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RESEARCH ARTICLE

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Eye ontogeny in *Pleurodema bufoninum*: A comparison with *Pleurodema somuncurense* (Anura, Leptodactylidae)

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Abstract

Vision is one of the main sensory systems in amphibians, and the eye structure is highly associated with habitat conditions. The ontogeny, as well as the adult structure, of the eye has been studied in only a few species. The life change after metamorphosis is accompanied by changes in the visual environment. The aim of this work is to describe the eye ontogeny of *Pleurodema bufoninum* and to compare it with that of *Pleurodema somuncurense*. Specimens of both *Pleurodema* species were processed for histology analysis at different stages of development, including the tadpole, postmetamorphic, and adult forms. Eyes in both *Pleurodema* species are composed of the 3 tunics, *tunica fibrosa*, *tunica vasculosa*, and *tunica interna*, and the lens. Additionally, in both, the iris presents a projection on its dorsal and ventral free ends that screens the cornea. This structure has been reported in the eye of several anuran species and is called the umbraculum, meniscus or pupillary nodule. Our results show that the structures related to light capture (retina and lens) appear early in larval life, while the components of the terrestrial-life eye (scleral cartilage, specialized cornea, eyelids, nictitating membrane, and Harderian's gland) do not develop until the metamorphic climax, when the tadpole leaves the water. The adult eyes of *P. bufoninum* and *P. somuncurense* are very similar in structure and development.

KEYWORDS
meniscus, umbraculum, visual system, development

1 | INTRODUCTION

The light spectrum underwater is different from that in air. Underwater, contrasts are attenuated and the light diffraction index is higher than in air. Animals that switch between aquatic and terrestrial environments, such as aquatic birds, can compensate for the loss of the refractive power of the cornea underwater by an exaggerated lens accommodation, among other mechanisms (Howland & Sivak, 1984; Sivak, 1980). The anuran metamorphosis is accompanied by changes in the visual environment (Hoskins, 1990); therefore, study of amphibians is useful for exploring differences in water-land transitions. The chemical composition of photopigments and wavelength absorption differ between terrestrial and aquatic amphibians and between tadpoles and terrestrial postmetamorphic individuals of the same species (Muntz, 1977).

The knowledge of amphibian eye ontogeny is limited to a few species, such as *Rana temporaria*, *Xenopus laevis*, and *Lithobates pipiens*. In the latter species, the lateral eyes are not functional until a few days after hatching (Nilsson, 1964; Saxen, 1954). However, tadpoles respond to light in this period using the pineal eye and dermal light sense (Hamasaki & Eder, 1977; Stevens, 1963). Saxen (1954) also established that while in *R. temporaria* the retina development is slow and the adult form arises after metamorphosis, in *X. laevis* the adult retina appears at earlier stages. According to Saxen (1954), this could be because the tadpoles and adults of *X. laevis* occupy the same environment and have developed the same adaptation.

Comparative study of vertebrates' eye morphology proposes the existence of "aquatic," "terrestrial," and "intermediate" eyes (Neagu & Petraru, 2015). The aquatic eye is characterized by a flat, thin cornea, and a spherical lens, while the terrestrial eye has a characteristic curved, thick cornea, and a flattened lens. The intermediate eye is adapted for aquatic and terrestrial vision and possesses both environment specializations, depending on the individual species (Neagu &

Petraru, 2015). For example, the epipelagic fish *Anableps anableps* possesses two different thicknesses of the cornea and a specific shape of the lens (González-Martín Moro, Gómez-Sanz, Sales-Sanz, Huguet-Baudin, & Murube-del Castillo, 2014). The study of the eye development of the terrestrial anuran *Pelobates syriacus* indicates that the larval stage has a fish-like eye, with a flat cornea and spherical lens, and the juvenile individual possesses a terrestrial vertebrate-like eye with a prominent refractive cornea and a flattened lens (Sivak & Warburg, 1983). Additionally, during metamorphosis, the dry weight of the lens and its protein content increase in *L. catesbeianus* (Smith-Gill & Carver, 1981). Therefore, it is thought that anuran metamorphosis allows the transition of an aquatic eye to a terrestrial or intermediate eye. Despite the common direction of changes in eye morphology during metamorphosis, the timing and rhythm of them in different species are related to the adult individual environment, the rate of metamorphosis and other unknown factors.

A peculiar structure in the eye of certain vertebrates has been described as a specialization of the iris and has been given many different names. The term "umbraculum" was first mentioned in mammals by Johnson (1901) to refer to a projection of the iris that screens the cornea. Later, Mann (1931) found a similar structure in two anuran species, *Hyperolius horstockii* and *Bombina bombina*, and proposed that the umbraculum is a modification of the upper iris that could be movable and play some role in the exclusion of the light from the eye. He also described *B. bombina* as having an umbraculum dorsally and a notch ventrally and *Bufotes viridis* as having a uveal dorsal and ventral proliferation, like *Anaxyrus cognatus*. Later, many authors described this structure in tadpoles and adult anurans (Ewer, 1952; Kruger, Weldon, Minter, & Du Preez, 2013; Van Dijk, 1966). Ewer (1952) observed that *Amietia vertebralis* (= *Rana umbraculata*) possesses a dorsal umbraculum and a ventral pupillary nodule that are different in composition. Duellman and Trueb (1986) described the amphibian eye morphology and mentioned that anurans with horizontal pupils possess dorsal and ventral pupillary nodules that are correlated with pupil contraction. Miranda-Ribeiro (1920, 1926) coined the term "meniscus" for a discoid lobule projecting from the upper margin of the iris, and this denomination was used by others to describe a similar structure (e.g., Barrasso, Cotichelli, Alcalde, & Basso, 2013; Basso, 1998; Cei, 1969, 1970; Izecksohn, 1983; Lutz, 1929, 1943). Nevertheless, not all anurans have the same type of meniscus. Cei (1969, 1970) reported that *Pleurodema somuncurense* has a dorsal and a ventral meniscus, but apparently, *Cyclorhampus* and *Zachaenus* possess only a dorsal meniscus of different sizes (Izecksohn, 1983; Lutz, 1929, 1943; Miranda-Ribeiro, 1920, 1926). Basso (1998) described the presence of an enlargement of the ventral pupillary nodule in *Atelognathus ceii*.

Van Dijk (1966) defined an ocular elygium [= umbraculum] as a pigmented layer arising from the margin of the iris distal to the pupil and an epidermal elygium as a pigmented layer in the skin above the eye and reported the elygium in tadpoles of *Leptopelis natalensis* and *Strongylopus wageri*. Years later, Kruger et al. (2013) analyzed the development timing of the elygium and the umbraculum and concluded that the elygium could be the larval way to screening of the pupil and the umbraculum the adult way.

In its original description, *P. somuncurense* was placed within the genus *Telmatobius*, and in this context, the presence of iris projections (among others) was highlighted as a diagnostic character (Cei, 1969). Following Lutz (1929) those structures were identified as meniscus. Lynch (1978) erected the monotypic genus *Somuncuria* for the species *S. somuncurensis*, but he was unable to specify its sister-group relationship. The most recent phylogenetic analysis (Faivovich, Ferraro, Basso, Haddad, Rodrigues, Wheeler & Lavilla, 2012) showed that *S. somuncurensis* nests within *Pleurodema* and is closely related to *P. bufoninum*. In this new phylogenetic context, some features of *P. somuncurense* tadpoles were revised and showed that the dorsal and ventral meniscus in the iris is a character shared with at least its sister species, *P. bufoninum* (Barrasso et al., 2013). However, a more detailed study of this structure and its ontogeny is necessary.

