Development of the Senegal sole, *Solea senegalensis* forebrain

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The present paper deals with the ontogeny of the forebrain of the flatfish Senegal sole, *Solea senegalensis*, through different developmental stages before and after to metamorphosis. A first approach was made by conventional histological techniques, which allowed the determination of the main ontogenetic events. A second approach was to analyze the proliferation zones (PZ) during development and their locations, as well as the relation between them and the telencephalic asymmetry of the Senegal sole.

The results show that before metamorphosis the Senegal sole goes through a fast development. The pituitary is visible 1 day after hatching (DAH), the inferior lobes of the hypothalamus appear 3 DAH, and the olfactory bulb and the differentiation between telencephalon and diencephalon are present around 4 DAH.

In addition, by applying proliferating cell nuclear antigen (PCNA) immunohistochemistry by means of a monoclonal antibody against the PCNA and ABC complex, we were able to determine the PZs in the forebrain of pre- and post- metamorphic specimens. Although in both cases the PZs were similar, in premetamorphic animals they were thicker. However, PZs were observed in the pallium and subpallium, preoptic region, pretectum, epithalamus, dorsal and ventral thalamus, posterior tuberculum and hypothalamus. In all cases the PZs, mainly focusing on the telencephalon, were symmetrical in both hemispheres.

Key words: forebrain, ontogeny, flatfish, metamorphosis, PCNA.

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It is known that Actinopterygians, a group that includes Teleost fishes, possess a different organization of the forebrain than land vertebrates and lungfish as a consequence of a different ontogenetic process. The forebrain is the result of an outward bending or eversion of the dorsal portion of the lateral wall of the neural tube. As a result of this, the telencephalon of adult specimens generally consists of two solid masses flanking a median ventricular space. By contrast, in all other vertebrates, as well as Dipnoos, the dorsal portion of the neural tube goes through an inward bending or inversion process resulting in a pair of hemispheres enclosing lateral ventricles (Gage 1893; Studnicka 1896; Johnston 1911; Holmgren 1922; Källen 1951; Nieuwenhuys 1962; Northcutt and Braford 1980; Schroeder 1980).

From a comparative point of view, these different ontogenetic processes have brought great confusion when comparing the forebrain neural nuclei among vertebrate classes. Indeed, it is considered that the forebrain is structurally and functionally the most complex region of the vertebrates' brain. How the different regions of the embryonic forebrain give rise to various adult structures is still not well understood. Moreover, many studies have been done to clear up how the forebrain develops, approaching the issue from a molecular point of view. In this sense, the expression patterns of several transcription factors and other molecules present in restricted regions of the telencephalon and diencephalon have been analyzed (Fishell *et al.* 1993, Tole and Patterson 1995, Grinblat *et al.* 1998, Kobayashi *et al.* 1998, Pedersen *et al.* 1998, Szele and Cepko 1998). The dorsal part of the telencephalon shows a progressive increase in the number of nuclei; this is likely related to the highest complexity and specializations of the Teleostean brain, in which there is an increment in the degree of eversion, cellular proliferation and posterior migration compared to polypteriforms, chondrosteans, holosteans, being the highest complexity

and specializations reached in the teleostean brain (Laudier and Liem 1983, Northcutt and Davis 1983).

The Senegal sole, *Solea senegalensis*, "left eyed" species we have focused our study on, is a species typical from warm climates and is well adapted to the Atlantic Spanish coast, and specially common in the vicinity of the Cadiz coast. This commercial species is interesting from the comparative point of view. These features have motivated several studies on the potential cultivation of this interesting species in Spain and Portugal (Dinis *et al.* 1999), as well as histological and histochemical studies on larvae ontogeny, which showed yolk-resorption, digestive tract, liver, pancreas, skin and gill development (Sarasquete *et al.* 1996, 2001, Ribeiro *et al.* 1999).

The main goal of the present paper was to first of all describe the most significant changes taking place in the *Solea senegalensis* forebrain during its development, from hatching until the specimens became asymmetrical. In addition, developmental stages before and after metamorphosis were analyzed by PCNA-immunohistochemistry. This allowed us to determine the location of the proliferation zones (PZs) and to relate those to the metamorphosis process.

