and firm texture [7]. In addition, the final product allows for an optimal elaboration and transformation to value-enhanced products due to the body size and shape. Further, the species has the biological characteristics required for commercial aquaculture using well-established culture technologies [8]. These characteristics include a fast growth of ~1 Kg per year [9] a low feed conversion rate of 0.9-1.2 [7,9] and resistance to stressful conditions that can occur during intensive aquaculture. Several studies have confirmed that larval rearing of meagre is relatively easy [10,11], the juveniles have high acceptance of existing commercial feed designed for other species, and the methods for their reproduction in captivity have been largely achieved [12].

1.3.1 Bottlenecks

In recent years, several fast growing species such as cobia (*Rachycentrom canadum*) (Joan Holt, Faulk and Schwarz, 2007), yellowtail (*Seriola sp*.) Atlantic Bluefin tuna (*Thunnus thynnus*) and other species of the family Serranidae [13] have come under consideration by aquaculture producers and therefore there is a need to research their biology to understand the requirements for domestication. Several bottlenecks have been identified among these fast growing species, and also in the case of meagre, especially in various aspects related to larval and juvenile rearing. Among the bottlenecks identified for meagre are the following:

- Adapting feeding protocols used by other species.
- Establish optimal nutritional requirements in terms of essential fatty acids during early stages of development.
- Eliminating cannibalistic behavior during the periods of weaning (from live prey to inert diets) and metamorphosis from larva to juvenile.
- Ameliorating the high variability of growth that occur during cultivation.
- Establish clear parameters in regard to the immune system in this species.

However, important achievements have been obtained especially in terms of reproduction under conditions of captivity, with good results in hatching and survival rates during larval rearing [10,12].

In meagre, successful larval rearing and live prey enrichment protocols have been established [10,11] using different commercial enrichment products. However, weaning and grow out with diets developed for other marine fishes (i.e., gilthead sea bream and European sea bass) are still bottlenecks that restrict the fast growing potential of this species and affects negatively their productive performance. The feeding protocol adopted for meagre larvae is based on the use of rotifers (*Brachionus* sp.) and *Artemia* nauplii and metanauplii fed to larvae in certain days after hatching, mimicking other species protocols (such as seabream) that has allowed an increase of 7 fold in larval production since 1997. Although, the precise requirements for essential amino and fatty acids are not completely known, the larvae show very good growth and survival rates using commercially available enrichment products for live prey. On the other hand, the

cannibalistic behavior and, as a consequence, the variable size distribution of the larvae after weaning are an important concern.

Moreover, suboptimal commercial feeds and feeding protocols result in direct economic losses through feed waste, poor growth and water quality deterioration. The lack of meagre-specific weaning and grow out diets will be addressed in this study, in order to maximize growth potential, enhance fry quality and promote health of this species.

1.4 Weaning Strategies

The larviculture of many marine finfish species is still dependent on live feed, such as the rotifer (*Brachionus spp*.) and brine shrimp (*Artemia sp*.) until metamorphosis [14,15]. Live feed supply is generally required past metamorphosis when larvae are weaned onto dry formulated diets. This can create significant financial barriers to the practical production of these larvae for commercial aquaculture. It is well documented that the cost and quality of *Artemia* can fluctuate over time as the supply is dependent on both the worldwide aquaculture demand. In addition to the unstable nature of *Artemia* supply, the percentage of hatchery feed cost attributed to *Artemia* is substantial, in some case in fish rearing the live food accounted for 79% of production cost for juvenile up to 45 days old [12] besides, the labour-intensive processes, high production costs and variable nutritional quality of the live prey [16]. The main objective in larval rearing is to replace or reduce live prey organisms, especially *Artemia*, by formulated diet. Substituting a compound diet for live prey was performed several weeks after hatching, depending on the species. In this sense, studies have reported improved growth and survival of several marine fish larvae from the combined feeding of live and manufactured diets (co-feeding). Person Le Ruyet et al., (1993) [17] formulated a diet adequate to sustain good growth and survival in European sea bass (*Dicentrarchus labrax*) from day 40 dph (days post-hatching), when the weaning of this species was usually conducted at day 55 in hatcheries. Cahu et al., (1998) [18], it has been reported that 35% of sea bass larvae, fed exclusively compound diet from mouth opening, survived at day 28. Concurrently, some survival was obtained in other marine species fed compound diet from mouth opening, such as sea bream *Sparus aurata* [19] while other species need a short period of the co-feeding, in a study by Hart and Purser, (1996) [20] achieved significantly higher survival in greenback flounder (*Rhombosolea tapirina*, Gunther) weaned prior to metamorphosis at 23 dph using a 10-day co-feeding regime, than was achieved weaning after metamorphosis (50

dph) [20]. In butter catfish, *Ompok bimaculatus* (Bloch) larvae was weaned onto microdiets after a short period of co-feeding when weaning takes place after 7 dph [21].

Generally, first-feeding marine fish larvae typically have a rudimentary digestive system which lacks a functional stomach and digestive enzyme activities are low, or in some cases undetectable, making it difficult for them to process and assimilate a complex microdiet [22]. The early introduction of dry formulated diets in marine fish larviculture protocols is crucial if larvae are to maximize growth and achieve high survival. In order to achieve this, many studies have suggested that combining live feed and dry formulated diets (co-feeding) from an early developmental stage seems to improve and stabilize the nutritional condition of the larvae and helps condition the larvae to more easily accept the dry diet when live feed is withdrawn, resulting in a shorter weaning period [23].

Overall, it is essential to ensure an adequate switch from *Artemia* to formulated microdiet because some risks such as decrease in larval weight, increase larva size variation, skeletal abnormalities and cannibalism behavior, occurred when the early weaning is unsuccessful. Some obstacles for an early weaning also exist associated with the lack of interest for immobile and perhaps unpalatable feeds on the part of the larvae [24]. Several authors has suggested that weaning success and efficient feeding can be improved by introducing new food sources at the appropriate stages of larval development using tools such as histological examination of the maturation stage of some digestive systems and processes such as the onset of pancreas secretory functions.

2. Nutrition (Fatty acids)

In recent years considerable research effort has been undertaken in order to develop aquaculture of these large pelagic migratory fish species [25]. Determining nutritional requirements, especially, at larval stages, will be a key task [25]. Although, the nutritional requirements during early larval development have been a relatively well-studied area in other marine fish, only a few comparative data exist on sciaenidae species. The importance of the lipids for growth and development of fish is based on their key roles as sources of metabolic energy and as essential components of tissue and cell membranes. Long chain polyunsaturated fatty acids (LC-PUFA) have well-established roles in membrane structure and function as well as energy sources during embryonic and early larval development of marine fish [26]. LC-PUFA, such as eicosapentaenoic acid (EPA;20:5n-3) docosahexaenoic acid (DHA; 22:6n-3) and arachidonic acid (ARA; 20:4

n-6), are essential for multiple physiological processes including the maintenance of structural integrity of the cell membrane and metabolic energy production, as precursors for eicosanoids, ligands for transcription factors and messengers in cellular pathways. LC-PUFA are also implicated in improving immunomodulatory activities. The composition and metabolism of lipids and fatty acids during embryogenesis and yolk-sac larval development can give useful information of the nutritional requirements during early larval stages [26]. This approach can provide insights that can be applied in the development of live feed enrichments and/or artificial feeds [25]. However, all marine fish studied to date have only very limited ability to biosynthesize LC-PUFA and so have an absolute requirement for dietary EPA and DHA [26].

LC-PUFA can be biosynthesized from short-chain (C18) PUFA in reactions catalyzed by fatty acyl desaturase (Fad) and elongase (Elovl) enzymes. Understanding the biochemical and molecular mechanisms of LC-PUFA biosynthesis in fish has advanced in recent years and several Fads have been characterized in various fish species. However, the biochemical study in fish cell lines [27,28] led to the hypothesis that some fish may be unable to biosynthesize LC-PUFA because they lack specific genes in the pathway [29]. Although, Δ6 Fad cDNAs have been cloned from all fish species so far [30–33], and all showed significant activity in heterologous yeast expression systems. However, Δ6 Fad expression and activity are very low in Atlantic cod (*Gadus morhua*) [31]. Monroig et al., [34] showed the first investigation of LC-PUFA biosynthesis from shorter chain PUFA in juvenile stages of *Argyrosomus regius* and reports the cloning of cDNAs for two important genes fatty acyl desaturase (*Fads2*) and elongase of very long-chain fatty acid (*Elovl5*). The increased activity of these Fad enzymes and elovl5 is important for the normal development and for providing sufficient supply of n-3 long-chain polyunsaturated fatty acids (LC-PUFA) in early developmental stages, such as was observed in tuna larvae (Δ6 Fad and elovl5) [35]. But, no studies have shown these enzyme activities in meagre larvae. Regardless there is a need as the concentration of n-3 long-chain polyunsaturated fatty acids (LC-PUFA) in neural tissues is crucial for effective prey capture from the time of first feeding in marine fish larvae, such as meagre.

3. Innate Immune System

The intensive culture of *A. regius* within the Mediterranean is at risk from a range of common disease agents that also impact on the production of other commercial species

[36]. There is increasing awareness in the aquaculture industry that knowledge about biology of new species contributes to strengthen economic sustainability. There are growing challenges associated with health and welfare of farmed fish. Diseases, pathogens and parasites as well as potentially stressful husbandry conditions and feed composition are key issues that need to be addressed in this species. A key factor in addressing the health and welfare challenges, and to allow timely and appropriate intervention, is the need for an increased understanding of the immune system in meagre. For that studying the larval development of the immune system will allow the establishment of the timeline for maturation of the immune system in meagre and the development of immune memory, which is required for optimal timing of vaccination. Expression and modulation of these genes will be monitored during larval development to be used as markers for comparison to histological landmarks of tissue development and establishment of potential functional developments of humoral cellular immune responses.

An immune system is a system of biological structures and processes within an organism that detect a wide variety of agents from virus particles to parasites, protects against disease and distinguishes them from the healthy tissue of the host organism. From an evolutionary point of view, fish are considered as the earliest class of vertebrates having both innate and adaptive immunity [37]. Fish are found in diverse and even extreme aquatic environments and they operate their defense mechanisms efficiently, through physical barrier as mucosal membranes of the skin, gills and gastrointestinal tract are in constant intimate contact with their surroundings and therefore continually encounter potential pathogens. Fish are able to protect themselves with the help of a complex innate defense mechanism that may be constitutive or responsive. In this sense, the pivotal role of the immune system is to participate in processes of maintaining homeostatic conditions during development and growth while also being responsive to inflammatory reactions or tissue damage.

The production of larvae in a new species usually represents the major bottleneck in aquaculture, where high mortalities are commonly experienced during the early larval stages, especially around the onset of exogenous feeding [38]. However, Roo et al., [10] and Suzer et al., [39] reported survival values between 36 and 54% but these authors, detected a strong cannibalistic behavior during larval metamorphosis and early juvenile

stage [10,11,39]. This inherent behavior should have more attention in future studies in this species.

On the other hand, it is well known that during the early production stages larvae are exposed to an environment that contains an abundance of bacteria that contributes to massive losses especially during the period prior to achieving full immune competence [40]. All carnivore species are known to ingest bacteria by drinking before active feeding of live prey. Live preys are offered during the first weeks of exogenous feeding, and they also carry a high diversity and abundance of bacteria [41] including some potentially pathogenic groups [42]. During this period, the larvae have to rely solely on innate mechanisms and possibly maternal antibodies until they develop a functional adaptive immune system, which may take several months [43,44]. However, the precise timing of this immune maturation, when mechanisms other than innate immunity are fully functioning depends upon the species under study as well as the number of degree days post hatch.

Substantial progress has recently been made in the characterization of the fish immune mechanisms and pathways in numerous species. The past decade has seen the rapid expansion of knowledge related to immune responses in fish, especially with regard to economically relevant species in aquaculture and fisheries, which is mainly salmonids (*Salmo salar*, *Oncorhynchus spp*.), cyprinids, flatfish (scophthalmids, pleuronectids), gadoids, ictalurids, moronids or sparids. More specific studies are available for some species, such as Mediterranean sea bass (*Dicentrarchus labrax*) [45] and eels (*Anguilla spp*.) [46]. Nevertheless, there are still important gaps in the knowledge of numerous immune mechanisms, and the available information varies according to the fish species.

Knowledge of the innate immune system in meagre and the mechanisms that control it are considered a promising approach for improving survival and controlling disease under culture conditions. The ontogeny of the lymphoid organs and mucous associated lymphoid tissues (MALT) and their immune functions in meagre has been studied in this thesis, using histochemistry and nucleic acid-based techniques. Studying the mRNA expression of immune-related genes will give more detailed information of the ontogeny of the immune system, supplement the histological description of the development of the immune tissues, and supply quantitative information of how different treatments/conditions would affect the expression of selected genes.

3.1 Development of meagre larvae

For most teleost species, the basic mechanisms that lead to development from egg to juvenile stage follow a similar pattern. The differentiation of cells, tissues, organs and organ systems process continuously from the early embryonic phase throughout metamorphosis in accordance with a gradual improvement of functionality. Apparently large differences exist between species with regard to egg quality, temperature and time of egg incubation, timing of development and functionality of organs and organ systems, and stage-specific environmental and nutritional differences at the early life stages [47]. The yolk quality and size, environmental condition, and genetic differences greatly affect the development, size at hatch, and the time of the shift to exogenous feeding [48]. Although, the basic mechanism of larval development does not differ greatly among teleosts, there is some interspecific variability in the timing at which the different ontogenetic events occur [49]. In *A. regius*, the hatching occurs from relatively small eggs of 0.99 ± 0.02 mm and the standard length of newly hatched larvae is 2.95 ± 0.01 corresponding to a dry weight of approximately 0.02 mg. The development process of meagre larvae from hatch to juvenile involves three distinct stages: a short yolk sac stages of 5 day-post hatching, larval stage beginning at the onset of exogenous feeding (3 dph) and metamorphosis when the larval characteristics are lost and the meagre transforms into a juvenile.

The first week of development in marine fish larvae is a critical period, particularly when the yolk sac is reabsorbed and exogenous feeding take places [50]. Although, the development of the digestive tract and accessory organs of meagre follow similar patterns as in others marine teleosts [51] the digestive system continues to change and develop during the first day of exogenous feeding which necessary for further digestion. Thus, as resorption of endogenous reserves (ie.- yolk) proceeds, the larva acquires morphological and enzymatic equipment (enterocytes, with microvilli, non-specific esterase and alkaline and acid phosphatase activities, hepatocytes with glycogen stores, pancreatic cells with first zymogen granules, formation of the duct connecting accessory gland to the gut), to complete the development of absorptive and digestive process [52]. In many fish species, an incipient stomach is distinguished soon after hatching at the transition between the esophagus and the intestine: this is the last organ of the digestive system to differentiate [53]. Following this, the appearance of gastric glands normally indicates the formation of a functional stomach, which is also a histological criterion to differentiate larvae from

juvenile [54]. In meagre, a fully differentiated and functional stomach was seen at stages 4 and 5 (15-33 dph) in accordance with other studies [8,39]. As development continue others complex structures are becoming formed, those include the respiratory system such as the gills where these are functional when lamellae develop. In meagre larvae, as in most marine teleosts, lamellae only appear in stages 2 (3-9 dph), contrarily to what occurs in the most freshwater species [55]. Although, osmoregulation rather than respiration would be the priority function of the gill during ontogeny.

The lymphoid organs of meagre develop in the sequence pronephric kidney (present at stage 1 = 1- 2 dph), thymus and spleen (first seen early at stage 2) as it occurs in other marine teleosts [56–58]. The exact timing of lymphocyte differentiation within these lymphoid tissues varies in different fish species and it is probably related to the rate of growth and general development.

3.2 The ontogeny of the innate immune system

The ontogenic development of the immune system of fish requires a coordinated development of the different lymphoid organs of the immune system (thymus, kidney, spleen). Studies of these organs have shown the appearance of specific immune parameters and acquired defense competence varies greatly between fish species, even between closely related species [44]. In general, the acquired immune system develops late in marine species compared with freshwater fish, which during the early stage of larval development rely on innate defenses for the first 2–3 months after hatching [40]. In older fishes, the lymphoid organs (primary and secondary) are involved in protection of the organism against potential pathogens. However, in fish culture, the question arises as to when young fish become immunocompetent and to understand the changing degree of vulnerability.

3.3 Primary lymphoid organs

3.3.1 *Kidney*

The distribution of the main lymphoid tissues in fish differs from that in mammals. The most evident example is the hematopoietic tissue that generates the white blood cells. In mammals the leukocytes originate from the bone marrow, but is lacking in fish. In teleost, the kidney is considered as an organ analogous to the bone marrow of mammals, in which erythropoiesis, granulopoiesis and lymphopoiesis occur [59]. The head kidney (pronephros) is aglomerular and bifurcates at the anterior part and penetrates beneath the

gills [60]. It is predominantly a lympho-myeloid compartment [61]. The form of the head kidney varies between species in some species there are two separate extensions in the most anterior part of the organs, while in salmonid species the kidney is present as a single organ.

Structurally, the anterior kidney is composed of a network of fibrous reticular structures that provide support for lympho-hematopoietic tissue, which is dispersed between sinusoid systems coated by reticuloepithelial cells [62,63] besides, a large number of macrophages, MMCs, and lymphoid cells in all their evolutionary stages, mainly Ig + (B lymphocytes) are also found [61,64–66] . Reticular cells play an important role by providing the interactions necessary for the functioning of lymphoid [61] and the endothelial cells of the sinusoids that represent the main blood filtration system functioning through endocytosis [62]. In teleosts, the head-kidney is considered the main macrophage producing tissue [67] through which cellular process such as phagocytosis and antigen processing, helping the formation of immunoglobulin M (IgM) and immune memory through melanomacrophage centers [62,68–70].

3.3.2 *Thymus*

The thymus is the first organ of the immune system to become lymphocytic during histogenesis of the lymphoid tissues that produces T lymphocytes and antibody generating B cells [71]. As in all other vertebrates, the fish thymus is a paired organ present in the dorsolateral region of the gill chamber. The thymus is delineated by a connective tissue capsule that projects trabecula within the thymic parenchyma. The fish thymus occupies a superficial position just beneath or within the gill chamber epithelium, although, some studies have pointed out the intraepithelial location of the gland in some teleosts [60,72,73]. From a comparative analysis, thymic cellular components are basically similar in all fish groups. The lymphoid tissues in general are composed of a reticular cell framework supporting nonlymphoid cell populations of epithelial origin that are coexisting with the pool of thymocytes. The stroma of the thymus is composed of epithelial-reticular cells. The gland is organized histologically in two distinct zones that can usually be distinguished, an outer layer or cortex and inner layer or medulla. Romano and colleagues [74] concluded that in carp the location of the two zones changes from juvenile to adult in association with changes in the thymus shape. Cortex and peripheral outer zones are highly lymphoid, whereas the medulla and deeper regions consist mainly of lymphocytes scattered within the mesh of a more abundant connective tissue network.

3.4 Secondary Lymphoid organs

3.4.1 *Spleen*

In fish, the spleen plays a secondary role compared to the head kidney in the specific and non-specific defense mechanisms. The spleen is located in the peritoneal cavity adjacent to the gut wall. The histological appearance of the spleen in meagre was consistently composed of a fibrous capsule and small trabeculae that extend into the parenchyma, which can be divided into a red and white pulp. The red pulp, consist of a reticular cell network supporting blood-filled sinusoids that hold diverse cell populations of macrophages and lymphocytes. The white pulp is divided in two compartment: the melanomacrophage accumulations and the ellipsoids. The melanomacrophage centres (MMC) of the spleen are considered major sites of erythrocyte destruction and retain antigens for long periods [61]. In teleosts, lymphocytes are widely scattered and show only a slight tendency to accumulate around blood vessels and melanomacrophage centers (MMC) [75]. The ellipsoids have the capacity to filter plasma and trap blood-borne substances [76].

3.4.2 *Mucosal Tissues Associated (MALT)*

Skin, gills and gut are the mucosal tissues associated with the immune system of fish. These tissues with their mucus layer and array of nonspecific immune defenses are directly exposed to the external environment. The gills of fish are mucosal tissues, serving to maintain fish homeostasis by the uptake of oxygen and ions, and by forming an active barrier that harbor macrophages, neutrophils, lymphocytes and mast cells/eosinophilic granulocytes protecting against the entry of pathogens. They contain a full complement of T cell-specific (Th1, Th2, Th17, and Treg) which these cells are known in other vertebrates and found at the interbranchial septum at the base of the gill filaments [77].

The gastrointestinal tract is an organ containing mucosal tissue that is responsible for nutrition as well as immunity. In teleost, the organization of the gut associated lymphoid system (GALT) is not as complex as that in mammals but has a more diffused pattern [78]. The gut mucosa contains populations of immune cells such as lymphocytes, plasma cells, eosinophilic (mast cell-like) granulocytes and macrophages. Intra-epithelial leucocytes are present in the *lamina propria* of the gut folds and luminal to the stratum compactum [79]. As mentioned, the gastrointestinal tract is an organ with multiple functions, the dynamics of nutrient transport and bacterial interactions in the intestine leads to a constant contact between the immune system and the luminal content. Immune

cells as well as intra epithelial cells (IECs) readily sample antigen from the lumen and present them to the immune system. Resident microbes (commensal or pathogenic) are in direct contact with the gut mucosa and the gut associated lymphoid tissue distinguishes between them to initiate either tolerance or immune response.

3.5. Innate Immune response in fish

The innate immune system is of prime importance in the immune defense of fish. It is commonly divided into 3 compartments: the epithelial/mucosal barrier, the humoral parameters and the cellular components. The epithelial and mucosal barrier of the skin, gills and alimentary tract is an extremely important barrier against disease in fish, being constantly immersed in media containing potentially harmful agents. As well as providing physical and mechanical protection the fish mucus contains several immune defense effectors including antimicrobial peptides, complement factors, lysozyme, interferons, C-reactive protein, transferrin, immunoglobulins and other components involved in defensive mechanisms [80,81].

The innate immune response is based on native recognition of harmful substances and instant activation of certain cell types and mediators. The recognition of non-self-motifs, i.e. pathogen-associated molecular patterns (PAMPs), is made by pattern recognition receptors (PRRs) such as toll like receptors (TLRs). The innate immune system recognizes pathogens or conserved pathogen derived structures like flagellin, lipopolysaccharides (LPS), lipoproteins, peptidoglycan (PGN) and nucleic acid by germ-line-encoded pattern recognition receptors (PRRs) that are distributed in extracellular, membrane and cytoplasmic compartments. Three major classes of PRRs have been identified: (a) the Toll-like receptors (TLRs) that recognize ligand on either the extracellular surface or within the endosome, (b) the NOD-like receptors (NLRs) that function as cytoplasmic sensors, and (c) RIG I-like receptors (RLRs) class of cytoplasmic PRR that recognize viruses[82,83]. Their activation by an antigen is followed by activation of the complement system acting to clear the threat by three primary immune functions: a) to cover pathogens and foreign particles to facilitate their recognition and destruction by phagocytic cells (opsonization), b) to initiate inflammatory reactions by stimulating the contraction of smooth muscle, vasodilation and chemoattraction of immune cells, c) to lyse pathogens through the perforation of their membranes [81]. The immediate innate immune response to the presence of a “foreign” agent includes inflammatory cytokines, acute phase proteins and antimicrobial peptides.

In the early innate immune response certain transcription factors control the expression of cytokines and effector proteins. Well studied transcription factors include nuclear factor κB (NF-κB) and, in the case of viral infection, interferon regulating factor (IRF) [84–86]. These transcription factors promote transcription and translation of cytokines that on binding to cytokine receptors in surrounding cells activate them and magnify and diversify the response to neutralize the threat at hand. In mammals, NF-κB is involved in many different responses, the immune response being one of the most frequent. Prior to activation, NF-κB is situated in the cytosol bound to its inhibitor (IκB). Upon activation, NF-κB is released and translocate to the nucleus where transcription of several genes is initiated, one of them being the gene for the inhibitor, IκB, leading to self-regulation of activation. The presence of this mechanism in fish has been reported [87]. The cytokines produced during the invasion of a microorganism propagates the response and if necessary facilitates the switch to a more specific response. Therefore, cytokines are important not only for activation but also for their timing in the response and the effect each cytokine has on different cells in the cascade, factors that all contribute to the sum response to a specific threat.

3.5.1 Complement

The complement system is composed of over 35 soluble plasma proteins and receptors that play key roles in both innate and adaptive immune responses. Upon initiation of the complement cascade, the resulting activation products play crucial roles in phagocytosis and cytolysis of pathogens, solubilization of immune complexes, and initiation of inflammatory responses. The complement system of teleost fish, like that of higher vertebrates, can be activated through all three pathways of complement activation: the alternative, lectin and classical [88]. The classical pathway is initiated by a complex between an antigen and an antibody. In the alternative pathway, the spontaneous activation of C3 is amplified upon the covalent binding of C3 (H2O) to various microbial surfaces (i.e., viruses, bacteria, fungi, parasites). The lectin pathway requires the interaction of lectins such as mannose-binding lectin (MBL) and ficolins, with sugar moieties found on the surface of microbes. In contrast to mammals, complement in teleosts is active at very low temperatures, and their alternative complement pathway titers are several orders of magnitude higher. The complement system plays a major role in the link between both innate and adaptive immune response in fish [89].

3.5.2 *Lyzozyme*

Lysozyme is a lytic enzyme acting on the peptidoglycan component of the bacterial cell wall, especially of gram-positive bacteria; it can also act as an opsonin. While in gram-negative bacteria its activity becomes effective after complement and other factor have disrupted the outer cell wall, exposing the inner peptidoglycan layer where lysozyme becomes effective. In fish, lysozyme is synthesized in the liver and the extra hepatic side (other tissues) [90]. Like mammals, the lysozyme in fish occurs mainly in neutrophils, monocytes and a small amount in macrophages. It is present in mucus, plasma as well as in the head kidney, which is rich in leucocytes, and at sites such as the gill, skin, gastrointestinal tract and eggs, where the risk of bacterial invasion is very high [91]. In fish, lysozyme, is released by leucocytes and has a broader activity than mammalian lysozyme [92] and has been frequently used as an indicator of non-specific immune functions.

3.5.3 *Antimicrobial peptides*

Antimicrobial peptides (AMPs) are an evolutionarily conserved component of innate immune response and recognized as a critical first-line defense against many pathogens. These are low molecular weight peptides with the ability to disrupt antigen like bacterial membranes. In general, AMPS are present in mucus and in cells lining epithelial surfaces, such as skin, gill as well as immune cells like mast cells, neutrophils, goblet and rodlet cells [93]**.** Several classes of these antimicrobial effectors are recognized as a broad category of innate immune effector molecules and, these small peptides (AMPs) are divided into different families. Several studies have found peptides in a wide variety of species belonging to the defensin, parasin, cathelicidin and hepcidin families, as well as piscidin, a family unique to teleost fish [94]. Fish AMPs are up-regulated in response to pathogen and appear to have direct broad-spectrum antimicrobial activity towards both human and fish pathogens. In addition, there appear to be interesting differences, specific to fish, that have evolved to address the unique aquatic environments and microbes encounters by these species. Many of the peptides identified have antibiotic activity as well as immunomodulatory functions. During the last times, there has been a great effort to study the potential of these peptides for applications in human medicine, and animal welfare [95].

3.5.4 *Cellular immune response*

The cell types involved in the innate immune response of fish includes monocytes (e.g. macrophages) and granulocytes (e.g. neutrophils) as well as eosinophilic granulocytes that resemble the mammalian mast cells. However, most mast cell-like cells in fish do not produce histamine [96] but, Mulero et al., (2007) [97] used immunohistochemistry (IHC) to describe the presence of histamine in the mast cells (MCs) of teleost fish belonging to the order Perciformes. At the cell level, a typical immune response starts with secretion of cytokines by tissue cells, as well as of prostaglandin and other compounds from mast cells. The secreted substances activate macrophages in the tissue and stimulate recruitment of neutrophils from the circulation [98]. Macrophages neutralize foreign antigen by phagocytosis and secrete cytokines and chemokines (chemotactic cytokines) that attract neutrophils to the area. The neutrophils take over and act on the threat by engulfing debris, clear the area of pathogens and undergo apoptosis. In a later stage a shift in chemokine action occurs and monocytes are recruited and differentiate into macrophages that then re-populate the tissue [99].

OBJETIVES

Objectives

Further development of the aquaculture industry requires diversification through the commercialization of new species. However, for new species to be commercialized there needs to be considerable effort made towards domestication of the proposed species. This means considerable research input to better understand their biology to enable completion of the life cycle in captivity. Although considerable progress has been made during the past few years, the production of larva and juveniles is still one of the main bottlenecks for several species. The problem regarding the poor reproducibility in terms of survival, growth and quality is the main point of interest. Based on the above, the production of fish larvae in European aquaculture sector is often hampered by high mortality rates due to infectious diseases that is believed to contribute most to economic loss.

Nutritional strategies of feeding and a better understanding of mechanisms related to innate immune responses are crucial for the development of effective approaches for growth and disease management. Advances in hatchery and research programs, suggest that bacteria normally present in hatcheries may be the principle cause of problems associated with production of juveniles. This is most likely due to opportunistic bacteria, since initial stages of development are exposed to numerous pathogens before their lymphoid organs have matured and adaptive immunity has developed. However, even though there exist extensive literature on nutritional studies and immune system functions in commercial species such as salmonid, pleuronectiforms, and cyprinids, each species show inherent differences that are likely species-specific, and relate to environmental conditions under which they are grown. Given the importance of nutrition and health to the successful culture of any animal, the main objective of this thesis was to evaluate the potential impact of the alternative strategies of feeding, understand the nutritional requirements, ontogeny of the organs and some relevant genes involved in the innate immune system, to improve captive rearing of meagre, (*Argyrosomus regius*) and thereby facilitate its introduction into commercialization. Knowledge gained regarding these parameters will also facilitate any future investigations with this or other similar species new to aquaculture.

For the purpose of addressing these questions, experiments combining a nutritional, physiological and molecular approach were performed during the development of the marine species meagre, *A. regius*. These have been presented and prepared as scientific manuscripts and presented in this format in the following chapters:

Chapter 2: **Early weaning in meagre *Argyrosomus regius*: Effect on growth, survival, digestion and skeletal deformities.**

This study was designed to establish a weaning protocol for meagre to reduce production losses due to cannibalism, study the changes in digestive enzyme activity when live preys are replaced by dry feed and describe possible skeletal deformities derived from early weaning onto artificial diets.

Chapter 3: **The effect of live food enrichment with docosahexaenoic acid (22:6n-3) rich emulsions on growth, survival and fatty acid composition of meagre (*Argyrosomus regius*) larvae.**

The objectives of this chapter of the thesis were to examine the effect on growth, survival and fatty acid composition of the meagre larvae after feeding live prey enriched with different emulsions, based on specialist oils with different DHA content and DHA/EPA ratio, and to check the ability of meagre larvae to elongate or desaturate fatty acids from precursors using an enrichment emulsion formulated with hemp oil.

Chapter 4: **Morphological and functional description of the development of the digestive system in meagre (*Argyrosomus regius*): An integrative approach.**

The objective of this study was to observe the changes in the organization and functionally of the digestive system from hatching to the juvenile stage and evaluate whether the acquisition of digestive capacities matched the morphological development of digestive organs.

Chapter 5: **Ontogeny of lymphoid organs and mucosal associated lymphoid tissues in meagre (*Argyrosomus regius*).**

The aim of this study was to describe the ontogeny of the thymus, kidney, spleen and mucosa-associated lymphoid tissue (gut and gill) from newly hatched larvae, and during larval development until juvenile stage in meagre.

Chapter 6: **Ontogeny and modulation after PAMPs stimulation, of β-defensin, hepcidin, and piscidin antimicrobial peptides in meagre (*Argyrosomus regius*).**

This study characterized the expression of the antimicrobial peptide genes for beta defensin (*defb*), hepcidin (*hep*) and piscidin (*pisc*) in meagre and describes the fluctuation in the expression patterns during larval growout to evaluate when in development these peptides appear. In addition, we have examined how their expression is modulated by pathogen-associated molecular patterns (PAMPs), both in vivo and in vitro as a proxy for actual infections [poly (I:C) as a mimic of virus; LPS as a mimic of bacteria, and beta glucan as a mimic of fungal pathogens] to understand how different pathogens potentially affect the levels of expression.

Chapter 7: **Ontogeny of the innate immune system in meagre (*Argyrosomus regius*) during early rearing.**

This chapter describes an experiment performed to determine and quantify ontogenic changes in innate immune-related gene expression during larval to juvenile transition of development in meagre.

CHAPTER 2

Early weaning in meagre *Argyrosomus regius*: Effects on growth, survival, digestion and skeletal deformities

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Early weaning in meagre *Argyrosomus regius*: Effects on growth, survival, digestion and skeletal deformities

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Abstract

Meagre *Argyrosomus regius* is considered a new species for the diversification of finfish aquaculture in the Mediterranean. Several bottlenecks have been identified by producers, and among them, the necessity to establish early weaning protocols to reduce production costs. In this study, two experiments were carried out with meagre larvae from 2 to 35 days post hatch (dph) using different weaning strategies, including the early introduction of artificial diets and the reduction of *Artemia* metanauplii to half of the normal amounts. A high frequency of cannibalism and high variability in growth rate and survival were obtained in one of the trials and several changes were introduced (reduction of light intensity, higher frequency of food distribution) in the second trial to increase the survival rate. In both trials, weaning started before the complete morphological and functional development of the stomach; thus, pancreatic enzymes, mainly trypsin and lipase tended to be more active in early weaned larvae compared to the control groups. Early weaning delayed the development of the stomach formation and secretion of acid proteases, which may explain the lower growth rates observed in our study. The effect of weaning on skeletal development was also studied and in this sense the results obtained showed no major influence of the early weaning on the incidence of skeletal deformities. Weaning of meagre larvae can be performed as early as 12 dph, but important aspects such as avoiding cannibalism and co-feeding live prey and artificial diets for at least 5 days were recommended.

KEYWORDS
cannibalism, digestive enzymes, growth, meagre, skeletal deformities, weaning

1 | INTRODUCTION

Larval rearing of meagre *Argyrosomus regius* is usually carried out following a protocol based in European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) larval rearing (Estévez, Treviño & Gisbert, 2007; Roo, Hernández-Cruz, Borrero, Fernández-Palacios & Schuchardt, 2007; Roo, Hernández-Cruz, Borrero, Schuchardt & Fernandez-Palacios, 2010), including the use of enriched live feeds (rotifers, *Brachionus* sp. and *Artemia* sp. metanauplii). However, larviculture practices based on live preys represent a high cost compared to inert diets, both in terms of production and labour costs. Different studies have revealed that these protocols need to be adapted to the biological demands of this species, as meagre larvae are quite sensitive to stress produced by high light intensity (>500 lux at water surface), long photoperiods or high densities of live prey (Roo et al., 2010; Suzer, Kamaci, Coban, Firat & Saka, 2013; Vallés & Estevez, 2013). Although the precise nutritional requirements for meagre have not been completely established, larvae show very good growth performance and survival rates using commercially available products for live prey enrichment and feeds

for weaning (Vallés & Estevez, 2015). Meagre producers do not consider larval rearing to be a major bottleneck for meagre culture (Lazo, Holt, Fauvel, Suquet & Quemener, 2010), and only cannibalism and variable size distribution in juveniles are considered the main concern in the intensive production of meagre fry, as they reduce production yield and increase the production costs. Therefore, advancing the early weaning of larvae from its dependence on *Artemia* onto a dry feed is a priority and the major focus of the current larval research on meagre. In this sense, a better knowledge of larval digestive physiology under a new feeding protocol may contribute to the optimization of diets (Zambonino-Infante et al., 2008) and may help to understand functions and limitations in the processing capacity of the digestive system, and consequently the delivery of nutrients to the rapidly growing larval tissues under an earlier weaning protocol (Rønnestad et al., 2013). Thus, establishing an adequate feeding protocol adapted to the digestive capacities and nutritional needs during early development while also addressing options to reduce cannibalism and size dispersion are of primary importance to improve survival and growth in meagre.

Skeletal deformities in cultured fish are a major factor that reduces production, suppresses growth and increases economic loss, as well as leads to high mortality rates. Most skeletal abnormalities appear during the larval and juvenile stages where several factors can interfere with the normal development of larvae. Existing literature clearly suggests that unfavourable abiotic conditions, inappropriate nutrition and genetic factors are the most possible causative factors of skeletal anomalies in reared fish (Boglione et al., 2013). In this regard, nutritional imbalances are known to play a key role in morphogenesis and skeletogenesis at early stages (Boglione et al., 2013; Person Le Ruyet, Alexandre, Thebaud & Mugnier, 1993). Thus, the efficiency of early weaning practices and its effects on skeletal development in meagre larvae needs also to be considered.

Thus, the objectives of the present study were (1) establish a weaning protocol for meagre to reduce production losses due to cannibalism, (2) study the changes in digestive enzyme activity when live preys are replaced by dry feed and (3) describe possible skeletal deformities derived from early weaning onto artificial diets.

2 | MATERIAL AND METHODS

2.1 | Larval rearing and experimental design

Fertilized eggs of meagre were obtained from a wild broodstock maintained in 4,000 l circular tanks connected to recirculation units (IRTAMar®, IRTA, Spain) at IRTA Centre of San Carles de la Rapita under controlled conditions and after hormonal induction (Duncan et al., 2012). Floating eggs were incubated in 35 L cylindrical PVC containers provided with air-lift systems and high aeration supply. On day 2 post hatching (dph), larvae were stocked into 100 L tanks at a density of 100 larvae/L and cultured from 2 to 35 dph on different dietary treatments. The 100 L tanks were connected to IRTAmar® units with 50% daily water renewal. Temperature (18.2 ± 0.5°C), salinity (35.4 ± 0.3 g/L), dissolved oxygen (7.9 ± 0.3 mg/L) and pH (7.9 ± 0.2) were checked daily, whereas nitrites (<0.25 mg/L) and ammonia (<0.07 mg/L) were measured once per week (Hach Colorimeter DR/890, USA). Light intensity was regulated with a manual potentiometer connected to each fluorescent lamp (Philips LPS100) and measured at the water surface in the middle of the tank with a luxometer (Lutron LX-101 LUX METER) and maintained at 500 lux at water surface, whereas the light regime was 12-hr light: 12-hr dark.

Larvae were fed enriched rotifers (*Brachionus sp*) from 2 dph until 14 dph at a density of 10 rotifers/ml and *Artemia* metanauplii (Sep Art Artemia, Inve, Belgium) from 9 dph starting with 0.5 metanauplii/ml, increasing the density up to 6 metanauplii/ml at 20 dph and decreasing the density down to 1.5 nauplii/ml at 25 dph, and keeping that density until the end of the trial. Both live preys were enriched using Red Pepper™ (Bernaqua, Belgium) for 12 hr at 28°C in the case of rotifers and 6 h at 25°C in the case of *Artemia*. Larvae were fed two doses of live prey (morning and evening) every day, whereas dry feed (Gemma Micro, Skretting, Norway) was administered by hand every morning at 9:00 hours and using automatic feeders every hour, from 9:00 to 20:00 hours. The amount of feed was adjusted to reach the level of apparent feeding satiation. Every day, the bottom of the tank was siphoned to remove dead fish, uneaten food and faeces.

Two experiments were carried out with meagre larvae. In Trial 1, the following experimental protocols were tested in triplicate:

Group A: weaning on dry feed started from 20 dph and it was completed at 30 dph (control group) following the standard protocol described above; Group B: weaning started from 20 dph and it was completed at 30 dph (the same as in Group A but using half the amount of *Artemia* metanauplii); Group C: weaning started from 15 dph and it ended at 25 dph using also half the amount of *Artemia* metanauplii; and Group D: weaning started at 12 dph and it was completed at 23 dph using half the amount of *Artemia* metanauplii.

Due to the high incidence of cannibalism (so-called coeval cannibalism; Folkvord, 1997) among similar-aged individuals observed in Trial 1, several changes in the rearing protocol were introduced in Trial 2. Thus, light intensity was reduced from 500 lux to 150–200 lux from 13 dph onwards, as well as the number of doses/meals of *Artemia* (given at 10:00, 13:00, 16:00 and 18:00 hours) and the artificial diet that were increased in order to provide enough food to larvae. This strategy was chosen according to Smith and Reay (1991) who reported that cannibalism is enhanced by low food availability, high fish densities, size disparity and lack of refuges. Thus, in Trial 2, only two treatments were tested (five replicates each): Group E: weaning started from 20 dph and it was completed at 30 dph (control group); and Group F: weaning started at 12 dph and it ended at 23 dph.

In both trials, 10 larvae from each tank were randomly collected to measure growth (standard length, SL and dry weight, DW) every week. Fish were previously anaesthetized with tricaine methanosulphonate (MS-222, Sigma-Aldrich, Spain) and SL measured under a stereomicroscope Nikon SMZ800 (Nikon, Tokyo, Japan) equipped with a digital camera Olympus DP70 (Olympus, Hamburg, Germany)

and an image analyser (AnalySIS, SIS Gmbh, Hamburg, Germany). The same larvae were used to estimate wet and dry weight, placing them, previously washed with distilled water, on preweighted coverslips, dried in an oven at 60°C for 24 hr, and weighted in a microbalance Mettler MX5 (Mettler Toledo, Barcelona, Spain).

Specific growth rate (SGR) was calculated using the following formula: SGR (%day^{-1}) = Ln DW_f − Ln $DW_i/t_f - t_i$; where DW_f and DW_i were the final and initial larval dry weight ant $t_f - t_i$ the days from the initial until the end of the sampling periods.

The coefficient of variation (CV, %) of weight was calculated according to the formula CV = standard deviation/mean × 100.

Larvae were randomly collected when the weaning started and the feed was changed from live to inert diets that was at 12 dph (Group D), 15 dph (Group C), 20 dph (Groups A and B) and at 24 dph in Trial 1 and at the end of Trial 2 (35 dph), to analyse the activity of digestive enzymes. In this case, sampled larvae were sacrificed with overdose of MS-222, rinsed in distilled water and conserved at −80°C until analysis. At the end of the trial, survival was evaluated by counting the survivors at the end of the experiment and calculated according to Buckley et al. (1984) that consider the number of sampled individuals during the experiment.

2.2 | Determination of digestive enzyme activities

Larvae in both Trials 1 and 2 (5–150 individuals depending on age and size) were collected for enzyme analyses at 12, 15, 20 and 24 dph in Trial 1 and at 35 dph in Trial 2. For quantifying the activity of pancreatic and gastric enzymes (total alkaline proteases, α-amylase, lipase and pepsin), samples were homogenized (Ultra-Turrax T25 basic, IKA©-Werke, Germany) in 5 volumes (v/w) of ice-cold Milli-Q water, centrifuged at 3,300 g for 3 min at 4°C, the supernatant removed for enzyme quantification and kept at −80°C until further analysis. For the quantification of the intestinal brush border (BB) enzyme, alkaline phosphatase samples were homogenized in cold 50 mM mannitol, 2 mM Tris-HCl buffer (pH 7.0) and intestinal BB membranes purified according to Crane et al. (1979).

Enzyme activities of pancreatic, gastric and intestinal enzymes were conducted as described in Gisbert, Giménez, Fernández, Kotzamanis and Estévez (2009) and Solovyev et al. (2016). In addition, spectrophotometric analyses were performed as recommended by Solovyev and Gisbert (2016) in order to prevent sample deterioration due to their short- and long-term storage. In brief, trypsin (E.C. 3.4.21.4) activity was assayed at 25°C using BAPNA (N-α-benzoyl-DL-arginine p-nitroanilide) as substrate. One unit of trypsin per ml (U) was defined as 1 μmol BAPNA hydrolysed per min per ml of enzyme extract at 407 nm (Holm, Hanssen, Krogdahl & Florholmen, 1988). Alpha-amylase (E.C. 3.2.1.1) activity was determined according to Métais and Bieth (1968), using 0.3% soluble starch as substrate, and its activity (U) was defined as the mg of starch hydrolysed during 30 min and ml of tissue homogenate at 37°C at 580 nm. Bile salt-activated lipase (BALT, E.C. 3.1.1) activity was assayed for 30 min at 30°C using p-nitrophenyl myristate as substrate. The reaction was stopped with a mixture of acetone: n-heptane (5:2), the extract centrifuged for 2 min at 6,080 *g* and 4°C and the absorbance of the supernatant read at 405 nm. Bile salt-activated lipase activity (U/ml) was defined as the nmol of substrate hydrolysed per min per ml of enzyme extract (Iijima, Tanaka & Ota, 1998). Regarding intestinal enzymes, alkaline phosphatase (E.C. 3.1.3.1) was quantified at 37°C using 4-nitrophenyl phosphate (PNPP) as substrate. One unit (U) was defined as 1 μg BTEE released per min per ml of brush border homogenate at 407 nm (Bessey, Lowry & Brock, 1946). Finally, pepsin activity (U) was defined as the μmol of tyrosine liberated per min at 37°C per ml of tissue homogenate at 280 nm (Worthington Biochemical Corporation, 1991). All enzymatic activities were expressed as specific activity defined as milliunits per milligram of protein (mU/mg protein). Soluble protein of crude enzyme extracts was quantified by means of the Bradford's method (Bradford, 1976) using bovine serum albumin as standard. All the assays were made in triplicate from each pool of larvae and absorbance read using a spectrophotometer (Tecan™ Infinite M200, Mannedorf, Switzerland).

2.3 | Analysis of skeletal deformities

To evaluate the impact of different weaning strategies on the incidence of skeletal deformities in meagre, 20 early juveniles aged 35 dph per tank were randomly sampled at the end of Trial 2. Fish were preserved in 10% buffered formalin and stored until double staining. Animals were stained with Alcian blue 8X and alizarin red (Sigma-Aldrich, Barcelona, Spain) to detect cartilaginous and bony tissues as described in Darias, Lan Chow Wing, Cahu, Zambonino-Infante and Mazurais (2010). Once stained, fish were individually examined under a dissection microscope by two independent observers. The incidence of skeletal abnormalities was determined in the cranium, vertebral column and caudal fin complex. Special attention was given to vertebral deformities, which were divided in two categories: "severe," which included the fusion and compression of adjacent vertebral bodies, deformation of vertebral bodies and changes in the anterior–posterior alignment of vertebrae (kyphosis and lordosis), and "light," including deformed haemal spines and neural spines, and changes in the osteological organization of the caudal fin complex. The nomenclature of skeletal elements was conducted according to the description of meagre skeletogenesis (Cardeira et al., 2012).

