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

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Abstract

This study investigates the development of lymphoid organs and mucosal tissues in larval and juvenile meagre, *Argyrosomus regius*. For this purpose, meagre larvae were reared from hatch to the juvenile stage, under mesocosm conditions at 18-19 ˚C, using standard feeding sequences with live prey and artificial food. The kidney was evident upon hatch and included a visible pronephros, with undifferentiated stem cells and excretory tubules at 1 dph (3.15 ± 0.1 mm SL). The thymus was first detected 8 dph (4.49 ± 0.39 mm SL) and was clearly visible 12 dph (5.69 ± 0.76 mm SL), 33 dph (15.69 ± 1.81 mm SL) an outer thymocytic zone and inner epithelial zone were visible. The spleen was present 12 dph, located between exocrine pancreas and intestine and by 26 dph (11.84 ± 1.3 mm SL) consisted of a mass of sinusoids filled with red blood cells. Melanomacrophage centers were found 83 dph (66.25 ± 4.35 mm SL) in the spleen. 14-15 dph (6.9 ± 1.1 mm SL), goblet and rodlet cells appear in the gill and intestinal epithelium. The lymphoid organs, which appear in the order of 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 activity of the immune response, the morphology of the lymphoid organs suggest that meagre is not immuno-competent until 83 dph.

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

1. Introduction

Within the last decade an extensive number of studies have been published on the organogenesis of commercially important teleost species (Jósefsson & Tatner, 1993; Santamaría *et al*., 2004; Falk-Petersen, 2005; Yúfera & Darias, 2007; Hachero-Cruzado *et al*., 2009). Studies addressing organogenesis of a species are essential for optimizing rearing conditions, developing larva-rearing techniques and ensuring the correct and healthy development of teleost fish (Padrós *et al.*, 2011). Generally, in rearing practice, the larval stage constitutes a critical period, during which, important structural and functional changes in fish tissues, organs and systems are occurring. c

In marine fish, major immune organs include the kidney, spleen and thymus, which appear during the later stages of ontogeny (Zapata *et al*., 2006a). Although the ontogeny of lymphoid organs is generally similar among teleost fish, the chronological appearance of organs and immune system components varies between species (Falk-Petersen, 2005). Knowledge of when tissue systems and lymphoid tissues develop is crucial in larviculture management for developing optimal rearing protocols and prophylactic measures, such as vaccination, in order to ensure optimal fish health. Furthermore, understanding tissue ontogeny is key for identifying immune memory can be attained, which in turn determines the timing of vaccination programs within hatcheries. In addition, if vaccination is to be successful it must not occur before lymphocyte differentiation is achieved or before immunoglobulins are being produced, as premature vaccination can induce tolerance to potential pathogenic antigens (Brown *et al*., 1996).

Meagre, *Argyrosomus regius*, is a newly emerging and important candidate for Mediterranean aquaculture. It has been selected for due to its fast growth, fillet quality, and low fat content (Monfort, 2010). In addition, this sciaenid species has shown many desirable characteristics for aquaculture, having a low feed conversion ratio 0.9-1.2

under standard dietary regimes (Duncan *et al*., 2013), viable reproduction in captivity (Duncan *et al*., 2012), tolerance to captive rearing conditions and thriving when subjected to established feeding protocols for production of juveniles. However, despite larval rearing protocols having already been established (Roo *et al*., 2010; Campoverde *et al*., 2017a), several factors that affect larval quality and survival rates need to be optimized, with the early developmental stages being the most critical rearing period. The majority of studies focused on larval development so far have been focused on describing the development of the digestive tract and its accessory glands (Papadakis *et al*., 2013; Suzer & Saka, 2013; Solovyev *et al*., 2016). However, there is no specific data available regarding the ontogeny of lymphoid organs and maturation of the immune system of meagre, especially during the late larval and juvenile stages. Therefore, this study aims to describe the ontogeny of the thymus, kidney, spleen and mucosa-associated lymphoid tissue (gut and gill), throughout larval development in meagre, in order to better understand the development of the meagre immune system and when vaccination can be carried out.

2. Materials and Methods

2.1. *Fish rearing*

Larvae used in this study were obtained by the hormonally induced spawning of 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 a controlled natural water temperature of 18 - 19 ºC and a photoperiod of 12 hr. of light and 12 hr. of dark using a recirculation aquaculture system (IRTAmar®). Batches of 50,000 eggs were incubated at 18 -19 °C in 35 L mesh-bottomed (300 µm mesh) incubators with aeration and gentle water exchange. Hatching

rate was determined by taking the average larval density of three 100 ml sub-samples of larvae and then extrapolating to tank volume.

Larvae were placed into 1.5 m^3 cylindro-conical tanks and reared using a mesocosm larviculture system. This system maintained a temperature of 22.1 °C (± 1.2 °C), salinity of 36.4 mg l^{-1} (± 0.77 mg l^{-1}), oxygen concentration of 7.8 mg l^{-1} (± 1.9 mg l^{-1}) and pH of 7.9 (± 0.1), which were checked daily. The concentration of nitrites was maintained at 0.05 (± 0.04) and the concentration of ammonia maintained at 0.14 (± 0.09) using a Hach Colorimeter (Hach Colorimeter DR/890, USA). The larval photoperiod consisted of 16 hr of light and 8 hr of darkness and the light intensity was set to 500 lux at the water surface. The larval feeding protocol was as follows: from 2 to 14 days post hatching (dph) larvae were fed enriched rotifers (*Brachionus plicatilis*) at a density of 10 rotifers ml^{-1}, from 9 to 31 dph, larvae were fed enriched *Artemia* metanauplii at a density of 0.5to 3 *Artemia* ml^{-1}. Both live prey were enriched using Red Pepper™ (Bernaqua, Belgium). Enrichment lasted 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 given 2 to 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.

2.2 *Sampling procedures*

Samples of whole larvae were taken, at random, at the following time 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, changes in rearing practices and changes in diet. Growth measurements were obtained from a pool of 10 larvae on the sampling days. Larvae were anesthetized using a high concentration (1 g/L) of MS-222 (Sigma-Aldrich, Spain). Data on standard length (SL) was collected during

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, using a Mettler A-20 microbalance (Mettler Toledo, Columbus, OH, USA), to the nearest ±1 µg after fish had been rinsed in distilled water, placed in weighted cover slides, and oven-dried at 60°C for 24 h. The animals used in the experiment were kept, sampled and euthanized following the rules of the ethic committee of IRTA.

2.3 *Histology*

Samples of larvae (n = 10) and organs (n = 3) from each sampling day were fixed in 4% formalin (Scharlab S.L, Spain) then dehydrated in a graded series of ethanol (Scharlab S.L, Spain) (70-96%), embedded in paraffin (Casa Alvarez, Spain) and cut into serial sagittal sections (2-3 µm) with a microtome (Leica RM2155, Germany). Sections were stained using the following established protocols: Harris´ hematoxylin and eosin (H&E); Mallory trichromic method (Mallory, 1900) and Periodic Acid Schiff - Alcian Blue (PAS-AB pH 2.5) (Yamabayashi, 1987). 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).

2.4 *Calculation of SL and W.Wt*

Meagre growth was described by the equation $SL = 23.69e^{0.01571x}$ ($R^2 = 0.9241$) 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).