Materials and Methods

Senegal sole, *Solea senegalensis* larvae, were supplied by CUPIMAR, S.A fisheries (San Fernando, Cadiz, Spain). Specimens were analyzed at the stages of 1,3,4,5,10,19 days after hatching (DAH), and processed and sacrificed in accordance with ethical standard procedures.

The larvae were anesthetized in 0.1% MS-222 and fixed with 4% paraformaldehyde in 0.1M phosphate buffer, pH7.4, and then processed for plastic embedding medium. The specimens were rinsed in phosphate buffer, dehydrated in ethanol and, after infiltration and polymerization, whole larvae of each stage were embedded in glycol methacrylate (LKB Historesin).

The plastic blocks were cut with a Jung Supercur Microtome in serial sections 3 μm thick. Sections were made in sagittal or transversal planes and stained with cresyl violet, toluidine blue or neutral red (Rodríguez-Gómez 1999).

A few specimens were processed according to the procedure for embedding in paraffin; their brains cut with a microtome in 5 μm thick sections and stained the same as the plastic sections. This material and the atlas of adult brain of *Solea senegalensis* and *Sparus aurata* (Rodríguez-Gómez, 1999; Muñoz-Cueto *et al.* 2001) have been used for morphological orientation.

PCNA immunohistochemistry

Specimens of *Solea senegalensis* at pre- (5-6 DAH) and post- (25 DAH) metamorphic developmental stages were analyzed for this part of the study. After anesthesia with MS-222, the whole animal, in the case of 6 DAH, or only the brain of post-metamorphic specimens were fixed in a mixed solution of ethanol and formaldehyde for 12 hours. After cryoprotection in 30% surcrose in PBS they were transversally cut with a cryostat at 6-10 μm.

For immunohistochemical staining, sections were washed in PBS treated with 3% H_2O_2 for 30 minutes to remove endogenous peroxidase activity, washed in PBS again, and incubated with 3% horse serum albumin in PBS-0.2% Triton for 3 hours. After several washes, sections were incubated in a primary monoclonal mouse antibody against rat PCNA (1:1000) overnight at 4° C. Then, after rinsing and protein blocking in a TBS/Triton solution with 1% normal goat serum, the sections were washed in PBS, and incubated for 1 hour in biotinylated goat-anti-mouse IgG (1:500). Finally, horseradish peroxidase conjugated Avidin Biotin complex (ABC complex) was applied for 30 min (1:250). The peroxidase colour reaction was started by incubation in 0.04% (w/v) diaminobenzidine (DAB), Tris-HCl (50 mM, pH 7.6) after adding 0.015% H_2O_2 for 5-15 min. All reagents and antisera were purchased from Santa Cruz Biotechnology (USA). Immunohistochemical black and white pictures were made as a negative film.

Results

The development of the Senegal sole, *Solea senegalensis*, forebrain is interesting due to the coincidence of several features. As for other Actinopterygian, the part of the neural tube rising the forebrain goes through the eversion process, with later migration and neural differentiation. On the other hand, the forebrain goes through a dramatic metamorphosis, changing from a symmetrical to an asymmetrical shape which implies brain transformations; this process mainly relay on the forebrain which undergoes significant shape change and is responsible for regulating such big modifications.

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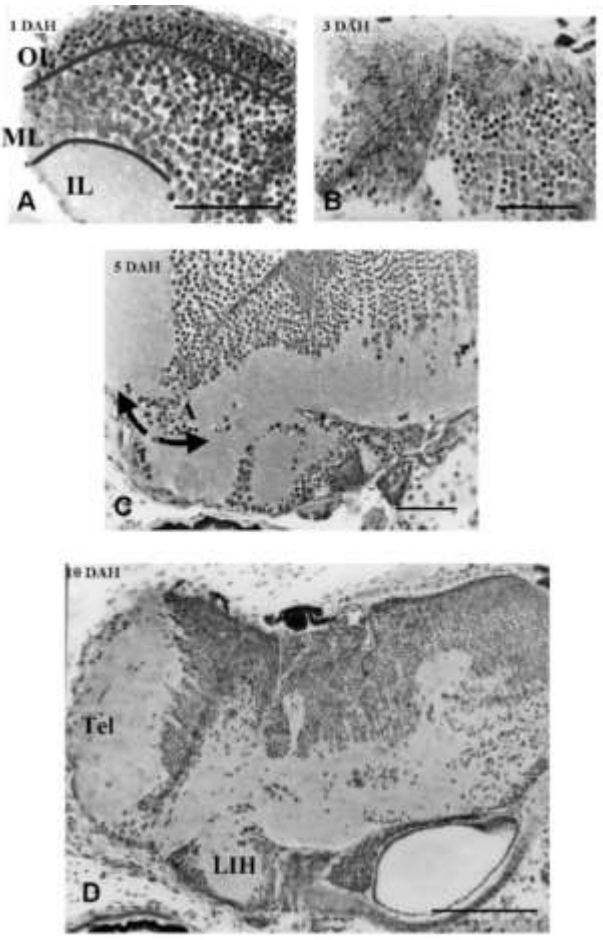

