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METHODS AND TECHNIQUES

Measuring metabolic rates of small terrestrial organisms by fluorescence-based closed-system respirometry

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ABSTRACT

We explore a recent, innovative variation of closed-system respirometry for terrestrial organisms, whereby oxygen partial pressure (P_{O_2}) is repeatedly measured fluorometrically in a constant-volume chamber over multiple time points. We outline a protocol that aligns this technology with the broader literature on aerial respirometry, including the calculations required to accurately convert O_2 depletion to metabolic rate (MR). We identify a series of assumptions, and sources of error associated with this technique, including thresholds where O_2 depletion becomes limiting, that impart errors to the calculation and interpretation of MR. Using these adjusted calculations, we found that the resting MR of five species of angiosperm seeds ranged from 0.011 to 0.640 ml g^{-1} h^{-1}, consistent with published seed MR values. This innovative methodology greatly expands the lower size limit of terrestrial organisms that can be measured, and offers the potential for measuring MR changes over time as a result of physiological processes of the organism.

KEY WORDS: Respirometer, Constant volume, Q_2 technology, Oxygen consumption, Metabolism, Seed biology

INTRODUCTION

One of the basic processes of life is the oxidation of substrates, termed metabolism (Kleiber, 1961). Simple measures of O_2 consumption to infer metabolic rate (MR) are difficult to compare across diverse species because patterns of ecology and evolution influence both MR (Isaac and Carbone, 2010; White and Kearney, 2014) and its method of measurement (Lighton, 2008; Vleck, 1987; Withers, 2001). Calculating MR has a long heritage from Lavoisier in the 1770s (Hulbert and Else, 2004; Lighton, 2008), and there are many methods for quantifying MR, but indirect calorimetry measuring O_2 consumption and/or CO_2 production has gained ascendancy owing to the ease of measuring gas exchange by living organisms (Kleiber, 1961; Lighton, 2008; Withers et al., 2016).

Two respirometry methodologies (closed and open system) are based on a simple rationale – the measurement of O_2 uptake and/or CO_2 excretion over a known time course. In a closed system, the organism is kept in a sealed container of known air volume and the change in the O_2 (and/or CO_2) composition of air is measured over a time interval (Lighton, 2008; Vleck, 1987). Typically, closed systems are constrained to one-off measurements, from the start to end of a trial, allowing only an average MR to be calculated. Closed systems can be optimised to measure small organisms, either by reducing chamber volume or by extending measurement time to maximise the amount of gas exchange. Conversely, open, flow-through systems allow continual, real-time measurement of MRs in changing states of metabolic activity (Withers, 2001). Measurement of small organisms in an open system, however, requires low flow rates to allow O_2 depletion levels to remain within detectable limits of the analyser, constraining open systems to a lower mass limit.

In both open and closed respirometry, the measurement of O_2 partial pressure (P_{O_2}; kPa) has traditionally been made using paramagnetic (Hill, 1972; Nunn et al., 1964), fuel-cell (Torda and Grant, 1972) or polarographic gas analysers (Clark et al., 1953), whereas CO_2 partial pressure (P_{CO_2}) measurements are determined using infra-red gas analysers (Fowler, 1949). More recently, a novel technique has emerged that measures P_{O_2} by predictable and repeatable fluorometric quenching of metal organic dye located inside the respirometry chamber (e.g. Köster et al., 2008; Szela and Marsh, 2005; Bradford et al., 2013). Such fluorescent analysers have been used for several years to measure MRs of small, aquatic organisms (Alton et al., 2012; Bywater et al., 2014; Köster et al., 2008; Szela and Marsh, 2005) but less commonly for small, terrestrial or diving organisms (Bradford et al., 2013; Matthews and Seymour, 2010).