2.4 | Statistical analyses

Data were expressed as mean ± standard deviation (SD) except for skeletal anomalies that were expressed in mean ± standard error of the mean (SEM) and tested by Student's t test (Trial 2, with only two treatments) or one-way ANOVA (Trial 1, with four treatments). When a significant difference was found between treatments, a Tukey's test was performed for multiple range comparisons with the level of significant difference set at $p < 0.05$. All the data were tested for normality, homogeneity and independence to satisfy the

assumptions of ANOVA, whereas data expressed as percentage were previously arcsine-transformed.

3 | RESULTS

3.1 | Growth and survival

The results in terms of larval growth in SL and DW from Trial 1 are shown in Figure 1. Growth in SL and DW of meagre aged 35 dph from Groups C, weaned at 15 dph with half the amount of *Artemia* metanauplii, and A, control, was higher than in the rest of the other groups ($p < 0.005$), whereas fish from Group B showed the lowest values in SL and DW among groups (data not shown). Specific growth rates of the different groups were 25.74% day^{-1} for Group A and 21.86%, 24.79% and 23.39% for Groups B, C and D respectively. Survival rates were significantly affected by the weaning strategy ($p < 0.05$), being higher in fish from the Group B (2.8 ± 0.6%), whereas meagre from Groups A, C and D showed similar and lower values (1.7 ± 0.1%, 1.2 ± 0.3% and 1.8 ± 0.3% respectively).

Large differences in size were detected among experimental groups at the end of the Trial 1, as indicated by the values of the coefficient of variation (CV) for SL (Figure 2). In particular, fish showing the largest size dispersion values were those from Group D (49.2 ± 20.2%), whereas fish from Groups A, B and C showed similar CV values (23.2 ± 3.4%, 24.1 ± 5.3% and 23.8 ± 8.1% respectively). Differences in DW among small, medium and large larvae are also shown in Table 1, with larvae weighing from 1.28 to 38.28 mg of dry weight depending on the group, such wide range of sizes might have enhanced larval cannibalism.

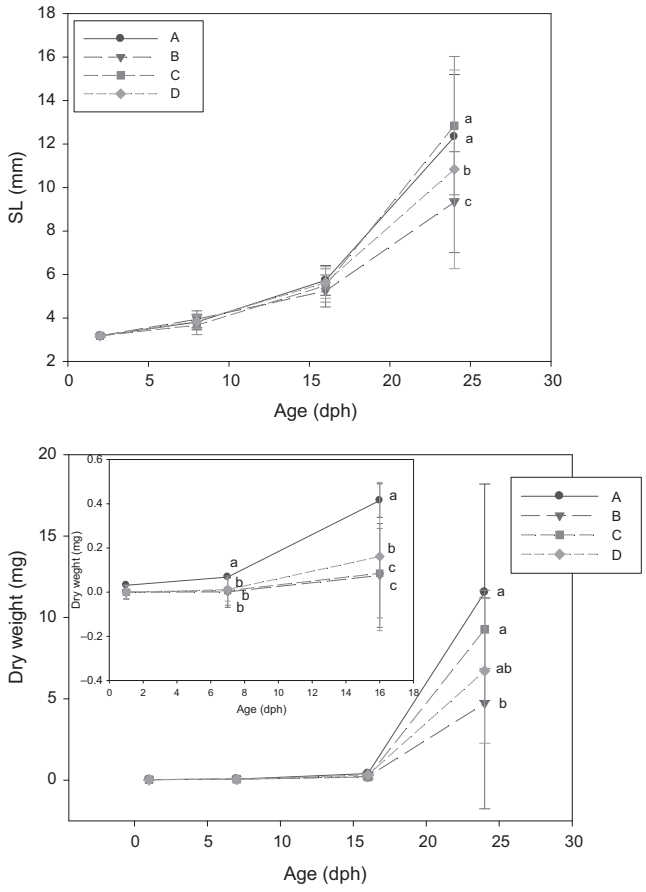

FIGURE 1 Growth in standard length (SL) (mm, mean ± SD) and dry weight (DW) (mg, mean ± SD) of meagre (*Argyrosomus regius*) larvae from the different groups at different sampling times in Trial 1. Different letters show significant differences (ANOVA, $p < 0.001$)

The results in larval growth in SL and DW from Trial 2 are shown in Figure 3a. Growth performance and survival rates were higher in meagre from the Group E, weaned at 20 dph and fed using the standard protocol, than those obtained in Group F (larvae weaned at 12 dph) ($p < 0.05$). Specific growth rates of larvae between 1 and 30 dph were 18.95%/day for the control group and 17.06%/day for early weaned larvae, values that were almost 1.5 times lower than those observed in Trial 1. In this case, the size between the groups was similar, with a CV value of 0.12 and 0.13 for groups E and F respectively (Figure 3b). Survival rate from Group E (4.9 ± 0.7%) was also higher than in the early weaned Group F (3.9 ± 0.5%), similarly to the results obtained in Trial 1. Measures adopted in Trial 2 to reduce cannibalistic behaviour (reduction of light intensity and higher feeding frequency) had positive results lowering the size differences among the larvae and improving the survival rate at the end of the trial.

3.2 | Activity of digestive enzymes

In both trials, digestive enzyme activities analysed in larvae at the end of live prey feeding period were the same for all the treatments (data not shown). The activity of the pancreatic and intestinal digestive enzymes was assessed in 24 dph larvae in the case of Trial 1 and 35 dph larvae for Trial 2; the results are shown in Figure 4. In Trial 1, trypsin activity was higher in meagre from Group C in comparison with larvae from Groups A and B, whereas fish from Group D showed intermediate values in trypsin activity ($p < 0.05$). Similarly, lipase activity was higher in larvae from Group B in comparison with the rest of the treatments ($p < 0.05$), whereas no statistically significant differences were found with regard to α-amylase activity between groups ($p > 0.05$). The activity of the brush border enzyme, alkaline phosphatase, was highest in fish from Group A, whereas the lowest values were observed in meagre from Group D ($p < 0.05$), and Groups B and C showed intermediate values. Similarly to Trial 1, there were no differences neither in α-amylase activity nor in trypsin regardless of the weaning strategy used in Trial 2 ($p > 0.05$), whereas lipase activity was higher in early weaned larvae (Group F, $p < 0.05$). The activity of alkaline phosphatase was higher in larvae from the control Group E than in Group F ($p < 0.05$). Regarding the activity of acid proteases, pepsin was only analysed in larvae from Trial 2; in particular, early weaned larvae (12 dph) showed a significantly lower activity of this acid protease (66.97 ± 20.03 U mg protein^{-1}) compared with larvae weaned at 20 dph (91.83 ± 10.76 U mg protein^{-1}) ($p < 0.05$).

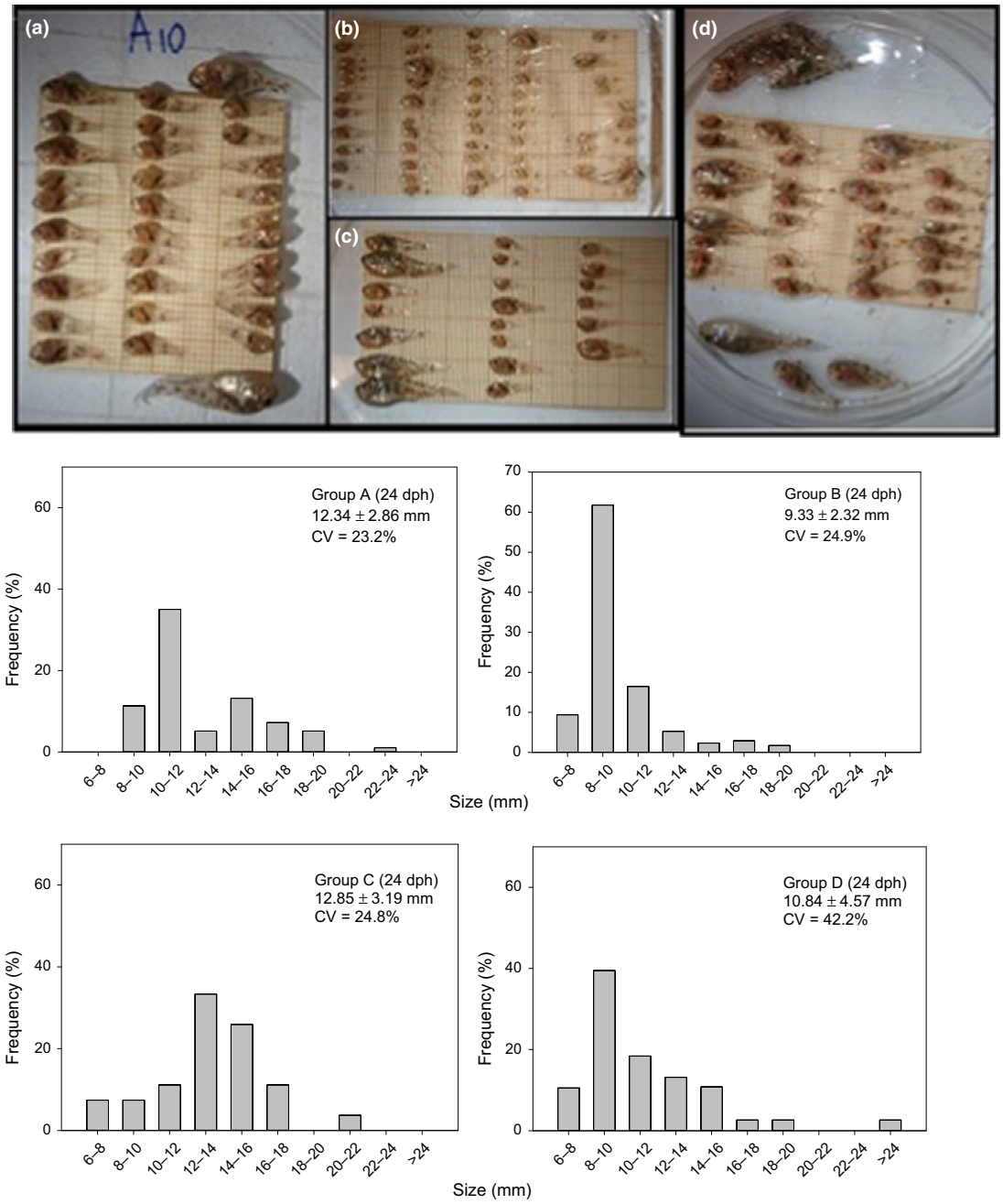

FIGURE 2 Photographs and frequency diagrams showing the differences in the size (SL) of the larvae (24 dph) of the experiment 1 in the groups a (left), b (centre, up), c (centre, down) and d (right), due to the high incidence of cannibalism. CV, coefficient of variation [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Differences in dry weight (mg, average ± SD) among small, medium and large larvae in the four feeding groups (A, B, C and D)

Group	Small	Medium	Large
A	4.52 ± 0.97	16.06 ± 9.58	38.28 ± 8.41
B	1.28 ± 0.16	8.00 ± 7.69	18.67 ± 9.75
C	3.73 ± 1.55	12.27 ± 3.79	23.17 ± 5.11
D	3.01 ± 1.29	8.91 ± 3.89	26.41 ± 2.77

3.3 | Skeletal deformities

In Trial 2, early weaning of the larvae did not have any effect on the incidence of total skeletal deformities in early juveniles of meagre ($p > 0.05$) with an average frequency of deformed fish ranging from 17.6% to 21.0% nor in the number of vertebral bodies (98.7% specimens with 25 vertebrae and 1.3% with 24). In addition, no differences were found in the incidence of light and severe skeletal deformities among groups ($p < .05$), being the light one the most

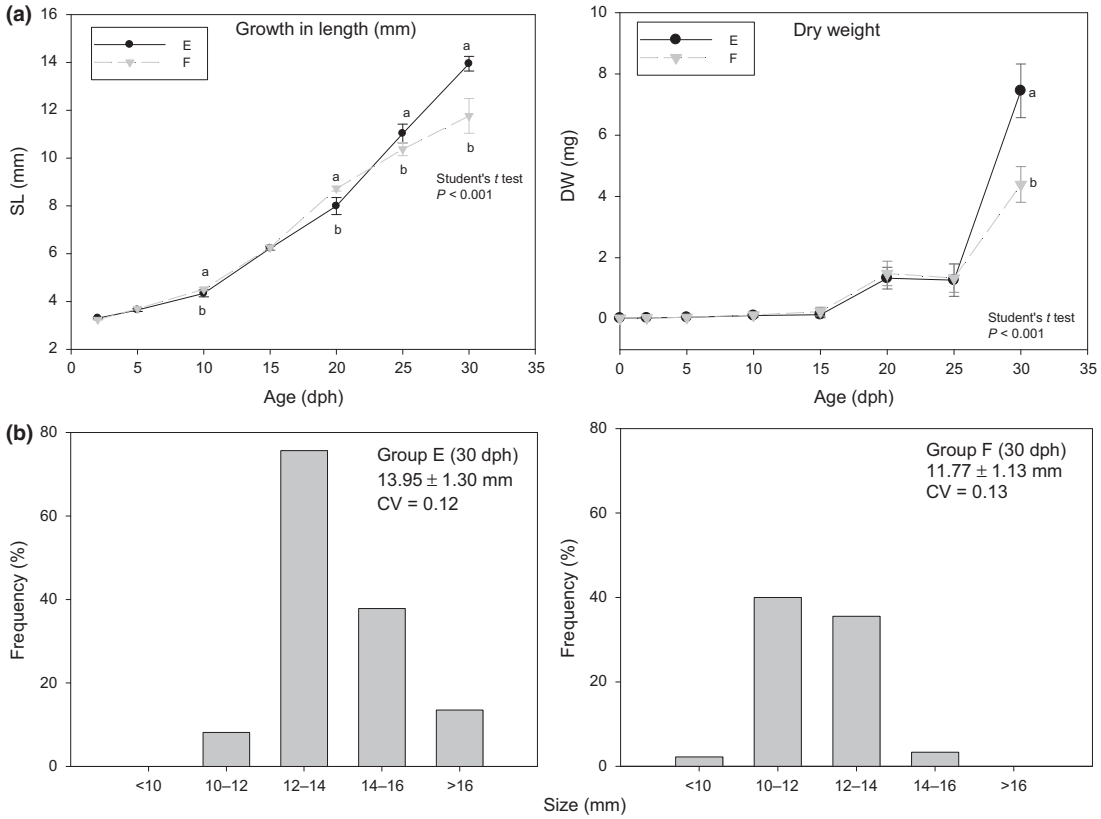

FIGURE 3 (a) Growth in standard length (SL) (mm, mean±SD) and dry weight (DW) (mg, mean± SD) of Trial 2 larvae, weaned at 20 dph (group E) and 12 dph (group F). Letters indicate significant differences (Student's *t* test, $p < 0.001$). (b) Frequency diagrams showing the differences in the size (SL) of the larvae (30 dph) in the groups E (left) and F (right) of Trial 2. CV, coefficient of variation

common among examined fish. In any of the groups examined, cranial deformities were observed, whereas most of skeletal abnormalities were detected in the vertebral column and caudal fin complex, particularly fusions between 20 to 24 haemal vertebrae, deformation of epurals 1 to 2 and in the last two haemal vertebrae before the urostile (Figure 5).

No significant differences in the frequency of skeletal abnormalities affecting the vertebral column (prehaemal and haemal regions) were observed, although when examining each type of vertebral deformity, the incidence of vertebral fusion in the haemal region was different between the two groups of Trial 2. Figure 5 shows some examples of the skeletal anomalies detected in both groups. Thus, most of the anomalies observed can be considered mild anomalies such as lordosis and scoliosis (0.4% for both groups). Fusion of haemal vertebrates was observed in 0.4 to 1.6% of the examined larvae in Group E and only in 0.4 to 0.8% in the larvae from Group F. Skeletal structures of the caudal fin complex were almost not affected showing some defects in ossification associated with the underdevelopment or absence of epurals (Group E = 3.6 ± 2.5% and Group F = 6.8 ± 2.2%).

The incidence of severe deformities (lordosis, kyphosis, scoliosis, deformed vertebral centra) was similar in both dietary treatments (4.25 ± 1.78% for group E and 4.65 ± 2.22% for group F), as well as in the case of light deformities (haemal spines and neural spines and modified epural) 12.38 ± 7.26% for Group E larvae and 16.80 ± 2.48% for Group F (Figure 6).

4 | DISCUSSION

Weaning, the transfer from live food to an artificial diet, is successful with most marine fish with a completely developed digestive tract (Person Le Ruyet et al., 1993). In the current study, weaning was carried out with a commercial diet (Gemma Micro, Skretting, Norway) using a gradual transfer of live prey to this artificial diet over a minimum of five days, although in some other marine species like European sea bass, there is an abrupt replacement (Person-Le Ruyet, 1990). Durán et al. (2009) using a weaning protocol for meagre, similar to the one used in Trial 2, obtained similar results in growth performance and survival rates than those obtained in the present study. Thus, early weaning can be carried out with meagre larvae if several measures to reduce cannibalism are in place.

In natural environments, cannibalism is regarded as an alternative feeding strategy, more likely to be adopted by larvae and early juveniles which are carnivorous, when resources become limiting (Hecht & Pienaar, 1993) or when the population is too crowded (Babbitt &

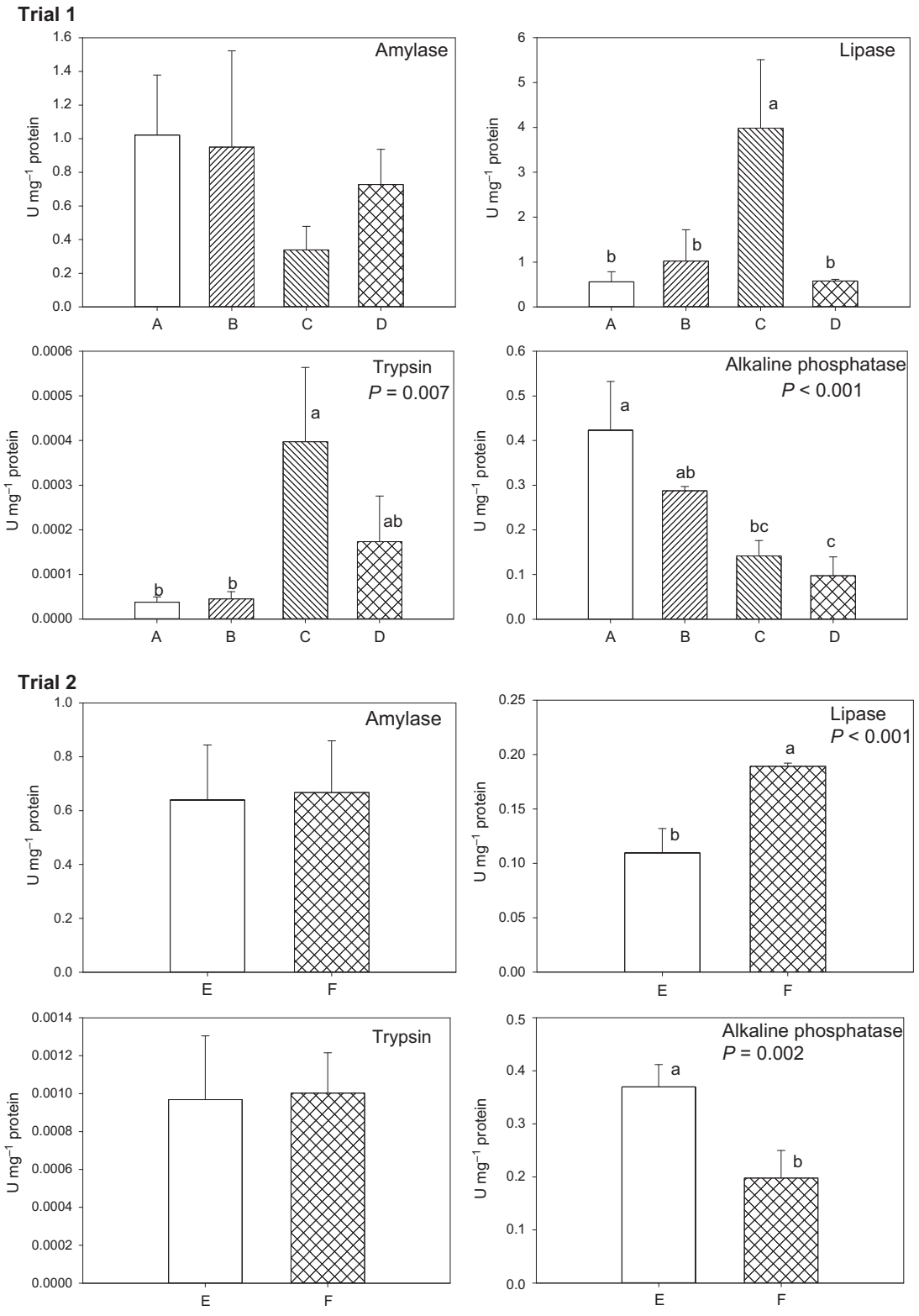

FIGURE 4 Results of digestive enzyme activity (mean± SD) measured in the larvae from Trials 1 and 2 at the end of the experiments. Letters indicate significant differences (ANOVA, data from Trial 1 and Student's *t* test, data from Trial 2, $p < 0.05$)

Meshaka, 2000). It is a major problem in the culture of many marine fish larvae because, being a size-selective form of predation, which has consequences on both the abundance and size structure of the population. Size heterogeneity is the primary cause of cannibalism in larval fish (Katavic, Jug-Dujakovic & Glamuzina, 1989), although other factors such as food availability, larval density, feeding frequency, light intensity, water clarity and shelter have been also identified (see review by Hecht & Pienaar, 1993). In particular,

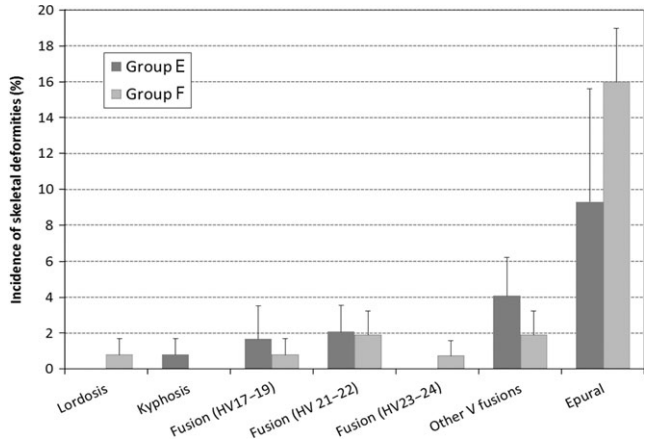

FIGURE 5 Different typologies of skeletal deformities (%) found in 37 dph meagre larvae in Trial 2 (E, control group; F, early weaned larvae), considering the number of abnormal skeletal elements per fish (mean and SEM in brackets). HV, haemal vertebrae; EP, epural

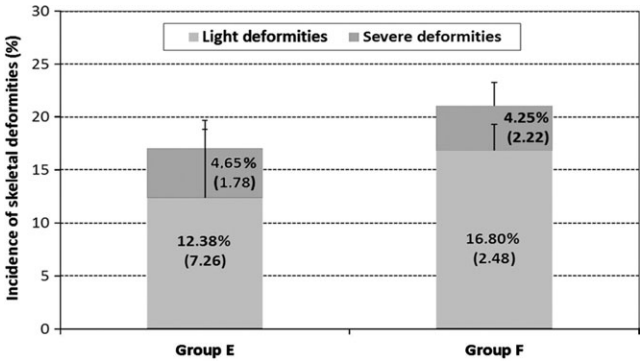

FIGURE 6 Skeletal deformities (in %, mean and SEM in brackets) in meagre from the control group (E) and early weaned (F), considering the degree of the abnormalities among treatments

Kestemont et al. (2003) considered size heterogeneity as the main cause for this behaviour, as the smallest fish are consumed by the largest ones, and considered the period of weaning in carnivorous species as one of the most important during larval rearing because the transition from live to dry diet is generally size dependent and fish that are slightly larger than others may gain a definite advantage while having access to more energetic diets. Depending on species behaviour and morphology, the resulting size advantage may result in more intense and frequent agonistic interactions in the context of dominance hierarchies, or in cannibalism. Cannibalism occurs in other cultured marine fish such as cod *Gadus morhua* (Puvanendran, Laurel & Brown, 2008), yellowtail *Seriola quinqueradiata* (Sakakura & Tsukamoto, 1996) or dusky kob *A. japonicus* (Timmer & Magellan, 2011), and authors attributed the aggressions to the high size variation within the cohorts with frequencies of cannibalistic behaviour increasing as the size differences between prey and predator increased. Density-dependent cannibalism has also been observed (Otterlei, Folkvord & Moller, 1994), being very rare when fish are cultured in extensive pond systems or mesocosm (N. Papandroulakis, pers. com.). In the present study, high cannibalistic behaviour in Trial 1 resulted in a high reduction in survival rates and high dispersion in fish size especially from 16 dph onwards when aggressions, fin napping and attacks from cannibal larvae were more frequent, which was completely unsuitable for commercial rearing purposes. Subsequently, several measures were adopted in Trial 2 to avoid this behaviour, such as increasing the feeding frequency and keeping the larvae in low light (150–200 lux) when food was unavailable or in short supply. The modification of feeding practices and the use of low light intensity before feeding in the morning increased the survival and reduced the size differences among the larvae of Trial 2 by reducing larval cannibalism.

Previous larval rearing studies carried out with meagre (Papadakis, Kentouri, Divanach & Mylonas, 2013; Roo et al., 2010; Suzer et al., 2013) reported higher survival rates (around 50% at 40 dph) and lower growth rates (SGR = 10.55%/day, Suzer et al., 2013) than those obtained in the present study. Present results in terms of growth performance and survival were similar to those recently reported by Solovyev et al. (2016), although the former authors had an initial rearing density of 200 larvae/L. Cannibalism was observed by these authors from 20 dph, but it was not considered as an important issue and other factors related to larval stress and hyperinflation of the swim bladder were considered as the main causes of mortality. Pastor et al. (2013) obtained similar results in growth rate and high incidence of cannibalism from 15 dph using a larval rearing density of 50 larvae/L; although the authors did not provide survival results, they considered size differences and larval density the main cause for this behaviour. Similarly, in the larval rearing protocols (Ballagh, Fielder & Pankhurst, 2010; Fielder & Heasman, 2011) developed for the mulloway (*A. japonicus*), weaning starts when the larvae are 10.5 mm length (approx. 20–22 dph), achieving higher survival rates (40%) although using low larval densities (20 larvae/L).

The weaning success of any finfish larvae from live feeds onto a formulated diet is partly dependant on the composition of the diet and the ability of larvae to digest it. Thus, stomach development and the production of gastric digestive enzymes (acid digestion) are generally regarded as the key indicators for the transition from live feeds to microdiets (Cahu & Zambonino Infante, 2001; Rønnestad et al., 2013; Watanabe & Kiron, 1994). In the present study, weaning was started before the complete morphological and functional development of the stomach (Solovyev et al., 2016); thus, protein digestion at early weaning stages was mainly based on alkaline proteolytic enzymes produced by the pancreas as it has been reported for European sea bass (Cahu & Zambonino Infante, 2001). Under current experimental conditions, pancreatic enzymes tended to be more active in the early weaned larvae, with a significantly higher lipase activity compared to the control groups (Groups A and E). Among different pancreatic digestive enzymes, proteolytic enzymes (alkaline proteases) are

regarded as being particularly significant in the early life stages of fish because of the absence of a functional stomach with its acid protease, pepsin (Rønnestad et al., 2013), as it has been recently demonstrated in meagre by means of histological and biochemical procedures (Solovyev et al., 2016). Lipase plays an active role in lipid digestion, especially in the breakdown of triacylglycerol to diacylglycerol and then to monoacylglycerol (Zambonino Infante & Cahu, 2001). In many fish species, including meagre, lipase is active during resorption of the oil globule and the complete transition to exogenous feeding, being relevant for the digestion of high levels of triacylglycerols present in the enriched live prey, as it was shown by Solovyev et al. (2016). On the contrary, the capacity to digest proteins by means of acid digestion (pepsin activity) was significantly lower in the early weaned larvae in Trial 2, also coinciding with the significantly lower growth rate achieved by this group (see results section and Figures 4 and 5). On the other hand, pepsin activity becomes apparent concurrently with formation of functional stomach. According to different authors, the stomach and gastric activity becomes functional in meagre between 15 dph (5.1–5.4 mm in SL; Suzer et al., 2013), 20 dph (6.6 mm in SL; Papadakis et al., 2013) and even at 31 dph (6.0–6.8 mm SL; Solovyev et al., 2016), which confirmed that the functional development of the digestive system in this species is a well-conserved process that generally occurs within a range of body size (notochord flexion) regardless of larval age and rearing conditions (Solovyev et al., 2016). The results of the present study indicated that 12 dph (weaning ages used in both trials) was probably a bit too early for weaning, especially having into account the reported differences in larval growth at the end of the weaning period. The weak ability of early weaned larvae for acid proteolytic cleavage of proteins from microdiets could be one of the reasons for the lower larval growth achieved. In a similar study with shi drum (*Umbrina cirrosa*), Papadakis et al. (2009) also observed lower growth in early weaned larvae and detected a short period of starvation during the adaptation to the artificial feed, without any influence on the timing of the appearance of the various components of the digestive system, although the differentiation or maturation of some organs might be delayed when inappropriate feeding protocols or diets are evaluated at early life stages of development (Gisbert, Ortiz-Delgado & Sarasquete, 2008).

Several studies have shown that nutrients are responsible for the appearance of skeletal deformities when their levels in the diet are inappropriate or unbalanced (Afonso et al., 2000; Boglione et al., 2013; Cahu, Zambonino Infante & Takeuchi, 2003; Lall & Lewis-McCrea, 2007). During early larval development as well as during weaning, a change to an inappropriate diet, or in the hydrodynamic conditions of the rearing tank, might cause problems in the skeleton. In the present study, malformation rate of the larvae was not affected by the feeding regime, which may indicate that the weaning protocol used, co-feeding for several days enriched Artemia metanauplii with commercial microdiets, can supply adequate nutrients for larvae without compromising the harmonious development of the skeleton, without affecting larval quality. As similar typology of deformities was observed in both treatments (larvae weaned at 20 dph or at 12 dph), we cannot discard other factors such as the rearing conditions (Sfakianakis, Koumoundouros, Divanach & Kentouri, 2004), genetic background (Afonso et al., 2000) or broodstock nutrition (Boglione et al., 2013; Cahu et al., 2003) as the main causative agents for such skeletal disorders.

5 | CONCLUSIONS

Based on these results, meagre larvae can be weaned from live feed to artificial diets at as early as 12 dph, but other important aspects for production success including larval performance and survival should be considered. Special care should be taken to avoid cannibalistic behaviour in the rearing tanks, either by reducing the light intensity at the water surface and increasing larval feeding rate and daily doses. Early weaning did not affect the incidence of skeletal deformities in meagre, which is of special relevance in terms of assuring fry quality for further on-growing purposes.

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CHAPTER 3

The effect of live food enrichment with Docosahexaenoic acid (22:6n-3) rich emulsions on growth, survival and fatty acid composition of meagre (*Argyrosomus regius*) larvae.

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The effect of live food enrichment with docosahexaenoic acid (22:6n-3) rich emulsions on growth, survival and fatty acid composition of meagre (*Argyrosomus regius*) larvae

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ABSTRACT

While spawning induction and larval rearing of meagre (*Argyrosomus regius*) have advanced as forcing factors to move this finfish species into the commercial aquaculture sector, larval nutrition still has unanswered issues to address, specifically in regard to live prey enrichment and fatty acid composition. In this study, two experimental trials for larval rearing of meagre utilizing different methods of live prey enrichment with emulsions of different specialist oils having distinct fatty acid composition, have demonstrated that docosahexaenoic acid (DHA) requirements may be species-specific, with a DHA supplement of 12–15% in live prey enrichment diets yielding optimum larval growth. Cannabilism in early life stages (post 20 dph) in this species also remains a challenge and requires stocks to be managed accordingly. Further, we found evidence suggesting meagre larvae are not capable of elongation or desaturation of fatty acids when precursors such as LA, GLA, LNA or SDA are offered in the live prey.

1. Introduction

The understanding of nutrition and feeding during early development is a major prerequisite to counter the challenges of marine fish larvae culture. The success of larval rearing is greatly influenced by first feeding regimes and the nutritional quality of starter diets, with dietary lipids being recognized as one of the most important nutritional factors affecting larval growth and survival (Watanabe, 1993).

Marine lipids are rich in saturated and monounsaturated fatty acids, which are a vital source of metabolic energy for the rapidly developing and growing fish larvae. In addition, they supply polyunsaturated fatty acids (PUFA) which are considered essential fatty acids (EFA) for marine fish since they cannot be biosynthesized and hence must be provided in the diet. Three long chain PUFA (LC-PUFA), namely docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (ARA, 20:4n-6) have a variety of vital functions in fish species, as in most vertebrates, being the main components of membranes and precursors of bioactive metabolites such as eicosanoids (Tocher, 2010). DHA plays an important role during larval development as it is incorporated into nervous and retina tissue (Mourente and Tocher, 1992; Bell et al., 1996), and when absent from the larval diet, leads to poor growth and high mortality as well as to several behavioral, physiological and morphological alterations (Lingenfelser et al., 1995; Tocher, 2010).

Meagre, *Argyrosomus regius*, is a valuable new finfish species for European aquaculture desired for its rapid growth during ongrowing and the quality of its meat (Poli et al., 2003; Grigorakis et al., 2011). At present, spawning induction (Duncan et al., 2012), larval development (Jimenez et al., 2007; Cardeira et al., 2012) and larval rearing protocols (Roo et al., 2010; Vallés and Estevez, 2013; Campoverde et al., in press) have already been established. However, nutritional requirements of the larva, including LC-PUFA, have not been established yet, although some preliminary information, using commercial enrichment products or microdiets for larval feeding, already exist (Vallés and Estevez, 2015; El Kertaoui et al., 2017). According to Monroig et al. (2013) they express at least one fatty acyl desaturase (Fads2) and one elongase (Elovl5) involved in the endogenous production of LC-PUFA.

Hemp seed oil has been dubbed “Nature's most perfectly balanced oil” (Callaway, 2004), due to the fact that it contains the perfectly balanced 3:1 ratio of Omega 6 (18:2n-6, linoleic, LA) to Omega 3 (18:3n-3, α-linolenic, LNA) essential fatty acids, determined to be the optimum requirement for long-term healthy human nutrition. In addition, it also contains smaller amounts of three other PUFA such as γ-linolenic acid (18:3n-6, GLA), oleic acid (18:1n-9, OA) and stearidonic acid (18:4n-3, SDA). This fatty acid combination is unique among edible oil seeds and can be used to check the capacity of marine

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Table 1
Formulation (g/100 g) and fatty acid composition (% weight of total fatty acids –TFA–) of the emulsions containing high (DHA-H), medium (DHA-M) and low (DHA-L) DHA concentrations, hemp oil and commercial product (Red Pepper) used in Experiments 1 (left) and 2 (right).

	Exp 1				Exp 2			
Ingredients (g)	DHA-H	DHA-M	DHA-L	Hemp	DHA-H	DHA-M	Hemp	
Cod liver oil[a]	10.7	45.3	71.4	5.7				
Incromega 500TG[b]	66.7	33.3	7.1	–	75.8	–	–	
Incromega 3322TG[b]					–	75.8	–	
Olive oil[e]	8.0	6.7	5.7	7.1	7.6	7.6	7.6	
Hemp oil[c]	–	–	–	71.4	–	–	75.8	
Soybean lecithin[f]	9.3	9.3	10.0	10.0	10.6	10.6	10.6	
α-Tocopherol[f]	5.3	5.3	5.7	5.7	6.1	6.1	6.1	
	DHA-H	DHA-M	DHA-L	Hemp	DHA-H	DHA-M	Hemp	Red Pepper[d]
Total lipids (mg/g DW)	705.2	650.8	703.5	680.3	703.6	641.7	672.9	359.8
Total FA (mg/g lipids)	644.7	686.5	711.4	712.0	684.8	789.8	849.5	821.3
Fatty acids (%TFA)								
14:0	3.1	2.9	2.6	2.1	0.1	0.1	0.1	3.6
16:0	3.2	7.2	11.1	2.9	3.6	6.0	6.7	32.3
18:0	3.5	3.3	3.2	2.6	3.1	2.8	2.6	1.3
Total SFA	10.0	13.6	17.1	7.8	6.7	9.0	9.4	37.4
16:1n-7	1.7	3.3	4.8	0.7	0.4	0.4	0.2	0.6
18:1n-9	18.5	21.5	24.5	19.1	12.3	14.2	14.6	2.3
18:1n-7	1.1	3.3	5.4	0.8	1.2	1.3	1.3	0.6
20:1n-9	2.8	6.3	9.5	0.9	1.8	1.0	0.0	0.2
Total MUFA	25.1	39.1	52.3	22.0	15.7	16.9	16.1	3.7
18:2n-6 (LA)	6.6	7.5	8.9	45.2	4.6	30.9	52.7	4.2
18:3n-6 (GLA)	0.1	0.1	0.0	2.9	0.2	2.1	3.3	0.2
20:4n-6 (ARA)	1.2	0.7	0.2	0.0	2.2	0.9	0.1	1.0
22:5n-6	2.2	1.2	0.3	0.0	3.0	0.2	0.0	12.2
Total n-6 PUFA	10.1	9.5	9.4	48.1	10.2	34.2	56.2	17.9
18:3n-3 (LNA)	0.2	0.4	0.6	12.5	0.5	9.5	16.2	0.3
18:4n-3 (SDA)	0.8	1.4	2.0	1.7	1.1	2.1	1.4	0.5
20:5n-3 (EPA)	8.2	7.2	6.1	0.9	11.5	13.5	0.0	2.0
22:5n-3	2.2	4.2	6.1	0.4	2.3	1.8	0.0	0.4
22:6n-3 (DHA)	40.2	22.8	5.9	1.2	50.7	11.9	0.7	37.2
Total n-3PUFA	52.1	36.4	20.9	16.7	67.5	40.0	18.3	41.0
Total PUFA	62.3	45.9	30.2	64.8	77.6	74.2	74.5	58.9
n-3/n-6	5.2	3.8	2.2	0.4	6.6	1.2	0.3	2.3
DHA/EPA	4.9	3.2	1.0	1.3	4.4	0.9	–	18.3

Totals include some minor components not shown.
[a] Cod liver oil.
[b] Incromega oil, Croda Iberica, Spain.
[c] Hemp oil, Hempoil Canada Inc., Canada.
[d] Red Pepper, Bernaqua, Belgium.
[e] Olive oil.
[f] Supplements: Soy lecithin, Laboratories Korott, Spain; α-tocopherol, Sigma-Aldrich, Spain.

fish larvae to elongate or desaturate fatty acids from precursors.

Thus, the objectives of the present study were (1) to examine the effect on growth, survival and fatty acid composition of the larvae of different enrichment emulsions for live prey based on specialist oils with different DHA content and DHA/EPA ratio and (2) to check the ability of meagre larvae to elongate or desaturate fatty acids from precursors using an enrichment emulsion formulated with hemp oil.

2. Materials and methods

2.1. Meagre larviculture and sampling

Newly hatched meagre (*Argyrosomus regius*) larvae were obtained after hormonal induction (Duncan et al., 2012) in the Centro de Aquicultura, Institut de Recerca i Tecnologia Agroalimentaries (IRTA, San Carles de la Rapita, Spain). Floating eggs were incubated in 35 L cylindrical PVC containers provided with air-lift systems and high aeration supply at 20 °C. Newly hatched larvae were distributed into sixteen 100 L tanks at a density of 50 larvae L^{-1}, in three replicated tanks per treatment. The tanks were connected to a recirculation unit (IRTAmar™). Water temperature was controlled every day, increased from 20 to 23 °C at 1 °C day^{-1} and maintained at 23.0 ± 1.7 °C, salinity at 35.82 ± 0.33 ppt, dissolved oxygen at 7.2 ± 1.0 mg L^{-1} whereas pH (7.97 ± 0.06) nitrities (0.02 ± 0.02 mg L^{-1}) and amonia (0.1 ± 0.05 mg L^{-1}) were checked 2 times per week (Hach Colorimeter DR/890, USA). Photoperiod was kept at 16 h light: 8 h darkness and light intensity was maintained at 500 lx at water surface following the recommendations of Vallés and Estevez (2013). At mouth opening, 2 days post-hatching (dph), larvae started being fed enriched rotifers until 14 dph twice daily (10:00 and 16:00 h) at a density of 10 rotifers mL^{-1}. Freshly enriched *Artemia* metanauplii were introduced at 8 dph and fed until 30 dph, in quantities ranging from 0.5 to 6 metanauplii mL^{-1}, and adjusted based on the increase of larval weight, in two to three daily rations (9:00–13:00–17:00 h). Larvae were fed metanauplii until the end of the experiment.

Samples of 10 larvae were collected from each tank at 0, 7, 16 and 30 dph in Experiment 1 and at 0, 14, 27 and 30 dph in Experiment 2 and killed with an overdose of anesthetic MS222 (1000 mg L^{-1}). Standard length (SL) was measured using a dissecting microscope and an image analyser (Analysis, SIS Gmbh, Germany). The same larvae were then washed on a mesh with distilled water, dry blotted to remove excess water and pooled onto pre-weighted coverslips. They were then oven-dried at 60 °C for 24 h and weighed to determine dry weight (DW) on a Mettler A-20 microbalance (Mettler Toledo, Columbus, OH, USA) to the nearest ± 1 μg. Specific growth rate (SGR) was calculated at the end of the trials using the formula:

$$SGR = (\ln Wf - \ln Wi \times 100)/t \ (\%day^{-1}).$$

where lnWf = the natural logarithm of the final weight; lnWi = the natural logarithm of the initial weight and t = time (days) between lnWf and lnWi.

At the end of the experiment, all the larvae remaining in the tanks were counted and, to evaluate survival, both these and the larvae sampled during the experiment were taken into account, using the formula developed by Buckley et al. (1984).

Two trials were carried out with meagre larvae in consecutive years using spawns from the same broodstock and under the same hormonal induction protocol. Briefly, in Experiment 1 three different levels of DHA were used to formulate the enrichment emulsions and compared to an emulsion formulated with hemp oil, whereas in Experiment 2 only two DHA levels, without any addition of cod liver oil, using only specialist oils, were assayed and the results were also compared to a commercial enricher (Red Pepper, Bernaqua, Belgium) generally used in meagre hatcheries (J. Carrillo, Pers. Com., 2016).

Samples of meagre larvae at the beginning and at the end of the trials and of the enriched live prey (at least 3 samples of each live prey throughout the duration of the experiments) were taken for lipid and fatty acid analysis. The larvae were previously anesthetized with MS-222, concentrated in a mesh sieve and washed with distilled water before freezing at − 20 °C for later analysis of lipids and fatty acids.

2.2. Experimental live prey enrichment emulsions

The emulsions to enrich the live prey used in the trials (Table 1), were prepared following the methodology described in Villalta et al. (2005) and Estevez & Gimenez (in press), mixing cod liver oil (Sigma-Aldrich, Madrid, Spain), Incromega DHA500 and Incromega DHA3322TG oil (Croda Iberica, Spain), high oleic olive oil (Borges, Lleida, Spain) and hemp oil (Hempoil Canada Inc., Ste. Agathe, Canada). The different oil mixtures were emulsified with warm (50 °C) distilled water, soy lecithin (Laboratorios Korott, Alicante, Spain) and α-tocopherol (Sigma-Aldrich, Madrid, Spain) by homogenising with an Ultra-turrax T25 at high speed for 60–90 s. The emulsions were then transferred to plastic syringes, the air removed, and kept refrigerated (4 °C) in an upright position until used for enriching the live prey.

2.3. Live food enrichment

2.3.1. Rotifers

Rotifers (*Brachionus* sp.) used for enrichment were cultured in 100 L conical fibreglass tanks at a salinity of 26 ppt, water temperature of 26 °C and 8 mg L^{-1} dissolved oxygen. The rotifer culture was aerated and daily fed microalgae (*Tetraselmis chuii)* at 4 × 10^5 cell mL^{-1} and yeast (Mauripan, Spain) at 0.7 g/million rotifers. The daily ration of rotifers was harvested and divided into four oxygenated 10 L containers filled with UV filtered seawater and enriched for 12 h with one of the four experimental emulsions at a density of 500 rotifers mL^{-1} and 28 ± 1 °C, using 0.6 g L^{-1} of each emulsion. After 12 h the rotifers were gently filtered and washed using a 40 μm mesh with UV filtered seawater and disinfected by further rinsing for 1 min with freshwater, before feeding to the larvae.

2.3.2. Artemia

Brine shrimp *Artemia* cysts (2 g L^{-1}, EG type; INVE, Belgium) originating from Great Salt Lake, USA, were incubated during 24 h in 100 L cylindrical-conical tanks and hatched at 28 °C in highly aerated seawater. The newly hatched nauplii were harvested and washed using a 150 μm mesh, transferred to clean seawater and their concentration was determined by counting the nauplii in 1 mL samples under a binocular microscope. *Artemia* nauplii were then enriched in 20 L containers, using 0.6 g L^{-1} of the emulsions for 12 h. The oil emulsions were blended with water (1:1) in a high-speed blender. Enriched metanauplii were harvested, washed and disinfected with hydrogen peroxide at 8000 ppm for 5 min, thoroughly washed for 15 min on 150 μm plankton nets with UV filtered seawater and counted.

