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Median Fin Patterning in Bony Fish: Caspase-3 Role in Fin Fold Reabsorption

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Median fin patterning in bony fish: caspase-3 role in fin fold reabsorption

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Abstract

Fish larvae develop a fin fold that will later be replaced by the median fins. I hypothesize that fin fold reabsorption is part of the initial patterning of the median fins, and that caspase-3, an apoptosis marker, will be expressed in the fin fold during reabsorption. I analyzed time series of larvae in the first 20-days post hatch (dph) to determine timing of median fin development in a basal bony fish - sturgeon- and in zebrafish, a derived bony fish. I am expecting the general activation pathway to be conserved in both fishes but, the timing and location of cell death to differ. The dorsal fin fold is the first to be reabsorbed in the sturgeon starting at 2 dph and rays formed at 6dph. This was closely followed by the anal fin at 3 dph, rays at 9 dph and only later, at 6dph, does the caudal fin start forming and rays at 14 dph. In zebrafish, reabsorption of the anal fin fold began to occur around 6 dph but, the dorsal fin did not begin until around 12dph, while in the caudal fin it did not occur until 18 dph. Contrary to the sturgeon larvae, there were no rays formed by day 19 dph, but all fins had started forming, and reabsorption of the fin fold was underway. In both species the timing of fin folding reabsorption is distinct and could be one of the drivers of differences in median fin shape. Zebrafish larvae were incubated with caspase-3 mouse antibody and then with a secondary antibody - fluorescent marker. Caspase-3 activity was seen at the edges of what would later become the anal fin at 6 dpf, in the same regions of the dorsal fin at 10 dph, and in the caudal fin edge at 6 dph. Caspase-3 and thus cell death can clearly be linked to fin patterning in fish. A histological analysis was performed on muscle fibers of bluegill sunfish fins to determine size and orientation of the fibers in the different locations. Collagen was

also observed in developing zebrafish larvae fins at 12 dph where actinotrichia was expected to be present.

Introduction

The class Osteichthyes is a vast group of fish with numerous morphologies amongst all fish within that class, which alludes to an evolutionary timeline of development for these fish. There are still many gaps in our understanding of fin development in fish and there is even less information and available research on the median fins (dorsal, anal, and caudal fins) (Figure 2). The vast majority of research that has been done involving fin development has neglected the median fins, and instead focused on the paired fins - pectoral and pelvic fins - and ultimately how the fish are using those fins to move about the water. The main reason for this is the fact that paired fins are homologous to limbs in tetrapods, thus it would make sense for them to be studied far more than median fins. The pectoral fins are used in appendicular-based locomotion of fish and in addition to stabilization and balance roles in fish that use body-caudal locomotion (Wilga & Lauder, 1999). In sturgeon, pectoral fins have been found to generate lift and balance out forces from the tail (Wilga & Lauder, 1999). The median fins are used in an undulatory fashion, or side-to-side movement, to propel the fish through the water (Chadwell et al., 2012). Median fins can have different shapes, numbers, sizes, and positions along the body. The dorsal and anal fins are positioned along the longitudinal axis of the fish, while caudal fin is the terminal fin of the fish (Figure 1). Although these fins have been studied in terms of functional morphology (Maia & Wilga, 2013), there is still much to learn on their function as well as their development.

The differences in fins allude to the evolutionary changes among taxa and by studying these fins the ancestry of fish species can be traced back and examined for evolutionary patterns. I focused the larval stages and median fin development from the median fin fold, a precursor of these fins (van den Boogaart et al., 2012). The median fin fold is made up of a double sheet of epidermis and is only present when the fish is developing their fins (van den Boogaart et al., 2012). From there, apoptosis should occur; causing reabsorption, and ultimately the destruction of the fin fold, exposing the newly made fins.

The superclass Osteichthyes, bony fish, is composed of fish that are made up of bone rather than cartilage (Chondrichthyes). Bony fish have two major classes, Sarcopterygii that gave rise to the Tetrapod lineages and Actinopterygii, referring to ray-finned fish, where my work will focus (Johanson et al., 2004). Amongst the Actinopterygii class, I have chosen the shovelnose sturgeon (*Scaphirhynchus platorynchus*) as a basal fish representative, and the zebrafish (*Danio rerio*) from the teleost lineage as a derived fish (Figure 2). Zebrafish are commonly studied due to how quickly they reproduce, their clutch size, as well as, their transparent embryo that develops outside of the mother (Dahm & Geisler, 2006). Due to the vast amount of research conducted on zebrafish, their whole genome has been sequenced allowing for continued research and comparison to other organisms.

In general, the dorsal and anal fins are symmetrically positioned along the anterior-posterior axis in Actinopterygii and are thought to be under similar regulatory mechanisms during development (Mabee et al., 2002). From sheer observations, it is not hard to notice discrepancies in the symmetry. For example, in

zebrafish, the dorsal fin is slightly more anterior than the anal fin. There is a boundary constraining how posterior the anal fin can be due to the position of the anus (Mabee et al., 2002). In comparison, the sturgeon's dorsal fin is set far more posteriorly than the zebrafish. A closer look at the fins and the differences in development between a basal and a derived ray finned fish will hopefully give insight into habitat differences that cause evolutionary divergence among taxa of fish. Phenotypic plasticity, where organisms have the ability to develop different morphotypes based on their environmental and ecological pressures yet have the same genotype, indicates to evolutionary factors having shaped these fish (Schneider, Li, Meyer, & Gunter, 2014). As form usually follows function, functional morphology can point to the relationship between the structure of the fins and how it helps the fish function in their environment (Lauder, 1983).

From a genetic standpoint, homeobox (*Hox*) and sonic hedgehog (*shh*) genes have primarily been seen to cause variation amongst fins due to differential expression of these genes. This is where there is a gap in the research, as it is not entirely clear how these genes play a role, only that when expressed in a specific area, fins begin to develop in those areas. It has been predicted that *Hox* genes are correlated with some aspects of positioning, and patterning of median fins, as well as growth and organization of the fins (Mabee et al., 2002). This has been hypothesized for various species of fish as *Hox* genes are correlated with spatial arrangement in other vertebrates such as amniotes (Ahn & Ho, 2008). In zebrafish, the position of the pectoral fin has been found to be correlated with *Hox* signaling, specifically with *HoxA* and *HoxD* (Grandel & Schulte-Merker, 1998). This indicates

that *Hox* gene signaling may also be involved in median fin patterning and thus needs to be researched in future studies (Mabee et al., 2002). In the classic *Drosophila* example, *Hox* genes seem to act along an axis as they give the organism the various segments of the body. If mutations occur, body segments are found in different places along the anterior-posterior axis (Duboule & Dollé, 1989). The same *Hox* genes found in organisms like drosophila have been seen in zebrafish acting on the same axis (Zakany & Duboule, 2007). It is believed that *Hox* genes play a role in the fin fold, which surrounds the growing fish larvae and serves as temporary locomotor structure so the developing fins underneath can grow properly (Dahn et al., 2007). The process in which the fin fold is reabsorbed back into the fish is likely by apoptosis, which is hypothesized to influence shape by changes in timing and location. Apoptosis in fish can be identified by immunohistochemistry assays of caspase-3 (Yabu et al., 2001). Understanding the timing of the genes that correspond to the development of the fins will help to better understand how these genes are working and where exactly they are expressed at different times during development.

Bone morphogenetic protein (BMP) is also involved in median fin development, specifically in the formation of fin radials and rays and in the zone of segmentation (ZS), which is where the proximal and distal part of the ray of the dorsal and anal fin separate to form a type of joint (Crotwell et al., 2004). *Hox* genes, *shh* gene, caspase-3, collagen forming genes, and *bmp* (*bmp2a* & *bmp2b*) all work in the development of the fins whether that is on the initial signaling, degeneration of the fin fold, or the formation of the rays in the median fins.