Figure 2. (A) Microphotograph showing the three-layer structure observed in the CNS of larvae 1 DAH. OL, outer layer; ML, medial layer; IL, internal layer. (B) Photomicrograph of the section of the CNS at larval stage 3 DAH showing the internal division of the outer (proliferation zone) layer. Arrows point out mitotic cells observed in that layer. (C) Photomicrograph of a section of the brain of Solea senegalensis at stage 5 DAH showing the nucleus "A". Arrows indicate the possible direction of movement of the cells from that nucleus. LIH, inferior lobe of the hypothalamus, Tel, telencephalon. (D) Photomicrographs of a sagittal section of the brain at stage 10 DAH. There are, at this stage, cells migrating into the rostral part of the dorsal telencephalic hemisphere (circle). Scale Bars=25μm.

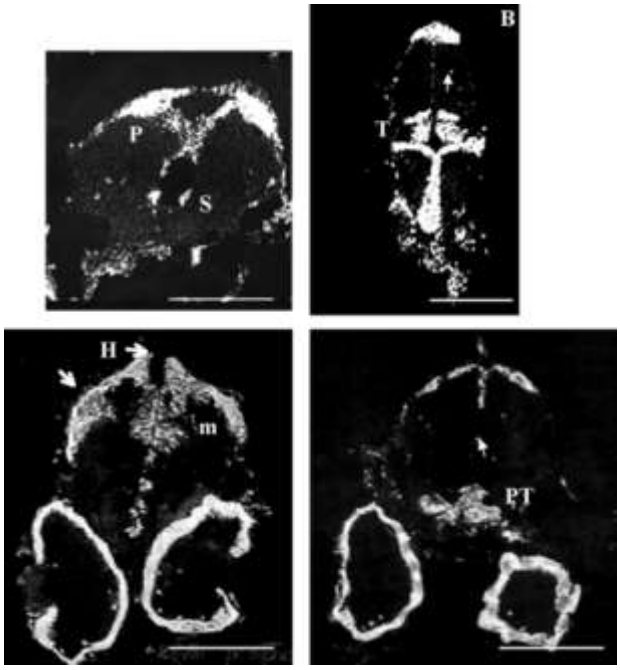

Figure 3. PCNA immunohistochemistry on transversal sections cross the brain of premetamorphic (5-6 DAH) Solea senegalensis larva. A: the most rostral section, through the telencephalon and B: the most caudal section through the *hypothalamus*. Abbreviations: H, *habenula*; Hi, *hypothalamus*; m, medial tectal proliferation zone; OT, optic *tectum*; P, *pallium*; PT, posterior tubercle; S, *subpallium*, T, *thalamus*. The arrows in B and C indicates PCNA immunoreactive cells far from a proliferation zone. Scale bars=100μm.

sal part of the telencephalon is observed with an increase in the number of telencephalic cells (Figures 1D, 2D). The preoptic area is quite well defined as is the torus lateralis (NLT). Close to the germinal zone and dorsal to the hypothalamus, there is a small cell group still indistinguishable as to whether it belongs to the thalamus or is part of the pretectum (full pointed nucleus in Figure 1D).

By 19 DAH, Senegal sole specimens have become asymmetric. The change in shape occurs from 12 DAH on. Both of the eyes are now located on the same side of the head and the brain shows a complexity similar to the adult (Figure 1E). Indeed, all the brain regions are well developed. Thus, mesencephalic areas are perfectly defined as optic tectum (OT) and torus semicircularis (tsc). Focusing on the telencephalon, both the area ventralis and dorsalis are easily distinguished. In the diencephalon, the preoptic area (PO), the thalamus, both the ventral (VT) and the dorsal (DT), subdivisions are found, and dorsally located the epithalamus (ET) as well as the pretectal area (Pr). The hypothalamus is present at this stage with all the nuclei and complexity that it displays in adults, and there is already a well developed glomerular complex. The pituitary has moved rostrally to the inferior lobes.

