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Histogenesis and cell differentiation in the retina of *Thunnus thynnus*: A morphological and immunohistochemical study

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ABSTRACT

This study examines the anatomical development of the visual system of Atlantic bluefin tuna, *Thunnus thynnus*, during the first 15 days of life at histological level, with emphasis in the immunohistochemical characterization of different cell types. As an altricial fish species, the retina was not developed at hatching. The appearance of eye pigmentation and the transformation of the retina from an undifferentiated neuroblastic layer into a laminated structure occurred during the first two days of life. At 16 days after hatching (DAH), the ganglion cells were arranged in a single row in the central region of the retina and the outer segments of the photoreceptors were morphologically developed. Furthermore, at this age, all the retinal cell types were immunohistochemically characterized. The presence of ganglion cell axons was confirmed with the TUJ1 antibody and the existence of functional synapses in the plexiform layers with antibodies against SV2. Cone opsins were immunostained with antibodies against visinin and CERN-922 immunoreactive rods were also identified. Different subpopulations of amacrine cells were immunostained with antibodies against αTH and PV. Highly GS-immunoreactive Müller cells were also detected at this age. These observations suggested that the *T. thynnus* retina was fully functional at the end of the second week of life. Basic studies on early morphology of the visual system and larval behavior are necessary to support applied research on larval rearing. Furthermore, they may have implications for understanding larval ecology in the wild.

1. Introduction

The fish retina has turned into an important model to study neurogenesis, regeneration, and ageing due to their persistent plasticity during development and growth, life history changes and response to injury (Álvarez-Hernán et al., 2019; Xu et al., 2020; Da Silva-Álvarez et al., 2020; Becker et al., 2021; Hernández-Núñez et al., 2021a; b). Because the retina of vertebrates is highly conserved (Kolb et al., 2001; Hoon et al., 2014; Bejarano-Escobar et al., 2015), the study of mechanisms that governs the persistent plasticity in teleosts has provided insights into some aspects of retinal neurogenesis in mammals after the embryonic period. Furthermore, during late embryonic stages and early stages of fish larvae, the accurate development of the visual system plays a very important role because it is involved in selection of the favorable habitat, detection of predators, orientation, and locating food. The detailed description of retinal cells differentiation and histogenesis of the retinal layers during these stages could provide information about beginning of the functionality of the eye. The timing of retinal differentiation varies depend on the altricial-precocial spectrum (Evans and Browman, 2004; Álvarez-Hernán et al., 2019). The eye of altricial fish species shows an undifferentiated retina and the lack of pigmentation at hatching (Doldan et al., 1999; Bejarano-Escobar et al., 2009, 2010; Mukai et al., 2010; Pavón-Muñoz et al., 2016). Retinal differentiation occurs during the first days of the larval life. In contrast, intense

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pigmentation is evident in the eye of precocial fish species at hatching and the retina is fully differentiated (Candal et al., 2005, 2008; Ferreiro-Galve et al., 2008, 2010a; b; Bejarano-Escobar et al., 2012a, 2013; Miranda et al., 2020). Therefore, the well-developed visual acuity of precocial fish species from its earliest stages must help it to detect food or predators, while the altricial larvae are specially vulnerable during the early stages of their life cycle because the low degree of maturation of their visual system.

In the case of tunas, the eye is considered to be the main sense organ greatly affecting their behavior (Kawamura et al., 1981). Their retina is highly developed, with abundant types of photoreceptors (Margulies, 1997). Furthermore, tunas have an extremely well developed optic tectum, underscoring the importance of vision in these fish species (Kawamura et al., 1981). Most of the aspects concerning retinal cell composition and adaptative responses to different environmental conditions in tunas has been most studied in the Southern bluefin tuna (*Thunnus maccoyii*) (Hilder et al., 2019), the Pacific bluefin tuna (*Thunnus orientalis*) (Kawamura, 2003; Torisawa et al., 2007; Miyazaki et al., 2008, Matsuura et al., 2010; Miyazaki, 2014; Ina et al., 2017), the Bigeye tuna (*Thunnus obesus*) (Somiya et al., 2000), and the Yellowfin tuna (*Thunnus albacares*) (Loew et al., 2002). However, similar studies on the Atlantic bluefin tuna (*Thunnus thynnus*, Linnaeus 1758) are absent. *T. thynnus* inhabits the North Atlantic Ocean and Mediterranean Sea is one of the most commercially valuable marine fish species (López-González, 2006; López-González et al., 2007; MacKenzie and Mariani, 2012). Farming of these tunas species has progressed during the last years becoming a relevant industry in Mediterranean countries (Aguado-Gimenez and Garcia-Garcia, 2005; Mylonas et al., 2010; De la Gándara et al., 2016), so efforts for successful aquaculture of this species have increased in urgency. Some studies are particularly important, like studies of gametogenesis or studies on reproduction and obtaining of fertilized eggs (Sarasquete et al., 2002; De Metrio et al., 2010; Gordoa et al., 2010; Zohar et al., 2016). Studies on larval life are rare and morphological descriptions of the larval stages of tunas, which are basic requirements for considering the evolutionary ecology and phylogeny of the family, are rather scarce (Miyashita et al., 2001). Some authors have described some aspects of the larval feeding selectivity patterns (Catalán et al., 2011), testicular development (Abascal et al., 2004), the early development of the posterior lateral line system (Ghysen et al., 2010, 2012), and the organogenesis of the digestive system (Yúfera et al., 2014). More recently, the larval transcriptome of *T. thynnus* has been assembled and characterized (Marisaldi et al., 2021).

The present study, therefore, aims to describe the histogenesis of the visual system of *T. thynnus* during the first days of larval life. Furthermore, we characterize immunohistochemically the main retinal cell types. Finally, we compare our results with those described for other scombrid species and other fish species. These data could be basic requirements for the design of more appropriate feeds and feeding protocols during these early stages of life, as well as for considering the evolutionary ecology and phylogeny of the family in more depth.