As previously mentioned, the zebrafish has been very well studied and the stages of their embryotic development have been identified. As all vertebrates start out, the single celled organism is a zygote until that one cell divides repeatedly over about a 72-hour period. Around 24 hours into the embryonic development, the median fin fold begins to form and at a point after that, fin development is triggered (Kimmel et al., 1995). Migration of precursor cells to muscle, skeletal, nervous tissue, and epidermal tissue is initiated. The ectoderm will push distally from the axial endoskeleton to aid in fin development by forming actinotrichia and the skin of the fish. The mesoderm will also begin to push distally to produce the other essential parts in the fin using undifferentiated mesenchymal cells (Hall, 2007). The mesoderm will produce the rays or lepidotrichia, as well as, the muscle in the fin (Sousa et al., 2011).

Although zebrafish and sturgeon share a common ancestor, their development and larval stages differ from one another. A sturgeon will develop from a prelarvae, to a larvae, to a juvenile, and finally an adult fish. Standard embryonic development occurs as the fertilized egg goes through cleavage, blastulation, gastrulation, neurulation and organogenesis. The sturgeon egg is globular and somewhat elongated, demersal, with a brown-gray color (Gisbert, 1999). The most important organ systems begin to form first as they are essential for the survival of the newly formed organism (Gisbert, 1999). Once the heart is beating and the muscles are contracting, the fish is able to move so the fins, and tail begin to grow. Once the embryo breaks out of the egg membrane, it is able to swim about the environment as a prelarvae (Dettlaff et al., 1993). It is during the

prelarvae stage that the position of the dorsal fin is determined. Morphogenesis and differentiation occur as well (Bolker, 1993). There is already an embryonic fin fold formed covering the developing fins as the embryo hatches from the egg casing. The prelarvae and larvae stages together are about a 40-day stage. At days 1 and 2 post-hatch (dph), the very beginning stages of the pectoral fins can be seen and at day 3, there is pigmentation in the dorsal fin fold indicating where the future rays will be located (Gisbert, 1999). The fin fold is slightly protruded on day 3 post-hatch, as well in the areas where the dorsal, anal, and caudal fins would develop. By days 5 and 6, there is evidence of the anal fin rays and between days 19-21 is when the caudal fin rays were seen to be completely formed (Gisbert, 1999). The sturgeon is different from teleosts in that they have this prelarval stage, which is an important characteristic of the sturgeon (Dettlaff et al., 1993). After the larval stage, the sturgeon is a juvenile, and will continue to grow until they mature into an adult fish (Gisbert, 1999).

The egg of the zebrafish is not as elongated as the sturgeon but will begin to elongate around 10-24 hours as the tail bud appears and begins to grow (Kimmel et al., 1995). The eggs are transparent, which make studying their development easy. The zebrafish embryo will hatch around 3 days after fertilization and at this time, the pectoral fins begin to develop. Once hatched, sturgeons are about 11 mm in length as opposed to zebrafish, which are 3 mm (Kimmel et al., 1995). The embryonic fin fold is visible at 20 hours post fertilization (Cole & Ross, 2001). From day 30-45 post-hatch, the median fins are fully developed and the fish will continue to grow until the adult size is reached.

Based on prior knowledge on paired fins and genes active in development in other fins, I expect *Hox* and *shh* genes to be active during development of the sturgeon and zebrafish (Figure 3). I expect caspase-3 to be present when there is reabsorption of the fin fold. I predict that caspase-3 expression will occur in the distal portion of the median fin fold and work towards the proximal portion up to the edges of the newly developing fins until complete reabsorption has occurred. I also predict the dorsal and anal fins will begin to develop first and occur almost simultaneously. If they are not symmetric, I predict the dorsal fin will begin to form first, as it is more anteriorly positioned on both fish species.

The ultimate goal of this research is to understand how the fins of these fish have developed over time due to evolutionary constraints and ecological pressures. A time map of when these genes are being expressed, as well as, where they are expressed (anterior or posterior portions of the developing fins) should help in determining how they are developed, and where they are positioned on the body. With this information, I can compare these developmental differences to other basal fish species, and incorporate other genes thought to be involved in development.

Methods

Study Animals

The zebrafish embryos were ordered from Carolina Biological Supply and stored in petri dishes around 28 ^{o}C in the Eastern Illinois Biology Department. The sturgeon larvae images were obtained courtesy of Dr. Robert Colombo and Dr. James Garvey from the Aquaculture program at Southern Illinois University.

Sturgeon Larvae

Shovelnose sturgeon larvae were analyzed from 0 days post hatch (dph) to 19 dph. Stages pertaining to fin development including complete median fin fold presence or absence, a dorsal, anal, or caudal fin fold, reabsorption of those fin folds, and when the rays of all three fins began to appear were documented and made into a time scale of events.

Zebrafish Larvae

Zebrafish larvae allowed to hatch, and preserved in paraformaldehyde (PFA) for 24 hours and then washed with (phosphate buffered saline with tween) PBST and put into methanol until all larvae were preserved. On each day, 4 larvae were extracted until the zebrafish began to hatch, then 4 larvae were preserved each day. Human/mouse activate caspase 3 antibody (source rabbit) was then added to the tubes and stored overnight at 0^{o} C. Once the antibody was removed, the larvae were washed with PDT, and then donkey anti-rabbit lgG NorthernLights NL557 conjugated antibody was added (Sorrells et al., 2013). They were shielded from light and imaged using a fluorescence microscope.

Trichrome Stain: Zebrafish

One zebrafish larvae at 12 dph was sent to the University of Illinois Urbana Champaign's histology lab to be sectioned and stained with trichrome, a specific stain for collagen (blue), as well as a Hematoxylin and eosin (H&E) stain to enhance muscle (red) (Garvey, 1984). Dorsal, anal, and caudal fins were stained to look for collagen fibers.

Staining of Bluegill Sunfish Fins

Bluegill sunfish (*Lepomis macrochirus*) were collected from Charleston Lake in Charleston, Illinois. Five bluegill sunfish, each about 16 cm, were used and, various sections of their median fins were cut and embedded into paraffin wax. A series of solutions containing water, 100% ethanol, and t-butyl alcohol were used in the dehydration process before being placed in paraffin wax and stored in an oven. The fin pieces in the paraffin wax were left to cool, and cut and placed on blocks for sectioning. Using a microtome, the blocks were sectioned at a width of 10 μm, and three ribbons were made from each block. Using Haupt's adhesive and formalin, the ribbons were placed on to slides. Finally, the paraffin was removed and the sections were stained using a series of limonene, 100% ethanol, 95% ethanol, deionized water, mordant, hematoxylin stain, and eosin counterstain. A coverslip was then placed over the sections using permount. The nuclei should be purple-blue, the cartilage and cytoplasm should be pink, and muscle fibers should be red (Fischer et al., 2008).

Results

Sturgeon Developmental Series

During larval development, a complete fin fold is present from day 0-2 dph. The dorsal and anal fin folds began to form first, followed by the caudal fin (Figures 4, 5 and 6a). The dorsal fin began developing slightly before the anal fin, and days later, the caudal fin began to form. The dorsal fin fold was present on day 1 dph, began reabsorption on day 2 dph, and the dorsal fin rays were present on day 7 dph. The anal fin fold was present on day 1 dph, began reabsorption on day 3 dph and on

day 9 dph, the anal fin rays were present. The caudal fin fold was present on day 3dph, began to be reabsorbed on day 6 dph, and the caudal fin rays were present on day 14 dph (Figure 6a). As the fin rays begin to form, so do the radials and the muscle elements surrounding the forming bone.

