

Effect of Temperature on Respiratory Responses to Increasing Hypoxia for Five Species of Nongame Stream Fishes

Nathan R. Hartline¹, School of Fisheries, Aquaculture, and Aquatic Sciences, Auburn University, AL 36849

Dennis R. DeVries, School of Fisheries, Aquaculture, and Aquatic Sciences, Auburn University, AL 36849

Russell A. Wright, School of Fisheries, Aquaculture, and Aquatic Sciences, Auburn University, AL 36849

James A. Stoeckel, School of Fisheries, Aquaculture, and Aquatic Sciences, Auburn University, AL 36849

Lindsay M. Horne, School of Fisheries, Aquaculture, and Aquatic Sciences, Auburn University, AL 36849

Abstract: Understanding the ability of fishes to tolerate low dissolved oxygen (DO) is important not only to our understanding of the ecology of aquatic systems, but also for flow management in regulated lotic systems. Historical flow management guidelines have been based on critical oxygen concentrations and incipient lethal levels from just a few species, and data on nongame fish species are lacking. Here we quantify respiration rate, critical DO concentration at routine metabolic rate, and regulatory capacity across temperatures for five nongame fish species. Oxygen consumption patterns represented a continuum between regulation and conformation, as ability to regulate was affected differently by temperature among species, declining with increasing temperature in blackbanded darter (*Percina nigrofasciata*) and increasing with temperature for banded sculpin (*Cottus caroliniae*). Further, oxygen consumption was not affected by temperature in bronze darter (*Percina palmaris*), greenbreast darter (*Etheostoma jordani*), and blacktail shiner (*Cyprinella venusta*). Critical oxygen levels increased with temperature for blacktail shiner, greenbreast darter, and blackbanded darter, but did not change with temperature for bronze darter or banded sculpin. Given this among-species variation, protections based on data from a limited number of taxa may not effectively protect habitat for entire fish communities.

Key words: regulation index, darters, minnows, respiration, oxyregulation

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Aquatic organisms are challenged by fluctuations in many interacting physical, chemical, and biological factors in the environment. Variation in these conditions can be both natural and human-induced. In lotic systems, dissolved oxygen (DO) and temperature, two of the most significant controlling factors, can vary dramatically due to fluctuations in flow. Below dams, variation in flow, often driven by reservoir operations, can sometimes lead to low DO events (i.e., hypoxia), particularly at high water temperatures. These conditions can be stressful given that fishes require oxygen for aerobic energy production, and rising temperature increases the respiration rate of poikilotherms while reducing available DO to support this increased metabolism (Pörtner 2010, Clark et al. 2013, Rogers et al. 2016).

Hypoxia tolerance has been well-studied in fish species that are recreationally and economically important (i.e., sport-fishes and cultured species; McBryan et al. 2013). Results have been important in understanding the basic physiology of fishes, specifically DO concentrations required by fishes to survive and thrive. This research has informed criteria for management of instream flow

and therefore management of DO and temperature in regulated rivers (Cech et al. 1979, Evans 1984). These regulatory criteria are then applied to development and implementation of hydroelectric dam operation protocols to ensure they meet minimum DO requirements (USEPA 1986a, 1986b). Dissolved oxygen criteria must provide protection for more than 600 freshwater species representing over 40 families in North America. Unfortunately, most pertinent data informing these criteria have come from studies of a relatively small number of species (USEPA 1986a, 1986b), and oxygen and temperature tolerance data specific to non-sport fishes inhabiting regulated rivers are lacking.

While nongame fishes may not have direct recreational or economic significance, their ecological roles as predators and prey are extremely important in aquatic food webs (DeVries and Stein 1990, Cambray and Pister 2002). Also, many are important hosts for the parasitic larval stage of freshwater mussels—a highly imperiled group (Williams et al. 1993, Haag and Williams 2014). Alteration of environmental conditions, such as temperature and DO, can greatly increase environmental stress on host fishes and often

1. Present Address: 430 George Wallace Dr, Gadsden, AL 35903

limits survival of both mussels and fishes (Moss and Scott 1961, Clarke and Johnston 1999). As such, the susceptibility of nongame fishes to warming temperatures and decreasing DO concentrations associated with modified flow regimes must be assessed.

Respirometry is commonly used to measure oxygen consumption and, therefore, metabolic rate in fishes. Standard metabolic rate is the minimum metabolic rate required for a fish to support aerobic metabolism in a rested, unfed state (Cech 1990, Wood 2018); however, this can be difficult to measure given that it is difficult to determine when a fish has reached a state of minimum respiration (Chabot et al. 2016). Routine metabolic rate (RMR) is often used to describe oxygen consumption in a quiescent state allowing for minimal spontaneous movements (Eliason and Farrell 2016). Because oxygen consumption rate and RMR can vary with changing abiotic conditions (e.g., temperature, pH, DO, etc.), respirometry can be used to determine the effects of temperature shifts and hypoxia similar to variation in conditions fish may experience *in situ*.

Historically fishes were thought to fit into one of two oxygen consumption groups: oxyregulators (Cech et al. 1979, Barnes et al. 2011) or oxyconformers (Tiffany et al. 2010, Barnes et al. 2011). Fish that maintained a constant oxygen consumption rate as oxygen concentrations declined were considered to be oxyregulators while those that reduced their oxygen consumption as DO decreased were considered to be oxyconformers. A decline in oxygen consumption with declining environmental DO has generally been assumed to incur some metabolic cost and thus would be associated with a reduced scope for growth (USEPA 1986a, Eliason and Farrell 2016). Given this, one would expect oxyregulators to be more tolerant of low DO than oxyconformers (e.g., Barnes et al. 2011) although regulators may also experience increased metabolic costs due to increased ventilation as DO decreases. One compromise approach to reduce the confusion caused by attempts to categorize organisms into one of two distinct metabolic patterns is the concept of a regulation index (RI; Mueller and Seymour 2011), which recognizes that there is a continuum of metabolic patterns. The RI quantifies the relative ability of an organism to regulate oxygen consumption (i.e., regulatory capacity) as DO declines to zero, rather than constraining an organism to be defined as either a regulator or a conformer.

Important to the discussion of oxyregulators and oxyconformers is the critical DO partial pressure (P_{crit}), saturation (S_{crit}) or concentration (DO_{crit}) which generally represents the DO metric below which the ability of an organism to obtain oxygen from its environment rapidly declines with decreasing oxygen availability (Jobling 1994, Valverde et al. 2006, Rogers et al. 2016). In this study, we use DO_{crit} as our preferred metric because concentration is typ-

ically measured in the field and readily available from monitoring data, lending itself to easy use in bioenergetics models examining effects of hypoxia on fish growth and habitat quality (i.e., Arend et al. 2011). Below DO_{crit} , regulators are no longer able to maintain a constant mass-specific oxygen consumption rate ($\dot{M}O_2$), and conformers exhibit an increasing rate of decline in $\dot{M}O_2$ (Pörtner et al. 1985, Mueller and Seymour 2011). Because a lower DO_{crit} is associated with a greater capacity to obtain oxygen in low DO environments, it is frequently used as an indicator of hypoxia tolerance in fishes (Speers-Roesch et al. 2012). The DO_{crit} will vary depending on whether standard, routine, or maximum metabolic rate is being estimated. Thus, some combination of RMR, RI, and DO_{crit} will likely provide the most complete view of effects of low DO on fishes (Mueller and Seymour 2011).

