Sources of variation in oxygen consumption of aquatic animals demonstrated by simulated constant oxygen consumption and respirometers of different sizes

M. B. S. Svendsen*†, P. G. Bushnell‡, E. A. F. Christensen* and J. F. Steffensen*

*Marine Biological Section, Department of Biology, University of Copenhagen, Strandpromenaden 5, DK-3000 Helsingør, Denmark and ‡Department of Biological Sciences, Indiana University South Bend, South Bend, IN 46634, U.S.A.

As intermittent-flow respirometry has become a common method for the determination of resting metabolism or standard metabolic rate (SMR), this study investigated how much of the variability seen in the experiments was due to measurement error. Experiments simulated different constant oxygen consumption rates ($\dot{M}O_2$) of a fish, by continuously injecting anoxic water into a respirometer, altering the injection rate to correct for the washout error. The effect of respirometer-to-fish volume ratio (RFR) on SMR measurement and variability was also investigated, using the simulated constant $\dot{M}O_2$ and the $\dot{MO}_2$ of seven roach Rutilus rutilus in respirometers of two different sizes. The results show that higher RFR increases measurement variability but does not change the mean SMR established using a double Gaussian fit. Further, the study demonstrates that the variation observed when determining oxygen consumption rates of fishes in systems with reasonable RFRs mainly comes from the animal, not from the measuring equipment.

INTRODUCTION

Measurements of oxygen consumption of fishes and other aquatic organisms have been made over time using a variety of respirometry techniques such as closed respirometry (Ege & Krogh, 1914; Scholander et al., 1943), flow-through (or open) respirometry (Niimi, 1978) and intermittent-flow (or stop-flow) respirometry (Forstner, 1983; Steffensen et al., 1984; Steffensen, 1989). Oxygen consumption using closed respirometry should, however, be avoided due to problems associated with the progressive hypoxia and simultaneous hypercapnia that inevitably develop during the experiment. Flow-through respirometry is also problematic due to issues of equilibration time, caused by the exponential washout effect of water in the respirometer (Keys, 1930a, b; Niimi, 1978; Steffensen, 1989; Eriksen, 2002; Svendsen et al., 2016). At present, intermittent-flow respirometry is probably the easiest method of accurately
determining oxygen consumption in aquatic animals at high frequency and for long durations, and it has become widely used.

Regardless of the technique employed, a source of variation in the measured oxygen consumption is noise from the data collection system. The type of oxygen sensors (e.g., polarographic, galvanic or optical), amplifiers and recording equipment can all potentially affect the quality of the data being recorded (Shannon, 1949). This can be seen as signal noise inherent in the calibrated probe and associated electronics, which can result in an over or under-estimation of the measured oxygen consumption of the animal and contribute to variability in the measurements. As far as is known, no previous studies have tried to examine and quantify such noise in aquatic respirometry systems. Instead, variation in oxygen consumption data has usually been attributed to differences among individual animals. A series of experiments were therefore undertaken using an artificial fish with a constant oxygen consumption to evaluate how much of the variability in metabolic rate measurements was biological in nature (i.e., the fish) and how much was inherent to the measurement system itself.

In addition to signal variability in the instrumentation, it is also interesting to assess the effect of the respirometer-to-fish volume ratio (RFR) on the scatter in measurements of oxygen consumption when using intermittent-flow respirometry. Compared with a large fish, a small fish in a large respirometer (high RFR) will produce a slower decline in oxygen content over time and hence a lower (flatter) slope, reduced coefficient of determination ($r^2$) and larger variation. The information provided in this study will be useful in aiding experimenters in the use of appropriately sized respirometers to obtain accurate standard metabolic rate (SMR) measurements with lower variation.