Proliferation activity – PCNA-

To study the proliferation activity, i.e. the PZs, a monoclonal antibody was used against PCNA.

This part of the study was focused on a premetamorphic stage (5-6 DAH) and postmetamorphic stage (25 DAH). Proliferation activity was very high at both stages; nevertheless, the proliferative layer was thicker in premetamorphic specimens. Although the mitoses are mostly observed in ven-

tricular position, some of the cells far from there kept their PCNA immunolabeling.

At 5-6 DAH, the proliferation activity was high in all the prosencephalic areas. In the telencephalon, PCNA-immunoreactive cells were located in the pallium and supallium (Figure 3A). The pallial PZ was especially pronounced and had two distinguished parts, the dorsal, thicker part and the medial, thinner part, whereas the subpallial PZ was unique and smaller. Caudally in the brain, proliferative cells were abundant in the thalamus, with two thalamic PZs, preoptic region as well as the habenula (Figures 3B, 3C). This mitotic activity was also observed in the hypothalamus and in the ventricular nucleus of the posterior tubercle (Figure 3D).

After metamorphosis, at 25 DAH, proliferation decreased in activity but not in the number of PZs. Two PZs, pallial and subpallial, were still visible in the telencephalon although the pallial PZ was in this case unique, being thinner and more dorsolaterally located, and the subpallial PZ, also thinner, extended along the periventricular layer (Figure 4A). On the other hand, PCNA-immunoreactive cells in the preoptic area were abundant, although mitotic activity was stronger in the ventral part (Figures 4A, 4B, 4C). Furthermore, the hypothalamus displayed strong PCNA immunoreactive cells in the periventricular part and in the inferior lobes (Figure 4D).

At both developmental stages, proliferation zones are separated by areas without mitotic activity, as occurs in the thalamus 25 DAH (Figure 4E). Indeed, in that part of the brain, we noticed two dif-

