

Physiological Plasticity and Resilience of Fundulidae Species to Aquatic Hypoxia

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Abstract:

Aquatic hypoxia is a naturally occurring phenomenon in waters subject to high primary production, eutrophication, and salinity-derived stratification. Fish utilize a suite of behavioral and physiological responses in order to cope with aquatic hypoxia. Species that regularly experience repeated bouts of hypoxia may rely more heavily on compensatory responses in order to survive. We hypothesize that species from hypoxia-prone environments such as estuaries are more tolerant to hypoxia than species living in more oxygen stable environments. We also hypothesize that all species will exhibit increased hypoxia tolerance following acclimation to either constant hypoxia or diurnal cycles of intermittent hypoxia (12 h normoxia: 12 h hypoxia). We assessed the resting oxygen consumption rates and several measures of hypoxia tolerance, including the critical oxygen tension, the oxygen tension at loss of equilibrium, and time to loss of equilibrium at 0.6 kPa hypoxia, in several species of Fundulidae that inhabit different ecological niches. For a subset of four species, we also examined the influence of acclimation to constant hypoxia or intermittent hypoxia on these parameters. Our results indicate that hypoxia tolerance for fish acclimated to normoxia is largely consistent between species and variation cannot be adequately explained in terms of the environment from which a species lives. Despite this, the most hypoxia tolerant species, *Fundulus confluentus*, is found in coastal estuaries with large oxygen fluctuations; the least hypoxia tolerant species, *Fundulus rathbuni*, is native to more oxygen-stable freshwater streams. Following an acclimation of 21-28 days to intermittent hypoxia or constant hypoxia, we observed significant reduction in resting oxygen consumption, significant reduction in the oxygen tension that resulted in loss of equilibrium, and a significant increase in time to loss of equilibrium in one or more of the four species of Fundulidae. Significant plasticity in hypoxia tolerance was observed in species from both oxygen stable and oxygen variable environments. Overall, the Fundulidae family is exceptionally tolerant to aquatic hypoxia, and demonstrates plasticity that enables its members to survive extreme fluctuations in oxygen availability. This study also suggests that the partial pressure of oxygen that results in loss of equilibrium may be a sensitive measurement of hypoxia tolerance despite its rare inclusion in the majority of aquatic hypoxia tolerance literature.

Introduction:

Aquatic hypoxia strongly influences the distribution of fish and the structure of marine communities in the environment (Chu & Tunnicliffe, 2015). Continuous hypoxia is commonly observed in ice-covered and stratified lakes, while intermittent hypoxia characterizes coastal marshes, estuaries, and tide pools. The frequency, severity and duration of hypoxic events can be enhanced by factors such as anthropogenic pollution and global climate change (Turner, Rabalais, & Justic, 2012). In regions like the northern Gulf of Mexico (nGOM), engineered freshwater diversions, used to reclaim eroded coastline, will likely promote hypoxia due to the introduction of nutrient-rich waters into marshes (Ashby). Given the prevalence of aquatic hypoxia in nature, there is a significant need to characterize hypoxia tolerance and the capacity for physiological adaptation in fish from hypoxia prone environments and environments that are likely to experience more frequent and severe episodes of hypoxia due to climate change. We expect that fish species adapted to oxygen-variable environments, such as estuaries, are physiologically more resilient to hypoxia than species living in more stable environments. The physiological plasticity of a species to environmental hypoxia is likely influenced, in part, by the relative stability of an organism's environment.

This research has assessed the magnitude of hypoxia tolerance of fish from the family Fundulidae. Within this family, there are at least six species that inhabit the nGOM and twelve species that inhabit the Gulf slope river drainages (Whitehead, 2010). Despite their evolutionary relatedness, Fundulid species differ in their tolerance to natural variations in temperature (Fangue, Hofmeister, & Schulte, 2006), salinity (Whitehead et al., 2012; Whitehead, Roach, Zhang, & Galvez, 2011), and dissolved oxygen (Everett & Crawford, 2010), and variations within certain species of *Fundulus* also exist (Duvernell, Lindmeier, Faust, & Whitehead, 2008; Whitehead, 2010; Williams, Brown, & Crawford, 2008). Although some species are relatively tolerant to low oxygen (Nordlie, 2006), including *F. grandis* (Virani & Rees, 2000), some populations of this species have a reduced ability to compensate for low oxygen (Everett & Crawford, 2010).

This research project has compared the physiological responses of Fundulid species derived from different habitats to determine hypoxia tolerance limits and the influence of hypoxia acclimation on hypoxia tolerance. Hypoxia tolerance is commonly quantified via the critical oxygen tension (P_{crit}). This value indicates the level of oxygen depletion at which an organism can no longer extract sufficient oxygen from the environment to meet its basal metabolic needs (e.g. physiological maintenance costs). Another measure of hypoxia tolerance is

the oxygen tension that results in loss of equilibrium (PO_2 at LOE), which is used as a proxy for acute survival at very low O_2 tensions.. In fish, LOE is characterized by failure to remain upright and a cessation of pectoral fin movement. Finally, time to loss of equilibrium at 0.6 kPa oxygen (time to LOE) is used to measure the ability of an organism to endure a set level of severe hypoxia. Resting oxygen consumption at normoxia (resting MO_2 rate), the rate at which O_2 is used by a fish to meet its basal energetic demands, is another relevant measurement, since organisms with lower routine O_2 demands would be expected to be more tolerant of low O_2 conditions such as aquatic hypoxia. We expect to see greater baseline hypoxia tolerance and a larger scope for phenotypic plasticity in species adapted to environments that are highly variable in dissolved oxygen such as estuaries.

Materials and Methods .

Collection.

Fundulus species were collected from various sites in the southeastern United States (Figure 1) and were held in the Galvez aquatic facility in the Department of Biological Sciences at Louisiana State University for at least 1 month prior to use. Fish were held in 30-gallon glass aquaria at 0.1 ppt or 12 ppt, depending on originating salinity of the species. In most cases, species were slowly acclimated to 0.1 ppt prior to experimentation to eliminate any salinity effects on metabolism and hypoxia tolerance.

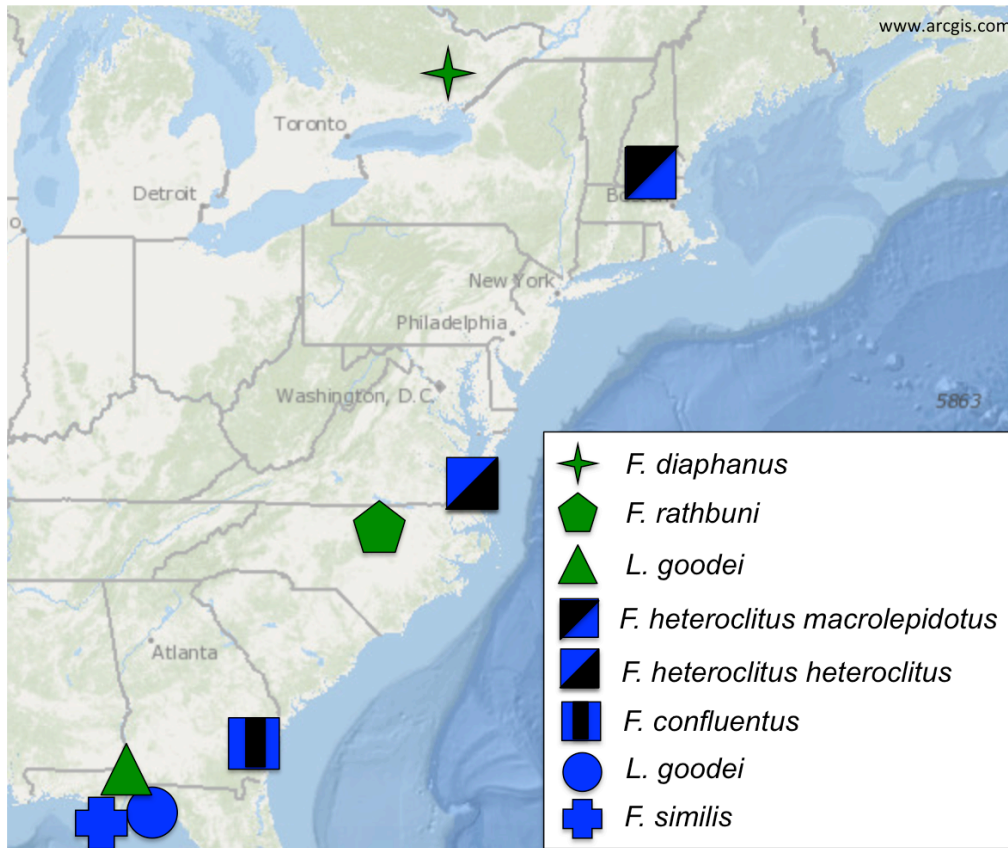


Figure 1. Collection sites of Fundulid Killifish included in this study. Green shapes denote stenohaline species from oxygen stable environments such as freshwater inland streams. Blue shapes represent euryhaline species from hypoxia prone environments like estuaries and costal marshes.