**MATERIALS AND METHODS**

**EXPERIMENTAL ANIMALS**

The experiments were conducted with roach *Rutilus rutilus* (L. 1756), caught in streams in Mølleåen, Denmark (55° 46′ 20″ N; 12° 29′ 47″ E) in December 2013. *Rutilus rutilus* was chosen as a test animal due to its availability and similar behaviour to the rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) in a respirometer. The fish, ranging in mass from 19.3 to 28 g (23.1 ± 3.6 g, mean ± s.d.), were kept in recirculated filtered fresh water and acclimated to 25°C for at least 2 weeks before being used in the experiment. Fish were fed Tetramin (United Pet Group; www.unitedpetgroup.com) flaked aquarium fish food every day. Food was withheld 2 days prior to experimentation to ensure that animals were in a post-absorptive state.

**EXPERIMENTAL PROCEDURE: THE EFFECT OF RESPIROMETER VOLUME ON *R. RUTILUS***

The experiments were carried out using intermittent-flow respirometers designed to measure SMR, defined as the oxygen consumption of a resting, non-digesting and non-reproducing animal, which thus reflects the minimum maintenance cost (Beamish & Mookherjii, 1964; Forstner, 1983; Schurmann & Steffensen, 1997; Svendsen et al., 2013; Chabot et al., 2016).

To test the effect of RFR on SMR measurements, experiments were carried out on *R. rutilus* ($n = 7$) in respirometers with a volume of 0.6 and 0.91. Although the metabolic rate measurements were done consecutively, the initial respirometer size was determined randomly to avoid any confounding effects of temporal habituation to the respiometers. The respirometer was immersed in a temperature-controlled water bath (301) connected *via* an overflow to a 401 sump in order to increase the total holding volume of water and maintain a constant pressure in the respirometer. Water in the bath and sump was vigorously aerated.
to maintain oxygen tension at normoxic levels and passed through a UV sterilizer (Tetra Pond UV10000; www.tetra-fish.com) to keep background respiration low. The temperature of the system was kept at 25±0.1°C, range ±0.1°C, by a programmable temperature regulator (PR 5714, PR Electronics; www.prelectronics.dk) with a pt-100 temperature probe (Ametek; www.ametek.com) and a submersible pump (Eheim 1004; www.eheim.com) which pumped warm water through a stainless steel coil in a thermostatted water bath (Heto CBN 8-30; www.holm-halby.dk) kept at 30°C. This pump was only activated when temperature in the holding tank fell below 25°C, thus maintaining constant temperature in the experimental setup. Oxygen content (% air saturation) was measured with a fibre optic oxygen sensor (Firesting O₂, Optical Oxygen Meter, 4 channel; www.pyro-science.com) calibrated to 0 and 100% air saturation using chemically oxygen-depleted water and air-saturated water. The experimental protocol used was similar to that described in Steffensen et al. (1984) with each measurement cycle consisting of flush, wait and measurement periods. During the flush period (300 s), the respirometer was flushed with fresh water using a pump (Eheim 1046) with a 51 min⁻¹ flow rate which effectively replaced 99-99% of the chamber water (Steffensen, 1989). At the end of the flush period, the pump was turned off and the water allowed to mix during a 30 s wait period. The measurement cycle concluded with 300 s measurement period where the linear decline in oxygen content was recorded every second. Flush-pump cycling and data collection were controlled via AquaResp (University of Copenhagen) a free programme for respirometry of aquatic animals available on the web (www.aquaresp.com).

OXYGEN CONSUMPTION

Oxygen consumption was calculated using the formula \( \dot{M}O_2 = \beta O_2 V_R W_F^{-1} \delta p O_2 \delta t^{-1} \), where \( \beta O_2 \) is the oxygen solubility (of the water) at the measurement temperature, salinity and atmospheric pressure, \( V_R \) is the respirometer water volume, \( \delta p O_2 \delta t^{-1} \) is the slope of the linear regression calculated from the decline in oxygen during the measurement periods and \( W_F \) is the mass of the fish (Ege & Krogh, 1914; Steffensen, 1989; Svendsen et al., 2016). Conversion from oxygen content or oxygen saturation to partial pressure was completed by using Henry’s Law (Svendsen et al., 2016).

Respirometer volume \( V_R \) was calculated using \( V_R = V_T - W_F \rho F^{-1} \), where \( V_T \) is the total volume of the empty respirometer, associated recirculating system tubing and pump and \( \rho \) is the density of the fish (for calculation purposes, the fish was assumed to be neutrally buoyant, \textit{i.e.} same density as the water).

