EARLY LENS ABLATION CAUSES DRAMATIC LONG TERM EFFECTS ON THE BONES OF THE CRANIOFACIAL SKELETON OF THE MEXICAN TETRA, *ASTYANAX MEXICANUS*

by

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DEDICATION

This thesis is dedicated to my family, without their endless love and support I wouldn't have had the courage to tackle my dreams.

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ABSTRACT

The Mexican tetra, *Astyanax mexicanus,* exists as two morphs of a single species, a sighted surface morph and a blind cavefish. In addition to eye regression, cavefish have an increased number of taste buds, maxillary teeth and have an altered craniofacial skeleton. I investigated the effect the lens has on the development of the surrounding skull by ablating the lens over early ontogeny. This unique long-term study sheds light on how early embryonic manipulations on the eye can affect the shape of the adult skull. The effects of lens ablation were analyzed using landmark based morphometric analyzes. Morphometric analyzes indicate that there is a significant difference in the shape of the supraorbital bone and suborbital bones four through six. These bones expand into the eye orbit exhibiting variability in their shape. Interestingly, the number of caudal teeth on the lower jaw is also affected by lens ablation. I compared these findings between morphs and across two teleost species. I conducted lens removal in the surface fish to determine if it would produce a cavefish phenotype. Lens removal in the surface fish only partially results in a cavefish phenotype, indicating that lens loss is not solely responsible for the phenotypic differences between the two morphs. The effects of lens removal were then compared in the Mexican tetra and zebrafish. Surprisingly, the results indicate that the same bones are variable in shape in both species, indicating that the variability of these bones is conserved across species. Finally, I compared laser lens damage and full lens removal, to investigate the capacity for both lens regeneration and healing in the Mexican tetra.

Together, the lens healing and regeneration studies indicate that lens absence in early development does not influence the shape of the skull. Lens absence during later development influences the mechanical forces in the skull resulting in the bones of the orbital region changing in size and shape. This study highlights the dynamic nature of the skull and sheds light on the influence the eyes (a soft tissue) have on the surrounding skull (a hard tissue) a topic which has been overlooked in the literature.

LIST OF ABBREVIATIONS USED

CNC- cranial neural crest

dpf- days post fertilization

dps- days post surgery

hpf- hours post fertilization

PC- principle component

PCA- principle component analysis

SL- standard length

SO- suborbital

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Chapter 1: Introduction

Extensive research has been conducted investigating genetic mutations leading to changes in the craniofacial skeleton, however, little investigation has examined how other factors (e.g. diet) of the environment can influence the development and final morphology of the skull and its components. Even less research has investigated natural variation present in the bones of the skull, and what characteristics of bone development and external influences might affect their variability.

The objective of this thesis research is to understand how altering soft tissues during early development can influence the development of the skull and how individual elements of the skull respond. I aim to expand on our current knowledge of how the development of the eye can influence the development of the craniofacial skeleton and to further investigate the variability present in the shape of the bones in the teleost skull. In addition this study makes use of a newly popular model species the Mexican tetra (Characiformes, *Astyanax mexicanus*). The Mexican tetra's two morphs (sighted and cavefish) make excellent models for this study to investigate the influence of eyes on the developing skull but also their influence on taste buds, and tooth number, which have been shown to vary between the two morphs.

This chapter will provide an introduction to the teleost skull, variations present in teleost skulls, and finally the Mexican tetra, the organism under study here.

1.1 The Teleost Skull

Since the Devonian period, over three hundred million years ago, the teleost skull has undergone extensive diversification leading to thousands of different morphologies (Gregory, 1933; Cooper et al., 2010). It has been estimated that there are over 28,000 species of teleosts or ray-finned fish (Santini et al., 2009).

Of the thousands of teleost skulls which exist today, few have been extensively described. One teleost has become a popular model species for fish skeletons, the zebrafish (Cypriniformes Cyprinidae, *Danio rerio*). The zebrafish skull has been extensively studied and has been well described morphologically and developmentally. As such, the zebrafish craniofacial skeleton is often used as a representative for teleost craniofacial studies. However, due to the vast variation amongst teleosts skulls, one

species cannot accurately represent an average of all teleost species. Despite this, and due to the limited information available describing teleost skull, a well described representative must often be selected. For the purpose of this section, the zebrafish skull will be used as a model for the teleost skull, for the following characteristics: developmental mode, cell origin, and bone locations. Figure 1-1 is a simplified schematic of the bones in the zebrafish craniofacial skeleton.

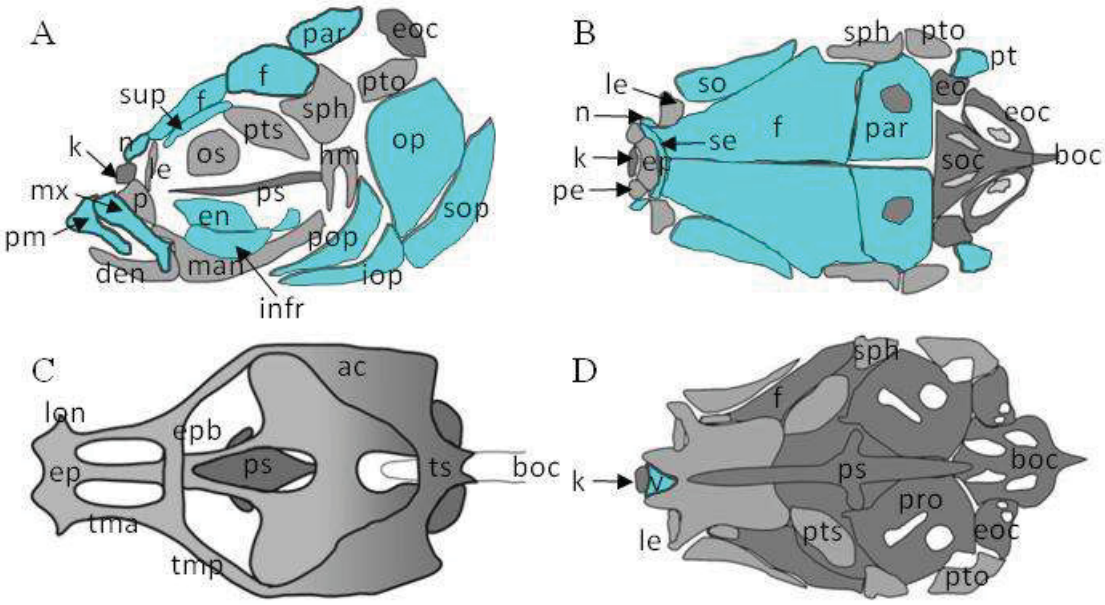


Figure 1-1: The craniofacial skeleton of the zebrafish. Bones in blue ossify via intramembranous ossification, bones in gray ossify via endochondral and perichondral ossification. A) Lateral view of the zebrafish skull, B) dorsal view of the adult zebrafish skull, C) dorsal view of the developing chondrocranium, D) base of the adult neurocranium. For clarity the pharyngeal skeleton, scleral ossicles, circumorbitals and the supraoccipital have been omitted from the diagrams. The quadrate, symplectic, and the reticular are depicted as one structure. ac, auditory capsule; boc, basioccipital; den, dentary; en, entopterygoid; eoc, exoccipital; ep, ethmoid plate; epb, epiphyseal bar; f, frontal; infr, infraorbitals; iop, interopercle; k, kinethmoid; le, lateral ethmoid; lon, lamina orbitonasalis; hm, hyomandibula, man, quadrate and reticular; mx, maxilla; n, nasal; op, opercle; os, orbitosphenoid; p, palantine par, parietal; pe, preethmoid; pm, premaxilla; pop, preopercule; pro, prootic; ps, parasphenoid; pt, posttemporal; pto, pterotic; pts, pterosphenoid; se, supraethmoid; so, supraorbital; sop, subopercule; sph, sphenotic; sup, supraorbital; tma, taenia marginalis anterior; tmp, taenia marginalis posterior; ts, tectum synoticum; v,vomer. Figure modified from Kague et al., 2012 and Cubbage and Mabee, 1996.

1.1.2 Ossification Modes of the Teleost Skull

In vertebrate skulls, there are typically two common modes of ossification, intramembranous and endochondral (Hall, 2005). During intramembranous ossification, bones form directly from a mesenchymal condensation located within a membrane, without a cartilage precursor (Hall, 2005). A large portion of the teleost skull develops through intramembranous ossification (Figure 1-1). During endochondral ossification, a mesenchymal condensation chondrifies into a cartilage precursor, which is later replaced by bone. A subtype of endochondral ossification often found in teleosts is perichondral ossification, in which ossification occurs in the perichondral connective tissue or an extension of it, surrounding the cartilage (Franz-Odendaal et al., 2006).

Figure 1 indicates the ossification mode of the bones of the zebrafish skull. For example, Meckel's cartilage ossifies through perichondral ossification, the frontal bones ossify through intramembranous ossification and the ethmoid ossifies through endochondral ossification (Cubbage and Mabee, 1996; Kague et al., 2012).

The teleost skeleton consists of the same building blocks as the mammalian skeleton, cartilage, and bone. The cells which form the skeleton are also shared between the two groups, including chondroblasts, chondrocytes, osteocytes and osteoclasts (Witten and Huysseune, 2009). In endochondral ossification, the first stage of ossification is the localized condensation of mesenchymal cells. The cells become closely packed forming prechondroblasts, which then differentiate into chondroblasts (Franz-Odendaal et al., 2006). The chondroblasts then mature through a series of transitional cell types including prechondrocytes, until they fully differentiate into chondrocytes. The chondroblasts secret extracellular cartilage matrix, containing largely collagen type II and glycosaminoglycans. In perichondral ossification, a bone collar begins to form around a cartilage element. This collar then extends surrounding and replacing the element (Franz-Odendaal et al., 2006). During endochondral ossification in fish, the center of the element can be retained as cartilage, in contrast to mammals where the cartilage template is fully replaced by bone.

In intramembranous ossification, the condensed mesenchymal cells differentiate into osteoblasts which secretet bone matrix (largely collagen type I and hydroxyapatite) (Witten and Huysseune, 2009). In mammals, osteoblasts secrete bone matrix, trapping

themselves within the matrix, the trapped cells then become osteocytes (Franz-Odendaal et al., 2006). This same process is observed in the bones of ancestral teleosts such as the osteoglossomorphs, while derived teleosts, (such as zebrafish and Mexican tetra) have acellular bones (Witten and Huysseune, 2009). During acellular bone development, osteoblasts do not become trapped by matrix secretion, resulting in mature bone void of osteocytes enclosed within (Witten and Huysseune, 2009).

The above processes of ossification occur in four phases (Hall and Miyake, 2000). The first phase is the migration of mesenchymal cells into the location of future skeletogenesis. The second is an epithelial-mesenchymal interaction. This interaction is critical in bone development and occurs just prior to the condensation of the mesenchymal cells. The third is condensation formation where the mesenchymal cells condense. In the third phase, mesenchymal cells condense in the future site of the bone undergoing rapid cellular division until a critical cell number is achieved. Finally when the number of cells present in the condensation is sufficient, the fourth stage of ossification occurs. In the fourth stage, mesenchymal cells undergo differentiation into chondroblasts in endochondral bones and osteoblasts in intramembranous bones. These cells then begin to secrete bone/cartilage matrix. Complex networks of genetic interactions control each one of these phases, each phase is responsible for setting up and determining the shape and size of the future structure (Hall and Miyake, 2000). The specific factors during early development that determine the shape and outgrowth of the skull bones is highly complex and has been described for only select bones in a few teleost species. Research indicates that the fate of cranial neural crest cells (CNC) may be determined before they even reach the location of their future condensations. Major signalling pathways involved in this induction include Wnts, Hedgehogs, BMPs and FGFs (Hall, 2005). Research also indicates that the condensations which give rise to endochondral elements are highly important in the shaping of the element (Hall, 2005; Hall and Miyake, 1992). In intramembranous ossification, epithelial-mesenchymal interactions in the location of the future bone are responsible for shaping that element. In fact, reciprocal signalling often takes places during the induction event beginning osteogenesis. For example, in the calvariae (the roof of the skull), the mesenchymal cells that will give rise to the bones interact with the overlying dura mater (the epithelium)

inducing ossification and shaping the future bones (Schowing, 1968, Opperman et al., 1993). In addition to early stages of ossification affecting bone shape, later stages of bone development, including bone outgrowth, can also affect the shape of the adult bone (Kimmel et al., 2010).

In a detailed analysis conducted by Kimmel et al. (2010), the authors studied the outgrowth of the opercle bone in zebrafish. The opercle is a large flat plate-like bone which protects the gills. It is an early forming bone which begins to ossify at approximately 3 days post fertilization (dpf). After the initial ossification of the condensation Kimmel et al., (2010) observed two forms of outgrowth in the bone, one increasing the bone size and the other controlling bone shape. The opercle bone undergoes major shape transitions from first ossification to adulthood. The shape of the bone is changed by altering the patterns of bone deposition. In this mode of bone outgrowth, bone spurs ossify in specific locations, the ossification of bone spurs is then followed by the ossification of veils of bone between the spurs. Spurs are linear outgrowth, which elongate at their ends, while veils are diffuse and lightly mineralized between the spurs. Increase in bone size occurs through the second method of bone outgrowth, in which ossification of incremental rings around the entire border of the bone occurs, evenly enlarging the entire element. The rate of the incremental banding controls the rate of bone outgrowth. This study is the only literature describing bone outgrowth in the head of a teleost species, as such, it is unclear if any other bones of the zebrafish skull grows in this same manner, or if the same bone ossifies in a similar manner in other fish species.

In summary, intraspecific variation exists in the teleost skull with respect to the mode of ossification (intramembranous, endochondral and perichondral) and the mode of bone outgrowth (e.g. appositional or veil and spur).

1.1.3 Cell Origins of the Teleost Skull

Bones of the vertebrate skull are derived from two cell origins, cranial neural crest cells, or prechordal mesoderm, or in some cases both (Hall, 2005; Kague et al., 2012). Neural crest cells are a population of migratory cells that migrate out of the closing neural tube during embryonic development. They are an extremely dynamic, plastic

population of cells capable of differentiating into a vast array of cell types (Sandell and Trainor, 2006). Plasticity is the vast ability to respond to environmental conditions during development. For example, cranial neural crest cells, which migrate into the head, are capable of differentiating into pigment, nerve ganglia, smooth muscle, bone and cartilage. Neural crest cells are capable of signalling each other, as well as giving and receiving signals from non-neural crest derived tissues (Sandell and Trainor, 2006). Schilling (2001) demonstrated the plastic nature of neural crest cells in the zebrafish, byremoving small or large groups of neural crest cells from one hox region (their normal location) and transplanting them into a different hox expressing region. Hox genes are a group of genes which determine the body plan of an embryo along the anterior to posterior axis. In small grafts, the cells were able to respond to the local environment, taking on the new hox regions specifications, indicating the plasticity of these cells; larger grafts respond in a less plastic nature. This study demonstrates the ability of transplanted neural crest cells to adopt the fate of its new location and their responsiveness to the local environment and thus their variability. As neural crest cells have a plastic nature, it would stand to reason that the structures formed from a plastic cell population would be more variable than structures that arise from non-plastic cells. Vast amount of literature is available on the plasticity of neural crest cells, which is easily demonstrated by a google scholar search. When a google scholar search is performed for “plasticity of neural crest cells” 288 hits are returned, while when a search for “plasticity of mesoderm” is performed only two hits are returned. Very little literature is available describing the plastic nature of mesoderm, suggestingthat mesoderm tends to be more stable in nature, giving rise to more stable structures.

Generally, bones which ossify through intramembranous ossification are neural crest derived and usually located in the skull, while endochondral bones are typically mesoderm derived and located in the posterior region of the skull, the axial skeleton (ribs and vertebrae) and the appendicular skeleton (girdles and fins/limbs) (Hall, 2005). More specific fate maps depicting cell origin of individual craniofacial skeletal elements have been published for mouse, and chicken (reviewed in Gross and Hanken, 2008). In chicken the facial region, including the orbital region of the skull is derived from neural crest cells, while the posterior portion of the skull is derived from mesoderm. The cell

origin of the frontal and parietal bones is still debated. In mouse, the anterior portion or facial region, including the orbital region, is derived from neural crest cells, while the posterior portion is derived from other cell origins. The mouse skull has yet to be fully mapped, including the jaws of which the cellular origins are not currently known. The consensus is that the cell origins of skull bones are likely conserved between birds and mammals (amniotes) (Reviewed in Gross and Hanken, 2008 and Kague et al., 2012).

Cell origins of bones in the teleost skull have only recently been fate-mapped in the zebrafish. The anterior portion of the zebrafish skull is largely neural crest derived (Kague et al., 2012), while the posterior half of the skull is largely mesoderm derived (Figure 1-2). The timing of cellular migration into the developing skull has only been mapped in one fish species, the zebrafish (Kimmel et al., 1995). Zebrafish were selected for a mapping study as transgenic zebrafish are easily made to analyze this relationship. In zebrafish, the cranial neural crest cells (CNC) begin migration into the developing skull at 14 hours post fertilization (Kimmel et al., 1995). Once CNC have reached their destination the post-migratory cells condense forming skeletogenic condensations.

Complex genetic networks determine the developmental pathway of a condensation to its skeletal structure. An enormously large suite of genes are responsible for ossification, from migration of mesenchymal cells to the site of ossification to ossification and bone outgrowth. A different suite of genes regulates each step in the ossification process controlling the initial and end of each stage (Hall and Miyake, 2000). These genes range from Bone Morphogenetic Proteins (BMPs) to genes controlling cell adhesions such has N-cadherins.

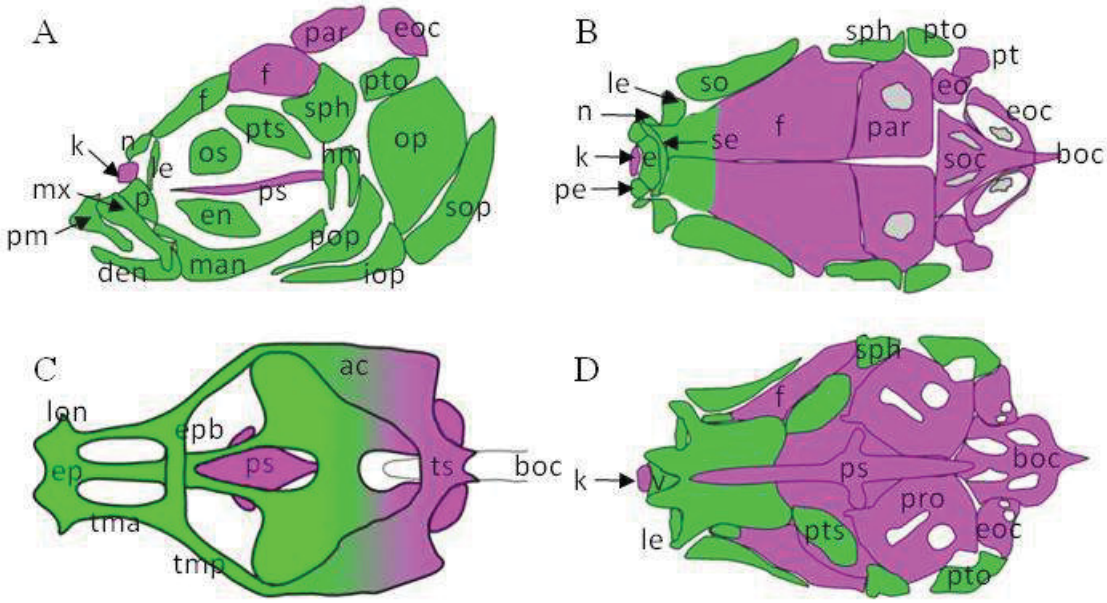


Figure 1-2: Cellular origins of the zebrafish skull. Green indicates structures derived from neural crest cells, purple indicates structures derived from other cell origin, presumed to be mesoderm, A) lateral view of the zebrafish skull, B) dorsal view of the adult zebrafish skull, C) dorsal view of the developing chondrocranium, D) base of the adult neurocranium. For clarity the pharyngeal skeleton, scleral ossicles, circumorbitals and the supraoccipital have been omitted from the diagrams. The quadrate, symplectic, and the reticular are depicted as one structure. Figure modified from Kague et al., 2012.

1.2 Variation in the Teleost Skull

This thesis aims to understand how altering soft tissues during early development can influence the development of the skull and how individual elements of the skull respond. Variation present in the teleost skull can be studied in a number of different manners.

Variations present in the teleost skull will be examined after three types of influences. Natural variation can be identified in the teleost skull with respect to the mode of ossification, ossification sequence, ossification timing, cell origin and bone shape. Induced variation may arise through influences from the environment. Finally, variation may arise after human perturbations. Some bones of the teleost skull exhibit variability, while others display constraint. These will be discussed under the headings of natural variation, effects of inbreeding and teratogens and finally, phenotypic plasticity.

As this thesis is largely focused on variations it is important to understand what variation is and it how arises. Variation is tendancy of an individual, structure, or genetic sequence to vary between individuals, or the potential to change in response to changes in the environment (Bateson and Gluckman, 2011). Three definitions which attempt to explain how variation arises can often be confusing. These terms include: phenotypic plasticity, adaptation and adaptability. Phenotypic plasticity is the ability of a genotype to produce more then one phenotype in response to the environment, while adaptability is the susceptibility of an organism to vary, or the capacity of an organism to respond to the environment. Finally, adaptation is the ability to respond to changes in the environment.. A large portion of this chapter is dedicated to describing the variability of the skull.

1.2.1 Natural Variation

In the following section, natural variation present in the bones of the teleost skull will be examined through timing of ossification, and variation in bone shape.

1.2.1.1 Variation in Onset of Ossification

The timing of ossification onset in the craniofacial skeleton has been fully described in a few teleost species (e.g. zebrafish and *Betta* (*Betta splendens*)) (Cubbage and Mabee, 1996; Mabee et al., 2000). However, both ossification sequence and variation

in the timing of ossification onset has been described for the zebrafish craniofacial skeleton, and will be discussed fully below (Cubbage and Mabee, 1996).

Natural variation can be observed in the onset of ossification in nearly all of the bones that constitute the zebrafish craniofacial skeleton. Each bone of the skull has a range of time in development during which the onset of ossification may occur. Some bones of the craniofacial skeleton show far more natural variation than others. For example, the infraorbital bones tend to vary relatively little, with the onset of ossification of this bone always occurs within a 24 hour period, while the ethmoid varies in onset of ossification over more than a few days (Cubbage and Mabee, 1996). Thus, when individuals of the same age are compared, variation can be observed with respect to which bones have begun to ossify between the individuals. Ossification of the zebrafish skull, as in other teleosts, occurs over a long period of development, over more than the first few months of life (Cubbage and Mabee, 1996). This long period of ossification may permit more variation to occur in ossification onset.

Greater variation is often observed in later forming structures (Waddington, 1957). Early forming structures are often stable as they can have a larger influence on the downstream development of other elements (Waddington, 1957). The variability in the timing of skull ossification was also studied in the fish species commonly known as the *Betta*. In *Betta* canalization (stability of early developing structures) in the bones of the skull was also observed (Mabee et al., 2000).

Due to the vast variation that has been demonstrated in the onset of ossification of skull elements in zebrafish and *Betta* (Cubbage and Mabee, 1996; Mabee and Trendler, 1996; Mabee et al., 2000) it is important to consider how natural variation may account for skull differences in studies describing effects on the juvenile skull. For example lack of ossification could be attributed to the effects of a manipulation performed on embryos, while in actuality the absence of ossification maybe natural variation in the onset of ossification and have no relevance to the study.

1.2.1.2 Natural Variations in the Shape of Skull Bones

Only one study is available in which intraspecific variation in bone shape was studied and described in a teleost skull. In the study, the author conducted an in depth

analysis of skull morphology in the Butterfly fish, *Pantodon buchholzi* (Pantodontidae Osteoglossiformes) (Kershaw, 1970). This author documented individual variation in the shape and fusions between bones. For example, the intertemporal bone varied in shape, while variations were present in fusions between the frontal and parietal bones and the prevomer with the parasphenoid.

As only one study has been conducted on intraspecific variation in teleost skull bones, information on shape variation must be gathered from other studies. Some information can be gathered from species comparisons, for example, Parsons et al. (2012) investigated differences in the oral region between cichlid species, and briefly commented on intraspecific variation. The study indicated that variation was observed in the shape of the mandible within the same species.

Finally, some literature is available on variation in the skulls of dimorphic fish species, such as the Mexican tetra, sticklebacks, cichlids and charr that can shed light on intraspecfic variation (Caldecutt and Adams, 1998; Proulx and Magnan, 2004; Stewart and Albertson, 2010). For example the cichlid, *Perissodus microlepis* (Perciforme Order Family Cichlidae) exhibits dimorphic jaw asymmetry (Stewart and Albertson, 2010). Some individuals have mouths which angle to the right, while others have mouths which angle to the left. As a result of the right/left handedness of the mouth, variations are present in the length of the jaw and jaw joint, thickness of the maxilla and premaxilla and curvature of the nasal bone (Stewart and Albertson, 2010). Some research has been conducted on the length differences of lower jaws in sexual dimorphic brook charr (*Salvelinus fontinalis*) males and females, as well as length differences in the jaws of trophic polymorphic charr (comparing littoral and pelagic individuals) (Proulx and Magnan, 2004). Sexual dimorphism as well as interpopulation variations in skull shape has been examined in the three spine stickleback, *Gasterosteus aculeatus* (Order Gasterosteiformes Family Gasterosteidae) (e.g. Caldecutt and Adams, 1998). The skull shape of four different populations were examined; anadromous, stream, lacustrine planktivorous and benthic-feeding populations, as well as between sexes. Variation was observed in the overall head depth, orbit size and the shape of the snout region between each different population. However, fewer changes were observed between sexes.

Beyond studies like the aforementioned, very little literature is available describing bone shape and skull variation between individuals of the same teleost species. However, these studies do demonstrate a trend. The bones of the oral region of the skull tend to most often vary in shape, indicating that the bones of the oral region are likely variable in nature and the least constrained. These bones may be least constrained as a result of their location and function. The bones of the oral region are subject to numerous changes in mechanical forces based on changes in diet. Iin addition, they are not constrained by neighbouring bones as the jaws articulate with the rest of the skull at a single major hinge.

1.2.2 Variation in the Teleost Skull After Human Perturbation

In the following sections the affects of human influences or perturbations on the shape of the bones in the teleost skull will be investigated. The effects of inbreeding and aquaculture, diet and teratogens will be examined.

1.2.2.1 Effects of Inbreeding and Aquaculture

Skeletal malformations in cultured marine fish are widespread and costly (Cobcroft et al., 2001). These malformations can negatively impact growth, survival and the marketability of cultured fish. The effects of aquaculture are thought to arise through differences in nutrition, inbreeding, temperature, salinity and environmental factors such as overcrowding and mechanical stress. The influences with the largest impact on the skull will be described here (Cobcroft et al., 2001). Although malformations do not express natural variation present in the skull they do demonstrate where constraints exist and where they do not.

The most common defects in the skulls of aquacultured fish are mouth malformations, these include cross-bite, pigheadedness, sucker mouthed, elongated jaws, double mouth and gaping mouth (Cobcroft et al., 2001). Intensive culturing of the striped trumpeter *Latris lineata* has demonstrated that aquaculture of this species results in ventro-lateral distortion of the jaw, premaxilla and hyoid, causing a permanently open mouth due to walling behaviour (repeatedly hitting the wall of the tank) (Cobcroft and Battaglene, 2009).

Inbreeding may also take place in aquaculture settings. Inbreeding has been demonstrated to have detrimental effects on the skeleton of the cichlid *Cichlasoma nigrofusciatum* (Winemiller and Taylor, 1982). Inbreeding performed over four or five generations resulted in malformations causing a sloping forehead, as well as changes in the mandible, hyoid and operculum. When similar studies were conducted in the zebrafish comparable findings were identified (Piron, 1978). One of the many advantages of zebrafish are the short generation time (six months), however after inbreeding for three generations in captivity severe malformations were also observed in the operculum and the angle of the head (Piron, 1978). In addition, survival and reproduction were also reduced (Piron, 1978; Mrakovic and Haley, 1979).

Both inbreeding and raising teleosts in hatcheries can have extreme detrimental effects on the development of their skeletons and cause a vast array of alterations in the shape of the skull bones. Amongst aquaculture studies, the majority of variations in bone shapes are again located in the oral region of the skull. The bones displaying variability are cranial neural crest derived and are of mixed ossification type.

In addition to habitat and inbreeding effects as a result of aquaculture, alterations in the administered diet can influence theshape of the bones of the oral region and thus influence the variability of the skull.

1.2.2.2 Dietary Effects on the Teleost Skull

Alterations in skull morphology of fish raised in aquaculture are thought to be partially attributed to differences in food quality, variety, size and shape (Meyer, 1987; Bouton et al, 2002; Ornsrud et al, 2004; Fernandez et al, 2008). In aquaculture largemouth bass, *Micropterus salmonides*, (Order Perciformes Family Centrarchidae) are fed a diet of inert pellet food, while wild individuals feed on elusive prey encouraging them to use many methods of prey capture (Wintzer and Motta, 2005). As a result, morphological differences can be observed in the length of the head, specifically the length of the lower jaw and the premaxilla between wild and cultured fish. Two studies conducted in the cichlid species (Order Perciformes, Family Cichlidae) *Chiclasoma managuense* and *Neochromis greenwoodi* similarly demonstrated the effects of diet on

the development of the skull. The authors divided a clutch of young into two groups and feeding the groups different diets during early development, it was determined that diet affects the shape and size of the mandible as well as the maxilla and premaxilla (Meyer, 1987; Bouton et al, 2002). Based upon the above studies it is clear that altering diet can affect the shape of the bones of the oral region of the skull. The bones displaying changes in morphology are all cranial neural crest derived, and are of mixed ossification type (Cubbage and Mabee, 1996; Kague et al., 2012). Diet can also affect bones located in the trunk region however this thesis is solely focused on variation in the skull and therefore variations in the trunk will not be discussed.

1.2.2.3 Teratogens

A teratogen is an agent that causes alterations during embryonic or fetal development which are permanent and results in changes outside of the normal range of morphology (Christian and Brent, 2001). Although teratogens are not used in my study, I will review their effects here in order to fully describe the common regions of variation.

One common teratogen is ethanol, the effects of which have been extensively studied in humans (Reimers et al., 2004). Zebrafish have recently been used to elucidate the negative impact of teratogens (including ethanol) on early development. Water soluble teratogens are easily studied in zebrafish by adding the teratogen to the tank water. Low levels of ethanol exposure in early development results in defects in the craniofacial skeleton (Carvan et al., 2006; Reimers et al, 2004). Studies investigating this relationship concluded that ethanol treatment in zebrafish juveniles resulted in alterations in the development and shape of the ethmoid plate, the trabeculae crania (contributes to the ethmoid), infraorbitals, basicranial commissure, auditory capsule, the length of the jaw, and the otoliths. Other teratogens that have been demonstrated to alter the developing bones of the skull of a number of fish species, including retinoic acid (Means and Gudas, 1995; Vandersea et al., 1998) and 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD) (Poland and Knutson, 1982).

These studies indicate that teratogens tend to mostly affect the ethmoid, dentary and ceratohyal. The majority of the bones influenced are located in the oral region and

positioned ventrally on the head. These bones displaying variability are all derived from the same cell origin, cranial neural crest cells, and are of mixed ossification type.

1.2.3 The Impact of the Environment on the Teleost Skull

The effect which the natural environment both within the animal (e.g. soft tissues) and outside the animal (e.g. diet) can have on the shape of skull will be examined. Clearly, while the internal environment is largely controlled and regulated by developmental processes, allowing for more mild changes, the external environment can have a broad and dramatic influence on the organism.

1.2.3.1 Phenotypic Plasticity in the Teleost Skull

The previous studies (1.2.2) examined human interference on variation. Phenotypic plasticity is naturally induced variation in populations as a result of influences from the external environment. The cichlids found in large lakes in rift valley, East Africa for example, have demonstrated vast adaptive radiation through phenotypic plasticity. These cichlids have evolved rapidly into new species enhanced by alterations in feeding morphology and availability of different foods (e. g. Komfield and Smith, 2000; Albertson et al. 2001; Parsons et al., 2012). Cooper et al. (2010) conducted an in-depth analysis of skull shape in closely related cichlids to determine how and what aspects of the skull have changed to allow for adaptive eating and radiation. Geometric morphometric analyses indicates that the bones that showed the most variation include, the mandible length, maxilla length, articular, retroarticular, dentary, premaxilla, nasal and palatine bones indicating that these structures display high levels of phenotypic plasticity. The variations in the bones of the oral region were found to be quite large, with dramatic changes in mandible shape and angle, indicating that the bones of the oral region are mechanically unconstrained.

The aforementioned studies (such as those described in the previous section) indicate that the bones of the teleost skull which are most likely to change (and thus more variable) are located in the oral, olfactory and dorsal regions. These bones ossify through both intramembranous and endochondral ossification, indicating that ossification type

may not be relevant to how variable or stable a bone is in shape. The bones which demonstrated variability are largely cranial neural crest in origin.

1.2.3.2 The Influence of Soft tissues on the Development of the Teleost Skull

The presence of the eye has been shown to have an important function in the proper migration of cranial neural crest derived structures surrounding the eye (Kish et al., 2008; Langenberg et al., 2011). Langenberg and colleagues demonstrated using an eyeless zebrafish mutant, *chokh/rx3*, that the eye is necessary for the proper migration of the anterior neural crest cells into the dorsal part of the eye. When the eye is absent CNC migration fails. Skeletal defects in this mutant include malformations of the lower jaw and the neurocranium. In addition, the author determined that when the lens vesicle was absent during early development CNC migration is arrested at the edge of the presumptive eye field. The authors did not describe the phenotype in adulthood.

Yamamoto et al (2003) investigated the effects of lens apoptosis and subsequent eye regression on the skull of the Mexican tetra, *Astyanax mexicanus,* Order Characiformes. Eye loss resulted in changes in both the shape and number of bones surrounding the eye orbit. These results will be discussed further in Chapter 2.

Other soft tissues present in the head which may influence the shape of the developing skull bones are the cranial muscles. The muscles present in the head of the zebrafish have been fully described in embryos and adults (Schilling and Kimmel, 1997; Diogo et al., 2008). There are over 20 paired and unpaired muscles present in the head of the zebrafish. The cranial muscles can be divided into six groups based on their location, including the extraocular, mandibular arch, hyoid arch, branchial arch, pharyngeal wall and the dorsal posterior groups. Each muscle of the skull has an origin and insertion point, allowing the muscles to exert forces on the bones to which they are attached. For example the protractor hyoideus originates on the dentary and inserts on the hyoid arch, this muscle is responsible for providing the force to depress the mandible and open the mouth. The cranial muscles play an important role in the forces exerted on the developing skull.

In zebrafish, the cranial muscles form early in development, the first muscle to form develops at 53 hours post fertilization and is located in the extraocular region. The

last cranial muscle to develop forms at 85 hours post fertilization and is located in the branchial region (Schilling and Kimmel, 1997). Based on this information it is clear that the cranial muscles form quickly and are present early in development when the skull is still developing and is largely unossified. For example, the ocular region of the skull consists oflate ossifying bones that do not ossify until well after the extraocular muscles develop (Cubbage and Mabee, 1996). The cranial muscles exert forces on the developing bones of the skull (Schilling and Kimmel, 1997) and thus may play a role is the shape development of these bones (e.g. Daly et al., 2004). Despite the fact that the extraocular muscles develop early in life, it is unknown when these muscles are innervated and begin to function, as such further resesarch is required to determine the impact of the extraocular muscles on orbital bone growth.

1.2.4 Summary of Variability in the Teleost Skull

The studies reviewed here indicate that the teleost skull is affected by physical manipulation, aquaculture and inbreeding, diet, teratogenic, physical, environmental influences and soft tissues. It is clear that while some bones of the teleost skull are constrained (e.g. back of the skull and the calvariae) while others are highly variable (e.g. oral region).The bones in the oral region are the least constrained, with each one of the types of influences examined in this study affecting the morphology of the bones in this region. However, the majority of studies in this areafocus on the bones of the oral region only and thus these studies may be missing variation in other areas of the skulls thereby skewing the results.

With regards to cell origins, the majority of elements that were determined to be variable in nature are neural crest derived bones. Very few mesoderm derived bones were identified as variable in this analysis. As described previously (1.1.3), some cell lines and germ layers are more plastic than others, as such, it is logical that bones derived from plastic cell lines would be more variable, while bones derived from stable cell lines would be less variable in nature.

In the studies reviewed here, it is clear that the variability of a bone's shape may be highly correlated to the cellular origin of the bone, as well as the location of the bone within the skull. However, it appears that ossification type does is not correlated with this

variability, as bones which ossify through both ossification types demonstrate variability and stability. Finally, more information describing the variation in timing of ossification of the bones of teleost skulls is required to determine how the timing of onset of ossification affects the variability of a bone's size and shape.

1.2.5 Impact of Variation

A large portion of the teleost skull is easily influenced by the external (natural and induced) environment. As fish biologists typically house their research specimens within a lab setting, in a limited breeding stock, and fed them a commercial diet, the effects that this type of life may have on the skeleton should be considered when using these animals for skeletal research. Variation or changes in the shape of skull bones has been observed in many different teleost species and can arise naturally or as a result of influences from the surrounding environment, or changes within the animal. There is a great need for research to better understand what level of natural variation exists in the shape and size of individual bones of the teleost skull.

By removing the lens, I aim to better understand how soft tissues can influence the development of the skull and its components.

1.3 The Mexican tetra, *Astyanax mexicanus*

The Mexican tetra, *Astyanax mexicanus,* belongs to the Order Characiformes. The Mexican tetra is unusual as it is a single species, which exists as two morphs, a surface morph and a blind cave morph. The sighted, surface morph is found throughout streams and rivers in north-eastern Mexico and southern Texas (Yoshizawa and Jeffery, 2011; Yoshizawa et al., 2012; Gallo, 2012). The approximately 29 different blind cavefish morphs are thought to have originated from the surface morph in the last one million years (Jeffery, 2001, 2005, 2008). These cavefish populations are found in limestone caves located in north-eastern Mexico (Jeffery,2001, 2005, 2008). Competing theories on the evolution of the cave morph hypothesize either multiple cave entries with evolutionary convergence of many features or alternatively, one cave entry event with later divergence into multiple cave systems (Jeffery, 2005, 2008). Many studies have investigated these hypotheses, based on interbreeding cave populations, genetic and

phylogeographical analysis (Jeffery, 2005, 2008). Currently, multiple cave entries with convergence appears to be the most accurate hypothesis. These investigations have lead to questions regarding the taxonomy of the species, specifically whether the Mexican tetra is one or two species.

1.3.1 Classification of the Mexican tetra

The classification of the Mexican tetra as one or two species is currently under debate. Upon initial discovery of the surface and cave populations they were believed to be separate species, and were thus given different species names (Espinasa and Borowsky, 2001). With further investigation, including genetic analysis and breeding experiments the cave and surface morphs were determined to be one species *Astyanax fasciatus*, later renamed *Astyanax mexicanus* (e.g. Wilkens 1988, Wilkens 2004, Wilkens 2007, Mitchell, 1977, Jeffery, 2001, Jeffery 2007, Porter, 2007, Yamamoto et al., 2009) . The original studies investigating the classification of the Mexican tetra explored the capacity of the surface and cave morphs to interbreed (Sadoglu, 1957; Mitchell, 1977; Wilkens, 1988). In these studies, the authors determined that the surface and cave morphs can easily interbreed, in addition, the cave morphs are also able to easily interbreed amongst themselves, giving rise to fertile young, of intermediate morphology, suggesting that they are two morphs of a single species. As such, a surface morph and multiple populations of blind cavefish has been the accepted taxonomy over the past 30 to 40 years (Wilkens, 1988; Jeffery, 2001, 2007, 2008). In the recent past, the classification of the Mexican tetra has again come under scrutiny. Two groups of researchers in the United States and one in the United Kingdom consider the Mexican tetra to be two morphs of a single species, the Jeffery group in Maryland, the Borowsky group from New York and the Yamamoto group in London (Jeffery 2001, 2007, 2008; Bradic et al., 2012), while the Wilkens group of Germany believes the Mexican tetra to be two species, the surface fish *Astyanax mexicanus* and the cave fish *Astyanax jordani* (Wilkens, 2004, 2007; Strecker et al, 2012).

The most recent analysis performed by the Borowsky group, (Bradic et al., 2012) demonstrated significant levels of gene flow between many of the surface and cavefish populations based on microsatellite analysis. They also note strong levels of migration

and interbreeding between the populations. Phylogenetic analysis conducted by the Wilkens group (Strecker et al., 2004) based on mitochondrial DNA cytochrome b indicates that the species organization is more complicated than previously reported and based on their results should be separated into different groups and require further investigation. As such they propose calling the group of fish in question *Astyanax fasciatus* until the taxonomy has been further elucidated. They do, however, agree that all the groups in question are able to interbreed; the ability to interbreed is a traditional way to identify a species.

Another group divided the fish into eight species (Ornelas-Garcia et al 2008) based on genetic analysis of cytochrome b. Others, for example the Wilkens group (Hausdorf et al., 2011) disagree with their findings due to different genetic patterns located in the nuclear, versus mitochondrial, DNA. The Wilkens group have recently performed analysis on the nuclear and mitochondrial DNA (Strecker et al, 2012), where they comment on a lack of gene flow between the populations. In addition, they use the differential species concept, in which features are compared between the two groups, in the case that exchanged features between groups would negatively impact fitness of either group, the populations are then considered to be different species. As such, the authors consider the Mexican tetra to be two species and thus should be renamed. At this time, it is still unclear how the Mexican tetra should be classified. With different groups having differing opinions, and all providing evidence for their argument, the literature as a whole does not provide a clear answer to this question.

Undoubtedly these morphs are closely related and still have the ability to interbreed. As such, for the clarity of this study I accept the more commonly agreed upon classification of two morphs of a single species.

1.3.2 Distinctive Features of the Mexican tetra

The most striking and most highly investigated feature of the cavefish is their lack of eyes in adulthood. Despite the fact that adult cavefish do not have eyes, eye development begins in the same manner in both the surface and cavefish morphs until 24 hours post fertilization (Jeffery, 2001, 2005, Yamamoto et al., 2009). In addition to eye degeneration, cavefish have other regressive changes including loss of pigmentation,

reduction in the size of the optic tectum, and a reduction in aggressive and schooling behaviour (Jeffery, 2001, 2005; Yamamoto *et al.,* 2009). Less commonly studied are the constructive changes, which include changes in body position while feeding, increased jaw size, increased number of taste buds, increased size and number of neuromasts, larger fat stores and increased number of maxillary teeth (Jeffery 2001, 2008). These regressive and constructive changes in the cavefish have intrigued scientists for many years.

1.3.2.1 Eye Regression in the Cavefish

The first sign of eye development is a bulge off of the diencephalon forming the optic vesicles. The optic vesicles then expand toward the overlaying head ectoderm. The overlying ectoderm then thickens forming the lens placode. The optic vesicle invaginates as the lens placode delaminates from the head ectoderm, forming the lens around 24 hours post fertilization. At this point in eye development the proliferative phase of eye development is ending, the eyes of the two morphs begin to undergo differentiation in the same manner, forming the retina (Jeffery, 2008).

The following processes of eye development and regression in the Mexican tetra are divided into 4 stages. In stage one, (>4.0 mm SL) the early eyes of both morphs begin to develop a retina, and are largely similar in structure, although the cavefish eye is smaller (Wilkens, 2007). During this stage of eye development the surface fish lens begins to develop fiber cells, while all lens differentiation fails in the cavefish. In stage two, (between 4.0 and 6.0 mm SL) eye growth is arrested in cavefish, while eye growth continues in the surface fish. Apoptosis can be observed in the retina and lens of the cavefish only. Apoptosis in the lens results in subsequent apoptosis in the retina, as a result the eye development is arrested. In stage three, eye growth resumes in the cavefish eye but at a much slower rate than that observed in the surface fish. The cavefish lens does not undergo any further differentiation and remains as a lens capsule surrounding undifferentiated cells. The surface fish lens is fully differentiated by this stage with fiber cells containing crystallin. By stage four, eye development at less than 15 mm SL, eye growth slows further in the cavefish (Wilkens, 2007). The main differences between the two eyes at this stage are the size of the optic cup and lens, which are smaller in the

cavefish. In the cavefish, the small optic cup is outgrown by the developing head causing it to sink within the orbit and become covered with fat and skin by adulthood.

1.3.2.2 Causes of Eye Loss in the Cavefish

The lens plays a central role in eye development (Yamamoto et al., 2000), as such, lens apoptosis in the cavefish is responsible for eye loss. If an embryonic surface fish lens is transplanted into an age matched cavefish eye, apoptosis is no longer observed in the new lens, as a result eye regression does not occur and the adult cavefish has an eye on the surgery side of the head. The lens is therefore also capable of rescuing the retina. Extensive apoptosis is normally present in the cavefish retina, however, when a surface fish lens is transplanted into the cavefish eye, apoptosis no longer occurs, allowing further development of the cavefish retina (Strickler et al., 2007). Additionally, as the iris, cornea and anterior chamber do not normally develop in the cavefish, lens transplantation of a surface fish lens into a cavefish eye also rescues these structures (Strickler et al., 2007).

The genetic changes in the cavefish resulting in eye loss have been studied extensively. It was hypothesized that eye regression would occur through the down regulation of key eye genes (eg. *pax6*), however when microarray analysis was conducted many eye genes were found to be up regulated (Strickler et al., 2007). The Mexican tetra has an overlapping midline expression of the genes *sonic hedgehog* (*shh)* and *tiggy winkle hedgehog* (*twhh*) also known as *shhα* and *shhβ* during early development. In the cavefish the midline expression of the hedgehogs are expanded outward from the midline. The hedgehog expression is six cells wide in surface fish and 10 cells wide in cavefish (Jeffery, 2004). The increased expression of the hedgehogs results in an decreased expression of a gene critical for eye development, *pax6*. *Pax6* is normally expressed in both of the ocular regions, as a result of the changes in expression of *pax6*, another key eye gene *pax2* is upregulated. Finally, as a result of the above genetic changes there is an up regulation of *hsp90α* in the lens. The molecular chaperone *hsp90α* is a proapoptotic factor only found to be expressed in the cavefish lens. *Hsp90α* expression cannot be identified in the lens for the surface fish. When *hsp90α* expression is inhibited in the cavefish lens, lens apoptosis is blocked, indicating that *hsp90α* strongly influences apoptosis in the cavefish lens which results in eye loss in this morph (Hooven, 2004). Finally, an anti-apoptotic factor *αa-crystallin* expression is strongly down regulated in the cavefish eye also contributing to the lens apoptosis in this morph

(Strickler et al., 2007). How lens transplantion alters this pathway was not been investigated.

1.3.2.3 Linked Traits in the Mexican tetra

Genetic studies which have examined the impact of *shh* expression in the Mexican tetra, identified its role in eye regression and increase in taste bud number (Yamamoto et al., 2009). It was hypothesized that the sensory organs of the Mexican tetra can be regarded as individual, yet linked modules (Franz-Odendaal and Hall, 2006). Some modules are expanded in the cavefish, while others are regressed. Modules exist as networks of gene expression, cell types and developmental processes; natural selection may act on modules at any of these levels (Franz-Odendaal and Hall, 2006). Experiments conducted on larval surface fish involved injecting *shh* mRNA to achieve a more cavefish-like level . In specimens where the level of *shh* was increased, eye loss as well as an increase in taste bud number was observed. In a reciprocal experiment, the *shh* level was reduced in cavefish larvae to surface fish like levels, in these specimens the eyes were rescued and the number of taste buds was reduced. This study indicates that there is a trade-off present in the cavefish in regard to eye size and taste bud number directly linked to the expression level of the gene *shh* (Yamamoto et al., 2009). It appears as though the gene *sonic hedgehog* (*shh*) has pleiotropic effects during early development and that a sensory module trade-off may exist between the loss of eyes (eye module), increase in taste buds (gustatory module) and increase in lateral line neuromasts, as described previously (Franz-Odendaal and Hall 2008; Yamamoto et al., 2009;).

Quantitative Trait Loci (QTL) analysis was performed in the Mexican tetra in order to determine which traits are genetically linked in the species. Features such as eye size, lens size, pigmentation, jaw length, maxillary teeth, and taste bud number were examined. Of the 26 correlations calculated by Protas et al., (2007), they determined that only nine of the relationships were correlated. Eye size was determined to be negatively correlated with melanophores, the number of maxillary teeth and positively correlated with lens size. Melanophore number was strongly correlated with the length of the dentary and the number of taste buds. Surprisingly, eye size was not correlated with dentary length, or taste bud number, indicating that the genetics responsible for eye

regression are not responsible for the increase in taste bud number found in cavefish. These results are in contrast to the genetic studies described above in which eye loss and taste bud number were determined to be linked via the level of *shh* expression.

1.3.2.4 The Orbital Region of the Mexican tetra

In the surface Mexican tetra the skeleton surrounding the eye consists of one supraorbital bone and six suborbital bones, collectively known as circumorbital bones, while the cavefish have a different skeletal morphology surrounding the eye differing from surface fish in number of elements, size, and position of suborbital bones (Yamamoto et al., 2003). Yamamoto et al., (2003) identified changes in the shape and number of bones surrounding the eye orbit afterreciprocal lens transplants between the surface fish and cavefish. The mechanisms responsible for the orbital differences between the two morphs have not been investigated, making this fish species an excellent model for this research.

1.4 Thesis Objectives:

The presence of the eye and the lens has been shown to influence both the cells which form the skull and the morphogenesis of the craniofacial skeleton. My focus was to determine if the lens influences the development of the skull in the Mexican tetra and how variable the skull is in shape.

Very little research has been dedicated to unstanding how a soft such as the eye and/or the lens can influence the development of a hard tissue, namely, the skull. This study aims to further unravel the complex interactions which shape the bones of the skull. In addition, this analysis aids in better elucidating at what time in development the eye has the greatest impact on skull development and ultimately its shape. Finally, this study aims to determine if the level of variability is conserved across species and morphs.

Objective 1: To determine if lens ablation performed in the embryonic surface Mexican tetra morph alters the craniofacial, skeleton and if so, whether the effects restricted to one time point, or if is there a wider developmental window in which the craniofacial

skeleton is affected. The changes in shape observed in the skulls were examined in juveniles and adults, over a number of surgery time points using morphometrics. I hypothesize that the shape of the bones in the eye region will be influenced by lens removal. Furthermore, I hypothesize that taste buds and teeth will be unaffected by lens removal, while the extraocular muscles may be affected. This data is presented in Chapter 2, which is the largest section of the thesis. This chapterconsists of Part A which deals with the direct effects on the skull, while Part B deals with the effects on other structures in close relation to the skull, such as muscles, taste buds and teeth.

Objective 2: To investigate if early partial lens removal or lens damage performed with a laser between 1 and 11 dpf can affect the adult skeleton. I hypothesize that the shape of the orbital bones will be most affected the earlier in life the lens is damaged. This objective is presented in Chapter 3A.

Objective 3: During the studies undertaken to investigate objectives one and two, evidence appeared suggesting that the Mexican tetra has the capacity to regenerate the lens. This objective is to determine if the surface Mexican tetra does in fact have the capacity for lens regeneration and if so over what time period regeneration occurs. I hypothesize that further analyses will reveal that the Mexican tetra has capacity for lens regeneration. These results will be presented in Chapter 3B. Identifying a new species with the capacity for lens regeneration would allow for future comparative lens regeneration studies.

Objective 4: To investigate if lens removal performed in objective one can transform a surface Mexican tetra into a cavefish morph. This research investigates the similarities between the cavefish skull and surface fish skull after surgery. Finally, the skulls of the surface surgery fish, cavefish and intermediate are compared to determine if a lens removal surface fish results in a more intermediate like skull. I hypothesize that the surgery skull will be more similar to the intermediate skull. This objective will provide further insight into the extent to which the lens can influence the development of the skull. Objective four is presented in Chapter 4.

Objective 5: To perform a comparative study in which lens removal was conducted in zebrafish in order to determine if lens removal results in similar changes to the skull across teleost species. In addition, the surgery zebrafish was compared to a zebrafish eye mutant which has an abnormal lens. I hypothesize that the effects of lens removal will be conserved between the Mexican tetra and zebrafish. These findings will be presented in Chapter 5.

Chapter 6: This chapter concludes the thesis and will discuss the findings of the thesis in a broader context.

Chapter 2: Early Lens Ablation Causes Dramatic Long Term Effects on the Shape of Bones in the Craniofacial Skeleton of *Astyanax mexicanus*

2.1 Introduction

This chapter of my thesis has been divided into two sections, Part A and Part B. The purpose of Part A is to further elucidate how the eye, a soft tissue, can influence the development of the hard tissues, namely the skull. In addition, this study aims to further investigate how variable in shape the bones surrounding the eye are and what capacity the bones have to change shape. Some of the findings of Part A have been previously published in PLoS ONE 7(11): e50308. Part B will investigate how lens removal can affect structures outside of the orbital region of the skull. The investigation will include how lens removal affects tooth and taste bud number.

The eye is an island of soft tissue surrounded by the neural crest derived tissues of the craniofacial skeleton. Studies investigating interactions between the soft eye tissues and the hard skull tissues have been rare until recently. Here, this relationship is explored. I investigate the links between the development of the eye and the surrounding craniofacial skeleton. To investigate this relationship between the eyes and skull, I conducted experiments on the Mexican tetra, *A. mexicanus*. I ablated the lens from surface tetra embryos and analyzed the effects on the adult skull and associated structures. I conducted lens ablation in surface fish at multiple time points and have examined the effects in adults; this relationship will be examined in Part A of this chapter. In addition, I carefully examined the shape changes in the circumorbital bones (the supraorbital and suborbital bones) surrounding the eye. In Part B, will examine how the lens can influence other structures of the Mexican tetra head, including the lens' influence on tooth and taste bud number, as well as the lens' influence on extraocular muscles.

Yamamoto et al. (2003) noted differences between the skulls of the two morphs of the Mexican tetra. The authors recorded differences in the orbital bones, specifically expansion in the supraorbital and small expansions in the suborbital bones. The ocular

skeleton of the surface morph consists of one supraorbital bone and six suborbital bones, while the cavefish differs in the number of elements, size, and position of suborbital bones (Yamamoto et al., 2003). The suborbital bones are intramembranous bones that form directly from mesenchymal condensations. These bones are late-forming bones that begin to ossify at approximately 22 mm body length, around the age when scales develop (Yamamoto et al., 2003). Skull development continues late into life, and is mature at approximately one year of age. In addition, the surface fish (as in many other teleosts) have two endochondrally formed scleral ossicles in the eye (Franz-Odendaal et al., 2007; Franz-Odendaal, 2008), while cavefish have a single cartilage element in the sclera (Yamamoto et al., 2003). The surface fish scleral skeleton development begins with a condensation forming first a solid ring of scleral cartilage, which is present at 11 dpf (Franz-Odendaal et al., 2007). At approximately 24 mm body length the anterior scleral ossicle begins to develop via perichondral ossification. As the anterior ossicle continues to lengthen, the posterior ossicle begins to ossify. The scleral skeleton of the cavefish has been reported to remain as a cartilage ring, as a result of a lack of ossification (Yamamoto et al., 2003). The craniofacial skeleton is considered to be mature at 35 mm standard length (SL), at approximately 9 months to 1 year of age.

Yamamoto and Jeffrey (2003) transplanted cavefish lenses into surface fish eyes at 24 hours post fertilization (hpf). This transplantation resulted in eye regression in the surface fish comparable to normal eye regression observed in the cavefish. After eye regression in the surface fish some of the craniofacial bones were affected, and are thus dependent on the presence of the developing eye (Yamamoto et al., 2003). Dependent elements include: the nasal bones, antorbital bones and olfactory pits, an ossified sclera (i.e. scleral ossicles), and the shape of both suborbital 3 and the supraorbital bone. Elements not altered by the presence of the eye (i.e. independent) include the maxillary teeth, the number of elements making up suborbital 3, the positions of suborbitals 4 through 6, and the shape of the opercular bone (Yamamoto et al., 2003). In a reciprocal lens transplant experiment with a surface lens transplanted into a cavefish eye, the cavefish eye was rescued and an eye developed. In the cavefish with a rescued eye, the eye developed an ossified sclera, which is not typically present in the cavefish sclera (Yamamoto et al., 2003). In the described study, lens transplantation was conducted and

therefore the effect of lens removal alone was not established. In addition, the skeletons were analyzed at approximately 6 months of age, when the Mexican tetra skull has yet to fully develop. Despite these shortfalls the study is valuable in first indicating a link between the eye and skull development.

2.1.1 Differential *shh* Expression in the Mexican tetra

The regressive and constructive changes in the cavefish have intrigued scientists for many years. In 2009, Yamamoto and colleagues investigated the relationships between the regressive and constructive changes through the over expression of *shh* in surface fish and inhibition of *shh* in cavefish embryos (Yamamoto et al., 2009). In surface fish there is small midline expression of *shh* present between the developing eyes, responsible for splitting the eye field into two, however in cavefish this midline expression of *shh* is expanded in width by 1 to 2 cells, prior to eye development. Hooven et al., (2004) proposed that the increase in *shh* in cavefish results in lens apoptosis and subsequent eye regression as a result of the decreased expression in the key eye gene *pax6* downstream of *shh* (Hooven et al., 2004). Yamamoto et al. (2009) investigated the other effects of altering *shh* expression. Following ectopic *shh* expression in surface fish the authors report an increase in taste bud number and mandible size. In addition, the authors concluded that the midline expression of the gene *sonic hedgehog (shh)* has pleiotropic effects during early development and that a sensory module trade-off may exist between the loss of eyes, increase in taste buds and increase in lateral line neuromasts (Franz-Odendaal and Hall, 2006). The concept of modularity has been applied to the various regressive and constructive traits of the Mexican tetra. Franz-Odendaal and Hall (2006) proposed that there is a regressed eye module and constructive taste bud module present in the cavefish, which may interact at the gene level. Yamamoto et al. (2009) confirmed that these two modules are linked through the expression of *shh*. Trade-offs between modules will be investigated in Section B of this Chapter.

2.1.2 The Teeth of the Mexican tetra

Other aspects of head development are different in cavefish, namely tooth number. Only one study has investigated tooth number in this species. In the Mexican tetra, early tooth development begins with a set of small unicuspid teeth which develop in the centre of the jaws and on the maxilla (Trapani et al., 2005). Tooth development begins at 3 to 4 dpf, while maxillary teeth do not develop until approximately 60 dpf. At 23-24 dpf the centrally located unicuspid teeth on the jaws are replaced with large multicuspid teeth. These multicuspid teeth undergo regular replacement cycles throughout life, which occur approximately every 50 days (Trapani et al., 2005). Teeth located on the caudal portion of the jaw are much smaller in size, and may be uni or multicuspid and follow different irregular replacement cycles compared to the central multicuspid teeth (Trapani et al., 2005). Unlike the central teeth the caudal teeth form outside of the bone (extraosseously) of the jaw and vary in number during adulthood, unlike the number of large teeth (Trapani et al., 2005). The teeth of the Mexican tetra can be divided into three groups, based on differences in location within the mouth, timing of development and pattern of replacement (Trapani et al., 2005). The three groups consist of the large multicuspid mandibular and premaxillary teeth, the small mandibular teeth, and the maxillary teeth (Trapani et al., 2005).

Differences in tooth numbers have been described between the two morphs of the Mexican tetra and as well as in variations between cave population (Yamamoto et al., 2003). Studies investigating tooth differences between cavefish and surface fish have largely focused on the maxillary teeth (Yamamoto et al., 2003). Surface fish have one multicuspid tooth per maxillary bone, while all cavefish populations have a minimum of two multicuspid teeth per maxillary bone (Yamamoto et al., 2003). Some cavefish such as the Tinaja cavefish population have as many as four multicuspid teeth per maxillary bone (Yamamoto et al., 2003). In studies where lens transplants were performed between cavefish and surface no changes were observed in the number of teeth present on the maxillary bone. Surface fish with a cavefish lens still had only one maxillary tooth, while cavefish with a surface fish lens still had at least two maxillary teeth. No studies have investigated differences in tooth numbers between the two morphs located outside of the maxillary bone.

