Spring 2019

Effects of Acute Hypoxia on Danio rerio

Karly Crail
kmc200@zips.uakron.edu

CRAIL

Effects of Acute Hypoxia on Danio rerio

Crail,Karly

University of Akron | kmc200@zips.uakron.edu

Introduction

A growing concern over significant areas of aquatic ecosystems is low environmental oxygen. It is predicted that average oxygen concentrations in oceans around the globe will decrease between 1-7% in the next century (Keeling et al., 2010). There are a number of natural and anthropogenic causes leading to oxygen depletion. Two natural sources include oceanic stratification and global warming. Stratification is achieved by the more buoyant, sunlit water floating on the denser, deep ocean water (Hain and Sigman, 2012). This forms a barrier in which passive mixing no longer occurs; therefore, stratification hinders oxygenated water from reaching greater oceanic depths. Global warming increases microbial growth as it reduces oxygen's solubility in water (Rogers et al., 2016). The absorption of sunlight by the photic zone allows for much warmer temperatures on the water's surface than the deep ocean. Anthropogenic drivers leading to hypoxia include agriculture, industry, and urbanization (Jean-Philippe et al., 2016). The ever-growing population results in an increased amount of fertilizer use and deforestation which result in increased run-off and nutrient loading (Pollock et al., 2007). When paired with the rising amount of greenhouse gas release leading to higher air temperatures naturally enhancing thermoclines and halocines, hypoxic waters will continue to proliferate (Pollock et al., 2007). Ultimately, hypoxic environments may lead to a rapid decline amongst aquatic populations. It is well known that animal behaviors correlate with their fitness, and with the prolonged exposure to hypoxic environments, survival rates amongst different species of fish will vary depending on the degree of hypoxia exposure and individual adaptation capacities (Barrionuevo et al., 2009). In addition, predation remains a strong selective force, and behavioral changes due to hypoxia may render aquatic species more vulnerable (Pollock et al., 2007). Some of these behavioral changes are specie-specific such as fish that begin traveling to show more

aquatic surface respiration in which they become prone to predation from aerial species. In addition, hypoxia can affect the schooling anti-predator ploy by impairing sensory functions and the overall synchronization of the school (Domenici et al., 2007).

Hypoxic environments can greatly affect the physiology and morphology of lower vertebrate embryos (Barrionuevo and Burggren, 1999). Several protective responses are elicited in order to improve oxygen uptake at the gills: increased red blood cell circulation, enhanced hemoglobin affinity, metabolic and heart rate reduction, and anaerobic regulation (Barrionuevo et al., 2009). Metabolic rates vary throughout development due to increasing size and the physiological processes of gas convection and diffusion (Adolf, 1983, Barrionuevo et al., 2009). Hypoxia challenges fish that are trying to maintain their standard metabolic rate. The ability to regulate metabolic rate decreases after fish reach their critical partial pressure of oxygen (P_{crit}) (Barrionuevo et al., 2009). P_{crit} can be thought of as the point in which oxygen consumption, via aerobic metabolism, can no longer be regulated at a constant level. Once metabolic end-products such as lactate build up in the body, anaerobic metabolism is utilized (Hochachka and Somero, 2002, Seibel, 2010). It is the partial pressure of oxygen that facilitates oxygen uptake by the tissues and the overall effect that hypoxia has on organismal function (Seibel, 2010). Organisms whose metabolic rates drop as the partial pressure of oxygen drops are known as oxyconformers (Seibel, 2010) as their metabolic functions are hindered due to complete dependence on the availability of environmental oxygen. Organisms with prolonged exposure to oxygen levels below the P_{crit} face an increased risk of death (Barrionuevo et al. 2010, Steffensen, 2006).

