

**NOVEL CHARACTERISTICS OF THE CULTURED
LUMPFISH CYCLOPTERUS LUMPUS EYE DURING POST-HATCH LARVAL
AND JUVENILE DEVELOPMENTAL STAGES**

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A thesis submitted to the School of Graduate Studies
in partial fulfillment
of the requirements for the degree of Master of Science

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Submitted October 2019

St. John's Newfoundland and Labrador

Abstract

The lumpfish *Cyclopterus lumpus* (*C. lumpus*), superorder teleostei, is an ocean fish that relies on healthy vision in the wild and in culture to see and eat prey or feed. Although the use of *C. lumpus* in aquaculture is growing and there is reason to believe that this fish can be used as a model system for visual research, the visual system of post hatch developing *C. lumpus* has not been studied in detail. To that end, we systematically analyzed the characteristics of the *C. lumpus* eye and retina during cultured post hatch developmental stages. Post hatch developing cultured *C. lumpus* eye and retinal tissues share a number of features typically conserved in other teleost fish. However, cultured *C. lumpus* possess some novel ocular and retinal features different from previous descriptions of other teleosts, including a prominent retractor lentis pigmented tissue closely associated with the vascular rete mirabile, peripherally located lobes of separate retinal tissue containing proliferative cells, extensive tapetum material of varying thickness, prominent fundus stripes and an elongated rod-shaped optic nerve stalk, all observed through gross observation, histology, fundus imaging and spectral domain optical coherence tomography (SD-OCT) retinal imaging. . Immunohistochemical staining using alpha smooth muscle actin and proliferating cell nuclear antigen revealed that post hatch developing cultured *C. lumpus* also developmentally regulate a protein homologous to alpha smooth muscle actin in strikingly dense continuous bands in the plexiform layers of the retina. The novel features of the eye and retina of cultured *C. lumpus* described here could contribute to our understanding of fitness and survival of *C. lumpus* in a widely ranging habitat and have implications in human visual health and disease.

Acknowledgments

I would like to thank my supervisors, Dr. Robert Gendron and Dr. H el ene Paradis, for guiding me, teaching me and mentoring me throughout my masters. They have helped me develop a better understanding of my own abilities and interests and have made this a great learning experience. I would like to thank Dr. William Driedzec for his guidance during the completion of the master's project and his valuable input on the thesis as a member of my Supervisory Committee. I would like to acknowledge the Faculty of Medicine for funding me through this project.

I would also like to thank my fellow student, Cassandra Davidson for keeping me company, guiding me and providing feedback and support in the lab every day. I would like to acknowledge Lidan Tao for helping me develop an understanding of High-Performance Liquid Chromatography and for her willingness to help me through the learning process.

Finally, I would like to thank my mother Raana Ahmad and my father Ahmad Raja for always supporting me, motivating me and pushing me to achieve my goals and to continuously work hard. I would also like to thank my siblings for always being a positive encouragement and support and for always being there for me.

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List of Abbreviations

3D	3-Dimension
ANOVA	Analysis of Variance
AP	Alkaline Phosphatase
ASMA	Alpha Smooth Muscle Actin
C. lumpus	Cyclopterus Lumpus
CDRF	Cold Ocean Deep Sea Research Facility
CMZ	Ciliary Marginal Zone
Dph	Days post hatch
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
FGF	Fibroblast Growth Factor
GCL	Ganglion Cell Layer
IACC	Institutional Animal Care Committee
IHC	Immunohistochemistry
JBARB	Joe Brown Aquatic Research Building
OD	Oculus Dextrous
OS	Oculus Sinister
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
RNA	Ribonucleic Acid
SD-OCT	Spectral Domain Optical Coherence Tomography

TBS	Tris Buffered Saline
TGF	Transforming Growth Factor
VIP	Volume Intensity Projection

CHAPTER 1

Introduction and Overview

1.1 Vision and the Eye

Vision is one of the most important of the five senses that allow organisms to interact and communicate with their surrounding environment. The ability to see greatly enhances an organism's survival as it allows for detection of danger from a distance and helps locate food and/or prey for consumption. The eye is the organ used for vision. In *Homo sapiens*, it is a small structure, of about 7g in weight and 2.5cm in diameter that performs this complex and important task (Kwitko & Ross, 1994).

1.1.1 Eyes

Eyes are the organ that process external visual stimuli and send it as electrical signals to the brain. The eyes are made up of several layers of tissue (Figure 1.1). The outermost layer of the eye, which is the initial site of entry of light, is known as the cornea. The cornea serves to protect the eye, maintain its shape and is the site where focusing of light begins. It also protects from damage and acts as a barrier to dust and other particles. It is a normally avascular structure made of five layers of tissue that contains small nerve fibers (Kwitko & Ross, 1994). Behind the cornea is the anterior chamber, which holds the aqueous humour. This functions to keep the anterior portion of the eye suspended through pressure, preventing collapse. The iris sits within the anterior chamber and is a muscular structure responsible for controlling the amount of light entering the eye by changing the size of the pupil. It is a vascular structure composed of

pigment cells, which result in differences in eye colour. The pupil is an aperture that lies at the center of the circular iris and is the site of entry of light into the posterior eye. The pupil's size adjusts to changes in light; when in bright light, the iris muscles relax, constricting the pupil to reduce the amount of light entering the eye. Conversely, in dim light, the iris muscle contracts, dilating the pupil to increase the amount of light entering the eye.

Once light passes through the pupil, it hits the lens. The lens is a round, transparent structure responsible for focusing light rays. It is made up of a lens capsule, a lens epithelium and lens fibers and is held in place by the ciliary body. In mammals, it focuses light when ciliary muscles contract or relax the lens, thereby changing its shape through a process known as accommodation. This focuses the image based on the distance of the object.

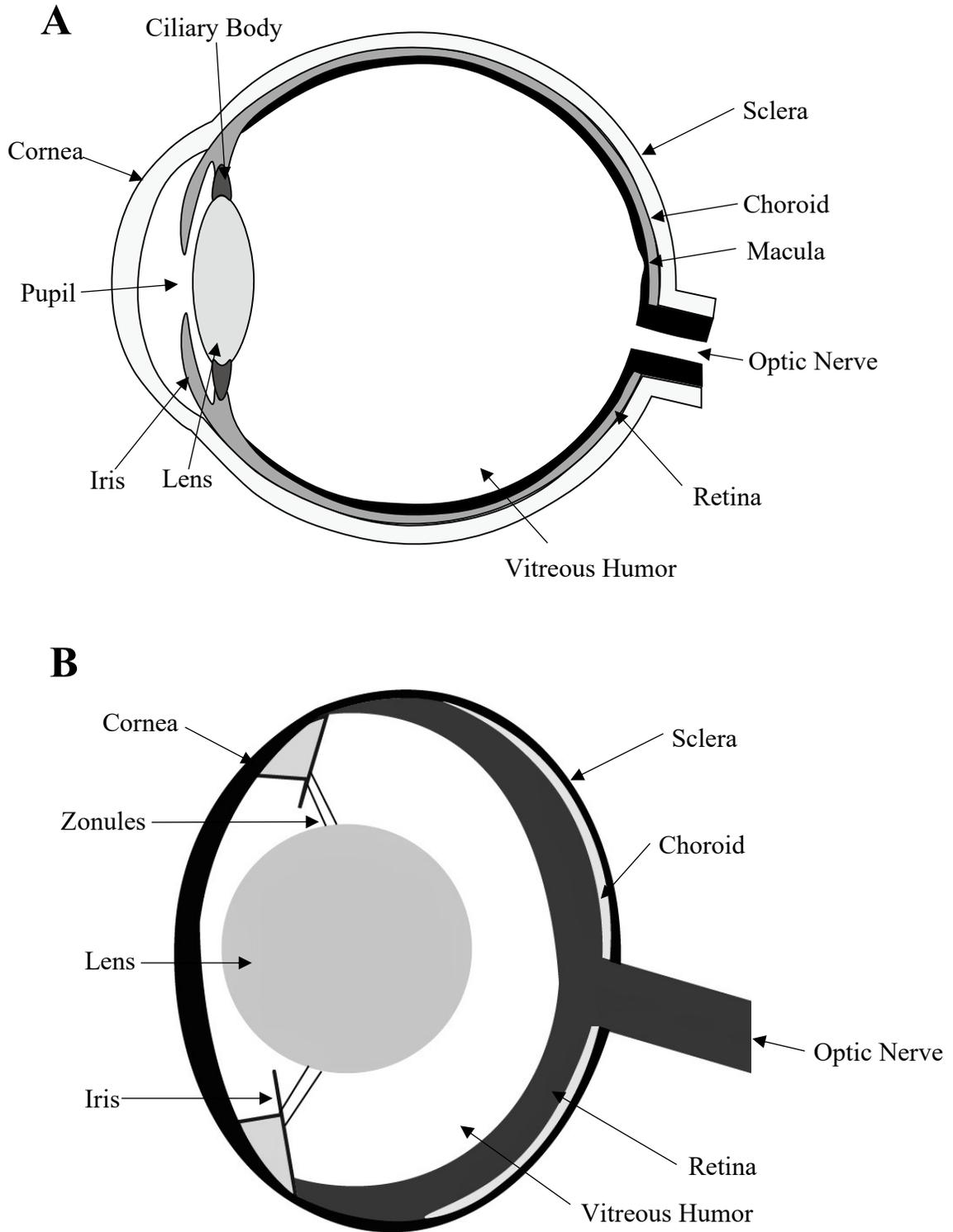


Figure 1.1 Gross anatomy of the (A) human eye versus the (B) zebrafish eye. Anterior structures in the human eye include the cornea, pupil, iris, lens and ciliary body while posterior structures include the vitreous humour, choroid, macula, optic nerve, retina and

sclera. Most anterior and posterior structures including the cornea, iris, lens, vitreous humor, retina, optic nerve choroid and sclera are conserved in the zebrafish eye, although the shape and relative sizes of each structure vary between organisms. The zebrafish possess zonules instead of ciliary bodies to hold the lens in place (original illustration).

After being focused through the lens, light is projected to the posterior retina. It travels through the vitreous humour, which is a transparent jelly like tissue that fills the cavity behind the lens, known as the vitreous body (Kwitko & Ross, 1994). The light then hits the retina, which contains cells known as photoreceptors harboring light sensitive pigments. These photoreceptors generate electrical signals upon receiving light, which are transmitted to the brain, creating an image. The retina is made up of several layers. The optic nerve sits between the retina and is made up of nerve fibers that transmit the electrical signals from the retina to the brain. Behind the retina is the choroid, a vascular layer that nourishes the retina. It is composed entirely of blood vessels and is integral in the blood retina barrier (Kolb et al., n.d.). In mammals, choroidal blood supply accounts for 80% of the blood source for the retina while the remaining 20% is accounted for by retinal vessels (Alm & Bill, 1972). The choroid also plays a role in maintaining a stable temperature in the macula within mammalian eyes (Parver, 1991). The sclera is the tough, white outer part surrounding the eye. It is made of collagen and elastin and serves a protective role in the eye. The conjunctiva sits on top of the sclera. It is a thin transparent film full of vessels that sits on the inside of the eyelid, protecting the sclera (Kwitko & Ross, 1994).

1.1.1.1 Retina

The retina is the light sensing portion of the eye derived from the neural tube, making it a part of the central nervous system. It is made up of seven distinct layers, and signals are transduced in the opposite direction of the movement of light. Light moves to the photoreceptor outer segment of the retina. This layer is made up of rods and cones, which contain photosensitive pigments (normally a vitamin A derivative) that absorb light and generate an electrical signal through the visual cycle (Kolb et al., n.d.). Posterior to the photoreceptor outer segment layer is the retinal pigment epithelium layer, which serves to support the photoreceptors by absorbing scattered light and maintaining the structural integrity of the retina (Strauss, 2011). The electrical signal moves down into the outer nuclear layer, which contains the nuclei of the photoreceptor cells. It then moves down into the outer plexiform layer, which contains the synapses between the end of the rod and cones and the dendrites of accessory cells such as the bipolar, horizontal and amacrine cells. The signal is then transmitted into these accessory cells, and moves to the inner nuclear layer, which is made up of the nuclei of the accessory cells. The signal is transmitted downstream to the inner plexiform layer, containing the synapses between axons of bipolar cells and dendrites of the ganglion cells. From here, the signal moves into the ganglion cell layer, composed of the cell bodies and nuclei of ganglion cells. The axons of the ganglion cells then converge at the optic nerve head, where the signal moves into the optic nerve and is sent to the brain (Kolb et al., n.d.).

The retina is a complex tissue, requiring constant nourishment, which it receives from two sources; the central artery and the choroidal blood supply. The central artery

enters the eye through the optic nerve and is responsible for nourishing the inner retina while the choroidal supply lies just peripheral to the retinal pigment epithelium, nourishing the outer layers of the eye (Kwitko and Ross, 1994).

1.1.2 Anatomical Development of Eye

Early development of the eye begins during gastrulation, with the formation of an eye field in the forebrain. Within the neural folds, two grooves, known as optic sulci, begin to develop on either side of the forebrain during neurulation (Sinn & Wittbrodt, 2013). These develop into out pocketing vesicles known as optic vesicles when the neural tube closes. The optic vesicles grow to form the optic stalks, which during blastulation, differentiate into the optic nerve. Upon interaction with the ectoderm, parts of the optic vesicle thicken to form a lens placode (Graw, 2010). Invagination of the lens placode causes the formation of the lens pit, which form the lens vesicle. The optic cup forms at the same time with the invagination of the optic vesicle, creating a double layered structure. A choroidal or optic fissure is a groove that forms on the inferior surface of the optic vesicle and optic stalk. This fissure provides access to blood vessels, including the hyaloid artery and branches of the ophthalmic artery and vein. Normally, the choroidal fissure fuses to allow the formation of a complete eye wall.

Ocular development relies on some key genes for normal formation of eyes. Pax6 is a critical transcription factor required in this process. During blastulation, sonic hedgehog (shh) suppresses the *PAX6* gene and upregulates *PAX2*, resulting in the division of the eye field into the two optic sulci. During gastrulation, budding of the optic vesicle

causes secretion of BMP4, inducing formation of the lens placode by differentiation of the ectoderm (Graw, 2010). Pax6 is essential for the induction effect of BMP4 on the ectoderm. Once the lens placode forms, it secretes growth factors such as FGF, which induce differentiation of the optic vesicle into the optic cup. Formation of the lens vesicle from the lens placode causes release of growth factors that induce formation of the neural retina in the optic cup wall. At this time, the ectoderm is also induced to form the cornea. Once formed, the neural retina secretes growth factors to induce cells of the lens vesicle to elongate into lens fibers. Simultaneously, secretion of transforming growth factor (TGF) from the mesenchyme around the optic cup induces formation of the retinal pigment epithelium, choroid and the sclera (Sinn & Wittbrodt, 2013).