2.1.3 The Taste Buds of the Mexican tetra

As described previously, there is a trade-off between eye loss and an increase in taste bud number in the Mexican tetra (Yamamoto et al., 2009). Taste buds are morphologically the same in both morphs of the Mexican tetra, i.e. pear-shaped intra epithelial sensory structures (Varatharasan et al., 2009). Each taste bud consists of one round basal cell surrounded by several pear-shaped receptor cells, located in the mouth on the surface of head, on the chin and on the gill arches. Taste buds on the lower jaw of both morphs are arranged in two rows, an inner and outer row (Varatharasan et al., 2009). Cavefish have a larger number of taste buds compared to surface fish, as much as a 5 to 7 fold increase in taste bud number by adulthood (Schemmel, 1967; Varatharasan et al., 2009). At 5 dpf both morphs possess only a single row of taste buds on each jaw, by 12 dpf a second row of taste buds have begun to form on the caudal portions of the jaws (Varatharasan et al., 2009). By 22 dpf the two full rows of taste buds are present. At 5 dpf the two morphs have approximately the same number of taste buds, indicating that initial taste bud patterning is the same in the two morphs. Following, the cavefish rapidly develop taste buds resulting in significantly more taste buds by 22 dpf (Varatharasan et al., 2009).

2.1.4 Extraocular Muscles

Vertebrates develop important networks of musculoskeletal connections during early development (Schilling and Kimmel, 1997). These musculoskeletal connections are important for producing the mechanical forces in the head and body. The muscles of the teleost head can be divided into five groups based on their location in the head. Once the muscles have the capacity to contract they produce strains on the surrounding skeleton, and therefore alterations in head muscles may result in influences on the bones on which they insert (Schilling and Kimmel, 1997). Unfortunately, no studies have described the head muscles in the Mexican tetra, but they have been described in the zebrafish. Adult zebrafish have six extraocular muscles, responsible for the movement of the eye (Schilling and Kimmel, 1997). The extraocular muscles develop early in ontogeny, between 66 and 72 hpf (Easter and Nicola, 1996). The superior and inferior oblique

muscles originate in the rostral orbit and insert on the dorsal and ventral surface of the eye. The superior and inferior rectus muscles originate in the caudal orbit and insert caudal to the oblique muscles. The medial and lateral rectus muscles are the last to form. In addition, there are muscles located in close proximity to the eye which are primarily associated with the mandible, but may also influence the orbital regions. These muscles include the adductor mandibulae, the elevator arcus palatine, and the dilator opercula (Schilling and Kimmel, 1997). Alterations in any of the muscles in contact with or in close proximately to the eye as a result of surgery may dramatically change the mechanical forces present on the bones within the skull.

This chapter of my thesis has been divided into two sections, Part A and Part B. The purpose of Part A of this study is to better understand how the eye, a soft tissue, can influence the development of the hard tissues, namely the skull, surrounding it. Further, to investigate how variable in shape the bones are surrounding the eye and to what extent the shape of bones are able to change. I hypothesized that the shape of the bones in the eye region would be influenced by lens removal, while tooth and taste bud number would not be. My analysis demonstrates that some bones are responsive to manipulations, while others are resistant. My results indicate that many (but not all) of the suborbital bones are affected by lens removal, highlighting the plasticity of some skull bones. This type of long-term study is unique and provides valuable insight into our understanding of how processes during early stages of eye development can affect the adult skull. Part B will investigate how lens removal can affect structures outside of the orbital region of the skull. The investigation will include, i) how lens removal affects skeletal structures outside of the orbital region, e.g. tooth number, and ii), how lens removal affects taste bud number. Finally this study builds on the evidence presented in Part A in order to determine the mechanism by which lens removal can influence bone shape, by investigating the effects of lens removal on the muscles located in the ocular region. Part A and B each have a Materials and Methods and a Results sections. There is one discussion in which the results from Parts A and B will be discussed together.

Part A:

2.2 Materials and Methods

2.2.1 Biological Material, Mexican tetra, *Astyanax mexicanus* Surface Fish

Second generation from wild surface Mexican tetra adults were obtained from Dr. R. Borowsky (New York University, New York City, USA) and Dr. W Jeffery (University of Maryland, Maryland). Adults were maintained at 21^{o}C on a 12 hour light, 12 hour dark cycle in a recalculating flow-through Aquatic Habitat housing system at Mount Saint Vincent University, Halifax Nova Scotia, Canada. To induce spawning, tank temperature was increased to 26^{o}C and two males were added to a tank containing one female. Eggs were collected the next morning. After collection embryos were reared in 200 to 500 ml of system water in a tumbler at 21^{o}C for two weeks. At 3 dpf to 2 weeks of age the larvae were fed hatched *Artemia fransicana.* At 2 weeks of age the juveniles were transferred to the rack system and fed crushed TetraMin staple flake. Adult cavefish of unknown origin were purchased from Pets Unlimited in (Bayers Lake, Halifax, Nova Scotia). Adults were maintained and bred in the same manner described above for surface fish. All juveniles used in this study were breed and collected in the Mount Saint Vincent University fish facility. All protocols follow the Canadian Council on Animal Care guidelines, which were annually reviewed by the SMU-MSVU Animal Care Committee.

2.2.2 Surgery Experiments

Lens removal was conducted unilaterally in surface fish at 24 (hpf), 2 dpf, 3 dpf, or at 4 dpf (n= 6, 7, 5, and 5 respectively) using tungsten needles and following the basic procedure outlined by Yamamoto and Jeffery (2002), by tearing the cornea and then removing the lens (Details in Appendix 3). Minor alterations to the protocol included incubating the specimens in 0.01% MS222 (Ethyl-3-aminobenzoate methanesulfonic acid salt, Sigma E10521) in calcium free zebrafish Ringer's solution for two minutes to anaesthetize the fish. After surgery, specimens were rinsed in zebrafish Ringer's solution three times, released from the agar mounting medium and returned to the fish facility

until adulthood. The non-surgically manipulated eye serves as a control for these experiments.

As an additional control, the cornea was torn but the lens was not removed (n=4). The maximum diameters of both eyes were measured along the anterior-posterior axis of each specimen that received a corneal tear, described further in section 2.2.6 (Figure 2-1). To determine if the corneal tear eye was different in size to the control eye statistical analysis was performed using an independent t-test in Minitab version 16.

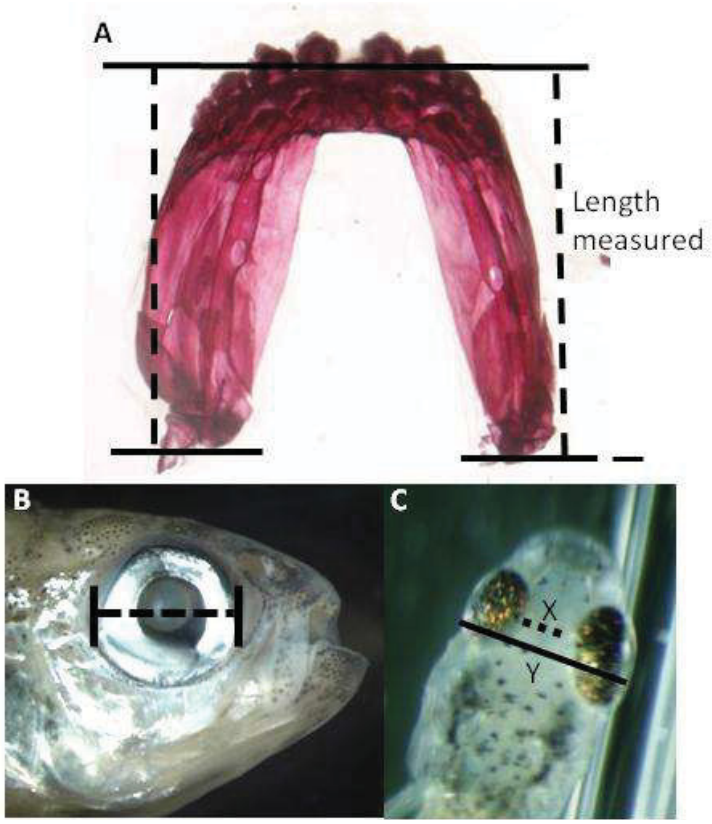


Figure 2-1: Jaw, eye and head width measurements of surgery surface fish. (A) An adult whole mount bone stained and dissected mandible. Dashed bars indicate the length of the surgery and control sides of the jaw that were measured; (B) measurement of eye diameter. Dashed bars indicate the diameters of adult and juvenile eyes were measured; measurements of head width between the eyes and total head width. Diagram depicting the distance measured between the eyes (X) and the measurement of total head width (Y) measured from the dorsal view of juvenile fish. Scalebar in A is 200µm.

2.2.3 Whole Mount Bone Stain

Adult surface fish between 9 months and 12 months of age (minimum standard length of 3.5 cm) were anaesthetized using 0.1% MS222, and then fixed in 10% Neutral Buffered Formalin (Fisher, 23-245-685). Alizarin red S (Sigma A5533) was used to whole mount bone stain the skeleton. The procedure was adapted from Franz-Odendaal et al., (2007). Adults were stained when the ocular skeleton is fully developed. An incision was made between the surgery and control sides of the head, along the sagittal suture during the staining process to allow for better stain penetration. Briefly, fish were bleached overnight in 3% hydrogen peroxide (Compliments, store brand) in 1% potassium hydroxide (Sigma 1767) solution. The following day, fish were rinsed in water, and then soaked in saturated sodium tetraborate (Sigma B9876) for eight hours. Fish were stained overnight in alizarin stain (1 mg/ml alizarin in 1% potassium hydroxide). Finally, specimens were rinsed in 1% potassium hydroxide (Sigma 1767) then cleared in 1% trypsin (Fisher Scientific 9002-07-7) and 2% Sodium tetraborate (Sigma B9876) in distilled water for three nights. The specimens were processed through an ascending series of glycerol in 1% potassium hydroxide solution then transferred to a storage solution of 100% glycerol. Further description of staining is provided in the Appendix 3.

Gross morphological analysis of the skull was then conducted on the surgery surface fish and control fish under a Nikon dissecting microscope.

2.2.4 Whole Mount Cartilage Stain

Juvenile surface fish were anaesthetized between 1.24 and 1.75 cm SL, using 0.1% MS222, and then fixed in 10% neutral buffered formalin (Fisher, 23-245-685). Alcian blue (Sigma A3157) was used to cartilage stain the skeleton of juveniles, during the timing when the ocular skeleton is known to be developing. The procedure was adapted from Klymkowski and Hanken, (1991). Briefly, fish were transferred from 70% ethanol (storage solution) to 0.015% alcian blue stain in 20% glacial acetic acid (Fisher A38212) in 100% EtOH overnight. The following morning samples were rinsed in 95% EtOH then placed in saturated sodium borax overnight. The following day the specimens were transferred to 2% sodium tetraborate and 1% trypsin for three nights, following the

specimens were bleached overnight in 3% Hydrogen peroxide in 1% Potassium hydroxide solution. The specimens were processed through an ascending series of glycerol in 1% Potassium hydroxide solution then transferred to a storage solution of 100% glycerol. Further description of staining is provided in the Appendix 3 .

2.2.5 Orbital Bone Outgrowth and Jaw Measurements

One-eyed juvenile surgery surface fish ranging from 0.9 cm SL to 3.5 cm SL were whole mount bone stained (as described above). Following staining, the fused supraorbital bones and frontal bones were dissected from each side of the head. Images of these bones were captured using a Nikon SMZ1000 microscope and NIS Elements software package, to allow for comparison of one side of the head to the other. Jaw measurements were conducted from the retroarticular joint of the dentary to the anterior most point of the dentary (Figure 2-1). Statistical analyses (one-tailed paired t-test) were conducted on measurements from both the surgery and control side of the lower jaw (n=12), using Minitab version 16.

2.2.6 Eye Regression Measurements

The maximum diameter along the anterior-posterior axis (as represented in Figure 2-1) of the eye was measured over early development (4 to 35 dpf, Appendix 2, Table 6 and 7). The differences in diameters between the control and surgery eyes were compared as a percentage. In addition, the width of the head in the eye region was also measured (in a dorsal view), following the distance between the eyes measured (Figure 2-1). To determine the ratio between normal head width and eye position within the head, the distance between the eyes was divided by the total width of the head including the eyes. Measurements were conducted on control surface fish, surgery surface fish and cavefish juveniles.

2.2.7 Morphometric Analyses

To compare the shape of the adult skull bones on the control side of the head to the surgery side images of the lateral, dorsal and ventral view were captured using a Nikon SMZ1000 microscope and NIS Elements software package. Independent analyzes

were conducted for each of the four time points of lens removal. Forty-two (x,y) landmarks were applied to the lateral view images of the head (Figure 2-2, Appendix 1, Table 1). Eleven landmarks were applied to each of the dorsal skull and ventral jaw views (Figure 2-2, Appendix 1, Table 2 and 3). Landmarks were applied using tspDIG2 software (F. James Rohlf, http://life.bio.sunysb.edu/morph/) and analysis was conducted using the IMP series of software (H. David Sheets, http://www3.canisius.edu/~sheets/morphsoft.html, version 6). The IMP software Coordgen was used to calculate Procrustes distance and the program TwoGroup was used to calculate the average Procrustes distances for each of the control and surgery eye groups. The statistical difference in shape between the surgery and the control lateral, dorsal and ventral views based on average Procrustes distances were analyzed using Goodall's F-test and F test, Procrustes. This tests the overall shape difference between Twogroups while taking into account variance within each group. In addition, the lateral view of the surgery side of the skull was compared to control adult tetras that had not undergone surgery on either side of the head. In addition, Twogroup was used to compare the control side of the surgery specimens head to control specimens that had not received surgery. Finally TwoGroup was used to determine if surgery at 1 dpf has a greater impact on the skull than surgery performed at 4 dpf, by comparing the 1 dpf to the controls and comparing the 4 dpf group to the controls and then comparing the distance between these two scenarios.

Vector analyses were conducted using the program tpsSuper written by F. James Rohlf (State University of New York). TpsSuper was used to create a consensus configuration of the landmarks for each of the four control groups and each of the surgery groups. The consensus of each surgery group was compared to its control group using tpsSplin (F. James Rohlf). Vector outputs depict the direction and magnitude of each surgery landmark is different from its corresponding average control. Thin-plate splines were calculated by comparing the average control to the average surgery in the tpsSplin program. Thin-plate splines allow visualization of shape changes in the landmarked locations based on deformation of a grid. PCAGen part of the IMP software (H. David Sheets, http://www3.canisius.edu/~sheets/morphsoft.html, version 6) was used to visualize patterns of variation based on principle component analysis (PCA). The first

principle component represents the axis with maximum variance, while the second principle component is orthogonal to the first (Webster and Sheets, 2010). PCA allows visualization of outliers or clusters of specimens. PCAGen was used to generate a PCA plot to compare adult skulls after surgery at 1 dpf, 2 dpf, 3 dpf, 4 dpf, and control specimens (without surgery). Finally, the morphometrics program Morphologika (written by Paul O'higgins) can be used to connect landmarks on a specimen in order to visualize bone shape, different specimens can then be overlaid to visualize differences.

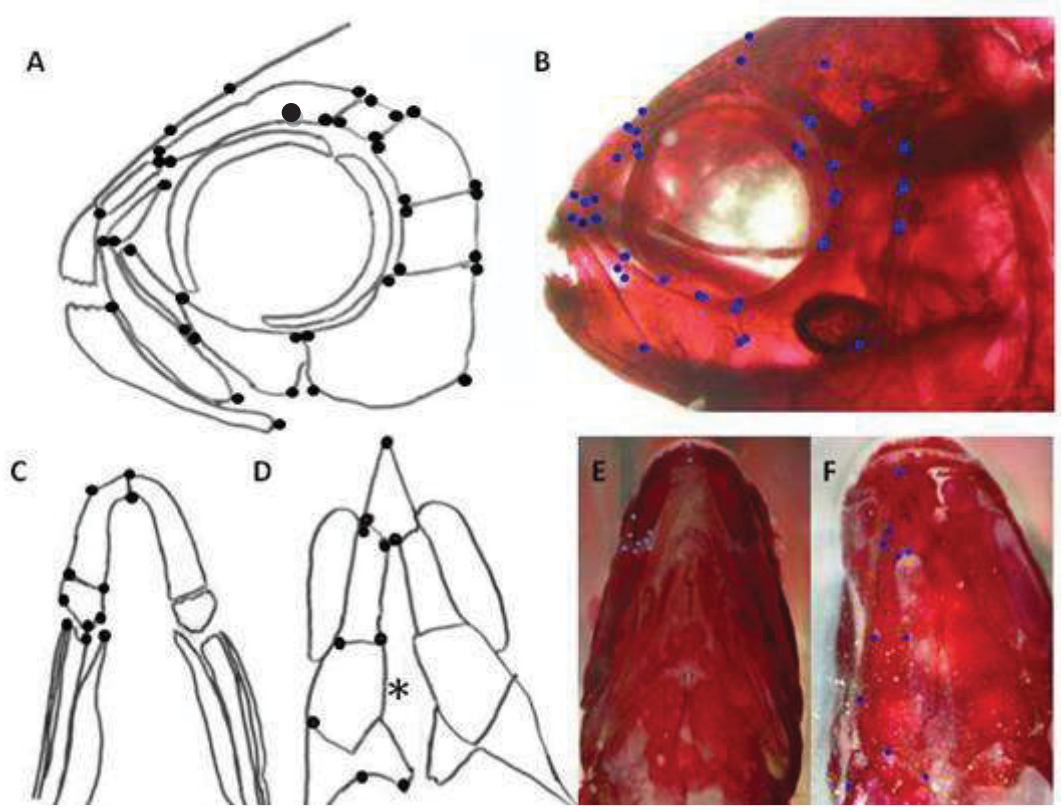


Figure 2-2: Schematic and whole mount bone stains showing morphometric landmark locations on adult surgery skulls. (A) Schematic of 42 lateral view landmarks locations; (B) the 42 lateral view landmarks applied to the tetra skull (C and E) 11 ventral jaw landmark locations; (D and F) 11 landmark locations on the dorsal view of the skull. The asterisk indicates an incision in the midline of the frontal and parietal bones. Landmark locations are identified in the Appendix 1.

2.3 Results

2.3.1 Eye Regression After Complete Lens Removal

Lens ablation in the surface Mexican tetra resulted in eye regression in approximately 50% of juveniles, regardless of the age of the embryo when surgery was performed (n=51). The 50% that did not exhibit eye regression undergo lens regeneration, which will be discussed in Chapter 3B. In the individuals that exhibited eye regression after lens removal at 1 dpf, the surgery eye was visibly smaller than the control eye 4 days after surgery (Figure 2-3). Over the first month of development the size (diameter) difference between the surgery eye and control eye becomes more dramatic (n=15). The diameters of the surgery and control eyes were measured and compared (Figure 2-4). For example, the diameter of the surgery eye was 17% smaller than the control eye by 4 days after surgery, by 14 days after lens removal the surgery eye was 28% smaller then the control eye and finally by 34 days after lens removal the surgery eye was 41% smaller then the control (Figure 2-4, Appendix 2, Table 6). A paired t-test confirmed that the surgery eyes were significantly smaller in diameter than the control eyes ($p<0.05$) in each of the time points examined. By adulthood, the surgery eye had completely sunken into the head and is covered with skin (Figure 2-3). By measuring the diameters of the eyes after surgery over early development it is clear that the difference in size between the surgery and control eyes occurs rapidly in early development (Figure 2-4). Between 15 and 20 days after surgery the surgery eye is between 35% and 55% smaller than the control. This difference in eye size is maintained and not increased by 34 days after surgery.

When the lens was removed at 3 dpf a difference in eye size was visible shortly after surgery (Figure 2-5, Appendix 2, Table 7). Eye regression was investigated by again measuring the diameter of the control and surgery eyes (n=7). By 4 days after lens surgery the surgery eye was 17% smaller than the control (Figure 2-6). By 6 days after lens removal the surgery eye was 42% smaller than the control, indicating that eye regression was more rapid when it was performed at 3 dpf (Figure 2-6). A paired t-test confirmed that the surgery eyes were significantly smaller in diameter than the control eyes after the lens was removed at 3 dpf ($p<.05$).

When lens removal is conducted at 3 dpf, eye regression was much more rapid than when lens removal was conducted at 1 dpf. Eye regression occurred in the same manner in the two surgery time points until 4 days after lens removal at which time both surgery eyes were 17% smaller, however by 6 days after the 3 dpf lens removal the surgery eye was 42% smaller indicating much more rapid eye regression (Figure 2-6). Later surgery resulted in much more rapid eye regression (Figure 2-4, 2-6).

To better understand how the regressing eye is positioned within the head, the distance between the back of the eyes and the total width of the head was measured on the dorsal view (Figure 2-1C). By graphing the ratio a trend can be observed of the timing of eye regression over early development. Based on the trends identified by the measurements it was determined that the lens removal and subsequent eye regression does not mimic natural eye regression observed in cavefish (Figure 2-7). Rather, in surface fish, eye growth was arrested and the head width outgrew the position of the regressing eye, rather than the surgery eye sinking into the head as was previously described (Jeffery, 2001, 2008) (Figure 2-7). In addition, the eye regression into the head occured much more rapidly in the cavefish and the eyes sunk more deeply into the head than it did in the surgery surface fish.

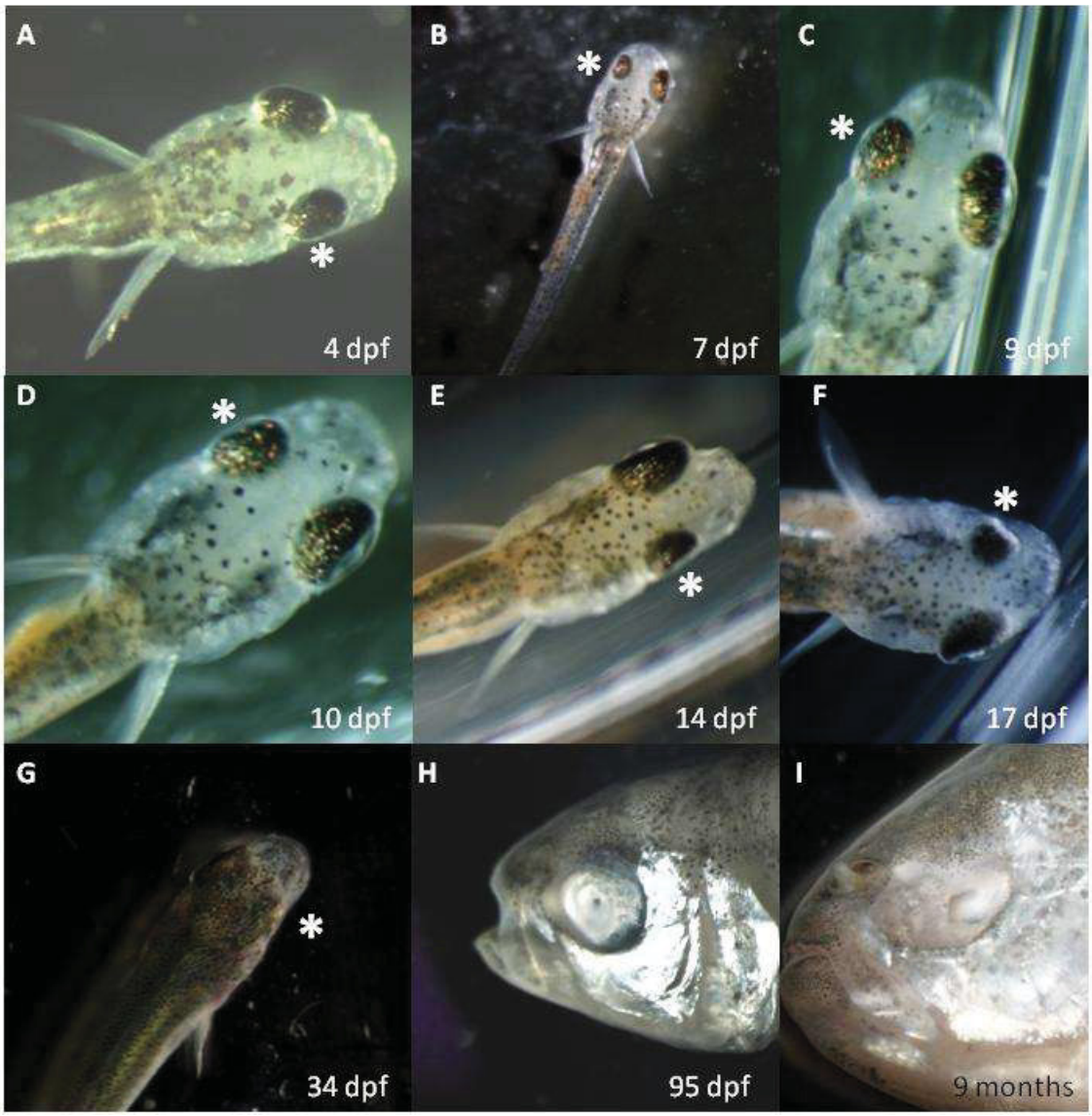


Figure 2-3: Juvenile to adult surgery tetras demonstrating the size difference between the surgery eye and the control eye. Affects lens removal at 1 dpf, the white asterisk indicates the surgery eye. (A) a live fish 4 days after surgery; (B) 7 days after surgery; (C) 9 days after surgery, surgery eye is 23.5% smaller than control; (D) 10 days after surgery, surgery eye is 24% smaller than control; (E) 14 days after surgery, surgery eye is 28.4% smaller; (F) 17 days after surgery, surgery eye is 41% smaller than the control; (G) 34 days after surgery, surgery eye is 41% smaller than the control; (H) surgery eye, 95 days after surgery; (I) surgery eye, 9 months after surgery.

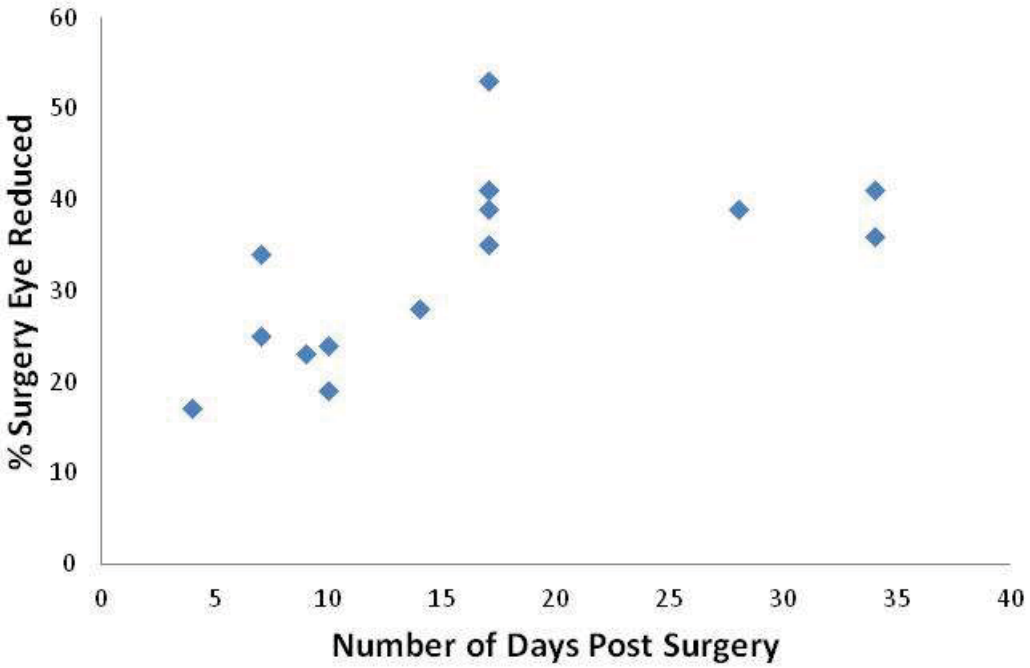


Figure 2-4: Percentage of eye reduction after lens removal at 1 dpf. The percentage the surgery eye is reduced in diameter compared to the diameter of the control eye, over the first 34 days after surgery, n= 14.

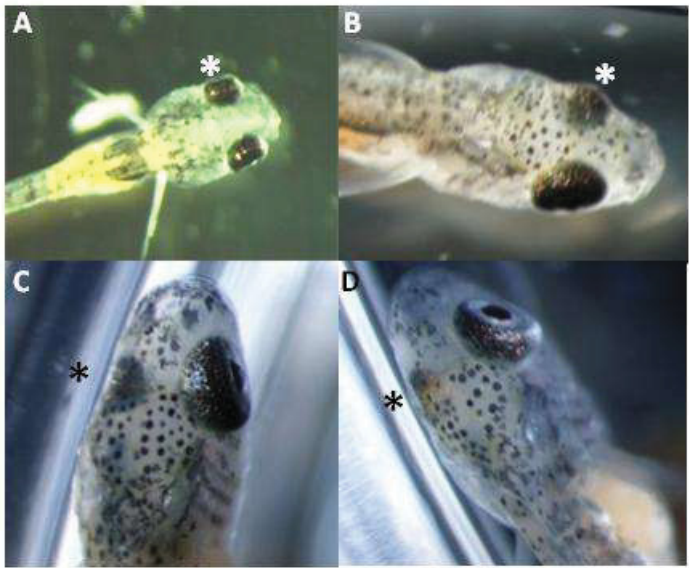


Figure 2-5: A comparison between the surgery and control eye size in juvenile tetras which received lens removal at 3 dpf. Manual lens removal was performed at 3 dpf, the white asterisk indicates the surgery eye. (A) live fish 3 days after surgery, surgery eye is 11.5% smaller than control; (B) live fish 12 days after surgery, surgery eye is 22% smaller than the control; (C) 13 days after surgery, surgery eye is 39% smaller; (D) 13 days after surgery, surgery eye is 44% smaller.

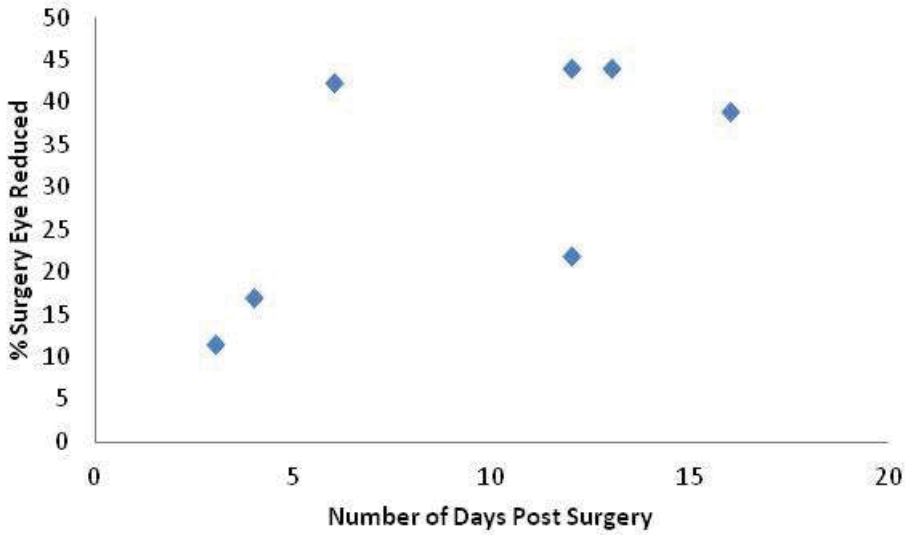


Figure 2-6: Percentage of eye reduction after lens removal at 3 dpf. The percentage the surgery eye is reduced in diameter compared to the diameter of the control eye, over the first 16 days after surgery, (n= 7).

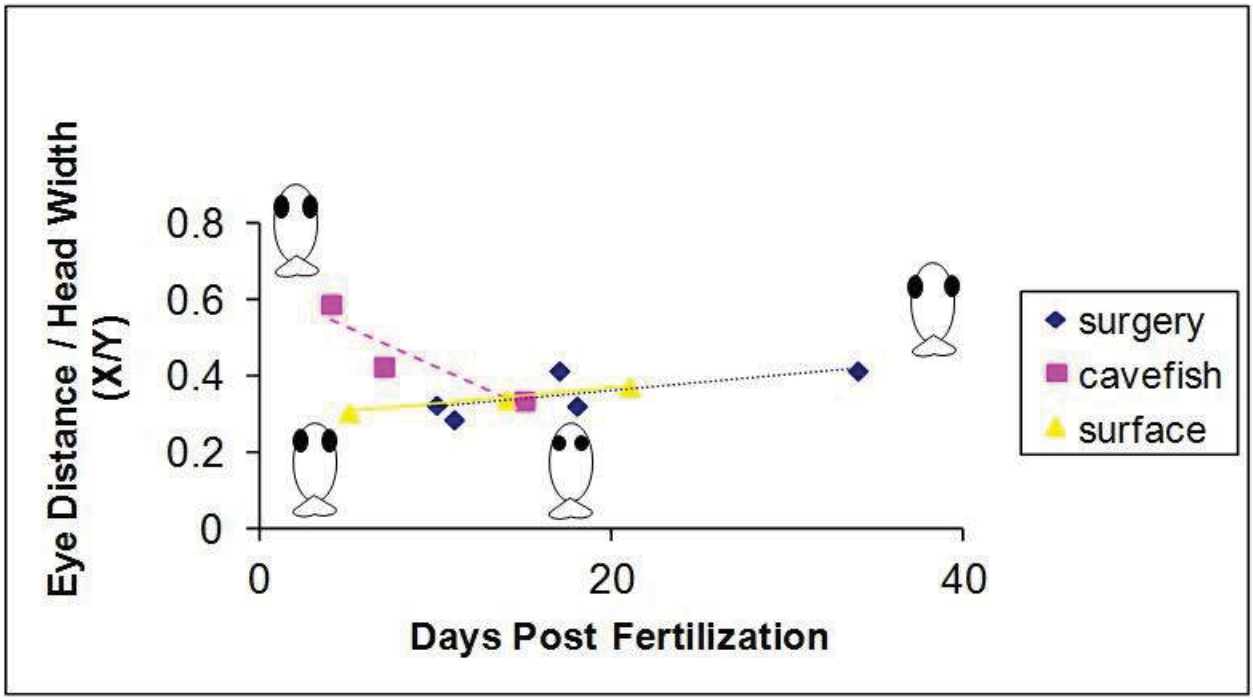


Figure 2-7: A comparison between the eye to head width ratio in surface, cavefish and surgery surface fish (1 dpf) over development. The small fish images depict the position of the eyes within the head and how the position changes in surface fish and cavefish over development.

2.3.2 Early Lens Ablation Affects the Size and Shape of Some Orbital Bones More Than Others

The surgery eye was approximately 30% smaller than the control eye by 30 days post surgery. As the skull grew in size, it appeared to outgrow the regressing eye (Figure 2-3). To determine how a small lens-less eye affects the surrounding skull, adult surgery specimens (3.5 cm SL) were whole mount bone stained.

Observation of the stained specimens showed that several bones surrounding the surgery eye are affected by lens ablation, and that the effects are less dramatic the later surgery was performed. These results are summarized in Table 2-1 and Figure 2-8. The supraorbital bone and suborbital bones four through six (the temporal group of suborbitals) were the most dramatically altered in size and shape following lens ablation. In general, these bones were expanded into the orbit. Orbit size decreases as a result of expansion of the bones surrounding the eye. The number of mandibular teeth was also altered as a result of lens ablation. Morphometric and statistical analyses of these differences are discussed below.

The supraorbital bone is the bone that was most influenced by lens removal (Figure 2-8). Lens removal caused the supraorbital bone to expand in all directions, resulting in a much larger bone, demonstrating itsvariability in shape. The element was expanded from a thin concave bone above the eye to a large flat plate with a flat ventral edge expanded into the orbit in adulthood. When surgery was performed at 1 dpf, the supraorbital bone also expanded into the normal position of suborbital six (SO6), overlapping this bone. However, when lens removal was performed later (2 to 4 dpf) the effects on the supraorbital bone were less dramatic, with very little expansion, and small changes in the size of the suborbital bones (Figure 2-8).

Suborbital 6 (SO6) was displaced by the supraorbital bone and by suborbital five after 1 and 2 dpf surgeries. On the control side SO6 was rectangular in shape and slightly overlapped the supraorbital bone, while on the surgery side of the head, SO6 had expanded into a triangular shape and was wedged adjacent to suborbital 5 and largely overlapped the supraorbital bone (Figure 2-8).

Suborbital 5 (SO5) was largely expanded into the orbit in adulthood, after surgery was performed at 1 or 2 dpf. The normally rectangular shaped bone expanded into a

larger triangular shaped bone with the longest axis extending into the orbit (Figure 2-8). Suborbital 4 (SO4) changed from a large square bone to a long thin rectangular bone, extended into the orbit (Figure 2-8). Similar to the supraorbital bone and SO6 the effects of lens removal on SO4 and 5 were most dramatic when performed at 1 and 2 dpf.

In contrast to the above however, suborbital 3 (SO3), the largest bone in the suborbital complex was minimally affected by lens ablation, indicating that the shape of SO3 was more stable, after lens removal. The dorsal edge of this bone had slightly expanded into the orbit, but otherwise remained relatively the same shape and size as the corresponding bone on the non-surgery side of the head. Suborbital bones 1 and 2 appear expanded into the orbit and are lengthened. The nasal region of the skull (anterior end of SO1, antorbital bone, nasal bone, frontal bone, and the maxilla) remained largely unaltered after embryonic lens removal, with only a minor expansion of each bone into the orbit.

To ensure the effects on the orbital bones were a result of lens removal a control experiment was performed where the lens removal procedure outlined previously was followed, with the exception that instead of removing the lens the cornea over the lens was torn. Tearing the cornea of one eye at 1 dpf resulted in 100% of the fish having two eyes in adulthood (n=7) (Figure 2-9). Gross morphological analysis showed that tearing the cornea did not cause eye regression as was observed in the lens removal specimens. The fish that received corneal tears resembled control fish rather than surgery fish (Figure 2-8 and 2-9). To ensure that the two eyes of the corneal tear fish were similar in size the diameters of both the right and left eyes were measured and the size difference was determined (Appendix 2, Table 10). The differences in eye diameters of tear specimens were then compared to the differences in eye diameter from right to left sides of control specimens using and independent t-test. The independent t-test results indicate that there was no significant difference between the diameter of the left and right eye of corneal tear specimens and the right and left eyes of control specimens ($p>0.05$). These results indicate that the corneal tear eye is not different in size from the control eye. In addition, the suborbital bones also appeared to be unaltered by the corneal tear. Two out of seven of those specimens have fused suborbitals 4 and 5, an event that occurs occasionally in the general population.

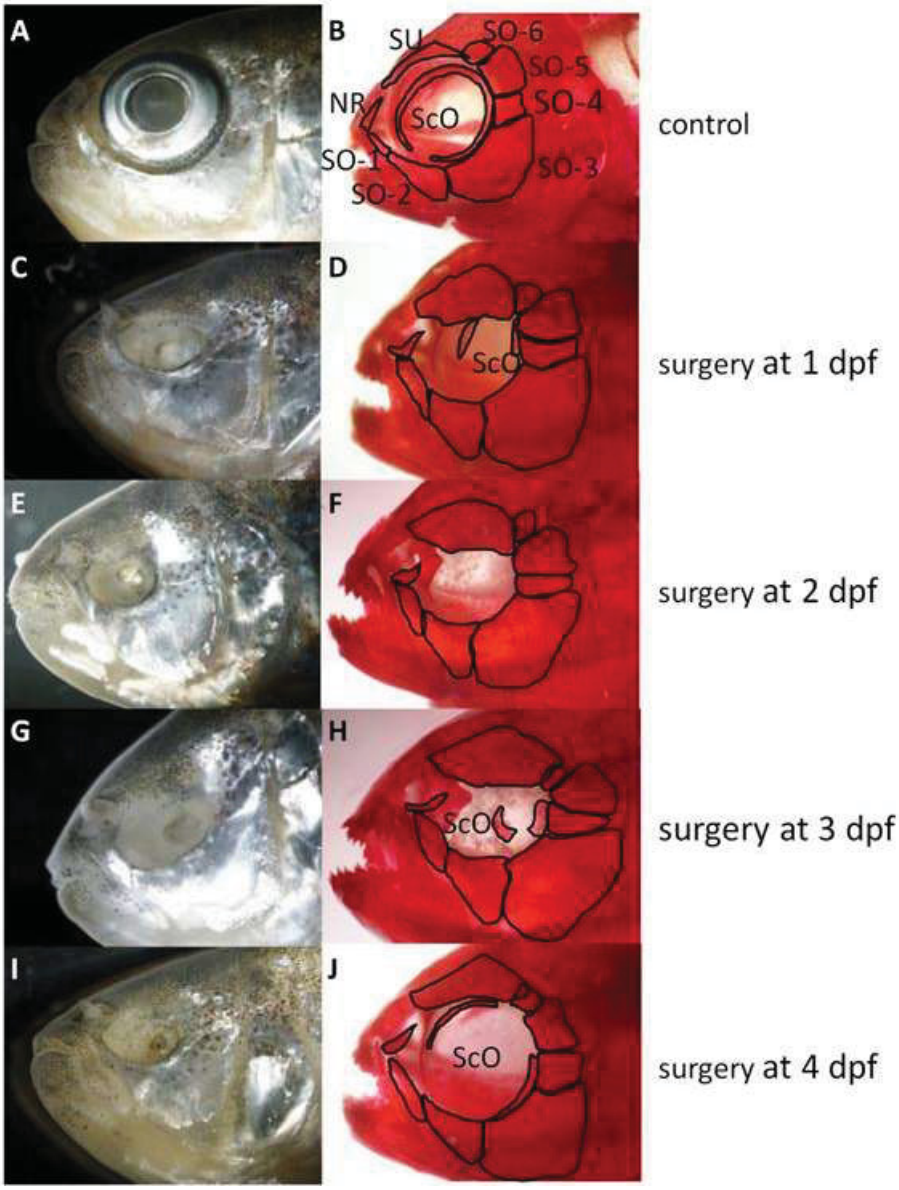


Figure 2-8: Alizarin red bone stained specimens showing effects after surgery. (A) Control specimen; (B) surgery performed at 1 dpf; (C) surgery performed at 2 dpf; (D) surgery performed at 3 dpf; (E) surgery performed at 4 dpf. NR nasal region, ScO scleral ossicles, SO suborbital bone 1 to 6, SU supraorbital bones.

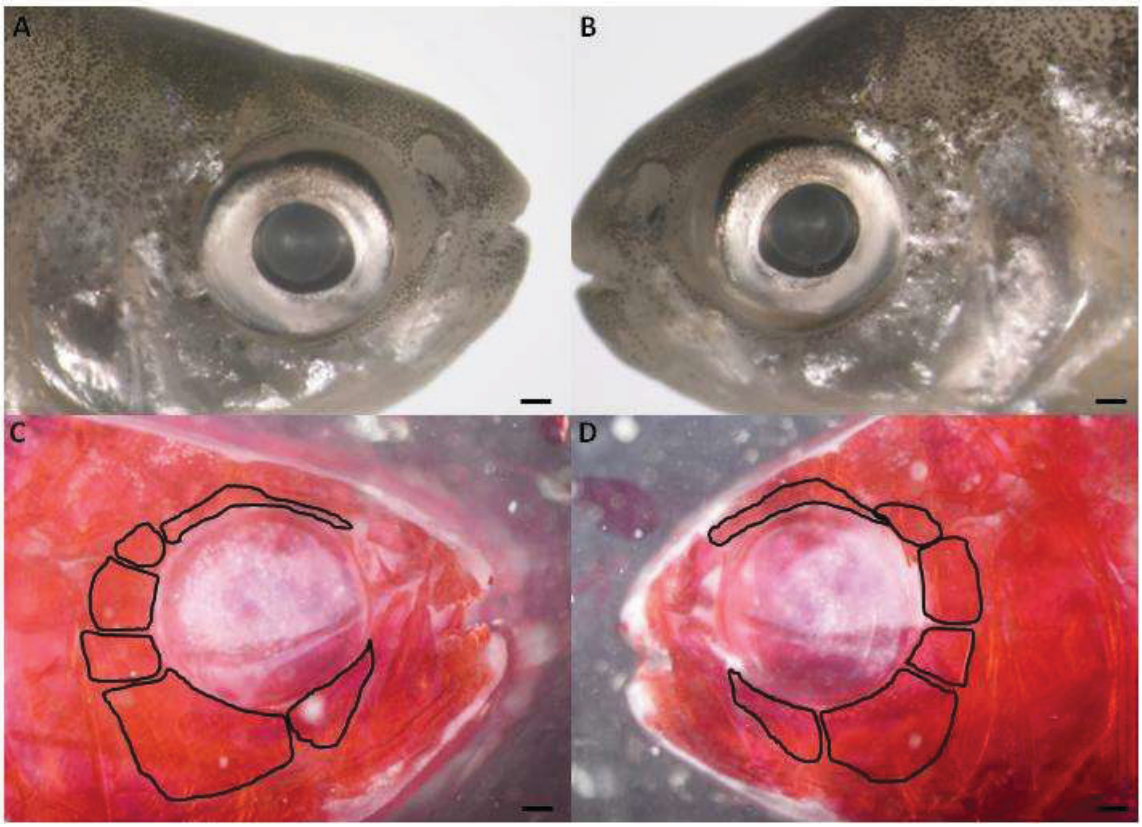


Figure 2-9: Unstained and whole mount bone stained corneal tear specimens. (A-B) show the unstained left and right sides of a surface fish which received a corneal knick on one side of the head; (C-D) show the whole mount bone stained left and right sides of a surface fish which received a corneal knick on one side of the head. Scale bars are 500 μm.

Table 2-1: The effect of lens ablation performed at 1, 2, 3 and 4 dpf on the bones surrounding the orbit. The number of individuals affected over total number of individuals analysed are included.

Region	1 dpf	2 dpf	3 dpf	4 dpf
Anterior scleral ossicle	absent in 3/6 individuals; highly reduced when present	absent in 5/8 individuals; reduced when present	absent in 3/5 individuals; normal when present	absent in 1/5 individuals; normal when present
Posterior scleral ossicle	absent in 1/6 individuals, but reduced when present	absent in 2/8 individuals; but large when present	absent in 0/5 individuals and normal in all others	absent in 0/5 individuals and normal in all others
Supraorbital bone	largely expanded in all directions	largely expanded in all directions	expanded in all directions	expanded in all directions
Suborbital 1	slightly expanded posteriorly and into the orbit	slightly expanded into the orbit and shifted posteriorly	expanded into the orbit and shifted anteriorly	expanded into the orbit and shifted anteriorly
Suborbital 2	slightly expanded into the orbit and shifted posteriorly	slightly expanded into the orbit and shifted posteriorly	expanded into the orbit and shifted anteriorly	expanded into the orbit and shifted anteriorly
Suborbital 3	expanded into the orbit and shifted posteriorly	expanded into the orbit and shifted posteriorly	expanded into the orbit and shifted anteriorly	expanded into the orbit and shifted anteriorly

Region	1 dpf	2 dpf	3 dpf	4 dpf
Suborbital 4	narrower and elongated; expanded into the orbit	narrower and elongated; expanded into the orbit	elongated and shifted into the orbit	elongated and shifted into the orbit
Suborbital 5	expanded into the orbit, wedge shaped, shifted ventrally	expanded into the orbit, wedge shaped, shifted ventrally	expanded into the orbit, wedge shaped, shifted ventrally	expanded into the orbit and into a wedge shape, shifted ventrally
Suborbital 6	displaced by the supraorbital	displaced by the supraorbital	displaced by the supraorbital	less displaced
Upper jaw	unaffected	unaffected	unaffected	unaffected
Lower jaw	unaffected	unaffected	unaffected	unaffected
Nasal region bones	slight shift posteriorly	slight shift posteriorly	shifted dorsally	shifted dorsally
N=	6	8	5	5

2.3.3 Morphometric Analyzes Shows Significant Changes in the Shape of the Suborbital Bones

In order to statistically compare the shape differences in the bones on the control side (no surgery) of the head to the surgery side (described above), morphometric analyses were conducted by applying 42 landmarks to the lateral views of both the control and surgery sides of the adult skull (Figure 2-2). Procrustes superimposition was used to align corresponding landmarks in order to analyze these changes in shape. Goodall's F-test (an analytical test) indicated that surgery performed at 1, 2, 3 or 4 dpf was significantly different from the control sides ($p<0.0001$, n=6, n=8, n=5 and n=6 respectively). These results agreed with the findings from the resampling test F test, Procrustes ($p<0.01$, F-score: 7.18, $p<0.01$, F-score: 9.36, $p<0.01$, F-score: 6.99, and $p<0.05$, F-score: 3.08, respectively). The surgery groups were also compared to controls that did not receive any surgery, all comparisons were significantly different ($p<0.01$, F-score: 14.88, $p<0.01$, F-score: 18.32, $p<0.01$, F-score: 16.22, and $p<0.01$, F-score: 9.65, for 1, 2, 3, and 4 dpf respectively). The morphometric program Twogroup indicated that the surgery side (of a 1 dpf surgery) was more similar to the control side of the head than it was to control fish without surgery (range= -0.0727 to 0.0395). The distance between the surgery side and the control side had a p value of 0.1688, while the distance between surgery side and control fish had a p value of 0.1855, indicating that the control side of the head also had minor affects of lens removal performed on the opposite side of the skull.

These results indicate large significant changes in skull shape, which cannot be accounted for by any minor natural right/left asymmetry that may be present in the skull. To test natural left and right asymmetry in the surface fish skull, the rights and left sides of 13 control fish were landmarked with the 42 landmarks described previously. Twogroup was used to compare the left group to the right group. F-test Procrustes analysis indicated that the right side of the skulls are not significantly different from the left sides ($p>0.05$, F-score: 1.84).

2.3.4 Vector Analysis of Effects of Manual Lens Removal

Vector analyses allow visualization of how and to what degree landmarks shift when comparing control versus surgery sides of the specimens. Vectors were created using a consensus or average for each surgery and each control group. Landmarks were grouped into clusters based on similar responses to lens removal. These groups are as follows; landmarks 1-10 and 39-42 (blue), landmarks 11-14 (red), landmarks 15-23 and 36-37 (yellow) and landmarks 24-30 and 33-36 (green) (Figure 2-10, Table 2-2).

The group 1 landmarks (1-10 and 39-42, blue in Figure 2-10) correspond to the bones directly above the orbit (i.e. the supraorbital bone and SO4-6 to the right of the orbit). These bones were shifted ventrally toward the center of the orbit as indicated by the vector directions (Figure 2-10). The more ventrally located landmarks in this group (landmarks 1-3, 5, 7, 9, 30, 39, 40) had vectors of larger magnitude than the dorsal landmarks (landmarks 6, 8, 10, 30, 41,42) indicating that these bones have expanded and shifted. At 1 dpf, the major change is in the size of the bones; the dorsal to ventral axis was expanded as a result of surgery. The directional effect of lens removal on group 1 did not appear to vary with age at the time of surgery. However, the length of the vectors in the group were different in earlier surgery fish and similar in later surgery fish (Figure 2-10). The vectors also had a greater magnitude the earlier surgery is performed, with the greatest effect at 1 dpf and the least at 4 dpf. The remaining three groups responded differently to lens removal based on the age at which the lens was removed.

Groups 2, 3 and 4 were less affected by lens ablation and thus will be described more briefly. Generally the groups tended to respond to surgery at 1 and 2 dpf in one manner and respond to surgery at 3 and 4 dpf in a different manner. When lens removal was conducted at 1 or 2 dpf, the groups respond in the following ways. Group 2 (Figure 2-10, red) vectors generally showed a movement into the orbit, with the anterior landmarks having greater magnitude indicating that SO4 (landmarks 9-12) had elongated into the orbit. Group 3 (Figure 2-10, yellow) vectors pointed in the posterior direction indicating that SO2-3, and the posterior portion of SO1 has shifted and expanded posteriorly. The vectors had small magnitudes indicating that there were small changes to those bones. Group 4 (Figure 2-10, green) vectors had a small magnitude and generally pointed in the posterior and downward direction. This indicates that lens removal has less

of an impact on group 4. Group 4 vectors indicated that the bones present to the anterior of the orbit (i.e. in the nasal region) shifted slightly in the posterior direction and with a slight expansion into the orbit.

Landmark groups 2, 3, and 4 responded differently to lens removal conducted at 3 and 4 dpf than they did to surgery at 1 to 2 dpf. When lens removal was performed at 3 or 4 dpf, group 2 landmarks responded by shifting in the anterior and slightly ventral direction, indicating that SO4 had shifted into the orbit in adulthood. After surgery at 3 dpf, the posterior landmarks in group 2 had moved less than the anterior landmarks indicating that SO4 had elongated. Group 3 landmark vectors point dorsally and slightly anteriorly after surgery at 3 and 4 dpf. The greater magnitude of the dorsal landmarks indicated that the SO1-3 had expanded into the orbit and had shifted anteriorly. Surprisingly, group 3 was more highly influenced by later surgery then earlier surgery, opposite to the effect that was observed in group 1. Group 4 landmarks were shifted in the dorsal direction as a result of lens removal performed at 3 or 4 dpf.

Overall my results indicate that group 1 landmarks (dorsal and posterior to the eye) were affected to the greatest extent with the greatest effects after lens ablation at 1 or 2 dpf. The effects of lens removal at 3 and 4 dpf were milder with the greatest impact to group 3 and 4. These results support my gross morphology findings described previously. In addition, using this method has allowed us to further understand in what direction the bones have expanded and in what direction they have shifted as a result of lens removal compared to the control side of the adult head.

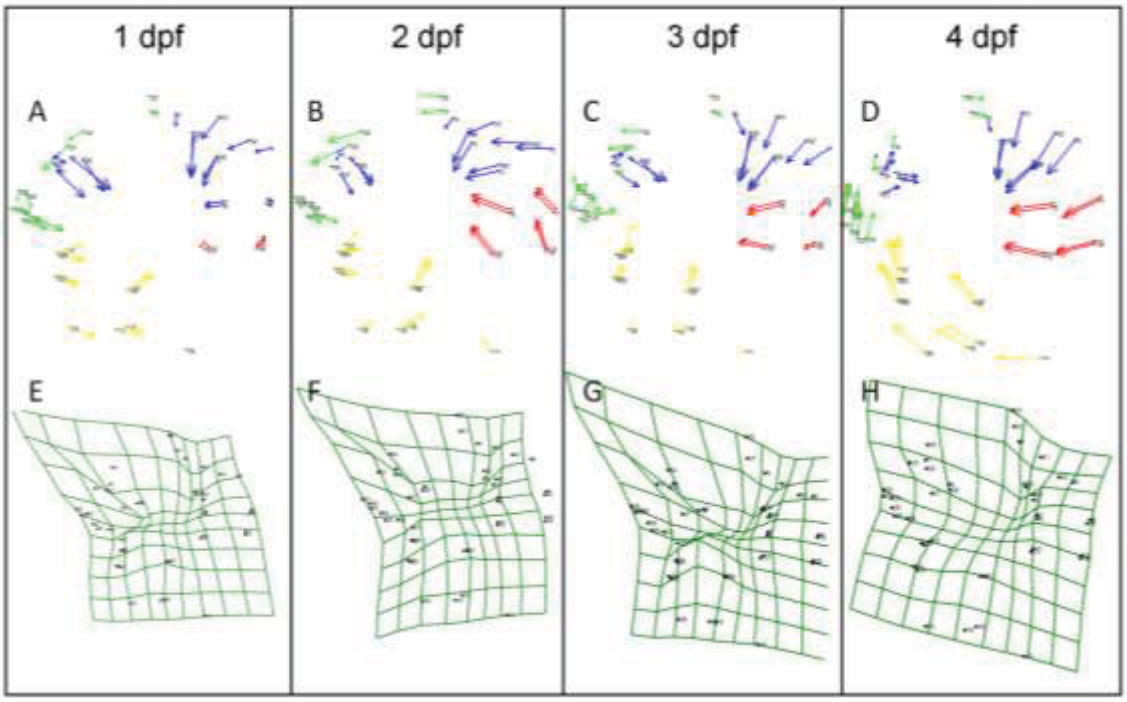


Figure 2-10: Vector analyzes and thin plates spline morphometrics of lateral view surgery adults.(A)-(D) vector analyzes comparing the control side of the head to the surgery side. Vectors are grouped based on their similar response to surgery. Group one consists of landmarks 1-10 and 39-42 (blue), group two has landmarks 11-14 (red), group three has landmarks 15-23 and 36-37 (yellow) and group four has landmarks 24-30 and 33-36 (green). (E-H) are thin plate splines of surgery conducted at 1 to 4 dpf.

Table 2-2: Landmark groups identified based on their similar response post surgery.

Group	Landmarks	Bones
1	1-10 and 39-42	Supraorbital, suborbital 6, suborbital 5 and the dorsal edge of suborbital 4
2	11-14	Ventral end of suborbital 4 and the dorsal edge of suborbital 3
3	15-23 and 36-37	Ventral part of suborbital 3, suborbital 2, posterior edge of suborbital 1 and the lateral ethmoid
4	24-30 and 33-36	Anterior edge of suborbital 1, antorbital bone, nasal bone, frontal bone, and the maxilla

2.3.5 Shape Change as a Result of Early Versus Late Surgery

Based on gross morphology and vector analysis (as stated previously) the effects of lens removal appeared to be more dramatic the earlier lens removal was preformed. To further investigate this observation, the morphometric program Morphologika, version 1 was used to outline bone shapes by connecting landmarks on adult skulls. An individual that received surgery at 1 dpf was overlaid with a control and a specimen which received surgery at 4 dpf was overlaid with a control (Figure 2-11). When surgery was performed at 1 dpf there were dramatic changes in the supraorbital bone and suborbital bones 4 to 6, as was described previously. When surgery was performed at 4 dpf the surgery specimen was much more similar in shape to the control than when surgery was performed at 1dpf. A 1dpf surgery specimen was overlaid with a 4 dpf surgery specimen, in this overlay large differences in shape between the two surgery time points could be observed in the supraorbital bone as well as suborbital 4 (Figure 2-11).

Principle Component Analysis was then used to begin to quantify these differences by statistically comparing the shape differences of the controls to 1 dpf, 2 dpf, 3 dpf, and 4 dpf specimens (n=42). PCAGen was used to produce the PCA plot comparing the shape differences between the four surgery time points. The PCA plot indicates that when surgery is performed at 1 dpf it is most different from the controls (Figure 2-12), while lens removal conducted at 2, 3, or 4 dpf had much less impact on the skull. The shape changes associated with the later three surgery time points is relatively similar. In addition, the latter three surgery time points were relatively close to the position of the controls on the plot. Based on the PCA and gross morphological findings, Twogroup was then used to determine if surgery at 1 dpf was more significantly different from the controls than surgery at 4 dpf. Results indicated that the 1 dpf group is not significantly more different from the controls than the 4 dpf group (distance between controls and 1 dpf means = 0.1688, distance controls and 4 dpf means = 0.1357, 95% range = -0.0420 to 0.0738). Surprisingly, this indicated that while visual differences can be observed they are not large enough differences to be significant differences in shape. This finding contradicted the observations made from the PCA analysis. PCA is used as a preliminary test to first determine if there were any differences worthy of further investigation, as such it amplifies small differences between groups for studies when only

small changes may be present. As a result the PCA analysis conducted on this data amplifies differences between the groups which were not significantly different.