Zebrafish Developmental Series

During larval development, a complete fin fold is present from 0-6 dph and the dorsal, anal, and caudal fin folds all were present at 3 dph. The dorsal fin fold was present until 12 dph when reabsorption of the fin fold began to occur. The anal fin fold was present until 6 dph when reabsorption of the anal fin fold began for the rest of the 20-day trial. The caudal fin fold was present until 18 dph when reabsorption of the caudal fin fold began (Figure 6b).

Developmental Differences between Species

The sturgeon developmental series differs from the zebrafish developmental series in that the sturgeon's fins began to develop much sooner than the zebrafish (Figure 6). Reabsorption occurred much later in zebrafish, and continued to happen for a longer period of time. The dorsal fin began reabsorption first in the sturgeon, and was followed by the anal fin fold reabsorption soon after. In the zebrafish, reabsorption of the anal fin began first and was followed by the dorsal fin fold reabsorption quite a few days afterwards. Between the 0-19 dph time frame, the zebrafish did not form fin rays in any of the fins, and we can conclude that fin ray formation occurs at some point after 19 dph (Figure 6). The sturgeon had a wider fin fold relative to fish size (Figure 4) as opposed to the zebrafish (Figure 7). The sturgeon's fully formed anal fin is set back in relation to the dorsal fin (Figure 2).

The zebrafish's dorsal and anal fins are more in line with one another. The zebrafish dorsal fin is larger in comparison to body size in adult fish. The sturgeon has a very long, exaggerated caudal fin, whereas the zebrafish has a symmetric caudal fin.

Role Apoptosis in Median Fin Development

I was able to localize caspase-3 expression to 6 dph and 10 dph in zebrafish. Caspase-3 was expressed near the developing anal fin and caudal fin at 6 dph and 10 dph (Figure 8). Caspase-3 was expressed on either side of the developing fins and occurred at the edges of the developing fins. There was no caspase-3 expression in areas where the developing fins were. The zebrafish were also imaged without any immunofluorescence markers as a control group to show there was no glowing affect without the marker (Figure 7).

Fin Ray Histology

Actinotrichia are also present in the very early stages of development, and remain for the entire duration of fin growth where the collagen fibers are stained blue and the muscle fibers are stained red (Figure 9). Fin rays can also be seen as the circle (Figure 9a), and hemitrichia (Figure 9b), which are the two-bottom portion of the developing lepidotrichia.

Bluegill Sunfish Fin Histology

The dorsal, anal, and caudal fins were sectioned and the muscle fibers were stained red (Figure 10). Depressor, erector, and inclinator muscles can be seen in the fins, which enable them to power through the water. The different shapes of the fibers show in which direction they are running along the body of the fish and the size of the fibers give light to where the largest muscles are on the fish.

Discussion

In sturgeon, the dorsal fin began to develop first, followed by the anal fin a few short days after, and finally the caudal fin. In zebrafish, the opposite happened with the anal fin preceding the dorsal fin in development and finally the caudal fin after that. Caspase-3 was found to be expressed in regions of the fin fold on either side of the developing fins but not expressed in the area of development. Apoptosis occurred away from the fin and proceeded toward the edges and stopped. I have confirmed that caspase-3 is part of fin patterning and that this apoptosis process is happening at different times in sturgeon and zebrafish.

The fin fold contains mesenchymal cells that occur from the mesoderm and are undifferentiated until they later make up the various cells within the fin (Hadzhiev et al., 2007). These are the cells that will later be killed off as apoptosis occurs. We observed that, as predicted, once the fins are completely formed, the median fin fold is reabsorbed using caspase-3 causing apoptosis or programmed cell death of the cells. The timing of development of all three fins is different, thus the local reabsorption of the fin fold has a distinct pattern. Fin fold reabsorption in general began earlier in the sturgeon before it did in the zebrafish and the same can be said for the fin ray formation. My observations in timing of development in sturgeon did differ from literature on sturgeon. I saw elements in the anal fin at day 10 dph, while prior observations place anal fin formation between days 5 & 6 (Gisbert, 1999). The variations in timing could be due to the temperature in which they are kept. I hypothesized that development of the dorsal and anal fins would

begin first and begin at the same time due to them being coupled fins. As evolution has occurred amongst actinopterygians, the patterning among the dorsal and anal fins have remained linked (Mabee et al.,2002). In sturgeon, the dorsal fin began first but the anal fin did not begin development until days later and finally the caudal fin began to develop. This could be due to the anal fin being set back posterior from the dorsal fin on the body of the sturgeon (Robins et al., 1986).

The caudal fin differs from the anal and caudal fin in the connection to the vertebral column (Arratia & Schultze, 1992). The caudal fin is made up of modified hypurals attached to the last caudal vertebrae to create a homocercal tail in zebrafish (Géraudie et al., 1995), although sturgeon retain a more primitive condition with a heterocercal tail. Homocercal refers to a symmetry amongst the dorsal and ventral lobes of the caudal fin whereas heterocercal refers to an asymmetric relationship where the dorsal lobe is a longer extension of the vertebral column (Liao & Lauder, 2000). Evolutionarily, the variations amongst the caudal fin affect the swimming of the fish whether that be speed, or the muscles used to move their tail (Lauder, 1989).

The zebrafish development differs from my hypothesis in that the anal fin began to form first and there was an even larger gap in between the timing of development of dorsal and anal fins. I did hypothesize that the sturgeon and the zebrafish would have different timing of fins, but I did not expect the anal fin to begin development before the dorsal fin in zebrafish. This could be due to the anal fin having more symmetry with the dorsal fin on the zebrafish body as opposed to the offset fins of the sturgeon. Both fish have symmetry in their dorsal/anal fin

positioning, however as evolution has occurred, the zebrafish has experienced a very small shift in axial level positioning where the dorsal fin is slightly anterior to the anal fin (Mabee et al., 2002). The sturgeon shows an even more exaggeration of this where their dorsal fin is set far more anterior to the anal fin giving the sturgeon less symmetry in their dorsal/anal fins. In general the zebrafish started reabsorption of the fin fold later than the sturgeon and did not have fin rays developing by day 19 dph.

It's important to note possible causes for evolutionary change as natural selection has helped these fish better adapt to the environment they occupy. The differences between these two species of fish could be due to their evolutionary histories. Variations in timing of fin development appear to be driving adult morphology. Larger fins tend to start developing earlier. The caudal fin of the sturgeon is much larger than that of the zebrafish and I saw the development of the sturgeon caudal fin begin before the zebrafish. The zebrafish being a more derived bony fish is evolutionarily different than a basal fish due to selective pressures that have helped the fish adapt to its environment (Furutani-Seiki & Wittbrodt, 2004). The zebrafish stays in shallow vegetated areas as they are susceptible to a wide variety of predation (Engeszer et al., 2007).The sturgeon however is a bottom feeder usually near sand or gravel in rivers and are not subject to predation once they reach a mature adult size (Spindler et al., 2012). As with the paired fins, the zebrafish uses the pectoral fins for high speed swimming and maneuvering. The zebrafish faces predation whereas a sturgeon will grow to great lengths and is not under as strong predatory pressures (Gerlai, 2010). The sturgeon will use the

pectoral fins for low speed maneuvering as they swim on the bottom and prey on food (Lauder & Drucker, 2004). These habitat and behavior differences could be the reason for the change in control of gene expression.

