










Research Article

Lymphoid organ system development and early window of immunological susceptibility in spotted rose snapper *Lutjanus guttatus*

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ABSTRACT. The lack of a lymphoid organ system in *Lutjanus guttatus* larvae may limit survival, the use of immunostimulants, and the implementation of vaccination during early juvenile stages. Identifying the timing of autologous immunoglobulin M (IgM) activity in lymphoid organs could improve progeny survival. Therefore, to examine maternal IgM depletion and the first IgM autologous production, samples were collected at multiple time points during the larval and early juvenile stages of *L. guttatus* development. The lymphoid organs (e.g. head kidney, spleen, gut-associated lymphoid tissue, and thymus) were described by histomorphology and immunohistochemistry, and we also analyzed the gene expression of immune-related markers. The primitive head kidney was evident at 2 days post-hatching (dph), the spleen and thymus appeared at 3 and 9 dph, respectively. Protection by IgM-dependent maternal transfer was depleted at 2 dph (1.8 mm total length, TL). The first IgM autologous production was detected in juveniles at 35 dph (15 mm TL), and an increase in immune-related gene expression was observed at 15 dph (~5.35 mm TL). This increased gene expression was associated with antigen-specific immune responses, such as helper T cell activity. *L. guttatus* presents an early window of immunological susceptibility (EWIS) during the larval period. Our data show how this window is covered by the innate response from 2 to 35 dph (1.8 to 15 mm TL). These findings represent essential knowledge for early immunostimulant or vaccination programs in *L. guttatus* culture schemes. Our results suggest that 35 dph (15 mm TL) may be the initial larval age-size for initiating vaccination programs at both experimental and commercial scales.

Keywords: *Lutjanus guttatus*; ontogeny; lymphoid organs; larvae; histomorphology; immunohistochemistry; gene expression

INTRODUCTION

The spotted rose snapper *Lutjanus guttatus* has significant aquaculture interest in several Latin American countries (Herrera-Ulloa et al. 2010, Alvarez-Lajonchère et al. 2012). However, the constant production of juveniles remains an important milestone to achieve despite recent significant efforts to develop a feasible, science-based full-cycle aquaculture technology (Ibarra-Castro et al. 2012, 2020a,b, Sandberg et al. 2014, Chacón-Guzmán et al. 2021, Martínez-Brown et al. 2023).

Unfortunately, as in other cultured marine fishes, frequent and unpredictable mortality episodes occur during the early life stages of larval development, which husbandry practices could influence (Kjørsvik et al. 2011), opportunistic microorganisms (Vadstein et al. 2013), and due to an incipient and poorly developed immune system in new larvae (Swain et al. 2002, Akbary et al. 2015), they are susceptible to getting infected easily. Thus, to evaluate the feasibility of immunoprophylaxis strategies to maximize *L. guttatus* juvenile production, we needed new insights into immune system development.

During the early life stages of teleost development, distinct immune-related patterns have been described, including hematological characterization (Zapata et al. 2006), onset of lymphoid organs (Swain & Nayak 2009), and the autologous production of immunoglobulin M (IgM) (Magnadóttir et al. 2005). The adaptive immune response is phylogenetically and ontogenetically conserved across teleost species (Mulero et al. 2007), suggesting that characterizing the timing and key events of immune ontogeny could inform the development of procedures for marine fish larviculture.

Most marine fish species with commercial aquaculture potential are oviparous; this implies that, from a physiological and nutritional perspective, free-floating fertilized eggs constitute a closed system (Kunz 2004). In this context, during the embryonic and yolk sac larvae stages, protection will depend on maternal immune molecules, such as IgM (Zhang et al. 2013), for a short period after hatching. Once this maternal protection is depleted, only the innate immune system is functional. Hence, in *L. guttatus*, the early window of immunological susceptibility (EWIS; Fig. 1) is defined by the absence of functional adaptive immunity, and it could represent an ontogenetic fragility window, and larvae can be infected (Vadstein et al. 2013), as previously observed in mammalian neonates (Chase et al. 2008). From a practical perspective, we believe that EWIS in *L. guttatus*

development can be described in two events: 1) when depletion of maternal IgM occurs; this event is species-specific, influenced by broodstock-specific conditions, and could occur after hatching, and 2) full functionality of the adaptive immune system; in marine fish, this event is delayed up to 8 to 12 weeks after hatch (Magnadóttir 2006). According to Ibarra-Castro et al. (2020a,b), vitelline reserve depletion in *L. guttatus* occurs at 2 days post-hatching (dph), and the adaptive immune system's functionality remains to be described.

In this sense, from early embryonic stages throughout metamorphosis, the differentiation of immune cells, tissues, organs, and organ systems follows a gradual improvement in functionality (Falk-Peterson 2005) (Tables 1-2). Hence, we hypothesize that immunity protection via maternal IgM is brief in *L. guttatus* larvae, and the adaptive immune responses may occur four weeks after hatch. Taken together, EWIS can occur from 3 dph through the end of complete metamorphosis. The end of maternal immune protection is relatively easy to determine; however, detecting activation of the adaptive immune response is more difficult. In vertebrates, the recombination-activating gene 1 (*rag1*) and IgM have been considered useful markers of the immune system's physiological maturity. Hence, thymus development and immune function have been linked to certain genes and protein (IgM) expression, including mature lymphocyte genes such as *rag1*, *rag2*, and macrophage genes such as *cd4.2*, and *cd11b* (Bowden et al. 2005, Bajic et al. 2013, Tokunaga et al. 2017, Ashfaq et al. 2019). Therefore, the goal of this study was to describe the immune organs during early ontogenetic events that have been investigated, rather than to present a comprehensive study of the immune system of *L. guttatus* larvae. We followed maternal IgM depletion, the onset of IgM-autologous detection, the development of lymphoid organs (e.g. head kidney, spleen, gut-associated lymphoid tissue (GALT), and thymus), and the temporal expression of immune-related genes (*rag1*, *rag2*, *cd4.2*, and *cd11b*). New knowledge on the ontogeny of the immune system and the identification of early defense mechanisms will provide useful tools for developing sustainable immunoprophylaxis and vaccination strategies for both experimental and commercial aquaculture.

MATERIALS AND METHODS

This study was conducted following the general provisions of the Guidelines for the Use of Fishes in Research (Jenkins et al. 2014).