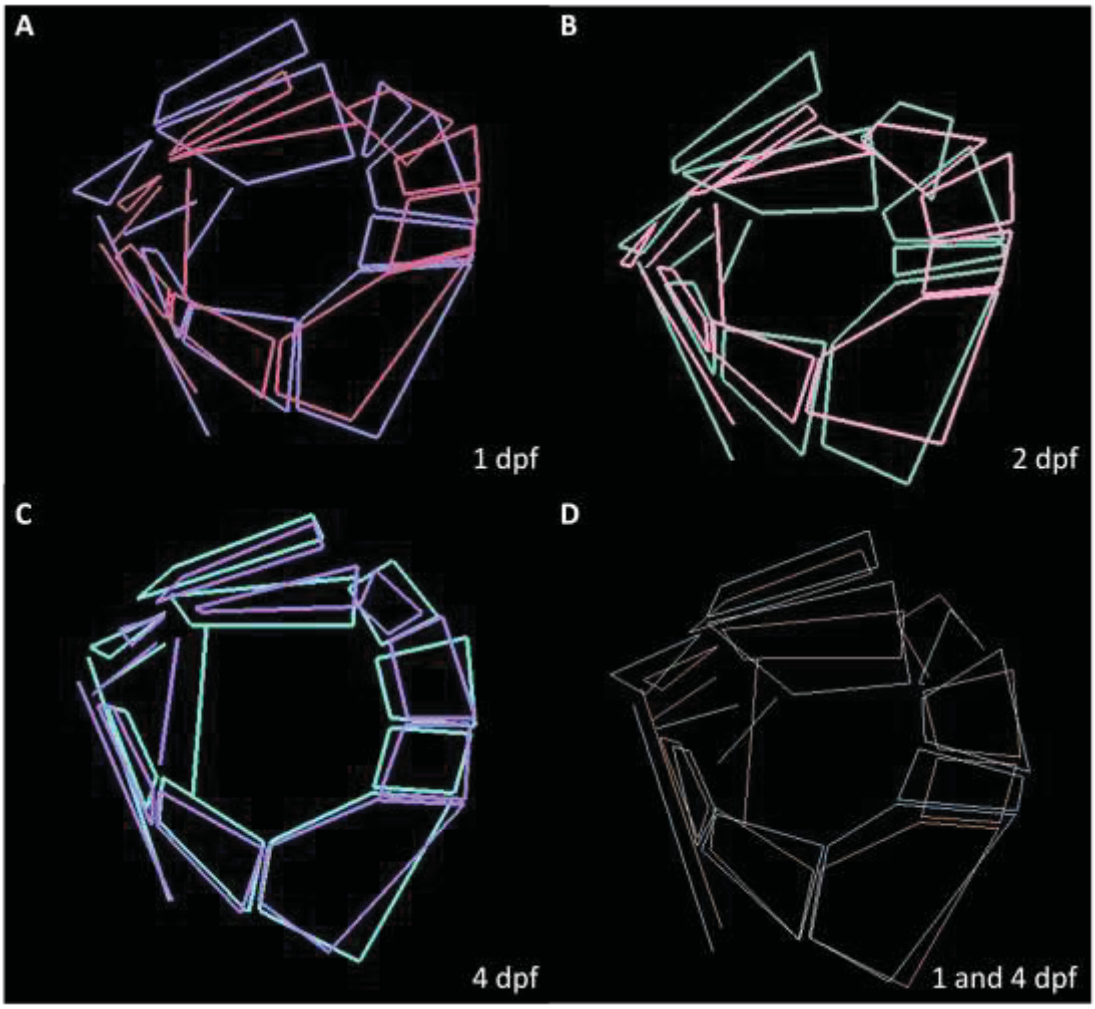


Figure 2-11: Morphologika wire frame shape comparisons between surgery and control. (A) Depicts overlapped wire frames of the control side of the head in pink and a 1 dpf surgery in purple; (B) depicts overlapped wire frames of the control side of the head in pink and a 2 dpf surgery in green; (C) depicts overlapped wire frames of the control side of the head in purple and a 4 dpf surgery orbital region in green; (D) depicts overlapped wire frames of surgery at 4 dpf in brown and a 1 dpf surgery orbital region in white.

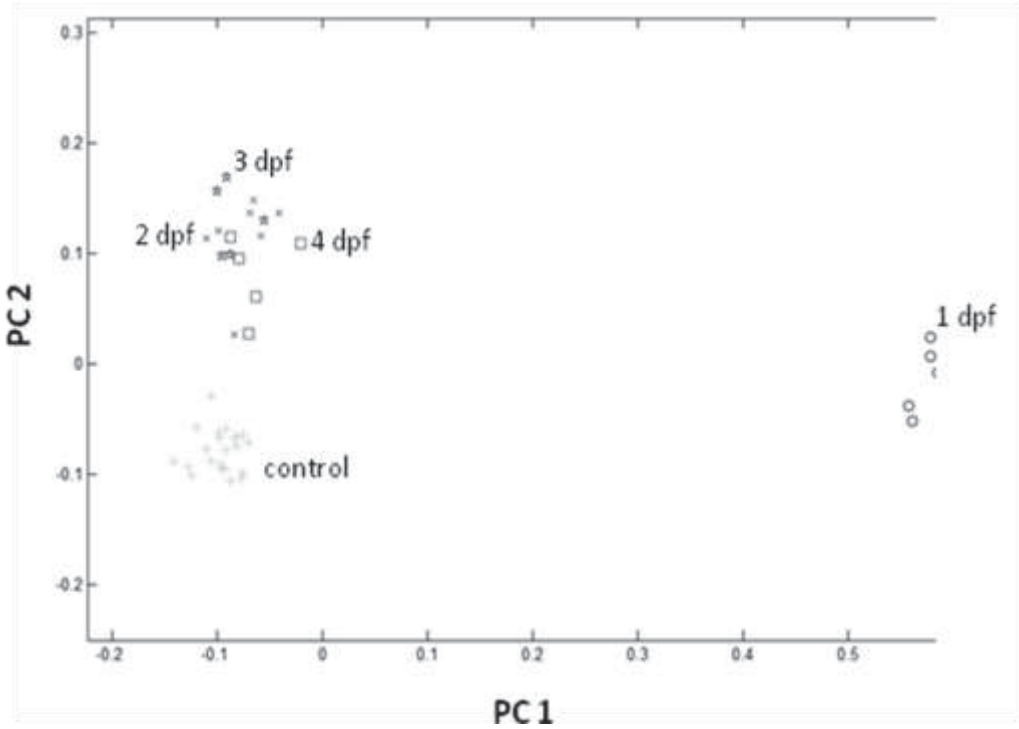


Figure 2-12: Principle component analysis comparing skull shape of control fish, 1, 2, 3, and 4 dpf surgery specimens. Circles represent the 1 dpf surgery group, x's represents the 2 dpf surgery group, stars represent the 3 dpf surgery group, squares represent the 4 dpf surgery group, and the crosses represent the controls.

2.3.6 Morphometric Analysis of the Affects of Lens Ablation on the Calvariae and Mandible

Gross morphological analysis of the adult surgery side of the mandible and calvariae did not reveal obvious changes in shape, however, morphometric analyzes was conducted to confirm these findings (Figure 2-13). Thin plate splines of specimens in which the lens was removed at 1 dpf showed that the calvariae and mandible were not influenced by lens removal (calvariae: Goodall's F-test, $p>0.05$ n=5, n=4, n=3, and n=2 for surgery at 1, 2, 3 or 4 dpf; mandible: Goodall's F-test, $p>0.05$, n=4 and n=3 for 1 day and 3 day respectively). Surprisingly, surgery performed at 2 and 4 dpf showed a significant difference in shape of the lower jaw on the surgery side compared to the control (Goodall's F-test, $p>0.01$, $p>0.03$, n=4 and n=3 respectively). No changes in jaw length were detected..

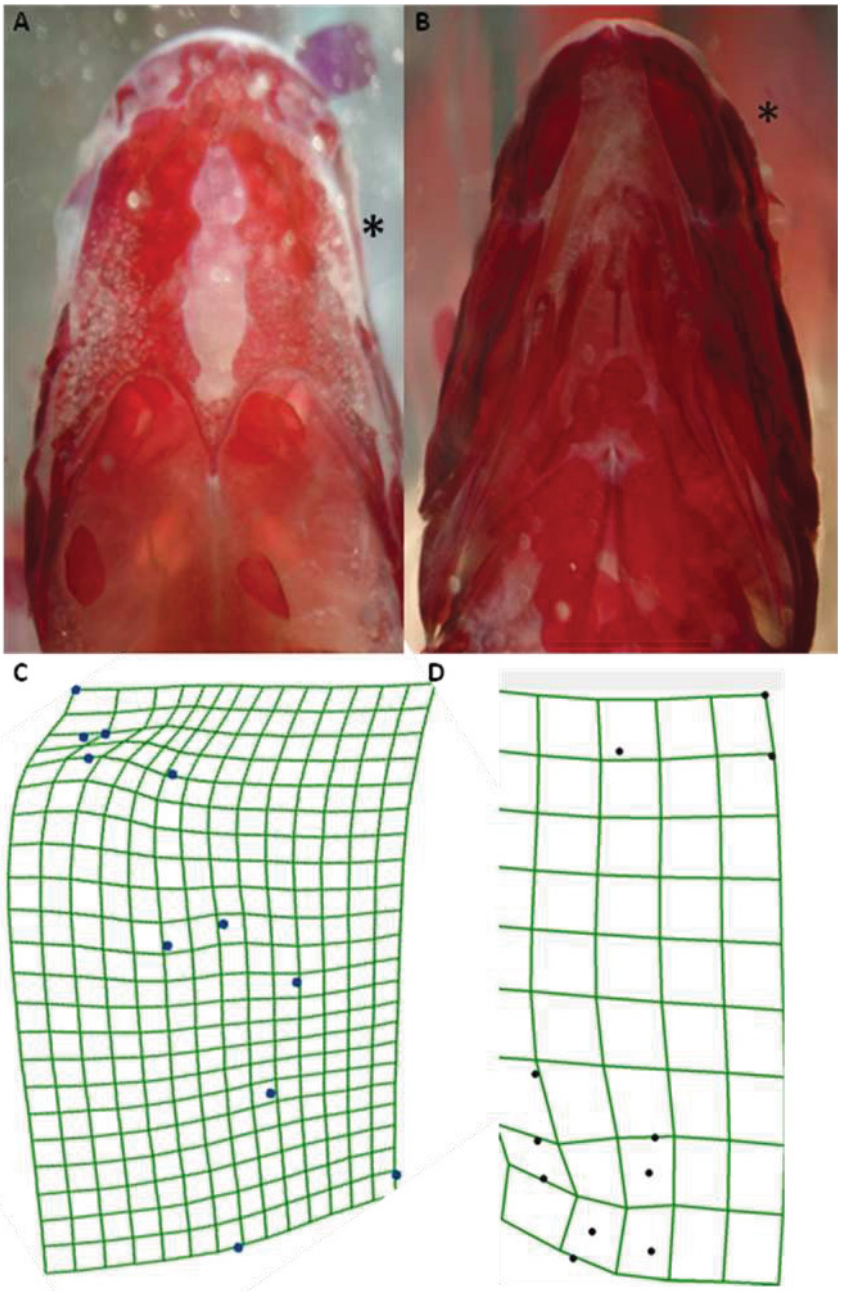


Figure 2-13: Dorsal and ventral view of whole mount stained surgery fish and corresponding thin plate splins. (A) Depicts the dorsal view, view of the calvariae of a specimen which received manual lens removal at 1 dpf, the surgery eye is identified with a red asterisks; (B) ventral view of a 1 dpf surgery specimen; (C) dorsal view thin plate splin morphometric analysis; (D) ventral view thin plate splin morphometric analysis.

2.3.7 Early Lens Ablation Results in Scleral Ossicles that are Reduced or Absent

Normally, sighted Mexican tetras have a large anterior and a large posterior scleral ossicle; these ossicles fuse into a solid bone ring during late adulthood (Franz-Odendaal et al., 2006). Lens ablation and subsequent eye regression causes a wide variety of changes in the ossicles (Table 2-1), ranging from large normal appearing elongated ossicles to small abnormal disk-like or completely absent elements (Figure 2-14).

When manual lens removal was performed at 1 dpf the majority of individuals had a small posterior ossicle. Fifty percent of surgery specimens with a regressed eye had an anterior ossicle, however, when present was highly reduced in size in three of sixspecimens examined (Figure 2-14). In contrast, lens removal at 2 dpf resulted in the majority of fish having a large posterior ossicle arching around the eye, while only half of the specimens (five of eight) had anterior ossicles. When anterior ossicles are present they tended to be larger than those present in 1 dpf surgery specimens. Lens ablation at 3 dpf resulted in all individuals developing posterior ossicles, the majority of which were large and normal in shape; 60% of these individuals also had reduced anterior ossicles (three of five). Lens ablation performed at 4 dpf results in 100% of the specimens having both a large posterior and a large anterior ossicle (n=5).

Overall, my results show that the anterior ossicle is more affected by lens ablation conducted at 1-3 dpf, while the posterior ossicle remains relatively unaltered. Lens ablation at 4 dpf results in minor changes to scleral ossicle development (Table 2-1).

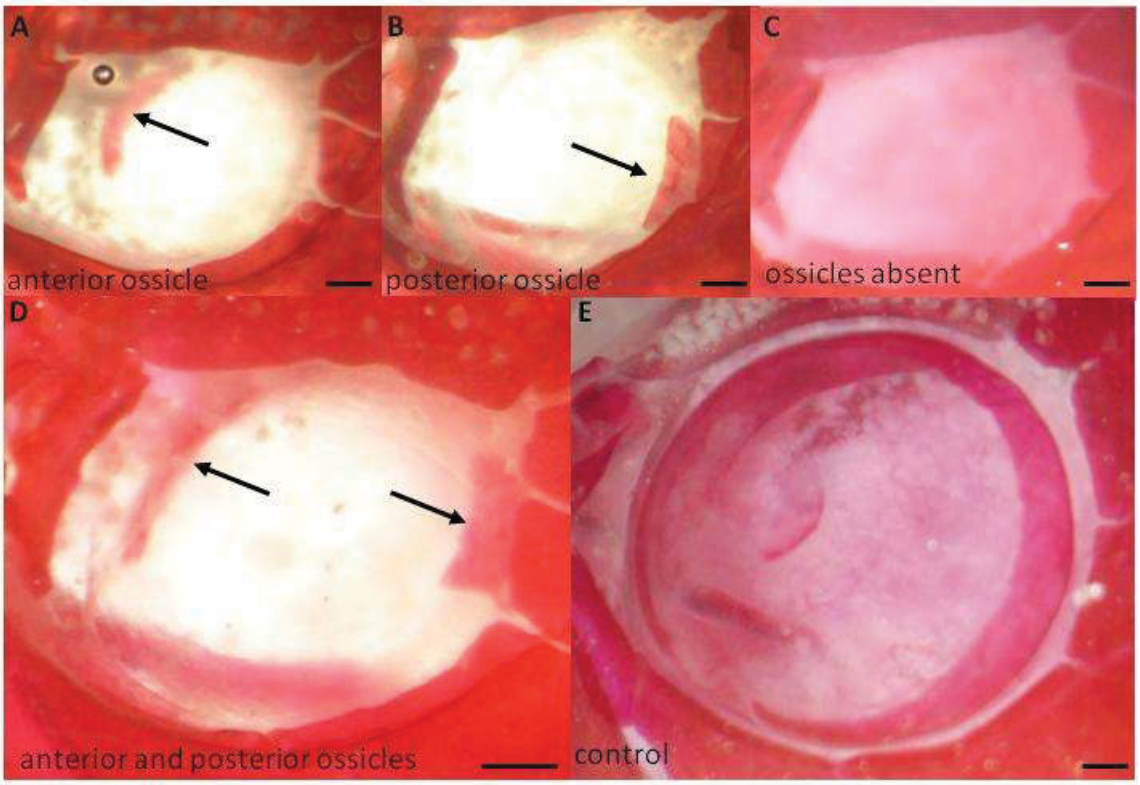


Figure 2-14: A magnified view of the orbital region of the skull of whole mount stained adult specimens. (A-C) Whole mount bone stained of adult surgery eyes, (D) is a whole mount bone stain of a control eye. (A) surgery eye with only and anterior scleral ossicle; (B) surgery eye with only a posterior scleral ossicle; (C) surgery eye with no scleral ossicles present; (D) control eye with fused scleral ossicle ring. Scale bars are 500 μm.

2.3.8 Orbital Bone Outgrowth

In order to determine if the first ossification of the supraorbital bone or bone outgrowth is affected by lens removal, orbital bone ossification was observed over development (n=21 surgery specimens, n=20 control specimens). I examined the supraorbital bone from onset of ossification until adulthood (adults reported previously in 2.3.2). The supraorbital bone was chosen as it was determined to be most affected by lens removal. This analysis is aimed at determining how those changes in bone shape occur. Control surface Mexican tetras between 1.24 and 1.75 cm SL were whole mount bone stained. In addition, one-eyed surface fish that had received manual lens removal at 1 dpf were also stained (Figure 2-15). The supraorbital bone begins to ossify around 1 cm SL, by 1.24 cm SL the supraorbital bone can be easily visualized with alizarin red stain. By 1.24 cm SL the supraorbital bone was marginally larger and slightly expanded into the orbit on the surgery side of the head (Figure 2-15). By 1.75 cm SL, the supraorbital bone on the surgery side of the head had rapidly expanded compared to the controls and control side of the head, the supraorbital bone was much larger in size in the dorsal to ventral axis. This indicated that rapid orbital bone outgrowth may contribute to the expansion of the supraorbital bone after lens removal and may be the major contributor to the expansion of the bone.

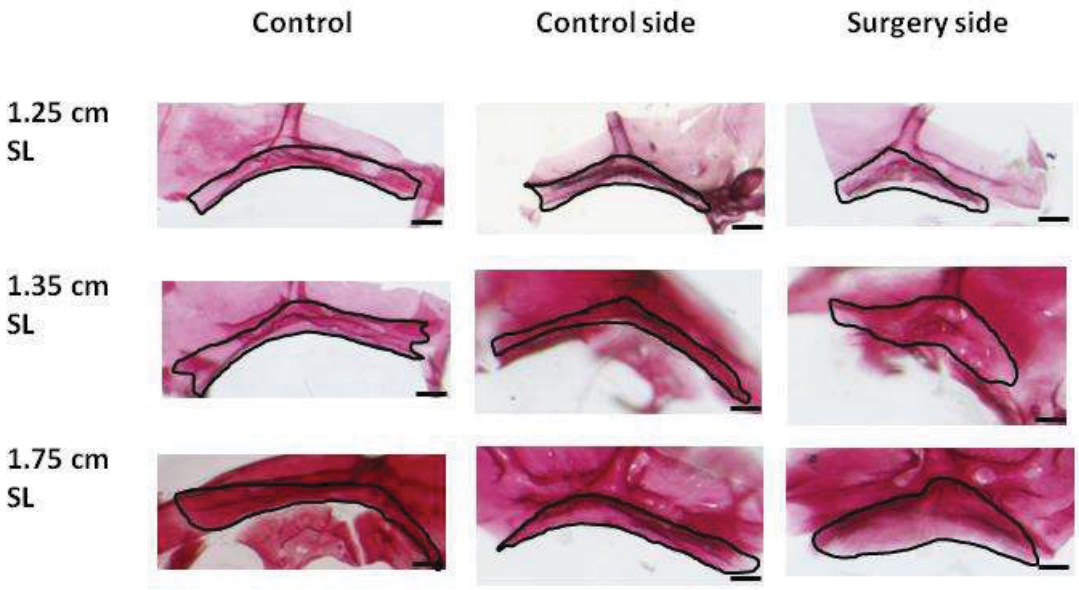


Figure 2-15: Outgrowth of the bones in the orbital region during development in the surface surgery eye, control eye and control fish. The supraorbital fused with the frontal bone and dissected from the rest of skull is depicted from 1.25 cm to 1.75 cm SL. At 1.25cm SL the supraorbital has just begun to ossify. At 1.75 cm SL the supraorbital bone of the surgery eye is much larger than that of the controls. Scale bars are 200 µm.

2.3.9 The Effects of Lens Removal on the Juvenile Cartilage Skeleton

After lens removal, the juvenile cartilage skeleton was stained with Alcian blue to visualize changes in the cartilage skeleton (Figure 2-16) (n=2). After lens removal at 1 dpf, very few differences were observed in the cartilage elements of the juvenile skull. In the two specimens that were fixed approximately four months after lens removal, minor changes were observed in the scleral cartilage ring within the eye. In the control eye the scleral cartilage was a large smooth ring around the equator of the eye (Figure 2-16). Not surprisingly, in the surgery eye the cartilage was highly reduced in size (due to the reduced eye size), but otherwise appeared normal in structure. The majority of the Mexican tetra skull does not form from a cartilage precursor. As such, stains for cartilage provide minimal insight into the early changes present in the juvenile skeleton after lens surgery.

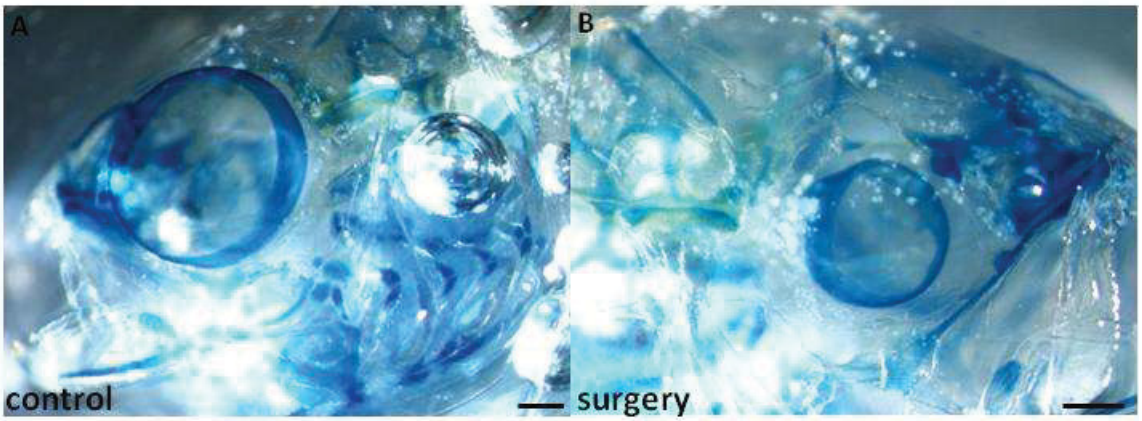


Figure 2-16: Whole mount cartilage stained juvenile surgery fish. (A) Control side of the surgery head, with a large scleral cartilage ring of a 1.25cm SL fish; (B) surgery eye with a small scleral cartilage ring. Scale bars are 500 μm.

Part B

2.4 Materials and Methods

2.4.1 Immunohistochemistry for Visualization of Taste Buds

Specimens in which the lens was removed at either 1 or 3 dpf were prepared for immunohistochemisty at 21-22 dpf. After fixation in 4% paraformaldehyde (Sigma P6148) in 0.01M phosphate buffered saline, pH 7.4, the immunohistochemistry procedure outlined in Varatharasan et al., (2009) was used to visualize receptor and basal cells of taste buds, using anti-calretinin and anti-serotonin respectively. Taste buds are visualized as one basal cell surrounded by multiple receptor cells. Isolated basal cells were identified but were not included in taste bud counts, similar to Varatharasan et al, (2009). Permeabilization was increased to four nights in 4% trition-x 100 (BDH Chemicals R06433) in 0.01M phosphate buffered saline. Two primary antibodies were used, a rabbit monoclonal anti-serotonin (Sigma, s5545) used at a concentration of 1:10000 to visualize basal cells and a mouse monoclonal anti-calretinin (Abcam, ab90632) at a concentration of 1:175 to visualize receptor cells. The primary antibodies were incubated at 4 ^{0}C for four nights, while secondary antibodies were incubated for 48 hours. The two secondary antibodies used as a cocktail of goat anti-rabbit Alexaflour 488 (Invitrogen, A11034) at a concentration of 1:500 and a bovine anti-mouse Texas red (Santa Cruz Biotechnology, sc-2788) at a concentration of 1:400. After staining, the jaws were removed and flat mounted. Taste buds were counted on both the right and the left side of the mandible. A one-tailed paired t-test was performed on these numbers of taste buds using Minitab version 16.

2.4.2 Counting of Teeth and Jaw Measurements

Teeth counts were conducted in specimens raised until adulthood (3.5 cm SL) then sacrificed, fixed and whole mount bone stained (as described previously, section 1.2.3).The number of small caudal teeth and large central multicuspid teeth were counted on each half of the lower jaw after manual lens ablation at 1, 2, 3, or 4 dpf. Small caudal teeth consisted of uni- and multicuspid teeth and were less than 50% smaller than the

large central multicuspid teeth. Statistical analyses (one-tailed paired t-test) were conducted on measurements from both the surgery and control side of the lower jaw; using Minitab version 16. Jaw measurements were conducted from the retroarticular joint of the dentary to the anterior most point of the dentary (Figure 2-1). Comparisons of jaw length on the surgery side of the head compared to the control were statistically analyzed (one-tailed paired t-test) using Minitab version 16. In addition, the average and standard deviations were calculated for each time point.

2.4.3 Whole Mount Phalloidin Skeletal Muscle Stain

Surface fish which received manual lens removal were anesthetized using 0.1% MS222, then fixed in 4% PFA in PBS at 4^{O}C overnight, and stored in PBS at 4^{O}C. Specimens were fixed at 15 dpf, between 6.3 and 7.7 mm SL, n=3. The specimens were incubated in Phalloidin tetramethylrhodamine B isothiocyanate (Sigma P-195) at a concentration of 1:500 in PBST (Tween) overnight in the dark at room temperature. Following the specimens were rinsed in PBS for 5 minutes. The specimens were then viewed under a Nikon SDi fluorescent microscope, using a CY3 cube.

2.5 Results

2.5.1 Later Lens Ablation Affects the Number of Small Caudal Mandibular Teeth

Previous investigation determined that cavefish have an increased number of maxillary teeth when compared with the surface fish (Yamamoto 2003). To determine if mandibular tooth number was altered by lens removal, tooth numbers on both the surgery and control side of the jaw were counted in adult specimens (Figure 2-17). A constant number of large multicuspid teeth was present in all samples (8 teeth per jaw) and no differences were detected on the surgery versus the control sides (Figure 2-17). However, after lens removal at 1 dpf the control side of the lower jaw had on average 1.5 ± 1.04 small (small multicuspid and unicuspid) teeth, while the surgery side had on average 2.8 ± 1.72 small teeth (Figure 2-18). This slight increase is not statistically significant (one-tailed paired t-test, $p>0.05$, n=6). One-tailed paired t-tests showed that lens removal conducted at 2, 3 or 4 dpf had a significant impact on the number of teeth ($p<0.05$ for

each time of surgery, n=7, n=5 and n=5 respectively) with more teeth on the surgery side (Figure 2-18). This indicates that lens ablation performed later in development has a greater impact on tooth number than when surgery is performed earlier (i.e. at 1 dpf).

After lens ablation performed at 1, 2, 3, and 4 dpf the length of the surgery side of the jaw and the length of the control side of the jaw was measured to determine if the increase in tooth number was due to an increase in jaw length. It was determined that the lens ablation did not significantly affect the length of the dentary (one-tailed paired t-test; $p>0.05$, n=4, n=3, n=3, n=2 respectively for each surgery time point).

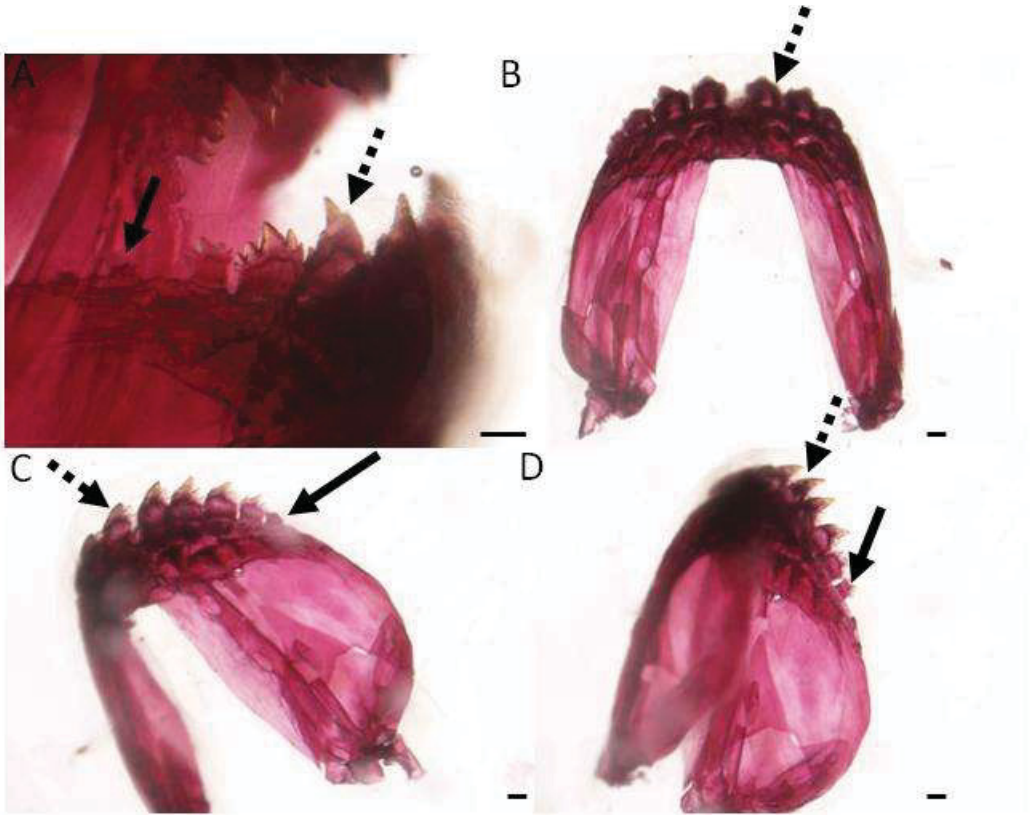


Figure 2-17: Whole mount bone stained mandible from an adult surface tetra.
(A) Lateral view of a surface fish mandible, the hashed arrow indicates the large mandible teeth, the solid arrows indicate small mandibular teeth; (B) Ventral view of dissected mandible, the hashed arrow indicates the large mandible teeth; (C) dorsolateral view of the dissected mandible, the hashed arrow indicates the large mandible teeth, the solid arrows indicate small mandibular teeth; (D) lateral view of the dissected mandible, the hashed arrow indicates the large mandible teeth, the solid arrows indicate small mandibular teeth. All scale bars are 200 μm.

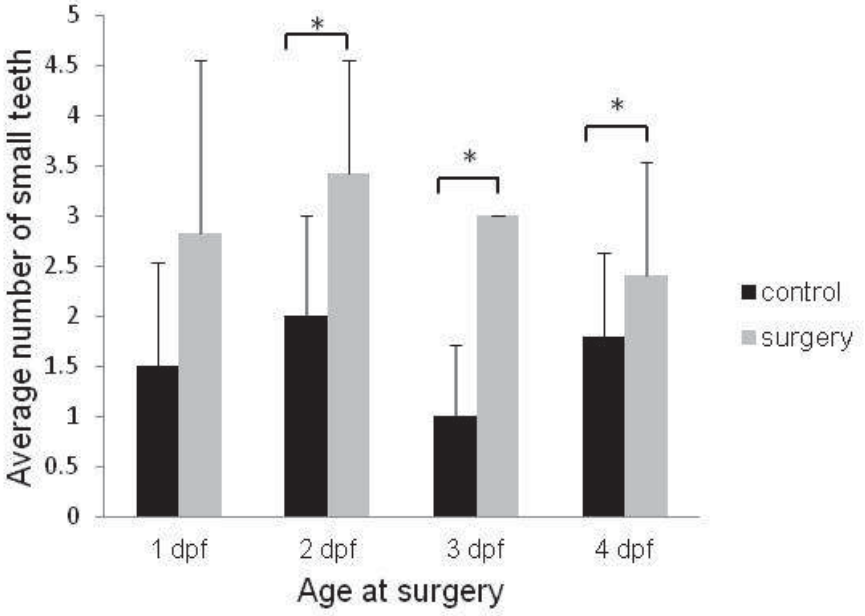


Figure 2-18: Number of small caudal teeth present in adults on the caudal portion of the lower jaw after surgery. The average number of small teeth on the surgery versus control side of the head is shown by the bar graph at each surgery time point (1, 2, 3, 4 dpf) (n=6, 7, 5, and 5 respectively). Standard deviation error bars are included. Asterisk indicates significance.

2.5.2 Lens Ablation Does Not Affect Mandibular Taste Bud Number or Pigmentation

In order to determine whether lens ablation affects taste bud number, taste buds were counted in surgery fish. After lens removal at 1 or 3 dpf the number of taste buds on the surgery side of the mandible were compared to the control side (Figure 2-19, 2-20). Results indicated that when lens removal was performed at 1 dpf (the earliest time point at which the lens can be removed) and 3 dpf (after taste bud development has begun) the difference in taste bud count on the control versus surgery side of the lower jaw was not significant (one tailed paired t-test, $p>0.05$, $n=19$ and $n=8$ respectively) (Figure 2-19). At 22 dpf, the surgery side did not have a two fold increase in the number of taste buds as expected in blind morphs (Varatharasan et al., 2009). Furthermore, the normal arrangement of taste buds in two rows was not altered in surgery specimens (100% of fish, $n=27$) (Figure 2-20). These results indicate that lens removal does not significantly impact the number and arrangement of taste buds on the lips of the lower jaw after lens removal (Figure 2-19, 2-20).

In addition, no obvious alterations were observed in the pigmentation pattern on the surgery compared to the control sides (Figure 2-21).

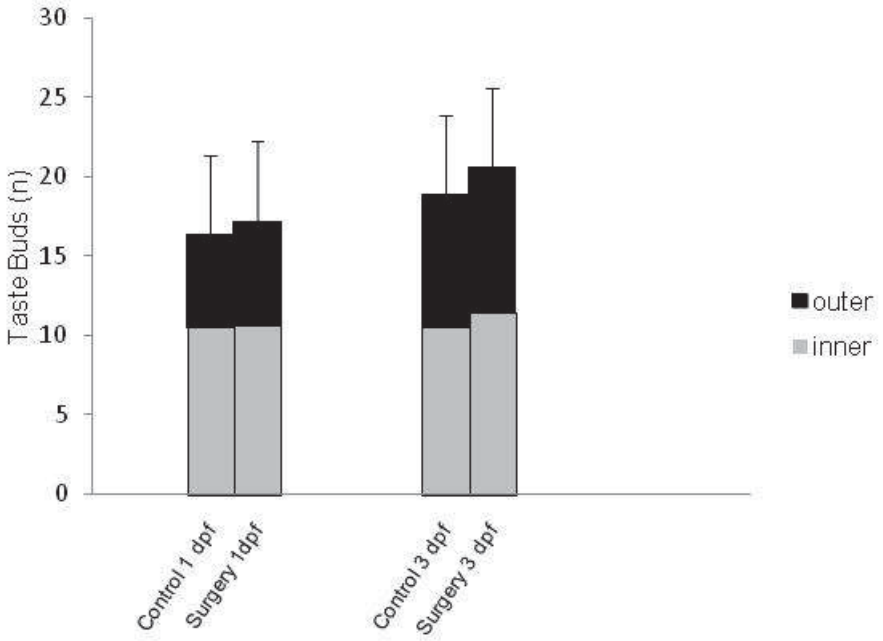


Figure 2-19: The effects of lens removal on taste bud number. The average number of taste buds on the inner and outer rows of the surgery side of the lower jaw compared to the control side of the lower jaw. Surgery was performed at 1 and at 3 dpf (n=19, and n=8 respectively). Taste bud counts were conducted at 21 dpf. A paired t-test anaylsis indicates that the number of taste buds are not significantly different.

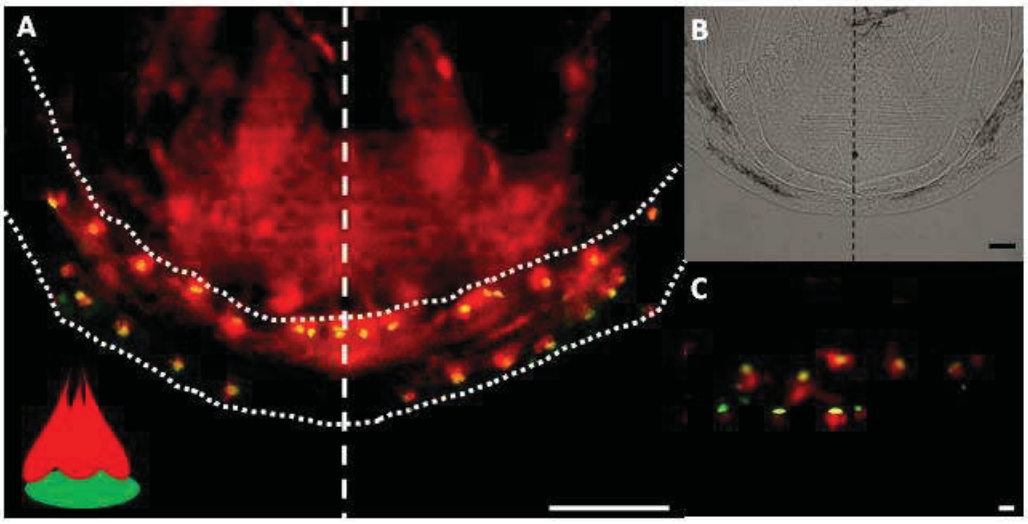


Figure 2-20: Immunohistochemical visualization of taste buds on the lower jaw at 21 dpf. (A) Flat mounted lower jaw (outlined by dotted line) showing two rows of taste buds. The dashed vertical line separates the control side from the surgery side. Each taste bud is visualized as one green basal cell grouped with one or more red receptor cells, as depicted in the schematic inset in (A). (B) Is a bright field image of the jaw in (A) at a lower magnification; (C) shows a higher magnification of the taste buds. All scale bars are 50 μm.

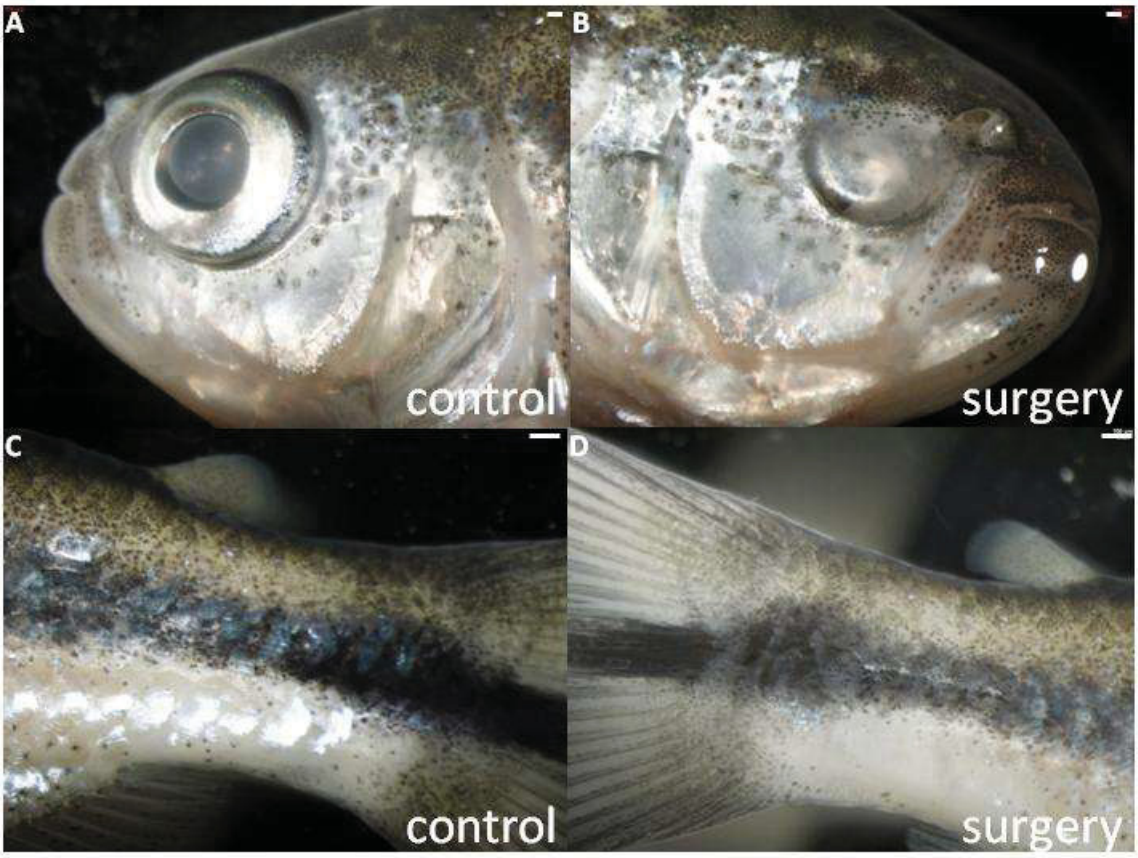


Figure 2-21: Pigment comparison in surgery surface tetras. (A) lateral control side of a surgery surface fish head; (B) lateral surgery side of the head; (C) control side of the surgery fish body; (D) surgery side of the body. Scale bars in A-B are 200 μm, scale bars in C-D are 500 μm.

2.5.3 Lens Ablation Affects Some Extraocular Muscles

Phalloidin antibody was used to visualize the extraocular muscles, as well as other muscles in the eye region. Surface fish which had received manual lens removal at 1 dpf and fixed at 15 dpf between 6.3 mm and 7.7 mm SL were stained and viewed. After staining, muscles that could be easily identified on both surgery and controls sides of the head were the adductor mandibulae, the elevator arcus palatine, and the dilator opercula (Figure 2-22). The adductor mandibulae muscle appeared large and flat positioned in the anterior to posterior direction ventral to the eye. It appeared to be in the same position and of the same size in both the surgery and control sides of the head. The elevator arcus palatine muscle and the dilator opercula muscle located posterior to the eye also appeared unaltered by lens removal. Based on the analysis it appears as though these muscles are not influenced by lens removal (Figure 2-22).

The extraocular muscles which insert on the eye were also analyzed. The superior rectus muscle was present from the equator to the anterior portion of the eye. On the control side of the head it could be easily visualized. However, the corresponding muscle could not be visualized on the surgery side of the head and appeared to either not be present, reduced in size or located deeper within the tissue not allowing for visualization (Figure 2-22). In addition, the superior oblique and the inferior oblique ocular muscles also appeared to be altered by lens removal. The superior oblique was present on the surgery side of the head, but appeared to have not formed its normal attachment to the eye, and appeared to be attached at a different location. The inferior oblique muscle also attached in a different location, rather than occupying its normal position attached to the ventral portion of the eye it appeared to be attached at the equator of the eye. Both of those muscles appeared to be elongated, extending into the eye orbit area where an eye of usual size eye would normally occupy, in order to maintain attachment to the small regressed surgery eye.

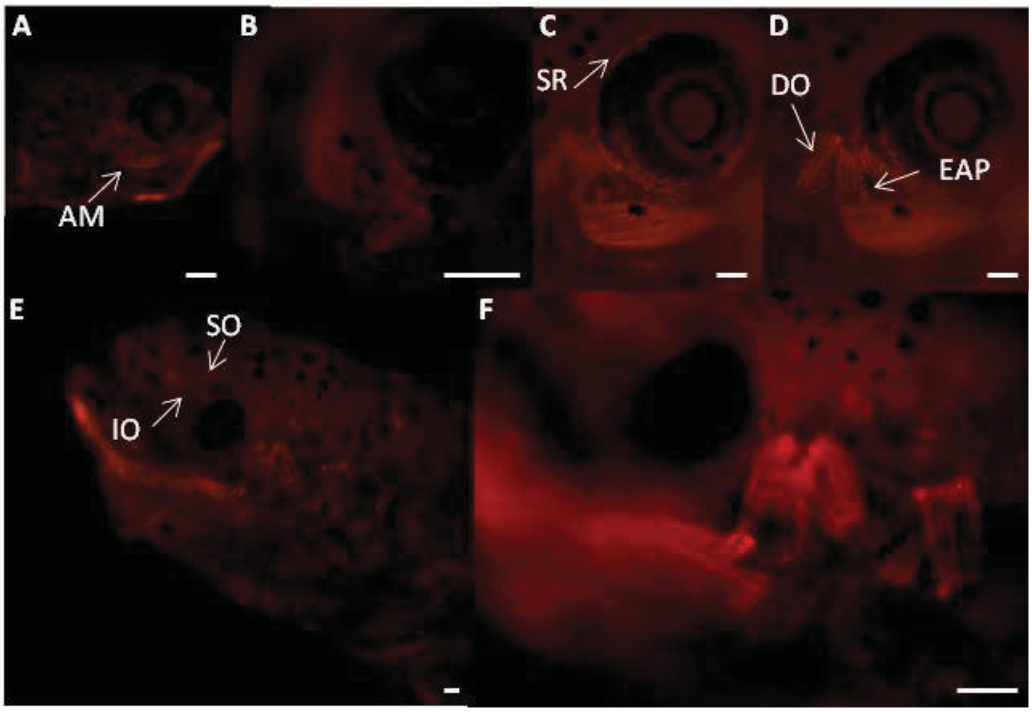


Figure 2-22: Phalloidin immunohistochemical analysis of the muscles surrounding the control eye and surgery eyes of a surgery surface fish. (A-D) lateral views of the control side of the head; (E-F) lateral view of the surgery side of the head. DO dilator opercula, EAP, elevator arcus palatine, IO inferior rectus, SO superior rectus, SR superior rectus. Scale bars are 100 μm.

2.6 Discussion (Parts A and B)

The objective of this study was to understand how lens removal and subsequent eye regression can influence the development of the craniofacial skeleton and to determine the developmental window during which lens ablation affects adult skull morphology in the Mexican tetra. In addition, I determined whether lens removal affects other structures outside the orbital region such as teeth, taste buds, extraocular muscles and pigment. Overall, the results of this study demonstrate that some bones of the craniofacial skeleton are more affected than others indicating that they are more variable. This data assists in beginning to unravel the mechanism by which lens removal affects the bones surrounding the eyes.

2.6.1 Eye Regression After Lens Removal

After the lens was removed at either 1, 2, 3, or 4 dpf eye development was arrested, and the surgery eye regressed into the head during further development. The diameter of the eyes, as well as the distance between the eyes over the width of the head was measured over the first few weeks of development in surface fish, cavefish and surgery surface fish. I hypothesized that the surgery eye would regress in the same manner as naturally observed in the cavefish however, that is not what was observed. The eye rudiment of the cavefish was engulfed by the head much earlier in development, than the surgery eye of the surface fish. Although the surgery eye is smaller in diameter, it appears to occupy the same position as the control eye shortly after surgery. By 9 months to a year after lens removal the surgery eye rudiment has been completely engulfed by the head. Eye regression in the cavefish is much more rapid, this may explain why early forming bones such as the calvariae (ossify before 1.5 cm SL) are unaffected by lens removal, as they ossify prior to the regression of the surgery eye. During the ossification of late forming bones such as the supraorbital bones (2.2 cm SL) the surgery eye no longer occupies its normal position within the head thereby allowing the expansion of the late ossifying bones, however, there do appear to be some differences between eye regression after surgery and eye regression in the cavefish. These differences will be discussed later.

2.6.2 Lens Ablation Affects Some Craniofacial Bones More Than Others

After lens removal and subsequent eye regression long term, permanent effects on the adult skeleton were observed. In general the teleost trunk [(De Schepper et al., 2004; Cloutier et al., 2010; Fiaz et al., 2010) and skull (Cooper et al., 2010; Parsons et al., 2011; Parsons et al., 2012)] are extremely dynamic structures that display great interspecific variability. Only one other study has conducted lens ablations and these authors similarly observed changes in the supraorbital and suborbital bones (Yamamoto et al., 2003). However, they minimally describe the effects of lens ablation, with only a partial analysis of skull affects. Lens removal was only conducted at 1 dpf, thus the effects over early development could not be determined. Finally analyses were conducted in a young adults (6 months of age) prior to the maturation of the skull. My analysis shows several expected and some surprising results.

First, I show that the bone most susceptible to change after lens removal is the supraorbital bone. This bone expands in all directions into a large plate covering much of the orbit. The second most affected bones were the SO4-6. Suborbital 4 and 5 are largely expanded, encroaching over the orbit and into the position normally occupied by SO6.

Second, for the entire orbital complex, dorsal orbital bones are more affected than ventral orbital bones (Figure 2-8) suggesting that the source of the upstream events leading to this variation might be located dorsally. My results also indicate that there are different levels of variability in the bones surrounding the eye, since some bones are affected by lens removal, while others are not.

Most intriguing is that lens removal conducted within the first 4 dpf has tremendous effects despite orbital bones not ossifying until the fish reach approximately 3-4 months of age (equivalent to 1.2- 2.2 cm standard length). Lens removal may affect the morphogenesis of skeletogenic condensations rather than the ossification process. I tracked the ossification and growth of the supraorbital bone (the most affected bone) over development in order to determine if early ossification was affected by lens removal. Specimens were whole mount bone stained just after the supraobital bone begins to ossify at 1 cm SL (Figure 2-15). At this stage the surgery fish's supraobital bone is slightly larger than the control. This indicates that the condensation which gives rise to the bone may also be increased in size. However, an analysis of condensation size using peanut

agglutinin lectin would be a more accurate investigation, and is required to confirm that the condensation is in fact altered by lens removal. Throughout the first few months of development the surpraorbital bone expands much more rapidly than the control, indicating that two different processes may affect the increase in size present in the adult surgery supraorbital bone, condensation size and outgrowth.

The presence of the eye has been shown to have an important function in the proper migration of cranial neural crest derived (CNC) structures surrounding the eye (Langenberg et al., 2008; Kish et al., 2011). Langenberg and colleagues demonstrated using an eyeless zebrafish mutant, *chokh*/rx3, that the eye is necessary for the proper migration of the anterior neural crest cells into the dorsal part of the eye; when the eye is absent CNC migration fails (Langenberg et al., 2008). Skeletal defects in this mutant include malformations of the lower jaw and the neurocranium (anterior CNC derived structures), indicating that there is a failure in the developmental event, rather than a pleiotropic affect. Furthermore, and most importantly, when the lens vesicle is absent during early development these authors found that CNC migration is arrested at the edge of the presumptive eye resulting in the failure of ossification of some skull bones and the malformation of others. My data similarly shows that the most affected bones are in the dorsal orbital region. If condensation size is in fact affected by lens removal there are a few different mechanisms which may be responsible for the affetcs. Lens ablation may affect the migration of neural crest cells into the orbital region. In zebrafish (Cypriniformes: *Danio rerio*) the orbital bones are neural crest derived (Kague et al., 2012), however, their origin has not been determined in the Mexican tetra (Characiformes: *Astyanax mexicanus*) but is presumed to be similar. Cranial neural crest cells (CNC) begin migration at 14 hours post fertilization (Kimmel et al., 1995) in zebrafish. Although the orbital bones are late forming bones, the cranial neural crest cells that give rise to them migrate to the area early in development, around the time of early lens removal (1 dpf). I hypothesize that in order for the suborbital bones to expand in size and shape an increase in cell number within the skeletogenic condensations may occur. This increase may arise through increased migration to the site or an increase in local cell proliferation.

Alternatively, lens removal may affect the size and shape of the bones surrounding the eye by direct or indirect signalling or via a mechanical influence during later bone development. The developing retina is a source of retinoic acid, which acts as a paracrine factor regulating important developmental processes surrounding the eye (Kish et al., 2011). The lens may also function in a similar manner. The lens has been shown to release paracrine factors (Schweigerer et al., 1988), these could have the capacity to affect orbital bone ossification later in development. Alternatively, lens ablation may affect orbital bone outgrowth as a result of reduced strain within the head. Mechanically the eye holds an important position within the head; when the eye is no longer present the bones of the skull are free to expand without constraint. In support of this alternative, the expansion of bones is always from the free edge of the bone (not adjacent to other bones) and is always directed towards the space previously occupied by the eye. As discussed previously, earlier forming bones may be less impacted by less removal due to the slow eye regression into the head. In chicken, frogs and some mammals the eye has been proven to produce important mechanical forces within the head (Coulombre and Crelin, 1956). In chicken, when the eye is removed in early life it can no longer exert mechanical forces on the surrounding skull, as such the orbit is reduced in size and the orbital bones expand into the orbit (Coulombre and Crelin, 1956). The results of eye removal in chicken very closely resemble the result of lens removal in the Mexican tetra, indicating that mechanical forces maybe the primary source of alterations in the skull of the Mexican tetra after lens removal, alternatively cell signalling networks may be strongly conserved between fish and chicken.

Furthermore, one needs to consider muscles, specifically, the ocular muscles. There are six extraocular muscles present in the zebrafish, which are thought to be conserved in all teleosts (Schilling and Kimmel, 1997). The extraocular muscles insert outside of the eye on the surrounding skeleton. Lens removal and subsequent eye regression may cause alterations in the development of these muscles which may in turn influence the development of the bones on which they insert, and will be discussed below.

Further investigation is required to determine how the lens is influencing the surrounding skeleton. Based on my observations of orbital bone outgrowth I hypothesise

that lens removal may influence condensation size either through proliferation and/or migration, alternatively, the lens may influence the skull through changes insignalling or mechanical influences. Evidence for two mechanisms affecting the orbital region includes the larger size of the supraorbital bones right after the onset of ossification of the bone, indicating an early effect, and later there is a much more rapid out-growth of the surgery orbital bone, indicating there may be a later mechanism affecting outgrowth. These findings indicate that there may be two factors affecting the shape of the orbital bones. As no data was collected on the size of the unossified elements, it is difficult to determine if the condensation or unossified element was affected by lens removal. Since the first ossified bone was slightly larger on the surgery side I hypothesized that the condensation maybe increased in size, however the enlargement of the early ossified bone may be a result of rapid appositional growth after ossification and may not involve any changes in the initial condensation and ossification of the structure.

2.6.3 Lens Removal Affects the Development of Extraocular Muscles

To further investigate the mechanical influences affecting the shape of the orbital bones phalloidin was used to visualize the skeletal muscles present surrounding the eyes of control surface fish and surface fish which received lens removal at 1 dpf. Extraocular muscles, as well as muscles in close proximately to the eye namely, the adductor mandibulae, the elevator arcus palatine, and the dilator opercula muscles. The latter are unaffected by lens removal.

Of interest were the extraocular muscles, the superior rectus muscle originates in the caudal orbit and inserts caudal to the oblique muscles (Schilling and Kimmel, 1997). The superior rectus muscle was present in its typical location on the control side of the head, however, it could not be visualized on the surgery side of the head, indicating that alterations may be present. In addition, the superior oblique and the inferior oblique ocular muscles appear to be altered by lens removal. This indicates that the extraocular muscles may play a role in altering forces within the head after lens removal, which may cause alterations in the orbital bones. The extraocular muscles develop early in development, long before the ossification of the orbital bones (Schilling and Kimmel, 1997; Yamamoto et al., 2003), thus alterations in these muscles are likely providing

different mechanical forces, likely increasing the forces on the orbital bones. Changes in the forces exerted in orbital region may be responsible for the alterations in the bone outgrowth, the increase in force may influence the expansion of these flat bones as a result of increased appositional secretion or mineralization of bone matrix. A study conducted in juvenile tennis players demonstrated that an abnormal increase in muscle strength during growth exerts greater forces on the bones in which they insert (Daly et al., 2004). The increased forces on the bones results in an abnormal increase in the size and mass of the associated bones, indicating that changes in the forces present on a bone can influence the size and shape of that structure (Daly et al., 2004). In addition to the forces of altered muscles affecting bone outgrowth, the small size of the surgery eye may reduce the forces which it would normally exert on the bones surrounding the eye, allowing them to expand unregulated by the normal forces. This is supported by the fact that the supraorbital is most affected by lens removal, as it occupies the largest space boarding the eye orbit, thus forces reduced as a result of eye loss would have the greatest impact on this bone.

Performing further phalloidin staining over multiply stages of development may provide a better insight into the origin and insertion points of the extraocular muscles and the role that extraocular muscles play on the developing skull. Specifically, it will allow for better identification of what muscles are associated with which bones, and whether the altered bones are infact associated with the altered muscles. Observing the extraocular muscles during the time period of orbital bone ossification and adulthood would allow for a better analysis of how the extraocular muscles are influence orbital bone outgrowth.

2.6.4 Earlier Lens Ablation has a Greater Effect on the Surrounding Skull

Most intriguingly, my results indicate that the earlier lens ablation is conducted the greater the effect on the surrounding skeleton. That is, the supraorbital bone and suborbitals 4 to 6 (SO4-6) were expanded to a much greater extent when surgery was performed at 1 dpf compared to when surgery was performed at 4 dpf (Figure 2-8). Principle component analysis demonstrated that lens removal at 1 dpf resulted in skulls that were very different in shape and much further from the control than when surgery was performed at 4 dpf. This finding supports my hypothesis of a cellular basis or

mechanical influence since an early surgery time point would provide a greater amount of time to influence CNC migration or a longer time period for mechanical changes to influence the skull. As stated above, zebrafish neural crest cell migration begins early in development at approximately 14 hours post fertilization and continues in waves through the first few days of development (Kimmel et al., 1995). The earlier the lens is removed the greater the time available to impact migration into the craniofacial region. When the lens is removed later, migration/proliferation may be nearly complete, thus allowing less time for lens removal to affect these cellular processes. This again supports my earlier conclusion that more than one mechanism of bone growth and development may have been influenced by lens removal. However, without examining the condensation size with a specific condensation marker we cannot confirm that the condensation was affected by lens removal. However, the data does indicate that changes in the mechanical forces on the bones during appositional growth may be responsible for the alterations observed in the shape of the bones of the orbital region.

2.6.5 Some Bones are Not Affected by Lens Removal

The frontal and parietal bones are not affected by lens removal, however, I hypothesize that this might be due to compensation by the bones encircling the eye. The bones in close proximately to the eye may absorb the impact of the lens removal and inhibit the propagation of detrimental changes in skull shape in other areas of the skull. Bones such as the supraorbital and the suborbital bones (SO4-6) may be able to absorb the effects of lens removal by expanding from their free edge, which other bones cannot do. As such the effects are not able to spread to the other more important areas of the skull such as the calvariae that protect the brain. In support of this, are the residual effects of lens removal present on the control side of the head. Significant differences in the shape of the orbital bones on the control side of the head compared to control fish were observed. The calvariae are located in much closer proximity to the surgery eye and to the altered bones on the surgery side of the head, than they are to the control orbital bones, despite this, the shape of the calvariae were not affected. Surprisingly, the bones studied on the control side of the head, which are not in close proximity to the eye were altered. This indicates that there is likely a mechanism constraining the shape of the

developing calvariae and not the orbital bones, demonstrating different levels of variability within these bones, this may be an adaptive responce to protect the brain.

2.6.6 The Scleral Skeleton is Affected by Lens Removal

In addition to observing the influence of lens removal on the bones surrounding the eye, I also observed an effect on the bones within the eye. The scleral skeleton (present in the sclera of the eye) of the surface fish consist of two ossicles joined by cartilage. The sclera of the cavefish is thought to contain only cartilage, as a result of a failure to form scleral ossicles (Yamamoto et al., 2003). However, I determined that some cavefish populations do have ossified structures within the sclera in adults (Figure 2-3). Scleral ossicles form through perichondral ossification, with the anterior ossicle ossifying first (Franz-Odendaal et al., 2007). The earlier I removed the lens, the greater the effect on the scleral ossicles; the greatest effect was always on the anterior ossicle which was either reduced or absent. This indicates, once again, that earlier forming structures are affected to a greater degree by lens removal. The mechanism by which the lens is influencing the scleral skeleton is unknown and requires further investigation. As in some surgery specimens scleral ossicles were completely absent, some had one ossicle absent, or had small irregular shaped scleral ossicles. I was unable to determine how the lens affects the ossification of these structures. The scleral cartilage ring which gives rise to the scleral ossicles was found to be present and unaltered by lens removal. Thompson et al., (2010) have suggested that scleral cartilage is induced by the pigmented retina, atleast in chicken embryos, while the scleral ossicles are known to be induced by the overlying conjunctival epithelium (e.g. Franz-Odendaal and Hall 2008). The induction mechanism of the ocular skeleton in teleosts is unknown. In my studies, the scleral cartilage was not influenced by lens removal, while the scleral ossicles were highly altered by lens removal. These findings suggest that the scleral cartilage and scleral ossicles are likely not induced by the samemechanisms in teleosts, which agrees with the findingsin chicken. That is, the scleral cartilage in teleosts may be induced by the pigmented retina, similar to the mechanism identified in chicken, while the scleral ossicles are likely not. The pigmented retina was not damaged by lens removal and thus may be still capable of influencing the scleral cartilage. As the scleral ossicles were altered by lens removal this

may indicate that the lens may play a direct role in directing ossification of the scleral cartilage.

