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The Effects of Sustained, High-Velocity Exercise on Gene Expression in California Yellowtail (*Seriola lalandi*)

A Thesis

Presented to

The Faculty and the Honors Program

Of the University of San Diego

By

Kelli Hatter

Biology

2015

Abstract:

California Yellowtail muscle fibers have been observed to exhibit two drastically different development patterns resulting from the speeds at which they are exercised. When fish are exercised at a moderate rate their epaxial fast-twitch muscle fibers grow in diameter—hypertrophy; when they are exercised at a fast speed, more new epaxial fast-twitch muscle fibers are produced—hyperplasia. To determine the underlying reason for this difference in muscle development, my summer research project and honors thesis exercised fish at: fast, moderate, and control speeds for a sustained amount of time to determine what is happening on a cellular level to cause the observed differences. Specifically, I am interested in the role of both IGF and HIF transcription factors in influencing the hyperplasia observed after sustained, high-speed exercise. My hypothesis is that an oxygen debt is incurred in the white muscle fibers and this leads to hypoxic conditions in the tissue. If HIF is found to be present in larger quantities in the tissues of fish that swam at fast speeds as opposed to the control and moderate speeds, it would suggest that there are very low levels of oxygen in the muscle fibers and that the HIF transcription factor is influencing various biochemical signaling pathways to induce a hyperplastic response.

Introduction:

Muscles are composed of red (aerobic, slow-twitch) and white (glycolytic, fast-twitch) muscle fibers. In contrast to mammals, these muscle fiber types are segregated into different muscle masses in fish. In teleost fish, muscle development is modulated by temperature and oxygen availability throughout maturation (van der Muelen et al, 2006). Many teleost fish species differ from humans, in that they exhibit indeterminate growth—where they will continue

to increase in size until death rather than reach a maximum size and cease growing. Mechanical constraints and the environment the fish are in greatly affect the dynamics of teleost muscle development. The mechanical constraints a fish is subjected to are often a reflection of the hydrodynamic flow and the current in their environment (Johnston, 2010). The rate of the flow around the fish has profound effects on the development of muscle tissues and on the rate of growth.

Different patterns of muscle development in teleost fish have been observed in response to various conditions of exercise. Mechanical load can be imposed on fish via endurance training and exercise (van der Meulen et al., 2006). A pattern of hypertrophy—or muscle fibers primarily increasing in size, has been observed in fish that were swum at moderate speeds that did not reach their highest aerobic capacities. Hyperplasia, or the development of new muscle fibers, has alternatively been observed in fish that are swum at high speeds, where the fish do reach their higher aerobic capacities (Buhr, 2002; Lowery et al., 2010). The fiber diameter measurements were made in fish reared at 60% Ucrit (based on their initial size) for a month, after which the hyperplasia was observed. The fish reared at 40% Ucrit (based on initial size) displayed primarily hypertrophy in response to the endurance exercise. This pattern was seen in two species—both White Seabass (Buhr, 2002; Lowery et al., 2010) and Yellowtail (Peters, Lowery, 2010). The mechanism, on a cellular level, leading to this phenomenon is not yet understood. Hyperplasia is an interesting muscle development patter because it relates to the indeterminate growth of fish and may help the fish grow much larger in size ultimately. This information could be very useful in the aquaculture industry.

Insulin-like-growth factors (IGFs) are transcription factors that are known to stimulate both hypertrophy and hyperplasia in muscle tissue. Originally the processes of proliferation and