The aim of this work is to describe the eye ontogeny of *P. bufoninum* and to compare it with that of *P. somuncurense* in order to propose new information that contributes to the understanding of the main eye structures shared by these sister species.

2 | MATERIALS AND METHODS

2.1 | Animals

Specimens of *Pleurodema bufoninum* (Bell, 1843) and *Pleurodema somuncurense* (Cei, 1969) were studied at different stages of development, including the tadpole, postmetamorphic and adult forms. A total of 20 individuals of *P. bufoninum* and 10 of *P. somuncurense* were processed for histological analysis. Tadpoles were classified by stages of development according to Gosner (1960). All specimens were fixed and stored in 10% formalin and housed at the herpetological collection of the Instituto de Diversidad y Evolución Austral (IDEAus-CONICET), Puerto Madryn, Argentina (see Appendix).

2.2 | Histological procedure

Tadpoles and postmetamorphic individuals were whole processed for light microscopy, and the ocular area in adults was dissected first. The specimens were dehydrated in an ethanol series and embedded in Paraplast, or glycol methacrylate (Historesin Leica, Mycrosystems Nußloch GmbH, Heidelberg, Germany). Tadpoles were sectioned in the transverse plane, and adult eyes were sectioned in the sagittal plane in reference to themselves, both 5 μm thick. The sections were stained with haematoxylin and eosin (H&E) or Masson's modified trichromic (MMT) for general cytology and histology. MMT is different from the original Masson's trichromic in that the acid fuchsin solution also contains orange G and xylidine ponceau. In addition, histochemical stains were performed on selected sections: Coomassie blue R250 technique (Coom; Kiernan, 1999) to characterize the presence of protein content in the lens and Periodic Acid-Shiff technique (PAS; Kiernan, 1999) to identify the basement membrane between the endothelium of the choroid capillaries and the pigmented retinal epithelium (Young, Lowe, Stevens, & Heath, 2006). The corneal layer of the epidermis and the goblet cells of the covering epithelium of the digestive system present

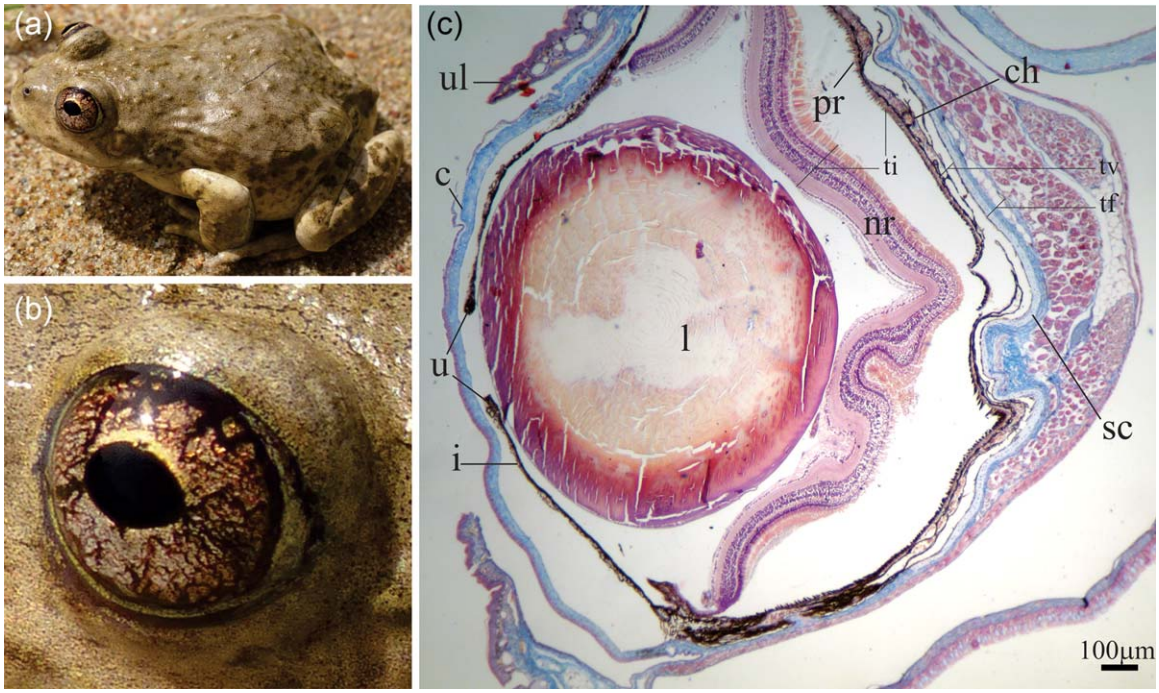

FIGURE 1 (a) *Pleurodema bufoninum*, adult individual. (b) Detail of the eye from a). (c) Sagital section of the eye from an adult individual (MMT). c: cornea, ch: choroid, i: iris, l: lens, nr: neural retina, pr: pigmented retina, sc: sclera, tf: *tunica fibrosa*, ti: *tunica interna*, tv: *tunica vasculosa*, u: umbraculum, ul: upper eyelid

in the section were considered to be positive controls of the Coom and PAS techniques, respectively. The stained sections were examined using a Zeiss Primo Star microscope, and images were captured using a Canon PowerShot A640 digital camera coupled with the software Axio Vision 4.8.2. (Zeiss).

3 | RESULTS

The amphibian eye is a complex organ, and for better understanding of its development in *Pleurodema*, it is essential to describe the adult eye histology first.

3.1 | Adult eye of *P. bufoninum* and *P. somuncurense*

Pleurodema bufoninum possesses prominent eyes in proportion to its head size and has a horizontal pupil, as shown in Figure 1a,b. For both *Pleurodema* species, the eyes are composed of the three tunics, *tunica fibrosa*, *tunica vasculosa*, and *tunica interna*, and the lens (Figure 1c). The *tunica fibrosa* is composed of the fibrous sclera and the substantia propria of the cornea (Figure 2a–d). The former is a continuous cup-shaped cartilage plate that extends to almost half of the eye cup and continues with a thick, dense connective tissue that gives place to the substantia propria of the cornea. A comparison of the two species shows that the *tunica fibrosa* is almost identical, although the substantia propria and the scleral cartilage are slightly thinner in *P. bufoninum* than in *P. somuncurense* (Figure 2a–d). The *tunica vasculosa* seems to have no differences between these two species. It is composed of a heavily pigmented loose connective tissue with numerous blood vessels and is called the choroid (Figure 3a). In the posterior eye, the choroid lies between the sclera and the pigmented retina. In the anterior eye, the choroid loses most capillaries and retains the pigmented connective tissue to conform the iris stroma (Figure 1c). Between the pigmented retinal epithelium and the endothelium of choroid blood vessels lies the Bruch's membrane. This structure is PAS positive since it is composed of the basement membranes of these two epitheliums plus intervening layers of collagen and elastic fibers (Figure 3b). The *tunica interna* is formed by the neural and pigmented retina and the iris epithelium. The pigmented retina is a simple cubic epithelium whose cells present pigmented projections that mingle with the outer segments of the photoreceptors in the neural retina (Figure 3c). The neural retina of *P. bufoninum* and *P. somuncurense* is divided into five distinct histological layers (Figure 3d,e). The outer segments layer is characterized by the presence of acidophilic structures corresponding to the rod and cone processes (Figure 3d). Below this lies the outer nuclear layer, which contains the densely packed nuclei of the photoreceptors. The outer plexiform layer contains the fibers of the photoreceptor cells and horizontal neurons, whose cell bodies belong to the inner nuclear layer along with the cell bodies of bipolar and amacrine cells. The inner plexiform layer is 3–5 times thicker than the outer plexiform layer, and here the projections of the inner nuclear layer cells make synaptic connections with the ganglionar cells. The cell bodies of the retinal ganglion cells constitute the ganglion layer, and its axons form the optic nerve.