2.4. Lipid and fatty acid analysis

Total lipids were extracted in chloroform:methanol (2:1, v:v) using the method of Folch et al. (1957) and quantified gravimetrically after evaporation of the solvent under a nitrogen flow followed by vacuum desiccation overnight. Total lipids were stored in chloroform:methanol (2:1, 20 mg mL^{-1}) containing 0.01% butylated hydroxytoluene (BHT) at − 20 °C prior to analysis. Acid catalyzed transmethylation was carried out using the method of Christie (1982). Methyl esters were extracted twice using isohexane diethyl ether (1:1, v:v), purified on TLC plates (Silica gel 60, VWR, Lutterworth, UK) and analyzed by gas–liquid chromatography on a Thermo TraceGC (Thermo Fisher, Spain) fitted with a BPX70 capillary column (30 m × 0.25 mm id; SGE, UK), using a two-stage thermal gradient from 50 °C (injection temperature) to 150 °C after ramping at 40 °C min^{-1} and holding at 250 °C after ramping at 2 °C min^{-1}. Helium (1.2 mL·min^{-1} constant flow rate) as the carrier gas and on-column injection and flame ionization detection at 250 °C were used. Peaks of each fatty acid were identified by comparison with known standards (Supelco Inc., Spain) and a well characterized fish oil, and quantified by means of the response factor to the internal standard, 21:0 fatty acid, added prior to transmethylation, using a Chrom-card for Windows (TraceGC, Thermo Fisher, Spain). Results of total lipids, total fatty acids and fatty acid content in the enrichment emulsions, enriched rotifers and enriched *Artemia* metanauplii for each treatment are shown in Table 2 and expressed as percentage of total fatty acids (TFA).

2.5. Statistics

Results in terms of larval DW, SL and survival of the larvae and the fatty acid profile of the larvae and live prey were compared by one way analysis of variance (ANOVA). Percentage values were arcsine transformed and the assumption of homogeneity of variances was checked using the Shapiro-Wilk test. Data was analyzed at a significance level of 0.05. When significant differences were found, the Tukey HSD multiple range test was performed, using SigmaPlot 12.0 software. All data is given as mean values and standard deviations (± SD).

3. Results

Tables 1, 2 and 3 show the formulation, lipid and fatty acid composition of the emulsions (Table 1) used for live prey enrichment and the fatty acid profile of the enriched live prey (Tables 2 and 3), used in both experiments. The main difference in the formulation of the emulsions was the use of cod liver oil mixed with Incromega 500TG oil to have the desired levels of DHA (Low –L–, Medium –M– and High –H–) in Experiment 1, whereas two different Incromega oils (500TG and 3322TG) with different content of DHA and EPA were used in Experiment 2 for the same purpose. Cod liver oil provided the emulsions with a lower content of EPA, and consequently a higher DHA/EPA ratio, and a higher content of oleic acid (18:1n-9), and consequently higher content of monounsaturated fatty acids (MUFA). The use of hemp oil in the emulsions resulted in a higher content of LA, GLA, total n-6 PUFA and LNA and very low content in n-3 PUFA and EPA and DHA.

The fatty acid composition of live prey (Tables 2 and 3, only mean values are shown for clarity) reflected the composition of the emulsions. Thus, rotifers enriched with DHA-H had the highest content of DHA 34.54% in Exp 1 and 42.59% in Exp 2 and DHA/EPA ratio of 7.36 in Exp 1 and 6.17 in Exp 2, whereas those enriched with DHA-L had the lowest DHA content and DHA/EPA ratio (8.7% and 1.44, respectively).

Table 2
Fatty acid composition (%TFA) of the enriched live prey enriched on experimental emulsions used in Experiment 1. Different letters indicate significant differences (ANOVA, $P < 0.05$).

	Rotifer				*Artemia*			
	DHA-H	DHA-M	DHA-L	Hemp	DHA-H	DHA-M	DHA-L	Hemp
Total lipids (mg/g DW)	138.3	122.2	149.6	167.5	191.3	169.3	123.8	221.9
Total fatty acids (mg/g lipids)	605.8	596.9	619.8	620.0	689.3	616.0	649.4	644.4
Fatty acid composition (% TFA)								
14:0	0.8	2.0	2.6	0.6	0.2	0.5	0.6	0.6
16:0	5.3	7.8	9.8	8.1	7.7	10.4	10.0	9.4
18:0	3.9	3.6	33.4	3.5	5.3	6.4	5.2	5.9
Total saturated	10.0c	13.4b	15.8a	12.2b	13.4	17.6	15.9	15.4
16:1n-7	5.4b	13.3a	12.2a	5.8b	0.6	2.0	2.1	0.6
18:1n-9	20.5	23.8	26.2	22.5	19.0	22.2	24.5	21.2
18:1n-7	7.8	7.3	4.4	2.3	8.6	6.3	7.6	3.9
20:1n-9	2.9	5.7	8.6	2.0	1.1	2.2	3.6	0.9
Total monounsaturated	38.1b	52.8a	56.3a	32.8b	29.7a	33.8ab	40.1b	26.8a
18:2n-6	5.0b	3.9b	5.7b	40.9a	6.0b	5.9b	7.5b	29.6a
18:3n-6	0.0b	0.0b	0.0b	2.0a	0.6b	0.8b	0.7b	1.5a
20:4n-6	1.0a	0.9a	0.4b	0.1c	1.2a	1.2a	0.6b	0.2c
Total n-6 PUFA	8.2b	6.6b	7.1b	43.4a	8.8b	8.7b	8.9b	21.4a
18:3n-3	0.6	0.6	0.9	9.45	21.7	22.3	19.1	22.2
18:4n-3	0.3	0.7	1.3	0.6	2.7	3.4	2.9	2.4
20:5n-3	4.7b	5.2ab	6.0a	0.7c	6.0a	5.1b	5.9a	1.0c
22:5n-3	1.3	1.0	0.8	0.1	0.6	0.5	0.5	0.0
22:6n-3	34.5a	16.1b	8.7c	0.7d	16.2a	7.6b	5.6c	0.5d
Total n-3 PUFA	42.1a	24.3b	18.5c	11.6d	48.0a	40.0b	35.0c	26.5d
Total PUFA	50.4a	31.0b	25.6b	55.0a	56.8a	48.6b	44.0b	57.9a
n-3/n-6	5.1a	3.7b	2.6c	0.3d	5.4a	4.6b	3.9c	1.2d
DHA/EPA	7.4a	3.1b	1.4c	1.0d	2.7a	1.5b	1.0c	0.5d

Rotifers enriched with the emulsion formulated with hemp oil had the highest levels of LA, GLA and LNA in both trials.

A similar trend was also observed in enriched *Artemia*, with DHA levels ranging from 16.22% to 5.61% when enriched with DHA-H and DHA-L, respectively, whereas *Artemia* enriched with hemp oil emulsion had the highest content of LA, GLA and LNA fatty acids. In experiment 2 the use of Incromega 3322TG gave as a result the highest EPA content in both live prey whereas the use of the commercial enricher, Red Pepper, produced rotifers and *Artemia* metanauplii with intermediate levels of DHA (23.83% and 6.95%, respectively) and the highest DHA/EPA ratio in the rotifers.

Larval growth is presented in Fig. 1. In experiment 1 larvae fed DHA-H enriched live prey had a statistically significant (ANOVA $P < 0.001$) better growth than the rest of the treatments, reaching

Table 3
Fatty acid composition (%TFA) of the enriched live prey enriched on experimental emulsions used in Experiment 2. Different letters indicate significant differences (ANOVA, $P < 0.05$).

	Rotifer				*Artemia*			
	DHA-H	DHA-M	Red Pepper	Hemp	DHA-H	DHA-M	Red Pepper	Hemp
Total lipids (mg/g DW)	180.4	194.3	170.9	196.0	122.4	138.3	113.2	130.6
Total FA (mg/g lipids)	574.5	535.2	524.4	693.9	678.3	723.0	687.7	720.1
Fatty acid composition (% TFA)								
14:0	0.2	0.2	1.9	0.2	0.4	0.4	0.7	0.4
16:0	4.1b	4.7b	18.6a	7.0b	9.0b	8.4b	14.7a	9.2b
18:0	3.5	3.7	3.1	3.2	5.9	5.7	7.0	5.1
Total saturated	7.8b	8.6b	23.9a	10.4b	15.8b	15.1b	23.2a	15.1b
16:1n-7	3.0b	3.4b	6.3a	2.4b	1.5	1.5	1.5	1.0
18:1n-9	16.2	17.3	12.4	17.8	22.2	21.7	19.1	20.6
18:1n-7	2.2	2.8	2.4	1.6	7.0	7.0	8.0	4.0
20:1n-9	2.4	2.9	1.8	1.0	1.3	1.5	0.7	0.7
Total monounsaturated	23.8	26.5	22.9	22.8	32.3	32.1	29.3	26.3
18:2n-6	5.7b	4.8b	7.1b	44.3a	7.2b	6.0b	6.2b	36.9a
18:3n-6	0.3b	0.3b	0.4b	3.1a	0.2b	0.2b	0.0b	1.6a
20:3n-6	0.3	0.5	0.6	0.0	0.1	0.1	0.2	0.0
20:4n-6	1.9a	2.1a	1.2b	0.5c	1.7a	1.6a	2.4a	0.4b
Total n-6 PUFA	11.2bc	8.6c	16.7b	48.1a	10.2b	8.2b	11.4b	38.9a
18:3n-3	1.0b	1.1b	1.3b	13.7a	18.2	16.8	20.5	16.8
18:4n-3	1.0b	2.0a	0.7b	1.8a	2.2	2.0	2.4	1.7
20:4n-3	1.2a	1.9a	1.9a	0.4b	0.4	0.9	0.5	0.0
20:5n-3	6.9b	25.6a	3.3c	0.6d	6.7b	16.3a	5.4b	0.9c
22:5n-3	1.8b	3.7a	0.7c	0.0d	0.8b	1.4a	0.2c	0.0d
22:6n-3	42.6a	18.5c	23.8b	0.9d	13.4a	6.8b	7.0b	0.4c
Total n-3 PUFA	55.1a	54.3a	32.4b	17.7c	41.8a	44.6a	36.0b	19.8c
Total PUFA	66.4a	62.9a	49.1b	65.8a	51.9a	52.8a	47.5b	58.6a
n-3/n-6	4.9b	6.3a	2.0c	0.4d	4.1b	5.4a	3.2c	0.5d
DHA/EPA	6.2a	0.7c	7.3a	1.5b	2.0a	0.4	1.3b	0.5c

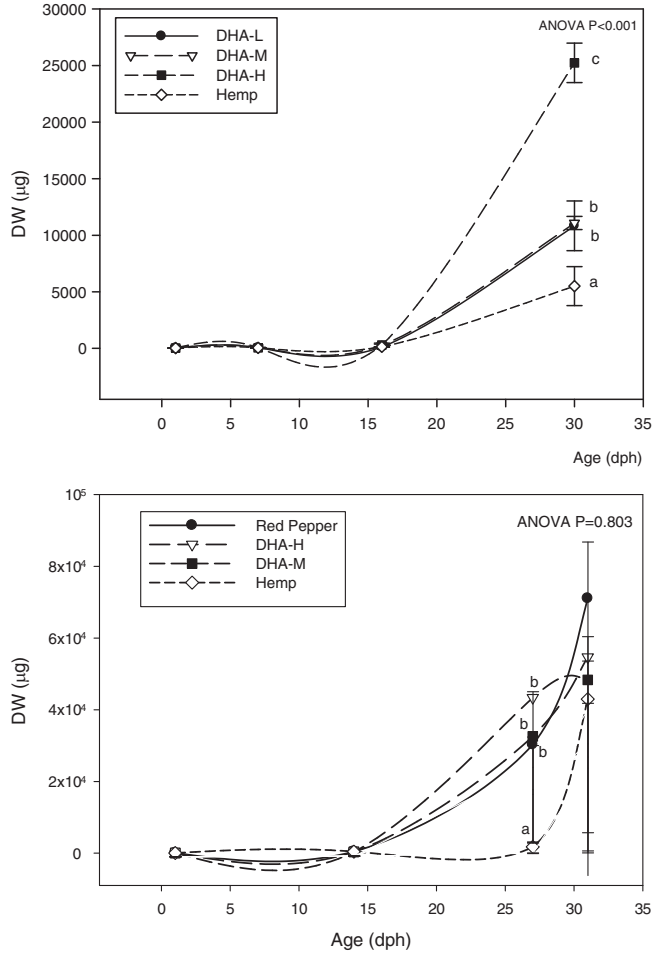

Fig. 1. Growth (dry weight, DW, µg) of the larvae in Experiments 1 and 2. Letters indicate significant differences (ANOVA, $P < 0.05$).

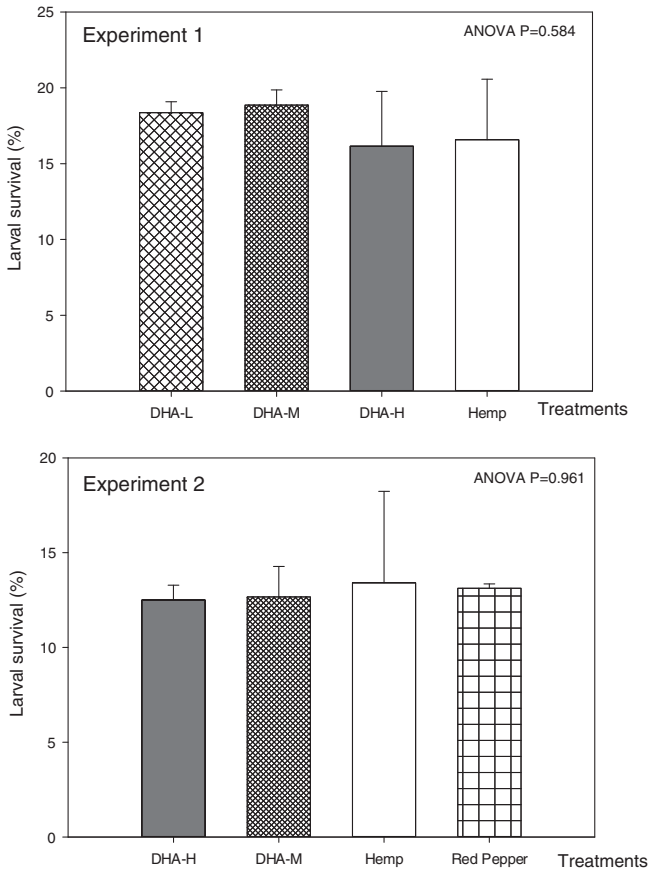

Fig. 2. Larval survival rate (%) obtained after 30 days feeding live prey enriched with the experimental emulsions. a) Experiment 1 and b) Experiment 2.

25.24 mg of dry weight at 30 dph and a specific growth rate (SGR) of 22.31% day^{-1} (Table 4). In experiment 2 no significant differences could be detected among the 4 groups of larvae due to the high variability of sizes and weights found among the larvae of each group. However, in this experiment larvae fed DHA-H enriched live prey attained 54.70 mg dry weight and SGR of 24.24% day^{-1} at 30 dph. One of the problems of experiment 2 was the high incidence of cannibalistic behaviour observed from 20 dph onwards that has already been described in the rearing of meagre larvae (Roo et al., 2010; Campoverde et al., in press) and in a closely related species *Argyrosomus japonicus* (O'Sullivan and Ryan, 2001; Timmer and Magellan, 2011). Having in mind this cannibalistic behaviour, at the end of Experiment 2, large (50 to 210 mg DW and 2.4 to 2.9 cm SL) and small (1.5 to 25 mg DW and 1.3 to 1.5 cm SL) larvae were separated to carry out the fatty acid analyses and detect possible differences in the fatty acid profile between them.

Larval survival rate is shown in Fig. 2, no significant differences could be found among the four larval groups in any of the experiments, with the survival rate being relatively higher (around 18%) in Experiment 1 compared to Experiment 2 (around 14%).

The fatty acid composition of the larvae at the end of the trials is shown in Tables 5, 6 and 7. Fatty acid composition of the larvae reflected the composition of the live prey. In both experiments larvae

Table 4
Dry weight (µg, mean ± SD) and specific growth rate (SGR. % day^{-1}) values obtained in Experiments 1 and 2.

Experiment 1					
	0 dph	7 dhp	16 dhp	30 dph	SGR (%)
DHA-H	31.3 ± 0.4	49.1 ± 6.5b	294.4 ± 172.4b	25,240.5 ± 1745.8c	22.3c
DHA-M	31.3 ± 0.4	63.8 ± 4.6d	259.0 ± 116.2b	11,089.8 ± 582.8b	19.6b
DHA-L	31.3 ± 0.4	57.8 ± 11.9c	109.9 ± 9.0a	10,838.0 ± 2199.4b	19.5b
Hemp	31.3 ± 0.4	41.5 ± 6.3[a]	138.0 ± 0.0a	5499.8 ± 1722.8a	17.2a
ANOVA *P*	< 0.001		< 0.001	< 0.001	< 0.001
Experiment 2					
	0 dph	14 dhp	27 dhp	30 dph	SGR (%)
DHA-H	29.8 ± 2.1	352.7 ± 115.9	32,586.8 ± 25,366.7b	54,702.5 ± 51,813.7	24.2
DHA-M	29.8 ± 2.1	314.4 ± 91.0	43,378.9 ± 36,515.7b	48,333.3 ± 46,421.0	23.8
Hemp	29.8 ± 2.1	388.5 ± 76.2	1755.9 ± 175.8a	42,980.0 ± 31,302.3	23.5
Red pepper	29.8 ± 2.1	424.2 ± 107.7	30,263.0 ± 23,595.1b	71,025.0 ± 81,238.5	25.1
ANOVA *P*	0.595 NS		0.03	0.803 NS	0.857 NS

Table 5
Fatty acid composition (%TFA, mean ± standard deviation) of meagre larvae at the beginning and end of the Experiment 1. Different letters indicate significant differences (ANOVA, $P < 0.05$).

	Day 0	Day 30			
		DHA-H	DHA-M	DHA-L	HEMP
Total lipids (mg/g DW)	86.0 ± 3.6	97.2 ± 16.9	75.3 ± 20.2	82.2 ± 4.1	109.0 ± 7.7
Total Fatty acids (mg/g lipids)	474.7 ± 1.5	455.7 ± 27.2	467.8 ± 38.9	476.3 ± 42.4	478.5 ± 21.5
Fatty acids (% TFA)					
14:0	1.1 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0
16:0	19.8 ± 0.3	12.3 ± 0.4	13.4 ± 0.5	14.1 ± 1.4	12.5 ± 0.2
18:0	7.9 ± 0.3	10.2 ± 0.4	10.4 ± 0.8	11.0 ± 1.7	10.6 ± 0.3
Total saturated	28.7 ± 0.6	22.9 ± 0.7	24.4 ± 1.3	25.5 ± 2.9	23.6 ± 0.3
16:1n-7	5.8 ± 0.5	0.7 ± 0.3b	0.9 ± 0.3b	2.2 ± 0.7a	0.6 ± 0.2b
18:1n-9	14.2 ± 0.1	20.9 ± 1.8	21.7 ± 1.0	21.1 ± 5.1	21.7 ± 1.2
18:1n-7	1.9 ± 0.0	4.6 ± 0.4	5.1 ± 0.7	2.7 ± 3.8	4.5 ± 0.5
20:1n-9	0.7 ± 0.1	1.0 ± 0.2	1.6 ± 0.3	2.0 ± 0.5	0.7 ± 0.6
Total monounsaturated	22.5 ± 0.4	27. ± 2.4	29.3 ± 1.3	28.0 ± 7.8	27.4 ± 0.9
18:2n-6	4.6 ± 0.0	5.8 ± 0.4a	5.6 ± 0.2a	7.7 ± 1.0b	17.8 ± 0.7c
18:3n-6	1.5 ± 0.4	0.6 ± 0.1b	0.5 ± 0.1b	0.5 ± 0.1b	1.2 ± 0.2a
20:4n-6	1.9 ± 0.1	2.3 ± 0.1	2.3 ± 0.2	1.9 ± 0.4	1.2 ± 0.1
22:5n-6	0.4 ± 0.0	1.2 ± 0.3	0.9 ± 0.2	0.5 ± 0.1	0.1 ± 0.1
Total n-6 PUFA	8.8 ± 0.3	10.0 ± 0.4a	9.6 ± 0.3a	10.8 ± 1.3a	20.8 ± 0.4b
18:3n-3	1.3 ± 0.1	13.4 ± 1.5	12.7 ± 1.5	13.2 ± 0.1	14.4 ± 1.6
18:4n-3	0.1 ± 0.2	1.6 ± 0.1	1.4 ± 0.2	1.1 ± 0.3	1.8 ± 0.2
20:4n-3	0.1 ± 0.0	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	1.2 ± 0.4
20:5n-3	4.5 ± 0.2	6.4 ± 0.8b	7.0 ± 0.4a	7.9 ± 1.5a	4.3 ± 0.4b
22:5n-3	1.6 ± 0.1	0.9 ± 0.1	1.0 ± 0.0	1.6 ± 0.8	0.5 ± 0.2
22:6n-3	27.7 ± 0.4	15.7 ± 4.4a	12.5 ± 1.7ab	7.5 ± 0.3bc	5.0 ± 2.6c
Total n-3 PUFA	35.4 ± 0.3	38.8 ± 2.2b	35.4 ± 0.2b	32.0 ± 1.2b	27.0 ± 1.3a
Total PUFA	44.2 ± 0.6	48.8 ± 1.8	45.0 ± 0.3	43.3 ± 1.5	47.8 ± 1.1
n-3/n-6	4.0 ± 0.1	3.9 ± 0.4b	3.7 ± 0.1b	3.2 ± 0.1b	1.3 ± 0.1a
DHA/EPA	6.2 ± 0.4	2.5 ± 1.0a	1.8 ± 0.3b	1.0 ± 0.8c	1.1 ± 0.5c

fed DHA-H enriched live prey had a significantly higher content of DHA, whereas larvae fed hemp oil enriched prey showed the lowest DHA content and the highest levels of LA and GLA. EPA levels were similar in the larvae reared in Experiment 1, whereas in Experiment 2 larvae fed DHA-M enriched live prey showed the highest EPA content, especially in the case of small larvae (Table 6).

Fig. 3 shows the differences found in the fatty acid profile between small and large larvae of Experiment 2. Large larvae had in all the cases

Table 6
Fatty acid composition of meagre larvae (% TFA, mean ± S D) at the beginning and at the end of Experiment 2 showing the fatty acid profile of the larvae classified as small. Different letters indicate significant differences (ANOVA, $P < 0.05$).

	Day 0	Small larvae day 30			
		DHA-H	DHA-M	Red Pepper	Hemp
Total lipids (mg/g DW)	112.4	108.0 ± 16.6	95.0 ± 2.6	93.2 ± 11.8	101.8 ± 10.5
Total fatty acids (mg/g lipids)	437.3	546.8 ± 34.4a	476.4 ± 24.8	504.2 ± 15.7ab	454.6 ± 20.0b
Fatty acids (% total FA)					
14:0	2.2	0.3 ± 0.0	0.4 ± 0.1	0.5 ± 0.0	0.3 ± 0.0
16:0	20.0	13.3 ± 0.2d	15.4 ± 0.8b	17.1 ± 0.2a	14.2 ± 0.3c
18:0	4.0	8.2 ± 0.1	10.0 ± 0.5	10.1 ± 0.4	9.9 ± 0.0
Total saturated	26.7	22.3 ± 0.4d	26.6 ± 1.2b	28.4 ± 0.5a	25.2 ± 0.3c
16:1n-7	7.7	1.6 ± 0.1	1.9 ± 0.2	1.6 ± 0.2	1.4 ± 0.2
18:1n-9	16.7	16.8 ± 0.3bc	17.5 ± 0.4b	14.9 ± 0.3d	18.0 ± 0.5ab
18:1n-7	3.6	7.4 ± 0.4	7.9 ± 0.4	7.2 ± 0.3	6.6 ± 0.1
20:01	1.1	0.9 ± 0.1	0.9 ± 0.0	0.7 ± 0.0	1.1 ± 0.1
Total monounsaturated	29.2	26.7 ± 0.1bc	28.3 ± 0.8b	24.4 ± 0.7d	27.2 ± 0.2ab
18:2n-6	6.4	5.8 ± 0.2b	5.1 ± 0.1c	4.6 ± 0.1d	23.6 ± 0.0a
18:3n-6	1.2	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	1.0 ± 0.0
20:4n-6	1.3	3.2 ± 0.2b	3.4 ± 0.2b	4.1 ± 0.3a	2.3 ± 0.1c
22:5n-6	0.2	1.3 ± 0.0	0.3 ± 0.1	4.4 ± 0.3	0.1 ± 0.0
Total n-6 PUFA	9.4	10.6 ± 0.4c	9.3 ± 0.2d	13.5 ± 0.6b	27.4 ± 0.0a
18:3n-3	2.1	10.2 ± 0.7b	10.8 ± 1.2ab	6.0 ± 0.8b	12.5 ± 0.6a
18:4n-3	0.9	1.2 ± 0.1	1.1 ± 0.2	1.1 ± 0.1	1.4 ± 0.0
20:4n-3	0.3	0.4 ± 0.1	0.6 ± 0.4	0.5 ± 0.0	0.5 ± 0.0
20:5n-3	4.0	5.9 ± 0.5b	12.8 ± 0.6a	4.3 ± 0.2c	3.2 ± 0.1d
22:5n-3	1.3	1.1 ± 0.1b	2.2 ± 0.3a	0.7 ± 0.1c	0.4 ± 0.0d
22:6n-3	20.4	20.5 ± 1.1a	6.6 ± 0.7c	16.6 ± 0.8b	1.0 ± 0.2d
Total n-3 PUFA	29.3	39.4 ± 0.4a	34.3 ± 0.8b	32.1 ± 1.0c	18.9 ± 0.3d
Total PUFA	38.7	50.0 ± 0.4a	43.6 ± 0.6c	45.6 ± 0.7b	46.3 ± 0.3b
n-3/n-6	3.1	3.7 ± 0.2a	3.7 ± 0.2a	2.4 ± 0.2b	0.7 ± 0.0c
DHA/EPA	5.1	3.5 ± 0.4a	0.5 ± 0.1b	3.9 ± 0.3a	0.3 ± 0.0b

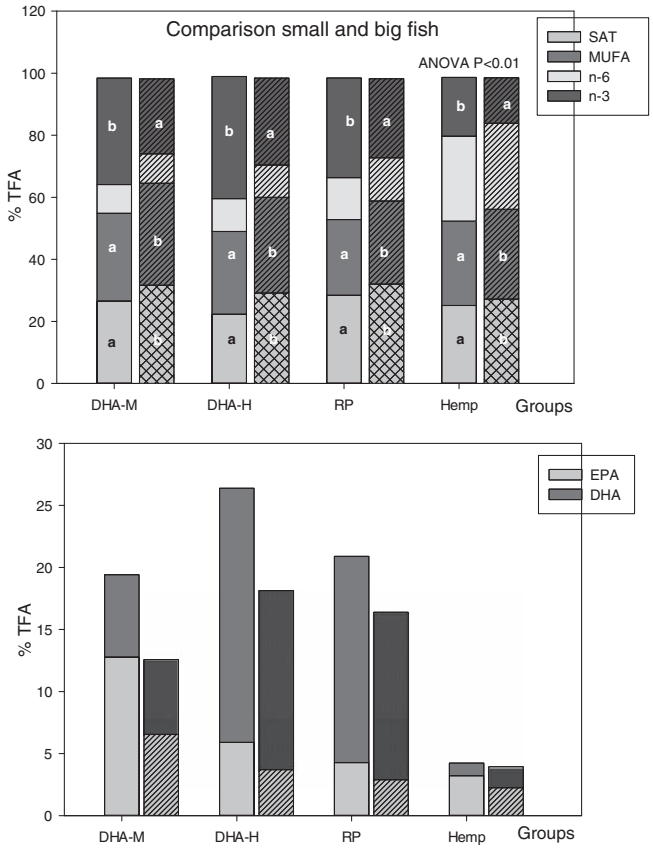

Fig. 3. Differences in the fatty acid profile between small (left hand bars without lines) and big (right hand bars with lines) larvae. Different letters indicate significant differences (ANOVA $P < 0.01$). Big larvae = 50–210 mg DW, 2.4–2.9 cm SL. Small larvae = 1.5–25 mg DW, 1.3–1.5 cm SL.

a significantly higher content of saturated (SAT, ANOVA $P = 0.022$) and monounsaturated (MUFA, ANOVA $P = 0.018$) fatty acids and a significantly lower content of n-3 PUFA (ANOVA, $P = 0.0015$) than small larvae. DHA and EPA content did not show statistically significant differences between small and large larvae, although in the case of EPA significant differences can be detected between the larvae fed diets DHA-H and Hemp (see Table 7). DHA levels were more conserved and similar between both larval sizes.

4. Discussion

The fatty acid composition of enriched live prey as well as the larvae reflected the profile obtained in the emulsions, as already published in previous reports (Dhert et al., 1993; Rainuzzo et al., 1994; Villalta et al., 2005).

Although differences exist between the two experiments carried out, there is a clear effect of the DHA level and DHA/EPA ratio in the live prey and emulsions on larval growth. Thus, the lower growth was obtained when the larvae were fed live prey with low levels of DHA and DHA/EPA ratio and n-3 PUFA content (Hemp and DHA-L groups) as already observed by El Kertaoui et al. (2017) in meagre larvae and in other marine fish (reviewed by Izquierdo and Koen, 2011). In the second experiment the differences in growth due to DHA content and DHA/EPA ratio in the live prey were clear until 27 dph, but not at the end of the trial due to the high cannibalistic behaviour of the larvae and the high differences and variability observed in size and weight between large and small larvae (see Table 4). Cannibalistic behaviour was also observed in experiment 1 but in this case this behaviour was observed later (day 28–29) than in experiment 2 due to the lower growth of the larvae (see Table 4 dry weight at 30 dph in Exp 1 is lower than the dry weight observed in 27 dph larvae in Exp 2) and lower differences in size and weight among the larvae. These differences in growth observed between the two trials were not related to differences in the rearing environment because the same protocols in terms of environmental parameters or live prey density were used for both.

Table 7
Fatty acid composition (% TFA, mean ± SD) of meagre larvae at the end of Experiment 2 showing the fatty acid profile of larvae classified as big. Different letters indicate significant differences (ANOVA, $P < 0.05$).

	Big larvae day 30			
	DHA-H	DHA-M	Red Pepper	Hemp
Total lipids (mg DW)	84.6 ± 9.0	97.8 ± 5.7	87.7 ± 8.2	98.5 ± 9.5
Total Fatty acids (mg)	441.7 ± 31.1	463.4 ± 43.6	453.6 ± 69.6	537.8 ± 28.4
Fatty acids (% total FA)				
14:0	0.3 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.3 ± 0.0
16:0	17.5 ± 1.7ab	19.5 ± 0.2a	19.7 ± 1.0a	15.6 ± 1.0b
18:0	10.8 ± 0.7	11.1 ± 0.8	11.3 ± 1.1	10.9 ± 0.5
Total saturated	29.2 ± 2.4ab	31.8 ± 0.8a	32.1 ± 1.2a	27.3 ± 1.4b
16:1n-7	2.1 ± 0.3	2.3 ± 0.3	1.8 ± 0.2	1.9 ± 0.1
18:1n-9	19.3 ± 1.0a	19.8 ± 0.1a	16.5 ± 1.1b	18.8 ± 0.6a
18:1n-7	8.4 ± 0.4	9.8 ± 1.6	7.7 ± 0.2	7.2 ± 0.1
20:01	1.0 ± 0.2	1.0 ± 0.0	0.8 ± 0.1	1.0 ± 0.0
Total monounsaturated	30.8 ± 1.0ab	32.9 ± 1.7a	26.8 ± 1.1c	29.0 ± 0.6bc
18:2n-6	5.7 ± 0.1bc	5.1 ± 0.2b	4.7 ± 0.1c	23.8 ± 0.8a
18:3n-6	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.5 ± 0.5
20:4n-6	3.3 ± 0.2b	3.3 ± 0.2b	4.4 ± 0.2a	2.6 ± 0.3c
22:5n-6	1.1 ± 0.1	0.7 ± 0.8	4.1 ± 0.1	0.2 ± 0.0
Total n-6 PUFA	10.4 ± 0.2c	9.5 ± 1.1c	13.9 ± 0.3b	27.7 ± 0.8a
18:3n-3	7.6 ± 0.9	8.3 ± 0.4	7.1 ± 0.2	8.7 ± 1.1
18:4n-3	0.9 ± 0.0	0.9 ± 0.1	0.8 ± 0.0	0.9 ± 0.1
20:4n-3	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
20:5n-3	3.7 ± 0.6b	6.5 ± 1.5a	2.9 ± 0.2b	2.3 ± 0.2b
22:5n-3	1.0 ± 0.1b	1.9 ± 0.3a	0.8 ± 0.0b	0.7 ± 0.1b
22:6n-3	14.4 ± 1.8a	6.0 ± 1.2b	13.5 ± 2.1a	1.7 ± 0.1c
Total n-3 PUFA	28.0 ± 3.5a	24.2 ± 1.5a	25.5 ± 2.5a	14.7 ± 1.0b
Total PUFA	38.5 ± 3.6ab	33.6 ± 1.4b	39.4 ± 2.3ab	42.4 ± 1.8a
n-3/n-6	2.7 ± 0.3a	2.6 ± 0.4a	1.8 ± 0.3b	0.5 ± 0.0c
DHA/EPA	3.9 ± 0.3b	1.0 ± 0.4c	4.7 ± 0.3a	0.7 ± 0.0c

DHA levels found in this study are similar to those reported by Vallés and Estevez (2015) using commercial enrichers and considered the most adequate for the proper growth and development of meagre larvae. DHA levels around 12–15%TFA in *Artemia* metanauplii, that correspond to around 40–50% of TFA in the emulsion, and around 260–300 mg of DHA per g of lipids in the *Artemia* can be considered as the required levels for this species. Similar levels of DHA are cited for gilthead sea bream (Salhi et al., 1994) and red porgy (Roo et al., 2009) for better growth and for preventing skeletal deformations in marine fish larvae.

Although there is a clear effect of DHA on larval growth, no effects on survival rate could be detected. In previous trials carried out with meagre, an effect of DHA and DHA/EPA ratio on survival was observed (Vallés and Estevez, 2015), although in that case commercial enrichers with other additives of unknown composition were used. Similar results not linking DHA levels with survival were observed in other species such as cod (Park et al., 2006; Garcia et al., 2008; Copeman and Laurel, 2010) or striped trumpeter (Bransden et al., 2005). These authors found that the length and dry weight of larvae at the end of the experiment were directly related to dietary DHA, whereas survival rate was not influenced by dietary DHA.

Other studies carried out with marine fish larvae suggested that higher levels of DHA (or n-3 PUFA) might reduce larval survival (Planas and Cunha, 1999). Izquierdo et al. (1992) showed that, in larval Japanese flounder (*Paralichthys olivaceus*), lower (or higher) DHA content in *Artemia* metanuplii did not affect survival rate, but larvae were significantly larger when fed *Artemia* containing a higher percentage of DHA (up to 3.5%). Thus, it seems that the requirement of dietary DHA levels of marine finfish larvae is species dependent as Sargent et al. (1999) suggested.

In experiment 2 large and small larvae were analyzed separately and a clear effect of the diet is observed in the case of the small larvae that show a fatty acid profile similar to the enriched *Artemia* metanauplii (and the emulsion used for enrichment), whereas the large larvae showed a similar composition among them, independent of the diet used, with a higher content of saturated and monounsaturated fatty acids, that are metabolic energy reserves (Tocher, 2003). This suggests that the fatty acid profile of large larvae is due to their cannibalistic behaviour where consumption of their smaller siblings provides another mechanism for accumulation of energy reserves, rather than the effect of live food enrichment, or the composition of the enrichment emulsions.

Having in mind the diadromy of meagre, that migrates from deep waters of the North Atlantic and Mediterranean Sea to coastal areas to spawn in river estuaries, similarly to Atlantic salmon, Monroig et al. (2013) studied the fatty acyl desaturase and elongase capacity of this species and found that meagre can express at least one fatty acyl desaturase with homology to Fads2 and one elongase, Elov15. According to these authors meagre is able to elongate a range of C_{18} PUFA to C_{20} PUFA and even towards C_{22} PUFA. However the results of the present study show that the use of hemp oil rich in 18:2n-6, 18;3n-6, 18:3n-3 and 18:4n-3 (the precursors and first desaturation product), as the main component of enrichment had a negative effect on the growth of the larvae, with the larvae reared using live prey enriched with hemp oil being the smallest and with the lowest content of n-6 and n-3 PUFA. A clear indication of the low or non-existent elongation or desaturation capacity of meagre larvae.

5. Conclusions

1.-It is advisable to include between 12 and 15% of DHA (260–300 mg/g lipids) in live prey enrichment diets used for meagre larval rearing to obtain optimal larval growth.

2.-Survival rate in meagre was not affected by the diet used for larval feeding, but it was affected by the cannibalistic behaviour of the larvae from 20 dph onwards. Cannibalistic behaviour should be avoided, either by reducing light intensity, increasing feeding frequency, or grading the larvae, to allow better survival rates. DHA levels did not show any effect on survival rate.

3.-Meagre larvae are not capable of elongation or desaturation of fatty acids when precursors such as LA, GLA, LNA or SDA are offered in the live prey.

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CHAPTER 4

Morphological and functional description of the development of the digestive system in meagre (*Argyrosomus regius*): An integrative approach

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ABSTRACT

The morphological and functional development of the digestive system in meagre (*Argyrosomus regius*, Asso, 1801) was described by means of histological and enzymatic approaches in order to provide insight into the digestive physiology of the species at early life stages of development and evaluate whether the acquisition of digestive capacities matched the morphological development of digestive organs. For this purpose, *A. regius* were reared from hatching to the juvenile stage at 18 °C using a standard feeding sequence of enriched rotifers (2–15 days posthatching, dph), *Artemia* metanauplii (14–51 dph) and microdiet (35–51 dph). Histological and enzymatic analyses indicated that *A. regius* larvae had at the onset of exogenous feeding (2 dph, 3.2 ± 0.1 mm in standard length, SL) a well differentiated exocrine pancreas, and bile salt-activated lipase and alkaline proteases being the main pancreatic enzymes involved in food digestion. Ontogenic changes in pancreatic enzymes occurred between 3.2 and 3.9 mm in SL, coinciding with the resorption of the oil globule and the complete transition to exogenous feeding, and between 4.2 and 5.8 mm in SL (20–25 dph). Regarding acid digestion, pepsin was not detected in *A. regius* until 6.0 and 6.8 mm in SL (31 dph), coinciding with notochord flexion and the progressive decrease in the activity of alkaline protease and leucine-alanine peptidase (intestinal cytosolic enzyme) activities, indicating a shift in the mode of digestion. The detection of pepsin activity in *A. regius* occurred after the appearance of the first gastric glands (4.6 and 5.8 mm SL). Thus, authors need to be cautious when extracting conclusions about the beginning of acid digestion and the onset of weaning of larvae just using histological data, since it has been shown that morphology does not match functionality with regards to the stomach. The comparison of present results with other studies conducted in this species using different rearing conditions (*e.g.* mesocosm technique, larval density, water temperature, feeding sequence) indicated that the functional development of the digestive system in *A. regius* assessed by the activity of alkaline and acid proteases is a well-conserved process that generally occurs within a range of body size regardless of larval age and rearing conditions. Thus, authors recommend using morphometric and/or morphological variables (*e.g.* length, developmental stage) together with the age of larvae expressed in days after hatching for comparative purposes among different studies.

Statement of relevance

The morphological and functional development of the digestive system in meagre (*Argyrosomus regius*) was described by means of histological and enzymatic approaches in order to provide insight into the digestive physiology of the species at early life stages of development. Combining histological and enzymatic analytical procedures we evidenced that stomach's functionality did not match its morphological organization, since pepsin activity was not detected until some days after the appearance of the first gastric glands. This indicates that authors need to be cautious when extracting conclusions about the beginning of acid digestion and the onset of weaning of larvae just using histological data.

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1. Introduction

Although the basic mechanisms of organ and system development are similar among teleosts, there has been a considerable increase in the number of studies dealing with the ontogeny of the digestive system in marine fish larvae during the last two decades. As there are considerable interspecific differences regarding their relative timing of differentiation, development, and functionality during early ontogeny (Treviño et al. 2011), these studies are a valuable tool for better understanding the digestive physiology of larvae, and can be used for improving fish larvae nutrition and culture (Ueberschär 1995; Gisbert et al. 2008; Zambonino-Infante et al. 2008; Lazo et al. 2011). In this sense, the histological development of the digestive system and its functionality are among the issues that have received most attention by researchers dealing with larvae, although these topics are generally assessed in separated studies. Recent data have shown that histological studies on the ontogenesis of the digestive tract and accessory organs need to be completed with biochemical analyses in which the activity of selected digestive enzymes from the pancreas, stomach and intestine are assessed in order to provide evidence of digestive system functionality, given that morphology does not always match functionality (Gisbert et al. 2013).

Meagre (*Argyrosomus regius,* Asso, 1891) is considered an emergent aquaculture species for the European aquaculture due to its large size, good processing yield, low fat content, excellent taste and firm texture (Monfort 2010). In addition, this scianid species possesses the biological characteristics required for commercial aquaculture using well-established gilthead sea bream (*Sparus aurata* L. 1758) and European sea bass (*Dicentrarchus labrax* L. 1758) culture technologies (Papadakis et al. 2013), a fast growth of ~1 kg per year (Duncan et al. 2013), a low feed conversion ratio of 0.9–1.2 (Monfort 2010; Duncan et al. 2013), a relatively easy larval rearing (Roo et al. 2010; Vallés and Estévez 2012; Pousão-Ferreira et al. 2013), established induced spawning protocols for the production of viable eggs (Duncan et al. 2012), and good tolerance to a wide range of salinity and environmental conditions of captivity (Jiménez et al. 2005). Although *A. regius* larval rearing is considered a relatively simple process in contrast to other marine species, regardless the use of mesocosm approaches (Papadakis et al. 2013) or intensive rearing systems (Roo et al. 2010; Vallés and Estévez 2012; Pousão-Ferreira et al. 2013), there are still important issues regarding larval nutritional requirements and cannibalistic behaviour that require further attention. In this context, understanding the morphological and functional changes of the digestive system occurring during ontogeny, under specific rearing conditions, may provide indicators of the nutritional status of the fish during early stages, as well as valuable information for *A. regius* larval culture and provide insight into its nutritional physiology. Thus, two recent studies have been focused on describing the histological development of the digestive tract in *A. regius* (Papadakis et al. 2013) and changes in digestive enzyme activities along larval development (Suzer et al. 2013). The above-mentioned studies were conducted under quite different rearing conditions and feeding protocols (mesocosm *vs.* intensive rearing procedures) and evaluated changes in the organization and functionality of the digestive system separately and their results are not directly comparable. Thus, given the evidence that morphology does not always match functionality (Gisbert et al. 2013), we decided to describe the histological and functional development of the digestive system in *A. regius* from hatching to the juvenile stage and evaluate whether the acquisition of digestive capacities (*e.g.* enzyme activity) matched the morphological development of digestive organs.

2. Material and methods

2.1. Fish rearing and sampling procedures

A. regius floating eggs (900 ml, fertilization rate = 92%) were obtained from a wild broodstock adapted to captivity at IRTA-SCR by induced spawning using GnRHa injection (20 μg kg^{-1} in females and 10 μg kg^{-1} in males) (Duncan et al. 2012). At hatching, eleutheroembryos were equally distributed among two cylindroconical 500 l tanks at the density of 200 larvae l^{-1}. Both tanks were connected to a recirculation unit (IRTAmar™) equipped with mechanical, biological and UV-filters and a temperature controller. Temperature (18.2 ± 0.5 °C, mean ± standard deviation, $n = 50$), salinity (35.4 ± 0.3 mg l^{-1}), oxygen concentration (7.9 ± 0.3 mg l^{-1}) and pH (7.9 ± 0.2) were checked daily, whereas nitrites (<0.25 mg l^{-1}) and ammonia (<0.07 mg l^{-1}) were checked once per week (Hach Colorimerter DR/890, USA; www.hach.com). Flow rate was adjusted to 6–12 l min^{-1} and water renewal from the recirculation unit was 5–10% of the total water volume. Photoperiod was 16 L:8 D and light intensity 500 lx at water surface. The feeding protocol was the following: from 2 to 15 dph (days post hatching) larvae were fed enriched rotifers (enriching conditions: 250 individuals ml^{-1}; 2 h at 19–20 °C, 0.15 g l^{-1} of Easy Selco©, INVE, Belgium) at a density of 10 rotifers ml^{-1}, and from 14 to 50 dph, larvae were fed enriched *Artemia* metanauplii (enriching conditions: 300 individuals ml^{-1}, 18 h at 19–20 °C, 0.6 g l^{-1} Easy Selco©) at a density of 1–3 individuals ml^{-1}. Co-feeding fish with *Artemia* metanauplii and an artificial diet (Gemma Micro 300, Skretting, Norway) was conducted from 35 to 51 dph (Fig. 1). Food items were distributed between 2 and 3 times per day in order to maintain as constant as possible live prey density in the rearing tanks, whereas inert diets were distributed by means of automatic feeders (EHEIM 3851; EHEIM GmbH & Co. KG).

Specimens were randomly collected from each rearing tank at 2, 5, 9, 13, 20, 25, 31, 38, 45 and 51 dph in the morning prior to larval feeding in order to minimize the potential effects of exogenous enzymes from undigested prey in larval fish gut (Kolkovski 2001). Sampled individuals for biochemical analyses were sacrificed with an overdose of tricaine methanosulphonate (MS-222, Sigma), rinsed in distilled water and conserved at −80 °C until their analysis. Growth measurements were obtained from a pool of 20 larvae on the sampling days. Specimens were anesthetised with MS-222, photographed under a dissecting microscope (Nikon SMZ800, Japan) and then sacrificed with an overdose of the above-mentioned chemical. The same individuals were gently rinsed with distilled water, and their dry body mass (M_D) measured after 24 h at 60 °C. Standard length (SL) measured at the nearest 0.01 mm on photographs (300 dpi) using an image analysing system (AnalySIS©, Soft Imaging Systems, GmbH, Germany).