EXPERIMENTAL PROCEDURE: THE EFFECT OF RESPIROMETER VOLUME ON SIMULATED FISH

Custom-made software, Artifishal, was used to simulate a constant and precise oxygen consumption of a fish by driving a voltage-controlled peristaltic pump (Minipulse 3, Gilson Inc.; www.gilson.com) which delivered anoxic water into the respirometer at a rate that was altered to maintain a constant decline in the oxygen content in the respirometer. The peristaltic pump flow rate was controlled with the analogue output of a digital-to-analogue converter (USB1208LS, Measurement Computing; www.mccdaq.com) connected to a PC.

Pumping anoxic water into the respirometer at a constant rate during the measurement period would result in an exponential decrease in oxygen content due to a decreasing difference in oxygen content between the injected anoxic water and the mixed water in the respirometer (Steffensen, 1989). As a linear decrease in oxygen in the respirometer was required, the injection rate was continuously increased to mimic both the oxygen consumption of a real fish during the measurement period as well as during the washout of the metabolic chamber during the flushing period.

The linear oxygen content decrease of a fish (Fig. 1) over time, \( f(t) \), will have the form \( f(t) = \delta CO_2 \delta t^{-1} = -RO_2 t + CO_2 \), where \( RO_2 \) is the oxygen consumption rate (mg O₂ h⁻¹), \( t \) is time and \( CO_2 \) is the total content of oxygen in the respirometer. The washout function, \( w(t) \), can be expressed as \( w(t) = \delta CO_2 \delta t^{-1} = CO_2 e^{\tau t} \), with \( \tau \) being the washout constant (Steffensen, 1989), determined by the infusion rate, \( r \), and the volume of the respirometer \( (V_f) = r V_f^{-1} \).
Fig. 1. A plot of air saturation during one measurement period (300 s) for (a) computer-controlled oxygen consumption ($\dot{MO}_2$), small $\dot{MO}_2$ in a large respirometer (average $r^2=0.975$), (b) computer-controlled high $\dot{MO}_2$ in a small respirometer (average $r^2=0.999$), (c) *Rutilus rutilus* in a large respirometer (average $r^2=0.998$) and (d) *R. rutilus* in a small respirometer (average $r^2=0.998$). There are four randomly selected slopes from the experiments on each part. Note that in (a) and (b), both from the Artifishal experiment, lines fall on top of each other, whereas in data from the *R. rutilus* experiments (c) and (d), lines from four random measurement periods do not fall on top of each other, and do not have similar slope. The $y$-values from where the slopes start is dependent on oxygen consumption, flush volume and mixing in the respirometer (Svendsen et al., 2016). Noise in the data, scatter around the slope, is considered the same in all experiments, and arise from non-instantaneous mixing and possibly from the oxygen sensor. Data are obtained from the Pyroscience Firesting (www.aquarespt.com) logger at 1 Hz.

To approximate the infusion rate to obtain a linear decrease in the respirometer, the following formula was used: $r = \int [f (t) CO_2^{-1}]^{1/1} - 1$, the experiments were carried out using the same respirometers as the *R. rutilus* experiments in order to imitate RFRs of 26:1 and 39:1 and simulate oxygen consumption rates of 1562, 4688 and 7500 μmol O$_2$ kg$^{-1}$ h$^{-1}$. Due to pump flow rate limitation and degassing capacity, it was not possible to produce an oxygen consumption rate that exceeded 7500 μmol O$_2$ kg$^{-1}$ h$^{-1}$.