3. Results

3.1 *Larval growth*

During early ontogeny, 0 – 40 dph, the wet weight (W.Wt) of the larvae steadily increased, while standard length (SL) remained similar throughout this period, it was not until 76 dph before a proportional increase of wet weight and SL was observed, as shown in figure 1. During this period, fish growth followed an exponential curve. New hatched larvae measured 3.15 ± 0.11 mm SL, reaching 109.5 ± 7.41 mm SL by the end of the period studied (118 dph). Growth, in terms of SL and W.Wt was least variable 0 – 40 dph, which corresponds to a live prey diet and was much more variable 76 - 136 dph, which corresponds to feeding with an artificial diet. The SL of meagre larvae was most variable from 90 – 118 dph and W.Wt was most variable between 104 -118 dph.

3.2 *Thymus*

The thymus is located at the dorso-posterior side of the branchial cavity, was first observed 8 dph (4.49 ± 0.39 mm SL) and was clearly seen 12 dph (5.69 ± 0.76 mm SL). At which time the organ was found within an epithelial capsule (**Fig. 2a**) and close to the pronephric (**Fig. 2b**). The thymus anlage consisted of two cell types, a population of reticular/epithelioid cells, which had large pale nuclei and long cytoplasmic processes, and scattered among them smaller cells with deeply stained nuclei could be observed. The morphologically larger cells correspond to the reticular cells and formed the reticulum within the thymic parenchyma. As the larvae developed, the smaller cells, with deeply stained nuclei, were identified as lymphoblasts or small lymphocytes, due to their small dense nucleus with high nuclear to cytoplasmic ratio. 15 dph (6.9 ±1.1 mm SL) the

number of blast cells 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 and a high nuclear to cytoplasmic ratio and two different areas in the parenchyma became apparent (**Fig 2c**). 36 dph (16.98 ± 0.74 mm SL), distinct regionalization in the thymus was observed, the two zones were differentiated into an outer cortex and packed with thymocytes. The inner medulla mainly consisted of lymphocytes with epithelioid-type cells scattered throughout, within the mesh of an abundant connective tissue network (**Fig. 2d**). At this stage, the thymus was ellipsoid in shape and extended across the dorso-posterior angle of the branquial cavity from the outer to the inner edge. Many small lymphocytes could be observed at this time as were larger cells with pale nuclei and acidophilic cytoplasm and epithelioid cells with long cytoplasmic processes.

3.3 *Kidney*

The kidney was present upon larval hatch (**Fig. 3a**), consisting of two straight primordial pronephric ducts running below the notochordal axis with a few renal tubules and haemocytopoietic cells clustered around them. 3 dph (3.3 ± 0.3 mm SL) larval renal tubules had increased in number (**Fig. 3b**) and the anterior kidney (pronephros) showed 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 12 dph (5.69 ± 0.76 mm SL) (**Fig. 3c**). 22 dph (6.9 ±1.1 mm SL) to 29 dph (14.41 ±1.62 mm SL) proximal renal tubules, hematopoietic components and erythrocytes could be observed (**Fig. 3 d, e**). By 33 dph, (**Fig. 3f**) the dorsomedial area had become lymphoid and fused with an extensive blood supply. Phagocytic reticular cells were found surrounding the vessels and within the walls of venous sinuses found in the trunk and cephalic kidney. 50 to 83 dph the

proportion of lympho-hemopoietic tissues had dramatically increased and become sinusoid, particularly within the pronephros, whereas the mesonephric region became primarily occupied by tubules and erythrocytes.

3.4 *Spleen*

The first evidence of the spleen anlage was seen during the pre-flexion stage, with the anlage comprising of only a few blast cells **(Fig. 4a**). As larval development progressed, the spleen changed from its original morphology of a loose ball of blast cells to a densely packed elliptical shape consisting of a variety of cells. 12 dph the spleen consisted of small spherical clusters of mesenchymal cells near to the exocrine pancreas and the wall of the midgut. As time progressed the spleen increased rapidly in size and acquired an elliptical shape by 19 dph (**Fig. 4b**). 29 dph sinusoids, with associated red blood cells, were evident and a capsule, composed of capsular fibroblasts, was clearly visible (**Fig. 4c**). 47 dph, the spleen showed distinct architectural zones divided into white pulp and red pulp, and differentiated ellipsoids were present (**Fig. 4d**). 66 dph the red pulp formed the majority of the spleen and appeared fully developed, whereas the white pulp (lymphoid areas) appeared less organized and less developed. The red pulp consisted of fibroblast-like cells intermingled with various types of cells found in the blood (**Fig. 4e**). Melano-macrophagic centers were visible at 83 dph (66.25 ± 4.35 mm SL) in the auxiliary of the ellipsoid branches (**Fig. 4f**).

3.4 *Gills*

The gill anlage were observed in the pharyngeal region of the larva upon hatch. 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**). By 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 and cartilage, covered with undifferentiated epithelium and at this time the pharynx opened (**Fig. 5b**). Primary filaments at this stage were observed for the first time as elongate proliferations of undifferentiated cells perpendicular to the gill arches (**Fig. 5c**). 10-14 dph the gills noticeably increased in length and the number of filaments and lamellae increased. The first mucus cells were observed in the gill filament epithelium 12 dph (5.69 ± 0.76 mm SL) and the first chloride cells at the base of the gill filaments 15 dph (6.9 ± 1.1 mm SL). 22 dph the lamellae, especially those at the base of gill primary filaments, showed the typical gill structure with pillar cells and vascular spaces where blood cells were evident (**Fig. 5d**). 29 dph, the number of mucus cells had increased and were located principally within the epithelium of the primary lamellae near the base the second lamellae (**Fig. 5e**). Rodlet cells were detected at 66 dph and increased in number during the subsequent stages (**Fig. 5f**). The gill structure of larvae at 33 dph was similar to that of a juvenile fish.

3.5 *Gut*

The digestive tract upon hatch appeared as a straight and undifferentiated tube located dorsally of the yolk sac. 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. 6a**). Upon hatch, the mouth and anus were still closed. 3 dph, the intestine bent further in the posterior region and the intestinal wall constricted, dividing the intestine in three distinct regions the anterior, middle and posterior intestine (**Fig. 6 b**). 12 dph, the *lamina propria* became distinct and contained leucocyte-like cells as well as granular and non-granular cells similar to small lymphocytes (**Fig. 6c**). Small lymphocytes with strong basophilic staining of the nuclei and with reduced cytoplasm were also present. The first mucous/goblet cells were observed in the posterior

buccopharyngeal cavity epithelium and stained with Alcian blue (AB) (pH = 2.5) indicating presence of carboxyl-rich acid mucins. As development continued, these cells increased in abundance along the buccopharyngeal epithelium. 12 dph (5.69 ± 0.76 mm SL), goblet cells were observed in the middle and posterior intestine (**Fig. 6d**). A mixture of acidic (AB pH = 2.5) and neutral (PAS positive) glycoproteins were found between the enterocytes. Mucus cells were detected in the anterior intestine 15 dph (6.9 ± 1.1 mm SL) and during development were proliferating extensively throughout the intestine (**Fig. 6e**). Rodlet cells were found in the intestinal epithelia 26 dph (11.84 ± 1.3 mm SL) (**Fig. 6f**). As development continued, leucocytes, mucous and rodlet cells proliferated rapidly along and throughout the intestine during larval development up to 83 dph (66.25 ± 4.35 mm SL) where the number of these cells present began to settle.