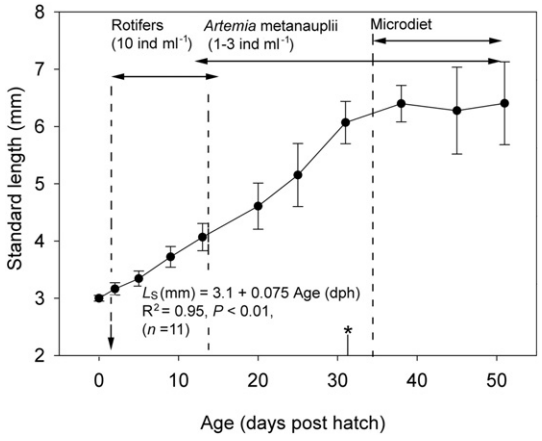

Fig. 1. Growth in standard length (SL) of *Argyrosomus regius* during larval and juvenile development. The different types of live preys and inert diet given to larvae at different stages of development are indicated between dashed lines. The arrow indicates the onset of exogenous feeding and the asterisk the beginning of notochord flexion at 31 days post hatch when larvae measured 6.2 ± 0.4 mm in SL.

2.2. Histology

Ten larvae from each sampling day were dehydrated in a graded series of ethanol, embedded in paraffin and cut into serial sagittal sections (3–5 μm thick). Sections were stained by Harris' Haematoxylin and Eosin (HE) for general histomorphological observations. Periodic acid-Schiff (PAS) was used to detect neutral glycoconjugates in mucous cells and glycogen deposits in the liver, whereas Alcian Blue (AB) at pH 2.5 and 1.0 were used to detect carboxyl-rich and sulphated glycoconjugates in mucous cells (Pearse 1985). Histological preparations were observed in a microscope Leica DMLB (Leica Microsystems, Spain; www.leica-microsystems.com) equipped with a digital camera Olympus DP70 (Olympus España SAU, Spain) and analysed using the digital image analysis software ANALYSIS™ (Soft Imaging Systems GmbH, Germany). The quantification of the areas occupied by fat in the hepatic parenchyma was conducted as described by Boglino et al. (2012), based on the analysis of six randomly chosen fields in five specimens for sampling point according to the formula HFa (%) = $(\Sigma AR_{HFa} / \Sigma AR_{Tt}) * 100$, where HFa corresponds to the level of fat accumulation in the liver, AR_{HFa} is the area of unstained fat deposits within hepatocytes (fat is generally removed during the paraffin embedding process of samples due to organic solvents) and AR_{Tt} the total area of the target tissue in the optical field. Values of hepatic fat accumulation were expressed as the mean ± the standard error (SE).

2.3. Determination of digestive enzyme activities

Larvae younger than 35 dph were completely homogenized for analytical purposes, since they were too small for being dissected, while older fish were dissected in order to separate the pancreatic and intestinal segments as described by Cahu and Zambonino-Infante (1994). Digestive enzyme activities were determined using the following number of specimens per biological replicate at each sampling age: 700–750 at 2 dph, 500–600 at 5 dph, 400–450 at 9 dph, 325–350 at 13 dph, 200–220 at 20 dph, 75–90 at 25 dph, 50 at 31 dph, 40 at 38 dph, 15 at 45 dph and 5 at 51 dph. Dissection was conducted under a dissecting microscope on a pre-chilled glass plate maintained at 0 °C. For quantifying the activity pancreatic and gastric enzymes (trypsin, chymotrypsin, total alkaline proteases, α-amylase, bile salt-activated lipase and pepsin), samples were homogenized (Ultra-Turrax T25 basic, IKA©-Werke, Germany) in 5 volumes (v/w) of ice-cold Milli-Q water, centrifuged at 3300 ×*g* for 3 min at 4 °C, the supernatant removed for enzyme quantification and kept at −80 °C until further analysis. Samples were homogenized in cold 50 mM mannitol, 2 mM Tris-HCl buffer (pH 7.0) in order to evaluate the activity of intestinal (cytosol and brush border membrane) enzymes. 1 ml of the supernatant was pipetted and stored at −20 °C for cytosolic enzyme (leucine–alanine peptidase) quantification. Then, the rest of the homogenate was used for brush border purification according to Crane et al. (1979).

Enzymatic determinations for pancreatic, gastric and intestinal enzymes were conducted as described in Gisbert et al. (2009). In brief, trypsin (E.C. 3.4.21.4) activity was assayed at 25 °C using BAPNA (*N*-α-benzoyl-DL-arginine p-nitroanilide) as substrate. One unit of trypsin per ml (U) was defined as 1 μmol BAPNA hydrolyzed per min per ml of enzyme extract at 407 nm (Holm et al., 1988). Chymotrypsin (EC. 3.4.21.1) activity was quantified at 25 °C using BTEE (benzoyl tyrosine ethyl ester) as substrate and its activity (U) corresponded to the μmol BTEE hydrolyzed per min per ml of enzyme extract at 256 nm (Worthington, 1991). Alpha-amylase (E.C. 3.2.1.1) activity was determined according to Métais and Bieth (1968) using 0.3% soluble starch. Amylase activity (U) was defined as the mg of starch hydrolyzed during 30 min per ml of tissue homogenate at 37 °C at 580 nm. Bile salt-activated lipase (BALT, E.C. 3.1.1) activity was assayed for 30 min at 30 °C using p-nitrophenyl myristate as substrate. The reaction was stopped with a mixture of acetone: n-heptane (5:2), the extract centrifuged for 2 min at 6080 ×*g* and 4 °C and the absorbance of the supernatant read at 405 nm. Bile salt-activated lipase activity (U ml^{-1}) was defined as the μmol of substrate hydrolyzed per min per ml of enzyme extract (Iijima et al. 1998). Regarding intestinal enzymes, alkaline phosphatase (E.C. 3.1.3.1) was quantified at 37 °C using 4-nitrophenyl phosphate (PNPP) as substrate. One unit (U) was defined as 1 μg BTEE released per min per ml of brush border homogenate at 407 nm (Bessey et al. 1946). The assay of the cytosolic peptidase, leucine–alanine peptidase (E.C. 3.4.11) was performed on intestinal homogenates using the method described by Nicholson and Kim (1975), using leucine-alanine as substrate in 50 mM Tris-HCl buffer (pH 8.0). One unit of enzyme activity (U) was defined as 1 nmol of the hydrolyzed substrate per min per ml of tissue homogenate at 37 °C and at 530 nm. Pepsin (E.C. 3.4.23.1) was quantified at 37 °C using 2% haemoglobin as substrate. Pepsin activity (U) was defined as the μmol of tyrosine equivalents liberated per min at 37 °C per ml of tissue homogenate at 280 nm (Worthington, 1991). All enzymatic activities were expressed as specific activity defined as units per milligram of protein (mU mg $protein^{-1}$). Soluble protein of crude enzyme extracts was quantified by means of the Bradford's method (Bradford 1976) using bovine serum albumin as standard. All the assays were made in triplicate from each pool of larvae and absorbance read using a spectrophotometer (Tecan™ Infinite M200, Switzerland) and data presented in total and specific activity units.

2.4. Zymogram characterization of digestive proteases

The characterization of proteases in SDS-PAGE zymograms was performed according to Garcia-Carreño et al. (1993) using 12% polyacrylamide and 8 × 10 × 0.075 cm gels, for samples from fish aged 2, 5, 9, 13, 20, 25, 31, 38, 45 and 50 dph. Volume of loaded samples was depended on protein concentration in every sample (5–10 μg of soluble protein per well). The samples were diluted (1:1) in sample buffer without 2-mercapto-ethanol and not boiled. The determination of different classes of alkaline proteases was conducted as follows: enzyme preparations (10 μl) were mixed with 5 μl of different inhibitor solutions: 10 mM in DMSO (dimethyl sulfoxide), ZPCK (*N*-CBZ-L-phenylanine chloromethyl ketone); 10 mM in HCl 2 mM, TLCK (tosyl-lysyl chloromethyl ketone); 250 μM in water, SBTI (soybean trypsin inhibitor); 0.5 mM in water; E-64 (L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino) butane). After incubation for 1 h at 25 °C, samples were mixed with 15 μl of buffer and loaded on SDS-PAGE plates. Electrophoresis was performed at a constant current of 15 mA per gel for 2 h at 4 °C. After electrophoresis, the excess of protease inhibitor was washed in 50 mM TRIS/HCl buffer (pH 8.5) for 15 min at room temperature. Then, gels were incubated in 50 mM TRIS/HCl buffer (pH 8.5) containing 3% casein Hammerstein for 30 min at 5 °C, and then the temperature was raised to 37 °C for 60 min without agitation. Thereafter, gels were washed, fixed in 12% TCA prior to staining with 0.05% Coomassie Brilliant Blue (BBC R-250) in a 40% methanol, 7% acetic acid solution. Destaining was carried out with the same solution without dye. Amersham Low Molecular Weight Calibration Kit for SDS electrophoresis (Code number: 17-0446-01, GE Healthcare Life Sciences, Spain) was used for determination of approximate molecular weight (MW) of proteases. Molecular weight of proteases was measured using log MW *vs.* migration plot and they were given in kDa.

2.5. Statistical analyses

Enzyme activity was presented as a mean ± standard error of the mean (SEM) of three samples of pooled larvae from each rearing tank. Enzyme activity (total and specific activity) and the level of fat accumulation in the hepatic parenchyma (HFa) between different ages were compared by means of the one-way ANOVA (all data were previously checked for normality and homogeneity of variances by means of the Kolmogorov–Smirnov test and Bartlett's test, respectively) using SigmaStat (SPSS, Richmond, USA) followed by a *post hoc* Holm-Sidak multiple-comparison test. Data expressed as percentage were arc sine

square root transformed. The level of significant difference was set at $P < 0.05$.

3. Results

At the end of the rearing period, *A. regius* survival was 27.3 ± 5.1% and fish measured 7.1 ± 0.8 mm in SL. Fish growth in SL is shown in Fig. 1.

3.1. Histological development of the digestive system

At hatching (3.0 ± 0.1 mm SL), the digestive tract appeared as a straight and undifferentiated tube located dorsally to a large eosinophilic yolk sac with the mouth and anus closed. At this stage, the undifferentiated digestive epithelium was pseudostratified, with central nuclei and evident apical microvilli. At 2 dph (3.2 ± 0.1 mm SL) coinciding with the onset of exogenous feeding, the mouth and the anus were open in all examined specimens, and the yolk sac was almost depleted, although a single oil globule was still present in the posterior part of the abdominal cavity (Fig. 2a). The buccopharynx, oesophagus and intestine were histologically identifiable. The buccopahrynx lined by a squamous epithelium communicated with the anterior intestine through a short oesophagus lined by a pseudostratified cubic epithelium devoid of mucous cells. The straight intestine bent 90° in its posterior region and the intestinal valve appeared as a constriction of the intestinal mucosa dividing the intestine in two regions, the prevalvular (anterior) and postvalvular (posterior) intestine. No histological differences were observed between the pre- and postvalvular intestine: both regions were lined by a simple columnar epithelium with basal nuclei, slightly basophilic cytoplasm and prominent eosinophilic microvilli. The exocrine pancreas was already visible at this age with prominent acini filled with eosinophilic zymogen granules.

At 5 dph (3.3 ± 0.1 mm SL), coinciding with the resorption of the oil globule and the complete transition to exogenous feeding, the oesophagus elongated, first mucous cells containing acid (AB pH = 2.5, 1.0) glycoproteins appeared spread along the oesophageal epithelium (2–3 mucous cells in 100 μm of epithelium) and longitudinal folds surrounded by a circular layer of striated muscular cells were also visible (Fig. 2b). At this age, the intestine grew in length, coiled, villi (20.0 ± 5.0 μm in length; $n = 5$) started to develop and large

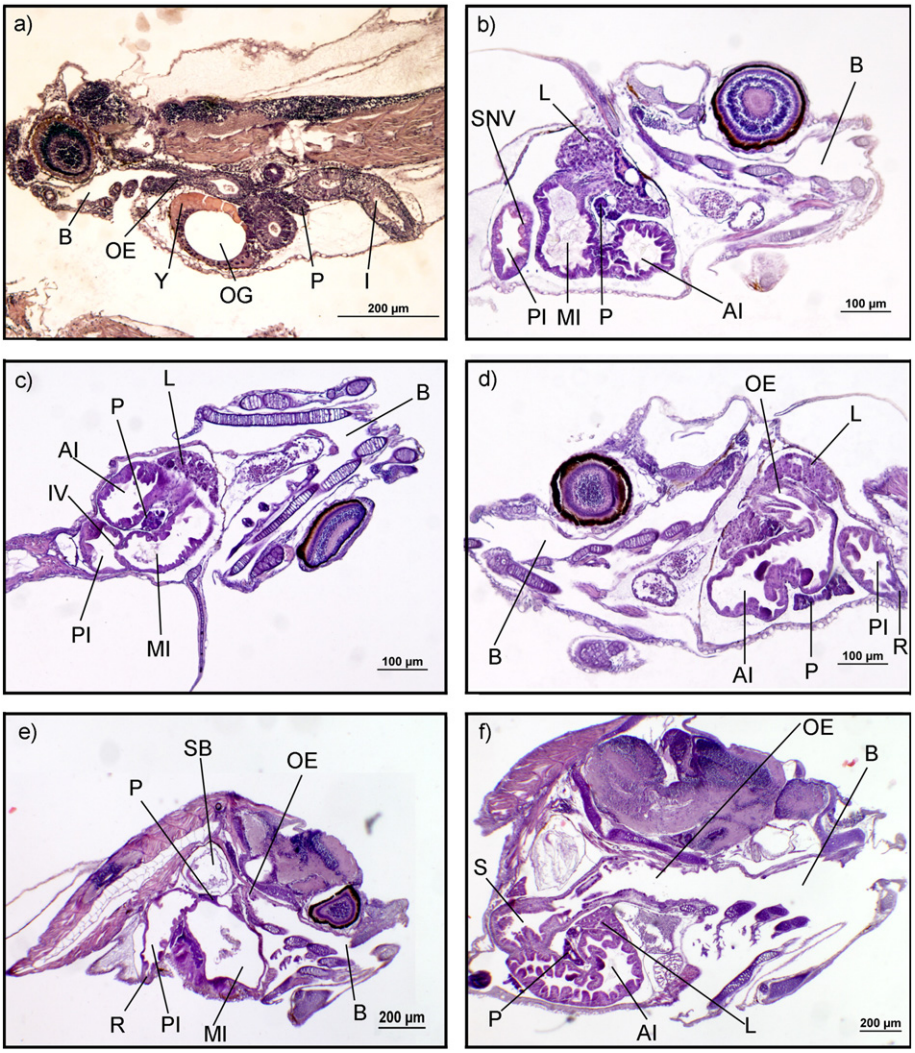

Fig. 2. Histological sections of the digestive system in *Argyrosomus regius* at different stages of development. a) Midsaggital plane section of a 2 days old larva (3.2 ± 0.1 mm SL) showing a large yolk sac and oil globule as well as the rest of the abdominal region; b) ventrally-oriented parasaggital plane section of a 5 days old larva (3.3 ± 0.1 mm SL) showing the complete resorption of the oil globule and the coiling of the intestine; c) ventral view of a frontal plane section a nine-days old larva (3.7 ± 0.2 mm SL) showing the general view of its digestive system; d) midsaggital plane section of a 13 days old larva (4.1 ± 0.2 mm SL) showing a prominent anterior intestine and the folding of the oesophageal mucosa; e) parasaggital plane section of a 20 days old larva (4.6 ± 0.4 mm SL) with a well differentiated digestive system, but still lacking of a stomach; f) parasaggital plane section of a 31 days old specimen (6.2 ± 0.4 mm SL) in which the formation of the stomach as a dilatation between the oesophagus and anterior intestine is clearly visible. *Abbreviations:* AI, anterior intestine; B, buccopharynx; I, intestine; IV, intestinal valve; L, liver; MI, mid-intestine; OE, oesophagus; P, pancreas; PI, posterior intestine; R, rectum; S, stomach; SB, swimming bladder; SNV, supranuclear vacuoles; Y, yolk sac. Staining: haematoxylin-eosin.

Table 1
Changes in the levels of fat accumulation in the hepatic parenchyma (HFa, %) along the early development of *Argyrosomus regius*. Data are shown as mean ± SEM calculated from the analysis of six randomly chosen fields in five specimens per age. Different letters denote statistically significant differences among ages (ANOVA, $P < 0.05$).

	Age (dph)							
	9	13	20	25	31	38	45	51
HFa (%)	0	0	17.5 ± 4.1 d	45.9 ± 8.2 c	37.5 ± 6.1 c	64.1 ± 4.9 b	77.4 ± 3.2 ab	89.6 ± 4.2a

supranuclear eosinophilic vacuoles were observed in the posterior intestine, which indicated the presence of pinocytotic absorption and intracellular protein digestion. No lipid accumulation was found in the posterior intestine or in the liver (Table 1).

At 9 dph (3.7 ± 0.2 mm SL), first mucous cells containing a mixture of acid (AB pH = 2.5, 1.0) and neutral (PAS positive) mucins were observed in the posterior part of the pharyngeal region, as well as the first taste buds. Regarding the oesophagus, it continued to develop and two layers of circular and longitudinal muscular fibres were visible, as well as a dramatic increase in the density of mucous cells (8–10 cells mucous cells in 100 μm of epithelium) containing a mixture of acid (AB pH = 2.5, 1.0) and neutral (PAS positive) glycoproteins. At this stage, the development of villi (40.5 ± 7.5 μm in length; $n = 5$) was the most singular feature dealing with morphogenesis of the intestine coupled with the disappearance of large supranuclear vacuoles in the posterior intestine (Fig. 2c). In this sense, the level of folding of the anterior intestine was more prominent than that of the posterior region, whereas first goblet cells did not appear in the anterior region of the intestine until 13 dph (4.1 ± 0.2 mm SL). At 13 dph, the level of fat deposits in the intestine was similar to that reported at 9 dph.

Between 13 and 20 dph (4.6 ± 0.4 mm SL), first mucous cells containing a mixture of acid (AB pH = 2.5, 1.0) and neutral (PAS positive) glycoproteins were detected in the anterior region of the buccopharynx and in both oral valves (Fig. 3d), whereas they were also firstly found in the anterior intestine (PAS positive; AB pH = 2.5, 1.0 negative). The density of goblet cells in the intestine was higher in the anterior and mid regions of the intestine (4–7 goblet cells in 100 μm of epithelium) than in the posterior segment (1–2 goblet cells in 100 μm of epithelium). Between 13 and 25 dph (5.2 ± 0.6 mm SL), no major histological changes were observed regarding the organization of the digestive tract and accessory glands in *A. regius* with the exception of the increase in size of digestive structures (Fig. 4a), and the presence of lipids in the posterior intestine (Fig. 2e) and liver (55.7 ± 3.3 μm^2; Fig. 3, Table 1). However, between 25 and 31 dph (6.2 ± 0.4 mm SL), the stomach

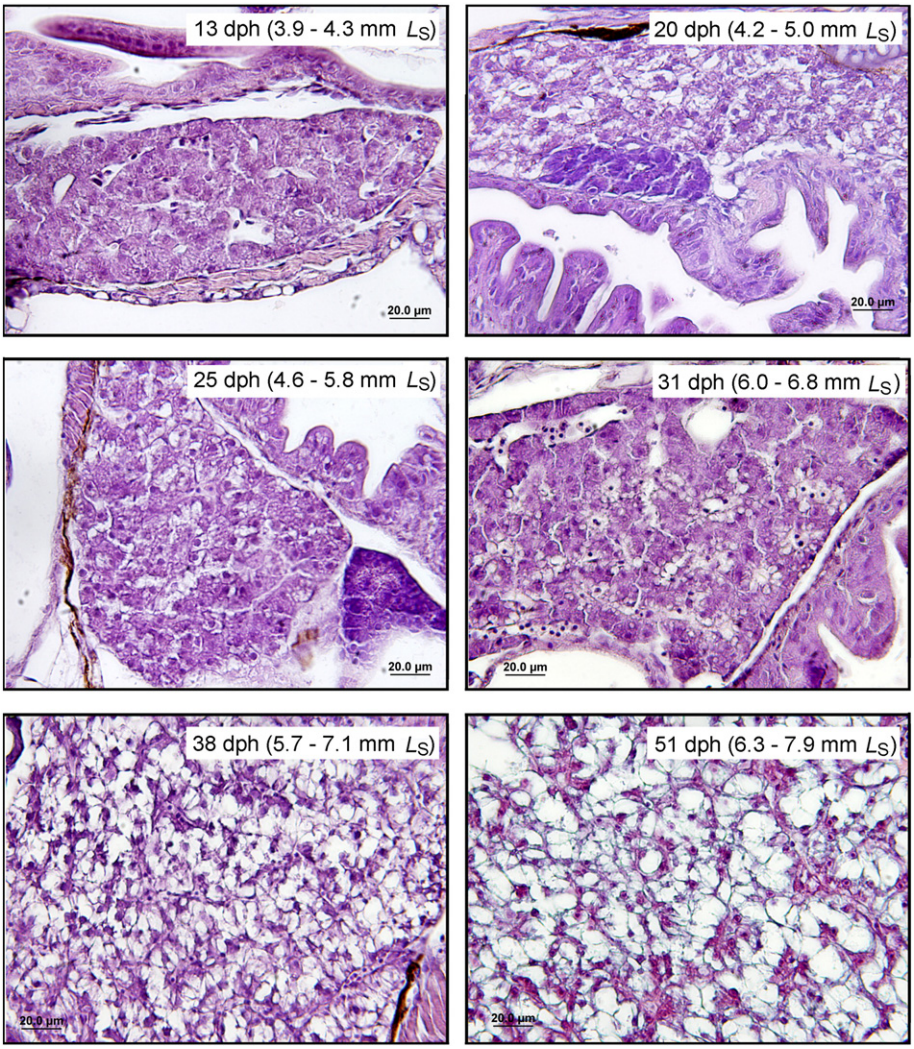

Fig. 3. Detail of the liver in *Argyrosomus regius* at different stages of development showing ontogenic differences in fat accumulation in hepatocytes. Staining: haematoxylin-eosin.

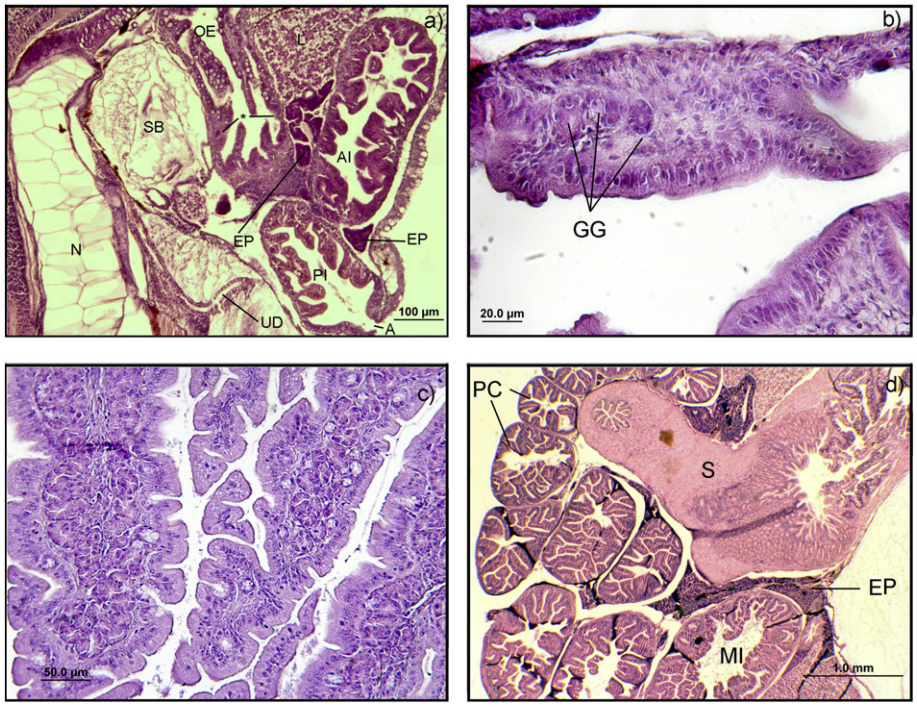

Fig. 4. Different images of the stomach in *Argyrosomus regius*. a) Parasaggital plane section of the abdominal cavity in a 25 day-old specimen (5.2 ± 0.6 mm SL) showing the zone where clusters of cubic cells are going to development into gastric glands at older ages (asterisk); b) detail of the appearance of first gastric glands in a 31 day-old specimen (6.2 ± 0.4 mm SL); c) detail of the fundic stomach in a specimen aged 38 dph (6.4 ± 0.7 mm SL); d) parasaggital plane section of the abdominal cavity in a specimen aged 45 dph (6.5 ± 0.5 mm SL) showing the large development of the pyloric caeca and stomach. *Abbreviations:* AI, anterior intestine; GG, gastric glands; L, liver; MI, mid-intestine; OE, oesophagus; EP, exocrine pancreas; N, notochord; PC, pyloric caeca; PI, posterior intestine; SB, swim bladder; UD, urinary duct. Staining: haematoxylin-eosin.

started to differentiate between the end of the oesophagus and the anterior intestine in 85% of the examined specimens, and first clusters of undifferentiated cubic cells were visible at 31 dph (Fig. 4b), coinciding with the flexion of notochord and the beginning of the formation of the caudal fin complex. Between 31 and 38 dph (6.4 ± 0.7 mm SL), gastric glands increased in number and were clearly distinguishable arranged along longitudinal folds, and surrounded by a thin layer of musculature and connective tissue (Figs. 2f, 4c). During this period, the size of fat deposits in hepatocytes slightly decreased (47.2 ± 2.1 µm^2; Fig. 3) and hepatocytes showed a large compact eosinophilic cytoplasm with small lipid inclusions; however, the area of the hepatic parenchyma with lipid deposits (HFa) did not vary with regards to younger ages (Table 1). At 38–45 dph (6.5 ± 0.5 mm SL), the stomach was completely differentiated into a short cardiac region with prominent folding and scattered gastric glands connecting the stomach with the oesophagus, a large fundic region with a thick circular layer of surrounding musculature and abundant gastric glands, and a funnel-shaped pyloric part with almost no gastric glands, prominent folding and separated from the anterior intestine by the pyloric valve (Fig. 4d). At this time, pyloric caeca were also distinguishable as finger-like projections with a mucosa similar to that of its intestinal counterpart (Fig. 4d), and lined by a simple columnar epithelium covered by prominent eosinophilic microvilli with scattered goblet cells containing a mixture of neutral and acid mucins (PAS and AB pH = 2.5, 1.0 positive, respectively). The size of fat inclusions substantially increased (277.8 ± 22.8 µm^2) as well as HFa values in comparison to former ages (Table 1), and hepatocytes showed large lipid inclusions with nuclei displaced to the periphery of the cell (Fig. 3). From this age to the end of the study at 51 dph (7.1 ± 0.8 mm SL), no major morphological changes were observed in the histological organization of the digestive system with the exception of the increase in size and complexity of the digestive organs, and the increase in fat accumulation in the liver due to the large size of lipid inclusions (491.6 ± 43.1 µm^2) as well as the area of the hepatic parenchyma covered by lipid deposits (Table 1).

3.2. Ontogenic changes in digestive enzyme activities

Changes in pancreatic and gastric digestive enzyme activities along larval development in *A. regius* are shown in Fig. 5. After hatching (3.0 ± 0.1 mm SL), trypsin specific activity progressively increased 16.2 times at peaked 5 dph (3.3 ± 0.1 mm SL), dropping 5.3 times at 9 dph (3.7 ± 0.2 mm SL) and increasing again between 9 and 25 dph (5.2 ± 0.6 mm SL) ($P < 0.05$) when dropped and remained stable between 31 and 51 dph (7.1 ± 0.8 mm SL) ($P > 0.05$) [Fig.5(a)]. Chymotrypsin activity doubled from 2 (3.2 ± 0.1 mm SL) to 9 dph, whereas they progressively dropped until 25 dph ($P < 0.05$) and became stable until 51 dph ($P > 0.05$) [Fig.5(b)]. The specific activity of total alkaline proteases increased between 2 and 9 dph when it reached maximum activity levels ($P < 0.05$), and it sharply decreased at 13 dph ($P < 0.05$), reaching minimum and stable values between 31 (6.2 ± 0.4 mm SL) and 51 dph ($P > 0.05$) [Fig.5(c)]. Pepsin was not detected in *A. regius* until 31 dph and it moderately increased until 45 dph (6.5 ± 0.5 mm SL), whereas it sharply increased at 51 dph when it showed maximum specific activity levels ($P < 0.05$) [Fig.5(d)]. Regarding the total activity of pancreatic proteases, all assayed enzymes followed a similar pattern characterized by a steady and gradual increase in activity between 20 and 25 dph, whereas maximum total activity values were recorded in larger fish at the end of the study ($P < 0.05$). After it first detection at 31 dph, pepsin total activity sharply increased during the following days, reaching maximum values at 51 dph ($P < 0.05$). Alpha-amylase specific activity increased 3.6 times between 2 and 9 dph and then showed a saw tooth profile ($P < 0.05$), and remaining stable between 45 and 51 dph ($P > 0.05$) [Fig.5(e)]. Total activity values for α-amylase remained stable until 31 dph, when they progressively increased between 38 and 45 dph, being maximal at 51 dph ($P < 0.05$). The specific activity of BALT moderately increased between 2 and 5 dph, whereas it dropped 2.0 times at 9 dph ($P < 0.05$). Between 9 and 13 dph, BALT slightly increased ($P < 0.05$) and remained stable between 13 and 51 dph ($P > 0.05$) [Fig.5(f)]. The total activity of BALT remained stable

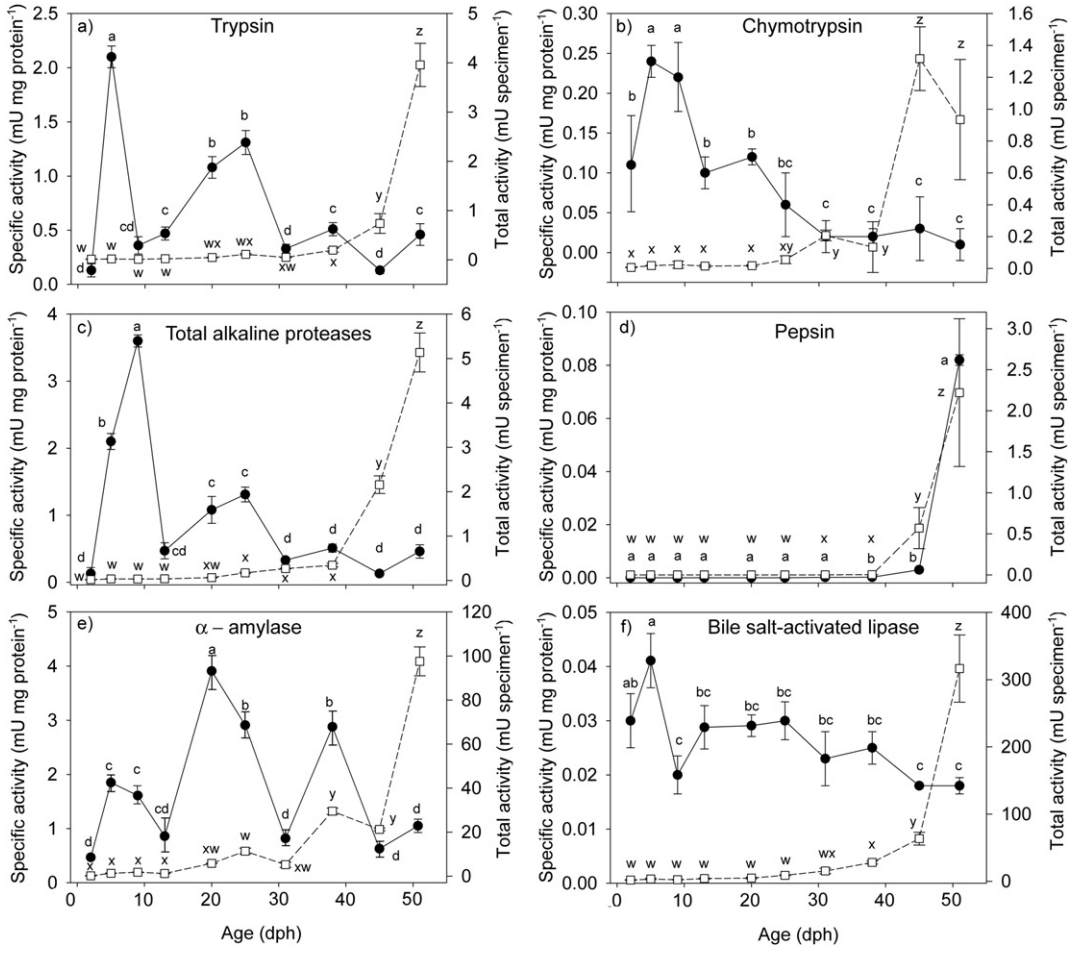

Fig. 5. Specific (●, mU mg protein^{-1}) and total (○, mU specimen^{-1}) activity of pancreatic enzymes (trypsin, chymotrypsin, α-amylase and bile salt-activated lipase) and pepsin from the stomach in *Argyrosomus regius* from hatching to the juvenile stage (51 dph). Different values of enzyme activity (mean ± SE, $n = 3$) with different superscript letters are statistically significant ($P < 0.05$).

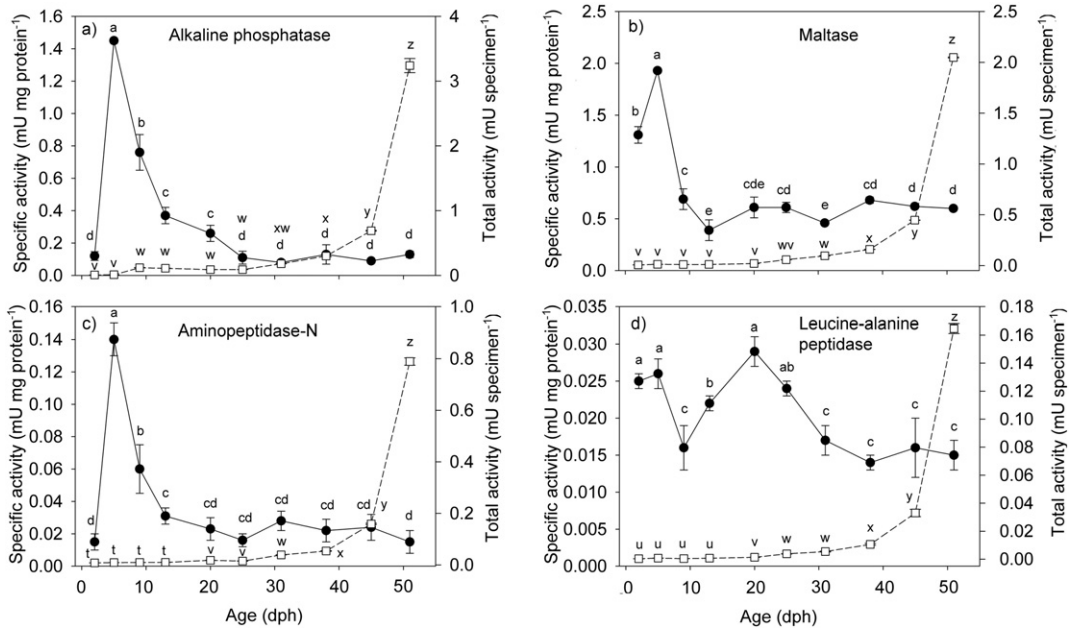

Fig. 6. Specific (●, mU mg protein^{-1}) and total (○, mU specimen^{-1}) activity of brush border (alkaline phosphatase, maltase and aminopeptidase-N) and cytosolic (leucine-alanine peptidase) intestinal enzymes in *Argyrosomus regius* from hatching to the juvenile stage (51 dph). Different values of enzyme activity (mean ± SE, $n = 3$) with different superscript letters are statistically significant ($P < 0.05$).

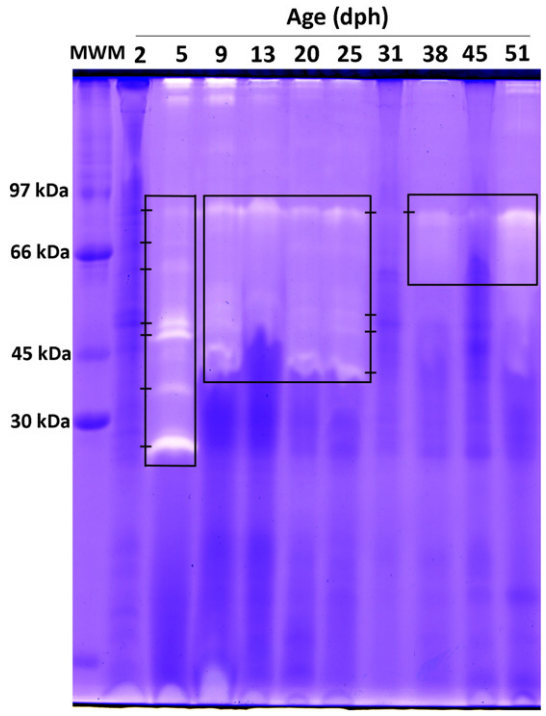

Fig. 7. Substrate-SDS-PAGE of homogenates of *Argyrosomus regius* specimens at different ages. Molecular weight markers (MWM) used were: phophorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (30 kDa). Different columns corresponded to different ages (days post hatch).

from hatching to 31 dph when it sharply increased until the end of the study ($P < 0.05$).

The activity of the three analysed brush border enzymes (alkaline phosphatase, maltase and aminopeptidase-N) followed the same pattern along *A. regius* ontogeny (Fig. 6). The specific activity of the above-mentioned enzymes peaked at 5 dph ($P < 0.05$) and then rapidly decreased until 20 and 25 dph when they remained constant ($P > 0.05$) until 51 dph. The specific activity of the cytosolic enzyme leucine-alanine peptidase dropped between 9 and 13 dph ($P < 0.05$), it recovered between 20 and 25 dph, and decreased and remained constant between 31 and 51 dph ($P > 0.05$). Values of total activity of intestinal enzymes followed a similar pattern characterized by a steady and gradual increase in activity between 13 and 20 dph, whereas maximum total activity values were recorded in larger fish at 51 dph ($P < 0.05$).

3.3. Zymogram characterization of digestive proteases

The zymogram of total alkaline protease activity during the larval stage of *A. regius* is shown in Fig. 7. A set of bands corresponding to the different proteases existing in 38 dph larvae (6.4 ± 0.7 mm SL) were already detected at the age of 5 dph (3.3 ± 0.1 mm SL). In particular, up to 7 characteristic bands were distinguished with an estimated molecular weight ranging from 25 to 78 kDa. Different protease band patterns were identified depending on the age of specimens; from 9 to 25 dph, four bands (MW = 42–78 kDa) were observed, but their staining intensity declined when compared to samples from fish aged 5 dph. Incubation of extracts prior to SDS-PAGE with specific inhibitors against trypsin-like (TLCK), chymotrypsin-like (ZPCK), serine (SBTI) and cysteine (E-64) proteases was conducted for determination of class of proteases. Thus, the comparison of zymograms obtained from 5 dph non-treated larval extracts allowed us to identify bands of 78, 50, 34 and 28 kDa as trypsin-like and chymotrypsin-like proteases. Similar comparisons could be done with 25 dph (5.2 ± 0.6 mm SL) non-treated larval extracts and bands of 78 and 28 kDa were also identified as trypsin-like and chymotrypsin-like proteases, whereas at 51 dph the band of 78 kDa was identified as trypsin-like proteases. All bands with the exception of that of 34 kDa corresponded to serine proteases. There was no inhibition of activity bands of enzyme extracts treated with E-64 (Fig. 8).

4. Discussion

Under present rearing conditions, *A. regius* growth performance was within the range of other studies on this species (Roo et al. 2010; Cardeira et al. 2012; Vallés and Estévez 2012), but much lower than that reported by Papadakis et al. (2013) and Suzer et al. (2013). Differences in growth between the present study and the above-mentioned ones may be due to differences in the feeding protocols and rearing conditions, especially water temperature (18 *vs.* 20–22 °C) and initial density of larvae (200 *vs.* 80 larvae l^{-1}, respectively), among other factors like the nutritional quality of the diet, initial egg size and/or larval quality (Fernández-Palacios et al. 2011; Pittman et al. 2013). As it was shown by Roo et al. (2010), *A. regius* larval density significantly affects

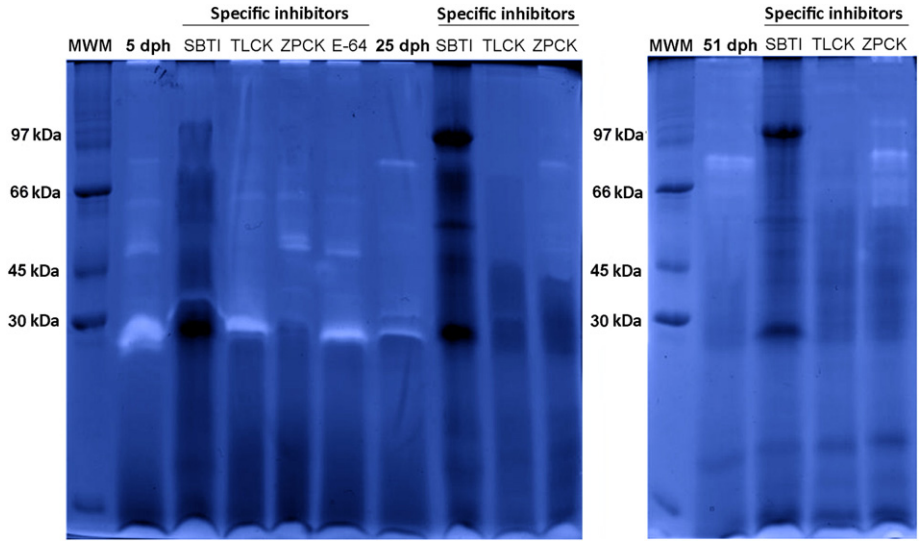

Fig. 8. Substrate-SDS-PAGE of an extract of *Argyrosomus regius* of different ages (5, 25 and 51 dph) treated with different protease specific inhibitors. Molecular weight markers (MWM) used were phophorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (30 kDa).

larval growth in length, being higher when larvae are reared under low density conditions; thus, it seems plausible that the lower growth rates observed in this study were mainly due to the high larval density conditions of this study (200 larvae l^{-1}). The survival rates were similar to those reported by Vallés and Estévez (2012) under similar environmental rearing conditions (*e.g.* feeding sequence, photoperiod, illumination), but much lower than those found by Roo et al. (2010) and Suzer et al. (2013) who reported survival values between 36 and 54%. The lower survival rates observed in our trial with regards to the former studies may be due to differences in larval rearing and husbandry protocols, in addition to the strong cannibalistic behaviour, typical of this species during larval metamorphosis and early juvenile stage (Roo et al. 2010; Vallés and Estévez 2012; Suzer et al. 2013), which might have been triggered by the high fish rearing density.

The first week of development in marine fish larvae is a critical period, particularly when the yolk sac is reabsorbed and exogenous feeding takes place (Gisbert et al. 2013). At the onset of exogenous feeding, *A. regius* larvae depended on the digestive enzymes produced by the exocrine pancreas and consequently, all major enzymes involved in the hydrolysis of the main nutrients (proteins, lipids and carbohydrates) were already detected. Hatchlings have a well-developed morphological and functional exocrine pancreas as indicated by histological (Papadakis et al. 2013; present study) and enzymatic (Suzer et al. 2013; present study) analyses. Similar to other species, alkaline proteases and BALT are the main pancreatic enzymes during the first days after hatching in *A. regius*, indicating that peptides and proteins, as well as lipids are the principal source of energy for sustaining larval growth and development. Among the two endopeptidases analysed in this study, trypsin seemed to have a predominant role in protein digestion in comparison to chymotrypsin; such differences in activity between these two alkaline proteases may reflect differences in protein composition and amino acid profile of live feed (Conceiçao et al., 2003). Regarding the ontogenic changes in the specific activity of alkaline proteases, two peaks in the trypsin and chymotrypsin specific activity were found: the first between 3.2 and 3.9 mm in SL coincided with the resorption of the oil globule and the complete transition to exogenous feeding; and the second of lesser magnitude than the former one, between 4.2 and 5.8 mm in SL, coincided with the completion of the histological development of the digestive tract and accessory glands. In contrast, Suzer et al. (2013) just reported a steady and continuous increase in trypsin specific activity from hatching to *ca.* 5.1–5.3 mm SL (15 dph; Suzer, pers. Com.), which corresponded to the second peak in activity found in our study. These differences in specific alkaline protease activity patterns between these two particular studies might be due to differences in feeding rates and growth performance rather than species-specific traits, since different patterns in trypsin activity (early peak followed by a steady increase *vs.* a gradual increase) have also been reported among different marine species (Gisbert et al. 2009; He et al. 2012; Tong et al. 2012; Hansen et al. 2013; Ma et al., 2014 among others).