Recognizing the ecological roles of nongame fishes and their potential importance in setting management criteria in systems where DO can be limiting due to hypoxic events, our objectives were four-fold. First, we quantified the respiration rate ($\dot{M}O_2$) of five nongame species native to southeastern U.S. rivers. Second, we quantified DO_{crit} at the RMR for each of these species. Third, we quantified the regulatory capacity of these five species using the RI. Last, we used a combination of these three metabolic endpoints to compare the effect of temperature on hypoxia tolerance among the five species tested.

Methods

Specimen Collection

Fishes were collected using a backpack electrofisher (SR 24 Electrofisher, Smith-Root, Inc., Vancouver, Washington) in combination with seines and nets, held in aerated coolers, and transported to a wet lab facility at Auburn University's E.W. Shell Fisheries Center. Banded sculpin (*Cottus caroliniae*) were collected from the Tennessee River basin during August 2012 and June 2013. Black-banded darters (*Percina nigrofasciata*), bronze darters (*P. palmaris*), greenbreast darters (*Etheostoma jordani*), and blacktail shiners (*Cyprinella venusta*) were collected from the Mobile River basin during January, March, and April of 2013. Fish were generally collected outside of or just prior to peak spawning periods (Boschung and Mayden 2004). All procedures were conducted in accordance with Auburn University IACUC PRN 2011–1990.

Experimental Design

Fish were acclimated to laboratory conditions for at least one week to appropriate treatment temperatures (20°, 22°, 24°, or 28° C) in 113-L tanks. Multiple species were collected in the field on each collection date and acclimated in the lab simultaneously. Water temperature was maintained within 1° C of the target tempera-

ture with a flow-through water chiller/heater (1/3-hp Aqua Logic Delta Star Research Chiller; AquaLogic Aquatic Design, Manufacturing and Integration, San Diego, California). During laboratory acclimation, fish were fed larval chironomids and *Chaoborus* until 48 h prior to initiation of experiments, allowing for complete gut evacuation (Beamish 1964). Fish were subjected to a 12-h light-dark daily photoperiod throughout acclimation and testing.

After the laboratory acclimation period, individual fish were placed in one of four appropriately sized acrylic respirometry chambers submersed within a 400-L polyethylene tank, while a fifth chamber contained no fish and served as a control. Chamber sizing was based on recommendations in the literature (Cech 1990) and was intended to limit movement and reduce water volume while minimizing stress on the fish. Chamber volumes ranged from 20–100 ml with an additional tubing volume of 30 ml, and fish wet mass (g) to chamber volume (ml) ratios averaged 37.5 (± 1.9 SE). All chambers were visually shielded from one another to reduce stress.

Because the time taken to recover from handling stress varies greatly among fish taxa, as does the relationship between metabolic rate and temporal position within a diel cycle (Voutilainen et al. 2011), determining a standard acclimation time across species is difficult. For our study, we sought to quantify RMR, given that fish in the field are likely not entirely acclimated to static conditions or expending only the minimum energy required for survival. As such, we used a constant start time (~ 1100 hours) and chamber acclimation time (~ 1 h; within the range of acclimation times seen in the literature, from 0.5–24 h; Mallekh and Lagardere 2002, Abogye and Allen 2013, Hovel et al. 2015). All fish across taxa became inactive within the 1-h acclimation time. Two subsequent flush cycles (see below) added an additional 30 min of acclimation time and allowed fish to become adjusted to the system, switching between open and closed before initiation of the final closed respirometry run when we began measuring respiration rates. Flow rates were similar between flush and closed cycles and were kept low enough so as to not cause fish to actively swim. Thus, our oxygen consumption measurements represent estimates of RMR of inactive fish more than 1.5 h after a disturbance event.

We quantified three metabolic endpoints using closed respirometry. Oxygen electrodes (model 1302, Strathkelvin Instruments, North Lanarkshire, Scotland) were calibrated with fully aerated water (maximum DO) and a saturated sodium sulphite solution (DO = 0) at the treatment temperature before each experiment. Chambers were plumbed so that fresh, oxygenated water could be flushed through each chamber at a constant rate via a head tank prior to closed respirometry runs. To initiate closed respirometry, flow of outside water from the head tank to a giv-

en chamber was halted via a shut-off valve. Water was then kept mixed in the chamber via a closed loop of tubing and a submersible pump (model 1046, Eheim, Deizsau, Germany). Dissolved oxygen in each chamber was measured via an oxygen probe fitted in a flow cell (meter model 929, flow cell model FC100, Strathkelvin Instruments) inserted in the tubing between the chamber and submersible pump. After an initial 1-h acclimation, a two-cycle, intermittent run was performed to further orient and acclimate individuals in chambers to the flushing water cycling on and off. During these cycles, the chamber was sealed for approximately 30 min and DO was allowed to be reduced by 1–2 mg L⁻¹ but DO concentration in chambers remained normoxic (> 6 mg L⁻¹). At the end of each cycle, freshly oxygenated water from the head tank was flushed through the chamber to bring it back up to $\sim 100\%$ saturation. These cycles were not used to calculate the RMR, RI or DO_{crit} estimates presented in this paper.

Following the second flush cycle, we again shut off the flow from the head tank and used closed respirometry to measure RMR, RI, and DO_{crit}. Oxygen consumption rate was calculated every 2–6 min (based on the rate at which individuals reduced oxygen while water recirculated in the closed chamber) using the formula:

$$\dot{M}O_2 = (O_{2(i)} - O_{2(f)}) \cdot V \cdot T^{-1} \cdot B_w^{-1}$$

where $\dot{M}O_2$ is the mass-specific oxygen consumption rate (mg O₂ g⁻¹ h⁻¹), $O_{2(i)}$ is oxygen concentration in water at the start of the measurement period (mg L⁻¹), $O_{2(f)}$ is the O₂ concentration at the end of the measurement period (mg L⁻¹), V is the volume of the chamber (L), T is the time elapsed during the measurement period, and B_w is the wet mass of the fish (Cech 1990). The $\dot{M}O_2$ estimates were corrected for background oxygen uptake by subtracting O₂ declines of the control chamber from each chamber containing fish. Each fish was allowed to deplete the O₂ in its chamber to < 1 mg L⁻¹ or to the point where fish began showing obvious signs of distress (e.g., loss of equilibrium), at which point fish were removed from the chambers. Trials ranged from 1.1 to 6.0 h (mean = 3.3, SE = 0.23). After each trial, all fish were measured (TL, mm) and weighed (g). Ammonia concentration (total ammoniacal-N = TAN) within each chamber was measured (LaMotte model 3304, LaMotte Company, Chestertown, Maryland) by testing the chamber water at the end of each trial to assess accumulation of metabolic wastes.

