"The Effects of Tail Regeneration on Betta splenden Behavior and Epigenetics"

An Honors Thesis

by

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Introduction:

Regeneration

Regeneration is a crucial part of life for all organisms. It ranges from cell renewal to organ regeneration using a range of processes of regrowth (Kawakami, 2010). In mammals, cells must regenerate to close wounds or repair organs, but some other species have harnessed regeneration so well that they can replace severed limbs and/or body parts. While these capabilities vary greatly between different species, several are widely known for their advanced regenerative capabilities. These include hydra, axolotl, freshwater planarians, and zebrafish. Because of their advanced capabilities, these species are probably the most widely used model organisms to study regeneration (National Institute, 2020; Tsai, 2020).

Planarian Regeneration

Planarians are members of a phylum of freshwater flatworms called the Platyhelminthes. This family of worms is not parasitic, but they do possess complex nervous, intestinal, musculature, and reproductive systems. In addition, they are symmetrical organisms that feed using the complex structure of a pharynx and use primitively developed eyes on the dorsal surface of their head to sense light in their environment (Ivankovic, et al., 2019). Despite all these advanced features in such primitive looking organisms, some species of planarians are known to have impressive regenerative abilities. Planarians only require $1/129^{th}$ of their original number of cells, or about 10,000 cells, to regenerate the rest of their body (Lobo, et al., 2012; Ivankovic, et al., 2019). In fact, planarians reproduce asexually by basically ripping themselves in half and regenerating the opposing half of their body with their own cells. Planarians consist of 20-30% neoblast cells, which are stem cells specific to these species. These neoblasts are key to their regenerative abilities and can differentiate into any kind of adult cell via the blastema that forms over a wound. What is uncertain is how those cells are signaled to migrate and differentiate into complex structures with specific functions (Ivankovic, et al., 2019).

The exact processes used in tissue regeneration has eluded scientists for years, but significant progress has been made. Once a wound is formed, planarians can harness the genes they used during embryonic development to begin expression again and reform the part of their body that has been injured (Pfefferli & Jaźwińska, 2015). After a small injury, a cascade of signals activates the localized cells to close and slightly regrow the wound. After a large injury, which resulted in a loss of the planarian's tissue, a global cascade of signals is initiated to begin wound closure and regeneration. Signaling of extracellular signal-related kinase (ERK) lead to blastema formation within only 30-45 minutes after the injury. Once the wound is closed and protected from the outside world, apoptosis is initiated at wound site to rid the body of cells that are too far damaged for repair. This lasts up to 4 hours after the initial injury, followed by neoblast mitosis throughout the planarian's body. Next, the neoblasts migrate to the wound site and begin mitosis again, but this time the mitosis would occur only locally for up to 72 hours after

the initial injury. Once again apoptosis occurs, but this time it is globally. Finally, the tissue orientation, regrowth, and patterning can begin and last for up to two weeks after the initial injury.

The regeneration orientation process is communicated throughout the body using morphogen and chemical gradients and pathways. Morphogens are proteins generally responsible for cell differentiation. The concentration of morphogen that a cell receives is the determining factor for what cell it should differentiate into. A global morphogen gradient is expressed throughout the entire organism, while a local morphogen gradient is expressed only in a certain location or type of cells, not the entire organism. Differences in morphogen gradient expression are regulated by developmental regulatory genes. Sometimes regeneration is due to one morphogen with a globalized gradient, other times it is due to multiple localized signals from different chemical pathways. Moreover, gap junctions can allow cell-to-cell signaling after being stimulated by the body's bioelectric or nervous system. Gradients have been found to be crucial in the orientation of the regenerating tissue.

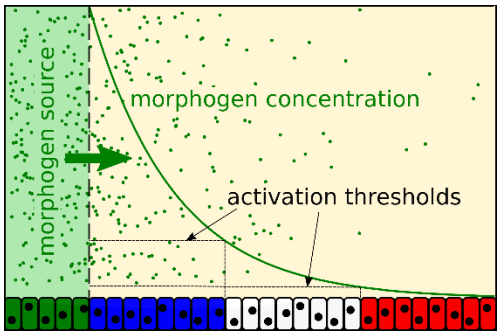


Figure 1. Example of a general morphogen gradient. The different colors of cells on the bottom represent the different concentration thresholds that will each produce a different cell type (Alnaif & Lander, 2017).

Anterior/posterior polarity is established using several different gradients at the opposing ends of the planarian. The posterior is established by Hedgehog, Wnt, and b-catenini pathways. Several studies have shown that if any of these pathways become inhibited, a head will develop in the posterior axis rather than a tail (Lobo, et al., 2012; Gurley, et al., 2008; Peterson & Reddien, 2011). Since head polarity is the global default, the Wnt and b-catenini inhibitor *notum* is needed at the head to block the Wnt and beta-catenin gradient signals to produce a head at the anterior axis. Exogenous application of retinoic acid has also been found to be a crucial part of establishing the anterior axis, and without either of these signals, a tail will develop anteriorly (Lobo et al., 2012; Iglesias, et al., 2011; Romero & Bueno, 2001). Another essential element for head formation is bioelectric stimulation. Scientists are unsure why, but several studies show that without this stimulation at the anterior axis, a tail will form, despite the signaling pathways still being complete (Dimmitt & Marsh, 1952; Lobo, et al., 2012; Marsh & Beams, 1952).

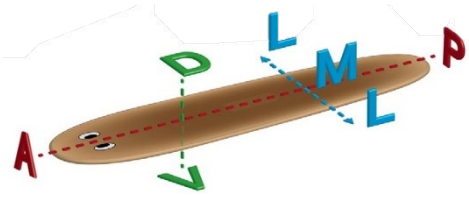


Figure 2. Axes of a planaria. (A) Anterior end. (P) Posterior end. (D) Dorsal side. (V) Ventral side. (M) Medial, middle body. (L) Lateral body. (Lobo, et al., 2012)

Similarly, dorsal/ventral polarity is also established due to several global protein gradients. The first is a bone morphogenetic protein (BMP) gradient. In vertebrates the origin of this gradient establishes the ventral axis, whereas in invertebrates, like planarians, the BMP gradient origin establishes the dorsal axis (Brown, et al., 2008; Lobo, et al., 2012; Lowe, et al., 2006). Anti-dorsalizing morphogenetic protein (ADMP) produces a gradient opposite of the BMP gradient to establish the proper opposing end. For vertebrates this would define the dorsal axis, and for invertebrates, like planarians, this would define the ventral axis. The ADMP gradient is the default dorso-ventral axis gradient within developing and regenerating organisms since it is inhibited by excess BMP molecules, and without them, neither end of the axis would be different from the other. For instance, without BMP inhibition in invertebrates, two ventral axes would form by default rather than one dorsal axis and one ventral axis. Moreover, the BMP inhibitor protein *noggin* is needed at the same end as the ADMP gradient to inhibit the BMP gradient and establish the proper end. Silencing any of these three genes disrupts dorso-ventral polarity throughout the organism (Gavino & Reddien, 2011; Lobo, et al., 2012; Molina, et al., 2011). Thus, the three molecules essentially work in a negative feedback loop to establish dorso-ventral axis polarity.