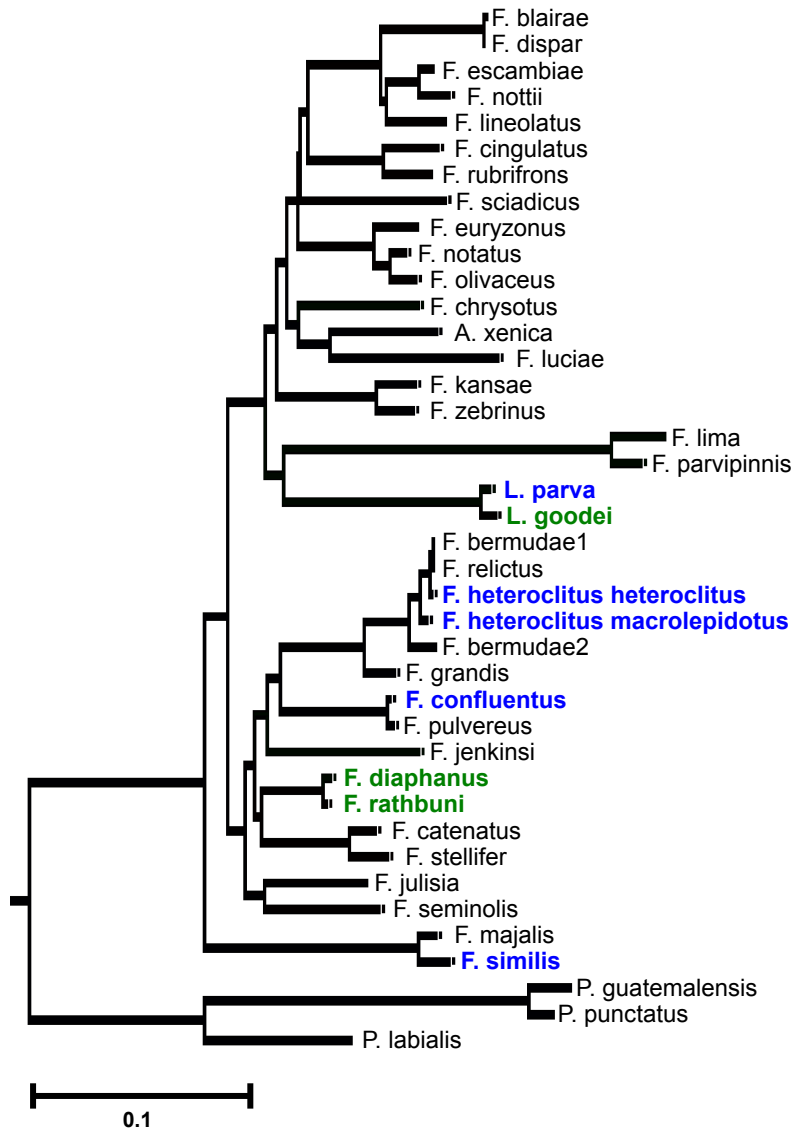
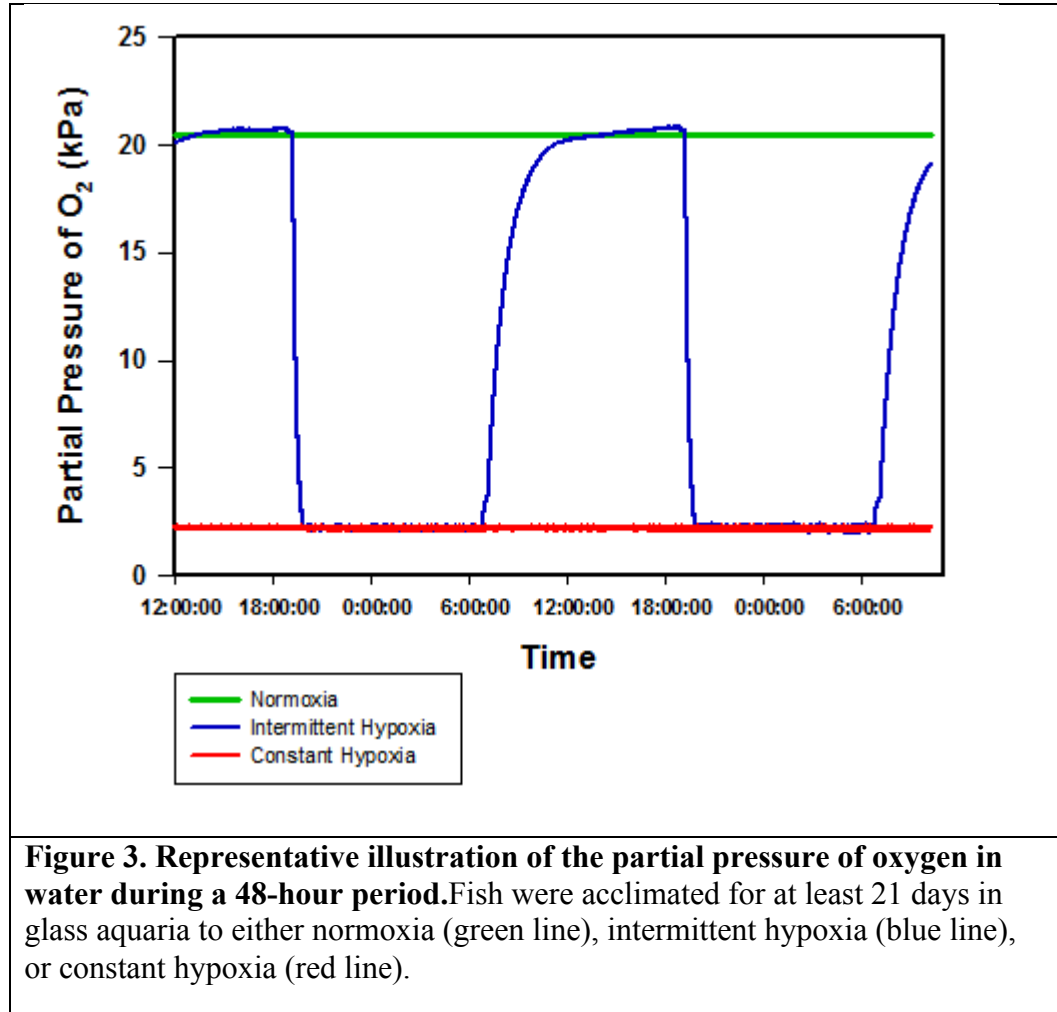


Figure 2. Maximum likelihood phylogeny for the Fundulidae family and out-groups based on nuclear and mitochondrial genes, with study organisms indicated. This figure is adapted from *The Evolutionary Radiation of Diverse Osmotolerant Physiologies in Killifish* (Whitehead, 2010). Studied species are bolded. Green denotes stenohaline species from oxygen stable environments while blue represents euryhaline species from less stable environments.

Acclimation to Oxygen Regimes.

Fish were acclimated for 21-28 days to one of three treatments, which included: a) normoxia (at 100% air-saturated water), b) intermittent hypoxia (12 h normoxia during the day and 12 h hypoxia each night at 11% air-saturated water), or c) constant hypoxia (at 11% air-saturated water) (Figure 3). During this acclimation period, fish were maintained in glass aquaria (water

temperature 20°C; salinity of 0.1 ppt) and fed daily with fish pellets until 24 h prior to experimentation. The intermittent pattern of hypoxia is designed to mimic the oxygen patterns in estuaries and coastal marshes where cessation of photosynthesis by aquatic plants and bacteria at night can result in hypoxic conditions (Tyler, Brady, & Targett, 2009).