Evolutionary Development

As humans, we have evolved from a common ancestor of fish, thus our entire existence has a connection to primitive fish (Stopper & Wagner, 2005). Numerous bones, organs, and even embryo development similarities can be seen throughout vertebrate evolution and fins are one of the best examples of homologous structures between humans and fish. The paired fins, the pelvic and pectoral fins, have direct homology to limbs in humans (Coates, 1995). The median fins do not have such homology to tetrapod limbs, however the little amount of research conducted on median fins suggests similarities in the two processes of developmental mechanisms even though they have different embryological origins (Freitas et al., 2006). Median fins evolved before paired fins, suggesting that paired fins and thus tetrapod limbs co-opted their genetic mechanisms from median fins (Mabee et al., 2002). Extensive research paired fins of zebrafish has revealed that genes such as *shh* gene, *Hox* genes, *col2a1a, col2a1b, col1a1a*, and *bmp* have been seen to be expressed during development (Ahn & Ho, 2008a; Crotwell et al., 2004; Dahn et al., 2007; Durán, Marí-Beffa et al., 2011; Laforest et al., 1998). *Shh* gene has been shown to contribute to patterning along the antero-posterior axis and is involved in the formation of skeletal elements (Avaron et al., 2013). Now that I know that apoptosis is helping to shape the position of the fins by selectively destroying the fin fold, I can focus on genes like *shh*, that would signal the proliferation of cells that will give rise

to fin structures such as bone, muscle, and fin rays. *Hox* gene expression is dependent upon *shh* signaling in the paired fins and the mechanisms that regulate *Hox* expression have been found to be seemingly unchanged throughout evolution (Ahn & Ho, 2008b). Future research will include looking at *shh* and *Hox* genes as well as collagen forming genes and bone morphogenesis proteins expression involved in median fin patterning.

Although the exact function of the fin fold is not entirely clear, many scientists have suggested that this structure is required for swimming in the developing larvae, it may help keep them afloat, reduce their chances of sinking, and it may also help with fin ray development (van den Boogaart et al., 2012). The median fins in teleosts (zebrafish) develop from the embryonic fin folds due to the expansion of the mesenchyme (Hadzhiev et al., 2007). The cells that surround the developing fins must be reabsorbed using apoptosis so the newly developed fins can be freed from the fin fold. Caspase-3 is thought to be responsible for the reabsorption in the fin fold. Caspase is an interleukin-1β-converting enzyme from the family of proteases and so far there have been 14 different caspases identified (Fan et al., 2005). All of the caspases share some sort of similarities such as their precursors are zymogens and they are all capable of autoactivating or activating other caspases (Fan et al., 2005). Caspase-3 is the active form of procaspase-3 and is activated by several different caspase or other various enzymes. Caspase-2 has been found to be the earliest caspase in mammals and one of the most important, where it appears to cause apoptosis (Fan et al., 2005). Mammal's digits originally have cells in-between and through the activation of caspase, apoptosis removes those cells,

which is a similar process to fish and their fin fold (Kumar, 2007). The caspase protein triggers apoptosis when caspase genes are expressed. We observed caspase-3 expression on either side of the developing fins and reabsorption occurred distally until the edges of the fins were reached, where apoptosis seized. Caspase-3 can thus be deemed part of fin patterning. Future research includes determining caspase-3 expression in sturgeon larvae to compliment our existing research on zebrafish. With this information, we can determine the timing of caspase-3 as well as where it is expressed in both basal and derived fishes and determine if these processes are conserved and how they drive fin diversity. A comparison of these fish will give us a better understanding of the genes that are in involved in median fin development.

Just as important as where the fin is placed, is the shape of the fin. The median fins are made up of bony fin rays (lepidotrichia) and radials (pterygiophores), which are the proximal and distal radials in zebrafish with a "joint" in between forming a zone of segmentation persisting for the fish's lifetime (Crotwell et al., 2004). Because sturgeon are a basal fish, they have proximal, middle, and distal radials. Zebrafish and other teleosts have reduced elements due to the fusion of the radials, which shows evolutionary changes between the two (Hall, 2007). The fin rays are distal to the radials and are made up of lepidotrichia formed from mesenchymal cells. These cells lead to lepidotrichia differentiation due to an interaction with the epidermis (Hadzhiev et al., 2007). They begin as acellular bone and are gradually thickened by osteoblasts constructing the bone (Hall, 2007). Lepidotrichia are made up of two hemirays or hemitrichia, that is they form as two half-moon shaped structures that fuse together distally as they get longer and can be

seen in our cross section of the zebrafish (Marí-Beffa & Murciano, 2010). The caudal fin is made up of expanded radials called hypurals with lepidotrichia being attached at the end making up the fin web (Mabee et al., 2002).

As the fish develops, the fin fold becomes more rigid as it is strengthened by the collagen fibers and eventually actinotrichia. Actinotrichia are rod-like structures that were thought earlier to be only made up of collagen secreted from the epidermal cells. It has been found that actinotrichia also have elastoidin, which is a mix of collagenous and non-collagenous proteins similar to collagen and elastin (van den Boogaart et al., 2012). Actinotrichia fibers are the first part of the fin skeleton that develops and gives the fin fold rigidity so mesenchymal cells can migrate into the fin fold and begin forming the new tissues (Durán et al., 2011). The actinotrichia come from blastemal cells and are synthesized by the ectoderm. As the fin continues to develop and mesenchymal cells begin to flood into the fin fold, actinotrichia separate from the ectoderm yet continue to grow due to the mesenchymal cells (Poleo et al., 2001). The exact function of actinotrichia has been questioned for some time and it is thought crucial for fin ray development, not just as secondary support in the developing fin (Durán et al., 2011). Trichrome stained cross sections of a zebrafish dorsal fin at 12 dph (Figure 9) show that collagen was present in the developing fins that I looked at. These findings support other research on collagen elements that aid in development and were seen as early as 12 dph.

As actinotrichia grow, they are replaced by lepidotrichia that are bony and connected to the axial skeleton by cartilaginous joints. Some actinotrichia will remain in the adult fin in the most distal portions of the lepidotrichia (Marí-Beffa &

Murciano, 2010). These elements will be surrounded by connective tissue, blood vessels, and nerves to make up the webbing of the fin (Becerra et al.,1983). When the larvae is entering the adult stage, scleroblasts from the mesenchyme secrete the bone matrix in between the actinotrichia and basement membrane to form the lepidotrichia (Avaron et al., 2013). The lepidotrichia can be branched and unbranched even within the same fish. The zebrafish has both branched and unbranched lepidotrichia at the end of the ray, but there is no branching in the caudal fin. The pterygiophores (radials) are originally cartilage and are later ossified into the bone that make them bony fish and separate them from cartilaginous fish (Hall, 2007). Sturgeon as well as zebafish exhibit these characteristics in that cartilage becomes ossified bone, leaving behind some cartilage elements (Dillman & Hilton, 2015). The median fin radials are made of hyaline cartilage, which is either surrounded by perichondral bone or undergo endochondral ossification where the cartilage is replaced by bone completely (Hall, 2007). I was able to photograph the radials and the lepidotrichia as they developed in the sturgeon fin fold. I was also able to see collagen in zebrafish as well as look at their radials. The stained muscle sections of the bluegill sunfish show the different sizes and shapes of the muscles in all three fins, likely red muscle based on fiber diameter (Nyack et al., 2007). The caudal had the largest size fibers, which are likely white muscle, important in accelerating fish through the water. The dorsal fin had larger muscle fibers than the anal fin did, again as expected, due to the muscle power needed in the dorsal fin more than in the anal fin. The different muscles, erectors, depressors, and inclinators can also be see in the sections as different shapes due to orientation.