While development of an optic cup is a complex process *in vivo*, recent studies have shown the possibility of optic cup morphogenesis in three-dimensional embryonic stem cell culture systems using serum free embryoid-body-like-aggregates, indicating that the multiplexity of this developmental process can be recapitulated, at least in part (Eiraku et al., 2011; Nakano et al., 2012).

1.2 Mammalian versus Teleost Eyes

Teleosts, a large diverse group of ray finned fishes that fall under the class Actinopterygii, are the most advanced bony fish that comprise about 96% of all fish (Mcmenamin & Parichy, 2013). Many teleosts are visually guided and rely on their vision for survival. While teleosts appear physically different from mammals, many anatomical structures, skeletal elements and developmental genes are highly conserved within these

two groups that fall under the kingdom animalia (Figure 1.1) (Witten et al., 2017). The most well studied teleost is the zebrafish (*Danio rerio*) as it is a good model organism for studying human growth, development and disease. The entire zebrafish genome has been sequenced to high quality and zebrafish share about 70% of human genes. Zebrafish models have been successfully used to study health diseases, some of which include diabetes, retinal degeneration, neurodegenerative diseases and cardiovascular disorder. (Meyer's, 2018). Thus, a vast majority of the current knowledge on teleost eyes comes from studies of zebrafish eyes. Many of the anterior and posterior structures including the cornea, lens, iris, vitreous, retina, choroid, sclera and optic nerve are conserved between teleosts and mammals (Figure 1.1). The retina is also comprised of the same seven layers described previously. These anatomical similarities, shorter life spans, larger growth spurts, and eyes that grow throughout life make teleosts a great model to understand the development and study the diseased states of eyes (Gestri et al., 2013). However, there are some differences in teleost eyes in comparison to mammalian eyes that allow them to adapt to and improve vision in their aquatic environment.

One major difference is that of the choroid and blood vessels in teleosts. The posterior retina of most teleost eyes is avascular, so teleosts rely on choroidal vasculature for nutrient and oxygen supply (Walls, 1942; Waser & Heisler, 2005). Teleosts possess a rete mirabile, which is a choroid body that sits behind the retina, below the sclera. In addition to acting as a blood supply for the retina, the rete mirabile also helps to elevate oxygen tension in the retina as it is a counter current system (Copeland, 1974).

Another structure present in teleosts not seen in mammals is a falciform process. The falciform process, found on the vitreal surface of the eye, is a pigmented tissue that is

a continuation of the choroid epithelium, which sits posteriorly on a region of the retina and serves to nourish the interior eye. The falciform process emerges as an invagination of the choroid epithelium during development and is associated with the embryonic fissure in several teleosts (James et al., 2016). In some species such as brown trout, both the embryonic fissure and falciform process persist into adulthood while others such as guppies lose the embryonic fissure during embryonic development (Kunz & Callaghan, 2011). The falciform process has been considered to impact visual orientation at Snell's window, which is a 97° wide circular field of light displaying the entire 180° view of the surface above water, as well as playing a role in detecting polarized light (Reckel & Melzer, 2004; Kunz & Callaghan, 2011; Lenz et al., 1996).

The retractor lentis muscle is another structure found in teleosts that is not present in mammalian eyes. The lens of teleost is round and heavy, and due to the large size of the pupil, it cannot be held by the iris alone. The lens is held in place by a tendon of a muscle known as the retractor lentis muscle in the equatorial plane and on the dorsal side by a suspensory ligament instead of the ciliary bodies as in mammals. The retractor lentis muscle is also responsible for accommodation in the teleost eye, wherein the relaxed lens state adjusts for near vision (Khorramshahi et al., 2008).

The ciliary marginal zone is a region of retinal progenitor cells found at the periphery of the mature retina of fish, amphibian and some bird eyes (Fischer et al., 2013). This ring of cells allows the eyes and retina of these organisms to grow continuously throughout life. This is the region of growth, which gives rise to most of the retina once the retinal neuroepithelium has differentiated. The ciliary marginal zone is

also important in regeneration of the retina by replacing damaged cells (Fischer et al., 2013). The peripheral retina of mammals also contains retinal stem cells, but they do not appear to have great regenerative potential, if any (Marcucci et al., 2016).

Finally, teleosts eyes possess a structure known as the tapetum lucidum, which is a guanine crystal structure present in the anterior eye that reflects light back into the eye once passed, allowing the retina to capture more light. This structure is present in many nocturnal animals as well as deep sea fish to improve vision in the dark. There are two types of tapeta characterized in teleosts; a retinal tapeta and a choroidal tapeta (Somiya, 1980).

1.2 Lumpfish

Lumpfish *Cyclopterus lumpus*, Linnaeus, 1758 (Scorpaeniforme, Cyclopteridae) are predatory teleosts that rely on their vision for survival. A growing industry in Newfoundland and across the world, lumpfish aquaculture provides an ever-increasing supply of lumpfish for their various uses, for the use of their roe as caviar and as biological cleaning agents in fish farms. Of these, their use as cleaner fish in salmon fish farms is becoming of utmost importance with the growth of the fish farming industry.

1.2.1 Habitat and Use

Lumpfish are an oceanwater teleost that can be found in the coastal waters of Newfoundland. Ranging from 40 to 60cm in width, these fish are short and hard, with tubercles protruding from their body (Fisheries and Oceans Canada, 1996). They vary in colour from blue-grey to green-yellow while breeding males become red during breeding

season. Their dorsal fin arises from a cartilaginous hump on the back. On the ventral side, lumpfish have a disc that helps them adhere to surfaces instead of ventral fins.

Lumpfish span a wide variety of habitats. In North America, they can be found on both sides of the North Atlantic from North America to Europe. In North America, their habitat extends from Hudson's Bay in the North and Chesapeake Bay in the South.

Lumpfish are semi pelagic fish that spend most of their lifetime in deep waters and a part of their life in shallow water. In the winters, they are found at depths greater than 300m, in the mesopelagic zone, where there is very little penetration of light (Fisheries and Oceans Canada, 1996). Between spring and summer, during the breeding season, lumpfish prefer shallow waters and are found in the epipelagic zone, where there is an abundance of light. In the fall, they return back to deeper waters until the next breeding season. They prefer rocky regions where they are able to adhere easily to the surface and hide below seaweed (Fisheries and Oceans Canada, 1996).

Lumpfish feed on jellyfish, shrimp, pelagic amphipods, copepods, worms, sea lice and small fish such as herring and sand lance. At certain stages, lumpfish can also become cannibalistic. Up to 25 days post hatch, cultured lumpfish are reared onto a pelleted diet by adding increased amounts with artemia into their diet (Personal Communication: Danny Boyce). As of November 2017, lumpfish have been listed as a threatened species by the government of Canada in the Atlantic Ocean and the Canadian range. They are not as yet classified as endangered as they are not at an immediate risk of endangerment and recolonization is deemed possible (Lorance et al., 2015; Committee on the Status of Endangered Wildlife in Canada [COSEWIC], Government of Canada).

1.2.2 Model Organism

With growing need for aquaculture and fish farming, the use of lumpfish as biological cleaning agents is increasing. The availability of lumpfish in aquaculture makes them easily available for research purposes. With a short life span of about 4 years and production of new egg masses of hundreds of fish, lumpfish are an easy model to study growth and development over their lifetime. To date, lumpfish research is relatively scarce, and their development and anatomy is largely uncharacterized in literature thus far. The recently discovered use of lumpfish as cleaner fish leads to questions about understanding how these fish grow and develop, particularly the anatomy and functionality of their eyes as they are visually guided organisms. Since many structural and developmental features in teleost and mammalian eyes are conserved, teleosts are often used as model organisms to study growth, development and disease. Understanding the structure of the lumpfish eye will provide insight into the previously unknown structure of lumpfish eye and may aid in restoring the population of this threatened species in the Atlantic as they are a visually guided species that rely on vision for survival (Ahmad et al., 2018). An in-depth analysis of few animals will help in understanding the structure of lumpfish at a wide range of ages and to see differences in visual structure and development in developmental and adult fish. For the scope of this project, using a small number of fish at various developmental stages will allow for identification of structures present in the eye and whether these structures change with development. Using techniques such as histology will provide the best possible way to visualize the anatomical and physiological structure of the lumpfish eye at a microscopic level to understand structures seen through observation of gross anatomy of the lumpfish eye

(Gartner, 2017). Immunohistochemical staining will be used to identify and localize specific proteins that, for the purpose of this study will determine the presence of specific structures (Gartner, 2017). Alpha smooth muscle actin (ASMA) is a cellular marker that can be used to label the smooth muscle cells within blood vessels (Wall et al., 2004). Immunohistochemical staining with this marker has been used for detection and localization of blood vessels within the eye in the past and will be used for blood vessel identification in this study (Wall et al., 2004). Another cellular marker that will be used in this study is proliferating cell nuclear antigen, which is a marker for cell growth and proliferation (Gendron et al., 2012). As the retina of many non-mammalian species appears to grow throughout development, use of this marker will determine whether the same is true of lumpfish eyes. Spectral-Domain Optical Coherence Tomography (SD-OCT) is a newer imaging technique which produces similar images as histological analysis of the retinal layers and also allows for visualization of blood vessels. This technique, which is much quicker than histological analysis, has not previously been used on lumpfish eyes. Using this technique will determine whether a faster method of visualizing the lumpfish eye is possible and will corroborate structure seen through histology. Fundoscopy will be used to determine the structure of the posterior eye.

Co-authorship Statement

All individuals listed on the manuscript have satisfied the criteria for authorship. A version of this manuscript has been published in *The Journal of Fish Biology* on December 19th, 2018 (J Fish Biol. 94(2):297-312. doi: 10.1111/jfb.13892.). The Journal of Fish Biology has granted permission for use of figures within this thesis. The roles of the authors were as follows: Raahyma Ahmad had primary responsibility for this thesis. Raahyma Ahmad participated in the intellectual development of the project, collaboratively designed the study, performed all histology and immunohistochemistry, collected and analyzed the data, prepared the manuscript and participated in final approval of the version to be published. Dr. Robert Gendron and Dr. H  l  ne Paradis, as co-investigators on the project, participated in the intellectual development of the project, collaboratively designed the study, performed and analysed ocular imaging, collected and analysed data and prepared and revised the manuscript. This project was in collaboration with Danny Boyce, Manager of the Dr. Joe Brown Aquatic Research Building, who was responsible for husbandry and care of fish used, assisting with sample collections, writing portions of the manuscript as well as providing valuable knowledge and expertise on the organism. James McDonald designed and produced a valuable instrument for the specific use of visual research in this project.

Chapter 2

NOVEL CHARACTERISTICS OF THE CULTURED

LUMPFISH *CYCLOPTERUS LUMPUS* EYE DURING POST-HATCH LARVAL AND JUVENILE DEVELOPMENTAL STAGES

INTRODUCTION

Lumpfish *Cyclopterus lumpus* L. 1758 is an ocean fish that relies on a healthy visual system for fitness, to hunt prey, or, in the case of aquaculture, to see and eat pelleted aquaculture diets. *Cyclopterus lumpus* eggs are harvested for human consumption and *C. lumpus* have more recently been used as a biological delousing agent in aquaculture (Imsland et al., 2014). Post-hatch *C. lumpus* are visually guided predators and feed on shrimp, crustaceans, jellyfish, worms and other fish (Ingólfsson et al., 2002).

Cyclopterus lumpus are known mainly as demersal bottom-dwelling fish but can also exist semipelagically and have been reported in Newfoundland trawler nets from mesopelagic depths of > 300 m at which sunlight levels are significantly low enough to prompt questions about the animal's visual capability in such conditions (Scott & Scott, 1988). *Cyclopterus lumpus*, which do not possess swim bladders, are now known to travel up from the bottom of the water column from day to night, respectively. Males are also now known to play a role in protecting eggs (Kudryavtzeva, 2008). In addition, *C. lumpus* spawning is thought to be dependent upon seawater temperature and optimal at 4C (Scott & Scott, 1988), suggesting that these fish might travel through a range of depths to reach optimal spawning zones. Some deep-sea fish that continuously live in very dark conditions display specific morphological ocular adaptations that confer advantages to

their visual abilities in low light (Partridge et al., 2014; Wagner et al., 1998, 2009). Since *C. lumpus* habitat might vary widely, including habitation of deeper water, one might expect that these fish have developed unique visual system adaptations that would confer specific survival advantages in the various habitats they frequent.

Eye anatomical structure in teleosts in general is very similar to mammalian eye anatomical structure. This similarity includes a multi-layered retina with photoreceptors and retinal pigment epithelium, intermediate neural retinal layers, ganglion cells converging on an optic nerve tract to the visual cortex of the brain, a lens, iris, ciliary body and anterior structures such as cornea. In fact, the zebrafish *Danio rerio* (Hamilton 1822) eye is considered a well-established model system for experimental studies relevant to mammalian and human eye (Hitchcock & Raymond, 2004). However, some teleosts have evolved novel ocular structures that probably optimise vision in the various habitats each of these species frequents (Kunz & Callaghan, 1989; Partridge et al., 2014; Wagner et al., 1998, 2009).

There is, to our knowledge, very little literature on eye and retinal morphology during post-hatch *C. lumpus* development at the histological or live imaging level. To that end, we undertook the present study as a first step to systematically analyse the characteristics of the cultured *C. lumpus* eye and retina during post-hatch developmental stages. The study herein also brings funduscopy into use on *C. lumpus* for the first time and spectraldomain optical-coherence tomography (SD-OCT) in which reflected infrared light and interferometry non-invasively recapitulates the inner surface of the retina (fundus) as well as individual layers of the retina in live animals at a resolution similar to histological resolution.

MATERIALS AND METHODS

Fish collection and processing

Animal work was performed under approval from the Institutional Animal Care Committee (IACC) of Memorial University. Hatched *C. lumpus* were cultured at the Dr. Joe Brown Aquatic Research Building (JBARB) at the Ocean Sciences Center, Memorial University, from eggs of cultured domesticated Newfoundland brood stock *C. lumpus*. Post-hatch *C. lumpus* were collected over several months in autumn from holding tanks held at 10C. *Cyclopterus lumpus* eye tissues were collected from a larval stage at around 10 days post hatch (dph) up to the end of their juvenile development at around 250 dph. Some animals were studied at later stages (early post juvenile 330 dph) and 4 years old in order to take advantage of the larger fully developed adult eye.