To quantify the relative ability to regulate oxygen consumption, we quantified an RI and DO_{crit} for each individual fish following Mueller and Seymour (2011). The RMR was plotted against DO for each closed respirometry run. The upper limit of the DO range for which RI was calculated was held constant at 6 mg O₂ L⁻¹ to avoid bias in cooler temperature runs where initial DO could be much higher than warmer temperature runs. Regressions relating

$\dot{M}O_2$ as a function of DO for each fish were generated using linear, quadratic, exponential, or sigmoidal functions. The relationship with the highest r^2 was then used in the Sigma Plot area under the curve (AUC) macro to calculate AUC for 1) the observed data, 2) a horizontal line that represented perfect regulation, and 3) a linear decrease that represented perfect conformation. The RI was calculated as:

$$RI = \frac{(\text{observed AUC} - \text{conformation AUC})}{(\text{regulation AUC} - \text{conformation AUC})}$$

The RI thus provided a quantitative measure of the degree to which fish were able to regulate oxygen consumption as ambient DO declined from 6 to <1 mg O_2 L^{-1} , with RI values approaching 1 or 0 indicating strong regulation or strong conformation, respectively. The DO_{crit} was calculated as the DO concentration showing the greatest distance between the observed $\dot{M}O_2$ and the perfect conformation line (Mueller and Seymour 2011) and was estimated for RMR.

To assess the effects of temperature on DO_{crit} , the previously described assays were run at three temperatures for each fish. All species were tested at 20° and 24° C. Greenbreast darters and blacktail shiners were also tested at 28° C. However, during acclimation to experimental temperatures, we found that temperatures above 24° C represented an upper lethal limit for bronze darter, blackbanded darters, and banded sculpins (Hartline 2013) so these species were tested at a third temperature of 22° C rather than 28° C. Given that RMR did not always remain constant with declining DO above DO_{crit} , we used the previously described regressions to predict RMR at 5 mg O_2 L^{-1} for each individual at each temperature tested (hereafter referred to as RMR_5); this allowed us to determine the effect of temperature on routine metabolic rate at a standardized dissolved oxygen concentration.

An ANCOVA (SAS Institute 2012) was used to test for interactions between temperature and mass of the individual fish (covariate) on RI, DO_{crit} , and RMR_5 of each species. If the interaction between temperature and mass was significant, pairwise comparisons were made among temperatures using LSMEANS with Tukey-Kramer adjustment. If the interaction between temperature and mass was not significant, an ANOVA followed by Tukey's honestly significant difference (HSD) post hoc test was used to determine if differences existed among specific temperature treatments. Logistic regressions (SAS Institute 2012) were run for those species with individuals exhibiting RI values equal to zero (e.g., the fish were oxyconformers) to test the effect of temperature and mass on the probability of getting zero RI. An ANOVA followed by Holm-Sidak was used to compare RI, DO_{crit} , and RMR_5 among species. In cases where the Shapiro-Wilk normality test failed, data were rank transformed and analyzed using Kruskal-Wallis followed by Dunn's Method. Results were considered significant at $P \leq 0.05$.

Table 1. The model selection results showing the number of individual fish (n) from each species at each temperature, that were best fit with a linear, quadratic, or exponential regression. "temp" is the temperature at which respiration was quantified, and n is the sample size for that species x temperature combination.

Species	Temp (°C)	n	Linear	Quadratic	Exponential
Blacktail shiner	20	10	0	0	10
	24	7	0	0	7
	28	7	0	0	7
Greenbreast darter	20	4	0	0	4
	24	7	1	1	5
	28	4	1	1	2
Blackbanded darter	20	5	0	0	5
	22	5	0	0	5
	24	6	0	2	4
Bronze darter	20	4	0	0	4
	22	10	0	1	9
	24	4	0	0	4
Banded sculpin	20	9	7	1	1
	22	7	2	1	4
	24	7	1	0	6

Results

Fish generally remained stationary throughout testing and showed no loss of equilibrium or increased movement, until reaching the lowest DO levels tested (<1 mg L^{-1}), supporting that RMR was measured. At the end of all experiments, total ammonium nitrogen concentrations in chambers were ≤ 0.5 ppm. Sample sizes for each species ranged from 15–24, with 4–10 individual replicates per temperature (Table 1).

Blacktail Shiner

The best fitting model for all blacktail shiner individual fish plots was a negative exponential model (Table 1, Figure 1). The ANCOVA analyses indicated a significant influence of temperature, no significant main effect of mass, and a marginally significant effect of the mass by temperature interaction on RI (Table 2). No pairwise comparisons for RI at different temperatures were significant in the ANCOVA analysis (Figure 2). The temperature by mass interaction was not significant for blacktail shiner DO_{crit} (Table 2). The DO_{crit} was marginally higher at 28° versus 24° C ($F = 3.25$; $df = 2, 21$; $P = 0.0590$; Tukey's, $P = 0.051$; Figure 2). Temperature, mass, and their interaction for blacktail shiner RMR_5 were all statistically significant with mass influencing respiration slightly positively at 24° C and negatively at 20° and 28° C (Table 2). The RMR_5 was higher at 28° C than at either 20° or 24° C (ANCOVA LSMEANS, $P = 0.0005$ and $P = 0.01$, respectively; Figure 2).

Table 2. Results of ANCOVA testing the effect of temperature, individual mass of the fish, and the interaction of these factors on the regulation index (RI), the critical dissolved oxygen concentration (DO_{crit}), and the routine specific respiration rate at dissolved oxygen of 5 mg O₂ L⁻¹ (RMR_5). Main effect results are presented when the ANCOVA temperature x mass interaction term was significant; otherwise, main effects were tested by ANOVA, with results presented in text. The range of fish mass for each species is included in parentheses.

