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METHODS AND TECHNIQUES

Short-duration respirometry underestimates metabolic rate for discontinuous breathers

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ABSTRACT

Metabolic rate is commonly estimated from rates of gas exchange. An underappreciated factor that can influence estimates is patterns of pulmonary respiration. Amphibians display discontinuous respiratory patterns, often including long apnoeas, in addition to cutaneous gas exchange. The contribution of cutaneous exchange increases at low temperatures when metabolic rate is low. Because of the relatively low permeability of skin, measurements that disproportionately capture cutaneous exchange can produce underestimates of metabolic rate. The permeability of amphibian skin to CO_2 is greater than that to O_2; therefore, calculating the ratio of whole-animal CO_2 emission to O_2 uptake (the respiratory exchange ratio, RER) can be used to avoid underestimates of metabolic rate by ensuring that observed values of RER fall within the normal physiological range (~0.7 to 1). Using data for cane toads, *Rhinella marina*, we show that short-duration measurements lead to underestimates of metabolic rate and overestimates of RER. At low temperatures, this problem is exacerbated, requiring over 12 h for RER to fall within the normal physiological range. Many published values of metabolic rate in animals that utilise cutaneous exchange may be underestimates.

KEY WORDS: Respiratory exchange ratio, Cutaneous, Gas exchange, Calorimetry, Cold, Amphibian

INTRODUCTION

Indirect calorimetry, commonly referred to as respirometry, is a technique used to measure the oxygen uptake ($\dot{V}_{O_2}$) and/or carbon dioxide emission ($\dot{V}_{CO_2}$) of animals and is used as a common proxy for energy expenditure/metabolic rate (MR) (Lighton, 2008). For ectotherms, perhaps the most common measurement of MR is standard metabolic rate (SMR), which is the MR of a non-reproductive, post-absorptive and inactive animal, measured at a known temperature during their resting phase (Careau and Garland, 2012). Some of these conditions are easy to satisfy, such as controlling the thermal environment or the reproductive status of the subject. Others are inherently difficult, such as determining whether the animal is truly inactive or 'at rest' for a sufficiently long period of time, though real-time (moment to moment) measurements of gas exchange (typically made using flow-through systems) and some method of monitoring activity can help to distinguish periods of rest from periods of arousal. If a given experimental design or protocol fails to meet or account for these conditions, then measurements will capture energy expenditure above that of maintenance metabolism. A potentially underappreciated factor that can compromise accurate estimates of energy expenditure from measurements of $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ is patterns of pulmonary respiration. While cellular respiration of an animal under SMR conditions may be relatively constant, respiratory gas exchange in many animals occurs via discrete pulmonary inspiration and expiration events. Thus, estimates of rates of energy expenditure derived from patterns of gas exchange must span multiple breaths over time to obtain a reasonable estimate of mean $\dot{V}_{O_2}$ or $\dot{V}_{CO_2}$. Among terrestrial vertebrates, mammals and birds often display continuous pulmonary respiration patterns whereas reptiles and amphibians often display patterns that are discontinuous (Milsom, 1991). For continuous breathers, breaths occur consecutively without pause, allowing an average measure of $\dot{V}_{O_2}$ or $\dot{V}_{CO_2}$ over a relatively short time period. For discontinuous breathers, breaths can be punctuated by long apnoeas. These long apnoeas require longer measurement times to be implemented, which can make accurate measurements of mean $\dot{V}_{O_2}$ or $\dot{V}_{CO_2}$ more challenging.

In general, amphibians derive a considerable proportion of consumed oxygen via cutaneous gas exchange (Lillywhite and Maderson, 1988). The degree of reliance upon pulmonary ventilation reflects the adequacy of cutaneous gas exchange to meet metabolic demands. This has been demonstrated in bullfrogs, where individuals with higher MRs derive a greater proportion of oxygen consumed from pulmonary gas exchange (Gottlieb and Jackson, 1976). As MR increases approximately exponentially with temperature in ectotherms, the reliance upon pulmonary ventilation is also thermally sensitive (Guimond and Hutchison, 1968; Kruhøffer et al., 1987; Whitford and Hutchison, 1965). As a result, ventilation rate and the length of apnoeas in amphibians are proportional and inversely proportional, respectively, to temperature (Kruhøffer et al., 1987). This can be easily observed in a flow-through respirometry system. Fig. 1 shows two typical O_2 and CO_2 traces in a flow-through respirometry system at 10 and 25°C. Fig. 1A shows traces recorded at 25°C, in which the regular peaks indicate relatively constant inspiration and expiration. Fig. 1B shows traces recorded at 10°C, and it can be seen that the peaks are less frequent and separated by significant apnoeas, with the highlighted section indicating a pulmonary apnoea of almost 3 h.

