

OCULAR HISTOLOGY IN THREE SOUTH CENTRAL TEXAS PAEDOMORPHIC
SALAMANDER SPECIES (*EURYCEA SOSORUM*, *EURYCEA NANA* AND
EURYCEA RATHBUNI) AND COMPARATIVE OCULAR
DEVELOPMENT OF TWO MORPHOTYPES

By

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A thesis submitted to the Graduate Council of
Texas State University in partial fulfillment
of the requirements for the degree of
Master of Science
with a Major in Biology
May 2014

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DEDICATION

This work could not have been accomplished without the support of my family. I specifically dedicate this thesis research in loving memory of my grandmother Carmen Uranga (1927-2013). My grandmother helped raise my sisters and me and always encouraged us to stay true to the most important virtue of her Christian religion, love. She taught us to love each other and always exemplified love to whomever she met. My grandmother was integral in shaping the person I am today, and I feel blessed to have had her in my life. To my sisters, Elisa and Gabriella Tovar, I hope to continue setting a positive example in both academics and in life. To my parents, Terrie and Roland Tovar, for the example they provide in both love and work ethic, I only hope to make you proud. To my extended family, and specifically my aunt Yolanda, for her continued perseverance in academia and for being a remarkable example of achievement in higher education, here's another book for your collection.

ACKNOWLEDGEMENTS

I would like to thank a number of people that counseled me through this research project. I would like to thank my committee members Dr. Caitlin Gabor and Dr. Glenn Longley for numerous reviews of my thesis and the project and helpful insights regarding this salamander system. My committee has been and continues to be an integral part of my maturation as a professional biologist. Thanks to my advisor Dr. Dana García for allowing me to pursue an independent project. Through her guidance I was able to attend a number of international meetings and obtain a number of grants. I am grateful to Dr. Benjamin Schwartz, Dr. David Hillis, Nate Bendik, Dr. William Jeffery, Dr. Andy Gluesenkamp, Dee Ann Chamberlain, and Dr. Dante Fenolio for meeting and discussing conceptual ideas regarding the project and its future.

Thanks to the U.S. Fish and Wildlife, San Marcos Aquatic Resource Center (SMARC) for their corporation and help in accomplishing this project, specifically Joe Fries, Valentin Cantu, Randy Gibson, Dr. Tom Brandt, Dr. Kenneth Ostrand and Justin Crow. Without the help of the SMARC this project would not have been accomplished. Helpful insight was also provided by Texas Parks and Wildlife herpetologist Andy Gluesenkamp, and the City of Austin biologist Nathan Bendik and Dee Ann Chamberlain.

Early conversations of this project started as an undergraduate and were specifically the result of a class taught by Dr. Andre Pires da Silva, who inspired me to pursue questions in evolutionary developmental biology. It was due to his mentorship that this project became reality. Equally, Dr. Paul Chippindale shared in early discussion of the salamanders, their natural history, and phylogenetics. I continue insightful conversations with faculty and graduate students at the University of Texas at Arlington studying herpetology. The faculty included Dr. Andre Pires da Silva (now at the University of Warwick), Dr. Jonathan Campbell, Dr. Paul Chippindale, and Dr. Eric Smith. Graduate students include Dr. Coleman Sheehy III, Dr. Jefferey Striecher, Dr.

Christian Cox, Dr. Corey Roelke, Dr. Brian Fontenot, Dr. Jyotiska Chaudhuri, Jacobo Reyes Velasco, David Sanches, Utpal Smart, and Carl Franklin.

Finally I would like to thank my funding sources which included: South Texas Herpetological Association, East Texas Herpetological Society, Southwestern Association of Naturalist Howard McCarley Grant, Herpetologists League Jones-Lovich Grant, Texas State Thesis Research Support Fellowship, Texas State Biology Department for travel funds, Two private donors for travel funds and accommodations in the dissemination of this research at the University of Oxford, University of Oxford Welcome Trust for accommodations and meals, Texas State Rising STAR Grant for travel funds.

Helpful conversations and moral support were provided by a number of graduate students and faculty from Texas State University and include: Kate Bell, Desirae Weyland, Dr. Chris Nice, Derek Wallace, Dr. Michael Huston, Dr. Vicente Lopes, Dr. James Ott, Rebecca Bracken, and Anjana Parandhaman. A special thanks to the García lab specifically those that participated in the research: Brian Fremaux, Clayton Cerdas, and Amanda Laird for the many hours contributed to the completion of these projects.

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LIST OF ABBREVIATIONS

Abbreviation	Description
<i>pax6</i>	A member of the Pax gene family, this gene encodes a conserved paired box domain.
<i>shh</i>	A gene which encodes for the protein sonic hedgehog (Shh), important during neural development and establishing symmetry in bilateral animals.
Shh	A major hedgehog family paracrine factor. Important during the neural development of vertebrates.
Pax6	A protein which acts as a morphogen and is important during neural development, specifically the development of the central nervous system.

ABSTRACT

The recent focus on conserved genes expressed through development has allowed for great headway in understanding the molecular mechanisms responsible for the variation seen among organisms. The expression of these integral developmental genes has implications with respect to evolutionary processes. The south central Texas *Eurycea* clade presents a unique continuum of karst phenotypes, having species representative of both subterranean and surface phenotypes. By describing the adult ocular morphology and the developmental processes leading to it, I hope to lay the foundation for better understanding the molecular mechanisms responsible for generating subterranean phenotypes in a karst salamander system.

Three species of salamanders (*Eurycea rathbuni*, *Eurycea nana*, and *Eurycea sosorum*) from the south central Texas *Eurycea* clade were obtained for examining adult ocular histology. All specimens were mortalities obtained from the U.S. Fish and Wildlife Service San Marcos Aquatic Resource Center. The adult histology revealed an underdeveloped eye in the subterranean species *E. rathbuni* and well-developed eyes in the surface species *E. nana* and *E. sosorum*. Interestingly, a prominent optic nerve was found in both surface and subterranean species. The optic nerve of the subterranean species *E. rathbuni* was further examined using transmission electron microscopy. A number of myelinated axons were observed, suggesting functional capability of the optic nerve.

I have described the adult ocular histology in three species of south central Texas *Eurycea*, and I am interested in describing the developmental processes leading to the divergent anatomy between the two morphotypes. Furthermore, I aim to understand how differences in gene expression influence the divergent outcomes of eye development between the two morphotypes; therefore, expression of genes involved in ocular development (*pax6* and *shh*) was examined in *E. rathbuni* embryos and *E. sosorum* embryos. The proteins Pax6 and Shh are conserved among all animals and share similar

expression patterns through development in species in which their expression has been examined. I found that both *E. rathbuni* and *E. sosorum* express Pax6 and Shh, but the time course and location of Pax6 and Shh expression in the developing eye of *E. rathbuni* differed from that in *E. sosorum*. Furthermore, I observed unexpectedly that the lens, which functions in inducing development of the retina in other organisms, persists in the latest stage of *E. rathbuni*, suggesting that they maintain a lens after hatching and potentially well into the juvenile state.

The two morphotypes examined share similar ontogeny, yet different spatial and temporal expression of Pax6 and Shh. Interestingly, a similar pattern can be seen by a cave adapted fish (*Astyanax mexicanus*), suggesting a degree of convergent evolution both through ontogeny and the expression of Pax6 and Shh. I conclude that these salamanders present an ideal system in which to study the evolutionary and developmental mechanisms that lead to the variation in subterranean morphotypes seen in the *Eurycea* clade. Moreover, this system represents an innovation from the fish system for understanding the evolutionary processes responsible for subterranean adaptation.

PREFACE

“There is grandeur in this view of life, with its several powers, having been originally breathed into a few forms or into one; and that, whilst this planet has gone cycling on according to the fixed law of gravity, from so simple a beginning endless forms most beautiful and most wonderful have been, and are being, evolved.” –Charles Darwin,
On The Origin of Species (1859).

The variation observed in organisms has long fascinated scientists and compelled them to understand the mechanisms underlying the incredible menagerie exhibited by organisms. As so eloquently stated by Charles Darwin in the Origin of Species, I set out to understand the underpinnings responsible for variation seen among organisms. From the seemingly simple beginnings of development to elaborate adult forms, I too set out to understand the variation seen among organisms. An organism’s adaptation to a niche via evolutionary processes can result in variable forms. Thus, the variation of a habitat into a number of microhabitats may facilitate the specialization of organisms to those specific conditions.

Multiple habitats are exemplified by the Balcones escarpment and fault zone, which is located in south central Texas, is a unique system derived from many geological events including carbonate deposition, uplifting, down faulting, and volcanic activity (Longley, 2003). Combinations of geological and hydrological events have contributed to the formation of a region above the Balcones escarpment known as the Edwards Plateau, which is composed of the carbonate rock, limestone. The Edwards Aquifer was formed after years of erosion via dissolution and is described as a porous, honeycombed, cavernous limestone region, and is a source of fresh water for millions of residents throughout south central Texas (Longley, 1981). The aquifer is also a source of fresh water to many endemic species of the region.

The south central Texas *Eurycea* clade is endemic to the Edwards Aquifer and its

associated environments (Baker, 1961). The aquifer provides a subterranean habitat for *E. rathbuni*, *E. waterlooensis*, *E. robusta* and *E. tridentifera*. Subterranean habitats differ dramatically from surface environments by having little or no light, consistent temperatures, and fewer food resources (Barr and Holsinger, 1985). Adaptations to subterranean living are exemplified by the Texas blind salamander (*Eurycea rathbuni*) which completes its entire life cycle in this aquatic, subterranean environment (Mitchell and Reddell, 1965). The species that have adapted to living under subterranean conditions have unpigmented skin, large heads, wide mouths, gracile limbs, eye and lung reduction, and vision loss - characteristics common to many subterranean organisms (Chippindale, 2000). Environments associated with the Edwards Aquifer include many spring systems, which provide habitat for surface species. These epigean species (*E. nana*, *E. troglodytes*, *E. tridentifera*, *E. spp.* Comal complex, *E. latitans*, *E. sosorum*, *E. tonkawae*, *E. chisholmensis*, *E. neotenes*, *E. naufragia*, *E. pterophila*) have contrasting characteristics, including pigmented skin and seemingly fully developed eyes (Chippindale and Price et al. 1998).