Species (range in mass, g)	RI			DO_{crit}			RMR_5		
	Temp (°C)	Mass	Temp x mass	Temp (°C)	Mass	Temp (°C) x mass	Temp (°C)	Mass	Temp (°C) x mass
Blacktail shiner (1.68–7.50)	$F = 4.51$ $df = 2,18$ $P = 0.026$	$F = 2.09$ $df = 1,18$ $P = 0.166$	$F = 3.37$ $df = 2,18$ $P = 0.057$	–	–	$F = 1.02$ $df = 2,18$ $P = 0.381$	$F = 3.88$ $df = 2,18$ $P = 0.04$	$F = 6.32$ $df = 1,18$ $P = 0.02$	$F = 4.32$ $df = 2,18$ $P = 0.029$
Greenbreast darter (0.51–1.85)	–	–	$F = 1.85$ $df = 2,9$ $P = 0.212$	–	–	$F = 2.97$ $df = 2,7$ $P = 0.117$	–	–	$F = 0.09$ $df = 2,9$ $P = 0.914$
Blackbanded darter (1.00–6.78)	–	–	$F = 0.19$ $df = 2,10$ $P = 0.827$	–	–	$F = 0.75$ $df = 2,10$ $P = 0.496$	–	–	$F = 0.36$ $df = 2,10$ $P = 0.704$
Bronze darter (0.54–5.01)	–	–	$F = 0.77$ $df = 2,12$ $P = 0.48$	–	–	$F = 0.36$ $df = 2,12$ $P = 0.76$	$F = 16.9$ $df = 2,12$ $P = 0.0003$	$F = 3.18$ $df = 1,12$ $P = 0.10$	$F = 8.47$ $df = 2,12$ $P = 0.005$
Banded sculpin (0.91–3.01)	–	–	$F = 0.57$ $df = 2,17$ $P = 0.575$	–	–	$F = 1.61$ $df = 2,7$ $P = 0.266$	–	–	$F = 1.30$ $df = 5,17$ $P = 0.298$

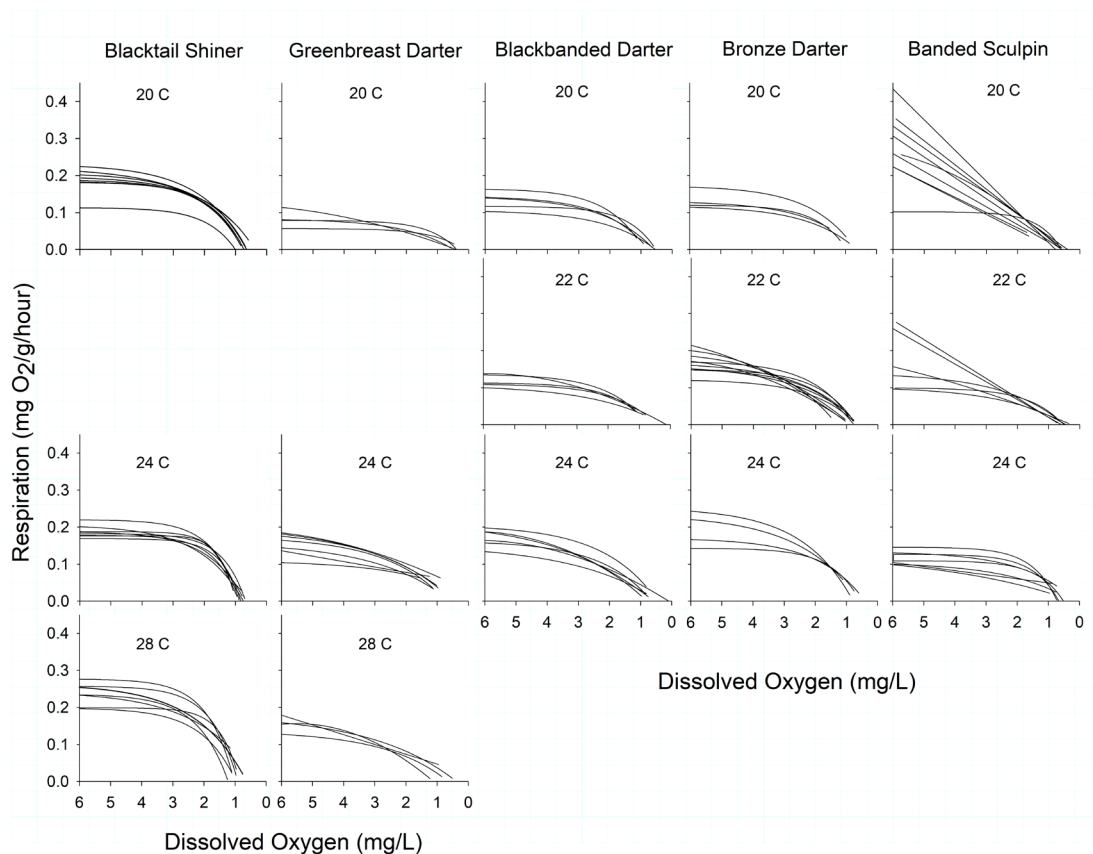


Figure 1. Relationship between mass-specific oxygen consumption rate (MO_2) and declining ambient dissolved oxygen concentrations at three temperatures for each fish species. Each regression line represents results of an individual fish. Bronze darter, blackbanded darter, and banded sculpin exhibited a lethal temperature $\leq 28^\circ\text{C}$ and therefore were assayed at a maximum temperature of 24°C .

