Rafaela Christine Perez

Padrões contrastantes de expressão gênica ao longo do desenvolvimento do olho entre girinos fossoriais e bentônicos

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Contrasting patterns of gene expression along eye development between fossorial and benthic tadpoles

Versão corrigida
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Prof. Dr. Taran Grant
Orientador

"I'm not trying to prove anything, by the way. I'm a scientist and I know what constitutes proof. But the reason I call myself by my childhood name is to remind myself that a scientist must also be absolutely like a child. If he sees a thing, he must say that he sees it, whether it was what he thought he was going to see or not. See first, think later, then test. But always see first. Otherwise you will only see what you were expecting. Most scientists forget that (...). So, the other reason I call myself Wonko the Sane is so that people will think I am a fool. That allows me to say what I see when I see it. You can't possibly be a scientist if you mind people thinking that you're a fool."

Douglas Adams
The Hitchhiker's Guide to the Galaxy vol 4: So Long, and Thanks for All the Fish

"Because science carries us toward an understanding of how the world is, rather than how we would wish it to be, its findings may not in all cases be immediately comprehensible or satisfying. It may take a little work to restructure our mindsets. Some of science is very simple. When it gets complicated, that's usually because the world is complicated - or because *we're* complicated. When we shy away from it because it seems too difficult (or because we've been taught so poorly), we surrender the ability to take charge of our future. We are disenfranchised. Our self-confidence erodes."

Carl Sagan
The Demon-Haunted World: Science as a Candle in the Dark

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1. Introduction

1.1. Origin and evolution of the vertebrate eye

When developing his theory of evolution by natural selection, Darwin famously worried about the origin of the eye, writing *"seems, I freely confess, absurd in the highest possible degree"* (Darwin, 1871). In his 'On the Origin of Species', he proposed an eye prototype composed of one photoreceptor cell and one pigment cell, as the basis for all more complex eyes found in nature (Gehring, 2014). However, given the morphological diversity of structures that can sense light and what is meant by "sensing light", it is challenging to provide a unified definition of the eye. By understanding the different functional needs imposed on animals by their environment, we can attempt to trace back the evolution and to understand the function of structures implicated in light sensing.

Based on the premise that the origin of eyes is a consequence of the evolution of visually-guided behaviors, Nilsson (2009) defines four classes of light-controlled behaviors: 1. Behaviors driven by non-directional monitoring of ambient light (control of circadian rhythms, response to shadow), 2. Behaviors based on directional light sensitivity (phototaxis, habitat orientation), 3. Visual tasks based on low spatial resolution (anti-collision responses, habitat selection), and 4. Visual tasks based on high spatial resolution (visual communication, predator detection).

Class 1 tasks require only a photoreceptor cell, while class 2 tasks require the addition of a pigment cell that will partially shade the photoreceptor and thus allow directional perception. Class 3 tasks require several photoreceptors organized in a cup surrounded by pigment so different directions can be simultaneously monitored, forming what we now define as pit-and-cup eyes. Class 4 tasks are accomplished by eyes with focusing optics and high spatial resolution, properties acquired with the evolution of lenses and brains able to process the increased volume of information coming from the eyes (Land & Nilsson, 2012; Nilsson, 2013). Evidence gathered from the fossil record and living forms suggests that classes 1, 2, and 3 gradually appeared before the Cambrian period. However, class 4 eyes arose only during the Cambrian explosion (520 to 515 Mya) when most of the major groups of animals first emerged in the fossil record (Lamb *et al.*, 2007; Nilsson, 2013; Parker, 2011). Class 4 tasks are undoubtedly the most demanding and complex, and eyes capable of performing them arose independently in the arthropods, cephalopod mollusks, and in the vertebrates (Land & Nilsson, 2012).

The first vertebrate eye belonged to the last common ancestor of jawless (agnathans) and jawed vertebrates (gnathostomes), which lived 500 Mya. The living representatives of jawless vertebrates, the hagfish and the lamprey, possess very distinct eyes: in the hagfish, they are small and conical, buried under translucent skin, and lack a lens, a cornea, an iris, and muscles. Their retina is composed of only two classes of neurons organized into two nuclear layers. In contrast, lamprey eyes are remarkably similar to those of jawed vertebrates, exhibiting a lens, a cornea, an iris, six pairs of extra-ocular muscles, and a retina with five classes of neurons organized into three nuclear and two plexiform layers. Their extra-ocular muscles and retinal

neurons are homologous to those found in jawed vertebrates (Gustafsson *et al.*, 2010; Lamb, 2013; Lamb *et al.*, 2007; Land & Nilsson, 2012). The eyes of jawed vertebrates differ from those of lampreys in retina organization and by possession of intra-ocular muscles, absent in lampreys. Even though the same classes of neurons are present in the retinas of both jawless and jawed vertebrates, lampreys lack a nuclear layer comprising only the cell bodies of the ganglion cells, resulting in their mixing with other retinal cells (Gustafsson *et al.*, 2010; Holmberg, 1977).

As vertebrates first originated underwater, the vertebrate eye evolved as a structure adapted to the aquatic medium. The first vertebrate invasion of land required profound restructuring of the eye to deal with a drastically different medium and especially with the differences in refractive index between water and air. In the case of aquatic vertebrates, such as fish, the refractive index on both sides of the cornea is very similar, limiting the role of this structure to providing a transparent window that protects the eyeball: the optical work of bringing light to a focus is done by the almost perfectly spherical lens. In contrast, for terrestrial vertebrates, each side of the cornea presents a different refractive index. This refractive index difference causes rays of light to bend, potentially distorting the image. A curved cornea brings them back into focus, while the elliptical lens complements the ray-bending power of the cornea and becomes responsible for adjusting of the focal length (J. G. Sivak *et al.*, 1999; Walls, 1963). The secondary invasion of aquatic environments that occurred in multiple groups independently (e.g., seals, penguins, and turtles) required novel adaptations such as an almost flat cornea and a change from the terrestrial elliptical lens to a more spherical one, similar to a fish lens. Alternatively, the ciliary muscles of the iris are recruited to squeeze the lens through the pupil effectively increasing its curvature and improving focus (Cronin *et al.*, 2014; Glaeser & Paulus, 2015).

1.2. The vertebrate eye structure

Even though vertebrates needed novel adaptations to successfully conquer different environments, the common plan over which the vertebrate eye is built remained conserved throughout evolution (Lamb, 2013; Land & Nilsson, 2012). This common plan consists of two optical lenses, three epithelial layers, one light-sensitive tissue, three chambers of fluid, intra- and extra-ocular muscles, and an optic nerve. The first optical lens is the cornea, an external transparent surface covering the front of the eye (Figure 1.1). Under the cornea, lies a circular muscle, the iris, with an aperture in the middle, the pupil. The iris controls the size of the pupil, determining how much light enters the eye and reaches the lens (the second optical lens, also known as crystallin lens). The lens is suspended by a set of ligaments attached to the ciliary body. The movement of the ciliary muscles leads to contraction or relaxation of these ligaments, changing the shape of the lens, a process known as accommodation (Figure 1.1) (Kolb *et al.*, 2012; B. Sivak & Sivak, 2000).

At the back of the eye is the retina, the light-sensitive tissue of the eye, composed of photoreceptors and neurons organized into three nuclear and two plexiform layers (Figure 1.1). The retina lays over the retinal pigment epithelium, a single-cell pigmented epithelial layer,

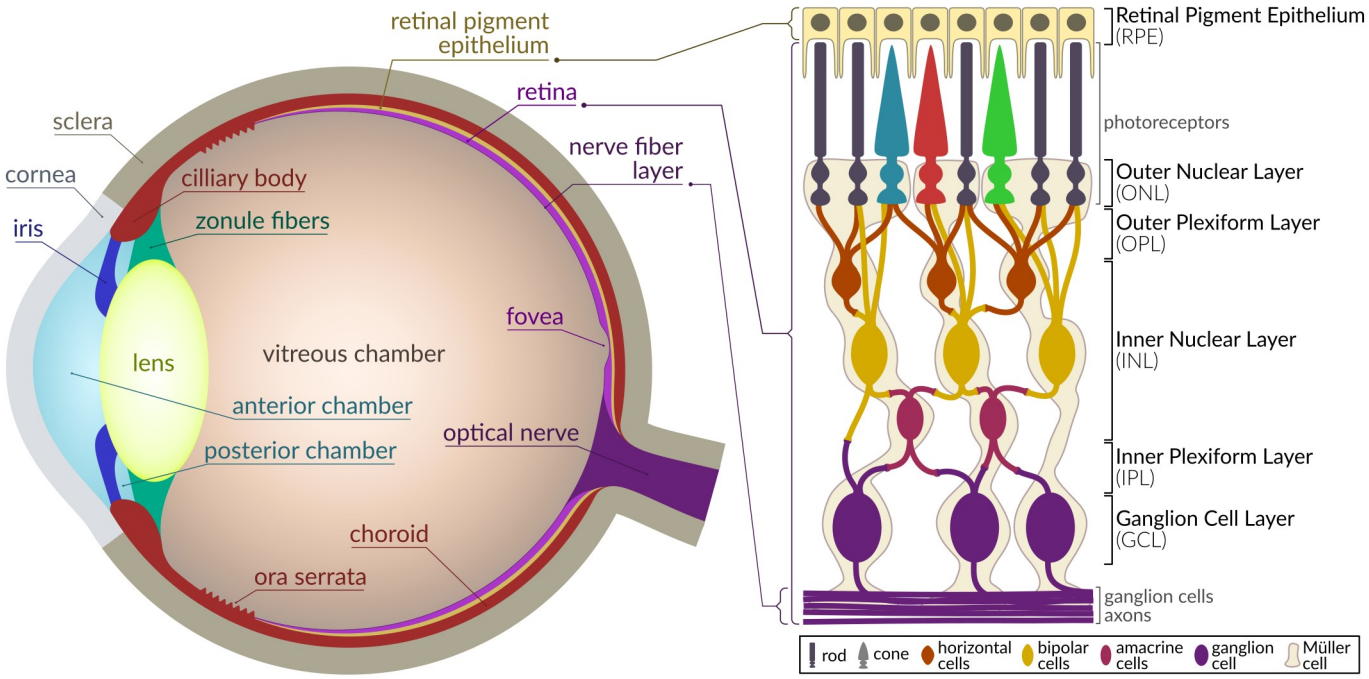

Figure 1.1: Vertebrate eye and retina structure. *Illustration by C. E. Amancio.*

which absorbs the scattered light, maintains ion homeostasis of the subretinal space, and recycles the photoreceptor cells and their chromophores. Behind the retinal pigment epithelium is the choroid, an epithelium responsible for retina blood supply. Finally, the sclera, an epithelium in continuity with the cornea, forms the supporting wall of the eyeball and holds the extra-ocular muscles responsible for rotating the eye inside its orbit. Also, at the back of the eye and continuous with the retina, is the optic nerve, formed by the axons of retinal ganglion cells and responsible for bringing the information captured by the eye to the brain.

The three eye chambers are filled with fluids and are responsible for maintaining the shape and pressure inside the eye. The vitreous chamber is placed between the lens and the retina and is filled with vitreous humor. Between the cornea and the iris is the anterior chamber, while the posterior chamber is positioned between the iris and the lens. (Figure 1.1). Both anterior and posterior chambers are filled with aqueous humor that, besides maintaining the intraocular pressure, provides nutrition to the lens and contributes to the maintenance of the correct curvature of the cornea and of the constant distance between the cornea and the lens (Kolb *et al.*, 2012; B. Sivak & Sivak, 2000).