2. Materials and methods

2.1. Atlantic bluefin tuna larvae rearing conditions

A total of 48 specimens (n = 48) larvae of Atlantic bluefin tuna (*Thunnus thynnus,* Linnaeus 1758) were included in the present study. Animals were provided by Spanish Institute of Oceanography (IEO), Planta Experimental de cultivos marinos de Mazarrón (Murcia, SE Spain). The larvae came from bluefin tuna eggs collected in a floating cage located in the Gorguel Bay (Cartagena coast, Murcia SE Spain) where broodstock were kept in captivity. Fertilized eggs were transported to the aquaculture facilities owned by IEO in Puerto de Mazarrón (Murcia, SE Spain), disinfected and place in 1400 L cylindrical tanks. The larvae hatched from these eggs were fed ad libitum from 2 DAH to 16 DAH with rotifers (*Brachionus rotundiformis*) and with Artemia from 13 to 16 DAH. Both preys were enriched with Algamac3050 ® before adding to tuna larvae. To maintain constant live prey concentration of rotifer and Artemia in the tanks, three water samples (10 mL) were counted twice per day before supplying new feed following the technique described in Ortega (2015) and De la Gándara et al. (2016). The salinity during the trial was 37 ppt, photoperiod was maintained at 14 h/10 h light/dark, and light intensity were about 500 lx at the water surface. Dissolved oxygen was kept above 90% saturation during the whole experiment and temperature was 24.8 ± 0.4 ºC. Tuna larvae were treated according to the recommendations of the European Union (Directive 2010/63/UE) and the Spanish Government (Real Decreto 53/2013). At appropriate times, larvae specimens were over-anaesthetized by immersion in a 0.04% solution of tricaine methanesulphonate (MS-222; pH7.4; Sigma Chemical). Animals were viewed under a Stereoscopic Microscope SMZ-1000 (Nikon). Digital images were captured with a Digital Camera DS-5Mc (Nikon), and processed in Adobe PhotoShop (vCS4). The larvae included in the present study ranged from the hatching day (0 day post-hatching, 0DAH) to 16DAH. Representative larval stages are shown in>Fig. 1.

2.2. Animals and tissue processing

Specimens were fixed by immersion in 4% paraformaldehyde (PFA) diluted in phosphate buffer saline (PBS) for 24 h and then washed several times in PBS. For histological analyses specimens were postfixed in 2% osmium tetroxide for 2 h and then, dehydrated in a graded series of acetone and propylene oxide. After that, samples were embedded in Spurr's resin. Serial sections of 5 μm were cut in a Reichert Jung microtome. Sections were stained with 0.5% toluidine-blue in 0.5% aqueous borax for 13 s in smallest larvae and 20 s in largest. For immunohistochemical analyses, samples were cryoprotected, immersed in embedding medium, frozen and freeze-mounted onto aluminum sectioning blocks. Cryostat sections of 14 μm and 20 μm were cut in a frontal plane. Sections were thaw-mounted on SuperFrost®-Plus slides (Menzel, Germany), air dried and stored at − 80 ºC.

2.3. Immunohistochemistry

Working solutions and sources of primary and secondary antibodies are summarized in Table 1. To ensure a better antigen-antibody join, cryosections were submitted to an antigen retrieval process before immunohistochemical analyses. Samples were washed two times in 0.05% Triton X-100 in PBS (PBS-T) and pre-blocked in 0.2% gelatin, 0.25% Triton X-100, Lys 0.1 M in PBS (PBS-G-T-L) for 1 h. Sections were incubated with primary antibody overnight at room temperature (RT) in a humidified chamber. Samples were washed several times in PBS-T and then incubated with secondary antibody for 2 h at RT in a dark humidified chamber. Sections were washed two times in PBS-T in darkness 10 min each one and the incubated with DAPI (4′,6-diamino-2-phenylindole) 10 min at RT in a humidified chamber. Slides were washed in PBS several times and mounted with Mowiol (polyvinyl alcohol 40–88, Fluka, Ref. 81386).

2.4. Image acquisition and processing

Toluidin-blue stained and immufluorescence sections were observed and photographed with a bright field and epifluorescence Nikon Eclipse 80i microscope attached to a Nikon DXM1200F digital camera. Graphical enhancement was performed in Adobe Photoshop CS4.

3. Results

3.1. Morphological study

At hatching day (0DAH), the retinal tissue of *T. thynnus* was composed of a neuroblastic layer (NbL) (Fig. 2A,B). At 1DAH, the

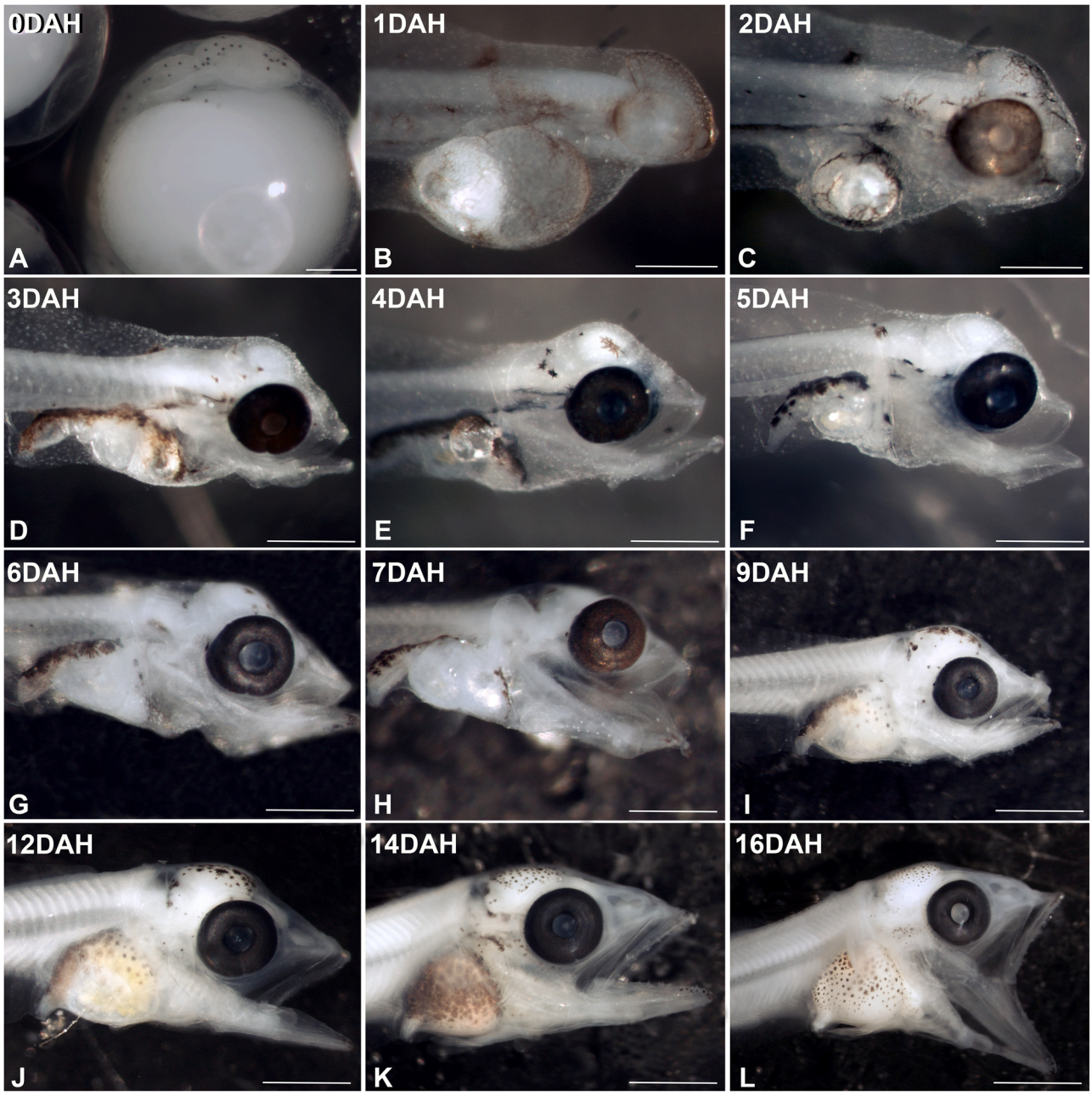

Fig. 1. Stereomicroscope images of *T. thynnus* specimens from the hatching stage (A) until 16DAH (L) showing the external gross anatomical changes in the eye. The optic anlagen is not pigmented at 0DAH (A). A faint pigmentation in the RPE is first observed at 1DAH) (B). At 2DAH, the eye is circled with pigmentation (C). From 3DAH (D) onward (E-L) a stronger pigmentation can be observed. *Scale bars:* 2 mm.