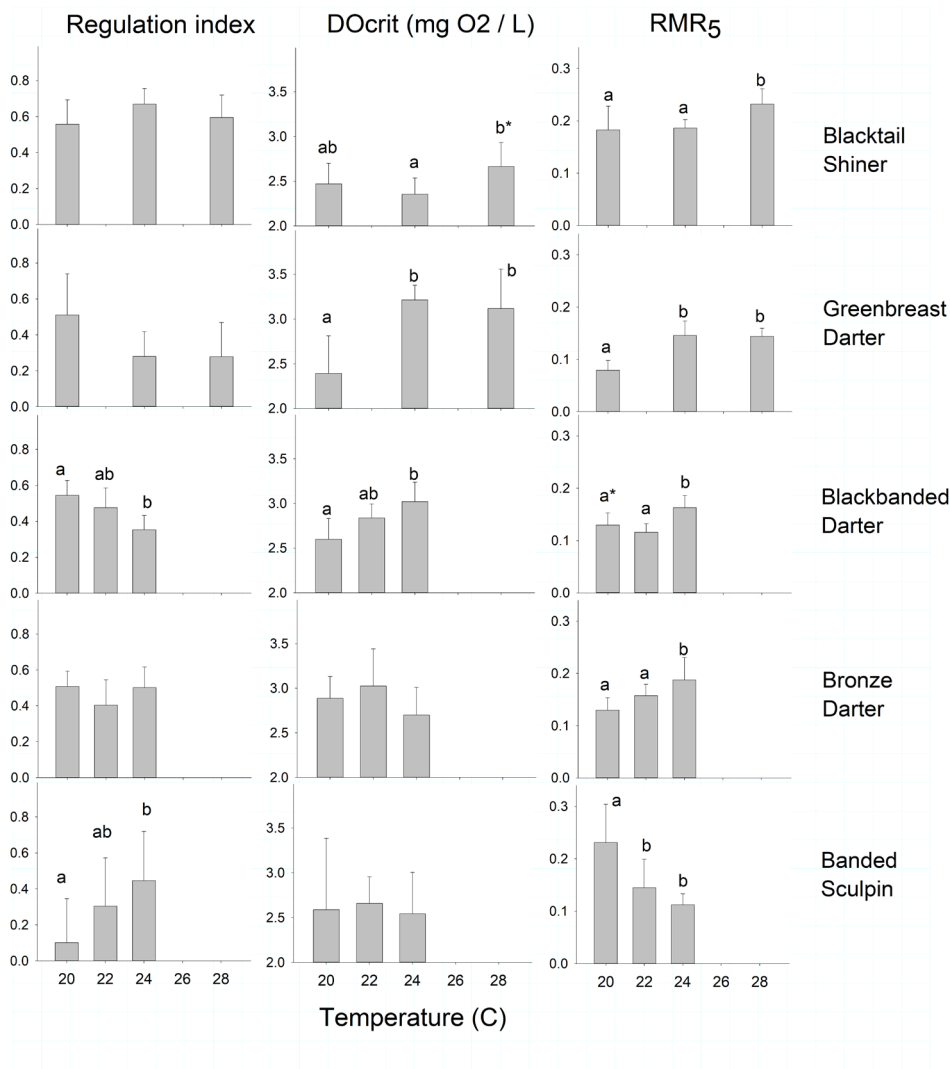


Figure 2. Regulation index (left panels), critical DO level (DOcrit; middle panels), and predicted MO_2 at 5 mg $O_2 \cdot L^{-1}$ (RMR_5) for five species of fish, each at three temperatures. Different letters within a panel indicate significant differences among temperatures. Letters with an asterisk indicate a marginally significant difference ($P = 0.059$). Error bars represent ± 1 SD.

Greenbreast Darter

The best fitting model for most greenbreast darter individual fish plots was a negative exponential regression, although two individuals exhibited a linear relationship between respiration rate and ambient DO (Table 1, Figure 1). No temperature by mass interaction was detected for greenbreast darter RI (Table 2). There were no differences in RI across temperatures ($F=2.49$; $df=2, 12$; $P=0.12$; Figure 2). No temperature by mass interaction was detected for greenbreast darter DO_{crit} (Table 2), with DO_{crit} at 24° and 28° C higher than at 20° C ($F=8.27$; $df=2, 10$; $P=0.0076$; Tukey's, $P=0.0074$ and $P=0.037$, respectively; Figure 2). There was no mass by temperature interaction detected for RMR_5 (Table 2), and greenbreast darter RMR_5 was higher at 24° and 28° C than at 20° C ($F=12.36$; $df=2, 12$; $P=0.0012$; Tukey's $P=0.0014$, $P=0.0044$, respectively; Figure 2).

Blackbanded Darter

The best fitting model for most blackbanded darter individual fish plots was a negative exponential model (Table 1, Figure 1). No temperature by mass interaction was detected for blackbanded darter RI (Table 2), and RI was lower at 24° C than at 20° C ($F=6.34$; $df=2, 13$; $P=0.012$; Tukey's, $P=0.010$; Figure 2). No temperature by mass interaction was detected for blackbanded darter DO_{crit} (Table 2), and DO_{crit} at 24° C was higher than at 20° C ($F=5.62$; $df=2, 3$; $P=0.017$; Tukey's, $P=0.013$; Figure 2). The temperature by mass interaction for blackbanded darter RMR_5 was not significant (Table 2), and RMR_5 was higher at 24° C than at 22° C and marginally higher at 24° C than at 20° C ($F=7.11$; $df=2, 13$; $P=0.0082$; Tukey's, $P=0.0079$, $P=0.059$; Figure 2).

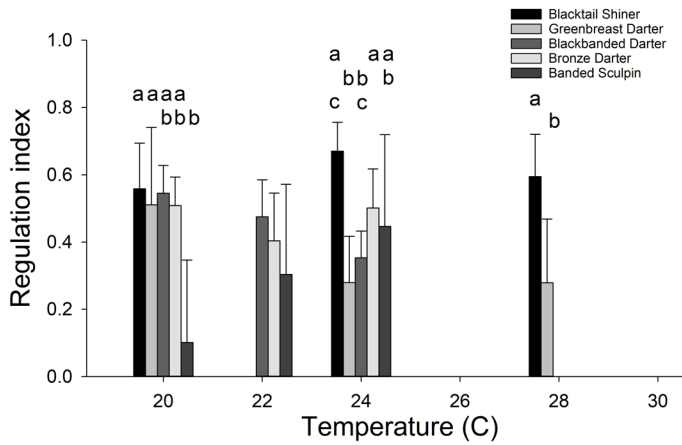


Figure 3. Regulation index for five species of fish, each run at three temperatures. Different letters above bars within a temperature indicate significant differences among species at that temperature. Error bars represent +1 SD.

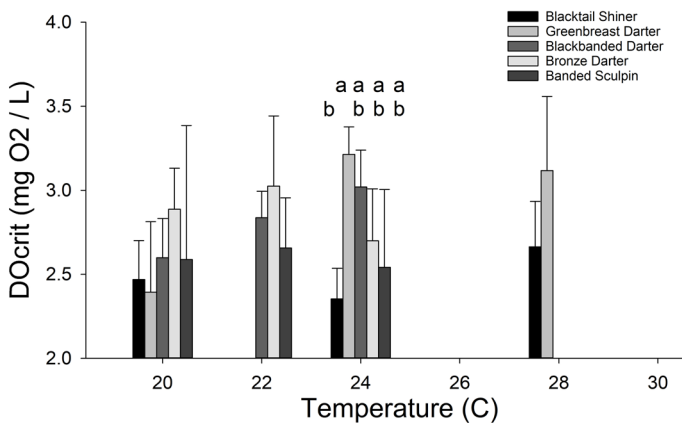


Figure 4. Critical DO level (DO_{crit}) for five species of fish, each run at three temperatures. Different letters above bars within a temperature indicate significant differences among species at that temperature. Error bars represent +1 SD.

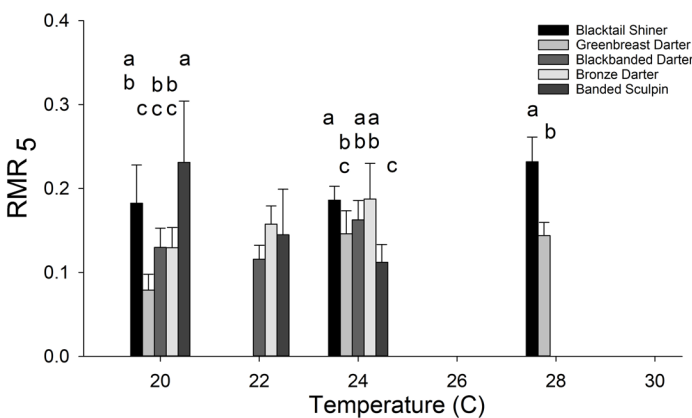


Figure 5. Predicted mass-specific oxygen consumption rate (MO_2) at $5 \text{ mg O}_2 \text{ L}^{-1}$ (RMR_5) for five species of fish, each run at three temperatures. Different letters above bars within a temperature indicate significant differences among species at that temperature. Error bars represent +1 SD.