Despite the striking morphological differences between the surface and subterranean species, phylogenetic analyses suggest a close relationship between epigean salamander species that possess functional eyes (e.g., *E. nana*) and stygobiont species that possess non-functioning eyes (e.g., *E. rathbuni*) (Fig. 1: Wiens et al., 2003). Genetic data also suggest multiple invasions of cave habitats by the central Texas salamanders, resulting in numerous species possessing a range of cave adaptations (Chippindale, 2000). Together, thirteen species in the genus *Eurycea* are recognized as constituting a monophyletic clade, which can be subdivided into three, genetically distinct groups associated with specific geographic regions (Chippindale et al., 2000). The Texas blind salamander and the San Marcos salamander are sympatric while the Barton Springs salamander is endemic to a spring system found in the city of Austin, Texas approximately 30 miles north of San Marcos. Together, these salamanders present themselves as an interesting group in which to study the developmental phenomena that

lead to the former having a reduced eye and the latter two as having fully developed eyes.

Evolutionary developmental biologists have identified several genes governing eye development in invertebrates and vertebrates (Carroll, 2005). Interestingly, comparisons of these genes across distantly related taxa have revealed that versions of the same genes appear to strongly regulate eye development in all animals (Gilbert, 2010). It appears that the master-coding gene *pax6* is integral to ocular development and lends itself to evolutionary inquiry when compared across taxa. When *pax6* is over-expressed, non-functional, or mutated in some ways, it can cause eye malformation or vision loss in many organisms, including humans (Prossor, 1998; Tzoulaki, 2005). Given the homology of master coding genes for eyes, it would be unexpected to see differences in eye development in two closely related species. On the other hand, the morphology exhibited by these salamanders does suggest developmental differences and an ideal opportunity to test the question “Does *pax6* expression differ through development between two polymorphic sister species in a way that could account for vision loss in the Texas blind salamander?” In order to address this question, a strong foundation must be built that describes the differences in morphology and charts the course of events during embryogenesis that leads to the deviations in ocular morphology.

Herein, I describe the adult ocular histology of three paedomorphic species of salamanders from the south central Texas *Eurycea* clade to better understand the ultimate result of development and to establish a foundation for future investigation of the system’s divergent morphotypes. Adult ocular histology was complemented with the examination of expression of genes integral to ocular development, specifically *pax6* and *shh*. These genes have been shown to be important for eye development (Gilbert, 2010). Furthermore, a previous study examining a cavefish population of the species *Astyanax mexicanus* has shown the importance of both *pax6* and *shh* in ocular development, and specifically lack thereof in the cave population exhibiting reduced eyes (Jeffery, 2008). We can therefore ask the question “Are the same molecular mechanisms responsible for subterranean phenotypes in this vertebrate, tetrapod system?”

CHAPTER I

Comparative Ocular Histology of Three South Central Texas Paedomorphic Salamander Species (*Eurycea nana*, *E. sosorum*, and *E. rathbuni*)

Introduction

The dissolution of limestone formed in what is now the Edwards Plateau in the Cretaceous period has led to the formation of subterranean habitats, which in turn have supported the evolution of stygobitic organisms, that is, organisms for which the entire life cycle is carried out underground in aquatic habitats. Stygobitic vertebrates are represented by two lineages; Teleost (boney fish) (Jeffery, 2012: Sket, 1996: Humphreys, 1993: Holsinger, 2000) and Caudata (salamanders). Importantly the order Caudata represents the only stygobitic tetrapods (Goricki et al., 2012).

The morphology of stygobionts is exemplified by the Texas blind salamander (*Eurycea rathbuni*), having a large head, wide mouth, gracile limbs, pigment reduction and vestigial eyes, characteristics widely accepted as cave adaptations (Mitchell and Redell, 1965). In contrast, the San Marcos salamander (*E. nana*) and Barton Springs salamander (*E. sosorum*) are epigean species and have pigmented skin, short robust limbs, and seemingly well-developed eyes. Importantly, phylogenetic analyses suggest a close relationship between epigean salamander species and stygobitic species (Wiens et al., 2003). The close relationship between the south central Texas *Eurycea* species and the tremendous divergence in morphotypes found in this genus makes it an ideal biological system in which to compare disparate ocular morphologies.

Ocular histology in Caudata has been examined in a number of taxa including the groups Cryptobranchidae, Ambystomatidae, Salamandridae, and Proteidae (Fite, 1976),

but not Plethodontidae. Most terrestrial caudate species have larger eyes than aquatic species. Moreover, the species representing neotenic or paedomorphic life strategies have small eyes considered to be in a state of degeneration (Walls, 1942). Differing degrees of ocular regression are especially obvious in the stygobitic genera *Eurycea*, *Typhlotriton*, and *Proteidae* (Walls, 1942; Moller, 1951; Eigenmann, 1900).

The largely inaccessible and fragile habitat of the Edwards Aquifer (Tovar and Solis, 2012) has doubtless contributed to the paucity of studies concerning this clade's ocular histology. Only one study describing the ocular histology of a species from this clade, *E. rathbuni* (Eigenmann, 1900) has been published to date. Moreover, *E. rathbuni* ocular histology was undertaken by Eigenmann because of its stygobitic morphology, and it cannot be considered representative of the majority of the species in the clade. The remaining species' ocular histology warrants a thorough review, particularly keeping their associated habitats in mind. Herein we present the ocular histology of three species of south central Texas *Eurycea* representing two morphotypes: the stygobitic *E. rathbuni*, and epigean *E. nana* and *E. sosorum*.

Material and Methods

The U.S. Fish and Wildlife Service Aquatic Resource Center San Marcos, Texas (SMARC) donated newly deceased specimens of Texas blind salamander (*Eurycea rathbuni*), San Marcos salamander (*E. nana*), and Barton Springs salamander (*E. sosorum*). The specimens' heads were removed and transported to Texas State University for further processing under scientific permit number SPR-0390-045, issued to Thomas

M. Brant (SMARC). General measurements along with tissue samples were taken of the remaining body, which was then preserved in 95% ethanol and cataloged at the SMARC. The heads of the specimens were placed in 4% buffered paraformaldehyde for approximately 24 hours and washed 3 times for 10 minutes for each wash with phosphate buffered saline. The heads were placed in a 30% sucrose solution prepared in phosphate buffered saline for cryoprotection and stored at 4° C for 24 hours or until sectioning took place. Sections (20 µm) were collected using a Shandon Cryotome at -28° C, mounted on a slide using 90% glycerol, and stored at -20° C (Saul et al. 2010). Images were acquired using an Olympus FV1000 equipped with differential interference contrast optics and a 10X objective.

Results

Examination of histological sections taken from two epigean species and a stygobitic species reveal markedly different histology between the stygobitic and epigean species. Features previously described by Eigenmann (1900) for *Eurycea rathbuni* were identified and included optic nerve (ON), ganglion layer (GL), inner reticular layer (IRL), outer and inner reticular layer (O/IRL), and pigment epithelium (PE). A well-defined optic nerve was observed emanating from the eyes of *E. rathbuni* (Fig. 4-C). The entire eyeball is surrounded by melanized tissue, both internal and external to the sclera and cornea.

Histological sections from the epigean species *Eurycea nana* and *E. sosorum* revealed well-defined retinal layers, corneal layers, iris, lens, and pigment epithelium. Furthermore, retinal layers were identified as pigment epithelium (PE), photoreceptors

(PR), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), and retinal ganglion layer (RGL). Importantly, a well-defined optic nerve was observed in both species. In the epigean salamanders, melanized tissue is restricted primarily to the PE, the choroid, the ciliary body of the iris; however, some dark pigmentation can also be observed outside the sclera and surrounding the optic nerve.

Discussion

This study provides a description of ocular histology comparing among three closely related species and two ecotypes, epigean and stygobitic. *Eurycea rathbuni* has drastically reduced optic anatomy, a characteristic widely accepted as reflecting cave-adapted morphology and exemplified by other stygobitic animals including other cave-dwelling salamanders (e.g., *Proteus anguis*), cave-dwelling fish (e.g., *Astyanax mexicanus*), as well as extremely phylogenetically divergent invertebrates (Romero, 2009). Furthermore, *E. rathbuni* exhibits a few vestigial retinal layers surrounded by pigment epithelium. These results suggest light would be unable to pass through the pigment epithelium to be utilized by photoreceptors if there were any. Interestingly, the optic nerve is still present in *E. rathbuni*, suggesting possible sensory function, but not necessarily vision.

Our results complement those reported by Eigenmann (1900). Upon close examination of *E. rathbuni* histology, the feature identified by Eigenmann as an optic nerve penetrating to the center of the eye resembles the hyaloid canal. The hyaloid canal provides vascularization to the developing lens during embryogenesis. I suspect the

hyaloid canal forms in the eye and progressively develops until the formation of the lens is either stunted or lost via apoptosis. In future studies, immunohistochemistry techniques will be used to stain for vascularization markers characteristic of the hyaloid canal to confirm its identity.

The epigean species *E. nana* and *E. sosorum* have well developed retinal layers including photoreceptors and pigment epithelium, thus exhibiting ocular anatomy expected of above ground species (Linke et al., 1986; Heatwole, 1998). The epigean species also exhibit a lens, cornea, and iris. Furthermore, *E. nana* and *E. sosorum* have a well-developed optic nerve. Thus it appears that all the ocular structures necessary to support vision are in place. Fundamental knowledge of ocular anatomy has implications to current research on these salamanders and their biology. The full extent of visual function in the epigean species has implications regarding mate choice and predator/prey recognition (Roth, 1987). Future quantification of photoreceptors including rods, cones, and their associated wavelength optima could elucidate the extent of light and dark adaption, and color perception (Roth, 1987).

In conclusion, our comparative examination of ocular histology suggests that full development of the retina in *E. rathbuni* is aborted during ontogeny and that the lens is lost. Furthermore, our results raise questions about the stage of development at which these events occur and the molecular processes that lead to this outcome.

