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The Cardiovascular Response to Acute Hypoxic Conditions in Danio rerio

Bryce Fetterman
baf61@zips.uakron.edu

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Bryce Fetterman

Department of Biology

Honors Research Project

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Approved:	Accepted:
Date:	Date:
Honors Project Sponsor (signed)	Honors Department Advisor (signed)
Dr. Brian Bagatto	Dr. Brian Bagatto
Honors Project Sponsor (printed)	Honors Department Advisor (printed)
Date:	Date:
Reader (signed)	Department Chair (signed)
Dr. Qin Liu	Dr. Stephen Weeks
Reader (printed)	Department Chair (printed)
Date:	
Reader (signed)	
Dr. Rolando Ramirez	
Reader (printed)	

Introduction

Humans have impacted the earth in many ways, which has created many obstacles that animals have had to overcome. Deforestation is one negative impact humans have had. Deforestation has led to an increase in carbon dioxide in the atmosphere, which has increased global warming (Bala et al., 2007). Not only does deforestation create many obstacles for animals, but global warming creates many other problems. One negative effect of global warming is the increase of water temperature. The increase in the temperature of water leads to stratification. Stratification happens when one layer of water has different properties than the layer below it. When the top layer of water is warmer than the bottom layer, this creates a barrier between them and prevents mixing. A more stratified ocean will lead to deoxygenation of the water (Gruber, 2011). If the layers of water can't mix, then the diffusion of oxygen from the top layer to the layers below won't occur (Turner et al., 1987).

With the increase of industrial agriculture, there has been an increase in the use of nitrogen fertilizers. The overuse of these fertilizers usually leads to run-off. The excess runoff ends up in lakes and estuaries and causes eutrophication. Eutrophication is the increase of organic material into an ecosystem (Nixon, 1995). The nitrogen and phosphorus from the fertilizer cause a decrease of silica in the water. These conditions help the formation of algae blooms in the water (Howarth et al., 2011). Algae blooms rapidly grow due to the excess nutrients, and eventually overpopulate the lake. Algae blooms cause problems for the aquatic life below because it depletes the water of oxygen and sunlight. Eutrophication is the most pressing matter in water pollution (Goldberg, 1995). Eutrophication is leading to dead zones, and the loss of aquatic life in many rivers, lakes, and oceans. When eutrophication and global warming are paired, this will cause a rapid increase in hypoxic water around the world (Pollock et al., 2007).

Not only will hypoxic conditions increase, but polluted water will continue to be a problem as well. Chemicals and hypoxic conditions have a synergistic effect, and decrease the fish's response to react to the conditions (Cypher et al., 2018). These two combinations will lead to a dramatic loss of aquatic life and biodiversity.

Hypoxic conditions can have profound effects on the distributions of aquatic life (Rahel and Nutzman, 1994). Many fish will have to migrate to more oxygenated water, if they cannot adapt to the hypoxic conditions. Fish could be more vulnerable because they have to adapt to a new environment. These fish will have to avoid new predators, which they have never associated with before. The fish that are able to adapt to the hypoxic conditions will have to overcome their own set of obstacles. There may be predators who can also withstand hypoxic conditions. They also may have never been associated with these predators either. Not all fish will be able to migrate and will die. All three situations provide their own set of obstacles. If hypoxic environments continue to increase, the aquatic population and diversity will decrease.

Some species of fish have adapted behavioral responses to low oxygen conditions (Dalla et al., 1998). The prolonged exposure to hypoxic conditions causes fish to change their behavior in response to the conditions (Wong and Candolin, 2014). If natural selection favors the behavioral response, then this will lead to an increased fitness amongst the species. This behavioral response will remain in the species until it is unfavorable. The survival rate varies among fish, which depends on their ability to withstand hypoxic conditions (Anjos et al., 2008). The hypoxic conditions have affected the physiology and morphology of the fish (Barrionueveo and Burggren, 1999). The fish react to the hypoxic conditions in many different ways. These reactions can be in the form of behavioral, molecular, and physiological. Some of these physiological responses are gill oxygen diffusing capacity, metabolic and heart rate reduction,