Regarding the activity of the two other pancreatic digestive enzymes assayed in this study, α-amylase showed different peaks in specific activity along larval development that matched the feeding sequence established in our trial. In particular, the first peak in activity recorded between 3.2 and 3.9 mm in SL (5–9 dph) corresponded to the complete transition to exogenous feeding and the foraging of *A. regius* larvae on rotifers, whereas the second one matched the change from the rotifer to the *Artemia* metanauplii feeding stage conducted between 4.2 and 5.8 mm in SL (20–25 dph) and differences in carbohydrate and glycogen content between these two types of live prey (Ma et al. 2005). The third peak in specific activity of α-amylase found in our study may correspond to the co-feeding larvae with the compound diet and its content in starch as discussed by Suzer et al. (2013). The above-mentioned changes in α-amylase specific activity in relation to SL closely paralleled those reported by the former authors with the exception of the change in α-amylase activity during the shift from the first to the second type of live prey. Indeed, there is a different rearing feeding schedule between both studies with a more progressive change from the rotifer to the *Artemia* metanauplii feeding stage under Suzer's et al. (2013) rearing conditions. Under culture rearing conditions, BALT specific activity in *A. regius* increased from the onset of exogenous feeding to the complete oil globule resorption, which is mainly composed of steryl and wax esters and triacylglycerides (Jaroszewska and Dabrowski 2011). Thus, the increase in BALT during this period may correspond to the high content of dietary triacylglycerides (Øie et al. 2011) in addition to those contained in oil globule (Jaroszewska and Dabrowski 2011), since they are the main substrates for this pancreatic enzyme. BALT specific activity remained constant during the *Artemia* metanauplii and co-feeding periods, which might be due to the similar total lipids and triacylglycerides levels in these types of feeds (Ma et al., 2004; Gisbert et al. 2005; Boglino et al. 2012; Tong et al. 2012). Ontogenic changes in BALT activity differed during the first days of exogenous feeding between the current study and that of Suzer et al. (2013), which might be due to differences in the rate of oil globule resorption, and total lipids and triacylglycerides levels of diets between both studies. Changes in BALT activity levels did not match with changes in fat deposition in the liver, the main tissue for lipid storage during the larval period (Gisbert et al. 2005, 2008). In this sense, lipid deposition in the liver of *A. regius* from this study was similar to that reported by Papadakis et al. (2013) when using mesocosm techniques. These results seemed to indicate that in addition to the type of food item considered (rotifer, *Artemia*, copepod or microdiet), the pattern of hepatic fat accumulation might be also due to the development of the digestive capacities and the involvement of the liver as one of the main organs for lipid storage during the early juvenile stage in *A. regius*.

The absence of a morphologically distinct and functional stomach during the first weeks after hatching is common to marine fish larvae. No acid digestion occurs before stomach development and digestion is mainly based on alkaline processes (Gisbert et al. 2013). In the present work, pepsin was not detected until 6.0 and 6.8 mm in SL (31 dph), coinciding with notochord flexion and the progressive decrease in the activity of alkaline proteases indicating a shift in the mode of digestion. According to Papadakis et al. (2013), the development of gastric glands in *A. regius* reared under mesocosm conditions was reported at 6.6 mm in SL (SL values estimated from L_T ones, 7.8 mm in L_T, since L_T values are *ca.* 15% higher than SL ones), which fails within the range of size values for SL reported in this study. In agreement with present results and regardless of the age of larval development, the onset of pepsin production and acid digestion in *A. regius* was assessed by Suzer et al. (2013) at *ca.* 5.1–5.4 mm in SL (15 dph), which confirmed that the functional development of the digestive system in *A. regius* is a well-conserved process that generally occurs within a range of body size regardless of larval age and rearing conditions. Deviations from these values obtained under standard rearing conditions may be indicative of problems in larval quality, development and/or rearing conditions as Zambonino-Infante and Cahu (2001) indicated. In the present study, the detection of pepsin activity in *A. regius* occurred some days later than the appearance of the first gastric glands that occurred between 4.6 and 5.8 mm in SL. This indicates that special care needs to be taken when extracting conclusions from histological descriptions regarding stomach functionality and acid digestion, especially when dealing with weaning strategies and early introduction of compound feeds in feeding sequences for larvae. Thus, considering histological and enzymatic data we suggest the onset of weaning in *A. regius* between 6.0 and 6.8 mm in SL, although the earlier presentation of dry feed in rearing tanks is advisable in order to start adapting larvae to this new type of diet.

The last group of enzymes considered in the present study are those from the intestine. In this sense, all the three brush border enzymes analysed, alkaline phosphatase, maltase and aminopeptidase-N, followed the same pattern of specific activity with a peak at 3.1–3.4 mm in SL (5 dph), followed by a sharp decrease in specific activity and constant values in fish larger than 4.2 mm in SL. The above-mentioned increase in specific activity found during the first days after hatching might be

correlated with the fast development of the intestinal mucosa characterized by the growth in length and folding (villi) of the intestine, its coiling and the enterocyte's development. Similar histological developmental features within the same period were reported in *A. regius* reared in mesocosms (Papadakis et al. 2013). The decrease and stabilization of specific activity values for brush border enzymes in fish longer than 4.2 mm in SL can be mainly explained by the increase of tissue proteins in growing larvae and does not correspond to a lowering in the amount of digestive enzymes or dietary shifts (Zambonino-Infante and Cahu, 2001). In addition, the decrease in the specific activity of leucine-alanine peptidase, indicated the progressive maturation of enterocytes (Zambonino-Infante et al. 2008), although the activity of this peptidase cytosolic enzyme was still present in early juveniles (>6.3 mm in SL) indicating that there was still intracellular protein digestion at this stage.

Molecular weight of alkaline protease bands are species-specific and are generally observed within the range of 18–90 kDa (Dimes et al. 1994; Alarcón et al. 1998; Chong et al. 2002). During larval development, the number of bands of alkaline proteases varies as different studies have pointed out (Rathore et al. 2005; Alvarez-González et al. 2010; Chakrabarti and Rathore 2010). Three major patterns of bands were detected in *A. regius* at different ages (5, 9–25 and 38–51 dph). A more complex set of bands was evident in specimens aged 5 dph, whereas the number of bands corresponding to different proteases progressively decreased to just one clearly visible band in early juveniles aged 51 dph. After treating samples from 5 and 25 dph with ZPCK and TLCK, the active bands of MW ~28 kDa could be classified as chymotrypsin- and trypsin-like enzymes. These results were in agreement with those obtained from various species of fish showing quite similar results concerning MW of trypsin and chymotrypsin enzymes (Asgeirsson and Bjarnason 1991; Kurtovic et al. 2006; Klomklao et al. 2007; Cai et al. 2011). The active band (MW = 36 kDa) found in *A. regius* specimens at 5 dph may belong to group of carboxypeptidase-like enzymes (Cohen et al. 1981). In addition, the presence of activity bands in the SBTI-treated samples showed the presence of some non-serine proteases in the enzyme extract. In our study the most conserved band had 75–78 kDa in MW and it was inhibited by TLCK (Kumar et al. 2007; Falcón-Hidalgo et al. 2011). The band patterning of alkaline proteases presented in the current study may serve as a reference for comparison with other studies conducted under different rearing protocols and feeding schemes, as it has been shown by García-Mielán et al. (2014a,b).

5. Conclusions

Ontogenic changes in digestive enzyme activities in *A. regius* followed the same pattern than in other marine fish species, being completely functional between 6.0 and 6.8 mm in SL, even though larvae were reared under very high density conditions resulting in very low growth performance. The present study combining histological and enzymatic analytical procedures evidenced that stomach's functionality did not match its morphological organization, since pepsin activity was not detected until some days after the appearance of the first gastric glands. This indicates that authors need to be cautious when extracting conclusions about the beginning of acid digestion and the onset of weaning of larvae just using histological data. In addition, present results indicated that regardless of the rearing conditions (*e.g.* mesocosm technique, larval density, water temperature and/or feeding sequence), the functional development of the digestive system in *A. regius* assessed by the activity of alkaline and acid proteases is a well-conserved process that generally occurs within the same range of body sizes independently of larval age. Thus, authors recommended using morphometric and/or morphological variables (*e.g.* standard or total length, and/or developmental stage) together with the age of larvae expressed in days after hatching for comparative purposes among different studies dealing with larval development.

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CHAPTER 5

Ontogeny of lymphoid organs and mucosal associated lymphoid tissues in meagre (*Argyrosomus regius)*

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Abstract

This study investigates the histological, histochemical and morphological development of the lymphoid organs and mucosal tissues in larvae and juvenile stages of meagre (*Argyrosomus regius*) For this purpose, larvae of meagre were reared from hatching to the juvenile stage at 18-19 ˚C under mesocosm conditions, using standard feeding sequences of enriched rotifers (2-14 dph), enriched *Artemia* metanauplli (9-31 dph) and commercial feed (21-118 dph). Ontogenic differentiation of early kidney evident at hatching included a visible pronephros, with undifferentiated stem cells at 2 dph (3.2 ± 0.1 mm SL), though the excretory tubules of the kidney were observed at 1 dph (3.15 ± 0.1 mm SL). The kidney developed under the ventral column along the peritoneal cavity, distinctly bi-lobed at its proximal end forming the head kidney and fused in the mid and tail kidney portions.The thymus was detected at 8 dph (4.49 ± 0.39 mm SL) and it was clearly visible at 12 dph (5.69 ± 0.76 mm SL); by 33 dph (15.69 ± 1.81 mm SL) an outer thymocytic zone and inner epithelial zone were visible. The spleen was present at 12 dph, located between exocrine pancreas and intestine, and by 26 dph (11.84 ± 1.3 mm SL) it consisted of a mass of sinusoids filled with red blood cells. Melanomacrophage centers were found by 83 dph (66.25 ± 4.35 mm SL) in the spleen. During 14-15 dph (6.9 ± 1.1 mm SL), goblet cells and rodlet cells appear in the gill and intestinal epithelium, while gill filaments and lamellae of the gills increase in number and length during this period. The lymphoid organs, which appear in the sequence pronephric kidney (1 dph), thymus (8 dph) and spleen (12 dph) remarkably increase in size during the post-flexion stage. While functional studies are needed to confirm the existence of active immune memory, the morphology of the lymphoid organs of meagre suggest that *A. regius*, is not completely immuno-competent earlier than 83 days of age (1834 degree day).

Keywords: histology, lymphoid tissues, meagre, development, thymus, kidney, spleen

1. Introduction

During the last decades an extensive number of studies have been published on organogenesis of commercially important teleost species [1–5]. Studies addressing organogenesis to species level are essential to optimize rearing conditions, develop larva-rearing techniques and ensure their correct and healthy development [6]. Generally, in every rearing practice, the larval stage constitutes a critical moment in which important structural and functional changes in the body tissues, organs and systems are occurring. During this period, the larvae rely entirely on innate mechanisms for immune defense until they develop a functional adaptive immune system [7], and for this reason they are more susceptible to diseases of varying etiology.

In marine fish, the major immune organs are the kidney, followed by the spleen and the thymus that ontogenically appear later [8]. Although the ontogeny of lymphoid organs is basically similar among teleost fishes, the order of appearance of the different organs and components of the immune system varies from one species to another [2]. Within this context, the timing of tissue systems becoming lymphoid is crucial in larviculture management in order to develop optimal rearing protocols and prophylactic measures such as vaccination. Success of larviculture depends on knowledge of larval developmental stages and methods applied to adjust culture conditions to the ontogenic status of the larva. Furthermore, knowledge of the time at which immune memory is attained plays an important role for successful vaccination programs within hatcheries. If this process of immune stimulation is carried out before lymphocyte differentiation is occurring and immunoglobulins are produced there can be an induced tolerance to potential pathogenic stimuli [9].

Meagre is an important candidate for Mediterranean aquaculture due to its fast growth, fillet quality, and low fat content [10]. In addition, this sciaenid species has showed outstanding characteristics for having a low feed conversation ratio 0.9-1.2 under standard dietary regimes [11], viable reproduction in captivity [12], tolerance to captive rearing conditions and the feeding protocols for production of juvenile are well established. Although, larval rearing protocols have already been established [13,14], several factors affect larval quality and survival rates, with the early developmental stages being the most critical rearing period. Most of the published studies so far have been focused on describing the histological development of the digestive tract and its accessory glands [15–17]. However, there are no specific data available regarding

ontogeny of lymphoid organs and maturation of the immune system of meagre, especially during the late larval and juvenile stages.

In this sense, understanding the immune system at early stages of development of meagre is essential to obtain information related to progression of immune-competence, which is necessary for designing vaccination protocols, to increase the capacity of the specific and /or the non-specific immune system. For all these reasons, the aim of this study was to describe the ontogeny of the thymus, kidney, spleen and mucosa-associated lymphoid tissue (gut and gill), along the larval development until juvenile stage in meagre, *Argyrosomus regius*.

2. Material and Methods

2.1. *Fish rearing and sampling procedures*

Larvae used in the present study were obtained by hormonally induced spawning of a meagre broodstock maintained in captivity at the Institut de Recerca i Tecnologia Agroalimentaries (IRTA) in San Carlos de la Rapita, Spain. The broodstock were maintained under controlled natural water temperature and photoperiod using a recirculation aquaculture system (IRTAmar®). Batches of 50,000 eggs were incubated (18 -19 °C) in 35 L mesh-bottomed (300 µm mesh) incubators with aeration and gentle water exchange using and air-lift that were placed in 2,000 L tanks. Hatching rate was determined by estimating the number of larvae (three 100 ml sub-samples) obtained from the stocked eggs.

Larvae were distributed into 1.5 m^3 cylindrical tanks under mesocosm larviculture system. Temperature (22.1 ± 1.2 °C), salinity (36.4 ± 0.77 mg l^{-1}), oxygen concentration (7.8 ± 1.9 mg l^{-1}) and pH (7.9 ± 0.1) were checked daily, whereas nitrites (0.05 ± 0.04) and ammonia (0.14 ± 0.09) were checked two times per week (Hach Colorimeter DR/890, USA). Photoperiod was 16 hL: 8hD and light intensity was 500 lux at the water surface. Feeding protocol was the following: from 2 to 14 dph (days post-hacthing) larvae were fed enriched rotifers (*Brachionus plicatilis*) at a density of 10 rotifers ml^{-1}, and from 9 to 31 dph, larvae were fed enriched *Artemia* metanauplii at a density of 0.5 - 3 individuals ml^{-1}. Both live preys were enriched using Red Pepper™ (Bernaqua, Belgium) for 12 h at 28 ºC in the case of rotifers and 6 h at 25 ºC in the case of *Artemia*. Artificial feeds were added progressively from 21 to 118 dph. Live food was distributed between 2 and 3 times per day in order to maintain live prey density in the rearing tanks, whereas inert diets were distributed by means of automatic feeders. Samples of larvae were randomly collected at

the following points 1, 3, 8, 12, 15, 22, 29, 33, 40, 50, 76, 90, 104 and 118 dph. At days 76, 90, 104 and 118 individual organs (kidney, spleen, gut and gill) were dissected aseptically. Time points were chosen as proxies for the specific periods of rapid changes in organogenesis and rearing practices, but also primarily changes in diet. Growth measurements were obtained from a pool of 10 larvae on the sampling days. Larvae were anesthetized using a high concentration of MS-222 (Sigma-Aldrich, Spain). Data on standard length (SL) were collected at each sampling by measuring to the nearest 0.01mm dimensions from digital photographs (300 dpi) using an image analyzing system (AnalySIS©, Soft Imaging Systems, GmbH, Germany). Additionally, dry weight (DW) was measured on a Mettler A-20 microbalance (Mettler Toledo, Columbus, OH, USA) to the nearest ±1 µg after fish were washed with distilled water, placed in weighted cover slides, and oven-dried at 60°C for 24 h.

2.2 *Histology*

Samples of larvae (n = 10) and organs (n = 2) from each sampling day were fixed in 4% formalin then dehydrated in a graded series of ethanol (70-96%), embedded in paraffin and cut into serial sagittal sections (2-3 µm) with a microtome (Leica RM2155, Germany). Sections were stained with the following protocols: Harris´ hematoxylin and eosin (H&E); Mallory trichromic method [18] and Periodic Acid Schiff - Alcian Blue (PAS-AB pH 2.5) [19]. In order to describe the ontogeny of lymphoid tissues all the sections were observed by microscopy using a Leica DMLB (Leica Microsystems, Spain) equipped with a digital camera Olympus DP70 (Olympus España SAU, Spain) and images (300 dpi) collected were later analyzed using the digital image analysis software ANALYSIS^{TM} (Soft Imaging Systems GmbH, Germany).

3. Results

Figure 1 shows the changes in SL and WWt of meagre larvae during first 118 days of life. Meagre growth was described by the equation LS = $23.69e^{0.01571x}$ (R^2 = 0.9241) and wet weight by the equation W.Wt = $850.57e^{0.0248x}$ (R^2 = 0.9255) between 1 and 133 dph of the experimental rearing. Newly hatched larvae measured 3.80 ± 0.1 mm SL, reaching 109.5 ± 6.6 mm SL by the end of the period studied (118 dph). The developmental stages during larval rearing of meagre in this study were divided into stage 1 = pre-larva (1-2 dph), stage 2 = larva (3-9 dph), stage 3 = pre-flexion (10-13 dph), stage 4 = flexion (14 - 15 dph), stage = 5 post-flexion (16-33 dph), and stage 6 = juvenile (34-118 dph).

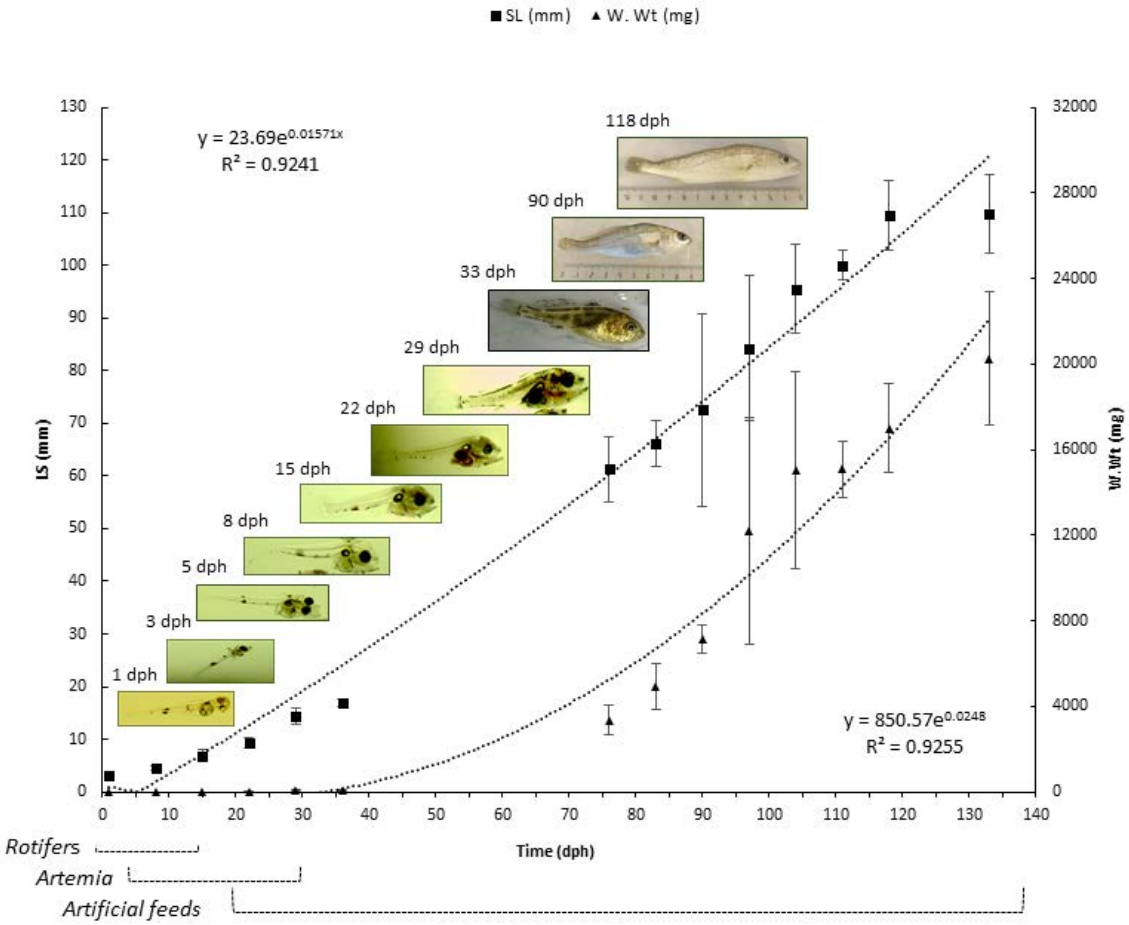

Fig. 1. Growth of meagre (*Argyrosomus regius*) larvae. The main stages during larval development and the larval feeding schedule are also shown. Meagre growth was described by the equation LS = $23.69e^{0.01571x}$ (R^2 = 0.9241) and wet weight by the equation W.Wt = $850.57e^{0.0248x}$ (R^2 = 0.9255) between 1 and 133 dph of the experimental rearing. The square symbol correspond to standard length (mm) and triangle symbol correspond to wet weight (mg).

3.1 *Thymus*

The thymus is located dorso-posteriorly from the branchial cavity. The thymus was clearly observed for the first time at day 8 dph (4.49 ± 0.39 mm SL). At this stage (larva), the organ is found within an epithelial capsule (**Fig. 2a**) and close to the pronephric (**Fig. 2b**). The thymus anlage showed two cell types, a population of reticular/epithelioid cells with large pale nuclei and long cytoplasmic processes, and scattered among them smaller cells with deeply stained nuclei were also detected. The large cells correspond to the reticular cells, forming a reticulum within the thymic parenchyma. As the larva developed, the smaller cells with deeply stained nuclei were identified as lymphoblasts, or small lymphocytes, considering their small dense nucleus with high nuclear to cytoplasmic ratio. At 15 dph (6.9 ±1.1 mm SL), the blast cell decreased and stained small lymphocytes could be distinguished. By 33 dph (15.69 ± 1.81 mm SL), small lymphocytes

were abundant, characterized by small, dense basophilic nuclei with high nuclear to cytoplasmic ratio and two different areas in the parenchyma were apparent (**Fig 2c**). By 36 dph (16.98 ± 0.74 mm SL), distinct regionalization in the thymus was observed, the two zones were differentiated into an outer cortex, packed with small thymocytes and inner medulla with epithelioid-type cells (**Fig. 2 d**). At this stage, the thymus was ellipsoid and extended across the dorso-posterior angle of the branquial cavity from the outer to the inner edge. High magnification showed many small lymphocytes and large cells with pale nuclei and acidophilic cytoplasm and epithelioid cells with long cytoplasmic processes.

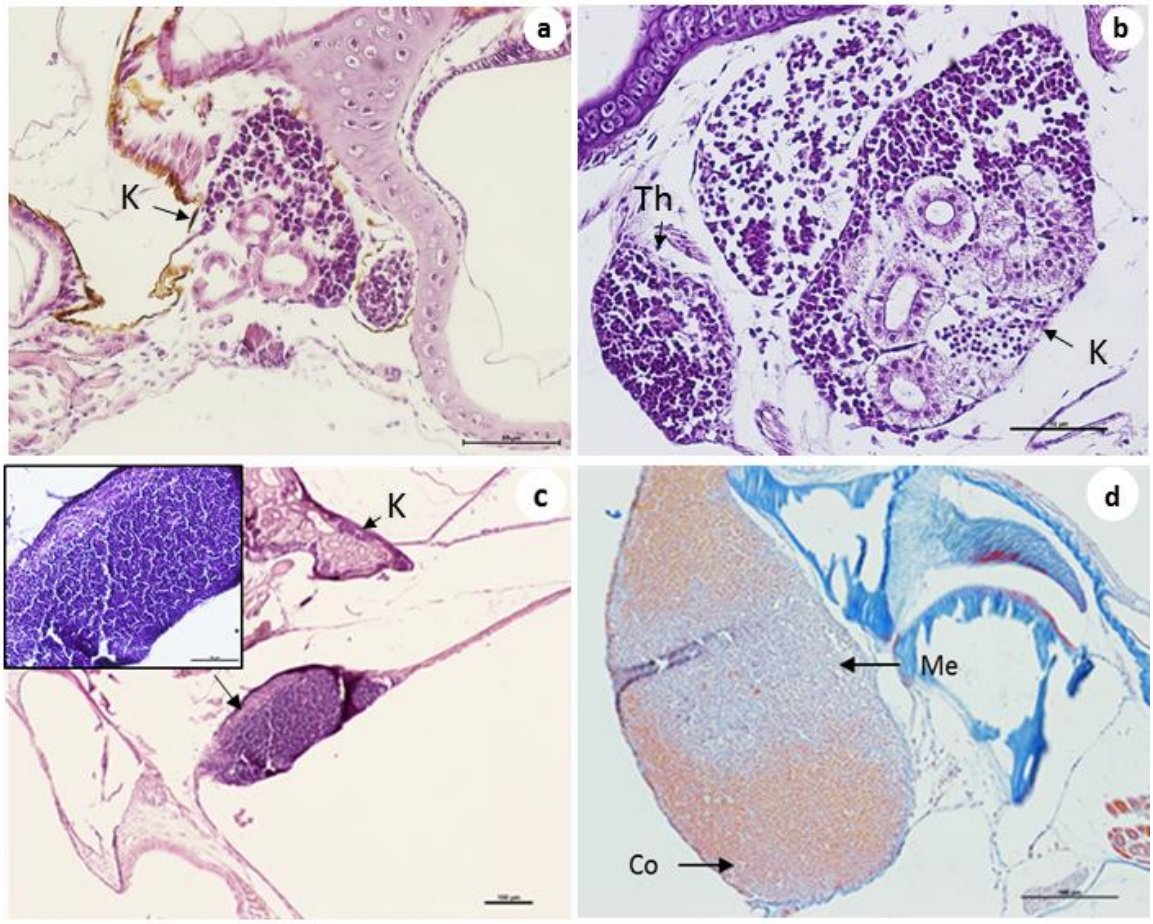

Fig. 2. Development of the meagre thymus. (a) Haematoxylin-Eosin staining at 8 dph undifferentiated basophilic cells in thymus (scale bar = 50 µm). (b) Haematoxylin-Eosin HES staining at 12 dph thymus close the kidney (scale bar = 100 µm) (c) Haematoxylin-Eosin staining at 33 dph (scale bar = 100 µm). (d) Mallory, staining longitudinal section at 36 (scale bar = 50 µm). Th = thymus; K = kidney; Co = cortex; Me = medulla.

3.2 *Kidney*

The kidney was already present at hatching (**Fig. 3a**), consisting of two primordial pronephric straight ducts running below the notochordal axis with a few renal tubules and haemocytopoietic cells. In 3 dph larvae (3.3 ± 0.3 mm SL), renal tubules increased in number (**Fig. 3b**) and the anterior kidney (pronephros) showed the accumulation of many undifferentiated hematopoietic stem cells within the pronephric tubules. As development continued, the blast cells become smaller and stained darker with a concomitant increase in hematopoietic cells at day 12 dph (5.69 ± 0.76 mm SL) (**Fig. 3c**). By 22 (6.9 ±1.1 mm SL) to 29 dph (14.41 ±1.62 mm SL), the proximal renal tubules, the hematopoietic components and blood cells were also observed (**Fig. 3d**). During the pre-flexion stage, the kidney was characterized by the appearance of the pronephros with abundant hematopoietic tissue and the mesonephros with rapid development of the renal tubules. By 33 dph, the dorsomedial area was lymphoid and fused and an extensive blood supply was evident. Phagocytic reticular cells were found outside the vessels and comprising the walls of venous sinuses within the trunk and cephalic kidney. The proportion of lympho-hemopoietic tissues increased, particularly at the pronephros, whereas the mesonephric region is occupied mainly by a large number of tubules and erythrocytes.

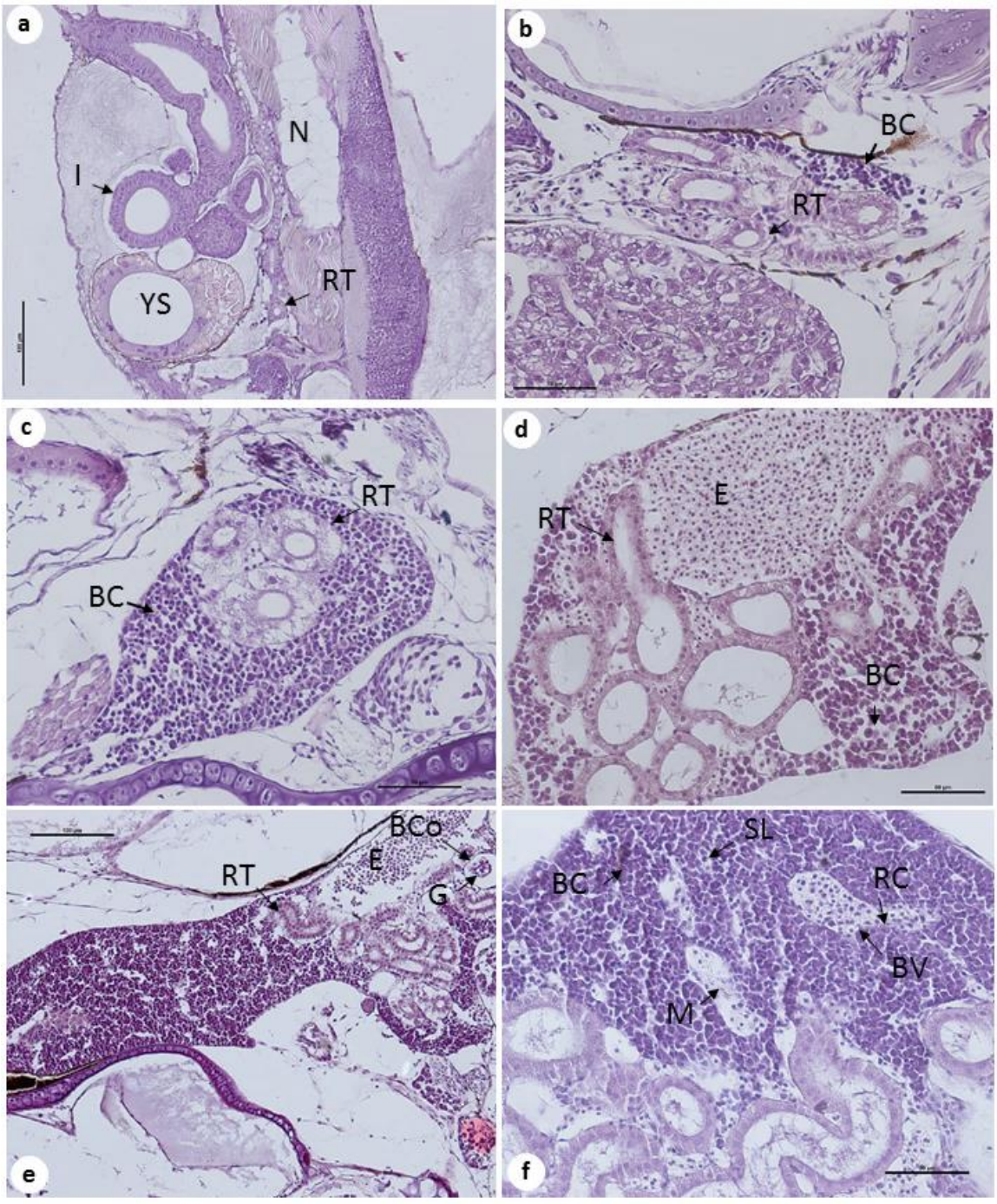

Fig 3. Microsections of the kidney of meagre larvae during stages 2, 3, 4. (a) Haematoxylin-Eosin staining at 1 dph: the excretory parts of the kidney in a larva showing the single tubule (arrow) (scale bar = 100 µm). (b) Haematoxylin-Eosin staining at 8 dph (scale bar = 50 µm). (c) Haematoxylin-Eosin staining at 12 dph (scale bar = 50 µm). (d) Haematoxylin-Eosin staining at 22 dph (scale bar = 50 µm). (e) Haematoxylin-Eosin staining at 26 dph (scale bar = 100 µm). (f) Haematoxylin-Eosin staining at 33 dph (scale bar = 50 µm). SL = small lymphocyte; M = macrophage; BC = blast cell; N = notochord; RT = reticular tubule; E = erythrocyte; YS= Yolk sac; BCo = Bowan´s capsule. RC = reticular cell; BV = blood vessel.

3.3 *Spleen*

The first evidence of appearance of the spleen anlage was seen during the pre-flexion stage (l2 dph), formed by a few blast cells **(Fig. 4a**). As larval development progressed, the spleen showed a change in its initial form of a loose ball of blast cells, and the red blood cells that were also present increase in number. At this stage, the spleen consisted of small spherical clusters of mesenchymal cells near to the exocrine pancreas and the wall of the midgut. The spleen increased rapidly in size and acquired an elliptical shape by day 19 dph (**Fig. 4b**). Sinusoids, with associated red blood cells were evident and the capsule, composed of capsular fibroblasts, was clearly visible by day 26 (**Fig. 4c**). By 29 dph, the spleen consisted of a mass of sinusoids filled with red blood cells. At 47 dph, the organ showed distinct architectural zones divided by white pulp and red pulp, and differentiated ellipsoids were present (**Fig. 4d**). Melano-macrophagic centers were visible at 83 dph (66.25 ± 4.35 mm SL) in the auxiliary of the ellipsoid branches (**Fig. 4f**).

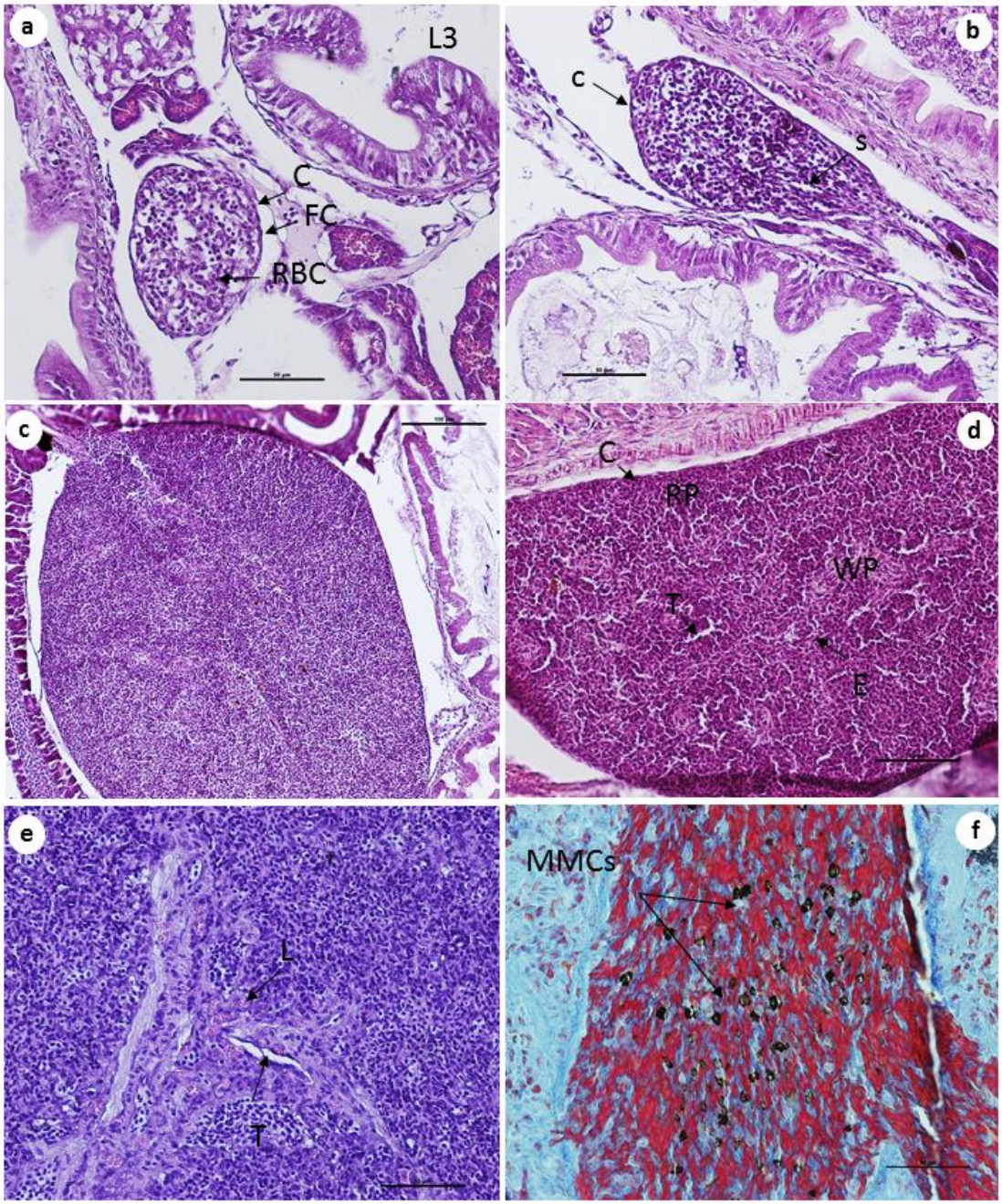

Fig 4 Histological organization and development of the spleen in meagre at various development stages. (a) Haematoxylin-Eosin staining at 12 dph (scale bar = 50 µm). (b) Haematoxylin-Eosin staining at 19 dph (scale bar = 100 µm). (c) Haematoxylin-Eosin staining at 26 dph (scale bar = 50 µm). (d) HES staining at 47 dph (scale bar = 50 µm). (e) HES staining at 66 dph (scale bar = 50 µm). (f) PAS-AB (pH =2.5) staining at 83 dph (scale bar = 50 µm). C = capsule; FC = fibrocytes forming capsule; RBC = red blood cell; S = sinusoid; RP = red pulp; WP = white pulp; T = trabecula; MMCs = center melanomacrophage.

3.4 *Gills*

The gill anlage was observed in the pharyngeal region at hatching. Four distinct ventral pairs of the primordial gill arches, formed by cores of chondroblasts covered with undifferentiated epithelium were observed at 1 dph (3.15 ± 0.1 mm SL) (**Fig. 5a**). At 2

dph (3.2 ± 0.1mm SL), these undifferentiated epithelial cells proliferated towards the pharyngeal cavity. From 3 dph (3.3 ± 0.3 mm SL), gill structures were observed that comprised cores of chondroblasts covered with undifferentiated epithelium, and the pharynx opened (**Fig. 5b**). During stages 3 and 4, the organ noticeably increased in length and the numbers of filaments and lamellae also increased. The first chloride cells at the base of the gill filaments were seen at 15 dph (6.9 ± 1.1 mm SL). The first mucous cells were observed in the gill filament epithelium at this time point. At 29 dph, the number of mucous cells had increased and they could be distinguished from the other cells by PAS positive staining, and were located principally within the epithelium of the primary lamellae near the base the second lamellae. Rodlet cells were detected at 66 dph and increased in number during the subsequent stages (**Fig. 5f**). The gill structure of larvae at 1 month was like that of a juvenile fish.

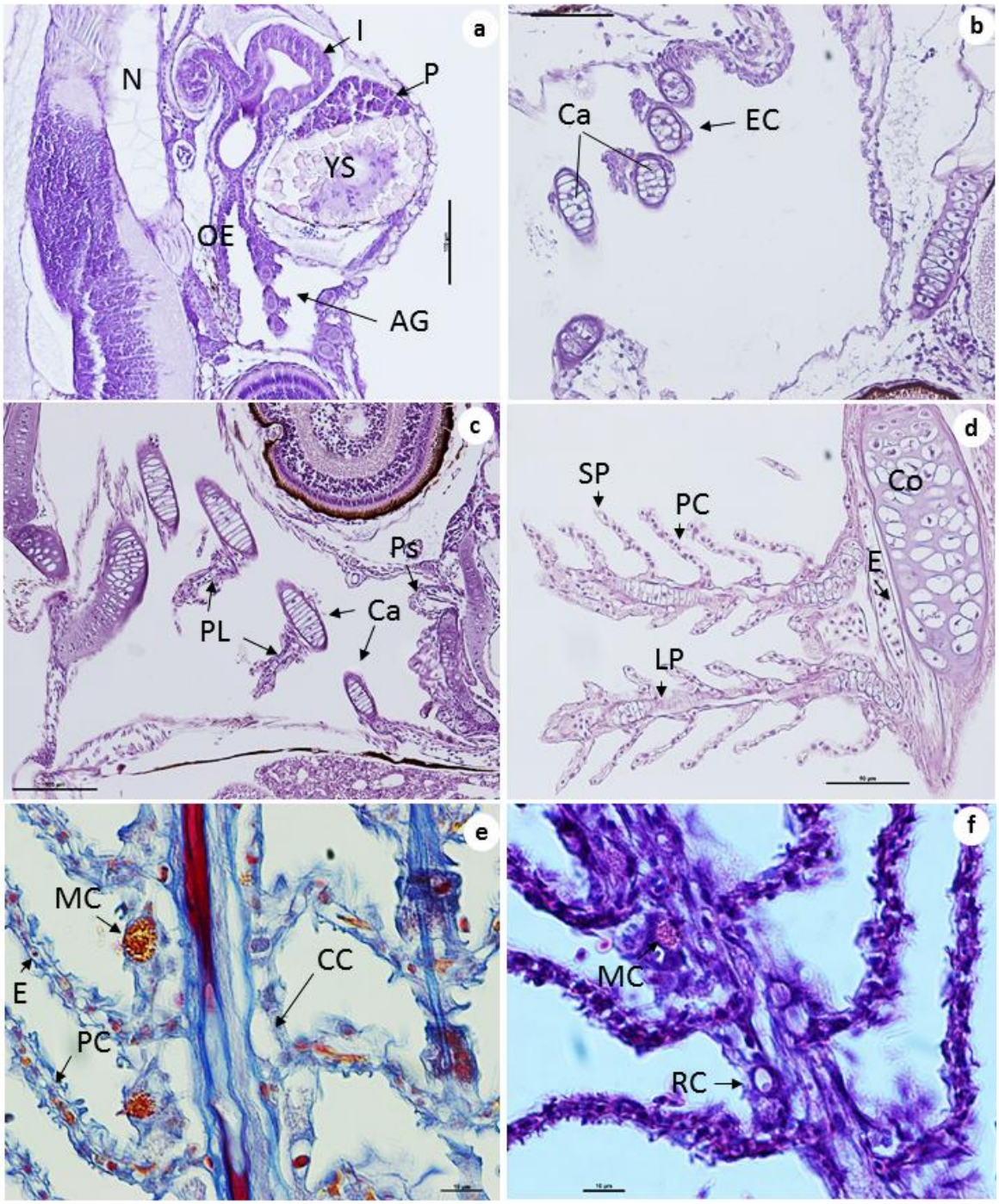

Fig 5 Microsections of *A. regius* larvae during stages 1, 2, 3, and 4. (a) Primordial gill filaments showing cartilaginous frameworks at 1 dph (scale bar = 100 µm). (b) Haematoxylin-Eosin staining at 3 dph (scale bar = 50 µm). (c) Haematoxylin-Eosin staining at 8 dph (scale bar = 50 µm). (d) Haematoxylin-Eosin staining at 22 dph (scale bar = 50 µm). (e) Mallory staining mucous cells at 33 dph (scale bar = 50 µm). (f) Haematoxylin-Eosin staining rodlet cells and leucocytes primary lamella at 66 dph (scale bar = 50 µm). AG = gill arches N = notochord; YS = Yolk sac; P = exocrine pancreas; OE = Oesophagus; EC = epithelial cells; Ca = cartilage; PL= primary lamellae Ps =

pseudobranch; E = erythrocyte; MC= mucous cells; RC = Rodlet cells; PC = pillar; CC = chloride cells.

3.5 *Gut*

The digestive tract at hatching appeared as a straight and undifferentiated tube located dorsally to the yolk sac. At 1 dph, the digestive epithelium was mono-stratified with basal nuclei and evident apical microvilli with the posterior portion of the digestive tube slightly bent (**Fig. 6 a**). At hatching, the mouth and anus were still closed. At 3 dph, the straight intestine bent in its posterior region and a constriction of the intestinal wall appeared dividing the intestine in three regions: anterior, middle and posterior (**Fig. 6 b**). The first mucous cells or goblet cells were observed in the posterior buccopharyngeal cavity epithelium and stained with AB (pH = 2.5) indicating presence of carboxyl-rich acid mucins. As development continued, these cells increased in abundance along the buccopharyngeal epithelium. At day 12 dph (5.69 ± 0.76 mm SL), goblet cells were observed in the middle and posterior intestine (**Fig. 6 d**). A mixture of acid (AB pH = 2.5) and neutral (PAS positive) glicoproteins were found between the enterocytes. By 15 dph (6.9 ± 1.1 mm SL), mucous cells were detected in the anterior intestine. At 12 dph, the *lamina propria* was distinct containing leucocyte-like cells, whereas granular and non-granular cells similar to small lymphocytes were also visible. Small lymphocytes were present with strong basophilic staining of the nuclei and with reduced cytoplasm. Rodlet cells were found in the intestinal epithelia at 26 dph (11.84 ± 1.3 mm SL) (**Fig. 6 f**). As development continued, leucocytes, mucous and rodlet cells proliferated rapidly along the intestine during juvenile stage up to 83 dph (66.25 ± 4.35 mm SL).

