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Correcting systematic error in PO_2 measurement to improve measures of oxygen supply capacity (α)

Alexander W. Timpe *, Brad A. Seibel

College of Marine Science, University of South Florida, 830 1st St. S., St. Petersburg, FL 33701, USA

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ABSTRACT

An organism's oxygen supply capacity, measured as a ratio of a metabolic rate to its critical oxygen partial pressure, describes the efficacy of oxygen uptake and transport. This metric is sensitive to errors in oxygen measurement, especially near anoxia where the magnitude of instrument error as a proportion of total signal is magnified. Here, we present a conceptual and mathematical method that uses this sensitivity to identify, quantify, and therefore correct oxygen measurements collected using inaccurately calibrated sensors. When appropriate, adding a small correction value to each oxygen measurement counteracts the effects of this error and provides results that are comparable to data from accurately calibrated oxygen probes. We demonstrate, using simulated, laboratory, and literature datasets, how this method can be used post hoc to diagnose error in, correct the magnitude of, and reduce the variability in repeat measures of traits relevant to oxygen tolerance.

1. Introduction

Low environmental oxygen is a common challenge for aquatic organisms. In large regions of the world's oceans, including in oxygen minimum zones (OMZs; Fuenzalida et al., 2009; Wyrtki, 1962), on continental margins (Helly and Levin, 2004), at the outflow of eutrophic river systems (i.e., "dead zones;" Rabalais et al., 2002), and in estuaries, tidepools and stagnant freshwater systems (Seibel, 2024a, 2024b), animals routinely experience oxygen partial pressures (PO_2) well below air saturation. Although there is abundant life in low-oxygen areas (Childress and Seibel, 1998; Levin, 2002), survival requires specific adaptations for oxygen uptake and transport. Thus, oxygen variability in the ocean is an important parameter that may limit growth, reproduction, activity, and biogeography (Breitburg et al., 2018; Richards, 2011; Seibel, 2024a). Moreover, due to anthropogenic influences, oxygen in the ocean is declining globally (Breitburg et al., 2018; Jenny et al., 2016; Keeling et al., 2010; Oschlies, 2021).

Oxygen uptake rate, determined using respirometry, is an indirect measure of whole-animal aerobic metabolic rate (MR) that is widely used to quantify an animal's metabolic response to changes in environmental conditions (Killen et al., 2021; Nelson, 2016; Steffensen, 1989). During a respirometry trial, the rate of oxygen consumption is calculated for a discrete period, defined by time or PO_2, and is often expressed in units of moles of oxygen consumed per unit time ($\dot{M}O_2$; Steffensen, 1989; Svendsen et al., 2016). The response of metabolic rate to changes in available oxygen informs physiological metrics such as the critical oxygen partial pressure (P_c; Farrell and Richards, 2009) and oxygen supply capacity (α; Kielland et al., 2019; Lindroth, 1942; Seibel and Deutsch, 2020). These indices can supplement measures of standard (SMR; Chabot et al., 2016; Claireaux and Chabot, 2016) and maximum metabolic rate (MMR; Norin and Clark, 2016) to address ecological and biogeographical questions.

Though there has been lively debate on the definition and utility of these metrics (Farrell et al., 2021; Seibel et al., 2021a, 2021b; Seibel and Deutsch, 2020; Wood, 2018), PO_2 must be determined with high precision and accuracy if they are to be of any utility. Killen et al. (2021) note that inconsistent and insufficient methods reporting cause challenges when interpreting respirometry data and evaluating their validity. Oxygen probe calibration details in published manuscripts are particularly lacking and, while the manufacturers of the wide range of oxygen sensors and probes report high accuracy and precision when properly calibrated (Appendix A), Helm et al. (2018) observed that standard laboratory practices during sensor calibration can easily introduce small amounts of systematic error in PO_2 measurement. This type of error can be difficult to diagnose but must be present if negative PO_2 values are recorded during an experiment or if a decrease in oxygen is recorded despite the apparent absence of oxygen.

The ratio of metabolic rate to PO_2 ($\alpha_0 = MR/PO_2$) is a measure of the

* Corresponding author.
E-mail address: timpe@usf.edu (A.W. Timpe).

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amount of oxygen being supplied per unit available in the environment (Seibel et al., 2021b). This ratio typically increases as oxygen declines during a respirometry trial and eventually peaks or reaches a plateau at the critical oxygen partial pressure (P_c). The P_c is typically defined as the lowest PO_2 that can support a given rate of oxygen consumption. The α_0 at the P_c is the oxygen supply capacity (α). As a ratio, α_0 is sensitive to error in the denominator that may result from imperfect instrument calibration. This sensitivity is particularly evident at low PO_2 where the ratio of error to environmental oxygen is amplified. Consequently, where PO_2 measurement error exists, α_0 may not reach a peak and, instead, continue upward indefinitely. In such instances, the calibration error may lead to unreasonable conclusions regarding animal low-oxygen tolerance. However, this sensitivity to calibration error provides an opportunity to identify, quantify, and therefore, correct it. Here we present a method for correcting PO_2 measurement error in respirometry data to improve measurements of metabolic traits related to oxygen tolerance.

2. Theory: Effect of calibration error on oxygen supply

The ratio of $\dot{M}O_2$ to the PO_2 for a given measure period is termed the instantaneous oxygen supply, α_0. Oxygen supply typically increases as PO_2 declines given a relatively constant, "regulated" oxygen consumption rate, until the critical oxygen level of that rate below which α_0 remains at capacity (α) despite a decrease in $\dot{M}O_2$ (Seibel et al., 2021b). This increase occurs because the oxygen uptake and transport rates increase as PO_2 declines to meet a given oxygen demand. Per unit of available oxygen, more oxygen is taken up as PO_2 declines because ventilation and heart rate increase. The oxygen supply capacity, α, is the maximum α_0 value and is reached at the P_c for the coincident metabolic rate. Supply capacity is not affected by changes in $\dot{M}O_2$ caused by diel cycles or spontaneous activity because α is independent of the rate at which it is determined.