increased circulation of red blood cells, enhancement of hemoglobin affinity, and anaerobic up-regulation (Barrionuevo et al., 2010). The fish will also up regulate transcription factors, like the hypoxia inducible factor (HIF-1α) (Semenza, 1998). While all the responses are important, it is most important for the fish to reduce its heart rate and metabolism. Most fish use oxygen to produce energy. During increasing hypoxic conditions, fish must try to maintain their standard metabolic rate. There is a point when the partial pressure of oxygen is too low, and the fish can no longer maintain their standard metabolism. This critical partial pressure point is referred to as P_{crit} (Barrionueveo et al., 2010). Once P_{crit} is achieved, the fish switches to a form of anaerobic metabolism for energy production. The partial pressure of oxygen is what determines the amount of oxygen uptake by the tissues (Seibel, 2011). The risk of death increases as the length of exposure to oxygen levels below P_{crit} increase (Barrionuevo, 2010).

There are many fish that can withstand hypoxia levels. One in particular is the zebrafish (*Danio rerio*). The zebrafish is a small-sized, Cyprinid teleost fish, and has been used in a laboratory setting for many reasons(Barrionuevo, 2010). A few reasons being that they are widely available, easy to care for, and the cost for these fish are very low. There are various reasons why these fish are used as a model. One main reason is because their cardiovascular system is one of the first developing organs (Bagatto, 2005). This allows for better resolution when studying the cardiovascular system. The purpose of this study is to investigate the cardiovascular system of *danio rerio* during low oxygen conditions, and to investigate the cardiac response when P_{crit} is reached. It is hypothesized that cardiac response will increase during the hypoxic conditions. This is because acute bouts of hypoxia are more stressful and cause more physiological impairment, than low O_2 environments (Robertson, 2014). A response

will be much more apparent in embryonic zebrafish in stressful conditions. This stress response will be seen when normal heart rate, cardiac output, and stroke volume change.

Methods

Breeding

Fish from The University of Akron Research Vivarium were used for breeding. Approximately, 50 adult wild-type zebrafish were placed into a 20 gallon tank. These fish were of mixed genders. Breeding baskets were placed into the tank. Plastic plants were placed within the baskets to facilitate breeding. 4 tanks were set up identically and used for breeding purposes. These breeding baskets were placed within the tanks at night and removed the next morning. If eggs were present, they were transported using a 100 mL glass beaker and placed in an incubator. They were incubated at 28°C (±1°C), and a 14 h:10 h light-dark cycle was used.

Treatment

After the 24-hour incubation period, the viable embryos were separated from non-viable using a pipette. The viable embryos (n=30) were separated into three 100 mL flasks. Each flask contained 10 embryos and 100 mL of dechlorinated water. The oxygen concentration of each flask was gradually decreased over an 8 hour period. The first measurement was taken at an oxygen concentration of 6.0 mg/L (±0.5). This was done by bubbling nitrogen or oxygen into the flasks and measuring the oxygen concentration using a YSI Clark electrode. Once the specified oxygen concentration was reached, flasks were capped. This prevented oxygen concentration fluctuations within the flask. The exposure lasted five minutes. Then flasks were uncapped and the oxygen concentration was measured again. This procedure was repeated for 4.0 mg/L (±0.5), 2.0 mg/L (±0.5), and 0.0 mg/L (±0.5) oxygen concentrations.

Two additional replicate experiments were performed. Fifty embryos were used and were divided into five 100 mL flasks. The treatments used for these flasks are the same as described above. These additional experiments were performed with Alysha Cypher (PhD), Jennifer Piechowski (PhD candidate).

Measurement

Once the five minute exposure to the given oxygen concentration ended, each embryo was removed from the flask. This was so their hearts could be observed. This was done using an inverted light microscope (Leica DMIRB) with a temperature-controlled stage (Harvard Apparatus, 28 ℃). The heart was then recorded using a high speed video camera (Red Lake MASD), which records at a speed of 125 frames/sec. This method of analysis was used to reduce the amount of error during measurement (Bagatto and Burggren, 2006). Each embryo was recorded for approximately five seconds. Once each embryo was recorded they were placed back into their flask and incubated.