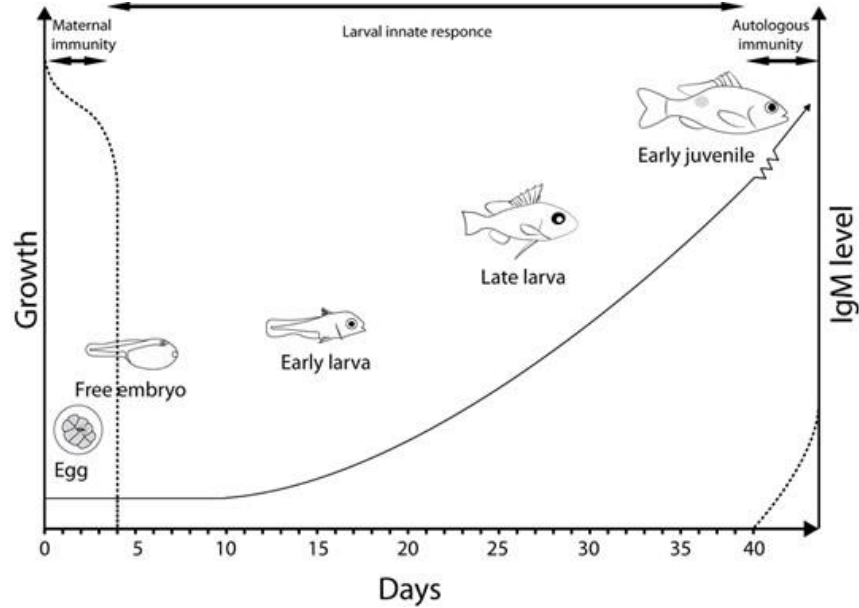


Figure 1. Immunological susceptibility window in *L. guttatus*. It is assumed that the maternal protection embryo stage involves immune molecules such as immunoglobulin M (IgM), and this typically occurs right after hatch and ends at 2 days post-hatching (dph) (1.8 mm TL). The autologous production of IgM in *L. guttatus* was first detected in juveniles at 35 dph (15 mm TL). Consequently, *L. guttatus* presents an early window of immunological susceptibility (EWIS) from day 4 to day 40, and the lymphoid organs appear in the early larval stage.

Table 1. Appearance of lymphoid organs of marine fish. dph: days post-hatching.

Species	Kidney	Spleen	Thymus	Reference
	dph			
<i>Lutjanus guttatus</i>	2	3	9	Present study
<i>Paralichthys olivaceus</i>	0	8	15	Liu et al. (2004)
<i>Gadus morhua</i>	0	5	28	Schroder et al. (1998)
<i>Scophthalmus maximus</i>	0	5-6	3-4	Padrós & Crespo (1996)
<i>Pagrus major</i>	<1	3	11	Chantanachookhin et al. (1991)
<i>Thunnus orientalis</i>	<1	2	5	Watts et al. (2003)
<i>Epinephelus bruneus</i>	<1	6	12	Kato et al. (2004)
<i>Seriola quinqueradiata</i>	1	3	11	Chantanachookhin et al. (1991)
<i>Harpagifer antarcticus</i>	1	3	3	O'Neil (1989)
<i>Lates calcarifer</i>	2	2	2	Azad et al. (2009)
<i>Lutjanus erythropterus</i>	2	6	14	Cui et al. (2018)
<i>Polyprion oxygeneios</i>	6	16	20	Parker et al. (2012)
<i>Paralichthys olivaceus</i>	7	8	10	Chantanachookhin et al. (1991)
<i>Dicentrarchus labrax</i>	10	18	21	Abelli et al. (1996)

Larviculture procedure

The study was carried out at the Pilot Marine Finfish Hatchery of the Research Center for Food and Development (CIAD, by its Spanish acronym) at Mazatlan, Mexico. Fertilized eggs were obtained from captive broodstocks during the 2017 reproductive season. The larval procedure protocol was described in

detail by Ibarra-Castro et al. (2020a,b). Briefly, egg incubation and larval rearing were conducted in a 7 m³ cylindrical fiberglass tank with black walls and a white bottom. The tanks were located indoors. The mean stocking density was 48 floating eggs L⁻¹ (100% fertilization), while egg and oil droplet diameters were 782 ± 23 and 123 ± 9.9 μm, respectively. The hatching

Table 2. First detection of immunoglobulin M (IgM) autologous antibodies and *rag1* gene peak expression in marine fish. dph: days post-hatching, TL: total length (mm), GALT: gut-associated lymphoid tissue.

Species	<i>rag1</i> (dph)	IgM (TL)	IgM (dph)				Reference
			Kidney	Spleen	GALT	Thymus	
<i>Lutjanus guttatus</i>	15	14.9	35	40	40	100	Present study
<i>Anarhichas minor</i>		25-86	7	14	56	91	Grøntvedt & Espelid (2003)
<i>Polyprion oxygeneios</i>			12	32	45		Parker et al. (2012)
<i>Seriola lalandi</i>	21-25						Peluso (2023)
<i>Latris lineata</i>	21-45						Covello et al. (2013)
<i>Paralichthys olivaceus</i>	35-40						Lee et al. (2014b)
<i>Melanogrammus aeglefinus</i>	40-50	6-7					Corripio-Miyar et al. (2007)

rate was determined by incubating 50 eggs with live embryos in each of six 1 L beakers, recording the number of newly hatched larvae in three 1 L beakers at 24 h. The same procedure was used for the rest of the beakers to estimate the rate of larval survival at 48-h post-hatch (hph). The 48-hph coincided with the first feeding stage (Fig. 3).

Before reaching the larval culture tanks, the seawater was filtered through 10, 5, and 1 μm multi-cartridge filters, then passed through a UV disinfection unit (dose: 60 mJ cm^{-2}). Seawater quality parameters during egg incubation and larval rearing were temperature $26 \pm 1.0^\circ\text{C}$, salinity 35 ± 1.0 , dissolved oxygen $5.1 \pm 0.6 \text{ mg L}^{-1}$ (saturation: $77.8 \pm 9\%$), and pH of 8 ± 0.2 . Daily husbandry activities included environmental control, cleaning, feeding, and behavioral observations as detailed by Ibarra-Castro et al. (2020b). The green-water technique (using the microalgae *Nannochloropsis* sp. and *Isochrysis* sp.) and co-feeding were employed in larviculture procedures (Ibarra-Castro et al. 2020a,b). Briefly, enriched rotifers *Brachionus rotundiformis* (Rojo-Cebreros et al. 2017) were added from 2 to 25 dph, *Artemia* sp. nauplii from 18 dph and enriched *Artemia* sp. metanauplii from 23 to 38 dph. Rotifers and *Artemia* metanauplii were enriched with DHA Protein Selco (INVE[®]) and Spresso (INVE[®]), respectively, according to the manufacturers' instructions. Micro diets (Otohime[®]) of different grain sizes were offered from 20 dph to the end of the juvenile period (100 dph).

Larval growth record

Weekly samples of 25 free embryos, 25 larvae, and 25 juveniles were randomly taken. Before morphometric measures were taken, larvae and juvenile (0-26 dph) were anesthetized (0.25 mL L^{-1} 2-phenoxyethanol at 9%; P1126, Aldrich) and photomicrographs were taken using an optical microscope (Olympus BX41, USA), and a stereomicroscope (Motic SMZ171, USA) equipped with a digital camera (Olympus DP27, USA)

and operated with image analysis software (SigmaScan Pro5, USA). Likewise, bigger fish (26-100 dph) were measured with a Vernier caliper (Scienceware[®], Z136115, USA).