To visualize the effect of systematic PO_2 measurement error (S) on α_0, we created a simulated dataset in which a known amount of error was added uniformly to all PO_2 values. The instantaneous oxygen supply with error (α_0') for each $\left(PO_2, \dot{M}O_2\right)$ pair was calculated as $\alpha_0' = \dot{M}O_2/(PO_2 + S)$. As PO_2 approaches zero, the influence of such error on the calculated α_0 increases dramatically. If measured PO_2 is an underestimate of reality (e.g., negative PO_2 values are measured due to a high zero calibration), α_0' diverges toward infinity as PO_2 approaches zero (Fig. 1). This increase is a problem because it makes animals appear to be able to support a given $\dot{M}O_2$ at a lower environmental oxygen level. The method we present here exploits that divergence to identify calibration error. Correcting calibration error relies on the assumption that, if the effect of error were removed by adding a small PO_2 correction value (C, defined below) to each point, two observations would provide equivalent estimates of α. To determine correction values that satisfy this assumption, we identify discontinuities in the second derivative of α with respect to PO_2 correction value. Even if oxygen supply falls immediately upon reaching P_c, this assumption can be met by adjusting the $\dot{M}O_2$ averaging period (bin size). Our assumptions impose no unique requirements on respirometry methodology. Here we demonstrate, using simulated, laboratory, and literature datasets, that this correction method successfully eliminates the effect of PO_2 calibration error on the calculated value of α. The following calculations can be performed using an R or Excel template available in the online supplementary materials for this manuscript. A summary reference for the notation used in this manuscript is provided in Table 1.

3. Calculation: Estimating calibration error using physiological principles

3.1. Part I: Two Datapoints

Assume a PO_2 measurement has some amount of systematic error (S) caused by inaccurate sensor calibration. Define the measured oxygen partial pressure, PO_2', as:

$$PO_2' = PO_2 + S \tag{1}$$

where PO_2 is the actual oxygen partial pressure without error. Here we assume error is invariant of PO_2. For a discussion of alternative cases, see Appendix B. An animal's aerobic metabolic rate ($\dot{M}O_2$) is calculated as the negative change in measured oxygen partial pressure over the change in time, t, and is adjusted for chamber size, animal mass, and the number of moles/l at maximum saturation to give final units of μmol/g/h (Steffensen, 1989). This adjustment is omitted below for simplicity. The change in oxygen is negative by convention to provide a positive consumption rate, interpreted as the "amount of oxygen consumed per gram of animal per unit time."

$$\dot{M}O_2 = -\frac{\Delta PO_2'}{\Delta t} = -\frac{PO_{2(1)}' - PO_{2(2)}'}{t_1 - t_2} \tag{2}$$

Equivalently, using eq. 1,

$$\dot{M}O_2 = -\frac{\left((PO_{2(1)} + S\right) - \left(PO_{2(2)} + S\right))}{t_1 - t_2} = -\frac{PO_{2(1)} - PO_{2(2)}}{t_1 - t_2} = -\frac{\Delta PO_2}{\Delta t} \tag{3}$$

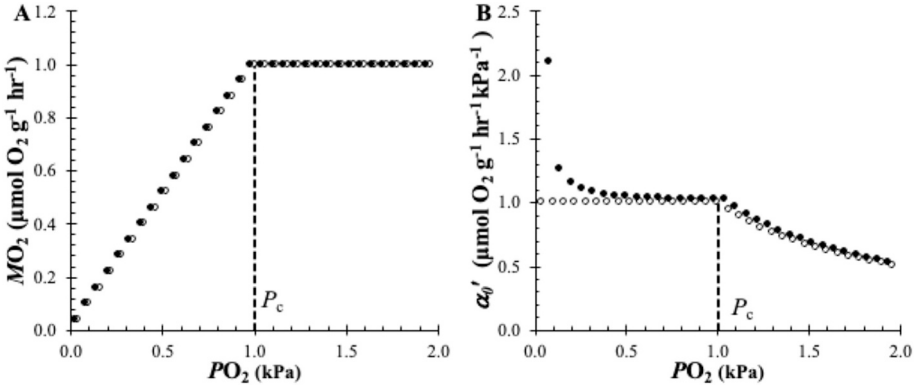

Fig. 1. Simulated respirometry dataset with calibration error. Filled points represent data with −0.1 % introduced systematic PO_2 measurement error (S = −0.0121 kPa). Data without systematic error (open points) are provided for comparison. This simulated dataset mimics the response of a resting animal to hypoxic conditions. **A)** $\dot{M}O_2$ as a function of PO_2. Oxygen uptake rate is constant as oxygen declines, until P_c (1 kPa; dashed line) below which it decreases proportionally to environmental oxygen. **B)** Oxygen supply (α_0) as a function of PO_2. Note the divergence between the reference (open points) and data with error (filled points) near anoxia. The input supply capacity value for this dataset is α = 1.0 μmol/g/h/kPa.

Table 1
Summary of Metrics and Variables. For each metric, we provide the symbol (or abbreviation), a brief description, and the typical units, when applicable.

Symbol	Description	Units
α	Oxygen supply capacity	μmol O_2 g^{-1} h^{-1} kPa^{-1}
α_0	Oxygen supply; ≤ α and is equivalent to α at P_c	μmol O_2 g^{-1} h^{-1} kPa^{-1}
α'_0	Measured physiological oxygen supply	μmol O_2 g^{-1} h^{-1} kPa^{-1}
α''_0	Corrected physiological oxygen supply	μmol O_2 g^{-1} h^{-1} kPa^{-1}
C	Correction value of PO''_2	kPa
C'	Potential correction value for PO'_2	kPa
C'_i	The i^{th} interval between $\pm C'$ of width ΔC_i	kPa
ΔC_i	Interval width between potential correction values	kPa
$f(C'_i)$	Maximum corrected oxygen supply at some C'	μmol O_2 g^{-1} h^{-1} kPa^{-1}
MMR	Maximum Metabolic Rate (maximal activity). Only the aerobic component is measured using respirometry.	μmol O_2 g^{-1} h^{-1}
$\dot{M}O_2$	Oxygen consumption rate, expressed in moles, and used as an indirect measure of aerobic metabolism	μmol O_2 g^{-1} h^{-1}
MR	Metabolic rate, nonspecific units	N/A
P_c	Critical oxygen partial pressure: the PO_2 below which a given $\dot{M}O_2$ is oxygen-limited and is calculated using α ($P_c = \dot{M}O_2/\alpha$). P_c for a specific MR is denoted P_{cMR} (e.g., P_{cSMR}).	kPa
PO_2	Environmental oxygen partial pressure. This value is estimated using oxygen probes.	kPa
PO'_2	Measured environmental oxygen partial pressure	kPa
PO''_2	Corrected environmental oxygen partial pressure	kPa
S	Systematic or calibration error in PO_2 measurement	kPa
SMR	Standard Metabolic Rate (resting, fasted animal)	μmol O_2 g^{-1} h^{-1}
t	Time	Hours

Thus, systematic error does not bias estimates of metabolic rate. However, each $\dot{M}O_2$ is paired with the average PO'_2 of all data used in the rate calculation, which retains calibration error. Seibel et al. (2021b) calculate the instantaneous oxygen supply (α_0) for a given aerobic metabolic rate and level of environmental PO_2 as:

$$\alpha_0 = \frac{\dot{M}O_2}{PO_2} \tag{4}$$

At the critical PO_2 for a given $\dot{M}O_2$, physiological oxygen supply reaches maximum capacity (α), $\dot{M}O_2$ is the maximum achievable rate at that PO_2, and $\alpha_0 = \alpha$.