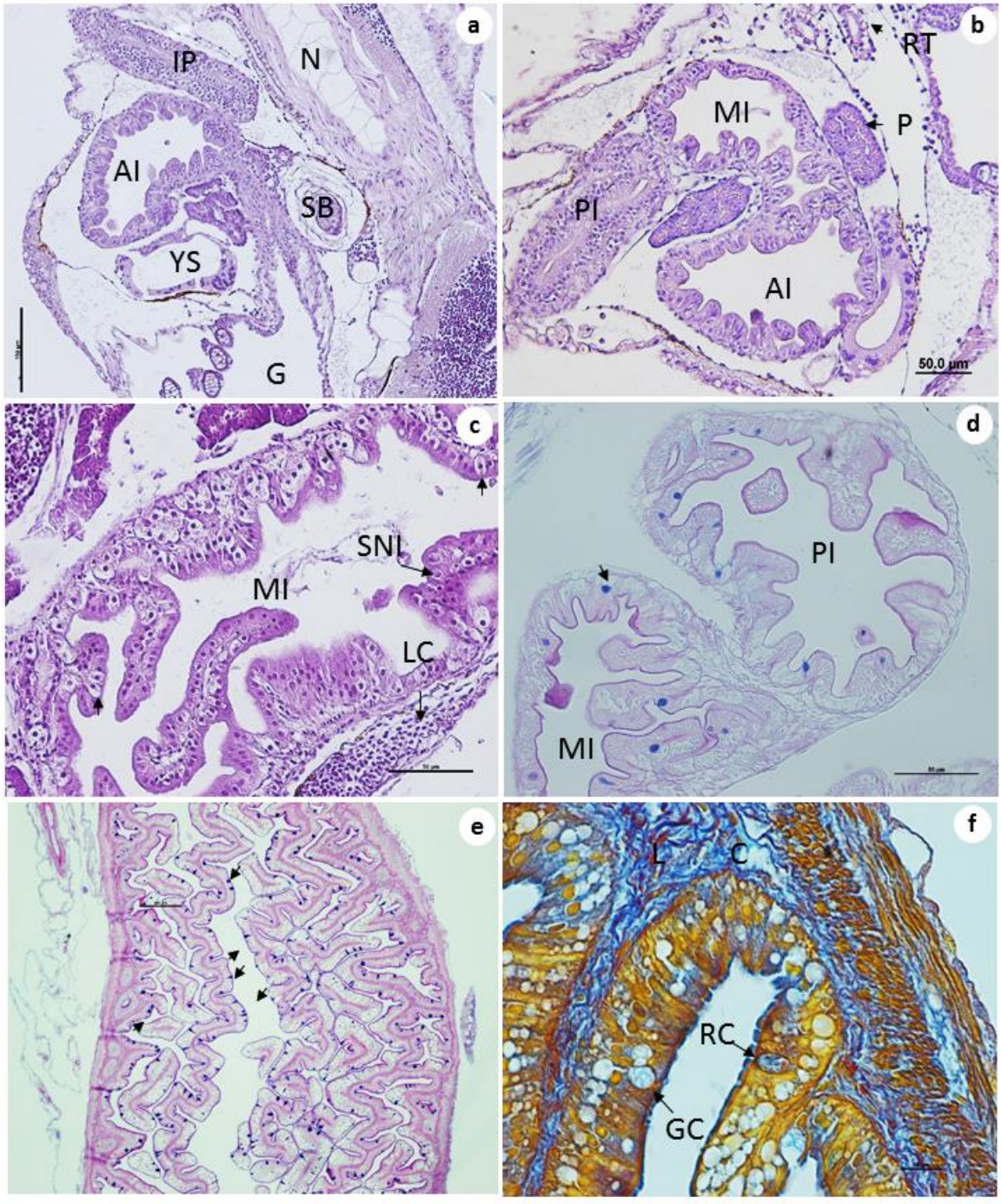

Fig. 6. Microsection of meagre larvae during stages 1-6. (a) Sagittal section of a 1 day old larva showing an anterior and posterior intestine as well as yolk sac (scale bar = 100 µm). (b) Day 3 larvae with three separated intestinal regions (scale bar = 50 µm). (c) Day 12 larvae. Note the intestinal loop and proliferation of lymphocyte cells (scale bar = 50 µm). (d) Middle and posterior region of day 15 larvae, arrowhead indicates goblet cells containing carboxyl-rich glycoproteins (AB positive, PAS negative) as indicated by on AB (pH =2.5) (e) Posterior intestine of day 66 larvae, arrowhead indicates goblet cells (scale bar = 100 µm). (g) Intestine section of 66 day larvae showing rodlet cells in the epithelial mucosa (scale bar = 50 µm). Haematoxylin-Eosin (a,b,c) PAS-AB (d,e) Mallory (f). Abbreviations: L = leukocytes; C = connective tissues.

Table 1. Organogenesis of the main immune organs of some teleost fish assessed histological procedures. The symbol indicates the emergence of the organs -, Days prehatch; +, Days post hatch;?, not known ; L, lymphocytes visible.

		Thymus		Kidney		Spleen		
Species	**T °C**	**Organ**	**L**	**Organ**	**L**	**Organ**	**L**	**Reference**
Marine species								
Argyrosomus regius	22	+8	?	+1	?	+12	?	In this study
Thunnus orientalis	27	+5	+7	<+1	+7	+2	>30	Watts *et al*., (2003)
Seriola quinqueradiata	22	+11	+20	+1	+26	+3	+36	Chantanachookhin *et al.,* (1991)
Cyprinus carpio	22	+2	+4 to +5	+1	+6 to +8	+5	+8 to +9	Botham & Manning (1981)
Pangrus major	20	+11	+22	<+1	+31	+31	+36	Chantanachookhin *et al.,* (1991)
Paralichthys olivaceus	20	10	21	7	28	8	30	Chantanachookhin *et al.,* (1991)
Sparus aurata	19-20	+22 to +29	+29 to +47	<+1	+45 to +47	+12	?	Josefsson & Tatner (1993)
Dicentrarchus labrax	16 - 20	+21	+21	+15	>21	?	?	Breuil *et al*., (1997)
Oncorhynchus mykiss	14	-5	+3	-8	+5	+3	+6	Grace & Manning (1980)
Harpagifer antarcticus	4	+21 to +28	?	<+1	?	+21 to +28	?	O´Neil (1989)
Salmo salar		?	-22	-23	-14	-42	?	Ellis (1977)
Freshwater species								
Ictalurus punctatus	25 - 27	+1	+10	+1	+5	+1	+5	Petrie-Hanson *et al.,* (2001)
Epinephelus bruneus	25	+12	+21	+1	+30	+6	+33	Kato *et al.,* (2004)
Oplegnathus fasciatus	23	+10	+15	-1	+10	+5	+21	Zhizhong *et al.,* (2013)
Tilapia mossambica		-2	+6 to +8	+1	+13 to +16	+1	>+32	Sailendri (1973) in Josefson & Tatner (1993)
Lates calcarifer	28-30	+2	+2	-2	+2	+2	?	Azad *et al.,* (2009)
Hippoglossus hippoglossus	6	+27	+49	+1	+66	+59	+80	Patel *et al.* (2009)

4. Discussion

The main purpose of this study was to describe the timing and morphological development of lymphoid organs in meagre. Our observations demonstrated that the anterior kidney is the first lymphoid organ to develop during the ontogeny. This is consistent with the pattern of development observed in other marine and freshwater species as summarized in Table 1. The immune organs of meagre larvae developed along the following sequence: the pronephric kidney was visible at 3.15 ± 0.1mm SL (1 dph), the thymus appeared at 8 dph (4.49 ± 0.39 mm SL), being clearly visible in larvae measuring 5.69 ± 0.76 mm SL (12 dph), whereas the spleen was detected at 12 dph in this study. Other marine fish have shown a different order of organ development as it is indicated in Table 1. The difference in the timing is influenced by the species' reproductive guild, environmental factors and the immune system evolution in each particular fish species [20]. In northern bluefin tuna (*Thunnus thynnus)* the lymphoid organs and small lymphocytes are present at a much earlier stage compared to meagre. In other species such as tilapia, the thymus anlage is evident as early as 24 hours after fertilization. Generally, in teleost, the thymus is the first organ of the immune system to become lymphoid [20]. However, the development and maturation of the thymus and differentiation into two areas (cortex and medulla) varies amongst fish species. In Japanese flounder, the medulla could not be demonstrated in the thymus, which went through total regression at 7 months [21]; however, two zones have been observed in other fish species like Atlantic cod [22], turbot [23] and channel catfish [24]. In the current study, the thymus in meagre developed in a short time (8-12 dph), while indistinct zones of differentiation were clearly observed at 36 dph (16.98 ± 0.74 mm SL). Although, the degree of involution of the thymus has been reported in other teleosts [25], this character of the thymus is usually achieved in the adult stage, which was not included in this study. Other studies have questioned if lymphoid stem cells become seeded in the thymus, or this organ was colonized by stem cells from the kidney through the cell bridge [26,27]. These cells (those forming a cell bridge) between the thymus and kidney have been described in several fish, such as sea bream, turbot, rainbow trout, and flounder [1,23,28,29]. Regardless of the fact that these "cell bridges" were also observed in meagre, it was not possible to confirm if these cells were originated from the kidney or thymus.

In the case of the kidney, this organ was already visible at hatching located close to the axial skeleton. Similarly to other teleost species, the kidney is divided into the anterior (pronephros) and posterior (mesonephric) compartments and consists of a simple tube. At 1 dph, undifferentiated stem cells and few renal tubules were observed. As the stages of development advanced, the darkly staining cells and undifferentiated hemopoietic stem cells were detected along the pronephric tubules at 8 dph. The proportion of the lympho-hematopoietic tissue increases, particularly at the pronephros at 12 dph, whereas the mesonephric region was occupied mainly by large numbers of tubules and erythrocytes. Similar observations have been made in other marine species [30]. With the advancement in the developmental stage, the pronephric tubules decreased in number, which evidenced their hematopoietic aspect, while the mesonephric regions displayed an increase in the number of tubules in agreement with their main role in filtration. These observations showed that from a morphological point of view the pronephros has less of an excretory function in contrast to other regions of the kidney, but it serves as an important hematopoietic organ and site for B cell maturation and phagocytosis of foreign matter [27,31].

The spleen initially consisted of a loose arrangement of mesenchymal stands located between the exocrine pancreas and the gut at 12 dph. At the early larval phase, the spleen exhibited active erythropoiesis and the principal components were mainly reticular cells and reticular fibers. At 47 to 66 dph, the spleen showed a high degree of vascularization and granular cells were evident around the splenic vein, suggesting the possibility that the early spleen may have a phagocytic function. White and red pulps were slightly different, while melano-macrophages center (MMCs) appeared at 83 dph, indicating that the non-specific defenses in meagre may be capable of handling invading foreign particles before the specific immunity is fully developed. Melanomacrophage centers of the spleen are also known to be major sites of erythrocyte destruction and retain antigens for long periods [27].

The mucosal-associated lymphoid tissues, the gut and gills, were also included in this study. The most striking structural and cellular developmental changes were observed during pre-flexion and flexion stages. There was a notable proliferation of mucous or goblet cells that were first seen at 3 dph (3.3 ± 0.3 mm SL) in the posterior part of the pharyngeal region, whereas mucous cells were detected in the intestine by 15 dph when larvae measured 6.9 ± 1.1 mm SL. Solovyev et al., (2016) [17] found the first goblet cell in pharyngeal region at 9 dph (3.7 ±0.2 mm SL) this results may be due to differences in

larval rearing and husbandry protocols. At 29-60 dph, the density of goblet cells increased in the buccopharynx and intestine. The secretion of mucous cells in fish species plays important roles in absorption of easily digestible substances, lubrication of the gut and protection from viral and bacterial attacks of the digestive mucosa [32,33]. Amongst the innate defense mechanisms present at mucosal surfaces, the mucus is one of the most important ones. The predominant molecules present in mucus are the mucins, which are highly glycosylated proteins that form a mesh-like barrier proximal to the gut epithelium. In fish, the secretions coating external and internal epithelial surfaces contain antimicrobial compounds, including lectins, complement, lysozyme, and antimicrobial peptides [34]. β-defensin (*defb*), hepcidin (*hep2*), piscidin (*pisc*), show high expression levels in larvae between 29 - 40 dph [35], and these observations could be correlated with increased mucous cells in these stages. In seabream gut mucus was demonstrated to change in response to a myxozoan parasite infection, with higher glycoprotein content and increased degree of glycosylation in the posterior intestine [36]. Under this context, intestinal goblet cells are responsible for the secretion of adherent mucus overlying epithelial cells, which constitutes a first line of innate immune defense against offending microorganisms, especially when the larvae do not yet have mature lymphoid organs. In this study, there was no accumulation of lymphoid cells in the gut up to 3 dph, whereas by 12 dph leukocytes appeared in the gut (Fig 6c). The gut was well differentiated with differentiated leukocytes like cells present in the *lamina propria* and also increased in the number of intraepithelial leukocytes from 12 dph to the adult stage.

Our results show that the rodlet cells (RCs) during *A. regius* early ontogeny first appeared in the posterior intestine in 3 dph larvae. Previous studies carried out with common carp (*Cyprinus carpio*) were focused on the migration of the RCs into the *bulbus arteriosus* that started at 10 dph [37], whereas in other species this was observed by 6 dph [38]. Calzada et al., (1998) [39] observed a few RCs in the posterior intestine of cultured sea bream *Sparus aurata* at 20 dph. Although, RCs may have a role as immune system cells, their function still remains poorly understood [40]. In some fish species, rodlet cells have been associated to the role of mast cells/eosinophilic granule cells [41], which are inflammatory cells in fish as well as in mammals. It is recognized that during the early ontogeny, gas exchange occurs primarily through the skin and the primary function of the larval gill is related to osmoregulation rather than respiration. During pre-larva and larva stages meagre had an incipient gill with small gill arches and primordial lamellae and filaments. At the pre-flexion stage (29 dph), the gill had completed full

morphological development and the larvae at this time had completed transition from cutaneous to functional branchial gas exchange. In contrast, most freshwater species [42] have a more developed branchial system during the first period of life. The granular cells that were seen in the spleen, were also present in the gut and gills in meagre larvae. Granular cells increased in abundance with development, especially at stage 5- 6 (16-118 dph). Granular cells are found throughout the body generally, but particularly in association with structures such as blood vessels and nerves, and in proximity to surfaces that interface with the external environment. In fish, granular cells are most abundant in the gills, gut and skin [33]. Granular cells and macrophages are mobile phagocytic cells found in the blood and secondary lymphoid tissues and are important in inflammation, which is the cellular response to pathogen invasion or tissues injury leading to the local accumulation of leukocytes and fluid. Granulocytes, termed eosinophilic granular cells (EGCs) are also involved in the host response to bacterial and helminth pathogens, particularly, at mucosal sites such as the gills and gut. These cells can degranulate, releasing substances involved in the inflammatory reaction in manner analogues to mammalian mast cells [43].

5. Conclusions

A conclusion this work is the sequence and morphological development of the different lymphoid tissues. The pattern of development of lymphoid organs of *Argyrosomus regius* is similar to the general pattern observed in other teleost fish species, but the timing of organ and system development exhibited interspecific differences, which were mainly due to different species' reproductive guilds (precocial *vs* altricial development) . Based on studies from other species on the relationship of the morphology of the lymphoid organs and their functional maturation, it is likely that meagre is not immuno-competent earlier than 83 days of age (66.25 ± 4.35 mm SL) (1834 degree day). However, functional studies should be carried out to demonstrate when they can mount an immune response on the basis of more specific methods such as immunohisto-staining against cell-specific surface receptors to identify and localize lymphocyte classes, or analysis of relative gene expression (q-PCR) from relevant genes involved in specific immune system.

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CHAPTER 6

Ontogeny and modulation, after PAMPs stimulation, of β-defensin, hepcidin, and piscidin antimicrobial peptides in meagre (*Argyrosomus regius*)

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Ontogeny and modulation after PAMPs stimulation of β-defensin, hepcidin, and piscidin antimicrobial peptides in meagre (*Argyrosomus regius*)

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ABSTRACT

Antimicrobial peptides (AMPs), components of innate immunity, play an important role in protecting fish. In this study we report the molecular cloning of full open reading frames and characterization of expression of three AMP genes (*β-defensin* (*defb*), *hepcidin* (*hep2*), *piscidin* (*pisc*) in meagre (*Argyrosomus regius*). A phylogenetic analysis of the expressed sequences obtained shows the *defensin* isoform forms a clade with the other members of the beta class of this family, *hepcidin* corresponds to *hepcidin 2*, and *piscidin* corresponds to class I of its respective family. Gene expression profiles of AMPs was investigated, by means of quantification of mRNA in nine development stages, from 8 days post-hatching (dph) to accomplishment of juvenile form (120 dph). During development it was demonstrated *defb, hep2, pisc* were expressed in all stages of larval development and in juvenile tissues (kidney, spleen gut and gill). Moreover, expression patterns suggest the expression levels of theses AMPs are influenced by live prey (rotifer, *Artemia*) and first intake of commercial diet. Induction experiments *in vivo* (24 h) and *in vitro* (4, 12, 24 h) with PAMPs (LPS, poly (I:C), β-glucan) revealed significant changes in gene expression of the three AMP genes, in kidney, spleen, gut and gill. However, expression profiles differed in magnitude and time course response. *defb* expression shows a similar trend *in vivo* and *in vitro* in kidney at 24 h after LPS and β-glucan stimulation. The *hep2* expression levels were up-regulated upon β-glucan challenge *in vivo*, more in gut and gills than kidney, while *in vitro hep2* expression was up-regulated in kidney cells by LPS, poly (I:C), β-glucan (4 h). *pisc* expression was up-regulated in kidney cells, splenocytes by β-glucan, but in gill cells by poly (I:C) and β-glucan *in vivo*. However, *pisc* expression was upregulated in kidney cells by β-glucan and gill cells by LPS at 4 post-stimulation *in vitro*. These data suggest that AMPs play an important role in defense against pathogens, with each AMP having differing efficacies against specific types of microorganisms, although follow-up studies focusing on the biological activities in fish are needed.

1. Introduction

Antimicrobial peptides (AMPs) are an evolutionarily conserved component of the innate immune response and recognized as a critical first-line defense against many pathogens. These innate effector molecules are present in both vertebrate and invertebrate life forms and play an important role in protection against a broad spectrum of pathogens including those of parasite, bacterial, fungal and viral aetiology [1] by molecular mechanisms of cellular disruption [2]. Fish is continuously fights against pathogens by secreting a wide range of AMPs [3] They are typically present in leukocytes (mast cells, neutrophils), mucus cells (goblet and rodlet cells), cells lining epithelial surfaces and in gill, skin intestine and other tissues [4–7].

As a broad category of innate immune effector molecules, AMPs are divided into different families. Several studies have found peptides in a wide variety of species belonging to the defensin, parasin, cathelicidin and hepcidin families, as well as piscidin, a family unique to teleost fish [8]. Many of the peptides identified have antibiotic activity as well as immunomodulatory functions.

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There has also been a recent effort to study the potential of these peptides for applications in human medicine, and animal welfare [3].

Fish AMPs are up-regulated in response to pathogen and appear to have direct broad-spectrum antimicrobial activity towards both human and fish pathogens. However, the regulation the AMPs transcripts in tissues in response to pathogen challenge is species and gene specific [9]. A major pathway of AMP production is via the recognition of pathogen associated molecular patterns (PAMPs) through the pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), triggering an intracellular signaling cascade involving MyD88, TRAF6, IRAK1 and IKK. This signal promotes the activation and translocation of NF-kB to the nucleus where it transcriptionally activates AMPs and other effector molecules.

Defensins are a family of small cationic, amphipathic, cysteine-rich AMPs found in plants, fungi, invertebrates, and vertebrates, including fish. These AMPs are involved in host-microbiota interactions, immunomodulation, and provide strong links between innate and adaptive immunity. In vertebrates three different Defensin subfamilies exist (α-, β- and θ-defensins) that differ on the basis of the disulfide bridges formed by the pairing and positioning of their six conserved cysteine residues [4,10]. However, α-defensins are only found in mammals whilst θ-defensins exist only in non-human primates [11]. In fish, β-defensin paralogue sequences have been isolated in zebrafish (*Danio rerio*), fugu (*Takifugu rubriges*), tetraodon (*Tetraodon nigroviridis*) and medaka (*Oryziaas latipes*) [12]. A recent study has also identified a novel group of anionic-defensins (fBDI-1 to −5) in flounder *Paralichthys olivaceus* that are expressed during the larval period [10]. Constitutive β-defensin expression varies between species and depends on the tissue type. Their biological functions have been investigated in relatively few fish species and have broad-spectrum antimicrobial, antiviral and chemotactic ability, as reported for gilthead seabream (*Sparus aurata*) and Atlantic cod (*Gadus morhua*) [13,14]. Remarkably, Defensins have also been implicated in some functions related to the reproductive system in orange-spotted grouper (*Epinephelus coioides*) [15].

Hepcidins are another family of cysteine-rich peptides, structurally stabilised by disulphide bridges. They were first identified in human liver [16] and named liver-expressed AMPs (LEAP-1 and LEAP-2). The biological functions of mammalian Hepcidin include antimicrobial activity and regulation of iron metabolism. Hepcidin (Hep) has been extensively documented in many species of fish [8], where it has been shown to respond to bacterial infections and to function in antimicrobial activity. Whilst the highest expression level of hepcidin may be in the liver [17–19], as in mammals, in some fish species [20] these peptides are also expressed in other organs cardiac stomach, esophagus [17] heart, gill, spleen, kidney, peripheral blood leucocytes [18] dependent upon species. In mammals, two hepcidin isoforms are known. However, many teleosts (e.g., Perciform and Pleuronectiform fishes) possess up to seven isoforms of *hep*, as seen in winter flounder (*Pleuronectes americanus*) [17], rockbream (*Oplegnathus fasciatus*) [21] and red-banded seabream (*Pagrus auriga*) [22]. The presence of diverse isoforms might reflect positive Darwinian selection during evolution, as a consequence of different environments and host-pathogen interactions [23].

Piscidins are AMPs that have cationic properties and an amphipathic α-helical structure. A total of 9 piscidins have now been identified in striped bass (*Morone saxatilis*) and 8 orthologous forms have been detected in hybrid striped bass [24]. They represent an evolutionarily conserved family of peptides unique to teleost fish and were first identified in the winter flounder with subsequent studies in hybrid striped bass (*Morone saxatilis* x *M. chrysops*) wherein the name was acquired [5,25]. They are widely active against Gram-positive and negative bacterial species, such as *Streptococcus, Pseudomonas, Bacillus* and *Vibrio* species [8], as well as being anti-fungal and anti-parasitic [26]. The primary mode of action is based on permeabilization of the plasma membrane of the pathogen. The immune-modulatory capacity of piscidins is another feature that has been widely assessed as they are able to modulate the expression of primarily pro-inflammatory genes as well as other immune-related genes, such as IL-1β, IL-10, TNF-α, NF-kb, NOS2, Myd88, TLR1, TLR3 and TLR4a through mechanisms involving a release of inhibition of NF-κB by down-regulation of IKB and, down-regulation of some anti-inflammatory signals such as IL-10, cAMP cascades and also indirect reduction of expression of inflammatory cytokines by elimination of pathogens [27,28]. Similarly, a study in orange-spotted grouper showed the immune-modulatory capacity of the *epinecidin-1* (*epi*) gene by electrotransfer of the *epi* gene in the absence of bacterial infection, increases in the levels of transcripts encoding MYD88, TNF-a, TNF2, and NACHT [29–31]. Studies based on peptide structure, phylogenetic analysis, gene expression and antimicrobial activity have demonstrated that there are three different groups of Piscidin each of which may have different functions.

Meagre (*Argyrosomus regius*) is considered a major candidate species for large-scale fish farming in the Mediterranean and many regions of Europe, due to its favorable characteristics related to fast growth rate and flesh quality. However, captive rearing of fish requires a well-developed larval rearing protocol and several factors affect the quality and survival rate of fish larvae, such as genetic background of the stock, diet, and the environmental conditions in which they are reared. This type of commercial growing necessitates intensive rearing in which crowding can augment skin abrasion/damage and lead to increased stress, facilitating transmission of infectious disease [32]. This study aims to elucidate one aspect of the innate response likely to be crucial in combating infectious bacterial diseases, namely some family members of AMPs that are present, and their level of expression in specific tissues. Target tissues were chosen for their significance as portals of entry of pathogens and their function in hematopoiesis, mucosal tissues of gills and intestine, and systemic tissues of kidney and spleen, respectively. We have characterized a *defb, hep* and *pisc* gene in meagre and describe their expression patterns during larval growout to evaluate when in development these peptides appear. In addition, we have examined how their expression is modulated by a variety of PAMPs, both *in vivo* and *in vitro*.

2. Materials and methods

2.1. Larvae production, and sample collection to determine AMP ontogeny

Larvae used in the study were provided from meagre broodstock held in IRTA San Carlos de la Rapita, Spain. The broodstock were maintained under controlled simulated natural water temperature and photoperiod using recirculation system (IRTAmar®). Breeders were fed frozen sardines (Clupeidae, fishmarket, Sant Carlos de la Rápita, Tarragona, Spain), frozen Patagonian squid (*Loligo gahi)* (from Falkland Islands, Congelados Marcos, Tarragona, Spain) and a broodstock diet (Vitalis Repro, Skretting, Burgos, Spain). During the natural reproductive period (April–June), mature fish were selected based on oocyte size (<550 μm) and ease to extract sperm. Pairs of mature fish (21.2 ± 3.7 kg females and 16.1 ± 2.6 kg males) were hormonally induced (15 μg/kg of des-Gly10, [D-Ala6]-gonadotropin-releasing hormone ethylamide, Sigma, Spain) to spawn spontaneously in 10,000 L tanks and spawned eggs were collected with a passive egg collector placed in the outflow of the tank. Egg number was estimated by counting the total number of

eggs in three sub-samples of 10 mL and fertilization by examining the development of 100 eggs. Batches of 50,000 eggs were incubated (18–19 °C) in 35 L mesh (300 μm mesh) bottomed incubators with aeration and gentle air-lift water exchange that were placed in 2000 L tanks. Hatching rate was determined by estimating the number of larvae (three 100 mL sub-samples) obtained from the stocked eggs.

Larvae were transferred and distributed into two 1.5 m^3 tanks under a mesocosm system. Water temperature was maintained at 20 °C. From two days post-hatching (dph), larvae were fed enriched rotifers until 11 dph. Freshly enriched *Artemia* metanauplii were introduced at 9 dph until 31 dph and a formulated artificial diet was incorporated at 21 dph until the end of the experiment (Fig. 1a). Samples of larvae were collected from post hatch until juvenile for growth measurements. Random samples of larvae and juvenile were taken at 8, 15, 29, 40, 43, 60, 85, 96, and 120 dph for each sample point collected in an Eppendorf tube on ice containing RNAlater™ (Ambion, Austin, Texas), then preserved at −80 °C until RNA extraction. Time points for analysis were chosen as proxies for the specific periods of change in rearing practices; primarily changes in diet, but also rapid changes in organogenesis, all of which increase stress and enhance susceptibly to diseases. The fish were euthanized using a high concentration of MS222 (1 g/L) (Aldrich, E10521) prior to sample collection. Larger larvae (post-29 dph) had excess tissue trimmed to reduce signal dilution from non-target tissues; the anterior section from the gills onwards, and the posterior part from the anus to the tail. The samples were collected from each time point for the study of AMP gene expression. At days

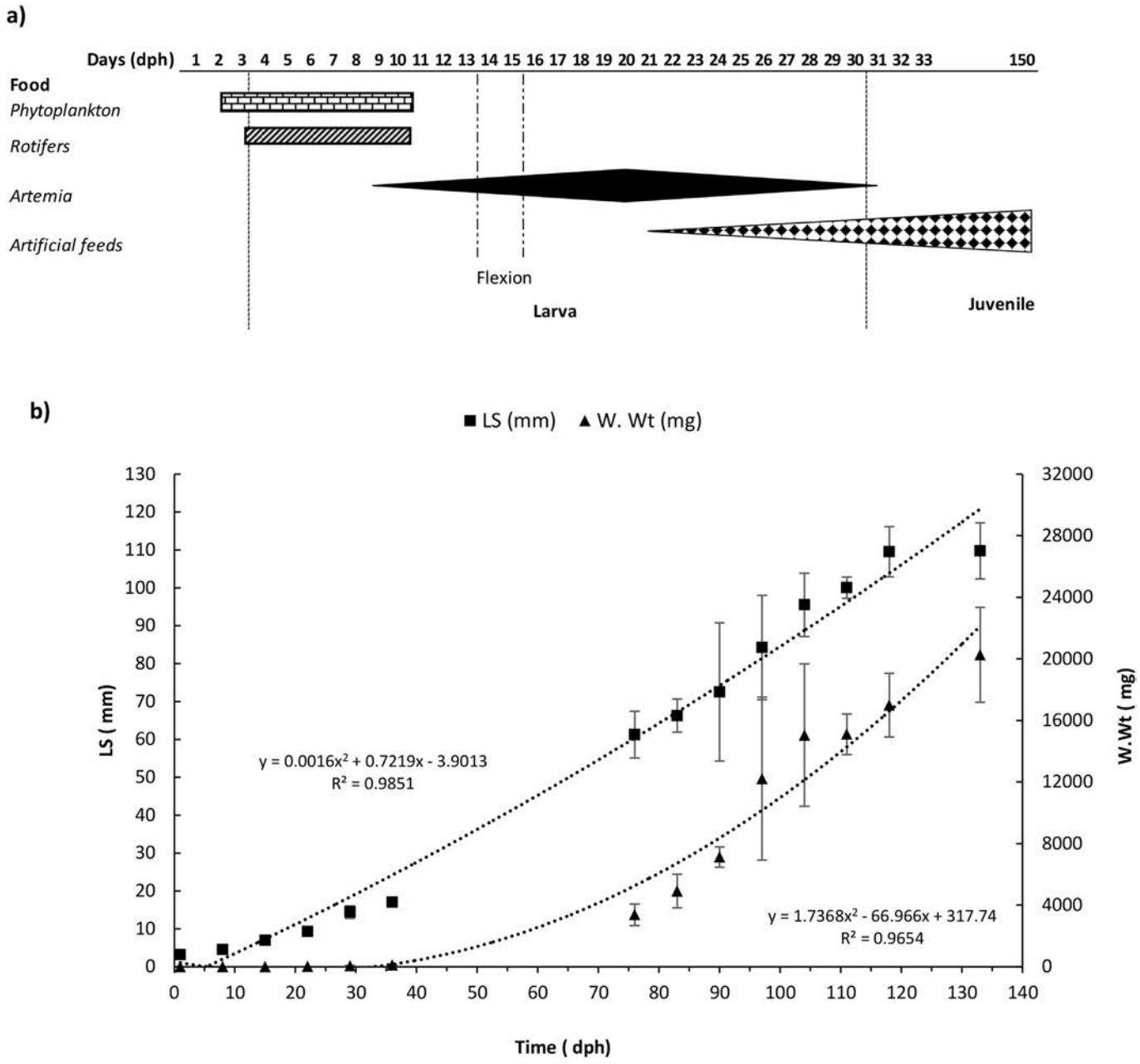

Fig. 1. Growth performance of meagre (*Argyrosomus regius*) larvae. a) The type and relative amount of different feed items offered during rearing are shown as an increase or decrease in thickness of the bar representing rotifers, *Artemia* metanauplii and artificial feeds, respectively. b) Total standard length and wet weight (mean ± SD) during larva rearing, in relation to time (days post-hatching). The regression lines describe the best-fit for growth and wet weight curves of meagre larvae.

85, 96, and 120 individual tissues (gill, kidney, spleen, and intestine) were excised aseptically.

2.2. Total RNA extraction and reverse transcription

Total RNA was extracted using Trizol reagent (Invitrogen) according to manufacturer's instruction. RNA concentration and purity was determined by spectrophotometry (NanoDrop2000, Thermo Fisher Scientific, Madrid, Spain) measuring the absorbance at 260 and 280 nm. The quality of extracted RNA was verified with visualization of the 28S and 18S ribosomal RNA bands by agarose gel electrophoresis. For preparation of cDNA total RNA was treated with DNase 1, Amplification Grade (AMPD1–1 KT, Sigma–Aldrich, Broendby, Denmark), according to manufacturer's instructions to remove possible contaminating genomic DNA. Total RNA was reverse transcribed in a 20 μL reaction volume containing 2 μg total RNA using the ThermoScript ™ Reverse Transcriptase (Invitrogen) with oligo (dT) $_{(12\text{-}18)}$ (0.5 μg/μL) and random hexamer primers (50 ng/μL) 10X RT buffer [200 mM Tris-HCl (pH8.4), 500 mM KCl] 1.5 mM $MgCl_2$, 800 mM dNTP mix, RNase inhibitor, SuperScript ™ II RT, followed by RNAse H (Invitrogen) treatment. Reverse transcription reactions were prepared, which were placed in a thermocycler (Mastercycle® nexus GSX1, Eppendorf AG, Hamburg, Germany) and run according to manufacturer's instruction. Negative controls (no RT enzyme) were included to confirm absence of genomic DNA contamination. The samples were then diluted 1:20 in molecular biology grade water and stored at −20 °C.

2.3. Gene isolation

Meagre were sampled for collecting data on specific growth rate of the chronological samples. The samples were collected in RNAlater at each time point, for extraction of RNA to be used in gene expression analysis. Sequences of target genes from the online database Genbank were chosen from extant marine teleost species that were available, such as large yellow croaker (*Larimichthys crocea*), mandarin fish (*Synchiropus splendidus*), Humphead snapper (*Lutjanus sanguineus*), European seabass (*Dicentrarchus labrax*), orange spotted grouper, but also tilapia (*Oreochromis* spp). The sequences were aligned, using CLUSTAL W incorporated in the package BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), for designing degenerate/consensus primers for amplification from cDNA of tissues. The fragments amplified were separated by gel electrophoresis and resulting bands of the expected length were excised, purified (QIAQuick PCR purification kit, Qiagen) and sequenced (Sistemas Genomicos, Valencia, Spain). The transcript sequences thus obtained were used as templates to design primers for specific q-PCR assays.

For some target genes, there are suspected to be paralogs that could create “noise” for the gene expression analysis if the gene specific primers targeted only the most conserved regions. To avoid this 5′-3′ RACE reactions were performed to obtain the full open reading frame using the method described by Zou et al. [33]. The RACE reactions followed the initial fragment isolation using degenerate-primed PCR with the primers: Pisc F - TGGTTGTTCTCATGGCTGAAC, Pisc R - GGTCA TAAGAAAGTGAACGT, Hep F - ATGAAGACATTCAGTGTTGC, Hep R-CAGCAACCGCAGCAAA, Defb F - GTGCTTCTCCTGATGCTCGC, and Defb R - CTGTATCTTCGAGGGCAAC.

2.4. Phylogenetic analysis

Confirmation of the relation of the AMP sequences obtained to extant marine teleost sequences was performed using a phylogenetic analysis. Evolutionary analyses were conducted in MEGA5 [34] utilizing only the coding portion of the open reading frames of each AMP. The nucleotide substitution model used was chosen utilizing the tool incorporated in MEGA5. The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model [35]. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was <100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise, the BIONJ method with MCL distance matrix was used. The analysis involved 33 nucleotide sequences. All codon positions were included in the analysis, however, positions containing gaps and missing data were eliminated. There were a total of 114 positions in the final dataset.

2.5. PAM stimulation

To investigate the effect of PAMP stimulation on the expression of *defb, hep* and *pisc*, 38 healthy juvenile individuals (30–40 g each) of meagre were held in a recirculating water tank (100 L) with seawater at 20 °C. The fish were fed with a commercial diet twice a day (9:00 h and 16: 00 h) for two weeks to acclimate them to the indoor culture environment. For primary cell culture and collection of samples, fish were killed after anesthesia with 50 mg/L MS-222.

2.5.1. AMPs expression after in vivo stimulation

Fish were injected intraperitoneally with 100 μL PBS containing 100 μg poly (I: C) (3.3 mg/kg) (Sigma, UK. P1530), 400 μg LPS (13.3 mg/kg) (Sigma, UK. L3129) or 100 μg β-glucan (3.3 mg/kg) (Sigma, UK. 89862). The control animals were injected with PBS only. After 24 h, the individuals were dissected and tissues (kidney, spleen, gut and gill) sampled. Total RNA was isolated and cDNA was prepared as described above. The mRNA expression level of *defb, pisc* and *hep* were determined by q-PCR. Relative expression was normalized to GAPDH expression and calculated as arbitrary units and converted to a proportion relative to the PBS control samples.

2.5.2. AMPs expression after in vitro stimulation

Tissues (kidney, spleen, gill, intestine) from apparently healthy fish were passed through a 100 μm nylon mesh cell strainer (SefarNytal PA-13xxx/100, Spain) in Leibovitz L15 medium (Gibco) containing penicillin/streptomycin (Gibco, #15140-122) at 1:1000 and 2% foetal calf serum (Gibco, #10270-098). The resulting cell suspension was collected and centrifuged at 400 × g for 10 min. The supernatant was removed and replaced with 10 mL of the previously described L15 media. The cell suspension was again centrifuged and supernatants removed and replaced with 30 mL of media. Cells were distributed to 12 well microtiter plates in 5 mL aliquots. Wells were stimulated using LPS (Sigma, #L3129-100 MG) at 50 μg/mL, poly (I:C) (Sigma, #P1530-25 MG) at 100 μg/mL, and β-glucan (Sigma, #89862-1G-F) at 50 μg/mL in triplicate. Control samples included 250 μL of PBS. Four, 12 and 24 h after stimulation the cells were harvested and centrifuged at 400 × g for 10 min, the supernatant discarded, and the pellet suspended in RNAlater. Total RNA was isolated and cDNA was prepared as described above.

2.6. Real-time q-PCR (RT-qPCR)

The q-PCR reactions for AMP gene expression were carried out in duplicate on a LightCycler® 480 Real-Time (Applied Biosystems, Roche). A master mix was prepared from: 6 μL SYBR Green Supermix (Life Technologies), 10 μM of each primer, diluted in molecular biology grade water (Sigma) and 4 μL of cDNA in a final volume of 10 μL. The real-time q-PCR cycling was carried out as follows: 10 min at 95 °C, 40 cycles of 95 °C for 25 s, followed by an annealing step of approximately 59 °C for 25 s (annealing temperatures were

adjusted for each specific primer pair), followed by 72 °C for 15 s, with a final melt curve stage of 0.5 °C increments from 75 °C to 95 °C. Each sample on the q-PCR plate had two technical replicates. Primer sequences used and amplicon lengths for each assay are shown (Table 1). The specificity of the primers was checked by running a q-PCR and confirming that only one melt peak was produced and also by running a subsample on an agarose gel (2%) to confirm the presence of a single band of the expected size. The efficiency of amplification (E %) of each primer pair was assessed from five serial ten-fold dilutions of cDNA from individual tissues, then calculated following the equation: $E\% = 10^{(-1/\text{slope})} - 1$ where the "slope" is that calculated from the regression line of the standard curve. Efficiencies of the gene expression assays ranged from 101.95% to 104.3% (102.85 ± 1.27) (Table 1). The absence of primer dimer formation in the NTC was also confirmed. Relative expression of genes from larvae, and each tissue from juveniles (ontogeny study), was normalized using the three endogenous controls glyceraldehyde phosphate dehydrogenase (GAPDH), beta-actin (β-actin), and hypoxanthine-guanine phosphoribosyltransferase (HPRT) as determined using geNORM (http://www.primerdesign.co.uk/products/9461-genormplus-kits/), while the relative quantification at 120 dph, showing the least expression, was used as the calibrator.

For analyzing the relative expression *in vivo* and *in vitro* after PAMP stimulation, data was normalized to GAPDH expression, as this endogenous control gene showed less variability under the conditions tested. After normalization to this endogenous control as arbitrary units, results were converted to a proportion relative to the control group (PBS injected fish).

2.7. Statistical analysis

All data were checked for homogeneity of variances using a Levene's test by univariate analysis in a general linear model, based on a Tukey HSD post-hoc test, with a sample size of $n = 10$ (larva) $n = 8$ (juvenile) to determine differences between time points ($P \leq 0.05$). The Kruskal-Wallis test was used for analyzing the expression for the *in vivo* ($n = 8$) and *in vitro* ($n = 6$) samples after PAMP stimulation, using the statistical software package SPSS 20.0 (SPSS Inc., US).

3. Results

3.1. Gene isolation

Specific gene products were obtained using degenerate or consensus-primed PCR assays. All sequences were analyzed using the BLAST utility via the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nig.gov/) to confirm their identity as proper orthologs of the intended target gene. Sequencing followed by BLAST analysis provided presumptive identification of AMP transcripts for *defb, hep* and *pisc*, reported for the first time in this species. Sequences were also obtained for GAPDH, β-actin and HPRT as endogenous control genes for gene expression assays for which sequences have been provided in GenBank under the following accession numbers MF186586, MF186587 and MF186588, respectively. The AMP sequences have been uploaded to GenBank under the following accession numbers and length the fragment *defb* = MF074072 (195 bp), *hep2* = MF074074 (255 bp) and *pisc* = MF074073 (216 bp).

3.2. Phylogenetic analysis

To verify the relationship of meagre AMPs to known AMP genes, the *A. regius* sequences were used to construct a phylogenetic tree with extant marine teleost sequences of AMPs (when available) (Fig. 2). Results indicate the meagre hepcidin gene is more similar to that of *hep 2* from *Pseudosciaena crocea* and species of the moronidae family (*Dicentrarchus labrax* and *M. chrysops*). The *defb* sequences appear to be most closely related to mugilidae (*Liza haematocheila*). Meagre *pisc* was included in the class I clade of piscidins according to the scheme of Salger et al. [24], with affinity to *Larimichthys crocea* piscidin supported by a significant bootstrap value (94).

3.3. Gene expression analysis

3.3.1. Ontogeny of AMPs

In the present study, AMP expression during larval development has been assessed. Meagre growth was described by the equation LS = 0.0016 dph + 0.7219 dph - 3.9013 ($R^2 = 0.9851$) and wet weight by the equation W.Wt = 1.7368 dph - 66.966 dph + 317.74 ($R^2 = 0.9654$) between 0 and 133 dph of the experimental rearing (Fig. 1b).

The expression of *defb* exhibited marked differences between the larval and juvenile stage, with a statistically significant increase in expression during transition from *Artemia* to standard commercial dry pellet feed at 29 dph, followed by a gradual decrease until 60 dph. Expression patterns of *defb* transcripts in individual tissues show significantly different expression profiles from 85 dph to 120 dph. The highest expression was seen in kidney compared to other tissues tested, however, in spleen there was no difference in expression over time. (Fig. 3a).

The *hep2* expression in larvae remained markedly low from day 8 to day 15, where the expression was increase at day 29, when the switch from feeding on *Artemia* to artificial feed is nearly complete. In the analysis of separate immune tissues a gradual increase of expression was observed over time (85, 96, or 120 dph) in spleen, gill and gut. Although the trend of increase in expression over time is similar in all three of these tissues, a difference in mRNA level was only statistically significant in gut, where an increase was seen at 96 to 120 dph. However, *hep2* gene expression was highest in kidney compared with the other tissues (gill, gut, spleen) (Fig. 3b).

The *pisc* transcripts showed a pattern of expression markedly different across developmental stages. The *pisc* gene expression remained low from 8 to day 29, where the expression increased at

Table 1
Primers used for gene expression analysis by real-time q-PCR, including the amplicon size and primer sequences. Abbreviations: *gapdh*; glyceraldehyde 3-phosphate dehydrogenase; *β-actin*, beta-actin; *hprt*, hypoxantine-guanine phosphoribosyltransferase; *defb*, β-defensin; *hep*, hepcidin; *pisc*, piscidin.

	Genes	E (%)	Size (bp)	Forward primer (5′- > 3′)	Reverse primer (5′- > 3′)
Reference genes	*gapdh*	100.00	109	CCAGTACGTGGTGGAGTCCACTG	AGCGTCAGCGGTGGGTGCAGAG
	β-actin	100.05	212	TGGGGGAGCAATGATCTTGATCTTCA	AGCCCTCTTTCCTCGGTATGGAGTC
	hprt	100.95	137	CATGGACTCATCTTGGACAGGACAGA	GCCTTGATGTAGTCCAGCAGGTC
Amp genes	*defb*	102.30	138	GGGAACGAAGATCCAGAGATGCAGTATTGGAC	CTAAGACCTCACAGCACAGCACCTG
	hep	104.30	140	CCGTCATGCTCGCCTTCG	CTCACGCATGTAATACGGAATCTTGCATG
	pisc	101.95	111	CAATGATCCATGGGCTTATCC	TTCAGTCTCGCCATTGAAGC

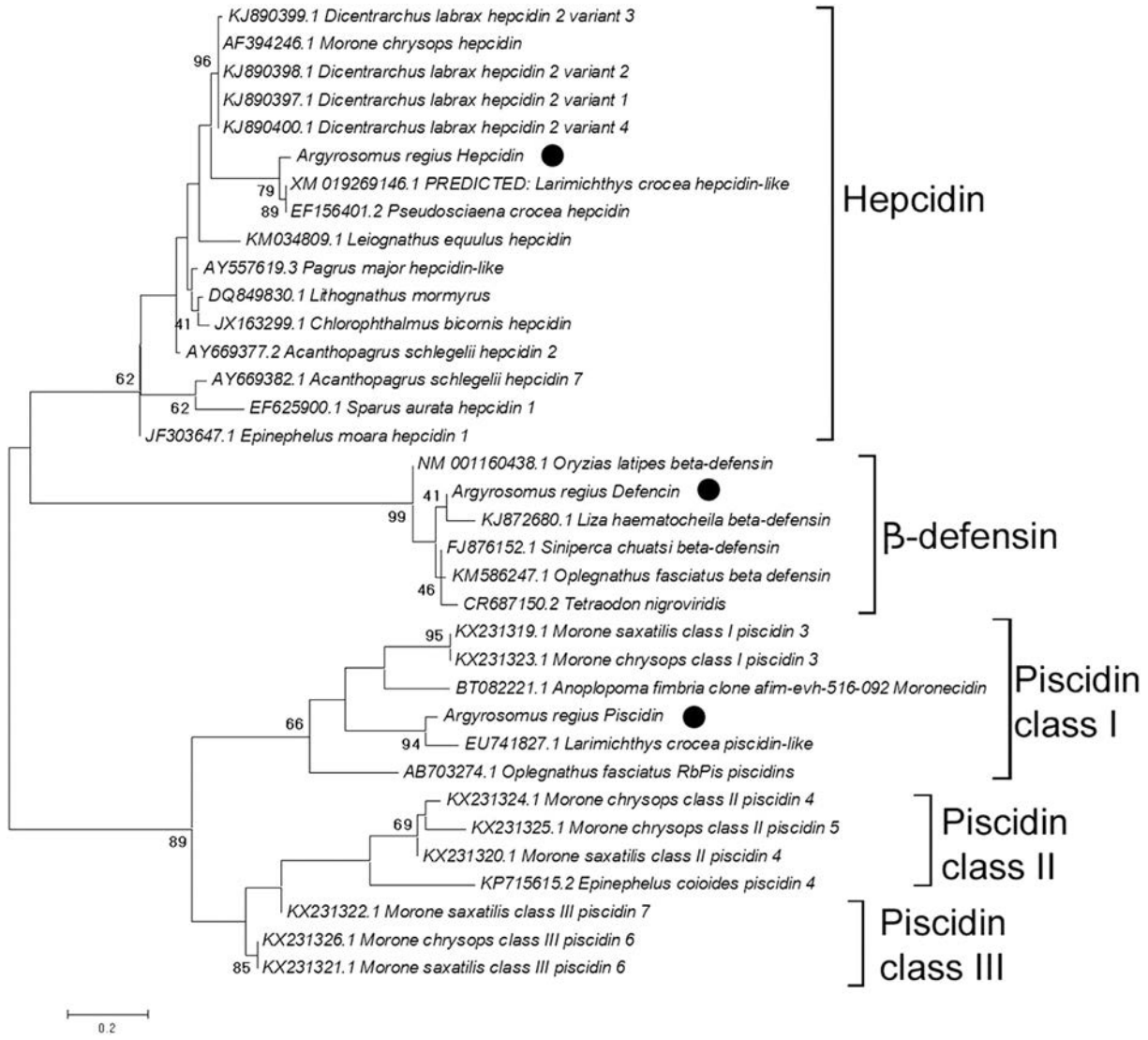

Fig. 2. Phylogenetic relationship among *defb, hep2* and *pisc* isolated from *A. regius* obtained by the Maximum Likelihood method. The tree with the highest log likelihood (−1611.2745) is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are indicated at nodes. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

day 40, when the larvae feed includes only artificial feed, followed by a gradual decrease thereafter. In isolated tissues, high *pisc* expression was detected in gills, but similar patterns of expression were found in kidney and spleen at 85, 96, 120 dph. However, gut tissue had consistently low levels of *pisc* expression during the time points examined (Fig. 3c).

