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METHODS AND TECHNIQUES

Standardizing the determination and interpretation of P_{crit} in fishes

Jessica E. Reemeyer and Bernard B. Rees*

ABSTRACT

The critical oxygen tension (P_{crit}) for fishes is the oxygen level below which the rate of oxygen consumption ($\dot{M}_{O_2}$) becomes dependent upon ambient oxygen partial pressure (P_{O_2}). We compare multiple curve-fitting approaches to estimate P_{crit} of the Gulf killifish, *Fundulus grandis*, during closed and intermittent-flow respirometry. Fitting two line segments of $\dot{M}_{O_2}$ versus P_{O_2} produced high and variable estimates of P_{crit}, as did nonlinear regression using a hyperbolic (Michaelis–Menten) function. Using nonlinear regression fit to an exponential (modified Weibull) function, or linear regression of $\dot{M}_{O_2}$ versus P_{O_2} at low P_{O_2}, and determining P_{crit} as the P_{O_2} when $\dot{M}_{O_2}$ equals standard metabolic rate (SMR) yielded values that were consistent across fish and among experimental trials. The magnitude of the difference in P_{crit} determined by alternative calculation methods exceeded the differences determined in closed and intermittent-flow respirometry, highlighting the need to standardize analytical as well as experimental approaches in determining P_{crit}.

KEY WORDS: Critical oxygen tension, Oxygen consumption, Aerobic metabolism, *Fundulus grandis*, Killifish, Hypoxia

INTRODUCTION

There is considerable interest in describing the oxygen dependence of aerobic metabolism of animals, especially for animals from aquatic habitats, where the oxygen concentration is much lower and more variable than in terrestrial habitats. Determination of this oxygen dependence is particularly relevant in the current context of human-induced environmental change, where increased nutrient input, warmer temperatures and changes in hydrology have increased the geographic scope and severity of aquatic hypoxia (Diaz and Rosenberg, 2008; Rabalais et al., 2010).

Perhaps the most common metric of the oxygen dependence of aerobic metabolism is the critical oxygen tension, P_{crit}. For animals, including most vertebrates, that can regulate aerobic metabolism over a broad range of oxygen levels (i.e. oxy-regulators), P_{crit} represents the oxygen partial pressure (P_{O_2}) at which the rate of oxygen consumption ($\dot{M}_{O_2}$) switches from being independent to being dependent on P_{O_2} with further decreases in ambient oxygen (Ultsch et al., 1981; Farrell and Richards, 2009; Rogers et al., 2016). P_{crit} has also been defined as the P_{O_2} below which an animal's basic metabolic needs, i.e. standard metabolic rate (SMR) in fishes, can no longer be sustained aerobically (Schurmann and Steffensen, 1997; Claireaux and Chabot, 2016; Pan et al., 2016; Snyder et al., 2016). This level of oxygen was originally described by Fry and Hart (1948) as the 'level of no excess activity'. Although related, these two concepts of P_{crit} differ: the former refers to an inflection point as $\dot{M}_{O_2}$ transitions between regulation and conformity, which depends upon the intensity of metabolism (Rogers et al., 2016; Wood, 2018), whereas the latter applies to the level of oxygen that limits a specific metabolic state (Claireaux and Chabot, 2016).

Recently, Wood (2018) questioned the usefulness of the P_{crit} concept based on two main concerns: uncertainty of its biological meaning and lack of standardization in its determination. The purpose of the present study is not to argue the biological relevance of P_{crit}, as this concern has been addressed (Regan et al., 2019). Rather, we aim to evaluate analytical methods used to determine P_{crit} from respirometric data. Traditionally, P_{crit} has been estimated as the intersection of two straight lines, one fit to a region where $\dot{M}_{O_2}$ is relatively independent of P_{O_2}, and a second describing the decrease in $\dot{M}_{O_2}$ at low P_{O_2} (Yeager and Ultsch, 1989; Rogers et al., 2016). Because respirometric data rarely conform neatly to two straight lines across a broad range of P_{O_2}, alternative linear or nonlinear regression solutions to determine P_{crit} have been proposed (Marshall et al., 2013; Claireaux and Chabot, 2016; Cobbs and Alexander, 2018).