The pigmented iris forms a diaphragm extending in front of the lens (Figure 1c); it is lined with a simple epithelium and an underlying loose connective tissue that is pigmented due to the presence of numerous melanocytes scattered in the stroma (Figure 4a). Within the

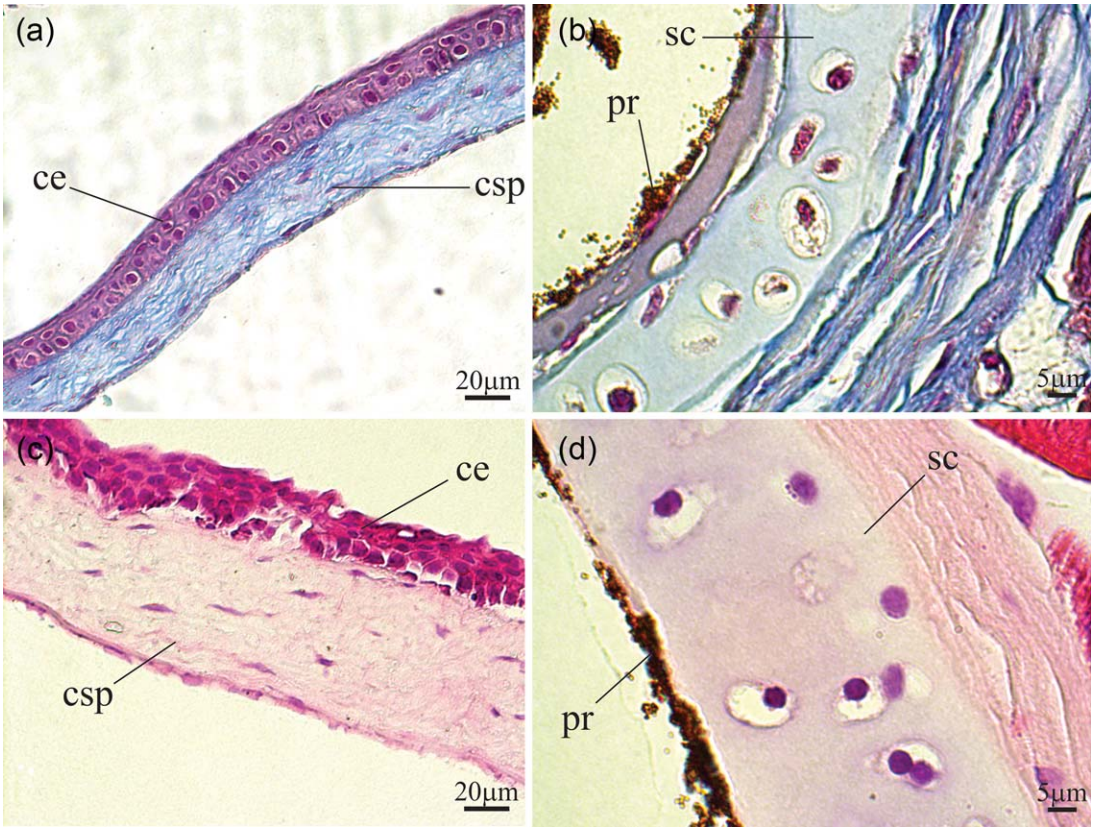

FIGURE 2 *Tunica fibrosa* of *P. bufoninum* (a, b; MMT) and *Pleurodema somuncurense* (c, d; H&E). (a, c): Detail of cornea. (b, d) Detail of scleral cartilage. ce: corneal epithelium, csp: corneal substantia propia, pr: pigmented retina, sc: scleral cartilage

proximal iris stroma, it is possible to identify the myoepithelial cells that conform the protractor lentis muscles involved in lens accommodation (Figure 4a). However, due to the presence of high quantities of melanin, it was not possible to identify the dilator pupillae muscles involved in pupil contraction. In both studied species, the iris presents an enlargement on its dorsal and ventral free ends: the umbraculum (Figure 1c). These structures are lined dorsally and ventrally by the iris epithelium and are composed of loose connective tissue with numerous melanophores (Figure 4b). The content of connective fibers and pigmented cells is variable, depending on the zone and the individual, without any particular arrangement.

The lens is a slightly flattened spherical structure covered by a single layer of cubical cells that retain their nuclei. The rest of the lens is constituted by cells that have lost their nucleus, called lens fibers, which are arranged in concentric layers. These fibers are prismatic in shape and extremely elongated, with an acidophilic Coom positive content, which means that they possess protein accumulation (Figures 1c,4c).

The adult eye has evident eyelids; these are skin projections that partially cover the eye. As skin components, they possess numerous dermal glands. The lower eyelid has an extra prolongation: the nictitating membrane. This structure is common in amphibians, and since it is movable, it can completely cover the eye outside the cornea. The adult cornea is separated from the lens by the aqueous body, which allows the cornea to have optical power. It is composed of a stratified epithelium underlined by the substantia propria that is the connective tissue of the *tunica fibrosa* in the anterior eye (Figure 1c,2a,c).