differentiation were thought to be mutually exclusive in terms of the proteins involved in their stimulation, so when IGF was discovered to play a role in both, the uniqueness of the protein was appreciated immediately. In myoblast cultures the increase in muscle development is related to the larger amount of myogenin mRNA available. Myogenin is a transcription factor from the MyoD family and it is best recognized for its role in increasing the terminal myogenesis (Florini, 1996). The process of stimulating hypertrophy that IGF influences is biochemically mediated via multiple signaling pathways. The increase in muscle mass as a compensatory adaptation is due to the downstream biochemical signaling of IGF. Specifically, IGF under this kind of exercise-induced stress stimulates the phosphatidylinositol-3 kinase (PI3K/Akt) pathway. This pathway has targets involved in muscle fiber development via protein synthesis (Glass, 2005). Lei et al. found that solely activating the Akt pathway was enough to induce an increase in overall muscle mass in mice via the activation of this specific protein synthesis pathway (2004). A plethora of studies have confirmed that IGF is sufficient to stimulate differentiation in skeletal muscle tissues, but it has recently been posed that IGF is involved in the inhibition of specific transcription factors that cause muscular atrophy as well. The ubiquitin ligases MuRF1 and MAFbx are down-regulated in response to the activities of IGF (Glass, 2005).

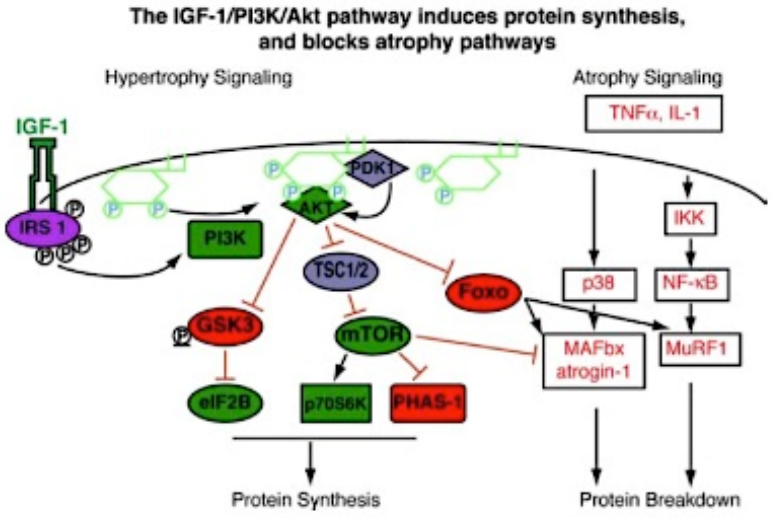

Figure 1: The down-stream signaling pathway of IGF via Akt/PI3K stimulates protein synthesis. When no IGF is present, protein breakdown, and atrophy is observed (Glass, 2004).

Ren et al. (2010) sought to find out how these two very different responses can be regulated by the same transcription factor and what differences cause the varying outcomes. What they found was that the proliferation of myoblasts was observed under hypoxic conditions, whereas the growth in diameter of myoblasts was only seen under normoxic conditions. The difference in the signaling pathways leading to this difference is related to the activation of the hypoxia-inducible-factor (HIF-1) transcriptional program. The activation of HIF in hypoxic conditions changes the mitogenic action of IGF into myogenic action (Ren et al., 2010). This suggests a mechanism that would explain why the fish have been observed to exhibit muscle fiber proliferation in response to hypoxic conditions if these are experienced during exercise at fast velocity.

HIF-1 is a transcription factor that is upregulated in response to low levels of oxygen. The HIF-1α monomer is built up in high enough concentrations in the cell and is then transported into the nucleus where it dimerizes with HIF-1β. In conditions of normoxia, the dimer of HIF forms, however it is quickly degraded. This degradation is due to the recognition of two proline

residues that are utilized to link the two monomers together. When these prolines are detected by a protein ubiquitin ligase complex and it is then tagged for degradation by the specific proteasome. Thus, in normal conditions HIF-1α cannot alter any biochemical pathways due to its rapid degradation. When low levels of oxygen are present in the cell, the dimer can be maintained and activate its down-stream targets (Bruick, 2003). The fully formed protein interacts with hypoxia response elements (HREs) on the genome and induces their activity (Kajimura, 2006). This pathway then converts the function of IGF under hypoxic conditions to differentially stimulate muscle growth (Ren et al., 2010). We don't know if HIF can be activated under condition of exercise, rather than exposing the fish directly to a hypoxic environment.