3.6 *Comparison of meagre organogenesis with teleost fish*

The appearance of the meagre thymus, kidney and spleen differs compared to a number of other teleost species, as seen in Table 1. In meagre the thymus was detected 8 dph while in other marine species, the thymus was detected much earlier, for example in channel catfish, *Ictalurus puntactus*, it is detected 1 dph, in rainbow trout, *Oncorhynchus mykiss,* it can be observed 5 days prior hatch and in Pacific bluefin tuna, *Thunnus orientalis*, it has been seen 5 dph. The thymus is also observed earlier in a number of freshwater species such as common carp, *Cyprinus carpio,* and barramundi *Lates calcifer*, in which, can be observed 2 dph. The first sighting of the meagre kidney was of a similar time to that of marine and freshwater species in that it was detectable upon hatch. However it did differ from a few notable species such as olive flounder, *Paralicthys olivaceus*, and European seabass, *Dicentrachus labrax*, whose kidneys were not detected until 7 and 15 dph respectively. In meagre the spleen was not detected until 12 dph, which is similar to sparids, such as gilthead seabream, *Sparus aurata*, which also

had the spleen appearing at this time. When the spleen is detected in other teleost species varies dramatically with it first being detected in channel catfish, *Ictalurus puntactus*, 1 dph and 59 dph in Atlantic halibut, *hippoglossus hippoglossus*. This puts the first detection of the meagre spleen somewhere in the middle when compared to other teleost species.

4. Discussion

The main purpose of this study was to describe the timing and morphological development of lymphoid organs in meagre. Meagre is a species with a fast growth rate 4.49 ± 0.39 mm SL by 8 dph, which becomes apparent when compared to other species, such as gilthead seabream (*Sparus aurata*) which only reaches 5.86 mm at 31 dph (Bessonart *et al.,* 1999), common seabream (*Pagrus pagrus*) which reaches an average SL of 7.59 mm 31 dph (Socorro, 2006). However, meagre growth is not the fastest of all the species currently cultured as it shows a slower growth rate than Pacific blue fin tuna, *Thunnus orientalis*, which reaches 17.1 mm SL by 9 dph (Seoka *et al.,* 2007). This fast growth rate is an important variable to consider when understanding the ontogeny of immune organs in meagre. Our observations demonstrated that immune organs differ in the timing and order of appearance with respect to species, environment and rearing condition. However, in this study, the kidney is the first major lymphoid organ to develop during early ontogeny, which is consistent with the development observed in a number of teleost species, as summarized in Table 1. The immune organs of meagre larvae appeared in the following order: first the pronephric kidney 1 dph (3.15 ± 0.1mm SL), next the thymus by 8 dph (4.49 ± 0.39 mm SL) which was clearly visible by 12 dph in larvae measuring 5.69 ± 0.76 mm SL and finally the spleen at 12 dph. Other marine fish have shown different sequential ontogenies regarding organ development, as indicated in Table 1. These differences in organ development chronology are influenced by the

species' reproductive guild, environmental factors and the immune system evolution particular to each fish species (Zapata *et al.,* 2006a). For example, in northern bluefin tuna (*Thunnus thynnus)* the lymphoid organs and small lymphocytes are present at a much earlier stage compared to meagre and in other species such as tilapia (*Tilapia mossambica)*, the thymus anlage is evident as early as 24 hours after fertilization. Generally, in teleost fish, the thymus is the first organ of the immune system to become lymphoid (Zapata *et al.,* 2006a). However, thymic development, maturation and differentiation into two regions (cortex and medulla) varies amongst fish species. In Japanese flounder (*Paralichthys olivaceus*), for example, the medulla could not be identified in the thymus at all during 7 months it is present before it regresses (Patel *et al.,* 2009), however, a district cortex and medulla have been observed in a number of fish species, such as, Atlantic cod (*Gadus morhua*) (Schrøder *et al*., 1998), turbot *(Scophthalmus maximus*) (Padrós & Crespo, 1996) and channel catfish (*Ictalurus punctatus)* (Petrie-Hanson & Ainsworth, 2001). In the current study, the thymus in meagre developed quickly, 8-12 dph and a compact unilobulate gland with distinct zones of differentiation is clearly visible 36 dph. A unilobulate gland is characteristic of shared by most teleost species (Chilmonczyk, 1992), however other morphologies have been noted in some species. Differing morphologies were reported in Japanese medaka, *Orzyzias latipes,* (Mohammad Momdooh Helal Ghoneum & Nobuo, 1982) where a bilobulated thymus was observed and a polylobulate shaped thymus has been described in the paddle fish, *Polyodon spathula*, (Good *et al*., 1966) and tilapia, *Tilapia mossambica*, (Sailendri & Muthukkaruppan, 1975). Although, a degree of thymic involution has been reported in other teleosts (Abelli *et al.,* 1998), this phenomena usually only occurs in adult fish and so was not observed in this study. In contrast with mammals, in which age-related involution is a rule, teleost thymic involution may also be the result

of cyclic and seasonal factors. However, the timing of thymic involution was not determined in meagre during as this study only covered 0 -118 dph and involution normally occurs in mature adult fish (Abelli *et al*., 1998). The origin of the thymus has been questioned by many authors and it has been suggested that the thymus is seeded with lymphoid stem cells, however, an alternative theory exists, which suggests that thymus is colonized by stem cells from the kidney through a cell bridge (Nakanishi, 1991; Press & Evensen, 1999). The cells that form the cell bridge between the thymus and kidney have been described in several fish, such as sea bream (*Sparus aurata* L.), turbot *(Scophthalmus maximus),* rainbow trout *(Salmo gairdneri),* and flounder (*Paralichthys olivaceus*) (Grace and Manning 1980; Jósefsson and Tatner 1993; Padrós and Crespo 1996; Liu *et al.*, 2004). Although these "cell bridges" were also observed in meagre, it was not possible to confirm if these cells originated from the kidney or thymus.

As for the kidney, this organ was visible upon meagre larval hatch, located close to the axial skeleton, which is similar to what has been observed in other teleost species, although the kidney was detected much later post hatch in *Paralichthys olivaceus*, at 7 dph, and *Dicentrarchus labrax,* at 15 dph (Table 1). In meagre the kidney is divided into anterior, pronephros, and posterior, mesonephric, compartments and consists of a simple tube-like structure upon hatch. 1 dph only undifferentiated stem cells and a few renal tubules were observed. As development advanced, 8 dph, darkly stained cells and undifferentiated hemopoietic stem cells were detected along the pronephric tubules. 12 dph the proportion of the lympho-hematopoietic tissue had increased, particularly in the pronephros, and the remaining mesonephric region was occupied mainly by a large number of tubules and densely packed with erythrocytes, similar observations have been made in other marine species, such as yellowtail (*Seriola quinqueradiata*), sea bream (*Pagrus major*) and Japanese flounder (*Paralichthys olivaceus*) (Chantanachookhin *et al*.,

1991). With the advancement of larval development, the pronephric tubules decreased in number, which suggests their hematopoietic nature, while the mesonephric regions displayed an increase in the number of tubules, which agrees with their expected involvement in filtration. These observations suggest that, from a morphological point of view, the pronephros has less of an excretory function compared to other regions of the kidney, however, it serves as an important hematopoietic organ, site for lymphocyte maturation and possibly phagocytosis of foreign matter (Bayne, 1986; Press & Evensen, 1999). From a functional point of view, the anterior kidney is mainly lymphoid and plays a pivotal role in immune function, behaving as the equivalent of the mammalian bone marrow, i.e. as a source of lymphoid stem cells, and a primary lymphoid organ (Watts *et al.*, 2003). From 64 dph the anterior region of kidney showed both hematopoietic and lymphoid qualities, while the posterior region only showed qualities of an excretory organ. Interestingly, melano-macrophages center (MMCs) were not detected in this organ during the time of the study.