Although median fins are not directly homologous to tetrapod limbs, they did evolve before paired fins and carry evolutionary connections to them. With that being said, it has been suggested that the median fin patterning mechanisms are ancestral to the paired fins and thus tetrapod limbs, giving significance to the study of the median fins (Mabee et al., 2002). It can be seen throughout the evolutionary history of bony fish that their fins have changed over time in various species. The driving forces for these changes stem from habitat differences and the need to better adapt to the environment in which the fish are placed. I saw different timing of caspase-3 expression that corresponded to changes between the sturgeon and the zebrafish as expected due to their varied fin size, shape, and location. Caspase-3 along with other known genes can be used to map various adaptations among fins as well as fish in general. Differences in timing and expression at the gene level have helped shape the animals we have today and will continue until the unforeseeable future. Future research will include looking at other genes previously mentioned as well as more basal fish to get a better look at how gene expression has changed and morphed the fins of these fish evolution.

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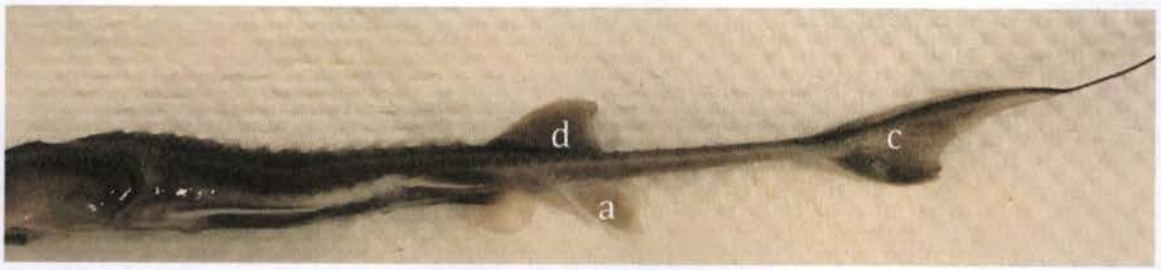