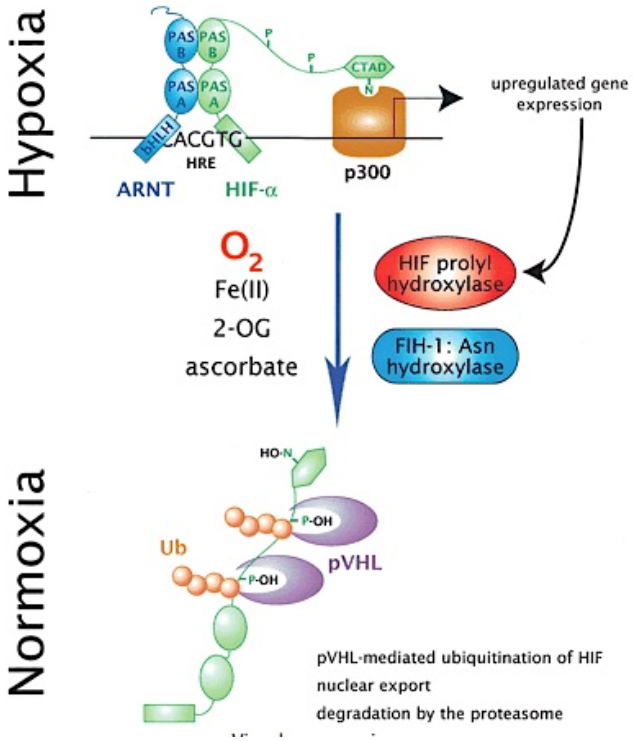

Figure 2: When normal oxygen conditions are present, HIF-1α is quickly degraded in the cell, and cannot interact with downstream targets. With low oxygen conditions, HIF-1α is not degraded by the proteasome

Proof of hypoxic conditions in fish's muscle fibers can be established in a few ways. By taking samples of fish axial muscle for lactate analysis, it is possible to demonstrate that the individual fish was utilizing anaerobic metabolism to meet the oxygen demands of the muscles

during heavy exercise. Previous electromyographic data shows that white axial muscles are stimulated right around the U_{crit} of the individual fish (Martinez, et al., 2004). The Ucrit of a fish is the velocity of current, which it can no longer propel itself forward due to exhaustion. Previous experiments with yellowtail in this lab have established a linear regression that allows us to solve for the U_{crit} of a fish using its body measurements (Peters, 2006). We believe low levels of hypoxia may be experienced transiently as the fish swim at high velocities in the flume.

My research sought to determine if the response of hyperplasia in fish reared at high velocity is due to an oxygen debt being incurred in the muscles—leading to hypoxic conditions in the muscle fibers. If hypoxia is induced in the muscle fibers it is reasonable to believe IGF would respond with myogenic action and that HIF-1 would be upregulated. This would feed multiple pathways through HREs. If these two transcription factors are part of the reason hyperplasia is observed in the fish we expect to see larger amounts of both proteins or their corresponding mRNA expressed in the tissues of fish that swam at higher speeds rather than slow and moderate speeds.

Materials and Methods:

Experimental Animals

The California Yellowtail (*Seriola lalandi*) were obtained from the Hubb's Seaworld Research Institute in San Diego, California. Each batch of fish was transferred to the aquarium at USD and given two days to adjust to their environment. The tanks were kept at 21°C while the fish were held there. They were held on a 12 hour light/dark cycle in the aquarium and fed two times daily. Ammonia levels were measured and adjusted on a regular basis. Tanks were cleaned daily.