Resting Oxygen Consumption, Critical Pressure of Oxygen, and Partial Pressure of Oxygen at Loss of Equilibrium.

Resting oxygen consumption rates (resting MO_2), critical oxygen tension (P_{crit}), and oxygen tension at loss of equilibrium (PO_2 at LOE) were measured in fish. Briefly, fish were placed in respirometry chambers and fasted overnight prior to experimentation in normoxic water. Measurements of O_2 consumption rate (MO_2) were taken at oxygen tensions between

100% and 10% using a stop-flow (intermittent) respirometry approach and AutoResp Software (Loligo Systems, Denmark) as described previously (Borowiec *et al.*, 2015). The PO_2 at LOE was measured by allowing fish to respire in a sealed chamber until LOE. Resting MO_2 was calculated by averaging the metabolic rates ($\mu\text{mol/g/hr}$) at 100% oxygen saturation. P_{crit} was determined using REGRESS software (Figure 4). Statistical analysis were performed in Microsoft Excel, Prism, or SAS.

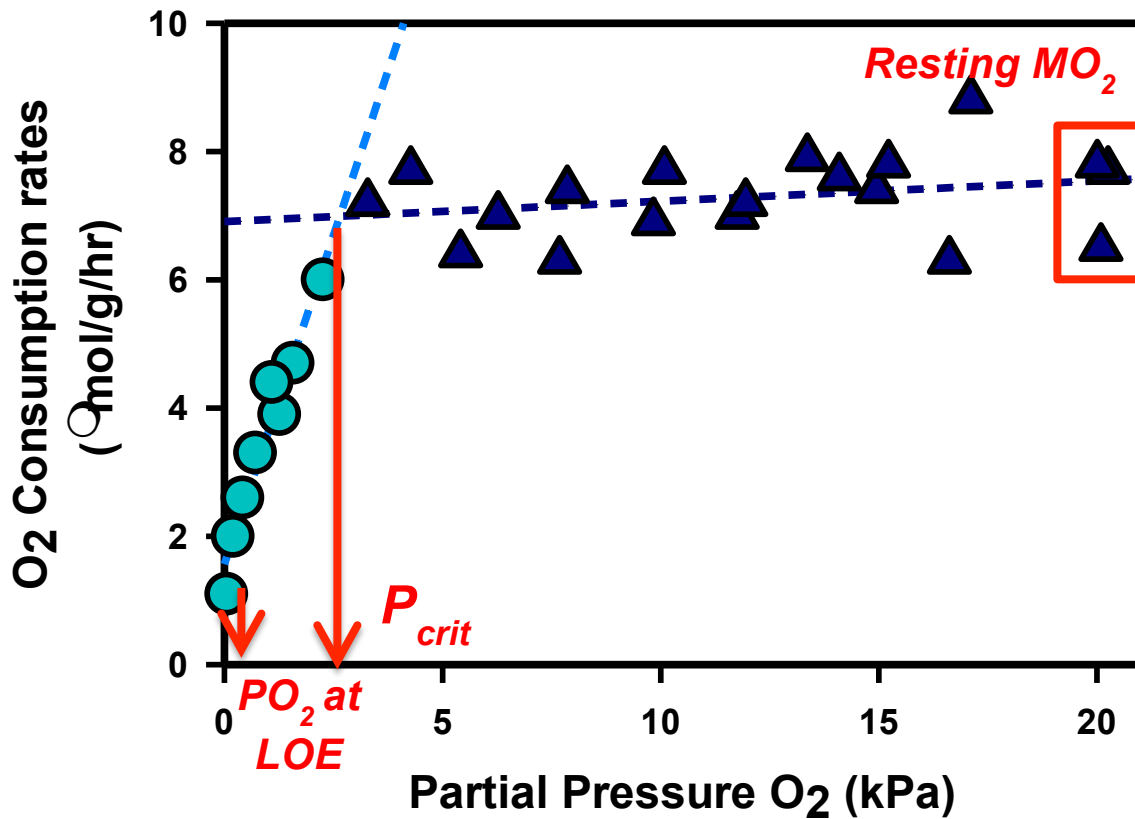


Figure 4. Simplified graphical representation of oxygen consumption rates (MO_2) in fish at different partial pressures of oxygen. MO_2 are measured continuously by intermittent respirometry. Resting MO_2 is obtained by averaging metabolic rates at 100% oxygen saturation or 20.5 kPa. Resting MO_2 rates are maintained nearly constant despite a large drop in water partial pressure of oxygen as depicted by the oxy-regulating line (dark blue hatched line). However, eventually an animal is unable to sustain MO_2 if the partial pressure of water is dropped too much. P_{crit} is the intersection of the oxy-regulating line (dark blue hatched line) and the oxy-conforming line (light blue hatched line). PO_2 at LOE is the partial pressure of oxygen that results in loss of equilibrium.

Time to LOE

The time required for fish to experience a loss of equilibrium to 0.6 kPa was used as another measure of hypoxia tolerance. At 12 h prior to experimentation, fish were placed in porous 500 ml chambers in order to prevent aquatic surface respiration during hypoxia exposures. At the start of experiments, water was bubbled vigorously with nitrogen 0.6 kPa saturation was achieved and then the time required for fish to lose equilibrium at 0.6 kPa oxygen monitored. The water surface was covered with plastic film to restrict gas exchange of air and hypoxia water and water was circulated with a flush pump to prevent stratification. Fiber-optic O₂ sensors connected to a Witrox 4 unit were used to monitor oxygen tensions (Loligo Systems, Denmark). LOE was characterized by the inability of fish to maintain equilibrium and was confirmed by gentle probing of the chamber.

Statistical Analysis

Microsoft Excel was utilized for all calculations of averages and standard error of the mean for P_{crit} , PO₂ at LOE, Time to LOE, and Resting MO₂ for each species. REGRESS software was used in the calculation of P_{crit} to find the intersection of the oxy-regulating line and the oxy-conforming line (Figure 4). A one-way ANOVA followed by Bonferroni post hoc test was used to determine significance for all comparisons of acclimation to normoxia, intermittent hypoxia, or constant hypoxia. These tests were performed in either SAS or Prism. Significance was determined by a P value less than or equal to 0.05. Figures were created in Microsoft Excel, Microsoft PowerPoint, or SigmaPlot. In figures, error bars represent standard error of the mean.

Results:

Hypoxia tolerance in Fundulus species acclimated to normoxic to water.

Hypoxia tolerance limits were measured in 6-8 *Fundulus* species following acclimation to normoxia for at least 27 days. The P_{crit} values varied only slightly between species and there was no relationship between P_{crit} and the ecological niche that fish were collected from in the field (Figure 5). PO₂ at LOE was similarly very consistent across species with the exception of *F. rathbuni* and *F. confluentus*. *F. rathbuni*, which were collected from oxygen-stable freshwater streams and rivers, lost equilibrium at a comparatively high oxygen content, suggesting comparably low hypoxia tolerance. In comparison, *F. confluentus* had a comparatively low PO₂ at LOE, indicating that this species had moderately better hypoxia tolerance (Figure 6). Time to LOE was similar across species, with the exception of *F. rathbuni* and *F. confluentus*. *F. confluentus* had a time to LOE approximately 27-fold greater than *F. rathbuni*, which also indicated greater hypoxia tolerance in the estuarine *F. confluentus* and reduced hypoxia tolerance in the freshwater *F. rathbuni* (Figure 7). Resting metabolic rates were also similar between species. No significant relationship existed between resting MO₂ and the ecological niche that fish were collected from in the field (Figure 8).