**STANDARD METABOLIC RATE**

SMRs were determined by fitting the sum of two Gaussian distributions to the histogram of the oxygen consumption ($MO_2$) data with a bin size of 25 μmol O$_2$ kg$^{-1}$ h$^{-1}$ (Fig. 2) as outlined by Steffensen et al. (1994). Fitting the sum of two normal distributions will give two mean values. The mean of the left (low values on x-axis) peaked distribution (green bell in Fig. 2) represents SMR and the width its s.d. The right peak (red bell) represents spontaneous activity (SA) defined as intermittent and prolonged bouts of higher oxygen consumption. The SMR and SA distributions each have an s.d., and thus variance that can be derived from the curve-fitting
algorithm. In this case, usage of two Gaussian distributions worked well, but up to four may be needed to establish SMR properly (Chabot et al., 2016).

The double Gaussian curve fit was carried out for both the real as well as the simulated fish using Python (Python Software Foundation, Python Language Reference, version 2.7; www.python.org).

**CORRELATION COEFFICIENT**

Partial pressure of oxygen, $pO_2$, of the water inside a respirometer containing a fish that consumes oxygen at a constant rate, will decline in a linear manner with respect to time to which a linear regression can be fitted with a correlation coefficient ($r^2$) that will range between 0 and 1 (Figs 1 and 3). When fitting a linear regression, the more points that are collected the more predictive it becomes, resulting in a higher $r^2$ and a more accurate measurement of metabolism (Fig. 3). The $r^2$ can be interpreted as the ratio between what the linear regression can explain $S_R$, and the total variation, $S_T$. The total variation is the sum of what the regression cannot explain (error, $S_E$) plus the regression, $S_R$:

$$r^2 = S_R \left( S_R + S_E \right)^{-1}$$  \hspace{1cm} (1)

In addition to the number of points used for the regression, another important factor is the slope. With time invariant noise, a steep slope provides a larger $r^2$, as the signal (slope) is relatively higher than the noise. When one has few measurements, the slope is more difficult to determine from the noise, so $r^2$ can be low (Cornell & Berger, 1987).

**RESPIROMETRY EXPERIMENTAL CONSTANT**

The amount of unexplained variance in a particular experiment can be found by plotting the decrease in per cent oxygen during the measurement as $x$-values and $r^2$ as $y$-values of...
Fig. 3. The change in $r^2$ (____) and regression slope ($\delta pO_2 \delta t^{-1}$, the change in oxygen tension per time; _____) over time. Stabilization of both variants occurs after c. 250 s. $r^2 = 0.95$. This example has a respirometer volume-to-fish volume ratio of 20:1.

all data from an experiment. A respirometry experimental constant (REC), describing the overall sensitivity of the system including the fish, can then be determined by plotting the points as a Michaelis–Menten process (Dowd & Riggs, 1965). The substratum concentration ($x$-axis) is replaced by the per cent reduction in oxygen and the reaction rate ($y$-axis) is replaced by the corresponding $r^2$ to each % drop in respirometer oxygen level. In this case, the Michaelis–Menten constant $K_M$ (the substratum concentration at 50% $V_{max}$) would represent the percentage decrease in oxygen needed to obtain an $r^2$ of 0.5 (the REC for the particular setup). As can be seen from Table I, doubling the REC will produce an $r^2$ of 0.80, tripling it an $r^2$ of 0.90 and so on. As the $r^2$ approaches one asymptotically, in order to achieve an $r^2 > 0.95$, it is necessary to produce a drop in per cent oxygen in the respirometer that is at least five-fold higher than the calculated REC.

**MEASUREMENT VARIATION**

The total variation from the ideal can be considered as the sum of the variations due to the fish ($v_F$) and the variation produced by the measuring equipment ($v_M$) $v_T = v_M + v_F$. The contribution of $v_M$ to total error expressed as a percentage error can be calculated as:

$$E\% = v_M (v_M + v_F)^{-1} 100$$

**Table I.** The relationship between selected error term level, multiple respirometry experimental constant (×REC) and the associated $r^2$ values needed to obtain this level using automated linear regression for oxygen consumption determination

<table>
<thead>
<tr>
<th>×REC</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>$r^2$</td>
<td>0.50</td>
<td>0.80</td>
<td>0.90</td>
<td>0.94</td>
<td>0.96</td>
<td>0.97</td>
</tr>
</tbody>
</table>
In order to gauge the magnitude of $v_M$ in response to different RFRs and measurement times, the measurement error was calculated using the average variance determined in the *R. rutilus* experiments as a proxy for $v_F$ and the average variance from the simulated fish experiments as an approximate value of $v_M$.