Histomorphology analysis

Random sampling of larvae and juveniles was conducted for histological analysis and to examine lymphoid organ development. The sampling protocol was designed to encompass critical developmental events from the egg to the juvenile stage. Sampling was conducted daily from 0 to 40 dph with a cohort of 50 larvae ($n = 50$). Subsequently, sampling took place every third day from 41 to 86 dph, involving 40 juveniles ($n = 40$). From day 87 to the conclusion of the experiment, at 100 dph, sampling occurred every five days, comprising 20 juvenile specimens ($n = 20$). All collected samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and, after 72 h, transferred to 70% ethanol. To facilitate proper penetration of the fixative solution after death, juveniles were injected into the visceral cavity. For histological processing, the modal size of each sample was utilized. Paraffin-embedded samples were sectioned at 5 μm and stained with hematoxylin-eosin (Sheehan & Hrapchak 1980). In addition, only samples from 20 dph were previously decalcified (Luna 1968) to streamline the cutting procedure.

Immunohistochemistry analysis

To determine the transition from the depletion of maternal IgM to the initiation of autologous IgM production, samples of eggs, free embryos, larvae, and juveniles were systematically collected. The fixation and histomorphology processing procedures mirrored those outlined in the histomorphology analysis. Immunohistochemistry assays were performed to detect and localize IgM. Specifically, a monoclonal anti-IgM antibody for fish was generated in a mouse (V53P3H2/B2, NOVUS), and a secondary anti-IgG-

HRP for mouse was generated in a rabbit (SIGMA A9044). Both antibodies were applied at a 1:100 dilution in PBS, as described by Ventura-Juárez et al. (2003).

RNA extraction and qPCR analysis

Triplicate samples, each containing 10 whole larvae, were collected at 5, 10, 15, and 20 dph. Samples were homogenized in TRIzol Reagent (Bioline) according to the manufacturer's recommendations to isolate total RNA. Afterward, RNA was treated with a TURBO DNA-Free kit (Thermo Fisher Scientific, Waltham, MA) to eliminate genomic DNA contamination (which was confirmed by direct amplification of RNA). cDNA synthesis was performed using Maxima Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA) and random hexamers (T4 Oligo, ADN Sintetico SAPI de CV, Ecuador) at 45°C for 1 h. The cDNA was stored at -20°C until used for qPCR analysis.

Primer sequences were obtained from *de novo*-assembled transcripts of *L. guttatus* (Escalante-Rojas et al. 2018) using PRIMER 3 (Untergasser et al. 2012). Briefly, gene sequences were retrieved from the NCBI database, and BLASTn was performed against the transcriptome (Table 3). Splicing variants were aligned, and primers were designed from conserved regions.

Primer sequences, as well as melting temperature, amplification efficiency, and amplicon size, are shown in Table 4. Samples were preamplified in a T100 thermal cycler (Bio-Rad, Hercules, CA) with 1 cycle of 2 min at 94°C, then 20 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. Amplification was carried out in a volume of 13 µL containing 0.13 µL of 10 µM of each primer, 0.66 µL of 2.5 mM deoxynucleotide mix, 0.75 µL of 25 mM MgCl₂, 1.25 µL of 10X buffer, 0.1 µL of Taq polymerase enzyme (5 U µL⁻¹), 8.98 µL of distilled water, and 1 µL of cDNA. Agarose gel electrophoresis (2%) with 1X TAE buffer (40 mM Tris base, 40 mM acetate, 1 mM EDTA) was used to examine the product of PCR. PCR products were diluted 1:100 in distilled water treated with diethyl pyrocarbonate (0.1%). Real-time PCR quantification was performed using the Master Mix qPCR kit (Thermo Fisher Scientific, Waltham, MA) in a CFX96 thermal cycler (Bio-Rad, Hercules, CA) under the following conditions: 1 cycle for 30 s at 95°C, then 40 cycles of 20 s at 95°C and 20 s at 60°C (Torres-Velarde et al. 2020). The geometric meaning of two reference genes (β -actin and 18S rRNA) was used to calculate gene expression according to Pfaffl (2004). The stability of both β -actin and 18S rRNA in this species was assessed in previous studies (Galaviz et al. 2012, Torres-Velarde et al. 2015).

Statistical analysis

The expression levels of *rag1*, *rag2*, *cd4.2*, and *cd11b* were statistically analyzed as follows: all data were tested for normality (Shapiro-Wilk) and homoscedasticity (Levene's test). Mean relative gene expression levels across sampling dates were compared using one-way ANOVA. The Tukey HSD test was used for multiple range comparisons at the $P < 0.05$ significance level. Non-parametric Kruskal-Wallis test and *post-hoc* Dunn's test were performed when the premises of the ANOVA were not met (Zar 2010). All statistical analyses were performed in R v 4.0.

RESULTS

Larval growth

In *L. guttatus*, the larval period started with the first feeding stage (3 dph) at a TL of 2.8 mm, and complete metamorphosis was observed at 35 dph (14.9 mm TL). The yolk and oil globule were depleted by 2 and 3 dph, respectively. Throughout the larval growth period and until 100 dph (83.3 mm TL) in the juvenile stage, an exponential growth rate was evident ($y = 7.8877e^{0.0253x}$, $R = 0.92$; Fig. 2).

Histomorphology and development of lymphoid organs

The immune system in teleost fish is shaped by two types of responses: innate and specific. The lymphoid organs associated with these responses include spleen, head kidney, GALT, and thymus (Fig. 3, Table 5). Key events in the early ontogeny maturation of lymphoid organs in *L. guttatus* occurred as follows: at 12-14 h post-fertilization, eggs hatched (0 dph), yolk depletion occurred (1.8 mm TL, 2 dph), oral first feeding initiated (2.8 mm TL, 3 dph), post-flexion stage was reached (6.6 mm TL, 20 dph), metamorphosis took place (14.9 mm TL, 35 dph), and weaning occurred (23.0 mm TL, 40 dph) (Table 5).

Head kidney

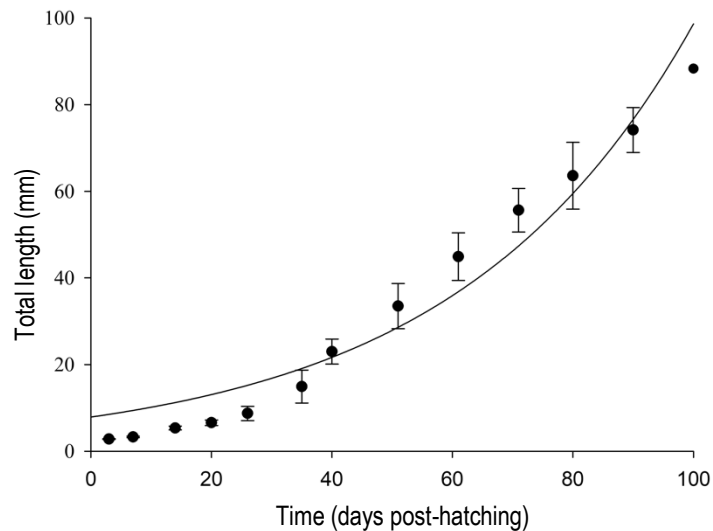
At 2 dph (1.7 mm TL), the kidney consisted only of the pronephros. The pronephros is composed of pronephric tubules and the ducts. The pronephric ducts run dorsally to the alimentary canal, from the post-cephalic region to the urinary bladder. At 3 dph (2.8 mm TL), the pronephric tubules were convoluted, surrounded by blast cells (Fig. 4a). By 25 dph (4 mm TL), the head kidney exhibited increased structural organization, characterized by a higher density of renal tubules and the presence of hematopoietic tissue surrounding them (Figs. 4b-c). At 25 dph (4 mm TL), head kidney devel-

Table 3. Primer sequences for de novo-assembled transcripts from *L. guttatus* (Escalante-Rojas et al. 2018).