Normoxic P_{crit}

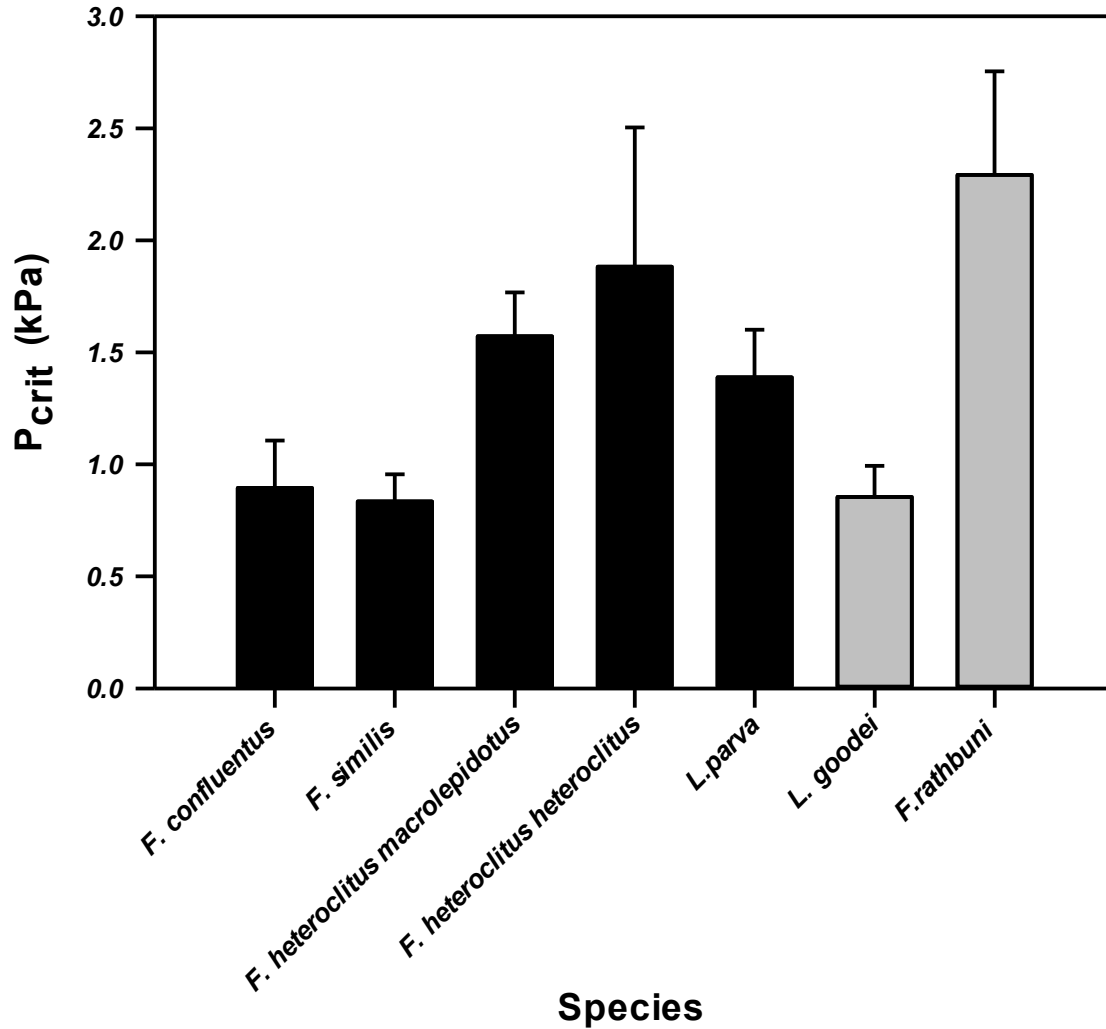


Figure 5. The critical partial pressure of oxygen (P_{crit}) values of Fundulid Killifish acclimated to normoxia. Black bars denote euryhaline species from hypoxia prone environments like coastal marshes and estuaries. Grey bars denote species from oxygen stable, inland freshwater streams. Error bars denote mean \pm s.e.m.

Normoxic PO₂ at LOE

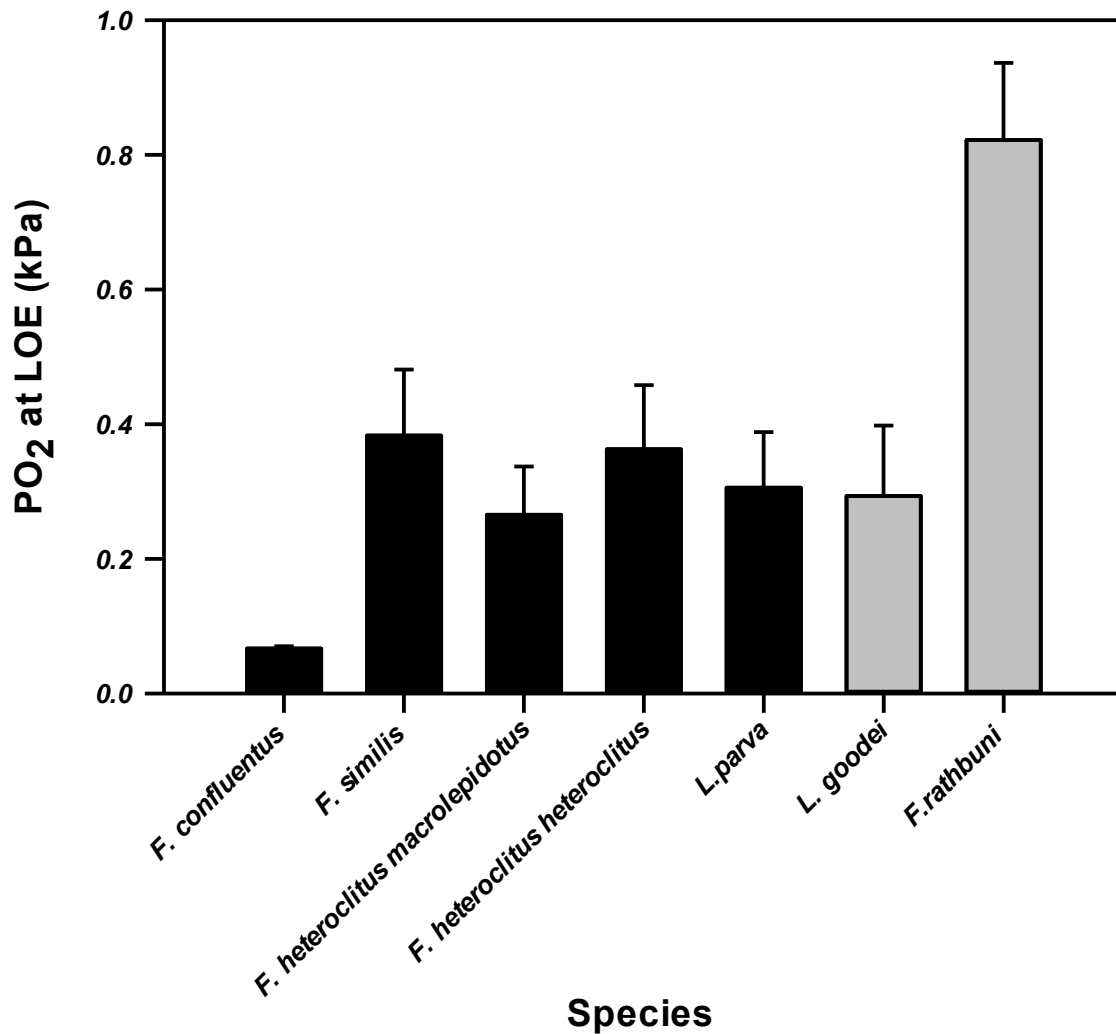


Figure 6. The PO₂ at LOE of Fundulid Killifish acclimated to normoxia. Black bars denote euryhaline species from hypoxia prone environments like coastal marshes and estuaries. Grey bars denote species from oxygen stable, inland freshwater streams. Error bars denote s.e.m.