3.2 | Ontogeny of the eye of *P. bufoninum*, with some considerations of *P. somuncurense*

Here, we describe the complete development of *P. bufoninum* and compare it with *P. somuncurense* at the stages for which we have information about the two species. The development of the eye of *P. bufoninum* and *P. somuncurense* is an early event in their ontogeny. It starts with the primary optic vesicle formation and its following invagination giving place to two folds. The internal fold is the nearest to the epidermis, and the external fold is the nearest to the brain. The two folds that emerge from the primary vesicle invagination will constitute the retina. Meanwhile, the lens vesicle arises from the epidermis (crystalline placode). By Stage G20, the secondary optic vesicle is evident; it is constituted by the optic vesicle and the lens vesicle (Figure 5a–c). At this stage, the external fold is a fully pigmented simple squamous layer, and the internal one becomes significantly thicker and stratified. All cellular types possess high quantities of yolk (Figure 5b,c). At Stage G22, the lens vesicle consists of a simple cylindrical epithelium, and the presence of a cell cumulus can be noted inside on the posterior side. We showed that the lens presents protein accumulation in the adult eye. However, little protein accumulation is visible at Stage G22, and yolk is not identifiable in the lens (Figure 5e,f). The neural retina starts to arrange into

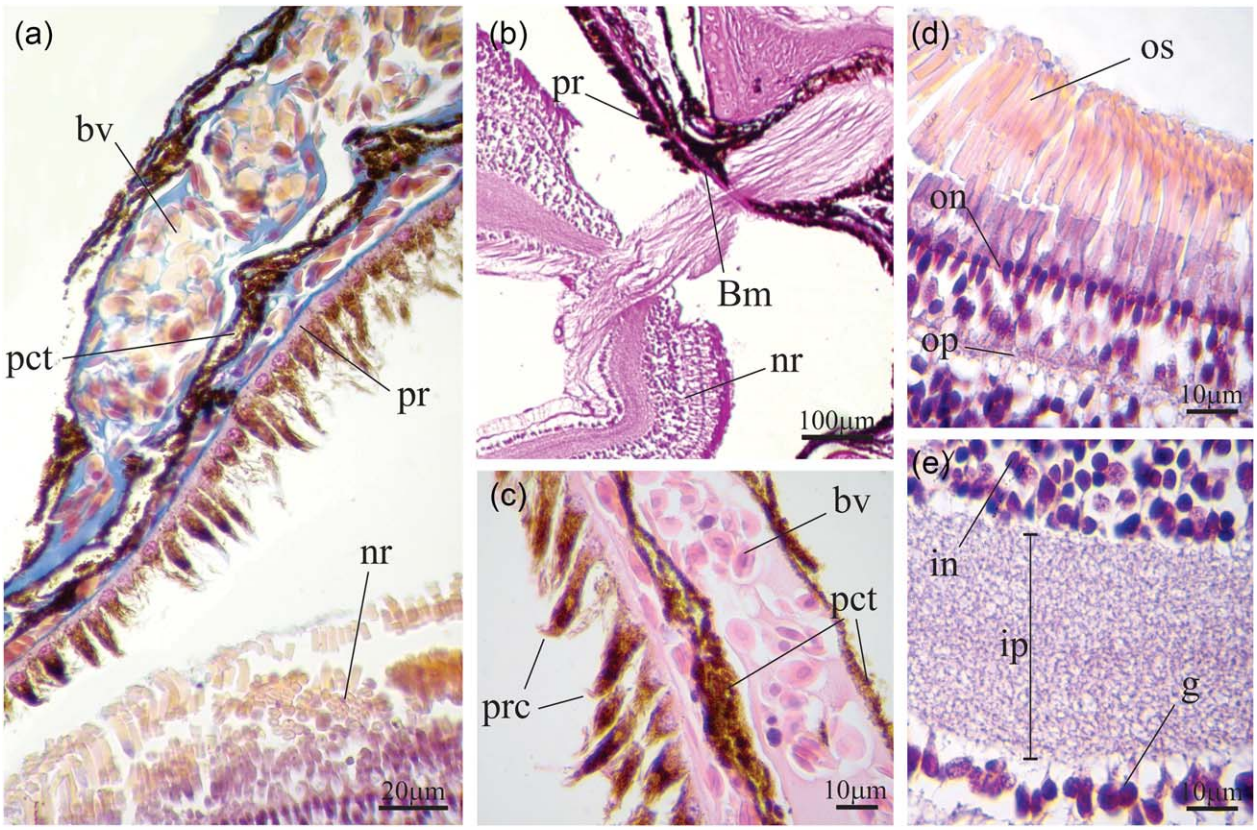

FIGURE 3 *Tunica vasculosa* and *tunica interna* of *Pleurodema bufoninum*. (a) Detail of choroid (MMT). (b) Note the PAS positive of Bruch's membrane. (PAS). (c) Detail of cytoplasmatic projections of pigmented retina cells (H&E). (d, e) Detail of neural retina layers (MMT). Bm: Bruch's membrane, bv: blood vessel, os: outer segments layer, g: ganglion cells layer, in: inner nuclear layer, ip: inner plexiform layer, nr: neural retina, on: outer nuclear layer, op: outer plexiform layer, pct: pigmented connective tissue, pr: pigmented retina, prc: pigmented retina cells

the adult organizational pattern in the margins, but the nuclear and plexiform layers are not differentiated in the middle. Although yolk remains in high quantities in this area (Figure 5f), developing optic nerve fibers can be seen (Figure 5e). The epidermis observed at the eyes position corresponds with the corneal epithelium, and the loose connective tissue observed surrounding the eye is part of the *tunica fibrosa* and corresponds with the corneal stroma.

At Stage G26, the neural retina organization is more defined; almost all neural retina layers are identified, but the outer segment layer is not yet established. Yolk deposits are significantly diminished but are still found in neural retina cells. The whole eyecup size and the lens size are augmented. Furthermore, the protein component in the interior of the lens is increased in comparison with Stage G22. The corneal epithelium has the same aspect as at previous stages (Figure 5d).

By Stage G28, the corneal epithelium and neural retina do not present significant changes. However, an enlargement of the pigmented retina that is in contact with the lens is observed. This enlargement will form the iris epithelium at the next stages. In addition, the lens has acquired a center of acidophilic protein accumulation in which there are no cells proliferating (Figure 5g).

From Stages G30–31, the neural retina is completely differentiated, and it is possible to identify a vitreous body between the retina and the lens (Figure 6a). The aspect of the lens is very similar to that of the adult one except for the size and spherical shape (Figure 6a). At this point, an incipient iris is evidenced (epithelium and stroma; Figure 6a); also, in *P. bufoninum*, an incipient dorsal and ventral umbraculum is observed that is identical in tissue composition to the adult one (Figure 6c). *P. somuncurense*, the umbraculum is well developed at this stage (Figure 6d). A comparison of the two species shows that from this stage until Stage G40, there are no evident changes in the histology and development of structures, but there is a change in the overall size of the eye.

At Stage G40, the aqueous body is evidenced. In addition, the corneal epithelium is stratified and starts to differentiate from the rest of the epidermis, which begins to develop dermal glands (Figure 6e,f). The substantia propria of the cornea is a thin, loose connective tissue in comparison with the thickness of the corneal epithelium. Additionally, the iris stroma and umbracula are more evident (Figure 6g). Nevertheless, it is not until the metamorphic climax that the eyelids develop in *P. bufoninum*. However, at Stage G41, eyelids and incipient nictitating membrane are developing in *P. somuncurense* (Figure 7a) but not in *P. bufoninum* (Figure 7b). At the end of this stage, the scleral cartilage of the *tunica fibrosa* begins developing in both species (Figure 7c). During the metamorphic climax, for both *Pleurodema* species, the eyelids