The spleen initially consisted of a loose arrangement of mesenchymal stands located between the exocrine pancreas and the gut, appearing 12 dph. As is the case the other meagre tissues (kidney and thymus), the spleen appeared at different time compared to other teleost species, as seen in Table 1. This organ exhibited signs of active erythropoiesis and principally consisted of reticular cells and reticular fibers. From 29 dph the white pulp and red pulp were differentiated and from 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 phagocytic activity. 83 dph white and red pulps became visibly apparent as a result of the differences in cellular makeup and MMCs could be observed, indicating that the non-specific defenses in meagre against bloodborne foreign particles may be functional at this time and relied

upon until specific immunity is fully developed. Splenic MMCs are known to be major sites of erythrocyte destruction and retain antigens for long periods of time, it would therefore, appear that the capability of storing antigens arises before the generation of a fully functional adaptive immune response in meagre (Press & Evensen, 1999).

Mucosal-associated lymphoid tissues, the intestine and the gills, were also included in this study. The most striking structural and cellular developmental changes were observed from 1 to 66 dph. 3 dph there was a notable proliferation of mucus/goblet cells in the posterior of the pharynx. However, mucus/goblet cells were not detected in the intestine until 15 dph when larvae measured 6.9 ± 1.1 mm SL. Previous studies in meagre found the first goblet cells in pharyngeal region were present 9 dph (3.7 ±0.2 mm SL) and intestine until 13 dph (4.1 ± 0.2 mm SL) (Solovyev *et al*., 2016), this may be the result of differences in larval rearing and husbandry protocols. 29-50 dph, the density of goblet cells increased in the buccopharynx and intestine. The secretion of mucus in fish species plays an important role in the absorption of easily digestible substances, lubrication of the gut and protection of the digestive mucosa from viral and bacterial infection (Gisbert *et al*., 2004; Gomez *et al*., 2013). Therefore, it is likely that the increase of goblet/mucus cells observed was to facilitate these functions. Mucus is predominantly made up from mucins, which are highly glycosylated proteins that form a mesh-like barrier proximal to the gut epithelium. Glyco-conjugates are known to exert a large variety of functions, from mechanical, to antimicrobial and anti-viral, to "osmotic", in that they may link and transport different ions, in nature (Allen, 1981). In teleost fish, the mucus based secretions coating external and internal epithelial surfaces contain antimicrobial compounds, including lectins, complement, lysozyme, and antimicrobial peptides (Lieschke & Trede, 2009). Previous studies in meagre have shown β-defensin, hepcidin, piscidin, (Campoverde *et al*., 2017b) complement and lysozyme to be highly

401 expression in larvae between 29 - 60 dph, these observations could be correlated with
402 the increase in mucus/goblet cells during this time period. In seabream, intestinal mucus
403 can alter in response to a myxozoan parasite infection, increasing glycoprotein content
404 and changing the degree of mucin glycosylation in the posterior intestine (Estensoro *et*
405 *al*., 2013). Intestinal goblet cells are responsible for the secretion mucus that forms a
406 layer over epithelial cells, which constitutes the first line of innate immune defense
407 against offending microorganisms, especially when the larvae do not have mature
408 lymphoid organs. In this study, there was no accumulation of lymphoid cells in the gut
409 until 3 dph and leukocytes did not appear in the gut until 12 dph, as seen in figure 6c. 12
410 dph the gut was well differentiated with an abundance of fully formed intraepithelial
411 leukocyte-like cells present in the *lamina propria*, which remained present during the
412 remainder of the study.
413 Rodlet cells (RCs) first appeared in the posterior intestine of meagre larvae during early
414 ontogeny (3 dph). This is much earlier than reported in other species, such as common
415 carp (*Cyprinus carpio*), however, these studies focused on the migration of the RCs into
416 the bulbus arteriosus, which did not develop until 10 dph (Mayberry *et al*., 1986).
417 Studies in other teleost species observed RCs 6 dph, which is later but closer to when
418 RCs were observed in this study (Leino, 1974). Contrastingly, in cultured sea bream
419 (*Sparus aurata),* RCs are not seen until 20 dph where they can be found in the posterior
420 intestine (Calzada *et al*., 1998). RCs are thought to have a role in the immune system but
421 their function still remains poorly understood (Mazon *et al*., 2007). Although, in some
422 fish species, RCs are thought to play a similar role to mast/eosinophilic granule cells
423 (Reite, 2005), which are inflammatory cells in vertebrates.
424 It is recognized that during early ontogeny, gas exchange occurs mainly through the skin
425 and the primary function of the larval gill is related to osmoregulation rather than

426 respiration. During pre-larval and larval stages meagre has an incipient gill with small
427 gill arches and primordial lamellae and filaments. By 29 dph the gills have completed
428 full morphological development and the larvae at this time have completed the transition
429 from cutaneous to branchial gas exchange. In contrast, most freshwater species develop
430 full branchial gas exchange much earlier (Li *et al*., 1995). Granular cells that were seen
431 in the spleen, were also present in the gut and gills in meagre larvae in accordance with
432 other studies (Gomez *et al*., 2013) where these cells are most abundant in these tissues.
433 Particularly in this study, these cells drastically increased in abundance in the gills
434 throughout development, especially as of 16 dph. Granular cells are found throughout
435 the body of vertebrates, but are commonly association with structures, such as blood
436 vessels, and found in close proximity to surfaces that interface with the external
437 environment. Granular cells and macrophages, which are mobile phagocytic cells found
438 in the blood and secondary lymphoid tissues, play an important role in inflammation,
439 which is a crucial response to pathogenic invasion or tissue injury leading to the local
440 accumulation of leukocytes and fluid. Granulocytes are also involved in the host
441 response to bacterial and helminth pathogens, particularly, in mucosal sites such as the
442 gills and intestine. Their primary mechanism of the action is degranulation releasing
443 substances involved in the inflammatory reaction, this behavior mimics that of
444 mammalian mast cells and so these cells may be the teleost analogues of these cells
445 (Reite, 1998).

446 **5. Conclusions**

447 This work describes the chronological appearance and morphological development of
448 the different lymphoid tissues in meagre, *Argyrosomus regius*. The pattern of
449 development of lymphoid organs in meagre is similar to the general pattern observed in
450 other teleost fish species. However, the timing of organ and system development

exhibited inter-tissue and interspecies differences, which can be primarily attributed to different species' reproductive guilds, ie., precocial *vs* altricial development. Based on previous studies in other species focused on the relationship of the morphology of the lymphoid organs and their functional maturation, it is likely that meagre is not fully immuno-competent earlier than 83 days of age (66.25 ± 4.35 mm SL, GDD 1834). However, functional studies should be carried out to demonstrate when they can mount different types of immune response using 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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634

Table

Table I. Organogenesis of the thymus, kidney and spleen of teleost fish, assessed using histological procedures. The symbol indicates the emergence of the organs, - = Days prehatch; + = Days post hatch; ? = not known; L = lymphocytes visible.