Gene	GenBank Accession	Species	Gene ID (<i>L. guttatus</i> transcriptome)	E-value
<i>rag1</i>	JN106042.1		DN69870_c0_g1_i1	3.48 e -27
<i>rag2</i>	JN106041.1	<i>Lutjanus sanguineus</i>	DN78597_c1_g1_i1	6.32 e -21
<i>cd4.2</i>	KY020127.1		DN71137_c0_g1_i1	0.0
<i>cd11b</i>	AM713180.2	<i>Oncorhynchus mykiss</i>	DN69540_c1_g1_i1	2.83 e -58

Table 4. Primer sequences from the genes analyzed in this study. AE: amplification efficiency, PS: product size, F: forward, R: reverse.

Gene	Sequence 5'-3'	T (°C)	AE (%)	PS (bp)
<i>rag1</i>	F ATGGAG(A/G)CTGTGGAGGTGGT	63.1	104.7	140
	R AGCTGGTCGGGGCACTCTTT	64.0		
<i>rag2</i>	F GCTGCAAAGAGAAAGAGCTGGTGG	64.4	91.4	109
	R CGACCGCCAAACAACACGCAA	65.0		
<i>cd4.2</i>	F CGCCCAACAGCTGCAGCAA	64.8	92.2	114
	R TCTGCTTTCTCAATCGGCCTTCA	63.2		
<i>cd11b</i>	F ACAACACCAACCTTTCGATGCACT	63.5	90.3	144
	R ACACAGGACGGCTGATACCACA	63.6		
β -actin	F CCACGCCATCCTGCGTCT	65.8	94.7	167
	R CCATCTCCTGCTCGAAGTCCAG	65.7		
<i>18S rRNA</i>	F CTGAACTGGGGCCATGATTAAGAG	68.4	93.1	165
	R GGTATCTGATCGTCGTCGAACCTC	68.7		

**Figure 2.** Growth of *L. guttatus* during development (total length mm \pm standard deviation, n = 25) from 0 to 100 days post-hatching. $y = 7.8877e^{0.0253}$; $R = 0.9278$.

opment persisted, with a significant number of lymphocytes observed between renal tubules (Fig. 4d). As larvae developed, these cells became darker and smaller, indicating lymphopoiesis, which was evident at the post-flexion stage (20 dph, 6.6 mm TL). At 29 dph, the basophilic kidney cells increased, and the number of tubules surpassed the initial stage (Fig. 4e).

Morphological development of the head kidney was complete between 35 and 40 dph (14.9 and 23 mm TL; Figs. 4e-f).

Spleen

The spleen anlage appeared at 6 dph (2.8 mm TL) and was surrounded by the pancreas (Fig. 5a). At this age,

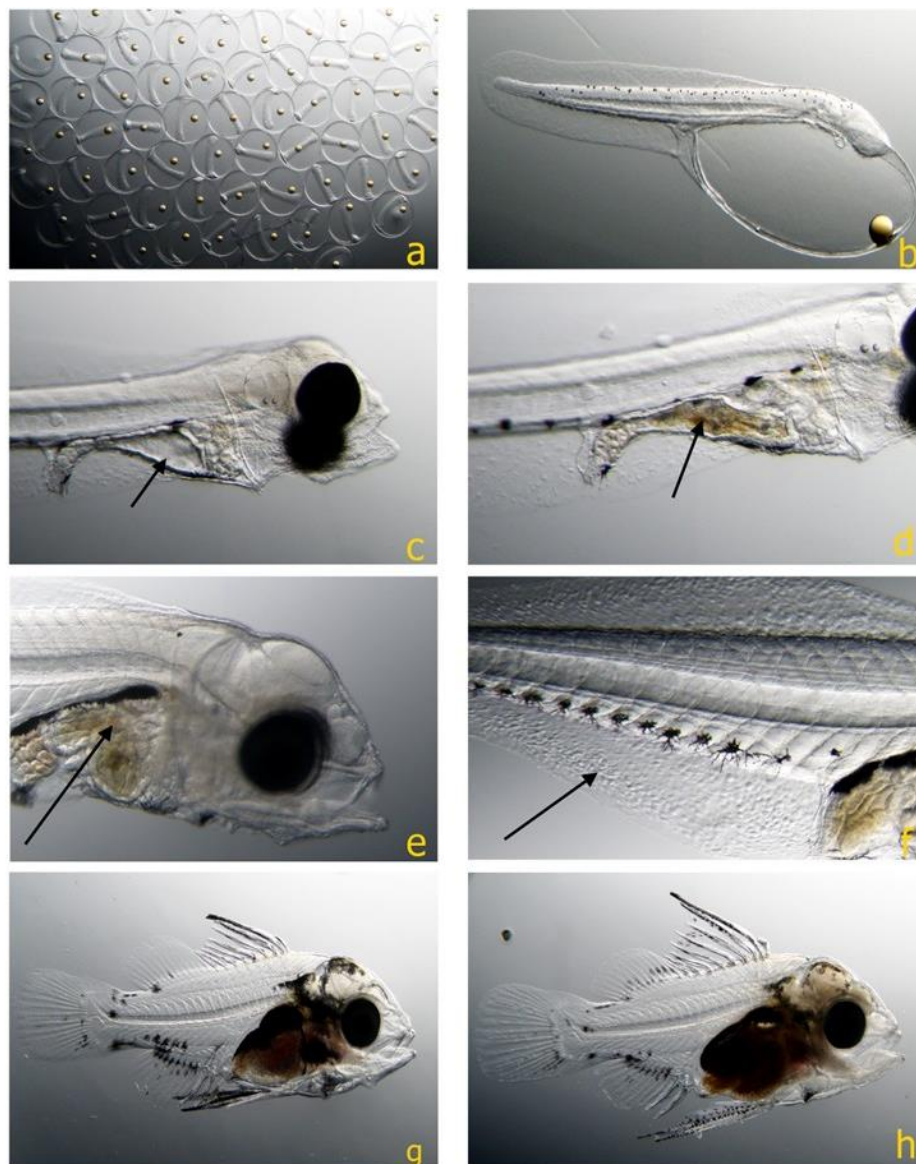


Figure 3. Early development of *L. guttatus*. a) Embryos at 12-14 hours post-fertilization, b) free embryo at 2 hours post-hatch; c) larvae with any endogenous reserve at 2 days post-hatching (dph) and empty intestine (arrow); d) larvae during the first feeding stage at 3 dph and the rest of the food in the intestine (arrow), e) the spleen (arrow) at 8 dph; f) production of mucus and globose cells in the fold of the fin (arrow) at 11 dph; g) larvae feeding *Artemia* nauplii at 20 dph; h) larvae metanauplius feeding *Artemia* at 26 dph.

the spleen manifested as a small group of dark stained hematopoietic cells, with numerous capillaries in the interior of the spleen anlage (Fig. 5b). At 21 dph (~6.5 mm TL), the spleen exhibited a distinct capsule and variably stained cell areas. By 23 dph (~7 mm TL), the spleen's cross-section became heart-shaped, increasing in size (Fig. 5c). The fully developed spleen (32 dph, ~13 mm TL) was characterized by the presence of red and white pulp and ellipsoids. The large vessels appeared on the spleen periphery, while reticular cells

and fibrous tissues were distributed inside the spleen (Fig. 5d).