Undoubtedly, the most complex component of the eye is the retina. Its intricate structure guarantees the ability to capture photons in different conditions and pre-process this information before sending it to the brain. All vertebrate retinas are composed of three layers of nerve cell bodies (nuclear layers) intercalated with two layers of synapses (plexiform layers) (Figure 1.1). The posterior-most layer—closest to the retinal pigment epithelium—contains the cell bodies of photoreceptors (cones and rods) and is called the outer nuclear layer (ONL). The next nuclear layer contains the cell bodies of bipolar, horizontal, amacrine, and Müller glial cells and is called the inner nuclear layer (INL). The anterior-most nuclear layer (ganglion cell layer, GCL), closest to the lens, contains the cell bodies of displaced amacrine cells and ganglion cells. Between the ONL and INL, the outer plexiform layer (OPL) contains the connections between photoreceptors, bipolar, and horizontal cells. Between the INL and GCL is

the inner plexiform layer (IPL), containing the connections between bipolar, amacrine, and ganglion cells.

Each type of cell that comprises the retina plays a specialized role. Rods and cones detect photons and conduct this information via chemical signaling. Bipolar, horizontal, and amacrine cells process and transmit information from photoreceptors to the ganglion cells. The axons of ganglion cells form the optic nerve, which carries visual information from the retina to the brain. The Müller glial cells support homeostasis and respond to retinal injury or disease by reentering the cell cycle. All the types of cells that comprise the retina have specific morphological and functional subtypes (except for Müller glial cells) (Kolb *et al.*, 2012; B. Sivak & Sivak, 2000).

1.3. Development of the vertebrate eye: morphogenesis and its underlying molecular mechanisms

1.3.1. Eye morphogenesis

Eye morphogenesis starts very early in the vertebrate embryo. During gastrulation, the ectoderm on the dorsal surface of the embryo thickens and forms the neural plate (Figure 1.2A). The neural plate begins to fold its lateral edges that upon fusion, give origin to the neural tube (neural ectoderm), a hollow structure that sits above the notochord and under the surface ectoderm. Cells at the anterior end of the neural tube give rise to the neural crest cells and form the earliest components of the brain: the forebrain, the midbrain, and the hindbrain.

A unified and centralized eye field is established in the forebrain at the late gastrula stage (Figure 1.2B–C) (Graw, 2010; B. Sivak & Sivak, 2000). This eye field begins to separate in two when some of its cells begin to invaginate, forming two optic grooves (sulci; Figure 1.2D–E). At the start of neurulation, the cranial neural folds bend inwards approaching each other and completing eye field separation (Figure 1.2F). The optic grooves continue to invaginate, growing outwards and towards the surface ectoderm. This results in the formation of the optic stalks (future optic nerves) and the optic vesicles, which will eventually reach the surface ectoderm (Figure 1.2G).

The contact between the optic vesicles and the surface ectoderm (Figure 1.2H) leads to the distal part of the optic vesicle folding inwards and forming the optic cup. It also determines the induction of the lens placode, a thickening of the surface ectoderm that invaginates into the optic cup, and its detachment from the surface to form a hollow spherical vesicle, the lens vesicle (Figure 1.2I–J) (Gilbert, 2010; Grainger, 1992; Graw, 2010; B. Sivak & Sivak, 2000).

The lumen of the lens vesicle is gradually filled by elongating cells (Figure 1.2K), the primary lens fibers, that form the embryonic nucleus of the lens. The primary fibers start to synthesize crystallins and the lens detaches from the anterior portion of the eye, generating a cavity, the anterior chamber. A single layer of cells, known as the lens epithelium, persists on the anterior portion of the lens vesicle. This epithelium is responsible for generating the new lens cells (which will also elongate and synthesize crystallins) throughout the life of the animal,

as fibers are continuously being laid down. As soon as the lens vesicle detaches from the surface ectoderm, the corneal epithelium originates from the surface ectoderm. The corneal

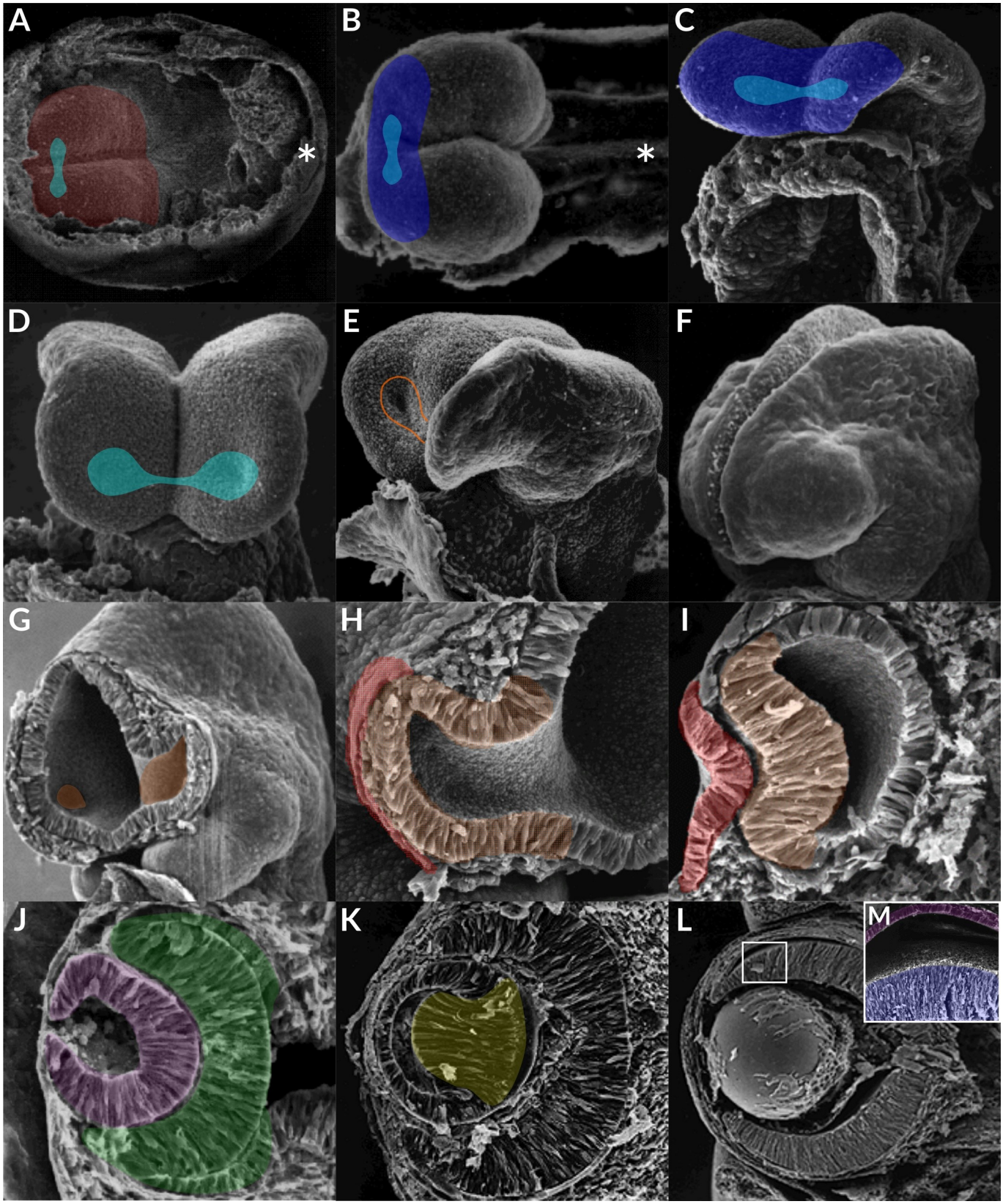

Figure 1.2: Eye morphogenesis in mouse. Neural plate (dark red) (**A**) and forebrain (blue) (**B**) formed. Forebrain growth carries this region forward, along with the eye field (cyan) (**C**). Formation of the optic grooves (red contour in **E**) occurs simultaneously with cranial folds bending inwards (**D–E**), completing eye field separation (**F**). Optic grooves give rise to optic vesicles (brown) (**G**). Contact between optic vesicle (brown) and surface ectoderm (red) (**H**) leads to invagination of optic vesicle (**I**), forming the optic cup (green) (**J**), and induction of the lens placode (red) (**I**), forming the lens vesicle (pink) (**J**). The lens vesicle is filled by lens fibers (yellow) (**K**). Outer and inner layers of the optic cup form the retinal pigment epithelium (light purple) and retina (dark purple), respectively (**L–M**). G–K, frontal cut; M, transverse cut; * marks posterior region of the embryo. *Modified from Sulik & Bream Jr. (n.d.).*

epithelium secrets a collagen-rich extracellular matrix that attracts mesenchymal cells from the neural crest into the space between the lens and the surface ectoderm. These cells condense to form several layers, dehydrate and originate the transparent stroma and endothelium of the cornea. Mesenchymal cells from the neural crest also originate the sclera, the choroid and the extra-ocular muscles of the eyes (Gilbert, 2010; B. Sivak & Sivak, 2000).

Concomitant with the process of lens and cornea formation, the optic cup gives raise to the iris, the ciliary body, the retinal pigment epithelium and the retina. The iris and the ciliary body are formed at the anterior edges of the outer and inner layers of the optic cup (Gilbert, 2010; B. Sivak & Sivak, 2000). The retinal pigment epithelium develops from the outer layer of the optic cup, while the retina develops from the inner layer (Figure 1.2L–M). The six retinal neuron classes (rods, cones, bipolar, horizontal, amacrine and ganglion cells) and the retinal glial cells (Müller) are derived from multipotent retinal progenitor cells, present in the optic cup. In vertebrates, the generation of retinal cell types follows an evolutionary conserved temporal order: ganglion cells are generated first, followed by cones and horizontal cells, amacrine cells, rods, bipolar cells, and Müller glia (Andreazzoli, 2009; Graw, 2010).

1.3.2. Molecular mechanisms underlying eye field specification

Eye organogenesis is a conserved process, tightly orchestrated to allow simultaneous development of different tissues and their cell types. This is possible due to finely tuned and coordinated expression of several genes, starting at the level of the neural tube. The anterior portion of the neural plate is specified by the expression of *Otx2* gene. At the end of gastrulation and beginning of neurulation, as the neural tube starts to form, *noggin* inhibits the *Tbx3* (ET) transcription factor at the anterior region of the neural tube, where Otx2 protein eventually accumulates, blocking noggin and resulting in the production of Tbx3 protein. In *Xenopus*, Tbx3 activates the transcription factor Rx1 (homologous to the murine *Rax* gene), which inhibits *Otx2* expression in the region where the eye field will be formed, and activates a cross-regulatory network, starting with *Pax6* expression, *Six3*, *Lhx2*, and further, *Xnr2e1* (also known as *tll*, and homologous to the murine *nr2e1* gene) and *Six6*. These genes—*Tbx3*, *Rx1*, *Pax6*, *Six3*, *Lhx2*, *Xnr2e1* and *Six6*—are known as eye-field transcription factors and are responsible for establishing of the eye field. They have overlapping but not identical expression domains that are maintained throughout eye field separation (Gilbert, 2010; Zuber *et al.*, 2003). The eye field separation is accomplished through the expression of *Shh* from the prechordal mesoderm. *Shh* suppresses *Pax6* in the center of the eye field, dividing the field in two (H. Li *et al.*, 1997; Macdonald *et al.*, 1995). Eye field specification and separation is a fairly well conserved process throughout vertebrates. The eye-field transcription factors described here were uncovered in *Xenopus* after pioneering studies in *Drosophila melanogaster* (Zuber *et al.*, 2003), but the same transcription factors appear to be involved in eye field specification across vertebrates (Graw, 2010; Kamijyo *et al.*, 2015; Zuber *et al.*, 2003; reviewed in Chow & Lang, 2001).