2.6.7 The Effects of Lens Ablation on the Number of Small Mandibular Teeth

The teeth of the Mexican tetra can be divided into three groups, based on differences in location within the mouth, timing of development and pattern of replacement (Trapani et al., 2005). In addition, there are differences in the number of maxillary teeth between the two morphs. Surface fish typically have one multicuspid maxillary tooth while the Pachon cavefish have two or more maxillary teeth (Yamamoto et al., 2003). Only one study has investigated whether the lens influences tooth number (Yamamoto et al., 2003). When reciprocal lens transplants were performed between the cavefish and the surface fish the number of maxillary teeth were not affected. Based on this evidence, Yamamoto et al., (2003) concluded that maxillary teeth are not influenced by the transplanted cavefish lens. However, this study did not investigate the effects on tooth number outside of the maxillary region. At present there are no studies which have investigated mandibular tooth number in cavefish. In my study, I demonstrate that lens removal does affect the number of small caudal teeth on the mandible in surface fish; these teeth are spaced apart from one another. In addition, I determined that the large multicuspid mandibular teeth are unaffected by lens removal. Since more jaw space typically means more teeth (Huysseune, 1995), I analyzed the differences in the length of the jaw and determined that the length of the mandible does not differ on the surgery side compared to the control side of the head and thus, jaw length cannot account for the observed changes in tooth number. Some studies also indicated that with more jaw space, teeth can become larger in volume over successive tooth generations (Huysseune and Sire, 1994; Huysseune, 1995), thus jaw width could be useful in my study to determine if it has influenced tooth number or size. Yamamoto et al., (2009) indicated that the cavefish have a wider mandible, providing more space for both taste buds and teeth. Further investigation of jaw width would strength this study to better elucidate if the space for teeth to form has changed. Although I did not analyze jaw width I did analyze jaw shape. In my analysis of jaw shape two of the four surgery time points (surgery at 2 dpf and 4 dpf) resulted in significant shape changes of the jaw. I hypothesize that the

shape changes in the jaw may provide more space to form additional teeth on the surgery side of the jaw. However, an increase in tooth number was present at three different surgery time points. This suggests that an increase in tooth number occurred without an alteration in jaw shape, indicating that increase in tooth number may be independent of changes in jaw shape. Alternatively, alterations in the mechanical forces on the jaw may influence tooth number.

To my knowledge only one study has investigated how changes in diet can influence the number and size of teeth in teleosts (Huysseune, 1995). In this study, the cichlids, *Astatoreochromis alluaudi,* were fed either hard food (snails) or soft food. Vast differences were observed between the two groups. Both jaw size, and tooth number and size differed between the two groups. Specimens fed hard food had significantly fewer teeth than the fish fed soft food; in addition to fewer teeth, the teeth that were present were larger in size. The soft food group had a larger quantity of small teeth. The amount of divergence in tooth morphology and number was dependant on the age of the fish when the diet was altered, the younger the specimen the greater the impact on the dentition. From this study, it is clear that tooth number is variable in nature and small influences can have a great impact on the development of the teeth. The hardness of the food may influence the forces applied to the jaw when eating, resulting in changes in jaw shape and tooth morphology. Lens removal also affected jaw shape (at two time points) resulting in changes in tooth number. Additionally, lens removal and subsequent eye regression may result in changes in mechanical forces around the eye and perhaps into the oral region. These changes in forces may affect the patterning of the teeth.

Due to the developmental differences between the caudal and central teeth in the Mexican tetra (age at development, and different replacement cycles) (Yamamoto et al., 2003; Trapani et al., 2005); it is likely that while the small caudal teeth appear to be influenced by the lens, the large multicuspid teeth are not susceptible to these influences. Interestingly, in the small eyed mouse mutant $Pax6^{Sey}/Pax6^{Sey}$, in which the lens fails to form; 80% of the mutants show an increase in anterior upper tooth number by 1 to 2 teeth [Huyssune, 1995; Kaufman et al., 1995]. Alterations in *Pax6* do have pleiotropic affects. In mice, it has also been demonstrated that a single tooth can inhibit both the size and development of neighbouring teeth (Kavanagh, 2007). In the tetra, the isolated nature of

the small teeth on the caudal portion of the mandible might enable them to be more variable in number compared to the large clustered multicuspid teeth in the centre of the jaw.

Although little is known about the development of the small caudal teeth on the mandible of the Mexican tetra, natural variation in tooth counts are reported within these teeth (Trapani et al., 2007). The small caudal teeth of the Mexican tetra are the only adult teeth that do not form within the jaw bones (extraosseously) (Trapani et al., 2007). Their small size, the timing and manner of their development and their location close to the optic cup, might make these teeth more susceptible to influences from the developing eye than the central larger multicuspid teeth. Further investigation is required to understand the underlying mechanism.

2.6.8 Variability of the Mexican tetra Skull

This study demonstrates a vast difference in the level of variability present in the bones of the Mexican tetra skull. Bones within the eye and dorsal to the eye are largely altered in shape in the absence of the lens, indicating that these bones are highly susceptible to their environment and are variable in nature. The roof of the skull, the calvariae, and orbital bones anterior and ventral to the eye were not influenced by lens removal indicating that these bones are much more constrained, less variable and less influenced by changes in their local environment. The literature reviewed in Chapter 1 suggests that there are differential levels of variability in the bones of the teleost skull and that natural variability can arise in different manners. This can range from timing of onset of ossification (Cubbage and Mabee, 1996) to diet affects, to cell origin, to ossification type and to bone location (Meyer, 1987; Bouton et al, 2002; Ornsrud et al, 2004; Fernandez et al, 2008).

Based on the studies reviewed in Chapter 1, no clear correlation could be determined between mode of ossification and variability in fish. In my study I determined that the bones which were most affected by lens removal formed via intramembranous ossification (the orbital bones), however all of the bones in close association with the eye ossify via intramembranous ossification. Thus, these data do not provide any further

evidence that ossification type influences the variability of a bone. There is a gap in the literature in this area addressing how mode of ossification can affect bone shape.

Chapter 1 indicated that in fish bones which are NCC in origin tend to be more variable in shape than those derived from mesoderm. In zebrafish, the bones of the orbital region are neural crest derived, while the calvariae are neural crest and mesoderm in origin (Kague et al., 2012). If we accept that the cell origins to be the same between zebrafish and the Mexican tetra, then cell origin maybe another factor influencing the vast variation present in the orbital bones. Both the CNC derived surgery side of the skull and the control side of the skull with residual affects where influenced by lens removal, while the mesoderm derived calvariae located between the affected bones remain unaffected. Cell origin of each bone may play a major factor in how lens removal is able to influence the shape of the bone.

As was discussed previously, timing of ossification may also play an important role in the variability of a bone. The calvariae ossify very early in development (relative to the orbital bones), prior to and during the development of many head muscles and prior to eye regression after surgery (Cubbage and Mabee, 1996; Schilling and Kimmel, 1997), while the orbital bones ossify late in development, long after the other structures of the head, including muscles and all other skeletal bones have ossified. The early ossification of the calvariae may result in the canalization of these bones, while the late forming orbital bones would not be canalized. In this chapter, I demonstrated that muscles in the orbital region were affected by lens removal, the calvariae are likely established before head muscles can exert forces on them, while the late formation of the orbital bones results in many forces already present and influencing their development. These finding indicate that timing of ossification may influence the variability of the bone.

Lastly, bone location may affect the variability versus stability of the calvariae over the orbital bones. As mentioned previously, the calvariae are located between other bones, giving them very few options for shape change. While the orbital bones border open space (the eye orbit) on one side allowing them space in which to expand. Additionally, the calvariae occupies a position which is crucial for the protection of the brain. As such it does appear, as was hypothesized in Chapter 1 that the location of the bone may play a critical role in the variability of the element.

This study identifies changes in the shape of skull bones after lens removal, however how and why some bones are more affected than others requires more investigation. The "why" has been addressed through investigating what factors make a bone variable in nature. Based on my findings I hypothesize that the "how" occurs through two mechanisms,in which both the condensation and bone outgrowth are responsible for changes in the adult bone shape, further investigation is required to determine the mechanisms influencing ossification and growth. Results indicate that perhaps both mechanical and signalling influences from surrounding structures are responsible for the changes in shape present in the skull after lens removal. Further to understanding how the lens is influencing the shape of select bones, research is required to determine how the variation of these bones impacts the development of other surrounding structures and tissues. Or if it is largely an indirect affect on the orbital bones as a result of changes in the mechanical forces present on the skull.

This study provides an excellent starting point and system in which to study the how and why of variability of bone shape in the teleost skull in the future.

2.6.9 The Effects of Lens Ablation on Taste Bud Development

In order to investigate the proposed links between the sensory modules (eyes and taste) (Franz-Odendaal et al., 2006), lens removal was conducted at 1 or 3 dpf in surface fish. My results show that the absence of the lens in surface fish does not affect the number or arrangement of taste buds, despite cavefish having a greater number. This analysis agrees with quantitative trait loci data which demonstrated that eye size is not significantly correlated to number of taste buds, despite cavefish having more taste buds (Protas et al., 2007). However, Yamamoto et al., (2009) hypothesized that an increase in midline expression of *sonic hedgehog* (*shh*) in the Mexican tetra cavefish has pleiotropic effects, which facilitates a trade-off between vision and taste. Yamamoto et al., (2009) experimented with levels of *hh* present in cavefish and surface fish during early development. The authors injected early developing cavefish with cyclopamine to block *hh* expression in the cavefish. Cavefish typically have a larger expression of *shh* in early development than their surface fish counter parts. When *hh* levels were reduced to surface fish levels in the cavefish embryos the cavefish took on a more surface fish

phenotype. Both the eyes and the taste buds were affected, eyes developed and taste buds numbers were reduced to a more surface fish-like number. When the opposite experiment was performed by injecting *shh* into the surface to give a more cavefish-like level of *shh* in early development the eyes were reduced and taste bud number was expanded. This study demonstrates the important trade-off present between eyes and taste driven by *shh* expression. However, Varatharasan et al., (2009) demonstrated that taste bud numbers could not be accurately determined until the specimens were 21 dpf. The previous study examined specimens which were 5 dpf to analyze the affects of *shh*, as such the study may over estimate the influence of *shh* in this trade-off and under estimate the role of the lens in eye regression. Despite the flaws of the previous study it appears as though genetic alterations between the two morphs are responsible for the differences in taste bud number, as lens removal did not impact this system.

2.7 Summary

I determined that lens removal conducted between 1 and 4 dpf influences the development of craniofacial bones surrounding the eye; most dramatically affecting the supraorbital bone and suborbital bones 4 through 6, while the calvariae are unaltered. Notably, lens removal had the greatest effect on the skull the earlier it was performed. The affected bones are more variable in their development than others in the skull and are more influenced by the eye, maybe due to their closer association with it. Scleral ossicles were either reduced or absent as a result of lens removal with the earlier developing anterior ossicle most affected. Additionally, I determined that lens removal does not affect the number of taste buds, but it does however affect the number of small caudal teeth present in the surface fish. The extraocular muscles appear to be altered by lens removal and may alter the normal forces present in the ossifying orbital bones, affecting their shape. Further investigation is required to unravel exactly what role orbital muscles may play in the shape of the orbital bones. With this long term study I have demonstrated that the lens, a soft tissue in the head, has the capacity to influence the development of particular bones that develop several months later. How and why certain bones are more affected than others has yet to be determined. Many factors relating to the bones origin, growth and location may influence the variability of the bone. How the lens influences

the skull also requires further investigation. Whether the lens influences the skull through a direct or indirect, mechanical or molecular manner has yet to be determined, but appears to occur through more than one mechanism. Overall, this research raises many questions regarding the role of the eye in directing development of the vertebrate head, including what role the eye plays in the development of the skull and the skeletal muscles.

Chapter 3: Laser Lens Damage and Lens Regeneration Results in Minimal Affects on the Craniofacial Skeleton

3.1 Introduction

This chapter of my thesis has been divided into two sections, Part A and Part B. In Chapter two I determined that permanently removing the lens in the first 4 dpf results in dramatic changes in some of the bones of the Mexican tetra skull. The objective of Part A of this chapter is to extend the time period in which the lens was damaged to determine how later lens damage affects the skull. Manual lens removal cannot be performed after 4 dpf, as the cornea becomes too thick to tear. In order to extend the timing of lens surgeries, laser ablation was used to damage the lens. To determine if later partial lens removal or lens damage can affect the skull, laser lens damage was performed between 1 and 11 dpf and affects were examined on the adult skeleton. This chapter aims to understand how damaging the lens in early development can affect the development of the adult skeleton. In these experiments, the lens was damaged during CNC migration. The objective of Part B is to determine if the juvenile Mexican tetra has the capacity to fully regenerate a lens, and if so, over what time period lens regeneration occurs.This data will be presented as a preliminary study.

The process of lens development has been described in detail in the zebrafish (Glass and Dahm, 2004), because it is often used as a model species. Lens development in the zebrafish is a rapid process, which occurs in four stages, competence, bias, specification and differentiation (Glass and Dahm, 2004). Competence is the ability of the head ectoderm to respond to inductive signals. Bias occurs when signals have influenced the ectoderm to a lens forming fate. Specification of the lens cell precursors then occurs, followed by the differentiation of the ectoderm into lens cells (Glass and Dahm, 2004). In zebrafish, by 21 hpf the lens placode has begun to form as a thickening of undifferentiated surface ectodermal cells. By 36 hpf the presumptive lens is no longer connected to the surface ectoderm. In teleosts, the solid lens vesicle forms from delamination and apoptosis separating it from the head ectoderm. Apoptosis also occurs in the anterior of the lens forming a single epithelium layer in the anterior region, while

the cells located in the center of the lens vesicle elongate in a circular manner forming primary lens fibre cells. Later the primary cells will undergo elongation at which time they will degrade their organelles. Between 60 and 84 hpf the lens develops and grows further as the single layer of lens epithelium undergoes proliferation giving rise to the secondary fibre cells. These cells enclose the primary cells and span from one pole of the lens to the other and will also degrade their organelles. By this time the fibre cells have produced large quantities of crystallin (Glass and Dahm, 2004). The adult structure of the lens consists of a single layer of cubodial epithelial cells, capable of giving rise to new fibres cells over the anterior pole of the lens and densely packed fibres cells in the cortex, responsible for reflecting light, all within a membranous lens capsule (Lovicu and Robinson, 2004). The lens maintains its polarity in the eye throughout life (Lovicu and Robinson, 2004).

Lens regeneration has been largely studied in urodeles (salamanders and newts) and anurans (Henry, 2003). In these groups lens regeneration occurs through transdifferentiation. The definition of transdifferentiation can vary between limb and lens regeneration studies. In lens regeneration transdifferentiation occurs in three stages, firstly, cellular dedifferentiation (a terminally differentiated cell re-entering the cell cycle and losing the characteristics of their origin), second, proliferation, and lastly transdifferentiation into lens cells from other cell populations (Henry, 2003). While the textbook definition of transdifferentiation (based on limb studies) is that it occurs in two steps, dedifferentiation followed by redifferentiation, in this thesis, I will consider the definition of Henry (2003) that transdifferentiation includes proliferation. In some amphibians, namely, salamanders and newts, dorsal iris pigmented epithelium dedifferentiate to form the regenerated lens, this is referred to as Wolffian lens regeneration (Henry, 2003). In other species corneal-lens transdifferentiation occurs. Lens regeneration occurs only through transdifferentiation of other (non-lens) cell populations. In some mammals (e.g. mice and rabbits) the lens is repaired without transdifferentiation and is thus considered lens healing (Call et al., 2005).

The location of the cells that undergo dedifferentiation in urodeles differs between the groups. In some urodeles the dorsal iris pigmented epithelium can dedifferentiate to form the regenerated lens; this is referred to as Wolffian lens regeneration (Henry, 2003).

In other amphibians transdifferentiation occurs in the outer corneal epithelium, this type of regeneration is termed corneal-lens regeneration.

Lens regeneration typically follows the same ontogenetic process as embryonic lens development, as described in Chapter 3. Wolffian regeneration takes place over 13 stages. Briefly, in the newt, regeneration begins with the dedifferentiation of pigmented iris cells, which lose their pigment, and alter nuclei shape. Proliferation of the newly dedifferentiated cells begins by four days post-surgery, the lens capsule then begins to form. Iris cells elongate by eight to ten days post lentectomy and a hollow epithelial lens vesicle is present approximately ten days after surgery was performed. The cells of the lens vesicle then differentiate in a very similar manner as is observed in lens development, forming the lens epithelial cells and fibre cells. By 12 to 16 days post lentectomy, the internal layer of the lens vesicle begins to produce crystallins, marking the onset of the differentiation of primary fibre cells. Crystallins are expressed specifically in the differentiated fibre cells of the lens, α, β, and δ are common in many animals. As such, they make an excellent marker for identifying a regenerating lens or lentoid cells (Tsonis et al., 2004). By 25 days post lentectomy the secondary fibre cells begin to develop, following which the regenerated cells appear to be fully formed and detach from the iris (Tsonis et al., 2004). The last stage of lens regeneration involves the loss of the nuclei from the fibre cells. Corneal-lens regeneration occurs in a similar manner as described above, with an exception of the site of the dedifferentiating cells (Henry, 2003). In corneal-lens regeneration, the regenerating lens forms from the inner layer of the outer corneal epithelium.

In newts, lens regeneration occurs in both the juvenile and the adult, however in *Xenopus laevis* regeneration only occurs in early life stages (i.e. tadpoles). The frogs lose the ability to regenerate their lenses as they approach metamorphosis. In Wolffian and corneal-lens regeneration, regeneration relies on signals from the neural retina. In some species, after further differentiation of the eye, signals from the retina can no longer reach the site of regeneration, inhibiting lens regeneration in adulthood (Henry, 2003).

Lens regeneration has been reported in only one teleost (Sato, 1961), *Misgurnus anguillicaudatus*, a common loach. The loach has the capacity to fully regenerate the lens in adulthood, via Wolffian lens regeneration (Sato, 1961). Sato surgically removed the

lenses from adult specimens, then observed the regeneration of the lens with histological analysis over 25 days. He was able to demonstrate that lens regeneration occurs from the iris and was first visible seven days after the surgery (Figure 3-1). By 25 days after lens removal, regeneration is nearly complete. At that time the lens is mature and ready to detach from the iris. This species of teleost is thought to have evolved the unusual capacity of lens regeneration as it encounters large numbers of parasites in its natural environment, which attack the lens of the fish (Sato, 1961). After observing two normal eyes in surface Mexican tetras which had received lens surgery, I hypothesize that the surface Mexican tetra may have the capacity for lens regeneration. This might be as a result of gene flow between the surface morph and the blind cavefish.

Lens healing in the absence of cellular transdifferentiation has been documented in mice, rabbits, fish, chicken and cats (Call et al., 2005). In mammals, when the lens was surgically removed or damaged a new lens will form if lens epithelial cells are left behind attached to the remaining lens capsule. If all of the lens epithelium and lens capsule are removed, lens healing does not occur (Call et al., 2005). Despite the fact that transdifferentiation does not occur in the aforementioned animals, it is clear that after substantial lens damage, the lens has the capability of healing (Henry, 2003). While few animals have the capacity to fully regenerate a new lens it appears that many animals may have the capacity to repair lens damage from the remaining lens cells.

Some studies have demonstrated that changes in response to manipulations (i.e. changes in diet, lens regeneration etc) performed in a juveniles may be corrected by adulthood, while others show that these changespersist throughout life. To determine if changes in early development can influence the morphology of the adult skull a clutch of cichlid (*Cichlasoma managuense*), young were randomly divided into two groups; the groups were then fed different diets for the first 8.5 months (Meyer et al., 1987). The results indicate that feeding different diets significantly influenced the shape and size of the bones in the preorbital region, including the mandible length and shape, as well as the maxilla length and shape. After 8.5 months the two groups were then fed the same diet until 16.5 months of age. At 16.5 months of age the skeletons of the two groups were examined for differences and determined that differences in the shapes of the bones in the preorbital region were no longer present. All alterations to the juvenile skeleton as a

result of different diets in early life were corrected with a normal diet later in life. This study demonstrates that alterations in early life can affect the juvenile skeleton, however when the altered diet was removed, the adult skeleton could responde and return to its normal morphology. This study demonstrates the plasticity of the oral region of the cichlid skull, in particular. In contrast, many other studies demonstrate the long lasting effects of treatments performed on juveniles on the adult skeleton. For example Mazurais *et al.* (2009) studied the effects of limiting the amount of vitamin A, in the diet of European sea bass larvae (*Dicentrarchus labrax*) in aquaculture. The authors determined that diets low in vitamin A fed to juveniles resulted in malformations in the maxilla, premaxilla, dentary and the operculum that persist intoadulthood.

To determine if lens absence in early or later life is responsible for the alterations present in the skull in Chapter 2 and to determine when the skull is most vulnerable to influence, lens damage was performed over 1 to 11 dpf. As was stated previously in zebrafish (Cypriniformes: *Danio rerio*) the orbital bones are neural crest derived (Kague et al., 2012). The cranial neural crest cells begin migration at 14 hours post fertilization (Kimmel et al., 1995), thus CNC are migrating just prior and shortly after the lens was removed. The orbital bones of interest do not ossify until 3-4 months of age, long after lens removal and CNC cell migration. Our results from Chapter 2 demonstrate that lens removal conducted within the first 4 dpf has tremendous effects on the shape of some of the orbital bones. As such the effects of lens removal may act on the CNC migration or later on the condensation, ossification and outgrowth of the affected bones. To address this I damaged the lens in early development (1 to 11 dpf) to inhibit the action of the lens during CNC migration. The lens then heals and is present during condensation and ossification. I examined the effects of damaging the lens in early development on the juvenile and adult craniofacial skeleton to determine if and when the lens is required for the proper formation of the skull (Part A). . I hypothesize that the shape of the orbital bones will be most affected the earlier in life the lens is damaged.

Part B consists of a preliminary study in which I will investigate the Mexican tetra's capacity for lens regeneration in juveniles and will determine over what time period lens regeneration occurs. I hypothesize that the Mexican tetra does have the capacity for lens regeneration. Part A and B each have a Materials and Methods and a

Results sections. There is one discussion in which the results from Parts A and B will be discussed together.

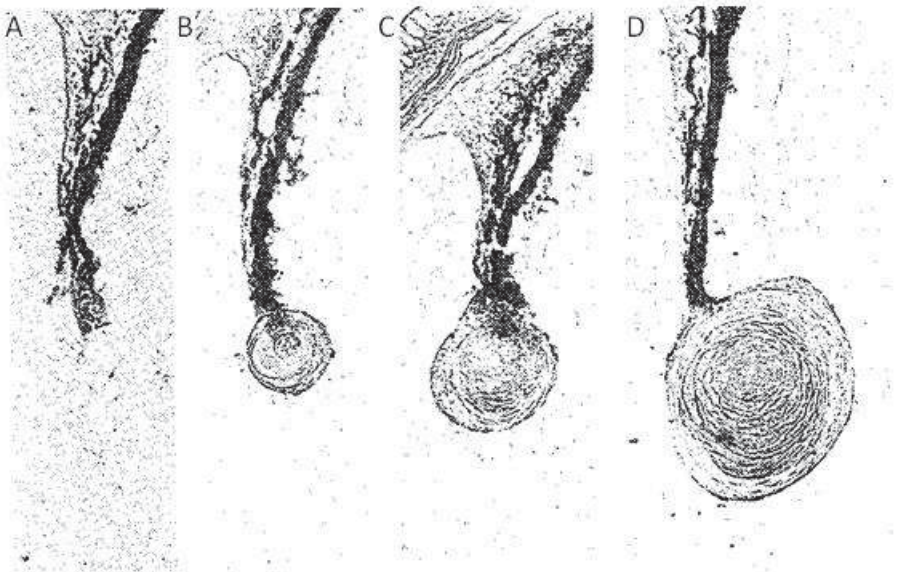


Figure 3-1: Stages of Wolffian lens regeneration in the the teleost, *Misgurnus anguillicaudatus*. (A) 7 days after lens surgery; (B) 13 days after surgery; (C) 17 days after surgery; (D) 25 days after surgery. Figure from Sato, 1961.

Part A

3.2 Materials and Methods

3.2.1 Partial Lens Ablation

Partial lens removal, or lens damage was conducted unilaterally in surface fish each day from 1 to 11 dpf, using a Nikon laser system by following the agar embedding process outlined by Yamamoto and Jeffery (2002). Manual lens removal conducted in Chapter 2 resulted in the complete removal of the lens, including the lens capsule. Laser treatment conducted in the current chapter results in lens damage, but does not fully ablate the lens. Minor alterations to the protocol described by Yamamoto and Jeffery (2002) included incubating the specimens in 0.01% Ms222 (Ethyl-3-aminobenzoate methanesulfonic acid salt, Sigma E10521) in calcium-free zebrafish Ringer's solution for two minutes to anaesthetize the fish and eliminating the 20 minute incubation in 0.2% EDTA as this step aids in manual lens removal only. Finally, fish were embedded in agar in a projector slide to allow for use of the laser under the compound microscope. The microscope was focused on the lens at various depths to ensure laser ablation of just the lens and ablation of lentoid cells at various levels as the fish lens is a sphere. Various amount of laser ablation can be selected when using the laser, Figure 3-2 demonstrates the varying amounts of laser ablation that can be conducted on a mirror coated slide. A small laser zap is approximately 10 μm in diameter, a medium zap is approximately 30 μm in diameter, and a large zap is approximately 100 μm in diameter. Medium to large size laser ablations were used in all experiments, I consider all ablations to be partial laser ablations. After surgery, specimens were rinsed in zebrafish Ringer's solution three times, released from the agar mounting medium and returned to the fish facility. The non-laser-treated eye serves as a control.

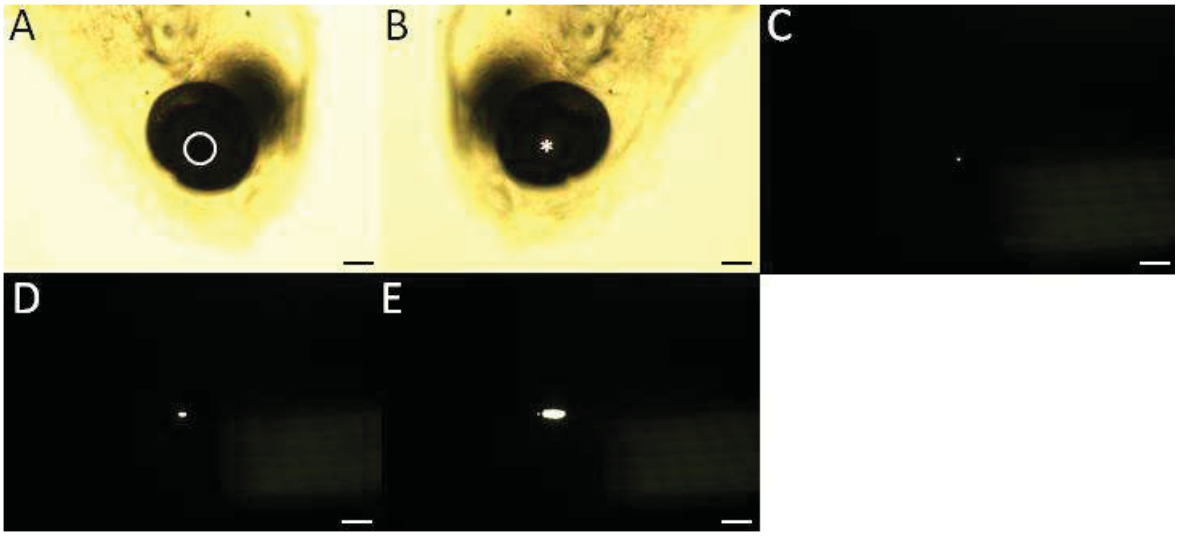


Figure 3-2: Size of laser ablation blast zones. (A) Specimen embedded in agar prior to laser ablation, lens outlined with white ring; (B) specimen after lens ablation, golden tone can be observed where fiber cells have been destroyed, the location of the damaged cells are identified with an asterisk; (C) small laser zap fired on a mirror slide; (D) medium laser zap fired on a mirror slide; (E) large laser zap fired on a mirror slide. Scale = 100 μm.

3.2.2 Laser Fish Growth Series and Adult Whole Mount Bone Stain

Adults at a minimum of 3.5 cm SL (9 to 12 months of age) were whole mount bone stained as described in Chapter 2. The maxium diameters of the both eyes of each adult fish were measured (Appendix 2).

Juvenile surface tetras ranging between 2.3 and 3.1 cm SL were whole mount bone stained (as described previously). Gross morphological analyses and images were captured of the lateral view of the speciemens using a Nikon SMZ1000 microscope and NIS Elements software package.

3.2.3 Laser Morphometric Analysis

During adulthood the laser treated eye cannot be distinguished from the control eye. Morphometric analysis was used to determine if the bones on one side of the skull was different in shape than the bones on the other side and if the changes were similar to alterations observed after full manual lens removal (described in Chapter 2). Specimens were grouped into nine groups based on age at surgery. Groups include the 1 dpf, 2 dpf, 3 dpf, 4 dpf, 5 dpf, 6 dpf, 7 dpf, 8 to 9 dpf combined and finally the 10 to 11 dpf combined.

Forty-two two-dimensional (x,y) landmarks were applied to the lateral view images of each fish (Figure 2-2). Landmarks were applied using tspDIG2 software (F. James Rohlf, http://life.bio.sunysb.edu/morph/) and analysis was conducted using the IMP series of software (H. David Sheets, http://www3.canisius.edu/~sheets/morphsoft.html). For each fish, the landmarked left side of the head was replicated 10 times and grouped, and for each fish the landmarked right side of the head was replicated 10 times and grouped. The IMP software Coordgen was used to calculate Procrustes distance of each of the left and right side groups for each specimen. The program TwoGroup was then used to calculate the average Procrustes distances for each of the left and right side groups. The statistical difference in shape between each fishes left and right side of the head was calculated in TwoGroup with both, Goodall's F-test and F test, Procrustes, analytical and resampling tests respectively. For each fish that demonstrated a significant difference in the shape of the bones on one side of the head compared to the other was selected for further analysis. Further analysis included testing for outliers. A group of controls, which had not received any surgery,

were assembled as described previously in manual lens ablation (Chapter 2). To visualize outliers the IMP software PCAGen was used to compare the control group to the left and right sides of the head of each fish independently. All specimens that showed that one side of the head was significantly different from the other and showed that one side of the head was an outlier compared to the controls was selected for the altered group. The side of the head that was not an outlier was placed in the unaltered group. Specimens were grouped based on the age at time of surgery as described above, thus all sides of the head that were altered in fish that had surgery at 1 dpf were group and all corresponding sides of the head that were unaltered after 1 dpf surgery were grouped, this was repeated for all surgery time points. Twogroup was then used to determine if there was a significant difference between the altered and unaltered group at each time point using Goodall's F-test and F-test Procrustes.

3.3 Results

3.3.1 No Eye Regression in Juveniles After Partial Lens Ablation

Lens damage was performed between 1 and 11 dpf. Based on gross morphological analysis it was clear that eye regression did not occur in the surgery eye (n=9), two eyes of approximately the same size were present. The size of the eyes were analyzed between 4 and 16 days after surgery.The diameter of each the right and left eye of each one of the juvenile surgery specimens were measured (Appendix 2, Table 8). The percentage difference in diameter between each specimens right and left eyes were calculated, size differences in eyes present between 4 and 16 days after surgery are shown in Figure 3-3 and 3-4 (n=9).The largest difference in the diameter of the eyes measured in an individual was 7.8%. The average difference in eye size in individuals was 3.1%.

To investigate the lack of eye regression, histological analysis was performed 24 hours after partial laser lens ablation. The histology showed that the laser treatment was not successful in ablating all lens cells (Figure 3-5). By 24 hours after laser lens ablation the majority of the fibre cells located in the core of the lens were damaged, absent, or improperly arranged. In the control lens the central fiber cells were arranged in the long

typical concentric rings, while the fibre cells of the other lens (surgery) were not visible as long thin cells in rings. The lens appeared smaller in size and more immature in its stage of development, with the lack of mature fibre cells. In addition, the cells located to the periphery of the control lens were present and appeared to be intact, including the epithelial cell layer on the anterior surface of the lens, whereas the opposite lens appears to have damage to the epithelial layer. Despite having these changes in the structure of the lens, eye regression did not occur. This most likely did not occur because of lens healing.The Mexican tetra's capacity for lens regeneration will be discussed in Part B.

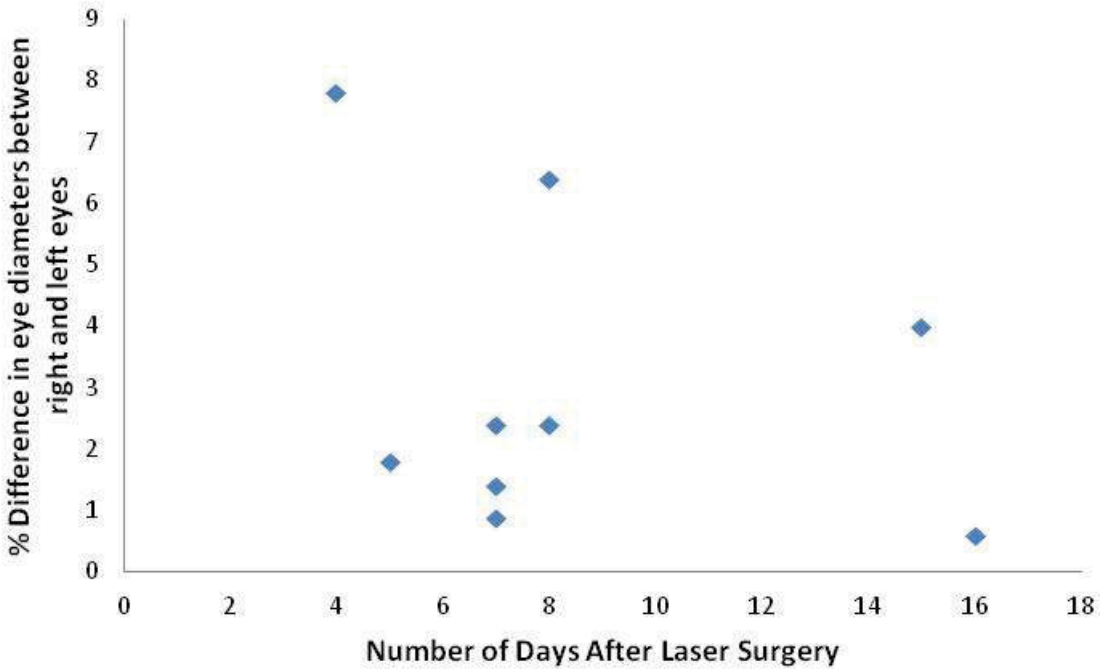


Figure 3-3: Difference in eye diameters between the right and left eyes after laser surgery. The difference in the diameters of the right and left eyes after laser surgery. Eye diameters are compared over the first 16 days after laser lens surgery performed over the first 11 dpf (n= 9). Specimens range between 5 and 27 dpf.

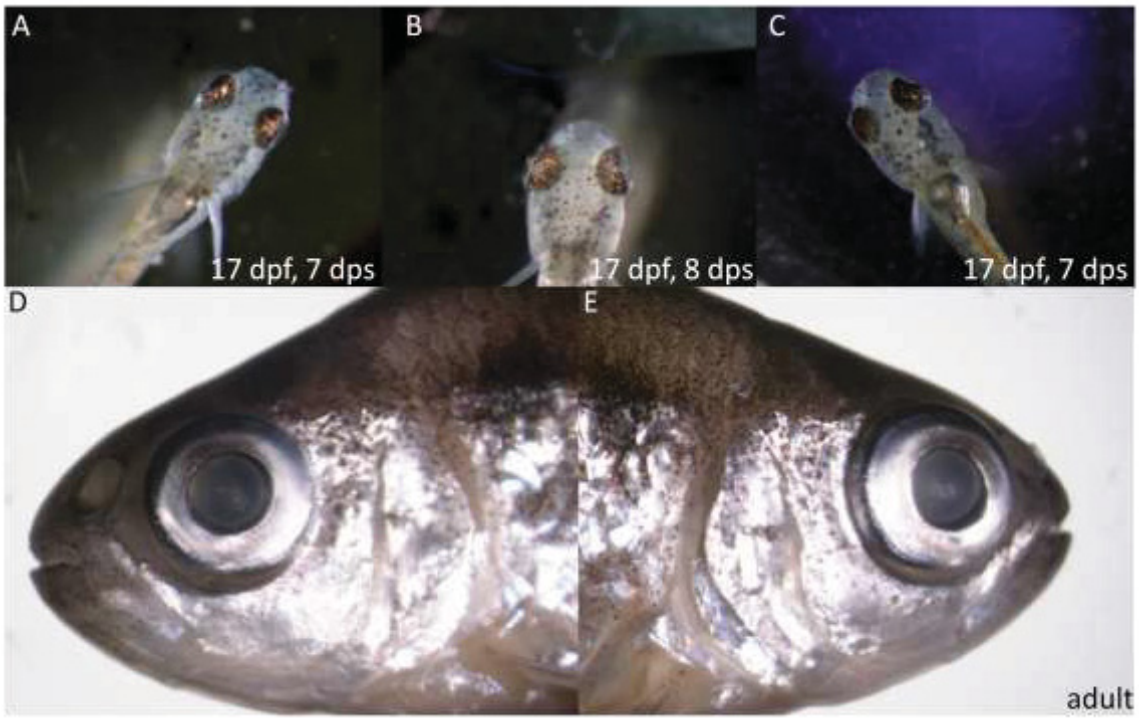


Figure 3-4: Juvenile and adult laser surgery specimens. (A) Laser surgery at 10 dpf and imaged 7 days after surgery; (B) laser surgery at 9 dpf and imaged 8 days after surgery (dps); (C) laser surgery at 10 dpf and imaged 7 dps; (D-E) right and left eyes of the same adult fish, one eye received laser surgery at 5 dpf and the other did not.

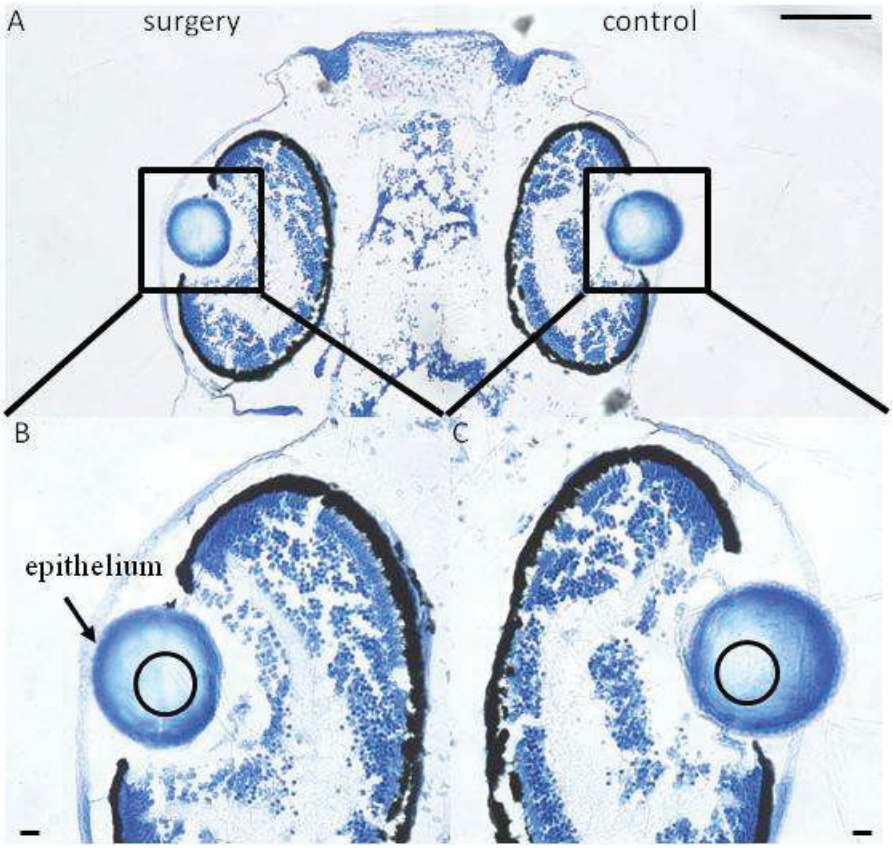
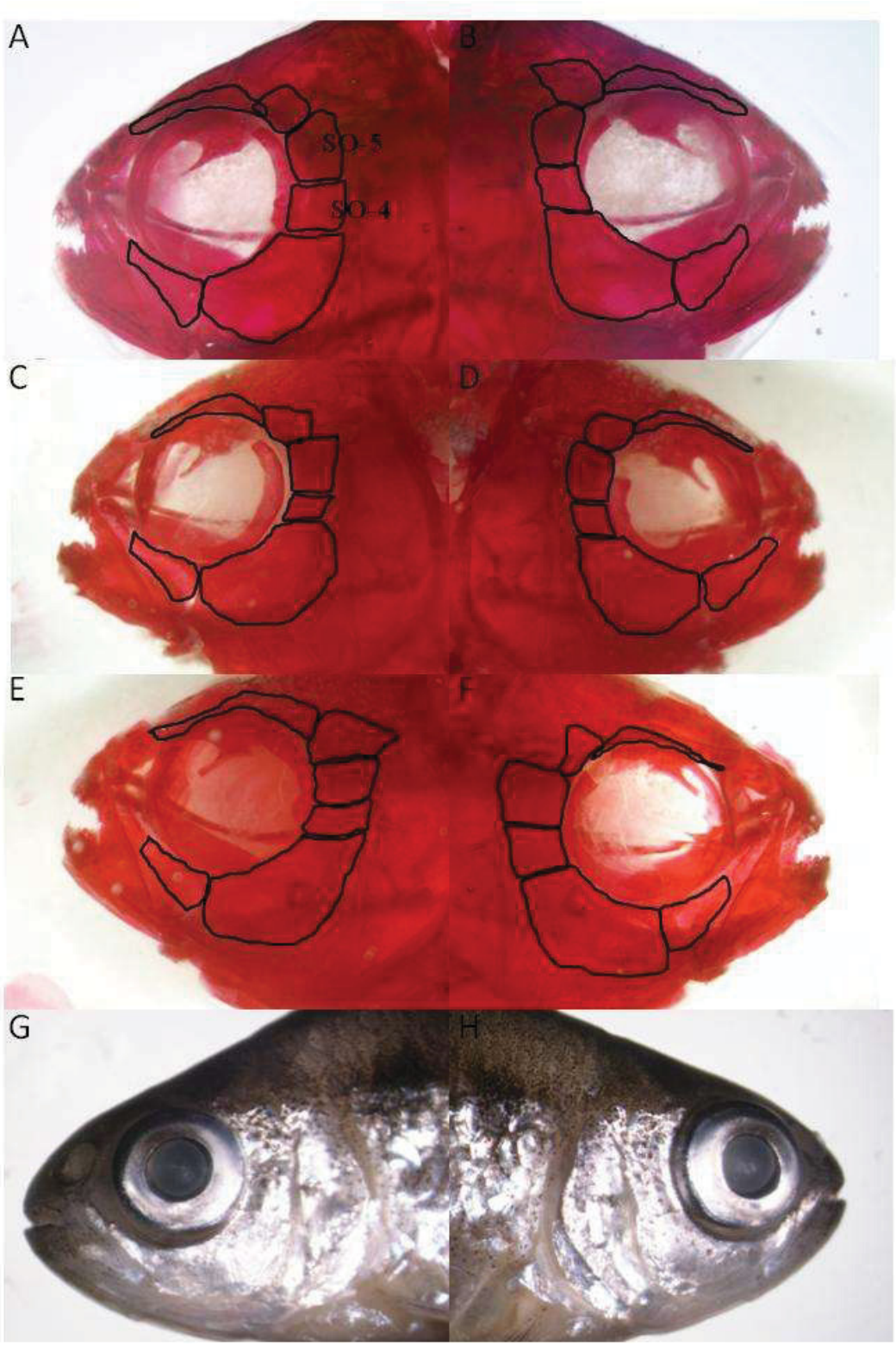


Figure 3-5: Histological sections through the control and laser surgery eye 24 hours after surgery. (A) the left eye is the surgery side with the lens lacking organized fibercells,the circle indicates area of disorganized fiber cells, and the right is the control eye with organized fiber cells, circle indicates area of highly organized fiber cells, scale = 10 μm; (B) higher magnification of (A) surgery eye; (C higher magnification of the control eye. Anterior is up. Scale = 100 μm.

3.3.2 Laser Ablation Does not Affect Adult Eye Size

As eye regression did not occur after lens damage and both eyes appeared normal in adulthood, the surgery eyes could not be discerned from the control eyes. To determine if partial laser lens ablation conducted between 1 and 11 dpf can influence eye size in adulthood and thus be used to discern the surgery eye from the control eye the diameter of both right and left eyes of the laser surgery fish were measured (Figure 3-6, Appendix 2, Table 9). Subsequently, the size differences between the right and left eye of each surgery specimen were determined. To determine if the surgery specimens had a greater difference in eye diameters between the right and left sides of the head than naturally found in the control specimens an independent t-test was performed on the eye diameter measurements. The independent t-test results indicated that there was no significant difference between the diameter of the left and right eye of adult surgery specimens and the right and left eyes of adult control specimens ($p>0.05$), indicating that the surgery eye is not different in size from the control eye in any of the surgery time point groups. Thus eye size in adult surgery specimens is not affected by partial lens ablation between 1 and 11 dpf. For this reason, eye diameter could not be used to determine which eye received surgery.

Figure 3-6: Alizarin red bone stained and unstained adult specimens after laser lens surgery. (A-B) stained right and left side of the head after laser lens surgery at 1 dpf, left side hypothesized to be surgery side; (C-D) stained right and left side of the head after laser lens surgery at 5 dpf, right side hypothesized to be surgery side; (E-F) stained right and left side of the head after laser lens surgery at 10 dpf, left side hypothesized to be surgery side; (G-H) unstained right and left side of the head after laser lens surgery. The hypothesized surgery sides are located in the left column. SO-4, suborbital 4; SO-5, suborbital 5.


A
SO-5
SO-4
B
C
D
E
F
G
H

3.3.3 The Effects of Laser Lens Ablation on the Shape of the Adult Skull

Gross morphological analysis of the adult whole mount stained skulls revealed that slight differences could be observed when comparing one side of the orbital skeleton to the other. The vast majority of specimens had slightly larger suborbital bone 4 and 5 on one side of the head compared to the other. When larger orbital bones were present they appeared to have expanded minimally into the orbit (Figure 3-6).

In order to statistically determine if one side of the head was different from the other and to determine if laser lens ablation had any impact on the skull, morphometrics analysis was conducted in two manners. First by using Principle Component Analysis (PCA) to compare the two sides of the head to a group of controls to determine if either side is an outlier from the controls, and thus different in shape and secondly to use TwoGroup to determine if there is a statistical difference between one side of the head and the other.

Firstly PCA was performed starting with samples that received laser ablation at 1 dpf. An individual PCA trial was run for each individual to determine if one side of the head was an outlier, an outlier was deemed a sample free from the cluster of controls and other side of the head. For the 1 dpf group of six individuals examined six were determined to have an outlier (Figure 3-7, 3-8, Table 3-1). Of the 2 dpf group 100% also had an outlier, in the 3 dpf group four of six had an outlier, in the 4 dpf group five of seven had an outlier, in the 5 dpf group five of six showed an outlier, in the 6 dpf group five of six had an outlier, in the 7 dpf group four of four showed and outlier, in the 8 and 9 dpf group three of five had an outlier and finally in the 10 and 11 dpf group five of eight had an outlier. PCA depicts fine differences in shape, to determine if these differences were statistically significant or not TwoGroup was used. TwoGroup analysis was conducted on samples which had an outlier present in the PCA analysis only, as they were the only samples that had any shape differences present. To conduct TwoGroup analysis, the left side and the right side of each head had to be replicated 10 times each in order to properly use the TwoGroup software. TwoGroup then made an average of each side of the head and compared it to the average of the other side of the head for each individual at each surgery time point. Goodall's F-test and Procrustes F-test were then performed to determine if the changes observed in PCA were statistically significant. In

the 1 dpf group of the six specimens four were determined to have a significant difference in shape from one side of the head to the other (an outlier), the total number of individuals that had significant changes can be observed in Table 3-1.

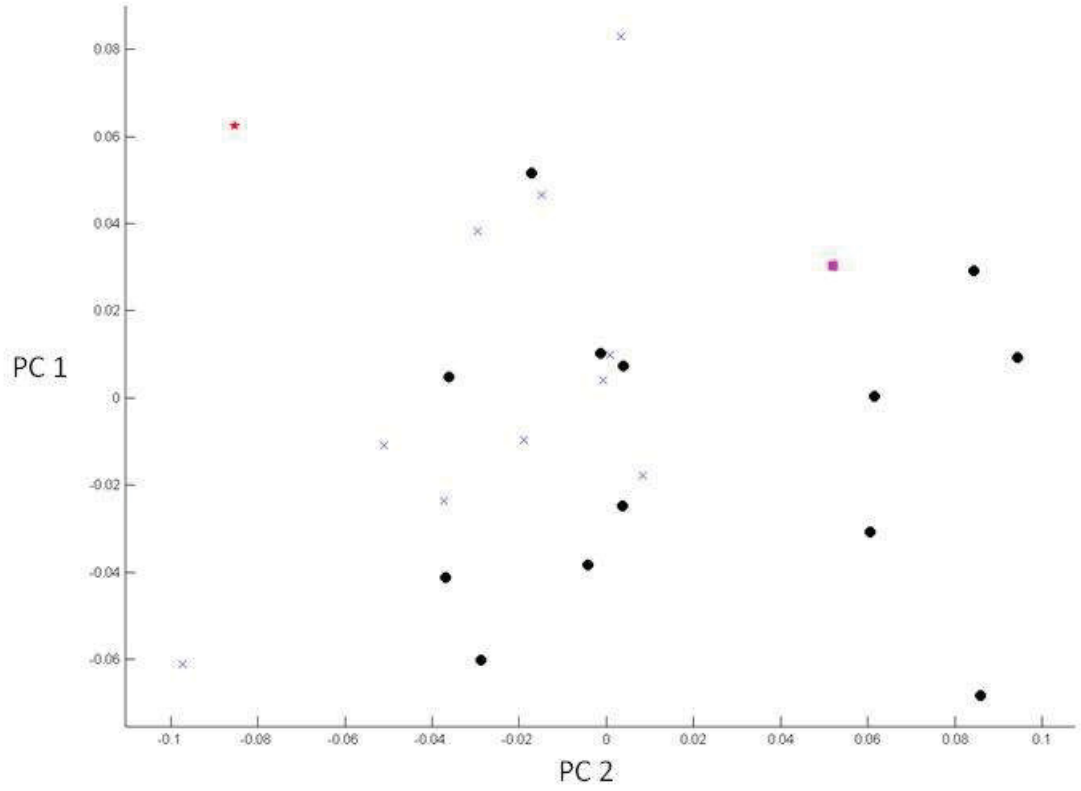


Figure 3-7: Principle component analysis comparing the right and left sides of the head of an 8 or 9 dpf surgery specimento controls. Blue crosses depict the right side of the head controls, black dots represent the left side of the head controls, the red star represents the right side of the surgery specimens head, and the purple square represents the left side of the surgery specimens head. No outliers are present.

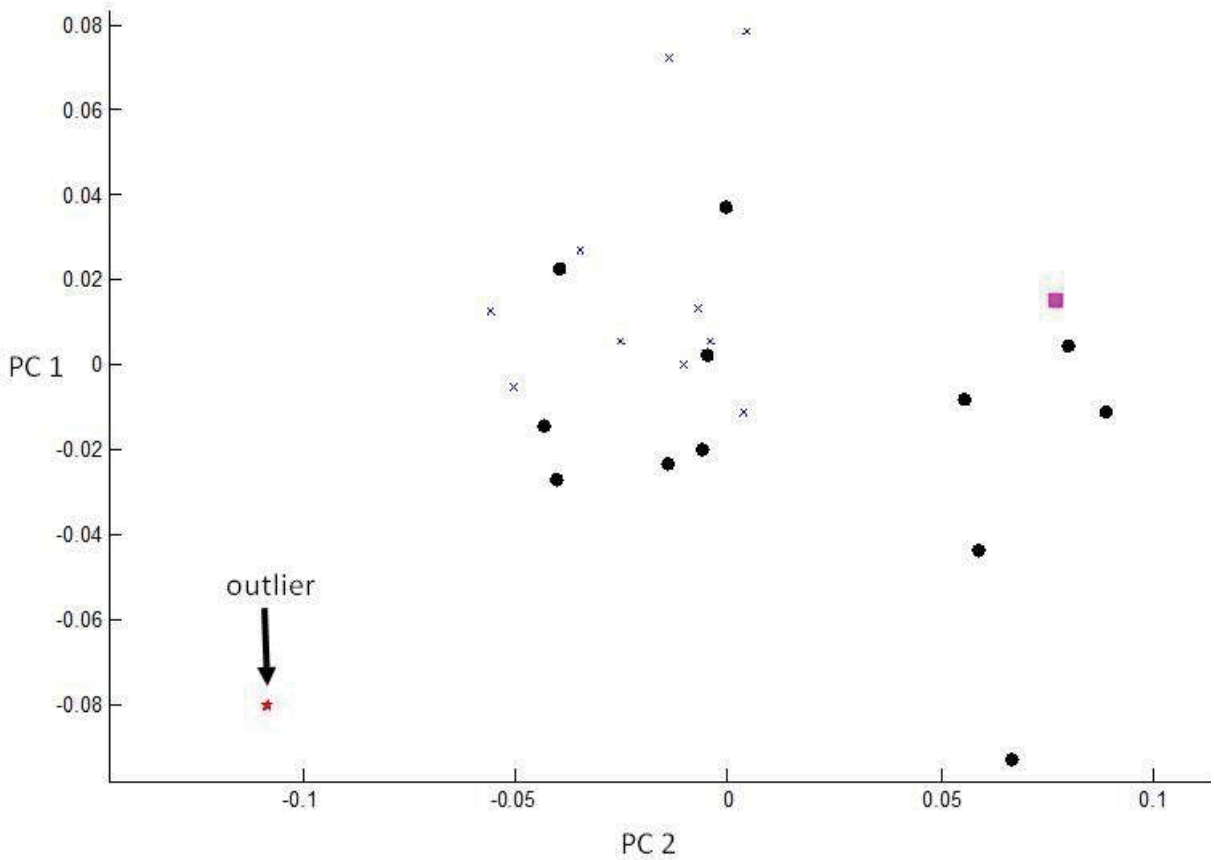


Figure 3-8: Principle component analysis comparing the right and left sides of a 3 dpf surgery specimens head to controls. Blue crosses represent the right side of the controls, black dots represent principle the left side of the controls, the red star represents the right side of the surgery specimens head, and the purple square represents the left side of the laser surgery specimens head. The red star (arrow) is an outlier.

Table 3-1: The effect of laser lens ablation performed at 1 to 11 dpf on the bones surrounding the orbit. Each individual was compared separately to determine if one side of the fish's head was significantly different from the other side of the fish's head using Goodall's F-test, F-test Procrustes and PCA.

	Goodall's F-test p value	Procrustes F-test p value	Outlier
1 dpf			
Fish # 1	p<0.001	p<0.01	yes
3	p<0.001	p<0.01	yes
4	p<0.001	p<0.01	yes
5	p>0.05	p>0.05	yes
6	p<0.001	p<0.01	yes
7	p>0.05	p>0.05	yes
n=6			
2 dpf			
Fish # 1	p<0.001	p<0.01	yes
2	p>0.05	p>0.05	yes
3	p<0.001	p<0.01	yes
4	p<0.001	p<0.01	yes
5	p<0.001	p<0.01	yes
6	p<0.001	p<0.01	yes
n=6			
3 dpf			
Fish # 1	p<0.001	p<0.01	yes
2	p>0.05	p>0.05	yes
3	p>0.05	p>0.05	yes
4	p>0.05	p>0.05	yes
5	p<0.001	p<0.01	yes
6	p<0.001	p<0.01	no

	Goodall's F-test p value	Procrustes F-test p value	Outlier
n=6			
4 dpf			
Fish # 1	p>0.05	p>0.05	yes
2	p>0.05	p>0.05	yes
3	p<0.001	p<0.01	yes
4	p<0.001	p<0.01	yes
5	p<0.001	p<0.01	yes
6	p>0.05	p>0.05	no
7	p<0.001	p<0.01	no
n=7			
5 dpf			
Fish # 1	p>0.05	p>0.05	yes
2	p<0.001	p<0.01	no
3	p>0.05	p>0.05	yes
4	p>0.05	p>0.05	yes
5	p>0.05	p>0.05	yes
6	p<0.001	p<0.01	yes
n=6			
6 dpf			
Fish # 1	p<0.001	p<0.01	yes
2	p>0.05	p>0.05	yes
3	p>0.05	p>0.05	yes
4	p>0.05	p>0.05	yes
5	p>0.05	p>0.05	yes
6	p<0.001	p<0.01	no

	Goodall's F-test p value	Procrustes F-test p value	Outlier
n=6			
7 dpf			
Fish # 1	p<0.001	p<0.01	yes
2	p<0.001	p<0.01	yes
3	p<0.001	p<0.01	yes
4	p<0.001	p<0.01	yes
n=4			
8 or 9 dpf			
Fish # 1	p<0.001	p<0.01	yes
2	p>0.05	p>0.05	no
3	p<0.001	p<0.01	no
4	p<0.001	p<0.01	yes
5	p<0.001	p<0.01	yes
n=5			
10 or 11 dpf			
Fish # 1	p>0.05	p>0.05	yes
2	p<0.001	p<0.01	yes
3	p<0.001	p<0.01	yes
4	p<0.001	p<0.01	no
5	p<0.001	p<0.01	no
6	p<0.001	p<0.01	no
7	p>0.05	p>0.05	yes
8	p<0.001	p<0.01	yes
n=8			

Further morphometrics analysis was conducted on surgery specimens that had an outlier present in PCA analysis and were determined to be significantly different in shape in Twogroup analysis, in order to determine how the right and lefts sides of the head varied in shape. One specimen from an early surgery time point, one specimen from a middle surgery time point, and one specimen from a late surgery time point were randomly selected to visualize the effects of lens damage using warp and vector analysis. Fish number 6 from surgery at 1 dpf was chosen as a representative for an early surgery time point. Based on warp analysis of this specimen (Figure 3-9 A-B) it is clear that there were some considerable differences between the right and left sides of the head. It appeared as though the orbital region is slightly constricted, while anterior to the eye has expanded. The anterior portion of the supraorbital bone is expanded while the posterior portion of the bone appeared to be smaller. The bones posterior to the eye appeared to be smaller in size. Based on the vector analysis it is clear that the bones anterior to the eye had shifted in the posterior direction and into the orbit, while the bones posterior to the eye were relatively unaffected. When partial lens ablation was performed at 6 dpf there was very little influence on the shape of the orbital bones (Figure 3-9 C-D). Warp analysis showed that the orbital region was unaffected by the ablation, minor differences could be observed between the right and left sides of the head anterior of the eye and in the region of the supraorbital bone. Based on vector analysis it appeared that the bones were shifted in the anterior direction, with the bones posterior to the eye being shifted to a greater extent. When partial lens ablation was performed at 10 or 11 dpf warp analysis showed that the orbital region was largely constricted (Figure 3-9 E-F). The bones anterior to the eye appeared to be expanded into the orbit, as well as expansion being present in the suborbital 3 bone. Vector analysis showed that the bones of the ocular region were shifted dorsally, with the greatest changes being present anterior to the eye. Surgery at 1 and 10 or 11 dpf showed the greatest impact of surgery, with 10 or 11 dpf surgery showing the greatest impact. Surgery at 5 dpf resulted in very little changes in the skull.