**STATISTICS**

Statistical analysis was conducted in SPSS (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0; www.ibm.com). Levene’s test (Brown & Forsythe, 1974) was used to test for equality of the variances and paired $t$-test for means between treatments. Tests were considered significant at $P < 0.05$. Values listed in texts and figures are mean ± s.d.

**RESULTS**

**EFFECT OF THE RESPIROMETER VOLUME ON SMR ESTIMATION**

Examples of metabolic rate measurements in both a real and a simulated fish, collected over a 2 day period, are shown in Fig. 4 and the effect of respirometer size on metabolic rate measurements in the *R. rutilus* experiments and the simulated fish experiments in Fig. 5.

![Fig. 4. Oxygen consumption measurements recorded in (a) *Rutilus rutilus* and (b) Artifishal experiment.](image)

Fig. 4. Oxygen consumption measurements recorded in (a) *Rutilus rutilus* and (b) Artifishal experiment. The shaded area is three times the s.d. obtained from the Artifishal experiment, thus approximating the 99th percentile. The slight variation (decline) seen in the Artifishal metabolism resulted from changes in atmospheric pressure, air temperature and mechanical wear on the rubber tubing used in the anoxic water pump.
Fig. 5. (a) The frequency distributions from Artifishal experiments where oxygen consumption rate was set to two different values and conducted in a small (respirometer-to-fish volume ratio, RFR, 26:1, _____) and large (RFR = 39:1, dotted line) respirometer. A single *Rutilus rutilus* experiment with similar SMR in a small (RFR = 20:1) respirometer (___) is included for comparison. Due to the overlapping graphics and differences in scaling between the lines, (b) is shown. In (b), the x-axis is changed to visualize the results between 4000 and 5000 μmol O₂ kg⁻¹ h⁻¹ range. The Artifishal experiments are small (RFR = 26:1, □) and large (RFR = 39:1, □□) respirometer. Due to the expanded x-axis scaling, the single *Rutilus rutilus* experiment appears as an apparently horizontal line (___).

While SMR (4979.6 ± 288.5 μmol O₂ kg⁻¹ h⁻¹) of the *R. rutilus* in the small respirometer (Table II) was not significantly different from SMR determined in the large chamber (4678.5 ± 445.3 μmol O₂ kg⁻¹ h⁻¹), the variance of the two group’s oxygen consumption measurements was statistically different (*P* < 0.001, *F*₁,₁₂ = 43.1, Levene’s test), with the latter having the largest variation. In addition, there was a significant difference in the means of the *r*² values between the two treatments (Table II), with the smallest respirometer having the highest values.

<table>
<thead>
<tr>
<th>Respirometer volume (l)</th>
<th>Mean ± s.d. <em>Ṁ</em>O₂ (μmol O₂ kg⁻¹ h⁻¹)</th>
<th><em>r</em>²</th>
<th>RFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6</td>
<td>4979.6 ± 288.5</td>
<td>0.997 ± 0.000</td>
<td>26.2</td>
</tr>
<tr>
<td>0.9</td>
<td>4678.5 ± 445.3</td>
<td>0.987 ± 0.004</td>
<td>39</td>
</tr>
<tr>
<td><em>P</em></td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Table III. Estimated measurement error ($E\%$), equation (2), in per cent as a function of measurement period time and different respirometer volume-to-fish volume ratios (RFR). Calculations were based on the $\mathrm{MO}_2$ of a quietly resting *Rutilus rutilus* (standard metabolic rate, SMR) in fresh water at $25^\circ$C. Values are not reported (___) for cases where prolonged measurement times resulted in hypoxic conditions ($<50\%$ air saturation) in the respirometer as they were assumed to be unrealistic. Numbers in bold were measured, the remainder were calculated.