Figure 1- Sturgeon fins, d: dorsal fin, a: anal fin, c: caudal fin

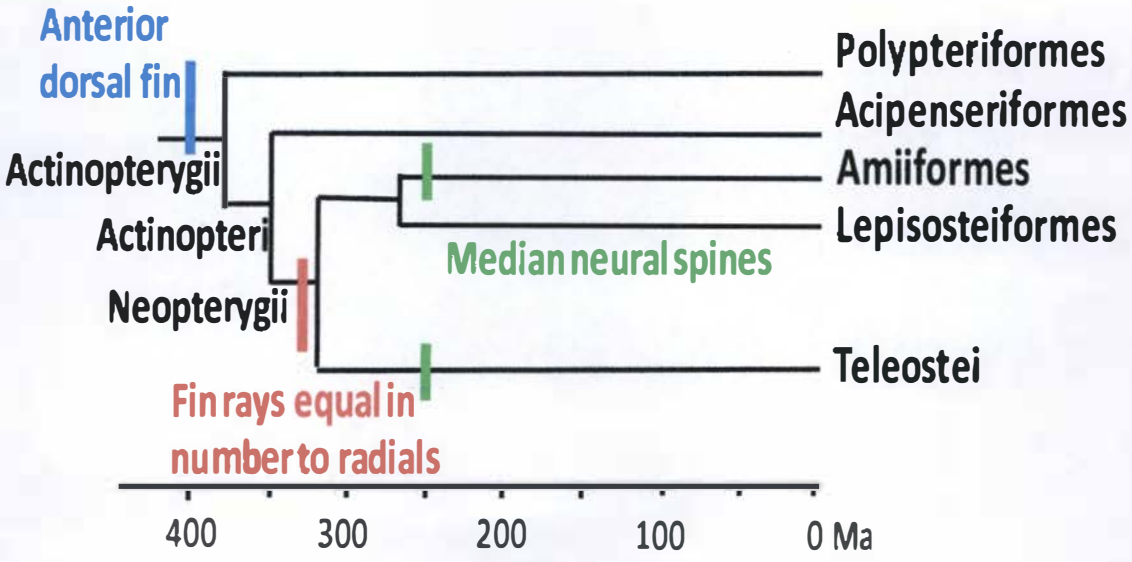

Figure 2- Cladogram of evolution in median fins

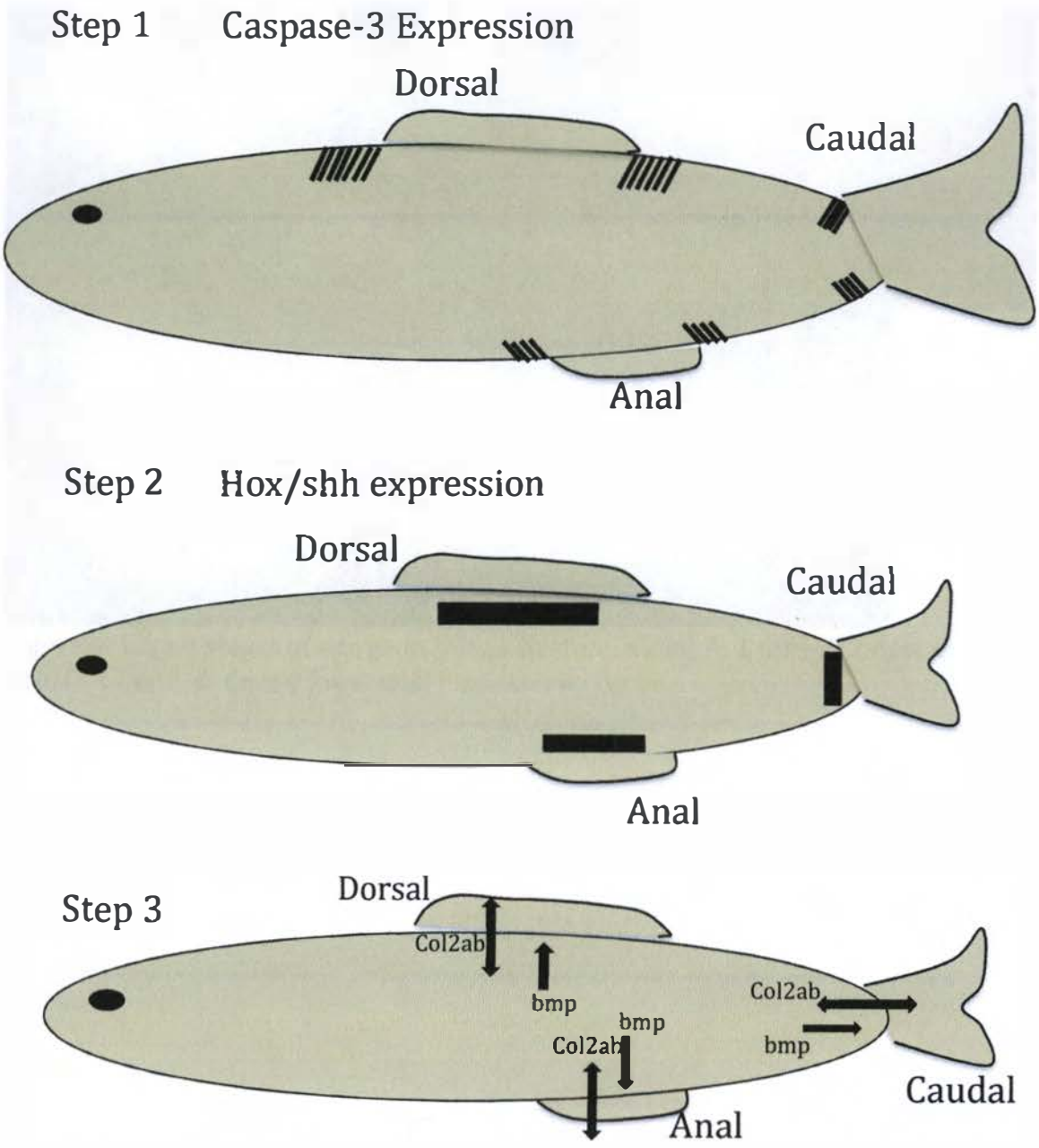

Figure 3- Expected gene localization

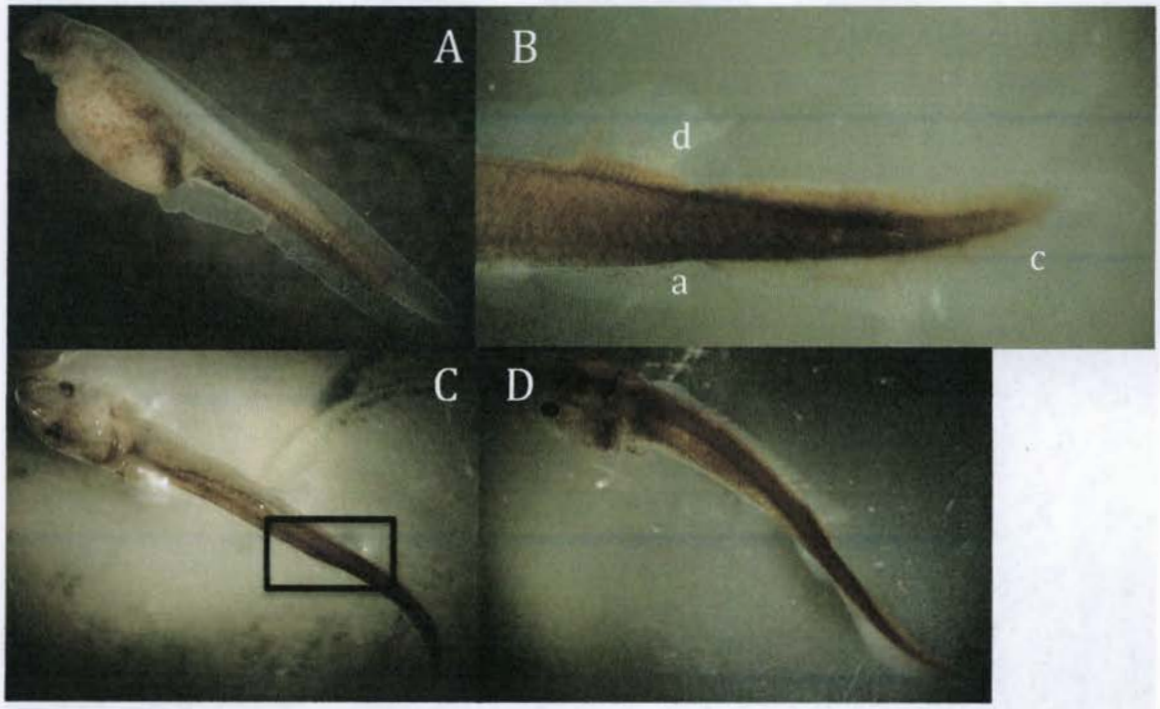

Figure 4- Larval stages of sturgeon (Magnification: 400x) A: 1 dph, B: 7 dph, C: 14 dph, D: 17 dph, d: dorsal fin, a: anal fin, c: caudal fin

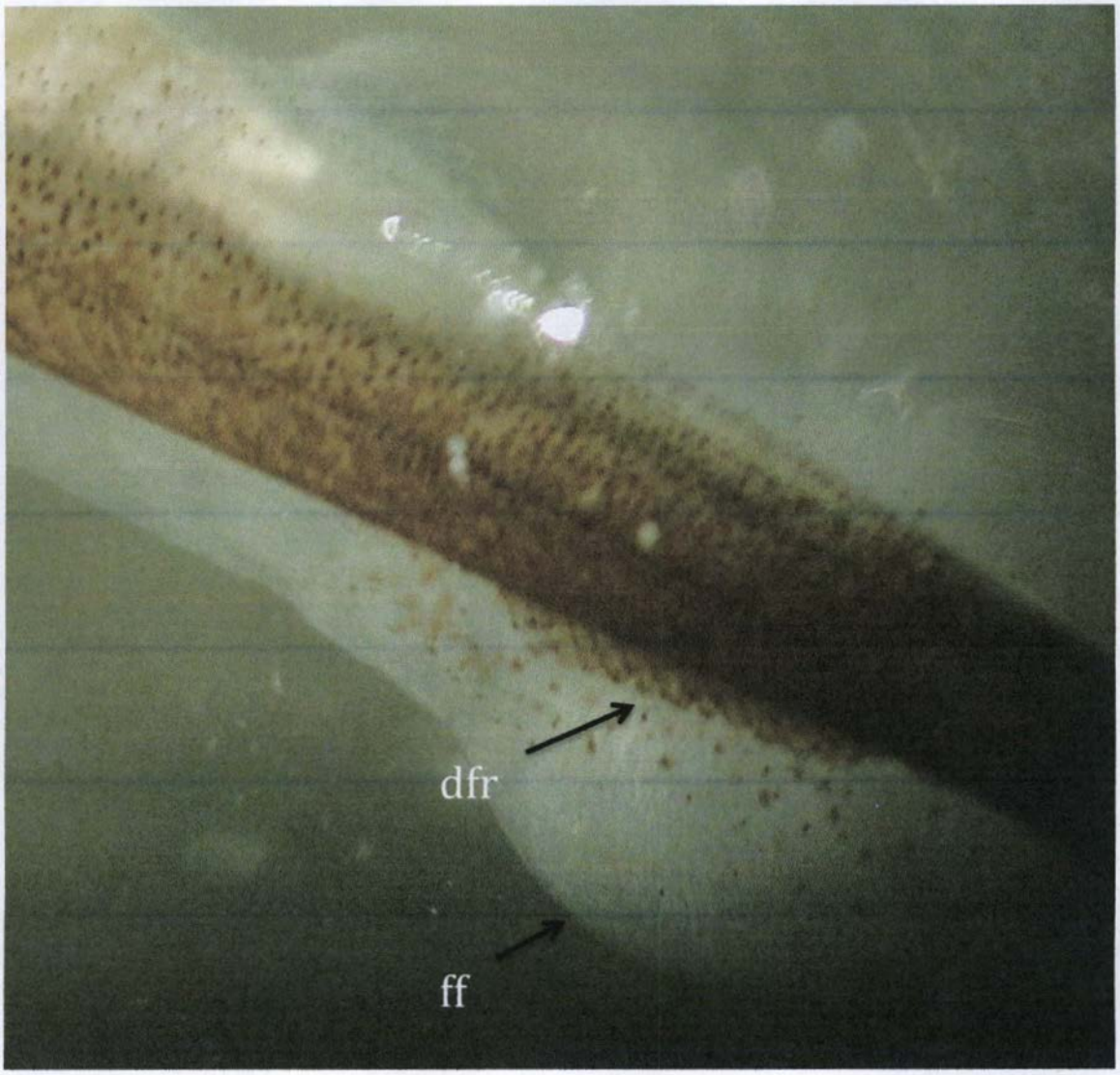

Figure 5- Higher magnification of dorsal fin rays (Magnification 400x) dl:developing lepidotrichia, ff: fin fold, dfr: dorsal fin rays