1.3.3. Molecular mechanisms underlying optic stalk, optic vesicle, and optic cup formation

The expression of the eye-field transcription factors persists throughout eye development and becomes increasingly regionalized as development progresses and new structures arise. While the eye field is separating, the optic grooves start to form, further originating the optic stalks and the optic vesicles. The optic vesicles then invaginate, originating the optic cups. Work on *Xenopus* has shown that expression of *Rx1* in the two eye fields leads to the formation of the optic grooves and their maintenance until the optic vesicles are completely formed (Mathers *et al.*, 1997). *Six3*, responsible for correct lens development in later stages of optic vesicle, is also necessary for optic groove formation, both in medaka fish and in mouse (Carl *et al.*, 2002; Yun *et al.*, 2009). Studies in these two model systems have revealed an interplay between *Pax2* and *Pax6* when establishing optic vesicle patterning. *Pax2* expression, promoted by *Shh* from the ventral midline and BMP7 from the ventral optic vesicle, determines the region where the optic stalk will form. At this stage, *Pax2* expression is restricted to the ventral region of the optic vesicle, including the ventral retina. At the same time, *Pax6* is expressed in the surface ectoderm and the optic vesicle in a dorsoventral gradient, with highest expression dorsally. A boundary is formed between the dorsal and ventral regions of the optic vesicle, where *Pax6* and *Pax2* expression mutually inhibit each other, ensuring the delimitation between the optic stalk and the retina (Heavner & Pevny, 2012; Macdonald *et al.*, 1995; Yun *et al.*, 2009).

Studies in mouse have shown that *Lhx2* is expressed in the whole optic vesicle, later becoming restricted to the retina. Failure to express *Lhx2* results in the arrest of eye development at the stage prior to optic cup formation (Porter *et al.*, 1997). Yun *et al.* (2009) proposed that *Lhx2* is responsible for coordinating the multiple pathways functioning simultaneously during the transition from optic vesicle to optic cup. Among its many functions, *Lhx2* regulates expression of *BMP4* in the dorsal area and *BMP7* in the ventral area of the optic vesicle, and those, in turn, regulate *Sox2*, an important transcription factor for lens induction expressed in the surface ectoderm. *Lhx2* also regulates the expression of Vsx2 protein which, together with FGF9 from the central optic vesicle and FGF1/2 from the surface ectoderm, marks the presumptive retina. On the dorsal side of the optic vesicle Wnt signaling induces the transcription factor *Mitf*, a retinal pigment epithelium marker that has its ventral boundary determined by the expression of Vsx2 (Heavner & Pevny, 2012; Yun *et al.*, 2009). Optic vesicle invagination is then ensured by *nr2e1* expression in the ventral region of the optic vesicle and retinoic acid signaling throughout the whole optic vesicle. Retinoic acid is produced by expression of a retinaldehyde dehydrogenase (*Aldh1a2*). Absence of *Xnr2e1/nr2e1* or *Aldh1a2* expression leads to failure in optic vesicle invagination, which results in no optic cup formation in both *Xenopus* and mouse (Hollemann *et al.*, 1998; Mic *et al.*, 2004).

The specific genes (such as BMPs, FGFs and HHs) and the many different pathways simultaneously activated during early eye organogenesis interact in complex ways and only a broad view of the main molecular mechanisms underlying this process was presented here. *Rx1*, *Xnr2e1, Pax6*, *Six3* and *Lhx2* appear to play central roles in vertebrate optic vesicle formation and maintenance, as regulators of other transcription factors that will contribute to establishing boundaries and axis patterning in the optic vesicle (Heavner & Pevny, 2012; Yun *et*

al., 2009). The expression of these eye-field transcription factors continues through optic vesicle invagination and establishment of the optic cup, with some variation in expression patterns in different regions and development stages. Regional differences characterize, for example, the expression of *Sox2* that starts to have a high expression in the central optic cup in opposition to *Pax6*, whose expression is greatly diminished in the central optic cup and increased in the peripheral areas, where the ciliary body will be originated (Gregory-Evans *et al.*, 2013; Heavner & Pevny, 2012).

1.3.4. Molecular mechanisms underlying lens and cornea formation

As the optic vesicle invaginates to give raise to the optic cup, the surface ectoderm thickens, forming the lens placode. The lens placode then invaginates towards the future optic cup, forming the lens vesicle that will mature and detach from the surface ectoderm, giving rise to the lens. The first step towards lens formation is then the specification and formation of the lens placode. In mouse, signals from the periocular mesenchyme that surrounds the optic vesicle inhibit the lens fate by expression of TGFβ ligands that induce Wnt/β-catenin activity and inhibit *Pax6* expression in the non-lens ectoderm. *Pax6* expression is then restricted to the cells that will start to thicken and become the lens placode, where it inhibits Wnt/β-catenin signaling known for its role inhibiting lens formation. Retinoic acid, previously expressed throughout the optic vesicle, is now encountered in the prospective lens placode and it is required for invagination of the lens placode and of the optic vesicle (Chow & Lang, 2001; Cvekl & Ashery-Padan, 2014). BMP4 and BMP7 expressed in the murine optic vesicle under the control of the *Lhx2* gene migrate to the future lens placode region, where BMP4 will activate *Sox2* (Furuta & Hogan, 1998). It was thought that BMP7 was responsible for the maintenance of *Pax6* expression levels (Wawersik *et al.*, 1999), but the current model proposes that *Six3* regulates the beginning of *Pax6* expression, which subsequently will regulate *Six3* expression. At this stage, BMP4 activates *Sox2* which is also regulated by *Six3*. *Sox2*, *Six3* and *Pax6* can self-regulate their own expression and the combination of their activity is what determines lens placode formation (*Pax6/Six3*) and its subsequent invagination (*Pax6*/*Six3*/*Sox2*; (Chow & Lang, 2001; Cvekl & Ashery-Padan, 2014).

Lens placode invagination forms the lens vesicle inside which anterior cells differentiate into a layer of epithelial cells that are the source of future lens fibers, while posterior cells will elongate and synthesize crystallins, originating the primary lens fibers. In mouse, induction of lens fiber cells differentiation is regulated by different FGF concentrations, eliciting proliferation, migration and differentiation. A lower concentration, associated with proliferation, occurs in the anterior cells, while a higher concentration, associated with differentiation, occurs in the posterior cells. Higher concentrations also correlate with the induction of cellular elongation and the accumulation of crystallins (Lovicu & McAvoy, 2005; McAvoy & Chamberlain, 1989). In addition to FGF, BMP also appears to be involved in lens fiber differentiation, as BMP inhibition through *noggin* leads to failure in fiber differentiation (Faber *et al.*, 2002). Finally, crystallin expression is controlled by *Maf*, a transcription factor expressed in lens fibers under *Pax6* regulation (Cvekl & Ashery-Padan, 2014).

Once the lens vesicle detaches from the surface ectoderm to give rise to the lens, the surface ectoderm forms the corneal epithelium, characterized by expression of *Pax6* and cornea-specific keratins K3/K12, with K12 expression dependent on *Pax6* (Grindley *et al.*, 1995; Liu *et al.*, 1999). The periocular mesenchyme located between the corneal epithelium and the lens originates from neural crest cells that migrate from the neural tube. In chicken, these cells express BMP inhibitors such as gremlin and noggin (Tzahor *et al.*, 2003) around the corneal epithelium, helping its specification. Following corneal epithelium formation, a first migration of neural crest cells to the space between the lens vesicle and the corneal epithelium originates the corneal endothelium. A second wave of neural crest cells migrates into this collagen-rich extracellular matrix lying between the epithelium and the endothelium, where cells will differentiate into fibroblasts or keratocytes, leading to the formation of the corneal stroma (Collomb *et al.*, 2013; Dhouailly *et al.*, 2014; Kao *et al.*, 2008). The endothelium and the corneal stroma are similarly regulated during development. Both express the transcription factors *Pitx2* and *Foxc1*, which appear to be essential for development, as failure in their expression causes a range of defective phenotypes in mouse (Gage *et al.*, 2005; Kidson *et al.*, 1999; Semina *et al.*, 1996). As pointed out by Kao *et al.* (2008) and Miesfeld & Brown (2019), relatively little is known regarding the molecular mechanisms and the genes involved in cornea morphogenesis. In addition, the signaling molecules upstream or downstream of *Pitx2* and *Foxc1* are yet to be identified.

1.3.5. Molecular mechanisms underlying retinal pigment epithelium and retina formation

During lens and cornea formation, the outer layer of the optic cup gives rise to the retinal pigment epithelium and the inner layer of the optic cup gives raise to the retina. As previously discussed, in mouse and chick the presumptive retinal pigment epithelium is marked by the expression of *Mitf*, a transcription factor induced by Wnt signaling (Heavner & Pevny, 2012; Yun *et al.*, 2009). *Mitf* is known for its conserved role in the development of melanin-producing cells and some of its isoforms, namely, Mitf-A, Mitf-D and Mitf-H, are known to be expressed in the retinal pigment epithelium. In mice and quail, loss of *Mitf* expression leads to conversion of the retinal pigment epithelium into retina, as cells fail to acquire pigmentation and start to hyper-proliferate. *Mitf* regulation appears to be intrinsically connected to *Otx1* and *Otx2* expression in the retinal pigment epithelium, since Otx mice mutants lose *Mitf* expression and Mitf mice mutants lose *Otx2* expression (Bäumer *et al.*, 2003; Nguyen & Arnheiter, 2000). Thus, *Mitf* and *Otx* appear to cooperate in the establishment of the retinal pigment epithelium instead of presenting a hierarchical relationship (Bharti *et al.*, 2006; Ramón Martínez-Morales *et al.*, 2004).

The observation that mouse mutants with non-functional *Pax2* and *Pax*6 genes showed normal expression of retinal markers and small optical vesicles without *Mitf* expression (Bäumer *et al.*, 2003) highlights a possible role for *Pax6* upstream of *Mitf* activation. *Pax6* is initially expressed broadly in the optic vesicle, being restricted to the retina by the late optic cup stage (Grindley *et al.*, 1995), indicating it is present at the beginning of the retinal pigment epithelium specification but absent in later stages. Studies in mouse, chick, and quail suggest that *Otx1*, *Otx2,* and *Pax6* initiate the specification of the retinal pigment epithelium. *Pax6* and

Wnt would then initiate *Mitf* expression which, in cooperation with Otx, would activate melanogenic genes. This would lead to the differentiation of the retinal pigment epithelium independently of *Pax6* activity (Ramón Martínez-Morales *et al.*, 2004). Additional signaling molecules appear to play important roles in retinal pigment epithelium differentiation, such as BMPs and Hh-related proteins, which upon blocking lead to expression of retinal markers and loss of pigmentation in both *Xenopus* and chick (Bharti *et al.*, 2006; Ramón Martínez-Morales *et al.*, 2004).

As previously discussed, the presumptive retina is marked by the expression of *Vsx2*, which is induced by FGF1 and FGF2 expressed in the surface ectoderm, both in mouse and chick (Bäumer *et al.*, 2003; Bharti *et al.*, 2006; Nguyen & Arnheiter, 2000). The dorsal boundary of *Vsx2* (retina→retinal pigment epithelium) and the ventral boundary of *Mitf* (retinal pigment epithelium→retina) are established by their relative expression, as they mutually inhibit each other (Heavner & Pevny, 2012; Yun *et al.*, 2009). The current model in mouse suggests that *Vsx2* activated by FGF might be capable of repressing *Mitf* in the retina, inhibiting retinal pigment epithelium specification in favor of retina specification (Bharti *et al.*, 2006).