Species	Culture T ˚C	Thymus			Kidney			Spleen			Reference
		Degree Days (GDD)	Organ present (dph)	Lymphocyte presence (dph)	Degree days	Organ present (dph)	Lymphocyte presence (dph)	Degree days	Organ present (dph)	Lymphocyte presence (dph)	
Marine species											
Argyrosomus regius	22	176	+8	?	22	+1	?	264	+12	?	In this study
Thunnus orientalis	27	135	+5	+7	27	<+1	+7	54	+2	>30	Watts *et al*., (2003)
Seriola quinqueradiata	22	242	+11	+20	22	+1	+26	66	+3	+36	Chantanachookhin *et al.,* (1991)
Pangrus major	20	220	+11	+22	22	<+1	+31	620	+31	+36	Chantanachookhin *et al.,* (1991)
Paralichthys olivaceus	20	200	10	21	140	7	28	160	8	30	Chantanachookhin *et al.,* (1991)
Sparus aurata	19-20	429 to 566	+22 to +29	+29 to +47	19.5	<+1	+45 to +47	234	+12	?	Josefsson & Tatner (1993)
Scophthalmus maximus	17.5	385 to 403	22-23	22-23	17.5	1	25	140 to 175	8 to10	25	Padros & Crespo (1996)
Dicentrarchus labrax	16-20	378	+21	+21	270	+15	>21		?	?	Breuil *et al*., (1997)
Harpagifer antarcticus	4	84 to 112	+21 to +28	?	4	<+1	?	84 to 112	+21 to +28	?	O´Neil (1989)
Hippoglossus hippoglossus	6	162	+27	+49	6	+1	+66	354	+59	+80	Patel *et al.* (2009)
Epinephelus bruneus	25	300	+12	+21	25	+1	+30	150	+6	+33	Kato *et al.,* (2004)
Oplegnathus fasciatus	23	230	+10	+15	-23	-1	+10	115	+5		Zhizhong *et al.,* (2013)
Oncorhynchus mykiss	14	-70	-5	+3	-112	-8	+5	42	+3	+6	Grace & Manning (1980)
Salmo salar				-22		-23	-14		-42	?	Ellis (1977)
Freshwater species											
Ictalurus punctatus	25-27	26	+1	+10	26	+1	+5	26.5	+1	+5	Petrie-Hanson *et al.,* (2001)
Cyprinus carpio	22	44	+2	4 to +5	22	+1	+6 to +8	110	+5	+8 to +9	Botham & Manning (1981)
Lates calcarifer	28.30	58	+2	+2	58	-2	+2	58	+2	?	Azad *et al.,* (2009)

Figure Captions

Figure 1. Growth of meagre larvae. This graph measures the SL (mm) ■ and W.Wt (mg) ▲ of meagre larvae against time (dph). The main stages of larval development and the larval feeding schedule are also shown.

Figure 2. Development of the meagre thymus. The histological organization and development of the thymus in meagre at various development stages. (a) Haematoxylin & Eosin staining 8 dph showing undifferentiated basophilic cells in thymus (scale bar = 50 µm). (b) Haematoxylin & Eosin staining 12 dph showing the thymus close to the kidney (scale bar = 50 µm). (c) Haematoxylin & Eosin staining showing the thymus 33 dph (scale bar = 100 µm). (d) Mallory stained longitudinal section showing the thymus 36 dph (scale bar = 100 µm). Co = cortex, K = kidney, Me = medulla, Th = thymus.

Figure 3. Development of the meagre kidney. The histological organization and development of the kidney in meagre at various development stages. (a) Haematoxylin & Eosin staining 1 dph: the excretory region of the kidney in a larva showing a single renal tubule (scale bar = 100 µm). (b) Haematoxylin & Eosin staining of larva 8 dph (scale bar = 50 µm). (c) Haematoxylin & Eosin staining of the kidney 12 dph (scale bar = 50 µm). (d) Haematoxylin & Eosin staining of the kidney 22 dph (scale bar = 50 µm). (e) Haematoxylin &Eosin staining of the kidney 26 dph (scale bar = 100 µm). (f) Haematoxylin & Eosin staining of the kidney 33 dph (scale bar = 50 µm). BC = blast cell; BCa = Bowan´s capsule; BV = blood vessel; E = erythrocyte; I = intestine; M = macrophage; Me = mesonephro; N = notochord; Pr = pronephro; RC = reticular cell; RT = reticular tubule; SL = small lymphocyte; YS = Yolk sac.

Figure 4. Development of the meagre spleen. The histological organization and development of the spleen in meagre at various development stages. (a) Haematoxylin & Eosin staining 12 dph (scale bar = 50 µm). (b) Haematoxylin & Eosin staining 19 dph (scale bar = 50 µm). (c) Haematoxylin & Eosin staining 29 dph (scale bar = 100 µm). (d) Haematoxylin & Eosin staining 47 dph (scale bar = 50 µm). (e) Haematoxylin & Eosin staining 66 dph (scale bar = 50 µm). (f) AB -PAS- (pH = 2.5) staining 83 dph (scale bar = 50 µm). C = capsule; E = erythrocytes; FC = fibrocytes forming capsule; GC = granular cells; L = leucocytes; MMCs = melanomacrophage center; M = macrophage; RBC = red blood cell; RP = red pulp; S = sinusoid; T = trabecula; WP = white pulp.

Figure 5. Development of the meagre gills. The histological organization and development of the spleen in meagre at various development stages. (a) Haematoxylin & Eosin staining showing the primordial gill filaments cartilaginous frameworks 1 dph (scale bar = 100 µm). (b) Haematoxylin & Eosin staining 3 dph (scale bar = 50 µm). (c) Haematoxylin & Eosin staining 8 dph (scale bar = 100 µm). (d) Haematoxylin & Eosin staining 22 dph (scale bar = 50 µm). (e) Mallory staining of mucous cells 29 dph (scale bar = 10 µm). (f) Haematoxylin & Eosin staining rodlet cells and leucocytes primary lamella 66 dph (scale bar = 10 µm). AG = gill arches Ca = cartilage; Co = chondorcytes; CC = chloride cells; E = erythrocyte; EC = epithelial cells; I = intestine; MC = mucous cells; N = notochord; OE = Oesophagus; P = exocrine pancreas; PC = pillar; PL= primary lamellae; Ps = pseudobranch; RC = Rodlet cells; SL = secondary lamella; YS = Yolk sac.

Figure 6. Development of the meagre intestine. The histological organization and development of the intestine in meagre at various development stages. (a) Haematoxylin & Eosin stained sagittal section of 1 dph larva showing the anterior and posterior intestine as well as yolk sac (scale bar = 100 µm). (b) Haematoxylin & Eosin stained 3 dph larvae showing three separated intestinal regions (scale bar = 50 µm). (c) Haematoxylin & Eosin stained 12 dph larvae. Note the intestinal loop and proliferation of lymphocyte cells (scale bar = 50 µm). (d) PAS-AB stained middle and posterior region of 15 dph larvae intestine, arrowhead indicates goblet cells containing carboxyl-rich glycoproteins (scale bar = 50 µm) (e) PAS-AB stained posterior intestine of 66 dph larvae, arrowheads indicate goblet cells (scale bar = 100 µm). (f) Mallory stained section of 66 dph larval intestine showing rodlet cells in the epithelial mucosa (scale bar = 10 µm). Abbreviations: AI = anterior intestine; C = connective tissues; G = gill; GA = gill arches; GC = goblet cells; L = leukocytes; LC = lymphocyte cells; MI = middle intestine; N = notochord; P = pancreas; PI = posterior intestine; RC = rodlet cells; RT = reticular tubule; SB = swimming bladder; YS = Yolk sac.