Methods of euthanasia depended upon developmental stage and were chosen in consideration of avoiding any type of retinal damage. There is no knowledge on the effects of central nervous system depressants such as MS-222 on retinal integrity of post-hatch developing *C. lumpus* but it has been described as toxic to retinal tissue in amphibians (Rapp & Basinger, 1982). Since little is known about retinal development in *C. lumpus*, there is no baseline from which to assume that central nervous system depressants have no effect on retinal integrity. Other usually accepted forms of physical euthanasia (blow to head or pithing) were not possible for larval stage *C. lumpus* and could cause retina damage in later stage developing fish. Therefore, fish of very small size (10–50 dph) were removed from the tanks using a beaker or net and placed directly into a labelled tube containing cold 4% buffered paraformaldehyde to induce rapid chemical euthanasia with minimal disturbance of the eyes. Larger *C. lumpus* were killed

rapidly by cervical dislocation using a scalpel blade to separate the head from the body without any physical damage to brain tissue, optic nerve tract or eyes immediately prior to immersion of the head in 4% buffered paraformaldehyde. For some larger fish, the eyes were removed from the separated head after sacrifice before placing in 4% buffered paraformaldehyde. Small whole fish were fixed for 48 h, while whole fish heads or eyes of larger fish were fixed for 72–96 h. After fixation, tissues were rinsed twice in phosphate buffered saline (PBS) and then placed in 14% Ethylenediaminetetraacetic acid (EDTA) solution to decalcify until flexible (11 to 20 days depending on fish size). The samples were inverted gently once each day to ensure even distribution of solution. Fish with larger eyes did not require decalcification as their eyes were removed from the head. After decalcification, fish were rinsed in PBS and placed in 70% ethanol for paraffin wax or methacrylate embedding.

Paraffin and methacrylate embedding, serial sectioning and microscopy

Some of the *C. lumpus* heads and eyes were processed for standard automated paraffin embedding by the histology core at Cold Ocean Deep Sea Research Facility of Ocean Sciences Center. Others for methacrylate embedding were dehydrated through an ascending series of 70%, 80% and 95% ethanol followed by an intermediate resin infiltration solution (50:50, v/v with 95% ethanol), each for 1 h on a shaker. Specimens were then placed in infiltration solution for 2 h on a shaker. The fish were oriented in historesin molds (such that the sectioning proceeded from the rostral end to the caudal end), covered with embedding medium and left at 4C overnight to harden. Serial sections (3 μm for resin and 5 μm for paraffin wax) were cut through the whole eye using a

microtome (Leica RM2255; www.leicabiosystems.com). Resin embedded eyes yielded up to c. 150 sections, all of which were stained with toluidine blue to microscopically map structures. Some of the paraffin sections were stained with haematoxylin and eosin while others were used for immunohistochemistry (IHC) as described below. The sections were imaged using brightfield and darkfield microscopy using a light microscope (Leica DM4000B). Some slides were digitally scanned at JBARB using an AxioScan.Z1 digital slide scanner (Zeiss; www.zeiss.com).

Photography, funduscopy (live fundus imaging) and SD-OCT imaging

Cyclopterus lumpus were photographed either in their tanks or immediately after removal from their tanks on a bench adjacent to the holding tanks using a sixth generation iPod Touch (Apple; www.apple.com) and the FiLMiC Pro App. For most photography including fundus imaging, the FiLMiC Pro App (www.filmicpro.com) was used with the torch set to low level. For fundus imaging, early post juvenile or adult *C. lumpus* were used since their eyes are large enough to allow informative fundus imaging using available optical tools. *Cyclopterus lumpus* were removed from the aquaculture housing tank and gently restrained by hand on a benchtop adjacent to the aquaculture housing tanks. Fundus imaging is an examination technique that passes light through a magnifying glass looking into the eye to create an image of the fundus (the back of the eye). This technique allows for capturing coloured images of the posterior eye, including the retina, retinal vasculature and optic disc (Ghosh et al., 2014). Fundus images were quickly and non-invasively obtained over the course of 1–2 min using FiLMiC Pro App in video mode with a clear 78 dioptre lens. Normal C57Black/6 J mice were lightly anaesthetised

with 100 mg kg⁻¹ ketamine–10 mg kg⁻¹ xylazine for comparative mouse fundus imaging. Anaesthetised mice were administered a drop of tropicamide (Alcon; www.alcon.ca) into each eye after which each eye was then treated with Tear Gel (Alcon) ophthalmologic lubricating gel prior to non-invasive fundus imaging using the same methodology and equipment as for *C. lumpus*. Since the 78D lens reverses images on both the vertical and horizontal plane, care was taken during all recordings to note and report the actual anatomical views.

Spectral-domain optical-coherence tomography (SD-OCT) technology uses reflected infrared light and interferometry to recapitulate the fundus and the layers of the retina. Juvenile *C. lumpus* are approximately the same size as adult mice, fit easily onto the rodent stage of the SD-OCT apparatus and therefore were imaged by SD-OCT in order to assess the characteristic of the optic stalk and individual retinal layers. For SD-OCT, *C. lumpus* near the end of the juvenile stage at 250 dph were collected at JBARB and brought to the SD-OCT imaging laboratory in a cooler box with seawater. The fish were left to quietly acclimate for 30 min and then lightly sedated with 40 mg l⁻¹ MS222 in seawater for c. 5 min until they were unable to correctly orient themselves to an upright position in the seawater. After removing the fish from the anaesthetic bath, a drop of tropicamide was applied to the eyes to dilate the pupil and Tear Gel was applied to keep the eyes moist. The fish, including the gill areas, were wrapped in gauze soaked with seawater to keep their skin surface moist and placed in a silicone cradle, with the imaged eye facing toward the bore lens of the SD-OCT instrument. The position of the cradle holding the fish was adjusted and the bore lens advanced toward the fish eye and focused until the inner retinal surface and optic stalk were visible in the volume intensity

projection and B-scan windows. SD-OCT images were acquired from the live anaesthetised fish using a Bioptigen Envisu R2210 SD-OCT instrument (Leica). Scans were acquired in rectangular volume, 0 degrees, $1 \times 1000 \times 100$, 1.8×1.8 mm mode. The optic nerve stalk was oriented at the bottom left quadrant of the en-face fundus image area. Scans were completed for both the right (oculus dextrus (OD)) and left (oculus sinister (OS)) eyes of each fish when possible. We also acquired the SD-OCT scans in Doppler mode, which, in mouse SD-OCT using the Bioptigen Envisu R2210, recapitulates live blood flow in blood vessels using red and blue colour rendering. Rectangular volume scan data was used with the 3-Dimension (3D) analysis tool of the Bioptigen Envisu R2210 InvivoVue software to produce 3D reconstructions of the *C. lumpus* retina.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on paraffin wax sections taken from central retinal regions of the developing eye to label expression of proliferating cell nuclear antigen (PCNA) in order to identify regions of proliferative cells (Gendron et al., 2012). IHC was also performed to label expression of alpha smooth-muscle actin (ASMA) to determine locations of muscle fibres and blood vessels (Wall et al., 2004). The anti-PCNA antibody was a rabbit polyclonal immunoglobulin (Ig)G antibody (FL-261, Santa Cruz Biotech, Dallas, TX, USA) raised against a recombinant PCNA protein (PCNA) corresponding to amino acids 1 to 261 of full length PCNA of human origin. The anti-ASMA antibody (ASMA, anti-smooth-muscle antibody) was a mouse IgG2a isotype derived from a hybridoma produced by the fusion of mouse myeloma cells and

splenocytes from an immunised mouse (A5691, Sigma; 222.sigmaaldrich.com). The sections were deparaffinised in xylene and then rehydrated slowly in increasing ethanol dilutions. The tissues were then postfixed in 4% paraformaldehyde and washed three times in Tris buffered saline (TBS). Sections were circumscribed with an Immedge pen (Vector; www.vectorlabs.com) and blocked with 2% enhanced chemiluminescence (ECL) advanced block (GE Healthcare Ltd.; www.gehealthcare.com) with Triton × 100 (Sigma) in TBS at room temperature. After 1 h in the blocking solution, the eye and positive control tissue sections were incubated overnight at room temperature in either a 1:100 dilution of rabbit polyclonal IgG anti-PCNA antibody in 2% ECL advanced block in TBS or a 1:200 dilution of mouse monoclonal 1A4 anti-ASMA antibody in 2% ECL advanced block in TBS. Negative control for anti-PCNA was 2% ECL block in TBS while that for anti-ASMA was an equivalent concentration of anti-UPC-10 isotype matched negative control antibody (Sigma) in 2% ECL block in TBS. The following day, the samples were washed three times in TBS before a 1 h antibody incubation with anti-rabbit IgG2a specific alkaline phosphatase (AP) conjugated antibody (Jackson ImmunoResearch Lab Inc.; www.jacksonimmuno.com) at a 1:200 dilution in blocking agent in TBS for PCNA or a 1 h antibody incubation in anti-mouse AP-conjugated antibody (Promega; www.promega.com) at a 1:200 dilution in blocking agent in TBS for ASMA. Sections were washed three times in TBS and subsequently developed for 15 min using Vector Red AP with Levamisole (Vector) and colour development was observed under an inverted microscope. Slides were air dried overnight and then mounted with Permount.

Quantitation and statistical analyses

Thickness measurements of the ganglion cell layer (GCL) over the whole retina were performed at selected stages in histological sections using Openlab 5 software (Improvision, Coventry, UK). Two fish per stage were used for a total of four eyes per stage. Three measurements were taken at each peripheral regions of the retina for a total of six measurements per eye. The measurements of four eyes at each stage were averaged and an Analysis of Variance (ANOVA) test (Tukey's test) was performed to compare the significance between all stages using GraphPad Prism 4 (www.graphpad.com). Thickness ratio were expressed as mean \pm SE. To quantify cell proliferation in the ciliary marginal zone (CMZ), PCNA stained sections of the eyes of three specimens at 50 dph were analysed. Using ImageJ (www.imagej.nih.gov), PCNA red staining levels were measured essentially as described previously (Ho et al., 2015) in the CMZ and in the central retina. Ten measurements were taken from each eye for the CMZ and the central retina. The negative control staining was subtracted from the PCNA staining values. Results were expressed as a percentage of the averaged PCNA staining of the CMZ. Student's t-test was performed between the staining percentages of the CMZ v. the central retina and reported as a P-value using GraphPad Prism. Statistical differences were considered significant when $P < 0.05$.

RESULTS

Animal behaviour can be predictive of use of environmental visual stimuli (Cronin & Douglas, 2014). When not feeding or swimming toward an observer, cultured *C. lumpus* remain mainly in a stationary position with head often oriented upwards. In holding tanks, many cultured 50 dph and 250 dph *C. lumpus* adhere to structures in their tanks, while some are in a horizontal position, with an eye either at the surface or protruding from the water, and others are deeper in the water column (Figure 2.1).

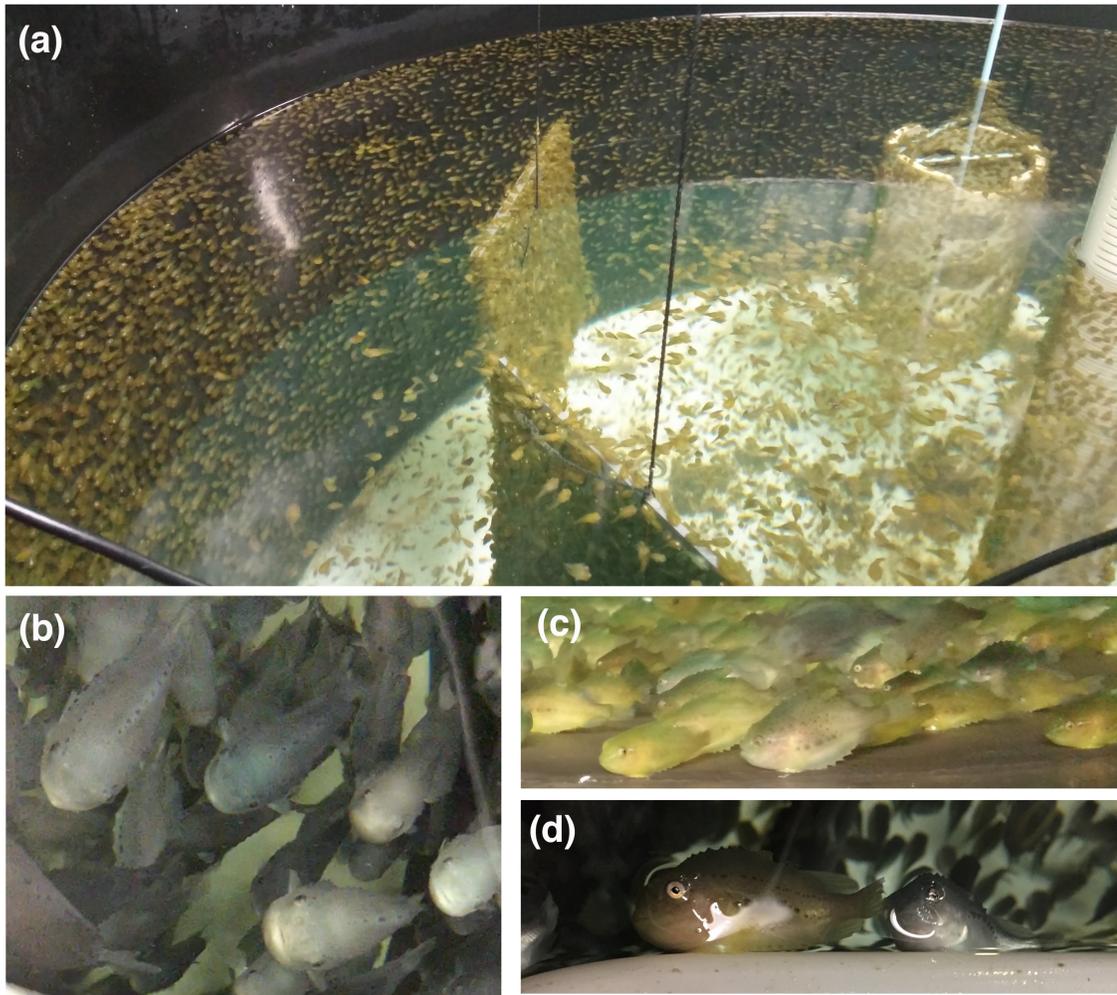


Figure 2.1 Photos of cultured *Cyclopterus lumpus* in aquaculture tanks at (a) 50 days post hatch (dph) and (b)–(d) 250 dph. (b) *Cyclopterus lumpus* swimming with heads oriented upwards toward an observer, (c) adhering to tank surfaces often with heads oriented upwards toward water surface and (d) adhering to tank objects with eyes protruding above water surface (n=1000).

Inspection of the gross anatomy of the dissected early post juvenile cultured *C. lumpus* eye and posterior structures using a stereomicroscope revealed the iris, lens, a prominent retractor lentis muscle structure, a rete mirabile and a neural retina (Figure 2.2). We noticed heavy pigmentation of the retractor lentis muscle tip area and the presence of pigmentation foci in the neural retina when stretched between the two portions of globe. It was noted that the ora serrata was very close to the ciliary body. The inner tip of the retractor lentis muscle structure was attached to the periphery of the lens by a fine filament while the outer portion seemed to be attached firmly to the ciliary body region (Figure 2.2).