With the retinal fate established, the multipotent retinal progenitor cells will start to proliferate and differentiate to give raise to the different retinal neurons and glial cells (C. L. Cepko *et al.*, 1996). In mouse, *Pax6* maintains multipotency of the retinal progenitor cells, while *Sox2* determines their differentiation into neurons, and *Vsx2* maintains their proliferative state (Bassett & Wallace, 2012; Zagozewski *et al.*, 2014). Once established, the retinal progenitor cells pass through a series of competence states to generate the various retinal cell types in an evolutionary conserved temporal order. Different models have been proposed to explain if one or more types of retinal progenitor cells exist, how and when the competence states are established, and what are the extrinsic and intrinsic factors involved (reviewed in C. Cepko, 2014). Currently, there is uncertainty regarding the existence of distinct types of retinal progenitor cells (distinct lineages, specified by a determined set of genes), established at the beginning of retinal development, each of which capable to produce specific types of progeny. However, it is well established that terminally dividing and specified retinal progenitor cells can produce specific progeny that is most likely patterned by gene expression (Bassett & Wallace, 2012; C. Cepko, 2014; Livesey & Cepko, 2001). Each type of retinal cell has its identity determined by the expression of a distinct set of genes (Ohsawa & Kageyama, 2008; reviewed in Andreazzoli, 2009). Further, species-specific differences in retinogenesis play a role in the timing of appearance of distinct progeny and in establishing the molecular mechanisms responsible for progeny fate (C. Cepko, 2014).

1.3.6. Molecular mechanisms underlying eye maintenance

The events described above covered all the steps in eye organogenesis, from morphogenesis of each structure to the molecular mechanisms underlying their formation. Once the eye is formed and its components are functional, as any other organ in the body, it needs to be maintained: cells dye and have to be replaced, aqueous humor turnover is necessary for lens nutrition and survival, outer segment of photoreceptors have to be recycled

to recover from photo-oxidation damage, among other maintenance needs. A system that can give us some insights into the molecular mechanisms determining eye maintenance is the blind cavefish *Astyanax mexicanus* (Characiformes), a species with both surface and cave populations. In cave phenotypes, the beginning of the embryonic eye development occurs as it would in any other vertebrate: the eye-field is established and further divided in two, the optical grooves are formed giving raise to the optic stalks and the optic vesicles. The distal part of the optic vesicles will form the optic cups, and the lens placode will form the lens vesicle (see 1.3.1, page 4). Up to this point, no major differences are noticeable when comparing the surface and cavefish embryonic eyes, except for smaller lens vesicles/optic cups in the cave phenotype. However, as development proceeds in the cave phenotype, primary and secondary lens fibers do not differentiate, nor does the majority of the retinal cell types, apart from ganglion and glial cells. Also, the cornea, iris and ciliary body do not develop, even though neural crest cells migrate into the eye region. The lens and the retina degenerate and the eye is buried underneath the skin (Jeffery, 2005; Yamamoto *et al.*, 2004).

Yamamoto *et al.* (2004) showed that the location of Hh expression is expanded in the anterior midline in different cavefish populations. As Hh proteins control the expression of *Pax6* and *Pax2*, its expanded expression leads to a reduction in *Pax6* expression in the domains of the future optic vesicles and in the lens placode. At the same time, *Pax2* expression at the base of the optic vesicle (where the optic stalks will form) expands. The reciprocal repression between *Pax6* and *Pax2* controls the patterning of the optic vesicles and their size, resulting in the big optic stalks and small optic vesicles characteristic of cavefish (Jeffery, 2005; H. Li *et al.*, 1997; Macdonald *et al.*, 1995). Overexpression of *Shh* prevents the development of the lens and alters the organization of retinal layers (Ekker *et al.*, 1995; Macdonald *et al.*, 1995) implying that *Hh* genes are able to control lens apoptosis, resulting in eye degeneration (Jeffery, 2005). Therefore, what we see in blind cavefish is that the same genes are expressed in both surface and cave populations of this species, instead of pseudogenization of genes in cave populations (Alunni *et al.*, 2007; Jeffery, 2005) and that the loss of eye function is brought about by changes in location and timing of gene expression (e.g. *Lhx2* and *fgf8*, Pottin *et al.*, 2011). These findings suggest that even tissues with an acquired identity can change their organization or revert their development as a result of changes in the correct spatial and temporal distribution and levels of gene expression, resulting in the degeneration of structures or even whole organs.

The morphogenesis of the vertebrate eye is a very well-studied process, observed and described from zebrafish to humans. But the molecular mechanisms underlying this process are still not fully understood. In this section, I presented a general overview of the main genes and signaling pathways known to be involved in vertebrate eye organogenesis, but this is far from representing an exhaustive scenario for the molecular bases of eye formation. Many transcription factors, morphogens and proteins remain unknown in this process, as well as functional interactions between classical signaling pathways. Once the eye is formed, its maintenance becomes crucial as any failure will most likely result in impaired or degenerated eye tissues, and yet, the molecular basis for understanding eye maintenance have just begun to be explored.

1.4. A heterochronic system in eye development

A valuable system to explore this gaps in our knowledge concerning the genetic basis and regulation of eye formation and maintenance would be one that exhibits heterochrony. Heterochrony is defined as differences in the relative timing of developmental events (Emerson, 1986). Heterochrony between related species allows the characterization and comparison of the role played by different genetic factors in establishing the temporal sequence of developmental events. A species with "regular" developmental timing is contrasted to a species that has experienced a shift in developmental timing, providing insights into the identity of genes acting as master "switches" throughout development and potential regulators that must be expressed to maintain cell/tissue identity after differentiation. This approach is particularly powerful to infer the nature of interactions and hierarchical relationships between genes and pathways. Furthermore, when relevant information regarding the life history of the species with different allochronic patterns is incorporated together with the genetic data, it is possible to gain insight in the ultimate evolutionary causes of heterochrony, providing a deeper understanding of eye development and evolution.

A model system with tremendous potential for studies of evolutionary developmental biology of the vertebrate eye is represented by the glass frogs of the family Centrolenidae. In glass frogs with fossorial tadpoles, such as *Centrolene savagei*, eyes are rudimentary throughout most of the larval phase, presenting the first significant morphological changes around stage 30 (Gosner, 1960) and a slow development of the external structures of the eye till the stages immediately preceding metamorphosis (Hoffmann, 2010a). In the phylogenetically related family Bufonidae, eye development follows a more conventional anuran timing. For example, in the bufonid *Rhinella ornata* tadpoles have discernible eyes at stage 21 (Gosner, 1960) that continue developing throughout the larval phase and appear to be morphologically similar to the adult eye in the pre-metamorphic stage. *R. ornata* (Bufonidae) and *C. savagei* (Centrolenidae) are separated by ~60 Mya (Feng *et al.*, 2017; Jetz & Pyron, 2018). In contrast to the fossorial habit of *C. savagei* tadpoles, *R. ornata* tadpoles are benthic. It is evident that these two groups develop at least the external features of their eyes at different times during the larval stage, raising the possibility that this apparent heterochrony might also involve differences in the timing and levels of expression of master switch and regulatory genes involved in eye formation. In this thesis, I compare the transcriptomic profile of the tadpole eye at three developmental stages across the two species of *C. savagei* and *R. ornata* in an attempt to characterize the developmental transcriptome and identify the major players of eye development and the timing of their expression. In the following section, I complete this introductory part of the thesis with a brief overview of amphibian vision and some information on the ecology of *C. savagei* and *R. ornata*.

1.5. Vision in Amphibia

In amphibians, the retina maintains the typical vertebrate design, with three nuclear layers and two plexiform layers. Depending on the species, the thickness of the nuclear layers and the width of the plexiform layers can vary significantly (Reyer, 1977; Smith-Gill &

Carver, 1981). Moreover, the retinal pigment epithelium is characterized by long cytoplasmic processes that extend between the photoreceptors, reaching the outer limiting membrane (Reyer, 1977). Despite the typical vertebrate configuration, amphibians uniquely possess, in addition to the usual green-sensitive rod, a second characteristic blue-sensitive rod. The visual palette is completed by two types of cones, violet and red-sensitive cones (Yovanovich *et al.*, 2017). The green-sensitive rods have large outer segments and short myoids, with maximum absorbance at 503–507 nm, whereas the blue-sensitive rods have similarly large outer segments with generally long and thin myoids, and maximum absorbance peaks at ca. 435–445 nm (K. O. Donner & Reuter, 1976; Keating & Kennard, 1976).

As in other vertebrates, cones can be single or double. The double cones have an accessory component and one principal component very similar in general aspect to a single cone. Single cones and the principal components of double cones are red-sensitive, peaking at 560–580 nm and generally feature a colorless oil droplet in the inner segment. The accessory component has no oil droplet and is usually green-sensitive, peaking at 500 nm (K. O. Donner & Reuter, 1976; Keating & Kennard, 1976; Walls, 1963). Blue-sensitive cones are rare, with maximum absorbance at the same wavelengths as blue rods, and so far have been described in only a few anurans, such as *Aquarana catesbeiana*, *Rana temporaria* and *Xenopus laevis*. Understanding the retinal distribution, function, and representation of violet and blue cones in the amphibian phylogenetic tree is hampered by their small size and extremely low abundance in the retina (Hisatomi *et al.*, 1998; Koskelainen *et al.*, 1994; Röhlich & Szél, 2000).

A dramatic functional change in the amphibian eye occurs in the retina during metamorphosis. Photoreceptors mediate light signals by making use of light-sensitive molecules, the visual pigments. These pigments are constituted by a transmembrane protein, the opsin, to which a chromophore (i.e. a photosensitive molecule) is covalently bound. This is retinaldehyde, a derivative of vitamin A. The visual pigment formed by an opsin and retinaldehyde is named rhodopsin and the aldehyde of retinal is often called vitamin A1. When retinal is substituted by 3,4-didehydroretinal, the resulting chromophore is termed vitamin A2 and the visual pigment is called a porphyropsin (Archer, 1995). In the anuran species studied so far for this character, tadpole retinas contain a mix of rhodopsin and porphyropsin. At the onset of metamorphosis, the retina undergoes a complete reorganization, and rhodopsin becomes the only visual pigment (Kollros, 1981; Wald, 1981). However, aquatic species such as *X. laevis*, retain a porphyropsin system in the adult (Smith-Gill & Carver, 1981). The bullfrog *A. catesbeiana* presents a very specific spatial distribution of chromophores, with a prevalence of porphyropsin in the dorsal region and rhodopsin in the ventral region of the retina in the adult eye (Muntz, 1977). Different relative proportions of rhodopsin/porphyropsin and their arrangement in the retina imply that spectral perception changes when tadpoles metamorphose into adults. In addition to drastic changes in the visual pigments in the retina, muscular modifications also take place during development: the extrinsic ocular muscles increase in size and change in position causing, at least partially, the bulging of the eyes of the post-metamorphic frog (Kollros, 1981; Smith-Gill & Carver, 1981).

Overall, tadpole eyes are highly similar to adult eyes in terms of structures and general morphology: organization of the retina is maintained through metamorphosis, during which we also have formation of new components such as the nictitating membrane and the upper and lower eyelids. By the time metamorphosis ends, the eyeball is almost a perfect sphere that increases in size by growth and mitosis of all layers (Smith-Gill & Carver, 1981; Walls, 1963). However, a few differences can be identified. Tadpoles have two corneas, an outer cornea continuous with the head epidermis and formed by connective tissue and epithelium; and an inner cornea that is very thin, separated from the outer cornea by intraorbital fluid, and continuous with the outer surface of the ciliary body. At metamorphosis, the two corneas fuse to form the adult cornea (Kollros, 1981; Reyer, 1977; Smith-Gill & Carver, 1981). The lens is spherical and lies close to the cornea. After metamorphosis, the lens becomes slightly flattened, more anteriorly than posteriorly, but maintains the typical vertebrate structure (Reyer, 1977; Walls, 1963).