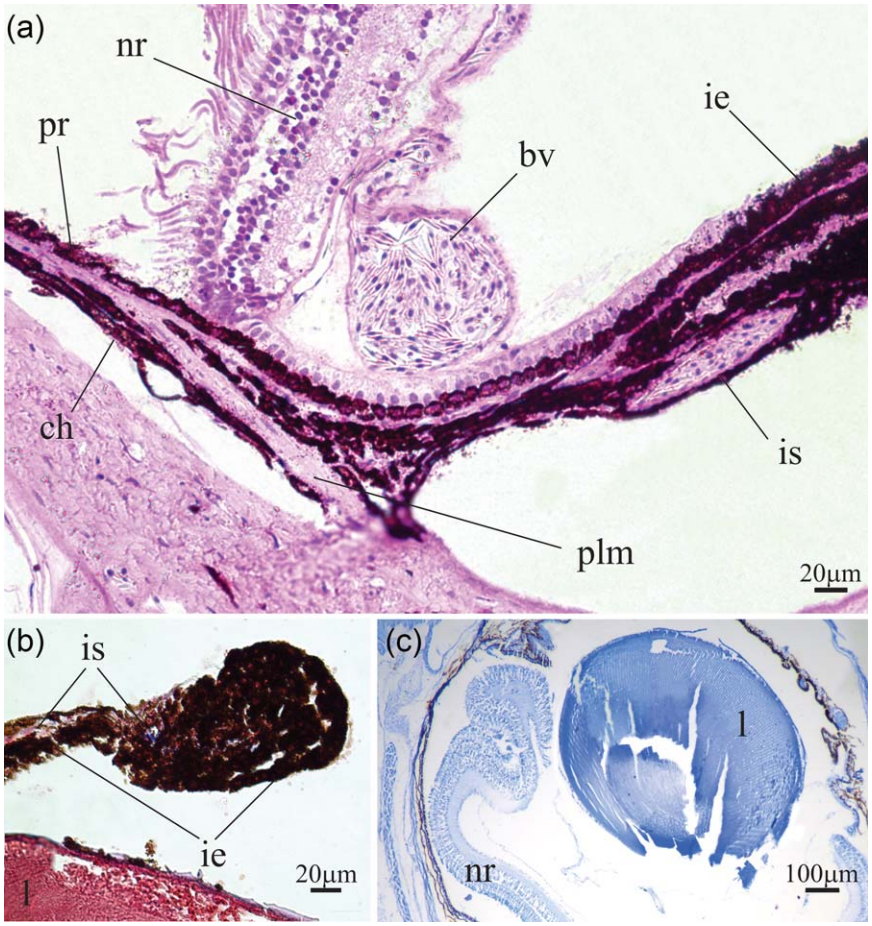

FIGURE 4 Iris and lens sagital sections of *Pleurodema* species. (a) Detail of iris epithelium and stroma of *P. somuncurense* (resin processed material, H&E). (b) Detail of umbraculum of *P. bufoninum* (MMT). (c) Eye of adult of *P. bufoninum* showing lens Coom positive reaction. bv: blood vessel, ch: choroid, ie: iris epithelium, is: iris stroma, l: lens, nr: neural retina, pr: pigmented retina, plm: protractor lentis muscle

develop completely and the corneal connective tissue becomes denser than at Stage G40 (Figure 7d). As mentioned before, the scleral cartilage formation occurs, and finally, the lens adopts a flattened shape (Figure 7d). In the juvenile individual, the Harderian's gland is visible, a structure that is not present in *P. bufoninum* or *P. somuncurense* until the end of the metamorphic climax (Figure 7e–g). From now until adult life, there is growth in the overall eyecup and lens size. The ontogenetic changes and timing of the appearance of the different structures of the eyes are summarized in Figure 8.

4 | DISCUSSION

The histological analysis of the eye of *Pleurodema bufoninum* and *Pleurodema somuncurense* at different stages of development allows us to understand how the adult eye structures evolve throughout metamorphosis and their characteristics. Here, we discuss the differences and similarities between the two species studied and the timing of the events in each of them.

P. bufoninum and *P. somuncurense* possess prominent eyes and horizontal pupils, as do other *Pleurodema* species (Cei, 1980). The umbraculum is difficult to observe in living animals due to its dark color, which is similar to the pupil. However, it is more evident after histology processing. No elygium from the skin, as was reported for *Amietia vertebralis* (Kruger et al., 2013), is seen either in living specimens or after processing.

The histologic study of the adults showed that both species possess "terrestrial" eyes, since the lens has a flattened shape and a curved, thick cornea with refractive power. The thickness of the cornea is homogeneous, and there seem to be no extra muscles or myoepithelial cells present in the iris that would be responsible for extreme accommodation, such as in diver birds (Howland & Sivak, 1984; Sivak, 1980). Thus, considering Neagu and Petraru (2015), *P. bufoninum* and *P. somuncurense* have no "intermediate" eye adapted to aquatic and terrestrial vision.

As mentioned before, the adult eye histology of both *Pleurodema* species is very similar. However, between the two species, some differences can be noted in the timing and size of structures during the eye development. The timing of emergence of structures such as the umbraculum is different: in *P. bufoninum*, umbracula start to develop at Stage G31, while in *P. somuncurense*, both umbracula are well developed at Stage G30. In addition, at the end of Stage G41, eyelids and