3.3.2. Response of meagre AMPs genes to in vivo PAMP stimulation

After injection of fish with PAMPs, the expression levels of *defb*, *hep2* and *pisc* were examined by q-PCR at 24 h post-stimulation. The *defb* transcription was up-regulated in kidney post-injection with LPS and β-glucan while a significant down-regulation was seen in spleen, gut and gills following LPS injection, and in spleen following poly (I:C) injection (Fig. 4a). In contrast, *hep2* was significantly up-regulated in gut and gills by β-glucan, and in gills by LPS (Fig. 4b). Lastly, the transcription of *pisc* showed significant up-regulation in kidney, spleen and gills by β-glucan treatment, and there was also significant up-regulation in gills by poly (I:C) stimulation (Fig. 4c).

3.3.3. Response of meagre AMPs genes to in vitro PAMP stimulation

After stimulation of isolated leucocytes *in vitro* with PAMPs, the kinetics of changes in expression levels of *defb, hep2* and *pisc* were examined by q-PCR. No change in expression of *defb* gene was found in the stimulated kidney cells at 4 h and 12 h, but at 24 h there was significant up-regulation ($P \leq 0.05$) using poly (I:C), LPS and β-glucan, greater than 20-fold in the latter case. With splenocytes no changes were seen at 4 h or 24 h, but at 12 h a small decline in the level was detected after stimulation with poly (I:C), LPS and β-glucan. The mRNA expression of *defb* in gill cells was significantly up-regulated at 4 h after stimulation by poly (I:C), but thereafter it showed a decline at 12 h - 24 h. Similarly, *defb* was up-regulated with LPS stimulation at 12 h, but declined at 24 h β-glucan stimulation resulted in down-regulation from 12 h onwards. Lastly, the mRNA expression of *defb* in intestinal cells was significantly up-regulated ($P \leq 0.05$) at 4 h and 12 h post-stimulation with poly (I:C), LPS and β-glucan, however an up-regulation at 24 h was only seen with LPS and β-glucan stimulation (Fig. 5a).

For *hep2*, transcription in kidney cells was significantly up-regulated at 4 h post-stimulation with poly (I:C), LPS and β-

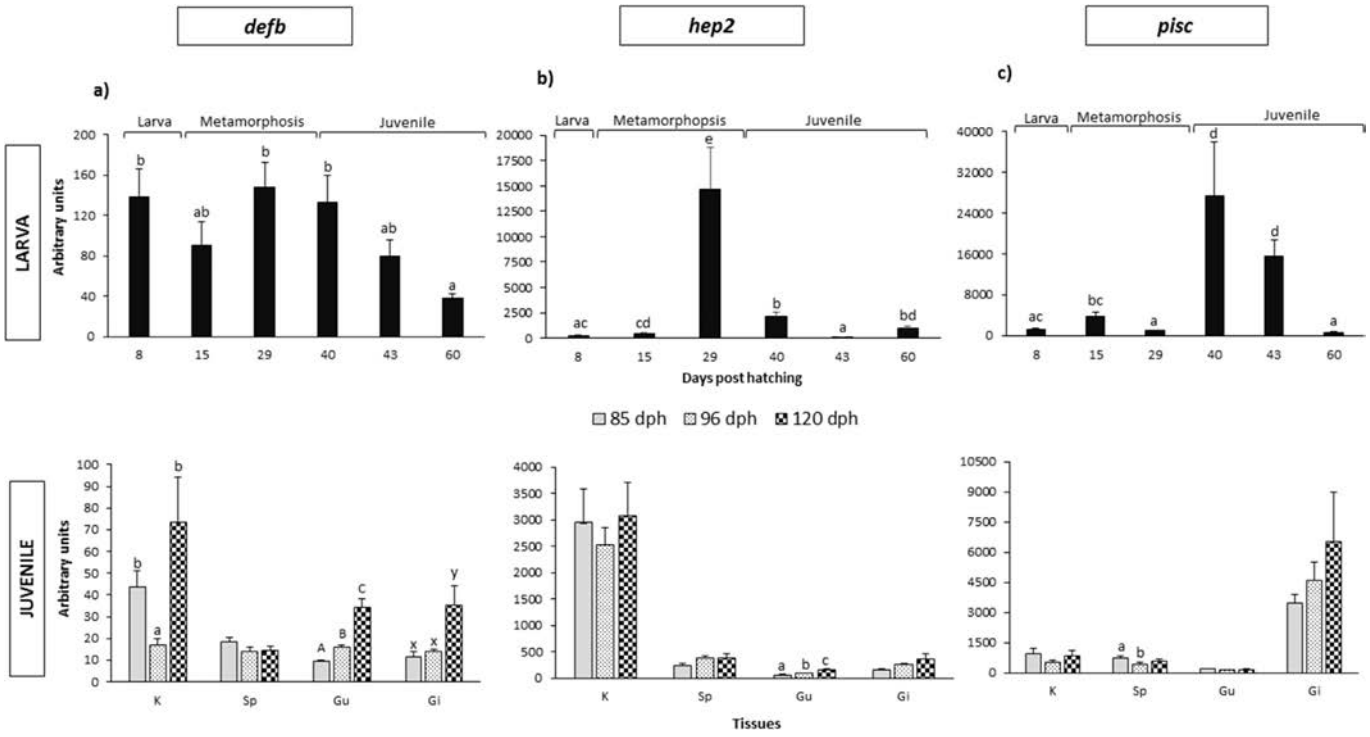

Fig. 3. Temporal change in gene expression levels (relative quantification), from larvae, juveniles and tissues. from 8 to 120 dph, of *A. regius* transcripts coding for *defb* (a), *hep2* (b) and *pisc* (c). Relative expression of *defb*, *hep2* and *pisc* transcripts were normalized using an arithmetic mean of three housekeeping genes: GAPDH, HPRT and β-actin. Transcriptional fold changes of *defb*, *hep2* and *pisc* at different time points were calculated by comparison to each previous timepoint. Different letters above the bars indicate significant differences among different time points (Tukey's test, $P \leq 0.05$). Results are expressed as the mean ± SEM. The biological samples were for larva ($n = 10$) and tissues, ($n = 8$) of gene expression. K = kidney, Sp = spleen, Gu = gut, Gi = gill.

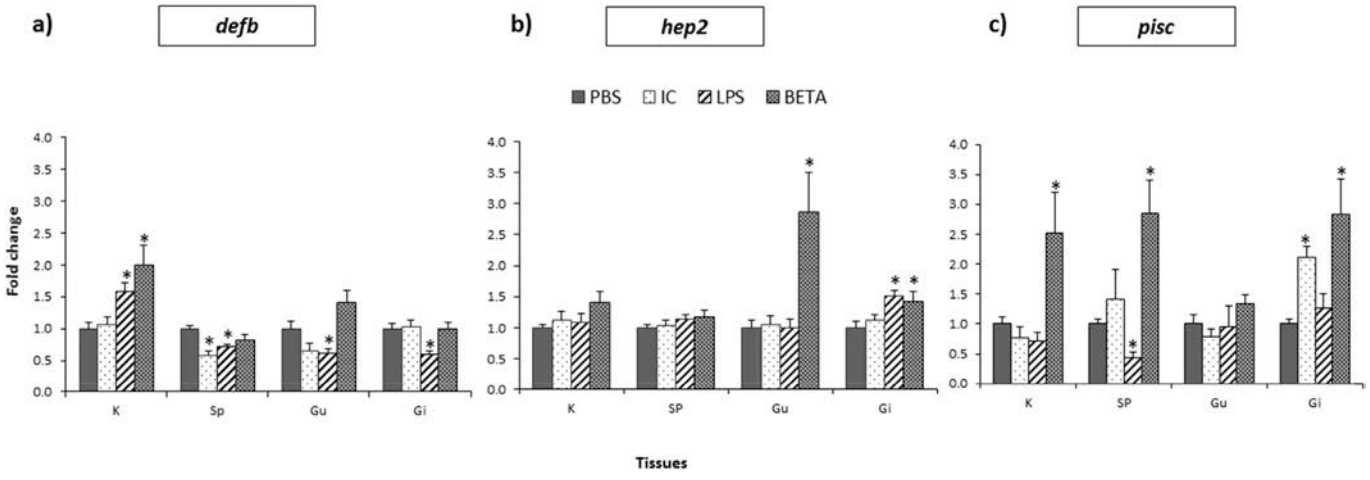

Fig. 4. Relative expression of *defb* (a), *hep2* (b) and *pisc* (c) in different tissues. Fish ($n = 8$) were injected intraperitoneally with poly (I:C), LPS and β-glucan and sampled at 24 h post-injection. GAPDH, was employed as an internal reference. Asterisks (*) mark significant differences between stimulated and control groups (Kruskal Wallis test, $P \leq 0.05$). Data are means ± SEM. K = kidney, Sp = spleen, Gu = gut, Gi = gill.

glucan and, thereafter, it showed a drastic decline at 24 h in the presence of these three PAMPs. In splenocytes only at 24 h after stimulation by PAMPs was a significant effect seen, with down-regulation apparent for all three PAMPs. In gill cells, the gene transcripts were rapidly and significantly up-regulated at 4 h after stimulation by poly (I:C), but showed a decline at 12 h, and later returned to basal levels at 24 h. In intestinal cells, the gene transcripts were significantly up-regulated at 4 h after LPS and β-glucan treatment, had declined at 12 h, but were again enhanced at 24 h after LPS stimulation (Fig. 5b).

A significant up-regulation of *pisc* expression was seen in kidney at 4 h and 12 h after stimulation by β-glucan and at 24 h after stimulation using poly (I:C). In splenocytes, up-regulated was seen at 24 h after stimulation by LPS. The gills cells displayed a higher expression level at 4 h and 12 h after stimulation with LPS, and at 4 h with β-glucan, compared with the control. Significant down-regulation of the expression of *pisc* transcripts was observed in the intestine, but only at 12 h post-stimulation with the PAMPs (Fig. 5c).

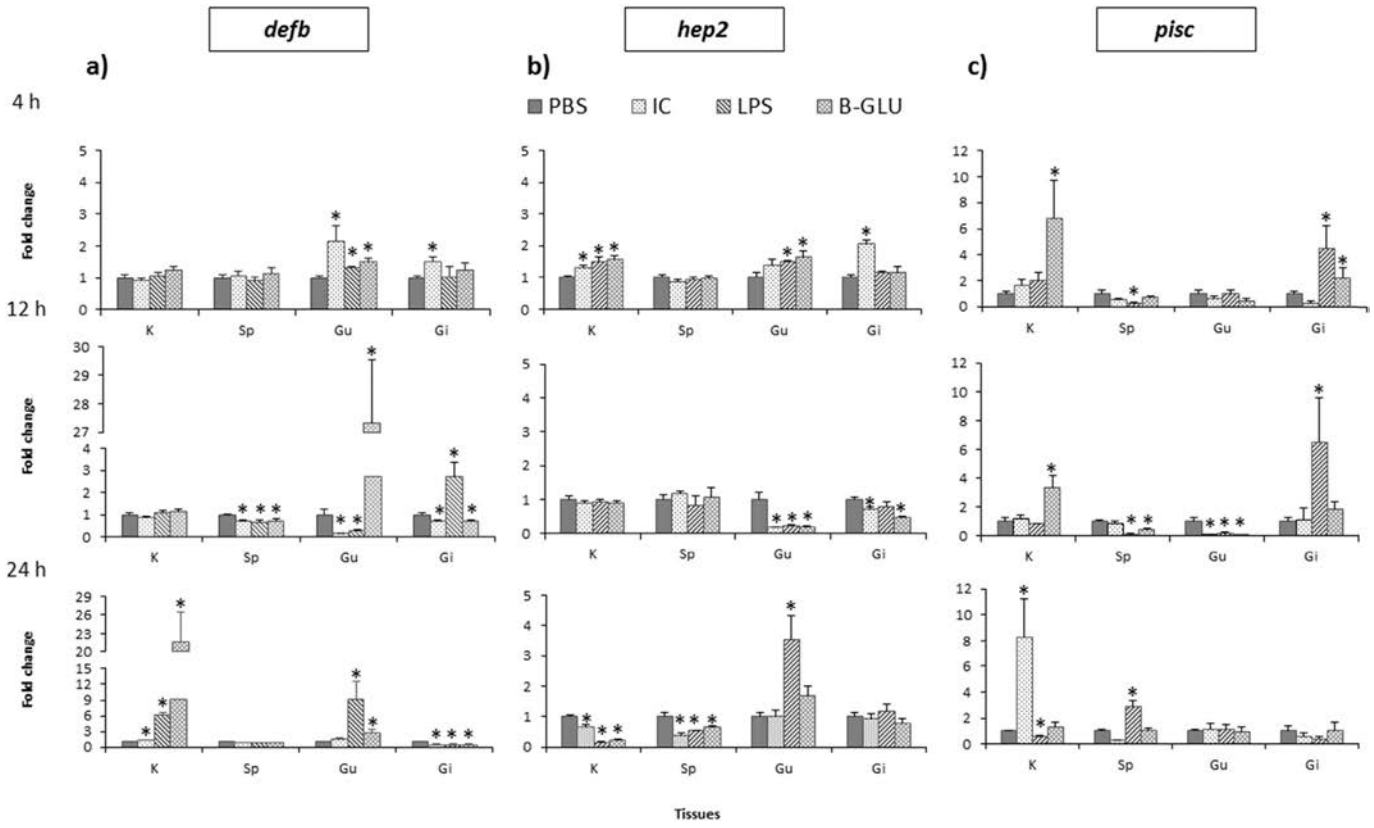

Fig. 5. Expression patterns of *defb* (a), *hep2* (b) and *pisc* (c) mRNA after PAMP stimulation of cell suspensions isolated from different meagre (*Argyrosomus regius*) tissues, extracted from healthy fish ($n = 6$). The cells were stimulated with poly (I:C), LPS and β-glucan, and sampled at different time points post-stimulation (4, 12 and 24 h), for RNA extraction and q-PCR. GAPDH, was employed as an internal reference. Asterisks (*) mark significant differences between stimulated and control groups (Kruskal Wallis test, $P \leq 0.05$). Data are means ± SEM. K = kidney, Sp = spleen, Gu = gut, Gi = gill.

4. Discussion

Antimicrobial peptides are a diverse group of small peptides that exhibit broad-spectrum antimicrobial activity towards pathogens (parasites, bacteria, viruses and fungi). They function as a part of the primary defense within the innate immune system protecting the host from attack by foreign particles and organisms in a non-specific manner [36]. Fish are free-living organisms from the initial stages of development and exposed to numerous pathogens before adaptive immunity has fully developed. It is known that some aspects of teleost immunological capacity are limited in early development and they rely on innate mechanisms such as AMPs for survival. This is the first description of the temporal appearance and flux of expression during larval development of representative members of three AMP families of immune effectors from *A. regius*, including Defensin, Hepcidin, and Piscidin. A phylogenetic analysis of the expressed sequences obtained (Fig. 2) shows that among the transcripts from *A. regius*, the *defensin* isoform forms a clade with the other members of the beta class of this family, *hepcidin* corresponds to *hepcidin* 2, and *pisc* corresponds to class I of its respective family.

The phylogenetic tree reveals that the isoform of *def* identified from meagre is *defb*, although no homolog from *L. crocea* was found in the GenBank database so the formation of a clade with *defb* from *Liza haematocheila* is likely an artifact of the database content. During growout the ontogeny of the expression of the identified AMPs from meagre was evaluated. In this study, *defb* was found expressed in all stages of larval development and in juvenile tissues examined in meagre. In larva, expression levels were identified after complete absorption of the yolk sac and were found to be significantly up-regulated around the time of *Artemia* feeding though decreased after weaning. This increased expression after the start of the exogenous feeding may reflect a need for efficient defense mechanisms against potential pathogens that can enter with the live feed or simply up-regulation due to PAMP stimulation from commensal organisms in the feed. The *defb* expression at early developmental stages has been demonstrated in vertebrates and invertebrate [4]. Two β-defensin genes were identified in snout bream (*Megalobrama amblycephala*) during early development of embryos and fry [37]. Investigation of the multiple DefB isoforms were found in olive flounder (*Paralichthys olivaceus*) after hatching, revealed a constitutive *FBD1* mRNA expression before and after hatching at 1–35 dph [10]. While only one isoform was identified in the current study, it also exhibited constitutive expression as seen with *FBD1* mRNA. In common sole (*Solea solea*) *defb* transcripts showed high transcription level across all larval stages [38]. In this context, expression patterns observed in this study might suggest that this peptide is implicated in innate conditioning of the early larval stages, preventing pathogenic assault, and conditioning of the gut microbiota [39].

The β-defensin gene expression in teleosts has been assessed in tissues from healthy fish, but the levels and patterns of expression varied for individual defensin genes [13,37]. In the present study with meagre, analysis of the isoform obtained revealed expression remained somewhat constitutive, but was highly expressed in kidney with lower levels in gill, gut, and spleen at 120 dph. The decrease in expression seen between 29 dph and 60 dph may be due to the expression signal being diluted as the larvae grew, as the

abundance of *def*-positive cells likely did not increase in the same proportion as the general growth and increase in musculature. However, the isoform under study can influence the expression pattern as well as the host species. In addition to viable microbial pathogens, the components of a fish's diet can act to regulate *defb* expression. In common carp, *BD1* and *BD2* in gills and *BD1* in skin were found significantly higher upon addition of β-glucan to the food [40]. As studies with Gilthead seabream fed microalgae (*Nannochloropsis gaditana,* and *Tetraselmis chuii*) show, an increase in the expression level of *defb* in head-kidney and gut can be accomplished via dietary supplement [41]. On the basis of the results obtained in this study (Fig. 3), further dietary studies to investigate how change of food from live prey (*Artemia*) to commercial feed may influence AMPs expression in larvae.

The potential to modulate the expression level of the AMPs (*defb, hep 2, pisc*) was also analyzed after *in vivo* and *in vitro* immune activation by PAMPs (LPS, poly (I:C), β-glucan). The baseline expression for all three types of AMPs was lowest in isolated gut tissue. This has biological sense when considering the importance of maintaining a healthy gut microbiota. A leaky promoter or slight defects in the secretion systems could have disastrous consequences for the health of the host [42]. Maintaining a tight regulation of control would help to avoid incurring inadvertent damage to the gut microbiota, therefore the control in the gut is more likely to be bi-model rather than multi-model [42]. Our results showed that the expression of *defb* was significantly up-regulated in the kidney after injection with LPS and β-glucan at 24 h. This result is in accordance with the idea that the kidney is an important hematopoietic organ and filters the blood where strong up-regulation of *defb* can be expected as a prophylactic measure to ensure pathogen elimination. Similar results have been seen in Atlantic cod, where *defb* is up-regulated in the kidney 48 h post-challenge with *Vibrio anguillarum* [43], and in the kidney in olive flounder 1 h after infection with *Edwardsiella tarda* [10]. Using cells isolated from individual tissues our results in meagre also show up-regulation of AMPs following PAMPs stimulation. The expression of *defb* was increased in kidney and gut cells at 4 and 24 h after stimulation by β-glucan, LPS and poly (I:C). These results suggest early responses (4–12 h) of *defb* in meagre are more dependent on the stimulus in mucosal tissue than in systemic tissues where significant differences were not observed. Although, later response (24 h) in the kidney were more pronounced. Interestingly, similar trends were found *in vivo* and *in vitro* with regard to *defb* expression in kidney at 24 h after LPS and β-glucan stimulation, possibly reflecting a large population of antigen presenting cells responsive to PAMPs in this tissue.

The phylogenetic analysis also reveals that meagre *hep2* and *pisc* were more similar to equivalent molecules in *Larimichthys crocea* as expected, since this is taxonomically a quite similar species of fish. Hepcidin is highly conserved from fish to humans [44,45]. In fish, it plays a major role in the innate immune response and iron regulation. In the present findings, meagre *hep2* gene expression patterns during larval development showed high expression levels between 8 dph and 15 dph, then increased significantly at 29 dph at the end of metamorphosis. Similar results were observed in *Solea solea* where *SsoHAMP* expression levels increased from 8 dph [38], as well as in Atlantic cod [46] and half -smooth tongue sole [47]. However, in the redbanded seabream and in *Pseudopleuronectes americanus hep* isoforms were found to peak earlier between 5 and 15 dph [17,22], whilst in Medium carp (*Puntius sarana*) a *hep* homologue had stable expression from 6 h post-fertilization onwards [48]. During these timings, numerous changes occur in embryogenesis and early larval development. Elevated expression at this time likely indicates a preparation of the larvae for dealing with a pathogen-laden environment after hatching, as well as conditioning the gut mucosa by stabilization of the normal commensal microbiota that prevent colonization of the gut by opportunistic pathogens through spatial exclusion [39]. Physical barriers of the innate immune response are not sufficient by themselves and when these barriers should be insufficient, additional mechanisms, such as the production of AMPs, are required. From the moment of hatching the fish larvae needs adequate defense mechanisms, with the commencement of external feeding potentially exposing larvae to viable biological agents contaminating the feed.

In this study, *hep2* was expressed in all tissues examined, and highly abundant in kidney followed by spleen and gills. Earlier studies from other species, have shown that *hep* transcripts are usually observed at a higher level in the liver in fish, although they can also be detected in other tissues [43–46]. Indeed, the expression patterns between different fish *hep* is highly divergent and may be related to the specific isoform(s) examined [18].

The i*n vivo* challenge results indicated, that *hep2* was also found to be up-regulated upon β-glucan stimulation, but in gut and gills rather than kidney. Further, in gills *hep2* was significantly up-regulated by LPS stimulation. Studies in fish species of Hep protein have shown multiple isoforms exist and these are classified into two groups: Hamp type 1 and Hamp type 2. One Hep isoform has iron regulatory roles sharing a high degree of homology with mammalian counterparts, and the second Hep copy has retained only the antimicrobial role [9,49]. Phylogenetic analysis in this study has shown the isoform identified in meagre is more similar to Hamp 2 and likely does not retain the iron regulatory functions. In this study the responsiveness of this isoform has only been demonstrated with the *in vitro* and *in vivo* PAMP stimulation indicating its antimicrobial functions. Additionally, there may exist other *hep* isoforms with different tissue-specific expression patterns related to distinct tissue-specific regulation via their promoters [9]. Indeed, different *hep* isoforms in a single species can have different levels of expression within the same tissue, and can be affected by different stimuli [21,22,50]. For example, in the olive flounder where multiple isoforms exist, Hep-JF1 was induced in a wide array of tissues (including liver, gill, kidney and spleen) by LPS, but another variant Hep-JF2 was unaffected [18]. However, whilst LPS is well known to modulate *hep* expression in teleost fish, in large yellow croaker (*Pseudosciaena crocea*) where spleen, heart, stomach, kidney, and liver tissues were examined, LPS stimulation significantly enhanced expression only in spleen, heart and stomach [20].

The in vitro challenge results indicated, *hep2* expression was up-regulated in kidney and gut cells at 4 h post-stimulation by β-glucan and LPS, and at the same time in kidney after poly (I:C) treatment (4 h), with later (24 h) stimulation of expression in intestinal cells by LPS. However, the changes were relatively small as compared to what might be expected in hepatocytes which were not analyzed in this study. The *hep* expression has been shown to be up-regulated *in vitro* by several PAMPs in other species [51]. Also in cell culture, in rainbow trout, where *hep* mRNA level was increased in a macrophage (RTS11) cell line treated with poly (I:C) [52]. In tilapia, Hep2 and Hep3 (TH2-3) showed inhibition of proliferation and migration of a tumor cell line (HT1080 cells) in a concentration-dependent manner [53].

Class I Piscidin has been shown to be more effective against prokaryotes and ciliated protozoan infections [24], as compared to class II and class III. Although in this study functional analyses have not been evaluated, on the basis of q-PCR results, meagre class I Piscidin showed up-regulation with all three PAMPs both *in vivo* and *in vitro*, which is suggestive of it being more responsive to a broader range of stimuli. Piscidin is generally expressed early during fish development and increases in expression over time. Our

results showed that *pisc* transcripts were detected in 8 dph larvae, with maximum levels seen at 40 dph when they have completed the transition to juveniles. There was a decrease after 40 dph, but as discussed above for *defb*, this decrease may in fact be related to a dilution effect due to rapid growth after the end of metamorphosis. In more developed juveniles where individual tissues were examined, *pisc* transcripts were detected at high levels in the gill, with lower expression in the kidney and spleen, and lowest expression in the gut. In studies of striped bass, Piscidin was observed by immunofluorescent labelling in post-vitellogenic oocytes, embryos or larvae from 14 dph [54]. European seabass Piscidin 3 was detected in cytoplasmic granules of inflammatory cells, and mast cells [55]. Until recently, piscidins had only been found in fish species from the superorder Acanthopterygii, but there has recently been *pisc* genes identified in Atlantic cod [56], where expression is seen in more diverse tissues including liver, blood, gall bladder, pyloric caeca, stomach, rectum, muscle, heart, and brain [56–58], as well as in mast cells and rodlet cells of the skin, gill and intestine [59].

In the present study *in vivo* stimulation also modulated *pisc* expression, with the highest level of expression found in kidney, spleen and gills at 24 h after injection with β-glucan, and in gills after poly (I:C) treatment. In other fish species *pisc* genes can be induced by a variety of stimuli such as LPS [60,61], parasite antigens, poly (I:C) [62] and β-glucan as a representative PAMP from fungi [58]. For example, in Mandarin fish (*Siniperca chuatsi*) a significant up-regulation of *pisc* was seen in most tissues, except the liver, after stimulation with LPS [63], while in large yellow croaker, *pisc* expression was up-regulated in response to the ectoparasite, *Cryptocaryon irritans* [26]. Furthermore, in hybrid tilapia (*Oreochromis* spp.) Piscidin showed a correlate with lower mortality after injection with *V. vulnificus* [64]. Regarding *pisc* expression *in vitro*, this was up-regulated in kidney cells by β-glucan and in gill cells by LPS at 4 and 12 h post-stimulation. A late induction in spleen and kidney cells was also seen in response to LPS and poly (I:C) at 24 h post-stimulation. Interestingly, *pisc* expression *in vitro* (4–12 h) and *in vivo* (24 h) showed up-regulation in the same tissues from whole animals and isolated cells (kidney and gills) by β-glucan stimulation. These results demonstrate that timing of induction from each stimulant has tissue-specific differences, although the *pisc* isoform identified can be induced by all three types of PAMPs.

In conclusion, the current study is the first identification of AMPs from meagre and demonstrates their expression modulation during development and in response to PAMPs. Further studies will be needed to elucidate whether additional isoforms exist and the antimicrobial activities of the peptide proteins. The *A. regius defb, hep2,* and *pisc* transcripts were expressed at different levels in early developmental stages and tissues of juvenile meagre, and the data hint that expression is regulated or influenced by dietary factors such as switching to live feed, or weaning onto artificial diets. This may allow larval feed composition for *A. regius* larvae to be manipulated to augment AMP gene expression to improve antimicrobial defenses as has been seen for *defb* expression in common carp [40] and gilthead seabream [41]. This work provides useful information for future investigations of the innate defense mechanisms in early development of meagre and studies focusing on the biological activities of these antimicrobial peptides for this fish species.

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CHAPTER 7

Ontogeny of the innate immune system in meagre (*Argyrosomus regius*) during early rearing.

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Abstract

The present study is the first report of some representative innate immune genes in meagre larvae and has focused specifically on a critical time in marine fish development when reliance on innate immune mechanisms are required for survival. We report molecular cloning of partial open reading frames and characterization of expression of some innate genes (*c3, cox2, met lyz, mxp, myd88, nod2, nod3)* in *A. regius*. A phylogenetic analysis of the expressed sequences obtained shows the *met* isoform form meagre is *met* II, and is an isoform more similar to a homolog described in *Larimichtthys crocea,* Lysozyme (*lyz*) corresponds to the c-type and Nod isoforms (*nod2*, *nod3)* separate into different clades confirming their distinct evolutionary history and their presumed identity. Gene expression profiles of innate genes were investigated, by means of quantification of mRNA in nine developmental stages, from 8 days post-hatching (dph) to completion of the juvenile form (120 dph). During development it was demonstrated *c3, cox2, met II, lyzc, mxp, myd88, nod2*, and *nod3* were expressed in all stages of larval development and in juvenile tissues (kidney, spleen gut and gill). Moreover, expression patterns suggest the expression levels of theses innate genes may be influenced by feed switching from live prey (rotifer, *Artemia*) and first intake of inert commercial diet. Modulation of gene expression experiments *in vivo* (24 h) with PAMPs (LPS, poly (I:C), β-glucan) revealed significant changes in mRNA levels of these innate genes in kidney, spleen, gut and gill. However, expression profiles differed in magnitude depending on stimulant and/or tissue.

Keywords: innate, immune system, *Argyrosomus regius*, meagre, larval growout, PAMP, ontogeny.

1. Introduction

The development of the aquaculture sector in recent decades has been driven by advances in culture techniques and the introduction of new species. To make the advances thus far gained with newly introduced species more efficient and enhance production further, there are issues of fish health that need to be addressed to reduce economic losses. Vaccine development is one solution to health issues related to infectious disease, however, in addition to significant investment in research and development for each infectious agent, their implementation requires an understanding of the particulars of each newly introduced species´ immune functions and the timing of their development.

Meagre (*Argyrosomus regius*) is currently a focal point for new species introduction in aquaculture for Europe. It is geographically distributed from the Mediterranean Sea and the Black Sea, throughout the European Atlantic and East African coast. However, as occurs in new species, the most important bottleneck is related to larval rearing with regard to survival and elevated fry production cost. Successful marine larva culture not only requires a feeding protocol based on the nutritional requirements and digestive capabilities of developing larvae, but also an effective and mature immune system. A successful productive industry depends on knowledge of their biology and improved rearing techniques. A major problem accompanying this production system are the occurrence of infectious disease, which cause high losses in cultures of fish, especially in early stages [1].

Fish larvae do not have the ability to develop specific immunity during the early stages of development. In this respect, the larvae are dependent on innate defenses against pathogens or opportunistic agents. While the skin provides an initial physical barrier, if this barrier is breached by a pathogen there is a first line of defense in the form of the innate immune system composed of various effector molecules. The innate immune system includes membrane-bound pathogen recognition receptors (PRRs) such as NOD-like receptors, and cytoplasmic proteins that bind pathogen-associated molecular patterns (PAMPs) expressed on the surfaces of invading microbes [2]. Innate immunity exerts its effect employing cells and a variety of proteins able to facilitate destroying or inhibiting the growth of the infectious microorganism [3]. The complement system is one of the first lines of immune defense as well as a modifier of acquired immunity. The complement system is activated through three different, but partially overlapping routes: the classical pathways, the alternative pathway and the lectin pathway [4]. Lysozyme (*lyz*) is also an important defense molecule of the fish innate immune system, which possesses lytic activity against microbial

invasion and is expressed in a wide variety of tissues [5]. The role of the Mx proteins (*mxp*) in resistance to negative-strand RNA viruses has been well established, although the actual mechanism for viral inactivation by Mxp is still not understood. Mx proteins are highly conserved in vertebrates, and are able to inhibit the virus life cycle in different phases whether they are localized in the cytoplasm or in the nucleus [6]. Cyclooxygenase (*cox2*) is responsible for synthesis of prostaglandins to maintain homeostatic functions, required for fish reproduction and inflammatory response in particular [7]. Metallothionein (*met*) is an important factor in resistance to heavy metal contamination in aquatic organism [8]. Metallothionein plays a relevant role in the detoxification of heavy metal ions such as Cd and Hg and providing a buffering action in cells by binding essential metals such as Cu and Zn which has an inhibitory effect on pathogens that have become systemic and provides protection against oxidative stress [9][10][11]. For these effector molecules to carry-out their function in a coordinated manner there is a need for intra- and intercellular signaling. For this, innate immunity relies on signaling by members of the TLR (toll-like receptor) family and associated adaptor molecules that are highly conserved in vertebrates, such as Myd88 (myeloid differentiation primary response gene). MyD88 (*myd88*) has been implicated in signaling by all TLRs with one possible exception (TLR3) [12] and implicated in defense against a variety of pathogens. This adapter, which contains TIR domains as do the TLRs, is required for activation of mitogen–activated protein (MAP) kinase family members as well as nuclear factor kB (NF-kB) translocation, which in turn activates transcription of pro-inflammatory cytokines [13]. Innate immunity additionally has the potential to respond to endogenous molecules [14] that are released by host cells as a result of necrosis, pathogen infection, and certain pathological conditions, which are directly or indirectly recognized by NOD-like receptors. The NOD-like receptors (NLRs) function as cytoplasmic sensors of pathogen presence. NOD2 (*nod2*) detects muramyl dipeptide (MDP) found in the peptidoglycan of the cell wall of both gram-positive and negative bacteria [14]. NOD2 in mammals is highly expressed in epithelial cells or macrophages associated with the intestine. Studies in zebrafish have detected NOD3 (*nod3*) has been shown to be an ortholog of mammalian NOD3 (NLR3) and found to have similar NACHT domains and an equal number of LRR domains [15]. It is a member of the largest family of NOD-like receptors but it specific ligand remains to be identified.

Within this study, in order to aid understanding of the limiting factors during larval rearing, as well as the maturation and onset of humoral immunity during development of meagre, this study (1) examines the ontogenic changes in immune-relate gene expression of these

innate immune genes during larval and juvenile transition development in meagre. (2) Evaluate when in development of meagre these genes appear. (3) How their expression is modulated by different PAMPs *in vivo*.

Materials and methods

2.1 *Larvae production, and sample collection to determine innate immune ontogeny*

Larvae used in the study were provided from meagre broodstock held in IRTA San Carlos de la Rapita, Spain. The broodstock were maintained under controlled simulated natural water temperature and photoperiod using recirculation system (IRTAmar®). During the natural reproductive period (April-June), mature fish were selected based on oocyte size (< 550µm) and ease to extract sperm. Pairs of mature fish (21.2 ± 3.7 kg females and 16.1 ± 2.6 kg males) were hormonally induced (15 µg/kg of des-Gly10, [D-Ala6]-gonadotropin-releasing hormone ethylamide, Sigma, Spain) to spawn spontaneously in 10,000 L tanks and spawned eggs were collected with a passive egg collector placed in the outflow of the tank. Egg number was estimated by counting the total number of eggs in three sub-samples of 10 mL and fertilization by examining the development of 100 eggs. Batches of 50,000 eggs were incubated (18-19 ºC) in 35 L mesh (300 µm mesh) bottomed incubators with aeration and gentle air-lift water exchange that were placed in 2,000 L tanks. Hatching rate was determined by estimating the number of larvae (three 100 mL sub-samples) obtained from the stocked eggs.

Larvae were transferred and distributed into two 1.5 m^3 tanks under a mesocosm system. Water temperature was maintained at 20 ºC. From two days post-hatching (dph), larvae were fed enriched rotifers until 11 dph. Freshly enriched *Artemia* metanauplii were introduced at 9 dph until 31 dph and a formulated artificial diet was incorporated at 21 dph until the end of the experiment. Samples of larvae were collected from post hatch until juvenile for growth measurements. Random samples of larvae and juvenile were taken at 8, 15, 29, 40, 43, 60, 85, 96, and 120 dph for each sample point collected in an Eppendorf tube on ice containing RNAlater™ (Ambion, Austin, Texas), then preserved at -80 ºC until RNA extraction. Time points for analysis were chosen as proxies for the specific periods of change in rearing practices; primarily changes in diet, but also rapid changes in organogenesis, all of which increase stress and enhance susceptibly to diseases. The fish were euthanized using a high concentration of MS222 (1 g /L) (Aldrich, E10521) prior to sample collection. Larger larvae (post-29 dph) had excess tissue trimmed to reduce signal dilution from non-target tissues; the anterior section from the gills onwards, and the

posterior part from the anus to the tail (Fig 1). The samples were collected from each time point for the study of AMP gene expression. At days 85, 96, and 120 individual tissues (gill, kidney, spleen, and intestine) were excised aseptically.

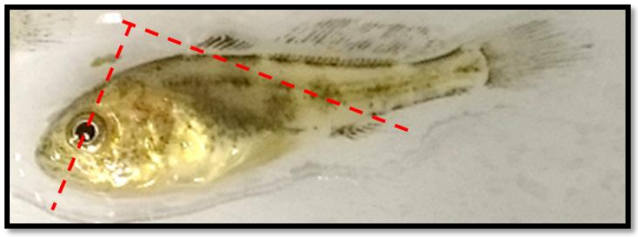


Fig. 1. Previous to the extraction of RNA from the samples, the rostrum and caudal portions were removed by cutting with scalpel as illustrated. Only the remaining ventral part was used for further analysis. The red line indicates the location of cutting each sample.

2.2 *Total RNA extraction and reverse transcription*

Total RNA was extracted using Trizol reagent (Invitrogen) according to manufacturer's instruction. RNA concentration and purity was determined by spectrophotometry (NanoDrop2000, Thermo Fisher Scientific, Madrid, Spain) measuring the absorbance at 260 and 280 nm. The quality of extracted RNA was verified with visualization of the 28S and 18S ribosomal RNA bands by agarose gel electrophoresis. For preparation of cDNA total RNA was treated with DNase 1, Amplification Grade (AMPD1–1KT, Sigma–Aldrich, Broendby, Denmark), according to manufacturer's instructions to remove possible contaminating genomic DNA. Total RNA was reverse transcribed in a 20 µL reaction volume containing 2 µg total RNA using the ThermoScript ™ Reverse Transcriptase (Invitrogen) with oligo (dT) $_{(12\text{-}18)}$ (0.5 µg/µL) and random hexamer primers (50 ng/µL) 10X RT buffer [200 mM Tris-HCl (pH8.4), 500 mM KCl] 1.5 mM $MgCl_2$, 800 mM dNTP mix, RNase inhibitor, SuperScript ^{TM} II RT, followed by RNAse H (Invitrogen) treatment. Reverse transcription reactions were prepared, which were placed in a thermocycler (Mastercycle ® nexus GSX1, Eppendorf AG, Hamburg, Germany) and run according to manufacturer´s instruction. Negative controls (no RT enzyme) were included to confirm absence of genomic DNA contamination. The samples were then diluted 1:20 in molecular biology grade water and stored at -20 ºC.

2.3 *Gene Isolation*

Meagre were sampled for collecting data on specific growth rate of the chronological samples. The samples were collected in RNAlater at each time point, for extraction of RNA to be used in gene expression analysis. Sequences of target genes from the online database

Genbank were chosen from extant marine teleost species that were available, such as large yellow croaker (*Larimichthys crocea*), mandarin fish (*Synchiropus splendidus*), Humphead snapper (*Lutjanus sanguineus*), European seabass (*Dicentrarchus labrax*), orange spotted grouper (*Epinephelus coioide),* turbot (*Scophthalmus maximus)* Asian sea bass (*Lates calcarifer*) and gilt-head (*Sparus aurata*). The sequences were aligned, using CLUSTAL W incorporated in the package BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), for designing degenerate/consensus primers for amplification from cDNA of tissues. The fragments amplified were separated by gel electrophoresis and resulting bands of the expected length were excised, purified (QIAQuick PCR purification kit, Qiagen) and sequenced (Sistemas Genomicos, Valencia, Spain). The transcript sequences thus obtained were used as templates to design primers for specific q-PCR assays.

2.4 *Phylogenetic Analysis*

Evolutionary analyses were conducted in MEGA5 [16] after alignment of all selected homologs using CLUSTAL W. Nucleotide substitution models were chosen for each analysis using the utility included in MEGA 5. The evolutionary reconstruction of the Nod isoforms was performed using the Kimura 2-parameter nucleotide substitution model [17] and Neighbor-Joining method [18]. The evolutionary history for metallothionein and lysozyme were inferred by using the Maximum Likelihood method based on the Jukes-Cantor model [19]. For these analyses the initial tree (s) for the heuristic search were obtained automatically as follows: when the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise Neighbor-Joining method with MCL distance matrix was used. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 200.0000)). Statistical robustness of data for all analyses were analyzed using the bootstrap method [20] with the bootstrap confidence values from 1000 replicates shown at branch nodes (values < 40 not shown). During analyses all ambiguous positions were removed for each sequence pair. The analysis of NOD isoforms involved 129 positions from 25 sequences. For metallothionein a total of 22 sequences were included in the final dataset each with 71 positions. The analysis of lysozyme included 25 sequences with 148 positions in each.

2.5 *PAMP stimulation*

To investigate the effect of PAMP stimulation on the expression of *met* , *c3*, *cox2*, *met lyz*, *mxp*, *myd88*, *nod2*, *nod3* , 38 healthy juvenile individuals (30 - 40 g each) of meagre were

held in a recirculating water tank (100 L) with seawater at 20 ˚C. The fish were fed with a commercial diet twice a day (9:00 h and 16: 00 h) for two weeks to acclimate them to the indoor culture environment. For primary cell culture and collection of samples, fish were killed after anesthesia with 50 mg/L MS-222.

2.5.1 *Innate immune expression after **in vivo** stimulation*

Fish were injected intraperitoneally with 100 µL PBS containing 100 µg poly (I: C) (3.3 mg/kg) (Sigma, UK. P1530), 400 µg LPS (13.3 mg/kg) (Sigma, UK. L3129) or 100 µg β-glucan (3.3 mg/kg) (Sigma, UK. 89862). The control animals were injected with PBS only. After 24 h, the individuals were dissected and tissues (kidney, spleen, gut and gill) sampled. Total RNA was isolated and cDNA was prepared as described above. The mRNA expression level of *met*, *c3*, *cox2*, *lyz*, *mxp*, *myd88*, *nod2*, *nod3* were determined by q-PCR. Relative expression was normalized to GAPDH expression and calculated as arbitrary units and converted to a proportion relative to the PBS control samples.

2.6 *Real-time q-PCR (RT-qPCR)*

The q-PCR reactions for innate immune gene expression were carried out in duplicate on a LightCycler® 480 Real-Time (Applied Biosystems, Roche). A master mix was prepared from: 6 µL SYBR Green Supermix (Life Technologies), 10 µM of each primer, diluted in molecular biology grade water (Sigma) and 4 µL of cDNA in a final volume of 10 µL. The real-time q-PCR cycling was carried out as follows: 10 min at 95ºC, 40 cycles of 95 ºC for 25 s, followed by an annealing step of approximately 59 ºC for 25 s (annealing temperatures were adjusted for each specific primer pair), followed by 72 ºC for 15 s, with a final melt curve stage of 0.5 ºC increments from 75 ºC to 95 ºC. Each sample on the q-PCR plate had two technical replicates. Primer sequences used and amplicon lengths for each assay are shown (Table 1). The specificity of the primers was checked by running a q-PCR and confirming that only one melt peak was produced and also by running a subsample on an agarose gel (2%) to confirm the presence of a single band of the expected size. The efficiency of amplification (E %) of each primer pair was assessed from five serial ten-fold dilutions of cDNA from individual tissues, then calculated following the equation: $E\% = 10^{(-1/slope)} - 1$ where the "slope" is that calculated from the regression line of the standard curve. Efficiencies of the gene expression assays ranged from 96.2% to 100.9% (100.27 ± 1.58) (Table 1). The absence of primer dimer formation in the NTC was also confirmed. Relative expression of genes from larvae, and each tissue from juveniles (ontogeny study), was normalized using the three endogenous controls glyceraldehyde phosphate

dehydrogenase (GAPDH), beta-actin (β-actin), and hypoxanthine-guanine phosphoribosyltransferase (HPRT) as determined using geNORM (http://www.primerdesign.co.uk/products/9461-genormplus-kits/), while the relative quantification at 120 dph, showing the least expression, was used as the calibrator.

For analyzing the relative expression *in vivo* after PAMP stimulation, data was normalized to GAPDH expression, as this endogenous control gene showed less variability under the conditions tested. After normalization to this endogenous control as arbitrary units, results were converted to a proportion relative to the control group (PBS injected fish).

Table. 1 Primers used for gene expression analysis by real-time q-PCR, including the amplicon size and primer sequences. Abbreviations: *gapdh*, glyceraldehyde 3-phosphate dehydrogenase; *β-actin*, beta-actin; *hprt*, hypoxantine-guanine phosphoribosyltransferase; *met*, methallothionein*; c3*, complement*; cox2*, cyclooxygenase; *lyz*, lysozymes; *mxp*, mx proteins; *myd88*, myeloid differentiation primary response gene 88; *nod2*, nucleotide-binding oligomerization domain-containing protein 2; *nod3*, nucleotide-binding oligomerization domain-containing protein 3.