If the rate of cutaneous oxygen uptake is sufficient to supply the oxygen consumed by cellular respiration, then pulmonary respiration is not required. If cutaneous uptake is insufficient to meet the demands of cellular respiration, i.e. a supply bottleneck, then pulmonary respiration is required and the presence of apnoeas within the measurement period may lead to underestimates of MR. We know that gas exchange is still occurring during this extended apnoea because the recorded levels of excurrent O_2 and CO_2 are relatively stable but are lower and higher, respectively, than the baseline levels established before the animal was added to the chamber. The fact that there are peaks at all, which occur when

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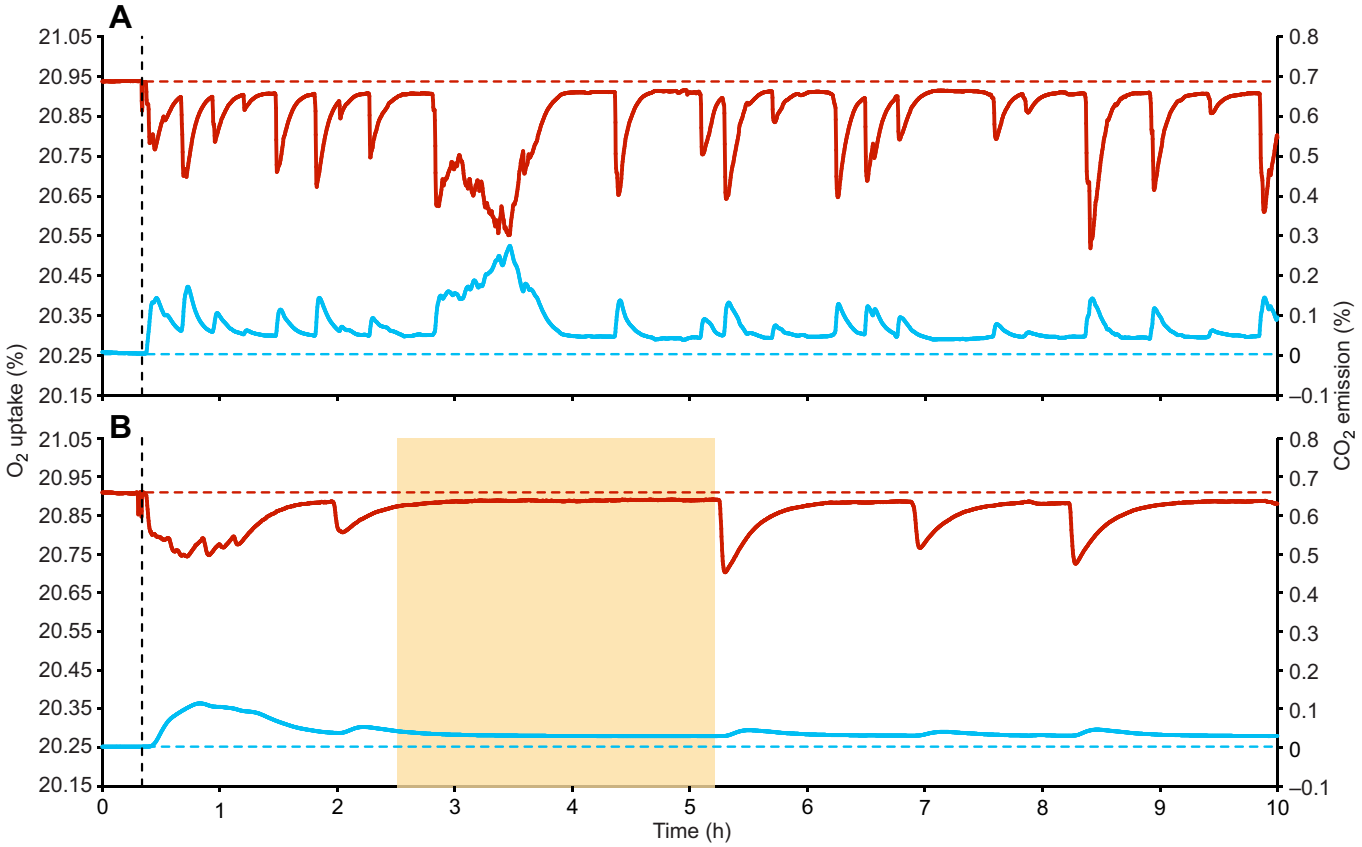