Thymus

The thymus is a paired organ positioned in the dorso-posterior area of the oropharyngeal cavity at a distal position, which emerges between the third and fourth gill arches, situated on the back of the gill cavity. The thymus anlage became apparent at approximately 9 dph (5.3 mm TL), consisting of several dark lymphoblast

Table 5. Summary of immune-relevant events and husbandry practices during the early ontogeny of rose spotted snapper (*L. guttatus*). dph: days post-hatching, IgM: immunoglobulin M, GALT: gut-associated lymphoid tissue.

Immune-relevant events	dph	Husbandry practices
Eggs and embryo IgM+	0	Hatch
	1	
Eggs and embryo IgM-	2	Vitellin reserve depleted
Sketch of the cephalic kidney	3	First feeding (rotifers)
Sketch of the spleen	4	
	5	First swim bladder inflation
	6	
	7	
	8	Appearance of the spleen
Sketch of the thymus	9	
	10	
	11	Abundant mucus
	12	
	13	
	14	
<i>rag1</i> , <i>cd4.2</i> and <i>cd11b</i> peak expression	15	
	16	Larvae flexion stage
	17	First feeding with artemia nauplii
	18	Final swim bladder inflation
	19	
	24	
	25	First feeding with artemia meta-nauplii
	26	
	27	Starting the weaning period
	28	
	34	
IgM cells+ in head kidney and GALT	35	
	36	
	39	
IgM cells+ in the spleen	40	
	41	
	99	
IgM cells ⁺ in the thymus	100	

cells (Fig. 6a). The pharyngeal epithelium with mucus cells at 32 dph was observed (Fig. 6b). By 40 dph (23.0 mm TL), the thymus exhibited distinct cortical and medullary zones (Fig. 6c) that were separated from the gill chamber by a thin layer of pharyngeal epithelium rich in mucous cells. At 51 dph (33.48 mm TL), structures resembling Hassall's corpuscle, with reticular margins, became observable. Thymus protrusion into the pharyngeal cavity was noted at 71 dph (55.64 mm TL), and full thymus development was observed (Figs. 6d-e).

Gut-associated lymphoid tissue (GALT)

At hatch, *L. guttatus* larvae had a straight, undifferentiated digestive tract. Differentiation started

before exogenous feeding onset (Fig. 7b); the esophagus, primordial stomach, anterior, medium, and posterior intestine were distinguished in larvae at 2dph (1.7 mm TL), including the mouth opening (Fig. 7c). Peristaltic movements were observed at 3 dph (2.8 mm TL) when the first feeding was implemented (Fig. 7d). At 6 and 8 dph, clear pre-stomach, anterior, and posterior intestine were observed (Fig. 7a-b). The initial formation of a loop gut was observed (Fig. 7c). The enterocytes presented microvilli, the buccopharynx epithelium had chloride cells, and there were ciliated cells in the foregut. Still, no mucous cells were present yet (Fig. 7d, 25 dph). The GALT was not clearly detected; only lymphoid cells (T and B) scattered along the guts were present (Fig. 7e, 28 dph), in the epithelium,

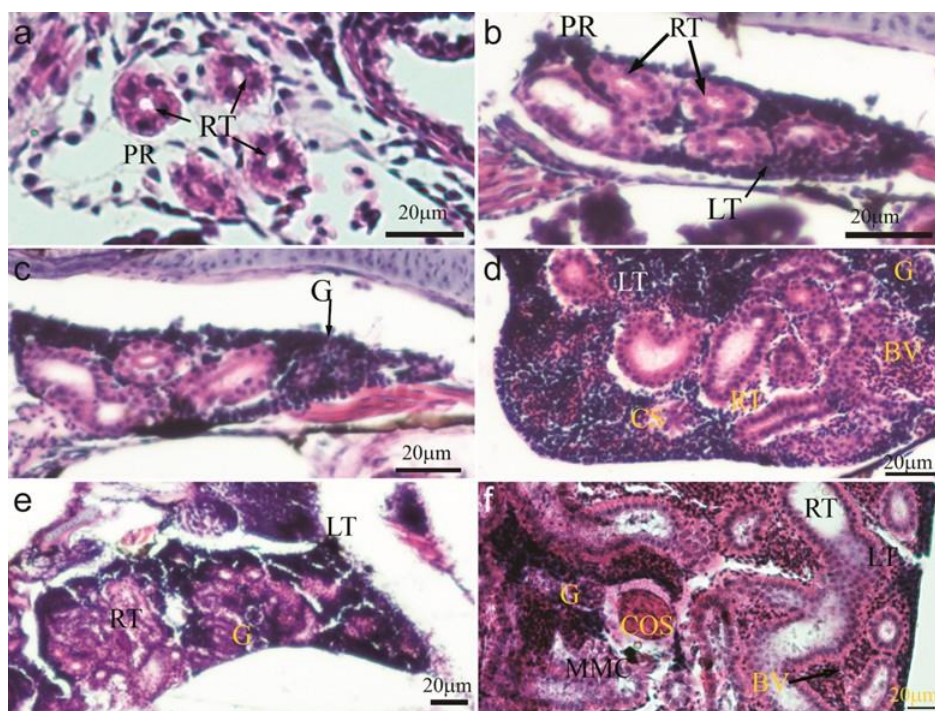


Figure 4. Early development of the head kidney (HK) in *L. guttatus* (photomicrographs of longitudinal sections, fixed in 4% PFA, 5 μ m sections, H&E staining. a) First pronephros (PR) at 6 days post-hatching (dph); b) development of pronephros structures and lymphoid tissues (LT) at 18 dph; c) first glomeruli present (G) at 21 dph; d) renal tubules (RT), capillary sinusoids (CS) and glomeruli at 25 dph; e) renal tubules, capillary sinusoids and glomeruli at 29 dph; f) hematopoietic tissue (HT) is distributed between renal tubules and blood vessels (BV), corpuscles of Stannius (COS) at 40 dph. PA: pancreas, LI: liver, PH: pharynx, ST: stomach, B: brain, N: notochord, T: thymus, BC: branchial cavity, BV: blood vessel, GB: gas bladders, PC: peritoneal cavity, MMC: melanomacrophage centers.

and numerous individual cells in the lamina propria (Fig. 7f, 28 dph).

Maternal and autologous IgM

Exhaustion of maternal IgM

Maternal IgM was detected in embryos but not in larvae after yolk consumption at 2 dph (1.8 mm TL).

Autologous production of IgM

In *L. guttatus*, autologous IgM production was first detected in the head kidney of juveniles at 35 dph (14.9 mm TL). Later, at 40 dph, in juveniles (23.0 mm TL), IgM was detected in the kidney, liver, and intestine. Finally, IgM was detected in the cortex zone of the thymus from juvenile fish at 100 dph (88.3 mm TL; Fig. 8a-b).