$$max\{\alpha_0\} = \alpha = \frac{\dot{M}O_2}{Pc} \tag{5}$$

When an organism is operating at oxygen supply capacity, oxygen uptake is dependent upon and linearly proportional to available environmental oxygen (Seibel et al., 2021a, 2021b). Using measured oxygen values, PO'_2 (eq. 1) that may be affected by calibration error, we define the measured physiological oxygen supply, α'_0, as:

$$\alpha'_0 = \frac{\dot{M}O_2}{PO'_2} = \frac{\dot{M}O_2}{(PO_2 + S)} \tag{6}$$

Next, we define a correction factor, C, that nullifies calibration error and

$$S + C = 0 \tag{7}$$

We define the corrected environmental oxygen partial pressure PO''_2 by adding the correction factor C to our observed oxygen value PO'_2

$$PO''_2 = PO'_2 + C \tag{8}$$

Thus, by eq. 1 and eq. 7, corrected PO_2 values are equivalent to data without calibration error:

$$PO''_2 = PO'_2 + C = PO_2 \tag{9}$$

In analogous fashion to our definition of PO''_2, we define the corrected physiological oxygen supply, α''_0:

$$\alpha''_0 = \frac{\dot{M}O_2}{PO''_2} = \alpha \tag{10}$$

Consider two observations measured at P_c, $\left(PO'_{2(1)}, \dot{M}O_{2(1)}\right)$ and $\left(PO'_{2(2)}, \dot{M}O_{2(2)}\right)$ that, if the effect of error were removed by adding a correction value C to both PO'_2, provide equivalent measures of α_0 and satisfy eq. 5. (i.e., $\alpha''_{0(1)} = \alpha''_{0(2)} = \alpha$). By eq. 9,

$$\frac{\dot{M}O_{2(1)}}{PO''_{2(1)}} = \frac{\dot{M}O_{2(2)}}{PO''_{2(2)}}$$

Equivalently,

$$\frac{\dot{M}O_{2(1)}}{PO'_{2(1)} + C} = \frac{\dot{M}O_{2(2)}}{PO'_{2(2)} + C}$$

Rearranging allows us to calculate C exactly if $\dot{M}O_{2(1)} \neq \dot{M}O_{2(2)}$ and $PO'_{2(1)} \neq PO'_{2(2)}$:

$$C = \frac{\left(\dot{M}O_{2(1)} * PO'_{2(2)}\right) - \left(\dot{M}O_{2(2)} * PO'_{2(1)}\right)}{\left(\dot{M}O_{2(2)} - \dot{M}O_{2(1)}\right)} \tag{11}$$

Because $PO'_{2(n)}$ and $\dot{M}O_{2(n)}$ are measured experimentally, if two points would without the effect of error be at capacity and therefore have equivalent α_0, we can determine a correction value, C, that satisfies this condition and negates the effect of error using eq. 11.

3.2. Part II: Correcting error in datasets with more than two points

In Part I, we show that systematic error (S) in the measurement of oxygen partial pressure (PO_2) does not affect the calculation of metabolic rate ($\dot{M}O_2$) but is retained in the averaged PO'_2 value paired with that rate. We also show how the magnitude of such error can be determined if the actual oxygen supply ($\alpha_{0(n)}$) of two measured points, $\left(PO'_{2(1)}, \dot{M}O_{2(1)}\right)$ and $\left(PO'_{2(2)}, \dot{M}O_{2(2)}\right)$, are, without error, at capacity and therefore equal. While this correction is straightforward with two points, the challenge in larger datasets is determining which points should be adjusted such that, after their correction, $\alpha''_{0(1)} = \alpha''_{0(2)} = \alpha$. In other words, we must identify observations that would give equivalent estimates of α if calibration error were removed. This is particularly important during trials where, for some duration, oxygen supply is below capacity (i.e., $\alpha_o < \alpha$ and $PO_2 > P_c$). The two points that should be used to precisely calculate calibration error in eq. 11 are identified by approximating the second derivative of corrected physiological oxygen supply (α''_0; eq. 10) with respect to potential correction value (C'). Discontinuities in the second derivative indicate correction values that give equivalent estimates of α for two corrected observations.

To correct for error in larger datasets, we create an array of potential correction values with a number, i, of discrete, equal intervals of width $\Delta C'$ between some maximum and minimum range of potential correction values ($\pm C'$). For example, one might choose an initial range that encompasses −10 % to 10 % of the saturation value (±2.1 kPa). In every test case, $i = 200$ intervals between these boundary values of C' provided sufficient resolution to identify the discontinuities used to determine C. Furthermore, it is possible to achieve higher resolution by narrowing the boundary conditions or increasing the number of intervals if the initial conditions are insufficient. Next, we calculate $\alpha''_{0(n)i}$, which describes the corrected oxygen supply for the n^{th} paired $\left(PO'_2, \dot{M}O_2\right)$ observation in a

respirometry dataset of length N and where C_i' is the value of C' at the i^{th} interval between $\pm C'$ of width $\Delta C'$:

$$\alpha''_{0(n)i} = \frac{\dot{M}O_{2(n)}}{PO'_{2(n)} + C'_i} \tag{12}$$

We then calculate $\alpha''_{0(n)i}$ for all $\left\{\left(PO'_{2(n=1,\ldots,N)}, \dot{M}O_{2(n=1,\ldots,N)}\right)\right\}$ and C'_i, and plot α''_0 as a function of C'_i. An idealized dataset where the animal always operates at capacity (i.e., $\alpha_0 = \alpha$ for all observations) provides the simplest example of the $\left\{\left(\alpha''_{0(n)i}, C'_i\right)\right\}$ relationship (Fig. 2). Since α_0 is maximized at α (Seibel et al., 2021b), only the maximum $\alpha''_{0(n)i}$ for any point(s) in the respirometry dataset $\left\{\left(PO''_{2(n=1,\ldots,N)}, \dot{M}O_{2(n=1,\ldots,N)}\right)\right\}$ for each C'_i are relevant for calculating α as only the highest $\alpha''_{0(n)i}$ could potentially be at capacity. Moreover, when the two highest $\alpha''_{0(n)i}$ are equal, the conditions of eq. 5 may be satisfied and, at that value of C'_i, by eq. 7, $C'_i = C = S$. Therefore, to determine where intersections between maximized $\alpha''_{0(n)i}$ occur, we define a new function $f(C'_i)$ using eq. 12 that describes the maximum $\alpha''_{0(n)i}$ as a function of C'_i:

$$f(C'_i) = max\left\{\alpha''_{0(n=1)i}, \ldots, \alpha''_{0(n=N)i}\right\} = max\left\{\frac{\dot{M}O_{2(1)}}{PO'_{2(1)} + C'_i}, \ldots, \frac{\dot{M}O_{2(N)}}{PO'_{2(N)} + C'_i}\right\} \tag{13}$$