At each surgery time point for the individuals that had an outlier and a significant change in shape, the outlier sides of the head were grouped into an outlier group, and the opposite sides of the head were grouped into a non-outlier group. For each surgery time

point, the outlier group was then compared to the non-outlier group, using TwoGroup, to determine if there was a significant difference in shape between the outliers and the non-outlier group. In addition the non-outlier group was also compared to a controls group to determine if the non-outliers are not significantly different from the controls. Finally, the control group was compared to the outlier group to ensure that the outliers were significantly different from controls. It would be expected that the outlier group would be significantly different from the non-outlier group, and significantly different from the control group, and finally that the control group would not be significantly different from the non-outlier group, however these relationships were not found at any surgery time points (Table 3-2). When laser lens ablation was performed at 1 dpf the outlier and non-outlier group were not found to be significantly different in shape (Table 3-2). At each surgery time point (2, 4, 7, 8 and 9, and 10 and 11 dpf) Goodall's F-test and F-test Procrustes resulted in p values greater than 0.05 which indicated that there was no significant difference between any outlier and non-outlier groups. Surgery time points at 3, 5, and 6 dpf had an insufficient number of outliers to run statistical analysis. When lens ablation was performed at 2 and 4 dpf there were significant differences in the shape of the skulls between the control group and the outlier group and no significant differences in shape between the control group and the non-outlier group. Assuming that the outlier groups were the sides of the head that received surgery, some mild residual effects of the early lens surgery may be present in the adult skull of these groups, despite rapid lens healing after laser treatment.

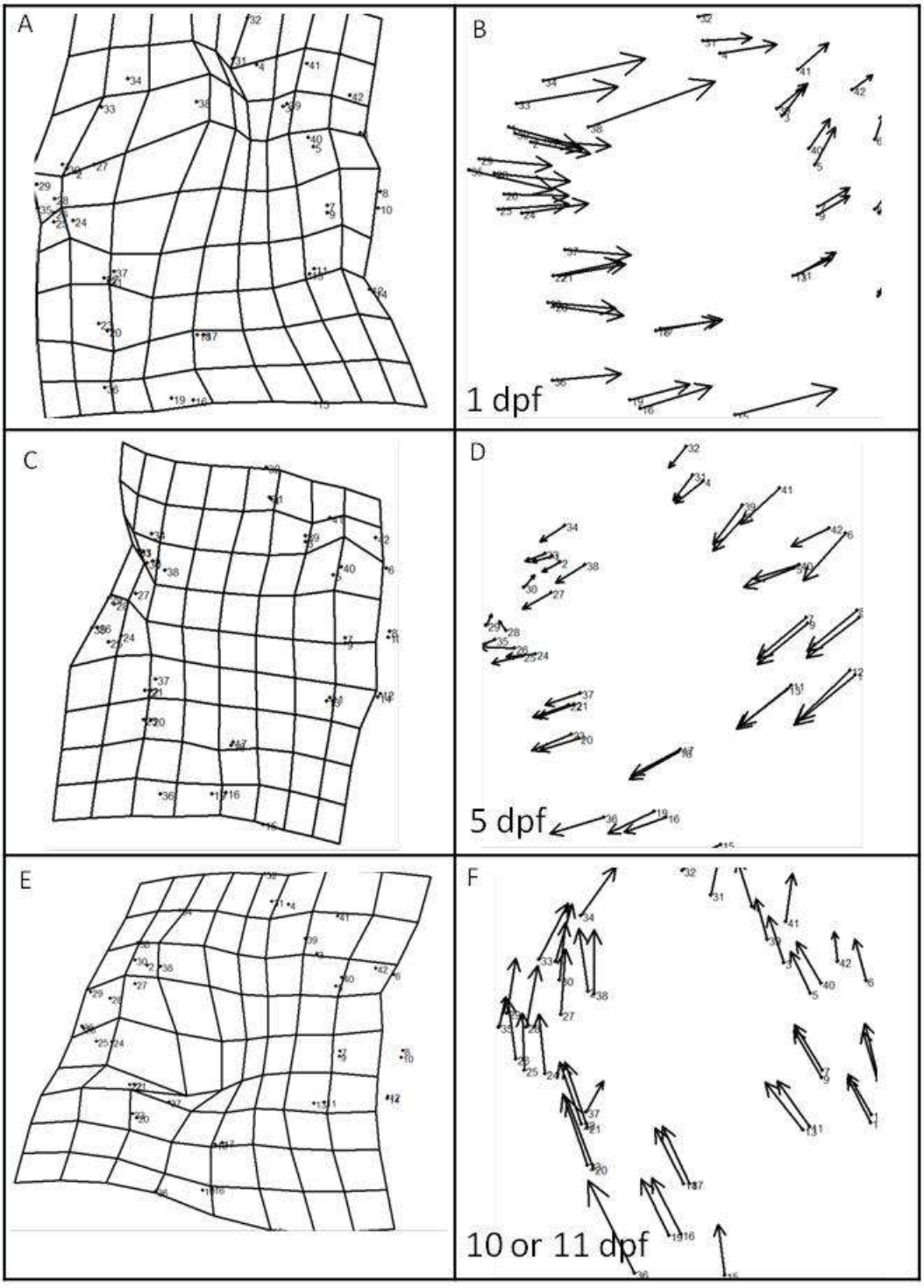


Figure 3-9: Vector and thin plate spline morphometric analysis comparing one side of the head to the other in individuals which received partial lens ablation. (A, C and E) thin plate splines. (B, D and F) vector analyzes. (A-B) spline and vector analysis after surgery at 1 dpf; (C-D) spline and vector analysis after surgery at 5 dpf; (E-F) spline and vector analysis after surgery at 10 or 11 dpf.

Table 3-2: Outlier analysis of the effect of laser lens ablation performed at 1, to 11 dpf on the bones surrounding the orbit. Individuals were grouped based on the day which they received surgery. Outlier sides were then compared to control sides, non-outlier sides and controls and non-outlier sides were statistically analyzed using Goodall's F-test and F-test Procrustes. The text "not sign." means that the data was not significant, and "sign" means that the data was significant.

Surgery Time point	Outlier group vs non-outlier group		Control group vs non-outlier group		Control group vs outlier group		Result
	Goodall's F-test	F-test Procrustes	Goodall's F-test	F-test Procrustes	Goodall's F-test	F-test Procrustes	
1 dpf	F= 0.63 p>0.05	p>0.05	F=2.58 p<0.001	p<0.01	F=3.03 p<0.001	p<0.01	not sign.
Result	not sign.	not sign.	sign.	Sign.	Sign.	Sign.	
2 dpf	F=0.83 p>0.05	p>0.05	F=1.62 p>0.05	p>0.05	F=2.44 p<0.001	p<0.05	not sign.
Result	not sign.	not sign.	not sign.	not sign.	Sign.	Sign.	
3 dpf	Insufficient number of outliers to perform analysis						
4 dpf	F=1.27 p>0.05	p>0.05	F=0.80 p>0.05	p>0.05	F=2.13 p<0.001	p<0.05	not sign.
Result	not sign.	not sign.	not sign.	not sign.	sign.	sign.	
5 dpf	Insufficient number of outliers to perform analysis						
6 dpf	Insufficient number of outliers to perform analysis						
7 dpf	F=0.64 p>0.05	p>.05	F=3.03 p<0.001	p<0.01	F=2.16 p<0.001	p<0.01	not sign.
Result	not sign.	not sign.	sign.	sign.	sign.	sign.	

Surgery Time point		Outlier group vs non-outlier group		Control group vs non-outlier group		Control group vs outlier group		Result
8 & 9 dpf	F=0.94 p>0.05	p>0.05	F=1.65 p<0.001	p>0.05	F=1.10 p>0.05	p>0.05	not sign.	
Result	not sign.	not sign.	sign.	not sign.	not sign.	not sign.		
10 & 11 dpf	F=1.19 p>0.05	p>0.05	F=1.74 p<0.001	p>0.05	F=2.47 p<0.001	p<0.05	not sign.	
Result	not sign.	not sign.	sign.	not sign.	sign.	sign.		

Warp analysis was conducted to compare the shape of the skull on outlier side of the skulls compared to the control side of the skulls for each surgery time point when there was a sufficient sample size. This analysis was conducted in order to examine the shape changes between the outlier group and the control group at each surgery time point in which a sufficient number of samples with significant differences were present. The warp for the 1 dpf surgery time point showed that there was very little difference in the shape of the skulls in the outlier group compared to the control (Figure 3-10). The orbit appeared to be slightly smaller, with a slight expansion in the bones anterior to and dorsal to the eye. When partial lens removal was performed at 2 dpf the skull is largely unaffected (Figure 3-10 B). The orbit region appeared to be slightly smaller than the controls, but otherwise appeared nearly normal, with a slight expansion ventral to the eye. When surgery was performed at 4 dpf there appeared to be a large affect on the shape of the orbital, however surprisingly not a significant one, as determine in two group (Figure 3-10 C). The orbital region was largely reduced in size, while the supraorbital bone was largely expanded. The bones located posterior to the eye SO4-6 also appeared to be expanded largely. When surgery was performed at 7 dpf, only minor changes were present between the control and outlier groups. The eye orbit was slightly smaller, while the bones anterior to the eye appeared to be slightly expanded, as well as a shape change present in the supraorbital bone. When surgery was performed at 8 or 9 dpf the orbit was again smaller in size and the anterior bones have expanded, similar to the changes observed after surgery at 7 dpf, only later surgery resulted in greater changes in the skull (Figure 3-10 D-E). Finally, when partial lens removal was performed at 11 dpf based on warp analysis (Figure 3-10 F) the orbit was reduced in size, while the bones anterior, dorsal and posterior to the eye appeared to have expanded. Early partial lens ablation appeared to have less impact on the shape of the skull bones in the orbital region as the warps representing those surgery time points were largely unwarped. The later surgery time points showed greater warping, and a greater expansion in the bones surrounding the eye, indicating that later partial lens removal has a greater impact on the skull.

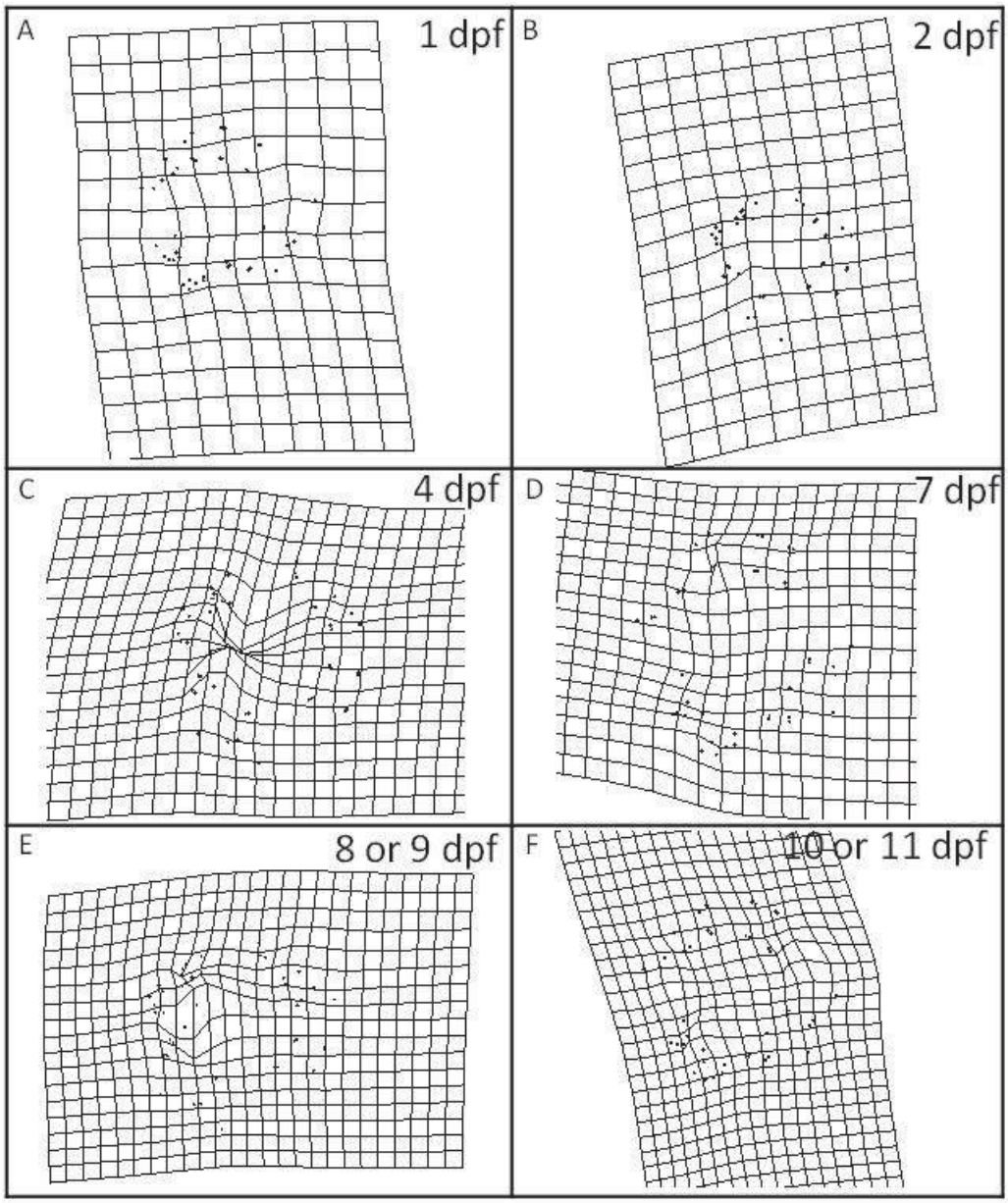


Figure 3-10: Thin plate spline analysis comparing the outlier groups to the control groups after partial lens ablation. (A) Thin plate spline analysis after surgery at 1 dpf; (B) thin plate splin analysis after surgery at 2 dpf; (C) thin plate splin analysis after surgery at 4 dpf; (D) thin plate splin analysis after surgery at 7 dpf; (E) thin plate splin analysis after surgery at 8 or 9 dpf; (F) thin plate splin analysis after surgery at 10 or 11 dpf.

3.3.4 The Effects of Partial Lens Ablation on the Shape of the Juvenile Skull

After partial laser ablation only minor alterations were observed in the adult skull. To determine if changes were present in the juvenile skull, but were then corrected by adulthood, the juvenile skull was examined. Seven specimens were whole mount bone stained. The specimens ranged between 2.3 and 3.2 cm in standard length and received partial lens ablation between 1 and 7 dpf (Figure 3-11). The right and left sides were compared. No differences in the shape of the bones in the orbital regions of one side of the head compared to the other were observed in any of the seven specimens, including the supraorbital bone and suborbital bones 4 and 5, which were demonstrated to be variable in shape in Chapter 2. Thus partial lens ablation does not influence the shape of the bones in the orbital region in the adult or juvenile skulls of the Mexican tetra. Morphometric analysis was not performed on this data due to insufficient sample numbers.

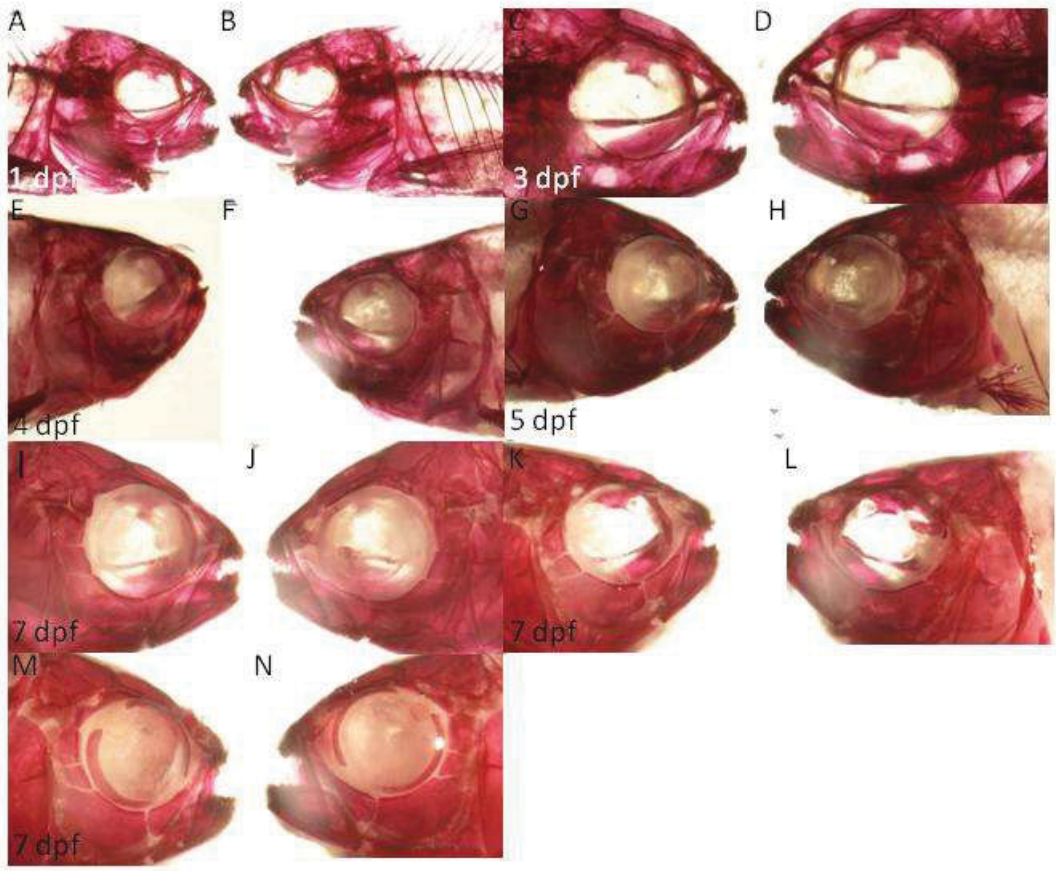


Figure 3-11: Whole mount bone stained, young adult laser specimens. (A-B) right and left sides of the head after surgery at 1 dpf, specimen is 3 cm SL; (C-D) right and left sides of the head after surgery at 3 dpf, specimen is 2.3 cm SL; (E-F) right and left sides of the head after surgery at 4 dpf, specimen is 2.6 cm SL; (G-H) right and left sides of the head after surgery at 5 dpf, specimen is 2.5 cm SL; (I-J) right and left sides of the head after surgery at 7 dpf, specimen is 2.3 cm SL; (K-L) right and left sides of the head after surgery at 7 dpf, specimen is 2.9 cm SL; (M-N) right and left sides of the head after surgery at 7 dpf, specimen is 3.1 cm SL.

Part B

3.4 Materials and Methods

3.4.1 Full Manual Lens Removal

Lens removal was conducted unilaterally in surface fish between 1 and 3 dpf with tungsten needles, as described in Chapter 2, section 2.2.2. After lens removal, specimens collected between one hour and one month after surgery. The specimens were then anesthetised in 0.1% Ms222 (Ethyl-3-aminobenzoate methanesulfonic acid salt, Sigma E10521). Specimens were fixed in 4% paraformaldehyde (Sigma P6148) in Phosphate Buffered Saline (PBS), and then stored in PBS (Appendix 3) at 4°C. The non-surgically manipulated eye serves as a control.

3.4.2 Histological Analysis of Lens Regeneration

Specimens were dehydrated from PBS to 100% EtOH, and then cleared overnight in CitriSolve (Fisher 22143975). The next day, specimens were transferred to low melting point paraffin wax (Fisher 23021401) for two hours, then transferred to fresh wax overnight. The following day embedding trays were used to embed the samples in wax and allowed to harden. Embedded samples were stored at -20°C until sectioning. Sectioning was performed on a microtome. Sections were cut between 8 and 10 μm in thickness. Sections were placed on aptes (Sigma A3648) coated slides (Appendix 3). Sections were incubated at 38°C overnight, and then stored at room temperature until staining. Slides were then either stained with toluidine blue staining protocol or for βcrystallin using immunohistochemistry. Touluidine blue was used as a general stain which allows for visualization of eye structures. *B* crystallin was used as a lens specific marker, the expression of β crystallins has been confirmed in the lens (of the Mexican tetra (Stricker and Jeffery, 2009).

3.4.3 β-crystallin Immunohistochemistry on Sections

Slides were dewaxed in CitriSolve for 30 mins, and then hydrated through an ascending series of ethanol to water. The slides were then rinsed in PBS, PBST, and a

final rinse in PBS for 15 mins each. The slides were then blocked in 10% goat serum (Sigma G9023) in PBS at room temperature for one hour. Following slides were incubated in primary mouse antibody β crystallin (Santa Cruz 48335) at a concentration of 1:150 in 10% goat serum in PBS overnight at 4°C. Following the slides were rinsed in PBS, PBST, and PBS for 15 minutes each at room temperature. Slides were then incubated in a secondary antibody of Alexaflour 488 goat anti-mouse (Invitrogen A11029) at a concentration of 1:100 in 10% goat serum and PBS for two hours at room temperature. Following the slides were rinsed in PBS, PBST, then PBS for 5 minutes each. To block auto-fluorescence the slides were then incubated 0.3% Sudan Black (Sigma 199664) in 70% EtOH for 20 minutes, then quickly rinsed in PBS five times. Cover slips were then mounted on the slides using aqueous gel mounting medium (Sigma G9018). No primary and no secondary controls were performed to ensure that the antibody was specifically binding to the lens.

3.4.4 Toluidine Blue Stain

Slides were dewaxed in CitriSolve for 5 minutes twice. Following, the slides were then hydrated from 100% EtOH to water through a descending series. Samples were then incubated in toluidine stain solution (Appendix 3) for 3 minutes. The slides were then rinsed for 1 minute three times in distilled water. The slides were then quickly dehydrated through an ascending series to 100% EtOH, following the slides were dewaxed in CitriSolve for two times for 3 minutes. Finally, the slides were then cover slipped with DPX mountant (Fluka 44581).

3.4.5 Eye and Lens Diameter Measurements

The maximum diameter was measured for each surgery and control eye. A series of sections were observed to identify the section through the maximum diameter of each lens. When the sections through the full diameter of the lenses were identified an image was captured and the diameters of the lenses were measured using the software program NIH Elements. The average and standard deviation was calculated.

The maxiumum diameters of the right and left eyes of adult surgery specimens were measured as described in Chapter 3, section 3.2.2. In addition the eye size of control specimens, of the same age, which had not received lens surgery were also measured.

3.4.6 Morphometric Analysis After Lens Regeneration

Adult specimens were whole mount bone stained (described previously), following lateral view images were captured using a Nikon SMZ1000 microscope and NIS Elements software package of each side of the head. Forty-two two-dimensional (x, y) landmarks were applied to the lateral view images of each fish (Figure 2-2, Appendix 1, Table 1). After manual lens removal and subsequent lens regeneration the surgery eye cannot be discerned from the control eye in adulthood (Part B regarding regeneration). Morphometric analysis was used to determine if the bones on one side of the skull were different in shape than the bones on the other side. Morphometric analysis was then completed as described in section 3.2.3.

3.5 Results

3.5.1 Regeneration After Lens Ablation

After manual lens ablation in the surface Mexican tetra, eye regression occurs in approximately 50% of juveniles, regardless of the age of the embryo when surgery is performed (n=51) (Chapter 2). This chapter investigates the other 50% of individuals that have two normal appearing eyes in adulthood.

Surgery specimens were fixed between 1 hour and 1 month after lens removal to determine the timeline and evidence for lens regeneration. A series of toluidine stained and β-crystallin stained consecutive sections were examined to ensure that one hour after lens removal, no lens or lens cells were present in the surgery eye (Figure 3-12, 3-13) (n=4). In the four stained samples no lens cells were present in the surgery eye. By observing toluidine stained sections and β-crystallin stained sections of specimens fixed 4 days after lens removal it was again determined that no lens cells were present in any of the surgery eyes (Figure 3-12) (n=4). Despite lens cells not being present, a small gap

was present in some samples near the cornea, suggesting that the process of lens regeneration may have started (Figure 3-12).

Crystallin stained sections of specimens fixed 5 or 6 days after lens removal show a crystallin positive stained lens in both the surgery and control eyes. The lens in the surgery eye appeared to be smaller in diameter than the lens present in the control eye. To analyze this finding, sections were studied to determine the maximum diameters of the control and regenerating lenses. The diameters of both of the lenses were then measured. The diameter of the small regenerating lens was compared to the diameter of the control lens. The diameter of the regenerating lens was only 58% of that of the control (n=4 individuals) (Figure 3-12, 3-13). By seven days after lens removal, approximately 50% of the specimens had two crystallin positive, normal appearing lenses, with one lens being marginally smaller in diameter. Thus lens regeneration was largely complete by 7 days after lens removal. The lens diameters of specimens fixed 7 days after lens removal were measured. In those specimens, the diameter of regenerating lens was 92% of that of the control lens (n=2) and appeared to be normal in cellular content, with a single epithelial layer covering crystallin fibre cells (Figure 6-13). After 7 days post surgery the surgery and control lens can no longer be discerned indicating that lens regeneration is complete. Lens regeneration appeared to begin at 4 days after surgery and is complete by 8 days after lens removal.

Based on histological analysis, the regenerating lens did not appear to be in close contact with the cornea. The regenerating lens did appear to be in close contact with the iris, as would be required if the process was one of Wolffian lens regeneration. However, further investigation is required to determine from which cell layer the lens is regenerated. Transmission electron microscopy would be highly useful in this analysis to confirm the source of regenerative material. Alternatively, corneal and/or iris markers could be used to identify the source of the regenerating lens.

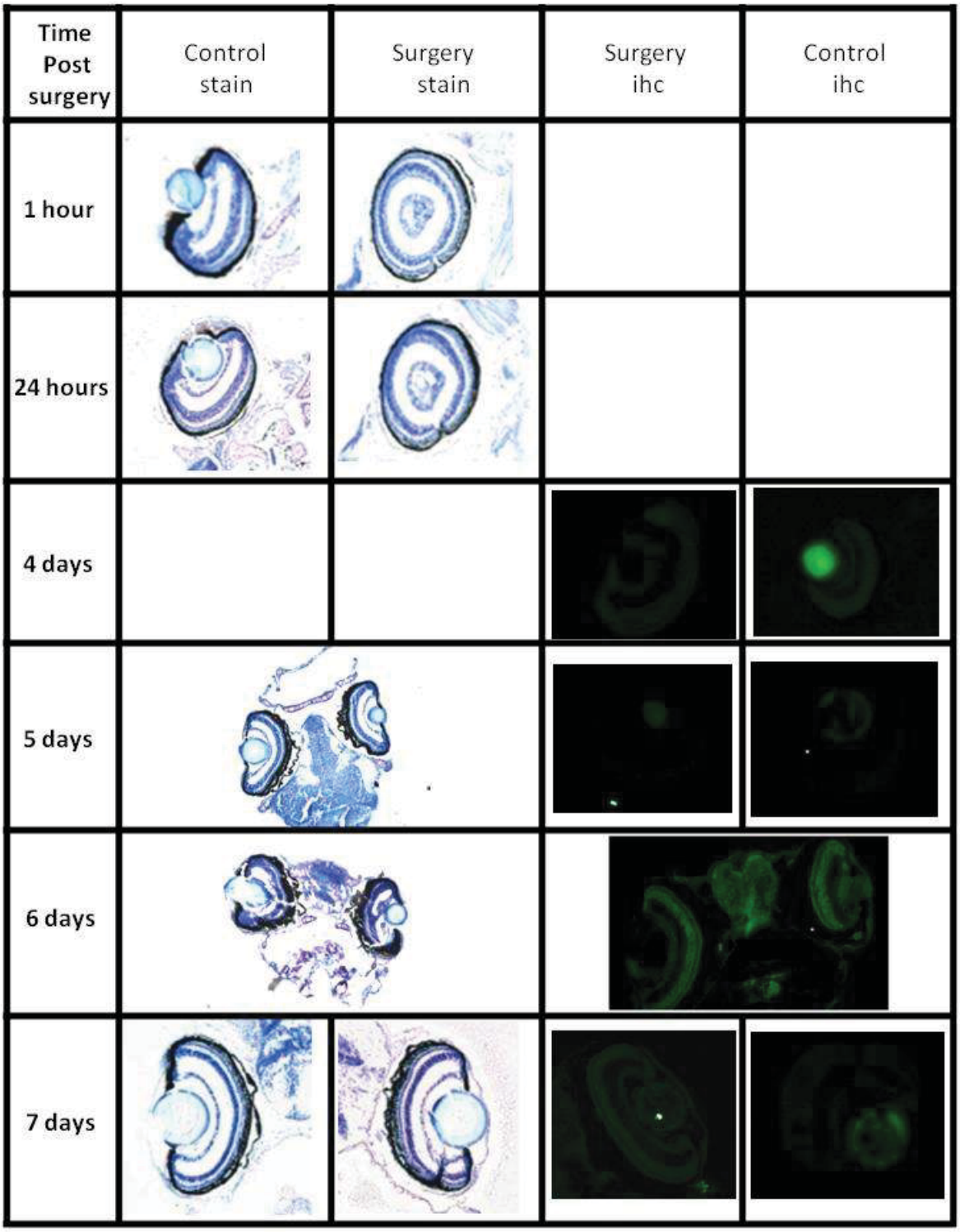


Figure 3-12: Histological and immunohistochemical analysis of lens regeneration in the Mexican tetra. Each row represents the analysis conducted over the six different time points examined after surgery. No lens was present on the surgery side at 1 hour, 24 hours, or 4 days after surgery. A small regenerating lens was visible at 5 and 6 days after surgery. Seven days after surgery the specimens have two normal appearing lenses.

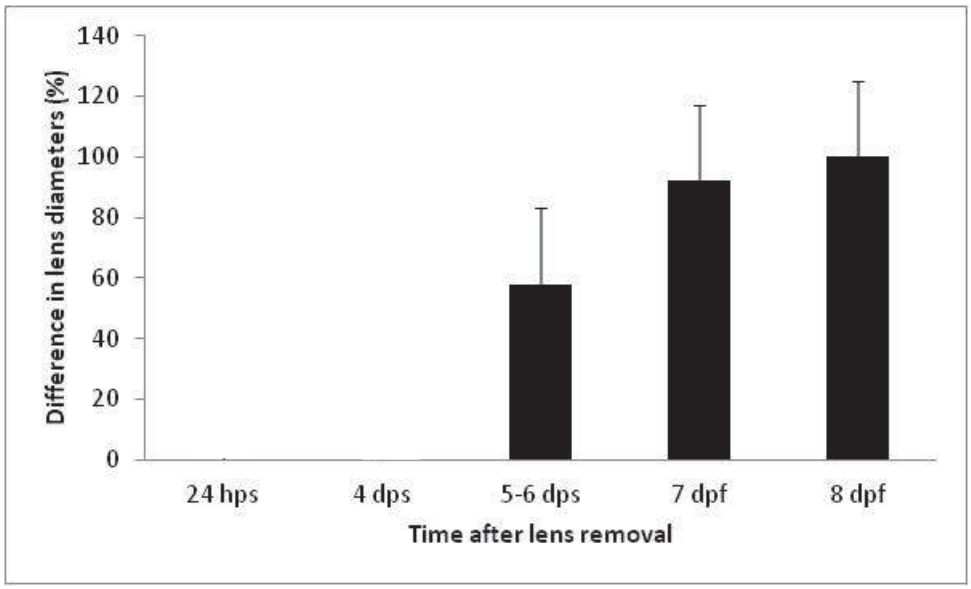


Figure 3-13: Percentage difference in the diameter of the control versus the surgery lens at various time points after surgery. Five to six days after lens removal the regenerating lens had a diameter that was 58% of the size of the control lens. Seven days after surgery, the diameter of the surgery lens was 92% of that of the control. Standard deviation is included.

3.5.2 Skeletal Analysis After Lens Regeneration

As described in Chapter 2, after lens removal which resulted in eye regression, dramatic changes were observed in the shape of the orbital bones surrounding the surgery eye, as well as minor residual effects on the opposite side of the head (Chapter 2). Fifty percent of the individuals that received lens surgery underwent lens regeneration. The orbital bones of specimens which exhibited lens regeneration were examined to determine if the absence of the lens between 1 dpf (day of lens removal) and 7 dpf (when the regenerating lens is nearly complete), impacts the shape of the bones in the orbital region in adult fish. Whole mount bone stains were examined in order to analyse the effects on the skull. Two out of ten specimens had suborbital bones four and five fused on one side of the head, similar to the fusions present in the corneal tear control fish (Chapter 2). The samples with fusions were excluded from morphometrics analyses (Table 3-3).

Gross morphological analysis was conducted to compare the right and left sides of the skulls of surgery specimens which did not undergo lens regression. The suborbital bones on opposite sides of the head appear to be symmetrical, with no obvious differences in shape (Figure 3-14). In two of eight specimens there was a slight expansion of the supraorbital bone on one side of the head. Overall, the skulls appeared to be unaltered by early lens removal and subsequent lens regeneration.

Morphometric analysis was performed on the remaining eight specimens (Table 3-3). Due to lens regeneration, the surgery eye could not be discerned from the control eye in adulthood, as both eyes appear to be normal eyes. As such the diameters of the left and right eyes were measured to determine if the surgery eye was smaller than the control eye as a method to discern the surgery eye from the control eye in adulthood (Appendix 2, Table 11). The difference in eye diameters were then compared to the differences in eye diameter from right to left sides in control specimens using an independent t-test to determine if there were any significant differences in the surgery specimens outside of natural left right asymmetry. The independent t-test results indicate that there is no significant difference between the diameters of the left and right eye of surgery specimens and the right and left eyes of control specimens ($p>0.05$, $n=5$), indicating that

the surgery eye was not different in size from the control eye. Thus eye diameter could not be used to determine which eye received surgery.

Morphometric analysis was then performed as described in Chapter 3, section 3.2.3. Briefly, in order to statistically determine if one side of the head was different from the other morphometrics analysis was conducted in two manners. First by using Principle Component Analysis to compare the two sides of the head to a group of controls to determine if either side is an outlier from the controls, and thus different in shape. Secondly, I used TwoGroup to determine if there is a statistical difference between one side of the head and the other.

Firstly PCA was performed, a PCA trial comparing the left to the right sides of each of the eight surgery specimens' heads, with controls which had not received surgery (Figure 3-15). Each specimen was colour coded individually in order to determine if one side of an individual's head was an outlier from the other side of the head, and the controls. An outlier was determined to be any specimen which did not cluster with the controls. Only three of the eight specimens were determined to have one side of the head outlying from the other side of the head and the controls (Table 3-3).

PCA depicts fine differences in shape. To determine if these fine differences were statistically significant TwoGroup was used. TwoGroup analysis was conducted on all eight samples, to determine if any significant differences in shape were present. Goodall's F-test and Procrustes F-test were then performed to test significance. Only two individuals were determined to have one side of the head significantly different from the other, neither of which were determined to have an outlier in PCA analysis, thus there are little to no detectable significant changes in the shape of the skull on the surgery side of the head compared to the control (Table 3-3). There are an insufficient number of samples with detectable outliers or significant differences to compare an outlier group to a non-outlier group statistically, indicating that lens removal, followed by rapid lens regeneration does not impact the shape of the bones of the adult skulls.

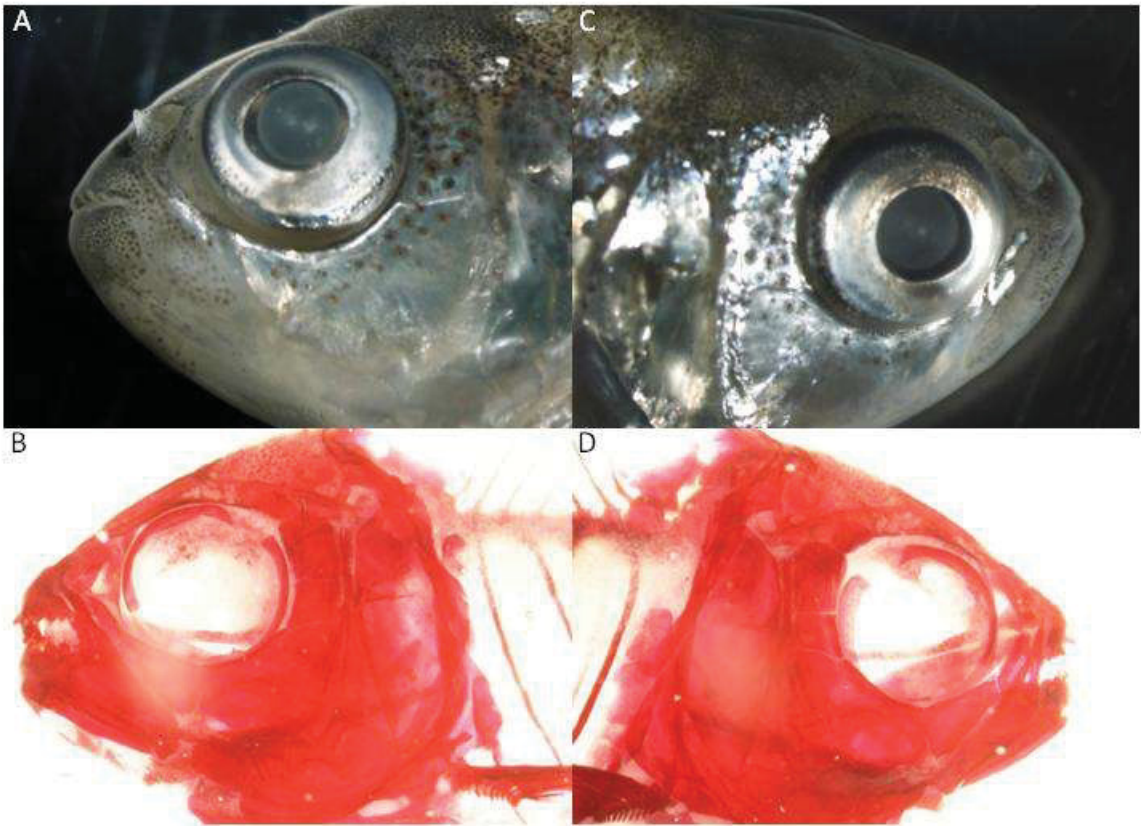


Figure 3-14: Unstained and Alizarin red bone stained adult specimens after lens regeneration. (A-B) left side of the head after surgery at 1 dpf; (C-D) right side of the head after surgery at 1dpf.

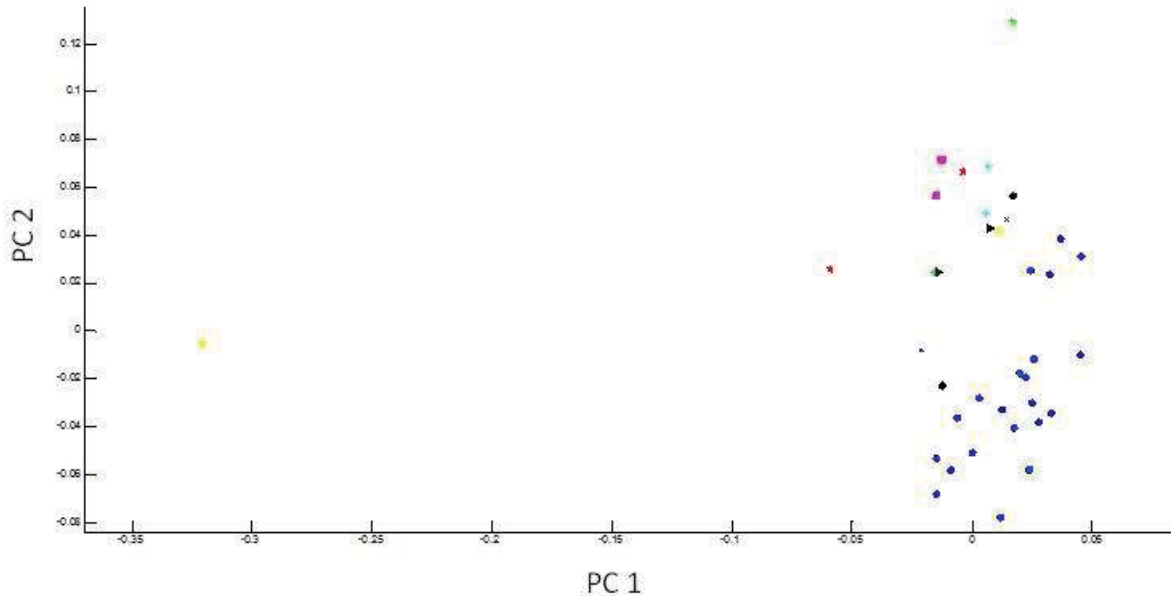


Figure 3-15: Principle component analysis comparing the right and left sides of the skull of control fish and fish which have regenerated a lens. Blue circles depict the right and left sides of control (non-surgery) fish. All other pairs of symbols represent the right and left sides of individual surgery specimens after lens regeneration. The yellow triangle pair represents a specimen with an outlier, one of the triangles of the pair does not cluster with any other points.

Table 3-3: The effect of manual lens ablation performed at 1, 2, or 3 dpf on the bones surrounding the orbit. The right and left sides of each individual's head were compared using twogroup, to determine if one side of the head is significantly different from the other using Goodall's F-test, F-test Procrustes. Principle component analysis was performed to determine if one side of the head is an outlier from the controls.

Adults with regeneration	Orbital bone fusions	Goodall's F-test p value	F-test Procrustes p value	PCA Outliers present
1 dpf				
1	fused			
2		$p<0.001$	$p<0.01$	
3		$p>0.05$	$p>0.05$	
4		$p>0.05$	$p>0.05$	outlier
5		$p>0.05$	$p<0.01$	
6		$p<0.001$	$p<0.01$	
7	fused			
2 dpf				
1		$p>0.05$	$p>0.05$	outlier
3 dpf				
1		$p>0.05$	$p>0.05$	outlier
2		$p>0.05$	$p>0.05$	

Although four individuals were identified in Twogroup as having significant differences in shape of their orbital bones when comparing the right and left sides of their heads, these individuals did not have an outlier in PCA analysis. As the PCA analysis indicated that both sides of the four individuals' heads were not different from the group it is likely that the differences observed in Twogroup are natural left-right asymmetries in the individuals which were amplified by replicating the same individual multiply times. If the differences in the two sides of the head were significant we would expect to see a significant result from the Twogroup analysis, as well as one side of the head outlying from the controls and the other side of the head clustered with the controls in the PCA analysis. Alternatively, there may be very mild shape changes on one side of the head as a result of lens regeneration in these individuals. As these changes are so mild, PCA analysis did not identify them.

Dramatic changes were observed in the shape of the orbital bones surrounding the eye after lens removal and subsequent eye regression. However, lens removal followed by rapid eye regeneration has little to no impact on the adult skeleton, despite one eye being lens-less for approximately 3 to 4 days in early development.

3.6 Discussion (Part A and Part B)

The objective of Part A of this chapter was to extend the time period in which the lens was damaged to determine how later lens damage affects the skull. After laser surgery, the lens likely healed and the eye did not regress, in addition there were little to no effects on the shape of the skull bones. However, after manual lens removal and subsequent eye regression there were dramatic effects on the skull (Chapter 2). These findings indicate that eye regression has a dramatic impact on the skull, but that lens damage and healing does not.

Manual lens removal could only be performed up to 4 dpf.In order to extend the timing of lens surgeries, laser ablation was used to damage the lens. This chapter aims to understand how damaging the lens in early development can affect the development of the adult skeleton. The objective of Part B of this Chapter is to investigate the Mexican tetra's capacity for lens regeneration and to determine how the absence of the lens for a

short amount of time, prior to lens regeneration can influence the development of the Mexican tetra skull.

3.6.1 No Eye Regression After Partial Lens Ablation

After partial lens ablation between 1 and 11 dpf, the surgery eye size is not reduced and the eye does not sink into the head later in development. This is unlike the results of early manual lens removal, in which the surgery eye was reduced in size, sunk into the head and was covered in skin (Chapter 2). The small differences found in the diameters of the laser surgery eyes may be due to slightly slowed eye growth after lens surgery, or natural variation, which may be present in eye size during early development. I examined natural variation present in the diameters of the left and right eyes of adults and determined that it was minimal, however, I did not investigate this relationship in juveniles. In manual lens removal, the entire lens was removed, and in contrast, laser ablation did not remove all of the lens cells in the surgery eye. Based on histological analysis laser ablation damaged the fibre cells in the center of the lens, but appears to have left the lens epithelial cells largely intact on the surface of the lens.

As stated in the introduction of this chapter lens regeneration occurs through the transdifferentiation of other cell types into lentoid cells and only occurs in a small select number of species (Henry, 2003). Lens regeneration or healing without transdifferentiation has been documented in mice, rabbits and cats (Call et al., 2005). In these animals the lens heals from remaining lens epithelial cells attached to the remaining lens capsule. In adult mice, after lens damage protrusion of damaged lens fibres occurs. Following, the epithelial cell layer begins to undergo cell proliferation by 18 hours after lens damage, beginning to heal the wound, entire lens healing is slow in mice requiring up to 4 months (Uga, 1981). In young mice, when lens fiber cells were damaged through transcorneal needle penetration all fiber cells were healed by 8 days after the surgery (Nelson and Rafferty, 1976). Studies in mice indicate that the epithelial cells undergo proliferation to heal the lens. As was stated previously, after partial lens ablation epithelial cells were still present in the laser surgery lens, as such it is likely that the remaining epithelial cells may be able to heal the lens through proliferation of new fibre cells, through a similar mechanism to that is seen in mice (Nelson and Rafferty, 1976).

Histological analysis conducted on the lens during the presumed healing process may be helpful to confirm that the lens does in fact heal as is described in the literature, in other vertebrates.

Due to the lack of eye regression after laser surgery, the surgery eye could not be discerned from the control eye. As such the shape of the skull was analyzed to determine if alterations from the normal skull morphology are present on one side of the head.

3.6.2 Lens Damage Mildly Affects the Adult Skull

After laser lens surgery the juvenile skull was examined for variations in the shape of the bones of the ocular region as a result of lens damage. There were no alterations in shape of the ocular region evident from gross morphological analysis of the juvenile skull. The adult skeleton was extensively analyzed for changes as a result of early laser lens ablation, minor changes were observed. Small expansions were observed in the suborbital 4, 5 and the supraorbital bone on one side of the skull in some specimens. Morphometric analysis indicated that after early partial lens ablation the shape of the bones in the ocular region on one side of the skull were statistically different in shape to the bones on the other side of the head based on an individual comparison.

In attempt to determine which side of the head was the surgery eye, the shape of the orbital bones on both sides of the head were compared to controls to determine if there was an outlier side of the head. When outliers were grouped no significant differences were found in shape, indicating that partial lens ablation results in small significant changes in the shape of the skull bones on an individual bases, however when the individuals with changes are grouped the small changes in shape are not large enough to be significant overall.

Partial lens ablation resulted in a lens which was damaged in early life, then healed later in development. As mentioned in the introduction of this chapter other studies have been conducted to examine how changing an aspect of a young fishes life, then removing it later in development, as is the case with the partial lens damage in this study, can influence the adult skeleton. In the study reviewed earlier conducted by Meyer et al., (1987), they demonstrated that feeding young fish different diets affected the shape of the juvenile skull, if the diets were then returned to “normal” the changes observed in

the juvenile skull were corrected by adulthood. This study demonstrates that alterations in early life can affect the juvenile skeleton, but the alterations may be corrected by adulthood if the influence, in this case diet has been removed. Other studies demonstrate that other alterations present during early development life only can have lasting effects on the shape of the skull, resulting in alterations in the shape of the adult bones. For example limiting vitamin A during early life affects the shape of the skull bones in adulthood (Mazurais et al., 2009).

Some influences on the juvenile skull have a lasting impact on the adult skull, while others do not. It appears that early partial lens ablation is in the middle of these options, with minor affects on the skull being present in adulthood. This may be due to the timing of when the bones of the orbital region ossify in relation to lens damage and healing.

The lens damage likely heals prior to orbital bone ossification. In juvenile mice in which the lens was damaged, a fully healed lens was present by 8 days after surgery (Nelson and Rafferty, 1976). In an adult newt complete lens regeneration occurs in 25 days (Henry, 2003). Due to the mild damage to the fibre cells after partial lens ablation performed in the Mexican tetra and the short timing of lens regeneration and healing in other animals and the lack if difference between the diameter of surgery and control eyes it is likely that the lens damage heals rapidly after surgery. The orbital bones of interest ossify in the Mexican tetra at 22 mm SL at approximately 3 to 4 months of age, thus the orbital bones ossify long after the lens damaged from the partial lens ablation has fully healed. As the lens damage has likely healed prior to orbital bone ossification it is less likely thatthe shape of the bones would be affected. Although it appears that partial lens ablation may not largely impact the ossification of the orbital bones, it may impact the cranial neural crest cell migration into the bones, resulting in the minor changes which were observed. Examining the sizes of the skeletogenic condensations which give rise to these alterated bones may be useful in further understanding the effects of lens damage on the shape of the orbital bones. For example, larger condensations would suggest an increase in CNC migration or proliferation in the condensation. Condensation size can be analyzed using peanut agglutinin lectin (atleast in amniotes).

As was stated in Chapter 2, in zebrafish the orbital bones are neural crest derived (Kague et al., 2012), this feature is thought to be conserved in the Mexican tetra. Cranial neural crest cells begin to migrate at 14 hpf (Kimmel et al., 1995) in zebrafish. CNC migration is presumed to occur around the same timing in the Mexican tetra. Partial lens ablation was performed between 1 and 11 dpf, thus the lens damage would be present during CNC migration. Due to the highly plastic nature of the neural crest it is likely that the migration of the cell population would be influenced by neighbouring structures. As was discussed in Chapter 2, section 2.6.2 the presence of the eye has been shown to have an important function in the proper migration of cranial neural crest derived (CNC) structures surrounding the eye (Langenberg et al., 2008; Kish et al., 2011). Thus the small alterations in the shape of the orbital bones of some of the surgery specimens could be due to the damaged lens's impact on CNC migration. Early lens damage may influence how many CNC migrate into their future condensations, or may influence proliferation of CNC in the condendation, thus affecting the shape of the orbital bones. Due to the small amount of damage which partial lens ablation exerted on the lens, the impact on the migration of the CNC is likely also mild, resulting in the minor changes, which were observed in the adult skulls. Although some evidence is available describing how the eye can influence the migration of CNC (Langenberg et al., 2008; Kish et al., 2011) these studies do not investigate which structure of the eye influences the migration. Further investigation is required to better understand the role the lens plays in the eye's influence on CNC migration. Based on this study it appears that the lens may play an important role in CNC migration, as mild lens damage impacted the shape of the same bones in the adult skull.

In Chapter 2, I hypothesized that orbital bone outgrowth after full manual lens removal maybe influenced by changes in the mechanical pressures within the skull as a result of the eye no longer being present. In specimens that received partial lens ablation, eye size was not significantly affected, indicating that the mechanical pressures within the skull were also unaltered. These findings indicate that the small changes observed in the skull after surgery were likely a result of changes in CNC migration and/or condensation size, rather than more global changes due toaltered mechanical forces.

3.6.3 Late Partial Lens Ablation has a Greater Impact on the Skull

Based on the thin plate spline analysis conducted comparing the outlier groups to the control groups at numerous time points (Figure 3-9) partial lens ablation appears to have more influence on the shape of the bones of the ocular region the later that surgery was performed. When partial lens ablation was performed at 1 and 2 dpf the thin plate splins showed very little warping, while surgery at 4, 7, 8 or 9 and 10 or 11 dpf showed a greater amount of warping in the grids, indicating a greater impact on the skull. Surgery performed later may result in a damaged lens being present and thus less able to exert its influence on the surrounding structures at a critical time in the development of the skull. When lens damage is performed at 1 dpf the lens is still undergoing final development and differentiation, while lens damage performed at 11 dpf damages a fully developed and differentiated lens (Glass and Dahm, 2004). Lens damage performed on a fully developed lens likely requires longer to heal, thus, extended healing time may differentially affect the developing skull. Additionally, condensations, which give rise to the bones of the orbital region may be developing during this time period. Despite the late ossification of the orbital bones of the Mexican tetra (Yamamoto et al., 2003) the cell populations which will give rise to the future bones are present in the site of ossification long before the first ossification events, making them available for influence from the damaged lens. After early lens damage, the lens may have had time to fully heal, prior to later developmental events which may require the lens, resulting in very little affect on the skull. As only mild affects were observed in the skull it is likely that the remaining undamaged lens cells present after surgery are able to largely compensate for the damaged portions of the lens, mostly maintaining the normal shape of the eye and the bones of the orbital region.

3.6.4 The Mexican tetra has the Capacity for Rapid Lens Regeneration

A preliminary study investigating the capacity for lens regeneration in the Mexican tetra was conducted in order to explain why 50% of the individuals with a lens removed manually (Chapter 2) has two normal appearing eyes in adulthood. Lens removal was conducted in the Mexican tetra at 1 dpf. After removal, lens regeneration was studied using histological and immunohistological analysis. β crystallin was used as

a lens specific marker to visualize the regenerating lens, and to ensure that the structure present within the eye was a lens. Crystallins have been demonstrated to be exclusively expressed in differentiated fibre cells of the lens, as such they make excellent markers for identifying lens cells (Tsonis et al., 2004). In addition, the expression of β crystallins has been confirmed in the lens of the Mexican tetra (Stricker and Jeffery, 2009). In addition, control experiments were conducted lacking primary antibody and others conducted using no secondary antibody to ensure that specific binding was occurring in the lens. Based on the cellular structure observed in the histological analysis, and the visible binding of β crystallin the new structure is a regenerating lens.

To confirm that the lens did in fact regenerate and was not a result of lens healing, specimens were examined 1 hour and 24 hours after lens removal to ensure that all lens material had been fully removed. In my study specimens were observed with histological and immunohistochemical analysis to ensure that all cells and lens capsule had been removed. In the specimens which were examined the capsule and lens cells had been fully removed and could no longer be visualized. In many studies in which the capacity of lens regeneration was proposed (e.g. in mice), later investigations determined that lens capsules had not been fully and cleanly removed in the initial studies, as such the species were later relabelled as species capable of lens healing, not lens regeneration (Henry, 2003). Further analysis in the Mexican tetra using a specific lens capsule and lens epithelial marker would strengthen the finding of regeneration over lens healing in this species.

Approximately 50% of the Mexican tetra which had their lenses removed underwent lens regeneration. Based on personal correspondence with Dr. Tsonis (University of Dayton, USA), a world expert on lens regeneration, largely unreported in the literature is the fact that species which have the capacity to regenerate their lenses only undergo lens regeneration in approximately 50 to 75% of individuals from which a lens has been removed. Lens regeneration is typically inhibited in an animal which has the capacity to regenerate the lens if other eye structures are damaged during the lens removal, specifically the retina (Henry, 2003).

Lens regeneration in the Mexican tetra was complete by 7 to 8 days after lens removal. Compared to some species, for example the loach regeneration occurs much

more rapidly in the Mexican tetra. In the adult newt lens regeneration occurs from the dorsal iris and takes 25 days to complete from the removal of the lens (Tsonis et al., 2004). Lens regeneration in the teleost, *Misgurnus anguillicaudatus*, also occurs through Wolffian regeneration and also takes 25 days to complete after the lens has been removed (Sato, 1961). Lens regeneration occurs in larval, *Xenopus laevis*. In this species lens regeneration occurs via transdifferentiation of cornea cells, corneal-lens regeneration and occurs over 8 days after the lens is removed (Freeman, 1963). Lens regeneration occurs in the juvenile frog, *Xenopus tropicalis* takes a greater amount of time, occurring over approximately 25 days (Henry, 2003). Literature states that the adult axolotl does not have the capacity for lens regeneration (Tsonis et al., 2004), however, in personal correspondence with Dr. Tsonis he indicated that the juvenile axolotl does have the capacity for lens regeneration, which occurs rapidly as observed in the Mexican tetra. Based on the aforementioned studies it appears as though lens regeneration can occur over a range of time periods in different species. The rapid lens regeneration which occurs in the Mexican tetra is more commonly found in juvenile animals, while slower lens regeneration occurs in adults.

Histological analysis of the regenerating lens in the Mexican tetra indicates that the regenerating lens is not in close association with the cornea. However, as the lens does appear to be in close contact with the iris, I hypothesize that lens regeneration occurs through the transdifferentiation of dorsal pigmented iris cells, Wolffian regeneration. Further in depth analyses is required to confirm what structure of the eye is giving rise to the new lens. Confirming the origin of the regenerating lens is important to determine prior to the publishing this data, and will vastly strengthen the study. Identifying the origin of the regenerating lens would confirm that the Mexican tetra lens can regenerate; without confirmation of trans- or dedifferentiation, regeneration cannot be proven. This finding would also allow for a better comparison to the lens regeneration present in the loachs.

The loach is the only other known teleost to have the capacity for lens regeneration, making the loach and the Mexican tetra highly exceptional species (Sato, 1961). The loach, like the zebrafish is a member of the Order Cypriniformes, while the Mexican tetra is a member of the Order Characiformes. As such the Mexican tetra and

the loach are largely closely related, however as the only members of their respective orders to have been identified to have the capacity for lens regeneration, and there level of relation it is likely that the capacity for lens regeneration in the two species evolved independently. In the loach the lens regenerates through Wolffian regeneration, as proposed for the Mexican tetra. The loach is thought to have evolved the capacity for lens regeneration as it encounters large numbers of parasites in its natural environment. The parasites specifically attack the lenses of the fish's eyes, as such it is proposed that this parasite-host relationship drove the evolution of the capacity for lens regeneration in this species. Although highly speculative, I hypothesize that the Mexican tetra may have evolved the capacity of lens regeneration through gene flow between the lens-less cavefish and the surface fish. This may have occured as a strategy for the surface fish to combat the genes carried by the cavefish which are detrimental to lens development. A high rate of gene flow has been documented to occur between select cave populations and the surface population (Wilkens 1988, 2004, 2007). In salmon, *Salmo salar* gene flow between the wild and captive bred salmon has been highly controlled (Youngson et al., 2001). Gene flow between the two populations has been demonstrated to influence the behaviour of the wild population, further exposing the group to predators (Youngson et al., 2001). Although, in this case gene flow results in negative changes it does provide evidence that gene flow between separated populations can influence characteristics of the populations.

5.6.5 Early Lens Regeneration has Little to no Affect the Shape of the Orbital Bones

Manual lens removal was conducted between 1 and 3 days post fertilization. Fifty percent of the specimens underwent lens regeneration between 4 and 8 days after lens removal. As a result of lens regeneration, eye regression was not observed in the surgery eye and the adult surgery specimens had two normal appearing eyes in adulthood. No changes in the shape of the suborbital bones were present after lens regeneration (based on gross morphological analysis), however a small expansion was observed in the supraorbital bones of two specimens. These findings indicate that lens regeneration has very little impact on the shape of the skull. Morphometric analysis agreed with these findings, indicating that there were no overall significant changes in the shape of the skull

after lens removal and regeneration. However, the morphometric analysis performed in this study may have amplified the small changes present in three specimens, as gross morphological observations indicated that there may be very minimal shape changes a few individuals. The larger proportion of specimens did not have any observable or statistically significant changes in shape, indicating that that lens regeneration has minimal influence on the shape of the skull.

3.6.6 Early Lens Damage and Lens Regeneration Have a Similar Impact on the Skull

Lens ablation was performed between 1 dpf and 3 dpf. If the lens regenerated there was little to no influence to the shape of the adult skull at any of the surgery time points. As stated previously, when lens damage was performed between 1 and 11 dpf there was very little impact on the shape of the adult skull. Due to the rapid process of lens regeneration and lens healing over a similar time period in early development it is likely that both processes would have a similar impact on the skull.