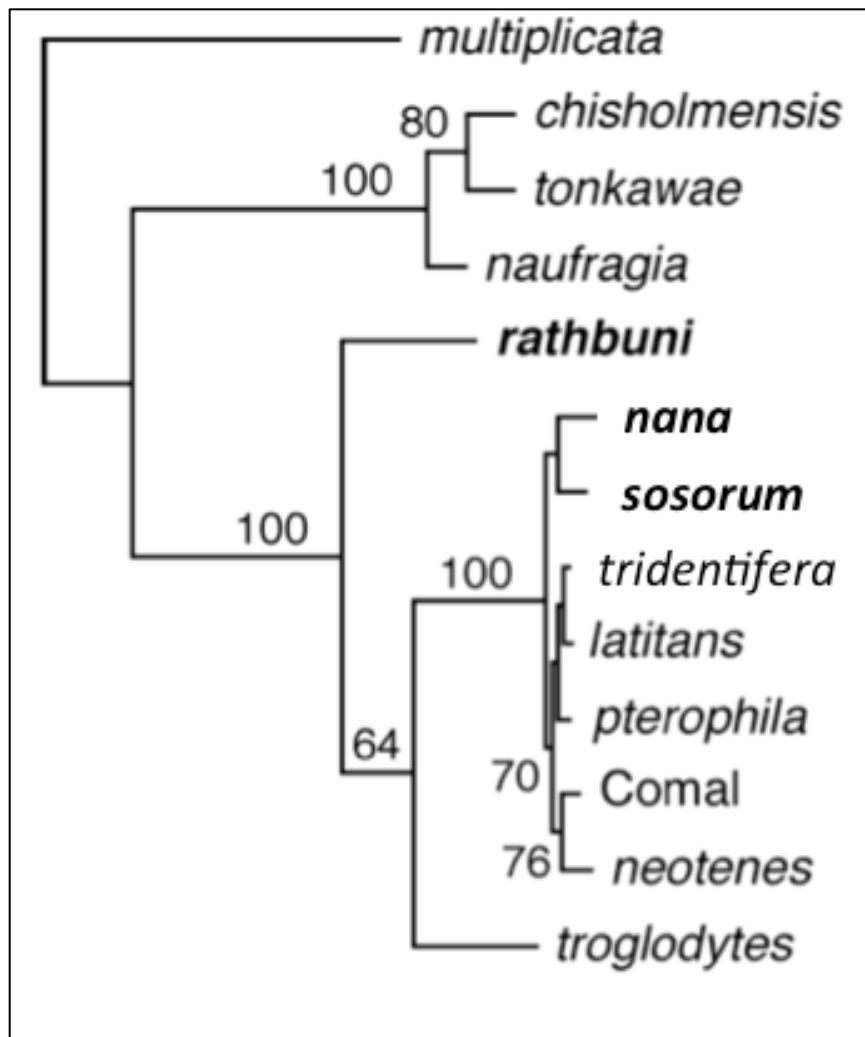


Figure 1. A consolidated phylogeny of allozyme, morphological, and mtDNA data for the south central Texas *Eurycea* clade. Modified from Wiens et al. 2003 to highlight (in bold) the species examined in this study. Furthermore, the phylogeny illustrates a monophyletic clade (*E. multiplicita* as the out group) and two different species within the clade, which exhibit stygobitic morphology suggesting multiple subterranean invasions.

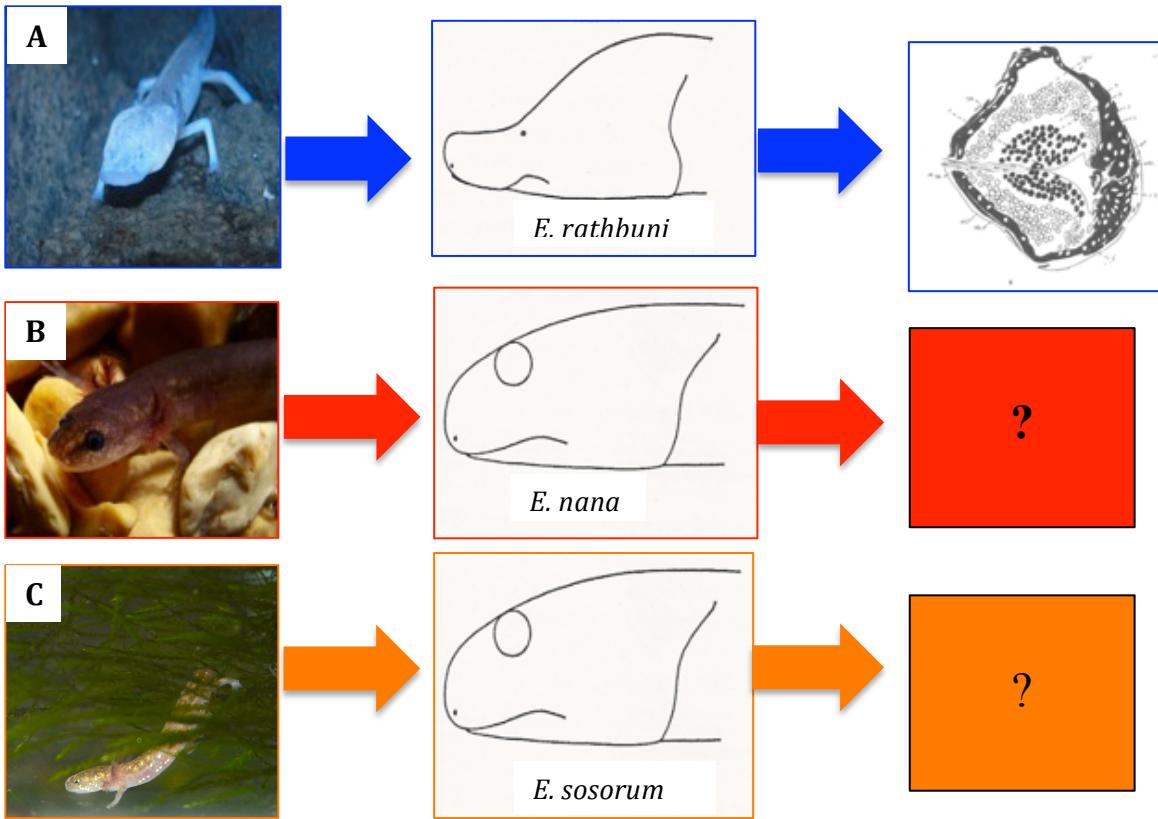


Figure 2. Photographs of adult *Eurycea rathbuni* (A), *E. nana* (B), and *E. sosorum* (C). Respective sketches of their heads modified from Mitchell and Reddell (1965), and a picture of the most current ocular histology (from left to right with each species boxed in a unique color). *E. rathbuni* ocular histology image taken from (Eigenmann, 1900). *E. rathbuni* is the only species for which ocular histology has been described.

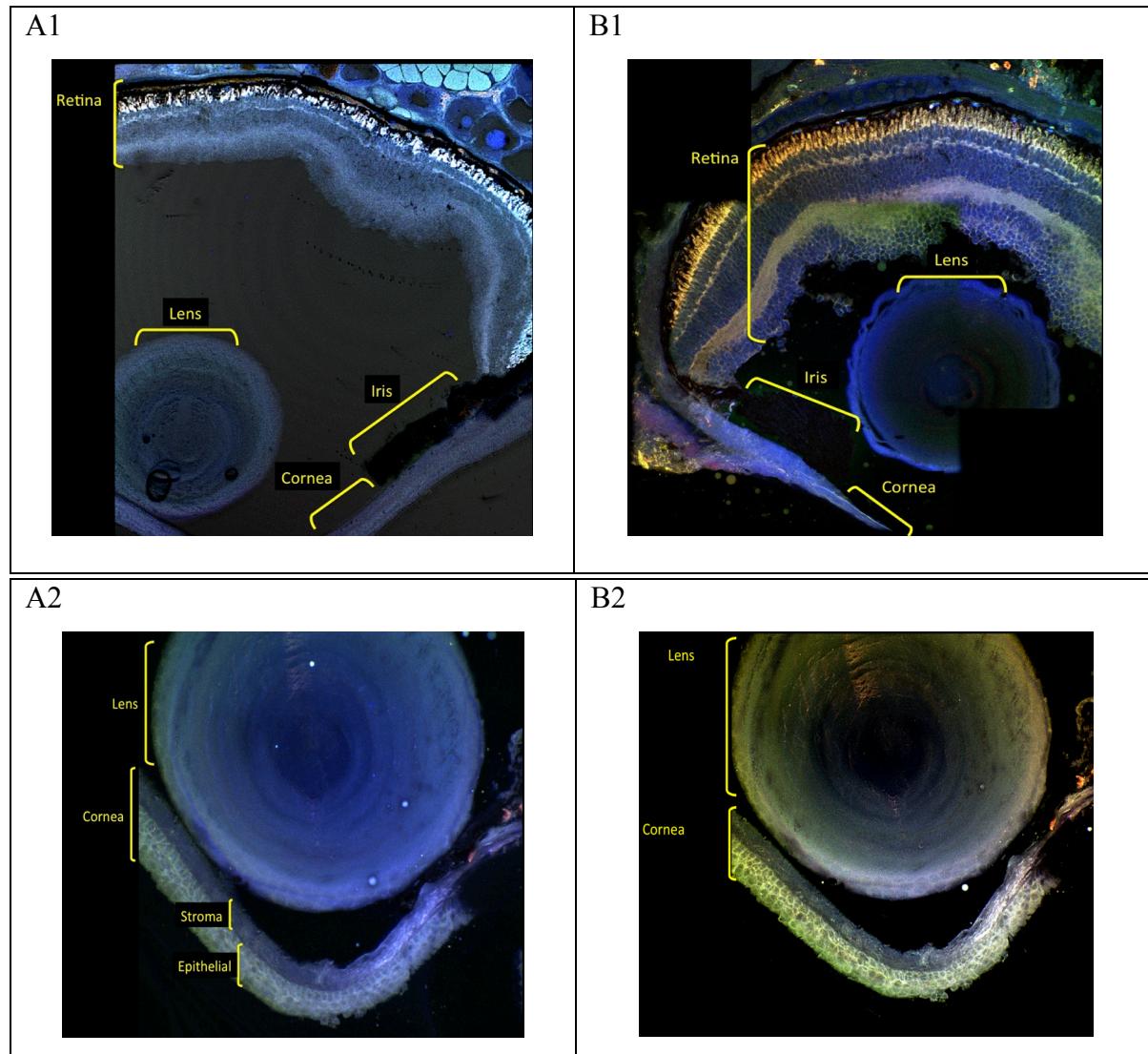


Figure 3. Sections of adult *E. nana* (A 1-4) and *E. sosorum* (B 1-4) eye. Illustrating regions of the posterior eye showing well-developed retinal layers and pigment (A1, B1). The lens, cornea, and iris are also visible (A2, B2).