Figure

1 Figure 1

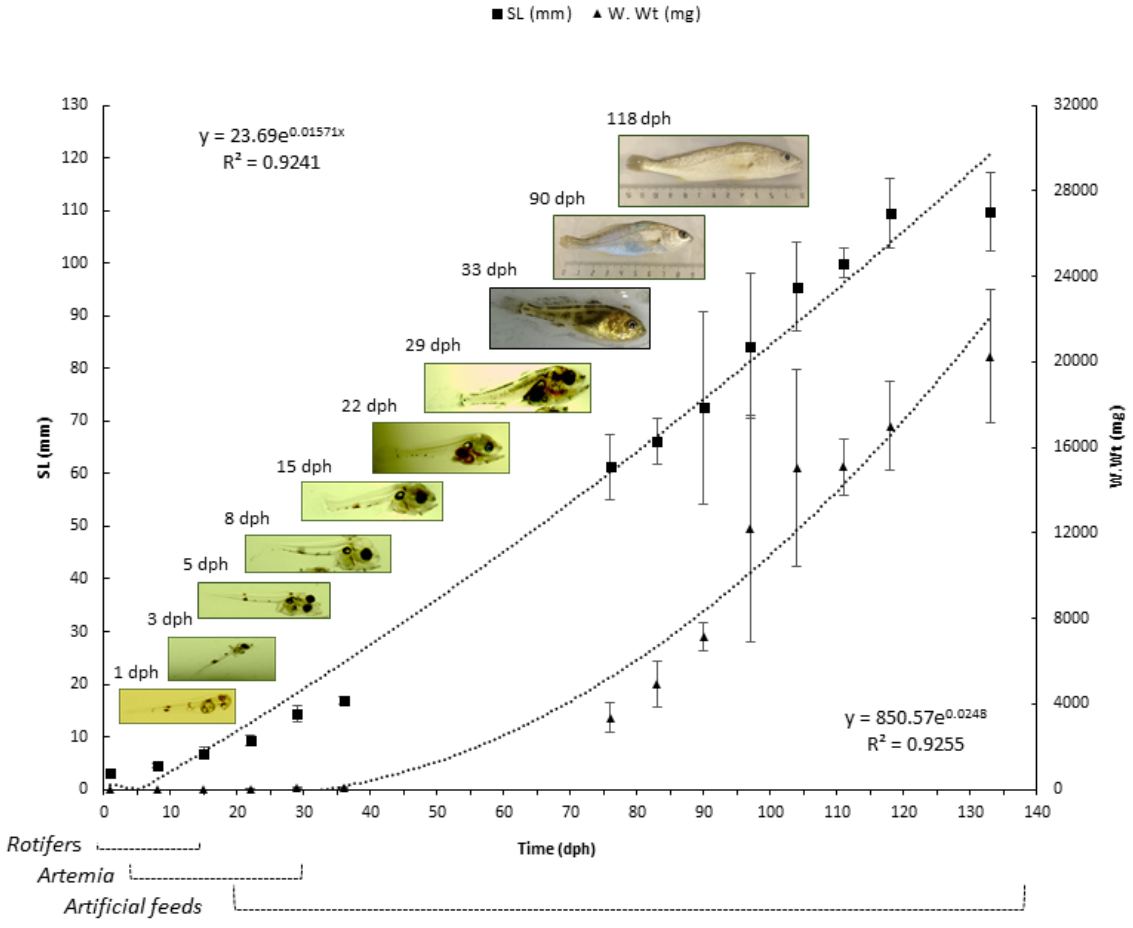

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Figure 2

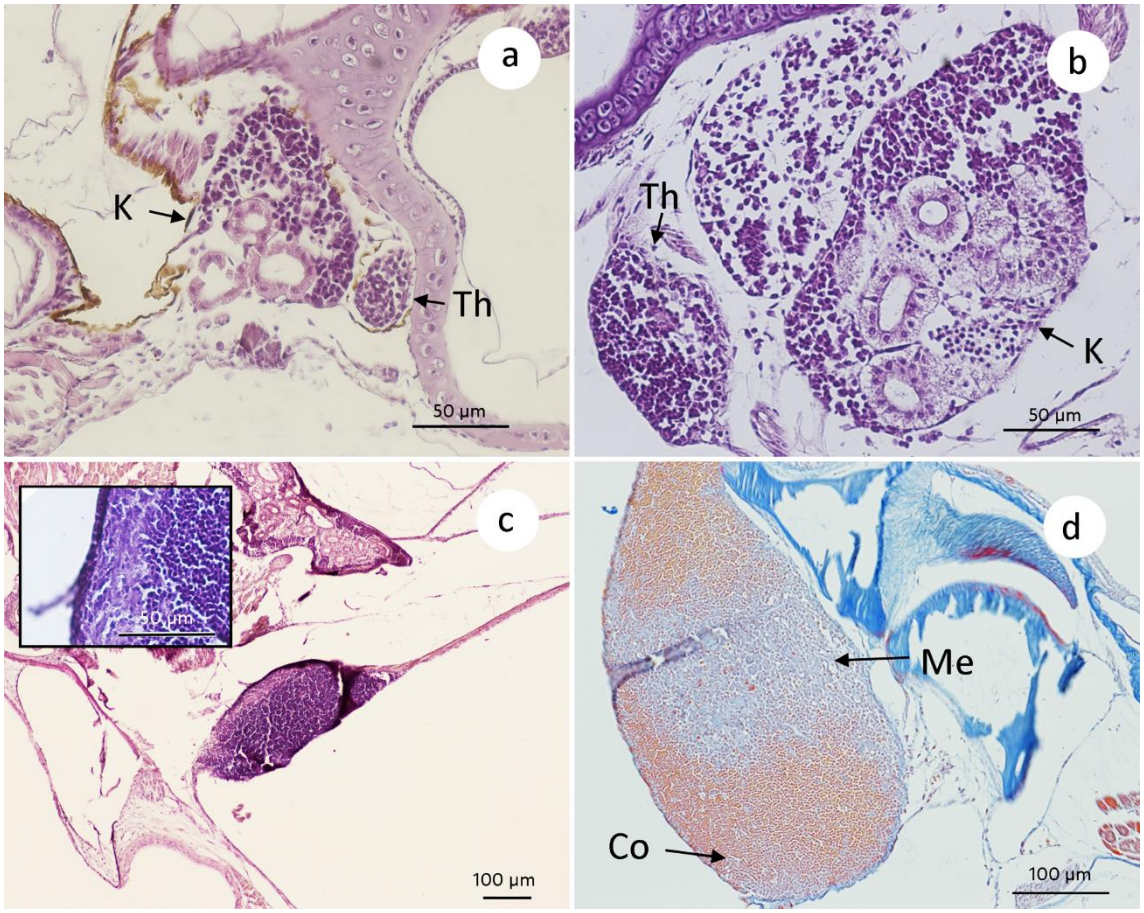
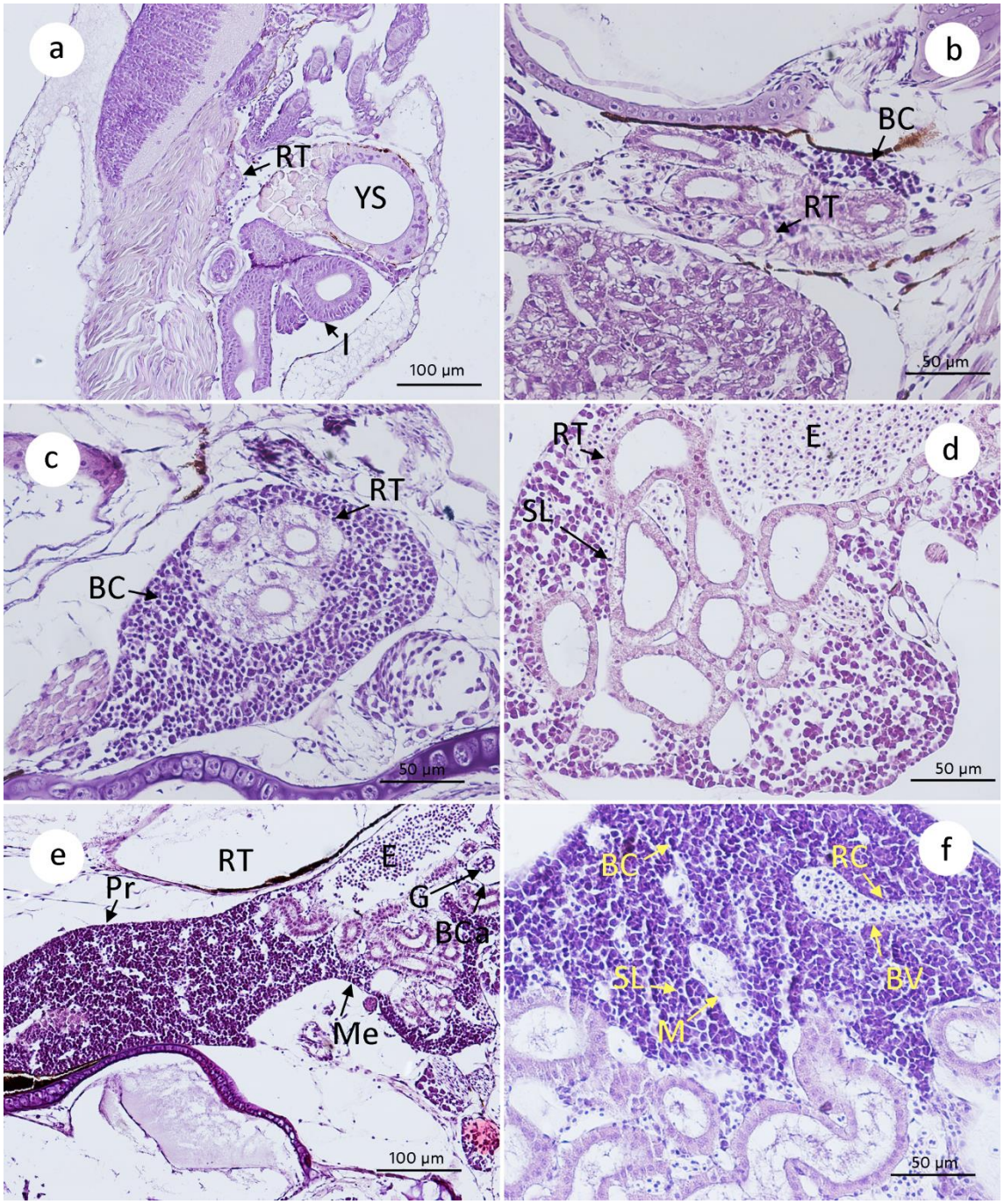