Gene expression

A significant increase of *rag1* expression was observed in whole larvae at 15 dph (~5.35 mm TL), with no significant changes at 20 dph (6.57 mm TL larvae; Fig. 9a). In contrast, *rag2* expression was barely detectable

across the different time points, with no significant increase (Fig. 9b). Expression levels of *cd4.2* increased significantly to 15 dph (~5.35 mm TL larvae). In comparison, a significant reduction was detected at 20 dph (6.57 mm TL larvae, Fig. 9c). Expression of *cd11b* also showed a significant increase at 15 dph (~5.35 mm TL larvae; Fig. 9d).

DISCUSSION

Understanding the immune system of teleost fish is relevant because they constitute a unique biological model that exhibits both innate and adaptive immune responses across life stages, making them important in the comparative immunology of vertebrate systems. Fish can exhibit only innate responses at certain stages of larval development, a condition essential for studies of trained immunity (Netea et al. 2016, Rojo-Cebreros et al. 2018). Later, depending on the species, fish can fully develop the adaptive response (Malmström et al. 2016). The efficiency with which the farm fish industry can immunize or immunostimulate fish depends on

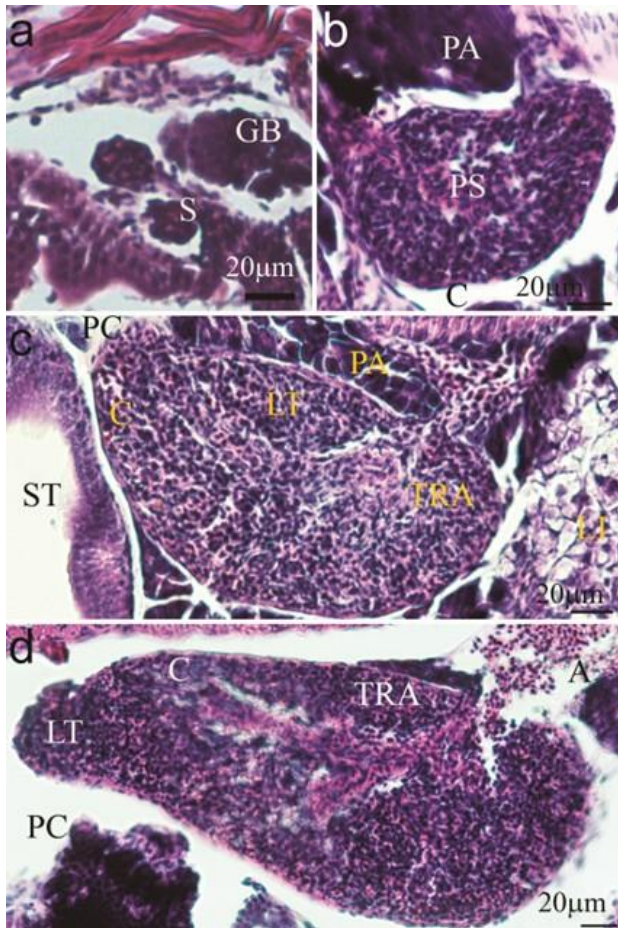


Figure 5. Early development of the spleen (S) in *L. guttatus* (photomicrographs of longitudinal sections, fixed in 4% PFA, 5 μ m sections, H&E staining). a) Anlage spleen; note the erythropoietic spleen with a stalk attached to the mesogastrium at 6 dph; b) primitive sinusoid (PS); capsule (C) at 21 dph; c) splenic artery at 23 dph; d) evident trabeculae (TRA) and abundant lymphoid tissues (LT) at 32 dph. B: brain, PA: pancreas, LI: liver, ST: stomach, BC: branchial cavity, GB: gas bladders, PC: peritoneal cavity.

knowledge and understanding of the immune system (Mulero et al. 2007). Immunization and/or immunostimulant strategies can be designed based on this basic knowledge.

The appearance sequence of lymphoid organs in *L. guttatus* agrees with that described by Mulero et al. (2007), where the head kidney and the spleen appear during the first week after hatching, as in other marine fish (Tables 1-2). However, species-specific differences may occur, and larval rearing temperature may determine growth rate, thereby affecting the timing of lymphoid organ maturation. The outline of the thymus

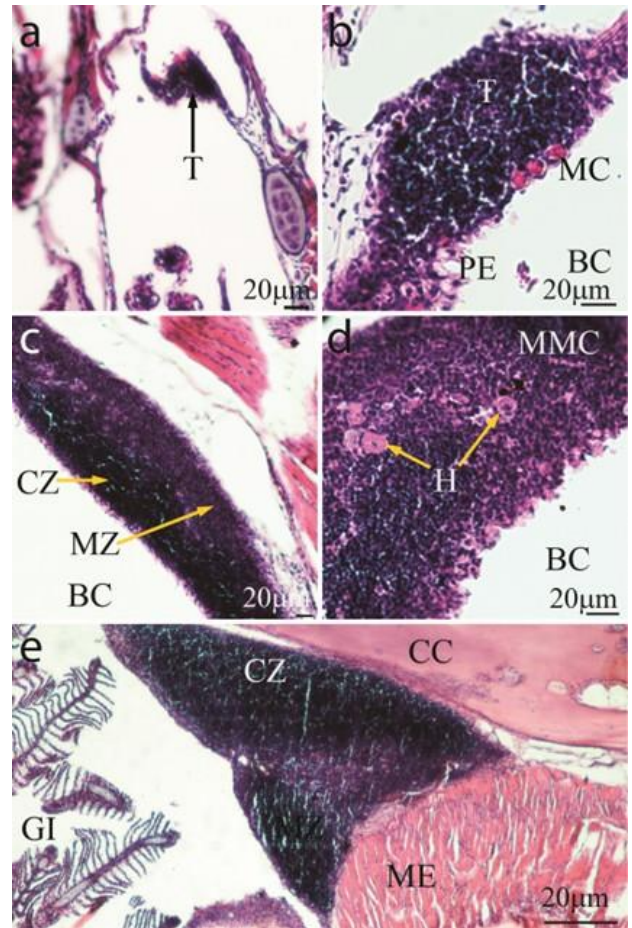


Figure 6. Early development of the thymus (T) in *L. guttatus* (photomicrographs of longitudinal sections, fixed in 4% PFA, 5 μ m sections, H&E staining). a) Anlage thymus (ventral sections) at 13 dph; b) note a thin layer of pharyngeal epithelium (PE) with mucus cells (MC) at 32 dph; c) differentiation of cortical (CZ) and medullary zones (MZ) in the fully developed thymus (arrows) at 40 dph; d) Hassall's corpuscle-like (H) (arrows) at 51 dph; e) protrusion of the thymus into the pharyngeal cavity at 71 dph. MMC: melanomacrophage centers, BC: branchial cavity, GI: gills, CC: cartilage cranial, ME: muscle skeleton.