The videos were then analyzed using Image Pro Software (version 4.5). This two dimensional analysis software was used to measure different parameters of the heart. The perimeter of the ventricle was manually traced during diastole and systole. Lines were then traced to measure length and width of the outline. This was repeated for a total of three times per video. Each measurement was taken at a different time frame. This was to get an average diastolic volume and systolic volume measurement for each embryo. End diastolic volume (EDV) and end systolic volume (ESV) was calculated using the average measurements and an equation. The equation used for the calculations was $Ventricular\ volume = 4\boldsymbol{\pi}ab^2/3$ (Bagatto and Burggren, 2006). The letter "a" represents the average diastolic/systolic length, whereas "b" represents the average diastolic/systolic width. Heart rate (HR) was also measured by counting

the number of frames between each heart contraction. This was done over the course of five contractions. This was so the beat per minute (BPM) could be calculated. The BPM was used to calculate heart rate. Stroke volume (SV), was calculated by taking the difference between the EDV and ESV per embryo. To calculate cardiac output (Q), stroke volume was multiplied by heart rate.

Statistics

A one-way analysis of variance (ANOVA) was used to check for significance between each cardiovascular parameter (EDV, ESV, HR, SV, Q) and each of the four oxygen concentrations. The ANOVA was done using JMP Pro 14 (SAS institute) software, with alpha set at $p<0.05$. If significance ($p<0.05$) was found, a *post hoc* Fischer's LSMeans Differences Tukey HSD test was performed. This was to test differences among sample means for significance.

Results

End Systolic Volume (ESV) and End Diastolic Volume (EDV) Once the EDV and ESV was calculated as described above, it was analyzed using an ANOVA to check for significance difference between each of the oxygen concentrations. There was a significant difference (ratio= 6.7252, $p=0.002$) in ESV amongst the four oxygen concentration exposures. The Tukey *post hoc* HSD Test showed significant difference in ESV when the 0.0 mg/L oxygen concentration exposure was compared to the other three oxygen concentration exposures. The most significant difference was between the 2.0 mg/L oxygen concentration exposure and the 6.0 mg/L oxygen concentration exposure. This shows at 0.0 mg/L, ESV significantly increased compared to its starting oxygen concentration (6 mg/L). A Significance difference (F ratio=13.2184, $p<0.0001$)

was found amongst the four oxygen concentration exposures for EDV. The Tukey *post hoc* HSD test showed a significant difference in EDV when the 0.0 mg/L oxygen concentration exposure was compared to all three oxygen concentration exposures. With the most significant difference being between the 0.0 mg/L oxygen concentration exposure and the 6.0 mg/L oxygen concentration exposure. This shows at 0.0 mg/L, EDV significantly increases compared to its starting oxygen concentration (6.0 mg/L). There was also some significant difference between the 2.0 mg/L oxygen concentration exposure and 6.0 mg/L oxygen concentration exposure.

Stroke Volume (SV) Stroke volume was analyzed using an ANOVA, and a log transformation was used. There was a significant difference (F ratio=15.5708 $p<0.0001$) amongst the oxygen concentration exposures. The Tukey *post hoc* HSD Test showed a significant difference in SV when the 0.0 mg/L oxygen concentration exposure was compared to the 4.0 mg/L and 6.0 mg/L oxygen concentration exposures. This shows that at 0.0 mg/L oxygen concentration, SV significantly increased compared to the other two oxygen concentration exposures (4.0 mg/L and 6.0 mg/L). There was also a significant difference in SV when the 2.0 mg/L exposure was compared to the 6.0 mg/L exposure.

Heart Rate (HR) There was a significant difference (F=ratio 37.7701, $p<0.0001$) between the oxygen concentration exposure groups. The Tukey *post hoc* HSD test showed there was a significant difference in SV when the 2.0 mg/L oxygen concentration exposure was compared to the 4.0 mg/L oxygen concentration exposure. This showed there was a significant increase in SV when the oxygen concentration exposure decreased from 4.0 mg/L to 2.0 mg/L. Unlike the previous *post hoc* tests, there was no significance between the 0.0 mg/L and 6.0 mg/L oxygen concentration exposures.