Medial/lateral axis polarity is established through protein gradient in regenerating tissue. The *slit* gene family is expressed along the medial line and the *Wnt5* gene family is expressed along lateral line to keep the *slit* gradient at bay (Adell, et al., 2009; Lobo et al., 2012; Gurley, et al., 2010). Inhibition of either of these gradients results in axis collapse as well as nervous system collapse. The medial/lateral protein gradient has been found to work in close association with the dorso-ventral gradient. Scientists are unsure why but disrupting the ADMP gradient also inhibits the Wnt5 expression gradient, disabling the lateral axis and producing multiple pharynxes in the planarian (Adell, et al., 2009; Lobo et al., 2012; Gavino & Reddien, 2011; Gurley, et al., 2010).

Following tissue orientation, tissue differentiation is initiated and orchestrated by cellular communications between the blastema, old tissues, and new tissues (Lobo, et al., 2012). Less is known about how tissue identity is determined than the tissue orientation pathways. One family of genes called *piwi* are thought to maintain of neoblast stem cells and prevent their differentiation throughout the body so that it is ready to repair itself whenever an injury occurs. Several different molecular markers have been identified in neoblasts before and after they have differentiated, suggesting that neoblasts are somewhat pre-determined before regeneration is initiated. Inhibition of this pathway results in a loss of regenerative abilities (Eisenhoffer, et al., 2008; Lobo et al., 2012; Oviedo & Levin, 2007; Palakodeti, et al., 2008; Reddien, et al., 2005). Additionally, the homologous proteins phosphatase and *tension* (PTEN) are present in planarians to regulate neoblast activity and prevent the stem cells from hyperproliferating. The proteins essentially act as tumor suppressors, as inactivation of either results in abnormal growths throughout the organism's body (Lobo et al., 2012; Oviedo & Levin, 2007). Otherwise, there is little sound evidence of how tissue identity is established in planarian regeneration.

Zebrafish Regeneration

In addition to planarian, the zebrafish, *Danio rerio*, is a common model organism for studying regeneration. Zebrafish are known to regenerate essential tissues in their bodies including their fins, heart muscle, and nervous system cells (Pfefferli & Jaźwińska, 2015; Qin, et al., 2009). Besides zebrafish being a great vertebrate model organism, they also share seventy percent of their genome with humans, meaning that many of the discoveries made about their genomic regulation is relevant to and has the possibility to be harnessed in humans as well (Gilbert, 2016). Unlike humans, zebrafish grow throughout adulthood, so their caudal fins would continue growth whether cut or not. Additionally, their bones grow and regenerate from the distal tip, not the proximal width like in humans (Pfefferli & Jaźwińska, 2015). In other words, their bones grow by adding new tissue to the farthest tip of the existing bone, whereas human bone tissue is produced and added to closest tip of the existing bone. The most studied part of the zebrafish is by far its caudal fin regeneration, in part because it regrows faster than any other fin out of necessity to the organism's survival (Pfefferli & Jaźwińska, 2015). This regeneration includes the orchestration of at least 3 different tissues, including bone. What sets zebrafish apart from other model organisms is the symmetrical morphology of their tail as well as its accessibility for amputation, tests, and photography. Like planaria, zebrafish regeneration is classified as epimorphic, meaning proliferation of tissue materials occurs before the new body part begins development (Kawakami, 2010; Pfefferli & Jaźwińska, 2015; Tsai, 2020).

A similar stepwise process of regeneration is used in zebrafish as in planaria. Directly after a tissue loss injury, fibroblasts, highly proliferative undifferentiated mesenchyme cells, begin proliferation and migration to the wound site. Dedifferentiated osteoblasts follow, and both cell types begin blastema formation. Within a day the blastema is formed to protect the wound from the external environment as well as cells organize regeneration between new and old tissues. Once the blastema is formed, fibroblast cells produce a protein called tenascin C to help organize and thicken its tissues (Jaźwińska, et al., 2007; Pfefferli & Jaźwińska, 2015). Directly under the blastema, a wound epithelium begins to form. The two layers are thought to work in conjunction to regenerate the severed tissues.

The wound epithelium and blastema work closely to organize mesenchymal and osteoblast cell proliferation and differentiation. The blastema has been shown to secrete proteins such as Fgf20a, Sdf1, Igf2b, and retinoic acid to organize the formation of the wound epithelium (Blum & Begemann, 2013; Bouzaffour et al., 2009; Chablais & Jaźwińska, 2010; Dufourcq & Vriz, 2006; Pfefferli & Jaźwińska, 2015; White et al., 2005; Whitehead et al., 1994). Once formed, the wound epithelium has been found to secrete proteins such as Sonic hedgehog (Shh), Wnt5b, and Fgf24 to help orient the blastema for proliferation (Laforest et al., 1998; Lee et al., 2009; Pfefferli & Jaźwińska, 2015; Poss et al., 2000; Quint et al., 2002). The feedback cues are thought to help create gradients for the proliferating cells to regenerate in the correct orientation along each

axis. This process is nearly the same pathway that is exhibited in planaria, showing the conservation of processes between species in nature.

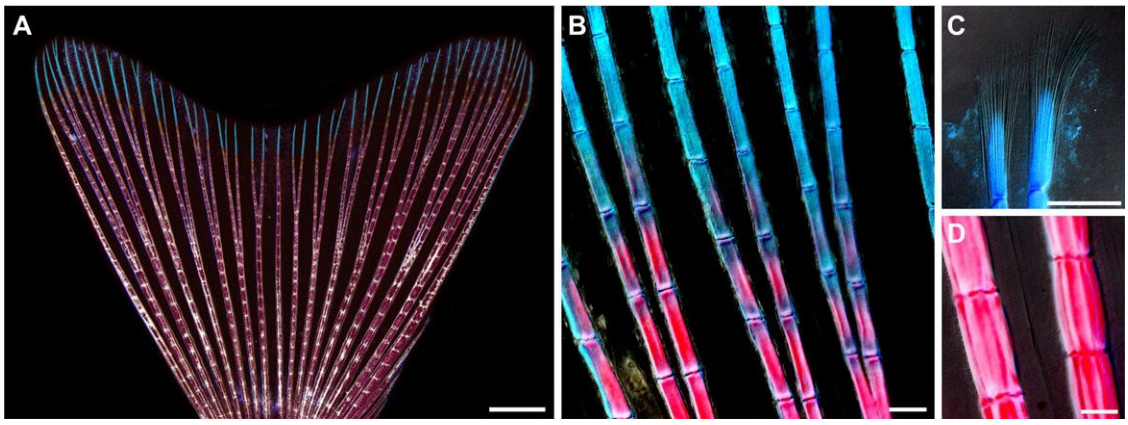


Figure 3. Pink staining shows the original tail while the blue staining shows the bone regrowth. (A) Regenerating zebrafish fin. (B) Close up of the transition area from original fin to new growth. (C) Brush-like spindles, actinotrichia, extending from the blue stained regrowth. (D) Thickened proximal bone of tail versus the thinner distal bone shown in (B) (Pfefferli & Jaźwińska, 2015).

The next two days are then defined by blastema outgrowth as fin skeleton formation begins. The fin skeleton can be seen in Figure 3 below. First, actinotrichia, non-mineralized spicule segments, are extended from the original bone tissue. These segments are organized in brush-like bundles at the tip of the old bone to act as architecture for the new osteoblasts (Duran´ et al. 2011; Kawakami, 2010; Knopf et al. 2011; Pfefferli & Jaźwińska, 2015). The protein secretions Shh and BMP from the wound epithelium have been found to be instrumental in guiding pre-osteoblasts to reform the pattern of mature bones (Laforest et al., 1998; Pfefferli & Jaźwińska, 2015; Smith et al., 2006; Quint et al., 2002; Zhang et al., 2012). One study has found that several actinotrichia genes are activated to direct the tissue extension, including the gene *actinodin1*. However, unlike in embryonic development or human bone, the growth is extended distally, rather than proximally. In addition, the surrounding tissues are also organized and extended as the fin becomes vascularized and innervated the farther the blastema extends (Bayliss et al. 2006; Pfefferli & Jaźwińska, 2015).