Raceway Experiment

The raceway flume at USD was filled with seawater and the water temperature was measured daily and recorded before the fish were put in. We randomly selected three individuals from the aquarium tanks for each experiment to be run at once since we decided this was the optimum number to minimize peripheral stress while maximizing the number of individuals exercised at once. We swam the fish at speeds relating to the aerobic capacities of interest to our research. The speeds were measured in cm/second and we aimed at specific rates relating to the fish's body lengths. We were trying to recreate the conditions of Chris Peter's MS thesis with Ucrit levels of 60% and 40%. The speeds we used were calculated using hypothetical U_{crit} values from a linear regression previously established for the U_{crit} of yellowtail. The graph is shown below. We wanted to transiently have the fish experiencing stress from exercising in the flume, and potentially being affected by hypoxic conditions in the muscle tissues. By using units of body lengths/second we were able to compare fish of different sizes along the same criteria. This was especially helpful because as time went on our batches turned out not to all be similarly sized. The fish were put into the flume and the speed of the current was increased over increments of time from 5-7 minutes until the desired speed was achieved. We made sure the fish were adjusting to the current speed through qualitative behavioral analysis. We made sure the fish were not caught against the grate at the back of the flume without swimming during the exercise period, as this would indicate exhaustion. The fish were then swum at the desired speed for 6 hours straight with observation and continued behavioral analysis.

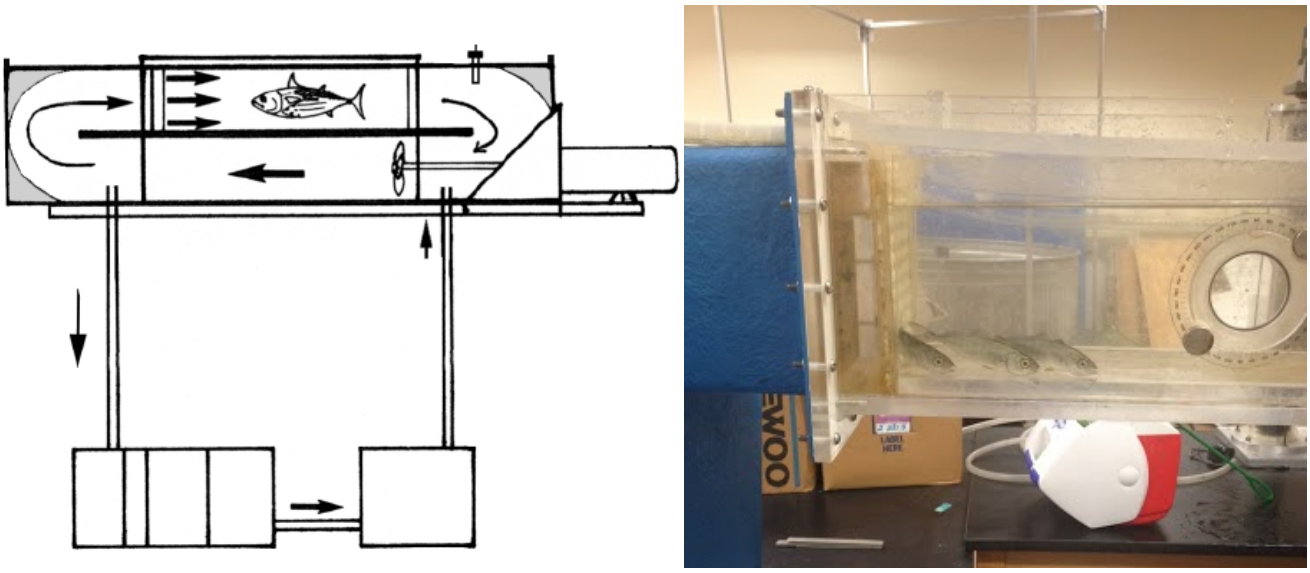

Figure 2: Left-The set up of the flume. Showing the flow of the current relative to where the fish were swimming through the apparatus. **Right**-Fish in the flume chamber.