Because there are discontinuities in $f(C_i')$, the function is not continuous for all C'_i. Additionally, at the C'_i where the datapoints that provides the solution to eq. 13 change, $f(C_i')$ may be continuous but not differentiable. Consequently, there are discontinuities in its derivative that identify C'_i of interest. For continuous segments of the function $f(C_i')$ from $C'_{i(n_1)}$ to $C'_{i(n_2)}$ where a single $\left(PO'_{2(n)}, \dot{M}O_{2(n)}\right)$ provides the solution to eq. 13, $f(C_i')$ is differentiable within that region and

$$\frac{d}{dC'_i} f(C'_i) = -\frac{\dot{M}O_{2(n)}}{\left(PO'_{2(n)} + C'_i\right)^2} \tag{14}$$

All solutions to eq. 14 must be ≤ 0 since oxygen consumption rate must be ≥ 0 in heterotrophic organisms and since $PO''_2 = PO'_2 + C$ (eq. 8) must be positive if $PO''_2 = PO_2$ and $\alpha''_0 = \alpha$. Differentiating again with respect to C'_i in that continuous, bounded region gives:

$$\frac{d^2}{dC'^2} f(C') = \frac{2*\dot{M}O_{2(n)}}{\left(PO'_{2(n)} + C'\right)^3} \tag{15}$$

Since the constraints on $\dot{M}O_2$ and PO''_2 enumerated above still apply, all valid second derivatives of $f(C')$ must be positive. While it would be sufficient to approximate the first derivative to identify discontinuities in $f(C')$, they are more obvious using the second derivative as it magnifies the y-axis difference between the continuous, bounded regions of $f'(C')$. Additionally, as discontinuities in the second derivative occur at $\frac{d^2}{dC'^2} f(C') > 0$, we identify them as a shift in $log_{10}\left(\frac{d^2}{dC'^2} f(C')\right)$ of typically

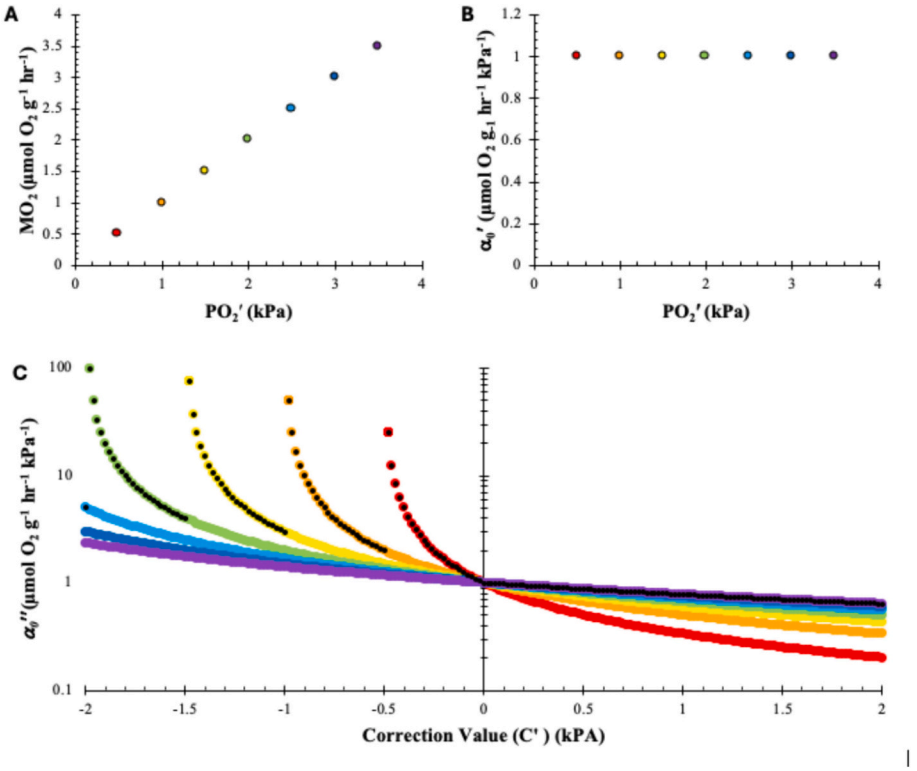

Fig. 2. A simulated maximum metabolic rate (MMR) dataset without calibration error. A) A maximum metabolic rate (MMR) trial $\left\{\left(PO'_{2(1)}, \dot{M}O_{2(1)}\right), \ldots, \left(PO'_{2(N)}, \dot{M}O_{2(N)}\right)\right\}$ where measured supply is always at capacity $\left(\alpha'_{0(n=1,\ldots,N)} = \alpha\right)$. The selected α for this dataset = 1.0 and there is no systematic error ($S = 0$). **B)** Measured supply (α'_0) as a function of measured oxygen pressure (PO'_2). **C)** Corrected oxygen supply (α''_0) as a function of potential correction value (C') at intervals $\Delta C' = 0.02$ kPa for all points in the dataset in panels **A** and **B**, and are color-coded to match. Superimposed black dots identify the points used to define the piecewise function $f(C'_i) = max\left\{\alpha''_{0(n=1)i}, \ldots, \alpha''_{0(n=N)i}\right\}$ (eq. 13), which isolates the highest corrected oxygen supply at each potential correction value. A corrected supply measurement $\alpha''_{0(n)}$ becomes negative when $C' + PO_{2(n)} < 0$ and is not displayed on a log-scale. Because all corrected PO_2 values should be positive, a C' that results in any $PO''_2 < 0$ is invalid. Note that, in this dataset where $S = 0$, the only solution to where the two highest corrected supply capacities are equivalent ($\alpha = \alpha''_{0(1)} = \alpha''_{0(2)}$; eq. 18) occurs at $C' = 0$ and therefore indicates no correction is needed ($C = -S = 0$). An example dataset with calibration error is provided in Appendix C.