Figure 3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Me
100 µm
BC
RC
f
BV
SL
M
50 µm

Figure 4

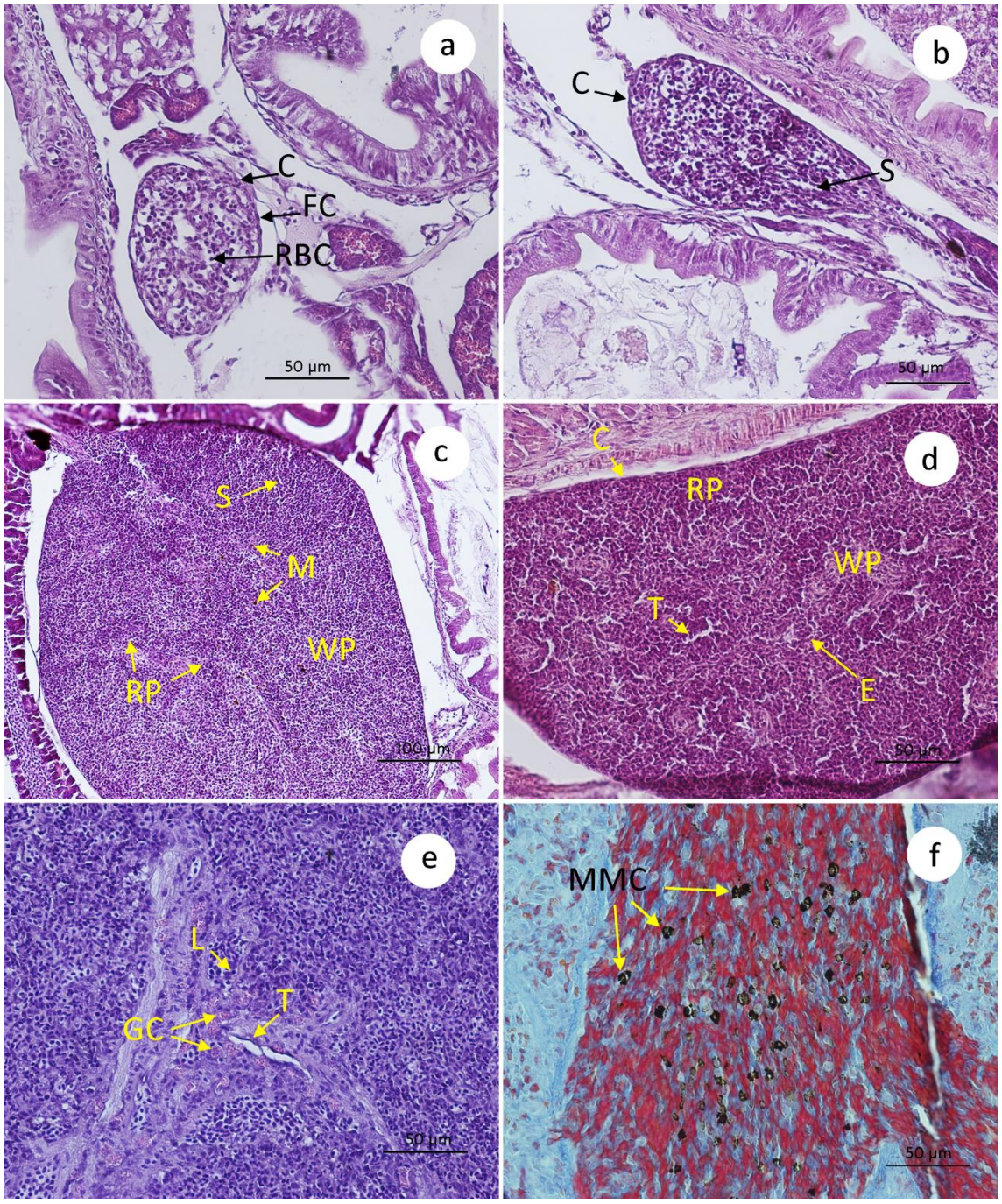

a
C
FC
RBC
50 µm
b
C
S
50 µm
c
S
M
WP
RP
100 µm
d
C
RP
WP
T
E
50 µm
e
L
T
GC
50 µm
f
MMC
50 µm