Here, we measured $\dot{M}_{O_2}$ as a function of P_{O_2} in closed and intermittent-flow respirometry with the Gulf killifish, *Fundulus grandis* Baird & Girard 1853, and applied multiple curve-fitting methods to estimate P_{crit}. Based upon our results, we recommend that P_{crit} be determined as the P_{O_2} at which $\dot{M}_{O_2}$ drops below SMR using linear regression of $\dot{M}_{O_2}$ versus P_{O_2} at decreasing P_{O_2} (Claireaux and Chabot, 2016). For this method to be general and reproducible, it is imperative that SMR be accurately determined by standardized methods (Chabot et al., 2016).

MATERIALS AND METHODS

Animals

Adult male *F. grandis* (n=11; mass=5.4–16.2 g) were purchased from local bait shops in the summer of 2018 and housed at The University of New Orleans under a 12 h:12 h (light:dark) photoperiod in aerated, filtered one-third strength seawater (salinity≈10) at ∼27°C. Fish were fed an amount of flake fish food equal to 1–1.5% of their body mass once per day. Fish were identified by unique passive integrated transponder (PIT) tags or housed individually. There were no differences in any metabolic variable between PIT-tagged and individually housed fish (Reemeyer et al., 2019; J.E.R., unpublished observations). Fish were maintained under these conditions for at least 1 month before experiments. Fish were starved for 24 h prior to respirometry. All procedures were approved by The University of New Orleans Institutional Animal Care and Use Committee (protocol no. 18-006).

Respirometry

$\dot{M}_{O_2}$ of each fish was determined in a sequence of three respirometry trials, described in detail below. Trials 1 and 2 employed intermittent-flow respirometry to estimate SMR and routine metabolic rate (RMR) (Svendsen et al., 2016; Reemeyer et al., 2019), followed by closed respirometry to estimate P_{crit}. In trial 3,

Department of Biological Sciences, University of New Orleans, New Orleans, LA 70148, USA.

*Author for correspondence (brees@uno.edu)

J.E.R., 0000-0002-0081-2573; B.B.R., 0000-0001-5636-1700

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List of symbols and abbreviations

BSR	broken stick regression
MLND	mean of the lowest normal distribution
MM	Michaelis–Menten function
$\dot{M}_{O_2}$	rate of oxygen consumption
LLO	linear function of $\dot{M}_{O_2}$ measured at low P_{O_2}
LMM	linear mixed model
low10	mean of lowest 10 data points
low10pc	mean of lowest 10% of data after removing the lowest 5 values
P_{crit}	critical oxygen tension
P_{O_2}	oxygen partial pressure
q	quantile
RMR	routine metabolic rate
SMR	standard metabolic rate
W	Weibull function

P_{crit} was determined by intermittent-flow respirometry. SMR and RMR were not determined in this trial because there were a limited number of $\dot{M}_{O_2}$ measurements at P_{O_2}>85% air saturation (see below). Trials were separated by approximately 1 week and they were performed at 27.0±0.5°C in one-third strength seawater.

For trials 1 and 2, fish were weighed (to the nearest 0.01 g) and placed into respirometry chambers between 14:00 and 15:00 h. For the first hour, the following intermittent-flow respirometry protocol was used: 60 s flush, 30 s wait and 120 s $\dot{M}_{O_2}$ measurement. At that point, the protocol was adjusted to 300 s flush, 60 s wait and 240 s $\dot{M}_{O_2}$ measurement, which was continued for approximately 14 h. Throughout the combined ∼15 h period, P_{O_2} was maintained at >85% of the air-saturated value. At 06:00 h the following morning, the flush pumps were turned off. At that point, the chambers, recirculating pumps and oxygen sensors formed closed systems, and the P_{O_2} declined due to $\dot{M}_{O_2}$ by the fish. During the closed period, $\dot{M}_{O_2}$ was measured over consecutive 60 s intervals until the fish were unable to maintain equilibrium for ≥3 s. At that point, the flush pumps were turned on to reoxygenate the chambers. The total time the chambers remained closed ranged from 45 to 108 min. All fish recovered upon reoxygenation, whereupon they were returned to their holding tank.

For trial 3, fish were weighed (to the nearest 0.01 g) and placed in respirometry chambers between 15:00 and 16:00 h. Chambers were flushed continuously with well-aerated water (>95% air saturation) until 21:00 h. At that time, the P_{O_2} was stepped down at 1 h intervals by introducing nitrogen gas via a computer-controlled solenoid valve. Target values of P_{O_2} were 20.75, 13.07, 8.30, 5.19, 3.32 and 2.07 kPa. Over the last 30 min at each P_{O_2}, $\dot{M}_{O_2}$ was measured in three cycles of 300 s flush, 60 s wait and 240 s measurement. Runs ended around 03:00 h, after which the water was reoxygenated with air. After 30 min recovery, fish were returned to their holding tanks. Importantly, all P_{crit} determinations were done during the dark phase of the photoperiod. The only illumination was that required to operate the computer (e.g. to start a closed respirometry trial or to activate nitrogen gassing in the intermittent-flow trial), from which fish chambers were shielded.