Fig. 1. Gas traces for two cane toads (*Rhinella marina*) at different temperatures in an open-flow respirometry system. Data were obtained over a 10 h period at (A) 25°C and (B) 10°C. The vertical dashed line indicates the time point at which the animals were placed within the respirometry chamber. The variable red line represents the concentration (%) of O_2 and the dashed horizontal red line aligns with the concentration (%) of O_2 in the chamber before the animal was placed within it. The variable blue line represents the concentration (%) of CO_2 and the dashed horizontal blue line aligns with the concentration (%) of CO_2 in the chamber before the animal was placed within it. The highlighted section indicates an extended pulmonary apnoea.

breaths are taken, demonstrates that cutaneous respiration is insufficient to supply demand. The question then is whether enough breaths were captured during the sampling period for the measurement of gas exchange to accurately reflect the mean rates of O_2 consumption and CO_2 production by cellular respiration.

Instead of making subjective assessments regarding the shape of the gas traces, we can determine whether our estimate of MR is distorted as a result of the presence of pulmonary apnoeas by measuring the respiratory exchange ratio (RER), which is calculated as the ratio of carbon dioxide produced to oxygen consumed. The RER for a post-absorptive animal fuelled by aerobic metabolism and measured in steady state should normally vary between 0.7 and 1.0, depending on the combination of substrates being metabolised (Schmidt-Nielsen, 1997), but RER values outside this normal physiological range are occasionally observed (Walsberg and Hoffman, 2005). The permeability of O_2 and CO_2 across the skin is not equal, being higher for CO_2 than for O_2 because of the greater solubility and diffusion rate of CO_2 through tissue and water (Feder and Burggren, 1984; Lillywhite and Maderson, 1988). With this information, we can infer that if RER is greater than 1, because oxygen uptake during the sample period is insufficient to meet the demands of cellular respiration, the measured rate of oxygen uptake underestimates the rate of cellular oxygen consumption. These points are clearly illustrated in Fig. 2, which depicts a segment of a trace that we recorded. The relative increase in $\dot{V}_{O_2}$ during peaks, which coincides with breaths, compared with $\dot{V}_{O_2}$ during apnoea is greater than the relative increase in $\dot{V}_{CO_2}$ during peaks compared with $\dot{V}_{CO_2}$ during apnoea. This indicates that the difference between the level of CO_2 released cutaneously during apnoea compared with the level of CO_2 produced by cellular respiration is relatively less than the difference between O_2 absorbed cutaneously and the O_2 demand of cellular respiration. The magnitude of this difference in O_2 uptake under the two conditions demonstrates the degree to which cutaneous uptake is insufficient to meet metabolic demands. If the sample for estimating MR is taken between peaks (Fig. 2, labelled a) we underestimate cellular respiration, which can also be inferred from the high value of the RER ($\dot{V}_{O_2}$ of 0.35 ml h^{-1}, RER of 1.4). Considering that the apnoea from which this sample is taken lasts approximately 1.5 h, it seems plausible that a measurement could be taken that captures cutaneous respiration exclusively. A sample that includes one of the peaks (Fig. 2, labelled b) returns a higher MR and a lower RER ($\dot{V}_{O_2}$ of 0.72 ml h^{-1}, RER of 0.7, i.e. higher and lower values, respectively, than sample a). It is common practice to take the lowest value from a longer measurement period under the assumption that this will give the best estimate of SMR. If measuring discontinuous breathers at low temperatures where significant apnoeas are present, the lowest value presumed to represent 'at rest' may fall between breaths and thus underestimate MR.