Bronze Darter

The best fitting model for all but one of the bronze darter individual fish plots was a negative exponential regression (Table 1, Figure 1). No temperature by mass interaction was detected for RI or DO_{crit} models for bronze darter (Table 2). There were no differences across temperatures in the RI ($F=1.41$; $df=2, 15$; $P=0.27$; Figure 2) or DO_{crit} ($F=1.14$; $df=2, 15$; $P=0.35$; Figure 2). Bronze darter RMR_5 was influenced by temperature and temperature by mass interaction (Table 2), and increased with mass at 20°C , but decreased with increasing mass at 24°C . The RMR_5 was higher at 24°C than at 20° or 22°C (ANCOVA LSMEANS $P=0.003$ and $P=0.04$, respectively; Figure 2).

Banded Sculpin

At 20°C the best fitting model for most individual banded sculpin was a linear regression; whereas a negative exponential regression was the best fitting model at 22° and 24°C (Table 1, Figure 1). No temperature by mass interaction was detected for banded sculpin RI (Table 2), and RI was greater at 24°C than at 20°C ($F=3.55$; $df=2, 20$; $P=0.048$; Figure 2). Logistic regression confirmed that as temperature increased, the probability decreased that the RI for a banded sculpin did not differ significantly from 0 (i.e., they were oxyconforming) (LOGISTIC: $X^2=5.98$; $df=2$; $P=0.05$); the probability that RI did not differ from 0 was only significant at 20°C (LOGISTIC: $X^2=5.04$; $df=1$; $P=0.025$). No mass by temperature interaction was detected for DO_{crit} (Table 2), and DO_{crit} was similar across temperatures ($F=0.09$; $df=2, 10$; $P=0.92$; Figure 2). No temperature by mass interaction was detected for banded sculpin RMR_5 (Table 2). In contrast to the other species that were tested, banded sculpin RMR_5 decreased with increasing temperature with values at 24° and 22°C , being lower than at 20°C . ($F=9.77$; $df=2, 20$; $P=0.011$; Tukey's $P=0.017$, $P=0.0012$, respectively; Figure 2).

Among-Species Comparisons

At 20°C , respirometry was conducted for all five species. Banded sculpin exhibited a lower RI than blacktail shiner or greenbreast darter (Kruskal-Wallis: $H=13.5$, $P=0.009$; Dunn's: $P=0.012$, $P=0.044$, respectively), but no significant differences in RI were found for any other species comparisons (Figure 3). There were no significant differences in DO_{crit} among species at 20°C ($F=1.728$; $df=4, 21$; $P=0.181$; Figure 4). Banded sculpin had a higher RMR_5 than greenbreast darter, blackbanded darter, and bronze darter; and blacktail shiner had a higher RMR_5 than greenbreast darter ($F=9.721$; $df=4, 28$; Holm-Sidak: $P=0.001$, $P=0.007$, $P=0.011$, and $P=0.004$, respectively; Figure 5). No differences in RMR_5 were found for any other species comparison.

At 22°C , respirometry was conducted for blackbanded darter-

er, bronze darter, and banded sculpin, and RI (Kruskal-Wallis: $H=0.78$, $P=0.68$), DO_{crit} ($F=1.96$, $df=2, 17$, $P=0.17$) and RMR_5 ($F=2.42$, $df=2, 19$, $P=0.12$) were similar among these species (Figures 3, 4, 5).

At 24° C, respirometry was conducted for all five species. Bronze darter RI was higher than greenbreast darter and blackbanded darter RI (Kruskal-Wallis: $H=20.3$; $P < 0.001$; Dunn's $P=0.003$, $P=0.007$, respectively), and blacktail shiner had a higher RI than greenbreast darter (Dunn's $P=0.042$; Figure 3). Blacktail shiner had a lower DO_{crit} than greenbreast darter (Kruskal-Wallis: $H=16.71$, $P=0.002$; Dunn's: $P=0.003$) but was similar between other species pairs (all $P > 0.05$; Figure 4). Banded sculpin had a lower RMR_5 than blacktail shiner, blackbanded darter, and bronze darter ($F=9.49$, $df=4, 26$, $P < 0.001$; Holm Sidak: $P \leq 0.001$, $P \leq 0.012$, $P \leq 0.001$, respectively), and greenbreast darter had a lower RMR_5 than blacktail shiner (Holm Sidak; $P=0.046$; Figure 5).

At 28° C, respirometry was conducted for only two species. Blacktail shiner had a higher RI ($F=11.29$; $df=1, 9$; $P=0.008$; Tukey's: $P=0.009$), and RMR_5 than greenbreast darter ($F=30.12$; $df=1, 9$; $P < 0.001$), but DO_{crit} did not significantly differ between species ($F=4.17$; $df=1, 8$; $P=0.075$; Figures 3, 4, 5).

Discussion

It has been recognized that many organisms do not fit solely into either of the metabolic categories of oxyregulators or oxyconformers as once believed (Mueller and Seymour 2011). Our results support the idea that fish metabolic response to declining DO across a range of species represents a continuum rather than being categorized as one of two distinct responses. For example, blacktail shiner exhibited a classic regulatory pattern at 24° C, with a stable respiration rate above a clear DO_{crit} , whereas the majority of banded sculpin showed classic conformation patterns at 20° C. These were exceptions, however, as the patterns exhibited by study species across the range of temperatures spanned the range of intermediate patterns and RI values.

In general, the species evaluated did not show consistent differences in metabolic endpoints with increasing temperatures. Thus, one species could not be described as being a consistently better regulator than another as temperature increased. This lack of consistent difference was due largely to interspecific variation in metabolic response to warming temperatures. For example, as temperature increased, RI declined for blackbanded darter; was similar for blacktail shiner, greenbreast darter, and bronze darter; and increased with temperature for banded sculpin. Likewise, as temperatures increased, DO_{crit} increased for blacktail shiner, greenbreast darter, and blackbanded darter, but did not change for bronze darter and banded sculpin. Predicted RMR_5 decreased with

increasing temperature for banded sculpin, but increased with temperature for blacktail shiner, greenbreast darter, blackbanded darter, and bronze darter.