Cardiac output (Q) Once cardiac output was calculated, it was analyzed using an ANOVA. A log transformation was used. There was a significant difference (F ratio= 17.7348, $p<0.0001$) amongst the oxygen concentration exposures. The Tukey *post hoc* HSD test showed a significant difference in Q when the 2.0 mg/L oxygen concentration exposure was compared to the 4.0 mg/L or 6.0 mg/L oxygen concentration exposures. This shows as the exposure decreased from 4 or 6 mg/L to 2mg/L, the cardiac output significantly increased.

A.

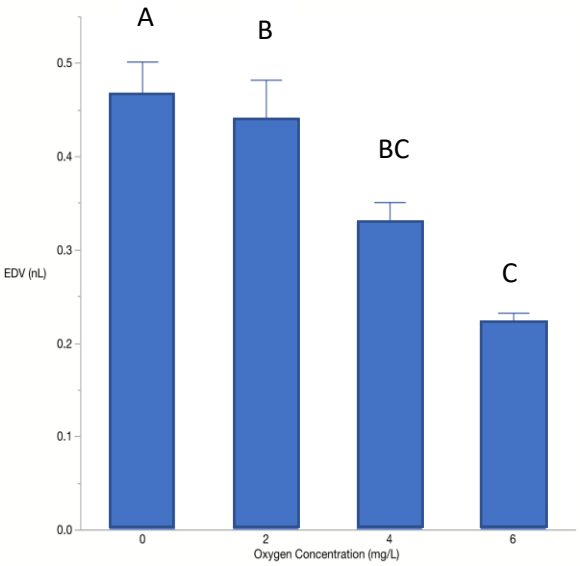
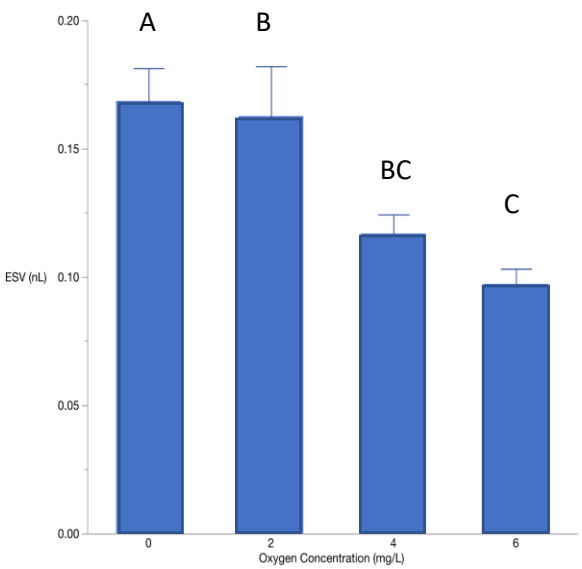
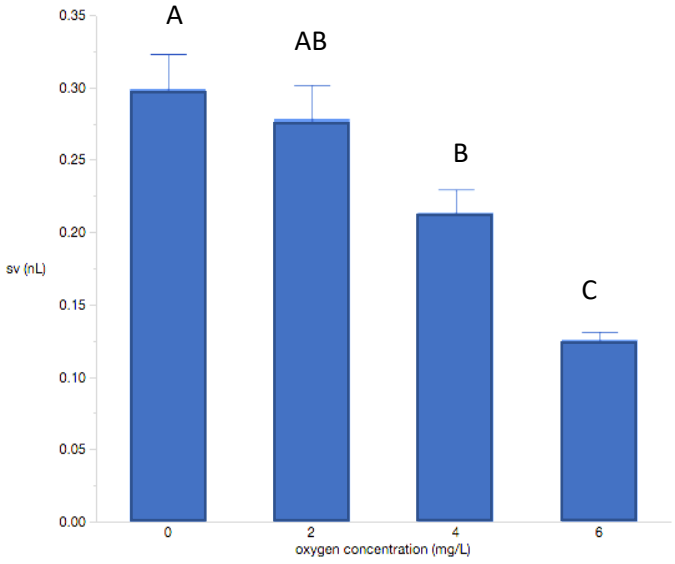


Figure 1. (A) End Diastolic Volume (EDV) **(B)** End Systolic Volume (ESV) (**C.)** Stroke Volume (SV) **(D.)** Cardiac Output (Q) **(E.)** Heart Rate (HR) at each of the four oxygen concentrations at 24 hpf. Approximately, 100 embryos were measured throughout the three experiments. A *post hoc* LSMeans Differences Tukey HSD was used to determine significance between each group, this is shown by the letters above each error bar. Each error bar is measuring standard error.