emergence of the IPL and the OPL occurred simultaneously and became recognizable as thin acellular zones (Fig. 2C,D). At this stage, sparse pyknotic nuclei could be observed in the undifferentiated neuroepithelium (Fig. 2C,D). Furthermore, lens delamination was complete and an anterior epithelial monolayer was clearly identified in the spherical lens (Fig. 2C,G). At 2DAH, the typical multi-layered structure of the vertebrate retina was clearly observable (Fig. 2E-G). However, signs of immaturity were still observed: the GCL was many cells thick, the plexiform layers were poorly developed, and the ONL showed photoreceptors with short outer segments (Fig. 2F). A typical ciliary marginal zone (CMZ) was clearly observable at the peripheral retina (Fig. 2E,G). A faint pigmentation was also detected in the pigment epithelium by this stage (Fig. 2G). A few pyknotic nuclei were observed in all three nuclear layers at this stage (not shown).

At 5DAH the *T. thynnus* retina increased in size (not shown) but it showed a similar aspect to that describe at 2DAH, both in the central (Fig. 3A) and in the peripheral (Fig. 3B) regions. At 7DAH the retinal layers in the ventral retina were not clearly distinguishable, but the dorsal halve showed well defined lamination (Fig. 3C). The stratification of the IPL could be distinguishable (Fig. 3D) and fusiform nuclei of the Müller cells, oriented in the vitreo-scleral axis and located in the INL could be also observable for the first time (Fig. 3D). At this stage, the ganglion cells were arranged in several rows in the GCL (Fig. 3D. At 16DAH, the last stage analysed, both the dorsal and ventral regions presented similar degree of lamination (Fig. 3E). The IPL became thicker and the ganglion cells were arranged in a single row in the central region (Fig. 3F). At this stage, the outer segments of the photoreceptors were larger than those observed at previous stages (Fig. 3F,G) and the CGZ persisted in the peripheral region (Fig. 3E).

The morphological study showed that the structure of the retina of

Table 1
Primary and secondary antibodies used in the present study, its dilution and sources.

Primary Antibody	Working dilution	Source
Mouse anti-glutamine synthetase monoclonal antibody	1:200	Chemicon (Ref. MAB302)
Mouse anti-parvalbumin monoclonal antibody	1:100	Millipore (Ref. MAB1572)
Mouse anti-proliferating cell nuclear antigen monoclonal antibody (PC10 clon)	1:200	(Ref. Ab29)
Mouse anti-SV2 monoclonal antibody	1:200	Developmental Studies Hybridoma Bank (DSHB)
Mouse anti-visinin monoclonal antibody (7G4 clon)	1:200	Developmental Studies Hybridoma Bank (DSHB)
Rabbit anti-phospho-histone H3 polyclonal antibody	1:200	Millipore (Ref. 06–570)
Rabbit anti-bovine rod opsine polyclonal antibody, Cern 922	1:200	Dr. Willem J. DeGrip donation, University of Iowa, U.S.A
Mouse anti-alphaTH monoclonal antibody	1:25	Developmental Studies Hybridoma Bank (DSHB), University of Iowa, U.S.A
Secondary Antibody	**Working dilution**	**Source**
Alexa Fluor 488 anti-mouse IgG	1:200	Molecular Probes
Alexa Fluor 594 anti-rabbit IgG	1:200	Molecular Probes

T. thynnus hatchlings was completely undifferentiated, but lamination occurred during the first two days of the larval life. The dorsal retina matured before than the ventral one. The GCL progressively decreased in width and at 16DAH, ganglion cells were arranged in a row. Therefore, the retinal maturation in *T. thynnus* is similar to that described for altricial fish species.

3.2. Immunohistological study

We used antibodies against PCNA and pHisH3 to identify niches of proliferative and mitotic cells in the developing *T. thynnus* retinal tissue. PCNA antibody labels cells in S phase (plus G2 and phase M) and pHisH3 antibody specifically labels cells in phase M. At 16DAH, the CMZ contained abundant PCNA-immunoreactive nuclei (Fig. 4A-C). pHisH3-positive mitotic figures were also restricted to the CMZ, but they were mainly located in the scleral surface of this region (Fig. 4B).

We also used cell-type-specific markers to characterize different populations of retinal cells. TUJ1, an antibody raised against neuron-specific class III β-tubulin, identifies early axons emerging from differentiating ganglion cells in the developing retina of sharks (Bejarano-Escobar et al., 2012a). At 5PDH, TUJ1-immunoreactivity was clearly detected in fascicles of axons that leave the eye through the optic nerve head and in the vitreal surface of the neuroretina (Fig. 4D-F). Functional synapses can be identified immunohistochemically with antibodies against the transmembrane synaptic vesicle glycoprotein SV2. In the 16DAH *T. thynnus* retina, both the OPL and IPL were heavily immunostained with the anti-SV2 antibody (Fig. 4G-I).

Several antibodies have been described to identify early steps of photoreceptor differentiation in fish (Bejarano-Escobar et al., 2009, 2010; Ferreiro-Galve et al., 2010; Álvarez-Hernán et al., 2019). Visinin, a cone homolog of S-modulin/recoverin, is a calcium binding protein expressed in differentiating and mature retinal cone cells in birds (Bruhn and Cepko, 1996; Fischer et al., 2008; Álvarez-Hernán et al., 2020). At 16DAH, visinin immunosignal was located in the ONL, mainly in the cell somata of photoreceptor cells, but also in the outer segments and in the pedicles of photoreceptors (Fig. 5B,D). A similar staining pattern in a less numerous population of photoreceptor cells was identified with the CERN-922 antibody (Fig. 5C,D). This antisera has been described to identify opsins in the developing retina of different fish species (Candal et al., 2005; Bejarano-Escobar et al., 2009, 2010, 2012a; b). In order to identify different amacrine cell subpopulations of the INL, anti-αTH and anti-PV antibodies have been used. At 7DAH, anti-αTH antibody stained barely a subpopulation of cells located in the inner region of the INL (Fig. 5E-G). In contrast, anti-PV antibody labeled abundant cell somata that were located in the proximal INL and in the outer region of the GCL (Fig. 5H-M). Furthermore, the processes of these populations of cells, distributed to two sub-laminae in the IPL, were also immunoreactive (Fig. 5H-M). Additionally, PV immunoreactived somata and cell processes were faintly labeled in the outer INL (Fig. 5H-M). Notice that PV staining pattern was detected from the central (Fig. 5H-J) to the most peripheral region of the retina that was in close contact with the CMZ (Fig. 5K-M).