1.6. *Rhinella ornata* and *Centrolene savagei* ecology

Haddad *et al.* (2013) described 33 species of Bufonidae in the Atlantic Forest, 7 of which occur in São Paulo, one of the states with the highest diversity of Brazilian amphibians (Jenkins *et al.*, 2015). Among these seven species is *R. ornata* (Figure 1.3B), a large-sized (61–95 mm) nocturnal and terrestrial frog found in forested areas in southeastern Brazil (Hartmann *et al.*, 2010; Heyer *et al.*, 1990). This species is an explosive breeder with intense calling activity at the beginning and at the end of the rainy season (Rodrigues & Bertoluci, 2002). Males call from ponds, swamps or rivers where, after the amplexus with a female, numerous eggs are deposited, external fertilization occurs and embryos development takes place. Tadpoles hatch around Gosner's (Gosner, 1960) developmental stage 17 (Candioti *et al.*, 2016; Del Conte & Sirlin, 1952; Limbaugh & Volpe, 1957) and there is no parental care. The tadpoles are benthic (i.e. they live at the bottom of ponds, spending little or almost no time in the water column) and are exotrophic (i.e. they feed by scraping substrates or filtering particulate matter, in contrast to endotrophic tadpoles that obtain energy from vitellogenic yolk (Haddad *et al.*, 2013; Wells, 2007). The tadpoles present a sightly depressed body, eyes situated dorsolaterally and directed laterally, ventral mouth with two upper and three lower tooth rows on an oral disc and a well-developed and finely serrated beak. An interesting characteristic of *R. ornata* tadpoles is their black pigmentation, which has been suggested as having an aposematic function (Haddad *et al.*, 2013). A second defense line against predators is the formation of aggregations when swimming, diminishing the chances of predation (Altig & McDiarmid, 1999; Haddad *et al.*, 2013; Heyer *et al.*, 1990; Wells, 2007).

In contrast to Bufonidae, Centrolenidae is a relatively small family of 157 described species (Frost, 2019), with Colombia being the hotspot of its diversity. They are distributed from Mexico to Bolivia, southeastern Brazil and northeastern Argentina, occurring in different forested habitats, such as evergreen forest, páramos and semideciduous, rain and cloud forests. Representatives vary in size from small (< 22 mm) to medium (22–35 mm) and large-sized (35–55 mm (Cisneros-Heredia & McDiarmid, 2007; Vitt & Caldwell, 2014). All species have a

Figure 1.3: *Centrolene savagei* (**A**) and *Rhinella ornata* (**B**) adults. *Photograph A by Marco Rada. Photograph B by Rachel Montesinos.*

partially or completely transparent venter from which the common name of "glass frogs" derives, while their dorsum varies in different shades of green, and so do their bones (Cisneros-Heredia & McDiarmid, 2007; Guayasamin *et al.*, 2009). They are nocturnal, epiphyllous, and arboreal. Egg clutches are deposited at the male calling site, out of the water, in vegetation overhanging streams (i.e., upper side of leaves, downside of leaves, mosses, branches) or attached to rocks above streams (Cisneros-Heredia & McDiarmid, 2007; Guayasamin *et al.*, 2009). Tadpoles are fossorial, burying themselves at the bottom of creeks immediately after hatching (Hoffmann, 2010b; Rada *et al.*, 2007; Terán-Valdez *et al.*, 2009).

Among the 78 centrolenids found in Colombia (Rada *et al.*, 2019), *C. savagei* (Figure 1.3A) is a small (21–24 mm), nocturnal and arboreal frog, endemic to Colombia, occurring in cloud forests on the western slopes of the Cordillera Occidental and eastern slopes of the cordillera central, between 1400 and 2400 m elevation (Díaz-Gutiérrez *et al.*, 2013; Vargas-Salinas *et al.*, 2014). Egg clutches comprise 15–27 cream-colored eggs deposited on the upper surface of small or medium-sized leaves. Parental care is provided by the male, who sits on or near the clutch and keeps the eggs hydrated, remaining near them during day and night for protection (Vargas-Salinas *et al.*, 2014). After hatching at stage 25 (Díaz-Gutiérrez *et al.*, 2013; Gosner, 1960), tadpoles seek refuge at the bottom of creeks, under leaf litter and detritus (Cisneros-Heredia & McDiarmid, 2007). Tadpoles have a fusiform (= vermiform) body with muscular tails, reduced tail fins and directed anteroventrally oral disc. They lack skin pigmentation but often appear to be bright red in life due to the abundant blood flow visible through the skin, a characteristic believed to improve the cutaneous gas exchange, in response to their fossorial condition (Altig & McDiarmid, 1999; Cisneros-Heredia & McDiarmid, 2007; Díaz-Gutiérrez *et al.*, 2013; Hoffmann, 2010b; Wells, 2007).

We suspect the contrasting benthic versus fossorial condition exhibited by the two studied species might affect eye development. The visual system is an expensive system to develop and maintain (Niven & Laughlin, 2008) and is very delicate one. Once the system is fully formed, it has to be maintained to ensure its functionality, a demanding task when there is a high risk of abrasion, during a fossorial stage. Furthermore, the adaptive value of a fully developed eye in a fossorial tadpole is questionable given the limited light reaching the eye and

a limited need for predator avoidance by visual cues. In contrast, *R. ornata* tadpoles, constantly exposed while swimming freely at the bottom of ponds, rely at least in part on vision for foraging and predator avoidance in a scotopic or photopic underwater environment with little scope for mechanical damaging of their eyes.

6. Conclusion

Anurans have been used as models in developmental biology for over a century, becoming well-known and wide-spread in embryology and developmental biology. The molecular basis of eye formation and maintenance are only starting to be understood with the aid of new technologies. Much research remains to be conducted to unravel transcription factors, morphogens, proteins and functional interactions between signaling pathways. Taking advantage of past extensive research in anurans and modern molecular biology techniques, we aimed to understand how the genetic expression timing of different factors influences the formation of amphibian eyes. Combining these new genetic data with knowledge of the ecology and life history of species is a powerful tool that can provide a deeper understanding of eye development and evolution.

Our data showed that different developmental stages of *Centrolene savagei* and *Rhinella ornata* tadpoles have different patterns of gene expression, and these patterns are connected. We also found that *C. savagei* tadpoles in the early and intermediate stages present increased GO terms associated with metabolism, energy generation, and general development processes, while decreased GO terms were primarily associated with the development of the eye and its components, visual perception, and sensory organ development. Conversely, *R. ornata* tadpoles presented increased GO terms associated with localization and locomotion, as well as responses to the environment and to other organisms.

We found evidence in *C. savagei* for a moderate increase in expression of all visual opsins throughout tadpoles development, linked to a period of over-expression of visual cycle genes in the early developmental stage, and low activity of the phototransduction cascade genes, suggesting retina development is triggered by light and possibly happens slower when compared to non-fossorial tadpoles. This is consistent with the species life history and ecology, since a growing retina in the absence of light requires constant replacement of visual pigments at the same time that it will not have activation of the phototransduction cascade. In *R. ornata* tadpoles there is a similar trend of (non-significant) increases in opsins expression, which suggests retina is gradually developing, a result that was expected in a species with benthic and exotrophic tadpoles that actively swim.

Notably, parietopsin gene was found to be expressed in the eyes of both frog species of this study, the first to report parietopsin in the eye of a jawed vertebrate. Parietopsin function is unclear in eyes, however given its role in parietal eyes, it might be mediating a heterochronic shift in development by altering the biological clock and its expression profile during development in *C. savagei*. The SWS2 pattern of expression in *C. savagei* and *R. ornata* brought out important questions regarding the function and location of this opsin in anurans, and more work on its role in scotopic and color vision is needed, in order to understand its pattern of expression.

By contrasting gene expression at the transcriptome level between frog species with different timing of organ development this work showed that there is difference in gene

expression between these species, and the differential gene expression is correlated to the frogs life history. The fossorial species, *C. savagei*, presented a delay in the expression of genes related to sensory organ development, with emphasis in eye development and its associated processes when compared to the benthic species, *R. ornata*. These findings open a new door for understanding how timing of expression of different genetic factors influences the formation of amphibian eyes, something that has only been explored in cavefish. From here, we can deepen our knowledge in the relationship among development and ecology throughout evolution of species that present biphasic life cycle.

7. Resumo

Olhos de anuros são um modelo particularmente interessante dado que os girinos se desenvolvem na água e passam a viver em terra após metamorfose. Este processo envolve adaptações morfológicas e genéticas para lidar com diferentes habitats. Apesar da morfogênese de olhos ser um processo bem conhecido, há uma lacuna no conhecimento referente à base genética por trás da formação e mantenimento dos olhos. Este estudo teve como objetivo caracterizar diferentes padrões de expressão gênica e entender como estes padrões interagem entre si ao longo do tempo para influenciar a formação de olhos de anfíbios. Nós selecionamos duas espécies de anuros que têm diferentes histórias de vida no período larval e tempo de desenvolvimento ocular concomitante. Os girinos de *Rhinella ornata* são bentônicos e têm olhos discerníveis em estágios iniciais de desenvolvimento, olhos estes que já são morfologicamente semelhantes aos olhos dos adultos nos estágios de desenvolvimento intermediário e tardio. Em contraste, os girinos de *Centrolene savagei* são fossoriais e têm olhos rudimentares durante a maior parte de sua fase larval, com complexidade começando a aumentar no estágio intermediário de desenvolvimento. Fizemos a montagem um transcriptoma *de novo* para cada espécie usando dados de RNA-seq de olhos de girinos extraídos em três diferentes estágios de desenvolvimento e realizamos análises de expressão gênica diferencial entre os estágios de cada espécie. *C. savagei* e *R. ornata* têm padrões diferentes de expressão gênica em diferentes estágios de desenvolvimento: os estágios intermediário e tardio de *C. savagei* são mais semelhantes entre si do que com o estágio inicial, enquanto em *R. ornata* os estágios inicial e intermediário são mais semelhantes entre si do que com o estágio tardio. Além disso, a expressão de genes relacionados ao desenvolvimento do olho, percepção de luz e ciclo visual é retardada em *C. savagei* em relação a *R. ornata*. Este estudo é o primeiro a comparar a expressão gênica diferencial e seu padrão temporal em espécies de sapos com e sem girinos fossoriais, permitindo elucidar a relação entre o desenvolvimento do olho, ecologia e evolução.

Palavras chave: *Centrolene savagei*, *Rhinella ornata*, Amphibia, transcriptoma *de novo*, expressão diferencial de genes, desenvolvimento de olhos, visão, opsinas, cascata de fototransdução, ciclo visual.

8. Abstract

The anuran eye is a particularly interesting model given that tadpoles develop in water and move to land after metamorphosis. This process involves morphological and genetic adaptations to deal with different habitats. Although eye morphogenesis is well understood, there is a lacuna in knowledge concerning the genetic basis underlying eye formation and maintenance. This study aimed to characterize different gene expression patterns, and how they interact with each other over time to influence the formation of amphibian eyes. We selected two anuran species that have different larval life histories and concomitant eye development timing. *Rhinella ornata* tadpoles are benthic and have discernible eyes in early developmental stages that are already morphologically similar to adult eyes in intermediate and late developmental stages. In contrast, *Centrolene savagei* tadpoles are fossorial and have rudimentary eyes throughout most of their larval phase, with complexity beginning to increase at an intermediate developmental stage. We assembled a *de novo* transcriptome for each species using RNA-seq data from eyes extracted at three different tadpoles' developmental stages, and performed differential gene expression analysis within species. We show that *C. savagei* and *R. ornata* have different patterns of gene expression across different developmental stages: *C. savagei* intermediate and late stages are more similar to each other than either is to the early stage, while in *R. ornata* the early and intermediate stages are more similar to each other than either is to the late stage. Additionally, expression of genes related to eye development, light perception, and visual cycle are delayed in *C. savagei* relative to *R. ornata*. This study is the first to compare differential gene expression and its timing in frog species with and without fossorial tadpoles, enabling the relationship between eye development, ecology, and evolution to be elucidated.