Upon the completion of 6 hours of exercise the fish were removed from the flume. The fish were put in excess MS-222 anesthesia to immediately sacrafice them with minimal amounts of stress incurred. Samples were taken from random individuals after recovery times of: 0, 1, 3, 8, 12, and 24 hours. After going through the primary literature we selected these times based on which ones had showed the most drastic changes in other experiments. We hypothesized that the effects would be more variable in the short term so we have quite a few short term recovery times and a few relatively long term to provide us with an overarching picture of the time course of gene regulation after being subjected to high velocity exercise. We ran duplicates of each current speed: fast, moderate, and control for each recovery time in order to give more statistical power. Fish for the various time points were selected from different days of high velocity treatment, so that sample points came from fish that had been at USD for a variety of different

times. This was taken into account and every day we ran a trial experiment we also did a control sample to monitor changes in the acclimation of the fish to the USD aquarium over time. Since aquarium conditions were not identical to the conditions at Hubbs SeaWorld Research Institute tanks, we wanted to randomize potential effects not related to exercise. See table one for a layout of the different treatments each fish experienced.

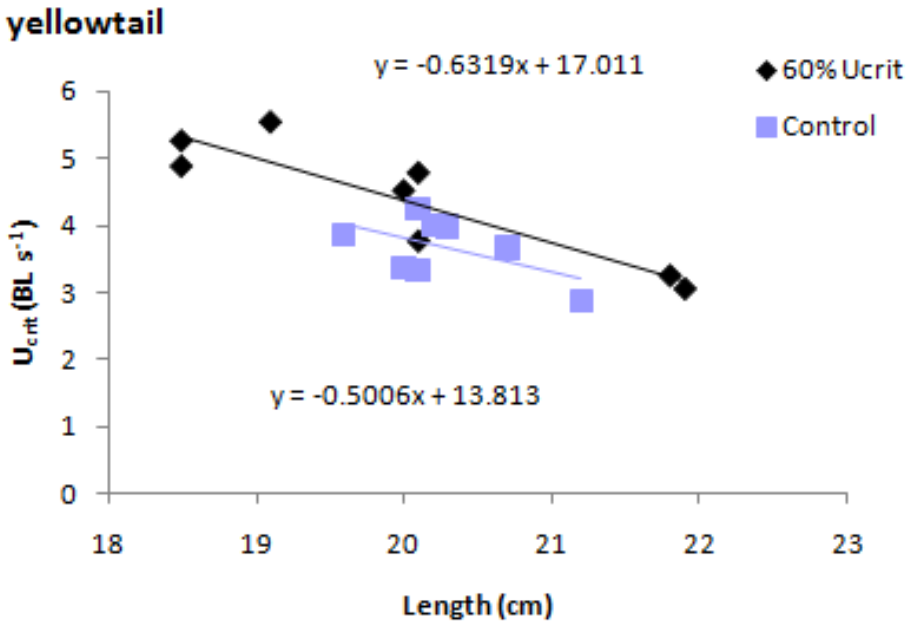

Figure 4: The linear regression expression we used to calculate hypothetical Ucrit values for our fish (Peters, 2008).

YellowTail Tracker							
Time	pre	0	1	3	8	12	24
Control 1	1	5	20	21	23	10	12
Control 2	2	22	25	26	28	24	27
Medium 1	18	3	5	6	9	4	7
Medium 2	19	40	35	37	40	39	41
Fast 1	32,33	11	14	15	17	13	16
Fast 2	34	29	36	38	42	30	31

Table 1: The numbers in the table correspond to individual fish: the speed they were swum at and the amount of recovery time they had before sampling.

"Pre" and "post" samples were taken. Pre samples relate to two fish from each batch that

were taken out of the tank immediately after their batch had adjusted for two days to the USD aquarium and sampled. Two fish were also sampled after the last run of their batch to demonstrate any changes in the conditions/adjustment of the fish over time in the USD tanks. This allows us to analyze how being in the tank for longer periods of time may have affected the fish—so we can draw the most accurate conclusions about fish that were swum in the flume later in the process as opposed to earlier while considering effects of their prolonged time in USD tanks.