$\dot{M}_{O_2}$ due to microbial respiration was measured for each chamber before and after each respirometry run. It was less than 6% of the average SMR of fish and independent of P_{O_2} across the range used. Thus, the $\dot{M}_{O_2}$ by each fish in each trial was corrected by subtracting a time-weighted value for background respiration (Reemeyer et al., 2019; Rosewarne et al., 2016). After background correction, $\dot{M}_{O_2}$ by fish was determined as μmol min^{-1} g^{-1} using standard equations (Svendsen et al., 2016). Oxygen concentrations were corrected for salinity, barometric pressure and temperature.

SMR and RMR determination

We evaluated seven methods of estimating SMR (Chabot et al., 2016) using $\dot{M}_{O_2}$ data collected between 20:00 and 06:00 h in trials 1 and 2, corresponding to 60 $\dot{M}_{O_2}$ measurements per fish per trial: (1) the mean of the lowest 10 data points (low10); (2) the mean of the lowest 10% of the data, after removing the five lowest values (low10pc); (3–6) quantiles that place SMR above the lowest 10–25% of the observations ($q_{0.1}$, $q_{0.15}$, $q_{0.2}$, $q_{0.25}$); and (7) the mean of the lowest normal distribution (MLND). SMR estimated by low10 was lowest, although not statistically different from low10pc, $q_{0.1}$, $q_{0.15}$ or $q_{0.2}$ (Table S1). In instances when cellular metabolism and gas exchange are not in steady state (e.g. hypoventilation), reliance upon a too few $\dot{M}_{O_2}$ measurements may lead to underestimation of SMR. This concern is greatest when averaging the lowest values (low10) and it is alleviated by methods that exclude outliers (low10pc) or are based upon quantiles. Another criterion in evaluating SMR calculation methods is whether the estimated value of SMR agrees with visual inspection of the raw data (Chabot et al., 2016). SMR values estimated by $q_{0.2}$ and $q_{0.25}$ best agreed with the distribution of $\dot{M}_{O_2}$ from more runs than any other estimate. The analytical method should also be reproducible when applied to data generated from multiple experimental runs with the same fish. SMR determined as low10pc, $q_{0.15}$ and $q_{0.2}$ were more highly correlated between trials 1 and 2 (Pearson's r>0.80) than SMR determined by other methods (Pearson's r<0.80). As a final test of the robustness of SMR determination, we pooled all the data from 22 runs on 11 fish to generate a frequency distribution of 1320 $\dot{M}_{O_2}$ values and then randomly sampled from this distribution to generate 1000 sets of 60 $\dot{M}_{O_2}$ data points (as in the experimental runs). When SMR was calculated from these randomly generated datasets, $q_{0.2}$ and $q_{0.25}$ produced the fewest statistical outliers (Fig. S1). Only the $q_{0.2}$ approach satisfied all the criteria: it generated a low estimate of SMR without undue influence by potentially spurious low values; it agreed with the distribution of raw $\dot{M}_{O_2}$ data; it was reproducible in repeated runs with the same fish; and it produced consistent values when applied to randomly generated datasets. Therefore, SMR determined by this approach was used for the remainder of these analyses. We also calculated RMR, which includes spontaneous, uncontrolled activity in an otherwise quiet, post-absorptive fish, by taking the average of all 60 $\dot{M}_{O_2}$ values collected between 20:00 and 06:00 h.

P_{crit} determination

We compared the following curve-fitting methods to describe $\dot{M}_{O_2}$ as a function of P_{O_2}: broken stick regression (BSR); nonlinear regression fit to a hyperbolic function, analogous to the Michalis–Menten equation (MM); nonlinear regression fit to an exponential function, the Weibull function (W); and a linear function of $\dot{M}_{O_2}$ measured at low P_{O_2} (LLO). BSR was performed using the Segmented package in R (Muggeo, 2003). The nls() function of the base R package (https://www.r-project.org/) was used to fit data to the MM and W functions. The MM function has the general form:

$$\dot{M}_{O_2} = \frac{aP_{O_2}}{b + P_{O_2}}, \quad (1)$$

where $\dot{M}_{O_2}$ is metabolic rate, P_{O_2} is oxygen tension, and a and b are constants (V_{max} and K_M, respectively, when applied to enzyme