Typically, closed-system respirometry has been used to measure the P_{O_2} change from the start to the end of a trial, by analysing an air sample from the chamber, and average MR is calculated (Vleck, 1987). A key advantage of fluorometric O_2 measurement, however, is that the non-invasive nature of the *in situ* fluorescence dye allows for sequential, repeated measurement of the same organism in the sealed metabolic chamber (e.g. in aquatic systems; Alton et al., 2012; Szela and Marsh, 2005). The automation of such repeated measurements has recently been specifically developed for measuring O_2 consumption in an aerial closed respirometry system of seeds (Bradford et al., 2013) and leaf tissue of *Arabidopsis* (Scafaro et al., 2017). Previous studies have used standardised O_2 depletion as a proxy for MR of seeds from highly selectively bred cultivars; for example, in relation to the seed quality of tomato and lettuce varieties (Bello and Bradford, 2016; Bradford et al., 2013). Here, we refine the handling and processing of closed-system fluorometric data to calculate MR in traditional units (e.g. ml O_2 h^{-1}, or ml O_2 g^{-1} h^{-1}), which allows the quantitative comparative analysis of MR variation expected for diverse, wild organisms.

Making repeated measurements of P_{O_2} and continuously calculating MR provides all the benefits normally ascribed to flow-through respirometry, including the ability to detect periods of altered metabolic states such as metabolic depression (Guppy and Withers, 1999). By aligning repeated-measurement fluorescence oxygen-sensing technology with broader respirometric theory and practice, we believe this represents a major advance in the

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List of symbols and abbreviations

ATPS	ambient temperature and pressure, saturated with water vapour
$F_{E_{O_2}}$	subsequent (end) fractional O_2 value
$F_{I_{O_2}}$	initial fractional O_2 value
$F_{S_{O_2}}$	fractional standardised atmospheric O_2
MR	metabolic rate
P_b	barometric pressure
P_{CO_2}	CO_2 partial pressure
P_{O_2}	O_2 partial pressure
RER	respiratory exchange ratio
RH	relative humidity
RMR	resting metabolic rate
STPD	dry standard temperature and pressure
SWVP	saturation water vapour pressure
T_a	chamber temperature
$\dot{V}_{O_2}$	volume of oxygen consumed (i.e. metabolic rate)
v_g	gas volume

measurement of MR for the great diversity of organisms too small for existing techniques. However, the location of the fluorescent sensor within a sealed aerial respirometry chamber is problematic, compared with conventional closed-system respirometry. Air sealed within the respirometric chamber is subject to P_{O_2} changes not only by organismal metabolism but also by changes in air pressure within the sealed chamber (e.g. owing to ambient temperature, water vapour pressure and P_{CO_2} changes). This substantially alters some of the assumptions and calculations of standard closed-system respirometry (Vleck, 1987).

Here, we have adapted several of the metabolic calculations of standard closed-system respirometry (Vleck, 1987) to align repeated-measurement fluorescence closed-system aerial respirometry with expectations of conventional closed system respirometry. Since repeated-measurement closed-system aerial respirometry has been exclusively used in the field of seed biology, we have used seeds as model species to apply these calculations for resting MR (RMR). Although the calculation of MR can provide insight into patterns and processes contributing to biological variation, miscalculation of MR can easily result from methodological and arithmetical errors (Lighton, 2008). We discuss these errors, and describe how they may be accounted for, eliminated or reduced in magnitude.

MATERIALS AND METHODS

Closed system respirometry

We used Q_2 technology (ASTEC Global, The Netherlands) for repeated-measurement fluorometric respirometry of seeds in air-filled 1800 µl closed plastic vials (Bradford et al., 2013; Scafaro et al., 2017). The fluorometric O_2 detector is suspended from a robotic arm above a series of vials laid out in a grid; the inner surface of the vial cap is impregnated with a fluorescent dye. Automated movement of the detector provides repeated measurement of internal P_{O_2} for vials at user-determined intervals (Scafaro et al., 2017). The fluorometer is calibrated before each set of measurements using standards containing ambient air (designated as fractional standard atmospheric O_2; i.e. $F_{S_{O_2}}$=1.0) and O_2-free air ($F_{S_{O_2}}$=0.0) above a saturated sodium dithionite solution (Scafaro et al., 2017). All organismal O_2 measurements are presented as standardised O_2 fractions.