GS is an enzyme usually restricted to Müller glia in the fish developing and mature fish retina (Sánchez-Farías and Candal, 2016; Hernández-Núñez et al., 2021b). At 7DAH, GS immunosignal was detected in radially oriented cells (Fig. 6A-C). The somata were arranged in a single row located in the INL and slender immunoreactive processes could be distinguished in the GCL (Fig. 6A-C). Intense bands of GS immunoreactivity were found in the vitreal surface of the retina, surrounding the photoreceptor nuclei and in the outer limiting membrane (OLM) (Fig. 6A-C). The same expression patterns were observed in the central (Fig. 6D-F) and peripheral retina (Fig. 6G-I) at 16DAH, but at this stage, the Müller cell somata and processes were quite thicker. Notice that GS immunoreactivity gradually decreased towards the periphery (Fig. 6G-I). The appearance of immunomarkers linked to cell differentiation followed central to peripheral and dorsal to ventral gradients in the *T. thynnus* developing retina (not shown).

The immunohistochemical analysis of the developing *T. thynnus* retina showed staining patterns similar to those observed in other fish species.

4. Discussion

4.1. Cell differentiation and histogenesis in the developing retina of T. thynnus

Newborn specimens of *T. thynnus* presented unpigmented eyes and a retina composed of a single NbL and the emergence of the retinal layers occurred during the first 2 days after hatching. These visual system features are typical of altricial fish species (for reviews, see Evans and Browman, 2004; Bejarano-Escobar et al., 2014; Álvarez-Hernán et al., 2019). Similar results have been described for the retina of the Pacific blue tuna (*Thunnus orientalis*) (Kawamura et al., 2003; Matsuura et al., 2010), in which the retina has no layered structure 12 h after hatching (HAH). Those authors also found that at 36 HAH the retina shows the typical laminated structure, in agreement with our results for *T. thynnus.* All these data coincide with those described for other altricial fish species such as the turbot (De Miguel Villegas et al., 1997; Doldan et al., 1999), the halibut (Kvenseth et al., 1996), the tench (Bejarano-Escobar et al., 2009), the chinese sturgeon (Chai et al., 2007), the African catfish (Mukai et al., 2008), the Senegalese sole (Bejarano-Escobar et al., 2010), the sutchi catfish (Mukai et al., 2010), the gilthead seabream (Pavón--Muñoz et al., 2016), and the West australian dhufish (Shand et al., 2001).

Most of the retinal cells were distinguished by both morphological and topographical features at 2DAH, with the exception of Müller cells, that were morphologically detected for the first time at 7DAH. These data agree with studies conducted with the fish retina that clearly demonstrate that Müller cells constitute the retinal cell type that differentiate last (Bejarano-Escobar et al., 2009, 2010, 2012a; Harahush et al., 2009; Pavón-Muñoz et al., 2016; Sánchez-Farías and Candal, 2016).

At 16DAH, the *T. thynnus* larvae retina presented features of maturity, such as ganglion cell perikaryal arranged in a single row, plexiform layers well developed, and an ONL that held a few rows of elongated