~0.3 log units or greater between two C'_i (Fig. 3). Because this function is not differentiable everywhere, it is possible to approximate the second derivative of $f(C'_i)$ (eq. 13) at the i^{th} interval between $\pm C'$ of width $\Delta C'$ by using the second-order central difference quotient:

$$\frac{d^2}{dC'^2}f(C'_i) \approx \frac{f\left(C'_{(i-1)}\right) - 2^*f(C'_i) + f\left(C'_{(i+1)}\right)}{(\Delta C_i)^2} \tag{16}$$

Equivalently,

$$\frac{d^2}{dC'^2}f(C') \approx \frac{max\left\{\alpha''_{0(1)(i-1)}, \ldots, \alpha''_{0(N)(i-1)}\right\} - 2^*max\left\{\alpha''_{0(1)i}, \ldots, \alpha''_{0(N)i}\right\} + max\left\{\alpha''_{0(1)(i+1)}, \ldots, \alpha''_{0(N)(i+1)}\right\}}{(\Delta C_i)^2} \tag{17}$$

The correction value C for the two points, $\left(PO'_{2(n_1)}, \dot{M}O_{2(n_1)}\right)$ and $\left(PO'_{2(n_2)}, \dot{M}O_{2(n_2)}\right)$, which are the solutions of eq. 17 that bound a discontinuity, is then precisely calculated using eq. 11 and, by eq. 10 and assuming eq. 5 is true for those two points,

$$\alpha = \alpha''_{0(n_1)} = \alpha''_{0(n_2)}. \tag{18}$$

In practice there will likely be more than one discontinuity in $f''(C')$, especially if $\Delta C'$ is large or the dataset contains many observations near or below P_c. We discuss methods for selecting the most appropriate correction value below.

4. Methods: Testing the correction method

4.1.1. Respirometry

To test the effects of probe calibration error on respirometry data empirically, we purposefully calibrated probes incorrectly, corrected respirometry data collected using those probes to account for calibration error and compared the results post-correction to properly calibrated control probes. Accurate zero calibrations were performed by immersing oxygen dipping probes (n = 2–4; Robust Oxygen Probe OXROB10 or Trace Range Robust Oxygen Probe TROXROB10, PyroScience GMBH, Aachen, Germany) for 30 min in a 500 ml flask containing a 1 %w/v solution of Na_2SO_3 in deionized water that was bubbled continuously with 300 cc/min of Nitrogen gas (Airgas USA, Clearwater, FL, USA) through a glass diffuser and regulated by a mass flow controller (Sierra Instruments, Monterey, CA, USA). The flask was covered with Parafilm™ (Amcor, Zurich, Switzerland), immersed in a LaudaE100 temperature-controlled water bath (Lauda-Brinkman LP, Marlton, NJ, USA), and stirred continuously. We performed inaccurate probe calibrations on a subset of probes (n = 1–2) by placing accurately calibrated probes in water bubbled with nitrogen until it reached the goal oxygen partial pressure (~0.05–0.2 kPa) at which point a new, inaccurate low calibration was recorded. We returned these inaccurately calibrated probes to the original anoxic solution and recorded error (negative oxygen partial pressure) for 10 min. At oxygen supply capacity, an animal's MR declines in proportion to the amount available ($MMR = \alpha^*PO_2$). This relationship between MR and PO_2 was simulated by displacing the oxygen in deionized water with nitrogen gas. Nitrogen bubbling rates across replicate trials ranged from 30 to 500 cc/min and were regulated by a mass flow controller (Sierra Instruments, Monterey, CA, USA). Nitrogen flow rates were kept constant during each trial to mimic an animal operating at MMR.

We analyzed oxygen traces from these experiments as if they were respirometry data from an animal using R (R Core Team, 2024) and the package respirometry v1.2.0 (Birk, 2020). We calculated the O_2 displacement rate, our $\dot{M}O_2$ analog, using sequential bins (Prinzing et al., 2021) and a bin duration that gave approximately the same number of observations (about 40–60) per trial. We assessed each trial using our correction method. Because N_2 flow rates differed between trials, we normalized data per trial by dividing the observed "oxygen supply capacity" for each probe with error by the mean "supply capacity" of control probes without calibration error. Thus, positive values indicate the apparent α from probes with calibration error is higher than accurately calibrated probes and a value of 1 post-correction indicates the method accounted for the effect of calibration error on α. An example oxygen trace from this experiment is provided in Appendix D.

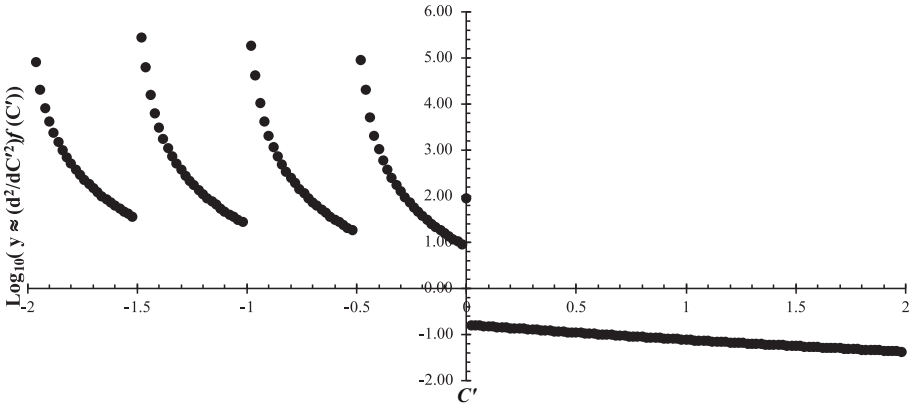

Fig. 3. Identifying discontinuities in a simulated dataset without calibration error. $\log_{10}$-transformed second-order difference quotient of $f(C')$ with respect to C' for the MMR dataset presented in Fig. 2. Discontinuities where potential $C' = C = -S$ are identified by a 0.3 log-unit or greater difference between $\frac{d^2}{dC'^2}f(C'_i)$ and $\frac{d^2}{dC'^2}f(C'_{i+1})$ or $\frac{d^2}{dC'^2}f(C'_{i-1})$, and where $PO'_2 + C'_i > 0$ for all PO'_2. For this dataset where $S = 0$, the only appropriate discontinuity occurs at $C' = 0$ and indicates no correction is needed. Discontinuities at negative C' would result in some $PO''_2 \leq 0$ and are therefore invalid.

4.1.2. Application to animal datasets

We evaluated several respirometry datasets to test the applicability of this correction method. These datasets include published curves for a diverse range of taxa that were extracted from manuscript figures using WebPlotDigitizer 4.7 (Rohatgi, 2024; https://automeris.io/WebPlotDigitizer/index.html; last accessed Feb 25, 2024), and several replicate trials from the Pacific oxygen minimum zone copepod *Megacalanus* spp. (Wishner et al., 2018). We also conducted experiments using bubbled N_2 to simulate an organism operating at maximum capacity (see above). Statistical analyses were conducted using the Paleontological Statistics (PAST) software package v. 4.15 (Hammer et al., 2001) and bootstrapped confidence intervals were calculated using R (R Core Team, 2024). Nonparametric tests were employed when the assumptions of parametric tests, such as normality or homogeneity of variance, tested using the Shaprio-Wilk test and Levene's test, respectively, were rejected. Significance for all tests was assessed at the 95 % confidence level.