As was stated in Chapter 1, in zebrafish the bones of the orbital region are neural crest derived (Kague et al., 2012). This developmental feature is thought to be shared with the Mexican tetra. Cranial neural crest cells begin to migrate at 14 hpf (Kimmel et al., 1995) in zebrafish. As such CNC migration would likely still be occurring during the timing of lens removal and during lens damage in the Mexican tetra. The lens would likely not regenerate by the end of CNC migration, in the case of lens healing the lens may have healed before the end of CNC migration at early surgery time points. In adults which did not regenerate a lens before the end of CNC migration, only very mild affects were observed in the shape of the bones of the orbital region. These alterations were similar to the mild shape changes present after lens healing prior to the end of CNC migration. This comparison indicates that the absence of the lens during CNC migration is not critical for the formation of the skull in the Mexican tetra.

The short timing of lens regeneration and lens healing results in a lens which appears to be normal in structure and normal size eye being present during skull development. As stated previously, the bones of the orbital region ossify in the Mexican tetra at 22 mm SL at approximately 3 to 4 months of age. Thus by the timing of orbital bone ossification and subsequent outgrowth the lens has long since been regenerated or

healed and as such maintained the normal size of the eye. As only mild changes in shape were present in the specimens which regenerated or healed their lenses, these data indicate that the presence of the lens during orbital region ossification and outgrowth is the key factor controlling the variability in shape of these bones.

Finally, due to the as the eye size is not affected by lens regeneration or lens healing, the mechanical forces would not be altered in these specimens and therefore do not influence the shape of the orbital bones.

3.6.7 The Mexican tetra may Hold the Key to Unravelling the Complex Processes of Regeneration

The discovery of a new species with the capacity for regeneration provides a new opportunity to attempt to unravel the complex nature of regeneration, which is currently not well understood. Different types of regeneration occur in different parts of the body. Types of regeneration are categorized based on the cell types which give rise to the regenerative material, for example transdifferentiation versus stem cells and the genetic factors which contribute to the process (Tsonis, 2000; Henry, 2003). (

Reforming a lost structure requires the activation of complex genetic cascades, many which appear to be shared through multiple sites of regeneration within the body (Tsonis, 2000). The processes of lens and retina regeneration require the transdifferentiation of only one cell type. While limb regeneration is much more complex, involving a specialized wound covering epithelium, while the underlying tissues undergo dedifferentiation, then later transdifferentiation into muscle and bone. Despite the differences in regeneration complexities FGFs and retinoic acids appear to be critical in the regeneration of all three (limb, lens and retina) of these structures (Tsonis, 2000; Henry, 2003). FGFs are expressed in the wound epithelium of the blastema, while retinoic acid establishes the distal proximal arrangements. Studying multiple structures that have the capacity for regeneration in the same species (typically the newt) allowed researchers to identify common factors in regeneration between the various structures, these commonalities indicate their importance in the process of regeneration (Tsonis, 2000). While the Mexican tetra does not have walking legs, they do have paired and unpaired fins. As Mexican tetras tend to have a naturally aggressive behaviour, animals

housed together often inflict injury on their tank-mate's fins. The fish are then able to fully regenerate the damaged fins. Comparing lens and limb regeneration in newts has provided insight into some key genetic factors involved in regeneration (Tsonis, 2000). The comparison between limb and lens regeneration in newts has allowed scientists to determine the common underlying genetic factors.. Investigating how fin and lens regeneration compare in the Mexican tetra may provide an exciting new system in which to expand our knowledge of regeneration.

3.7 Summary

In conclusion, damaging the lens with laser surgery between 1 and 11 dpf did not result in eye regression. Histological analysis indicates that the fiber cells of the lens were damaged by the surgery, however the epithelial cells remained intact, as such rapid lens healing likely occurs after surgery. Early lens damage did not affect the skull bones in a dramatic manner, however, mild effects of surgery are present in the adult skulls. The lens cells still present after laser surgery may be capable of influencing the skull during development. Additonally, this study demonstrated that the Mexican tetra has the capacity for lens regeneration as a juvenile. After lens ablation, lens cells were no longer present in the surgery eye. By 5 days after surgery a regenerating lens, approximately 42% smaller than the control lens was present in the surgery eye. Lens regeneration is nearly complete by 7 days after surgery. The regenerating lens was positively identified by performing immunohistochemistry with β crystallin. The adult skull was analyzed to determine if early lens regeneration affected the shape of the bones of the adult skull. Gross morphology and morphometrics analysis determined that there was little to no effects of lens regeneration on eye size or the shape of the bones in the orbital region of the adult skull. The capacity to both heal and regenerate the lens in a single species is exceptional and rarecharacteristic.

Chapter 4: Does Lens Removal in a Surface Fish Result in Cavefish Morphology?

4.1 Introduction

The objective of this chapter is to investigate if the absence of the lens in cavefish is responsible for the morphological differences between the cavefish and surface fish skulls. Namely, can simple lens removal performed in surface fish result in a cavefish-like skull morphology.

While there is only one known sighted, surface morph of the Mexican tetra, there are approximately 29 different blind cavefish morphs (different phenotypes of the same species) found in a small region of limestone caves located in north-eastern Mexico (Mitchell et al., 1977). Amongst the 29 morphs is the Tinaja cavefish, which will be investigated in this study. The most highly investigated feature of the cavefish is their lack of functioning eyes in adulthood. As described previously, in addition to eye degeneration, cavefish have other regressive changes including loss of pigmentation, reduction in the size of the optic tectum, loss of schooling behaviour, changes in body position while feeding, increased jaw size, increased number of taste buds, increased size and number of neuromasts, larger fat stores and increased number of maxillary teeth (Yamamoto and Jeffery, 2000; Jeffery, 2005). Each cave population has a level of regression in the aforementioned characteristics. For example some cave population such as the Micos cave population have maintained body pigment, while others such as the Pachon cavefish completely lack pigment. In addition, there can be differences in the amount of eye regression in each morph (Wilkens, 2004). By crossing two different cave populations a more surface fish-like phenotype in both eyes and pigment has been achieved (Wilkens, 2004). The offspring often have small eyes and a small to moderate amount of pigment, indicating that eye and pigment loss results from different mechanisms in the different cave morphs.

Very little research has been focused on the Tinaja cavefish population, however, some descriptions of the Tinaja cavefish can be gathered from the general Mexican tetra cavefish studies. The Tinaja cave is a long continuous cave system located in the

southern region of the limestone network in Mexico. The Tinaja cave population has regressed, non-functioning eyes and have minimal body pigment during adulthood (Jeffery, 2005; 2008). A very brief comparison between the surface fish and the Tinaja cavefish was conducted by Yamamoto et al., (2003). In the study the authors indicated that the width of the olfactory pits, growth of the orbital bones and position of suborbital 3 through 6 differ between the two populations (Yamamoto et al., 2003).

In this thesis, I investigate how the presence of the eye can influence the development of the skull. The two morphs of the Mexican tetra have unique skeletal morphologies surrounding the eye differing from surface fish in number of elements, size, and position of suborbital bones (Yamamoto et al., 2003). In addition, the surface fish have two scleral ossicles in the eye joined by cartilage (Franz-Odendaal et al., 2007; Franz-Odendaal, 2008), while cavefish have a single cartilage element in the sclera (Yamamoto et al., 2003). Yamamoto and Jeffrey (2000) transplanted a cavefish lens into a surface fish eye at 24 hours post fertilization in order to cause eye regression in the surface morph. When eye regression was induced some craniofacial bones were affected, such as the distance between the nasal bone, antorbital bones and olfactory pits, an ossified sclera, and the shape of both suborbital 3 and the supraorbital bone (Yamamoto et al., 2003). I hope to better understand morphologically, how changes in the surface fish skull compare to the cavefish skull, and to further explore what mechanisms influence the phenotypes.

The Mexican tetra can be easily bred in the laboratory (Jeffery, 2001; 2005; 2008), giving rise to cavefish and surface fish hybrids, which will be referred to as intermediates. Wilkens and Strecker (2003) conducted experiments in which they interbred the populations of Mexican tetras, in order to determine if all cave populations had the same eye and pigment genes responsible for the loss of these structures. The authors determined that when they interbred different cavefish populations the offspring had small eyes and had a greater level of pigment than their parents, indicating changes in different genes cause eye and pigment loss in different cave populations (Wilkens and Strecker, 2003). However, more pertinent to my study are the crosses that were performed between surface and cavefish. When a cavefish was bred with a surface fish the offspring fall morphologically in the middle of the two morphs (Wilkens and

Strecker, 2003). The surface fish-cavefish intermediates have small sized eyes and a light to medium amount of body pigmentation. How a small eye, such as present in the intermediates, influences the shape of the surrounding circumorbital bones has not been studied.

The objective of this study is to determine if simple lens removal in a surface fish results in a more cavefish-like phenotype. I hypothesize that the surgery skull will be more similar to the intermediate skull. Intermediates allow me to determine how an intermediate sized eye and a small lens can influence the shape of the orbital bones. Specifically, does lens removal in a surface fish, result in a more cavefish-like skull, or does it produce a phenotype more similar to an intermediate? Is the lens the main structure responsible for transitioning the surface morph to a cavefish morph?

Additionally, there is a gap in the literature demonstratingthe ways in which eye size can affect the shape and size of the orbital orbital bones, this study allows us to compare the surface fish skull, surgery fish skull, and for the first time, describe and compare the Tinaja cavefish and intermediate skulls in the same species of fish with large eyes, small eyes, and no eyes. This study allows us to gain better insight into whether and how eye size correlates to orbital bone growth and shape.

4.2 Materials and Methods

4.2.1 Biological Material, Mexican tetra, *Astyanax mexicanus* Cavefish and Intermediate Fish (Cave/Surface)

Adult Tinaja Cavefish were received in 10% Neutral Buffered Formalin from Dr. R. Borowsky (New York University, New York City, USA). These Tinaja cavefish were a second generation from wild populations. Wild specimens were caught in the Tinaja cave, Mexico (n=8). Intermediate fish were offspring (F1) of aTinaja cavefish and surface fish cross (n=5). Intermediates were received in 10% NBF from Dr. R. Borowsky. All cavefish and intermediates were a minimum of 3.5 cm SL. All specimens were whole mount bone stained (as described previously in Chapter 2).

4.2.2 Surgery Fish and Intermediate Morphometrics

As described in Chapter 2, to compare the manual lens removal surgery surface to intermediates images of the lateral view of the adult skull were captured using a Nikon SMZ1000 microscope and NIS Elements software package. A reduced subset of the landmarks used in Chapter 2, were used in this study, in order to concentrate on the orbital region. Twenty-nine two-dimensional (x,y) landmarks were applied to the lateral view images of the head (Figure 4.1, Appendix 1, Table 4). The morphometric analyses were then conducted as described in Chapter 2.

4.2.3 Tooth Counts

The number of small teeth present in the caudal region of the lower jaw in intermediates (n=7) and Tinaja cavefish (n=7) were counted as described in Chapter 2, section 2.4.2.

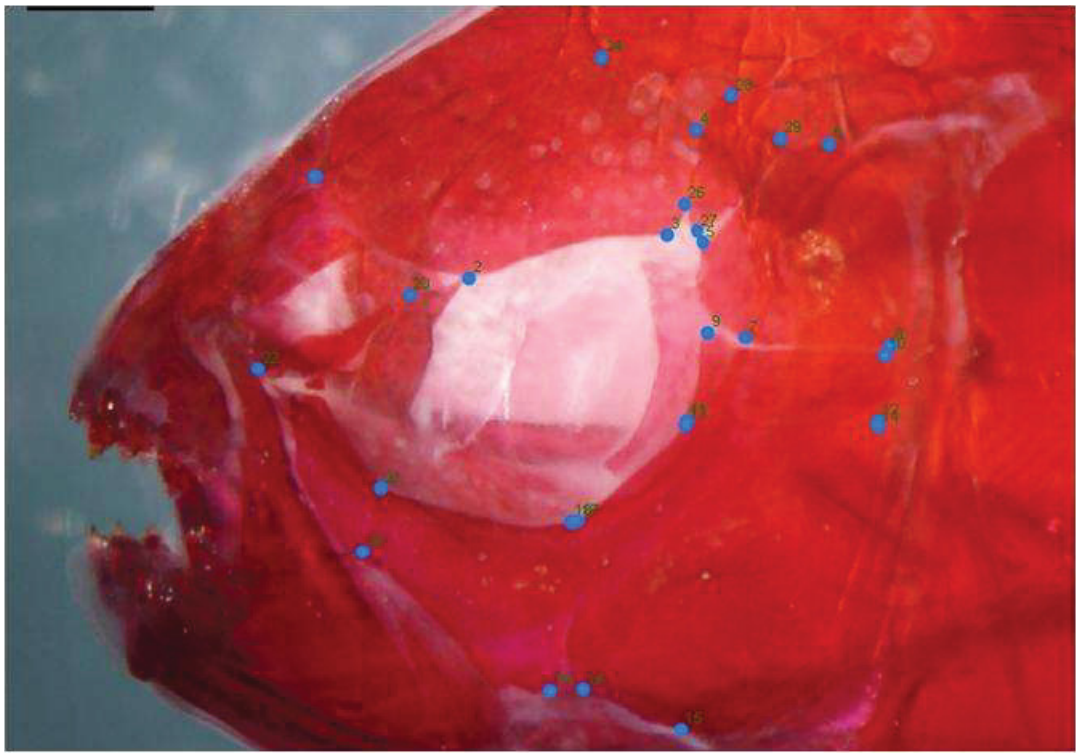


Figure 4-1: Whole mount bone stained surface fish skull showing 29 morphometric landmark locations.

4.3 Results

4.3.1 Morphology of the Bone of the Tinaja Cavefish Orbital Region

The orbital region of the Tinaja cavefish skull consists of five to six suborbital bones and a supraorbital bone bordering the eye. In adulthood the supraorbital bone consists of a flat plate like bone, which is expanded into the eye orbit. The ventral edge of the bone is highly variable in shape, a different shape in each of the 8 specimens (Figure 4-2). Suborbital 1 is also highly variable (it may consist of two to seven elements) generally occupying the normal location and shape of the corresponding bone in surface fish. Suborbital 2 is also variable, in the cavefish this bone is variable in shape, it may take on a number of different morphologies, from a large flat fan shaped bone to a small triangular shaped bone. Suborbital 3 is both variable in shape and the number of bones, which make up the element. Some individuals have a large fan shaped bone, while others have a large rectangular bone, with one to two additional small bones located ventral to the large bone making up the element. Suborbital 4 tends to be more stable in shape and location, generally consisting of a small square shaped bone. Suborbital 5 is stable in the number of bones making up the element, however, it is variable in shape. In some specimens, the bone is small and square in shape, while in others it is larger and more rectangular, with an irregular dorsal border. Finally, suborbital 6 is present in some individuals and not present in others. Additionally, it may be present on one side of an individual's head and not present on the other. When present it is small, or a large wedge shape.

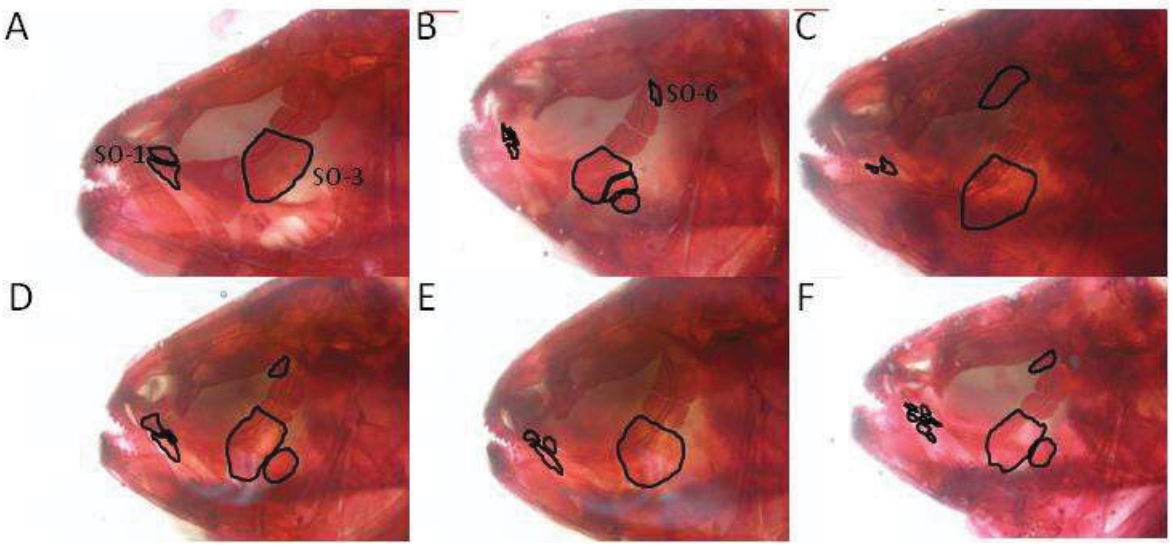


Figure 4-2: Natural variation present in whole mount bone stained adult Tinaja cavefish skulls. (A-F) Lateral views of adult Tinaja cavefish. Suborbital bones 1, 3 and 6 are outlined in black depicting vast variation in their morphology in different fish..

4.3.2 A Comparison Between the Cavefish and Surgery Fish Skulls

To determine if manual lens ablation in the surface fish results in a cavefish-like skull morphology, the adult skull after surgery at 1 dpf was compared to the Tinaja cavefish skull (n=8). After lens removal many morphological changes occur in the skull of the surface fish (Chapter 2). All of the morphological changes, which were observed were alterations in bone shape and bone number, not in bone location or the presence or absence of bones. When the surgery skull is compared to a cavefish skull there are obvious differences in the number of bones present around the eye, such as multiple small bones, which make suborbital 1 (1 to 5 small bones) in the cavefish. Due to these differences in the number of bones and the large variations present in bone shape, morphometrics is not possible for analyzing the differences between these two groups.

Despite differences in the number of elements that make up some bones (e.g. suborbital 1 and 3), other bones can be closely compared (Table 4-1). The supraorbital bone for example is very similar in size and shape between the cavefish and surgery fish (Figure 4-3). The bone is large and plate-like in both of the groups. In comparison, the supraorbital of a normal surface fish is much smaller and curved over the top of the eye. In addition, suborbital two is also similar in shape between the two groups, it is larger and more triangular in shape then the typically present in the surface fish. Scleral ossicles are present in some surgery fish (Chapter 2), when present they strongly resemble the scleral ossicles present in the regressed eyes of the cavefish.

In the surgery specimens, suborbital one consists of one small bone, in cavefish the bone is expanded in the dorsal ventral direction, when present as one structure. However, in cavefish it most often consists of a complex of many small jagged bones, ranging from one to five elements (Table 4-1, Figure 4-2 and 4-3). Suborbital three consists of a large flat bone in the surgery fish, however in cavefish the element is small and shifted further into the orbit of the eye, when present as one element. In cavefish, suborbital 3 may consist of one to three elements, with one large element and one to two smaller smooth round elements located ventral to the large element. In the cavefish suborbital 4 and 5 are highly variable, both in shape and structure. Some individuals have suborbital 4 and 5 fused, while others have two separate structures (Figure 4-2). Some individuals have fused SO 4 and 5 on one side of the head and separate elements on the

other. The elements also range widely in shape, from small square bones, to large wedge shaped bones. All surgery specimens have large wedge shaped suborbital 5 and a smaller rectangular shaped suborbital 4. Finally, suborbital 6 is a small wedge shaped bone in surgery fish, when present in cavefish it ranges widely in shape, from a small rectangle to a large wedge shaped bone. Suborbital 6 is only present in approximately half of the cavefish and maybe present on one side of the head only.

In conclusion, despite the vast changes in the skull of the surgery surface fish from the wild-type surface fish, as described in Chapter 2, these alterations do not result in a cavefish skull, however, the changes in shape observed in the supraorbital bone and SO-2 as a result of lens removal does result in bones more similar to those found in the cavefish. The surgery fish is more similar to an intermediate, based on gross morphology, as hypothesized.

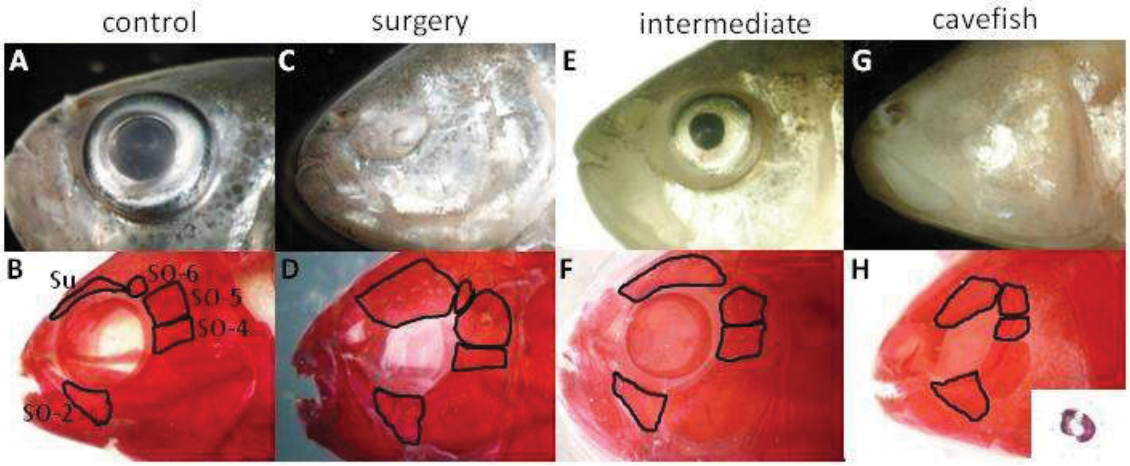


Figure 4-3: Adult skull of surface morph, surgery surface morph, intermediate and cavefish. A, C, E, G are unstained adults. B, D, F, and H are alizarin bone stained adult skull. (A-B) are controls; (C-D) are surgery fish; (E-F) adult intermediates (G-H) are cavefish; (H) scleral ossicle inset. The supraorbital bone, and suborbital bones 2, 4, 5, and 6 are outlined in the images of stained skulls. SO suborbital bone 2, 4, 5 and 6, SU supraorbital bones.

Table 4-1: A comparison between the shape and number of elements present in the orbital regions of the Tinaja cavefish and surgery surface fish.

Bone	Surgery surface fish	Tinaja Cavefish	Comparison
Supraorbital bone	large and expanded	large and expanded	similar in shape and number
SO-1	1 large element	variable, 1 to 5 small elements	dissimilar in shape and number
SO-2	large and expanded	large and expanded	similar in shape and number
SO-3	1 large element	variable, 1 to 3 elements	dissimilar in shape and number
SO-4	rectangular bone	variable, maybe fused to SO-5	dissimilar in shape and number
SO-5	wedge shaped bone	variable, maybe fused to SO-4	dissimilar in shape and number
S0-6	small wedge shape	variable, present or absent	dissimilar in shape and number
Scleral ossicles	1-2 small arch shaped bones	small arch shaped bones	similar in shape and number

4.3.3 Morphology of the Orbital Region Bones of the Intermediates

The intermediates examined in this study are the F1's of a cross between a Tinaja cavefish and a surface fish. The intermediates have intermediate size eyes that are smaller than surface fish eyes but larger than cavefish eyes in adulthood. They also have an intermediate amount of pigment. The intermediates have a skull morphology different from both the surface fish and the cavefish. The orbital region of the intermediate skull consists of six suborbital bones, each consisting of only one element and a supraorbital bone over the dorsal portion of the eye (Figure 4-3).

The supraorbital bone of the intermediate is a large flat bone expanded into the orbit. It is longer in the dorsal to ventral axis than that present in the surface fish. The shape and size of SO 1 and 2 are similar between surface fish and intermediates with no obvious differences. Suborbital 3 is larger in the intermediate, slightly expanded into the orbit, and is mainly larger in the dorsal to ventral axis. Suborbital 4 and 5 are slightly expanded into the orbital, resulting in bones which are larger in the anterior to posterior axis. Finally, SO-6 is displaced from the position in which it is typically located in surface and is crowded by the supraorbital bone. When comparing the intermediate skull to the surface fish skull major differences are seen in the shape of the supraorbital bone, SO-3 and the position of SO-6, but is otherwise similar in morphology.

4.3.4 Comparison Between the Skulls of the Surgery Surface Fish and Intermediates

The adult skulls of 1 dpf surgery fish and intermediates (a F1 hybrid of a surface fish and a cavefish cross) were compared to determine if lens removal in the surface fish results in a skull more similar to an intermediate, than a cavefish. Unlike the cavefish skull the intermediate skull has the same number of bones as the surgery surface fish and thus can be compared morphologically and statistically using morphometrics.

Based on gross morphological analysis, the skulls of the intermediate fish and the surgery fish are much more similar than either group is to the cavefish. The shape of the supraorbital bones of the intermediate and surgery fish are very similar, the only difference occurs in the variation observed in the intermediate supraorbital bones, with some of the bones more expanded into the orbit than others (Figure 4-3). Suborbitals 4 and 5 tend to be large in the anterior to posterior axis in the surgery specimens then the

intermediates, with a greater expansion into the eye orbit. Suborbital bones 1, 2, 3 and 6 appear to be relatively the same shape in the surgery and intermediate skulls.

Twenty-nine landmarks were chosen to focus on the orbital region of the skull around the eye. In Chapter 2, 42 landmarks were selected, many of which did not identify variation in their corresponding bones. As such, the number of landmarks was reduced in this study to focus on the potentially variable areas of the skull.

Landmarks were applied to the surgery side of 1 dpf surgery specimens heads, to four left and four right sides of intermediates heads and to control surface fish skulls. Warp analysis conducted using the 1 dpf surgery consensus as the reference and the intermediate consensus as the data showed very little warping in this comparison (Figure 4-4). The majority of the warping occurs due to size differences in the orbit between the two groups, the orbit is larger in the intermediate group than the surgery group. Small alterations can also be observed in the shape of suborbital 5 and 6. Vector analysis confirms these observations with vectors pointing away from the orbit, with the largest vectors and changes associated with suborbital 5 and 6. TwoGroup was then used to determine if the small differences in shape observed between the surgery and intermediate groups are significant. Goodall's F-test and F-test Procrustes indicates that the difference in shape between the two groups is significant ($F= 15.92$ $p<0.0001$, and $p<0.01$, respectively, $n=6$).

Principle component analysis was then used to determine if the surgery group or the intermediate group is most similar to the control surface tetras. PCA analysis shows three distinct groups, a surgery group, control group and intermediate group (Figure 4-5). On the PC1 the intermediate group lies between the surgery and control group, while on PC2 the surgery group lies more closely with the control group. Principle component 1 accounts for approximately 35% of the variation, while PC2 accounts for approximately 20% of variance (Figure 4-5), thus the intermediate group and the surgery group are approximately the same distance from the control group, with the surgery group being slightly more different in shape from the control than the intermediate. TwoGroup was then used to compare the difference in shape between the intermediate and control, and the surgery group and controls statistically. The distance between the surgery group and control is 0.1969, while the distance between the intermediate group and the control

group is 0.1363 and the 95% range between these groups is 0.0307 and 0.0958. Thus, the shape differences between the surgery group and the control, and the intermediate group and the control are not significantly different. These statistics confirm that the intermediate group and the surgery group are close in shape and have a similar degree of changes in shape compared to the controls. Based on these results, and the descriptive comparisons between the cavefish and surgery specimens, this data suggests that lens removal performed in the surface tetra results in a skull which is more similar to an intermediate skull than a cavefish skull.

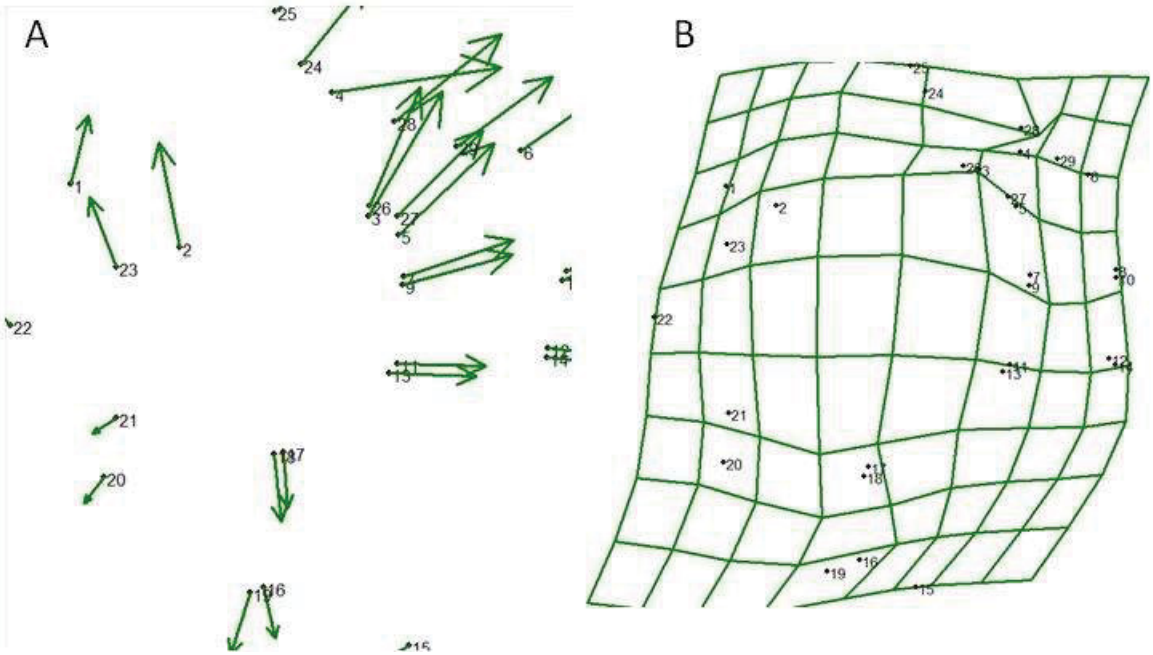


Figure 4-4: Thin plate splin and vector analysis of comparison between the surgery surface fish and intermediate. (A) Vector analysis, the origin of the vectors is the surgery specimen and the arrow head is the location of intermediate fish landmark; (B) deformation grid.

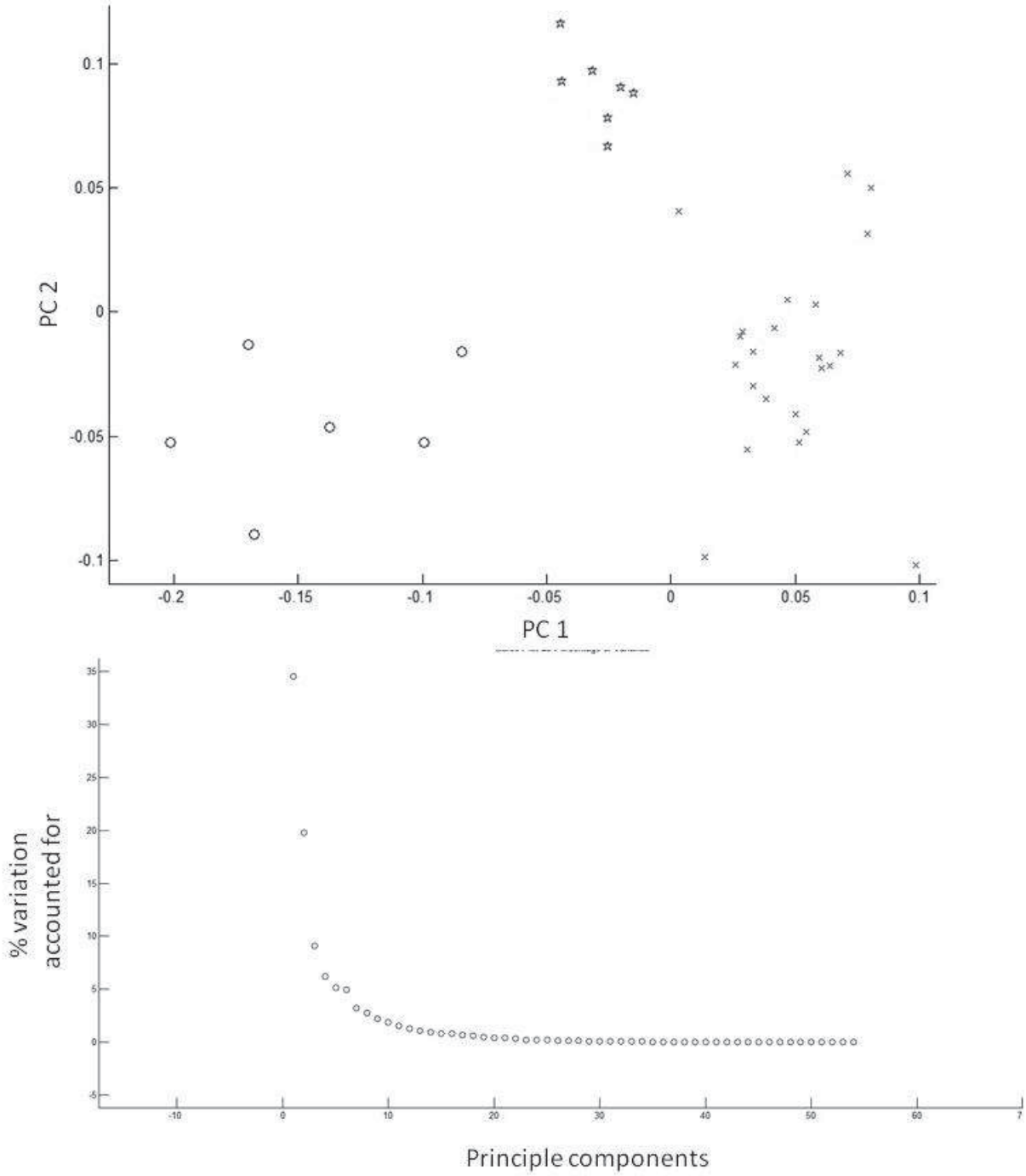


Figure 4-5: Principle component analysis comparing intermediate, surgery surface fish and control surface fish. (A) Principle component 1 (x) and 2 (y), black circles represent the surgery surface fish, red stars represent intermediates and the crosses represent the surface fish controls; (B) graphical representation of the percentage of variation accounted for by each principle component.

4.3.5 Cavefish Have a Greater Number of Small Caudal Teeth Than Surface Fish

The numbers of small caudal teeth were counted on the control side of the head in surface fish, the surgery side of the head after surgery at 1, 2, 3, and 4 dpf, in intermediates and in Tinaja cavefish adults (Figure 4-6). On average, the control side of the head has on average 1.6 ± 0.9 small caudal teeth (n=6). Surgery at 1 dpf resulted in 2.8 ± 1.7 small caudal teeth (n=6). After surgery at 2 dpf there were 3.4 ± 1.1 teeth (n=7), after surgery at 3 dpf ± 0.7 there was on average 3 teeth (n=5) and finally after surgery at 4 dpf there was on average 3.1 ± 1.1 small caudal teeth (n=5). Intermediates have on average 5.7 ± 1.5 small teeth (n=6), while Tinaja cavefish 6.4 ± 1.4 small caudal teeth (n=8). In Chapter 2, it was determined that lens removal in the surface fish resulted in significantly more small caudal teeth than the control side of the head. Here, it is clear that Tinaja cavefish and intermediates have a greater number of teeth than surface fish and surface fish after surgery.

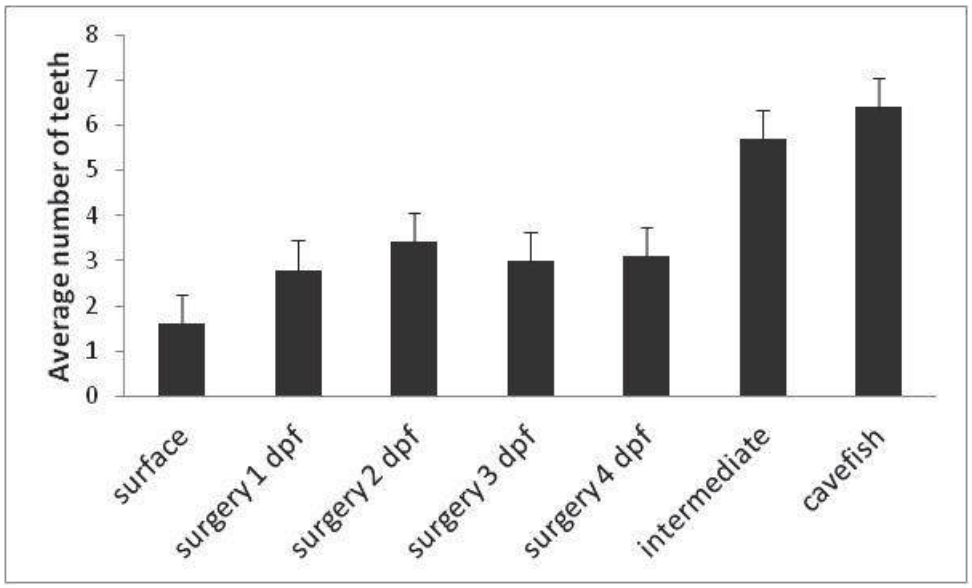


Figure 4-6: The number of small caudal teeth present in adult surface fish (no surgery), surface fish after surgery (at 1-4 dpf), intermediates and cavefish. The sample size for each group is as follows: for surgery at 1- -4 dpf) (n=6, 7, 5, and 5 respectively), for intermediates (n=6) and for cavefish (n=7).

4.3.6 Lens Ablation in the Sighted Tetra Partially Resembles the Cavefish Phenotype

I removed the lens in the surface morph embryos to determine whether lens removal in the surface fish would resemble the cavefish phenotype in adulthood. I determined that there are some skeletal similarities shared between the surgery surface fish and the Tinaja cavefish, specifically the supraorbital and suborbital 2 bones (Figure 4-3). In addition, the orbit size is smaller in the surgery fish, similar to the cavefish. Some traits are shared between the surgery surface fish and the Tinaja cavefish that are not shared with a non-surgery surface fish. Lens removal in the surface fish also resulted in an increased number of small caudal teeth.

The skull of the intermediate fish also shares some of the same features of the cavefish skull, however, the intermediates share more features with the surgery group. It was determined that the surgery skull and the intermediate skull have roughly the same level of changes in shape from the control skull. This indicates that lens removal may be responsible for some of the skull differences between the cavefish and surface fish but other important and yet to be determined factors are likely also involved.

4.4 Discussion

To determine if the absence of the lens in cavefish is responsible for the differences in the surface fish and cavefish skull, the lens was removed from the eye of the surface fish and the surgery skull and cavefish skulls were compared.

4.4.1 Lens Removal in the Surface Fish Only Partially Resembles the Cavefish Skull

Comparisons between the surface fish skull after lens removal at 1 dpf and the Tinaja cavefish skull show that the surgery skull does share some similarities with the cavefish skull. Namely the shape of the supraorbital bone, suborbital bone 2 and the scleral ossicles, however, suborbital 1, 3, 4, 5, and 6 are not similar are in shape. Although the surgery skull is more similar to the cavefish than the control surface fish skull, simple lens removal does not fully transition the surface fish skull to the cavefish skull, indicating that the lens is not solely responsible for transitioning a surface skull to a cavefish skull. In addition to shape differences present in the bones of the two groups there are also difference in the number of elements present. The cavefish were

determined to be highly variable in nature, both in the shape of the orbital bones, and in the number of elements that make up each structure. The number of the small elements that make up suborbital bones 1, 3, 4, 5, and 6 is highly variable, for example suborbital 1 may consist of one to five elements, or any number in between. A large amount of natural variation is present within these structures of the cavefish. Although the corresponding bones do not vary in number in the surface fish they are variable in nature. The variability of these bones is in the shape of the bones in surface fish rather than the number of bones making up an element in cavefish. These differences may be a result of the mechanisms that divide the groups into two morphs. In a single species I would expect that the same physical change, in this case the absence of a lens would yield roughly the same impact on all the animals, however in this case we see that the absence of the lens affects the cave and surface morphs differently. This finding indicates that the morphs are stable, independent groups, indicating that there may be more separation between the two morphs than is proposed by some groups, in particular the Jeffery group from Maryland (Jeffery, 2001, Jeffery 2007, Porter, 2007, Yamamoto et al., 2009). The differences in variation between the morphs also indicates that the lens is not solelyresponsible for the differences in the skull between the two morphs indicating that other factors maybe different between the two morphs controlling the development of these bones, including genetic differences.

Yamamoto et al., (2003) studied the effects of transplanting a surface fish lens into a cavefish eye at 24 hpf. When the surface fish lens was placed in the Pachon cavefish eye, the eye did not follow normal cavefish eye regression, rather a medium sized eye formed. When the adult skull was examined on the surgery side of the head, vast differences could be observed in the skull. The skull took on a much more surface fish like phenotype in bone shape, however, the number of small elements making up each orbital bone was still variable in number and did not match the surface fish orbit. This study again demonstrates that the presence of the lens has dramatic effects on the shape of the orbital bones, however, it does not influence the number of elements present, indicating other factors (i.e. genetic influences) are also highly important in the formation of these bones.

4.4.2 What Other Factors Might Influence the Differences Between the Cavefish and Surgery Fish Skulls?

I have shown that lens ablation conducted at 1- 4 dpf in the surface fish affects the adult cranial skeleton and tooth number, but not taste bud number (Chapter 2). I asked whether this phenotype resembles a more cavefish-like phenotype. Overall, morphologically, the surgery surface fish share some of the craniofacial features of the cavefish, however many differences such as the number of suborbital bones, still exist between the skulls. The surgery phenotype may more closely resemble an intermediate morph (a F1 hybrid of a surface fish and a cavefish cross), however, the details of the intermediate phenotype has not been documented. This comparison would be useful to gain a better understanding of the developmental mechanisms which result in the morphological changes present between the surface and cavefish. As was hypothesized, my study demonstrates that the regressing lens is one player in a much more complex system that led to the phenotypic changes in the derived cavefish. Increased midline expression of *shh* in the cavefish leads to a decrease in *pax6* expression (a key eye development gene) (Yamamoto et al., 2009). Downstream effects of this increased expression include an up-regulation of the following genes *patched, vax1*, and *pax2A* in the head region and the eye (Mitchell et al., 1977; Schwarz, 2000; Strickler, 2001; Take-uchi, 2003). These genetic changes result in alterations in the lens, including a decrease in anti-apoptotic factors and an increase in pro-apoptotic factors (Schwarz, 2000). According to Yamamoto et al. (2009) the changes in gene expression levels of *shh* might be responsible for a decrease in cavefish eye size, increase in taste bud number and an increase in jaw size. This study clearly demonstrates that there are genetic differences between the two morphs, with many downstream effects. As a result I hypothesize that the observed phenotype of cavefish are unique characteristics of its genome that are not replicable in surface fish lacking an eye. The upstream signalling events that direct lens apoptosis are likely also responsible for the associated constructive and regressive changes observed in cavefish.

4.4.3 The Surgery Surface Fish Skull More Closely Resembles the Intermediate Skull

Comparisons between the surgery surface fish skull and the cavefish skull indicates that there are some similarities between the skulls of the two groups, however, there are more discrepancies between the skulls than there are similarities. I then hypothesized that the surgery skull maybe more close in shape to an intermediate skull, to investigate this relationship I examined the gross morphology and statistically compared the surgery skull and intermediate skulls.

My results show that the intermediate skulls are very similar to the surgery surface fish skull. The supraorbital bone as well as suborbital bones 1, 2, 3, and 6 were determined to be very similar in shape between the two groups. The supraorbital bone of the intermediates appears to be highly variable in nature as it takes on many different shapes in these specimens. Principle component analysis demonstrated that the intermediate skull is and the surgery skulls have approximately the same amount of shape differences from the controls. However, the intermediates are much closer to the controls in orbit size. Orbit size appears to be highly correlated with the size of the eye.

Investigations conducted by Wilkens, (2003, 2004 2007) demonstrated that the F1's of cavefish and surface fish most often have medium size eyes and a medium amount of pigment, which can be highly variable. This was also the case for the Tinaja intermediate specimens, which were investigated in this study. The intermediates have medium size eyes, light body pigment and each eye containing a small lens. With an intermediate size eye there is an intermediate amount of expansion of the orbital bones, indicating that eye size and orbital bone outgrowth are highly correlated. With a large eye (surface fish) the orbit size is large, while the orbital bones are small, with the intermediate eye size the orbital bones are mildly outgrown, when the eye and lens are absent (surgery fish and cavefish) the orbit is small and the orbital bones are large and expanded. While this study indicates that the surgery skull is similar to the intermediate skull it does not further explain how the bones are affected. The small lens present in the intermediates maybe providing a smaller amount of signals to the surrounding skull, than the larger control lens, alternatively, the medium sized intermediate eye may result in minor changes in the amount of mechanical forces within the skull. Finally, Yamamoto et al., 2009 determined that there is an increased midline expression of *shh* in the head of

the cavefish, which has many downstream affects, the levels of *shh* expression in the intermediates has not been studied and thus may also play a role in shape changes found in the intermediate skull. Studying the orbital region of the intermediate skull demonstrates again the highly variable nature of these bones, with the multitude of shapes that each element can take on, however, it does not provide further evidence for whether the eye is influencing the skull through mechanical or signalling mechanism.

4.5 Summary

In summary, I have determined that simply removing the lens from the eye of a surface fish does not fully produce the cavefish skull phenotype, indicating that other factors outside of the lens and eye determine the shape and number of elements present as orbital bones. Finally, lens removal in the surface fish appears to result in a skull more similar to that of an intermediate with a medium sized eye than that of a cavefish, indicating that there is a complex combination of lens signals and genetic influences not related to the lens giving rise to the cavefish phenotype. Overall, this study again demonstrates the highly variable nature of the Mexican tetra's skull, as with simple manipulations a large number of highly variable phenotypes can be produced.

Chapter 5: How Does Lens Removal Affect the Craniofacial Skeleton of Zebrafish?

5.1 Introduction

The objective of this chapter is to determine if lens removal conducted in the zebrafish influences the shape of the bones of the orbital region. This study compares the variability observed in the shape of the bones in the orbital region of the Mexican tetra, to that present in the skull of the zebrafish, *Danio rerio*. I hypothesize that lens removal will have a similar impact on the shape of the orbital bones across species.

Zebrafish are small freshwater fish, which have become a highly investigated model species for vertebrate embryogenesis. Zebrafish have been used as a model species for studying cell movement, cell lineages and neurogenesis (Kimmel and Law, 1985; Kimmel, 1989; Warga and Kimmel, 1990). Other features of the zebrafish that make them a particularly useful model species include their ease to obtain, ease to breed and production of large numbers of young. In addition, zebrafish develop rapidly and have a short generation time making them an excellent model species (Meyer et al., 1993). In the past 15 years zebrafish have become a popular model species for investigating the developing skeleton, including investigation of pharyngeal arch development, neural crest cell contribution to the skeleton and deposition and resorption of the skeleton (for example, Schilling and Kimmel, 1994; Connolly and Hall, 2008; Edsall and Franz-Odendaal, 2010). The zebrafish genome has been fully sequenced. In addition, numerous mutant strains can be easily obtained and a multitude of material is available for easy genetic manipulation, making them an excellent model for genetic studies. Finally, zebrafish are a derived member of the family Cyprinidae, a large family, which consists of approximately 1600 species making them ideal models for comparative studies (Meyer et al., 1993).

The skull and orbital region of the zebrafish is relatively similar compared to that of the Mexican tetra, in the arrangement and shape of bones present. The orbital complex consists of five suborbital bones, often referred to as infraorbital bones, with one supraorbital bone (Cubbage and Mabee, 1996). As in the Mexican tetra these elements

ossify directly from skeletogenic condensations, without a cartilage precursor. The orbital bones ossify relatively late in development, similar to the Mexican tetra. Suborbital bone 1 ossifies first, followed by 5, 3, 4, and lastly 2. Suborbital 1 begins to ossify when the fish are 6.8 mm SL, suborbital 3 ossifies at 9.8 mm SL, while suborbital 2, the smallest suborbital ossifies much later at 19.5 mm SL. The supraorbital ossifies at 7.4 mm SL. In zebrafish the suborbital 2 bone is the last bone to ossify in the skull (Cubbage and Mabee, 1996). Due to the numerous similarities between the orbital region of the Mexican tetra skull and the zebrafish skull, the zebrafish makes an excellent candidate for a cross species comparison.

Zebrafish are members of the Order Cypriniformes, Family Cyprinidae, while the Mexican tetra is a member of the Order Characiformes, Family Characidae (Meyer et al., 1993; Jeffery, 2008) (Figure 5-1). In addition to the fact that the zebrafish is a widely accepted model species they also make an excellent comparison species for this study due to the fact that they belong to a different Order to the Mexican tetra. Finally, a lens-less zebrafish mutant, named *bumper* exists for a "cavefish-like" comparison.

The *bumper* mutant was first identified in a large-scale mutagenesis screening (Heisenberg et al., 1996), however it was not described until 14 years later, by Schonthaler et al. (2010). Histological analysis conducted by Schonthaler et al. (2010) on the homozygous null mutant (*bum*$^{-/-}$) described abnormalities in the early lens development, including abnormal proliferation in the lens epithelium and also in the secondary fibre cells. Malformations in the lens begin at 3 dpf, as a result of the increase in proliferation of the lens epithelium. The increase in proliferation results in a multilayered lens epithelium rather than the typical monolayer, resulting in the lens becoming disorganized. The cells take on a "tumour-like" growth. Later the tumour-like cells undergo rapid cell death, eliminating the excess number of epithelial cells. The secondary fibre cells then fail to differentiate properly. By 5 dpf the lens is highly reduced in size and maybe located ectopically. By adulthood, a lens cannot be located within the eyes.

Despite the dramatic changes in the structure of the lens in the *bumper* mutant all other eye tissues remain normal in structure, indicating that the mutation present in the *bumper* mutant is lens specific. The normal development of other eye structures is likely

due to the fact that the abnormalities in the lens do not begin until 3 dpf, at which time most of eye development is complete. However, due to the small lens the eye is highly reduced in size in *bumper* adults. Histological analysis performed on the adult *bumper* eye indicates that there is no lens present in the eyes of the adults. The authors also noted alterations in the shape of the adult bumper skull (Schonthaler et al., 2010). The skull was reduced in the anterior posterior axis, lateral axis and the dorsal ventral axis, resulting in the skull length, width and depth being reduced. The dentary, maxilla and premaxilla showed mild changes, while the orbital bones were altered in shape, size and position. The authors noted that the supraorbital was most affected by lens removal, resulting in the expansion of the bone into the orbit. Alterations observed in the skull were hypothesized to be the result of the reduced eye size.

In this chapter, I examine the effects of lens removal on the zebrafish fish skull, in order to how lens removal affects the shape of the bones in the orbital region of the skull. Additionally, I investigate the morphology of the *bum*$^{-/-}$ mutant skull to determine if gradual lens loss over development affects the shape of the orbital bones, and whether it has the same influences as an early lens removal. Finally, I determine if lens removal conducted on the zebrafish has the same impact on the skull as I observed after lens removal conducted in the Mexican tetra (Chapter 2).

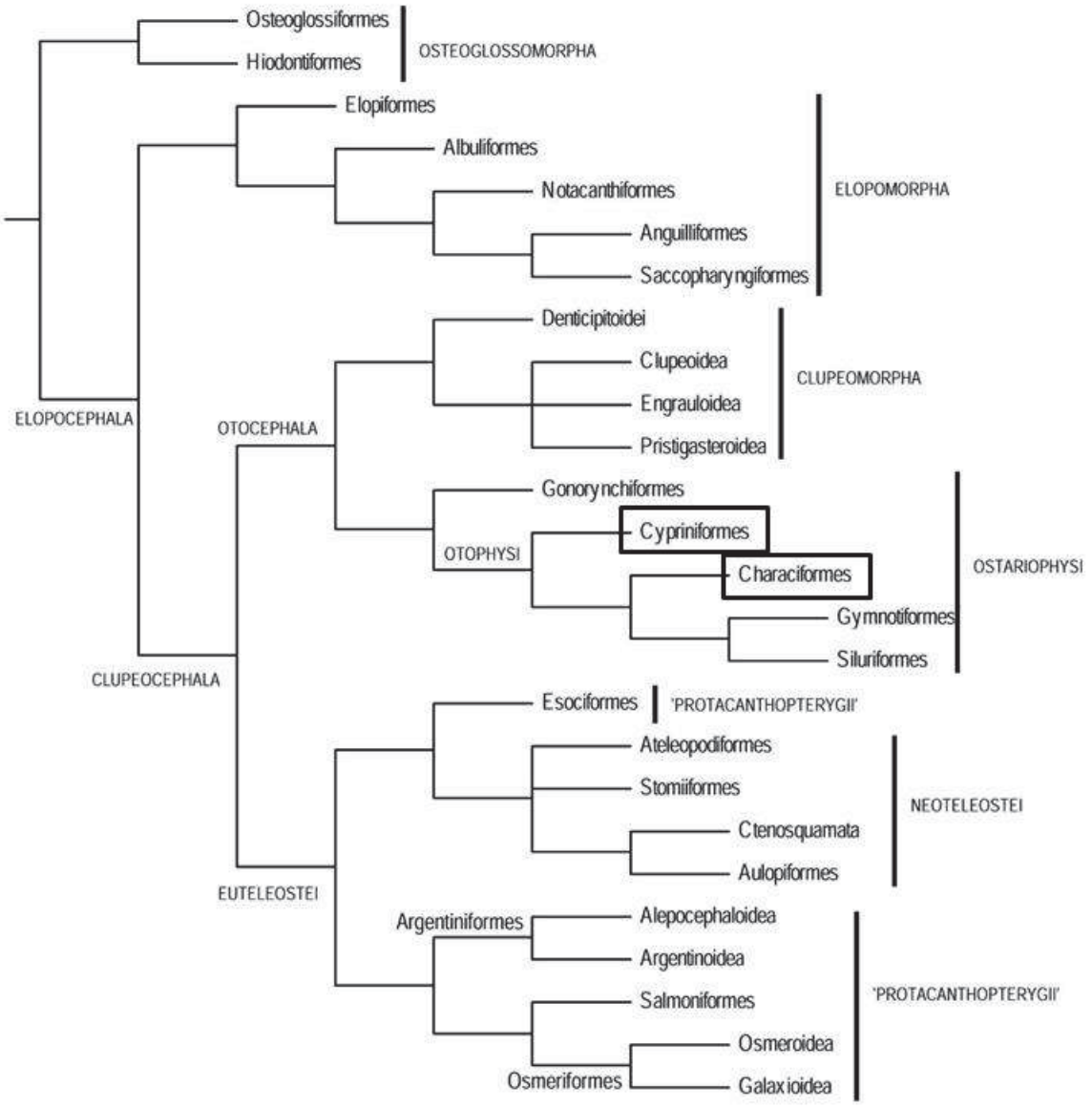


Figure 5-1: Teleost phylogeny. The phylogeny was modified from Diogo et al., (2008). Boxes indicate the location of the species of interest.

5.2 Materials and Methods

5.2.1 Zebrafish Husbandry and Complete Manual Lens Ablation in Zebrafish, *Danio rerio*

Zebrafish adults were obtained from the National Research Council fish facility from Dr. K Soans' laboratory (Halifax, Nova Scotia). The origin of these samples as well as the strain is unknown. Adults were then housed in the Mount Saint Vincent University fish facility in a recirculating flow-through Aquatic Habitat fish rack system. Adults were maintained at 28 ^{0}C on a 12 hour light, 12 hour dark cycle. To induce spawning, male and females were transferred to an off system tank. A container of marbles was added to the tank. Eggs were then spawned over the container of marbles. Eggs were collected the following morning. After collection embryos were reared in 200 to 500 ml of system water in a tumbler at 28 ^{0}C for two weeks. Embryos were staged based on the approximate number of hours since the embryos were fertilized. At 5 dpf the larvae were fed a mixture of yeast, AP2000 and TetraMin staple fry food. At 2 weeks of age the juveniles were transferred to the wrack system and fed crushed TetraMin staple flake and *artemia fransicana*. Lens removal was conducted unilaterally in zebrafish at 4 dpf (n=3) using tungsten needles and following the procedure outlined in Chapter 2, section 2.2.2. The early development of the zebrafish is slower than that of the Mexican tetra as suchfour dpf was selected as the surgery time point in an attempt to align with the same stage of eye development as when the Mexican tetra surgery was performed (ie. 1 to 2 dpf). This staging was based on the stages of eye development (Hinaux et al., 2011). After surgery, specimens were rinsed in zebrafish Ringer's solution three times and incubated at 28 ^{0}C, released from the agar mounting medium and returned to the fish facility. Only a small sample size of three was successfully raised to adulthood, as the zebrafish had a very poor survival rate after surgery compared to the Mexican tetra. The non-surgically manipulated eye serves as a control in all experiments. In addition, corneal tear control experiments were also performed as described in Chapter 2, section 2.2.2.

5.2.2 Homozygous Zebrafish Mutant, *Bumper (bum$^{-/-}$)*

The homozygous zebrafish *bumper* mutants (*bum$^{-/-}$)* were received in 70% EtOH from Dr. Ralf Dahm (Spanish National Cancer Research Centre, Madrid) (n=8). The *bumper* mutant was originally referred to as tg413b in a large-scale ENU mutagenesis screen conducted by Max Planck Institute for Developmental Biology in 1994-1995 (Heisenberg et al., 1996). Further description of the *bum$^{-/-}$* mutant conducted by Schonthaler et al. (2010) described malformation in the development of the secondary lens fibre cells, ultimately resulting in lens degeneration. Adult mutants ranged between 1.7 and 2.2 cm SL. *bumper* mutants were whole mount bone stained (as described previously in Chapter 2, section 2.2.3).

5.2.3 Comparative Morphometric Analyses

To compare the control eye to the surgery eye of lens ablated zebrafish, to the *bumper* mutants and to the control zebrafish, images of the lateral view of adult skulls were captured using a Nikon SMZ1000 microscope. Twenty-four two-dimensional (x,y) landmarks were applied to the lateral view images of the head (Figure 5-2, Appendix 1, Table 5). The landmarks are the same landmarks that were used in Chapter 2, however fewer landmarks were placed on the zebrafish skull (compared to Chapter 2) because the orbital complex has one less bone than that of the Mexican tetra. In addition, landmarks were only placed within the orbital region, unlike in the Mexican tetra to allow for a concentrated investigation of the orbital area. Landmarks were applied using tspDIG2 software (F. James Rohlf, http://life.bio.sunysb.edu/morph/). The IMP series of software was used for morphometric analysis as described in Chapter 2, section 2.2.7. The statistical difference in shape between the surgery side and the control side of surgery zebrafish was analyzed. In addition, the surgery side of the head was compared to the *bumper* mutant as well as the unoperated zebrafish controls. Landmarks were applied to the right side of four *bum$^{-/-}$* mutants and to the left sides of four *bum$^{-/-}$* mutants (n=8). The individuals were randomly selected in order to represent the natural variation present in the skulls of the mutants.

Principle Component Analysis was performed to compare lens-ablated zebrafish, the control side of the head, unoperated controls and *bumper* mutants, as performed in Chapter 2.

Vector analyses were conducted to compare the average control to the average surgery in the tpsSplin program, as performed in Chapter 2.