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kinetics). The W function is:

$$\dot{M}_{O_2} = a\left(1 - e^{-\left(\frac{P_{O_2}}{b}\right)^c}\right) + d, \quad (2)$$

where $\dot{M}_{O_2}$ is metabolic rate, P_{O_2} is oxygen tension, a, b, c and d are constants, and e is the natural base. In preliminary analyses, the W function failed to converge for five of 11 runs of intermittent-flow respirometry (trial 3). Setting c=1 allowed the function to converge in all cases without appreciably affecting results from closed respirometry (trials 1 and 2). Thus, we set c=1 for all fits to the W function. As such, this function is analogous to the 'exponential rise to a maximum' function used by Bilberg et al. (2010) with the addition of an intercept (d). Because neither the MM nor W functions have a parameter equivalent to P_{crit}, we used the derived equations to determine the P_{O_2} at which $\dot{M}_{O_2}$ equaled SMR for each fish. The last method (LLO) used the lm() function of the R base package to fit a linear relationship between $\dot{M}_{O_2}$ and P_{O_2} to data collected after $\dot{M}_{O_2}$ fell below and remained below that individual's SMR. From this relationship, we determined P_{crit} as the P_{O_2} at which $\dot{M}_{O_2}$ equals SMR for that fish.

Importantly, SMR and RMR were determined during a previous overnight (~10 h) intermittent-flow respirometry experiment, rather than from $\dot{M}_{O_2}$ determined during the P_{crit} measurement, when fish might become agitated and display increased $\dot{M}_{O_2}$. In addition, BSR, MM and W used all the $\dot{M}_{O_2}$ data collected during a given experimental run without subjective data elimination; LLO used only a subset of data determined below the P_{O_2} when $\dot{M}_{O_2}$ fell and remained below SMR. Because SMR and RMR were not determined during trial 3 (see above), the mean SMR or RMR from trials 1 and 2 was used for P_{crit} determination by the MM, W and LLO methods in trial 3. For all methods, the P_{O_2} for a given $\dot{M}_{O_2}$ was calculated as the mean P_{O_2} over the measurement period (1 min