5. Results and application

5.1. Appropriate use of the correction method

The critical oxygen partial pressure (P_c) for any metabolic rate can be calculated using the oxygen supply capacity ($\alpha = \frac{MMR}{P_c}$; Seibel and Deutsch, 2020). However, small negative calibration errors in oxygen probes (i.e., less oxygen is measured than is really in solution) can cause the estimate of α to be exaggerated and increase variability among replicate respirometry trials. Therefore, these calibration errors must be recognized and corrected for to accurately measure α. The following profile morphologies will illustrate the criteria for appropriate use of the correction method.

The effects of negative PO_2 error on α_0 may manifest in the (PO_2', α_0') profile two different ways. First, negative oxygen pressures recorded during a respirometry trial indicate calibration error and consequently such trials require correction. These trials can produce extremely variable and high α_0' values that result in ecologically irrelevant estimations of α and, thus, P_c (Fig. 4). Second, if oxygen is not consumed until $min\{PO_2'\} < S$, negative oxygen will not be measured despite the presence of calibration error. If S is small relative to P_c, oxygen supply may increase dramatically at $PO_2' < P_c$. This appears graphically as a plateau in α_0' near P_c, followed by an increase in α_0' at very low PO_2' (Fig. 5). We can offer no satisfactory physiological explanations for this oxygen supply response and, therefore, recommend employing the correction method for profiles of this type.

Additional (PO_2', α_0') curve morphologies include those in which α_0' increases continuously throughout the entire trial (Fig. 6) or peaks and then decreases at low PO_2. These types of profiles may either be generated by calibration error or by valid physiological processes. A decrease in α_0 at low PO_2 likely indicates some physiological failure or metabolic suppression in a hypoxic environment and not calibration error. If S is sufficiently large relative to P_c, the plateau in α_0' described earlier may be obscured and correcting the data would produce a better estimate of α compared to the uncorrected value.

Alternatively, a continuously increasing α_0' may indicate that the animal never reached supply capacity during the respirometry trial or, for the species tested, the relationship between α and P_c is nonlinear (e.g., oxyconforming animals without a complex circulatory system or clear P_c). In these cases, applying a nonzero correction value to the data is inadvisable as it would fail to generate a physiologically meaningful result (i.e., $\alpha''_{0(n_1)} = \alpha''_{0(n_2)} \neq \alpha$). Due to this ambiguity, the decision to correct trials with profiles of this type is left to the researcher's best judgement. If one is confident that α was reached, or if α is unrealistically high, correction may be appropriate. If a trial does not exhibit compelling evidence of calibration error, no correction is warranted and $C = 0$.

Many datasets will have multiple correction values that provide two equivalent estimates of α, especially if there are negative oxygen pressures recorded. In these cases, select the smallest correction value such that, post correction, the trial no longer satisfies a correction criterion as described above. For example, consider a respirometry trial for which some $PO_2' < 0$. If the smallest correction value results in α_0'' that plateau at intermediate PO_2'' and subsequently increase at the lowest-recorded PO_2'', the next-largest solution may be appropriate. Additional examples of the correction method are available in the supplementary materials of this manuscript.

5.2. Nitrogen test experiments

We bubbled N_2 gas through deionized water to mimic an animal operating at MMR and allow us to compare corrected data to a control, recorded without calibration error. Because gas flow and stirring rates varied between trials, we normalized all α-values per trial to the control probes without calibration error. An example curve demonstrating the effect of correction is provided in Appendix D. Applying the correction method as described above substantially improved the pseudo-α calculated from probes with calibration error (Fig. 7). Because many improperly calibrated probes recorded negative PO_2' during trials, α in the uncorrected dataset is elevated and highly variable compared to the control. However, post-correction the mean α is on average only 1 % higher than the control values from accurately calibrated probes. There is no significant difference between corrected values and α measured

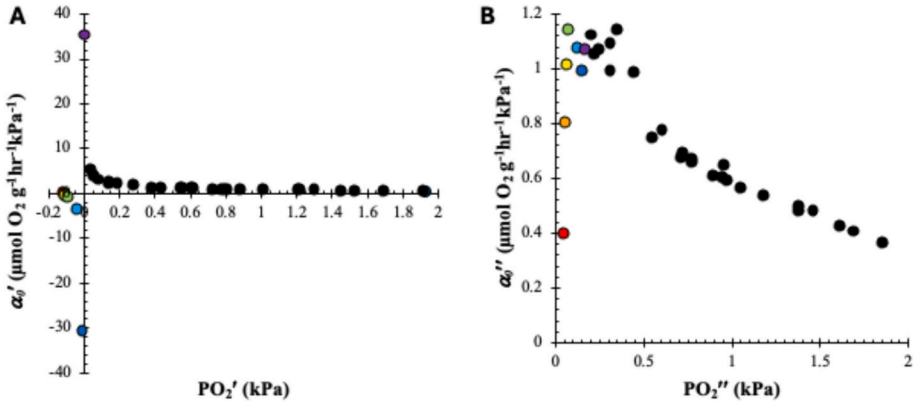

Fig. 4. The effect of correcting a respirometry trial in which negative oxygen was recorded. Respirometry data (PO_2, α_0) from the OMZ copepod genus *Megacalanus* (Wishner et al., 2018). **A)** Without correction, α_0' is highly variable and results in an extremely low P_c estimate for its routine metabolic rate ($P_c = 0.024$ kPa). **B)** Post-correction ($C = 0.1663$ kPa), all PO_2 are positive and α_0'' has an interpretable relationship with respect to PO_2. Datapoints in panels **A**, **B** are color-coded to match.

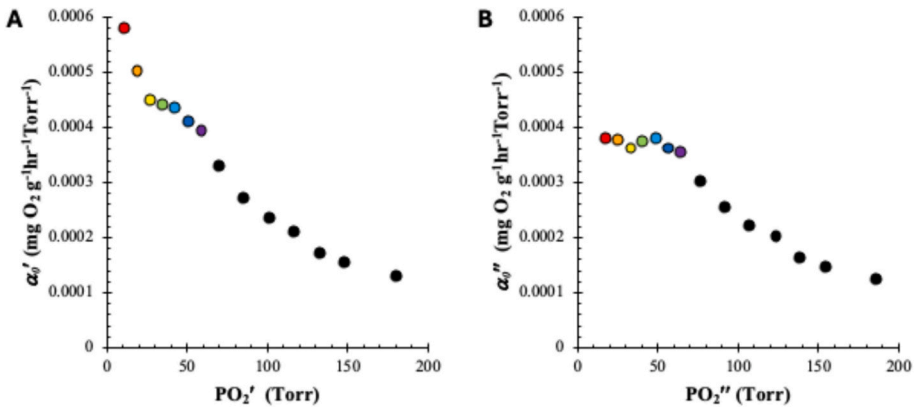

Fig. 5. The effect of correcting a respirometry trial in which oxygen supply diverges near anoxia. Respirometry data (PO_2, α_0) from the southern rock lobster *Jasus edwardsii* (Crear and Forteath, 2000) illustrating **A)** a plateau in α_0' at intermediate PO_2 followed by an increase at the lowest-recorded values, and **B)** how correcting the dataset ($C = 6.4$ Torr) reduces the α_0'' of values closest to anoxia and simplifies its physiological interpretation. Data are presented in the originally published units and the points in panels **A** and **B** are color-coded to match.