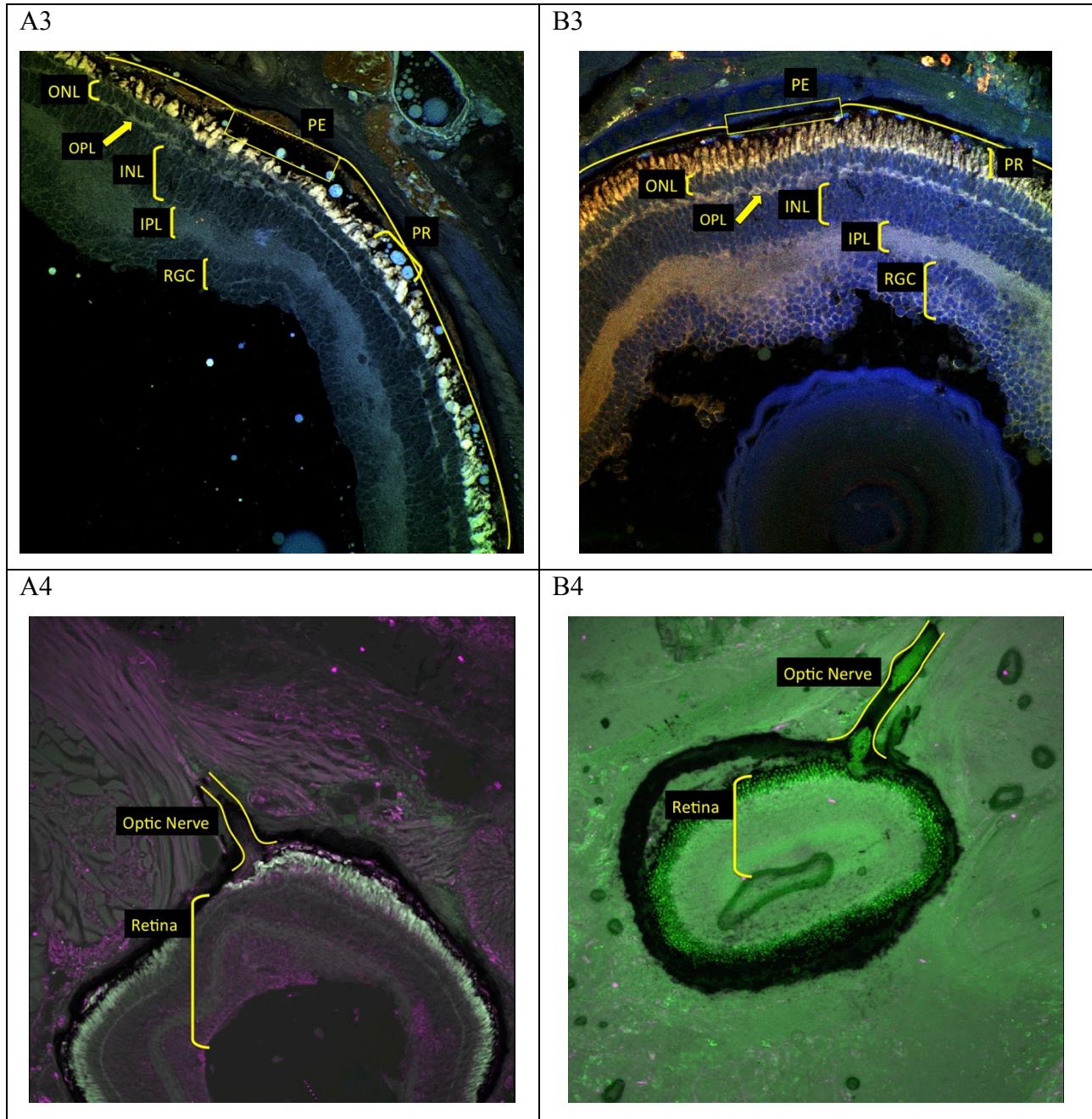


Figure 3. Ocular sections of adult *E. nana* (A 1-4) and *E. sosorum* (B 1-4) eye. Continued: Identification of retinal layers is as follows: retina ganglion cell layer (RGC), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), photoreceptor (PR), lens, iris, cornea (A3, B3). The optic nerve is pigmented and visible stemming from the posterior region of the pigment epithelium (A4, B4), optic nerve, pigment epithelium (PE).

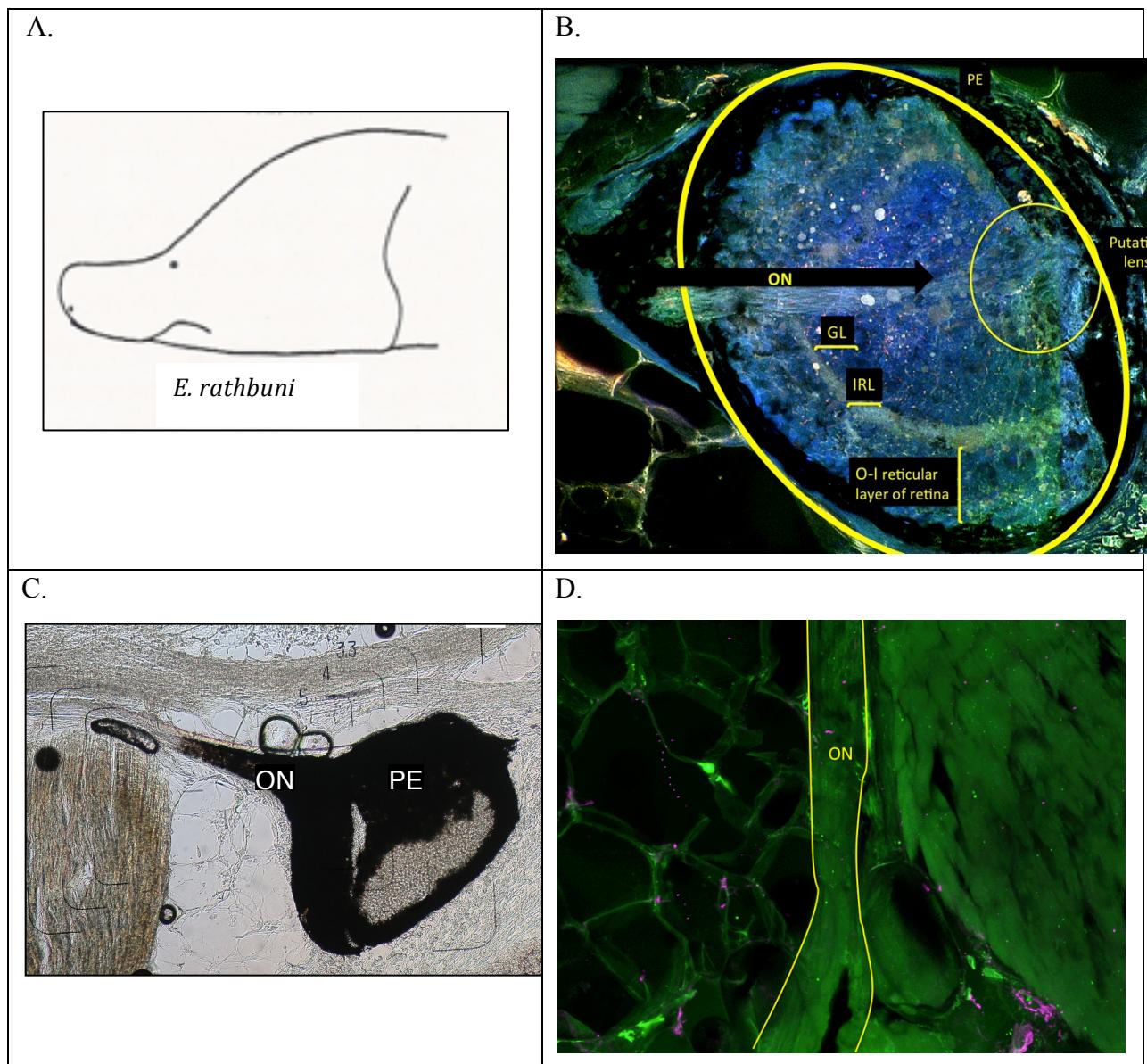


Figure 4. Adult *E. rathbuni* ocular sections. Sketch modified from Mitchell and Reddell, 1965 (A). Showing undifferentiated tissue layers surrounded by pigment epithelium (B&C). Evidence of optic nerve also attached to the posterior region of the vestigial eye (C), and an optic nerve image taken at higher magnification and outlined in yellow (D). Identification of labels is as follows: optic nerve (ON), pigment epithelium (PE), ganglion layer (GL), inner reticular layer (IR), outer and inner reticular layer of the retina (O/I).

CHAPTER II

Embryogenesis and Examination of *pax6* and *Shh* in Eye Development of Two Salamander Morphotypes (*Eurycea rathbuni* and *E. sosorum*)

Introduction

Together, *Eurycea rathbuni* and *E. sosorum* represent two extremes in a continuum of phenotypes exhibited by the south central Texas *Eurycea* clade of salamanders living in a karst environment. The Texas blind salamander (*E. rathbuni*) is considered a stygobiont because it completes its life cycle in the aquatic subterranean habitat of the Edwards Aquifer where it lives in perpetual darkness (Fenolio, 1999). Consequently, *E. rathbuni* exemplifies what is widely accepted as subterranean features, including a broad head, gracile limbs, limited pigmentation and highly reduced eyes (Fig. 2-A). In contrast, the Barton Springs salamander (*E. sosorum*) is considered epigean and is endemic to surface habitats; it exhibits small robust limbs, pigmentation, and well developed eyes (Fig. 2-C). Based on phylogenetic studies the species of the south central Texas *Eurycea* clade share close relationships (Chippindale et al. 2003, and Fig. 1). The two species (*E. rathbuni* and *E. sosorum*) provide a platform for comparing the sequence of events during development that lead to such disparate phenotypic outcomes. These differences are expected to be subtle because the two species are closely related.

Studies carried out on the Mexican blind cavefish (*Astyanax mexicanus*) by William Jeffery and colleagues provide an excellent model for understanding the development of morphotypes with and without eyes (Jeffery, 2009; Alessandro et al., 2007; Jeffery, 2008 and 2005; Yamamoto et al. 2004). The *Astyanax* system comprises a surface morphotype with pigmented skin and fully developed eyes and a cave morphotype lacking pigment in

the skin and an obvious eye. Through their pioneering work, they have shown that development of the eye in the cavefish proceeds to a fairly advanced stage until the developing lens undergoes apoptosis, at which point the retinal development stops and the eye regresses (appropriate references). These changes are accompanied by progressive decreases in the expression of the Pax6 protein and increases in Shh proteins as compared to the expression levels observed at similar stages in development in the surface-dwelling fish (appropriate references). To determine whether similar events occur in *E. rathbuni*, I will track ocular development and the expression of genes integral to ocular development (*pax6* and *shh*) through embryogenesis.

Materials and Methods

Specimens and Staging

Eggs laid by the two species of salamanders were collected from the U.S. Fish and Wildlife Service, San Marcos Aquatic Resource Center (SMARC). Incubation time was recorded and developmental staging followed Duellman and Trueb (1994).