The zebrafish *(Danio rerio)* is a small-sized, Cyprinid teleost fish that has become a widely used model organism to study cardiovascular development due to their rapid breeding habits and transparent embryos which leads to high resolution viewing of the heart during the

developmental stages (Stanier and Fishman, 2004). In addition, the cardiovascular system is one of the first functioning systems amongst developing fish (Glickman and Yelon, 2002). While anatomic differences persist between human hearts and the model organism, many of the heart formation processes and molecular mechanisms underlying vertebrate heart development remain similar amongst vertebrates. In addition, early developmental processes can continue for a duration of time in the absence of a functional circulation, allowing for a thorough evaluation of altered cardiovascular development (Lohr and Yost, 2002).

It is generally accepted that vertebrates in the embryonic and larval stage are more prone to stressful environments as opposed to adults since organogenesis and histogenesis require a series of planned processes (Barrionuevo et al., 2009). Early environmental conditions are critical for developing organisms because they are used to assess the likely conditions they will encounter in the future and will adjust their developmental track accordingly to yield traits best suited for those perturbations (West-Eberhard, 2005). While it is known that the phenotypic effects due to early onset hypoxia are developmental stage-specific in mammals, birds, and invertebrates, the mechanisms driving developmental plasticity in fish embryos exposed to early bouts of hypoxia are not well understood (Robertson et al., 2014). The purpose of this study is to determine the cardiac response due to reduced oxygen concentrations on *Danio rerio* in the embryonic stage, specifically the concentration of oxygen at which the functionality of the organism becomes compromised. It is hypothesized that hypoxia will stimulate cardiac activity, because in early developmental stages, tissues are supplied with oxygen through bulk diffusion. Embryonic zebrafish are rather proficient at gas exchange as the partial pressure of oxygen between blood and ambient water increases gas diffusion capacity (Barrionuevo et al., 2010).

In regards to the heart rate, while chronic exposure may cause bradycardia due to myocardial depression as well as vasodilation by direct actions of the tissues (Marshall 1998), the acute effect should provoke a tachycardia. The acute onset of hypoxia is thought to be more stressful during the embryonic stage causing greater physiological impairment than compared to chronic environments with low O_2 (Robertson et al., 2014). Lowering oxygen concentration would allow me to determine the point at which the organism's cardiovascular functions are diminished.

Methods

Breeding

Adult, wild-type zebrafish were used from The University of Akron Research Vivarium to obtain embryos used for the exposure. Breeding took place by placing breeding baskets with plastic plants into four, 20-gallon tanks which contained approximately 50 fish of mixed genders. Breeding baskets remained overnight and were removed the following morning to check for eggs. The eggs were then transported and placed in a 100 mL glass container to be incubated at 28°C (±1°C) and kept on a 14 h light:10 h dark cycle.

Treatment

After the 24-hour incubation period, the non-viable eggs and embryos were removed. The remaining embryos (n=30) were separated into three 100 mL flasks that were filled with dechlorinated water. All three flasks underwent a series of decreasing oxygen concentrations over the course of 8 hours. Oxygen concentrations started at normoxic conditions of 6 mg/L O_2 (±0.5) then decreased to 4 mg/L O_2 (±0.5), 2 mg/L O_2 (±0.5)., and 0 mg/L O_2 (±0.5). Different

oxygen concentrations were achieved by extracting oxygen by bubbling liquid nitrogen into the flasks and monitoring with a YSI Clark electrode. The flasks were sealed with rubber stoppers after exposure to reduce O_2 concentration changes from arising. They were then placed back in the incubator. After five minutes, a second O_2 recording was taken before measurements occurred. This process was repeated for every flask at each concentration.

In addition to this 24-hour post fertilization (hpf) experimental run, two replicate experiments were conducted by Alysha Cypher (PhD), Jennifer Piechowski (PhD candidate), and Bryce Fetterman (undergraduate student). Fifty embryos were divided into five, 100 mL flasks for both experiments.

Measurement

After a group of embryonic zebrafish within their respective flask experienced oxygen concentration (0-6 mg/L O_2) for 5 minutes, their hearts were observed underneath an inverted light microscope (Leica DMIRB) with a temperature-controlled stage (Harvard Apparatus, 28°C). A high speed video camera (Red Lake MASD) capturing 125 frames/sec was used to record approximately five second videos of each embryo. After a video was taken of each embryo, they were returned to their original flask and placed in incubation until the next round of decreasing oxygen concentration.