Following bone regeneration, the apical blastema begins extension. Notch signaling organizes undifferentiated mesenchymal cells during the blastema outgrowth (Grotek et al., 2013; Munch et al., 2013; Pfefferli & Jaźwińska, 2015). The blastema itself is thought to be an upstream organizer for the tissues, influencing cell proliferation, epidermal patterning, and cell redifferentiation with Wnt signaling. Moreover, Fgf and BMP signals from the blastema are believed to be responsible for coordinating secondary osteoblast maturation (Pfefferli & Jaźwińska, 2015; Wehner et al. 2014).

Figure 4. Progression of tail regeneration in a zebrafish in days post amputation (dpa). 1dpa shows the formation of the wound epidermis. The thick white end of the tail shown 3dpa is the fully formed blastema. The blastema outgrowth is shown at 6pda and 12dpa as more advanced. 20dpa shows the fully regenerated zebrafish tail (Pfefferli & Jaźwińska, 2015).

The next three days, the blastema begins to shrink and ends up covering only the distal most tip of the tail. This whitish tip will remain on the zebrafish's' tail for the rest of its life as a scar would on human skin. The proximal most undifferentiated cells begin differentiation into their destined cell fates. Several studies have shown that, like planarian neoblasts, undifferentiated cells have morphogenetic markers in their DNA that can be traced to their future, differentiated, cell type. This growth and repatterning of new and old tissue continues for up to two to three weeks until the zebrafish fin is fully regenerated and functional (Knopf et al. 2011; Pfefferli & Jaźwińska, 2015; Singh et al. 2012; Sousa et al. 2011; Stewart & Stankunas 2012; Tu & Johnson 2011).

Epigenetic Influence

For years, the mechanisms behind the variation in regenerative abilities between species has eluded the scientific community. However, recent findings show that the variation in regenerative abilities may be linked to different epigenetic modifications found in each species' genome. Epigenetic modifications are defined as "a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence" (Berger et al., 2009). These changes in the chromosome can come in several different forms including DNA acetylation and methylation. DNA acetylation is considered the default state of chromatin where acetyl groups are attached to the histone proteins chromatin is wrapped

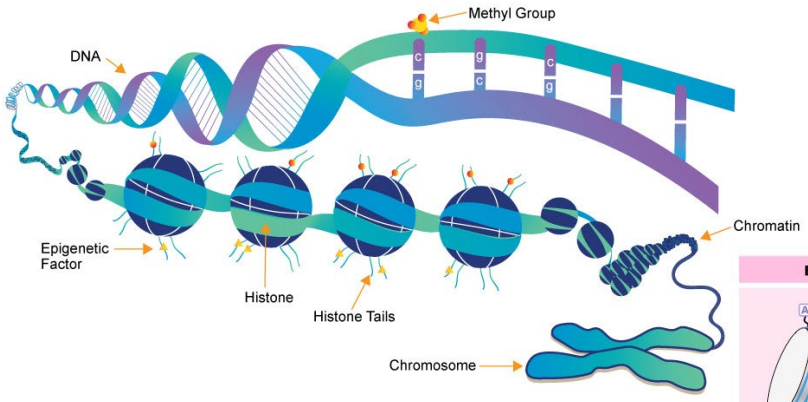


Figure 5. Chromatin structures where epigenetic modifications are applied (What is epigenetics, n.d.).

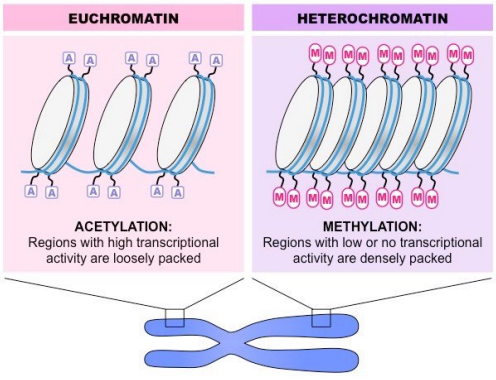


Figure 6. Visual difference between euchromatin and heterochromatin (Cornell, 2016).

around (Sharon, 2017). This type of chromatin is loose, easily transcribable, and called euchromatin (Javaid & Choi, 2017). Conversely, DNA methylation is defined as the attachment of a methyl group to a histone protein or a nucleotide base in the DNA sequence (Mulligan, 2016; Sharon, 2017). The addition of this methyl group changes how the DNA is packaged. Hypermethylation causes tightly packed, harder to transcribe chromatin called heterochromatin (Javaid & Choi, 2017). Both can result in a difference in how the DNA is transcribed and can be detrimental to an organisms' health depending on the gene(s) that are methylated (Dincer, 2016).

Based on one study of gene 5-methyl-cytosine and 5-hydromethylcytosine comparisons, early phase regrowth in regenerative species is characterized by DNA demethylation and expression of repair-related genes (Hirose et al. 2013; Pfefferli & Jaźwińska, 2015). Additionally, it theorized that these genes are upregulated in the presence of an injury, and downregulated once regeneration has ceased (Rodriguez & Kang, 2020). One study provides evidence that histone modifications at specific loci, like the demethylation of the H3K27me3 gene, can re-activate the genes necessary to regenerate living structure like all organisms once did in embryonic development (Pfefferli et al., 2014; Pfefferli & Jaźwińska, 2015; Stewart, et al., 2012). Further, several genes related to the nucleosome remodeling and deacetylase (NuRD) complex were found to be upregulated during blastema proliferation. Several of the genes required for upregulation to complete this complex are the *chd4a*, *hdac1*, *rbb4*, and *mta2* genes. Failure of this complex to activate results in no formation of the actinotrichia bristles, and thus, no architecture for the undifferentiated cells to regenerate upon (Pfefferli et al., 2014; Pfefferli & Jaźwińska, 2015). Hence, epigenetic variation plays a profound role in variation of regenerative abilities between organisms.

Regenerative Abilities of Fish

Overall, the regeneration process differs greatly between different types of organisms. Nevertheless, many kinds of fish have been used as model organisms for studying regeneration since the 1700s. The regeneration process between many kinds of fish is remarkably similar (Kawakami, 2010). As most research on fish fin regeneration has been focused on defining the specific steps of the regeneration process, how well the regrowth matches the original fin in shape, size, and coloration has often not been examined. Most studies that have been conducted utilize fish with simple fin shape and coloration, such as zebrafish. The aim of this experiment was to examine fin regeneration in more elaborate fish fins to see how the regrowth compared to fish with a simpler fin shape and coloration. Thus, the subject of study chosen for this experiment was the species of *Betta splendens*. This species is known for its elaborate tail shapes and coloration, but not necessarily its research value. While there is little to no research on regeneration in this species, these fish do possess the regenerative properties necessary to regrow large parts of their fins after an injury. However, they are often used as a model organism to study behavior and provide an opportunity to also examine whether fin regeneration affects behavioral displays and their epigenetics.