Samples mainly consisted of three three-day intervals post oviposition starting with day 6 (stage 21). Many eggs were preyed upon (mainly by snails found in the aquaria containing adults); therefore, not all stages are represented for the two species. Embryos were preserved in 4% paraformaldehyde, cryoprotected in 30% sucrose, frozen, mounted and sectioned at 10 μm using a Shandon Cryotome at -19°C.

Antibodies, Immunohistochemistry, and Imaging

Immunohistochemistry was accomplished by blocking with bovine serum albumin (Sigma Aldrich, A7030-10G) for two hours then washed three times at ten

minutes each. Each primary and secondary antibody was incubated separately as follows, Pax6 primary, Pax6 secondary, Anti-Shh primary, Anti-rabbit IgG (Anti-Shh secondary). Two fifteen-minute washes were implemented between each incubation period. Finally, the nuclear stain Hoechst was applied last and incubated for one hour, after which the samples were given two fifteen minute washes. All washes were accomplished using PBST (0.05% tween).

Images were obtained using an Olympus FV-1000 scanning confocal microscope. Each of the three lasers were optimized and continuously used to acquire each image, with a 20X water emersion lens. The only exception to this is the image seen in Fig. 7, in which settings were manipulated for a 60 X oil emersion lens.

Results

Development

A description of the development and staging was accomplished using specimens provided by the SMARC. The staging scheme by Duellman and Trueb (1986) was used to identify embryo morphology, and the developmental stages of embryos obtained as well as the days post-oviposition at which the stage was achieved are listed in Table 1. I obtained specimens of *E. rathbuni* at stages including Stage 21 at day 6 post-oviposition. The Stage 21 embryos were defined by having neural folds closed to form neural tube (white line) (Fig. 5, A). Stages 22-23 at day 9 were characterized as having a head, an optic vesicle (star) and a hyomandibular groove (arrow) (Fig. 5, B). Stages 25-26 at day 11 are defined as having a prominent head, ear spot dorsal to the hyomandibular groove (arrow), and 9-10 somites (white outline) (Fig. 5, C). Stage 27 embryos at day 13 have a

visible stomodeum (arrow), mandibular arch (star), and optic vesicle (star) (Fig. 5, D). Stage 29 at day 15 is defined as having the same features at the Stage 27 embryo plus a nasal vesicle (star) and hyoid arch (star) which occurs between the mandibular arch and maxillary arch (Fig. 5, E). Stage 31 at day 17 adds distinct gill folds (white lines) to the features observed in the Stage 29 embryo (Fig. 5, F). At Stage 37-38 at day 34 prominent gill folds, forelimb buds, and pigment migration from neural tube become evident (Fig. 5, G and H). Additionally, a sagittal view of late stage development (Stage 37-38) exhibits a prominent forelimb and hind limb bud, elongated and laterally compressed tail, pigmentation, and prominent eye spot. The spread of pigmentation and concentrated pigmentation of the eye suggest some degree of ocular development (Fig. 5, I1 and I2). I obtained *E. sosorum* embryos at the stages shown in Table 2. Embryonic *E. rathbuni* and *E. sosorum* were obtained at various stages and at various times post-oviposition as detailed in Table 2. In these embryos we observed the hallmark features for the various stages described by Duellman and Trueb (1986) and detailed in the table. Although this study was not designed to compare rates of development between the two species, we observed that *E. sosorum* appeared to reach its respective stages earlier than *E. rathbuni*.

Pax6 and Shh Expression

Expression of Pax6 and Shh proteins is observed in both morphotypes represented by *E. rathbuni* and *E. sosorum*. Five developmental stages of *E. sosorum* were identified and labeled for the gene expression study. Specimens consisted of embryos from stages 21-46, and juveniles (from *E. sosorum* only). Pax6 and Shh protein is observed during early development of *E. sosorum* (Stages 25-26, & 31) with prominent labeling of Shh

protein in the forebrain and in some surface ectoderm cells and Pax6 protein labeling mainly concentrated in three regions of the developing prosencephalon, mesencephalon, and optic vesicle (Fig. 7, A-B). The expression of these genes continues through latter developmental stages 34-46, as defined by day's 25-30 post oviposition (Fig. 7, C-D). At stage 34, Pax6 is heavily expressed in the optic cup while faint expression of Shh is observed (Fig. 7, C). Interestingly, a lens could be identified at this stage and has the highest expression of Pax6 relative to the rest of the eye. The lens is maintained through stage 37-38, and continues to heavily express Pax6 while continued expression in the optic cup, diencephalon, and mesencephalon are observed (Fig. 7, D). During this stage, cells expressing Shh are clearly identified mainly adjacent to the forebrain and the surrounding area. Finally, in a newly hatched juvenile expression of Pax6 is maintained mainly in the lens, and is absent in the remaining developing retina where Shh is observed (Fig. 7, E).

The expression of Pax6 and Shh can also be observed in the specimens representing six developmental stages of *E. rathbuni*. In early development (stage 22-23) expression of Pax6 and Shh is faint and with the exception of Shh not easily seen (Fig. 8, A1-4). At stages 25-26, both Shh and Pax6 are observed with expression of Pax6 greatest in the developing regions of the brain, including the diencephalon, mesencephalon, and the cells migrating to form the optic vesicle (Fig. 8, B1-4) and expressed at a higher level than was observed at Stage 22. The labeling of Pax6 is mainly concentrated in the nuclei of the cells as can be better appreciated when observed at higher magnification (Fig. 9, A-D). Shh protein seems ubiquitous in the early developmental stages, yet specific cells are not heavily labeled, contrasting with late stage development (Fig. 8, E3 and F3). At

stage 27, Pax6 protein is observed maintaining the same spatial expression as in stage 25-26 (Fig. 8, C4). Stage 29, Pax6 expression is increased in the developing brain and optic cup (Fig. 8, D4). During the late stages (37-38, & 40) of development, Pax6 expression has decreased in spatial distribution relative to the above earlier developmental stages (Fig. 8, E4, F4, & G4).

Discussion

The ontogeny, and expression of *pax6* and *shh* during ocular development of two salamander morphotypes differed in that *E. sosorum* maintained expression of Pax6 and Shh through embryogenesis and into a juvenile stage. Decreased labeling of Pax6 was observed during later stages of *E. rathbuni* development, while Shh labeling was increased in a select subset of cells in the head. These results parallel the two morphotypes explored in the *A. mexicanus* (Jeffery 2009). Moreover, this suggests that the salamanders examined in this study, and the teleost fish examined by Jeffery (2009) share a degree of convergent evolution in development and the molecular mechanisms (*pax6* and *shh*) responsible for the final state of vestigial eyes.

Pax6 and Shh Expression

When compared spatially, the expression of Pax6 and Shh proteins through development of *E. rathbuni* and *E. sosorum* is similar and follow what is expected during vertebrate neurulation. Specifically, the genes are expressed in the developing central nervous system, including the brain and eye (Gilbert, 2010). The continued expression of *pax6* and *vax1* genes is important as transcription factors which bind the enhancer

sequence of the δ -*crystallin* gene, which encodes the crystalline proteins found in the lens (Gilbert, 2010). If *pax6* gene expression is down regulated during the development of the lens, the lens will cease to develop. In *A. mexicanus*, the down regulation of *pax6* gene expression contributes to apoptosis of the lens. The elimination of the lens via apoptosis stunts further retinal differentiation and results in the formation of vestigial remnants of retina found in *A. mexicanus* and described by Jeffery (2005, 2008, 2009).

The histology of the adult eye suggests that *Eurycea sosorum* develops a well-organized, functional eye, suggesting the continued expression of Pax6 protein well into the late stages of development. Moreover, in newts, *Cynops pyrrhogaster*, that *pax6* gene expression is persistent through adulthood and plays an important role in regeneration when the animal is subjected to retinal injury (Del Rio-Tsonis et al., 1995). The expression of the *pax6* gene in *E. sosorum* persists through all developmental stages to the latest stage acquired (juvenile), at which point it is concentrated in the lens. The expression of the *pax6* gene in *E. sosorum* follows the canonical developmental expression of a sighted vertebrate. In particular its expression is continued in the juvenile anticipating proper retinal development. The expression of the Shh protein is also observed in *E. sosorum*, as expected in vertebrate development, yet it does not appear to be highly expressed, as would down regulate *pax6* gene expression and impede lens and retinal development.

In *E. rathbuni* the expression of Pax6 protein is noted in early development and is spatially distributed in the developing brain and eye in a pattern similar to that seen in *E. sosorum*. The expression of Pax6 protein strongly persists through most stages with the exception of the later stage at day 34. Moreover, labeling of Pax6 is almost unidentifiable

at day 37, suggesting little expression. Interestingly, during these late stages Shh is highly expressed when compared to the earlier stages of conspecifics. The expression of Shh at days 34 and 37 is associated with select cells with high expression relative to adjacent cells. Some of these cells that express Shh are in close proximity to the developing eye (Fig. 8, E3, F3, & G3). The high expression of Shh during late stage development in *E. rathbuni*, particularly its concentration in specific cells surrounding the eye and forebrain, plus the reduced expression of Pax6, is consistent with down regulation of the *pax6* gene by Shh protein. Thus, the pattern seems to parallel that observed in cave fish (Jeffery, 2008), raising the possibility that down regulation of the *pax6* gene leads to apoptosis and a stunted development of the retina in *E. rathbuni*. I plan to address this question in future studies.

Development

The ontogeny of both species is relatively similar including migration of pigmented cells, cranial development, and limb bud development. Interestingly, the tempo of *E. rathbuni* development seems to be slower than *E. sosorum*. Moreover, organisms that exhibit neotenic or paedomorphic life strategies, such as *E. rathbuni* and *E. sosorum*, develop at a slower rate (Armstrong and Malacinski, 1989). Therefore, it is not surprising that the development of the two species (*E. rathbuni* and *E. sosorum*) is slower than *Ambystoma* as illustrated by Duellman and Trueb (1986). Interestingly, pigment migration through ontogeny is similar between the two species, specifically regarding the cranium, eye, and neural tube. Pigment migration begins soon after the neural tube closes signifying the end of gastrulation and the beginning of neurulation.