Each fish was weighed, measured, labeled and photographed before the dissections. Samples were taken from the axial muscle for lactate measurements and RNA isolation. Gill samples were taken and the hearts of the fish were removed for comparison of cardiac muscle and axial muscle changes due to exercise. The tissue samples were immediately frozen at -80°C and stored until processing.

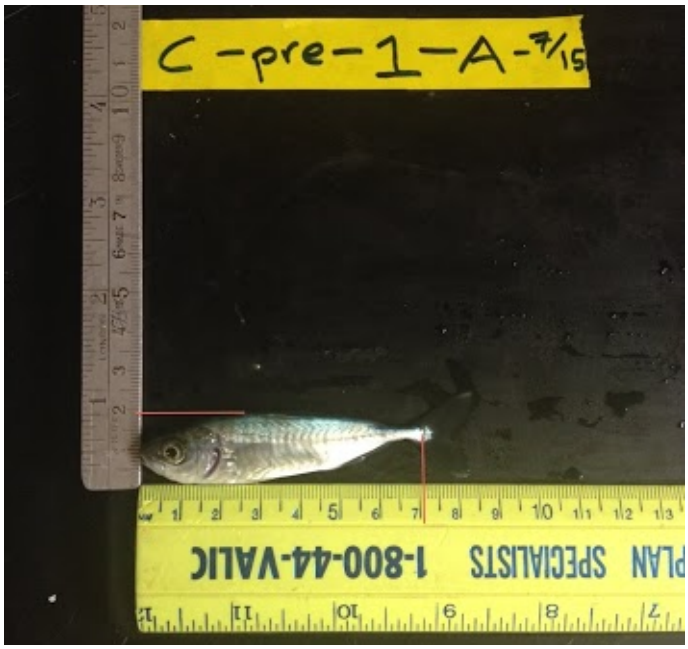

Figure 5: How the fish were individually photographed and labeled for documentation and analysis after each run.

RNA Isolation

Isolation of mRNA from the muscle samples of each fish was performed according to the protocol provided in the Qiagen RNeasy kit. RNA samples were frozen at -80°C until they were converted into cDNA.

cDNA Production

The mRNA samples from the fish were converted to cDNA using the Invitrogen kit. The stable cDNA strands were stored at -80°C until further processing took place.

PCR

Primers utilized for PCR are outlined above. The heat cycle amplification utilized was: 95°C, 60s; 95°C, 30s; 59°C, 30s; for 45 cycles. The cDNA from the samples were separated by 1.5% agarose gel electrophoresis, stained with cybermix DNA stain, and photographed under UV light.

Western Blots

Western blot techniques were utilized to analyze and quantify the presence of specific proteins in the fish muscle tissues. Polyacrylamide gels were polymerized. Muscle tissue homogenates were added to the gels and run through the gel to separate based on size. The proteins were then transferred to a membrane with the help of an electrical current. The membranes were then developed using antibodies specific to the HIF and IGF proteins. The amount of protein was visualized using 50X stock BCIP/NBT obtained from Santa Cruz Technology. Higher levels of HIF proteins in the tissues of fish swam at higher velocities would suggest that hypoxic conditions were established at least transiently in the muscle fibers.

Results:

Qualitative Observations of the Fish in the Flume

Fish swam at the control speeds tended to adjust quickly to their time in the flume. Usually they maintained their position within the group swimming relatively the same. The striped pattern on these fish tended to stay relatively the same as it had been when the fish were in the holding tank, during the run The noise level produced by the flume was notably lower at the lower speeds. Noise from the motor increased substantially as the speed was increased, which is important to note in relation to the possible stress of the run and its effects on the fish.

The fish swum at the moderate pace adjusted to their max speed after roughly 2-3 minutes for each run. The individuals maintained a pretty uniform position within the group. We noticed the fish sometimes utilized a burst and glide method of swimming during the six hours. This is when they would accelerate forward into the current and then glide backwards again into their position. The colors of the fish decreased and most of them became noticeably whiter during the six-hour run. After 2 or more hours of recovery though their coloring returned.