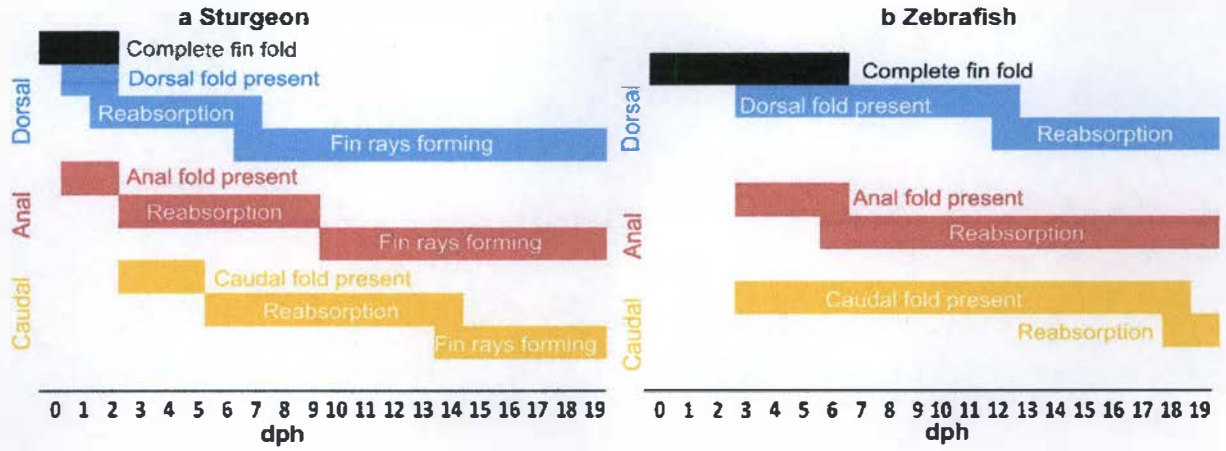

Figure 6- Timelines of sturgeon(a) and zebrafish(b) fin development

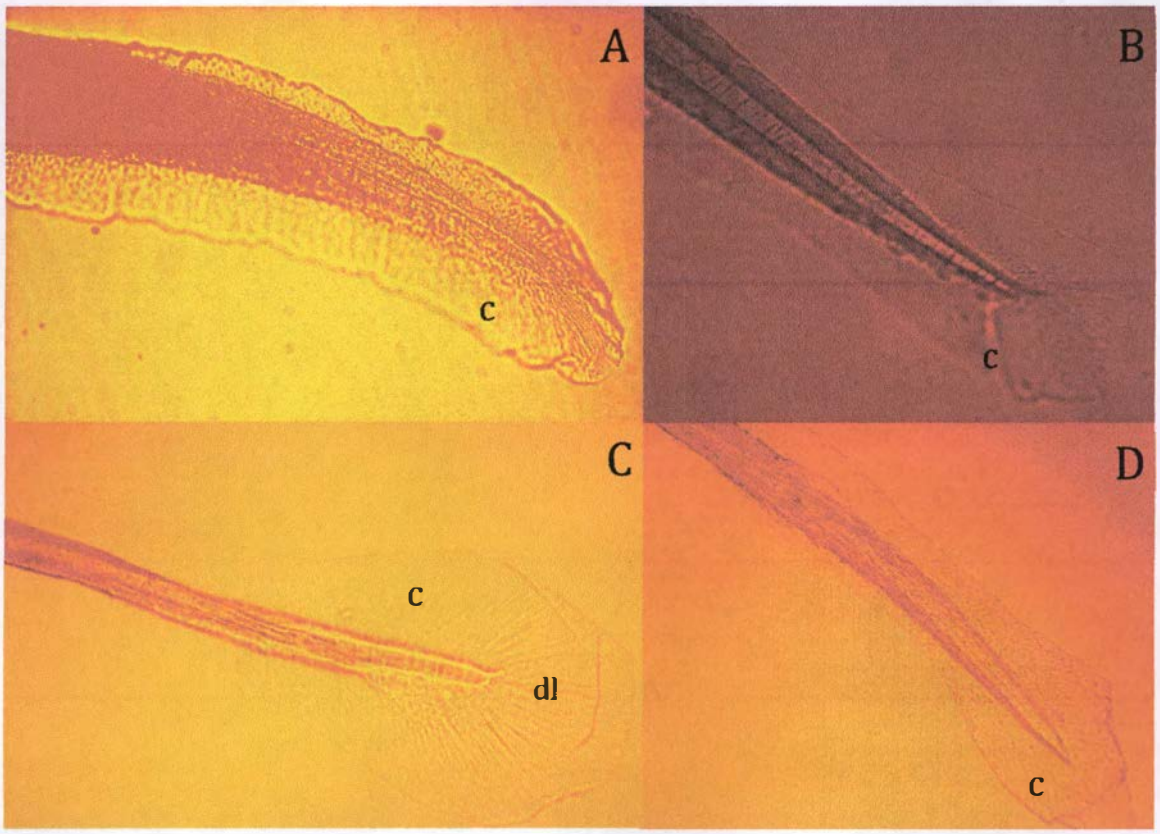

Figure 7- Larval stages of zebrafish without immunofluorescence (Magnification: 400x) A: 4 dph, B: 8 dph, C: 13 dph, D: 18 dph, c: caudal fin, dl: developing lepidotrichia

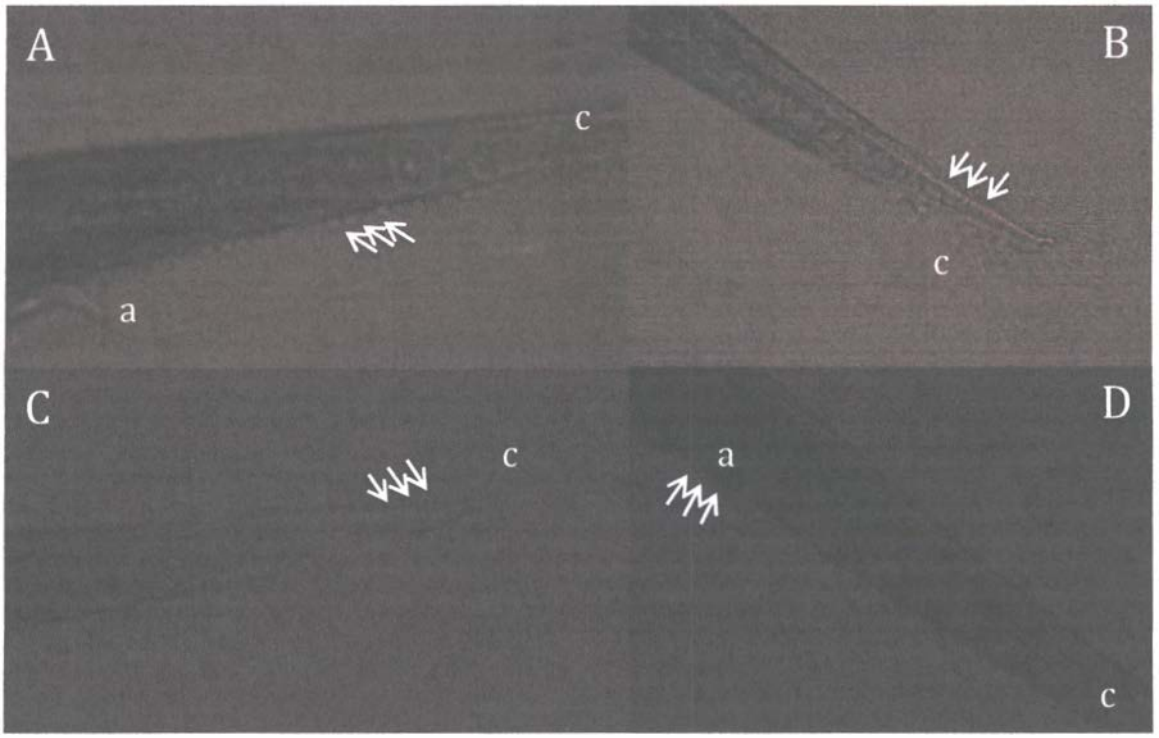

Figure 8- Larval stages of zebrafish with immunofluorescence (Magnification: 400x) A:6 dph, B: 10 dph, C: 6 dph, D: 10 dph, c: caudal fin, a: anal fin, arrows: caspase expression