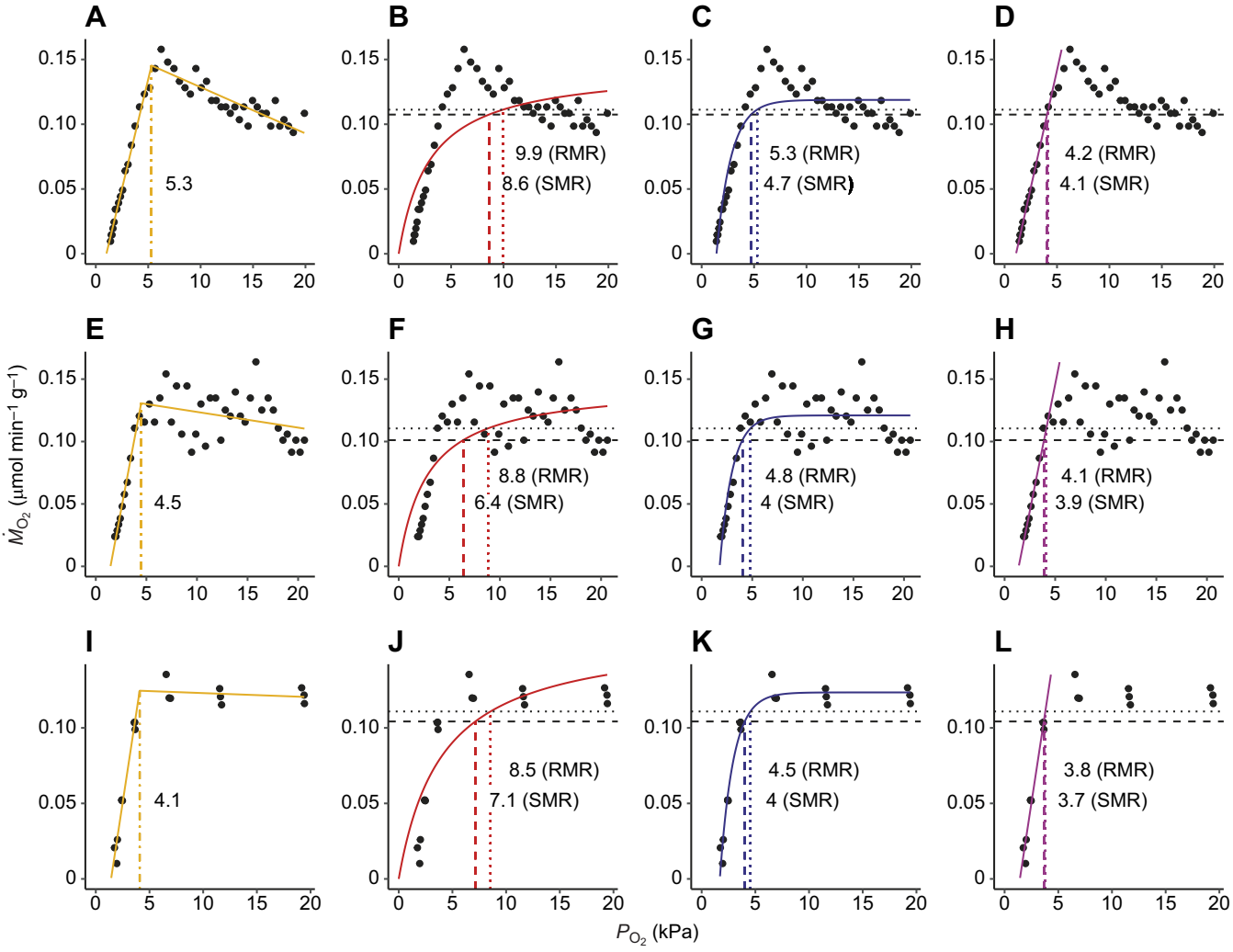

Fig. 1. Model fits of each P_{crit} calculation method for a single *Fundulus grandis* used in three respirometry trials. Each row represents one experimental run: (A–D) trial 1 (closed respirometry); (E–H) trial 2 (closed respirometry); (I–L) trial 3 (intermittent-flow respirometry). Each column represents one P_{crit} calculation method: (A,E,I) broken stick regression (BSR), where two linear segments were fit to the data (solid orange lines) and P_{crit} is the P_{O_2} at their intersection (dashed orange line); (B,F,J) nonlinear regression using the Michaelis–Menten (MM) function (solid red line), and P_{crit} is the P_{O_2} when $\dot{M}_{O_2}$ equals standard metabolic rate (SMR; dashed red line); (C,G,K) nonlinear regression using the (W) function (solid blue line) and P_{crit} is the P_{O_2} when $\dot{M}_{O_2}$ equals SMR (dashed blue line); (D,H,L) linear regression of $\dot{M}_{O_2}$ versus P_{O_2} at $\dot{M}_{O_2}$≤SMR (LLO method, solid purple line) and P_{crit} is the P_{O_2} when $\dot{M}_{O_2}$ equals SMR (dashed purple line). For comparison, P_{crit} values determined using routine metabolic rate (RMR) are shown for the MM, W and LLO methods (dotted lines). P_{crit} estimates are shown in the respective panels.

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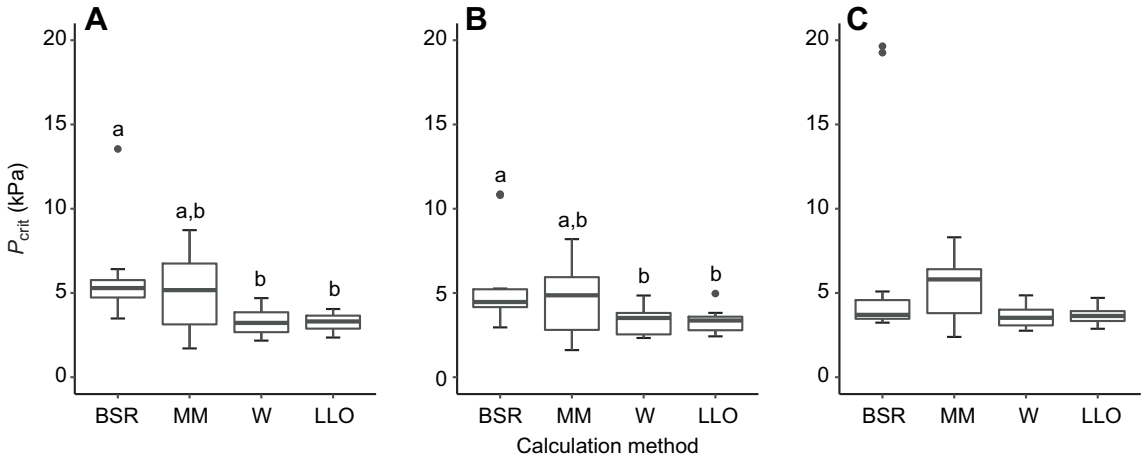