Here, we demonstrate the relationship between measurement time, temperature, MR and RER in the cane toad, *Rhinella marina* (Linnaeus 1758). To do this, we measured gas exchange for cane toads in a flow-through respirometry system at 10 and 25°C for 22 h and calculated mean $\dot{V}_{O_2}$, $\dot{V}_{CO_2}$ and RER over multiple durations

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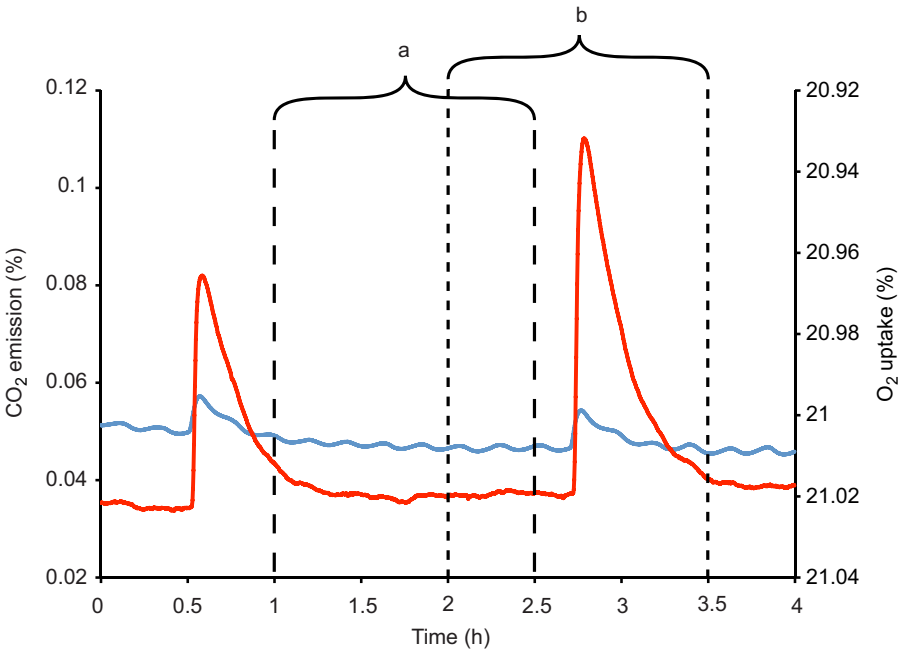

Fig. 2. Section of gas traces for a cane toad at 10°C in an open-flow respirometry system. The red line represents the concentration (%) of O_2 and the blue line represents the concentration (%) of CO_2. The *x*-axis crosses both *y*-axes at baseline (empty chamber) values. The axis for O_2 has been flipped so that the size of the peaks and troughs relative to baseline concentrations can be compared between the two gases. Labels a and b represent two different samples which produce very different values for O_2 uptake and respiratory exchange ratio (RER) if used. Sample a produces a $\dot{V}_{O_2}$ of 0.35 ml h^{-1} and a RER of 1.4, and sample b produces a $\dot{V}_{O_2}$ of 0.72 ml h^{-1} and a RER of 0.7 (see Introduction for more details).

increasing in 1 h intervals. We argue that it is critical to measure RER in discontinuous breathers that utilise cutaneous respiration in order to determine the measurement time that will be sufficient to avoid underestimating MR, and that this is particularly important for low-temperature measurements.

MATERIALS AND METHODS

All experimental procedures were approved by the University of Queensland Native and Exotic Wildlife and Marine Animals ethics committee (certificate SBS/350/12/ARC).

For 10 cane toads, MR was estimated from measurements of resting oxygen uptake at 25 and 10°C. Measurements were taken for 22 h between 13:00 h and 11:00 h the following day. Prior to being measured, toads were fasted for a minimum of 5 days (Halsey and White, 2010; Secor and Faulkner, 2002; Winwood-Smith et al., 2015). The first 2 h of measurement time were discarded to allow for gas washout and settling time of the animal. Mean $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ were calculated for measurement windows of 1–20 h (10°C) and 1–8 h (25°C) increasing in 1 h increments, according to standard equations (Lighton, 2008). The lowest mean $\dot{V}_{O_2}$ observed during the 20 h respirometry period was calculated for each measurement window duration, and was used as an estimate of MR.