Normoxic Time to LOE

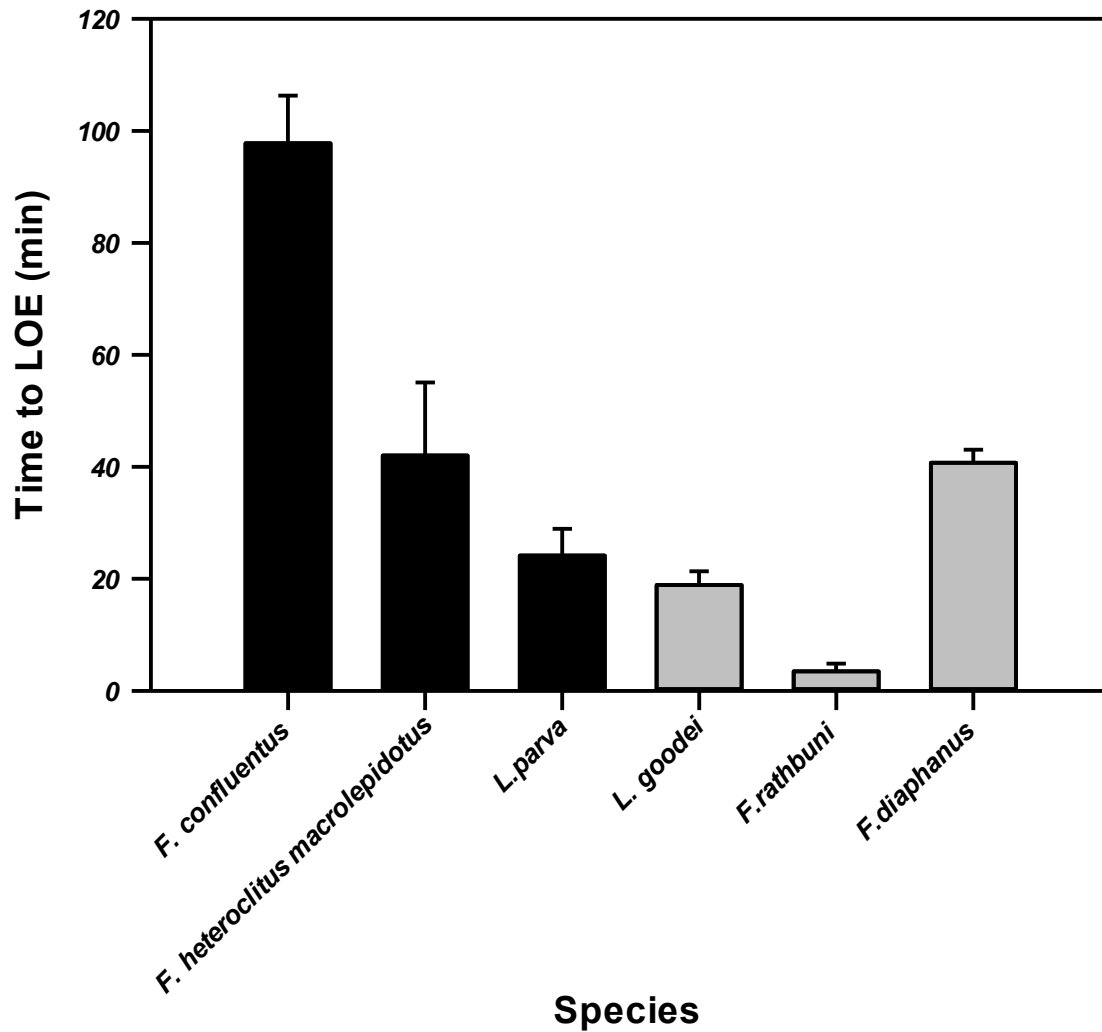


Figure 7. The time to LOE at 0.6 kPa hypoxia of Fundulid Killifish acclimated to normoxia. Black bars denote euryhaline species from hypoxia prone environments like coastal marshes and estuaries. Grey bars denote species from oxygen stable, inland freshwater streams. Error bars denote s.e.m.

Normoxic Resting MO_2

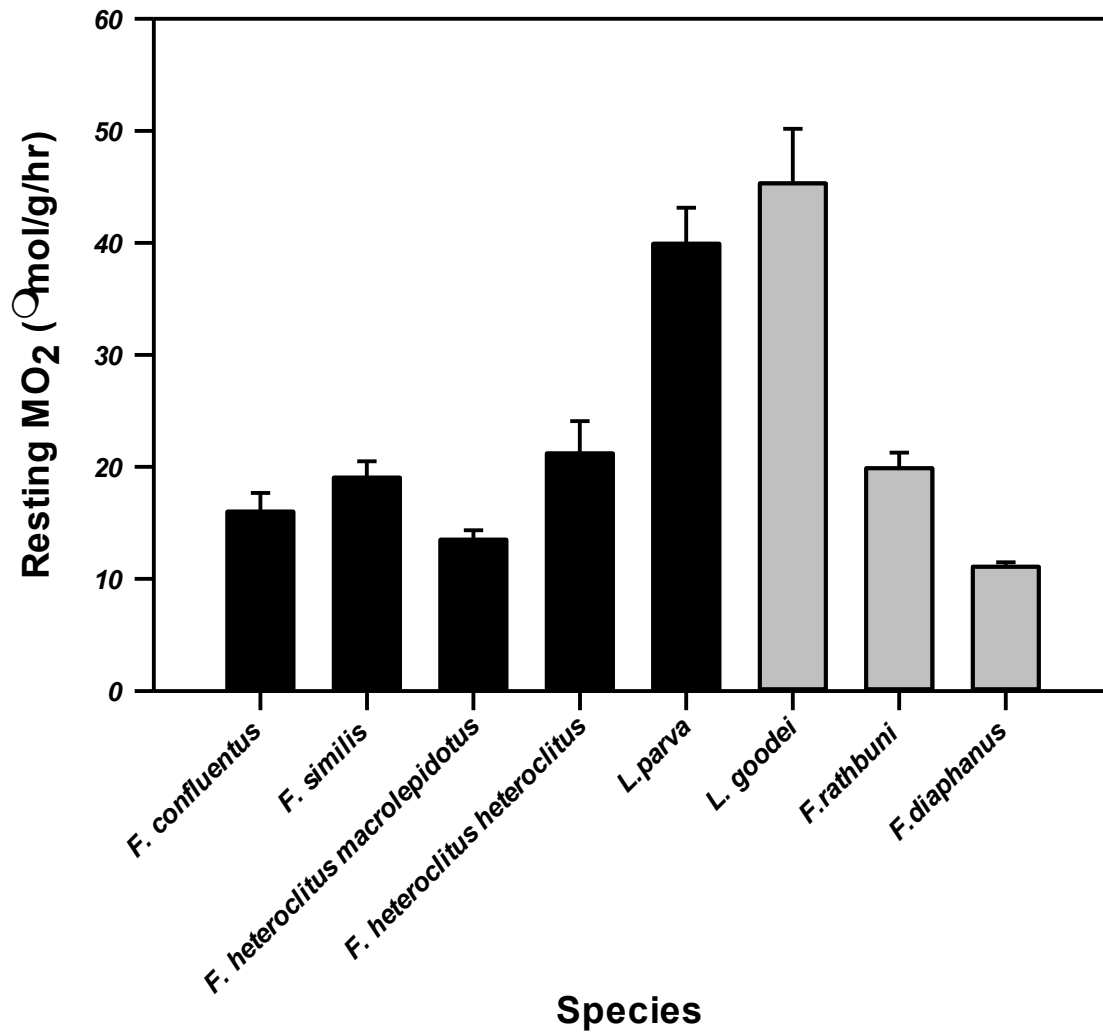


Figure 8. The Resting MO_2 of Fundulid Killifish acclimated to normoxia. Black bars denote euryhaline species from hypoxia prone environments like coastal marshes and estuaries. Grey bars denote species from oxygen stable, inland freshwater streams. Error bars denote s.e.m.

Acclimations to Oxygen Regimes.

F. rathbuni and *Lucania goodei*, which inhabit oxygen-stable freshwater streams, and *F. confluentus* and *L. parva*, which usually inhabit oxygen-variable estuaries, were acclimated to normoxia, intermittent hypoxia, and constant hypoxia for 21-28 days (Figure 9). Acclimations to intermittent and constant hypoxia significantly reduced PO₂ at LOE for *F. rathbuni* (p=0.0002), *L. goodei* (p=0.0453), and *L. parva* (p=0.0374). For *F. rathbuni*, significant pairwise differences were observed between normoxia and intermittent hypoxia (p=0.0002) and normoxia and constant hypoxia (p=0.0038). For *L. parva*, significant pairwise differences were observed only between fish acclimated to normoxia and constant hypoxia (p=0.041). *L. goodei* displayed no significant pairwise differences when acclimated to normoxia or intermittent hypoxia, and between normoxia and constant hypoxia (Figure 10). Acclimation to intermittent and constant hypoxia usually, but not always, increased time to LOE. Significant effects of acclimation were observed only in *L. goodei* (p=0.0001) with both intermittent hypoxia (p<0.0001) and constant hypoxia (p<0.0001) significantly differing from normoxia in pairwise comparisons (Figure 11). Resting oxygen consumption was significantly reduced by acclimation only in *F. rathbuni* (p=0.0181) with both intermittent (p=0.0342) and constant hypoxia (p=0.0405) differing from normoxia in pairwise analysis (Figure 11). Only *F. rathbuni* exhibited reduced resting metabolism as measured by resting oxygen consumption under normoxic conditions (Figure 12).