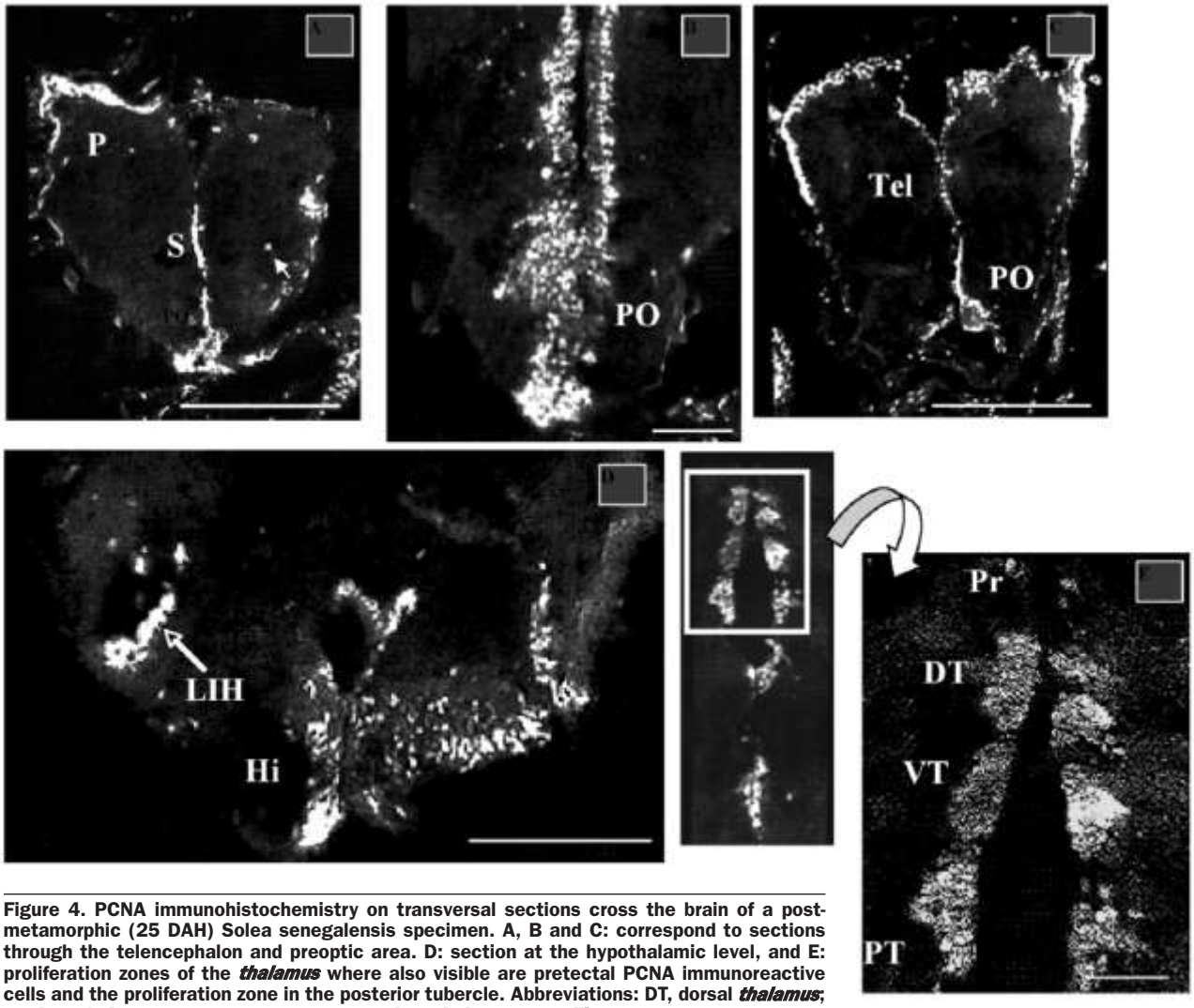

Figure 4. PCNA immunohistochemistry on transversal sections cross the brain of a post-metamorphic (25 DAH) Solea senegalensis specimen. A, B and C: correspond to sections through the telencephalon and preoptic area. D: section at the hypothalamic level, and E: proliferation zones of the *thalamus* where also visible are pretectal PCNA immunoreactive cells and the proliferation zone in the posterior tubercle. Abbreviations: DT, dorsal *thalamus*; Hi, *hypothalamus*; LIH, inferior lobes of the *hypothalamus*; P, *pallium*; Pr, *pretectum*; PT, posterior tubercle; S, *subpallium*, T, *thalamus*, VT, *ventral thalamus*. The arrows in A indicates PCNA immunoreactive cells far from a proliferation zone. Scale bars=50μm.

ferent PZs, a dorsal PZ corresponding to the dorsal thalamus (DT), and a ventral PZ which gives rise to the ventral thalamus (VT). Ventral to the PZ of the ventral thalamus is located the proliferation zone of the posterior tubercle (PT), and dorsal to the dorsal thalamus a discrete cell group displaying PCNA immunoreactivity is seen and may be the pretectum.

Nevertheless, after analysing the proliferation zones in pre- and post- metamorphic specimens of *Solea senegalensis*, we could determine that the asymmetry in the telencephalon of this species is not produced apparently by difference in the mitotic zones in both hemispheres, and it may be only caused by the adaptation of this brain part to the new shape of the animal. On the other hand, in the Senegal sole, the PZs are maintained long after metamorphosis.

Discussion

Usually Teleostean species have dorso-ventrally-symmetrically oriented bodies with symmetrical brains. During their development, most fishes become juveniles after either a dramatic metamorphosis or rather moderate transformational changes in external and internal morphology (Youson 1988). During this period, both internal and external organs are changed into a form that is ecologically suitable for life as juveniles. Flatfish are an example of development with dramatic metamorphosis. As larvae, flatfish look very similar to all other fishes. Thus, they have a symmetrical shape and they live a planctonic life. As they continue growing, they swim in the normal pattern for some time, until metamorphosis begins in the open water, whereby the larva is transformed into a very small, flatfish-shaped fish (Fukuhara 1986). The most striking aspect of this metamorphosis is the migration of one of the eyes, left or right depending on the species, from one side to the other. Finally, both of the eyes lie on the same side, while the eyeless side, blind, becomes the under side. The eye migration occurs shortly before the fish begin their bottom-dwelling life. The migration also implies changes in the brain shape and a rearrangement that mainly applies to the rostral part of the brain. It is assumed that most ethological and ecological changes seen during the postembryonic, transitional stages occur in parallel with appropriate neural innervation developments and commands and, obviously these changes are reflected in the brain. After a few months, when the transformation to flatfish shape has been concluded, the young take up an exclusively bottom dwelling existence (Youson 1988).