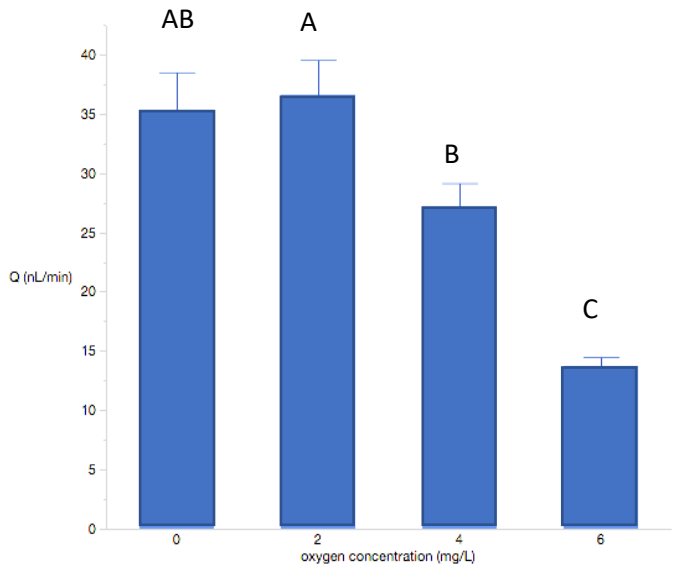
B.


A
B
BC
C
ESV (nL)
0.20
0.15
0.10
0.05
0.00
0
2
4
6
Oxygen Concentration (mg/L)

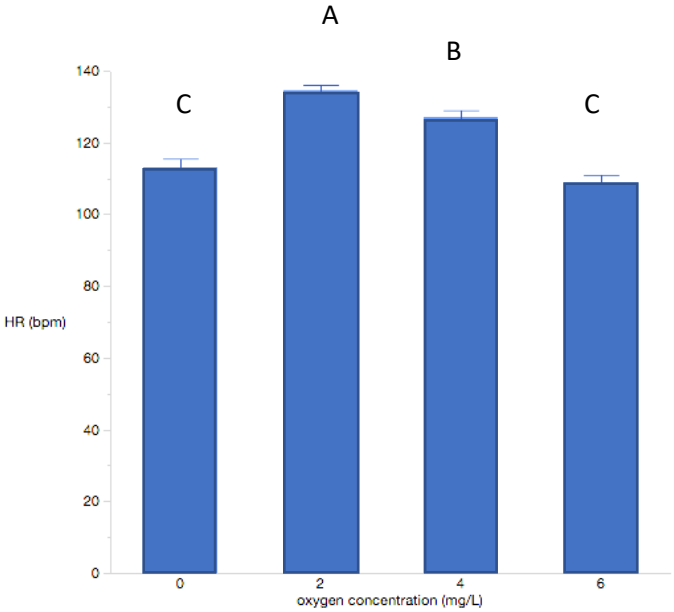
C.


A
AB
B
C
sv (nL)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
2
4
6
oxygen concentration (mg/L)

D.


AB
A
B
C
Q (nL/min)
0
5
10
15
20
25
30
35
40
0
2
4
6
oxygen concentration (mg/L)

E.