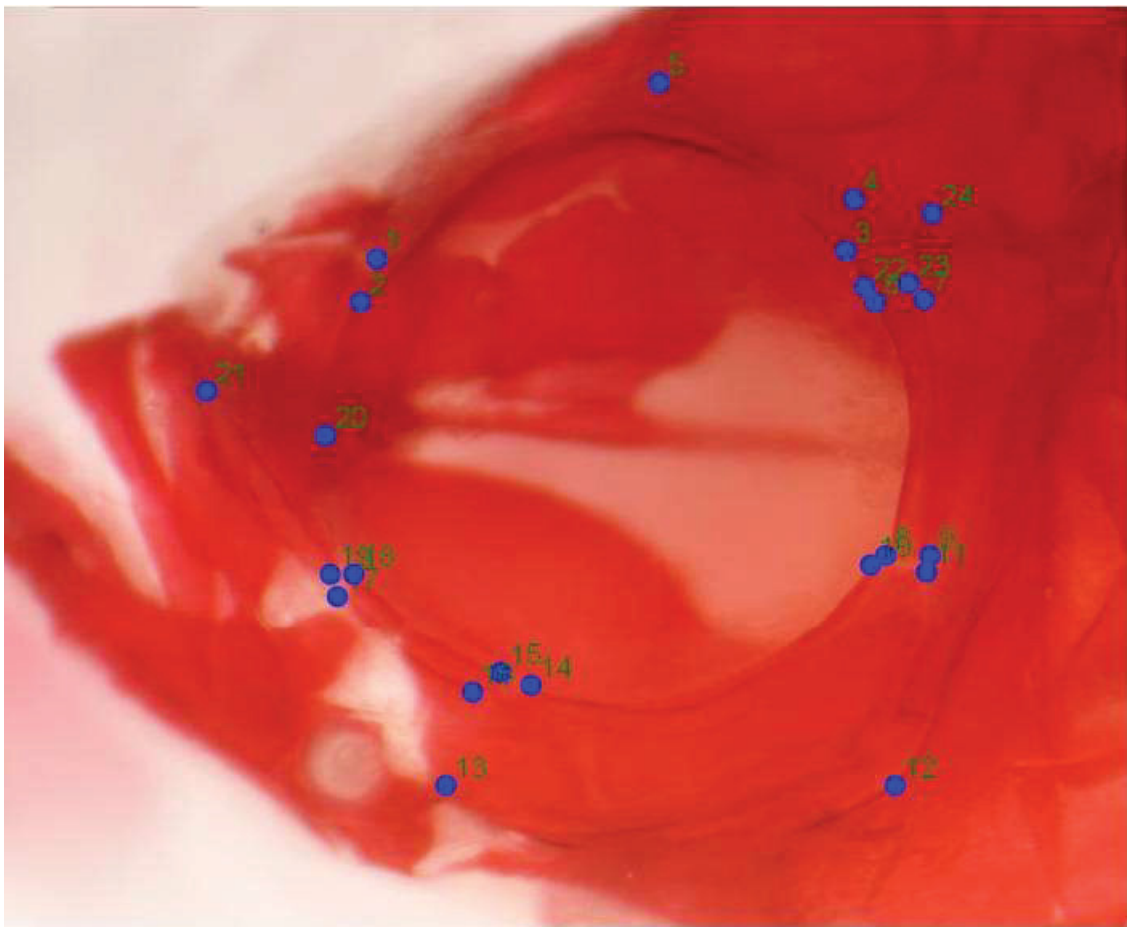


Figure 5-2: Whole mount bone stained zebrafish showing morphometric landmark locations on an adult skull. Lateral view of the skull with 24 landmarks.

5.3 Results

5.3.1 Supraorbital Bone Most Affected by Lens Removal in the Zebrafish

Manual lens removal was performed in wild type zebrafish at 4 dpf, in order to determine how lens removal affects the shape of the surrounding skull. Eye regression was observed in 100% of all zebrafish, which received lens removal (n=3) (Figure 5-3, C-D). The specimens were grown until approximately 2.0 cm SL or adulthood, then whole mount bone stained.

Based on gross morphological analysis conducted on the adult skull it appears that the supraorbital bone was most affected by lens removal. The supraorbital bone was largely expanded into the orbit, normally it exists as a small concave bone dorsal to the eye, after surgery and eye regression the supraorbital bone expanded into a large flat bone covering the dorsal half of the orbit (Figure 5-3). The ventral edge of the supraorbital bone is normally smooth, after surgery it was uneven and rough.

Gross morphological analysis indicated that the suborbital bones 4 and 5 located posterior to the eye were also largely expanded into the orbit (Figure 5-3). Suborbital 4 normally consists of a long narrow bone, which is positioned lengthwise in the dorsal-ventral axis, after lens removal the bone expanded largely into the orbit, becoming much wider than normal. Suborbital 5 expanded in a similar manner to suborbital 4, widening into the orbit. Suborbital 3, the largest bone in the suborbital series expanded along its dorsal edge, expanding into the orbit. The expansion appeared smooth and regular in shape despite its increase in size. Suborbital 2, normally a small thin bone also expanded into the orbit, thickening the element. Suborbital 1, to the anterior of the eye showed very little change from the control fish.

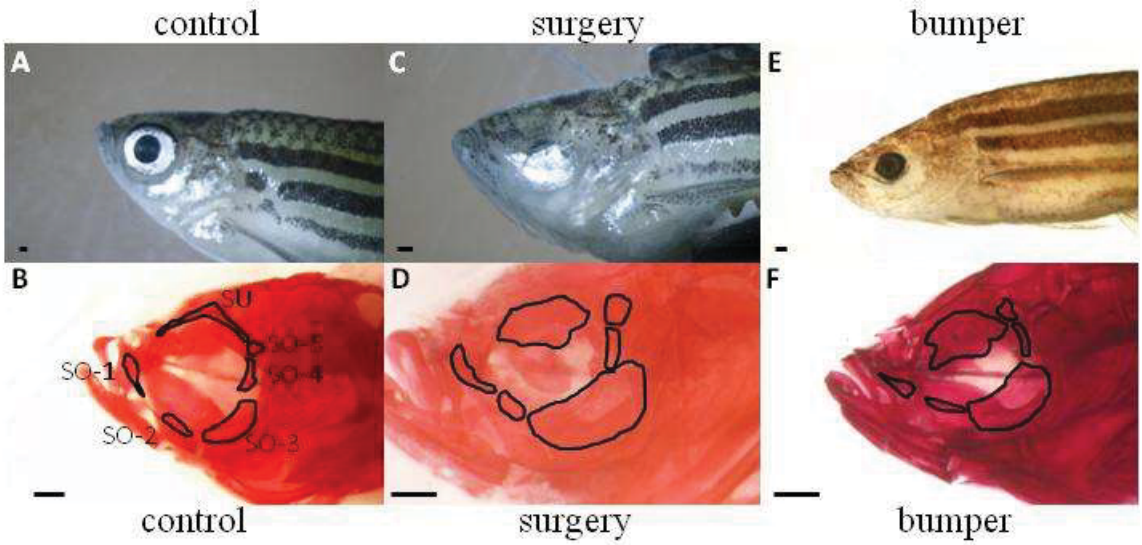


Figure 5-3: Adult skull of control zebrafish, surgery zebrafish, and the zebrafish *bumper* homozygous mutant. A, C, and E are unstained adults. B, D, and F are alizarin bone stained adult skulls. (A-B) are control zebrafish; (C-D) are surgery zebrafish fish; (E-F) *Bumper* mutant. The supraorbital bone (SU), and suborbital bones (SO) 1, 2, 3, 4, and 5 are outlined in the images of stained skulls. Scale bars A, C= 100 µm, B= 200 µm, D, F= 500 µm, E= 200 µm.

5.3.2 Tearing the Cornea Does not Affect the Shape of the Orbital Bones

To ensure the effects on the orbital bones were a result of lens removal, a control experiment was performed where the lens removal procedure was followed with the exception that the cornea overlaying the lens was torn, but the lens was not removed. The specimens were allowed to grow to adulthood, then, whole mount bone stained. Gross morphological analysis of these specimens determined that tearing the cornea does not cause eye regression, 100% of the fish had two normal sized eyes (n=3) and the supraorbital bone and all suborbital bones remain unaltered.

5.3.3 Morphometric Analysis of the Orbital Region After Manual Lens Removal

Twenty-nine landmarks were applied to each the control and surgery sides of the adult head. A consensus of each the control and surgery sides of the head were made using tpsSuper. Shape analysis was then conducted of the consensus using tpsSplin. Warp analysis comparing the control and surgery sides indicated that the eye orbit was largely constricted in the surgery specimens, due to the expansion of the bones above and to the posterior of the eye (Figure 5-4A). Little to no changes in shape were present in the bones below and anterior to the eye. In addition, tpsSplin was used to perform vector analysis (Figure 5-4B). Vector analysis results agreed with the findings from the warp analysis, landmarks and their corresponding bones located to the dorsal and posterior of the eye (supraorbital and suborbital 4 and 5) had large vectors into the orbit, demonstrating that the bones were both expanded and shifted into the orbit. While the landmarks and bones ventral and anterior to the eye (suborbital 1 to 3) had very small vectors, the vectors do not point toward the orbit.

TwoGroup was used to statistically analyze the changes in shape between the surgery and controls sides of the zebrafish, which had their lenses removed. TwoGroup was used to conduct both Goodall's F test and F-test Procrustes. Using Goodall's f-test the surgery and control sides of the same head were found to be significantly different in shape ($F= 2.82$, $p<0.0001$). In addition, F-test Procrustes was performed, however, the two sides were not found to be significantly different ($p> 0.05$). F-test Procrustes is a resampling test, which is hindered by a small sample size, in this study the sample size was limited to only 3 specimens, which is likely confounding the results of the

resampling test, as the analytical Goodall's f-test found strongly significant changes in shape. A low number of zebrafish surgery specimens were available for analysis as zebrafish specimens with one eye did not often survive until adulthood, a much higher mortality rate was present in the zebrafish surgery specimens than the Mexican tetra after the same surgery. Finally, the control side of the head was compared to controls that had not received surgery in TwoGroup. It was determined that the control side was significantly different in shape from the controls without surgery ($f= 4.31$ Goodall's F-test $p<0.0001$, F-test Procrustes $p<0.01$) which indicated that the impact of lens removal affected the shape of the bones on the surgery side of the head and residual effects were also present on the control side of the head.

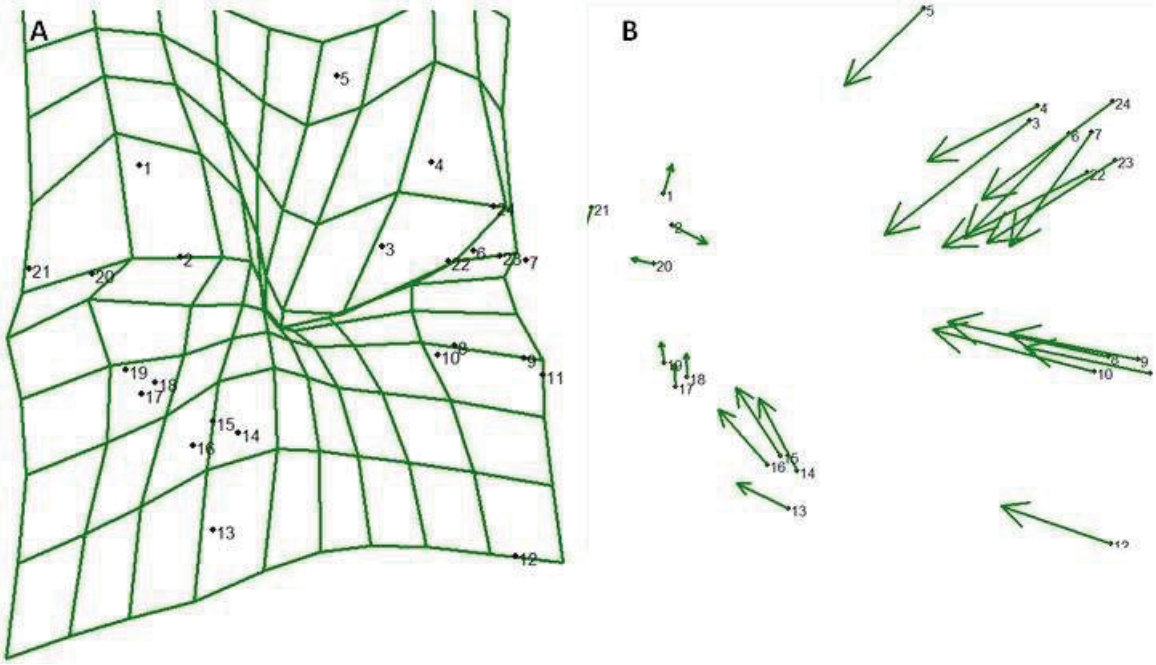


Figure 5-4: Thin plate splin and vector analysis of comparison between the surgery zebrafish and control zebrafish. (A) Deformation grid; (B) vector analysis. The anterior is to the left.

5.3.4 Gross Morphological Analysis of the *Bumper* Mutant Skull

Adult *bum*$^{-/-}$ mutants were whole mount bone stained. Gross morphological analysis demonstrated that the supraorbital bone appeared to be the most different from wild type control zebrafish (Figure 5-3). The supraorbital bone was largely expanded into the eye orbit, with the ventral edge of the supraorbital bone expanded and jagged in the eye orbit, in contrast to the wild type supraorbital bone which is small, smooth, concave (Figure 5-3). The supraorbital bone of the *bumper* mutant is very similar to the surpraorbital bone present around the orbit of the one eyed surgery zebrafish. The supraorbital bone appeared to be the only bone dramatically altered in the *bum*$^{-/-}$ mutant. Unlike after lens removal the suborbital bones 1 to 5 remain normal in this mutant. Small changes could be observed in suborbital 5, this bone appeared to be shifted ventral to its normal position and elongated in the dorsal to ventral axis. In some individuals (2 of 8) suborbital 5 is expanded in the dorsal ventral axis and suborbital 6 is reduced in the same axis, this may be a result of their close proximity.

5.3.5 Morphometric Shape Analysis of the *Bumper* Mutant

Twenty-nine landmarks were applied to the right side of four adult *bum*$^{-/-}$ mutants. The left sides of four different *bumper* mutants' heads were also landmarked, resulting in a totally of eight specimens being used in this study (n=8). A consensus for the *bumper* group was constructed using tpsSuper. The *bumper* consensus was then compared to the control zebrafish consensus using tpsSplin. Warp analysis comparing the controls and *bum*$^{-/-}$ indicated that the area above the eye occupied by the supraorbital bone was expanded, while the eye orbit was constricted (Figure 5-5 A). In addition, the area posterior to the eye, the location of suborbital 4 and 5 was also marginally expanded. Anterior to the eye appeared to be constricted. Little to no changes in shape were present in the bones below the eye (Figure 5-5 A). Vector analysis was also conducted using tpsSplin. The vector plot comparing zebrafish controls to *bum*$^{-/-}$ mutants showed that landmark numbered 1 through 5 have large vectors in the ventral direction, indicating that the supraorbital bone was largely expanded into the orbit (Figure 5-5 B). In addition, surprisingly the vectors indicated that all other landmarks and their associated bones were shifted ventrally and posteriorly, below and away from the orbit with a large magnitude,

rather than toward the orbit, which has been the case in all other comparisons (i.e., the Mexican tetra and surgery zebrafish).

TwoGroup was used to statistically analysis the changes in shape between the *bumper* mutants and control zebrafish. TwoGroup was used to conduct both Goodall's F test and F-test Procrustes. Using Goodall's f-test the *bumper* mutants and control zebrafish were found to be significantly different in shape ($F= 5.07$, $p<0.0001$). F-test Procrustes also determined that the shape differences between the *bumper* mutants and the control zebrafish were significantly different in shape ($p<0.01$).

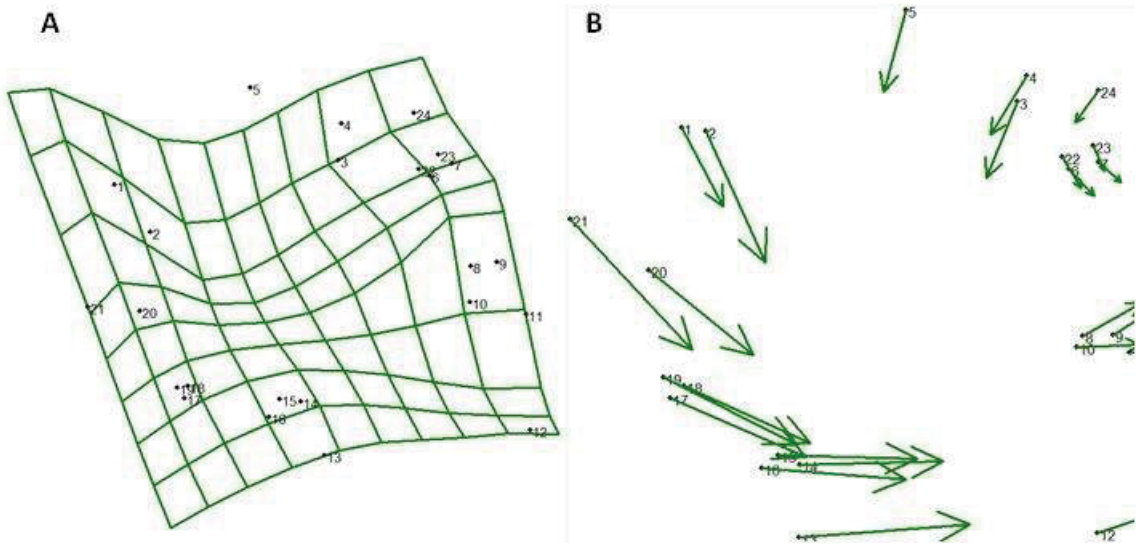


Figure 5-5: Thin plate splin and vector analysis of comparison between the *Bumper* mutant and control zebrafish. (A) Deformation grid; (B) vector analysis. The anterior is to the left.

5.3.6 *Bumper* Mutant to Surgery Fish Comparison

The shape of the adult *bum*$^{-/-}$ mutant skull was compared to the shape of the adult zebrafish skull after early lens removal. Gross morphological analysis of the two groups demonstrated similarities in the shape of the supraorbital bones, but differences in shape in the suborbital bone 1 to 5 (Table 5-1). TpsSplin was used to compare the shape differences between the two groups, in which the surgery group was used as the reference. Based on warp analysis it appears that the orbit of the surgery specimens are much smaller and covered by the surrounding suborbital bones, compared to the size of the orbit in the in *bum*$^{-/-}$ mutants, based on the orbit region of the grid being largely expanded (Figure 5-5 B). In vector analysis, the largest differences between the surgery and *bum*$^{-/-}$ groups were located in suborbital bones 1 to 5. Vectors representing those bones point in the ventral and posterior direction indicating a shift to the more wild-type position of the bones of the *bum*$^{-/-}$ group (Figure 5-6 A, landmarks 6 to 24). Statistical analysis conducted using TwoGroup indicated that differences observed in shape between the mutant zebrafish and the surgery zebrafish were significant ($F= 2.27$, Goodall`s F-test $p<0.0001$, F-test Procrustes $p<0.04$), despite falling in close proximately to the surgery specimens on the PCA plot (Figure 5-7).

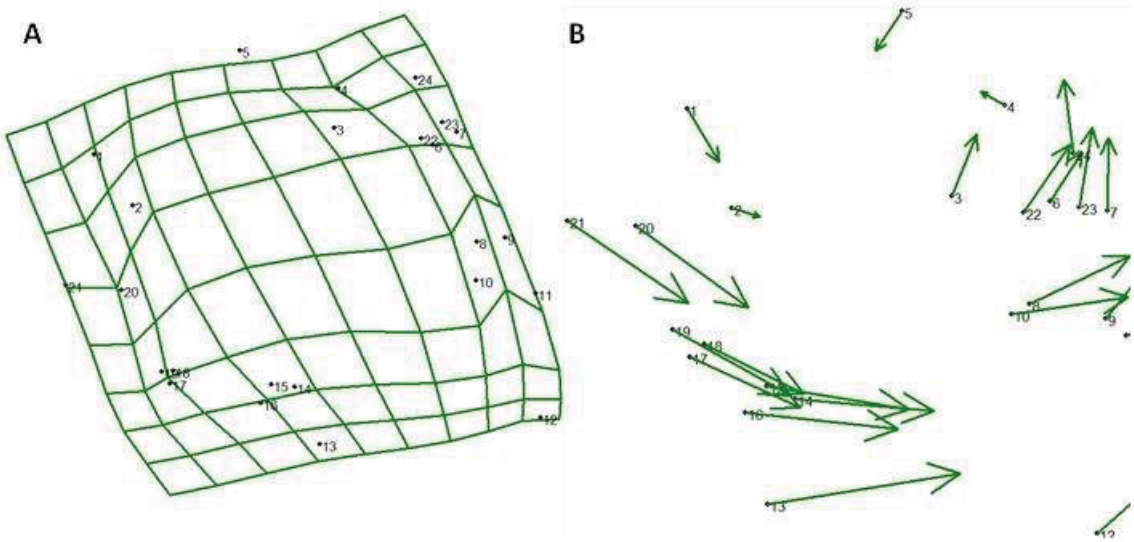


Figure 5-6: Thin plate splin and vector analysis of comparison between the surgery zebrafish and *bum*$^{-/-}$ mutants. (A) Deformation grid; (B) vector analysis. The anterior is to the left.

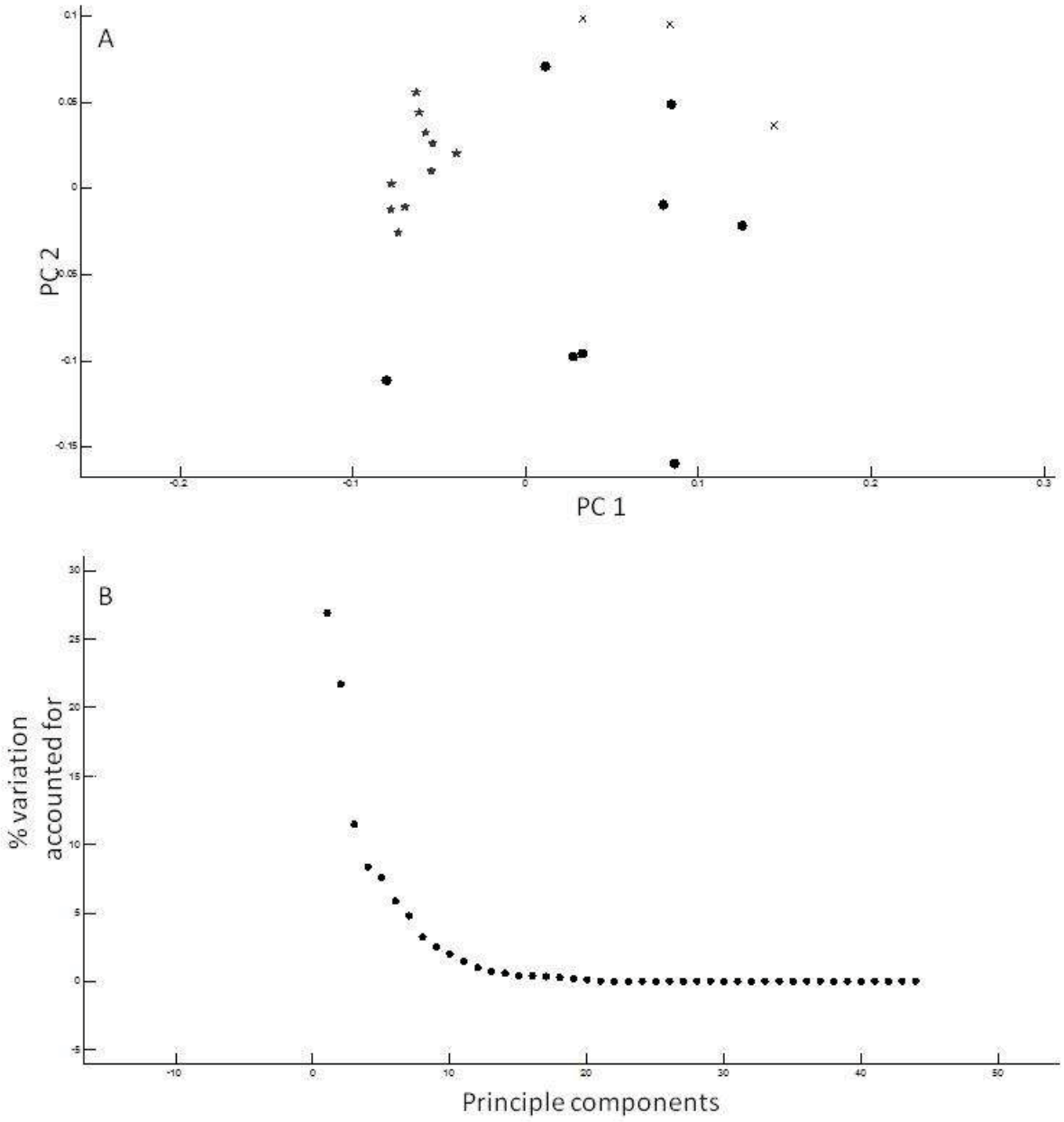


Figure 5-7: Principle component analysis comparing *bum*$^{-/-}$ mutants, surgery zebrafish and control zebrafish. (A) Principle component analysis showing PC 1 and 2, black dots represent the *bum*$^{-/-}$ mutants, stars represent the controls and the crosses represent the surgery zebrafish; (B) graphical representation of the percentage of variation accounted for by each principle component. Each black dot represents one principle component.

Table 5-1: A comparison of orbital bone shape between wild-type, surgery and *bumper*$^{-/-}$ mutant zebrafish.

Bone	Wild-type	Surgery zebrafish	*Bum*$^{-/-}$
Supraorbital bone	small and concave	large and expanded	large and expanded
Suborbital 1	small	slightly expanded	small
Suborbital 2	small	expanded	slightly expanded
Suborbital 3	1 large element	1 large element	1 large element
Suborbital 4	small	slightly expanded	slightly expanded
Suborbital 5	wedge shaped bone	expanded	expanded
Orbit size	large	reduced	slightly reduced

5.3.7 Shape Comparison Between Zebrafish Control, *bum*$^{-/-}$ Mutant and Zebrafish After Lens Removal

A PCA analysis was conducted using PCAGen to compare the shape differences between adult control zebrafish, *bumper* mutants and zebrafish after early lens removal. The generated PCA plot depicted that the *bumper* fish were closer in shape to the wild-type zebrafish, than surgery zebrafish are to the control zebrafish (Figure 5-7). The *bum*$^{-/-}$ specimens fell between the control and surgery points on the PCA plot (Figure 5-7), on both the principle component 1 and principle component 2 axis. The graphical representation of the percentage of variation accounted for by each principle component demonstrated that principle component 1 accounted for approximately 27% of the variation, while principle component 2 accounted for approximately 22% of the variation. Principle component 3 only accounted for approximately 11% of the variation, indicating that principle component 1 and 2 accurately represented the variation present.

TwoGroup was used to determine if the surgery zebrafish were significantly more different in shape from the controls than the *bum*$^{-/-}$ mutants are to the controls. The partial Procrustes distance between the surgery and controls was 0.1826, while the partial Procrustes distance between the *bumper* mutants and the control was 0.1304 the 95% range between the two pairings is -0.0048 to 0.1118, meaning that the *bum*$^{-/-}$ mutants were significantly closer in shape to the controls than the surgery specimens were to the controls. Overall, this datademonstrates that while both the *bum*$^{-/-}$ mutants and the surgery zebrafish are significantly different with respect to the shape of the orbital bones surrounding the eye, the *bum*$^{-/-}$ mutant were closer to the controls.

5.4 Discussion

The objective of this study was to determine if lens removal conducted in zebrafish in early development affects the shape of the bones in the orbital region of the skull. Additionally, this study has provided the opportunity to examine the affects of slow natural lens loss on the skull over the first few months of development. The effects of early lens removal and slow lens loss on the skull were then compared. Finally, a cross species comparison was conducted, in order to determine if lens removal has the same impact on the zebrafish skull as it does on the Mexican tetra skull. This study attempts to

understand if the variability present in the orbital bones is a conserved feature across teleost species.

5.4.1 The Supraorbital Bone is Most Largely Affected by Lens Removal

Manual lens removal performed at 4 dpf in zebrafish embryos has a dramatic impact on the shape of the bones in the ocular region of the skull on the surgery side of the head. The supraorbital bone was most largely affected by lens removal, resulting in a larger surpraorbital bone expanded into the orbit with ventral edge being rough and abnormal in shape. This indicates that the supraorbital bone is the most variable. The bones located to the anterior of the eye were largely unaffected by lens removal, and are likely more constrained.

The variability of bone shape does not appear to be related to the age at which the bones ossify. The orbital bones ossify in the following order 1, 5, 3, 4, and lastly 2 (Cubbage and Mabee, 1996). The most affected bones, including the supraorbital bone, fall in the middle of the ossification sequence, which occurs over a long period of time, indicating that timing of ossification may not be a factor influencing the bones variability in this study. A more likely cause of the changes present in the orbital bones after lens removal are alterations in the orbital bone outgrowth. The ventral edge of the supraorbital bone is highly abnormal, uneven, and jagged, indicating that the outgrowth of the ventral edge of the bone occurs uninhibited and unregulated as a result of lens removal. Lens removal and subsequent eye regression likely changes the mechanical forces within the head. The reduced force on the supraorbital bone, as a result of the small regressed surgery eye, likely results in uninhibited orbital bone outgrowth. The bones located to the dorsal and posterior of the eye may be more variable (to be discussed below), as such they absorb the effect of the reduced eye size, allowing the bones anterior to the eye to maintain their normal shape.

5.4.2 Eye Loss in the *bum*$^{-/-}$ Mutant Results in Similar Skull Changes as Manual Lens Removal in Zebrafish

Tumour-like proliferation, followed by excessive apoptosis in the lens of the *bum*$^{-/-}$ mutant results in a lens-less, small eyed adult zebrafish (Schonthaler et al., 2010). I

examined the orbital bones surrounding the eye of the *bum*$^{-/-}$ mutant to determine if the shapes of the bones are altered by lens loss. Some changes present in the skull of the *bum*$^{-/-}$ mutants are similar to those identified in the surgery zebrafish. Similar changes were identified in the supraobital bone. The supraorbital bone is the most largely affected in both of the groups of fish. In the *bum*$^{-/-}$ mutant, the shape of all other bones of the orbital complex appear to be relatively unaltered by the lens loss, however, the anterior bones appear to be shifted largely in the ventral posterior direction, which was not observed in the surgery zebrafish. Overall, the *bumper* mutant skull is less altered from wild type than the surgery skull. This indicates that lens removal at 4 dpf and gradual lens loss over the first few weeks of development do not have the same effects on the shape of the orbital bones.

Although the skulls of the surgery zebrafish and the *bum*$^{-/-}$ mutant both differ from the control zebrafish, they do not vary from the controls in the same manner as each other, as was hypothesized, likely a result of the difference in lens loss. Differences in the impact of lens removal versus lens loss on neural crest cell migration, timing of lens loss, and differences in the mechanical forces on the skulls will be discussed below.

Removing the lens at 4 dpf ensures that the lens capsule, and the lens containing the epithelial layer, primary fibres cells and secondary fibres have been fully removed relatively early in development. However, cranial neural crest cells begin migration at 14 hours post fertilization in zebrafish (Kimmel et al., 1995), therefore lens removal conducted at 4 dpf likely occurs after cranial neural crest cell migration is complete. This indicates that the effects of lens removal in the zebrafish are likely not a result of effects on neural crest cell migration. In the *bum*$^{-/-}$ mutants, abnormalities in the lens begin at 3 dpf, becoming severe by 5 dpf (Schonthaler et al., 2010), therefore the *bum*$^{-/-}$ lens is likely present and functioning until after neural crest cell migration. Despite the fact that abnormalities are present in the lens epithelium and secondary fibere cells in early development, the primary fibre cells of the *bum*$^{-/-}$ lens form normally and are still present over early development. The normal primary fiber cells maybe capable of fulfilling any required signalling to the migrating CNC cells, thus changes observed in the shape of the *bum*$^{-/-}$ skulls is likely not a result of changes in CNC migration. As a result, a lens is still present within the eyes in both the surgery and *bum*$^{-/-}$ mutant eyes during the critical

times of neural crest cell migration. Further investigation is required to determine if the *bum*$^{-/-}$ lens is capable of cell signalling prior to its loss.

Eye regression in the surgery fish may not occur in the same manner as in the *bum*$^{-/-}$ mutant. Eye regression could not be observed over development of the *bum*$^{-/-}$ mutant as the specimens were received as fixed adult samples. However, due to the extended period in which the lens in present in the mutant eye, eye regression likely occurs over a longer time period (Schonthaler et al., 2010). The differences in eye regression likely result in differences in mechanical forces present in the two skulls over orbital bone outgrowth. As mechanical forces present during orbital bone outgrowth has been proposed to be important in shaping of these bones (Chapters 2 and 4) it is likely that the force disparity between the two skulls influences the skulls in different manners resulting in the differences observed between the skulls.

In summary, as lens removal and eye regression occurs in these two groups after CNC migration, alterations observed in the adult skulls are likely not due to changes in the lens's influences on CNC migration. A more likely cause of the changes observed in the skull, is alterations in the mechanical forces present within the skulls after eye regression. Differences in the shape of the skull bones of the two groups may result from differences in eye regression causing differences in mechanical forces on the bones over development. Despite the differences in the surgery and *bum*$^{-/-}$ skulls, there was one similarity, namely the effect on the supraorbital bone. The supraorbital bone was expanded in the same manner in both groups indicating that as in the Mexican tetra, the supraorbital bone is highly susceptible to change (i.e. not constrained).

5.4.3 Does Lens Removal Have the Same Effect on the Zebrafish Skull as it Does the Mexican tetra Skull?

In Chapter 2, full manual lens removal was conducted between 1 and 4 dpf in the Mexican tetra. In zebrafish lens removal was conducted at 4 dpf. Early development of the Mexican tetra is more rapid than in zebrafish (Hinaux et al., 2011). Due to the differences in timing of development between the two species lens removal conducted at 1 to 2 dpf in the Mexican tetra may be similar in developmental timing to zebrafish lens removal at 4 dpf. All staging was estimated by hours post fertilization, rather than staging

based on morphological features, and thus is an approximation of stage. Unfortunately, the first staging table for the Mexican tetra was not published until late 2011 by Hinaux et al., long after I had performed the lens removal experiments. Having this staging table earlier would have been useful when choosing the surgery time points to compare between the zebrafish and the Mexican tetra.

When the shape changes in the orbital region of the Mexican tetra (Chapter 2) after lens removal are compared to the shape changes that occur after lens removal in the zebrafish, it is clear that similar alterations are present in both fish species (Figure 5-8). In both species the most affected bones are located dorsal and posterior to the eye, specifically the most affected bone, the supraorbital bone, which expands largely into the orbit. Additionally, the suborbital bones located posterior to the eye are also largely expanded into the eye orbit. Finally, the largest suborbital bone located ventral to the eye is also affected in both species. In both the Mexican tetra and the zebrafish very little to no changes are present in the bones located anterior to the eye. Remarkably similar changes in the orbital region of both fish species are present after lens removal. A further analysis to compare the size changes in the bones may be useful. For example, by measuring the areas occupied by each bone of the orbital region one could determine the percentage of area that changes after surgery, and then compare these changes were across species.

The orbital regions develop in a similar manner in the two species, with respect to ossification type and timing. As similar changes were observed in the skulls of the two species after lens removal it suggests a conserved nature of the bones. There is a high level of variability in the supraorbital bone and the suborbital 4 and 5 bones in each species, similarly, suborbital 1 to 3 are relatively stable in shape in both species indicating a conserved nature of these traits between these species of fish.

Due to the similar nature of the response to lens removal between the two species, I hypothesize that the same mechanism is acting in both species to cause the similar shape changes in the orbital region. In Chapter 2, I hypothesized that lens removal may likely affect the shape of the orbital bones through signals from the lens either increasing neural crest cell migration or increased local cell proliferation. Alternatively, I suggested that removing the lens and subsequent eye regression changes the mechanical forces

within the head allowing expansion of some bones into the orbit. Examining the effects of lens removal in the zebrafish provides further insight into which mechanism is more likely to have influenced the shape of the orbital bones. In zebrafish lens removal was conducted at 4 dpf, while CNC migration begins at 14 hours post fertilization, indicating that the majority of migration has likely completed by the time the lens was removed. I therefore hypothesize that the observed changes in the orbital region of the two species is not likely a result of alterations in cell signalling influencing CNC migration and/or proliferation. Changes in mechanical pressures within the head are more likely mechanism influencing the shape of the orbital bones.

These findings indicate that the changes in the mechanical forces on the supraorbital bones of both the Mexican tetra and zebrafish may be responsible for the dramatic changes in shape, which were observed in Chapters 2, and the present study.

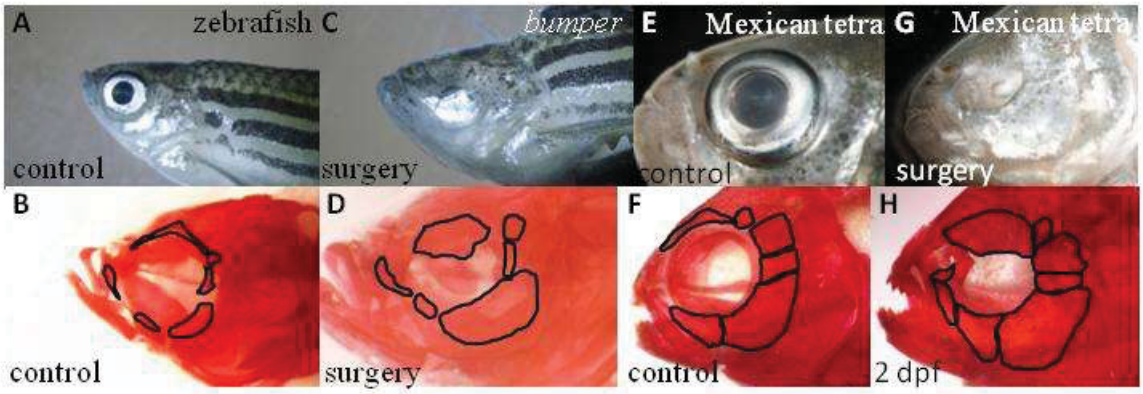


Figure 5-8: A comparison between surgery zebrafish and surgery Mexican tetras. (A-D) adult zebrafish; (E-I) adult Mexican tetra. (A) whole mount control zebrafish; (B) whole mount bone stain control zebrafish; (C) whole mount surgery zebrafish; (D) whole mount bone stained surgery zebrafish; (E) whole mount control Mexican tetra; (F) whole mount bone stained control Mexican tetra; (G) whole mount surgery Mexican tetra, surgery at 2 dpf; (H) whole mount boned stained surgery Mexican tetra, surgery at 2 dpf.

5.5 Summary

Lens removal conducted in zebrafish resulted in eye regression and shape changes in the supraorbital bone and bones located to the posterior of the eye. The skulls of lens-less *bum*$^{-/-}$ mutants were examined in order to determine if lens removal in early development has the same affect on the skull as lens loss in the *bum*$^{-/-}$ mutant. In both the surgery group and the *bum*$^{-/-}$ group the supraorbital bone was most affected. In both groups the supraorbital bones were highly expanded into the eye orbit. Differences in the shape of the other orbital bones were present between the two groups. Overall, the changes observed in the zebrafish skulls, which lack a lens are highly similar to those observed in the lens-less Mexican tetra. In both the zebrafish and the Mexican tetra the supraorbital bones were most affected by lens removal, in addition, the suborbital bones located posterior eye were also influenced. Based on this analysis it appears as though the the ability of an orbital bone to vary is conserved across species. The findings in this chapter indicate that lens removal or a lack of a lens effects the orbital bones by changing the mechanical forces present on the bones, influencing them to expand into the orbit. Finally, these studies suggest that the highly variable nature of the supraorbital bone maybe a result of the position it occupies within the head.

Chapter 6: Discussion and Conclusions

6.1 Discussion

The objectives of this thesis are to investigate in what way altering soft tissues during early development can influence the formation of the skull, and secondly, to investigate the ability of the skull and its elements to vary in shape. Upon performing these investigations a number of other fascinating relationships were identified, including the lens's influence on the number of teeth, taste buds, and a highly unusual capacity for lens regeneration in the Mexican tetra.

6.1.1 The Variability of the Teleost Skull

In Chapter 2, the lens was removed from the surface fish eye to determine how lens removal influenced the skull. In Chapter 4, I compared the skulls of the surgery surface fish to the lens-less cavefish and an intermediate (F1 between cross of surface fish and cavefish) to determine if natural lens loss and manual lens removal influence the skull in the same manner. Finally in Chapter 5 lens removal was performed in the zebrafish and a lens-less zebrafish mutant was observed in order to determine if lens removal has the same impact on the skull across species.

Based on the results from the aforementioned studies it is clear that some bones of the teleost skull are highly adaptable, as they were observably variable in shape while others were not. Surprisingly, in the surgery surface fish, intermediate, cavefish, surgery zebrafish and the *bumper* mutant mostly the same bones were determined to be variable in shape as a result of lens absence or the presence of a small lens (i.e. intermediates). Each study demonstrated largely the same findings, that the shape of the supraorbital bone was most influenced by lens removal, and thus the most variable in shape. Smaller changes were observed in the shape of other bones present in the orbital region, namely the bones located posterior to the eye, while no large alterations in shape were present in any of the studied groups in the bones located anterior to the eye. These studies indicate that the lens has a similar influence on the shape of the bones of the orbital region across both morphs and species. This indicates that some bones of the teleost skull are variable, while others are constrained, likely a conserved factor amongst teleosts.

While many studies demonstrate the vast capacity for the variability of the shape of the bones in the oral region of the skull (Meyer, 1987; Bouton et al, 2002; Ornsrud et al, 2004; Fernandez et al, 2008), very little literature has documented the variable nature of the bones in the orbital region. The studies that have documented variation in the orbital region study the effects of treating juvenile teleosts with harsh teratogens such as ethanol and high levels of retinoic acid. The studies do not provide an in-depth description of the effects on these bones, but simply state that the bones are altered in shape (Vandersea et al., 1998; Carvan et al. 2004).

Yamamoto et al., (2003) were first to report how manipulation of the lens could affect the shape of the bones of the orbital region in the Mexican tetra. This study sparked our lab's interest in this area of the skull and the vast differences in variation present in these bones. The authors first described differences present between the cavefish and surface fish skulls. Yamamoto et al., (2003)performed experiments in which they transplanted a cavefish lens into surface fish eyes at 24 hours post fertilization. When the specimens reached 6 months of age they were stained to analyze the effects on the skeleton. Yamamoto et al., (2003) determined that some features were dependant on the lens, while others were determined to be independent from the lens. The following structures were determined to be independent from the lens: maxillary tooth number, the number of suborbital 3 (SO-3) elements, the shape of SO-3, and the shape of the opercular bone. A number of structures were determined to be dependent on the presence of the lens including: size of the nasal and antorbital bones, width of the olfactory pits, the shape of supraorbital bone and SO-3, scleral ossicles and the SO 4-6 bones were determined to be weakly dependant. Unfortunately, the authors performed their analysis on specimens which they presumed to have a fully developed skull, however, the specimens were too young and skull development was incomplete. This error resulted in inaccurate analysis of the scleral and some of the suborbital bones. No further investigation had been conducted after the Yamamoto et al., (2003) study, leaving a gap in the literature describing the variable nature of the bones of the orbital region of the skull and how the shape of these bones can be influenced. Despite the shortcomings of the previous study, it provided a reference point for beginning to unravel the interactions between the lens and the skull. My findings largely agree with their analysis, specifically,

that the supraorbital bone is most influenced by lens removal, the independence of SO-3 elements and maxillary tooth number, however they conclude that SO 4-6 are only weakly affected by lens removal, while I determined that there was a significant impact to the shape of the bones. In addition the authors identified alterations in the nasal and antorbital bones, while I did not. The different findings are likely a result of Yamamoto et al. (2003) analysis having been conducted on young adults in which the suborbital bones had yet to fully ossify. Overall the studies have complimentary findings, indicating that some bones are highly influenced by the lens, while others are not.

My study has demonstrated that the lens does in fact play an important role in the development of the skull and we now know which bones of the skull are variable in shape, allowing us to investigate how the lens is influencing the skull, and why some bones are more variable or show more changes after treatment than others.

6.1.2 How Does the Lens Influence the Skull?

The data described in Chapter 2 indicates that the earlier the lens was removed the greater affect it had on the shape of the bones in the orbital region. There are many different factors which influence the size and shape of bones. In Chapter 2, I hypothesized that changes is the shape of the orbital bones after full lens removal was a result of an early increase in condensation size, either from an increase in CNC migration into the condensations and/or an increase in cell proliferation. An increase in CNC migration is a more likely cause, as little evidence exists indicating that proliferation influences condendation size, while evidence of CNC migration into condensations has been well documented (e.g. Hall and Miyake, 2000; Franz-Odendaal, 2008). Alternatively, alterations may be a result of changes in signalling normally provided by the lens. In addition, I hypothesized that there may also be a late effect influencing the degree of orbital bone outgrowth. Later effects likely entail alterations in mechanical forces within the eye region. The mechanical forces may be altered after lens removal and subsequent eye regression as the eye no longer held its normal position within the skull, exerting forces on the surrounding bones. In addition, I observed changes in the extra-ocular muscles after lens removal indicating that lens removal affected eye size, in turn affecting the development of the ocular muscles, and thus the shape of the bones on

which they insert. Although there are a number of different mechanisms that control and influence bone shape, I have focused on a few of the possible influences which my data relates to. Throughout Chapters 2 to 5 evidence on the variability of the bones of the orbital region was presented. In addition, this evidence enabled me to suggest what mechanism is most likely responsible for the alterations in bone shape.

Evidence for elucidating the mechanism by which the lens influences the bones of the orbital region was gained by comparing the data from Chapters 2, 3, and 5. In Chapter 5 the lens was removed from the eye of the zebrafish a 4 dpf. As was stated previously cranial neural crest cells begin migration at 14 hours post fertilization in zebrafish (Kimmel et al., 1995), therefore lens removal conducted at 4 dpf likely occurs after cranial neural crest cell migration. This indicates that the affects of lens removal are likely not a result of effects on neural crest cell migration, as was hypothesized in Chapter 2.

In Chapter 2, I examined the shape of the supraorbital bone just after it had ossified, in both surgery and control specimens. In the surgery specimens the supraorbital bone was slightly larger than the control supraorbital bone. This data suggests that there likely was not a large increase in migration into the supraorbital bone condensation or a large increase in local cell proliferation, if either of these two alterations had occurred I would have expected to see a much larger bone on the surgery side of the head just after ossification. These results suggest that early lens absence does not influence CNC migration or local cell proliferation, as was proposed after Chapter 2, however, this assumption requires further investigation.

Alternatively, the lens may be providing direct or indirect signalling to the surrounding skeleton which was removed with lens removal. In Chapter 3, I demonstrated that early laser lens damage (between 1 and 11 dpf) does not impact the shape of the orbital bones in adulthood. Additionally, I demonstrated if the lens is absent in early life (between 1 and 8 dpf) and then regenerates by 8 dpf, and is maintained through to adulthood there is little to no effect on the shape of the bones in the orbital region. This data from Chapter 3 therefore demonstrates that the alterations which were observed in the shape of the bones of the orbital region after permanent lens removal is not a result of lens absence during early life. Overall, the data indicates that the absence

of the lens during early life only, does not affect the shape of the bones of the orbital region in adulthood. As such the absence of the lens or the presence of a small eye later in life, during orbital bone ossification and outgrowth is responsible for the shape changes observed after lens removal. Thus, any signals from the lens to the surrounding skeleton during early life may not be critical for the development of the surrounding skull, alternatively, there may not be any signals from the lens. These findings indicate that there is a critical developmental window in which the lens can influence the development of the skull.

As was stated previously, the circumorbital bones of the Mexican tetra are late forming bones, which do not ossify until approximately 22 mm body length, at approximately three to four months of age (Yamamoto et al., 2003). Skull development continues late into life, and is mature at approximately one year of age. I hypothesize that the absence of the lens during the timing of ossification onset and outgrowth results in changes in the shape of some of the bones of the orbital region, due to alterations in mechanical forces.

Mechanically the eye holds an important position within the head; when the eye is no longer present the bones of the skull are free to expand without constraint. The expansions observed in the circumorbital bones were consistently from the free edge of the bone and were always directed towards the space previously occupied by the eye. This indicates that the forces which the eye would normally exert on the surrounding skeleton are removed by the reduced eye size, thus allowing the outgrowth of the circumorbital bones to occur without constraint. In Chapters 2, 4 and 5, specimens which did not have a lens present and had a regressed eye during orbital bone ossification and outgrowth had numerous alterations in the shape of the circumorbital bones. Mechanical forces may act directly on the circumorbital bones from the eye or indirectly through the alteration of extra-ocular muscles after lens removal.

In chicken and some mammals, the eye produces important mechanical forces within the head (Coulombre and Crelin, 1956). When the eye is removed in early life in chicken, it can no longer exert mechanical forces on the surrounding skull, as such the orbit is reduced in size and the orbital bones expand into the orbit (Coulombre and Crelin, 1956). In other studies conducted in the chick, the lens was removed from the eye

primordial at four days of incubation, the effects on the skeleton were then observed at 5, 7 or 9 days after surgery. Abnormal proportions develop in the skull, with a shorter depressed head, as well as alterations in the cartilages surrounding the eyes. In some individuals the effects of lens removal could be detected in the upper beak, resulting in deviations in beak shape (Tonneyck-Muller, 1976). Alternatively, when the size of the eye is increased the size of the orbit increases as the orbital bones become smaller. In rabbits, glaucoma was induced in young individuals, resulting in an increase in eye size. The increase in eye size resulted in an enlarged eye orbit (Wesseley, 1920). These studies indicate that eye size has an important role to play in the shape and size of the surrounding orbital bones, a relationship which appears to be highly conserved. In summary, mechanical forces have a dramatic impact on the surrounding skull, they can influence the orbital bones to either expand or regress based on the size of the eye. This also indicates that the bones surrounding the eyes are largely variable in shape, and can be easily modified by changes in the neighbouring structures. The results of eye or lens removal in chicken closely resemble the result of lens removal in the Mexican tetra, this indicates that mechanical forces maybe the primary source of alterations in the skull of the Mexican tetra after lens removal.

One must consider how the eye might influence the size and shape of the orbital bones other than the direct exertion of forces on the surrounding bones. My data indicates that the reduced eye size also affects the muscles associated with the eye, specifically, the ocular muscles. There are three extraocular muscles in the zebrafish, which are thought to be conserved in all teleosts (Schilling and Kimmel, 1997). The extraocular muscles insert outside of the eye on the surrounding skeleton. Lens removal and subsequent eye regression may cause alterations in the development of these muscles which may in turn influence the development of the bones on which they insert, thus eye size may directly and indirectly affect the size and shape of the orbital bones.

To determine if the mechanical influence on the skull is a result of a direct or indirect effect, further studies will be required. A more in depth analysis of the alterations present in the extraocular muscles, such as extraocular muscle analysis over development and during orbital bone ossification, is necessary. Origin and insertion points, and muscle length should be analyzed over development and in adulthood, to determine how the

muscles are altered. Additionally, muscle analysis should be conducted in the other groups examined in this study including, surgery zebrafish, *bumper* mutants, cave fish and intermediates to better understand if and how eye regression influences the extraocular muscles. Or alternatively, does simply providing more space influence the shape of the bones, allowing expansion into open areas. Regardless of the mechanism which leads to the shape changes in the bones of the orbital region it is likely that epigenetic changes facilitate the alterations observed in bone shape.

6.1.3 Why are Some Circumorbital Bones More Variable Than Others?

In Chapter 1, I summarized a number of factors which can affect the variability of a bone. I deduced that timing of ossification, ossification type, cell origin and the location of the bone within the skull may influence the adaptibility of a bone. Each of these influences will be discussed below and how they relate to the variations observed in this study.

In all of the fish groups examined in Chapters 2 to 5, the supraorbital bone was most affected by lens manipulations. The supraorbital bone ossifies fairly late in development, after the vast majority of the skeleton outside of the orbital region has already ossified (Cubbage and Mabee, 1996; Yamamoto et al., 2003). Eye regression after lens removal in both the Mexican tetra (Chapter 2) and the zebrafish (Chapter 5) appears to be a relatively slow process with the surgery eye being 30 to 40% smaller than the control eye by 15 to 20 days after lens removal (Chapter 2). By 15 to 20 dpf in both the Mexican tetra and in the zebrafish a large portion of the skull has already begun to ossify (Cubbage and Mabee, 1996; Yamamoto et al., 2003). Due to the slow process of eye regression after lens removal the early forming bones of the skeleton may not be affected by lens removal, while the later forming bones such as the orbital bones, which ossify after the eye has regressed, may be more impacted by lens removal. If this true, it would be expected that the most affected bone would be the last bone to ossify. Within the orbital complex the supraorbital bone ossifies around the middle of the ossification sequence of the orbital bones in both species (Cubbage and Mabee, 1996; Yamamoto et al., 2003), thus timing of early versus late ossification may be relevant to the variability

present in the orbital bones, however, if a bone ossifies in the middle of a sequence of late forming bones well after eye regression, may not influence the variability of a bone.

In all fish examined in this study, the bones of the orbital complex ossify through intramembranous ossification (Cubbage and Mabee, 1996; Yamamoto et al., 2003). In the review of bone variability (Chapter 1) it was unclear if there is a link between ossification type and bone variability. As all of the bones of the orbital complex and the vast majority of the Mexican tetra skull ossify via intramembranous ossification, ossification type does not provide further evidence for the high level of variability present in some bones and not in others. These findings agree with the studies reviewed in Chapter 1, in which the data indicated that ossification type may not influence the variability of aa bone.

In the fish examined in this thesis, the bones of the orbital complex and a large portion of the skulls are presumed to be neural crest derived (Kague et al., 2012). The literature reviewed in Chapter 1, indicated that cell origin has a strong influence on the variability of bone shape. If cell origin was largely responsible for the variability in the shape of the bones I would expect that the bones of a similar origin to be similarly affected. In particular, I would expect to observe the same level of variation present in all of the circumorbital bones since all of these bones are CNC derived. However the vast shape alteration observed in Chapters 2, 4, and 5 in surgery surface fish, cavefish, surgery zebrafish and the lens-less zebrafish mutant were solely located dorsally and posteriorly to the eye. Therefore, this data demonstrates that some bones which are CNC derived are variable in shape, while others are not, indicating that in this case cell origin may not impact the variability of all bones.

In Chapter 3, I hypothesized that alterations in mechanical forces on the bones are likely responsible for the changes in shape after lens manipulations. Thus the characteristic of a bone which may most influence the variability of the structure are mechanical forces exerted on it and how easily the mechanical forces can be altered. Therefore, bone location appears to play a vital role in variability of the bone. In each of the fish groups examined the supraorbital bone occupies an important position within the head. Although it is the same distance from the regressing eye as all of the other orbital bones, it is unique in the length of the orbit in which it borders. The supraorbital bone holds the position in the complex, which occupies the greatest space on the border of the

eye orbit, while the other orbital bones have minimal free edges bordering the orbit. The supraorbital makes up the entire length of the dorsal border. Particularly the bones located anteriorly to the eye are small with very little free edges falling on the eye orbit. With the decrease in forces on the orbital edges of the bones after eye regression, it would reason that the bones with the largest free edge now uninhibited by the eye would expand the greatest into available space. As such the large plate-like supraorbital bone expanded over the orbit. In some individuals (Chapter 2) the supraorbital bones expanded until they nearly covered the entire orbit. The other circumorbital bones are largely sandwiched by each other leaving the supraorbital bone largely uninhibited on the orbital and anterior edges. Thus, the location of a bone, in this case the supraorbital bone, may strongly play a role in the variability of this bone.

The calvariae were determined to be highly stable in shape after lens removal (Chapter 2) this is likely a result of location. The calvariae are largely surrounded by other bones of the skull, with very little to no free edges in which to expand. Based on the location the calvariae there is likely a constant level of mechanical forces present constraining them, due to the bones' location wedged in between other bones of the skull. In addition, the calvariae also form sutures with the adjacent bones, further constraining the shape and size of these elements (Quarto and Longaker, 2005). This constrained nature may be a feature which evolved to protect the delicate brain, which lies directing below these bones. It would stand to reason that it would not be advantageous to have bones which are highly variable in shape protecting a delicate structure which is vital for life.

In addition to the changes in mechanical forces as a direct consequence of eye regression, the location of a bone may also play an important role in the variability of a bone as a result of indirect mechanical changes. Adult zebrafish have six extraocular muscles which insert on the bones surrounding the eyes (Schilling and Kimmel, 1997). The extraocular muscles form early in development, prior to the ossification of the orbital complex (Schilling and Kimmel, 1997). In Chapter 2, I demonstrated that some of the extraocular muscles were affected by lens removal. Therefore, the bones on which the altered muscles insert likely experience changes in the mechanical forces which the extra-ocular muscles exert on them. As was stated previously, changing the muscle force on a

bone i.e. the arm of a tennis player, can largely impact the size of the bones (Daly et al., 2004). Thus, again the location of a bone may play a major role in thevariability of the bone, as the location they occupy maybe be largely associated with muscles which are influenced by lens removal.

One alternative that has been largely overlooked in this study is bone remodeling. Bone remodeling (bone reabsorption and deposition) has been demonstrated to play and important role in the shaping of the skeleton (e.g. Daly et al., 2004; Albertson and Yelick, 2007;Witten and Huysseune, 2009; Edsall and Franz-Odendaal, 2010). Changes in the mechanical forces excerted on a bone can also influence the amount of remodeling; as such bone remodeling may be acting in conjunction with alterations in mechanical forces to produce the dramatic changes observed in some of the bones in the orbital region after lens removal. Further investigation to visualize bone remodelling, such as comparing the remodelling present in a control versus surgery supraorbital bone will behighly informative in unraveling the mechanism(s) responsible for the alterations observed after lens removal.

These findings indicate that the location of a bone may be the key factor in determining the variable nature of a bone. The more constrained a bone is by surrounding structures the less likely it is to vary in shape, given the space and opportunity many bones may be variable in shape. To further investigate this finding it may be useful to ablate a bone during early development to remove its forces on the neighbouring bones and then determine if the neighbouring bones expand into the open space. A further investigation is required to determine if some bones are variable enough in shape, that they simply expand into available space.

6.1.4 What We Have Learned About the Surface Versus Cavefish

This study provides some interesting insight on the similarities and differences between the two morphs of the Mexican tetra and what influences have led to these differences. Namely, I investigated alterations in skull shape, number of skull bones, and the number of taste buds and teeth.

The first aspect of the cavefish which drew my attention was the differences present in the shape of the skull bones surrounding the eyes of the two morphs.

Yamamoto et al., (2003) reported that placing the lens from the cavefish eye into the eye of a surface fish resulted in the surface taking on a more cavefish-like skull morphology. In Chapter 2, I removed the lens from the eye of the surface fish to determine how lens loss would affect the development of the skull. In Chapter 4, I investigated if lens removal in the surface fish resulted in a more cavefish-like skull. Based on these investigations, I determined that lens removal conducted in the surface fish does result in a more cave-fish-like skull, however, it does not fully resemble the cavefish morphology. While the shape of the circumorbital bones in the surface fish took on a more cavefish-like morphology the number of elements present in the orbital region did not take on a cavefish-like morphology or pattern. As stated in Chapter 4, the orbital region of the cavefish skull is highly variable in nature, both in the shape of the bones in the orbital region, and the number of elements that make up each bone of the orbital region. In the cavefish the number of the small elements that make up suborbital bones 1, 3, 4, 5, and 6 are highly variable, for example suborbital 1 may consist of one to five elements, or any number in between. In the surface fish these bones only consist of one element, after lens removal this did not change. This indicates that although the lens is involved in influencing the morphology of the cavefish skull, it is not involved in other changes such as the number of elements present in the orbital region. Thus, the lens is not solely responsible for transitioning a surface skull to a cavefish skull. I hypothesize that either genetic differences between the two morphs, alternatively, developmental events which occur prior to lens removal are responsible for the differences in the skulls. In a single species, I would expect that the same physical change, in this case the absence of a lens, would yield roughly a similar impact, however, the absence of the lens affects the cave and surface morphs differently. If genetic changes are responsible for the skull differences, then these findings suggest that the morphs are stable, independent groups, with genetic differences perhaps pushing the Mexican tetra morphs toward speciation. As discussed in Chapter 4, a candidate gene approach identified alterations in the midline expression of *shh* and the decrease in *pax6* expression, the alterations in expression may influence the development of the cavefish skull (Yamamoto et al., 2009). I propose that the changes in the skull may be a result of other genetic changes which have yet to be identified. At this time, the genome of only one cavefish population (the Pachon

population) has been sequenced, the surface fish genome has yet to be sequenced, but is scheduled for the near feature. This will allow for the better identification of additional genetic differences between the two morphs. I hypothesize that the number of orbital elements present in the orbital region of the cavefish are exclusive characteristics of its genome that are not replicable in surface fish lacking a lens.