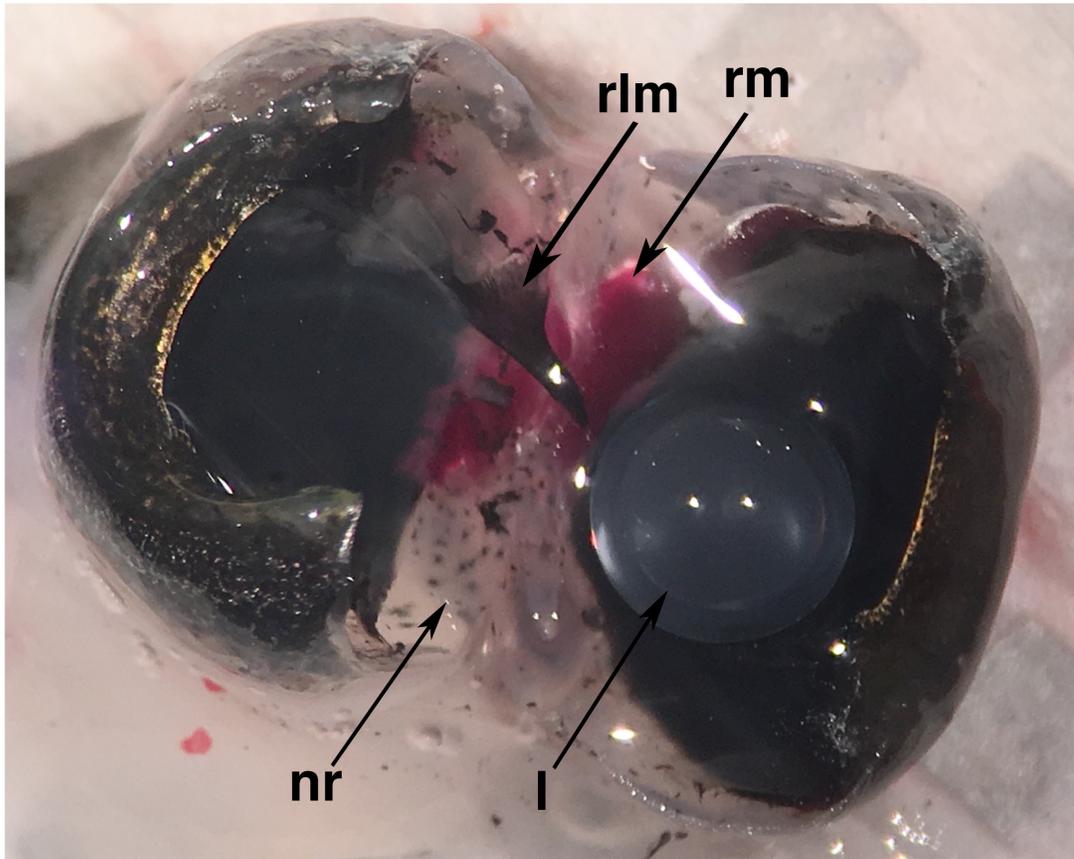


Figure 2.2 Stereomicroscopic photograph of the gross anatomy of *Cyclopterus lumpus* eye and posterior structures 330 days post hatch (dph): eye cut in half on an anterior to posterior plane showing lens (l), retractor lentis muscle (rlm), rete mirabile (rm) and neural retina (nr). Note the heavy pigmentation of the retractor lentis muscle tip and the neural retina, which has been stretched between the two portions of globe, and the ora serrata very close to the ciliary body. The conjunctiva and cornea were removed prior to dissection and photography (n=3 animals).

Histological sections taken from central retinal regions of the globes of advancing developmental stages of post-hatch *C. lumpus* revealed overall eye growth with advancing stage, a spherical lens typical of teleosts, a small vitreous:retina ratio, a retina with a pronounced and thick inner plexiform layer, a prominent optic tract and a continuous scleral cartilage shell (Figure 2.3). The ganglion cell layer (GCL) in early stage fish is significantly thicker compared with more advanced stages (Figures 2.3 and 2.4). The inner nuclear layer in developing stage fish appeared to be subdivided in two distinct layers. The more anterior nuclear layer displays larger nuclei than the more posterior layer. This pattern is not observed in adult (cf. Figure 2.3 vs. Figure 2.8), (n=3 animals).

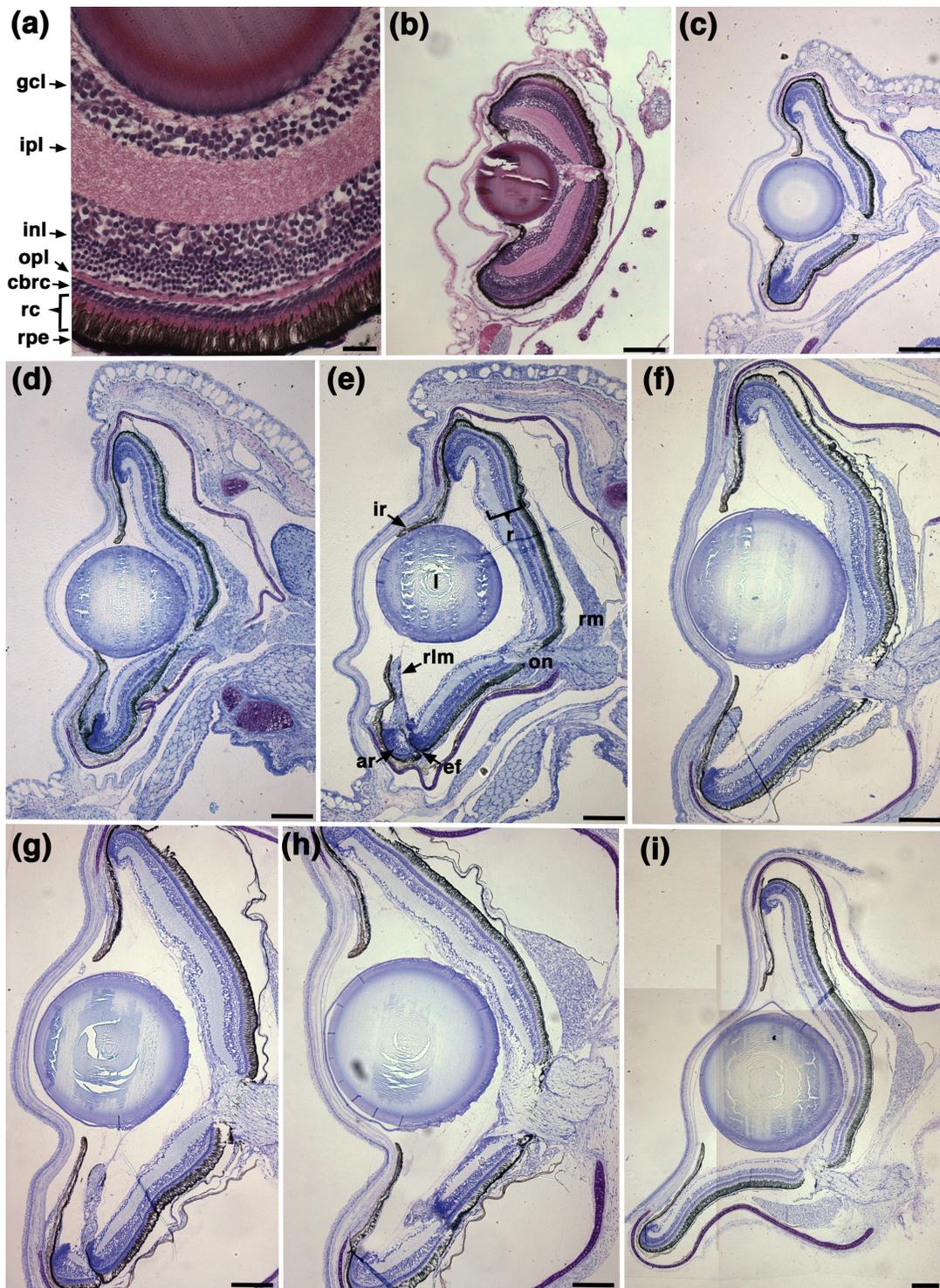


Figure 2.3 Histological sections of representative developing post-hatch cultured *Cyclopterus lumpus* eye and retina: (a) labelled retinal layers at 10 days post hatch (dph) showing ganglion cell layer (gcl), inner plexiform layer (ipl), inner nuclear layer (inl), outer plexiform layer (opl), cell bodies of rods and cones (cbrc; also commonly known as outer nuclear layer), rods and cones (rc), retinal pigment epithelium (rpe). Scale bar 20 μm . (b) Eye 10 dph mapped from 86 sections per eye of 12 eyes ($n = 6$ animals). Scale bar 100 μm . (c) Eye 50 dph mapped from 434 sections per eye of 6 eyes ($n = 3$ animals). (d) Eye 60 dph mapped from 572 sections per eye of 2 eyes ($n = 1$ animal). (e) Eye 70 dph mapped from 462 sections per eye of 2 eyes ($n = 1$ animal). showing iris (ir), lens (l), retina (r), optic nerve (on), rete mirabile (rm), embryonic fissure (ef), accessory retinal structure (ar), retractor lentis muscle (rlm); (f) Eye 85 dph mapped from 597 sections per eye of 2 eyes ($n = 1$ animal). (g) Eye 95 dph mapped from 615 sections per eye of 2 eyes ($n = 1$ animal). (h) Eye 105 dph mapped from 732 sections per eye of 1 eye ($n = 1$ animal). (i) Eye 118 dph eye collage mapped from 805 sections per eye of 1 eye ($n = 1$ animal). Scale bar (c)–(i) 200 μm .

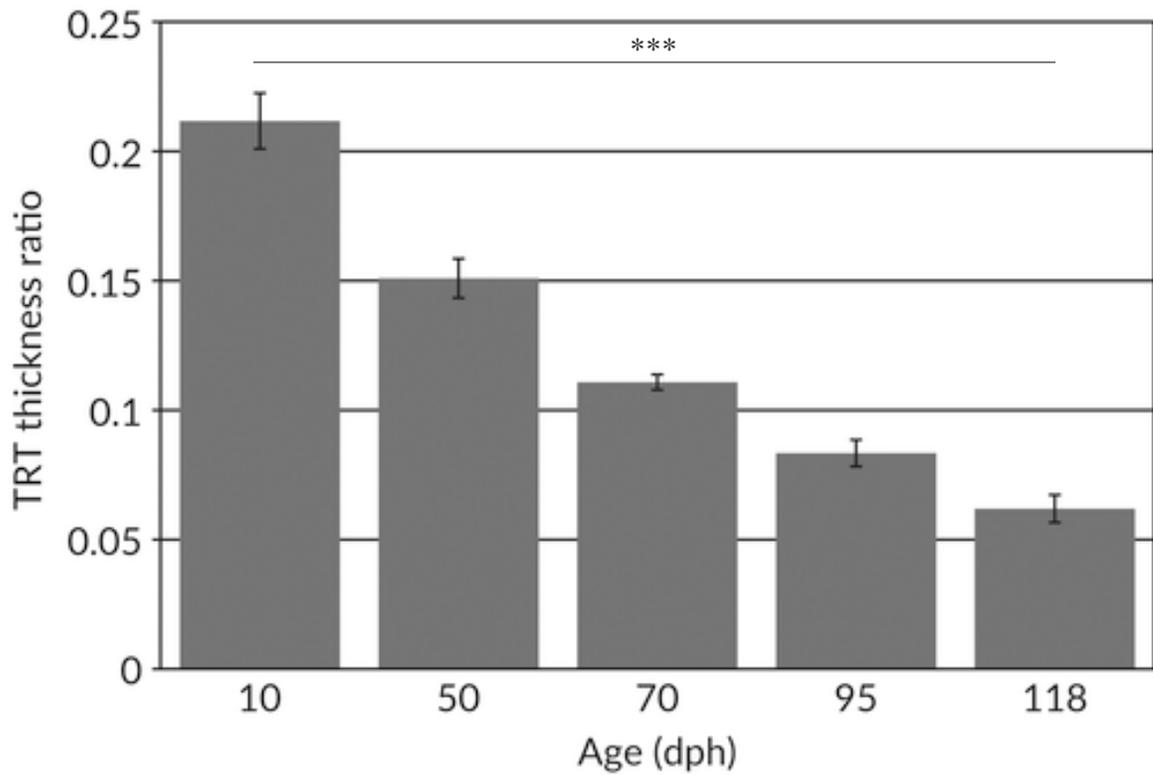


Figure 2.4 Changes in mean (\pm SE) relative ganglion cell layer (GCL) thickness (measured as the GCL layer thickness per the total retinal (TRT) thickness) of *Cyclopterus lumpus* eye at 10, 50, 70, 95 and 118 days post hatch (dph). Measurements were taken from four eyes at each stage, with 10 measurements from each eye ($n=4$ animals). A significant decrease in GCL:TRT with age was observed in 10 and 50 dph compared with later stages ($P < 0.0001$)

At all stages, the peripheral regions of the retina were prominent and well developed. Histological serial sections revealed that ventral regions of retina contain a physically separate region or lobe of retinal tissue circumscribed by pigmented epithelium reminiscent of an accessory retina in some deep-sea fish (Partridge et al., 2014; Wagner et al., 1998, 2009; Figures 2.5 and 2.6). The accessory retinal structure became more prominent with advancing developmental stage and is peripherally located within the eye. Serial sectioning also revealed a very prominent rete mirabile consisting of tightly packed vascular tissue highlighted by the presence of red blood cells in the vessels lumens (Figure 2.5(a)). The rete mirabile extended anteriorly from the posterior portion of the globe to retractor lentis muscle tissues projecting from a region adjacent to a structure resembling the embryonic fissure in brown trout *Salmo trutta* L. 1758 (Kunz & Callaghan, 1989) and the accessory retinal structure (Figures 2.3(g) and 2.4(b)). This retractor lentis muscle tissue extended almost to the pupil. This structure appeared and disappeared in serial sectioning indicating that it projects into the vitreous from one side of the eye.