A
B
C
C
HR (bpm)
0
20
40
60
80
100
120
140
0
2
4
6
oxygen concentration (mg/L)

Discussion

It was hypothesized cardiac responses would increase as more stress occurred. This stress came in the form of decreasing oxygen concentrations, creating hypoxic conditions. There were some significant increases in cardiac response as oxygen concentration decreased. There was a significant increase in ESV and EDV in the embryonic fish were exposed to 0.0 mg/L of oxygen compared to when they were exposed to 4.0 mg.L or 6.0 mg/L of oxygen. Stroke volume significantly increased in the embryonic zebrafish when exposed to 0.0 mg/L compared to when they were exposed to 4.0 mg/L or 6.0 mg/L of oxygen. There was an increase in heart rate when the embryonic zebrafish were exposed to 2.0 mg/L of oxygen compared to when they were exposed to 4.0 mg/L of oxygen. It was interesting that there was no significant increase in heart rate when the embryonic zebrafish were exposed to 0.0 mg/L oxygen compared to any of the other concentrations.

It was interesting that there was no cardiac dysfunction observed. This is because extreme hypoxia plays a main role in cardiac dysfunction (Yaqoob and Schwerte, 2010). It was hypothesized cardiac dysfunction would occur once P_{crit} was reached. This could be due to the developmental plasticity embryonic zebrafish possess (Bagatto 2005). Many factors can play a role in this plasticity. If zebrafish are reared under hypoxic conditions have higher breathing frequencies at rest compared to zebrafish reared in standard conditions (Vulesevic and Perry, 2006). Hypoxia may activate plasticity mechanisms in embryonic zebrafish. This could be the mechanism that protects the cardiovascular system. This change in physiology early in life will continue to affect them in adult life. Hormones could also activate a mechanism for plasticity. Nitric oxide modifies and enhances blood vessel formation in embryonic zebrafish (Pelster 2002). An increase in blood vessel formation would lead to an increase in oxygen

transportation. This could be a way the fish protect themselves from damage or death from hypoxic conditions.

The activation of transcription factors could be another reason there was no cardiac dysfunction. Hypoxic conditions activate hypoxia inducible factor-1 (HIF-1α). This transcription factor mediates the plasticity to the low oxygen conditions (Robertson 2014). It upregulates vascular endothelial growth factor (VEGF), which promotes angiogenesis (Pelster, 2002). This growth factor along with nitric oxide could increase the transport of oxygen. This would protect organs during low oxygen conditions, and is most likely the reason there was no cardiac dysfunction observed. HIF-1α also upregulates glycolysis enzymes, allowing ATP to be produced independently of oxygen. Metabolic reactions will continue if these enzymes are increased. This may be a reason P_{crit} was not observed. The metabolic reactions continued through the exposure even in severe hypoxic conditions. Five minutes exposures may not be enough time to trigger the switch from aerobic metabolism to anaerobic metabolism. The zebrafish heart is fully functional at 24 hpf, but it is a tube. At 36 hpf, this is when the atrium and ventricle morphology can be distinguished (Reifers et al., 2000). This could have been another reason why P_{crit} was not observed, since the heart was still developing.

Conclusion

The EDV, ESV, Stroke volume, and cardiac output all significantly increased as the oxygen concentration decreased. Unfortunately, P_{crit} was not observed throughout the experiment. This could be due to many reasons. The fish were observed at 24 hpf, at this point the heart is a tube like structure. When measuring it was hard to distinguish the ventricle and atrium. Throughout the experiment the heart was developing, which could have had an impact on the results. If the experiment was replicated, the zebrafish should be exposed after 36 hpf. This is so

the heart is fully developed and the atrium and ventricle can be distinguished. This along with human error when measuring could lead to very inaccurate measurements.

Another way the experiment could be improved is the length of time exposed. Five minutes was not enough time to observe a change in metabolic reactions (P_{crit}). If this experiment was replicated, exposures should last twenty or more minutes. This should be sufficient time for P_{crit} to be observed. To record the fish, they had to be removed from the beaker. This would allow reoxygenation of the water, and could affect the results. The time taken to record should be minimized as possible, so reoxygenation can't occur. This experiment could be expanded with more fish and more groups per experiment. If this were to occur, many more researchers would be needed. This would ensure the experiment would run smoothly, and make sure all exposures were equal.