<table>
<thead>
<tr>
<th>Measurement time (min)</th>
<th>26</th>
<th>39</th>
<th>200</th>
<th>400</th>
<th>800</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1.7</td>
<td>3.4</td>
<td>20.9</td>
<td>39.2</td>
<td>60.7</td>
</tr>
<tr>
<td>10</td>
<td>0.6</td>
<td>1.2</td>
<td>5.6</td>
<td>12.8</td>
<td>25.6</td>
</tr>
<tr>
<td>20</td>
<td>—</td>
<td>0.4</td>
<td>2.0</td>
<td>4.3</td>
<td>9.1</td>
</tr>
<tr>
<td>40</td>
<td>—</td>
<td>—</td>
<td>0.7</td>
<td>2.4</td>
<td>3.1</td>
</tr>
<tr>
<td>60</td>
<td>—</td>
<td>—</td>
<td>0.4</td>
<td>0.8</td>
<td>1.4</td>
</tr>
</tbody>
</table>

**MEASUREMENT ERROR**

Under the measurement paradigm from the *R. rutilus* experiments (5 min measurement period), the contribution of measurement variation to total variation as calculated using equation (2) in the small (RFR = 26:1) and large respirometers (RFR = 39:1) was 1.7 and 3.4%, respectively. Table III also shows the effects of increasing respirometer size and increasing measurement time.

Respirometry experiment constants for the Artifishal experiments using measured $r^2$ values were 0.28 and 0.51% for the small and large chambers, and the values were 1.07 and 1.30% for the *R. rutilus* experiments.

**DISCUSSION**

**SOURCES OF VARIATION**

Using the custom-made software, Artifishal, and a computer-controlled peristaltic pump hooked up to a supply of deoxygenated water, trials designed to simulate constant oxygen consumption of a fish, provided an estimate of measurement variation, with the higher variation having the lowest $r^2$. It follows, therefore, that the best option for decreasing measurement variation is to design the experiment with a high $r^2$ in mind. This can be accomplished by ensuring that the RFR in static respirometry is low ($<30$), and by controlling the measurement time so that the $r^2$ is maximized (Fig. 3). It should also be kept in mind that experiments carried out at low temperatures, where the dissolved oxygen content of the water is higher and metabolic rate is lower, will result in reduced rate of oxygen depletion (flatter slope). It may therefore be necessary to extend the measurement period significantly to achieve a satisfactory slope and $r^2$ (Svendsen et al., 2016).

The measurement distributions for the two simulated oxygen consumption rates, as well as the SMR portion of the Gaussian curve fit of one *R. rutilus* experiment are depicted in Fig. 5 for comparative purposes. It is clear that when comparing the two curves of similar SMR, the variance of the *R. rutilus* distribution is much wider than...
that of the simulated fish. This indicates that, in this case, the animal is the major source of variation in oxygen consumption and not the experimental setup.

It can also be seen from Figs 4 and 5 that there is nonetheless a certain error in the Artifishal setup, as $\dot{M}O_2$ decreased slightly over time, although initial testing of the system showed no change in injected water mass and oxygen content over the course of the experiment. Over prolonged test periods, the tygon tubing on the peristaltic pump wears down, which introduces variation in the amount of injected water. Small changes in room temperature and barometric pressure will also alter the solubility of oxygen in the injected water and thus create a certain variance, which in this experiment was not quantified. Given the sources of variation in the Artifishal setup, the real measurement error must therefore be even lower.

Since SA and behaviour are not experimental variables that can be controlled fully, they can only be minimized by reducing external sources of disturbance. As is reflected in Fig. 4, the *R. rutilus*, similar to *O. mykiss*, is a fairly calm fish that settles down in the respirometer and remains so, with relatively infrequent bouts of SA.