Strong interspecific variation in the trajectories of metabolic endpoints suggested strong interspecific variation in tolerance of declining oxygen availability with increasing water temperature. A decreased ability to regulate oxygen consumption and an increasing DO_{crit} have traditionally been considered indicators, though imperfect, of decreasing tolerance for low DO (Barnes et al. 2011, Rogers et al. 2016). The use of DO_{crit} alone as a metabolic endpoint to predict hypoxia tolerance has been recently called into question by Wood (2018), who suggests that RI is a more informative alternative to DO_{crit} . Considering the combination of metabolic endpoints measured, greenbreast darter and blackbanded darter would become increasingly intolerant of hypoxia under warming temperatures, exhibiting increasing oxygen demand, variable ability to regulate, and increasing DO_{crit} . Tolerance of blacktail shiner and bronze darter would remain fairly stable as oxygen demand increased due to an ability to maintain a stable RI with little to no increase in DO_{crit} .

Banded sculpin were unique among the suite of fish species we tested. Respiration rate of banded sculpin decreased with increasing temperature. Furthermore, the ability to regulate oxygen increased with increasing temperature while DO_{crit} did not increase. Taken together, these patterns would suggest that banded sculpin exhibited reduced stress with increasing temperature. However, this is not likely to be the case. Sculpins are a cool to coldwater species (Baltz et al. 1982, Brown 1989) and in this study, banded sculpin did not survive acclimation to 28° C.

It is likely that metabolic rates of banded sculpin respond differently to thermal stress at the range of temperatures tested than the other fish species in this study. Previous studies have found that rather than RMR increasing with increasing temperature, the RMR of various freshwater sculpin species either remains stable (Brown 1989) or declines (Walsh et al. 1997) as temperatures rise above a certain threshold, suggesting that sculpin have a maximum metabolic rate and that greatly elevated temperatures may cause metabolic depression in some species. Respiration declines at high temperature in other fish species have been shown to be linked to hypoxia and reduced activity rate in the test chambers (Crocker and Cech 1997). Metabolic depression at high temperatures has also been demonstrated for some invertebrates (e.g., Anestis et al. 2007), suggesting it can occur across a range of taxa (*sensu* Thornton and Lessem 1978).

Banded sculpin were also unusual in that they exhibited a strong pattern of oxygen conformation at 20° C as DO declined below 6 mg O₂ L⁻¹. Most teleost fish have been thought to be oxyregulators

(Ultsch et al. 1978, Perry et al. 2009), but some fish species such as the inanga (*Galaxias maculatus*) and the skilletfish (*Gobiesox strumosus*) are oxyconformers (Tiffany et al. 2010). It is possible that sculpin fall within this group at low temperatures and that the apparent increase in RI with increasing temperature was an artifact of metabolic depression at high temperatures as described previously. However, conformation for the inanga and skilletfish was hypothesized to be an adaptation for warm, hypoxic habitats, and both species are also thought to be capable of cutaneous breathing (Tiffany et al. 2010). In contrast, sculpin are typically found in cool, oxygenated waters, and not thought to be capable of cutaneous breathing. Further research is needed to determine why conformation is shared among these widely divergent species.

Management Implications

Results of this study supports the U.S. Environmental Protection Agency recognition that the warmwater fish DO criteria are based on a relatively small number of fish species and may not be sufficient to describe (and potentially protect) all warmwater species (USEPA 1986a, 1986b). In our study, five stream fish species, often coexisting in similar habitats, differed in the trajectory of the response of metabolic endpoints to warming temperatures approaching their upper lethal limit. This indicates differences among species in sublethal stress thresholds associated with increasing temperature and decreasing DO. Given that regulations involving minimum DO requirements of water released downstream of dams are typically based on temperature and DO requirements of a few focal species, they may not adequately reflect requirements of other ecologically important species. In certain cases where flow from impoundments is reduced, temperatures and DO concentrations could quickly exceed the tolerances of some stream species although still remaining within limits that are sufficient to protect other (including those few focal) species.

Obviously, it is not practical to assess oxygen tolerances of all warmwater species individually. Future research to determine factors (e.g., taxonomic groupings, habitat preferences, feeding guilds) that could accurately predict metabolic characteristics such as degree of oxygen conformation and DO_{crit} in response to increasing temperatures would be extremely valuable. Furthermore, additional research is needed to relate results of standard respirometry assays to hypoxia tolerance. For example, Tiffany et al. (2010) provide evidence that oxygen conformation in fish is an adaptation for tolerance of hypoxia while other studies (e.g., Barnes et al. 2011) use oxygen conformation as evidence for low tolerance of hypoxia. Similarly, a relatively low DO_{crit} is associated with increased hypoxia tolerance in some species (e.g., Speers-Roesch et al. 2012), while other hypoxia-tolerant species exhibit a relatively

high DO_{crit} (Scott et al. 2008). Results of our study support the contention that interspecific variation in metabolic responses to declining DO must be considered in setting water quality criteria.

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Literature Cited

- Aboagye, D. L. and P. J. Allen. 2013. Metabolic and locomotor responses of juvenile paddlefish *Polyodon spathula* to hypoxia and temperature. *Comparative Biochemistry and Physiology, Part A* 169:51–59.
- Anestis, A., A. Lazou, H. O. Pörtner, and B. Michaelidis. 2007. Behavioral, metabolic, and molecular stress responses of marine bivalve *Mytilus galloprovincialis* during long-term acclimation at increasing ambient temperature. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 293:911–921.
- Arend, K., D. Beletsky, J. DePinto, S. Ludsins, J. Roberts, D. Rucinski, D. Scavia, D. Schwab, and T. Hook. 2011. Seasonal and interannual effects of hypoxia on fish habitat quality in central Lake Erie. *Freshwater Biology* 56:366–383.
- Baltz, D. M., P. B. Moyle, and N. J. Knight. 1982. Competitive interactions between benthic stream fishes, riffle sculpin, *Cottus gulosus*, and speckled dace, *Rhinichthys osculus*. *Canadian Journal of Fisheries and Aquatic Sciences* 39:1502–1522.
- Barnes, R., H. King, and C. G. Carter. 2011. Hypoxia tolerance and oxygen regulation in Atlantic salmon, *Salmo salar* from a Tasmanian population. *Aquaculture* 318:397–401.
- Beamish, F. W. H. 1964. Influence of starvation on standard and routine oxygen consumption. *Transactions of the American Fisheries Society* 93:103–107.
- Boschung, H. T., Jr. and R. L. Mayden. 2004. *Fishes of Alabama*. Smithsonian Books, Washington, D.C.
- Brown, L. R. 1989. Temperature preferences and oxygen consumption of three species of sculpin (*Cottus*) from the Pit River drainage, California. *Environmental Biology of Fishes* 26:223–236.
- Cambray, J. A. and E. P. Pister. 2002. The role of scientists in creating public awareness for the conservation of fish species: African and American case studies. Pages 414–423 in M. J. Collares-Pereira, I. G. Cowx, and M. M.