Aggressive Behavior of Male Betta splendens

Male *Betta splendens*, also known as Siamese fighting fish, are widely known for their displays of aggressive behavior. Whether exhibiting courtship behavior or general aggressive behavior towards another male, *Betta splendens* have been used for scientific behavioral studies for a variety of reasons. For instance, despite having to be kept in separate tanks, they are relatively easy to care for, and their behavioral aggressions are easy to count and identify in behavioral test (Todd, et al., 2008).

In the wild, a *Betta splendens'* fitness relies heavily upon dominance over other males in its species. Often, wild male *Betta splendens* form hierarchies as ranking of the fittest individual to the least fit individual (Jameson, et al., 1999). Fitness hierarchies are developed based on female sexual selection as well as aggressive competition between males (Jameson, et al., 1999; Milinski, 2014). Further, aggressive behavior is also used to defend territory and nests to help ensure their offspring survive (Forsatkar, et al., 2017; Todd, et al., 2008). Male *Betta splendens* aggressive behaviors are used scare off and fight their opponents since the behaviors generally make them look larger and fiercer (Todd, et al., 2008; Qvarnström and Forsgren, 1998). *Betta splendens* aggressive behaviors generally consist of frontside or broadside movements. Frontside movements include operculum and branchiostegal membrane flaring, biting, and pectoral fin beating (Glesener, 2001; Simpson, 1968; Todd, et al., 2008). Broadside movements include tail beating, pelvic fin flickering, and tail flashing (McGregor, et al., 2001; Simpson, 1968; Todd, et al., 2008). Other movements that are also considered aggressive, but are not categorized as either frontal or broadside, include chasing and charging the opponent (Halperin, et al., 1997; Simpson, 1968; Todd, et al., 2008). Though not as common, similar aggressions have been examined in female *Betta splendens* (Simpson, 1968; Todd, et al., 2008). Thus, it makes sense that both sexes display the same behaviors in captivity because they have evolved so that their reproductive success depends on it.

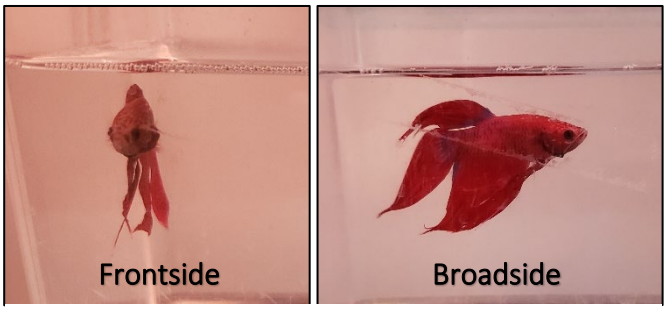


Figure 7. Visual orientations of *Betta splendens*

Numerous studies have shown that male *Betta splendens* aggressive behaviors are affected by the presences of a female (Forsatkar, et al., 2017; Lück, 2014; Milinski, 2014; Todd, et al., 2008). Further, there is some research available presenting numerous effects of stress on fish behavior. For example, salmonoids generally present proactive, aggressive behaviors or reactive, shyer behaviors when they are stressed (Laursen et al., 2011). In zebrafish, stressed behavior is presented as swimming or sitting on the bottom of the tank (Valvarce, et al., 2020). However, there is little to no research looking at how aggressive behaviors in male *Betta splendens* are affected by stress and whether regenerated fins are functionally normal in behavioral displays. As part of this

experiment, behavioral tests were conducted with male bettas that did and did not have their tails cut for regenerative study.

Experimental Aims

This study aims to determine whether fin regeneration affects male *Betta splendens* aggressive behavioral displays. Tail amputation is thought to affect either the number of times or the amount of time aggressive behaviors are displayed because aggressive behavioral displays are heavily reliant on the use of their tail. Since external environment can also shape epigenetics, this study also aims to examine whether tail regeneration results in epigenetic changes to DNA methylation.

Materials/Methods:

Experimental Setup & Maintenance

Unforeseen circumstances forced this experiment to be conducted from a home setting rather than in a laboratory. *Betta splendens* and the materials for maintenance, care, and experimentation were dropped off at my house by my professor periodically when it was time for me to move to the next step in my experiment.

Ten, red, male *Betta splendens* were bought from a local pet store and delivered to my house over the course of three weeks. Each was labeled with a number 1-10 to for organizational data purposes. To set up their tanks, one gallon of tap water was treated with 5mL of Aqueon, Betta Bowl Plus to dechlorinate the water. It was left to sit open in an empty bedroom for at least 24 hours to remove any excess minerals in the water, and to allow it to reach room temperature. Then, in a bathroom, an empty one-gallon tank was filled with the dechlorinated water and a *Betta splenden*, still in its small transportation bowl, was placed in the tank to acclimate. After at least an hour, the fish was gently submerged into the tank and the small transportation bowl was removed. This process was repeated nine times as new fish were acquired.

Eight of the nine tanks used were small, clear, rectangular tanks with an individual fish in them. The nineth tank was slightly larger and housed two *Betta splendens*, one of which had its tail cut, and the other of which did not. Five of the nine tanks were then placed on its own shelf on a shelving unit in the corner of my bedroom for storage, to prevent the tanks from shaking, and to prevent the fish from seeing each other. The other four tanks were placed by twos on another, smaller shelving unit in a corner right outside of my bedroom. Pieces of cardboard were placed in between the two tanks on each shelf to prevent the fish from constantly seeing each other and displaying aggressive behaviors towards its neighbor.

The *Betta splendens* were fed 2-3 TopFin color enhancing betta bits at approximately 8AM and 8PM, five days a week. On the weekends, they were not fed at all as to help prevent overfeeding. Once a week, debris and 20-30% of every tank's water was removed and replaced with the same dechlorinated tap water as the tank was started with.

Anesthetization & Amputation

After three weeks of adapting to the tanks and house, 5 of the 10 *Betta splendens* had their tails cut. The 5 fish amputated were randomly picked from the 10 males in the experiment. While their tail lengths were not precisely measured, all males and their tales were relatively the same size. To amputate, my professor dropped off Tricaine-S (MS-222), 1 pair of forceps, sterile razor blades, a petri dish, a box of slides, and a box of slide covers. A folding table in an empty living room of the house next to a window so that there was sufficient light. It was covered with a sheet of industrial plastic for sterility and easy clean-up. One fish, in its tank, was set on the card table as well as the rest of the supplies. The MS-222 was poured into one of the small transportation bowls, and the fish was caught in its tank using another. The fish was then gently transferred to the bowl of MS-222 and allowed in the solution until just limp and anesthetized. This process took significantly longer, up to 2-3 minutes, than for the zebrafish model organisms because of how much larger the *Betta splendens* are in size. A new, sterile, plastic spoon was used to scoop the limp fish out of the anesthetic and gently place on the petri dish. Then, the tail was spread using forceps, and a cut of approximately one third of the tail was made using a new, sterile razor blade. Using the same spoon, the fish was then gently placed back into its tank and water wash pushed over its gills to help it recover. Once the fish began showing signs of movement again, forceps were used to transfer the sample from the petri dish to the microscope slide. Once again, forceps were used to position the sample and place the cover slip over it. This process was repeated for each of the 5 *Betta splendens* with the same equipment.