Figure 5

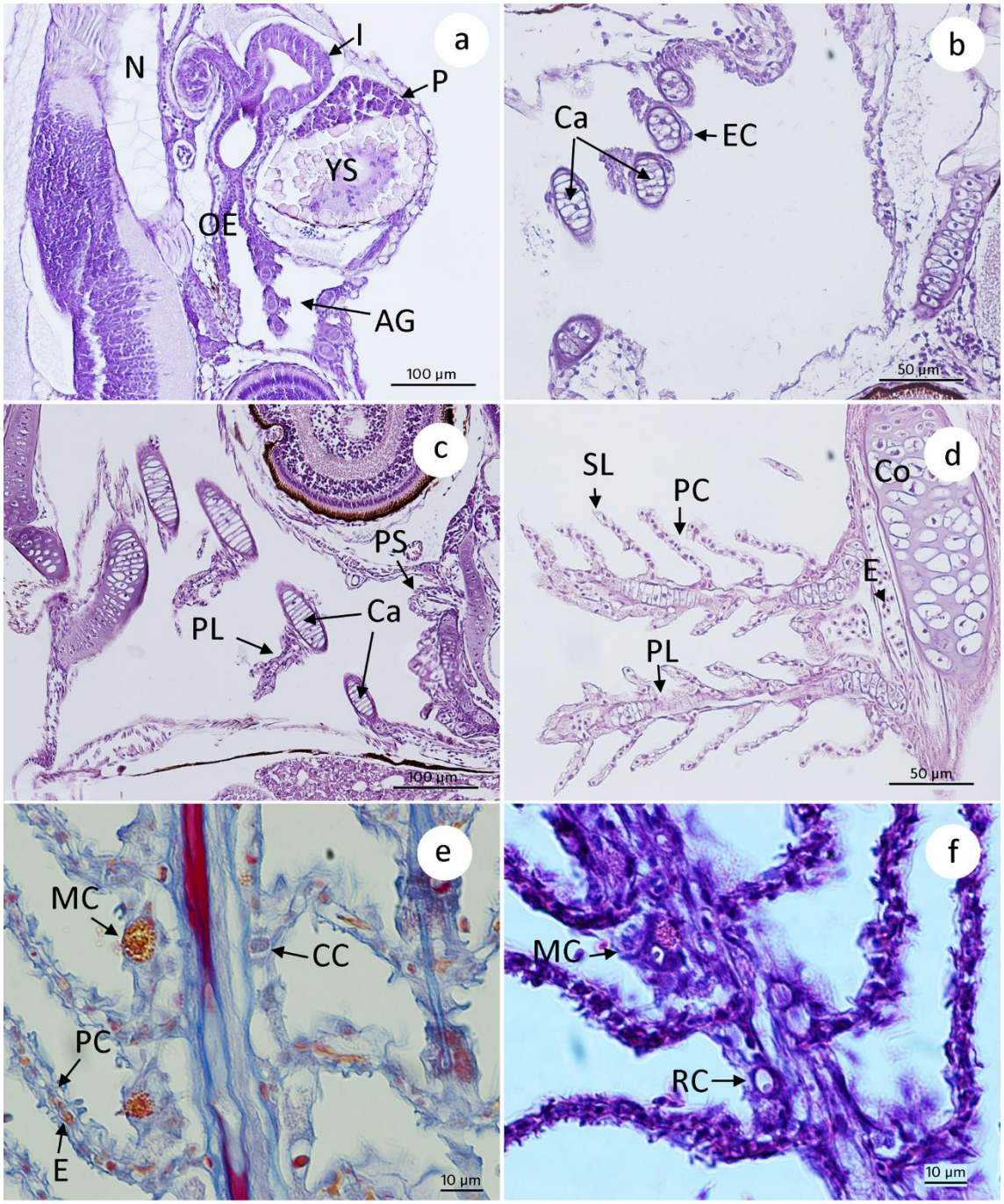
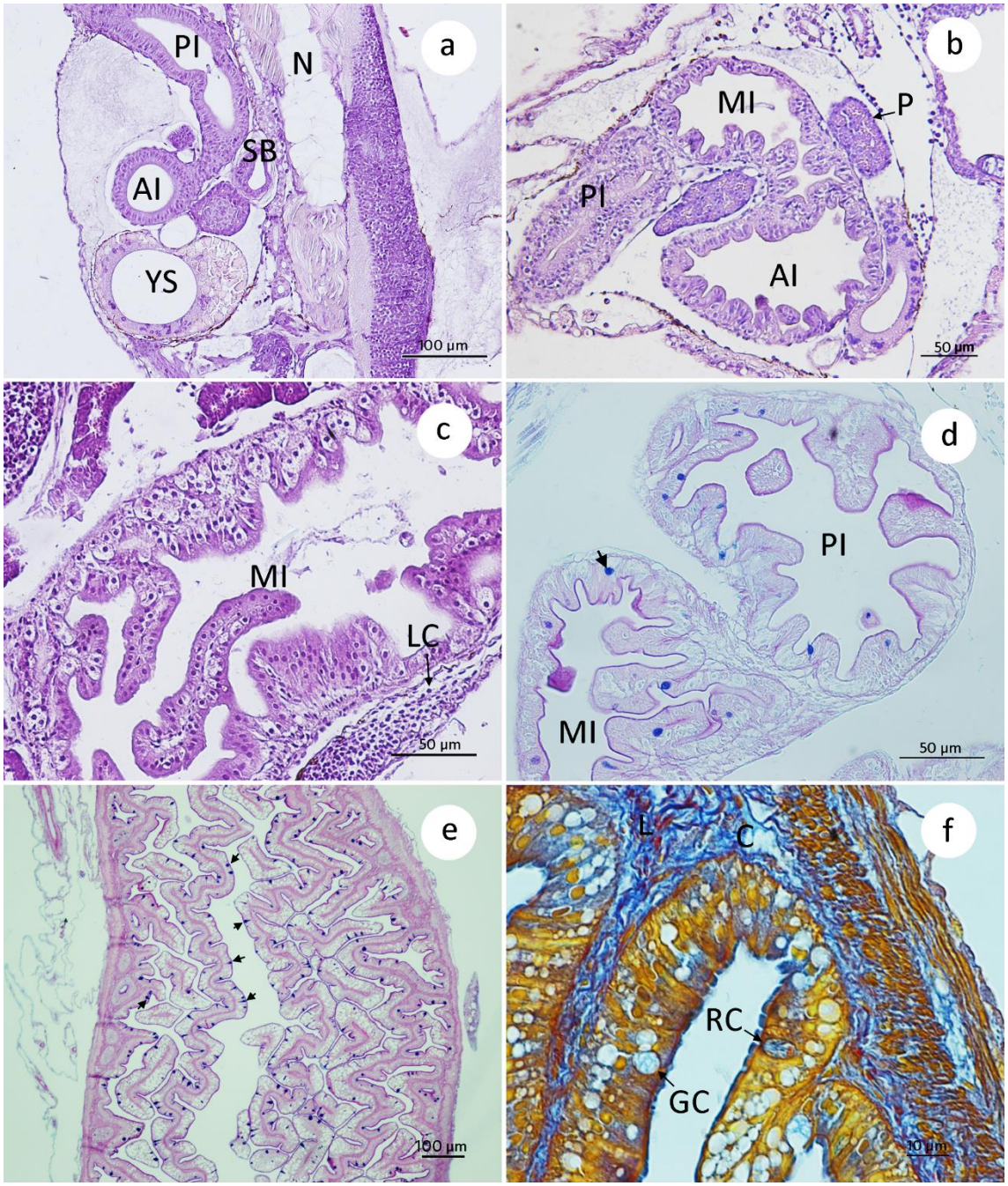

a
N
I
P
YS
OE
AG
100 µm
b
Ca
EC
50 µm
c
PS
PL
Ca
100 µm
d
SL
PC
Co
E
PL
50 µm
e
MC
CC
PC
E
10 µm
f
MC
RC
10 µm

Figure 6


a
PI
N
SB
AI
YS
100 µm
b
MI
P
PI
AI
50 µm
c
MI
LC
50 µm
d
PI
MI
50 µm
e
100 µm
f
L
C
RC
GC
10 µm