Conclusions

I found that the expression of Pax6 declined earlier in *E. rathbuni* and Shh was maintained compared to *E. sosorum*. The expression of these genes suggests their potential for being the mechanisms driving differences in ocular development. Observing Pax6 and Shh proteins in later stages of *E. sosorum* and *E. rathbuni* is needed to understand the completion of retinal development in *E. sosorum* and lens degeneration in *E. rathbuni*. Moreover, later stages would allow understanding of the molecular underpinnings in lens degeneration and specifically address the potential of apoptosis as a means to eye regression as seen in *A. mexicanus*. Importantly, the overall ontogeny and expression of Pax6 and Shh proteins during ocular development of two salamander morphotypes share a parallel with the two morphotypes explored in the *A. mexicanus* (Jeffery 2009). This parallel suggests that the salamanders examined in this study, and the teleost fish examined by Jeffery (2009) share a degree of convergent evolution in development and the molecular mechanisms (*pax6* and *shh*) responsible for the ultimate state of vestigial eyes.

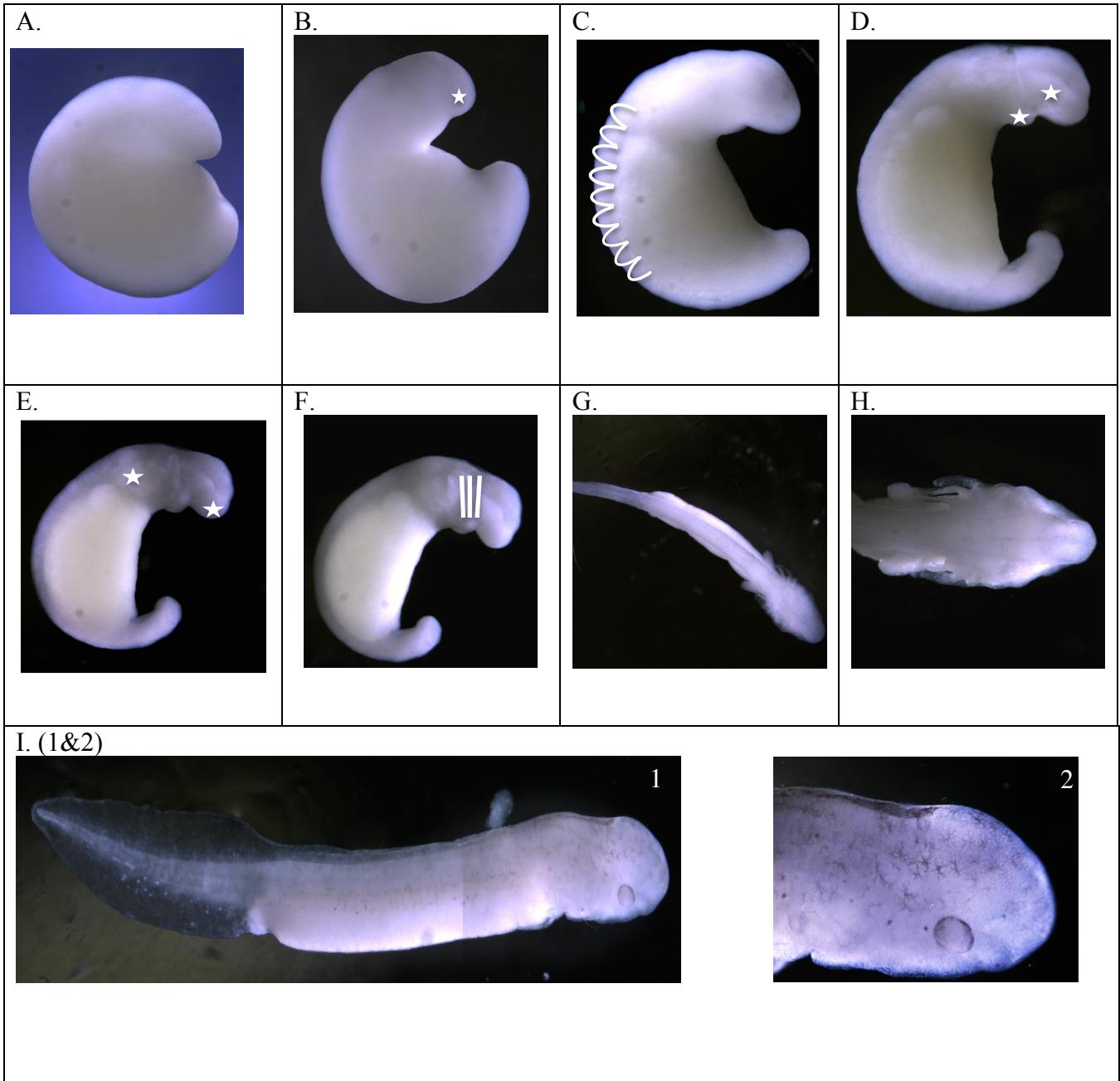


Figure 5. Staging of the Texas blind salamander (*E. rathbuni*). Stages are assigned via morphology, and follow descriptions by Duellman and Trueb (1986). Stages are as follows: Stage 21: Day 6 (A). Stage 22-23: Day 9 (B). Stage 25-26: Day 11 (C). Stage 27: Day 13 (D). Stage 29: Day 15 (E). Stage 31: Day 17 (F). Stage 37-38: Day 34 (G). Stage 37-38: Dorsal view of the head at day 34 (H). Stage 40: Sagittal view of late stage development at day 37 (I1). Stage 40: Sagittal view of cranium at late stage development at day 37 (I2).

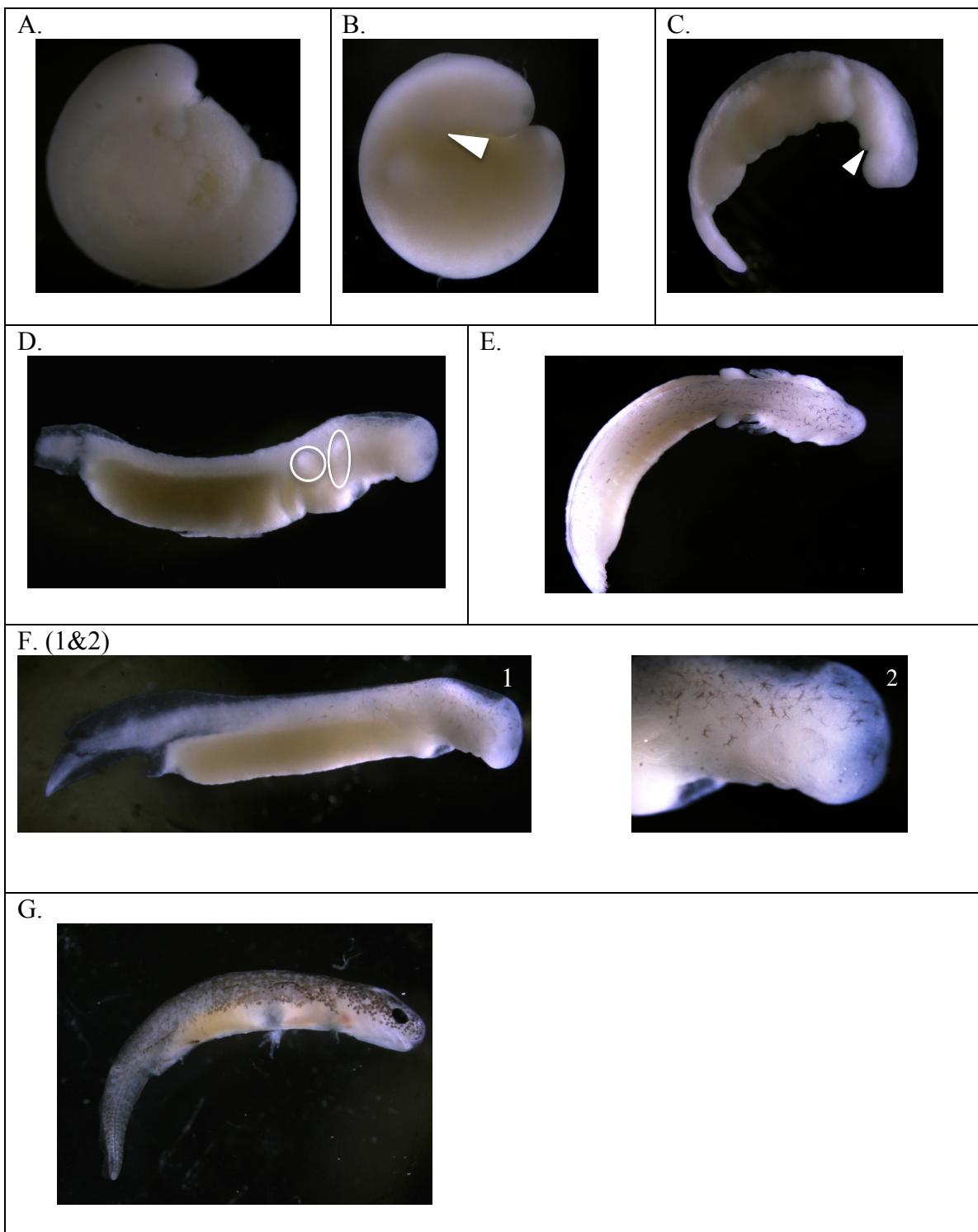


Figure 6. Staging of the Barton Springs salamander (*E. sosorum*). Stages are assigned via morphology, and follow descriptions by Duellman and Trueb (1986). Stages are as follows: Stage 21: Day 4 (A). Stage 25-26: Day 10 (B). Stage 31: Day 13 (C). Stage 34: Day 25 (D). Stage 37-38: Day 30 (E). Stage 40: Day 35 (F1). Stage 40: Sagittal view of the head at day 35 (F2). Stage 46: Yolk completely absorbed; fourth finger bud distinct; juvenile hatchling (G).