Tail Amputation & Behavioral Test Schedules

Since the fish were received in three staggered groups, their tails were cut at staggered timeframes. This meant that the final tail cuts of all 5 fish showed 3 different weeks of the regrowth process. The final tail cuts were made 6 weeks after the first fish's tail was cut, meaning the samples show tail regrowth of 4, 5, and 6 weeks. There were two additional samples taken from fish whose tails were not previously cut to use as a control. The tail cut schedule can be seen in Figure 8.

Tail Cut Schedule		
	Date First Cut:	Date Second Cut:
Cut Tails		
R2'	9/25/2020	10/30/2020
R3	9/18/2020	10/30/2020
R5	9/25/2020	10/30/2020
R8	10/2/2020	10/30/2020
R9	10/2/2020	10/30/2020
Uncut Tails		
R1	~	~
R4	~	~
R6	10/30/2020	~
R7	~	~
R10	10/30/2020	~

Figure 8. Tail cut schedule.

Behavioral tests were conducted 3 weeks after the tails were cut. Even at this stage, the tail regrowth in the bettas was not fully complete but needed to be conducted for the rest of the data to be collected within the semester. Figure 9 shows the schedule of when each fish was tested against each group. Each group of fish was tested against another of its group and another of the opposite group. For each test, the fish were placed next to each other in their separate, clear tanks for 5 minutes. The tests were recorded and later analyzed for the number of times and the amount of time, in seconds, each aggressive behavior was displayed. Tail beating, tail flashing, flaring gills, extending the gill membrane, raising the dorsal fin, lowering the head, darting toward the opponent, and nipping at the opponent were all considered aggressive displays. Nipping at the opponent was only examined for the number of times it occurred and not the amount of time it occurred because nipping happens so quickly it is impossible to count the length of time that it occurred. The number of aggressive displays between the beginning and ending each test were not accounted for.

Behavioral Test Schedule	
Dates:	Uncut: Uncut
10/9/2020	R1:R4
10/16/2020	R1:R6
10/23/2020	R7:R10
	Uncut: Cut
10/9/2020	R4:R3
10/16/2020	R1:R5
10/16/2020	R6:R2’
10/23/2020	R7:R8
10/23/2020	R10:R9
	Cut: Cut
10/9/2020	R3:R2’
10/16/2020	R2’:R5
10/23/2020	R8:R9

Figure 9. Behavioral test schedule. Red indicates which fish were analyzed in each test.

DNA Isolation

The second set of tail samples taken from the group of males with cut tails as well as the only tail samples taken from the males with previously uncut tails were frozen to 40°C within an hour after amputation. The samples were chopped into the smallest pieces possible on the slides they were stored on using a sterile razor blade for each sample to not contaminate any samples. DNA was extracted using the DNEasy DNA extraction kit (Quiagen). The procedure was modified to extend the 56°C incubation to two hours, and the final eluded in 100µl rather than the time and amount described in the kit. This was to ensure the thicker sample fully broke down and to increase the DNA concentration.

DNA Purification & Analysis

The original samples of extracted DNA were prepared and tested for the amount of DNA in each sample using spectrophotometer. However, only incorrect readings from the machine were acquired because all the transmittance levels were near 3, which is too high of a reading for just a blank sample containing deionized water, let alone samples containing DNA. An ethanol precipitation with Sodium Acetate was performed on the samples with DNA, to further purify the DNA, in case contamination was an issue. While this slightly decreases the amount of DNA that will be in each sample, it ensure the DNA is much purer. After re-testing the machine, the same results, that were too high, were given again. Originally an epigenetic analysis of the amount of methylation in each sample was supposed to be conducted. However, since the spectrophotometer malfunctioned, so the amount of DNA in each sample was not able to be obtained,

meaning the epigenetic tests could not be run to find the percentage of methylation in each.

Statistical Analyses

The data collected from the behavioral tests was first analyzed using a Shapiro-Wilk Test of Normality. Four of the sixteen groups of the number of times aggressive behavior was displayed were found to be statistically significant from normal as shown in Table 1. The rest of the data sets, in both the number of times and the amount of time groups, were found to be of normal distribution and can be found in Tables 1 and 2. To perform a uniform test on all the data sets in both groups, a nonparametric, two-tailed, Independent Samples Mann-Whitney-U Test was conducted.

Epigenetic tests were originally supposed to be conducted on the average amount of methylation in males with both cut and uncut tails. Those groups were planned to be tested with the Fisher's exact test to see if there was a correlation between tail cuts and amount of epigenetic methylation.

Results:

Aggressive behaviors were examined in a control group of male *Betta splendens* with uncut tails, and in an experimental group of males three weeks after caudal fin amputation. Behaviors were quantified in individual fish, in separate displays when compared to other control males and when compared to other experimental males. Table 3 below shows the average number of times fish displayed tail beating, tail flashing, flaring gills, extending the gill membrane, raising the dorsal fin, lowering the head, darting toward the opponent, and nipping at the opponent. Table 4 below show the average time fish displayed tail beating, tail flashing, flaring gills, extending the gill membrane, raising the dorsal fin, lowering the head, and darting toward the opponent. Normality tests were conducted on each data set within each group to determine the type of statistical test that should be used to compare data sets. Since several of the data sets were statistically significant from normal, with a p-value < 0.05, and several others were close to being statistically significant from normal, with p-values of 0.08, a nonparametric Mann-Whitney U test was used to compare them. Of the fifteen comparisons conducted, only two showed a statistically significant difference. The amount of time the dorsal fin was raised in males with cut tails was significantly higher than the amount of time the dorsal fin was raised in males with uncut tails, as seen by a p-value of 0.008 and the bar graph in Figure 19. Similarly, the amount of time males with cut tails spent lowering their head was significantly greater than the amount of time males with uncut tails spent lowering their head, shown by a p-value of 0.03 and Figure 21. The rest of the comparisons showed no statistically significant difference in the number of times or the amount of time aggressive behavior was displayed between males with cut and uncut tails, as can be seen in Figures 10-18, 20, and 22-24.

Normality Test Results				
Aggressive Display:	Tail:	Test Statistic:	Degrees of Freedom	P-value
Tail Beating	Uncut	0.96	5	0.78
	Cut	0.89	5	0.37
Tail Flashing	Uncut	0.95	5	0.75
	Cut	0.96	5	0.80
Flaring Gills	Uncut	0.93	5	0.57
	Cut	0.77	5	0.04
Extending Gill Membrane	Uncut	0.93	5	0.60
	Cut	0.75	5	0.03
Raising Dorsal Fin	Uncut	0.84	5	0.18
	Cut	0.88	5	0.30
Lowering Head	Uncut	0.93	5	0.61
	Cut	0.92	5	0.56
Darting Toward Opponent	Uncut	0.80	5	0.08
	Cut	0.85	5	0.20
Nipping at Opponent	Uncut	0.95	5	0.71
	Cut	0.80	5	0.08

Table 1. Normality Test Results of the number of times each aggressive behavior was displayed in males with uncut and cut tails. Yellow highlighting indicates a p-value calculated from data that is significantly different from a normal distribution. Orange highlighting indicates a p-value calculated from data that is almost significantly different from a normal distribution.

Table 2. Normality Test Results of the amount of time each aggressive behavior was displayed in males with uncut and cut tails. None of the data sets showed a distribution that was statistically significant from normal.