Fig. 2. P_{crit} estimated by different analytical methods for *Fundulus grandis*. (A,B) Closed and (C) intermittent-flow respirometry. For the MM, W and LLO methods, P_{crit} was determined as the P_{O_2} at $\dot{M}_{O_2}$ equals SMR. For each box, the center line indicates median, boundaries are upper and lower quartiles, and whiskers are the full data range without outliers. Outliers, defined as being 1.5 times the interquartile range outside of the box, are shown for graphical purposes (solid circles). All P_{crit} estimates were included in statistical comparisons of analytical methods (see Table 1). Calculation methods with different letters yielded significantly different P_{crit} within a trial (*t*-test, $P<0.05$, false discovery corrected). Sample sizes=11.

for closed respirometry; 4 min for intermittent-flow respirometry). Data for a representative fish, along with the methods for determining P_{crit}, are shown in Fig. 1.

Statistics

All statistical analyses were performed in R v3.3.3 (https://www.r-project.org/). The effects of analytical method (i.e. method used to calculate SMR or P_{crit}) were determined within a given trial using linear mixed models (LMM) with analytical method as a fixed factor and fish as a random factor. LMMs were fit using the lmer() function of the lme4 package (Bates et al., 2014) with *P*-values generated by the lmerTest package (Kuznetsova et al., 2017). All possible *post hoc* pairwise comparisons were made with *t*-tests on model fit means and employed *P*-values adjusted for false discovery (Benjamini and Hochberg, 1995) using the emmeans package in R (https://CRAN.R-project.org/package=emmeans). Paired *t*-tests were used to compare P_{crit} values based upon SMR and RMR within the MM, W and LLO methods. The effects of respirometry method (closed versus intermittent flow) on the value of P_{crit} determined by a given analytical method were evaluated with LMM with respirometry method as a fixed factor and fish as a random factor. Correlations of values determined by a single analytical method in different respirometry trials were evaluated with Pearson's correlation coefficient (*r*). Variation in body size was accounted for by including fish as a random factor in our statistical models, or by comparing values for a given fish across trials or analytical technique. Therefore, body mass was not included as a variable in these analyses. Data and R script used in this study are available at figshare.com (https://doi.org/10.6084/m9.figshare.8869253.v1).

RESULTS AND DISCUSSION

Models used to estimate P_{crit}

The pattern of $\dot{M}_{O_2}$ versus P_{O_2} among fishes and other aquatic vertebrates has traditionally been modeled by the intersection of two straight lines (Yeager and Ultsch, 1989). In the present study, P_{crit} values estimated by BSR were among the highest and most variable estimates, including at least one value >10 kPa (50% air saturation) in each respirometry trial (Fig. 2, Table 1). In addition, P_{crit} values estimated by BSR were poorly reproducible between respirometry trials conducted with the same individuals under identical (closed respirometry) conditions, as well as between closed and intermittent-flow respirometry (Table S2). These results are likely due to the variability of $\dot{M}_{O_2}$ at levels of P_{O_2} that do not limit oxygen uptake (i.e. at $P_{O_2}>P_{crit}$), as well as the tendency in some individuals for $\dot{M}_{O_2}$ to increase as P_{O_2} decreased from 20 to 5 kPa, resulting in a poor linear fit of $\dot{M}_{O_2}$ data at high P_{O_2} and influencing the intersection of two line segments. This variability occurred even though P_{crit} measurements were made after >24 h fasting, after 8–12 h since transferring fish to the respirometer, and during the dark phase of the photoperiod, when this species is less active. Owing to the variability of $\dot{M}_{O_2}$ at high P_{O_2}, the use of BSR is frequently coupled with removal of $\dot{M}_{O_2}$ data points that fail to meet certain criteria (see Claireaux and Chabot, 2016 and Wood, 2018 for examples). This practice has raised concern over the rationale and validity of applying data selection criteria (Claireaux and Chabot, 2016; Wood, 2018). In addition, direct comparisons of BSR with various nonlinear regression approaches have shown that BSR is seldom the best model to fit $\dot{M}_{O_2}$ data across a range of P_{O_2} (Marshall et al., 2013; Cobbs and Alexander, 2018). In a recent meta-analysis,

Table 1. Comparison of analytical method and respirometry format in the determination of P_{crit} (kPa; means±s.d.) of the Gulf killifish, *Fundulus grandis*