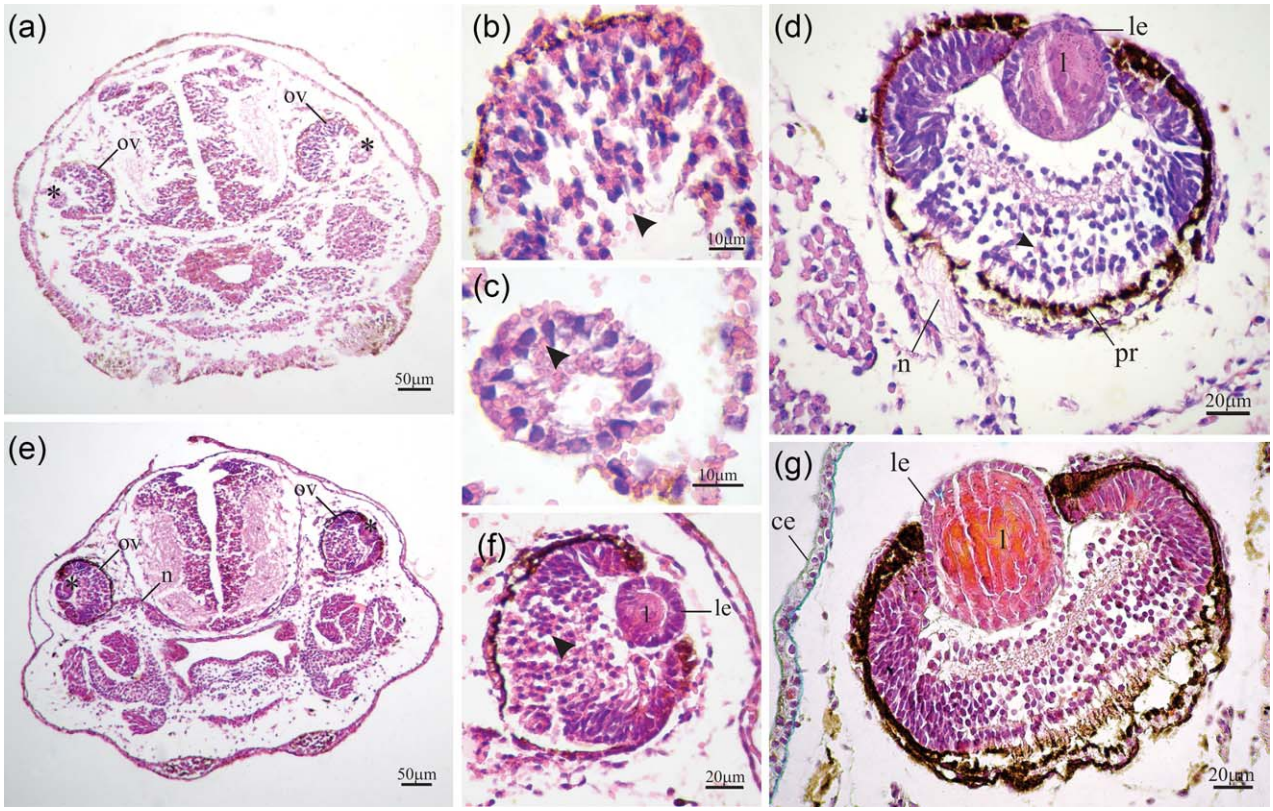

FIGURE 5 (a) Transversal sections of *P. bufoninum* tadpoles (G20; H&E). (b, c) Detail of the optic vesicle and lens vesicle at G20. (d) Detail of the eye at G22 (H&E). (e) Transversal sections of *P. bufoninum* (G22; H&E). (f, g) Detail of the eye at G26 (H&E) and G28 (MMT), respectively. Asterisks: lens vesicles, arrowheads: yolk drops, ce: corneal epithelium, l: lens, le: lens epithelium, n: optic nerve, ov: optic vesicle, pr: pigmented retina

incipient nictitating membrane are developing in *P. somuncurense* but not in *P. bufoninum*. From the time that it arises, the umbraculum seems to be larger and more prominent in *P. somuncurense* than in *P. bufoninum*, and this character remains in the adult eyes. Nevertheless, the timing and size are small and punctual differences that are probably due to the different environment of precedence; to asseverate that, it is necessary to take demographic measures. In general, *P. somuncurense* and *P. bufoninun* have similar eye ontogeny and adult histology.

Unlike Johnson's description of the mammalian umbraculum (Johnson, 1901), Miranda-Ribeiro (1920, 1926) proposed the term "meniscus" for the amphibian structure. In both cases, the authors referred to the macroscopic structure, but neither made detailed histological observations. Our results suggest that the meniscus described by Miranda-Ribeiro (1920, 1926) and also observed by Cei (1969, 1970) in *P. somuncurense* is the same structure termed "umbraculum" in other amphibians. We suggest that these terms refer to a projection of the stroma of the middle iris screening the pupil, which has high variability between phylogenetic groups and within species. We refer to this structure as the "umbraculum" in accordance with the first reference to it reported by Johnson (1901). Our results suggest that in *P. bufoninum* and *P. somuncurense*, dorsal and ventral pupil projections are not different structures, as in *A. vertebralis* (Ewer, 1952). In that species, Ewer (1952) found that the dorsal projection (umbraculum) is significantly larger than the ventral projection (pupillary nodule) which, in addition, possesses higher quantities of collagen fibers. In *P. bufoninum* and *P. somuncurense*, pupil projections are very similar in size, and their collagen composition is also comparable. Another difference is that while in *A. vertebralis*, the umbraculum is composed of multiple vesicles, in *P. bufoninum* and *P. somuncurense*, the dorsal and ventral umbracula do not have a multivesicular arrangement. The umbraculum that is present in the adult eye develops early in the larval life, at Stage G31 in *P. bufoninum*. One interesting comparison is that many authors have noted the contractile capacity of the umbraculum/meniscus (Lutz, 1943; Mann, 1931; Miranda-Ribeiro, 1920, 1926). Our observations indicate that the umbraculum may have neither muscle cells nor myoepithelial cells. Instead, it can be moved as a consequence of pupil contraction through the myoepithelial cells present in the iris. It may also be possible that the umbraculum muscles are hidden by the high pigment content.

Unlike Van Dijk's observations (Van Dijk, 1966), neither *P. bufoninum* nor *P. somuncurense* seems to have ocular elygium. Externally, there is no such skin pigmentation as epidermal elygium visible, and internally, in the iris, it is not possible to identify a punctual pigmentation apart from the normal melanophores that conform the pigmented retina layer and iris stroma. Regarding the ontogeny, in the studied species, there is no larval elygium, as occurs in *A. vertebralis* (Kruger et al., 2013). Instead, the umbraculum appears at early larval Stages (G30–31), grows until the end of the metamorphosis and remains in adult