Acclimation P_{crit}

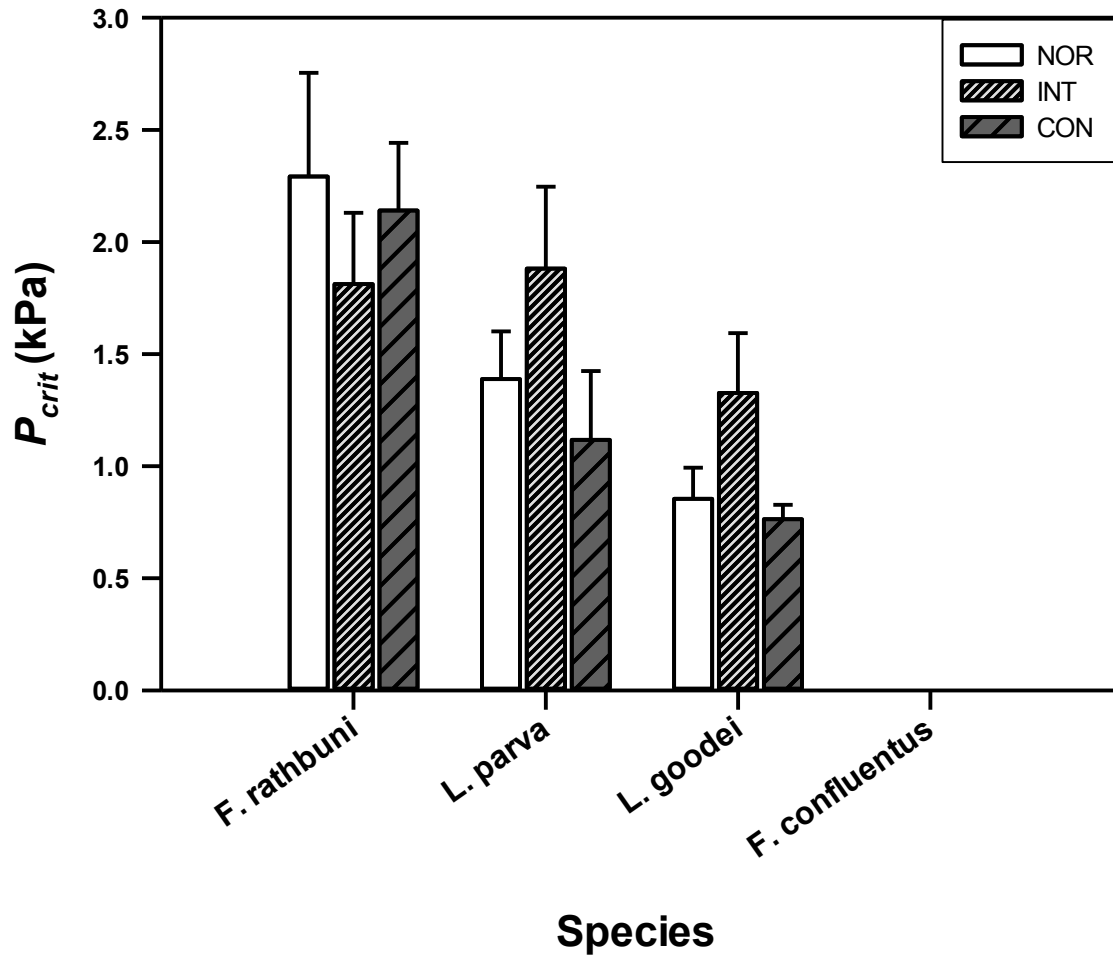


Figure 9. Influence of acclimation to 21-28 days of normoxia, intermittent hypoxia, or constant hypoxia on P_{crit} of Fundulid Killifish. No significant results ($p < 0.05$) were observed. *F. confluentus* data are pending.

Acclimation PO_2 at LOE

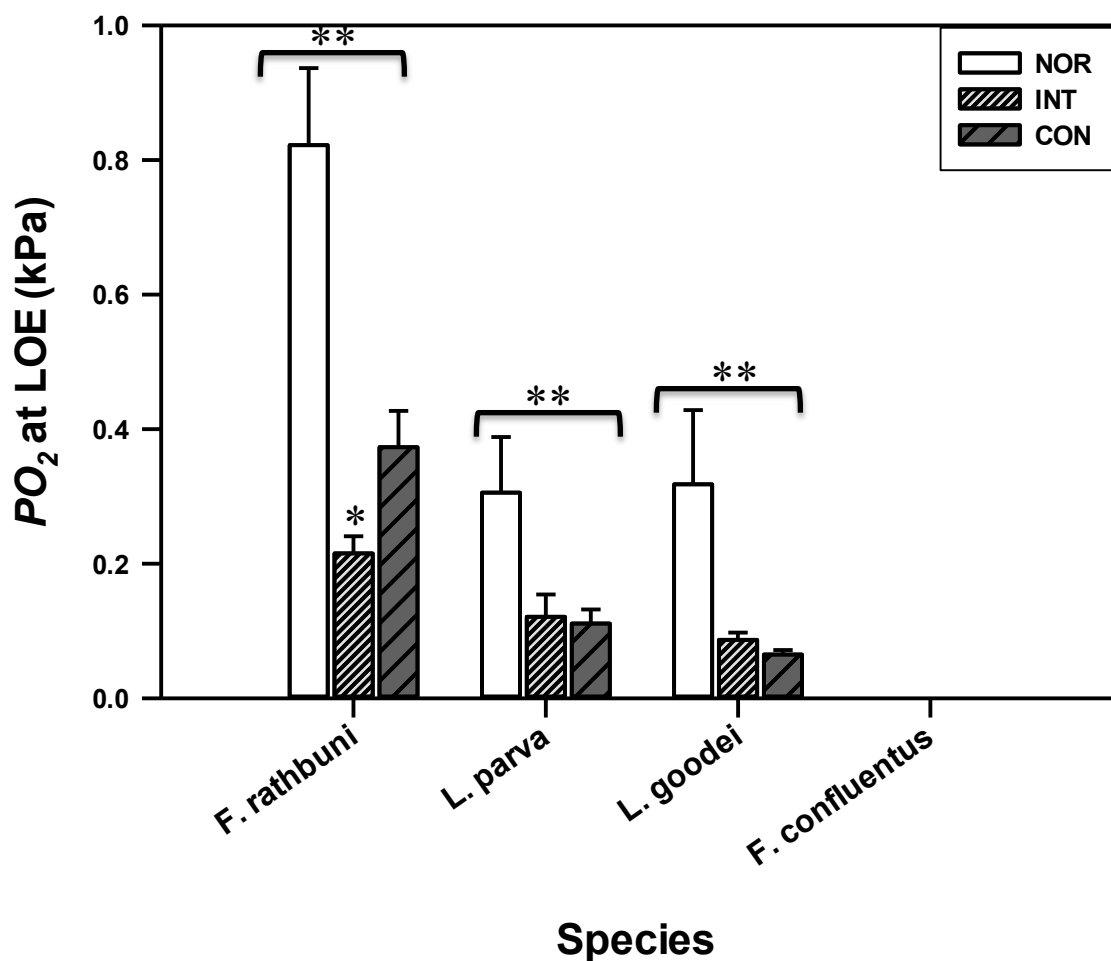


Figure 9. Influence of acclimation to 21-28 days of normoxia, intermittent hypoxia, or constant hypoxia on PO_2 at LOE of Fundulid Killifish. Significant main effects of acclimation ($p < 0.05$) are denoted by ** and significant pairwise differences ($p < 0.05$) to the normoxic control are denoted by *. *F. confluentus* data are pending