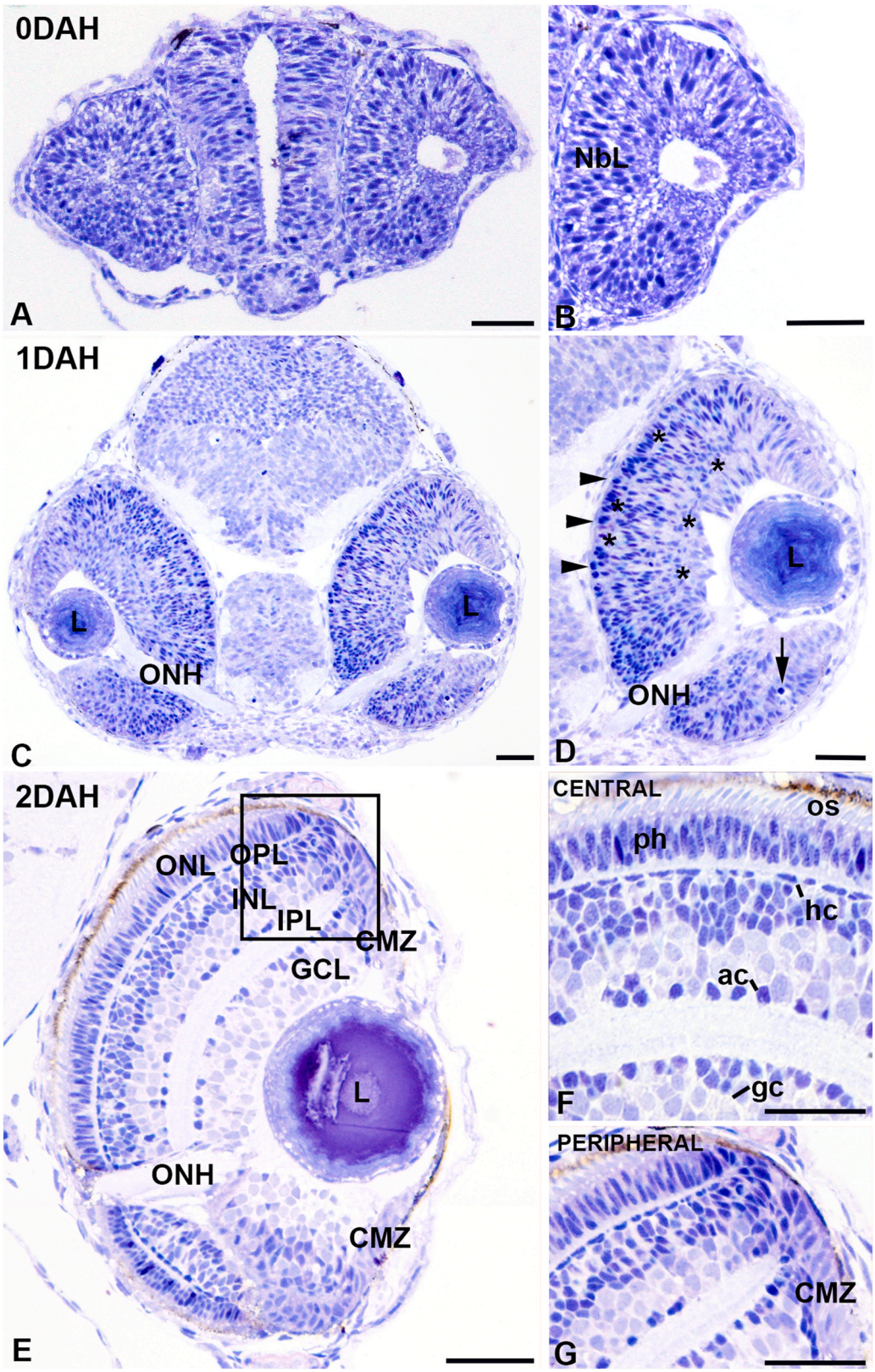

Fig. 2. Toluidine blue-stained semi-thin transverse sections showing emergence of the retinal layers in the developing *T. thynnus* retina. At 0DAH the neural retina is composed of a retinal neuroepithelium (A,B). At 1DAH (C,D) occurs the emergence of the plexiform layers (asterisks in D). Some mitosis in the ONL are detected (arrowheads) as well as sparse pyknotic nuclei in the ventral retina (arrow). At 2AH the typical cytoarchitecture of the mature retina was clearly observable (E,F), with the exception of the peripheral-most region, occupied by the CMZ (E,G). photoreceptors show the presence of short outer segments and the GCL is composed by multiple layers of ganglion cells (F). *ac, amacrine cells; CMZ, ciliary marginal zone; gc, ganglion cells; GCL, ganglion cell layer; hc, horizontal cells;* INL, inner nuclear layer; IPL, inner plexiform layer; L, lens; ONL, outer nuclear layer; ONH, optic nerve head; OPL, outer plexiform layer; os, outer segments; ph, photoreceptors. Scale bars: 100 μm.

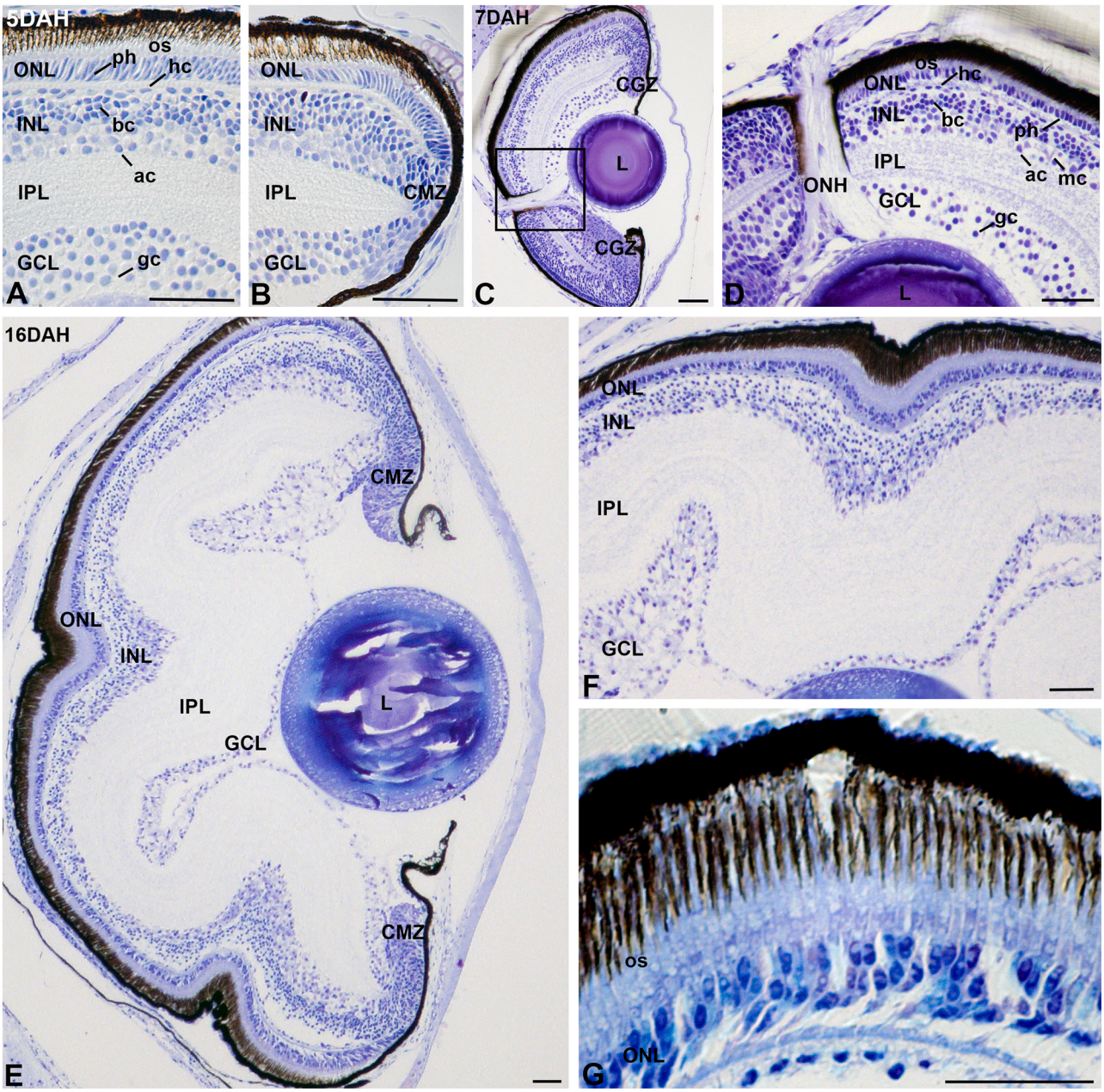

Fig. 3. Toluidin blue-stained semi-thin transverse sections of the middle eyeball of *T. thynnus* showing the retinal histology in the retina during selected posthatching stages. A thick IPL can be observed at 5DAH and 7DAH. The GCL shows a multi-layered structure (A,C,D). The CMZ can be observed at peripheral regions (B). At 16DAH the thickness of the IPL is higher and the ganglion cells tended to be distributed in a single row in the central tissue (E,F). photoreceptors show long, slender outer segments (G). *ac, amacrine cells; bc, bipolar cells; CMZ, ciliary marginal zone; gc, ganglion cells; GCL, ganglion cell layer; hc, horizontal cells;* INL, inner nuclear layer; IPL, inner plexiform layer; L, lens; ONL, outer nuclear layer; ONH, optic nerve head; os, outer segments; ph, photoreceptors. Scale bar: 100 μm.

photoreceptors with long outer segments. Furthermore, the main retinal cell types were immunohistochemically characterized at this age (see below). These observations coincide with those reported in *T. orientalis,* in which vision may be improved between 15 and 21DAH due to the morphological development of the photoreceptor outer segments (Matsuura et al., 2010). These features of maturity are observed during the first two weeks of life in other altricial fish species studied (De Miguel Villegas et al., 1997, Doldán et al., 1999, Mukai et al., 2008, 2010; Bejarano-Escobar et al., 2009, 2010; Pavón-Muñoz et al., 2016).