The Senegal sole, *Solea senegalensis*, has two phases of development. On the one hand, it goes through the usual embryonic process as fish (symmetric shape), and on the other, it goes through a posterior metamorphic process (ending as asymmetric shaped).

Developmental process and comparative aspects

It may be expected that this species has a longer embryological time since goes it through more events, but in reality there is a 1st phase, just before they become asymmetric, which is shorter-lasting than in other species. Indeed, the olfactory bulb and the differentiation between telencephalon and diencephalon are present 4 DAH; the pituitary is already visible at 1 DAH, the inferior lobes of the hypothalamus at 3 DAH. Whereas in the sea bream, *Pagrus major*, the olfactory bulb and the division telencephalon-diencephalon are seen on day 28, the pituitary is present on day 18 and the inferior lobes of the hypothalamus between day 18 and 28 (Toyoda and Uetmatsu 1994).

In this sense, it has been reported in another flatfish, the turbot *Scophthalmus maxima* (Briñón *et al.* 1993), that the metamorphic process starts approximately on the third week after hatching as occurs in the Senegal sole. Furthermore, our observations suggest that even the biggest transformations regarding the telencephalon take place in quite a short time, the complete metamorphosis ends much later, perhaps after three months, as has been described in the Turbot.

Proliferation zones –PCNA-

PCNA immunohistochemistry has been shown to be a useful tool to analyze proliferative cells in different tissues since PCNA probably is involved in one of the last steps preceding DNA synthesis (Bravo and Macdonald-Bravo 1984, 1985; Cappello *et al.* 2003) being essential for binding DNA polymerase-δ during leading strand DNA synthesis (Fairman 1990, Lee and Hurwitz 1990). Furthermore, it has been successfully applied on fish brain (Wulliman and Puelles 1999). Among the antibodies against PCNA, we have utilized PC10 which, as Wulliman and Puelles (1999) mention, is established for differentiating cells with pro-

liferative potential from quiescent cells.

Our results show that indeed, PCNA immunohistochemistry visualized PZs in the central nervous system. These PZs are seen in the specimens just after hatching as a continuous periventricular layer, also visible by conventional histological techniques. Nevertheless, soon after that early stage, the PZs appear as discrete zones separate by non-mitotic areas.

The proliferation zones described in specimens 5-6 DAH are conserved until late in the development, long after metamorphosis, although some of those are thinner, probably with less mitotic cells. This may happen in this flatfish species as a special feature since Wulliman and Puelles (1999) found out that in zebrafish only some of the PZs that appear in larvae 5 days after fertilization go beyond day 10. Furthermore, earlier studies performed on adult zebrafish by autoradiography did not detect the PZ either in the pallium or in the preoptic region (Rahmann 1968). Moreover, the bromodeoxyuridine technique in adult electric fish, Apeteronoutus leptorhynchus, visualized the PZ in the subpallium, whereas in the pallium, preoptic region, pretectum, dorsal and ventral thalamus and hypothalamus, only a few labeled cells were seen (Zupanc and Horschke 1995). The proliferation zones we observed in the Senegal sole 5-6 and 25 DAH are closely similar to those described in the zebrafish 5 days after fertilization. Thus, the PZs are seen in the pallium, separated zones for the sub-pallium and preoptic region, pretectum, habenula, dorsal thalamus, ventral thalamus, two PZs in posterior tubercle, one periventricular and the other more caudal and the hypothalamus.

These results suggest that the variances observed in the number of PZs in the different Teleostean species may be related not only to the developmental stage, but mainly to the technique employed to analyze them. Dispersed PCNA-labeled cells far from the periventricular layer were often seen. According to Bravo and Macdonald-Bravo (1987), this may be explained by the fact that cells leaving the mitotic cycle keep 30-40% of their PCNA for about 24 hours.

Acknowledgments

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