The resting rate of oxygen uptake ($\dot{V}_{O_2,rest}$, ml O_2 h^{-1}) of an individual toad was measured using positive-pressure flow-through respirometry, as previously used for this species (Winwood-Smith et al., 2015). Atmospheric air was drawn from outside using a pump (TR-SS3, Sable Systems, Las Vegas, NV, USA) and scrubbed of CO_2 using soda lime and water vapour using Drierite before passing through a mass-flow controller (GFC17, Aalborg, Orangeburg, NY, USA) that regulated flow rate to a nominal value of 50 ml min^{-1}. Mass-flow controllers were calibrated using a NIST-traceable bubble film flow meter (1-10-500 ml, Bubble-O-Meter, Dublin, OH, USA). After passing through the mass-flow controller, air was rehumidified using a 1 l gas washing bottle (Schott, French's Forest, NSW, Australia) before passing through the respirometry chamber (460 ml plastic container). The humidifying gas washing bottle and respirometry chamber were housed inside a temperature-controlled cabinet (ERI140, ProSciTech, Thuringowa, QLD, Australia) that regulated the test temperature to 10±0.5 or 25±0.5°C. The air was then scrubbed of water vapour using Drierite before passing through a CO_2 analyser (LI-7000, LI-COR, Lincoln, NE, USA) and an O_2 analyser (Oxzilla II, Sable Systems). The CO_2 and O_2 analysers were interfaced with a PowerLab 8/30 A/D convertor (ADInstruments, Bella Vista, NSW, Australia), which recorded fractional concentrations of CO_2 and O_2 in the excurrent air at a frequency of 10 Hz. The CO_2 analyser was calibrated with dry CO_2-free air and a certified gas mix (0.386±0.008% CO_2 in N_2, BOC Gases, Wetherill Park, NSW, Australia), and the O_2 analyser was calibrated using dry, CO_2-free air.

RESULTS AND DISCUSSION

In the present study, we measured $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ of cane toads in a flow-through respirometry system at 10 and 25°C. Here, we present mean values of $\dot{V}_{O_2}$ and RER at both temperatures for sample durations ranging from 1 to 20 h. At both treatment temperatures, we found a relationship between measurement window duration and MR, with shorter measurement windows producing lower estimates of MR (Fig. 3, top panels) coupled with higher estimates of RER (Fig. 3, bottom panels). The take-home message from this is that when measurement times are short, mean RER is high, and this high and physiologically improbable estimate of RER is a clear indicator that MR is being underestimated. This effect is exaggerated at lower temperatures where longer measurement times are required to obtain reasonable values for RER and thus accurate estimates of MR. This occurs as a result of the long apnoeas that occur at low temperatures when MR is lower (an approximately 70% reduction in MR at 10°C compared with 25°C) and the reliance upon cutaneous respiration is greater. At this low temperature, apnoeas lasting as long as 3–4 h were regularly observed in the present study.

Generally, we expect a post-absorptive animal at rest to exhibit a RER between approximately 0.7 and 1.0, depending on the combination of substrates being metabolised (Ferrannini, 1988), though more extreme values are occasionally observed (Walsberg and Hoffman, 2005). In the case of the present study, RER plateaus occurred at values of approximately 1 and 0.85 in the 10 and 25°C treatments, respectively. This may reflect an effect of temperature on

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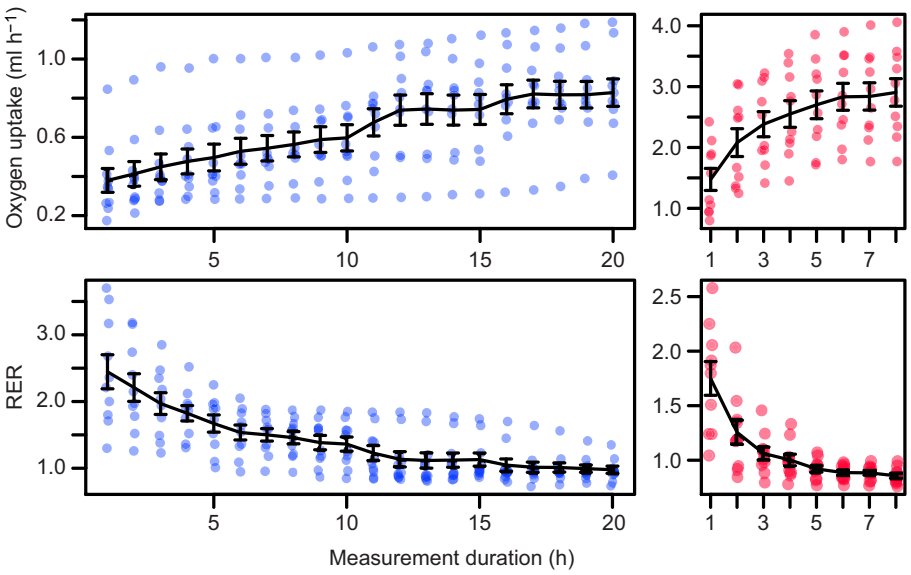