	Genes	E (%)	Forward primer (5' → 3´)	Reverse primer (5' → 3´)	Size (bp)
Reference genes	*gapdh*	100.0	CCAGTACGTGGTGGAGTCCACTG	AGCGTCAGCGGTGGGTGCAGAG	109
	β-actin	100.0	TGGGGGAGCAATGATCTTGATCTTCA	AGCCCTCTTTCCTCGGTATGGAGTC	212
	hprt	100.9	CATGGACTCATCTTGGACAGGACAGA	GCCTTGATGTAGTCCAGCAGGTC	137
Immune genes	*met*	102.2	GATCCTGCAATTGCAAAGACTGTTC	CCGGATGGGCAGCATGGGCAG	70
	c3	100.1	AACCCATACGCTGTTGCCATGACG	CACGTCCTTTAGGTACTGGGCCAG	120
	cox2	100.5	GGAAGTTGGTGTTGACATGCACTAC	CAATCAGGATGAGCCGTGTGGTC	211
	lyz	100.0	GATGGATCCACTGACTACGGCATC	AAGCTGGCTGCACTGGATGTGGC	100
	mxp	96.2	AGTCAGTGGTTGACATTGTTCATAATG	AACAGTGGCATGACCGTCATTGTAG	187
	myd88	102.2	GCTACTGCCAGAGTGACTTCGAGT	TCCATACACACGAACCCGGGAGG	120
	nod2	100.1	CTCAATACTGTGCTGATGTCCATGG	CAAGTGTAACCTTTGGAGTAAGGTAG	145
	nod3	100.8	CAGCTTGGTGGAACTTGTTCATCAC	TAACATCAGTCAGGATCTCAGTGTTG	130

2.7 *Statistical analysis*

All data were checked for homogeneity of variances using a Levene's test by univariate analysis in a general linear model, based on a Tukey HSD post-hoc test, with a sample size of n =10 (larva) n = 8 (juvenile) to determine differences between time points ($P \leq 0.05$). The Kruskal-Wallis test was used for analyzing the expression for the *in vivo* (n = 8) samples after PAMP stimulation, using the statistical software package SPSS 20.0 (SPSS Inc., US).

3. Results

In the present study, innate genes expression during larval development has been assessed. Meagre growth was described at day 8 dph (4.49 ± 0.39 mm SL), 15 dph (6.9 ±1.1 mm SL), 29 dph (14.41 ± 1.62 mm SL), 40 (18.40 ± 0.74 mm SL), 85 (66.25 ± 4.35 mm SL) 96 (84.25 ± 13.7 mm SL) 120 dph (109.5 ± 6.60 mm SL).

3.1 Gene *Isolation*

Specific gene products were obtained using degenerate or consensus-primed PCR assays. All amplified fragments were sequenced bi-directionally and analyzed using the BLAST utility via the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nig.gov/) to confirm their identity as proper orthologs of the intended target gene. Sequences for transcripts from *met*, *c3*, *cox2*, *lyzc*, *mxp*, *myd88*, *nod2*, *nod3*, are reported for the first time in this species. These sequences have been uploaded to GenBank under the following accession numbers: *met* = MF281966, *c3* = MF281960, *cox2* = MF281967, *lyzc* = MF281968, *mxp* = MF281965, *myd88* = MF281964, *nod2* = MF281970, *nod3* = MF281969. Sequences were also obtained for GAPDH, β-actin and HPRT as endogenous control genes for gene expression assays and have been entered into in the GenBank database under the following accession numbers MF281962, MF186587 and MF186588, respectively.

3.2 *Phylogenetic Analysis*

Homologs of metallothionein, nod 2, nod 3, and lysozyme from meagre were further characterized as to class or isoform to give clarity to resulting gene expression analyses. To achieve this extant sequences from GenBank were aligned using CUSTAL W and edited manually. For all analyses, the percentage of trees in which the associated taxa clustered together (bootstrap values) is shown next to the branch nodes. Phylogenetic analysis of representative homologs of *nod2* and *nod3* sequences from GenBank were

compared to that obtained from meagre resulting in an optimal tree with a sum branch length = 1.89832313. The two different Nod isoforms clearly separate into different clades confirming their distinct evolutionary history and their presumed identity. Analysis of metallothionein II confirmed the segregation from metallothionein I and resulted in a tree with the highest log likelihood of -157.7290. The tree resulting from the analysis of lysozyme had highest log likelihood value of -2526.8685 and demonstrated its membership in the clade with *lyzc*.

3.2 *Gene expression analysis*

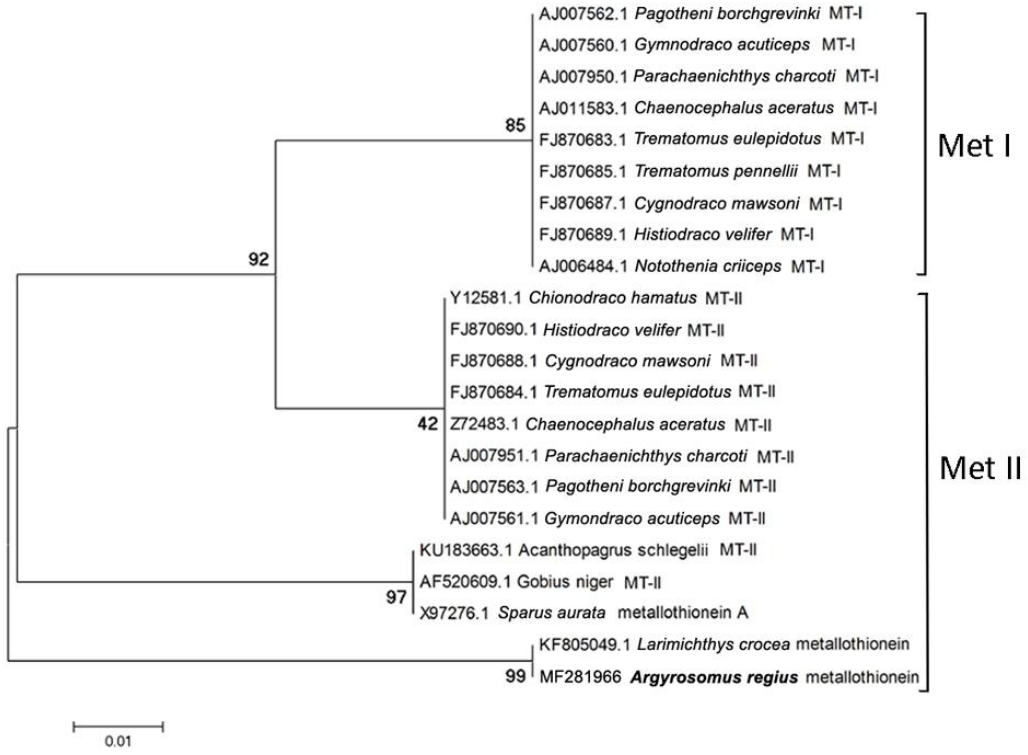
3.2.1 *Gene expression during ontogeny*

In order to better understand the biological role of relevant innate immune genes in meagre, the expressions of mRNA for *met II*, *c3*, *cox2*, *lyzc*, *mxp*, *myd88*, *nod2*, *nod3* were analyzed by quantitative real-time PCR from 8 until 120 dph (Fig 2). The expression of genes under study herein were dynamically variable throughout development and growth of the fry. However, three general patterns of expression were evident. For some genes (*cox2*) there was a general decrease over time between 8 and 60 dph. For a second group composed of *mxp*, *myd88*, *c3*, the pattern showed an initial low expression that increased with time with a dramatic peak at 60 dph. A third group *lyzc*, *nod2*, *nod3* was discernable which showed low levels initially with peaks at 29 dph and 60 dph. The pattern of *met II* and *c3* expression was clearly at a high level at day 8, and then it decreased to a moderate level at day 29 and followed by a substantial increase at day 60. However, a different expression pattern was found for *mxp* and *myd88* transcripts. They were detected at day 8 and subsequently the expression level increased gradually until day 60. In contrast, the expression of *cox2* was gradually decreasing during the study period, possibly due to the expression signal being diluted as the fish larva grew. A comparison of *nod2* and *nod3* show their expression profiles are similar. In fact, the levels of expression of both transcripts was higher at day 29 and then, decreased to moderate levels (40-43 dph), followed by an increase at day 60. The pattern of expression seen with lysozyme was similar but with greater differences in expression at 29 and 60 dph. Interestingly, the expression of these genes seemed regulated by diet in the growing fry (Fig 3).

As larvae increased in size in the latter part of the ontogeny study (85, 96, or 120 dph) the levels of expression of the eight transcripts were in separate tissues (kidney, spleen, gill and gut). As shown in Figure 4, the eight genes exhibited different patterns of tissue

expression. In contrast, *cox2* shows a significant increase in gill, gut and kidney at day 120 in comparison with 85-96 dph. Expression of *myd88* transcripts shows a significant increase in the kidney at 96 dph and then maintaining high and stable expression until 120 dph. However, *met* II and *mxp* transcripts show significant increases in the gut during 85-96 dph. Finally, *nod3* transcript levels increased significantly ($P \leq 0.05$) in spleen (85 dph) and gut (120 dph) in comparison with other preceding time points (Fig 4).

a)



b)

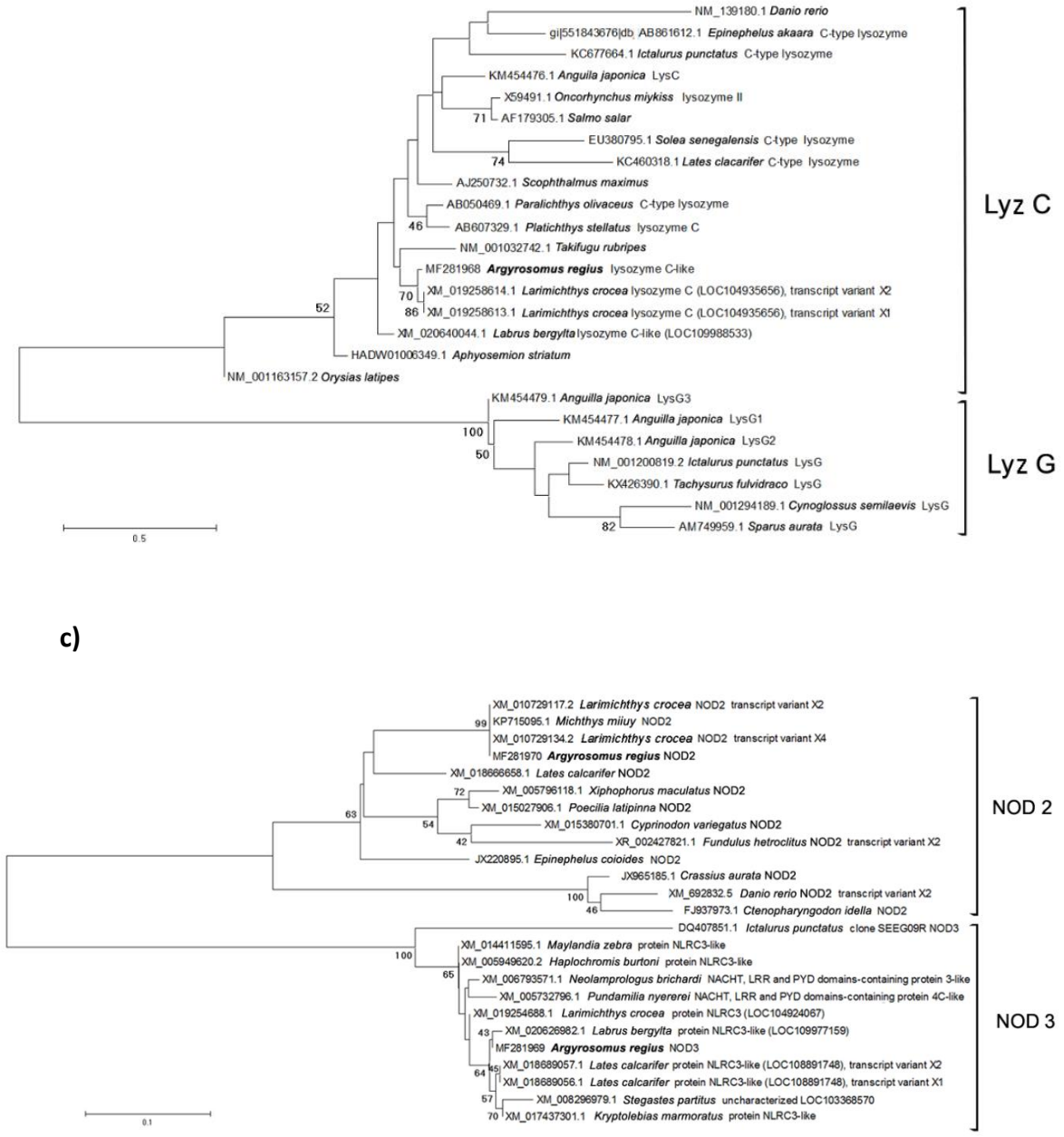


Fig. 2. The evolutionary history for (a) *met II*, (b) *lyzc* (c) *Nod2*, *Nod3* from meagre (shown in bold) was inferred using the Maximum Likelihood (a), (b) and Neighbor-Joining (c) method conducted in MEGA5. All coding positions were included and values for the bootstrap test values (1000 replicates) are shown at branch nodes.

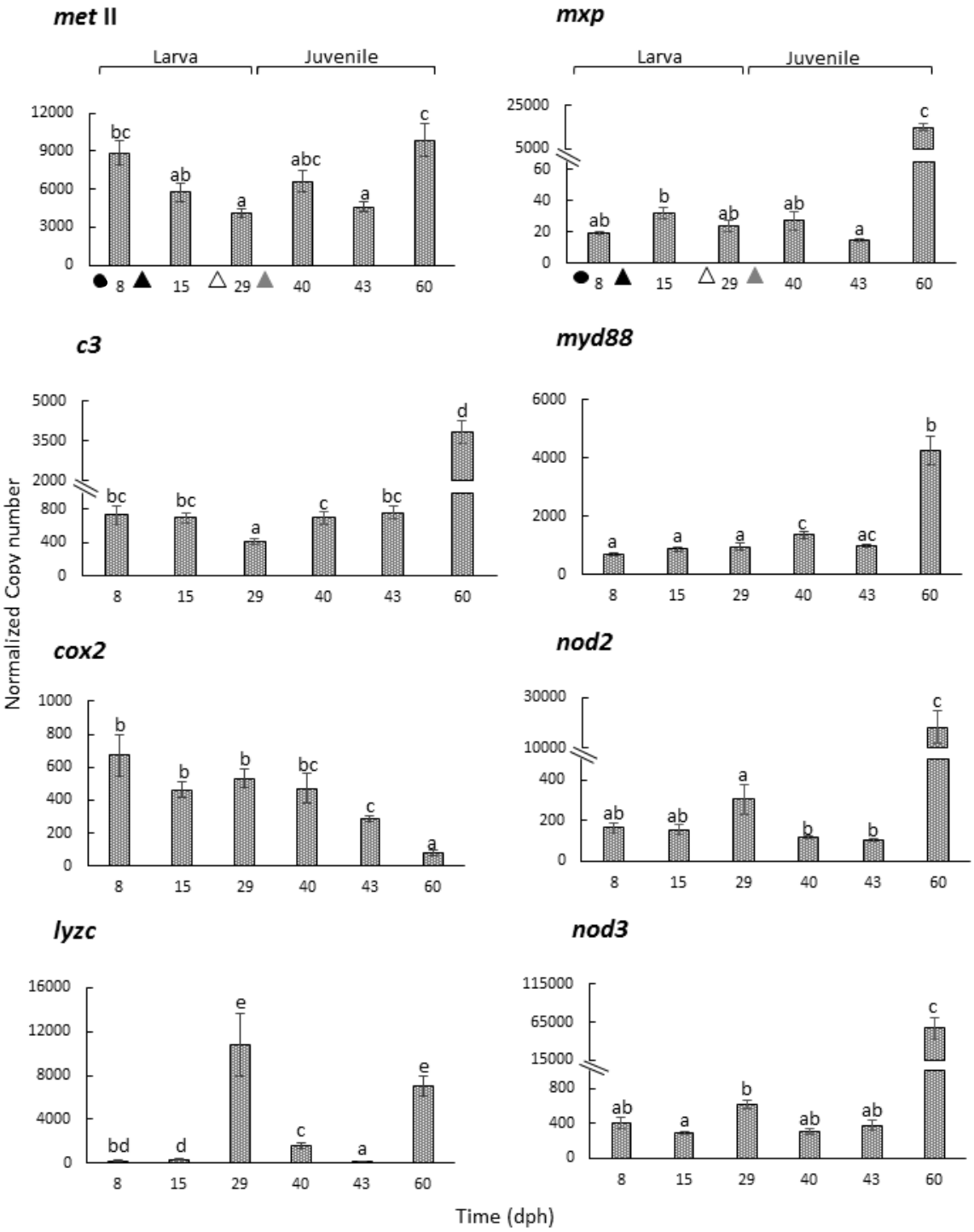


Fig. 3. Temporal change in gene expression levels (relative quantification), from larvae and juvenile (n = 10) from 8-60 dph, of *A. regius* transcripts coding for *met II*, *c3*, *cox2*, *lyzc*, *mxp*, *myd88*, *nod2*, *nod3*. Relative expression *met II*, *c3*, *cox2*, *lyzc*, *mxp*, *myd88*, *nod2*, *nod3* transcripts were normalized using an arithmetic mean of three housekeeping genes: GAPDH, HPRT, and β-actin. Transcriptional fold changes of these genes at different time points were calculated by comparison to each previous time point. Different letters above the bars indicate significant differences among different time points (Tukey´s test, $P \leq 0.05$). Results are expressed as the mean ± SEM. Different symbols represent different diets offered ● rotifers ▲ *Artemia* △ *Artemia*-artificial diet ▲ artificial diet.

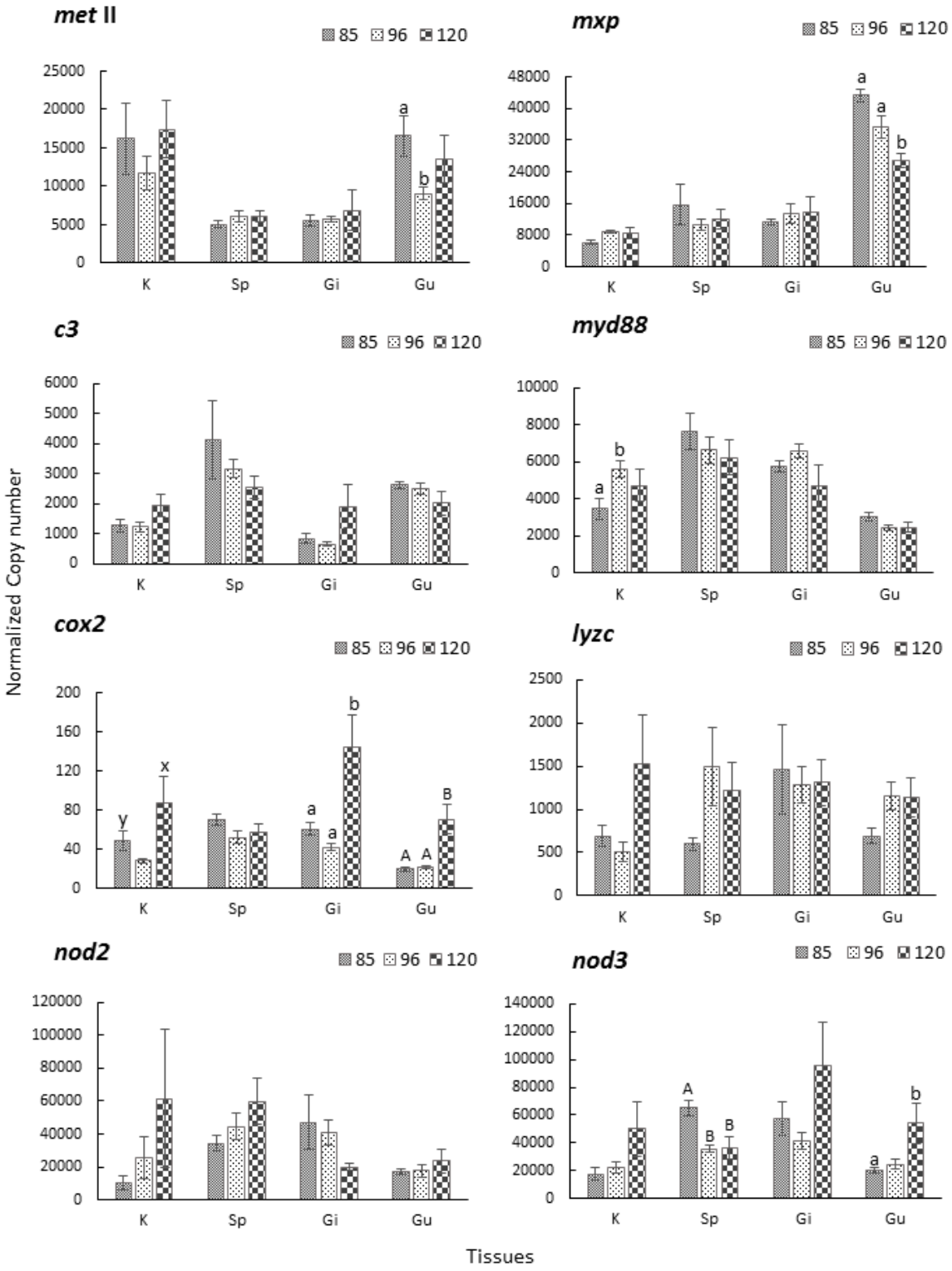


Fig. 4. Temporal change in gene expression levels (relative expression), from tissue (n = 8) from 8-120 dph, *of A. regius* transcripts coding for *met II*, *c3*, *cox2*, *lyzc*, *mxp*, *myd88*, *nod2*, *nod3*. Relative expression *met II*, *c3*, *cox2*, *lyzc*, *mxp*, *myd88*, *nod2*, *nod3* transcripts were normalized using an arithmetic mean of three housekeeping genes: GAPDH, HPRT, and β-actin. Transcriptional fold changes of this genes at different time points were calculated by comparison to each previous time point. Different letters above the bars

indicate significant differences among different time points (Tukey´s test, $P \leq 0.05$) Results are expressed as the mean ± SEM. K = kidney, Sp = spleen, Gu = gut, Gi = gill.

3.2.*2 Response of meagre innate immune genes to in **vivo** PAMP stimulation*

To gain knowledge of how different pathogen stimuli influence changes in the expression of these genes, an *in vivo* challenge with various PAMPs was performed, with samples collected for analysis after 24 h. Post-infection, *c3* mRNA expression most highly up-regulated in kidney, spleen, gut and gill by β-glucan. The LPS seemed to be a more potent stimulator of expression in gut and gill. Lysozyme was significantly up-regulated in kidney, spleen, gut and gill by LPS; curiously, there was significant down-regulation in mucosal tissues (gut and gill) by poly (I:C) while there was significant up-regulation in systemic tissues (kidney and spleen) by β-glucan stimulation. The expression of *met* II was potently induced in spleen, gut, and gill by LPS stimulation, whereas in spleen and gut it was also significantly up-regulated by poly (I:C) and β-glucan stimulation. The expression of *myd88* was significantly up-regulated in gut using all the PAMP stimulants. The abundance of *mxp* transcripts were increased in all the tissues tested when stimulated by poly (I:C), while in gut there was significant up-regulation ($P \leq 0.05$) using LPS and β-glucan. The expression level of *cox2* was significantly up-regulated in kidney and spleen by LPS. The expression of *nod2* transcripts showed the highest up-regulation in spleen and gut by LPS and β-glucan. Different expression profiles were evidenced for *nod3*; there was significant up-regulation ($P \leq 0.05$) in kidney and spleen by β-glucan, while the other PAMP stimulants had little significant effect (Fig 5).

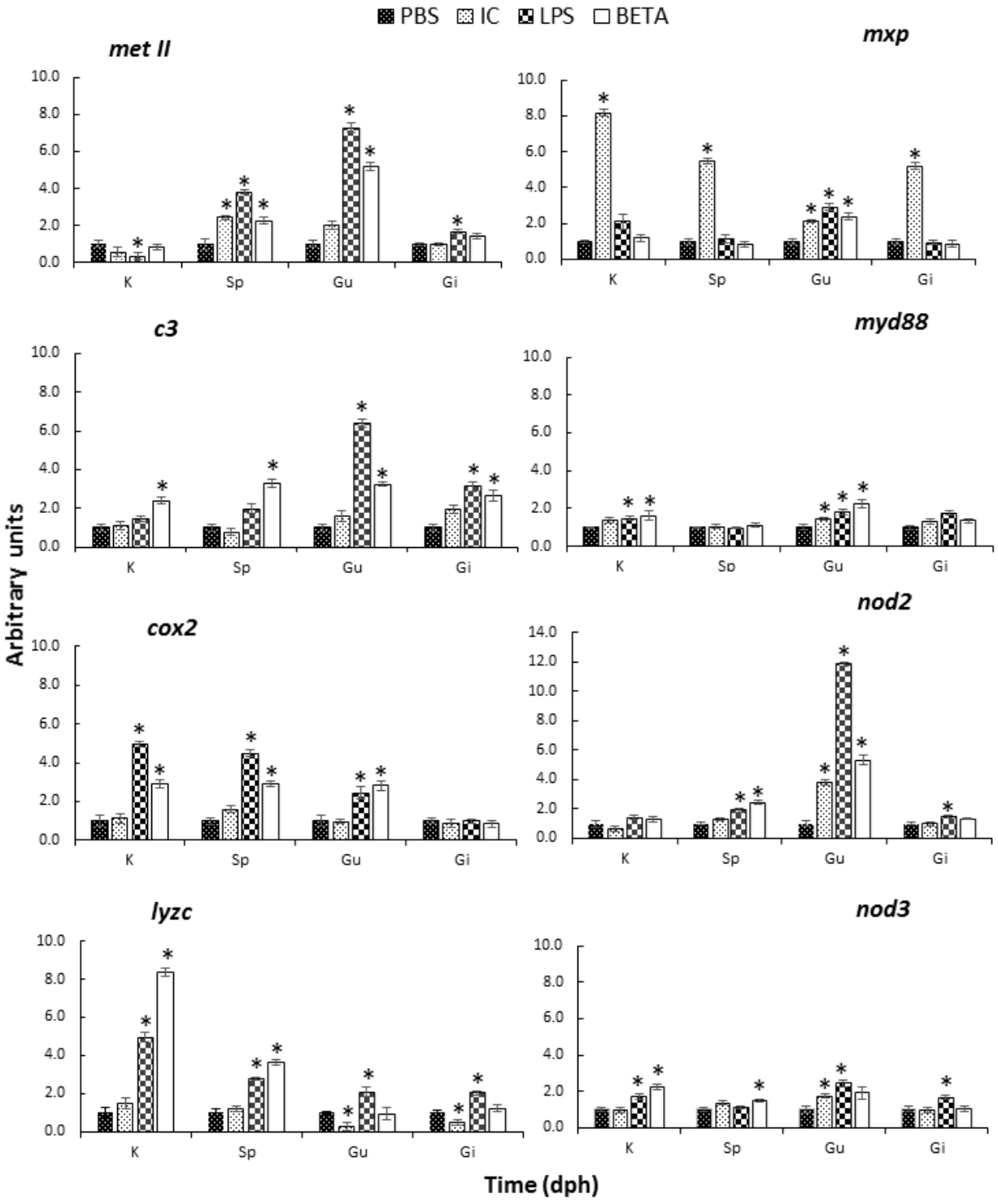


Fig. 5. Relative expression of *met II*, *c3*, *cox2*, *lyzc*, *mxp*, *myd88*, *nod2*, *nod3* in different tissues. Fish (n = 8) were injected intraperitoneally with poly (I:C), LPS and β-glucan and sampled at 24 h post-injection. GAPDH, was employed as an internal reference. Asterisks (*) mark significant between stimulated and control groups (Kruskal Wallis test, $P \leq 0.05$). Data are means ± SEM. K = kidney, Sp = spleen, Gu = gut, Gi = gill.

4. Discussion

The present study is the first report of some representative innate immune genes in meagre larvae and has focused specifically on a critical time in fish development when reliance on innate immune mechanisms are required for combatting infectious disease. Although previous studies have focused on identifying immune genes and /or characterizing their response to certain pathogens, there are few comparative data about their temporal appearance during development as a whole. In this study, the temporal appearance during larval development and tissues of relevant members of the innate immune repertoire has been described.

Phylogenetic analysis was used to confirm the specific identity of some genes where closely aligned isoforms are known that could confound gene expression analysis. The phylogenetic analysis of *met* reveals that the isoform identified from meagre is *met II*, and is an isoform more similar to a homolog described in *Larimichtthys crocea* as expected, since this is taxonomically a quite similar species of fish. Metallothionein has been shown to be a potential biomarker for metal contamination but also has an important role in Zn regulation during development [21]. Zinc is one of the major trace elements indispensable for cell division and multiple biological functions, particularly where zinc-finger transcription factors are implicated. In this study *met II* showed a higher expression level from 8 dph, but slowly decreased during development in meagre. In specific tissues during later development, the abundance of *met* transcripts increased, particularly in kidney and gut. In these tissues metallothionein is especially important in development due to cellular signals that are required during cell growth and differentiation, which require nuclear localization of this intracellular zinc regulator [22].

Complement is known as an essential humoral system of innate immunity, also providing a link with the adaptive immune response and has been shown to be involved during pattern formation of developing embryos/larvae [23]. In this study, the expression of *c3* was detected from the initial timepoint of the study (8 dph), but at 29 dph transcript levels of *c3* reached their lowest, coinciding with the end of the weaning (*Artemia* to artificial diet), and their highest at 60 dph. As larvae matured, the expression level of *c3* in tissues increased in spleen and gut tissues, probably acting to facilitate hematopoietic development [24] stem-cell differentiation [25] and defense against invading pathogens ingested with feed [23]. These results suggest that complement is not only important in the immune defense, but may play a role in the formation and generation of different organs during

development [26]. Cyclooxygenase is the enzyme responsible for the initial rate-limiting conversion of arachidonic acid to prostaglandins (PGE2 and PGH_2). Mammals contain two *cox* genes, cox1 and *cox2*, and homologs of these genes have been found in many fish including zebrafish (*Danio rerio*) [27]. Although, it is known that both isoforms are similar, *cox1* is generally assumed to be constitutively expressed performing homeostatic and maintenance functions, while *cox2* is induced by inflammation, cytokines, endotoxin and other pathophysiological processes [28]. In the present study, *cox2* displayed a decrease in expression between 8 dph and 60 dph which may be due to the expression signal being diluted as the larvae grew, as the abundance of *cox2*-postive cells likely did not increase in the same proportion as the general growth and increase in musculature. Although, Ishikawa *et al*. [29] found *cox2* in zebrafish, but did not see evidence of a link to processes of development. In Atlantic salmon (*Salmo salar*) [28] and mummichug (*Fundulus heteroclitus*) [30] *cox2* also was observed, possibly leading to an up-regulated production in total PGs. In the present study high *cox2* expression was seen in kidney, gill and gut at 120 dph, although the relation of this expression to PGE production in these tissues was not evaluated. High *cox2* expression and consequent high production of PGs in tissues such as gill possibly aid the process of osmoregulation [7] and ion transport in euryhaline species [30], and in the gut to maintain the integrity of the mucosal barrier [31].

Myeloid differentiation factor 88 (Myd88) is an important adaptor protein in the Toll-like receptor (TLR) signaling pathway and is used by all TLRs except for TLR3. Orthologs of *myd88* have been found in multiple fish species [12,32,33] demonstrating the ubiquitous nature of this intracellular immune mediator due to its crucial role in host innate immunologic surveillance as an accessory protein to ligand receptors. The gene for Myd88 was found expressed continuously in all stages of larval development with a sharp increase at 60dph. In more developed juveniles where individual tissues were examined, *myd88* transcripts were detected in all tissues in meagre, while high levels of transcripts were observed in the gill, spleen and kidney at 85, 96 and 120 dph.

The induction of the gene *mxp* in mammals is specifically induced by type I-IFN [34]. The antiviral effect of type I IFN is exerted through binding to the IFN-α/β receptor, which triggers signal transduction through the Jak-stat signal transduction pathway resulting in expression of *mxp* and other antiviral proteins. During early development the peak in expression was observed at 60 dph and it was also expressed in all tissues examined during later stages of development, with a prominent increase in the gut. The gut epithelium is a continuation of the interface between the external and internal host environment, therefore

requires defense mechanisms to be present continuously to act rapidly for limiting infection and maintaining gut homeostasis. Mxp is among those proteins that responds quickly to viral attack, but as the immune system matures might be maintained at a constitutively higher level in the gut relative to other tissues.

The lysozyme isoform initially identified in meagre was shown to be c-type, which has been reported previously in many teleost species during ontogenesis [35–37]. Lysozyme possesses lytic activity against both Gram-positive bacteria and Gram-negative bacteria. For this reason it is an important defense molecule of the immune system, widely present at host /environment interfaces including gills, and gastrointestinal tract, but also present in lymphoid tissues, serum, mucus, and other fluids [24]. Besides an antibacterial function, it promotes phagocytosis by directly activating polymorphic nuclear leucocytes and macrophages, or indirectly by an opsonizing foreign bodies. In fish, there exist two variants of lysozyme: c and g- types. At 29 dph, transcript levels of *lyzc* were found to be significantly up-regulated around the time of weaning (transfer to *Artemia* and dry feed). Possibly, this increased expression during the transition of diet may reflect certain nutrients or exposure to new cohorts of bacterial species in different feed sources are stimulating expression of this immune effector. It may also be that specific components supplemented in the feed modulate the lysozyme expression [5,38]. Lysozyme gene expression in teleost has been assessed in tissues from healthy fish, but the levels and pattern of expression varied for individual species, the isoform under study, and tissues examined [36,39]. In the current study, analysis of *lyzc* in tissues showed an increase in kidney, spleen and mucosal tissue (gut and gill) at 85 dph and a much larger increase at 120 dph, in accordance with other studies in teleosts [35–37,40]. These authors [40] found c type lysozyme in rainbow trout to be specifically expressed in the liver and kidney [41] whereas in japanese flounder (*Paralichthys olivaceus*) the c-type lysozyme gene is expressed in the head kidney, spleen, brain and ovary, and in brill it was observed in liver and stomach [35]. Therefore, differences in the tissue distribution of Lyzc between fish species might suggest some variation in function, and potentially multiple roles.

The phylogenetic analyses of NOD isoforms from meagre demonstrated the two isoforms identified reside hierarchically in different clades, consistent with their presumed separate functions. The genes *nod2* and *nod3* belong to the subfamily of NOD-like receptors (NLRs) characterized by CARD-containing effector-binding domains. Nod2 is a member of the cytoplasmic pattern recognition receptors (PRRs) family which recognizes muramyl dipeptides derived from peptidoglycan, present both in Gram-positive and negative

bacteria. Upon ligand recognition, Nod2 induces the activation of the NF-kB and MAPK pathways. Activation of NFkB and Mapk induce transcription and production of inflammatory cytokines, chemokines and antimicrobial peptides which mediate the antimicrobial response. Nod3 (NLRC3) belonging to the NLR-C subfamily, plays a role in the innate immune response against bacteria and virus. Studies have also suggested that it has a role in modulating T cells and inhibiting inflammatory mechanisms, and although studies in species such as catfish have shown *nod3* is present in many tissues [42],

Little is known about the precise mechanism, activation and signaling cascades of members of the NLRC subfamily. During the first 60 days of this study, Nod2 and Nod3 genes showed similar expression patterns during larval development, with the transcripts exhibiting abrupt up-regulation at 29 dph and 60 dph, which could suggest a coordinated activity between the two peptides these genes encode. In contrast, differences were observed in the individual tissues examined. Expression of *nod2* increased in the kidney and spleen while *nod3* transcripts were highly expressed in gill and gut at 120 dph. Similar results for *nod2* expression in the kidney were obtained from grass carp (*Ctenopharyngodon idella)* [43], and grouper [44], while in rainbow trout, *nod2* isoforms (*nod2a* and *nod2b*) were detected highly expressed in muscle [45].

In vivo

Although information on the expression of genes of the immune response is growing, the totality of data is somewhat limited and comparative studies on molecular effectors of the innate immune system often rely on mammalian literature as a starting point. We examined whether the expression of these genes could be modulated by PAMPs. An *in vivo* study was performed with three individual PAMPs injected intraperitoneally and measured the immune gene expression responses 24 h later.

Metallothionein is a protein usually rich in cysteine residues that coordinate multiple zinc and copper atoms under physiological conditions. In our study, we found in the spleen an increase in expression of *met II* by stimulation using the three different PAMPs, while in gut the expression of *met II* was up-regulated by LPS and β-glucan, but in gill was only stimulated slightly by LPS. These results suggest the spleen is somewhat more responsive with regard to *met II* activation where recruitment of lymphocyte lineages are an important response for preventing systemic infections. The response in the gut is much more pronounced using LPS and β-glucan as stimulants, demonstrating this tissue is functioning as a first line of defense with a much faster, stronger response against invading pathogens.

Interestingly, there was a slight, but statistically significant, decrease in expression in the kidney under stimulation by LPS.

A relatively high *mxp* expression was observed in all tissues by poly (I:C) stimulation, but *mxp* expression was also stimulated to a lesser degree in the gut by LPS and β-glucan. High expression of *mxp* transcripts in mucosal tissues (gill and gut) are expected as these are the main entrance route for fish viral infections, while systemic tissues (kidney and spleen) have a high content of lymphocytes that respond to viral attack and produce Mx protein.

The expression of *myd88* was found to be up-regulated in gut by all PAMPs tested. The expression of *myd88* is ubiquitous as it functions as an intracellular immune mediator. Increased expression in the gut may facilitate improving the integrity of the intestinal barrier by PAMPs recognition, TLR (toll –like receptor) can recruit adaptor molecules Myd88 for signal transduction to activate nuclear factor-kappa B (NF-kB).

The complement cascade of the innate immune system functions to eradicate pathogenic microbes by either the classical pathway, or after hydrolysis and initiation of the alternate pathway, leading to cell lysis. In our study, *c3* transcripts were up-regulated in all the tissues tested by β-glucan. Interestingly, Campoverde et al. (2017) [46] detected a similar response by piscidin, an antimicrobial peptide, in meagre in all the tissues tested (kidney, spleen, gut and gill) after β-glucan stimulation, which could suggest a common response mechanism under β-glucan stimulation such as regulation by pro-inflammatory cytokines such as IL-1. However, in a study with rainbow trout changes in the expression level of *c3-1*, *c3-3*, *c3-4* mRNAs were detected in liver, spleen and head kidney after stimulation with LPS, but β-glucan stimulation resulted in a moderate down-regulation of *c3-4* in all tissues studied [47]. .

Multiple genes encoding the c-type lysozyme are found in different species, and in some species different isoforms perform distinct functions, or have tissue-specific expression patterns [36]. In the case of meagre *lyzc* transcripts were found to increase significantly in kidney and spleen after injection with LPS and β-glucan, while in gill and gut expression was up-regulated by LPS stimulation, but down-regulated by poly (I:C) stimulation. The higher levels of expression detected in the gill are due to the epithelium of this tissue providing the primary barrier against a hostile external environment that harbors a multitude of potential pathogens, and therefore a quick and rapid response is the best way to obviate any ingression of invaders. These data from meagre are consistent with other studies from teleosts, but contrasting results have been obtained; studies of c-type lysozyme in brill (*Scophthalmus rhombus*) showed no significant change in mRNA levels after

bacterial challenge [36] as this isoform might possess a digestive function, whereas g type lysozyme likely has a defensive role.

With regard to *cox2* gene expression, significant difference was observed in the kidney, spleen, and gut after injection with LPS and β-glucan, but there were no changes in gill tissue. This makes sense physiologically as there is a need to maintain respiratory functions and avoid unnecessary damage to respiratory epithelia from an excessive inflammatory response and therefore more stimulation might be needed to elicit significant up-regulation of *cox2* and the concomitant increase in prostaglandin synthesis. These results demonstrate that *cox2* is not constitutive, but can be induced by PAMPs binding to PRRs in kidney, spleen, and gut.

In the case of *nod2*, transcripts were observed up-regulated in spleen after injection by LPS and β-glucan, while in gut all the PAMPs tested resulted in up-regulation. In gills there was a weak response when fish were injected with LPS, which can again be explained as a protective measure to avoid an unregulated pro-inflammatory response leading to tissue trauma. The aquatic habitat is a rich source of bacterial LPS and the immune responses activated by the presence of LPS would need to be under tight control to differentiate presence of LPS liberated from natural bacterial cell death from an active invasion. . Gene expression levels for *nod3* were generally weak in all tissues. The expression of *nod3* was not high, but significantly up-regulated, in kidney by LPS and β-glucan, in spleen by β-glucan, in gut by poly (I:C) and LPS, and in gill tissue by LPS. Gene expression was effectively modulated in response to PAMPs in all the studied genes, while the induction of gene expression by each stimulate demonstrated tissue-specific differences.

In conclusion, the current study is the first identification in meagre of these genes and describes their modulation of expression during development and demonstrates their response to PAMP stimulation. The data from the ontogeny portion of this study in *A. regius* implicates diet (ie. - switching from live feed onto artificial feed) as one factor that influences the observed fluctuations in expression of these innate immune genes. This work provides information for future investigations of the innate defense mechanisms during early development of meagre. Further studies in larval nutrition and identifying changes in the components in the diet that can beneficially augment gene expression of the immune system will benefit larval rearing protocols by reducing mortalities.

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Conclusions

Conclusions

Fish Larval Nutrition

1. *A. regius* larvae can be weaned from live feed to artificial diets as early as 12 dph (5.14 mm SL), but other important aspects for production success, including larval performance and survival, should be considered.
2. Early weaning at 12 days post-hatch did not affect the incidence of skeletal deformities in meagre, which is of special relevance in terms of assuring fry quality for further on-growing purposes.
3. It is advisable to include between 12 - 15% of DHA (260-300 mg/g lipids) in the live prey enrichment diet for meagre larval rearing to obtain good growth and survival.
4. The survival rate in meagre was not affected by DHA levels in the diet used for larval feeding, but it was affected by the cannibalistic behavior of the larvae from 20 dph (7.9 ± 0.35 mm SL) onwards.
5. Cannibalistic behavior should be avoided and rearing parameters such as light intensity (reduce), feeding frequency (increase), and differences in larval size (grading), need to be controlled to enhance larval survival rates.
6. Meagre larvae are not capable of elongation or desaturation of fatty acids when precursors such as LA (18:2n-6), GLA (18:3n-6), LNA (18:3n-3) or SDA (18:4n-3) are offered in the live prey.

Ontogeny

Digestive system

7. Ontogenic changes in digestive enzyme activities in *A. regius* followed the same pattern than in other marine fish species, being completely functional between 6.0 and 6.8 mm in SL.
8. The histological and enzymatic analytical procedures evidenced that stomach's functionality did not match its morphological organization, since pepsin activity was not

detected until some days after the appearance of the first gastric glands and coincident with notochord flexion and the progressive decrease in the activity of alkaline proteases indicating a shift in the mode of digestion.

9. The functional development of the digestive system in *A. regius*, assessed by the activity of alkaline and acid proteases, is a well-conserved process that generally occurs within the same range of body sizes independently of larval age.

Innate Immune system

10. The pattern of development of the lymphoid organs thymus, kidney, and spleen of *Argyrosomus regius* is similar to the general pattern observed in other teleost fish species, but the timing of organ and system development exhibited interspecific differences, which were mainly due to different species' reproductive guilds (precocial vs altricial development).

11. The immune organs of meagre larvae developed along the following sequence: the pronephric kidney was visible at 3.15 ± 0.1mm SL (1 dph), the thymus appeared at 4.49 ± 0.39 mm SL (8 dph), being clearly visible in larvae measuring 5.69 ± 0.76 mm SL (12 dph), whereas the spleen was detected at 12 dph in this study.

12. While functional studies are needed to confirm the existence of active immune memory, the morphology of the lymphoid organs of meagre suggest that *A. regius* is not completely immuno-competent earlier than 66.3 ± 4.3 mm in SL that correspond to 83 days of age (1834,3 degree days) in this study.

13. This study presents the first identification of antimicrobial peptides (AMPS: beta defensin [*defb*], hepcidin 2 [*hep2*], and piscidin [*pisc*]) in meagre. The abundance of mRNA varied during ontogeny by developmental stage, and in later juvenile stages, by tissue type. The phylogenetic analysis of the sequences obtained demonstrate that these newly described AMPs are more similar to those homologs already described from taxonomically related species, such as *Larimichthys crocea*.

14. The modulation of AMPs expression was observed under conditions mimicking pathogen infection using pathogen associated molecular patterns (PAMPs): LPS, β-glucan, and poly (I:C), and this confirmed some of the expected modes of stimulation.

15. *β-defensin, hepcidin, piscidin*, expression seems to be influenced by dietary factors such as the switch from rotifers to *Artemia* nauplii, or the weaning onto artificial diets that were used in the current rearing protocols.

16. Melano-macrophages center (MMCs), important in the recruitment of phagocytes appeared in spleen at 83 dph, indicating that the non-specific defenses in meagre may be capable of handling invading foreign particles before the specific immunity is fully developed.

17. The immune gene transcripts *met* II (metallothionein II), *c3* (complement), *cox2* (cyclooxygenase 2), *lyzc* (lysozyme C-type), *mxp* (mx protein), *myd88* (myeloid differentiation primary response gene 88), *nod2* (nucleotide-binding oligomerization domain-containing protein 2), and *nod3* (nucleotide-binding oligomerization domain-containing protein 3) were expressed at different levels in gene-dependent manner during early developmental stages and in a tissue-specific manner in later juvenile stages of meagre.

18. The phylogenetic analysis of the sequences obtained demonstrate these newly described *met* II, *lyzc, nod2* are more similar to those homologs already described from taxonomically related species, such as *Larimichthys crocea*.

19. The transcripts for *c3, met II, lyzc, mxp, myd88, cox2 nod2* and *nod3* displayed expression patterns consistent in response to three different PAMPs [LPS, β-glucan, poly (I:C)].

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