The central to peripheral and dorsal to ventral gradients of retinogenesis in the developing retina of *T. thynnus* were clearly observable, as in the retina of other fish species (Vecino et al., 1993; Doldan et al., 1999; Bejarano-Escobar et al., 2009, 2010; 2012). It has been described for Chondrichthyes the OPL evolves later in development than the IPL, suggesting an additional vitreal to scleral gradient of histogenesis (Harahush et al., 2009; Bejarano-Escobar et al., 2012a). However, the emergence of both plexiform layers occurred simultaneously in the retina of *T. thynnus*, in concordance with data from other teleosts (Vecino et al., 1993; Doldan et al., 1999; Bejarano-Escobar et al., 2009, 2010).

Pyknotic nuclei in the developing *T. thynnus* retina were found in toluidine-blue stained sections during the first 2 days after hatching and was restricted to sparse cells with no clear concentration or pattern, in concordance with data obtained from other altricial fish species (Bejarano-Escobar et al., 2010). In contrast, dying cells have been shown to be clustered in degenerating areas during early stages of the visual system development (Candal et al., 2005; Bejarano-Escobar et al., 2013). These studies also shown that the sequence of cell death during retinal

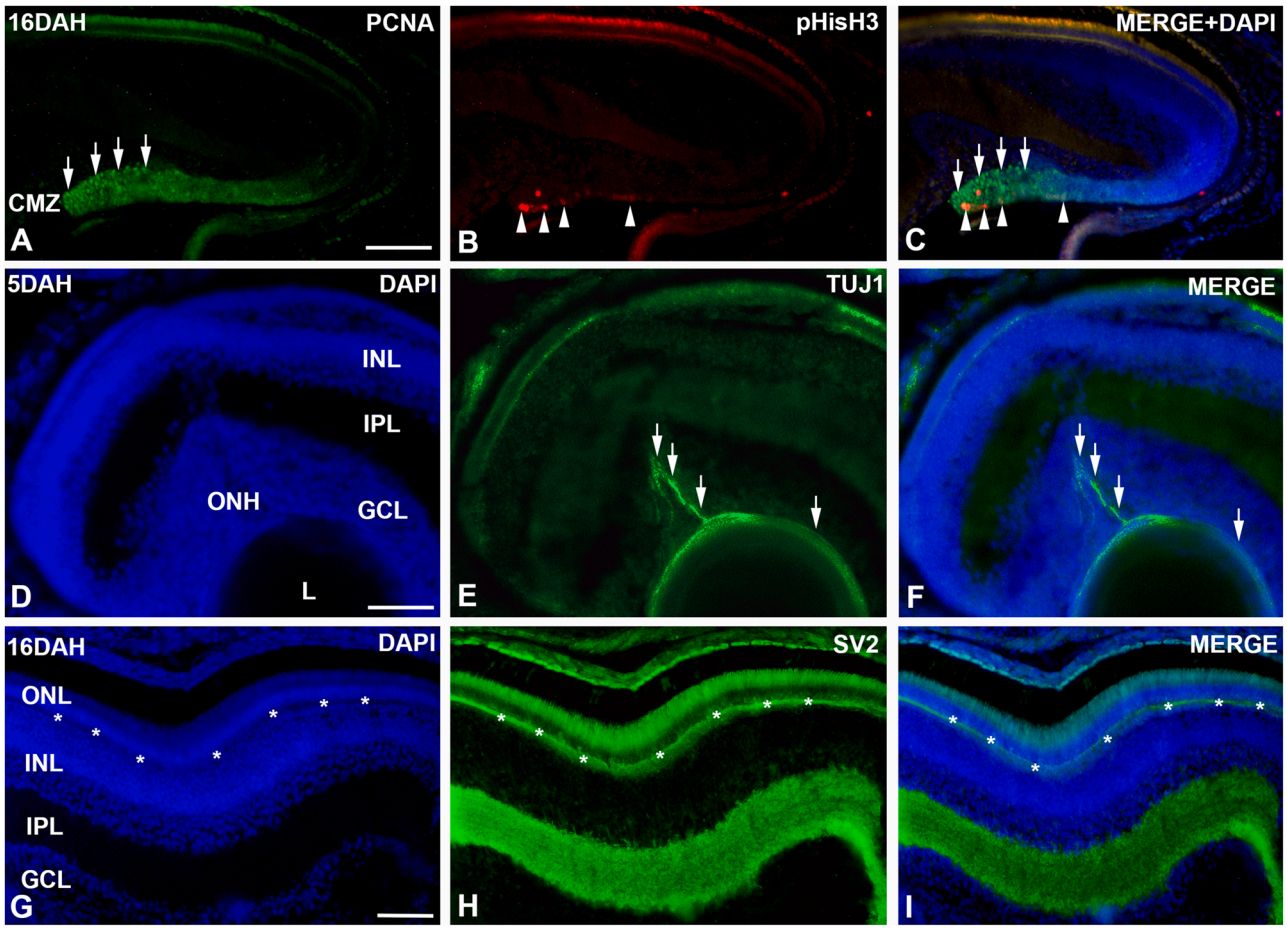

Fig. 4. Patterns of PCNA-pHisH3 (A-C), TUJ1-immunoexpression (D-F) and SV2-immunoreactivity (G-I) in retinal cryosections of *T. thynnus* specimens of selected posthatching stages. At 16DAH a strong PCNA (arrows in A,C) and pHisH3 (arrowheads in B,C) reactivity, located in the CMZ, is detected (A-C). At the same stage, SV2 immunoexpression is present in the OPL (asterisks in H,I) and in a thicker IPL (G-I). By 5DAH a faint TUJ1 signal is detected in the axons that leave the retina through the ONH (arrows in E,F). *CMZ, ciliary marginal zone; gc, ganglion cells; GCL, ganglion cell layer; hc, horizontal cells;* INL, *inner nuclear layer; IPL, inner plexiform layer; L, lens; ONL, outer nuclear layer; ONH, optic nerve head. Scale bars:* 100 μm.

histogenesis recapitulates the sequence of retinal cell differentiation. However, further studies are necessary to elucidate the distribution of dying cells in the developing *T. thynnus* visual system, using more specific techniques for detecting apoptosis.

4.2. Immunohistochemical cell markers in the developing retina of T. thynnus

Retinal cell markers constitute powerful tools in studies of retinal development because they allow characterization of the cell types as they differentiate in the neural tissue. Proliferative cells (PCNA-positive/pHisH3-positive) were detected immunohistochemically in the CMZ of the *T. thynnus* retina. These proliferating cells, located in the most peripheral region of the retina, are the source for the growth of the teleost retina, even at posthatching stages (Marcucci et al., 2016; Álvarez-Hernán et al., 2019; Xu et al., 2020; Hernández-Núñez et al., 2021a; b).

TUJ1 antibody labeled the ganglion cell axons at early stages of retinogenesis in birds (Kim and Sun, 2012; de De Mera-Rodríguez et al., 2019, 2021; Álvarez-Hernán et al., 2020) and mammals (Sharma and Netland, 2007). Furthermore, it is known as an excellent marker of pioneer retinal axons in the developing retina of sharks (Bejarano-Escobar et al., 2012a, 2014). Here we show that this antibody is also an excellent marker of early axons that emerge from the developing ganglion cells in the *T. thynnus* retina. The IPL and the OPL contained abundant synapses in the *T. thynnus* retina and, therefore, appeared strongly immunostained with the anti-SV2 antibody, as it has been described for other fish species (Bejarano-Escobar et al., 2010, 2012a).