Keywords: *Centrolene savagei*, *Rhinella ornata*, Amphibia, *de novo* transcriptome, differential gene expression, eye development, vision, opsins, phototransduction cascade, visual cycle.

9. References

Altig, R., & McDiarmid, R. W. (1999). Body plan: Development and morphology. In R. W. McDiarmid & R. Altig (Eds.), *Tadpoles: The Biology of Anuran Larvae* (pp. 24–51). University of Chicago Press. https://press.uchicago.edu/ucp/books/book/chicago/T/bo3643050.html

Alunni, A., Menuet, A., Candal, E., Pénigault, J.-B., Jeffery, W. R., & Rétaux, S. (2007). Developmental mechanisms for retinal degeneration in the blind cavefish *Astyanax mexicanus*. *Journal of Comparative Neurology*, *505*(2), 221–233. https://doi.org/10.1002/cne.21488

Andreazzoli, M. (2009). Molecular regulation of vertebrate retina cell fate. *Birth Defects Research Part C: Embryo Today: Reviews*, *87*(3), 284–295. https://doi.org/10.1002/bdrc.20161

Archer, S. (1995). Molecular biology of visual pigments. In M. B. A. Djamgoz, S. N. Archer, & S. Vallerga (Eds.), *Neurobiology and Clinical Aspects of the Outer Retina* (pp. 79–104). Springer Netherlands. https://doi.org/10.1007/978-94-011-0533-0_4

Babraham Institute. (n.d.). *FastQC: a quality control tool for high throughput sequence data*. Retrieved October 8, 2019, from https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Bassett, E. A., & Wallace, V. A. (2012). Cell fate determination in the vertebrate retina. *Trends in Neurosciences*, *35*(9), 565–573. https://doi.org/10.1016/j.tins.2012.05.004

Bäumer, N., Marquardt, T., Stoykova, A., Spieler, D., Treichel, D., Ashery-Padan, R., & Gruss, P. (2003). Retinal pigmented epithelium determination requires the redundant activities of Pax2 and Pax6. *Development*, *130*(13), 2903. https://doi.org/10.1242/dev.00450

Bharti, K., Nguyen, M.-T. T., Skuntz, S., Bertuzzi, S., & Arnheiter, H. (2006). The other pigment cell: Specification and development of the pigmented epithelium of the vertebrate eye. *Pigment Cell Research*, *19*(5), 380–394. https://doi.org/10.1111/j.1600-0749.2006.00318.x

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*(15), 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Callery, E. M. (2006). There's more than one frog in the pond: A survey of the Amphibia and their contributions to developmental biology. *Seminars in Cell & Developmental Biology*, *17*(1), 80–92. https://doi.org/10.1016/j.semcdb.2005.11.001

Candioti, F. V., Grosso, J., Haad, B., Pereyra, M. O., Bornschein, M. R., Borteiro, C., Costa, P., Kolenc, F., Pie, M. R., Proaño, B., Ron, S., Stanescu, F., & Baldo, D. (2016). Structural and Heterochronic Variations During the Early Ontogeny in Toads (Anura: Bufonidae). *Herpetological Monographs*, *30*(1), 79–118. https://doi.org/10.1655/HERPMONOGRAPHS-D-16-00004.1

Carl, M., Loosli, F., & Wittbrodt, J. (2002). Six3 inactivation reveals its essential role for the formation and patterning of the vertebrate eye. *Development*, *129*(17), 4057.

Cepko, C. (2014). Intrinsically different retinal progenitor cells produce specific types of progeny. *Nature Reviews Neuroscience*, *15*(9), 615–627. https://doi.org/10.1038/nrn3767

Cepko, C. L., Austin, C. P., Yang, X., Alexiades, M., & Ezzeddine, D. (1996). Cell fate determination in the vertebrate retina. *Proceedings of the National Academy of Sciences*, *93*(2), 589–595. https://doi.org/10.1073/pnas.93.2.589

Chow, R. L., & Lang, R. A. (2001). Early eye development in vertebrates. *Annual Review of Cell and Developmental Biology*, *17*(1), 255–296. https://doi.org/10.1146/annurev.cellbio.17.1.255

Cisneros-Heredia, D. F., & McDiarmid, R. W. (2007). Revision of the characters of Centrolenidae (Amphibia: Anura: Athesphatanura), with comments on its taxonomy and the description of new taxa of glassfrogs. *Zootaxa*, *1572*, 3–82.

Collomb, E., Yang, Y., Foriel, S., Cadau, S., Pearton, D. J., & Dhouailly, D. (2013). The corneal epithelium and lens develop independently from a common pool of precursors: Multistep induction of corneal epithelium. *Developmental Dynamics*, *242*(5), 401–413. https://doi.org/10.1002/dvdy.23925

Cronin, T. W., Johnsen, S., Marshall, N. J., & Warrant, E. J. (2014). *Visual ecology*. Princeton University Press. https://press.princeton.edu/books/hardcover/9780691151847/visual-ecology

Cvekl, A., & Ashery-Padan, R. (2014). The cellular and molecular mechanisms of vertebrate lens development. *Development*, *141*(23), 4432–4447. https://doi.org/10.1242/dev.107953

Darwin, C. (1871). *On the origin of species* (5th ed.). D. Appleton and Co. https://doi.org/10.5962/bhl.title.28875

Davies, W. I. L., Tamai, T. K., Zheng, L., Fu, J. K., Rihel, J., Foster, R. G., Whitmore, D., & Hankins, M. W. (2015). An extended family of novel vertebrate photopigments is widely expressed and displays a diversity of function. *Genome Research*, *25*(11), 1666–1679. https://doi.org/10.1101/gr.189886.115

Del Conte, E., & Sirlin, J. L. (1952). *Pattern series of the first embrionay stages in Bufo Arenarum*.

Dhouailly, D., Pearton, D. J., & Michon, F. (2014). The vertebrate corneal epithelium: From early specification to constant renewal. *Developmental Dynamics*, *243*(10), 1226–1241. https://doi.org/10.1002/dvdy.24179

Dias, P. H. dos S. (2018). *Evolution of larval characteres in Dendrobatoidea Cope, 1865 (Amphibia; Anura; Dendrobatidae & Aromobatidae)* [Doutorado em Zoologia, Universidade de São Paulo]. https://doi.org/10.11606/T.41.2018.tde-20082018-141408

Díaz-Gutiérrez, N., Vargas-Salinas, F., Rivera-Correa, M., Rojas-Morales, J. A., Escobar-Lasso, S., Velasco, J. A., Gutiérrez-Cárdenas, P. D. A., & Amézquita, A. (2013). Description of the previously unknown advertisement call and tadpole of the Colombian endemic glassfrog *Centrolene savagei* (Anura: Centrolenidae). *Zootaxa*, *3686*(2), 289–296. https://doi.org/10.11646/zootaxa.3686.2.9

Do, C. B., & Batzoglou, S. (2008). What is the expectation maximization algorithm? *Nature Biotechnology*, *26*(8), 897–899. https://doi.org/10.1038/nbt1406

Donner, K. O., & Reuter, T. (1976). Visual pigments and photoreceptor function. In R. Llinás & W. Precht, *Frog neurobiology: A handbook* (pp. 251–277). Springer. https://doi.org/10.1007/978-3-642-66316-1_8

Donner, K., & Yovanovich, C. A. M. (2020). A frog's eye view: Foundational revelations and future promises. *Seminars in Cell & Developmental Biology*, *106*, 72–85. https://doi.org/10.1016/j.semcdb.2020.05.011

Ekker, S. C., Ungar, A. R., Greenstein, P., von Kessler, D. P., Porter, J. A., Moon, R. T., & Beachy, P. A. (1995). Patterning activities of vertebrate hedgehog proteins in the developing eye and brain. *Current Biology*, *5*(8), 944–955. https://doi.org/10.1016/S0960-9822(95)00185-0

Emerson, S. B. (1986). Heterochrony and frogs: The relationship of a life history trait to morphological form. *The American Naturalist*, *127*(2), 167–183. https://doi.org/10.1086/284476

Faber, S. C., Robinson, M. L., Makarenkova, H. P., & Lang, R. A. (2002). Bmp signaling is required for development of primary lens fiber cells. *Development*, *129*(15), 3727.

Feng, Y.-J., Blackburn, D. C., Liang, D., Hillis, D. M., Wake, D. B., Cannatella, D. C., & Zhang, P. (2017). Phylogenomics reveals rapid, simultaneous diversification of three major clades of Gondwanan frogs at the Cretaceous–Paleogene boundary. *Proceedings of the National Academy of Sciences*, *114*(29), E5864–E5870. https://doi.org/10.1073/pnas.1704632114

Frost, D. (2019, June 10). *Amphibian species of the world 6.0: An online reference*. https://amphibiansoftheworld.amnh.org/

Furuta, Y., & Hogan, B. L. M. (1998). BMP4 is essential for lens induction in the mouse embryo. *Genes & Development*, *12*(23), 3764–3775. https://doi.org/10.1101/gad.12.23.3764

Gage, P. J., Rhoades, W., Prucka, S. K., & Hjalt, T. (2005). Fate maps of neural crest and mesoderm in the mammalian eye. *Investigative Opthalmology & Visual Science*, *46*(11), 4200. https://doi.org/10.1167/iovs.05-0691

Gehring, W. J. (2014). The evolution of vision. *Wiley Interdisciplinary Reviews: Developmental Biology*, *3*(1), 1–40. https://doi.org/10.1002/wdev.96

Gilbert, S. F. (2010). *Developmental biology* (9th ed). Sinauer Associates. http://www.devbio.com/

Glaeser, G., & Paulus, H. F. (2015). *The evolution of the eye*. Springer International Publishing. https://doi.org/10.1007/978-3-319-17476-1

Gordon, J., & Hood, D. C. (1976). Anatomy and physiology of the frog retina. In K. V. Fite (Ed.), *The Amphibian Visual System* (1st ed., pp. 29–86). Elsevier. https://doi.org/10.1016/B978-0-12-257450-4.50008-2

Gosner, K. L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica*, *16*(3), 183–190.

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., di Palma, F., Birren, B. W., Nusbaum, C., Lindblad-Toh, K., … Regev, A. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology*, *29*(7), 644–652. https://doi.org/10.1038/nbt.1883

Grainger, R. M. (1992). Embryonic lens induction: Shedding light on vertebrate tissue determination. *Trends in Genetics*, *8*(10), 349–355. https://doi.org/10.1016/0168-9525(92)90280-H

Graw, J. (2010). Eye development. In P. Koopman (Ed.), *Organogenesis in Development* (Vol. 90, pp. 343–386). Elsevier. https://doi.org/10.1016/S0070-2153(10)90010-0

Gregory-Evans, C. Y., Wallace, V. A., & Gregory-Evans, K. (2013). Gene networks: Dissecting pathways in retinal development and disease. *Progress in Retinal and Eye Research*, *33*, 40–66. https://doi.org/10.1016/j.preteyeres.2012.10.003

Grindley, J. C., Davidson, D. R., & Hill, R. E. (1995). The role of Pax-6 in eye and nasal development. *Development*, *121*(5), 1433.