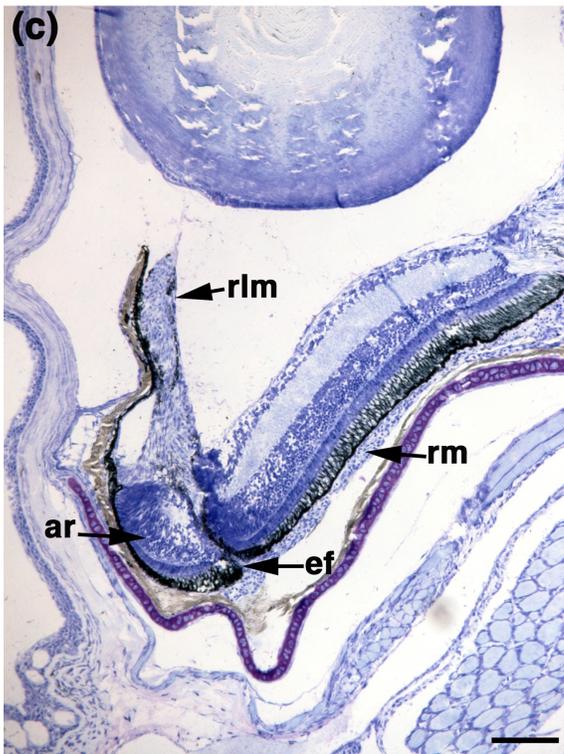
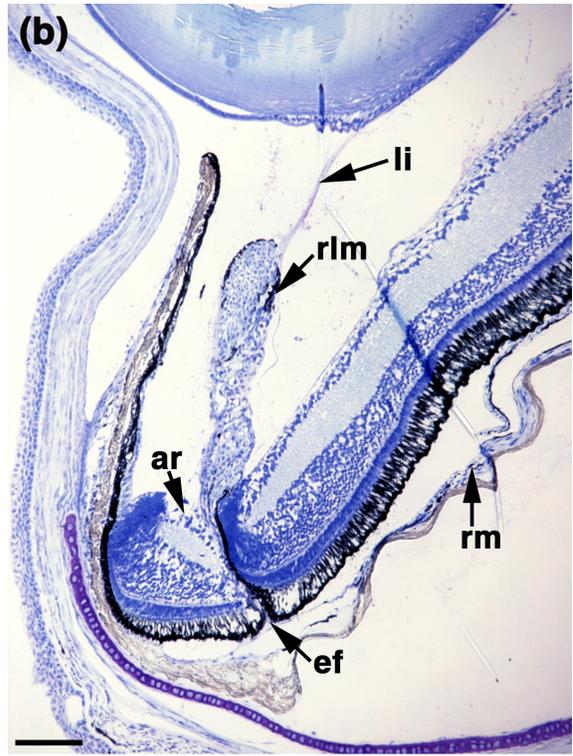


Figure 2.5 Histological sections of rete mirabile and retractor lentis muscle structures of the developing post-hatch cultured *Cyclopterus lumpus* retina T: (a) 50days post hatch (dph; scale bar 100 μm) (n=3), (b) 95 dph (scale bar 100 μm) (n=3 animals), (c) 70 dph (scale bar 100 μm) (n=1 animal) and (d) 60 dph (scale bar 50 μm) (n=1 animal) showing a well-developed retractor lentis muscle (rlm) with pigmented tip and highly vascularized rete mirabile (rm) structures attaching the peripheral retina to the lens *via* thin ligaments (li). The retractor lentis muscle is continuous with the rete mirabile (rm) and projects from the embryonic fissure (ef), anatomically defining the accessory retinal structure (ar). All panels show ventral at bottom.

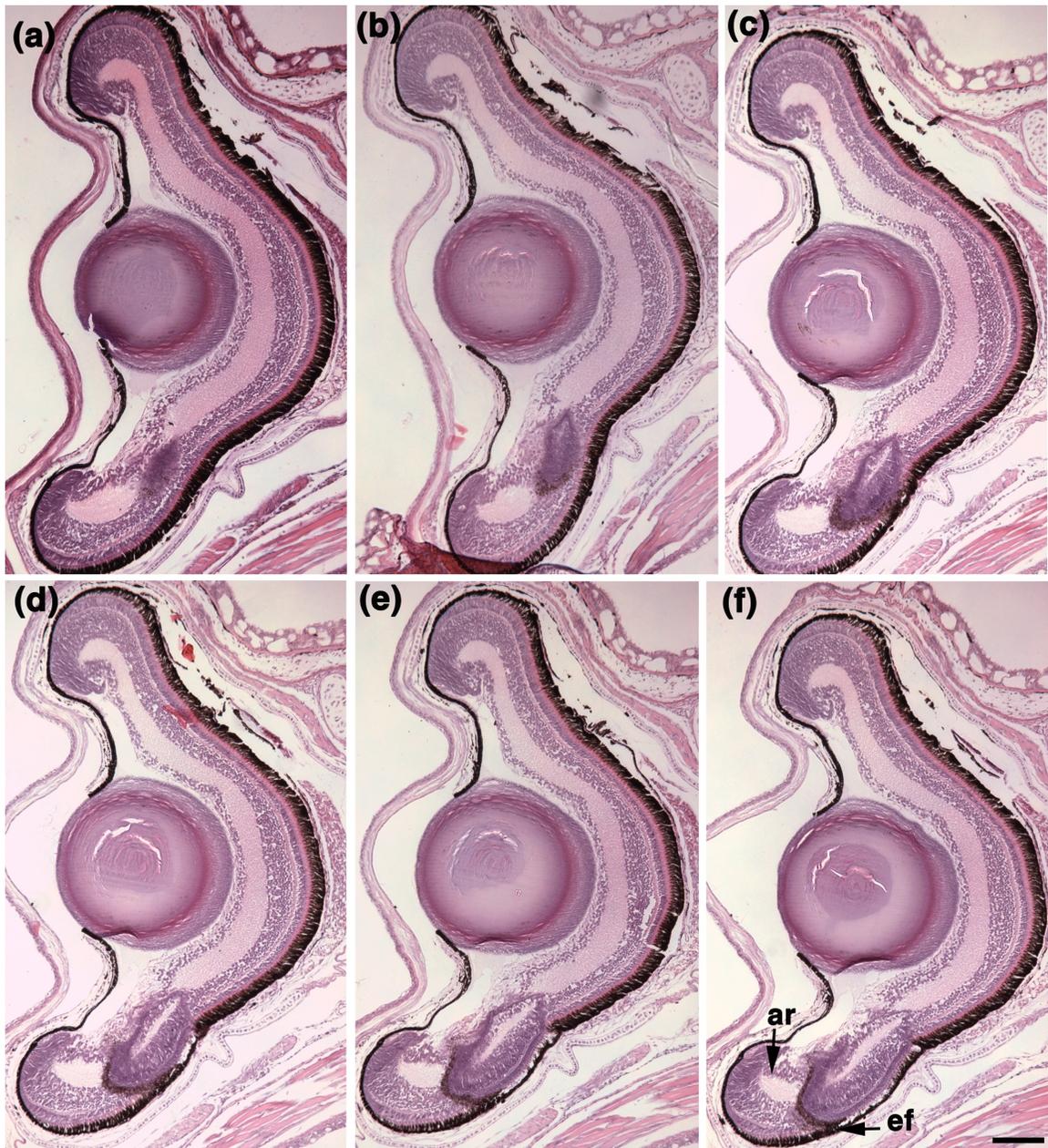


Figure 2.6 Histological sections of serial sections of a 50 days post hatch (dph) cultured *Cyclopterus lumpus* eye from (a) caudal to (f) rostral showing the histological emergence of (f) the embryonic fissure (ef) and accessory retina (ar) at a peripheral ventral position. Scale bar 100 μ m. All panels show ventral at bottom (n=3 animals).

Dark-field microscopy revealed reflective tapetum-like material of varying thickness observable at most locations surrounding the sclera, iris and retina, including the separate lobe of retina (Figure 2.7). The rete mirabile of 4-year-old adult *C. lumpus* was robust (Figure 2.8(c)) and fed into an extensive choroidal vasculature (Figure 2.8(a)). Aged adults also retained the peripheral accessory retinal tissues (Figure 2.8(b)) but the retractor lentis muscle structure was not as prominent as during developmental stages (not shown).

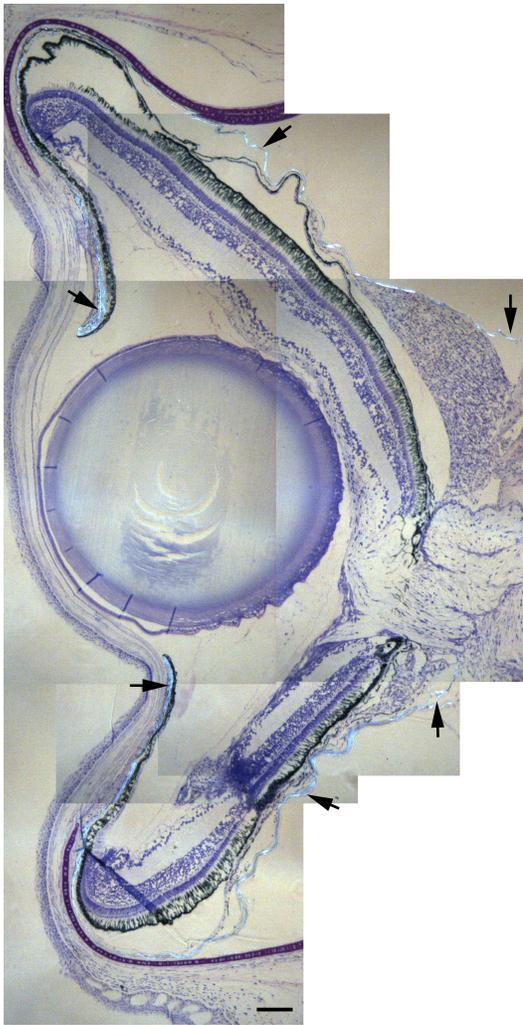


Figure 2.7 Darkfield image of a 105 days post hatch (dph) cultured *Cyclopterus lumpus* eye showing the tapetum-like structure (→) surrounding the entire eye. Tapetum is thin in posterior retinal areas but very thick in peripheral retinal areas and around the accessory retinal tissues associated with the embryonic fissure. Darkfield images show that, depending on plane of section, the tapetum can transmit light. Separate images were superimposed and aligned in a collage in order to show whole eye (n=2 animals). Scale bar 100 μm . Ventral at bottom.

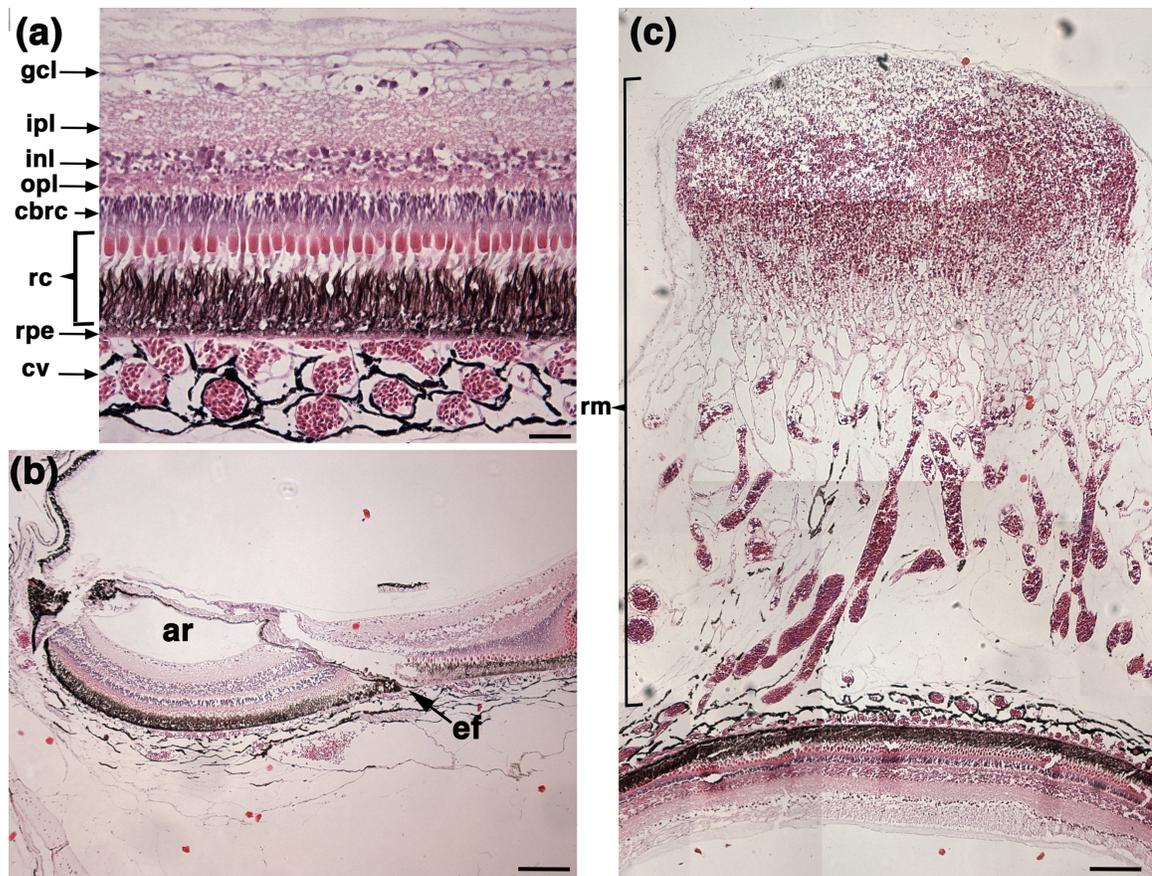


Figure 2.8 Histological analysis of morphology of a representative 4 year old adult *Cyclopterus lumpus* retina, choroid and rete mirabile: (a) retinal and choroid layers of adult fish (ganglion cell layer (gcl), inner plexiform layer (ipl), inner nuclear layer (inl), outer plexiform layer (opl), cell bodies of rods and cones (cbrc), rods and cones (rc), retinal pigment epithelium (rpe), choroidal vasculature (cv)), scale bar 20 μm ; (b) the accessory retina (ar) tissues associated with the remnants of the embryonic fissure (ef); (c) the rete mirabile (rm), a large mushroom like mass of vascular tissue posterior to retina, is robust and feeds into an extensive choroidal vasculature (cv). Results shown were mapped from 317 serial sections from normal adult eye. (n=2 animals) Scale bars (b), (c) 200 μm .