For the two-dimensional data analysis, Image Pro Software (version 4.5) was used to measure the cardiac parameters including heart rate, stroke volume, and cardiac output using the digital motion technique (Schwerte and Pelster, 2000). The 5-7 second videos were analyzed in order to determine heart rate, where the number of frames between each contraction was counted

for five beats. The perimeter of the ventricle in end systole and end diastole was manually outlined with the computer mouse followed by the measurements of greatest width and length. This was repeated for three different contractions in order to obtain an average systolic and diastolic area, width, and length for each embryo. The values collected were used to calculate end systolic volume (ESV) and end diastolic volume (EDV) using the equation provided by Bagatto and Burggren (2006): $Ventricular\ volume = 4\pi ab^2/3$

Where a represents the length and b represents the width at either systole or diastole.

The difference between the mean end diastolic volume (EDV) and mean end systolic volume (ESV) per embryo were used to calculate mean stroke volume (SV). The stroke volume was multiplied by heart rate (HR) to calculate overall cardiac output (Q).

Statistics

A one-Way analysis of variance (ANOVA) was conducted between the four decreasing oxygen concentration groups (6, 4, 2, 0 mg/L O_2) and each cardiovascular parameter (EDV, ESV, SV, HR, Q). Upon finding a significant difference ($p< 0.05$) in any of the concentration groups, a *post hoc* Fischer's LSMeans Differences Tukey HSD test was performed to assess the source of significance from the four groups. Statistics were completed using JMP Pro 14 (SAS institute) with alpha set at $p < 0.05$.

Results

The results were taken from a combination of three data sets.

End Systolic Volume (ESV) and End Diastolic Volume (EDV) After utilizing the aforementioned equation to calculate ESV and EDV for the zebrafish embryos exposed to decreasing oxygen concentrations, the four concentration groups were found to differ significantly for ESV (F ratio=6.7252, p=0.002). The post hoc test showed a significant increase in ESV in the 0 (mg/L) concentration group compared to the 4.0 (mg/L) and 6.0 (mg/L) exposures. Embryonic zebrafish exposed to 2.0 (mg/L) had a significant increase compared to the starting oxygen concentration of 6.0 (mg/L). There was a significant difference amongst the four groups while measuring EDV as well (F ratio=13.2184, p<0.0001) with the greatest difference seen in the starting concentration of 6.0 mg/L compared to the highest EDV in hypoxic conditions (0.0 mg/L).