Normality Test Results				
Aggressive Display:	Tail:	Test Statistic:	Degrees of Freedom	P-value
Tail Beating	Uncut	0.84	5	0.16
	Cut	0.92	5	0.54
Tail Flashing	Uncut	0.89	5	0.38
	Cut	0.92	5	0.54
Flaring Gills	Uncut	0.93	5	0.56
	Cut	0.97	5	0.86
Extending Gill Membrane	Uncut	0.91	5	0.49
	Cut	0.98	5	0.91
Raising Dorsal Fin	Uncut	0.84	5	0.18
	Cut	0.9	5	0.39
Lowering Head	Uncut	0.93	5	0.61
	Cut	0.87	5	0.28
Darting Toward Opponent	Uncut	0.82	5	0.12
	Cut	0.9	5	0.43

Mann-Whitney U Test Results			
Aggressive Display:	Test Statistic:	Degrees of Freedom:	P-value:
Tail Beating	-0.94	10	0.42
Tail Flashing	1.16	10	0.31
Flaring Gills	-1.15	10	0.31
Extending Gill Membrane	-1.15	10	0.31
Raising Dorsal Fin	-0.63	10	0.55
Lowering Head	-0.11	10	1.00
Darting Toward Opponent	-1.19	10	0.31
Nipping at Opponent	0.31	10	0.84

Table 3. Statistical results from the Mann-Whitney U test on the number of times aggressive behaviors were displayed.

Mann-Whitney U Test Results			
Aggressive Display:	Test Statistic:	Degrees of Freedom:	P-value:
Tail Beating	-0.73	10	0.55
Tail Flashing	-0.94	10	0.42
Flaring Gills	-0.52	10	0.69
Extending Gill Membrane	-0.52	10	0.69
Raising Dorsal Fin	2.61	10	0.008
Lowering Head	2.20	10	0.03
Darting Toward Opponent	-1.26	10	0.22

Table 4. Statistical results from the Mann-Whitney U test on the amount of time aggressive behaviors were displayed.

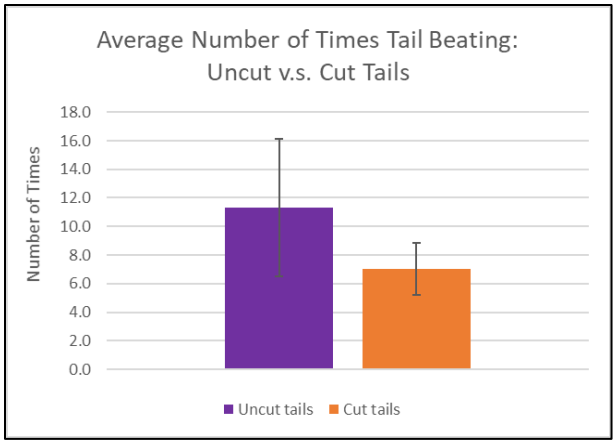


Figure 10. Average number of times tail beating. Error bars show standard error. t=-0.94, df=10, p=0.42

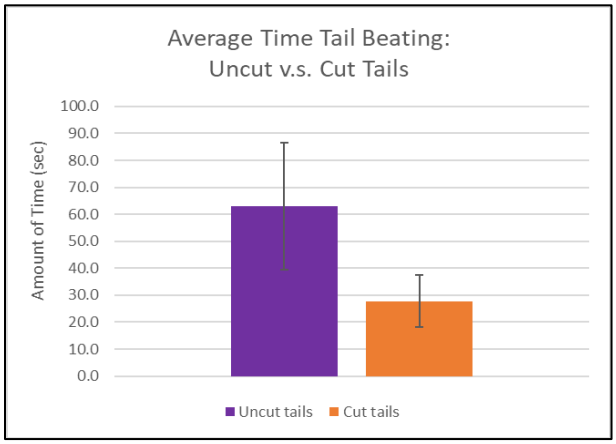


Figure 11. Average amount of time tail beating. Error bars show standard error. t=-0.73, df=10, p=0.55

Figure 12. Average number of times tail flashing. Error bars show standard error. t=1.16, df=10, p=0.31

Figure 15. Average amount of time flaring gills. Error bars show standard error. t=-0.52, df=10, p=0.69

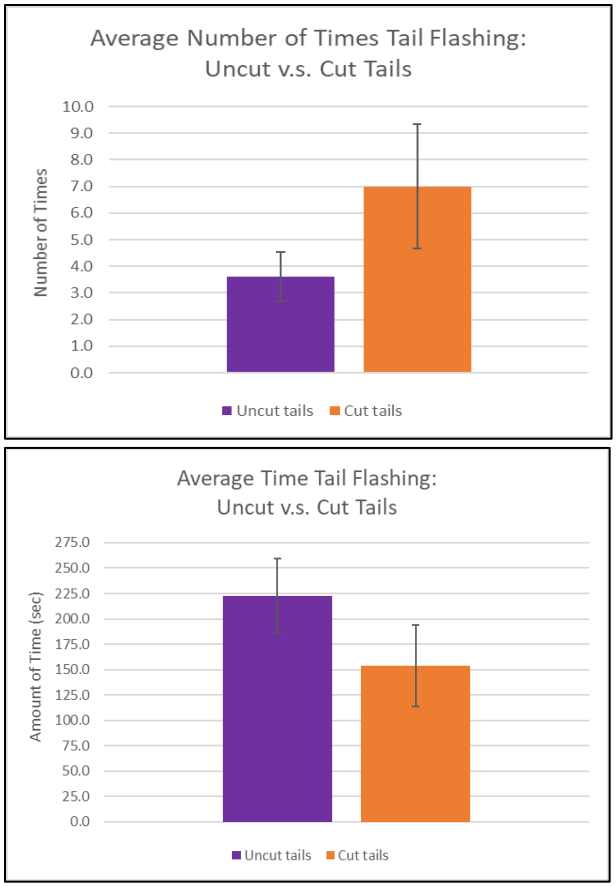


Figure 14. Average number of times flaring gills. Error bars show standard error. t=-1.15, df=10, p=0.31

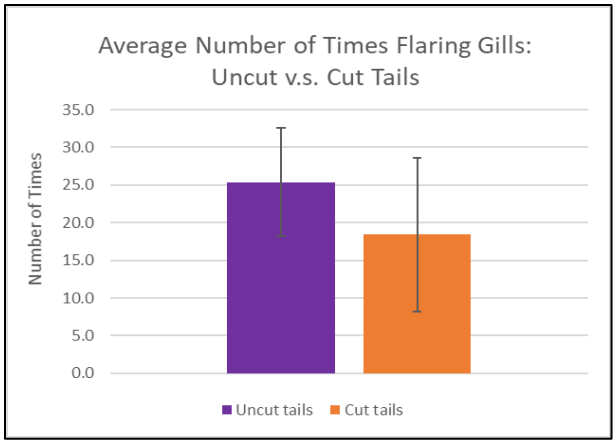
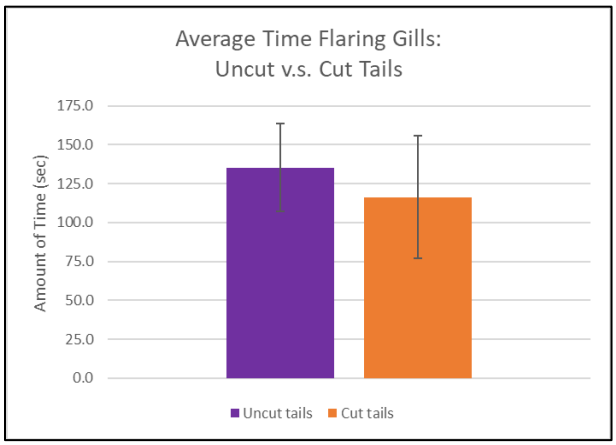