	Trial 1 (closed)	Trial 2 (closed)	Trial 3 (intermittent flow)
BSR	5.8±2.7^{a}	5.5±2.6^{a}	6.6±6.4
MM (SMR)	4.9±2.3^{a,b}	4.5±2.1^{a,b}	5.3±1.9
W (SMR)	3.3±0.8^{b}	3.3±0.9^{b}	3.6±0.7
LLO (SMR)	3.3±0.6^{b}	3.3±0.7^{b}	3.7±0.5*
MM (RMR)	8.5±4.6	9.9±5.4	8.2±2.7
W (RMR)	4.5±1.4	4.5±1.0	5.1±1.5
LLO (RMR)	3.6±0.7	3.8±0.7	4.3±0.7*

BSR, broken stick regression; MM, Michaelis–Menten; W, Weibull function; LLO, linear function of $\dot{M}_{O_2}$ measured at low P_{O_2}; SMR, standard metabolic rate; RMR, routine metabolic rate. Sample size=11. Means with different superscript letters are significantly different within a trial (*t*-test, $P<0.05$, false discovery corrected). For MM, W and LLO functions, P_{crit} estimates based upon RMR were significantly higher than estimates based upon SMR (paired *t*-test, $P<0.05$). *Significantly higher than values determined by the LLO method during closed respirometry (linear mixed model, $P<0.05$).

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BSR was the best model in only one of 68 datasets fit with various statistical models (Cobbs and Alexander, 2018).

With the advent and accessibility of nonlinear regression methods, it is possible to fit a variety of nonlinear functions to $\dot{M}_{O_2}$ data. Here, we focused on two nonlinear models, a hyperbolic function, analogous to the Michaelis–Menten equation for enzyme kinetics, and an exponential function, the Weibull function. Although the relationship between $\dot{M}_{O_2}$ and P_{O_2} in biological material as diverse as mitochondria to fishes can be hyperbolic (Tang, 1933; Gnaiger, 1993; Marshall et al., 2013), $\dot{M}_{O_2}$ by *F. grandis* was poorly described by a hyperbolic function (Fig. 1). In contrast, the W function generally fit the $\dot{M}_{O_2}$ data well, especially at low P_{O_2} (Fig. 1). This observation agrees with Marshall et al. (2013), who found that the W function fit respirometric data better than other nonlinear functions, including the MM function. Neither the MM nor W functions, however, have a parameter equivalent to P_{crit}. For the MM function, the parameter b is the P_{O_2} when $\dot{M}_{O_2}$ is half of the extrapolated maximum $\dot{M}_{O_2}$ in that run. In the earliest attempts to model respirometric data with a hyperbolic function, however, there was no reliable, quantitative relationship between b and P_{crit} (Tang, 1933). Also, it is not clear that this parameter has any meaning when applied to whole-animal $\dot{M}_{O_2}$, unlike its meaning in enzyme kinetics (Regan et al., 2019). Marshall et al. (2013) suggested that P_{crit} of a nonlinear function be estimated as the P_{O_2} at which the slope of the function approaches zero. In their analysis, the value of 0.065 was chosen as the slope giving a P_{O_2} that 'best approximates P_{crit}'. This is a circular argument and requires prior knowledge of P_{crit}, presumably based upon BSR.

Alternatives to inflection points to determine P_{crit}

Rather than estimate an inflection point, we used the derived MM and W equations to determine the P_{O_2} at which $\dot{M}_{O_2}$ equaled SMR for each fish. Other studies have similarly determined P_{crit} as the value of P_{O_2} when $\dot{M}_{O_2}$ equals SMR based upon linear or nonlinear functions (Schurmann and Steffensen, 1997; Bilberg et al., 2010; Thuy et al., 2010; Snyder et al., 2016; Claireaux and Chabot, 2016). For *F. grandis*, using the MM function to estimate the P_{O_2} when $\dot{M}_{O_2}$ equals SMR resulted in high and variable estimates of P_{crit} (Figs 1, 2, Table 1), owing to the poor fit of the data to the hyperbolic relationship. In contrast, using the W function yielded values of P_{crit} that were reproducible within and among trials (Figs 1, 2, Table 1). At low P_{O_2}, the decline in $\dot{M}_{O_2}$ by *F. grandis* was essentially a linear function of ambient oxygen, during both closed and intermittent-flow respirometry (Fig. 1), as it is for numerous fish species (Schurmann and Steffensen, 1997; Thuy et al., 2010; Pan et al., 2016; Snyder et al., 2016; Wong et al., 2018). When P_{crit} was determined as the value of P_{O_2} when $\dot{M}_{O_2}$ equals SMR using linear regression of $\dot{M}_{O_2}$ versus P_{O_2} at low P_{O_2} (LLO method), values were similar to those generated by the W method (Fig. 2, Table 1), reproducible for a given respirometry format (closed respirometry, Table S2), and agreed with previously published values for *F. grandis* (Virani and Rees, 2000). This method is also straightforward and easy to implement, given that SMR is accurately determined.