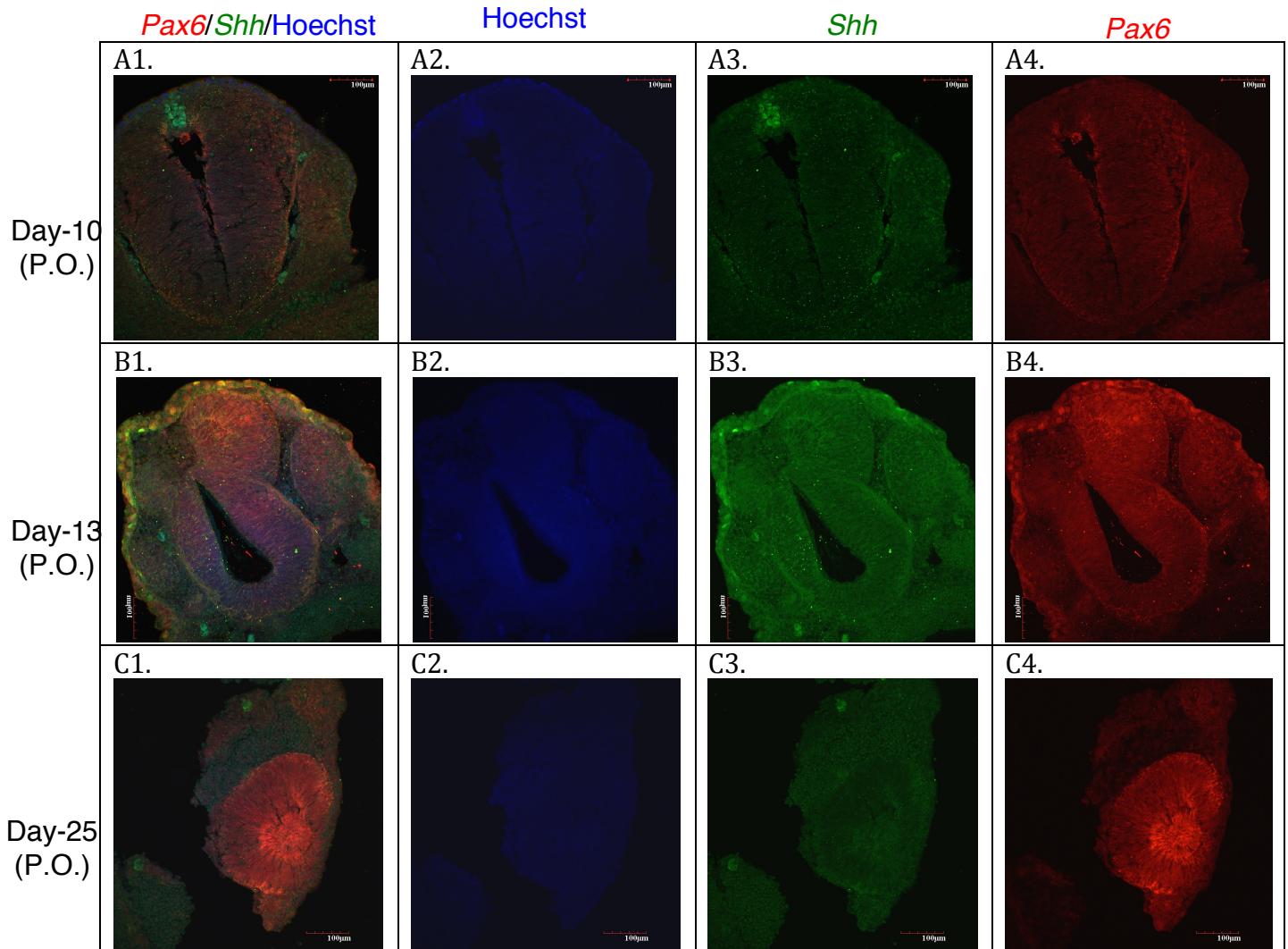


Figure 7. Five stages of *E. sosorum* ocular development with Pax6 and Shh labeling. One stain and two antibodies were used to visualize genes integral to ocular development (A1-E1), and included; Hoechst, nuclear stain (A2-E2), Shh (A3-E3), and Pax6 (A4-E4). Respective days post oviposition (P.O.) on the left and each wavelength split to show labeling.

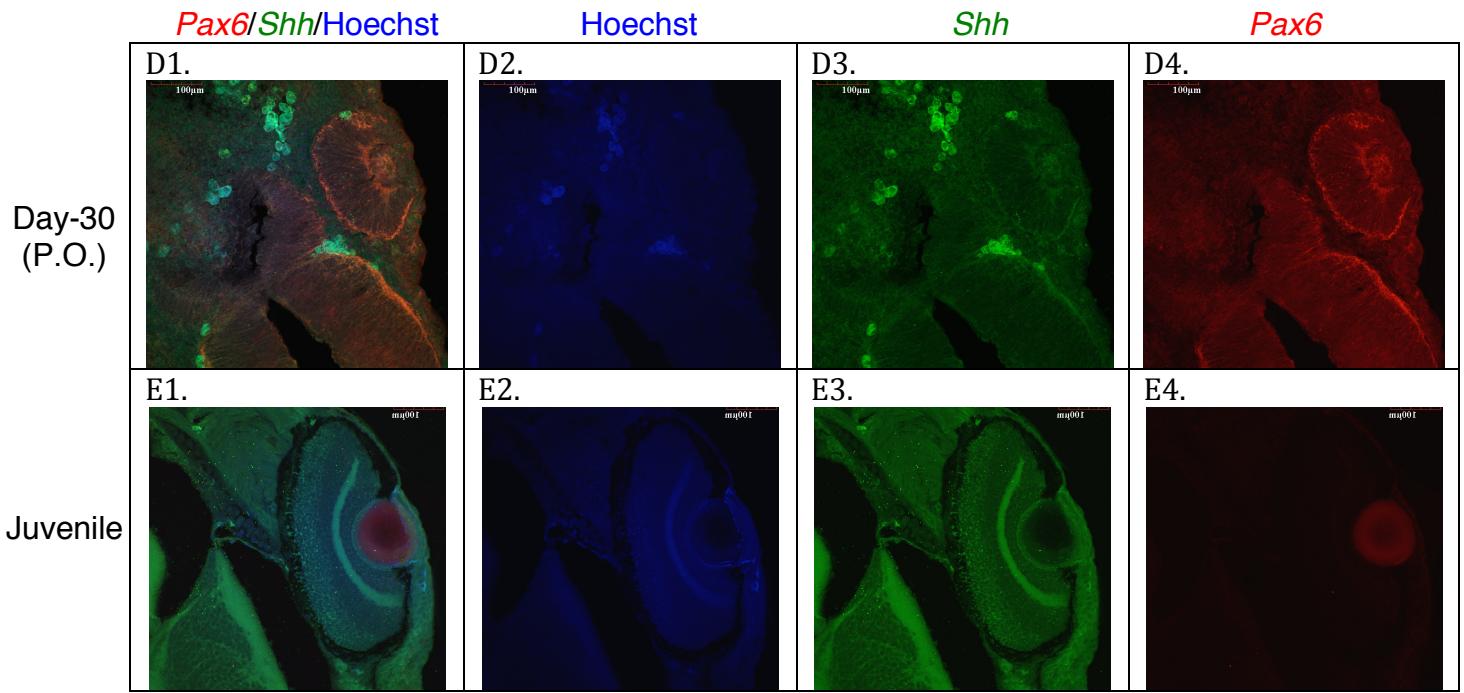


Figure 7. Five stages of *E. sosorum* ocular development with Pax6 and Shh labeling.

Continued: One stain and two antibodies were used to visualize genes integral to ocular development (A1-E1), and included; Hoechst, nuclear stain (A2-E2), *Shh* (A3-E3), and *Pax6* (A4-E4). Respective days post oviposition (P.O.) on the left and each wavelength split to show labeling.

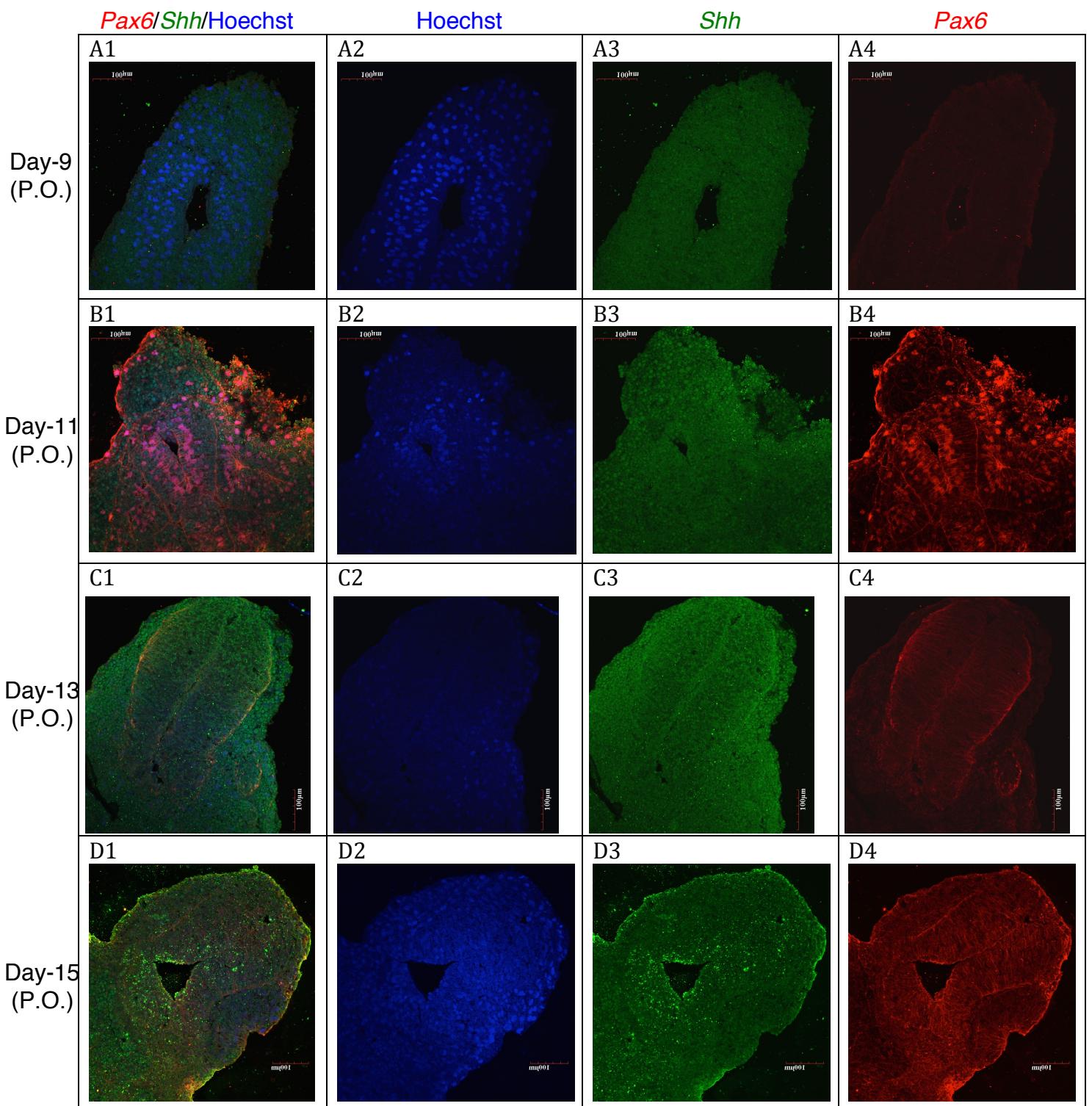


Figure 8. Six stages of *E. rathbuni* ocular development in with Pax6 and Shh labeling. One stain and two antibodies were used to visualize genes integral to ocular development (A1-G1), and included; Hoechst, nuclear stain (A2-G2), Shh (A3-G3), Pax6 (A4-G4). Respective days post oviposition (P.O.) on the left, and each wavelength split to show expression.