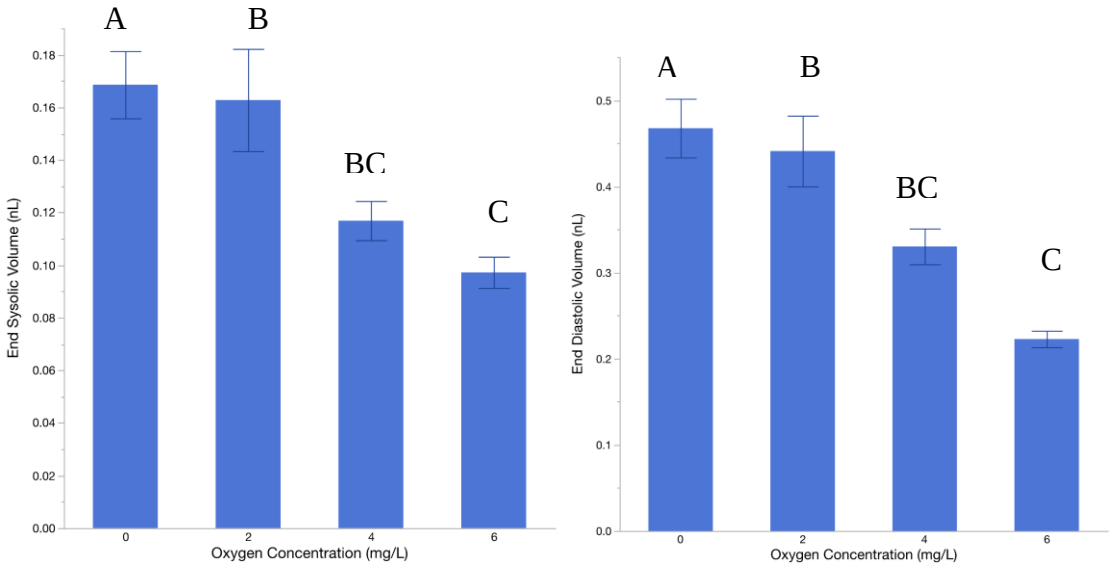
Stroke Volume (SV) Stroke volume was determined by subtracting ESV from EDV at approximately 24 hpf for all treatment groups. The ANOVA test showed that there were differences between at least two of the oxygen concentration groups (F ratio=15.5708, p<0.0001). The *post hoc* Tukey HSD Test was conducted which showed a significant increase in stroke volume at a concentration of 0 (mg/L) compared to both 4.0 (mg/L) and 6.0 (mg/L). The oxygen concentration at 2.0 (mg/L) showed a significantly greater stroke volume than when exposed to an oxygen concentration at 6.0 (mg/L). The embryonic zebrafish exposed to the normoxic conditions (6.0 mg/L) had a significantly lower stroke volume than when exposed to the three decreasing concentrations (4-0 mg/L). The above results were summarized in figure 1.

Heart Rate (HR) Heart rate was measured in beats per minute (BPM) at approximately 24 hpf for all treatment groups. The ANOVA test showed that there is a significant different amongst two groups or more (F ratio=37.7701, p<0.0001). The *post hoc* test did not show any significant differences in the 6.0 (mg/L) exposure group compared to the 0 (mg/L) group, however, there

was a significant increase in heart rate in the 4.0 (mg/L) and 2.0 (mg/L) exposure groups. Embryonic zebrafish exposed to an oxygen concentration of 2.0 (mg/L) had a significantly increased heart rate from the other three exposure groups.

Cardiac Output (Q) A significant difference was found amongst the four concentrations (F ratio= 17.7348, p<0.0001). The *post hoc* test showed a significant increase in overall cardiac output in an oxygen concentration of 2.0 (mg/L) compared to 4.0 (mg/L) and 6.0 (mg/L). The starting concentration of 6.0 (mg/L) was significantly lower than the other three concentration groups. The mean value for 6.0 (mg/L) was 13.73 (±2.55) compared to 36.60 (± 2.36) in the 2.0 (mg/L) group. The results for heart rate and cardiac output were summarized in figure 2.

A. **B.**



C.

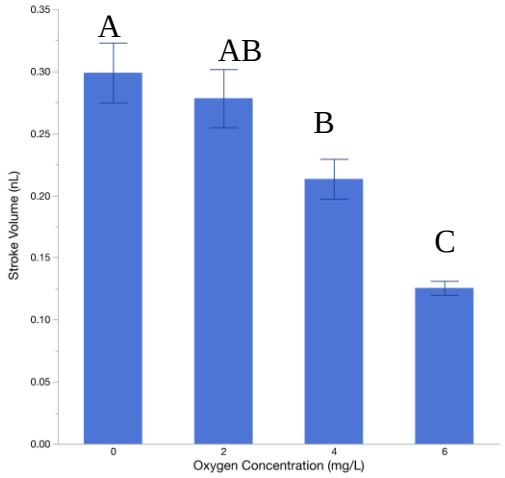
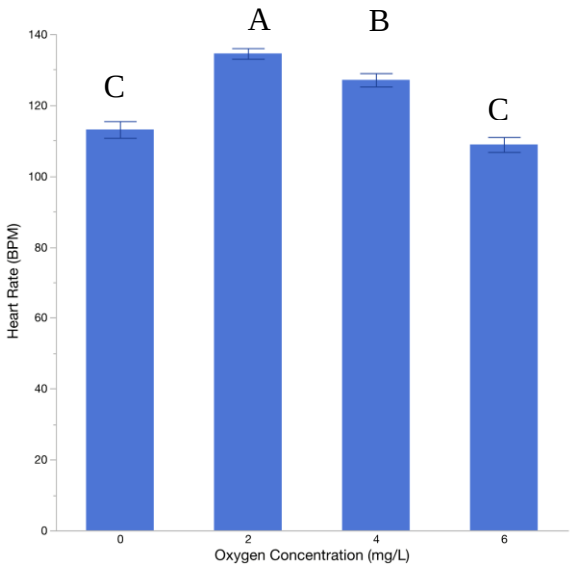