Acclimation Time to LOE at 0.6 kPa

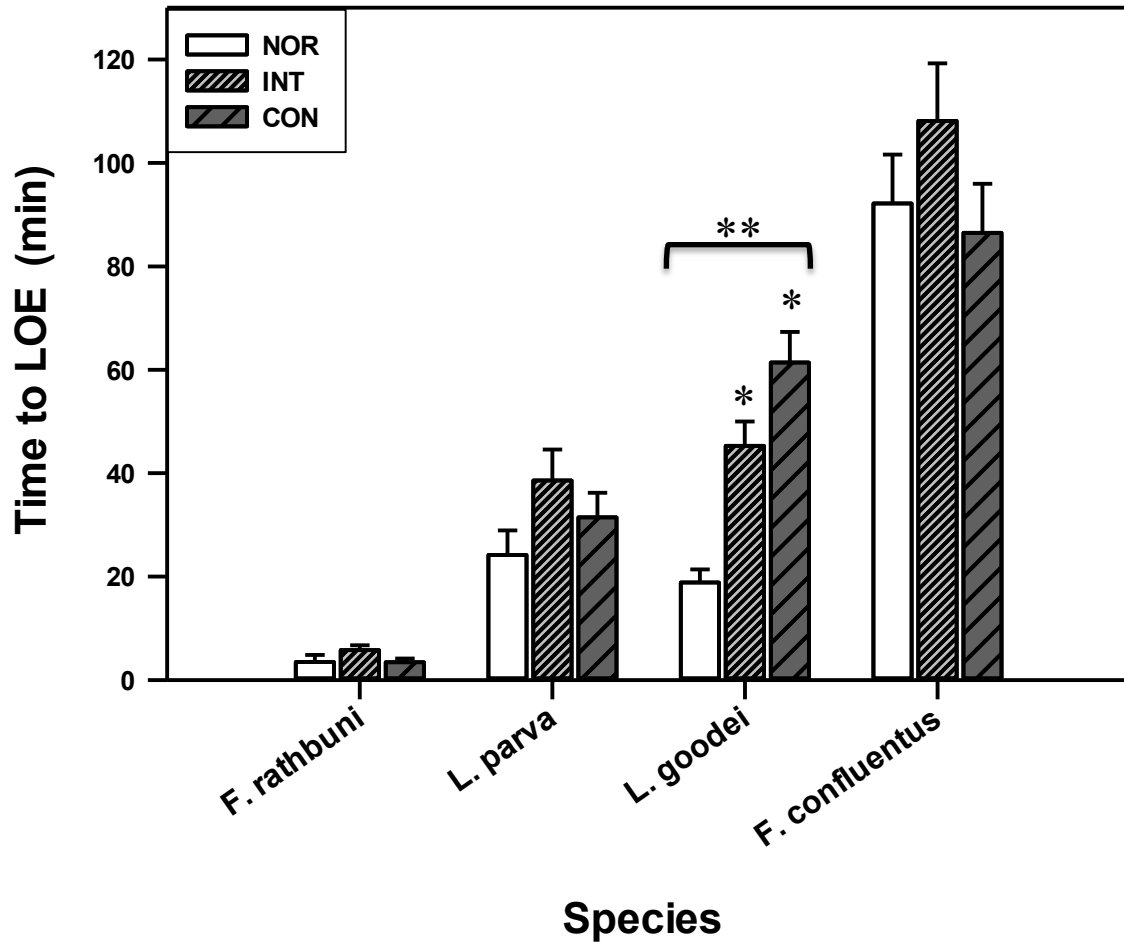


Figure 9. Influence of acclimation to 21-28 days of normoxia, intermittent hypoxia, or constant hypoxia on Time to LOE at 0.6 kPa hypoia for Fundulid Killifish. Significant main effects of acclimation ($p < 0.05$) are denoted by ** and significant pairwise differences ($p < 0.05$) to the normoxic control are denoted by *.

Acclimation Resting MO₂

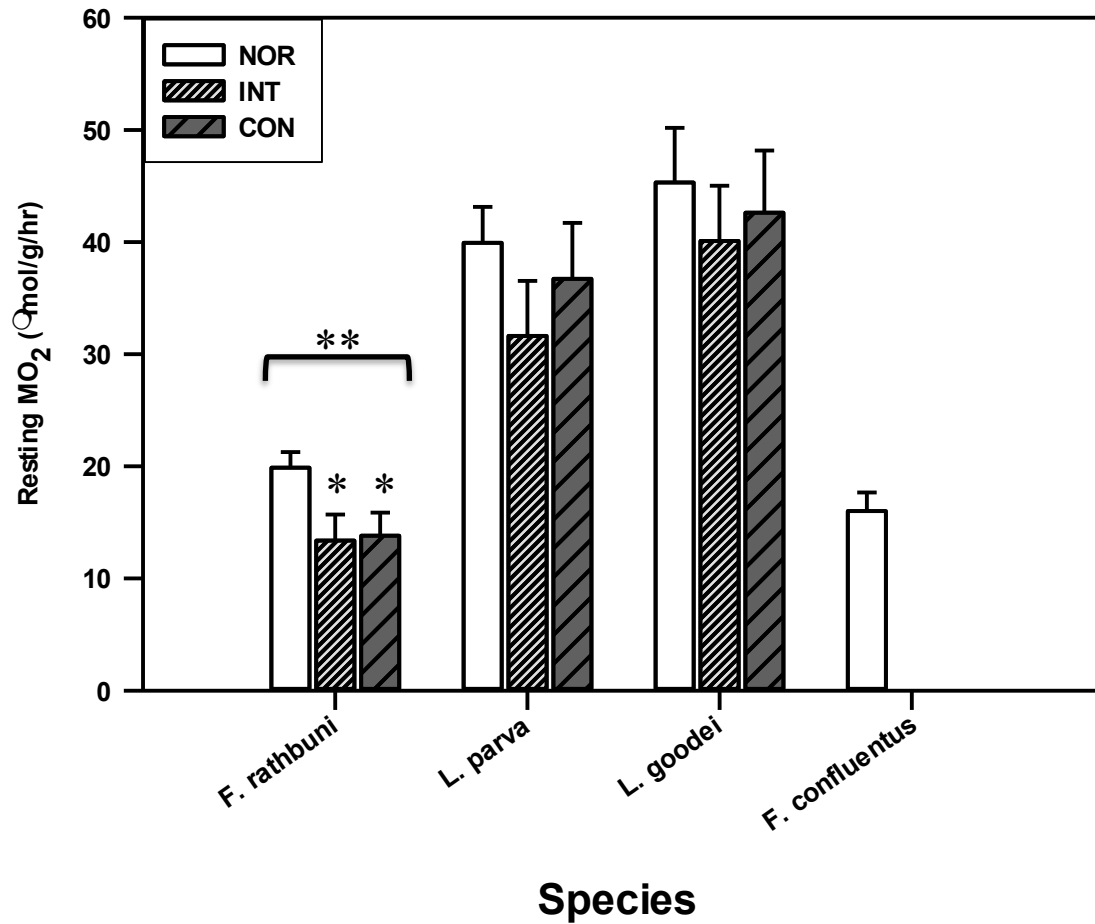


Figure 9. Influence of acclimation to 21-28 days of normoxia, intermittent hypoxia, or constant hypoxia on Resting MO₂ of Fundulid Killifish. Significant main effects of acclimation ($p < 0.05$) are denoted by ** and significant pairwise differences ($p < 0.05$) to the normoxic control are denoted by *. Missing *F. confluentus* data are pending.

Discussion:

Variation in Normoxic Hypoxia Tolerance

The family Fundulidae contains killifish species that have adapted to survive in various environments from inland rivers and creeks, to hypoxia-prone coastal estuaries. Despite variation in native habitats, salinity tolerance, thermal tolerance, and physiology, the Fundulid killifish included in this study demonstrate little variation in hypoxia tolerance. The observed variation cannot be consistently explained in terms of a species environment. Based on both the time to LOE data and the PO₂ at LOE data (Figure 6 and 7), the marsh killifish, *Fundulus confluentus*, is the most hypoxia tolerant of the species included in this study. The same data sets shows that *Fundulus rathbuni*, which inhabits oxygen-stable freshwater streams, is the least hypoxia tolerant species studied. Future work will seek to explain the variation in hypoxia tolerance between these species in terms of the physiological response mechanisms to hypoxia. Alterations of enzymatic activity in the brain, heart, gills, liver, intestine, and spleen as well as morphological alterations of the muscle and gill could explain the observed differences in hypoxia tolerance.