Although the number of elements present in the cavefish orbital region does not appear to be solely controlled by the presence of the lens, it does appear as though tooth number is largely influenced by lens presence. When the lens was removed from the eye of the surface fish (Chapter 2) the number of small caudal teeth increased on the surgery side of the head. In Chapter 4, it was determined that the cavefish have significantly more small caudal teeth than the surface fish. By comparing the data from Chapters 2 and 4 it appears as though simple lens removal dramatically impacts the number of small caudal teeth, resulting in a more cavefish-like phenotype in the surface fish. The effects of lens removal on tooth number will be discussed further below.

Lens removal does not influence the number of taste buds. In Chapter 2, I investigated how lens removal conducted in the surface fish affected the number of taste buds. I hypothesized that lens removal in the surface fish would result in an increase in taste bud number on the surgery side of the head, as the cavefish have been documented to have as much as a five to seven fold increase in taste bud number compared to the surface fish (Schemmel, 1967; Varatharasan et al., 2009). After lens removal the numbers of taste buds present on the surgery side of the head were counted. No significant differences were present in the number of taste buds on the surgery side of the head after lens removal compared to the control. This finding indicates that lens removal alone does not impact the number of taste buds. As described previously, the concept of modularity has been applied to the various regressive and constructive traits of the Mexican tetra. Franz-Odendaal and Hall (2006) proposed that there is a regressed eye module and constructive taste bud module present in the cavefish, which may interact at the gene level. Yamamoto et al. (2009) confirmed that these two modules are linked through the expression of *shh*. My data agrees with these studies, as simple lens removal did not impact the number of taste buds, indicating that taste bud number has a more complex control, likely genetic.

Clearly, while some of the morphological differences between the cavefish and the surface fish are a direct consequence of lens loss, such as the shape of the orbital bones, and perhaps the number of teeth, other differences, such as taste bud number and orbital bone number are not directly linked to lens loss. I hypothesize that they are a result of genetic changes between the two morphs. Yamamoto et al. (2009) propose that the increased midline expression of *shh* in the cavefish has pleiotropic effects, influencing both taste bud number and initiating lens loss. The authors propose that many of the morphological differences in the two morphs are a result of this alteration in *shh* expression. I hypothesize that genetic differences between the two morphs requires further investigation to fully understand what genetic changes are influencing differences in the morphologies.

Having the genome of the surface fish and multiple cavefish populations sequenced will provide great insight as to forces driving the morphological changes between the morphs. The small amount of molecular analysis which has been conducted on this fish species was performed as a candidate gene approach. Based on the studies which largely focused on describing lens loss, and eye rescue experiments conducted by interbreeding different cave populations, it is clear that there are some significant genetic differences between the two morphs. Although there are genetic differences between the two morphs they are still able to interbred, indicating that they are two morphs of a single species, if you consider the biological species concept, isolation species concept, or the recognition species concept (de Queiroz, 1998). According to the three aforementioned species concepts, due to the vast amount of gene flow that is able to occur between the surface population and many of the cave populations, speciation has not fully occurred. These concepts indicate that the two morphs may represent a species still undergoing a speciation event, an event which has not completed due to the presence of occasional gene flow between the populations. However, if you consider the evolutionary species concept, which states that even if populations can interbred, if the populations are separate lineages from an ancestral population, which maintain their identity from other lineages and have their own evolutionary tendencies they are separate species (de Queiroz, 1998) then the Mexican tetra maybe considered to be multiple species. If you consider my data in relation to the Evolutionary species concept my findings provide

further evidence to demonstrate the divergence of the populations into two species. My research demonstrates additional phenotypic differences between the populations which were previously unknown, such as caudal tooth number, suborbital bone number and shape, and differences in the capacity for lens regeneration and healing, in addition these results indicate that there are likely more genetic differences between the morphs than were previously hypothesized. These newly discovered phenotypic differences between the morphs strengthen the support for two separate species as they demonstrate the individual identities of one morph from the other lineage.

6.1.5 Lens Removal Influences the Number of Caudal Teeth

Lens removal conducted in the surface fish (Chapter 2) yielded a very surprising result, lens removal conducted in early life influenced the number of small teeth present on the caudal portion of the lower jaw. Previous research determined that cavefish have an increased number of maxillary teeth when compared with the surface fish (Yamamoto et al., 2003), however, the differences in the number of teeth present on the mandible has not been documented. In my research I determined that both the surface fish and the Tinaja cavefish have eight large multicuspid teeth located on the central portion of the lower jaw, while variations in tooth numbers arise on the caudal portion of the jaw. In contrast to the centrally located teeth, the caudal teeth are much smaller in size and may be uni or multicuspid. The number of small caudal teeth present appears to be highly variable in nature, while the number of large centrally located multicuspid teeth is not variable in adulthood.

In Chapter 2, I investigated the average number of teeth present on the lower jaw on the control side of the head. On average the control side of the head has 1.6 ± 0.9 small teeth located in the caudal region. In Chapter 4, I determined that on average the Tinaja cavefish have 6.4 ± 1.4 small caudal teeth, this data indicates that cavefish have significantly more small caudal teeth than surface fish. In Chapter 2, I determined that when lens removal was conducted in the surface fish, the number of small caudal teeth present on the surgery side of the head significantly increased. This data indicates that lens removal in the surface fish results in a more cavefish-like number of teeth. In Chapter 4, I also investigated the number of small caudal teeth present in the surface fish

Tinaja cavefish intermediate. I determined that the intermediates have on average 5.7 ± 1.5 small teeth.These results indicate that the lens has a significant impact on the number of caudal teeth present in the Mexican tetra.

As stated previously the teeth located in the central portion of the jaw (unaffected by lens removal) and the teeth located in the caudal portion are very different in nature. The teeth located on the caudal portion of the jaw are much smaller in size, and follow different irregular replacement cycles (Trapani et al., 2005). Additionally, different from the central teeth the caudal teeth form outside of the bone (extraosseously) of the jaw and thus would be differentially affected by alterations in bone formation. In addition, there is natural variation in the number of caudal teeth during adulthood, unlike the number of large teeth (Trapani et al., 2005). These features of the caudal teeth may allow them to be variable in nature. The number of small caudal teeth are influenced by the lens, while large central teeth are not, this may be due to the developmental differences between the teeth in the Mexican tetra (age at development, and different replacement cycles). The caudal teeth form much later in development than the centrally located teeth. The caudal teeth do not form until after eye regression in the surgery surface fish, while the centrally located teeth develop prior to eye regression. Additionally, the caudal teeth develop outside of the jaw bone, while the large central teeth develop within the bone, thus they would likely be affected differently by changes in the bone. These differences may influence why one group of teeth are influenced by lens removal and the other is not.

I hypothesize that the alterations in caudal tooth number may be influenced by changes in the space available for teeth, alternatively, changes in the mechanical forces on the skull or direct signalling from the lens to the developing teeth may influence tooth number. Each of these influences will be discussed below.

Direct signals may be sent from the lens to the developing teeth. In the small eyed mouse mutant *Pax6*^{Sey}, in which the lens fails to form; 80% of the mutants show an increase in anterior upper tooth number by 1 to 2 teeth (Huysseune, 1995; Kaufman et al., 1995). This study is consistent with or not at variance with my hypothesis that direct signals from the lens may impact the number of teeth that develop. However, the study does not consider the fact that the mutant mouse eye is reduced in size. As was determined in the Mexican tetra, changes in eye size can lead in changes in the

mechanical forces located within the head. It may be the alterations in mechanical forces which influence the tooth number in the mutant, rather than direct signals from the lens. Further investigation is required to determine if the lens does in fact produce diffusible signals which can reach the caudal region of the jaw.

One study which supports my hypothesis of alterations of mechanical forces within the head resulting in the changes of tooth, was conducted in the cichlid *Astatoreochromis alluaudi*. The fish were fed either hard food (snails) or soft food (Huysseune, 1995). Both jaw size, and tooth number and size varied between the two groups. The amount of divergence in tooth morphology and number was dependant on the age of the fish when the diet was altered. From this study it is clear that tooth number is variable in nature and small influences can have a great impact on the development of the teeth. Changes in diet may have also resulted in changes in the jaw. The literature reviewed in Chapter 1 demonstrated the highly variable nature of the shape of the bones in oral region. In addition, a number of studies described changes in jaw shape as a result of alterations in diet, as such, it is likely that although jaw shape changes were not described in the study that they were present. Changes in jaw space may influence tooth number rather than changes in the mechanical forces present on the jaw or it could be a combination of the two. Lens removal in the Mexican tetra also affected jaw shape (at two time points).

Since more jaw space typically means more teeth (Huysseune, 1995), I analyzed the differences in the length of the jaw on the surgery and control sides of the head. I determined that the length of the mandible does not differ on the surgery side compared to the control side of the head and thus, jaw length cannot account for the observed changes in tooth number. However, some studies also indicate that with more jaw space tooth number may increase or teeth can become larger in volume over successive tooth generations (Huysseune and Sire, 1994; Huysseune, 1995), thus jaw width could be useful in this study to determine if it has influenced tooth number. Yamamoto et al., (2009) indicated that the cavefish have a wider mandible, providing more space for teeth. Unfortunately, the method of measurement used in the study does not accurately capture differences in jaw shape, thus a more accurate analysis of jaw shape, using for example, morphometrics analysis is required to accurately determine differences in jaw shape.

Investigating jaw size on the surgery side of the head compared to the control maybe a useful study in the future to determine if more space is available for teeth after lens removal.

In mice, it has also been demonstrated that a single tooth can inhibit both the size and development of neighbouring teeth (Kavanagh et al., 2007). In the tetra, the caudal teeth tend to be isolated compared to the central teeth. The teeth in the caudal region are spread out over a large portion of jaw space with large gaps between each of the teeth, in contrast to the centrally located teeth which are present in a small portion of the jaw and are crowded without any space between the teeth. The isolated nature of the small teeth on the caudal portion of the mandible might enable them to be more variable in number compared to the large clustered multicuspid teeth in the centre of the jaw. Although jaw width was not analyzed in my study, jaw shape was. In my analysis of jaw shape, two of the four surgery time points resulted in significant shape changes of the jaw, these shape changes may provide more space to form additional teeth on the surgery side of the jaw. However, an increase in tooth number was present at three different surgery time points, thus an increase in tooth number was present without an alteration in jaw shape. A limited number of landmarks (11) were used in the morphometrics analysis of jaw shape. The 11 landmarks were placed over the whole jaw and did not focus on the caudal region. The landmarks selected may not have accurately described the shape changes that were present, specifically in the caudal region. In addition, the sample sizes were limited and may not have been able to capture shape differences accurately. A future study using morphometric shape analysis exclusively on the caudal portion of the jaw would better describe how, and if, this area of the jaw was influenced by lens removal. This analysis would then allow for a better understanding of how jaw shape and space may have influenced the number of caudal teeth after lens removal.

The intermediates have a medium size lens, a medium size eye and a larger number of caudal teeth than the surface fish and surgery fish, but fewer than the cavefish. This indicates that the absence of the lens may not be directly responsible for the increase in tooth number, as the intermediates have both a lens and an increased number of teeth; in fact more teeth than the lens-less surgery fish. This indicates that there may be a more complex cascade of events which begins with changes in the lens and eye and ends in an

increased number of caudal teeth, unfortunately, the intermediate fish do not provide further evidence to aid in unravelling this complex process.

Based on the above analysis, it is clear that determining how the lens is influencing the number of teeth is a complicated interaction to unravel. The studied reviews in Chapter 1, demonstrated the highly variable nature of the oral region of the skull. It was determined that the bones of the oral region could be easily influenced by a number of different factors. As the oral region is so variable from a number of different influences it is difficult to determine if lens absence is the primary influence on the number of teeth or if the increase observed in tooth number is a downstream affect or indirect affect of lens removal. This finding of the lens influencing the number of caudal teeth is a highly surprising result, one that requires further investigation to determine the mechanism by which the lens is influencing caudal tooth number and to explain why the caudal teeth are influenced and the large central teeth are not. Molecular analysis of Mexican tetra tooth development and the differences present between the tooth groups may be helpful in unravelling this complex question.

6.1.6 Lens Regeneration and Lens Healing

In Chapter 3A, the lens was damaged with laser ablation, while in Chapter 3B the lens was fully removed. Comparing the data gathered in each section of this chapter demonstrates the versatility of the Mexican tetra lens and provides further evidence for the capacity of lens regeneration in this species.

When laser lens damage was performed between 1 and 11 dpf, 100% of the specimens which received laser treatment had two normal appearing lenses in adulthood (Chapter 3). To investigate the lack of eye regression, histological analysis was performed 24 hours after partial laser lens ablation. The histology performed one day after laser treatment shows that the laser treatment did not ablate all of the lens cells. The fibre cells located in the core of the lens tended to be damaged, absent, or improperly arranged, while the epithelial cell layer on the anterior surface of the lens, appear to be intact. The lens appears to heal rapidly as minor to no size difference was present between the surgery and control eyes.

In Chapter 2, manual lens removal was conducted in surface fish. Approximately 50% of juveniles underwent lens regeneration and had two normal appearing eyes in adulthood. In Chapter 3B, the entire lens including the lens capsule was removed from the eye using a tungsten needle. Twenty-four hours after the lens was removed, no lens or lens cells could be detected with β crystallin. By 8 days after lens removal the surgery eye had completely regenerated a lens.

While lens healing occurs in a number of vertebrate species, including mice, rabbits, fish, chicken and cats (Call et al., 2005), lens regeneration is not a common feature of vertebrates, occurring in only newts, frogs and one species of fish. The main difference between lens healing and lens regeneration is the underlying mechanism involved. As stated previously, lens regeneration occurs through the transdifferentiation of other cell types into lentoid cells. Transdifferentiation involves dedifferentiation, followed by redifferentiation into a new cell type (Henry, 2003). This was observed in Chapter 3B after the lens way fully removed from one eye of a surface Mexican tetra. Lens healing occurs without transdifferentiation, the lens likely heals from remaining lens epithelial cells attached to the remaining lens capsule. Lens healing was observed in Chapter 3A after the lens was damaged with laser ablation. Having the capacity to both heal and regenerate a lens is highly uncommon, thus with the Mexican tetra's capacity to perform both make them an outstanding model for investigating the mechanism involved in lens regeneration. As many vertebrates including humans have some capacity to heal the lens, comparing lens healing and lens regeneration in the same species may allow for better insight into how a species with the capacity for lens healing can be manipulated to induce lens regeneration. The discovery of lens healing and/or regeneration in the Mexican tetra will allow for an expansion of knowledge on lens regeneration, and provide a new model species in which to study lens regeneration. Since the eye is largely the same between humans and fish, a better understanding of lens regeneration in fish will provide insight into the potential for lens regeneration in humans. Thus the Mexican tetra may be a useful organism to model human lens disorders, such as cataracts and for developing new therapies for treating such disorders.

The loach is the only other known teleost to have the capacity for lens regeneration (Sato, 1961). The loach is a member of the Order Cypriniformes, while the

Mexican tetra is a member of the Order Characiformes. The loach is hypothesized to have evolved the capacity for lens regeneration as it encounters large numbers of parasites in its natural environment (Sato, 1961). The parasites specifically attack the lens of the fish eye, as such, it is proposed that this parasite-host relationship drove the evolution of the capacity for lens regeneration in this species (Sato, 1961). It is unknown what capacity this species has for lens healing. Unfortunately, the primary literature available on lens regeneration in the loach is not written in English, however the images included in the manuscript include images of an old degenerating lens in the same image as a newly regenerating lens. This indicates that the loach does not have the same capacity for lens healing as is found in the Mexican tetra, but rather the capacity for regeneration. This confirms how valuable the Mexican tetra maybe in the future for unravelling the unknown molecular controls for lens regeneration and healing.

I propose that the surface Mexican tetra may have evolved the capacity of lens regeneration and lens healing as a result of gene flow between the lens-less cavefish and the surface fish. Lens Due to the ability of the two morphs to interbreed in the wild, gene flow can easily occur. I hypothesize that the surface fish have evolved the capacity to regenerate the lens in order to counter-act the genetic influences which enhance lens-loss in the cavefish after gene flow between the morphs. As stated previously, lens loss occurs in the cavefish as a result of apoptosis. Surface fish may have evolved the capacity to heal a lens damaged by apoptosis which may occur in the surface fish lens after the populations have had the opportunity to interbreed.

Studies which conducted breeding experiments between various cave populations demonstrated that interbreeding two cave populations rescued the eyes in the offspring. This finding indicates that different genetic changes have resulted in eye loss in different cavefish populations, and thus eye loss in the cave populations is similar due to convergence (Wilkens, 2004). The expansion of *shh* expression leading to lens-loss is specific to the Pachon cave population however much of the literature disregards this fact and presumes that the same mechanism results in lens loss in all of the cave populations, despite breeding experiments indicating otherwise (e.g. Jeffery, 2004; 2008). The molecular mechanisms responsible for lens loss in other cave populations likely involve changes in key eye and lens genes which have not been investigated. The surface morph

has likely undergone gene flow with multiple different cave populations (although poorly described in the literature) with multiple different genetic changes which result in lens-loss. This extreme influence toward lens loss may have predisposed the surface fish toward evolving the exceptional capacity to both heal and regenerate the lens.

6.2 Final Conclusions

While many studies have investigated how environmental changes such as alterations in food affect the shape of the teleost jaws, very little attention has been given to how variable the skull is in general and how soft tissues of the head can influence the shape of the skull. Removing the lens from the eye of the surface Mexican tetra resulted in shape changes in a number of the bones located in the orbital region of the skull. While some bones demonstrated a great level of shape variability, such as the supraorbital bone, others were highly stable in shape, such as the calvariae. Largely the bones which were determined to be variable in shape are variable across the morphs of the Mexican tetra as well as in another teleost species, the zebrafish. The variability of a bone appears to be controlled by a number of different factors, for example bone location and the mechanical forces present on the bone. In addition, I determined that the absence of the lens had the greatest impact on the skull when it was absent later in development, during suborbital bone ossification and outgrowth. Surprisingly, I determined that the lens does influence the number of small caudal teeth present on the lower jaw, but does not affect the number of oral taste buds. By removing the lens in the surface Mexican tetra, I was able to demonstrate that while some features arise through the loss of the lens in the cavefish other factors are involved in transitioning a surface fish into a cavefish. Finally, I was able to demonstrate that the Mexican tetra has the capacity for both lens healing and lens regeneration, providing an excellent new model system for studying lens regeneration.

This type of long-term study demonstrates that the removal of the lens, a soft tissue in the head, during early development has the capacity to influence the development of some of the bones of the orbital region, which develop several months later. Alterations in bone shape as a result of lens removal where present in the adult phenotype. Furthermore, this study highlights interactions present between sensory systems during early development and sheds light on the cavefish phenotype. Overall,

this research raises many questions regarding the role of the eye in directing development of the vertebrate head.

References

Albertson CR, Kocher TD (2001) Assessing morphological differences in an adaptive trait: A landmark-based morphometric approach. *J Exp Zool* 289:385-403.

Albertson, R.C., and Yelick, P.C. (2007) Fgf8 haploinsufficiency results in distinct craniofacial defects in adult zebrafish. *Dev Biol* 306: **505-515.**

Bateson A, Gluckman P (2011) Plasticity, robustness, development and evolution. Cambridge University press.

Bouton N, Witte F, Van Alphen JJM (2002) Experimental evidence for adaptive phenotypic plasticity in a rock-dwelling cichlid fish from Lake Victoria. *Biol J Linn Soc* **77**:185-192.

Bradic M, Beerli FJ, Garcia-de Leon F, Esquivel-Bobadilla S, Borowsky RL (2012) Gene flow and population structure in the Mexican blind cavefish complex (*Astyanax mexicanus*). *BMC Evol Biol* 12:9 doi:10.1186/1471-2148-12-9.

Brockes JP (1997) Amphibian Limb Regeneration: Rebuilding a Complex Structure. *Science* 276(5309): 81-87.

Caldecutt W, Adams DC (1998)Morphometrics of Trophic Osteology in the Threespine Stickleback, *Gasterosteusa culeatus*. Copeia (4): 827-838.

Call MK, Grogg MW, Tsonis PA (2005) Eye on Regeneration. *The Anat Rec (Part B: New Anat)* 287:42-48.

Carvan MJ, Louchks E, Weber DN, Williams FE (2004) Ethanol effects on the developing zebrafish: neurobehaviour and skeletal morphogensis. *Neurotoxicol Teratol* 26:757-768.

Cloutier R, Caron A, Grunbaun T, Le Francois NR (2010) Effect of water velocity on the timing of skeleogenesis in the Artic charr, Salvelinus alpinus (Salmoniformes: Teleostei): An empirical case of developmental plasticity. *Int J Zool* 2010: 1-15.

Cobcroft JM, Battaglene SC (2009) Jaw malformation in striped trumpeter *Latris lineata* larvae linked to walling behaviour and tank colour. *Aquaculture* 289:274–282.

Cobcroft JM, Pankhurst PM, Poortenaar C, Hickman B,Tait M (2004) Jaw malformation in cultured yellowtail kingfish (*Seriola lalandi*) larvae. *NZ J Mar Freshwat Res* 38(1):67-71.

Connolly M H, Hall BK (2008) Embryonic heat shock reveals latent hsp90 translation in zebrafish (*Danio rerio*). Int J Dev Biol (52):71–79.

Cooper JW, Parsons K, McIntyre A, Kern B, McGee-Moore A, et al. (2010) Bentho-pelagic divergence of ciclid feeding architecture was prodigious and consistent during multiple adaptive radiations within African rift-lakes. *Ann Rev Evol Syst* 31: 163-196.

Coulombre AJ, Crelin ES (1956) The role of the developing eye in the morphogenesis of the avian skull. *Am J Phys Anthropol* 16(1): 25-37.

Cubbage CC, Mabee PM (1996) Development of the cranium and paired fins in the zebrafish (*Danio rerio*) (Ostariophysi, Cyprinidae). *J Morph* 229:121-160.

Daly RM, Saxon L, Turner CH, Robling AG, Bass SL (2004) The relationship between muscke size and bone geometry during growth and in response to exercise. *Bone* 34(2): 281-287.

De Queiroz K (1998) Endless forms: Species and speciation. Oxford Uni Press p57-75.

De Schepper N, Adriaens D, Teugel GG, Devarere S, Verraes W (2004) Intraspecific variation in the postcranial skeleton morphology in African clariids: a case study of extreme phenotypic plasticity. *Zool J Linn Soc* 140(3): 437-446.

Diogo R, Doadrio I, Vandewalle P (2008) Teleostean phylogeny based on osteological and mycological chatacters. *Int J Morphol* 26(3): 463-522.

Diogo R, Hinits Y, Hughes SM (2008) Development of mandibular, hyoid and hypobranchial muscles in the zebrafish: homologies and evolution of these muscles within bony fishes and tetrapods. *BMC Dev Biol* 8(24): 1-22.

Easter SS, Nicola GN (1996) The development of vision in the Zebrafish (*Danio rerio*). *Dev Biol* 180:646-663.

Edsall S, Franz-Odendaal TA (2010) A whole-mount protocol for staining osteoblasts and osteoclasts in Teleosts. *Zebrafish* 7:3:275-280.

Espinasa L, Borowsky RB (2001) Origins and Relationship of Cave Populations of the Blind Mexican Tetra, *Astyanax Fasciatus*, in the Sierra De El Abra. Envir Biol Fishes 62(1)233-237.

Fernandez I, Hontria F, Ortiz-Delgado JB, Kotzamanis Y, Estevez A, Zambonino-Infante JL, Gisbert E (2008) Larval performance and skeletal deformities in farmed gilthead sea bream (*Sparus aurata*) fed with graded levels of vitamin A enriched rotifers (*Brachionus plicatilis*). *Aquaculture* 283:102–115.

Fiaz AW, van Leeuwen JL, Kranenbarg S (2010) Phenotypic plasticity and mechano transduction in the teleost skeleton. *J Appl Ichthyol* 26: 289-293.

Franz-Odendaal TA (2008) Scleral ossicles of Teleostei: Evolutionary and developmental trends. *Anat Rec* 291(2): 161-168.

Franz-Odendaal TA, Hall BK (2006) Modularity and sense organs in the blind cavefish, Astyanax mexicanus. *Evol Dev* 8(1): 94-100.

Franz-Odendall TA, Hall BK, Witten PE (2006) Buried alive: how osteoblasts become osteocytes. *Dev Dyn* 235(1):176-90.

Franz-Odendaal T, Ryan K, Hall B (2007) Developmental and morphological variation in the teleost craniofacial skeleton reveals an unusual mode of ossification. *J Exp Zool* 308B (Mol Dev Evol): 709-721.

Freeman G (1963) Lens regeneration from the cornea in *Xenopus laevis*. J Exp Zool 154: 39-66.

Gallo N, Jeffery WR (2012) Evolution of space dependent growth in the teleost *Astyanax mexicanus. Plos One* 7(8): e41443.

Gregory WK (1933) Fish Skulls: A study of the evolution of natural mechanisms. Florida: Krieger Publishing Company.

Gross JB, Hanken J (2008) Review of fate-mapping studies of osteogenic cranial neural crest in vertebrates. *Dev Biol* 317:389-400.

Hall BK, Miyake T (1992) The membranous skeleton: the role of cell condensations in vertebrate skeletogenesis. *Anat Embryol* 186: 107–124.

Hall BK, Miyake T (2000) All for one and one for all: condensationsand the initiation of skeletal development. *BioEssays* 22:138–147.

Hall BK (2005) Bones and Cartilage: Developmental skeletal biology. Academic Press 1ed.

Hausdorf B, Wilkens H, Strecker U (2011) Population genetic patterns revealed by microsatellite data challenge the mitochondrial DNA based taxonomy of Astyanax in Mexico (Characidae, Teleostei). *Mol Phylogenet Evol* 60(1): 89-97.

Heisenberg CP, Brand M, Jiang YJ, Warga RM, Beuchle D, van Eeden FJ, Furutani-Seiki M,Granato M, Haffter P, Hammerschmidt M, et al. (1996) Genes involved in forebrain development in the zebrafish, *Danio rerio. Development* 123:191-203.

Henry JJ (2003) The cellular and molecular bases of vertebrate lens regeneration. *Intern Rev of Cytol* 228:195-265.

Hinaux H, Pottin K, Chalhoub H, Pere S, Elipot Y, Legendre L, Retaux S (2011) A Developmental Staging Table for *Astyanax mexicanus* Surface Fish and Pachon Cavefish. *Zebrafish* 8(4):155-165.

Hooven TA, Yamamoto Y, Jeffery WR (2004) Blind cavefish and heat shock protein chaperones: a novel role for hsp90α in lens apoptosis. *Int J Dev Biol* 48:731–738.

Huysseune A (1995) Phenotypic plasticity in the lower pharyngeal jaw dentition of *Astatoreochromis alluaudi* (Teleostei: Cichlidae). *Arch Oral Biol* 40(11): 1005-1014.b

Huysseune A, Sire JY, Meuniew FJ (1994) Comparative study of lower pharyngeal jaw structure in two phenotypes of *Astatoreochromis alluaudi* (Teleostei: Cichlidae). *J Morph* 221:25-43.

Jeffery WR (2001) Cavefish as a model system in evolutionary developmental biology. *Dev Biol* 231: 1-12.

Jeffery WR (2005) Adaptive evolution of eye degeneration in the Mexican blind cavefish. *J Hered* 96: 185-196.

Jeffery WR (2008) Emerging model systems in evo-devo: cavefish and microevolution of development. *Evol Dev* 10(3): 265-272.

Kague E, Gallagher M, Burke S, Parsons M, Franz-Odendaal T, et al, (2012) Skeletogenic Fate of Zebrafish Cranial and Trunk Neural Crest. *PLoS ONE* 7(11): e47394 .

Kavanagh KD, Evans AR, Jernvall J (2007) Predicting evolutionary patterns of mammalian teeth from development. *Nature* 427: 427-433.

Kaufman MH, Chang HH, Shaw JP (1995) Craniofacial abnormalities in homozygous small eye (Sey/Sey) embryos and newborn mice. *J Anat* 186: 607-617.

Kershaw DR. 1970. The cranial osteology of the 'Butterfly Fish', *Pantodon buchholzi*. *Zool J of the Linn Soc* 49:5-19.

Kimmel CB (1989) Genetics and early development of zebrafish. Trends *Genet* 5:283-288.

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995) Stages of embryonic development of the zebrafish. *Dev Dynam* 203(3): 253-310.

Kimmel CB, DeLaurier A, Ullmann B, Dowd J, McFadden M (2010) Modes of Developmental Outgrowth and Shaping of a Craniofacial Bone in Zebrafish. *PloS ONE* 5(3): e9475.

Kimmel CB, Law RD (1985) Cell lineage of zebrafish blastomeres I. Cleavage pattern and cytoplasmic bridges between cells. *Dev Biol* (108):78-85.

Kish PE, Bohnsack BL, Gallina D, Kasprick DS, Kahana S (2011) The eye as an organizer of craniofacial development. Genesis 49: 222-230.

Klymkowsky MW, Hanken J (1991) Whole-mount staining of Xenopus and other vertebrates. *Methods Cell Biol* 36:419–441.

Smith PF (2000) African Cichlid Fishes: Model Systems for Evolutionary Biology. *Ann Rev of Ecol Syst* 31:163-182.

Langenberg T, Kahana A, Wszalek JA, Halloran MC (2008) The eye organizes neural crest cell migration. *Dev Dynam* 237: 1645-1652.

Mabee PM, Olmstead KL, Cubbage CC (2000) An experimental study of intraspecific variation, developmental timing, and heterochrony in fishes. *Evolution* 54(6):2091-2106.

Mabee PM, Trendler T. 1996. Development of the cranium and paired fins in *Betta splendens* (Teleostei: Percomorpha): Intraspecific variation and interspecific comparisions. *J Morph* 227(3)249-287.

Means AL, Gudas LJ (1995) The roles of retinoids in vertebrate development. *Ann Rev Biochem* 64:201-233.

Meyer A (1987) Phenotypic plasticity and heterochrony in *Cichlasoma managuense* (Pisces, Chichlidae) and their implications for speciation in cichlid fishes. *Evol 41*(6):1357-1369.

Meyer A, Biermann CH, Orti G (1993) The phylogenetic position of the zebrafish (*Danio rerio*), a model system in developmental biology: an invitation to the comparative method. *Proc Biol Sci* 252(1335):231-236.

Mitchell RW, Russell WH, Elliot WR (1977) Mexican eyeless Characin fishes, genus Astyanax: environment, distribution, and evolution. *Spec Publ Mus Texas Tech Univ* 12: 1–89.

Mrakov M, Haley LE (1979) In breeding depression in the zebrafish Brachy *Danio rerio* (Hamilton Buchanan). *J Fish Biol* 15:323-327.

Nelson KJ, Rafferty NS. 1976. A scanning electron microscopic study of lens fibers in healing mouse lens. *Exp Eye Res* 22(4)335-346.

Parsons KJ, Cooper JW, Albertson CR (2011) Modularity of the oral jaws is linked to reapted changes in the craniofacial shape of African cichlids. *Int J Evol Biol* 2011: 1-10.

Parsons KJ, Marquez E, Albertson RC (2012) Constraint and opportunity: The genetic basis and evolution of modularity in the cichlid mandible. *Amer Nat* 179(1)64-78.

Piron RD (1978) Spontaneous skeletal deformities in the zebra danio (Brachy *danio rerio*) bred for fish toxicity tests. *J Fish Biol* 13:79-83.

Poland A, Knutson JC (1982) 2,3,7,8-tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: Examination of the mechanism of toxicity. *Annu Rev Pharmacol Toxicol* 22:517-554.

Porter ML, Dittmar K, Perez-Losada M (2007) How long does evolution of the troglomorphic form take? Estimating divergence times in *Astyanax mexicanus*. *Acta Carsol* 36: 173–182.

Protas M, Conrad M, Gross JB, Tabin C, Borowsky R (2007) Regressive evolution in the Mexican cave tetra, *Astyanax mexicanus. Curr Biol* 17(5): 452-454.

Proulx R, Magnan P (2004) Contribution of phenotypic plasticity and heredity to the trophic polymorphism of lacustrine brook charr (*Salvelinus fontinalis M.). Evol Ecol Res* 6:503–522.

Opperman LA, Sweeney TM, Redmon J, Persing JA, Ogle RC (1993) Tissue interactions with underlying dura mater inhibit osseous obliteration of developing cranial sutures. *Dev Dyn* 198(4): 312-322.

Ornelas-García CP. Domínguez-Domínguez O. Doadrio I (2008) Evolutionary history of the fish genus Astyanax Baird & Girard (1854) (Actinopterygii, Characidae) in Mesoamerica reveals multiple morphological homoplasies. *BMC Evol Biol* (8):340.

Ornsrud R, Gil L, Waagbo R (2004) Teratogenicity of elevated egg incubation temperature and egg vitamin A status in Atlantic salmon, *Salmo salar L. J Fish Dis* 27:213-223.

Quarto N, Longaker MT (2005) The zebrafish (*Danio rerio*): A model system for cranial suture patterning. *Cells Tissues Organs* 181:109-118.

Reimers MJ, Flockton AR, Tanguay RL (2004) Ethanol and acetaldehyde mediated developmental toxicity in zebrafish. *Neurotoxicol Teratol* 26:769-781.

Sadoglu P (1957) Mendelian inheritance in hybrids between the Mexican blind fish and their overground ancestors. *Verh Dtsch Zool Ges* 57:432–439.

Sandell LL, Trainor PA (2006) Neural crest cell plasticity: Size matters. Landes Bioscience and Springer Science.

Santini F, Harmon LJ, Carnevale G, Alfaro ME (2009) Did genome duplication drive the origin of teleosts? A comparative study of diversification in ray-finned fishes. *BMC Evol Biol* 9: 194.

Sato T (1961) Über bei der Cobitiden-Fischen. I. *Misgurnus anguillicandatus* (Cantor). *Embryologia* 6:251-290.

Schemmel C (1967) Vergleichende untersuchungen an den hautsinnesorganen ober-and unter-irdisch lebender *Astyanax-foramen. Z Morph Tiere* 61: 255-316.

Schilling T (2001) Plasticity of zebrafish Hox expression in the hindbrain and cranial neural crest hindbrain. *Dev Biol* 231:201-216.

Schilling TF, Kimmel CB (1994) Segment and cell type lineage restrictions during pharyngeal arch development in the zebrafish embryo. *Development* 120:483-494.

Schilling TF, Kimmel CB (1997) Musculoskeletal patterning in the pharyngeal segements of the zebrafish embryo. *Development* 124: 2945-2960.

Schonthaler HB, Franz-Odendaal TA, Hodel C, Gerhring I, Geisler R, Schwarz H, Neuhauss SCF, Dahm R (2010) The zebrafish mutant bumper shows a hyperproliferation of lens epithelial cells and fibre cell degeneration leading to functional blindness. *Mech Dev* 127(3)203-219.

Schowing J (1968) Demonstration of the inductive role of the brain in osteogenesis of the embryonic skull of chicken. *J Embryo Exp Morphol* 19:83-94.

Schwarz Mea, (2000) Spatial specification of mammalian eye territories by reciprocal transcriptional repression of Pax2 and Pax6. *Development* 127: 4325-4334.

Schweigerer L, Ferrara N, Haaparanta T, Neufeld G, Gospodarowicz D (1988) Basic fibroblast growth factor: Expression in cultured cells derived from corneal endothelium and lens epithelium. *Exp Eye Res* 46(1): 71-80.

Stewart TA, Albertson RC (2010) Evolution of a unique predatory feeding apparatus: functional anatomy, development and a genetic locus for jaw laterality in Lake Tanganyika scale-eating cichlids. *BMC Biology* 8(8):1-11.

Strecker U, Hausdorf B, Wilkens H (2012) Parallel speciation in Astyanax cavefish (Teleostei) in Northern Mexico. *Mol Phyl Evo* 62(1):62-70.

Strecker U, Faundez VH,Wilkens H (2004) Phylogeography of surface and cave Astyanax (Teleostei) from Central and North America based on cytochrome b sequence data. *Mol Phylogenet Evol* 33:469–481.

Stricker A, Jeffery WR (2009) Differentially expressed genes identified by cross-species microarray in the blind cavefish Astyanax. *Integ Zool* 4: 99-109

Strickler AG, Yamamoto Y, Jeffery WR (2001) Early and late changes in Pax6 expression accompany eye degeneration during cavefish development. *Dev Genes Evol* 211: 138-144.

Strickler AG, Yamamoto Y, Jeffery WR (2007). The lens controls cell survival in the retina: evidence from the blind cavefish Astyanax. *Dev Biol* 311: 512–523.

Take-uchi M, Clark JD, Wilson SW (2003). Hedgehog signaling maintains the optic stalk-retinal interface through the regulation of Vax gene activity. *Development* 130: 955-968.

Tonneyck-Muller I (1976) Growth of eye and orbit in chick embryo IX. *Acta Morphol Neerl Scand* 14(2):139-64.

Trapani J, Yamamoto Y, Stock DW (2005) Ontogenetic transition from unicuspid to multicuspid oral dentition in a teleost fish: *Astyanax mexicanus*, the Mexican tetra (Ostariophysi: Characidae). *Zool J Linn Soc* 145(4): 523-538.

Tsonis PA (2000) Regeneration in Vertebrates. *Dev Biol* 221: 273-284.

Tsonis PA, Madhavan M, Tancous E, Del Rio-Tsonis K (2004) A newt's eye view of lens regeneration. *Inter J Dev Biol* 48:975-980.

Uga S (1981) Wound healing in the mouse lens. *Exp Eye Research* 32(2):175-186.

Varatharasan N, Croll R, Franz-Odendaal T (2009) Taste bud development and patterning in sighted and blind morphs of *Astyanax mexicanus. Dev Dynam* 238(12):3056-3064.

Vandersea MW, McMarthy RA, Fleming P, Smith D (1998) Exogenous retinoic acid during gastrulation induces cartilaginous and other craniofacial defects in *Fundulus heteroclitus. Biol Bull* 194:281-296.

Waddington CH (1957) The strategy of genes. George Allen and Unwin Ltd., London.

Warga RM, Kimmel CB (1990) Cell movements during epiboly and gastrulation in zebrafish. *Development* 108:569-580.

Webster M, Sheets HD (2010) A practical guide to landmark based geometric morphometrics. http://geosci.uchicago.edu/~mwebster/Webster_and_Sheets_2010.pdf

Wesseley K (1920) Ueber Korrelationen des Wachstums (nach Versuchen am Auge). *Zeit f Augenheilk* 43:654-681.

Wilkens H (1988) Evolution and genetics of epigean and cave *Astyanax fasciatus* (Characidae, Pisces). *Evol Biol* 23: 271–367.

Wilkens H (2001) Convergent adaptations to cave life in the *Rhamdia laticauda* catfish group (Pimelodidae, Teleostei). *Environ Biol Fish* 62: 252–261.

Wilkens H (2004). The Astyanax model (Teleostei): neutral mutations and directional selection. *Mitt Hamb Zool Mus Inst* 101: 123–130.

Wilkens H (2007). Regressive evolution: ontogeny and genetics of cave fish eye rudimentation. *Biol J Linnean Soc* 92: 287–296.

Wilkens H, Strecker U (2003). Convergent evolution of the cavefish Astyanax (Characidae, Teleostei): genetic evidence from reduced eye size and pigmentation. *Biol J Linnean Soc* 80: 545–554.

Winemiller KO, Taylor DH (1982) Inbreeding depression in the convict cichlid, *Cichlasoma nigrofasciatum* (Baird and Girard). *J Fish Biol* 21: 399–402.

Wintzer AP, Motta PJ (2005) Diet-induced phenotypic plasticity in the skull morphology of hatchery-reared Florida largemouth bass, *Micropterus salmoides floridanus*. Ecol *Freshwater Fish* 14:311-318.

Witten EP, Huysseune A (2009) A comparative view on mechanisms and functions of skeletal remodelling in teleost fish, with special emphasis on osteoclasts and their function. *Biol Rev* 84(2):315-346.

Yamamoto Y, Jeffery WR (2002) Probing teleost eye development by lens transplantation. *Methods* 28: 420-426.

Yamamoto Y, Espinasa L, Stock DW, Jeffery WR (2003) Development and evolution of craniofacial patterning is mediated by eye-dependent and -independent processes in the cavefish Astyanax. *Evol Dev* 5(5): 435-446.

Yamamoto Y, Byerly M, Jackman W, Jeffery WR (2009) Pleiotropic functions of embryonic sonic hedgehog expression link jaw and taste bud amplification with eye loss during cavefish evolution. *Dev Biol* 330(1): 200-211.

Yoshizawa M, Ashida G, Jeffery WR (2012) Parental genetic effects in a cavefish

adaptive behavior explain disparity between nuclear and mitochondrial *DNA*. *Evol* 66(9): 2975-2982.

Yoshizawa M, Jeffery WR (2011) Evolutionary tuning of an adaptive behavior requires Enhancement of the neuromast sensory system. *Commun Integr Biol* 4(1): 89-91.

Youngson, Dosdat, Saroglia, Jordan (2001) Genetic interactions between marine finfish species in European aquaculture and wild conspecifics. *J Appl Ichthyol* 17(4): 153-162.

Appendix 1: Landmark Locations

Table 1: The 42 landmark locations used on the lateral skull of the Mexican tetra.

Landmark Number	Location of landmark
Landmark 1	anterodorsal corner of the supraorbital bone
Landmark 2	anteroventral corner of the supraorbital bone
Landmark 3	posterodorsal corner of the supraorbital bone
Landmark 4	posterodorsal corner of the supraorbital bone
Landmark 5	anterodorsal corner of suborbital 5
Landmark 6	posterodorsal corner of suborbital 5
Landmark 7	anteroventral corner of suborbital 5
Landmark 8	posterodorsal corner of suborbital 5
Landmark 9	anterodorsal corner at suborbital 4
Landmark 10	posterodorsal corner of suborbital 4
Landmark 11	anteroventral corner of suborbital 4
Landmark 12	posterodorsal corner of suborbital 4
Landmark 13	anterodorsal corner of suborbital 3
Landmark 14	posterodorsal corner of suborbital 3
Landmark 15	the notch on the ventral edge of suborbital 3
Landmark 16	anteroventral corner of suborbital 3
Landmark 17	anterodorsal corner of suborbital 3
Landmark 18	posterodorsal corner of suborbital 2
Landmark 19	posterodorsal corner of suborbital 2
Landmark 20	anteroventral corner of suborbital 2
Landmark 21	anterodorsal corner of suborbital 2
Landmark 22	posterodorsal corner of suborbital 1
Landmark 23	posterodorsal corner of suborbital 1
Landmark 24	anterodorsal corner of suborbital 1
Landmark 25	anteroventral corner of suborbital 1
Landmark 26	the ventral tip of the antorbital bone
Landmark 27	the dorsal tip of the antorbital bone
Landmark 28	posterodorsal corner of the nasal bone
Landmark 29	anteroventral corner of the nasal bone
Landmark 30	the center of dorsal edge of the nasal bone
Landmark 31	ventral posterior corner of the frontal bone
Landmark 32	the posterior midline corner of the frontal bone
Landmark 33	anterior edge of the frontal bone
Landmark 34	dorsal edge of the frontal bone where a drastic downward slope begins
Landmark 35	top most point of the maxilla
Landmark 36	ventral most point at the bottom of the maxilla
Landmark 37	center of the ventral edge of the lateral ethmoid
Landmark 38	center of the dorsal edge of the lateral ethmoid
Landmark 39	anterodorsal corner of suborbital 6

Landmark Number	Location of landmark
Landmark 40	anteroventral corner of suborbital 6
Landmark 41	posterodorsal corner of suborbital 6
Landmark 42	posterodorsal corner of suborbital 6

Table 2: The 11 landmark locations used on the dorsal skull of the Mexican tetra.

Landmark Number	Location of landmark
Landmark 1	anterior tip of the supraethmoid
Landmark 2	center of posterior edge of the supraethmoid
Landmark 3	lateral posterior corner of the supraethmoid
Landmark 4	midline anterior corner of the frontal bone
Landmark 5	center anterior edge of the supraorbital
Landmark 6	center posterior edge of the supraorbital
Landmark 7	lateral posterior corner of the frontal bone
Landmark 8	midline posterior corner of the frontal bone
Landmark 9	lateral posterior corner of the parietal bone
Landmark 10	center of the posterior edge of the parietal bone
Landmark 11	midline posterior corner of the parietal bone

Table 3: The 11 landmark locations used on the ventral jaw of the Mexican tetra.

Landmark Number	Location of landmark
Landmark 1	dorsal edge of the mandibular symphysis
Landmark 2	ventral edge of the mandibular symphysis
Landmark 3	anterior corner of the mandible
Landmark 4	midline posterior corner of the coronoid process
Landmark 5	center of posterior edge of the coronoid process
Landmark 6	lateral posterior corner of the coronoid process
Landmark 7	midline anterior corner of the coronoid process
Landmark 8	midline anterior corner of the coronoid process
Landmark 9	anterior most point of the preopercle
Landmark 10	anterior most point of the quadrate
Landmark 11	anterior most point of the interopercle

Table 4: Twenty-nine landmarks applied to the lateral view of the intermediate skull

Landmark Number	Location of landmark
Landmark 1	anterodorsal corner of the supraorbital bone
Landmark 2	anteroventral corner of the supraorbital bone
Landmark 3	posterodorsal corner of the supraorbital bone
Landmark 4	posterodorsal corner of the supraorbital bone
Landmark 5	anterodorsal corner of suborbital 5
Landmark 6	posterodorsal corner of suborbital 5
Landmark 7	anteroventral corner of suborbital 5
Landmark 8	posterodorsal corner of suborbital 5
Landmark 9	anterodorsal corner at suborbital 4
Landmark 10	posterodorsal corner of suborbital 4
Landmark 11	anteroventral corner of suborbital 4
Landmark 12	posterodorsal corner of suborbital 4
Landmark 13	anterodorsal corner of suborbital 3
Landmark 14	posterodorsal corner of suborbital 3
Landmark 15	the notch on the ventral edge of suborbital 3
Landmark 16	anteroventral corner of suborbital 3
Landmark 17	anterodorsal corner of suborbital 3
Landmark 18	posterodorsal corner of suborbital 2
Landmark 19	posterodorsal corner of suborbital 2
Landmark 20	anteroventral corner of suborbital 2
Landmark 21	anterodorsal corner of suborbital 2
Landmark 22	the ventral tip of the antorbital bone
Landmark 23	the dorsal tip of the antorbital bone
Landmark 24	ventral posterior corner of the frontal bone
Landmark 25	the posterior midline corner of the frontal bone
Landmark 26	anterodorsal corner of suborbital 6
Landmark 27	anteroventral corner of suborbital 6
Landmark 28	posterodorsal corner of suborbital 6
Landmark 29	posterodorsal corner of suborbital 6

Table 5: Twenty-four landmarks applied to the lateral view of the zebrafish skull

Landmark Number	Location of landmark
Landmark 1	anterodorsal corner of the supraorbital bone
Landmark 2	anteroventral corner of the supraorbital bone
Landmark 3	posterodorsal corner of the supraorbital bone
Landmark 4	posterodorsal corner of the supraorbital bone
Landmark 5	apex of the supraorbital bone
Landmark 6	anterodorsal corner at suborbital 4
Landmark 7	posterodorsal corner of suborbital 4
Landmark 8	anteroventral corner of suborbital 4
Landmark 9	posterodorsal corner of suborbital 4
Landmark 10	anterodorsal corner of suborbital 3
Landmark 11	posterodorsal corner of suborbital 3
Landmark 12	the notch on the ventral edge of suborbital 3
Landmark 13	vental anterior corner of suborbital 3
Landmark 14	posteriordorsal corner of suborbital 3
Landmark 15	posterodorsal corner of suborbital 2
Landmark 16	posteroventral corner of suborbital 2
Landmark 17	anteroventral corner of suborbital 2
Landmark 18	anterodorsal corner of suborbital 2
Landmark 19	posterior end of suborbital 1
Landmark 20	dorsal notch of suborbital 1
Landmark 21	anterior end of suborbital 1
Landmark 22	anteroventral corner of suborbital 5
Landmark 23	posteroventral corner of suborbital 5
Landmark 24	posterior notch of suborbital 5

Appendix 2: Raw Measurements

Table 6: Reduction in surgery eye size after lens removal performed at 1 dpf.

Days Post Surgery	% Reduced	Surgery Eye Diameter (µm)	Control Eye Diameter (µm)
4	17	298	358
7	34	341	452
7	25	316	461
9	23	267	349
14	28	394	549
17	53	272	566
17	41	191	324
17	41	397	667
17	39	920	1510
17	35	409	629
28	39	827	1348
34	41	795	1342
34	36	842	1323

Table 7: Reduction in surgery eye size after lens removal performed at 3 dpf.

Days Post Surgery	% Reduced	Surgery Eye Diameter (µm)	Control Eye Diameter (µm)
3	11.5	272	307
6	42.4	160	278
12	33	440	565
13	44	368	660
16	39	428	702

Table 8: Reduction in surgery eye size after lens removal performed at 3 dpf.

Days Post Surgery	% Reduced	Surgery Eye Diameter (µm)	Control Eye Diameter (µm)
4	7.8	691	749
5	1.8	454	462
7	1.4	980	967
7	0.9	456	460
7	2.4	450	461

Table 9: A comparison between the diameters of the adult right and left eyes after laser surgery.

Sample Number	Right Eye Diameter (mm)	Left Eye Diameter (mm)
1	3.49	3.52
2	3.85	3.81
3	3.59	3.67
4	3.70	3.72
5	3.70	3.73
6	3.37	3.37
7	3.85	2.78
8	3.60	3.53
9	3.55	3.57

Table 10: A comparison between the diameters of the adult right and left eyes after corneal tear surgery.

Sample Number	Right Eye Diameter (mm)	Left Eye Diameter (mm)
1	3.00	3.14
2	4.24	3.31
3	3.18	3.10
4	3.68	3.60

Table 11: A comparison between the diameters of the adult right and left eyes after lens regeneration.

Sample Number	Right Eye Diameter (mm)	Left Eye Diameter (mm)
1	3.99	3.90
2	4.01	3.94
3	3.89	3.84
4	3.67	3.57
5	3.38	3.37

Appendix 3: Protocols

Manual Lens Ablation Protocol

Complete lens ablation was completed at 1, 2, 3, or 4 dpf for surface tetras and at 4 dpf for zebrafish. The fish are processed as follows prior to ablation:

1. 1 x 5mins wash in calcium free zebrafish ringer on a bed of 2% agar (Sigma A7002) in calcium free zebrafish ringer
2. 1 x 12mins wash in fresh calcium free zebrafish ringer on a bed of 2% agar in calcium free zebrafish ringer
3. 1 x 2mins anaesthetize in 0.001% MS222
4. 1 x 1mins wish in calcium free zebrafish ringer on a bed of 2% agar in calcium free zebrafish ringer
5. 1x 20mins incubate in 0.2% EDTA
6. 1x 1mins calcium free zebrafish ringer at 38^{O}C in a water bath
7. 1 x 2mins 1.2% agar in calcium free zebrafish ringer 38^{O}C in a water bath
8. Prewarm a glass petri dish in water bath (38^{O}C). Transfer embryos in 1.2% agar in calcium free zebrafish ringer to the glass petri dish
9. petri dish was then removed from the water bath
10. allow agar to harden
11. cut embryos into blocks and placed in a petri dish
12. use 1.2% agar in calcium free zebrafish ringer to glue blocks containing embryos in place
13. flood petri dish with 0.2% agar in calcium free zebrafish ringerlenses were then removed using a tungsten needle
14. 3x 20mins zebrafish ringer
15. remove specimens from agar and return to system water in the fish facility

Calcium Free Zebrafish Ringer Recipe

- 6.78g NaCl (Sigma SX0420-3)
- 0.22g KCl (Sigma P217-10)
- 0.2g $CaCl_2$ (EMD CX130-1)
- 1.19g HEPES (Sigma H4034)
- 1L of dH_2O
- pH 7.2

Zebrafish Ringer Recipe

- 6.78g NaCl
- 0.22g KCl
- 1.19g HEPES (Sigma H4034)
- 1L of dH_2O
- pH 7.2

Whole Mount Bone Stain

From storage solution (70% EtOH) specimens were hydrated through an ascending series

1. 1 x 1hr 50% EtOH
2. 1 x 1hr 25% EtOH
3. 1 x 1hr dH_2O
4. specimens skinned and gutted
5. 1 x overnight bleach solution- 5% H_2O_2 in 1% KOH
6. 1 x 5 mins dH_2O
7. 1 x 7hr saturated sodium borate
8. 1 x overnight 1 mg/ml Alizarin Red (Sigma-Aldrich, A5533) in 1% KOH
9. 1 x 5 mins 1% KOH
10. 3x overnight 2% borax and1% trypsin in dH2O
11. specimens processed through and a 1%KOH and glycerol series, with one night in each solution.
12. samples stored at room temperature in 100% glycerol

Whole Mount Cartilage Stain

From storage solution (70% EtOH)

1. 1 x overnight 0.015% Alcian blue stain (Sigma A3157) in 20% glacial acetic acid, in 100% EtOH
2. 1 x 5 mins 95% EtOH
3. 1x overnight saturated sodium borax (Sigma L4390)
4. 3x overnight 2% borax and1% trypsin (Fisher Scientific T360) in dH2O
5. 1 x overnight 3% H_2O_2 IN 1% KOH (Sigma P1767)
6. specimens processed through and a 1%KOH and glycerol series, with one night in each solution.
7. samples stored at room temperature in 100% glycerol

Laser Lens Ablation (Partial Removal)

Complete lens ablation completed at 1, to 11 dpf for surface tetras

1. 1 x 5 mins calcium free zebrafish ringer on a bed of 2% agar (Sigma A7002) in calcium free zebrafish ringer
2. 1 x 12 mins fresh calcium free zebrafish ringer on a bed of 2% agar in calcium free zebrafish ringer
3. 1 x 2 mins 0.001% MS222
4. 1 x 1 mins calcium free zebrafish ringer on a bed of 2% agar in calcium free zebrafish ringer
5. 1x 1 mins calcium free zebrafish ringer at 38^{O}C in a waterbath
6. 1 x 2 mins 1.2% agar in calcium free zebrafish ringer 38^{O}C in a water bath
7. float glass petri dish in water bath
8. transfer embryos in 1.2% agar in calcium free zebrafish ringer to a glass petri dish
9. remove dish was then removed from the water bath
10. allow agar to harden
11. cut embryos into blocks and placed in a plastic projector slide
12. use 1.2% agar in calcium free zebrafish ringer to glue blocks containing embryos in place
13. flood petri dish with 0.2% agar in calcium free zebrafish ringer
14. lenses were then laser ablated
15. 3x 20 mins zebrafish ringer
16. remove specimens from agar and return to system water in the fish facility

Immunohistochemistry for Taste bud Visualization

Specimens were placed in an eppendorf with 8 to 10 fish per tube

1. fix in 4% PFA (Sigma P6148)in PBS overnight at 4 ^{O}C
2. wash 3x for 15 mins in 0.1M PBS

Day 1

1. permeabilze in 4% triton x-100 (BDH Chemicals R06433) in 0.01M PBS for 4 nights at 4^{O}C

Day 2

1. wash 3x for 15 mins in 0.01M PBS at room temperature
2. add primary antibodies diluted with 0.5% triton x-100 in 0.01M PBS
    a. rabbit monoclonal anti-serotonin (Sigma, s5545) 1:10000
    b. mouse monoclonal anti-calretinin (Abcam, ab90632) 1:175
3. gently rotate tubes (in the dark) for 4 days at 4^{O}C

Day 3

1. remove primary antibody and rinse 3 x 30 mins in 0.01M PBS
2. add secondary diluted with 0.5% trition x-100 (catogue number)in 0.01M PBS
    a. anti-rabbit Alexaflour 488 (Invitrogen, A11034) 1:500
    b. bovine anti-mouse Texas red (Santa Cruz Biotechnology, sc-2788) 1:400
3. put in dark on gentle spin for 2 days at 4^{O}C

Day 4

1. rinse 3 x 45 mins with 0.01M PBS
2. mount in 3:1 glycerol (VWR CABDH1172)in 0.1M Trizma base (Sigma 93362) with 2% N-Propyl gallate (Sigma P3130), pH 8.0

0.1M PBS Stock Solution Recipe

- 80g NaCl (Sigma SX0420-3)
- 2g KCl (Sigma P217-10)
- 11.2g Na_2HPO_4 (Sigma SX0720-1)
- 2g KH_2PO_4 (Sigma P5655)
- in 1000mL of dH_2O
- pH 7.4

Aptes Coated Slides

1. dip slides in 100% EtOH
2. dip slides in tap H_2O
3. dry in incubator at 38^{O}C overnight
4. allow slides to cool
5. dip slides in 2% Aminopropyl triethoxy-silane (APTES) (Sigma A3648) in acetone (Fisher A18-4)
6. dip in 100% acetone (Fisher Scientific A18-4) twice

7. dry in incubator at 38^{O}C overnight
8. store at room temperature

B-Crystallin Immunohistochemistry Protocol

Day 1

*The below performed at room temperature in coplin jars on a table top shaker

1.	2 x	15 mins	Citrisolv
2.	1 x	2 mins	100% EtOH
3.	1 x	2 mins	90% EtOH
4.	1 x	2 mins	80% EtOH
5.	1 x	2 mins	70% EtOH
6.	1 x	2 mins	50% EtOH
7.	1 x	2 mins	30% EtOH
8.	1 x	2 mins	dH_2O
9.	1 x	15 mins	PBS
10.	1 x	15 mins	PBST
11.	1 x	15 mins	PBS
12.	1x	1 hr	10% goat serum in 0.01M PBS at room temperature without shaking
13.	1x	overnight	in primary antibody at $4^{O}C$

Primary mouse β crystallin concentration of 1:150 in 10% goat serum in PBS

Day 2

*The below performed at room temperature in coplin jars on a table top shaker

1.	dip in fresh PBS		
2.	1 x	15 mins	PBS
3.	1 x	15 mins	PBST (tween-20)
4.	1 x	15 mins	PBS
5.	1 x	2 hrs	secondary antibody in the dark at room temperature

Secondary Alexaflour 488 goat anti-mouse (Invitrogen A11029) concentration of 1:100 in 10% goat serum (Sigma G9023)and PBS

After Secondary

*The below performed at room temperature in coplin jars on a table top shaker

1.	dip in fresh PBS		
2.	1 x	5 mins	PBS
3.	1 x	5 mins	PBST
4.	1 x	dip	PBS

Block

5.	1x	20mins	0.3% Sudan Black (Sigma S-4131)in 70% EtOH
6.	rinse quickly in PBS five times.		

Mount

Coverslips mounted using gel mounting medium (Sigma G9018).

Toludine Blue Stain

*The below performed at room temperature in coplin jars

1. 2 x 5 mins Citrisolv
2. 1 x 1 mins 100% EtOH
3. 1 x 1 mins 90% EtOH
4. 1 x 1 mins 80% EtOH
5. 1 x 1 mins 70% EtOH
6. 1 x 1 mins 50% EtOH
7. 1 x 1 mins 30% EtOH
8. 1 x 2 mins dH_2O
9. 1 x 3 mins toludine stain solution
10. 3 x 1 mins dH_2O
11. 10 dips 30% EtOH
12. 10 dips 50% EtOH
13. 10 dips 70% EtOH
14. 10 dips 80% EtOH
15. 10 dips 90% EtOH
16. 10 dips 100% EtOH
17. 2 x 3mins Citrisolv
18. coverslip with DPX mountant (Fluka 44581)

Toludine Stain Solution Recipe

- 0.25g Toludine Blue (Sigma T3260)
- 25mL of 70% EtOH
- 2.25g Sodium chloride
- 225mL dH_2O
- pH 2.4

Phalloidin Skeletal Muscle Stain

1. Specimens were fixed in 4% PFA in PBS overnight, then store in PBS until staining
2. Specimens were placed in a 1:500 dilution of phalloidin (Sigma P-195)in PBST overnight at room temperature
3. Rinse in PBS for 5 mins
4. mount in methylcellulose (Sigma M0387, 4%) to view