Figure 1. (A) End Systolic Volume (ESV), **(B)** End Diastolic Volume (EDV), and **(C)** Stroke Volume (SV) after undergoing four decreasing oxygen concentrations at 24 hpf. Error bars represent standard error. Bars not connected by the same letter are significantly different (determined by *post hoc* LSMeans Differences Tukey HSD). Both **(A)** and **(B)** were affected significantly amongst the four concentration groups. **(C)** showed an increase in stroke volume as oxygen concentration decreased.

D.



E.

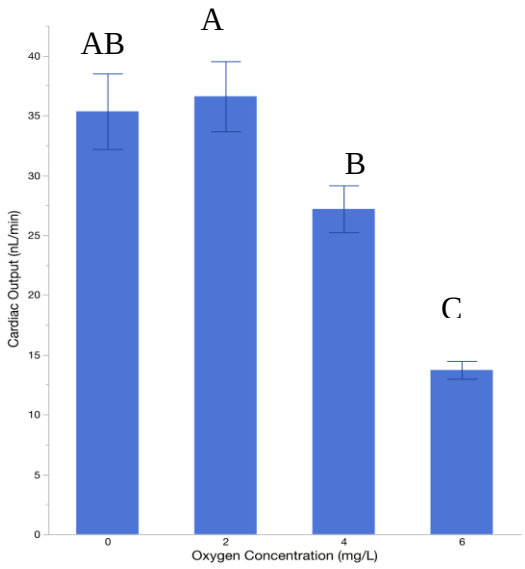


Figure 2. (D) Heart rate, **(E)** Cardiac Output (Q) after undergoing four decreasing oxygen concentrations at 24 hpf. Error bars represent standard error. Bars not connected by the same letter are significantly different (determined by *post hoc* LSMeans Differences Tukey HSD). **(D)** was affected significantly with the greatest increase seen in an oxygen concentration of 2.0 (mg/L) compared to 6.0 and 0 (mg/L). **(E)** Showed a significant increase in overall cardiac output in the 4.0, 2.0 and 0 (mg/L) groups compared to the starting conditions at 6.0 (mg/L).

Discussion

The cardiovascular parameters were not shown to have any significant detrimental effects when exposed to decreasing oxygen concentrations. In fact, there were significant increases in ESV, EDV, stroke volume, and cardiac output from the 2.0 and 0 (mg/L) exposure groups and increases in stroke volume, heart rate, and cardiac output in 4.0 (mg/L) compared to the beginning concentration of 6.0 (mg/L). Heart rate did have a significant decrease when exposed to an oxygen concentration of 0 (mg/L). During early developmental stages, lower vertebrates are commonly found to experience a sharp rise in their heart rate, approach an apex, and then decline (Barrionuevo and Burggren, 1999). Therefore, this decrease could be explained by a cardiac depression by tissue hypoxia instead of a reflexive slowing of the heart (Burggren and Pinder,1991). While heart rate changes during developmental stages are specie-specific and not fully understood, a second possibility for the bradycardia seen in the 0 (mg/L) group could be a result of cholinergic and adrenergic receptors that function when cardiac nerves reach the heart. Changes in the membrane permeability of the myocytes alters frequency of the pacemaker action potentials thus leading to changes in cardiovascular control systems (Bagatto, 2005, Burggren and Warburton, 1994).