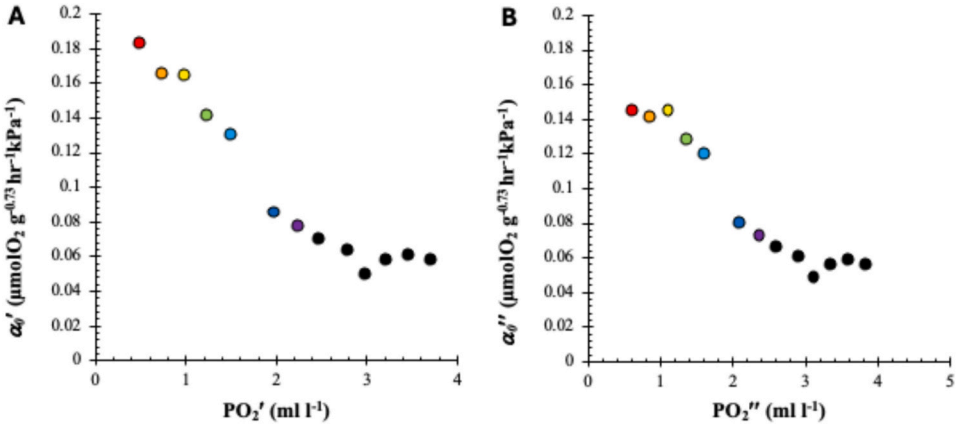

Fig. 6. The effect of correcting a respirometry trial in which oxygen supply continuously increases. Respirometry data (PO_2, α_0) from the goldfish *Carassius auratus* (Prosser et al., 1957) illustrating **A)** a continuous increase in α_0' with falling PO_2 and **B)** the result of correction ($C = 0.106$ ml l^{-1}). Profiles of this type are possible if error is sufficiently large relative to P_c, if the animal did not reach P_c during the trial, or if there is a nonlinear relationship between α and P_c for the species being investigated. Thus, correcting profiles of this type is at the researcher's discretion. For trials of this type, correcting the data may not improve estimates of α. Data in panels **A** and **B** are color-coded to match and are presented in the originally published units.

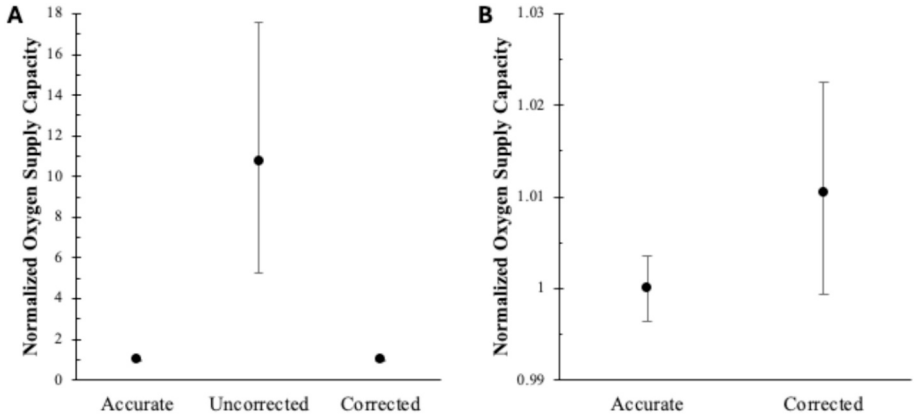

Fig. 7. The effect of correction on nitrogen test experiments. A) "Accurate" values are the oxygen supply capacity of control probes normalized by the mean α of control probes in each trial. The low variance of this value indicates probes without error give consistent results. "Uncorrected" values are the calculated α of probes with calibration error normalized by the mean of control probes. "Corrected" values are the α of the improperly calibrated probes post-correction, normalized by the control value. All values represent the mean ± 95 % bootstrapped CI with $n = 100{,}000$ iterations. **B)** The same data in **A**, with uncorrected data removed to improve visualization. Note the y-axis variation is only 0.04.

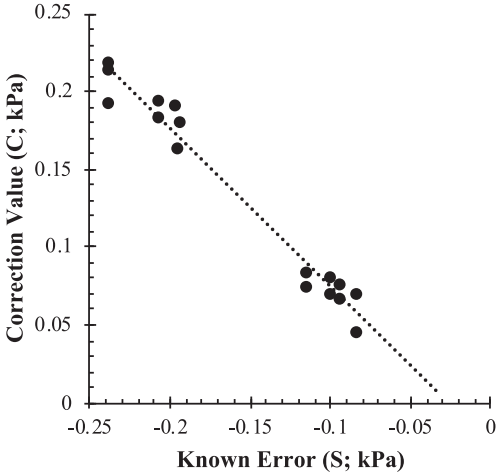

Fig. 8. Correction value as a function of known error in nitrogen test experiments. The relationship is between known error (S) and correction value (C) can be described using the linear regression ($C = -1.015 * S$ -0.027; $R^2 = 0.97$; $p < 0.0001$). The slope of the regression is negative because C = -S (eq. 7).

using control probes without calibration error, (Welch test; $t = 1.63$; $df = 34$; $p > 0.05$), though the variance is higher in corrected results (F-test; $df = 15, 19$; $F = 8.75$; $p < 0.005$). Correction values are significantly correlated to known error (Spearman's D; $p < 0.001$; Fig. 8), but the absolute magnitudes are different. For the range tested, correction values underestimate error by an average by 19 % but provide similar estimates of α post-correction. This difference is not surprising, as random error in probe readings may add additional complexity to estimating the "true" correction value C. While any synergistic effects of random and systematic error on α appear to be accounted for post-correction, further investigation is warranted.

5.3. Evaluation using animal data

To evaluate the proportion of respirometry trials where a correction for systematic error may be necessary, we conducted a survey of the literature for datasets that could be extracted for reanalysis. This resulted in 152 respirometry trials from 81 species spanning five phyla (Appendix E). Of the trials surveyed, 16 % ($n = 24$) met a definite correction criterion; either a plateau in α_0' at intermediate PO_2' values followed by an increase at low PO_2' ("Plateau, increase" in Table E1) or negative PO_2'. For another 21 % ($n = 33$), α_0' increased continuously with falling PO_2' ("Cont. increase" in Table E1) and may either indicate calibration error or, alternatively, that the animal never reached α (or P_c) or has a nonlinear relationship between α and P_c. The remaining 63 % ($n = 95$) did not meet any criteria for correction. The number of trials requiring correction is likely inflated due to inconsistent treatment of $\dot{M}O_2$ averaging period among experiments. For many trials, data are either sparse with very few observations reported below P_c (i.e., long averaging periods or very short experiments), or had abundant data calculated using relatively small averaging periods that are more susceptible to random probe error. Additionally, many manuscripts report only averaged $\dot{M}O_2$ values from several trials at common PO_2 values, complicating the interpretation of C if many calibration errors are averaged together or if the reported PO_2 values are not the average oxygen concentration of data used to calculate each $\dot{M}O_2$.