Figure 13. Average amount of time tail beating. Error bars show standard error. t=-0.94, df=10, p=0.42



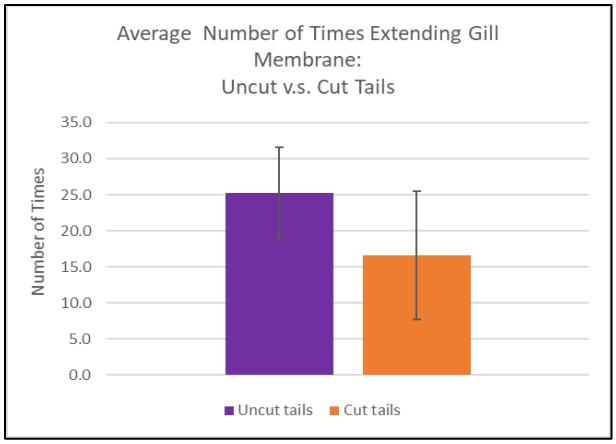


Figure 16. Average number of times extending the gill membrane. Error bars show standard error. t=-1.15, df=10, p=0.31

Figure 17. Average amount of time extending gill membrane. Error bars show standard error. t=-0.52, df=10, p=0.69

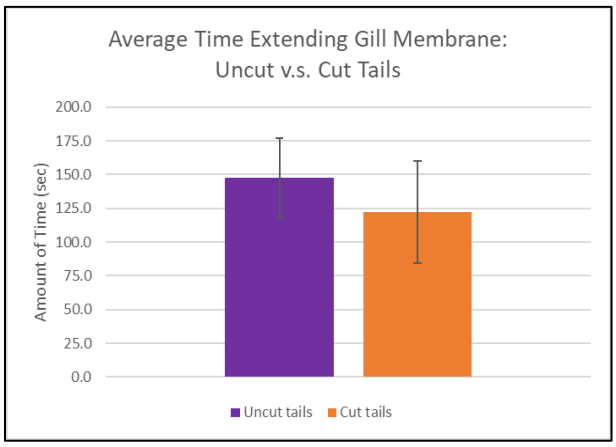


Figure 18. Average number of times raising the dorsal fin. Error bars show standard error. t=-0.63, df=10, p=0.55

Figure 19. Average amount of time raising the dorsal fin. Error bars show standard error. t=2.61, df=10, p=0.008

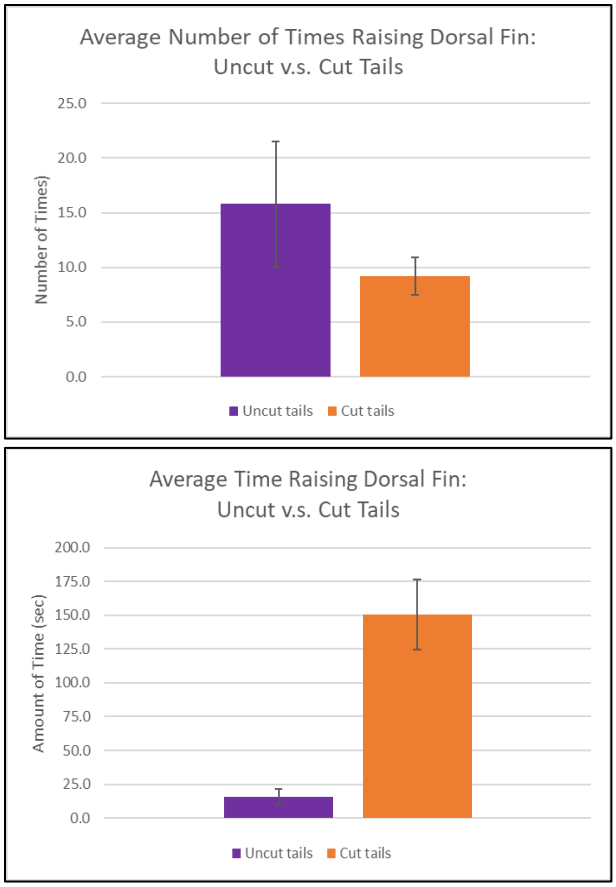


Figure 20. Average number of times lowering head.
Error bars show standard error. t=-0.11, df=10, p=1.00

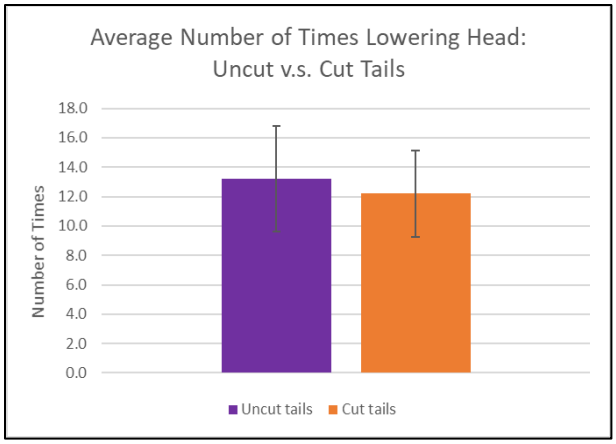
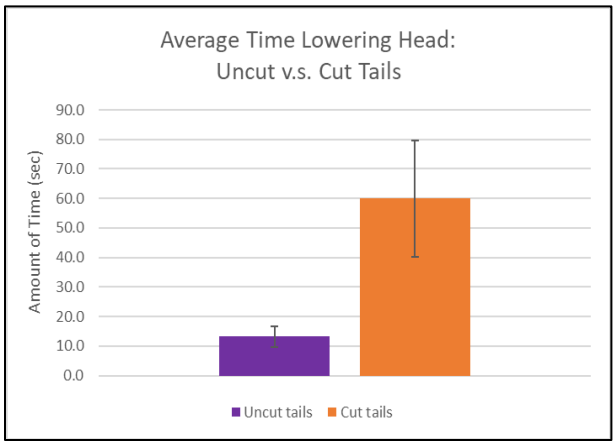


Figure 21. Average amount of time lowering head. Error bars show standard error. t=2.20, df=10, p=0.03



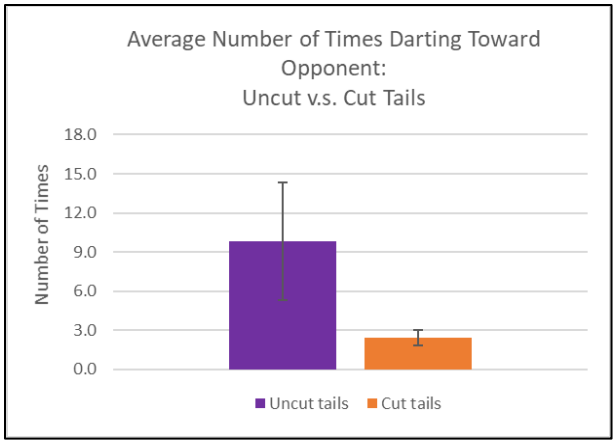


Figure 22. Average number of times darting toward opponent. Error bars show standard error. t=-1.19, df=10, p=0.31