We have tested two antibodies to identify photoreceptors in the developing *T. thynnus* retina. Anti-visinin antibody is expressed in cone cells in the developing and mature bird retina (Bruhn and Cepko, 1996; Fischer et al., 2008; Álvarez-Hernán et al., 2020). In the *T. thynnus* retina, it labeled a numerous population of photoreceptors. However, the CERN-922 immunoreactivity was detected in a less numerous population of photoreceptors with elongated external segments. This antibody has been considered as an excellent marker of rods in the developing and mature fish retina (Candal et al., 2005; Bejarano-Escobar et al., 2009, 2010, 2012a; b; Pavón-Muñoz et al., 2016). Other tuna species possess tightly packed cone photoreceptors during the first days of life, coinciding with the acquisition of feeding ability (Kawamura et al., 2003; Matsuura et al., 2010; Hilder et al., 2019). Rod differentiation occurs during the second week of life in *T. orientalis* and this population of photoreceptors is less numerous than the population of cones. According to these data, our results suggest that anti-visinin antibody could be identifying cones in the *T. thynnus* retina, while CERN-922 antibody is recognizing rod cells, as in the rest of teleosts studied (see above).

Different subpopulations of amacrine cells were identified with anti-αTH and anti-PV antibodies. αTH-immunoreactive amacrine cells were sparse and always located in the inner region of the INL, as has been described in other teleosts such as zebrafish (Arenzana et al., 2006), the trout (Candal et al., 2008), the tench (Bejarano-Escobar et al., 2009), the

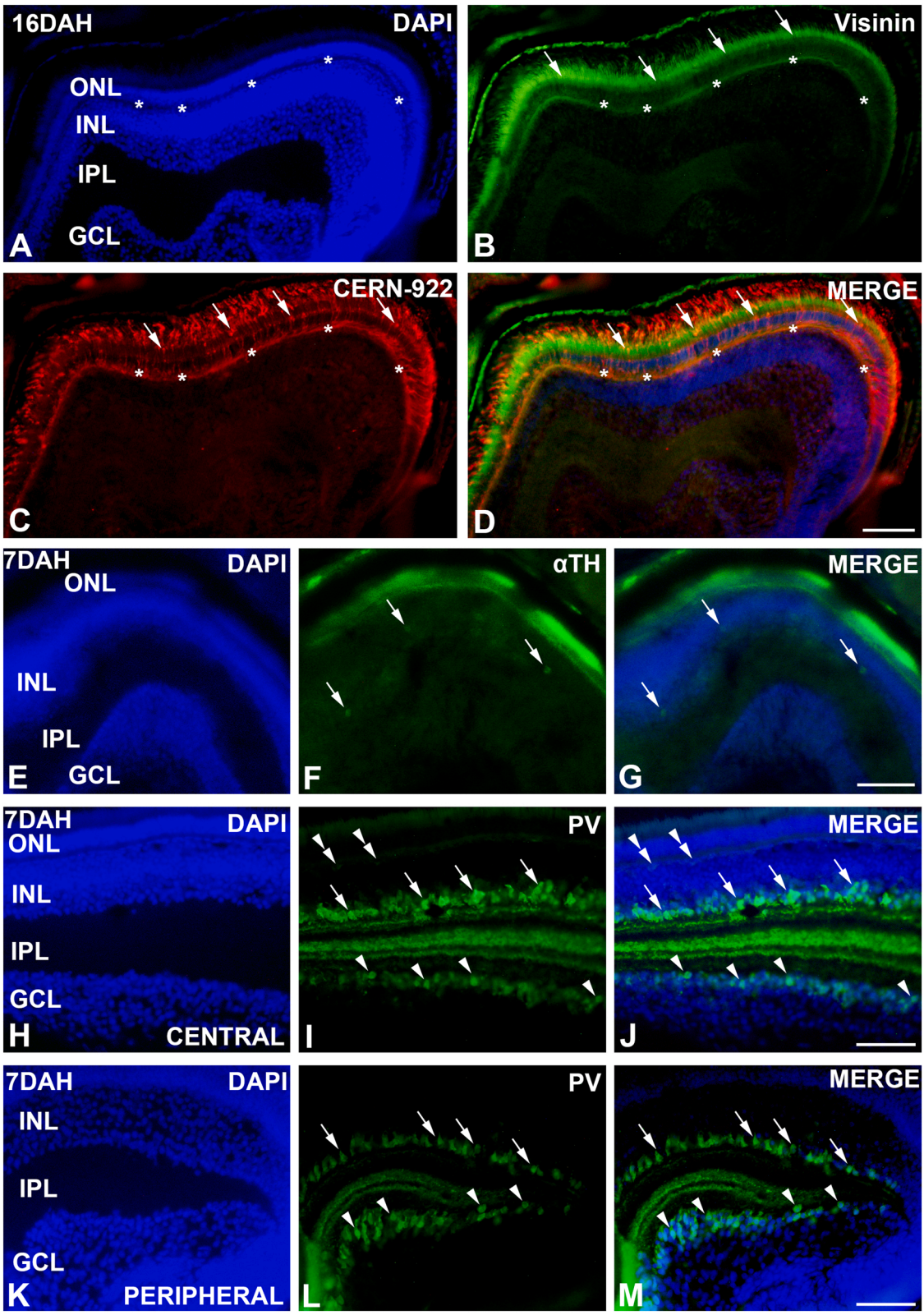

Fig. 5. Expression of different cell markers in retinal cryosections of *T. thynnus* specimens of selected posthatching stages. At 16DAH a strong visinin (B,D) and opsin (C,D) immunoreactivity is detected in the outer segments, cell somata (arrows in B-D), and the pedicles (asterisk in B-D) of the photoreceptors. By 7DAH αTH-containing neurons located in the inner half of the INL are sparse and are small in size and rounded, without stained prolongations (arrows in F,G). In the central neuroretina the immunoreactivity of PV is strong in the innermost region of the INL (arrows in I,J) and the outermost region of the GCL (arrowheads in I,J) as well as in two IPL sub-laminae (I,J). A faint PV expression is located in the outermost region of the INL (double arrowheads in I,J). Peripheral regions exhibit a similar PV immunoreactivity pattern (L,M).*GCL, ganglion cell layer*; INL, *inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer. Scale bars:* 100 μm.