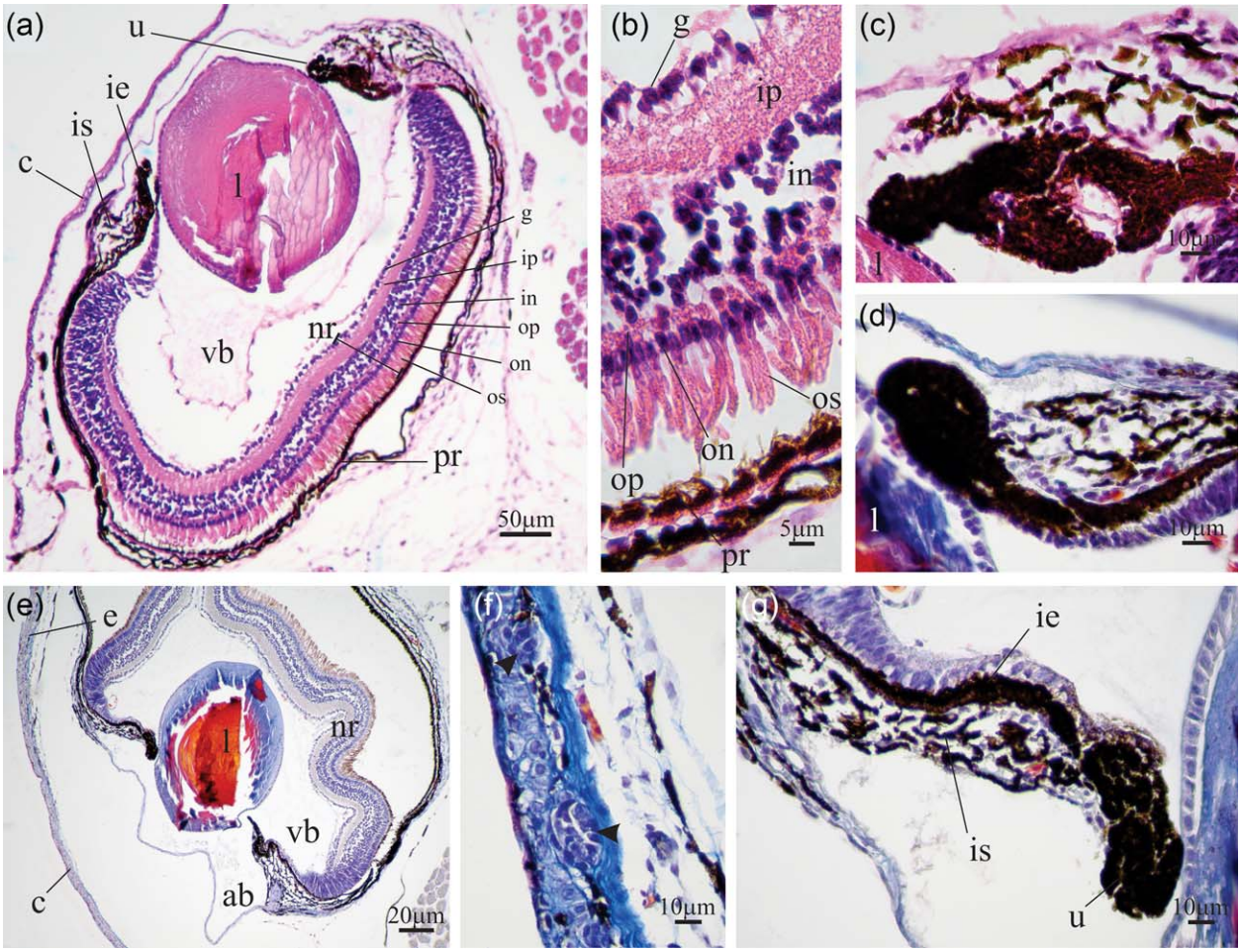

FIGURE 6 (a–d) Detail of the eye of tadpoles of *P. bufoninum* (a–c; G30-31; H&E) and *Pleurodema somuncurense* (d; G30-31; MMT). (a) Topographic aspect of the whole eye. (b) Detail of retina. (c, d) Detail of umbraculum. (e–g) Transversal sections of *P. bufoninum* tadpole (G40; MMT). (e) Topographic aspect of the whole eye, (f) Detail of epidermis with developing dermal glands. (g) Detail of umbraculum. Arrowheads: developing dermal glands, ab: aqueous body, c: cornea, e: epidermis, g: ganglionar layer, ie: iris epithelium, in: inner nuclear layer, ip: inner plexiform layer, is: iris stroma, l: lens, nr: neural retina, on: outer nuclear layer, op: outer plexiform layer, os: outer segments, pr: pigmented retina, u: umbraculum, vb: vitreous body

life, emphasizing the idea that the elygium and the umbraculum are structures that possess great diversity in different groups. Since the larval outer cornea is actually the epidermis, it is difficult to affirm that melanophores found in that area do not belong to normal skin pigmentation. However, between Stages G36 and G38, there is clearly no elygium layer in *P. bufoninum* or in *P. somuncurense*.

The eye development of *P. bufoninum* is an early event in larval life and occurs mainly at early larval stages (G20 onwards). The retina differentiation also occurs at early stages (before G30), which is similar to what occurs in *Xenopus laevis* but different from what occurs in *Rana temporaria* (Saxen, 1954). As shown in Figure 8, before the metamorphic climax, the eye of *P. bufoninum* and *P. somuncurense* is composed of a flat, thin cornea; a spherical lens with protein accumulation; and a differentiated iris and retina. These morphological features were reported in eyes of aquatic vertebrates (Neagu & Petraru, 2015; Sivak & Warburg, 1983) and, according to Sivak and Warburg (1983), can be related to underwater vision. During the climax and early juvenile life, when the transition from water to land occurs, certain structures are modified, such as the flattening of the lens and the thickening of the cornea. Additionally, terrestrial vision specializations develop as dermal glands, the Harderian's gland and scleral cartilage (Figure 8).

However, as in fact occurs in *R. temporaria*, yolk deposits remain in cells while they are differentiating into the neural retina (Glücksmann, 1940). Although Glücksmann's (1940) observations do not reference the eyes changes with body development, some aspects can be compared. "Stage 7" implies crystalline vesicle detached from the epidermis, as occurs at Gosner's Stage 20 in *P. bufoninum*. In *R. temporaria*, the eye development finishes when the iris completes its formation ("Stage 12"), and until the end of the metamorphosis, changes in size occur only because of cell multiplication. However, after the iris formation of *P. bufoninum*, the dorsal and ventral umbraculum arises, and it is not until the metamorphic climax that the eyelids and the adult cornea develop (Figure 8).

Perusal of the literature indicates that anuran tadpoles have an inner and outer cornea that fuse during metamorphosis (Kruger et al., 2013; Reyer, 1977). Our observations indicate that at early stages, the