- Coelho, editors. Conservation of freshwater fishes: options for the future. Blackwell Science, Oxford, U.K.
- Cech, J. J., Jr. 1990. Respirometry. Pages 334–362 in C.B. Schreck and P.B. Moyle, editors. Methods for fish biology. American Fisheries Society, Bethesda, Maryland.
- _____, C. G. Campagna, and S. J. Mitchell. 1979. Respiratory responses of largemouth bass (*Micropterus salmoides*) to environmental changes in temperature and dissolved oxygen. Transactions of the American Fisheries Society 108:166–171.
- Chabot, D., J. F. Steffensen, and A. P. Farrell. 2016. The determination of standard metabolic rate in fishes. Journal of Fish Biology 88:81–121.
- Clark, T. D., E. Sandholm, and F. Jutfelt. 2013. Aerobic scope measurements of fishes in an era of climate change: respirometry, relevance and recommendations. Journal of Experimental Biology 216:2771–2782.
- Clarke, A. and N. M. Johnston. 1999. Scaling of metabolic rate with body mass and temperature in teleost fish. Journal of Animal Ecology 68:893–905.
- Crocker, C. E. and J. J. Cech, Jr. 1997. Effects of hypoxia on oxygen consumption rate and swimming activity in juvenile white sturgeon, *Acipenser transmontanus*, in relation to temperature and life intervals. Environmental Biology of Fishes 50:383–389.
- DeVries, D. R. and R. A. Stein. 1990. Manipulating shad to enhance sport fisheries in North America: an assessment. North American Journal of Fisheries Management 10:209–223.
- Eliason, E. J. and A. P. Farrell. 2016. Oxygen uptake in Pacific salmon *Onchorhynchus* spp.: when ecology and physiology meet. Journal of Fish Biology 88:359–388.
- Evans, D. O. 1984. Temperature independence of the annual cycle of standard metabolism in the pumpkinseed. Transaction of the American Fisheries Society 113:494–512.
- Haag, W. R. and J. D. Williams. 2014. Biodiversity on the brink: an assessment of conservation strategies for North American freshwater mussels. Hydrobiologia 735:45–60.
- Hartline, N. R. 2013. Differences in oxygen consumption and critical oxygen levels of five stream fishes, Master's Thesis, Auburn University, Alabama.
- Hovel, R. A., D. A. Beauchamp, A. G. Hansen, and M. H. Sorel. 2015. Development of a bioenergetics model for the threespine stickleback. Transactions of the American Fisheries Society 144:1311–1321.
- Jobling, M. 1994. Fish Bioenergetics. Chapman and Hall, London, U.K.
- Mallekh, H. and J. P. Lagardere. 2002. Effect of temperature and dissolved oxygen concentration on the metabolic rate of the turbot and the relationship between metabolic scope and feeding demand. Journal of Fish Biology 60:1105–1115.
- McBryan, T. L., L. Antilla, T. M. Heally, and R. M. Schulte. 2013. Responses to temperature and hypoxia as interacting stressors in fish: implications for adaptation to environmental change. Integrative and Comparative Biology 53:648–659.
- Moss D. D. and D. C. Scott. 1961. Dissolved-oxygen requirements of three species of fish. Transactions of the American Fisheries Society 90:377–393.
- Mueller, C. A. and R. S. Seymour. 2011. The regulation index: a new method for assessing the relationship between oxygen consumption and environmental oxygen. Physiological and Biochemical Zoology 84:522–532.
- Perry, S., M. G. Jonz, and K. M. Gilmour. 2009. Oxygen sensing and the hypoxic ventilatory response. Pages 193–253 in J. G. Richards, A. P. Farrell, and C. J. Brauner, editors. Fish Physiology, Volume 27. Academic Press, New York, New York.
- Pörtner, H. O. 2010. Oxygen-and-capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. Journal of Experimental Biology 213:881–893.
- _____, N. Heisler, and M. K. Grieshaber. 1985. Oxygen consumption and modes of energy production in the intertidal worm *Sipunculus nudus* L: definition and characterization of the critical P_{O_2} for an oxyconformers. Respiration Physiology 59:361–377.
- Rogers, N. J., M. A. Urbina, E. E. Reardon, D. J. McKenzie, and R. W. Wilson. 2016. A new analysis of hypoxia tolerance in fishes using a database of critical oxygen level (P_{crit}). Conservation Physiology 4:1–19.
- SAS Institute. 2012. SAS user's guide: statistics, version 12.1 edition. SAS Institute, Inc., Cary, North Carolina.
- Scott, G. R., C. M. Wood, K. A. Sloman, F. I. Iftikar, G. D. Boeck, V. M. F. Almeida-Val, and A. L. Val. 2008. Respiratory responses to progressive hypoxia in the Amazonian oscar, *Astronotus ocellatus*. Respiratory Physiology and Neurobiology 162:109–116.
- Speers-Roesch, B., J. G. Richards, C. J. Brauner, A. P. Farrell, A. J. R. Hickey, Y. S. Wang, and G. M. C. Renshaw. 2012. Hypoxia tolerance in elasmobranchs. I. Critical oxygen tension as a measure of blood oxygen transport during hypoxia exposure. The Journal of Experimental Biology 215:93–102.
- Thornton, K. W. and A. S. Lessem. 1978. A temperature algorithm for modifying biological rates. Transactions of the American Fisheries Society 107:284–287.
- Tiffany, B. N., L. A. Enzor, and W. A. Bennett. 2010. Responses of skiffetfish *Gobiosox strumosus* to high temperature and low oxygen stress. Journal of Fish Biology 76:556–563.
- Ultsch, G.R., H. Boschung, and M.J. Ross. 1978. Metabolism, critical oxygen tension, and habitat selection in darters (*Etheostoma*). Ecology 59:99–107.
- U.S. Environmental Protection Agency (USEPA). 1986a. Ambient water quality criteria for dissolved oxygen. EPA 440/5-86-003, Washington, D.C.
- _____. 1986b. Quality criteria for water. EPA 440/5-86-001, Washington, D.C.
- Valverde, J., F. Lopez, and B. Garcia. 2006. Oxygen consumption and ventilator frequency responses to gradual hypoxia in common dentex (*Dentex dentex*): basis for suitable oxygen level estimations. Aquaculture 256:542–551.
- Voutilainen, A., E. Seppanen, and H. Huuskonen. 2011. A methodological approach to measuring the oxygen consumption profile of six freshwater fish species: implications for determination of the standard metabolic rate. Marine and Freshwater Behaviour and Physiology 44:239–250.
- Walsh, S. J., D. C. Haney, and C. M. Timmerman. 1997. Variation in thermal tolerance and routine metabolism among spring- and stream-dwelling freshwater sculpins (Teleostei: Cottidae) of the southeastern United States. Ecology of Freshwater Fish 6:84–94.
- Williams, J. D., M. L. Warren, K. S. Cummings, J. L. Harris, and R. J. Neves. 1993. Conservation status of the freshwater mussels of the United States and Canada. Fisheries 18(9):6–22.
- Wood, C. M. 2018. The fallacy of P_{crit} are there more useful alternatives? Journal of Experimental Biology 221(22):1–9.