At the fastest speeds the fish adjusted slower to the increased velocity. Once they were adjusted they did not maintain their position within the swimming group as much as the one swam at slower speeds. They also changed to a white color more often than the fish swam at the moderate and control speeds. Their original color returned upon recovery. We noticed the fish utilized the burst and glide swimming technique more often at the fast speed level in the flume. We hope that this behavior is an indicator of transient levels of hypoxia in the axial muscle tissues.

Choosing Exercise Velocities Based on Estimated Ucrit

We have estimated the U_{crit} for each fish we exercised in the flume using the regression line seen in figure 2. We were primarily interested in looking at the effects of the exercise on axial muscle tissue, therefore we needed to establish a method of identifying whether these fibers

were in use. Using the linear regression pictured in Figure 2 we calculated U_{crit} for our fish. We hope to indicate that we were simulating speeds relating to 40 and 60% of the fish's Ucrit.

Range of Fish Lengths (cm)	Range of Ucrit (m/s)	Speed of Flume (cm/s)
5.5-8	62-80	Fast-28.5 Moderate-22.5 Control-5

Table 2: Summary of the average fish length range and Ucrit range corresponding to specific speeds utilized in the flume experiment.

RNA Isolation

RNA samples have been isolated from white epaxial muscle of every fish run in the flume. These samples are currently frozen and stored at -80° Celsius. Analysis with the nanodrop has shown good yield of RNA in the isolates. RNA isolation was performed because we want to convert the mRNA to cDNA and then use these strands to see how much mRNA of interest to us is present. Through the use of primers for IGF and HIF we will be able to quantify the amount of mRNA coding for these proteins present in each fish. This will require qPCR. We expect to see a greater amount of HIF mRNA in the fish swam at higher speeds if they experienced hypoxic conditions in their axial muscle fibers.

Discussion:

This summer research experience for my honors thesis has helped me to master techniques required for physiological research. I worked on techniques in the disciplines of molecular biology, biochemistry, and physiology including: western blotting, agarose gel electrophoresis, homogenizing tissues, PCR, and RNA isolation. These techniques are vital for

the continuation of this research project as a whole. I have learned about other techniques unrelated to my specific project including: compound microscopy, histology, cryostat use, and light microscopy. I have written my own protocol for western blotting and homogenization that has allowed me to optimize conditions specifically for my experiment and get the best results for what I am interested in.

I learned a lot about setting up an experiment. It's not just something you can set up and run very quickly, it takes weeks of planning and perfecting the methods to be used. I've found research isn't a linear process and that it takes time, especially with such a large experiment like ours where we ran upwards of 40 fish in a flume for 6 hours at a time each. It took time and dedication from multiple people to complete the experimental portion of the project. The analysis will be more individualized so it was very important for me to master the techniques I will need to use when I began this project in the summer of 2014.

Over the summer we collectively spent two full weeks running the fish in the flume at the end of July through the beginning of August. The fish were sampled and frozen immediately. This is to ensure all metabolic processes are halted that are not in relation to our experimental conditions. They are stored at -80°C for further analysis. If ever there is an interest in looking at other effects of the exercise experiments we conducted, the fish will be there to be used. There is interesting work being done on the comparison of adaptations in muscle fibers in response to exercise, contrasted with how cardiac muscle adapts in response to endurance exercise.

The project has gone as planned. We were able to exercise the fish at the expected % U_{crit}. Based on this, we hope to see that the axial muscles were activated transiently during extra burst and glide activity and utilizing anaerobic metabolism during the time the fish were in the flume. It would be ideal to finish work on the Western blot analysis to see if HIF protein was

elevated at the higher speeds. The hope is that through more conclusive western blotting and qPCR (which examines mRNA for HIF and IGF) we will see that both HIF and IGF were up-regulated as a response to the endurance exercise in our fish, indicating that hypoxia was induced due to the strenuous exercise in the axial muscle and potentially affected IGF regulation of growth patterns, resulting in two distinct muscle fiber compositions.

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