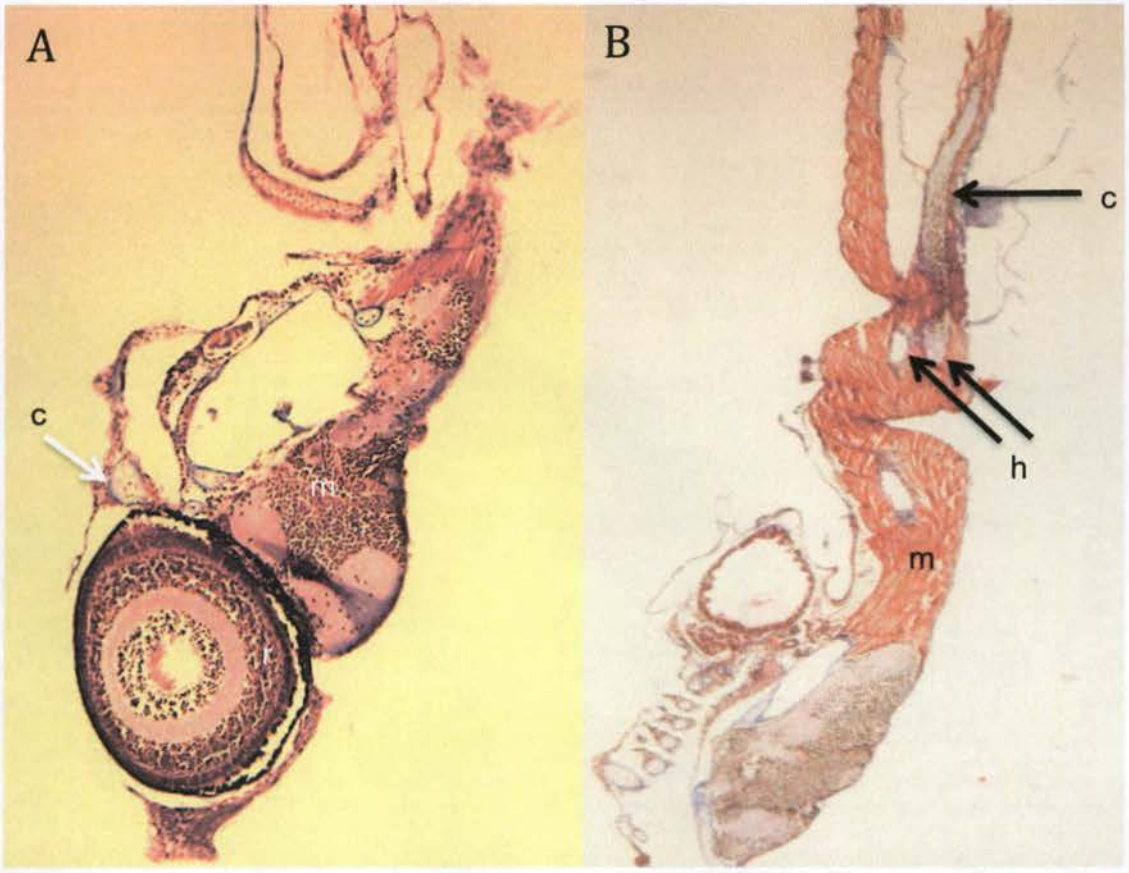

Figure 9- Zebrafish cross section of dorsal fin at 12 dph (Magnification: 400x) H: hemitrichia, m: muscle fibers, c: collagen fibers, r: radials

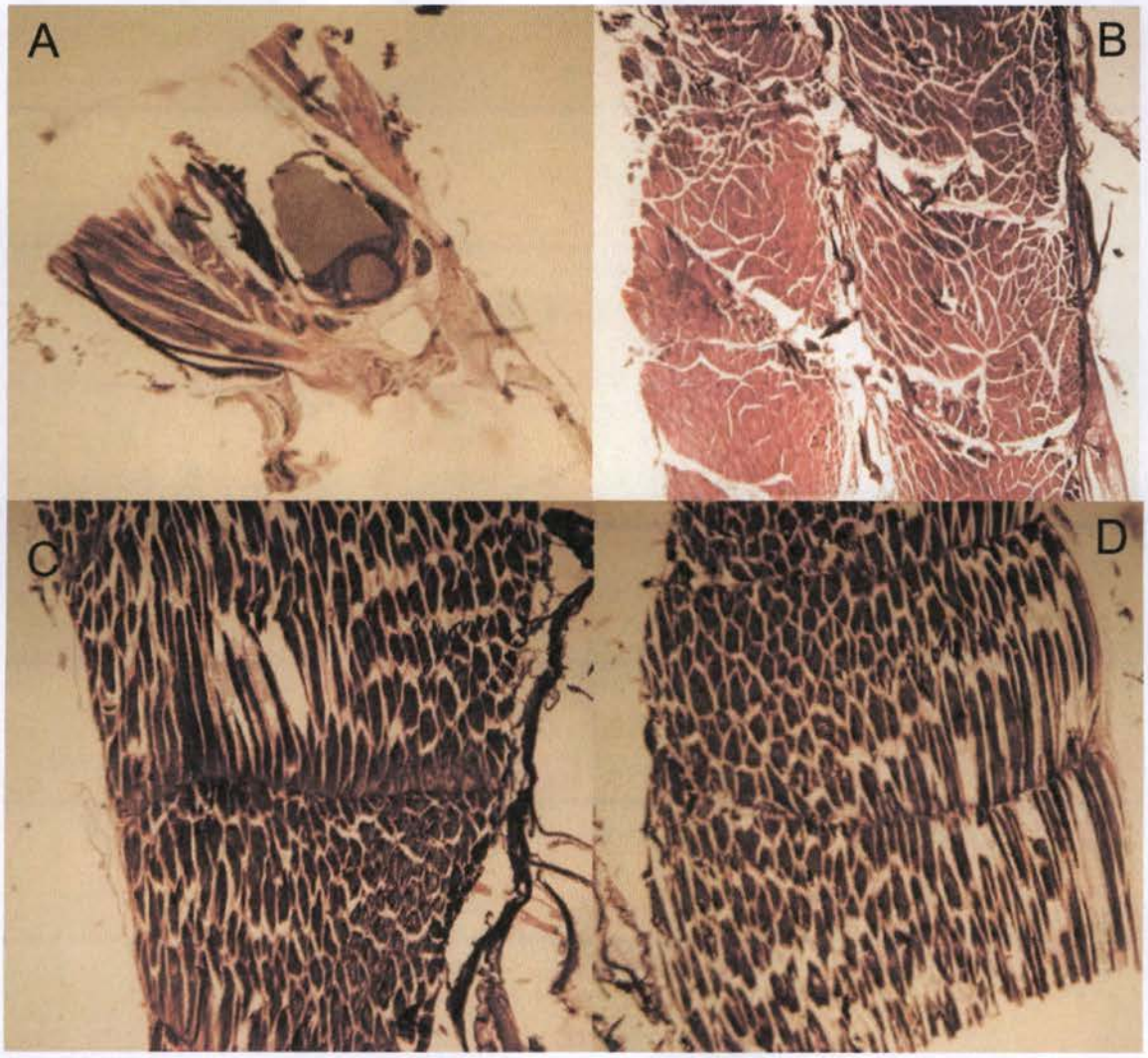

Figure 10- Bluegill Sunfish sections (Magnification: 400x) A: Anal fin muscle, B: Caudal fin muscle, C: End of dorsal fin, D: Middle of dorsal fin