of *L. guttatus* appeared at 9 dph (~5.3 mm TL) and was evident from the second week after hatching. The structural and functional development of the thymus in *L. guttatus* was similar to that of other marine fish species (Table 5). IgM-positive lymphocytes in marine fish are generally detected between 1 and 10 weeks post-hatch (Magnadóttir et al. 2005; Table 5). However, in *L. guttatus*, autologous IgM production was detected until 40 dph (23 mm TL; Table 5), highlighting the importance of fish size (rather than age) in immune system development (Mulero et al. 2007). For instance, Magnadóttir et al. (2005) reported

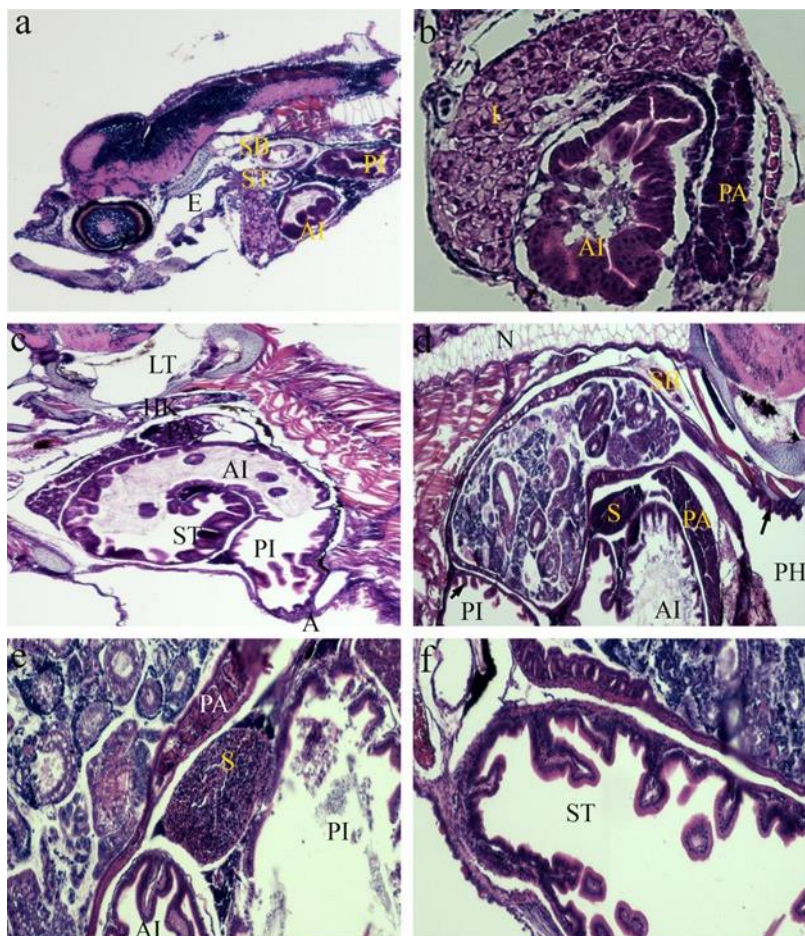


Figure 7. Early development of gut-associated lymphoid tissue (GALT). (photomicrographs of longitudinal sections, fixed in 4% PFA, 5 μ m sections, H&E staining). a) Side view of the main organs (esophagus, pre-stomach, anterior intestine and posterior intestine) of the digestive system at 6 dph; b) liver and pancreas surrounding the foregut at 8 dph; c) a gut loop is observed at 21 dph; d) the enterocytes present microvilli, the buccopharynx epithelium has chloride cells, there are ciliated cells in the foregut, but no mucous cells are present yet at 25 dph; e) lymphoid cells (T and B) scattered along the guts in the epithelium at 28 dph; f) numerous individual cells in the lamina propria at 30 dph. A: anus, E: esophagus, LI: liver, ST: pre-stomach, PH: pharynx, HK: head kidney, PA: pancreas, SB: swim bladder, PI: posterior intestine, AI: anterior intestine.

that IgM is produced for the first time in fish at 20-30 mm TL.

The adaptive immune response is phylogenetically and ontogenetically conserved in teleost fishes (Mulero et al. 2007). Nevertheless, the ability to generate an adaptive response does not necessarily correlate with the first detection of IgM-producing cells (Petrie-Hanson & Ainsworth 2001, Magnadóttir et al. 2005). Therefore, the time lag between cellular IgM detection and full immunological functionality should be determined in future work for *L. guttatus*.

Together with IgM, the gene complex *rag1/rag2* is considered a valuable marker of the physiological maturity of the immune system due to the onset of expression during the maturation of the lymphoid

organs, making *rag1* gene expression a reliable marker for studying the development of immune organs (Dunham 1999, Lee et al. 2014). In this study, the *rag1* gene showed a significant expression at 15 dph (~5.35 mm TL), indicating the onset of maturation in lymphoid organs. The *rag1* gene has been reported to be expressed in other fish species after 1-5 dph (Peluso 2023, Lee et al. 2014; respectively) with peaks at 21-25 dph (*Seriola lalandi*; Peluso 2023), 21-45 dph (*Latris lineata*; Covello et al. 2013), 35-40 dph (*Paralichthys olivaceus*), and 40-50 dph (*Melanogrammus aeglefinus*; Corripio-Miyar et al. 2007). By contrast, *rag2* expression showed only a slight increase at 20 dph (6.57 mm TL). This non-synchronized expression of *rag* genes has been reported to occur occasionally

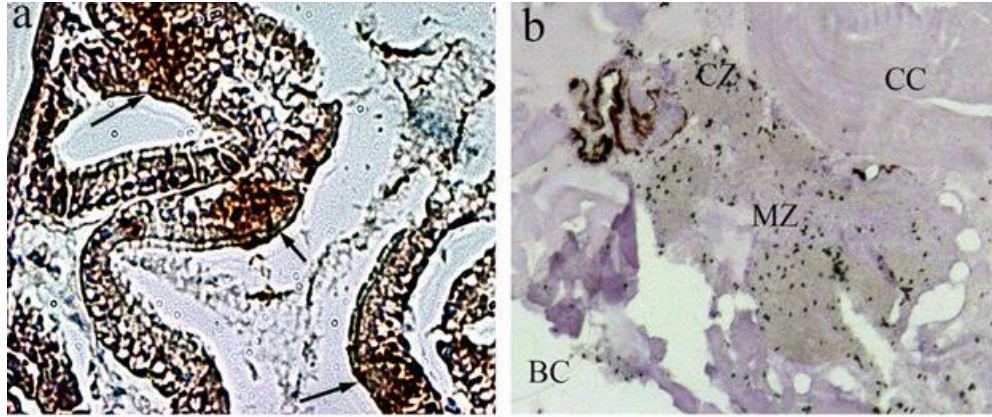


Figure 8. Immunodetection of autologous immunoglobulin M (IgM) in *L. guttatus* (photomicrographs of longitudinal sections, fixed in 4% PFA, 5 μ m sections). a) Detection of IgM in the intestine of juvenile fish at 40 days post-hatching (dph) (arrows), b) detection of IgM in the cortex zone of the thymus of juvenile fish at 100 dph (arrows). CC: cortex zone, MZ: medullar zone, BC: branchial cavity.