The genus *Lucania* is phylogenetically nested within the genus *Fundulus* and two representative species were included in this study (Figure 2). The sister taxa *L. goodei* and *L. parva* have evolutionarily diverged along a salinity gradient (Fuller, 2008). Our data shows *L. goodei* and *L. parva* have very similar resting O₂ consumption, PO₂ at LOE, and time to LOE. Only slight differences in P_{crit} are observable between these species. Taken together, these data suggest that, despite divergence based on a salinity gradient, these closely related species have not diverged in hypoxia tolerance (Berdan, 2012).

Interspecies comparisons along a phylogeny are limited unless the comparison incorporates the level of evolutionary divergence between species. Phylogenetic Independent Contrast (David, Tanguy, Pichavant, & Moraga) methods allows for statistical analysis based on both physiological endpoints and the phylogenetic distance between species (Felsenstein, 1985). Software such as “Mesquite” is now available to preform PIC comparisons and statistical analysis. Future work will incorporate PIC analysis to better understand the evolutionary aspects of hypoxia tolerance that may be evident in the results of this experiment.

Fundulidae Hypoxia Tolerance

In the present study, we investigated the hypoxia tolerance limits of 7 species of *Fundulus*. These species are known to inhabit diverse ecological niches, some of which are known to experience bouts of hypoxia. We found that P_{crit} values ranged between 0.89 kPa O₂ and 2.3 kPa O₂. In comparison to a recent meta-analysis of P_{crit} values of 93 fish species tested within a similar temperature range (20-36 °C), the 7 Fundulid killifish species included in this study all fall within the lowest ten percent of P_{crit} values (Rogers, UrbinaLt, Reardon, McKenzie, & Wilsonl, 2016). This comparison, made across a diverse selection of species, does not control for life stage, salinity, phylogenetic contrast, or experimental methodology. Additionally, our experimental P_{crit} values for both *F. heteroclitus macrolepidotus* and *F. heteroclitus heteroclitus* are not consistent with the P_{crit} values from two other studies. As a result, this validity of this comparison is limited, yet it suggests that the Fundulidae family has extremely high tolerance to hypoxia compared to most fish species. This is encouraging as the frequency and severity of

hypoxic events is forecasted to increase globally (Rabalais, 2010). Our data suggests that resident *Fundulus* species in areas like the northern Gulf of Mexico, where the increased use of freshwater diversions and further nutrient loading due to recent floods may increase the prevalence of hypoxia, should not be considered to be at risk as these species have evolved to be exceptionally hypoxia tolerant.

Phenotypic Plasticity

A subset of four species were acclimated to either normoxia, intermittent hypoxia, or constant hypoxia for 21-28 days (Figure 3). None of the three species tested (*F. confluentus* data pending) exhibited a significant ($p < 0.05$) reduction in P_{crit} (Figure 9). Acclimation to intermittent hypoxia slightly increased P_{crit} while constant hypoxia slightly decreased P_{crit} relative to normoxic fish in *L. parva* and *L. goodei*. Based on these results, acclimation to 21-28 days of either intermittent or constant hypoxia does not significantly increase the ability of these fishes to extract oxygen from hypoxic waters. *F. rathbuni* differed from *L. goodei* and *L. parva* in that intermittent hypoxia slightly reduced P_{crit} in this species. It is interesting that the *Lucania* species demonstrate a similar response to each other but differ from the response of the more distantly related *F. rathbuni*. This potentially indicates the importance of phylogenetic analyses in addition to these metrics. Our observation of differential plasticity in some species between fish exposed to intermittent hypoxia and fish exposed to constant hypoxia is consistent with previous work demonstrating unique physiological alterations that *F. heteroclitus* utilizes to cope with these varying patterns of hypoxia (Borowiec, Darcy, Gillette, & Scott, 2015).

In all three species tested (*F. confluentus* data pending), acclimations significantly reduced PO_2 at LOE. An increase in anaerobic pathways for energy production could explain this observation. Some fish species, such as hypoxia tolerant sculpins, show increased activity of enzymes involved in anaerobic metabolism in the brain when exposed to hypoxia (Mandic, Speers-Roesch, & Richards, 2013). Another explanation could be the level of metabolic depression. If fish acclimated to hypoxia are better able to reduce metabolic demands, then they would be able to endure a more severe level of oxygen depletion before succumbing to loss of equilibrium (G. E. Nilsson, 1992). Metabolic depression during hypoxia involves reduction of protein turnover and ion gradient maintenance (Podrabsky & Hand, 2015).

Acclimation to intermittent and constant hypoxia increased time to LOE at 0.6 kPa hypoxia in most, but not all, cases. These trends can also be explained by a possible increase in anaerobic metabolism. For *F. confluentus* and *F. rathbuni*, acclimation to constant hypoxia either decreased or had no observable effect on time to LOE. During acclimations, these species exhibited an observable reduction in appetite. The acclimation process may have reduced glycogen and lipid stores in these fish as caloric intake decreased. Without the necessary energy stores, these fish would not be better suited to survive hypoxic events, even if they alter their enzymatic levels to better utilize glycolysis pathways for the production of ATP in hypoxic environments by anaerobic metabolism.

Acclimation to intermittent and constant hypoxia decreased the normoxia resting metabolism of oxygen in all species studied (*F. confluentus* data pending). Significant reduction in resting MO_2 were observed for *F. rathbuni* only. These data suggest that hypoxia induced alterations to enzyme levels may be effecting metabolic processes even in normoxic conditions. If enzymatic activity was altered to enhance anaerobic metabolism during hypoxia, this could

explain the lower oxygen consumption at normoxia. Also, if metabolic depression occurs in hypoxic conditions, this metabolic depression may be sustained for some amount of time after a return to normoxia.

PO₂ at LOE as a comparative measure of hypoxia tolerance.

Several measures exist for the quantification of hypoxia tolerance. By far the most commonly used measure in the field of fish physiology is the critical pressure of oxygen (P_{crit}). The P_{crit} represents the oxygen concentration at which an organism cannot extract enough oxygen from its environment in order to maintain standard metabolic rate. Below P_{crit} , fish species utilize metabolic depression to varying extents to prolong survival (van Ginneken & van den Thillart, 2009). In this way, P_{crit} is a measure of a fish's ability to abstract oxygen efficiently, but a low P_{crit} does not always correlate to better hypoxia tolerance (Speers-Roesch, Mandic, Groom, & Richards, 2013). For example, the large brained eel *Gnathonemus petersii* has an exceptional ability to extract oxygen from hypoxic waters, and a low P_{crit} , yet this species does not demonstrate exceptional hypoxia tolerance as its large brain is oxygen demanding (G. Nilsson, 1996). Another common physiological endpoint of hypoxia tolerance is time to loss of equilibrium. This measurement tests the duration of an organisms ability to maintain essential biological processes in severely hypoxic conditions. In this way, time to loss of equilibrium is a test of endurance. The practicality of this measurement for interspecies comparisons is limited in that comparisons can only be made between species subjected to the same level of hypoxia. Time to LOE can be utilized if the level of hypoxia is consistent, as it usually is within a single study, but methodology varies largely between different studies on varying species. The PO₂ at LOE measures the minimal oxygen concentration required for a fish to maintain essential biological processes. As a measure of hypoxia tolerance, it is potentially useful in that it lends itself to interspecies comparisons (Borowiec et al., 2016). As we have demonstrated in this study, some experimental designs allow for PO₂ at LOE measurement in conjunction with P_{crit} measurement. PO₂ at LOE is a test of physiological endurance and efficiency that incorporates both an organism's ability to obtain oxygen in hypoxic conditions and its ability to utilize anaerobic metabolism to maintain ATP production. Despite its relevance as a physiological endpoint, PO₂ at LOE is incorporated in very few physiological studies. Our results demonstrate both the sensitivity and the effectiveness of PO₂ at LOE as a physiological measurement of hypoxia tolerance. Considering the large variation in hypoxia tolerance across many fish species, it is imperative to include multiple measures of hypoxia tolerance in experimental design (Rogers et al., 2016).

Future Directions

F. grandis from Cocodrie LA were collected but prior to experimentation they were mixed with a closely related species of *F. heteroclitus*. The two species cannot be reliably distinguished on a visual basis. This mixture of fish was included in experimentation but DNA extraction from fin clippings and genotyping will be required to separate the data by species. Future work will separate these data or repeat the experiment for these species to quantify hypoxia tolerance of Louisiana populations of Killifish species.

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