With respect to the value of $\dot{M}_{O_2}$ to use to solve for P_{crit}, we and others advocate the use of SMR (Claireaux and Chabot, 2016). If oxygen drops below this level, the fish cannot sustain its minimal metabolic requirements via aerobic metabolism, thus representing a clear physiological limitation. Among fishes, however, RMR is more commonly used to determine P_{crit} (Rogers et al., 2016). This metabolic state includes routine, spontaneous activity, which has been argued to be more ecologically relevant than SMR (Fry and Hart, 1948; Rogers et al., 2016; Wood, 2018). Thus, we also determined P_{crit} based upon RMR using the MM, W and LLO functions (Fig. 1). As expected, estimates of P_{crit} based upon RMR were significantly higher and more variable than those based upon SMR using all calculation methods (paired t-tests, $P<0.05$; Table 1). Because RMR includes an uncontrolled and usually undetermined level of activity, behavioral differences among individuals or species may confound comparisons of P_{crit} based upon RMR, and potentially obscure fundamental differences in oxygen extraction capacity. Indeed, Wong et al. (2018) found differences in P_{crit} among multiple species of triggerfishes when using SMR to calculate P_{crit}, but not when using RMR.

Recommendations

Based upon our results with *F. grandis* and the foregoing discussion, we propose that P_{crit} be defined as the P_{O_2} at which $\dot{M}_{O_2}$ equals SMR during declining ambient P_{O_2}. This recommendation requires that SMR be determined with high accuracy and using robust analytical techniques that yield a value that is insensitive to occasional low outliers, agrees with the distribution of raw $\dot{M}_{O_2}$ data, and is reproducible across multiple trials (Chabot et al., 2016). In the current experiments, the $q_{0.2}$ method satisfied these criteria. Once SMR is determined, P_{crit} may then be determined in a continuation of the same experiment or in a different experiment if SMR is repeatable over time (Reemeyer et al., 2019). We recommend that P_{crit} be estimated as the P_{O_2} at which $\dot{M}_{O_2}$ equals SMR based upon a linear relationship of $\dot{M}_{O_2}$ and P_{O_2} at low P_{O_2} (i.e. the LLO method). Using an exponential function (the W function setting c=1) yielded comparable results and may provide better fits for species where the relationship between $\dot{M}_{O_2}$ and P_{O_2} is not linear at low oxygen (see Bilberg et al., 2010).

There was a trend of lower P_{crit} estimates from closed respirometry compared with intermittent-flow respirometry, which was statistically significant when using the LLO method to calculate P_{crit} (LMM, $P<0.05$; Table 1). Also, even though P_{crit} values were highly correlated between replicate trials of closed respirometry, they were not correlated between either of the trials of closed respirometry and the single trial of intermittent-flow respirometry (Table S2). The two respirometry formats differ in the accumulation of metabolic wastes and, potentially, the rate at which hypoxia develops, both of which can influence P_{crit} (Snyder et al., 2016; Regan and Richards, 2017). Importantly, the magnitude of the difference in P_{crit} determined by alternative calculation methods (e.g. BSR and LLO) exceeded the differences determined in closed and intermittent-flow respirometry. Hence, the method used to calculate P_{crit} is as important as respirometry format, highlighting the need to standardize analytical as well as experimental approaches in assessing the oxygen dependence of metabolism.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.E.R., B.B.R.; Methodology: J.E.R., B.B.R.; Software: J.E.R.; Validation: J.E.R.; Formal analysis: J.E.R.; Investigation: J.E.R.; Resources: B.B.R.; Data curation: J.E.R.; Writing - original draft: J.E.R., B.B.R.; Writing - review & editing: J.E.R., B.B.R.; Visualization: J.E.R.; Supervision: B.B.R.; Project administration: B.B.R.; Funding acquisition: B.B.R.

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Data availability

Data and R script used in this study are available from figshare: https://doi.org/10.6084/m9.figshare.8869253.v1.

Supplementary information

Supplementary information available online at
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