Fundus videography of the *C. lumpus* fundus using the camera's torch set continuously on at low setting was completely unlike that of a mouse fundus (Figure 2.9(a)) and showed several main features. The first was a prominent elongated rod-shaped optic nerve stalk and the overall bluish colour of the fundus (Figure 2.9(a)). The second was the presence of white radial stripes on the inner retinal surface projecting from the optic nerve stalk into the peripheral retina (Figure 2.9(a)–(c)). The third was very strong reflections of light composed of various visible spectral wavelengths especially from the portion of the retina ventrocaudal to the optic stalk of both early post juvenile and older animals (Figure 2.9(b),(c)). Fourth, images of a structure likely to be the retractor lentis muscle, could be captured when the 78 D lens was held at a level closer to the animal's lens (Figure 2.9(b), panel 1). When the 78 D lens was held at a position further from the retinal surface, the retractor lentis muscle was in focus and could be seen in its position close to the lens (Figure 2.9(b), panel 1). With the gradual changes in focus at different depths of the eye upon moving the 78 D lens gradually closer to the retinal surface (Figure 2.9(b), panels 2 to 5), the retractor lentis muscle structure became magnified and faded out of view and the retinal surface and optic nerve stalk came into focus. Reflections of visible spectrum light could be observed in the distinct areas of panels 1, 2 and 5. Additionally, while mouse fundus images clearly displayed the red and blue coloured inner retinal blood vessels, *C. lumpus* fundus imaging using the same method and equipment did not reveal the presence of coloured blood vessels (Figure 2.9(a)).

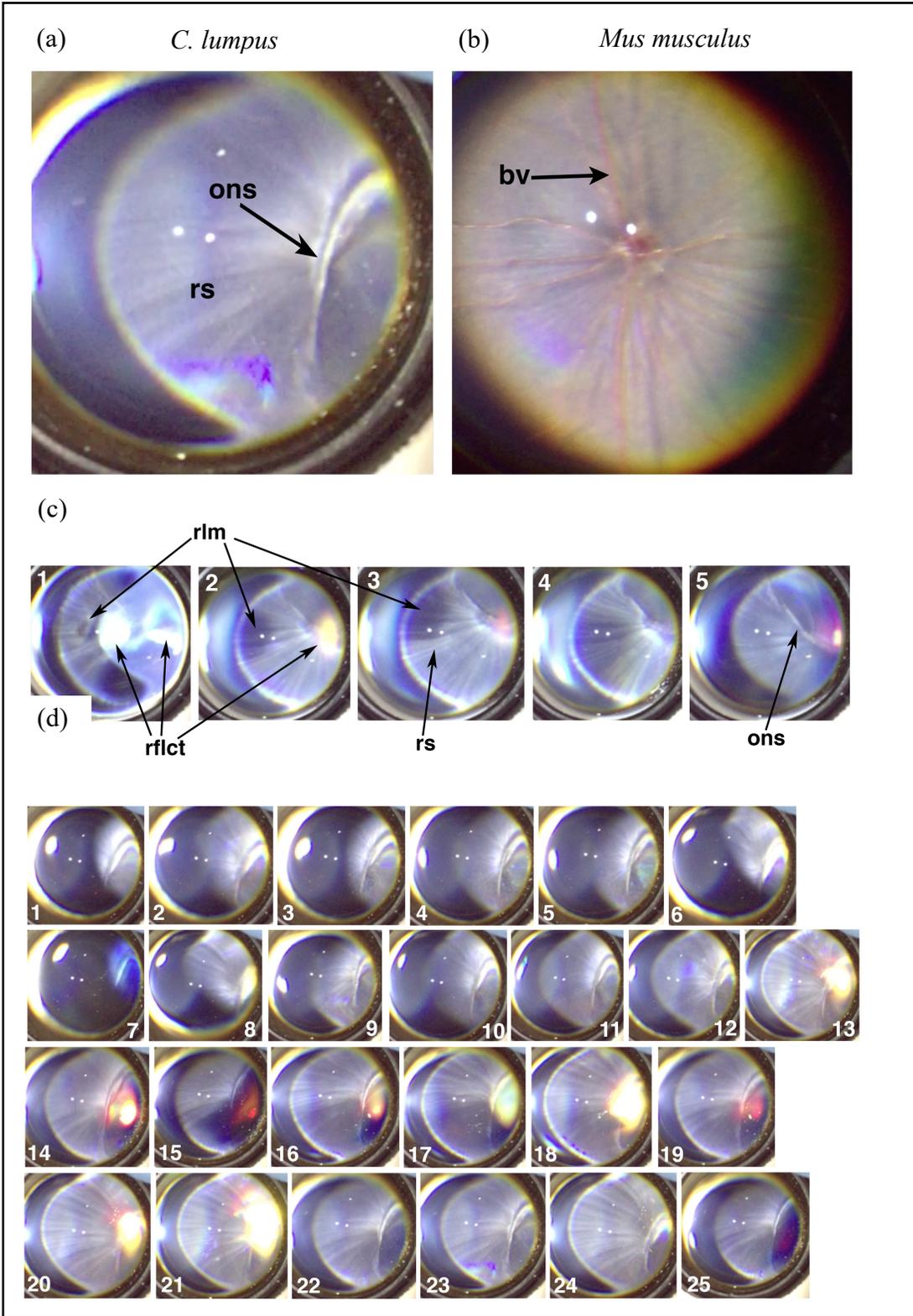


Figure 2.9 (a) Comparison of live 330 days post hatch (dph) *Cyclopterus lumpus* fundus videograph image with that of an adult mouse (*Mus musculus*) fundus image using the same methodology and equipment. Note the elongated rod-shaped optic-nerve stalk (ons), retinal stripes (rs) and the overall bluish colour of the *C. lumpus* fundus. Blood vessels (bv) coloured red or blue are easily visible in the mouse fundus. (b) Separate videograph frames (panels 1 to 5) of fundus images over *c.* 30 s of the early post juvenile 330 dph cultured *C. lumpus* fundus magnified through a 78 D lens placed between the eye and the camera. Panels 1 to 5 correspond to gradual changes in the position of the 78 D lens to enable gradual changes in focus at different depths of the eye. Note the retractor lentis muscle (rlm) coming in and out of focus; and the strong reflections of light (rflct) especially from the portion of the retina ventrocaudal to the optic nerve stalk (panels 1 and 2). (c) Separate videograph frames (panels 1 to 25) over *c.* 1 min of a 4 year old adult cultured *C. lumpus* fundus magnified through a 78 D lens held at different positions to enable focus at different depths of the eye. Note the strong reflections of visible spectrum light in the ventrocaudal quadrants of panels 8, and 13–21. Representative images from 6 animals. (b) and (c) Bottom right of each panel is an approximately ventro-caudal view of the inside of the animal's left eye

High quality SD-OCT images revealing the retinal layers of live *C. lumpus* were obtained but were consistently dimmer than those obtained from mice (Figure 2.10(a)). The SD-OCT images revealed a rod shaped optic-nerve stalk connecting to the various retinal layers including a prominent inner plexiform layer. Stripes projecting from the optic-nerve stalk into the peripheral retina were observed in SD-OCT en face volume intensity projection (VIP) recapitulating the fundus images. Three-dimensional (3-D) reconstruction of the rectangular volume scans displayed the *C. lumpus* retina as convex rather than concave as it is usually observed in mammals (Figure 2.10(e)). Doppler mode imaging did not reveal evidence of blood flow in the stripe like structures of the *C. lumpus* fundus. Foci of hyper-reflectivity were observable at the retinal surface on the B-scans that corresponded to positions of the stripes projecting from the optic-nerve stalk into the peripheral retina observed in the en face SD-OCT VIP images of the fundus.

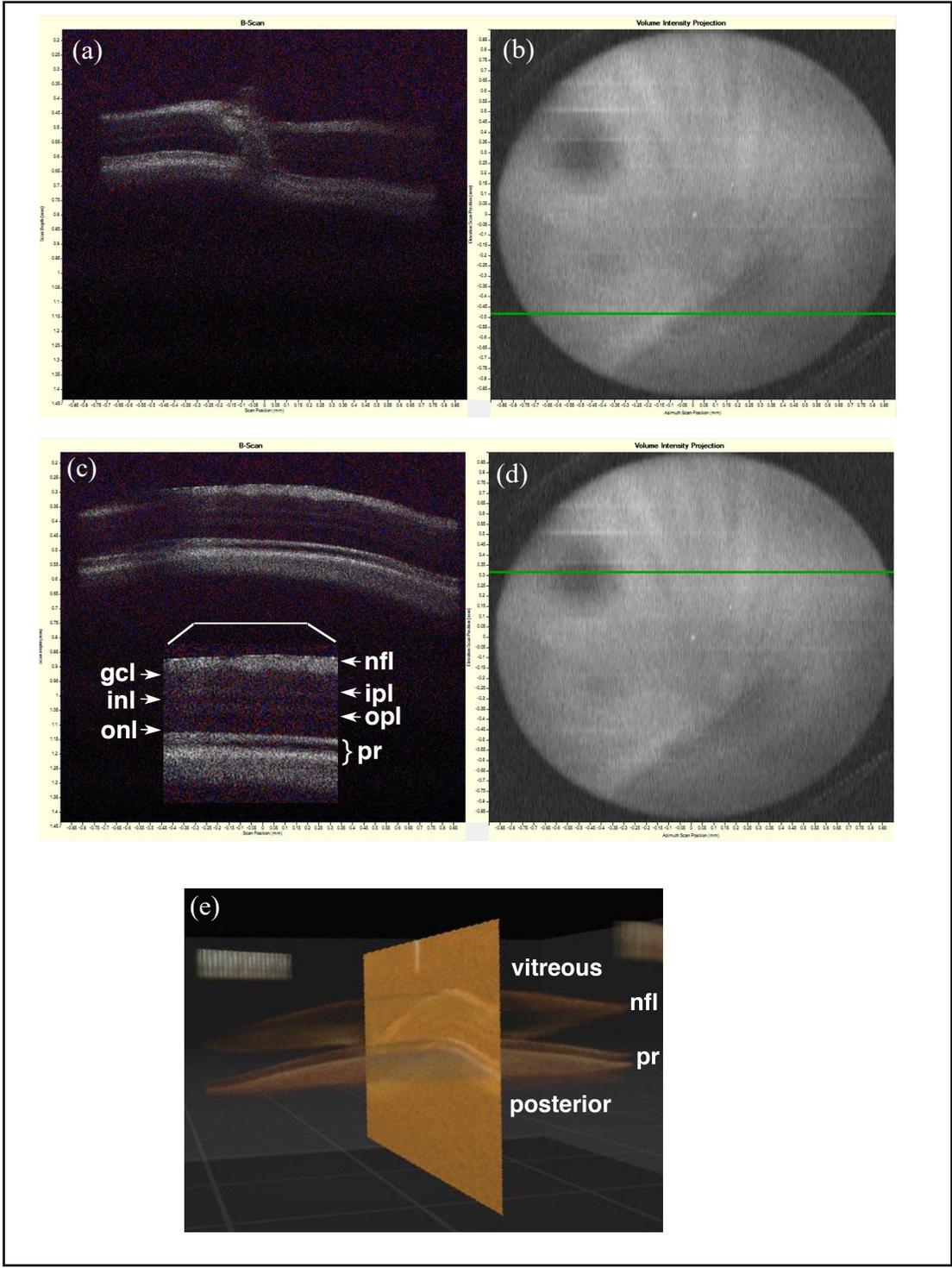


Figure 2.10 Representative images of spectral-domain optical-coherence tomography (SD-OCT) of juvenile cultured *Cyclopterus lumpus* retina. (a) B-scan (cross sectional plane of OCT image data composed of sequences of A-scans where A-scans are single lines of OCT data along axial (longitudinal) direction of the image). Note the rod shaped optic-nerve stalk. (b) Enlargement of B-scan to show retinal layers and lumps of hyperreflectivity in the ganglion-cell layer that correlate with the position of the stripes present in the corresponding volume intensity projection (VIP). Nfl, Nerve fibre layer; gcl, ganglion cell layer; ipl, inner plexiform layer; inl, inner nuclear layer; opl, outer plexiform layer; onl, cell body of rods and cones; pr, rods and cones. (c), (d) VIP (*en face* view of inner retinal surface) for (a) and (b), respectively, in which the position of the B-scan images are shown (—). The dark round spot in the upper left quadrant of (c) and (d) could be shadow created by the retractor lentis muscle structure. (e) Three-dimensional view of the rectangular volume scan. Positions of vitreous and posterior of retina, nerve fibre layer (nfl) and rods and cones layers (pr) are shown. The apparently empty space between the nerve-fibre layer and the photoreceptor layer corresponds to the same layers seen in the B-scan that are the dimmest in the image. The 3D reconstruction represents the *C. lumpus* retina, including the inner surface of the retina, as convex rather than concave. SD-OCT was performed on 3 individual animals.

To gain further knowledge of the characteristics of developing *C. lumpus* eyes analysis of markers expression for PCNA and ASMA using specific antibodies were undertaken. Anti-human PCNA antibody can be used to identify areas of proliferation. To evaluate the potential cross reactivity of this antibody with *C. lumpus* PCNA sequences of other teleosts, *D. rerio* and salmon *Salmo salar* L. 1758, were compared with that of human. Both teleosts showed greater than 90% identity to the human PCNA protein. As the anti-ASMA antibody was raised against a mouse ASMA amino-acid sequence, the ASMA protein sequence of another teleost, *D. rerio*, was compared with that of mouse to determine the level of identity of the ASMA sequences. *Danio rerio* ASMA showed 97% identity to the mouse ASMA protein. Therefore, we have a high level of confidence that the antibodies utilised were suitable. PCNA and ASMA immunohistochemistry was performed on *C. lumpus* eye paraffin wax sections from 50 dph fish. Paraffin wax sections of pellets of mouse internal elastic membrane (IEM) endothelial cells (Gendron et al., 1996) grown exponentially, were used as positive controls as they exhibit significant levels of PCNA. Mouse liver paraffin wax sections were used as positive controls for ASMA since ASMA is expressed in the smooth muscle cells of the media of blood vessel walls (Wall et al., 2004).

PCNA IHC in *C. lumpus* retina revealed significantly higher levels of PCNA expression in regions resembling a ciliary marginal zone (CMZ) as compared with the main retina (Figures 2.11(a),(b) and 2.12). Ciliary marginal zone is a region of fish peripheral retina where cells are added to support lifelong retinal growth (Hitchcock & Raymond, 2004; Wehman et al., 2005; Song et al., 2017). PCNA expression was also observed in the separated portions of retina, or what we refer to here as accessory retinal

structure, in *C. lumpus*. ASMA was expressed in continuous tight bands within the plexiform layers in *C. lumpus* retina (Figure 2.11(d),(e)). We noticed that such banding was noticeable in toluidine blue stained histological sections and in live SD-OCT images of *C. lumpus* retina (not shown and Figure 2.10, respectively). The positions of the inner and outer plexiform layers in B-scans from SD-OCT imaging of *C. lumpus* retina appeared to correspond to areas of ASMA expression (Figure 2.10). The plexiform layer bands of ASMA were also observable, albeit at a much lower level of expression, in central regions of adult retina (Figure 2.11 (g)). Peripheral regions of adult retina expressed the bands of ASMA in the plexiform layers at levels similar to that seen in the earlier developmental stage animals (not shown). In all areas of adult retina, high levels of ASMA expression were present in strikingly tightly defined square shaped areas between the outer segments and cell bodies of the rods and cones (Figure 2.11(g)). Such square-shaped areas of ASMA expression between the outer segments and cell bodies of the rods and cones was not observed in developmental stage animals.