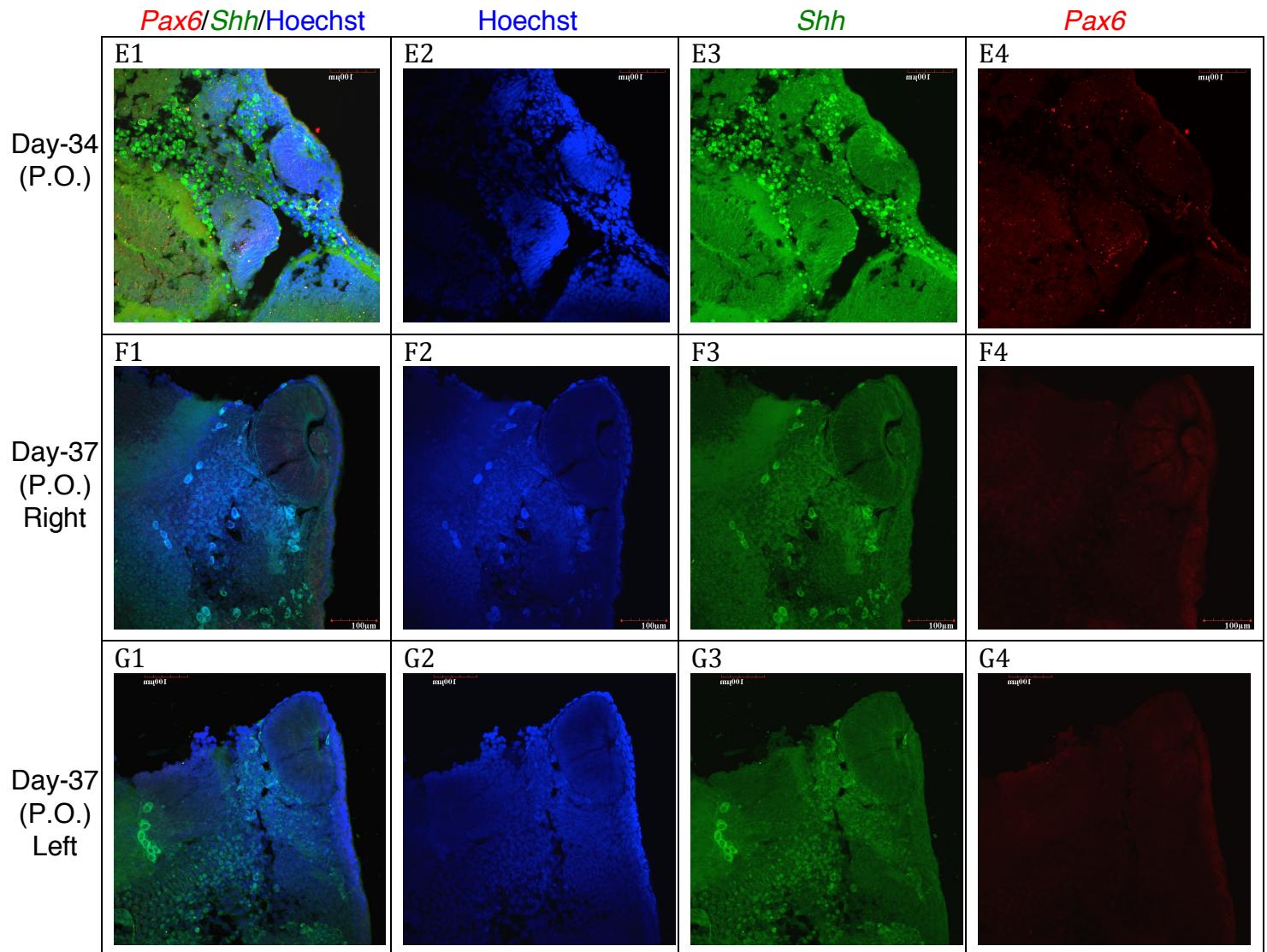


Figure 8. Six stages of *E. rathbuni* ocular development with Pax6 and Shh labeling.

Continued: One stain and two antibodies were used to visualize genes integral to ocular development (A1-G1), and included; Hoechst, nuclear stain (A2-G2), Shh (A3-G3), Pax6 (A4-G4). Respective days post oviposition (P.O.) on the left, and each wavelength split to show expression.

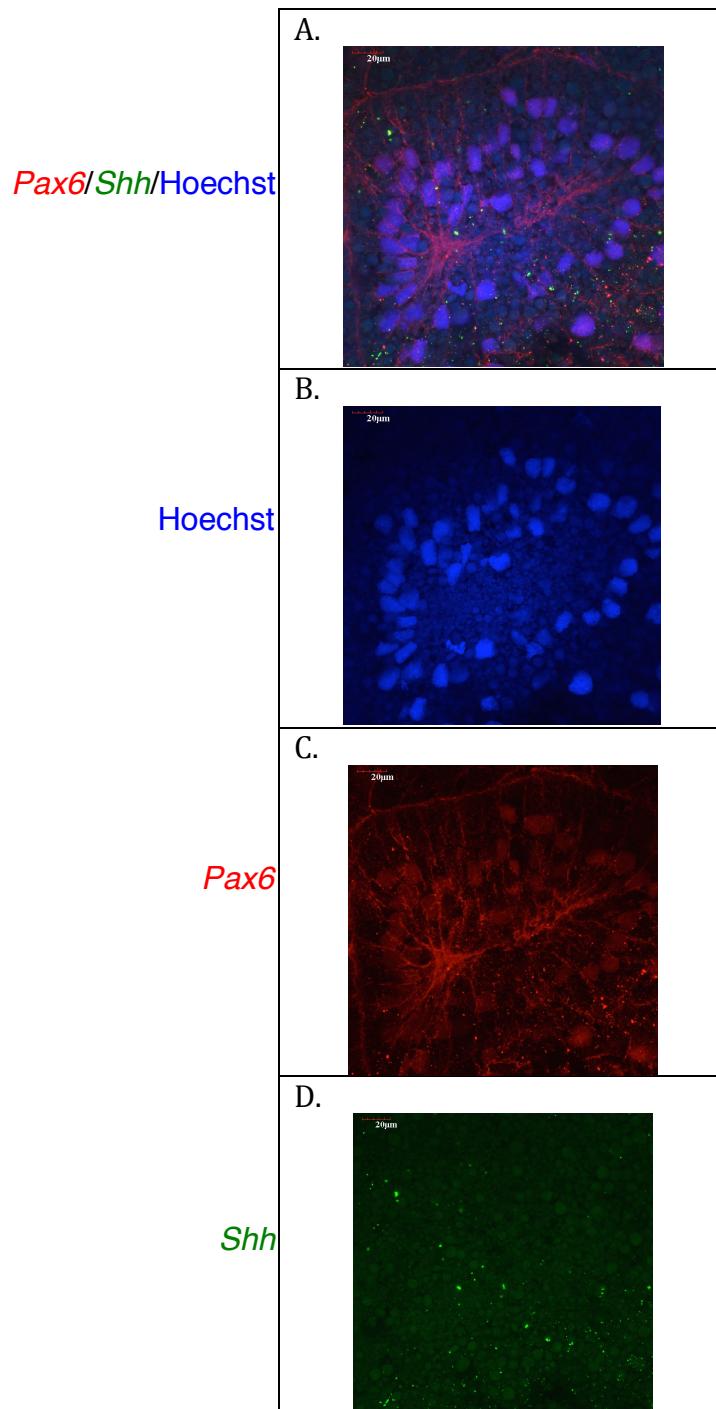


Figure 9. Day 11, stage 25-26 of ocular development in *E. rathbuni* with Pax6 and Shh labeling. One stain and two antibodies were used to visualize genes integral to ocular development, and included; Hoechst, a nuclear stain (B), Pax6 (C), and Shh (D). Respective days on the left and each wavelength split to show expression. Note the co-localization of Pax6 and Hoechst (A), suggesting Pax6 expression in the nucleus and reduced labeling of Shh (D).

Table 1. Stage description and number of specimens. Stage number as reference to Duellman and Trueb (1986), corresponding morphological description, and number of days post oviposition (p.o.). Note that representative stages were not acquired for every species due to difficulty of breeding and paucity of adult specimens.

Stage, and corresponding figure	Description	Number of days post oviposition (p.o.)	
		<i>Eurycea sosorum</i>	<i>Eurycea rathbuni</i>
21 (Fig. 5, A) (Fig. 6, A)	Neural folds close to form neural tube, and the beginning of neurulation.	4 (n=4)	6 (n=5)
22-23 (Fig. 5, B)	Head with optic vesicle, hyomandibular groove.	N/A	9 (n=3)
25-26 (Fig. 5, C) (Fig. 6, B)	Prominent head with; ear spot dorsal to hyomandibular groove, 9-10 somites.	10 (n=3)	11 (n=5)
27 (Fig. 5, D)	Stomodeum appears, mandibular arch, optic vesicle.	N/A	13 (n=5)
29 (Fig. 5, E)	Stomodeum,mandibular arch, optic vesicle + nasal vesicle, hyoid arch.	N/A	15 (n=4)
31 (Fig. 5, F) (Fig. 6, C)	Stomodeum, mandibular arch, optic vesicle, nasal vesicle, hyoid arch + gill folds becoming distinct.	13 (n=6)	17 (n=3)
34 (Fig. 6, D)	Prominent gills and forelimb buds	25 (n=6)	N/A
37-38 (Fig. 5, G and H) (Fig. 6, E)	Prominent gills and forelimb buds, and pigment migration from the neural tube becomes apparent.	30 (n=5)	34 (n=15)
40 (Fig. 5, I) (Fig. 6, F)	Forelimb bud prominent, hind limb bud prominent, elongated and laterally compressed tail, pigment migration ventrally and prominent around the eye.	35 (n=6)	37 (n=2)
46 (Fig. 5, G)	Yolk completely absorbed; fourth finger bud distinct; Newly hatched juvenile.	40 (n=3)	N/A

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