Interestingly, one study founded little to no effects on overall cardiac output within the first 24 hpf in hypoxia-induced environments (Jacob et al., 2002). This result was somewhat supported by findings of another study showing that it is not until 30 days post fertilization that

Danio rerio can respond to acute hypoxia by utilizing anaerobic metabolism (Barrionuevo, et al., 2010). In the early stages of development, the metabolic demands and cardiac activity are not thought to be coupled, and little to no changes are seen in cardiac activity in response to hypoxia (Jacob et al., 2002).

The increase in cardiac activity in response to hypoxia could be representative of regulatory responses to environmental perturbations. One adaptive countermeasure could be found from the alteration of gene expression. The master regulator hypoxia-inducible factor-1 (HIF-1) is known to mediate the cellular response to hypoxic environments. While HIF-1 protein extraction was out of the realm of this project, the results from prior research experiments suggest that 24 hpf is the start of the critical development window as embryos begin initiating HIF-1 signaling in response to hypoxia to increase O_2 uptake while decreasing the demand (Robertson et al., 2014). In addition, increases in cardiac activity early on in development can be explained by activities of receptors that sense hypoxic conditions (Jacob et al., 2002). The reduction in oxygen diffusion between the environmental waters and tissues promotes the receptors to stimulate convective oxygen transport as a way of compensation (Jacob et al., 2002). Convective oxygen transport is crucial for conducting aerobic metabolism, but this mechanism may need to be further studied because it is typically not needed until two weeks after fertilization in larvae living in normoxic conditions (Jacob et al., 2002). With that being said, exposure to low amounts of oxygen may result in increased external convection (Burggren and Pinder, 1991) to compensate for the lowered amount of internal diffusion of oxygen. When exposed to low oxygen, the metabolic demands naturally increase, which means that blood flow to the surrounding tissues must increase too in response to the higher demands of the body. As increased blood flow stretches the ventricle, a greater force of contraction is exerted.

Hypoxia can selectively accelerate certain developmental aspects, which could be representative of why multiple embryos hatched throughout the experiment (Burggren and Pinder, 1991). It is found that if environmental oxygen is lowered close to the critical partial pressure, hatching occurs sooner than expected. This is thought to occur in order increase the uptake of oxygen by removing the resistance of the egg membrane (Burggren and Pinder, 1991). Individual adaptations and genetics are fundamentally important as well, as cardiac performance is affected by a family-specific developmental response (Moore et al., 2006).

The P_{crit} of the embryonic zebrafish could not accurately be assessed, as metabolic function persisted throughout exposure to various oxygen concentrations. The small exposure time of 5 minutes to each of the decreasing oxygen concentrations could be an insufficient amount of time to elicit a loss of metabolic function. Since the heart develops rapidly in zebrafish with the heart tube functioning by 24 hpf producing peristaltic contractions and distinct sequential contractions by 36 hpf, the heart is simply able to continually progress from the embryonic tube to its final form (Glickman and Yelon, 2002). This could be a possibility because cellular hypoxia should result in insufficient amounts of ATP which would damage cardiac muscle contractions and relaxation (Kalabde, 2012). Knowing what oxygen concentration and exposure amount results in a compromised metabolic function is important in understanding consequential environmental hypoxia. Overall, the physiological processes in the embryonic stages need further attention in order for us to truly understand the complex mechanisms regulating cardiovascular and respiratory performance in developing species, especially in regards to the onset on physiologic functions.

Conclusion

As the results show, increased hypoxia leads to an increase in a number of cardiac parameters including ESV, EDV, stroke volume, and cardiac output. Heart rate increased in a normal fashion until the final exposure to 0 (mg/L) in which could be explained as a cardiac depression due to tissue hypoxia. As the demand for oxygen increased, overall cardiac output increased to effectively pump blood to the surrounding tissues.