Guayasamin, J. M., Castroviejo-Fisher, S., Trueb, L., Ayarzagüena, J., Rada, M., & Vilà, C. (2009). *Phylogenetic systematics of Glassfrogs (Amphibia: Centrolenidae) and their sister taxon* Allophryne ruthveni. https://kuscholarworks.ku.edu/handle/1808/13694

Gustafsson, O. S. E., Ekström, P., & Kröger, R. H. H. (2010). A fibrous membrane suspends the multifocal lens in the eyes of lampreys and African lungfishes. *Journal of Morphology*, *271*(8), 980–989. https://doi.org/10.1002/jmor.10849

Haddad, C. F. B., Toledo, L. F., Prado, C. P. A., Loebmann, D., Gasparini, J., & Sazima, I. (2013). *Guia dos anfíbios da Mata Atlântica: Diversidade e biologia*. Anolis Books. https://www.anolisbooks.com.br/produtos/detalhes/489/

Hartmann, M. T., Hartmann, P. A., & Haddad, C. F. B. (2010). Reproductive modes and fecundity of an assemblage of anuran amphibians in the Atlantic rainforest, Brazil. *Iheringia. Série Zoologia*, *100*(3), 207–215. https://doi.org/10.1590/S0073-47212010000300004

Heavner, W., & Pevny, L. (2012). Eye development and retinogenesis. *Cold Spring Harbor Perspectives in Biology*, *4*(12), a008391–a008391. https://doi.org/10.1101/cshperspect.a008391

Heyer, W. R., Rand, A. S., Cruz, C. A. G. da, Peixoto, O. L., & Nelson, C. E. (1990). Frogs of Boracéia. *Arquivos de Zoologia*, *31*(4), 231–410.

Hisatomi, O., Kayada, S., Taniguchi, Y., Kobayashi, Y., Satoh, T., & Tokunaga, F. (1998). Primary structure and characterization of a bullfrog visual pigment contained in small single cones. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *119*(3), 585–591. https://doi.org/10.1016/S0305-0491(98)00032-7

Hoffmann, H. (2010a). *The glass frog tadpoles of Costa Rica (Anura, Centrolenidae): A study of morphology*.

Hoffmann, H. (2010b). *The glass frog tadpoles of Costa Rica (Anura: Centrolenidae): A study of morphology*. Schweizerbart Science Publishers. http://www.schweizerbart.de//publications/detail/isbn/9783510613960/Abhd_Senckenb_Gesf_Naturforschung_5

Hollemann, T., Bellefroid, E., & Pieler, T. (1998). The *Xenopus* homologue of the *Drosophila* gene tailless has a function in early eye development. *Development*, *125*(13), 2425.

Holmberg, K. (1977). The cyclostome retina. In F. Crescitelli, C. A. Dvorak, D. J. Eder, A. M. Granda, D. Hamasaki, K. Holmberg, A. Hughes, N. A. Locket, W. N. McFarland, D. B. Meyer, W. R. A. Muntz, F. W. Munz, E. C. Olson, R. W. Reyer, & F. Crescitelli (Eds.), *The Visual System in Vertebrates* (pp. 47–66). Springer. https://doi.org/10.1007/978-3-642-66468-7_2

Jeffery, W. R. (2005). Adaptive evolution of eye degeneration in the Mexican blind cavefish. *Journal of Heredity*, *96*(3), 185–196. https://doi.org/10.1093/jhered/esi028

Jenkins, C. N., Alves, M. A. S., Uezu, A., & Vale, M. M. (2015). Patterns of vertebrate diversity and protection in Brazil. *PLOS ONE*, *10*(12), e0145064. https://doi.org/10.1371/journal.pone.0145064

Jetz, W., & Pyron, R. A. (2018). The interplay of past diversification and evolutionary isolation with present imperilment across the amphibian tree of life. *Nature Ecology & Evolution*, *2*(5), 850–858. https://doi.org/10.1038/s41559-018-0515-5

Kamijyo, A., Yura, K., & Ogura, A. (2015). Distinct evolutionary rate in the eye field transcription factors found by estimation of ancestral protein structure. *Gene*, *555*(2), 73–79. https://doi.org/10.1016/j.gene.2014.10.003

Kanitz, A., Gypas, F., Gruber, A. J., Gruber, A. R., Martin, G., & Zavolan, M. (2015). Comparative assessment of methods for the computational inference of transcript isoform abundance from RNA-seq data. *Genome Biology*, *16*(1), 150. https://doi.org/10.1186/s13059-015-0702-5

Kao, W. W.-Y., Xia, Y., Liu, C.-Y., & Saika, S. (2008). Signaling pathways in morphogenesis of cornea and eyelid. *The Ocular Surface*, *6*(1), 9–23. https://doi.org/10.1016/S1542-0124(12)70102-7

Keating, M. J., & Kennard, C. (1976). The amphibian visual system as a model for developmental neurobiology. In K. V. Fite (Ed.), *The Amphibian Visual System* (pp. 267–315). Academic Press. https://doi.org/10.1016/B978-0-12-257450-4.50013-6

Kidson, S. H., Kume, T., Deng, K., Winfrey, V., & Hogan, B. L. M. (1999). The Forkhead/Winged-Helix Gene, Mf1, is necessary for the normal development of the cornea and formation of the anterior chamber in the mouse eye. *Developmental Biology*, *211*(2), 306–322. https://doi.org/10.1006/dbio.1999.9314

Kolb, H., Nelson, R., Fernandez, E., & Jones, B. (2012, January 25). *Webvision: The organization of the retina and visual system* [Online book]. https://webvision.med.utah.edu/

Kollros, J. J. (1981). Transitions in the nervous system during amphibian metamorphosis. In L. I. Gilbert & E. Frieden (Eds.), *Metamorphosis: A Problem in Developmental Biology* (pp. 445–459). Springer US. https://doi.org/10.1007/978-1-4613-3246-6_13

Koskelainen, A., Hemilä, S., & Donner, K. (1994). Spectral sensitivities of short- and long-wavelength sensitive cone mechanisms in the frog retina. *Acta Physiologica Scandinavica*, *152*(1), 115–124. https://doi.org/10.1111/j.1748-1716.1994.tb09790.x

Kumar, G., Ertel, A., Feldman, G., Kupper, J., & Fortina, P. (2020). iSeqQC: A tool for expression-based quality control in RNA sequencing. *BMC Bioinformatics*, *21*(1), 56. https://doi.org/10.1186/s12859-020-3399-8

Lamb, T. D. (2013). Evolution of phototransduction, vertebrate photoreceptors and retina. *Progress in Retinal and Eye Research*, *36*, 52–119. https://doi.org/10.1016/j.preteyeres.2013.06.001

Lamb, T. D., Collin, S. P., & Pugh, E. N. (2007). Evolution of the vertebrate eye: Opsins, photoreceptors, retina and eye cup. *Nature Reviews Neuroscience*, *8*(12), 960–976. https://doi.org/10.1038/nrn2283

Land, M. F., & Nilsson, D.-E. (2012). *Animal eyes*. Oxford University Press. https://doi.org/10.1093/acprof:oso/9780199581139.001.0001

Law, C. W., Chen, Y., Shi, W., & Smyth, G. K. (2014). voom: Precision weights unlock linear model analysis tools for RNA-seq read counts. *Genome Biology*, *15*(2), R29. https://doi.org/10.1186/gb-2014-15-2-r29

Lê, S., Josse, J., & Husson, F. (2008). FactoMineR : an R package for multivariate analysis. *Journal of Statistical Software*, *25*(1), 1–18. https://doi.org/10.18637/jss.v025.i01

Li, B., & Dewey, C. N. (2011). RSEM: Accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics*, *12*(1), 323. https://doi.org/10.1186/1471-2105-12-323

Li, H., Tierney, C., Wen, L., Wu, J. Y., & Rao, Y. (1997). A single morphogenetic field gives rise to two retina primordia under the influence of the prechordal plate. *Development*, *124*(3), 603.

Limbaugh, B. A., & Volpe, E. P. (Erminio P. (1957). *Early development of the Gulf Coast toad, Bufo valliceps Wiegmann. American Museum novitates ; no. 1842*. https://digitallibrary.amnh.org/handle/2246/4877

Liu, J. J., Kao, W. W.-Y., & Wilson, S. E. (1999). Corneal epithelium-specific Mouse Keratin K12 Promoter. *Experimental Eye Research*, *68*(3), 295–301. https://doi.org/10.1006/exer.1998.0593

Livesey, F. J., & Cepko, C. L. (2001). Vertebrate neural cell-fate determination: Lessons from the retina. *Nature Reviews Neuroscience*, *2*(2), 109–118. https://doi.org/10.1038/35053522

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, *15*(12), 550. https://doi.org/10.1186/s13059-014-0550-8

Lovicu, F. J., & McAvoy, J. W. (2005). Growth factor regulation of lens development. *Developmental Biology*, *280*(1), 1–14. https://doi.org/10.1016/j.ydbio.2005.01.020

Macdonald, R., Barth, K. A., Xu, Q., Holder, N., Mikkola, I., & Wilson, S. W. (1995). Midline signalling is required for Pax gene regulation and patterning of the eyes. *Development*, *121*(10), 3267.

Martin, J. A., & Wang, Z. (2011). Next-generation transcriptome assembly. *Nature Reviews Genetics*, *12*(10), 671–682. https://doi.org/10.1038/nrg3068

Mathers, P. H., Grinberg, A., Mahon, K. A., & Jamrich, M. (1997). The Rx homeobox gene is essential for vertebrate eye development. *Nature*, *387*(6633), 603–607. https://doi.org/10.1038/42475

McAvoy, J. W., & Chamberlain, C. G. (1989). Fibroblast growth factor (FGF) induces different responses in lens epithelial cells depending on its concentration. *Development*, *107*(2), 221.

Mic, F. A., Molotkov, A., Molotkova, N., & Duester, G. (2004). Raldh2 expression in optic vesicle generates a retinoic acid signal needed for invagination of retina during optic cup formation. *Developmental Dynamics*, *231*(2), 270–277. https://doi.org/10.1002/dvdy.20128

Miesfeld, J. B., & Brown, N. L. (2019). Eye organogenesis: A hierarchical view of ocular development. In D. M. Wellik (Ed.), *Organ Development* (Vol. 132, pp. 351–393). Elsevier. https://doi.org/10.1016/bs.ctdb.2018.12.008

Muntz, W. R. A. (1977). The visual world of the Amphibia. In F. Crescitelli, C. A. Dvorak, D. J. Eder, A. M. Granda, D. Hamasaki, K. Holmberg, A. Hughes, N. A. Locket, W. N. McFarland, D. B. Meyer, W. R. A. Muntz, F. W. Munz, E. C. Olson, R. W. Reyer, & F. Crescitelli (Eds.), *The Visual System in Vertebrates* (pp. 275–307). Springer. https://doi.org/10.1007/978-3-642-66468-7_5

Nguyen, M., & Arnheiter, H. (2000). Signaling and transcriptional regulation in early mammalian eye development: A link between FGF and MITF. *Development*, *127*(16), 3581.