Fig. 3. Mean resting oxygen uptake and mean RER for cane toads at different temperatures. Data were obtained at 10°C (left panels, blue markers) and 25°C (right panels, red markers) over different measurement periods. Data points represent values for individual animals and n=10 for each time period. The solid line connects mean values and error bars represent s.e.m.

substrate metabolism. Without detailed investigation to determine the combination of substrates being metabolised, we cannot necessarily be sure of exactly what value of RER to expect. While the reason for the temperature effect on RER in the present study is unknown, what is important is that these data show clearly that certain conditions must be satisfied to obtain accurate estimates of MR using respirometry: measurement length must be sufficient for values of RER to plateau, and values of RER should plateau within normal physiological range. If these conditions are met, we can be confident that we are not underestimating MR. It is important to acknowledge that long measurement times via flow-through respirometry measuring $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ present a significant logistical burden. The full setup used for the measurements in this study is limited to measuring two animals simultaneously, and so obtaining a large sample size across multiple treatments would require a considerable amount of time. If this is limiting, we suggest that a precision flow-through system be used to establish what measurement time is required to obtain accurate estimates of MR at the desired test temperatures. Once this is known, other methods can be used to increase throughput, such as a fibre-optic oxygen microsensor (Klimant et al., 1995) in conjunction with closed respirometry chambers large enough to avoid significant CO_2 build up during the measurement period, or with intermittent flushing to allow for long measurement times (e.g. Reilly et al., 2013) which can efficiently measure a high number of animals simultaneously at relatively low cost. Such systems allow the use of a single fibre-optic sensor to take recordings from a multitude of chambers in sequence, repeatedly over time, as opposed to a flow-through system, which requires a dedicated channel on an analyser for a single chamber for the duration of the measurement period. Alternatively, one could also eschew indirect calorimetry entirely in favour of direct calorimetry and avoid the challenges of estimating energy expenditure, or oxygen consumption, from measurements of oxygen uptake.

Here, we have demonstrated the importance of measuring RER for accurately estimating $\dot{V}_{O_2}$ via indirect calorimetry in discontinuous breathers that utilise cutaneous respiration. This is particularly significant at low temperatures where longer measurement times are required to obtain accurate estimates. It is crucial to note that in the data we have presented, the differences in $\dot{V}_{O_2}$ vary by more than twofold from the shortest to the longest measurement times used at each temperature, and underestimate $\dot{V}_{O_2}$ by different amounts for the same measurement time at different temperatures (e.g. a 7 h mean would underestimate $\dot{V}_{O_2}$ by approximately 50% at 10°C while producing an accurate estimate at 25°C; see Fig. 3). The problem we identify is therefore not simply a matter of shaving small levels of error from otherwise reasonably accurate estimates. Failures to address this problem can produce large inaccuracies in measurements of $\dot{V}_{O_2}$ and effect sizes across experimental conditions, leading to false positive or false negative results. These concerns are not limited to amphibians. Discontinuous breathing patterns associated with non-physiological RERs have been observed in other taxa with different respiratory physiology (Lighton, 1988; Lighton and Garrigan, 1995; Morris et al., 1994). The complications and solutions we have described may be of benefit to a broad range of study systems.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: C.R.W.; Investigation: H.S.W.-S.; Data curation: H.S.W.-S.; Writing - original draft: H.S.W.-S.; Writing - review & editing: C.R.W.; Visualization: H.S.W.-S.; Supervision: C.R.W.; Project administration: H.S.W.-S.; Funding acquisition: C.R.W.

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