One downfall to this experiment was that the zebrafish embryos were only exposed to each decreasing oxygen concentration for 5 minute periods. This most likely was not a long enough exposure time which allowed the embryonic cardiac functions to persist and continue to develop in a normal fashion. In addition, recording of embryos took up to one hour for multiple concentration groups. This could have provided an ample amount of time for some zebrafish to reoxygenate before being observed and recorded under the inverted microscope.

A second limitation to this experiment was the digital image analysis. Manual outlinings of the ventricle in end systole and end diastole were subject to human error, as certain videos did not elicit clear images, which would in turn affect abilities to properly outline the ventricle. While three measurements were taken at three separate beats per embryo to make up for human error as best as possible, there still remains limitations to accurately identify and measure the ventricle in each video.

This study could be improved by lengthening not only the exposure time, but also expanding the experiment to observe the hypoxic affects at 48 hpf and 72 hpf. In addition, coupling hypoxia exposure with variations on rearing temperature may be more suitable for

understanding how aquatic ecosystems react to hypoxia as temperatures often fluctuate in their environments. It is found that temperature and dissolved oxygen control ectothermic functionality and behavior, with temperature setting the pace for metabolism and oxygen availability serving as a limiting factor (Claireaux and Chabot, 2016).

Aquatic systems and their response to increasing amounts of hypoxia are of rising concern due to the ever-growing population and changes in agricultural practices. Human effects upon aquatic ecosystems continue to propagate. Oxygen is required for metabolism to convert food into energy reserves, but this oxygen must be obtained from the environment. It has been noted that sub-lethal levels of hypoxia can increase embryonic fish malformation by 77.4% (Shang and Wu, 2004). This will ultimately worsen species' fitness and lead to a natural decline in aquatic populations. Further research should be conducted to simulate real-life environmental perturbations in order to create awareness on the negative implications for our aquatic ecosystems.

Acknowledgements

I would like to thank Dr. Bagatto and Jen Piechowski for all of their help in learning about the various experimental procedures and feedback. I would also like to thank Bryce Fetterman for his assistance in performing the experiment and data collection. Finally, I would like to thank The University of Akron Biology Department for granting me the opportunity to conduct research at the undergraduate level.

References

Adolph, E. F. (1983). Uptakes and uses of oxygen, from gametes to maturity: An overview. *Respiration Physiology,53*(2), 135-160.

Bagatto, B. (2005). Ontogeny of cardiovascular control in zebrafish (Danio rerio): Effects of developmental environment. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,141*(4), 391-400.

Bagatto, B., & Burggren, W. (2006). A Three-Dimensional Functional Assessment of Heart and Vessel Development in the Larva of the Zebrafish (Danio rerio). *Physiological and Biochemical Zoology,79*(1), 194-201.

Barrionuevo, W. R., & Burggren, W. W. (1999). O2 consumption and heart rate in developing zebrafish (Danio rerio): Influence of temperature and ambient O2. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology,276*(2).

Barrionuevo, W., Fernandes, M., & Rocha, O. (2010). Aerobic and anaerobic metabolism for the zebrafish, Danio rerio, reared under normoxic and hypoxic conditions and exposed to acute hypoxia during development. *Brazilian Journal of Biology,70*(2), 425-434.

Fritsche, R., & Burggren, W. (1996). Development of cardiovascular responses to hypoxia in larvae of the frog Xenopus laevis. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology,271*(4).

Burggren, W., Pinder, Alan. (1991). Ontogeny Of Cardiovascular And Respiratory Physiology In Lower Vertebrates. *Annual Review of Physiology,53*(1), 107-135.

Burggren, W., & Warburton, S. (1994). Patterns of form and developing hearts contributions from non-mammalian vertebrates. *Cardioscience,5*(3), 183-191.

Claireaux, G., & Chabot, D. (2016). Responses by fishes to environmental hypoxia: Integration through Frys concept of aerobic metabolic scope. *Journal of Fish Biology,88*(1), 232-251.