The $r^2$ value of the linear regression provides a useful tool for assessing quality of oxygen consumption determinations. In published papers where it has been noted, it is often the case that only measurements with $r^2$ higher than 0.90 and 0.95 are used in calculations, or that measurement times have been adjusted to obtain a given decrease in oxygen content (Behrens & Steffensen, 2007; Casselman et al., 2012; Svendsen et al., 2013). Forstner (1983) proposed that measurement times should be set to produce a drop in oxygen content of 10%. Using this as a rough guide, it can be seen from Fig. 6(a) that a 10% drop in oxygen content would result in a $r^2$ value of at least 0.98 at the highest calculated error level (1% unexplained error line). Thus, using this decrease in oxygen content as a set point will provide determinations of high $r^2$ even with a fair amount of noise. At lower noise levels, a smaller decrease in oxygen content can be used, as long as the $r^2$ remains high. Rather than choosing an arbitrary per cent drop in oxygen as a basis for setting the measurement time of the data collection cycle, an $r^2$ value should be chosen that is acceptable, 0.95 perhaps, and the data collection interval set for a time period sufficient to achieve that goal. By calculating a system’s REC after the first experiment, this can be done. An REC value of 0.28% for small respirometers and 0.51% for the larger respirometers reflects the fact that a larger RFR produces increased variation, which will therefore require a more substantial drop in per cent oxygen to achieve the same $r^2$ (i.e. 0.28% reduction in oxygen for an $r^2$ of 0.5 in the small respirometer v. 0.51% for the larger respirometer). Multiplying the REC by 5 (c.f. Table I) will produce a reduction in per cent oxygen large enough to produce an $r^2 > 0.96$ so that the measurement period should be long enough for the decrease in oxygen to exceed this threshold. As exemplified in Table III and Figs 3 and 6, however, extending the measurement time to produce a further reduction in oxygen in the chamber will only nominally increase the $r^2$.

As unexplained variance of the regression is determined by the slope of the regression and the noise around the mean, a regression can be obtained with a low slope and low noise that has the same unexplained variance as a determination with a high slope with high noise (see Figs 1, 3 and 6). Thus, oxygen consumption determinations with low $r^2$ values should not be used. A minimum threshold for $r^2$ should be set (at least 0.95) and all $\dot{M}O_2$ values below the $r^2$ threshold discarded. Likewise, using long running means on raw oxygen data during or after an experiment can make $r^2$ appear larger by smoothing both data and noise (moving points upwards in parallel to the y-axis in
Fig. 6. The relationship between the decrease in oxygen and $r^2$. Symbols representing *Rutilus rutilus* (stars) and Artifishal (circles) experiments are superimposed for comparative purposes. $\star$ and $\circ$, small respirometer; $\star\star$ and $\bigcirc$, large respirometer. When the percentage measurement error (total noise) $E\%$, is high, a larger decrease in oxygen will be necessary to achieve a satisfactory $r^2$. (a) The 0-15% error (---) and 1% error (----) and best fits of equation (1) (and ) for the Artifishal experiments are given. The REC value is the respirometry experimental constant. The REC value is specific to the experimental setup, in part describing the amount of noise inherent to the system. (b) The $r^2$ from all measurements collected over a 12 h standard metabolic rate (SMR) experiment, plotted against the resulting decrease in oxygen during each 10 min measurement period. , the numerical fit of equation (1), which can be used to calculate the REC value ( on the $x$-axis) (cf. Table I). Multiplying the REC by a factor of 5 will produce a value for decrease in oxygen necessary to achieve an $r^2 > 0.95$ (cf. Table I).

Fig. 6). This, however, does not necessarily provide more accurate oxygen consumption determinations because the unexplained variance remains the same.

In general, three things affect the $r^2$ when measuring oxygen consumption. First, if a leak were present, the change in oxygen level would not be linear and the $r^2$ would become smaller because the data would not fit a linear regression. A longer measurement period in this case would reduce the $r^2$ as well as the slope, because the oxygen content would continue to decline in an exponential manner approaching a new equilibrium, whose level would depend on size of the leak and oxygen consumption of the fish (Svendsen *et al.*, 2016). Secondly, the $r^2$ can be low if the measurement noise is large.
compared with the decline in oxygen level (i.e. a low signal-to-noise ratio). In Fig. 1 for instance, the linear decline in oxygen content was due to the fish and the noise was the variability in the oxygen trace itself. How the $r^2$ and linear regression slope of the experiment in Fig. 1 changed as the measurement period progressed is plotted in Fig. 3. Clearly, as the measurement time increased, the decline in oxygen content increased thus increasing the signal-to-noise ratio. Along with the higher drop in oxygen, more data points are sampled, both improving the $r^2$, up to a point. Lastly, low $r^2$ values can occur owing to random periods of short-term increases in oxygen consumption due to SA. This will result in a non-linear change in oxygen depletion and therefore a smaller $r^2$. Unfortunately while there are some steps the experimenter can take to reduce the external stimulation of the fish in the metabolic chamber, SA is often a characteristic of the fish itself.