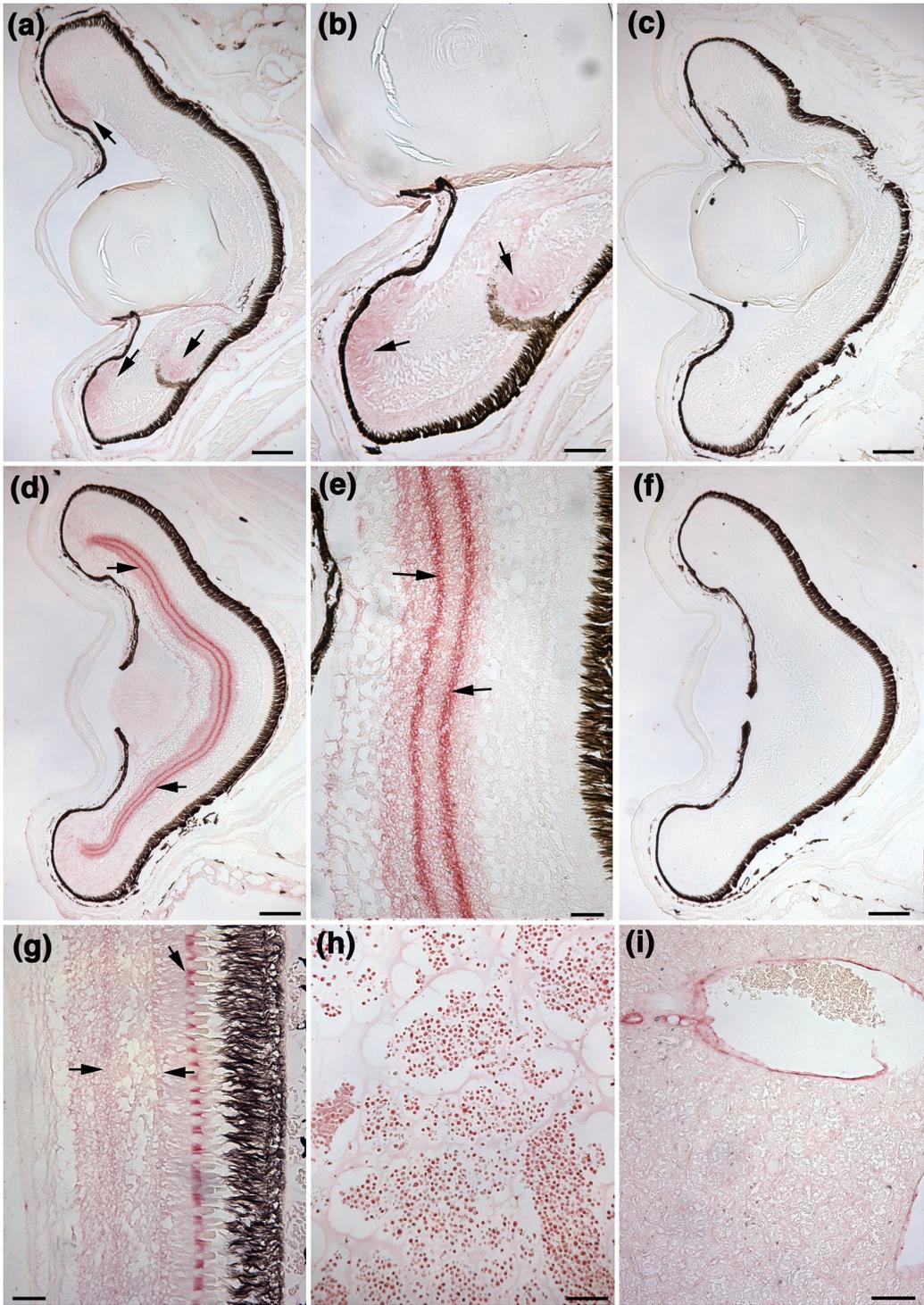


Figure 2.11 Expression pattern of proliferating cell nuclear antigen (PCNA) and anti-smooth-muscle antibody (ASMA) in cultured *Cyclopterus lumpus* eye. (a) Representative PCNA immunohistochemical staining (red colour) at 50 days post hatch (dph; $n = 3$ animals; scale bar 100 μm) and (b) the same image at a higher magnification (scale bar 50 μm), showing staining in the peripheral retina and in separate retinal tissue associated with the embryonic fissure, for comparison with (c) negative antibody control *C. lumpus* retina section (scale bar 100 μm) and (h) PCNA positive tissue control internal elastic membrane (IEM) cell pellets (scale bar 100 μm). (d) Representative ASMA immunohistochemical staining (red colour) was performed on 50 dph *C. lumpus* ($n = 7$ animals scale bar 100 μm), with (e) the same image at a higher magnification (scale bar 20 μm), showing a striking pattern in the plexiform layers compared with (f) negative antibody control *C. lumpus* (scale bar 100 μm) and (i) mouse hepatic blood vessels as a positive tissue control (scale bar 50 μm). (g) Immunohistochemistry for ASMA was also performed on a representative 4 year old *C. lumpus* retina showing a striking pattern of staining of ASMA in the photoreceptor cell layer (\longrightarrow) and persistence, at a lower level of the pattern of ASMA in the plexiform layers (\longrightarrow ; \longleftarrow) ($n = 1$ animal; scale bar 20 μm). All panels show ventral at bottom.

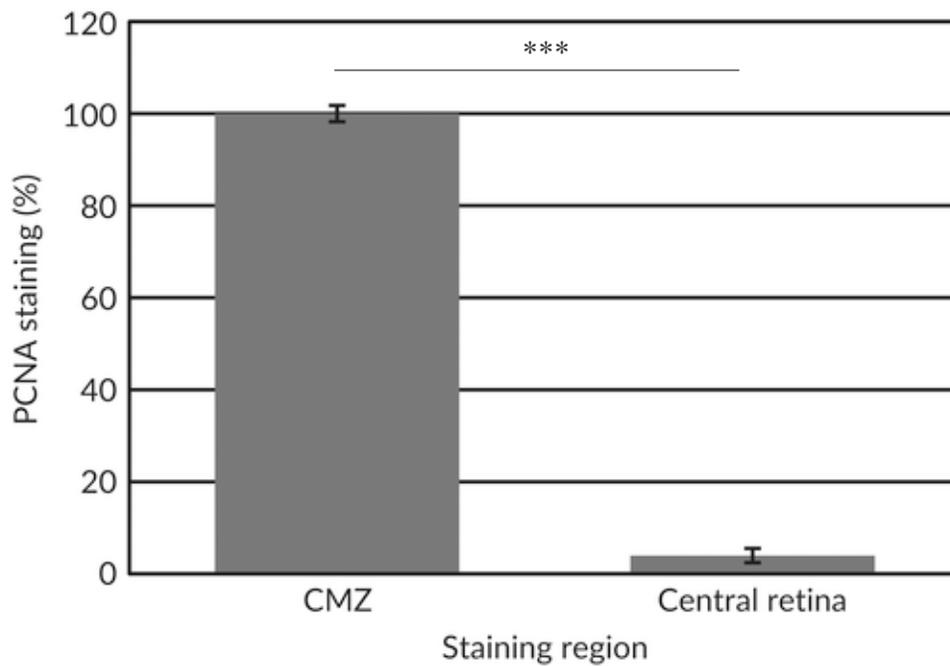


Figure 2.12 Proliferating cell nuclear antigen (PCNA) expression in the ciliary marginal zone (CMZ) compared with the central retina. Significantly higher PCNA expression in the CMZ (mean \pm SE = 100.0 \pm 1.8) compared with the central retina (mean \pm SE = 3.973 \pm 1.553; n = 3 animals; P < 0.001).

DISCUSSION

We used serial histological sectioning to completely map cultured *C. lumpus* eye and retinal structures. Histological sections from central retinal regions of advancing developmental stages of cultured posthatch *C. lumpus* revealed continuous eye growth over time which is well known in teleosts (Hitchcock & Raymond, 2004). The spherical lens was also typical of teleosts. The scleral cartilage shell we observed is consistent with the wide variability of scleral ossicle tissues in teleosts (Franz-Odenaal, 2008). The two distinct sizes of nuclei we observed in the inner nuclear layer in developing stage fish may represent either different stages of differentiation or different types of retinal neural cells as found in other teleosts, and this distinction can be made with further studies using light and electron microscopy (Hitchcock & Raymond, 2004).

At all stages, the peripheral regions of the retina were prominent and well developed, but, as revealed by serial sectioning, also contained some novel structures which, to our knowledge, were not previously described. Firstly, a very prominent rete mirabile consisting of tightly packed vascular tissue extended from the posterior portion of the globe, where it is expected for teleosts, to project from a region adjacent to what we interpret as a peripheral embryonic fissure (Kunz & Callaghan, 1989) and what appeared to be a separate retinal lobe. This peripherally located, pigment epithelium circumscribed portion of retina that was physically separated from the main retina, might represent a *C. lumpus* version of an accessory retina, reminiscent of that described previously in deep sea fish (Partridge et al., 2014; Wagner et al., 1998, 2009). A previous study in the fresh water teleost *S. trutta* described the persistence of the embryonic fissure

as a retinal structure containing specifically oriented photoreceptors that might be specialised for detecting polarised light (Kunz & Callaghan, 1989).

Histological analysis suggests that the accessory retinal structure in *C. lumpus* contains most layers of normal retinal tissue including photoreceptors and that the photoreceptors in this region of retina are oriented differently than in the main retina (Figure 2.5). The retractor lentis muscle with a pigmented tip appeared to be projected from the rete mirabile, extended nearly to the pupil and was physically attached to the lens by a clearly defined ligament. This structure appears and disappears in serial sectioning indicating that it projects into the vitreous from one side of the eye. Retractor lentis muscle is a characteristic of teleosts but to our knowledge the presence of large amounts of rete mirabile tissue associated so closely with it has not been described before in other fish (Khorramshahi et al., 2008). Whether the prominence of rete mirabile vascular tissue associated with the retractor lentis muscle is related with nourishment or support of vitreal structures will require further study.

While melanin is thought of mainly as playing a light absorbing protective role, some have theorised that melanin in pigmented structures such as the avian pecten might have light mediated metabolic relevance (Goodman & Bercovich, 2008). The retractor lentis muscle pigmentation observed in histology during post-hatch development was consistent with a prominent pigmented structure visible in dissected early post juvenile *C. lumpus* eye which connected the ciliary body region to the lens (Figure 2.2) via ligaments and with a clearly visible lens-associated structure captured during live funduscopy imaging of early post juvenile animals (Figure 2.9(b)), indicating that the structure probably persists throughout adulthood.

Tapetum like material of varying thickness at most locations surrounding the sclera, iris and retina, including the separated portion of retina, is reminiscent of the guanine crystal material that mediates reflective optic properties in deep-sea fish eye (Wagner et al., 2009). Fundus videography supports the notion that the tapetum-like material in *C. lumpus* eye serves to reflect light within the retina since strong reflections of light composed of various visible spectral wavelengths were captured, especially from the portion of the retina ventrocaudal to the optic stalk (Figure 2.9). These reflections did not seem to be a methodological artefact since such reflections were not observed in fundus of mouse using the same methodology. It is possible that the tapetal material surrounding various portions of retinal tissue (Figure 2.7) serve to redirect or amplify light rays within the *C. lumpus* eye's globe. This could conceivably support a low acuity but high sensitivity directionally specific peripheral vision system in the low-light levels of deeper water. Sections from 4-year-old adult *C. lumpus* verified the importance of the development of the rete mirabile since this structure was surprisingly robust in these aged animals, feeding into an extensive choroidal vasculature (Figure 2.8). Adults also retained the accessory retinal structure but the retractor lentis muscle was not as prominent as that observed during developmental stages.

The inner retinal surface of cultured *C. lumpus* fundus was completely unlike that of mouse (used as a methodological control; Figure 2.9(a)) or even of the teleost *D. rerio* fundus (Tschopp et al., 2010) and revealed prominent white stripes or wrinkles radiating outwards from a rod-shaped optic nerve stalk. SD-OCT images were easily obtained from live *C. lumpus* but consistently seemed dimmer than those from the methodological control animal mouse, possibly due to the additional anterior tissues present in teleosts

(Figure 2.10(a)). The dimness of the SD-OCT images also might suggest that the *C. lumpus* eye can absorb some degree of infrared light since SD-OCT utilises reflected infrared. Nevertheless, the SD-OCT images of retinal layers was of high enough quality to distinguish the various retinal layers and confirmed our histological evidence that the plexiform layers were very prominent and thick in *C. lumpus* retina, consistent with what has been described previously in *D. rerio* (Bailey et al., 2012).

White retinal surface stripes observed by both fundus imaging and SD-OCT could be either arborising blood vessels or other non-vascular structures. Previous studies in *D. rerio* proposed that these stripes are retinal blood vessel arborisations (Bailey et al., 2012) but we were not able to visualise coloured blood vessels in *C. lumpus* fundus as we observed in the methodological control animal mouse (Figure 2.9(a)), arguing that the stripes might contain tissue other than blood vessels. It remains possible that any blood vessels present in the *C. lumpus* inner retina are of too small a calibre to visualise with these techniques. Acquisition of the SD-OCT scans in Doppler mode, which recapitulates blood flow in blood vessels, did not reveal any evidence of blood flow in the stripe like structures of the *C. lumpus* fundus. However, foci of hyperreflectivity on the B-scan images corresponded to hyperreflectivity of stripes in the en face VIP recapitulation of the fundus images. The rod-like shape of the optic stalk coupled with convergence of the stripes from periphery to optic stalk might suggest that ganglion-cell axon bundles radiate inwards to form a narrowing ribbon from the optic stalk. Fundus images clearly support the rod-like or even cord-like morphology of the optic stock (Figure 2.9). Some teleosts have evolved unique anatomical pathways transmitting retinal visual information to brain

(Schiller, 2010). Further studies are needed to reveal if the retinal stripes and rod-like optic nerve head are relevant in any way to the neural transmission of visual information.

Both the SD-OCT B scans and the 3D reconstruction image of the retina suggest a slightly convex curvature (Figure 2.10). If this slightly convex appearance on SD-OCT is not due to actual convexity of the retinal tissue, it may be due to extreme infrared ray refraction artefact from the spherical shape of the *C. lumpus* lens or unavoidable imposition of rodent specific pre-set instrument variables. However, no degree of possible instrument reference-arm adjustment overcame the convexity. The shape of the juvenile *C. lumpus* lens is similar to that of the methodological control adult mouse lens, which is also spherical but does not create an SD-OCT artefact of retinal convexity. Moreover, histology consistently showed slightly convex portions of the central *C. lumpus* retina.