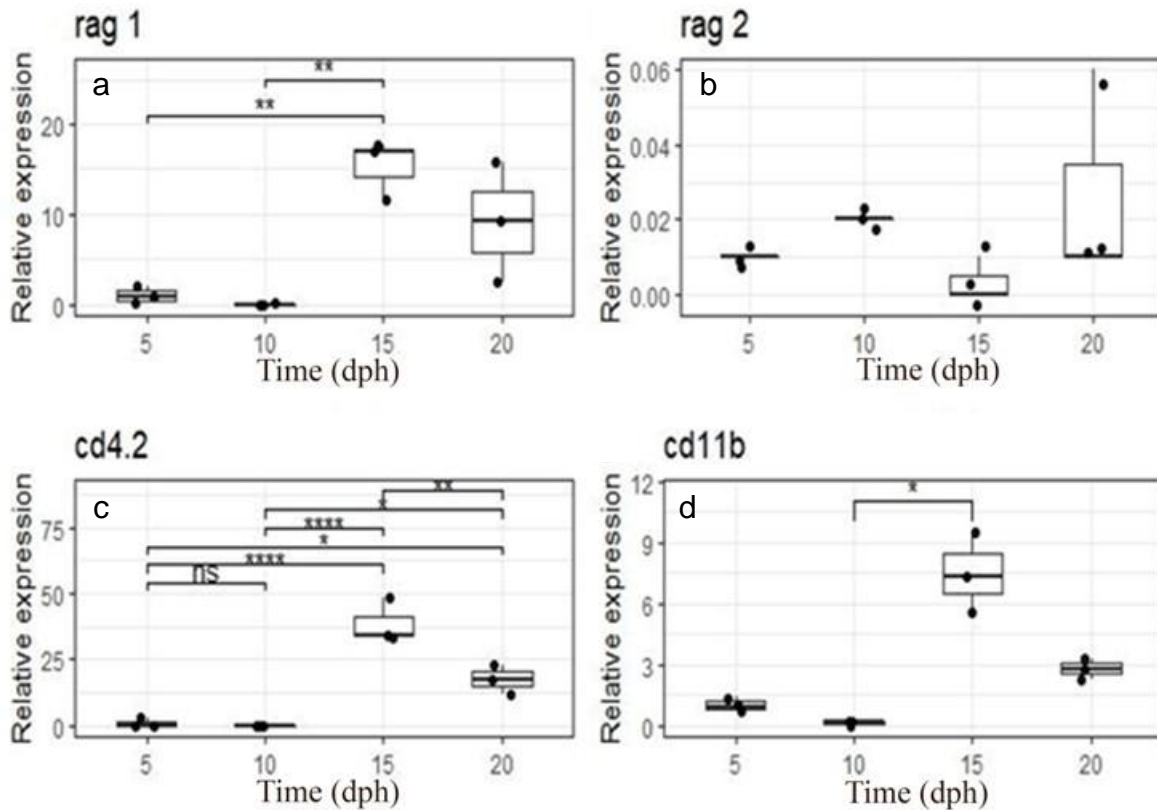


Figure 9. Expression of immune-related genes in *L. guttatus* larvae at 5, 10, 15, and 20 dph. The data are presented as the mean \pm standard error (n = 3). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ by ANOVA-Tukey HSD or Kruskal-Wallis-Dunn tests.

(Greenhalgh et al. 1993, Peluso 2023), although *rag2* may show higher levels of expression at later time points (>20 dph); further research is needed to confirm this hypothesis.

Consistent with *rag1*, the *cd4.2* and *cd11b* genes also showed higher expression levels at 15 dph (~5.35 mm TL). These genes are associated with functional immune responses, including helper T cell activity

following antigen exposure (Ashfaq et al. 2019) and the activation of natural killer cells, dendritic cells, macrophages, monocytes, and neutrophils (Bajic et al. 2013, Takizawa et al. 2016). In general, teleost fish have two CD4 genes: *cd4.1* contains four Ig-like domains, while *cd4.2* contains two or three Ig-like domains. For example, *cd4.2* has been linked to CD4+ T cells that perform several functions in fish, including stimulating macrophages to enhance microbicidal activity, stimulating B cells to produce antibodies, and enhancing cell-mediated immunity (Ashfaq et al. 2019). The CD11b gene encodes a transmembrane protein, a component of complement receptor 3 (CR3), which is mainly expressed on myeloid cells and acts to clear iC3b-opsonized doddery/apoptotic cells and pathogens (Bajic et al. 2013).

The *rag1* gene is associated with the differentiation of immature B and T cells through V(D)J recombination (Nagaoka et al. 2000). V(D)J recombination is crucial for diversifying antibodies produced by B- and T-cells. Because *rag1* is indicative of immune development, it has been used to determine the early-life stage at which fish initiate adaptive immunity (Willett et al. 1997, Lee et al. 2014). Expression of the *rag1* gene during early life stages of *L. guttatus* can indicate activation of adaptive immunity and may suggest the optimal timing for vaccination.

According to our results, the maturation and activation of the adaptive immune response in *L. guttatus* occurred after metamorphosis (35-40 dph, 15-23 mm), as reported in other fish species (Blazer 1992, Cordero et al. 2016). Hence, the completion of metamorphosis serves as a reference point for the activation of the adaptive immune response in marine fish with indirect larval development. The susceptibility window in mammals is the period during which animals are most susceptible to disease, driven by various factors. For example, the neonatal susceptibility window is the period during which the level of inherited antibodies is insufficient to protect against natural infection but high enough to interact with vaccination (Chase et al. 2008). In *L. guttatus*, an EWIS is covered by the innate response. During this period, maternal antibodies are depleted. Immune protection by maternal IgM begins at hatching and continues until the yolk reserve is depleted at 2 dph (1.8 mm TL). The adaptive immune response is activated (as evidenced by autologous IgM production) at 35 dph (15 mm TL). The end of the susceptibility period (35-40 dph, 15-23 mm) may be an appropriate time to evaluate future vaccination programs in *L. guttatus*. Additionally, administering immunostimulants (using rotifers and *Artemia* nauplii as vehicles) to *L. guttatus* larvae may be beneficial, as maturation of the head kidney, spleen,

and GALT could elicit an innate immune response before vaccination is effective.

CONCLUSION

In the spotted rose snapper *L. guttatus*, an EWIS occurred at 2 dph, when the larvae reached 1.8 mm TL, and ended after metamorphosis at 35 dph (15 mm TL); this EWIS was covered only by the innate response. Hence, this window could be used to develop immunoprophylactic strategies for *L. guttatus* larvae within the framework of trained immunity. The maturation and activation of the adaptive immune response of *L. guttatus* early juveniles could be reached at 35-40 dph (>15 mm TL); this time could be appropriate for planning future vaccination programs.

Credit author contribution

A.H. Rojo-Cebreros: conceptualization, validation, methodology, formal analysis and writing-original draft; L. Ibarra-Castro: conceptualization, validation, methodology, formal analysis, writing-original draft, funding acquisition, project administration, supervision, review and editing; M. Mirabent-Casals: methodology, validation and review; A. García-Gasca, J. Ventura-Juárez, J. Fonseca-Madrigal, C. Martínez-Chávez, J.M. Martínez-Brown and J. Navarro-Flores: methodology, data curation, formal analysis, review, and editing. All authors have read and accepted the published version of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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