Domenici, P., Lefrançois, C., & Shingles, A. (2007). Hypoxia and the antipredator behaviours of fishes. *Philosophical Transactions of the Royal Society B: Biological Sciences,362*(1487), 2105-2121.

Gamperl, A. K., Gillis, T. E., Farrell, A. P., & Brauner, C. J. (2017). *The cardiovascular system: Development, plasticity and physiological responses*(Vol. 36, B). Amsterdam: Elsevier.

Glickman, N. S., & Yelon, D. (2002). Cardiac development in zebrafish: Coordination of form and function. *Seminars in Cell & Developmental Biology,13*(6), 507-513.

Hain, M., Sigman, D., & Haug, G. (2014). The Biological Pump in the Past. *Treatise on Geochemistry,*485-517.

Hochachka, P.W. and Somero, G.N. (2002) Biochemical adaptation: Mechanism and process in physiological evolution. Oxford University Press, New York.

Jacob, E., Drexel, M., Schwerte, T., & Pelster, B. (2002). Influence of hypoxia and of hypoxemia on the development of cardiac activity in zebrafish larvae. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology,283*(4).

Jenny, J., Normandeau, A., Francus, P., Taranu, Z. E., Gregory-Eaves, I., Lapointe, F., . . . Zolitschka, B. (2016). Urban point sources of nutrients were the leading cause for the historical spread of hypoxia across European lakes. *Proceedings of the National Academy of Sciences,113*(45), 12655-12660.

Keeling, R. F., Körtzinger, A., & Gruber, N. (2010). Ocean Deoxygenation in a Warming World. *Annual Review of Marine Science,2*(1), 199-229.

Lohr, J. L., & Yost, H. J. (2000). Vertebrate model systems in the study of early heart development:Xenopus and zebrafish. *American Journal of Medical Genetics,97*(4), 248-257.

Moore, F., Hosey, M., & Bagatto, B. (2006). Cardiovascular system in larval zebrafish responds to developmental hypoxia in a family specific manner. *Frontiers in Zoology,3*(1).

Patterson, A. J., & Zhang, L. (2010). Hypoxia and Fetal Heart Development. *Current Molecular Medicine,10*(7), 653-666.

Pollock, M., Clarke, L., & Dubé, M. (2007). The effects of hypoxia on fishes: From ecological relevance to physiological effects. *Environmental Reviews,15*(NA), 1-14.

Robertson, C. E., Wright, P. A., Koblitz, L., & Bernier, N. J. (2014). Hypoxia-inducible factor-1 mediates adaptive developmental plasticity of hypoxia tolerance in zebrafish, Danio rerio. *Proceedings of the Royal Society B: Biological Sciences,281*(1786).

Rogers, N. J., Urbina, M. A., Reardon, E. E., Mckenzie, D. J., & Wilson, R. W. (2016). A new analysis of hypoxia tolerance in fishes using a database of critical oxygen level (Pcrit). *Conservation Physiology,4*(1).

Seibel, B. A. (2010). Critical oxygen levels and metabolic suppression in oceanic oxygen minimum zones. *Journal of Experimental Biology,214*(2), 326-336. doi:10.1242/jeb.049171

Shang, E. H., & Wu, R. S. (2004). Aquatic Hypoxia Is a Teratogen and Affects Fish Embryonic Development. *Environmental Science & Technology,38*(18), 4763-4767.

Schwerte, Thorsten & Pelster, Bernd. (2000). Digital Motion Analysis as a Tool for Analyzing the Shape and Performance of the Circulatory System in Transparent Animals. *The Journal of Experimental Biology*, 203(11), 1659-1669.

Stainier, D. Y., & Fishman, M. C. (1994). The zebrafish as a model system to study cardiovascular development. *Trends in Cardiovascular Medicine,4*(5), 207-212.

West-Eberhard, M. J. (2005). Developmental plasticity and the origin of species differences. *Proceedings of the National Academy of Sciences,102*(Supplement 1), 6543-6549.