**RESPIROMETER VOLUME-TO-FISH VOLUME RATIO**

Using an excessively large volume of water compared with the oxygen consumption, i.e. a large RFR, combined with an insufficiently long measurement period would decrease the signal-to-noise ratio, and is the primary cause of a low $r^2$. The large volume of water acts as an oxygen reservoir or bank that the fish has to consume in order for the decline in oxygen to be large enough for the experimenter to reliably determine metabolism. The *R. rutilus* experiment showed that a minor increase in respirometer volume had a negative effect on the precision of the SMR measurement, thus using excessively large static respirometer volumes (e.g. RFR > 200) should be avoided whenever possible.

The results demonstrate that less than a doubling of RFR from 26 to 39 has significant effects on the variation and $r^2$ in measured metabolic rates in the *R. rutilus* experiment. With this in mind, caution must be taken about interpreting literature reports where RFRs are extremely large [e.g. RFR c. 3.4–3499 (Graham & Baird, 1984) and RFR c. 110–999 (Urbina *et al.*, 2011)]. For instance, based on the reported oxygen consumption by Wardle *et al.* (1996), it can be calculated that a measuring period of >10 h would be needed to reach a decline of 10%, leading to a conclusion that the measurements can only reflect routine oxygen consumption. In theory, measurement error associated with large RFR can be reduced by increasing the measurement time to achieve a substantial enough decrease in oxygen content and a measurement with high $r^2$. A single measurement point that is calculated over a period of 1 h or more, however, may not be representative for a resting state of a fish, but rather expresses a combination of resting and routine metabolic rates. Thus, to get improved temporal resolution, it is important to achieve an appropriate RFR. Trying to correct for a large RFR with a long measurement period will presumably reduce scatter in $\dot{M}_{O2}$ associated with SA during the measurement period (Nyquist, 1928; Shannon, 1949).

Design constraints inherent to swimming respirometers (Wardle *et al.*, 1996) make it difficult to produce systems with RFRs lower than 150–200. As active metabolic rate is typically five to ten times higher than SMR, determination of oxygen consumption rates at higher swimming speeds is more precise. At low swimming speeds, however, it is necessary to use extended measurement periods to obtain satisfactory $r^2$ values due to the lower metabolism. It is important to note, therefore, that most swimming respirometry protocols (Brett, 1964) utilize identical measurement periods at all swimming speeds. As a consequence, metabolic rates determined at low swimming speeds
in large respirometers will result in lower $r^2$ and thus less precise oxygen consumption values. For these reasons, RFR values of higher than c. 500 should be avoided in swim tunnel respirometry [e.g. RFR c. 630 (Wardle et al., 1996), RFR c. 720 (Payne et al., 2015), RFR c. 5100 (Dewar & Graham, 1994) or RFR c. 17 000 (Graham et al., 1990)].

In summary, whenever possible, it is advisable to avoid large respirometer-to-fish volumes, as detecting a decrease in oxygen tension requires a longer measurement period. As a single measurement period will provide the average metabolism during this time, the longer the period, the more likely SA can occur. Further, the larger the respirometer used, the smaller the signal-to-noise ratio ($O_2$ decrease-to-noise) due to the oxygen reservoir problem. In order to ensure the veracity of the oxygen consumption measurement, therefore, the coefficient of determination, $r^2$, should be used. As a rule of thumb, this measure should be above 0.95 (REC × c. 5) to be certain of the precision of the determined oxygen consumption (slope of the linear regression).

References