Nilsson, D.-E. (2009). The evolution of eyes and visually guided behaviour. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *364*(1531), 2833–2847. https://doi.org/10.1098/rstb.2009.0083

Nilsson, D.-E. (2013). Eye evolution and its functional basis. *Visual Neuroscience*, *30*(1–2), 5–20. https://doi.org/10.1017/S0952523813000035

Niven, J. E., & Laughlin, S. B. (2008). Energy limitation as a selective pressure on the evolution of sensory systems. *Journal of Experimental Biology*, *211*(11), 1792–1804. https://doi.org/10.1242/jeb.017574

Ohsawa, R., & Kageyama, R. (2008). Regulation of retinal cell fate specification by multiple transcription factors. *Brain Research*, *1192*, 90–98. https://doi.org/10.1016/j.brainres.2007.04.014

Parker, A. R. (2011). On the origin of optics. *Optics & Laser Technology*, *43*(2), 323–329. https://doi.org/10.1016/j.optlastec.2008.12.020

Patro, R., Duggal, G., Love, M. I., Irizarry, R. A., & Kingsford, C. (2017). Salmon provides fast and bias-aware quantification of transcript expression. *Nature Methods*, *14*(4), 417–419. https://doi.org/10.1038/nmeth.4197

Porter, F. D., Drago, J., Xu, Y., Cheema, S. S., Wassif, C., Huang, S. P., Lee, E., Grinberg, A., Massalas, J. S., Bodine, D., Alt, F., & Westphal, H. (1997). Lhx2, a LIM homeobox gene, is required for eye, forebrain, and definitive erythrocyte development. *Development*, *124*(15), 2935.

Pottin, K., Hinaux, H., & Rétaux, S. (2011). Restoring eye size in *Astyanax mexicanus* blind cavefish embryos through modulation of the Shh and Fgf8 forebrain organising centres. *Development*, *138*(12), 2467. https://doi.org/10.1242/dev.054106

Rada, M., Dos Santos Dias, P. H., Pérez-Gonzalez, J. L., Anganoy-Criollo, M., Rueda-Solano, L. A., Pinto-E, M. A., Quintero, L. M., Vargas-Salinas, F., & Grant, T. (2019). The poverty of adult morphology: Bioacoustics, genetics, and internal tadpole morphology reveal a new species of glassfrog (Anura: Centrolenidae: Ikakogi) from the Sierra Nevada de Santa Marta, Colombia. *PLOS ONE*, *14*(5), e0215349. https://doi.org/10.1371/journal.pone.0215349

Rada, M., Rueda-Almonacid, J. V., Velásquez-Álvarez, Á. A., & Sánchez-Pacheco, S. J. (2007). Descripción de las larvas de dos centrolénidos (Anura: Centrolenidae) del noroccidente de la Cordillera Oriental, Colombia. *Papéis Avulsos de Zoologia*, *47*(21), 259–272. https://doi.org/10.1590/S0031-10492007002100001

Ramón Martínez-Morales, J., Rodrigo, I., & Bovolenta, P. (2004). Eye development: A view from the retina pigmented epithelium. *BioEssays*, *26*(7), 766–777. https://doi.org/10.1002/bies.20064

Reyer, R. W. (1977). The amphibian eye: Development and regeneration. In F. Crescitelli, C. A. Dvorak, D. J. Eder, A. M. Granda, D. Hamasaki, K. Holmberg, A. Hughes, N. A. Locket, W. N. McFarland, D. B. Meyer, W. R. A. Muntz, F. W. Munz, E. C. Olson, R. W. Reyer, & F. Crescitelli (Eds.), *The Visual System in Vertebrates* (pp. 309–390). Springer. https://doi.org/10.1007/978-3-642-66468-7_6

Ritchie, M. E., Phipson, B., Wu, D., Hu, Y., Law, C. W., Shi, W., & Smyth, G. K. (2015). Limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Research*, *43*(7), e47–e47. https://doi.org/10.1093/nar/gkv007

Robinson, M. D., & Oshlack, A. (2010). A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biology*, *11*(3), R25. https://doi.org/10.1186/gb-2010-11-3-r25

Rodrigues, M. T., & Bertoluci, J. (2002). Seasonal patterns of breeding activity of Atlantic Rainforest anurans at Boracéia, Southeastern Brazil. *Amphibia-Reptilia*, *23*(2), 161–167. https://doi.org/10.1163/156853802760061804

Röhlich, P., & Szél, Á. (2000). Photoreceptor cells in the *Xenopus* retina. *Microscopy Research and Technique*, *50*(5), 327–337. https://doi.org/10.1002/1097-0029(20000901)50:5<327::AID-JEMT2>3.0.CO;2-P

Semina, E. V., Reiter, R., Leysens, N. J., Alward, W. L. M., Small, K. W., Datson, N. A., Siegel-Bartelt, J., Bierke-Nelson, D., Bitoun, P., Zabel, B. U., Carey, J. C., & Murray, J. C. (1996). Cloning and characterization of a novel bicoid-related homeobox transcription factor gene, RIEG, involved in Rieger syndrome. *Nature Genetics*, *14*(4), 392–399. https://doi.org/10.1038/ng1296-392

Simão, F. A., Waterhouse, R. M., Ioannidis, P., Kriventseva, E. V., & Zdobnov, E. M. (2015). BUSCO: Assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics*, *31*(19), 3210–3212. https://doi.org/10.1093/bioinformatics/btv351

Sivak, B., & Sivak, J. (2000). Vertebrate eye development and refractive function: An overview. In M. E. Fini (Ed.), *Vertebrate Eye Development* (pp. 1–14). Springer. https://doi.org/10.1007/978-3-540-46826-4_1

Sivak, J. G., Andison, M. E., & Pardue, M. T. (1999). Vertebrate optical structure. In S. N. Archer, M. B. A. Djamgoz, E. R. Loew, J. C. Partridge, & S. Vallerga (Eds.), *Adaptive Mechanisms in the Ecology of Vision* (pp. 73–94). Springer Netherlands. https://doi.org/10.1007/978-94-017-0619-3_4

Smith-Gill, S. J., & Carver, V. (1981). Biochemical characterization of organ differentiation and maturation. In L. I. Gilbert & E. Frieden (Eds.), *Metamorphosis: A Problem in Developmental Biology* (pp. 491–544). Springer US. https://doi.org/10.1007/978-1-4613-3246-6_15

Smith-Unna, R., Boursnell, C., Patro, R., Hibberd, J. M., & Kelly, S. (2016). TransRate: Reference-free quality assessment of de novo transcriptome assemblies. *Genome Research*, *26*(8), 1134–1144. https://doi.org/10.1101/gr.196469.115

Srivastava, A., Malik, L., Sarkar, H., Zakeri, M., Almodaresi, F., Soneson, C., Love, M. I., Kingsford, C., & Patro, R. (2020). Alignment and mapping methodology influence transcript abundance estimation. *Genome Biology*, *21*(1), 239. https://doi.org/10.1186/s13059-020-02151-8

Sulik, K. K., & Bream Jr., P. R. (n.d.). *Embryo images: Normal and abnormal mammalian development*. Retrieved August 2, 2020, from https://syllabus.med.unc.edu/courseware/embryo_images/

Supek, F., Bošnjak, M., Škunca, N., & Šmuc, T. (2011). REVIGO summarizes and visualizes long lists of Gene Ontology terms. *PLoS ONE*, *6*(7), e21800. https://doi.org/10.1371/journal.pone.0021800

Terán-Valdez, A., Guayasamin, J. M., & Coloma, L. A. (2009). Description of the tadpole of *Cochranella resplendens* and redescription of the tadpole of *Hyalinobatrachium aureoguttatum* (Anura, Centrolenidae). *Phyllomedusa*, *8*(2), 105–124.

Tzahor, E., Kempf, H., Mootoosamy, R. C., Poon, A. C., Abzhanov, A., Tabin, C. J., Dietrich, S., & Lassar, A. B. (2003). Antagonists of Wnt and BMP signaling promote the formation of vertebrate head muscle. *Genes & Development*, *17*(24), 3087–3099. https://doi.org/10.1101/gad.1154103

Vargas-Salinas, F., Quintero-Ángel, A., Osorio-Domínguez, D., Rojas-Morales, J. A., Escobar-Lasso, S., Gutiérrez-Cárdenas, P. D. A., Rivera-Correa, M., & Amézquita, A.

(2014). Breeding and parental behaviour in the glass frog *Centrolene savagei* (Anura: Centrolenidae). *Journal of Natural History*, *48*(27–28), 1689–1705. https://doi.org/10.1080/00222933.2013.840942

Vitt, L. J., & Caldwell, J. P. (2014). *Herpetology: An introductory biology of amphibians and reptiles* (Fourth edition). Academic Press. https://doi.org/10.1016/C2010-0-67152-5

Wald, G. (1981). Metamorphosis: An overview. In L. I. Gilbert & E. Frieden (Eds.), *Metamorphosis* (pp. 1–39). Springer US. https://doi.org/10.1007/978-1-4613-3246-6_1

Walls, G. L. (1963). *The vertebrate eye and its adaptive radiation* (Reprint of 1942 Edition). Hafner Publ. http://www.archive.org/details/vertebrateeyeits00wall

Walter, W., Sánchez-Cabo, F., & Ricote, M. (2015). GOplot: An R package for visually combining expression data with functional analysis. *Bioinformatics*, *31*(17), 2912–2914. https://doi.org/10.1093/bioinformatics/btv300

Wawersik, S., Purcell, P., Rauchman, M., Dudley, A. T., Robertson, E. J., & Maas, R. (1999). BMP7 acts in murine lens placode development. *Developmental Biology*, *207*(1), 176–188. https://doi.org/10.1006/dbio.1998.9153

Wells, K. D. (2007). *The ecology and behavior of amphibians*. The University of Chicago Press. https://press.uchicago.edu/ucp/books/book/chicago/E/bo5298950.html

Wickham, H. (2016). *ggplot2: Elegant graphics for data analysis*. Springer-Verlag New York. https://ggplot2.tidyverse.org

Wingett, S. W., & Andrews, S. (2018). FastQ Screen: A tool for multi-genome mapping and quality control. *F1000Research*, *7*, 1338. https://doi.org/10.12688/f1000research.15931.2

Yamamoto, Y., Stock, D. W., & Jeffery, W. R. (2004). Hedgehog signalling controls eye degeneration in blind cavefish. *Nature*, *431*(7010), 844–847. https://doi.org/10.1038/nature02864

Young, M. D., Wakefield, M. J., Smyth, G. K., & Oshlack, A. (2010). Gene ontology analysis for RNA-seq: Accounting for selection bias. *Genome Biology*, *11*(2), R14. https://doi.org/10.1186/gb-2010-11-2-r14

Yovanovich, C. A. M., Koskela, S. M., Nevala, N., Kondrashev, S. L., Kelber, A., & Donner, K. (2017). The dual rod system of amphibians supports colour discrimination at the absolute visual threshold. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *372*(1717), 20160066. https://doi.org/10.1098/rstb.2016.0066

Yun, S., Saijoh, Y., Hirokawa, K. E., Kopinke, D., Murtaugh, L. C., Monuki, E. S., & Levine, E. M. (2009). Lhx2 links the intrinsic and extrinsic factors that control optic cup formation. *Development*, *136*(23), 3895. https://doi.org/10.1242/dev.041202

Zagozewski, J. L., Zhang, Q., & Eisenstat, D. D. (2014). Genetic regulation of vertebrate eye development: Review of eye development. *Clinical Genetics*, *86*(5), 453–460. https://doi.org/10.1111/cge.12493

Zhang, C., Zhang, B., Lin, L.-L., & Zhao, S. (2017). Evaluation and comparison of computational tools for RNA-seq isoform quantification. *BMC Genomics*, *18*(1), 583. https://doi.org/10.1186/s12864-017-4002-1

Zuber, M. E., Gestri, G., Viczian, A. S., Barsacchi, G., & Harris, W. A. (2003). Specification of the vertebrate eye by a network of eye field transcription factors. *Development*, *130*(21), 5155. https://doi.org/10.1242/dev.00723