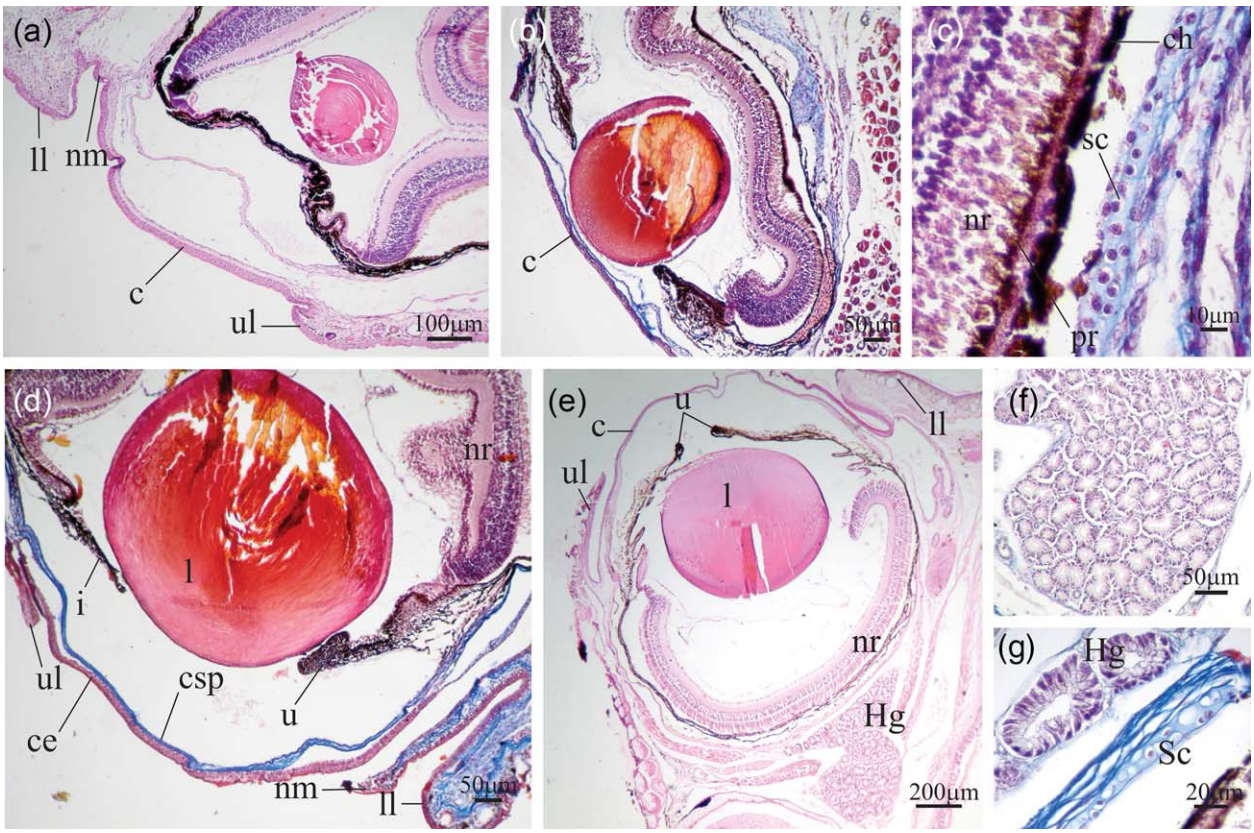

FIGURE 7 (a-g) Detail of the eye of tadpoles and juvenile individuals of *Pleurodema* species. (a) Tadpole of *P. somuncurense* showing eyelids development (G41; H&E). (b) Tadpole of *P. bufoninum* showing no eyelids development (G41; MMT). (c) Detail of scleral cartilage development in *P. bufoninum* tadpole (G41; MMT). (d) Juvenile individual of *P. bufoninum* (MMT). (e) Topographic aspect of the whole eye of juvenil (H&E). (f,g) Detail of Harderian's gland at different magnifications (MMT). c: cornea, ce: corneal epithelium, ch: choroid, csp: corneal substantia propia, Hg: Harderian's gland, i: iris, l: lens, ll: lower eyelid, nm: nictitating membrane, nr: neural retina, pr: pigmented retina, Sc: scleral cartilage, u: umbraculum, ul: upper eyelid

cornea appears to be constituted by two different structures: the epidermis, or corneal epithelium, and the loose connective tissue of the *tunica fibrosa*. With the advance of the metamorphosis, the loose connective tissue becomes denser and thicker, and by the end of the metamorphosis, the cornea is similar to the adult one. Because of the different origins and technical procedures, the two components of the

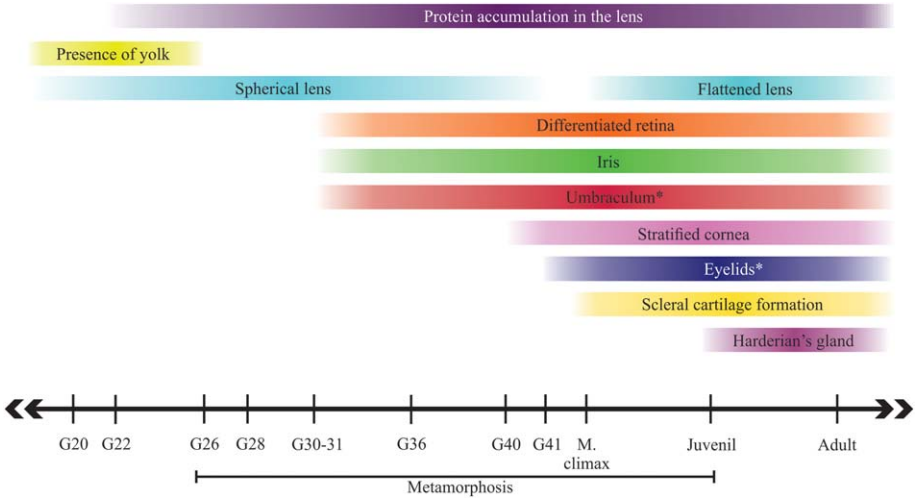

FIGURE 8 Summary of ontogenetic changes in eye development of *Pleurodema bufoninum* and *Pleurodema somuncurense*. Asterisks: since the timing differences found in both species could be probably due to the different environment of precedence as we mentioned above they were not taken into account in this summary

cornea appear to be separated at early stages. However, this is not what occurs in the individual, and it could be inaccurate to mention them as outer cornea and inner cornea. Instead, they are the epithelium and the underlying connective tissue of the cornea.

Our observations also indicate that the optic nerve appears in the eyes before crystalline maturation and retina differentiation; thus, it is possible that light information is being captured by the eyes and processed in the brain beginning with the hatching, prior to codifying images. Hamasaki and Eder (1977) and Stevens (1963) determined that early light capture occurs in the eyes and pineal gland; consequently, it could be interesting to approach the study of pineal light capture at early larval stages.

In summary, our results showed that those structures related to light capture (retina and lens) appear early in larval life, while the components of the terrestrial-life eye (scleral cartilage, specialized cornea, eyelids and nictitating membrane, and Harderian's gland) do not develop until the tadpole leaves the water at the metamorphic climax.

Considering the adult morphology, the eyes of *P. bufoninum* and *P. somuncurense* are very similar in structure and development. Furthermore, both species possess a unique prominent dorsal and ventral umbraculum that has not been reported until now. Future studies are needed to elucidate the biological function of these structures as well as their diversity within Anura, and it could be interesting to evaluate in detail the eye histology as well as the presence and type of umbracula in other anurans.

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APPENDIX

CNP.A, Herpetological Collection of Instituto de Diversidad y Evolución Austral (IDEAus-CONICET, Puerto Madryn, Chubut, Argentina); **MLP**, Herpetological Collection of Museo de La Plata (Buenos Aires, Argentina); RP, provincial route; RN, national route.

Pleurodema bufoninum

Total of 20 individuals were processed for histological analysis: **CNP.A 2975** ($n = 4$; larval series bred at laboratory), one Stage 20, one Stage 22, one Stage 26, and one Stage 28, from RP N° 8, 19km NE crossing RP N° 67, 25 de Mayo department, Chubut Province, Argentina. **CNP.A 3101** ($n = 2$), one Stage 30 and one Stage 31 from 40 Km SE of Gastre, Gastre department, Chubut Province, Argentina. **CNP.A 3110** ($n = 3$), one Stage 33, one Stage 34, and one Stage 35 from Guajalaina, Cushamen department, Río Negro Province, Argentina. **CNP.A 3081** ($n = 3$) two Stages 36 and one juvenile individual, from Caceres small farm, Gastre department, Chubut Province, Argentina. **CNP.A 3817** ($n = 2$), one Stage 40 and one Stage 42, from RP N°17, 34km E Corcovado, Futaleufú department, Chubut Province, Argentina. **CNP.A 3103** ($n = 2$), one Stage 41 and one Stage 44, from Gan Gan, Telsen department, Chubut Province, Argentina. **CNP.A 2553,** adult, from 18km S to Sarmiento, Sarmiento department, Chubut Province, Argentina. **CNP.A 3251**, adult, 21.4km NW Telsen, Telsen department, Chubut Province, Argentina. **MLP 4050** ($n = 1$), Stage 38 from crossing between RN N° 40 and Rayhuan creek, Ñorquinco department, Río Negro Province, Argentina. **MLP 4052** ($n = 1$), Stage 38 from crossing between ex-RN N° 40 and Las Bayas creek, Pilcaniyeu department, Río Negro Province, Argentina.

Pleurodema somuncurense

Total of 10 individuals were processed for histological analysis. **CNP.A 1980** ($n = 3$), one Stages 30–31, one Stage 36, and one Stage 37 from Chipauquil, Valcheta department, Río Negro Province, Argentina. **CNP.A 1981** ($n = 4$; larval series bred at laboratory), one Stage 41, one Stage 43, one Stage 44, and one juvenile, from Chipauquil, Valcheta department, Río Negro Province, Argentina. Three adults (**CNP.A 1986, CNP.A 2013, CNP.A 2042**) from Chipauquil, Valcheta department, Río Negro Province, Argentina.