In addition to the literature survey, we assessed the effect of the correction method on replicate respirometry trials from the OMZ copepod *Megacalanus* (Wishner et al., 2018) measured at 5 °C and 10 °C. Due to these animals' low P_c, all trials approached anoxia and therefore probe calibration error would likely have an outsized effect on α. Of the 16 trials evaluated, 12 showed evidence of calibration error and 9 of those recorded negative PO_2'. Applying the correction method reduced the mean α and variance of both datasets, and post-correction there is a significant difference in oxygen supply capacity at 5 °C and 10 °C (ANOVA; $df_t = 15$, $F = 103.7$, $p < 0.001$; Fig. 9).

As observed with the nitrogen validation experiments, the correction method reduced the magnitude of α in copepod trials that definitively contain error to values approximating those from trials without evidence of calibration error. This correction method has implications for data interpretation by providing a conceptual framework that explains extreme outliers in some datasets. Moreover, overestimating α (or underestimating P_c) may lead researchers to conclude that species are more tolerant of low oxygen than they are. For example, using the uncorrected oxygen supply capacity, we would calculate a P_c for *Megacalanus* that is lower other plankton in its OMZ community (Wishner et al., 2018). Elevated intraspecific variability in α and P_c caused by calibration error may also obscure the effect of environmental variables such as salinity and temperature, or unduly reduce the predicted impact of changing environmental conditions on a population by exaggerating apparent phenotypic diversity.

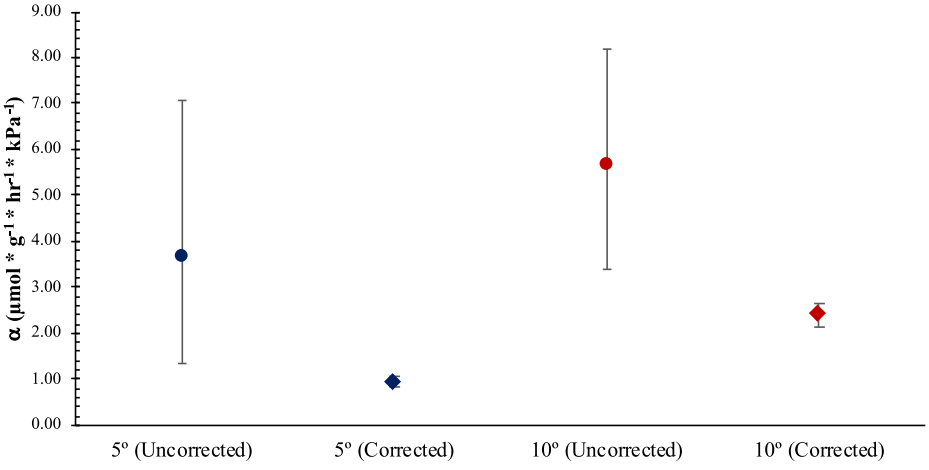

Fig. 9. Oxygen supply capacity (α) for the OMZ copepod *Megacalanus* at 5 °C ($n = 9$) and 10 °C ($n = 7$) pre- (Uncorrected) and post-correction (Corrected). Data are from Wishner et al. (2018). Values represent the mean ± 95 % bootstrapped CI with $n = 100{,}000$ iterations. One significant outlier in the 5° uncorrected dataset ($\alpha \cong 1.5 * 10^{14}$) was removed before calculating the mean and CI (Dixon Q, $p < 0.001$). No corrected values at 5 °C were identified as significant outliers (Dixon Q, $p > 0.05$). For trials that showed evidence of calibration error ($n = 7$ at 5 °C; $n = 5$ at 10 °C), correction values ranged from 0.005 to 0.19 kPa (overall mean = 0.10 kPa).

6. Conclusions

The method of Seibel et al. (2021b), used to determine the oxygen supply capacity (α), facilitates the identification and correction of systematic error in P_{O_2} measurement caused by inaccurate oxygen probe calibration. There are two definitive scenarios in which this correction method should be employed: 1) if negative oxygen values are recorded, or 2) if there is a plateau in measured oxygen supply (α_0') at intermediate P_{O_2} followed by a dramatic increase at the lowest-recorded oxygen levels. Alternative relationships between oxygen supply and environmental P_{O_2} may represent valid physiological processes and not error. If no calibration error is suspected given the criteria listed above, no correction is merited and $C = 0$.

Validation respirometry experiments using nitrogen showed that, post-correction, inaccurately calibrated oxygen probes provide comparable estimates of α to properly calibrated control probes. Our literature survey found that approximately 16 % of published datasets meet a proposed correction criterion, though this proportion is potentially inflated. Correcting a dataset from the OMZ copepod *Megacalanus* where some trials showed evidence of calibration error reduced both the mean and variance of α, primarily by reducing extreme high outliers.

The need for data correction does not indicate shoddy, improper, or haphazard work. It is entirely possible, and indeed probable, for small amounts of systematic error to appear in datasets despite a researcher's diligent efforts and use of best practices. While accurate calibration and high-precision equipment can reduce the magnitude of such error, it is impossible to eliminate completely and should thus be accounted for when analyzing respirometry data to determine an animal's oxygen supply capacity or critical oxygen partial pressure.

7. Summary

1. This correction method successfully mitigates the effect of systematic P_{O_2} error caused by inaccurate probe calibration on some measured metabolic traits.
2. Small amounts of calibration error can affect measures of oxygen supply capacity (α) and P_c and must be accounted for, especially in species that live in low-oxygen environments where the effect of this error is magnified.
3. When multiple, valid potential correction values are identified, we recommend using the smallest one such that, after correction, the trial no longer meets a correction criterion. Valid correction values are typically positive and reflect erroneous calibration at anoxia.
4. For trials that do not show evidence of calibration error, the correction value $C = 0$.
5. For the data analyzed here, the correction method reduced variability among replicate observations of oxygen supply capacity (and consequently P_c calculated as $P_c = \mathrm{MR}/\alpha$).
6. Based on a literature survey, approximately one in six published respirometry curves meet a correction criterion.

CRediT authorship contribution statement

Alexander W. Timpe: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Brad A. Seibel:** Writing – review & editing, Writing – original draft, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data and code is available in the data supplement

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Appendix A. Supplementary data

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