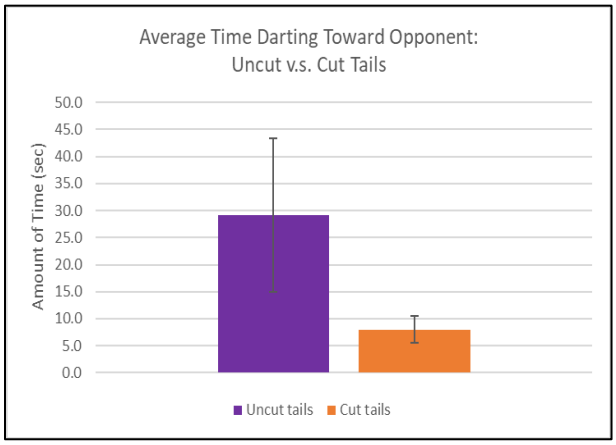


Figure 23. Average amount of time darting toward opponent. Error bars show standard error. t=-1.26, df=10, p=0.22

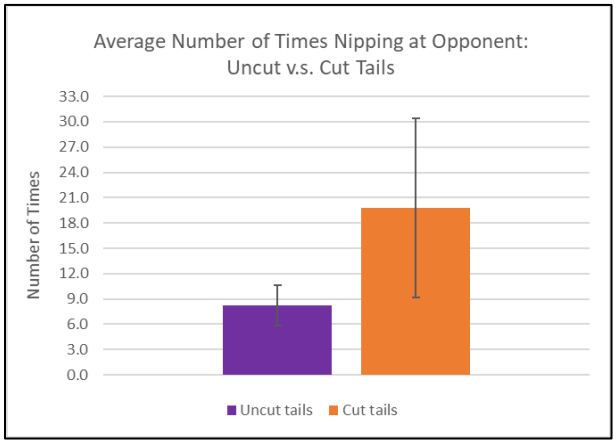


Figure 24. Average number of times nipping at opponent. Error bars show standard error. t=0.31, df=10, p=0.84

Discussion:

The lack of statistical significance in male *Betta splendens* with cut tails compared to male *Betta splendens* with uncut tails in both the amount and number of times aggressive behaviors were displayed suggests that tail regeneration generally does not affect their aggressive behavior towards other males.

This study shows that the regenerated tissue is functionally normal. Additionally, this information is consistent with findings from zebrafish tail amputation research, suggesting that complex fin regeneration is very similar to simple fin regeneration. Moreover, this lack of significance on aggressive behavioral displays indicates that the *Betta splendens* must not have experienced enough psychological harm to change their behavior due to the amputation.

Tail amputation was thought to affect either the number of times or the amount of time aggressive behavior was displayed in male *Betta splendens* since many of their aggressive behaviors include their tail. Amputation would decrease the size of their tail, generally even when regrown, and the size they could make themselves look towards other opponents, which would theoretically make them back off from fighting other male *Betta splendens*. However, this hypothesis suggests that *Betta splendens* are aware of the size of their tail, which is yet to be determined.

The two behaviors, raising the dorsal fin and lowering the head, that showed a statistically significant higher display in males with cut tails suggests male *Betta splendens* are in some way aware of their tail size, since they resort more frequently to aggressive behaviors that do not depend on their tail. They may have used longer dorsal fin and head-lowering displays to make up for that new weakness. The data set used in this experiment was the smallest possible though, so the results could change drastically if a larger sample size is used. Future studies should conduct the same experiment with a larger sample size to verify the results.

The lack of statistical significance found also suggests that wild *Betta splendens* would still fight to the death to increase their own fitness, despite a substantial tail injury that may make them look weaker or smaller when compared to their uninjured opponent. This evidence suggests that the cost of tail regeneration is less than the cost of not displaying aggression. If the male *Betta splendens* do not display aggression, they forgo their chances of passing on their genes to the next generation, which significantly lowers their fitness. Also, multiple sources describe that behavioral displays are a way for the males to "resolve the conflict without costly escalated fighting" (Castro, et al., 2006; Caryl, 1979; Maan et al., 2001; Neat et al., 1998; Zahavi, 1977). Thus, it makes sense that males who experienced regeneration still display aggressive behaviors since they are less costly than physically fighting for their fitness. However, since tail regeneration was not examined in the presence of a female *Betta splendens*, no conclusions can be made about how this might affect their fitness in terms of finding a partner to mate with. Future studies could focus on how if length after amputation changes how aggressive *Betta splendens* are towards their opponent, or even how tail amputation might affect a male *Betta splendens* fitness.

Unfortunately, epigenetic data could not be tested to see if the tail amputation affected the epigenetic in the males with cut tails. Thus, it is difficult to conclude how the *Betta splendens* may have been physically stressed due to this experience. If epigenetic changes occurred, this would indicate that there are long-term changes in the gene expression associated with amputation and regeneration.

As a result, *Betta splendens* could act as a model organism, if need be, since they show more ability than zebrafish in recognizing other males and forming social hierarchies (Forsatkar, et al., 2017; Jameson, et al., 1999). Their development could be further studied to find how simulated traumatic injuries may trigger regeneration and how, when applied in humans, it could affect their epigenetics and behavior in the future.

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APPENDIX:

Average Number of Occurances of Various Behaviors					
Behavior	**Uncut vs. Cut**	**Average Number of Occurances**	**Std. Dev.**	**Std. Error**	**Normal Data?**
Tail Beating					
	Uncut tails	11.33	10.78	4.82	Yes
	Cut tails	7.00	4.06	1.82	Yes
Tail Flashing					
	Uncut tails	3.60	2.07	0.93	Yes
	Cut tails	7.00	5.24	2.35	Yes
Flaring Gills					
	Uncut tails	25.40	16.10	7.20	Yes
	Cut tails	18.40	22.85	10.22	No
Extending Membrane					
	Uncut tails	25.20	14.34	6.41	Yes
	Cut tails	16.60	19.83	8.87	No
Raising Dorsal Fin					
	Uncut tails	15.80	12.76	5.70	Yes
	Cut tails	9.20	3.83	1.71	Yes
Lowering Head					
	Uncut tails	13.20	8.04	3.60	Yes
	Cut tails	12.20	6.53	2.92	Yes
Darting Toward Opponent					
	Uncut tails	9.80	10.08	4.51	No
	Cut tails	2.40	1.34	0.60	Yes
Nipping at Opponent					
	Uncut tails	8.20	5.40	2.42	Yes
	Cut tails	19.80	23.83	10.66	No

Average Length of Time in Seconds that Various Behaviors Occur					
Behavior	**Uncut vs. Cut**	**Average Length of Time in Seconds**	**Std. Dev.**	**Std. Error**	**Normal Data?**
Tail Beating					
	Uncut tails	63.00	52.55	23.50	Yes
	Cut tails	27.80	21.78	9.74	Yes
Tail Flashing					
	Uncut tails	222.40	82.48	36.89	Yes
	Cut tails	153.60	89.75	40.14	Yes
Flaring Gills					
	Uncut tails	135.40	62.95	28.15	Yes
	Cut tails	116.40	87.85	39.29	Yes
Extending Membrane					
	Uncut tails	147.40	66.09	29.56	Yes
	Cut tails	122.20	85.19	38.10	Yes
Raising Dorsal Fin					
	Uncut tails	15.80	12.76	5.70	Yes
	Cut tails	150.60	57.87	25.88	Yes
Lowering Head					
	Uncut tails	13.20	8.04	3.60	Yes
	Cut tails	60.00	44.12	19.73	Yes
Darting Toward Opponent					
	Uncut tails	29.20	31.77	14.21	Yes
	Cut tails	8.00	5.43	2.43	Yes