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References

Anjos, M., De Oliveira, R., & Zuanon, J. (2008). Hypoxic environments as refuge against predatory fish in the Amazonian floodplains. *Brazilian Journal of Biology, 68*, 45-50. Retrieved from http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1519-69842008000100007&nrm=iso

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. doi:https://doi.org/10.1016/j.cbpb.2005.07.002

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. doi:10.1086/498185

Bala, G., Caldeira, K., Wickett, M., Phillips, T. J., Lobell, D. B., Delire, C., & Mirin, A. (2007). Combined climate and carbon-cycle effects of large-scale deforestation. *Proceedings of the National Academy of Sciences, 104*(16), 6550-6555. doi:10.1073/pnas.0608998104

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), R505-R513. doi:10.1152/ajpregu.1999.276.2.R505

Cypher, A. D., Fetterman, B., & Bagatto, B. (2018). Vascular parameters continue to decrease post-exposure with simultaneous, but not individual exposure to BPA and hypoxia in zebrafish larvae. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 206-207*, 11-16. doi:https://doi.org/10.1016/j.cbpc.2018.02.002

Goldberg, E. D. (1995). Emerging problems in the coastal zone for the twenty-first century. *Marine Pollution Bulletin, 31*(4), 152-158. doi:https://doi.org/10.1016/0025-326X(95)00102-S

Gruber, N. (2011). Warming up, turning sour, losing breath: ocean biogeochemistry under global change. *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences, 369*(1943), 1980-1996. doi:doi:10.1098/rsta.2011.0003

Howarth, R., Chan, F., Conley, D. J., Garnier, J., Doney, S. C., Marino, R., & Billen, G. (2011). Coupled biogeochemical cycles: eutrophication and hypoxia in temperate estuaries and coastal marine ecosystems. *Frontiers in Ecology and the Environment, 9*(1), 18-26. doi:10.1890/100008

Nixon, S. W. (1995). Coastal marine eutrophication: A definition, social causes, and future concerns. *Ophelia, 41*(1), 199-219. doi:10.1080/00785236.1995.10422044

Pelster, B. (2002). Developmental plasticity in the cardiovascular system of fish, with special reference to the zebrafish. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 133*(3), 547-553. doi:https://doi.org/10.1016/S1095-6433(02)00194-0

Pollock, M. S., Clarke, L. M. J., & Dubé, M. G. (2007). The effects of hypoxia on fishes: from ecological relevance to physiological effects. *Environmental Reviews, 15*(NA), 1-14. doi:10.1139/a06-006

Rahel, F. J., & Nutzman, J. W. (1994). Foraging in a Lethal Environment: Fish Predation in Hypoxic Waters of a Stratified Lake. *Ecology, 75*(5), 1246-1253. doi:10.2307/1937450

Robertson, C. E., Wright, P. A., Köblitz, 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), 20140637. doi:10.1098/rspb.2014.0637

Seibel, B. A. (2011). Critical oxygen levels and metabolic suppression in oceanic oxygen minimum zones. *The Journal of Experimental Biology, 214*(2), 326. doi:10.1242/jeb.049171

Semenza, G. L. (1998). Hypoxia-inducible factor 1: master regulator of O2 homeostasis. *Current Opinion in Genetics & Development, 8*(5), 588-594. doi:https://doi.org/10.1016/S0959-437X(98)80016-6

Turner, R. E., Schroeder, W. W., & Wiseman, W. J. (1987). The role of stratification in the deoxygenation of Mobile Bay and adjacent shelf bottom waters. *Estuaries, 10*(1), 13-19. doi:10.2307/1352020

Via, J. D., Van den Thillart, G., Cattani, O., & Cortesi, P. (1998). Behavioural responses and biochemical correlates in Solea solea to gradual hypoxic exposure. *Canadian Journal of Zoology, 76*(11), 2108-2113. doi:10.1139/z98-141

Vulesevic, B., & Perry, S. F. (2006). Developmental plasticity of ventilatory control in zebrafish, Danio rerio. *Respiratory Physiology & Neurobiology, 154*(3), 396-405. doi:https://doi.org/10.1016/j.resp.2006.01.001

Yaqoob, N., & Schwerte, T. (2010). Cardiovascular and respiratory developmental plasticity under oxygen depleted environment and in genetically hypoxic zebrafish (Danio rerio). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 156*(4), 475-484. doi:https://doi.org/10.1016/j.cbpa.2010.03.033