Lack of information on the *C. lumpus* genome precludes a range of experimental approaches relying on homology with mammals. However, by comparing protein sequences of mammal and fishes with known genomes (*D. rerio* and *S. salar*) we were able to predict that sequences used to raise two antibody reagents (anti-human PCNA and anti-mouse ASMA) with relevance to investigation of retinal biology, had sufficient homology with teleosts that they might cross react with *C. lumpus* protein homologues. PCNA IHC in *C. lumpus* retina revealed expression in a ciliary marginal zone consistent with that seen in *D. rerio* (Hitchcock & Raymond, 2004; Song et al., 2017; Wehman et al., 2005). Moreover, PCNA expression was also observed in the separated, accessory retinal portions of retina in *C. lumpus*. Since PCNA marks cell proliferation, these results are novel and suggest that both the ciliary marginal zone as well as the accessory retinal

structure contain proliferative cells and that *C. lumpus* might also utilise a ciliary marginal zone in both these retinal locations for supporting lifelong retinal growth.

The expression of ASMA in continuous tight bands within the retinal plexiform layers in developing *C. lumpus* retina was completely unexpected since our rationale for ASMA IHC was to label smooth muscle cells in blood vessels in order to localise *C. lumpus* retinal vasculature (Franke et al., 1980). These results indicate that tightly banded physical structures in the plexiform layers express a protein with homology to ASMA. They also suggest that blood vessels and any other structures in *C. lumpus* retina possibly containing smooth muscle, such as the retractor lentis muscle, might express different isoforms of smooth-muscle actin. Previous work has described actin filaments in the photoreceptor layers of teleost retina, but the isoform of actin was not characterised in these studies (Burnside, 1976, 1978; Hitchcock & Raymond, 2004; Liepe & Burnside, 1993; Nagle et al., 1986; O'Connor & Burnside, 1981; Pagh-Roehl et al., 1992a, 1992b). Actin expression as revealed by phalloidin staining of *S. trutta* retina has been reported by others but does not seem to be in the same patterns and locations as the ASMA we observed in *C. lumpus* retina (Lin-Jones et al., 2009). Whether the unique banding expression pattern of an ASMA-like protein in the plexiform layers of *C. lumpus* retina is related to any contractile properties or not will require further study. However, ASMA is also known to be expressed by neural-crest cell derived lineages including cells found in nervous tissues (Jahed et al., 2007; Sugimoto et al., 1991). Interestingly, ASMA expression in the plexiform layers was higher in peripheral regions v. central regions of adult *C. lumpus*. In adult, ASMA also appeared in striking square shaped foci around the photoreceptors in a staggered pattern reminiscent of corrugation. Further work is needed

to define if the ASMA-like expression in *C. lumpus* retina is related to a contractile apparatus or to other properties of nervous tissue.

Scott and Scott (1988) discuss observations that provide some evidence that *C. lumpus* can dwell in the upper levels of the mesopelagic zone (200–1000 m depth), where light levels are low. *Cyclopterus lumpus* might not be entirely pelagic (Kudryavtzeva, 2008) as they move around extensively to spawn and live on the ocean bottom, in intertidal pools or, semipelagically, sticking to a range of different objects near the water surface such as kelp. *Cyclopterus lumpus* in the culture housing tanks at JBARB often swim toward a human observer above the surface of the water. Such behaviour is probably feeding conditioning but exemplifies their obviously effective vision even for above-surface interests. JBARB *C. lumpus* also often adhere to surface structures with an eye close to or protruding from the surface of the water. This behaviour also suggests some level of out-of-water visual interest and capability for seeing features in a vertical plane. JBARB staff have also observed that cultured *C. lumpus* will often move through the water or remain stationary with their heads slightly vertical in the water rather than horizontally like other fish. These behaviour patterns prompt questions about whether *C. lumpus* might utilise their vision, including the novel structures in the ventral portions of their retinas for perception of vertical or other lines of sight in their environments.

To conclude, this study provides a systematic analysis of the anatomy, histology, imaging findings and molecular expression patterns of the eyes of developing cultured *C. lumpus*. The novel features of the eye and retina of cultured *C. lumpus* described here including accessory retina, robust retractor lentis muscle, robust rete mirabile, extensive tapetum, as well as novel imaging findings and protein expression characteristics could

contribute to our overall understanding of fitness and survival of *C. lumpus* in a widely ranging habitat.

ACKNOWLEDGEMENTS

We thank W. Driedzic, Department of Ocean Sciences, Memorial University and A. Oldford, Henderson Optometry, St. John's NL, for critical review of the manuscript. We thank W. K. Stell of University of Calgary, Department of Cell Biology and Anatomy, for advice. We thank Department of Ocean Sciences staff members Stephen Hill and N. Smith of Cold Ocean Deep Sea Research Facility (CDRF) and JBARB staff members J. Fry, J. Monk, D. Tucker and K. Jeannot for expert technical assistance. We also thank the reviewers for helpful comments and suggestions which strengthened the manuscript.

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Chapter 3

Discussion and Summary

Lumpfish are a poorly characterized teleost that provide many emerging opportunities for basic discovery and for use in research and aquaculture. Primarily, our results indicate that lumpfish and mammals share the same basic characteristics of the eye structure, similar to the zebrafish that are used as a model organism to study human development and disease. Our research revealed the presence of several structures in the lumpfish eyes, some of which are characteristic of other teleosts. We also identified several novel structures in the lumpfish eye that have not previously been reported in fish present in shallow waters.

The accessory retina is a unique structure in the lumpfish eye as it is normally seen only in deep sea fish that do not frequent shallow waters. The accessory retina has been suggested to play a role in extending the field of view into lateral visual space for detection of unresolved light (Collin and Partridge, 1996). A structure similar to the accessory retina has only previously been reported in one teleost, the brown trout (*S. trutta*). It was suggested that this accessory retina contains photoreceptors at a specific orientation and may be involved in detecting polarized light (Kunz & Callaghan, 1989). To date, little is known about the function, optics and the regulation of the accessory retina, and further genomics and proteomics studies could reveal the significance of this structure in fish such as lumpfish and brown trout (*S. trutta*) that are not always present in deep dark waters. This structure, once fully understood, may have implications in human visual health and contribute to our understanding of structures adapted for dark vision that might play an important role in finding treatments for night blindness. The accessory

retina likely requires separate and novel genetic programming in addition to normal retinal development that allows for the development of such a structure in lumpfish. Understanding the genes and mechanisms involved in the development of a separate retinal lobe that also appears to grow continuously will provide novel insights into the mechanism of such development and the purpose of such a structure. This could also provide relevant information on how development of an accessory retina in organisms such as lumpfish can be applied to better understanding retinal regeneration in mammals. These studies could lead to possible treatments for retinal degenerative diseases in humans.

Another key structure present in the lumpfish eye is the ciliary marginal zone (CMZ), composed of retinal progenitor cells, that continually replace old and damaged retina present in the periphery of the eyes as well as in the accessory retina. According to research on zebrafish (*D. rerio*), retinal stem cells are present in a niche at the extreme periphery of the CMZ and those that divide and form retinal progenitor cells move to a more central position, with only one daughter retinal stem cell remaining in the niche (Wan et al., 2016). Additional research on the lumpfish CMZ will be required to determine whether growth of the retina occurs in a similar manner in the lumpfish eye, given the presence of an additional ciliary marginal zone in the accessory retina. The presence of a potential CMZ in the accessory retina strongly suggests that the accessory retina exhibits continuous growth over time in lumpfish eyes and is an additional region of the eye with retinal progenitor cells. In previous teleost studies, the CMZ has only been described in the peripheral retinas. This novel result of proliferative cells in the accessory retina requires further studies, particularly molecular studies that will lead to an

understanding of the genes involved in the development and regulation of a CMZ in the accessory retina. Also, it would be worth noting that within a small region of the eye where the accessory retina is present, there is additional growth of the eye on the lateral but not dorsal surface of the retina, which may indicate the need for additional repair mechanisms on one side on the dorsal surface of the retina compared to the ventral. Understanding of the mammalian CMZ and whether it contributes retinal cells during embryogenesis is limited, with no knowledge of whether this loss of proliferative potential occurs during embryogenesis or postnatally (Marcucci et al., 2016). However, recent studies on mammalian eyes have found that retinal stem cells, which have the potential to divide into self-renewing cells and thereby possibly for retinal regeneration in mammals, have been isolated from mammalian ciliary epithelium, which is the mammalian equivalent of a CMZ (Balenci & Kooy, 2014; Coles et al., 2004; Tropepe et al., 2000). The understanding and implementation of a CMZ with proliferative potential in fish indicates an adaptive mechanism to a harsh environment that requires continuous retinal regeneration into adulthood. Lumpfish can be used to study and understand CMZ regulation in non-mammalian species, to help form a better understanding of what key regulators may be involved in mammalian CMZs. The presence of a CMZ in the accessory retina may be further studied for the possibility of its use in the treatment of degenerative diseases of the retina, which are currently exploring stem cell therapies as treatment (Mead et al., 2015).

The expression of smooth muscle actin seen in the inner plexiform layers of the lumpfish eye suggests either a novel role of smooth muscle –like tissue within the retina or the existence of a molecule homologous to the smooth muscle actin in the lumpfish

eye. Based on this, functional genomics studies that map the lumpfish genome may be beneficial for understanding what this structure may be and for identifying its significance in the eye. Ribonucleic acid (RNA) sequencing of lumpfish eye tissues will provide the opportunity to study a large variety of genes that are present and involved in development of novel lumpfish structures, such as that seen through ASMA immunohistochemical staining. Additionally, proteomics analysis can be used to detect and identify this protein. The smooth muscle staining was also prominent in adult fish around the photoreceptors. Domains of actin filaments have previously been described in the photoreceptor cilium of rat retina, with a suggestive role in photoreceptor outer segment disc morphogenesis (Chaitin, 1991). Actin expression in photoreceptors has also been previously described in teleost retina (Burnside, 1978). Teleosts are known to shorten their photoreceptors in light and lengthen them in darkness by the action of actin-like and myosin-like filaments in the inner segments of the photoreceptors (Burnside, 1978; Liepe & Burnside, 1993; O'connor and Burnside, 1981). However, these actin-like filaments were not identified as ASMA, indicating that different isoforms of actin may be present in various teleost species. Further studies are required in lumpfish to determine whether the actin filaments present in the lumpfish eye play a similar role or whether they are involved in accommodation by moving the photoreceptors.

Teleosts have a spherical crystalline lens that is too heavy to be held in place by the iris and they do not possess a ciliary body (Copeland, 1982). Previous research indicates that the teleost lens is held in place dorsally by a suspensory ligament and ventrally by the tendon of a muscle in the campanula of Haller, called the retractor lentis muscle (Walls 1942, Duke-Edler, 1958). We believe that the structure projecting from the

rete mirabile at the peripheral retina near the iris towards the pupil, which is physically attached to the lens by a clearly defined ligament, is the retractor lentis muscle. Dissection of the lumpfish eye as well as fundus imaging confirmed the presence of such a structure. To our knowledge, no previous research has described a pigmented retractor lentis muscle or one associated with rete tissue. We speculate that the pigmented tip is an invagination of the pigmented aspect of the choroid, along with rete mirabile tissue and may play a role in nourishing the retractor itself and / or vitreous structures of the eye, but further study will be required to confirm this. Pigmentation has previously been described in the falciform process, which is positioned near the optic disc (Reckel & Melzer, 2004). Our results only showed presence of the retractor lentis muscle attaching to the lens in the lumpfish eye, but we did not identify a falciform process or suspensory ligaments. These might be destroyed by fixation or dissection. Further studies using more advanced methods of detecting more delicate tissues, such as transmission electron microscopy, may be required to determine whether additional structures are present in lumpfish eyes, holding the lens in place.

The rete mirabile is a choroidal structure present behind the retina in the eyes of many avascular teleosts. The rete mirabile in lumpfish eyes was of an extensive size, especially in adults, and extended from the posterior retina all around the globe. As lumpfish eyes grow to an extremely large size and are present in deep waters, the large sized rete may be necessary for increased nutrient and oxygen requirement with size in the adult lumpfish eye. Mammalian eyes have both retinal vasculature and a choroidal blood supply nourishing the interior retina. Thus, only 80% of the mammalian eye circulation is choroidal while 20% is through retinal vasculature. However, since

lumpfish appear to have an avascular retina, 100% of their eye circulation might be choroidal, thus owing to the large sized rete (Alm & Bill, 1972). There is large scope for research to be performed on the rete and how it is involved in regulating nutrient influx /efflux into and out of the eye. Studies can be performed to understand whether genes present in the vascular mammalian eyes are expressed in the rete or choroidal layer and how regulation and maintenance of substances into and out of the eye is controlled.

This study reveals the wide scope of visual research that can be performed using lumpfish eyes due to the presence of many novel and unique structures. However, our study emphasizes the need for genomics studies as essential in lumpfish as it would increase our knowledge and understanding of what genes and mechanisms are involved in allowing lumpfish to adapt to their environment and develop these novel structures. RNA sequencing would be a useful method of identifying genes regulating the development of many of the novel structures seen in lumpfish. It may also provide information on other specialized pathways and structures that may be present in lumpfish eyes that may not have been identified in this study. Further, RNA sequencing may help identify differentially expressed transcripts in lumpfish under different environmental conditions or those showing diseased states.

To conclude, lumpfish eyes share the same basic structures of the eye as mammalian species, providing the premise to conduct further research on the genetic similarities between lumpfish and mammals to frame the foundation for use of lumpfish as a model organism for vision research. As a threatened species, the need to study and understand this organism and factors that affect survival and fitness become increasingly important to learn how to help preserve this organism. Previously characterized as well as

novel structures in the lumpfish eye provide the opportunity to study and understand adaptive mechanisms lumpfish possess that may be useful for human visual research in disease and treatment. Characterization of the lumpfish eye has laid the groundwork for further studies that may be conducted on this species. This includes studies to understand how vitamin levels effect visual function in lumpfish, which may possess some adaptive mechanisms to control for deficiency and toxicity due to their fluctuating environmental conditions. Additionally, this research only characterized the eye of North Atlantic lumpfish. A study by Pampouile et al. (2014) suggests that there may be three genetically distinct populations of lumpfish, based on geographical location; one from the North Atlantic-Main-Canada Greenland region, the second from the Iceland-Norway region and a third from the Baltic Sea. Comparative studies looking at structural differences in the eyes of these three genetically distinct lumpfish might reveal further adaptations specific to environment and may provide insight on the evolution of teleost eyes. Our findings will also aid in understanding of basic lumpfish biology as this threatened species is largely uncharacterized. It will also support future work on understanding and improving the aquaculture of lumpfish optimizing aquaculture conditions.

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