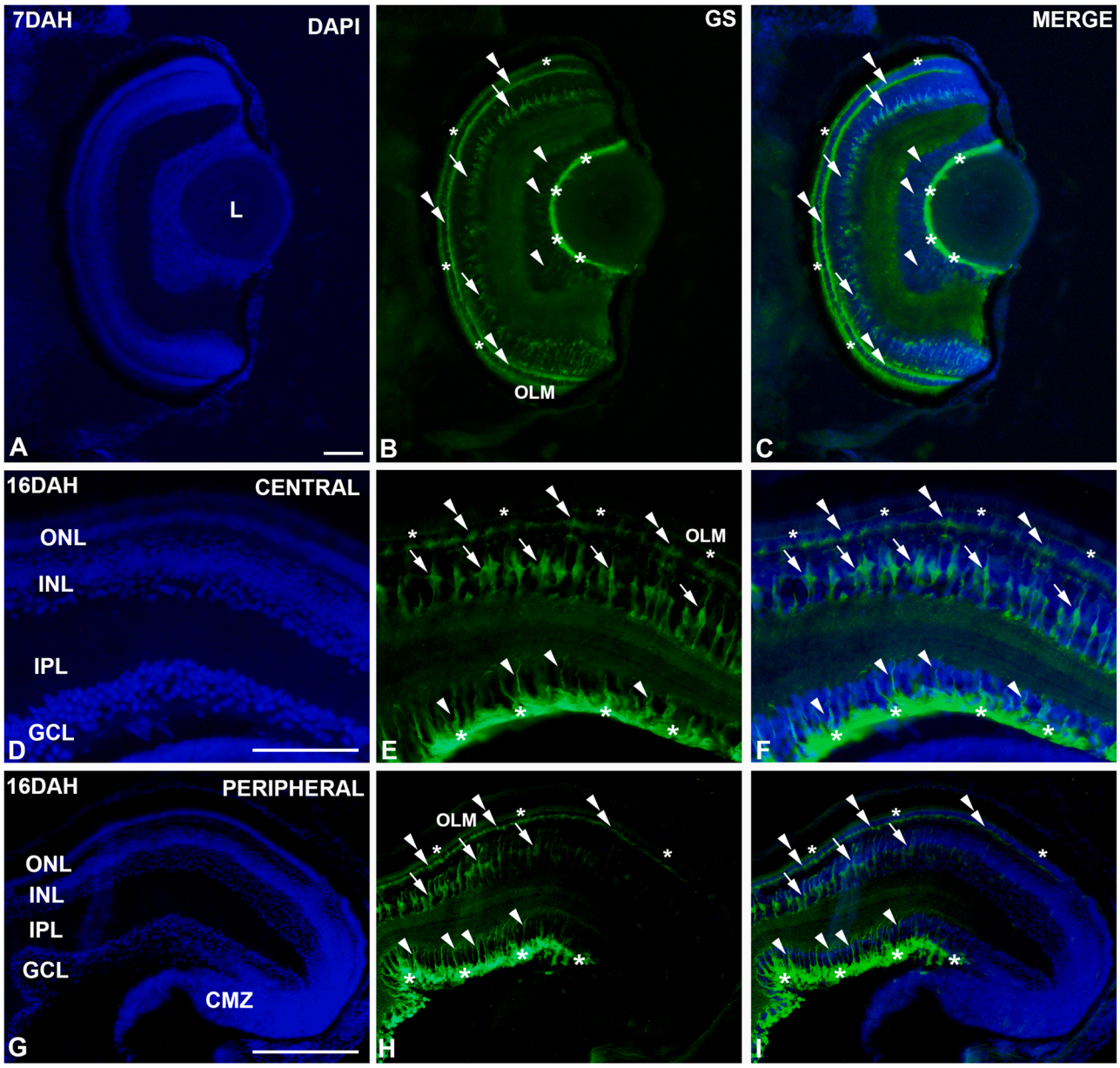

Fig. 6. Expression pattern of GS in retinal cryosections of *T. thynnus* at 7DAH (A-C) and 16DAH (D-I). GS immunoreactivity is mainly located in the soma (arrows in B,C,E,F,H,I) and the processes (arrowheads in B,C,E,F,H,I) of the Müller cells at 7DAH and 16DAH. GS immunoexpression reveals the presence of the OLM and the ILM (asterisks in B,C,E,F,H,I) and the scleral processes of the Müller cells branched at the level of the photoreceptor nuclei (double arrowheads in B,C,E,F,H,I). Notice that GS expression is absent in the most peripheral retina (H,I). *CMZ, ciliary marginal zone; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; L, lens; OLM, outer limiting membrane; ONL, outer nuclear layer. Scale bars:* 100 μm.

Senegalese sole (Bejarano-Escobar et al., 2010), and in elasmobranchs (Ferreiro-Galve et al., 2012). In the case of PV, it has been described that it is found in the cell bodies and dendrites of a subpopulation of amacrine and displaced amacrine cells (Weruaga et al., 2000; Yeo et al., 2009).

Finally, a marker of Müller glia has also been tested in the retina of *T. thynnus*. GS is the most useful marker for Müller glia identification and it is expressed by immature and differentiated Müller cells (Mack et al., 1998; Peterson et al., 2001; Bejarano-Escobar et al., 2012a; b). It was expressed in the Müller cell bodies, their scleral and vitreal processes, and their glial endings located in the ILM and the OLM. Furthermore, GS-immunoreactive cell processes branched in the region that surrounds the nuclei of photoreceptor cells, in concordance with results obtained in the rest of fish species studied (Bejarano-Escobar et al., 2014).

In the present study, it has been found that PV-expressing amacrine cells were present in a region located near the CMZ, but GS immunoreactive Müller cells were found far from the most peripheral region. Similar results have been described in the developing *S. canicula* retina, where late glial markers such as GFAP or GS were found in differentiating and mature Müller cells (Sánchez-Farías and Candal, 2015, 2016). However, radial glia (Müller glia precursors), located in the most peripheral region of the retina, do not express these late markers. In contrast, these radial progenitors can be identified with early markers of neurogenesis, such as doublecortin (DCX), whose immunoreactivity has been detected in the cell bodies of radially oriented cells located bordering the CMZ (Sánchez-Farías and Candal, 2015, 2016). Therefore, GS was expressed in young and mature Müller cells in the *T. thynnus* retina, but not in early radial glia distributed near the CMZ.

5. Conclusions

The ontogenetic development of visual system in *T. thynnus* is similar to that described for other teleost with altricial larvae (Álvarez-Hernán et al., 2019). The eyes of *T. thynnus* were pigmented and the retina was completely layered and therefore functional enough between 2 and 3 DAH, coinciding with the opening of the mouth (Yúfera et al., 2014). Furthermore, it has been described that the differentiation and maturation of the digestive system, thyroid gland, swim bladder, kidneys, and heart occur in the very early larval ontogeny (Yúfera et al., 2014), as it has been described for *T. orientalis* (Kawamura et al., 2003; Matsuura et al., 2010). At the end of the second week of life, all retinal cell types can be characterized in the retina of *T. orientalis* (Matsuura et al., 2010), coinciding with the results obtained in the present study, stages at which all retinal cells can be characterized immunohistochemically. These data suggest that this rapid transformation during the early larval life permits the larvae to acquire a degree of development that enables the defense against predators, efficient predation, and digestion of complex feeds. These basic studies on early morphology of the visual system and larval behavior are necessary to support applied research on larval rearing.

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