The calculations we report here account for chamber size. The Q_2 system has standardised chamber types and sizes, ranging from 5500 µl chambers on a 24-well plate (384 chambers for 16 plates) to 500, 1800 and 2200 µl chambers on a 48-well plate (768 chambers for 16 plates), and 515 µl chambers on a 96-well plate (1536 chambers for 16 plates); we used 24- and 48-well plates. We make the general assumption that the respirometry chamber is sealed, impermeable to gases, and rigid (constant volume).

Calculation of MR

We outline here the calculation of absolute MR ($\dot{V}_{O_2}$; ml O_2 h^{-1}) and highlight some assumptions that currently remain untested for repeated-measures closed-system fluorometric respirometry, modifying conventional closed respirometry calculations (Vleck, 1987). Automated repeated and long-duration measurements possible with fluorometric respirometry result in large volumes of raw, 'noisy' data for calculation. We have developed an R script that automates our calculations, constructed around the specific data file structure of the Q_2 software, but these should be readily translatable to other data from other systems. The R script is available in the supplementary material, and is heavily annotated to provide guidance to its use (Script 1).

Respirometry chamber air volume

The initial chamber volume at dry standard temperature and pressure (STPD) conditions must be known, and corrected for the space occupied by the organism(s) in each chamber (Vleck, 1987).

The initial gas volume of the metabolic chamber (v_g; ml) at ambient temperature and pressure, saturated with water vapour (ATPS), can be calculated from the total chamber volume (v_0; ml), the mass of the organism(s) (M), the number of individuals (n) and their density (ρ; g ml^{-1}):

$$v_g(\text{ATPS}) = v_0 - \left(\frac{M}{\rho} \times n\right). \quad (1)$$

The chamber v_g contains O_2, CO_2, 'N_2' ('N_2' representing nitrogen and the other inert gases in atmospheric air) and H_2O vapour:

$$v_g(\text{ATPS}) = v_{O_2} + v_{CO_2} + v_{N_2} + v_{H_2O}. \quad (2)$$

We assume initial v_{CO_2} is 0 (see below) and that initial v_{H_2O} corresponds to saturation. The v_g(ATPS) is converted to STPD air (P_b=101.3 kPa; T_a=273°C) volume as:

$$v_g(\text{STPD}) = v_g(\text{ATPS}) \times \frac{273}{273 + T_a} \times \frac{P_b - \text{SWVP}}{101.3}, \quad (3)$$

where T_a is the initial chamber temperature (°C), P_b is the initial barometric pressure (kPa) and SWVP is the saturation water vapour pressure (kPa).

Changing air constituents and partial pressures

The measurement of P_{O_2} by fluorometry occurs inside the sealed respirometry chamber, unlike conventional closed-system respirometry, where an air sample is removed from the chamber for analysis. Simplistically, the P_{O_2} is dependent on barometric pressure inside the closed chamber at the time of measurement (P_b; kPa), but P_b inside the respirometry chamber is not known (or measurable) over repeated measurements. The calculation of P_{CO_2} depends on the percent CO_2 content of chamber air (which the Q_2 does not measure), and chamber pressure. The partial pressure of N_2 inside the respirometry chamber is not known, but we assume that the molar N_2 content of the air inside the respirometry chamber remains constant, i.e. it is metabolically inert and does not

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exchange with N_2 inside the organism(s). The water vapour pressure of the air inside the respirometry chamber is determined by the SWVP, which we account for in calculating v_g(STPD) (Eqn 3).

To calculate MR, P_{O_2} (from FS_{O_2}) must be converted to the fractional O_2 content (F_{O_2}; $\%O_2/100$). In a conventional closed respirometry system (e.g. Vleck, 1987), a dry CO_2-free gas sample is analysed at the start (i.e. initial FI_{O_2}) and at the end (i.e. final FE_{O_2}) of the respirometry period, and the difference is used to calculate $\dot{V}_{O_2}$ (i.e. the P_{O_2} is measured and is converted to F_{O_2} at STPD). The F_{O_2} can also be measured for a second dry sample that still contains CO_2 to calculate CO_2 production, and it can be measured for a further moist sample to calculate evaporative water loss (Vleck, 1987). In essence, conventional closed-system respirometry is considered an ambient pressure system, with initial and final P_{O_2} values measured at atmospheric pressure and converted to FI_{O_2} and FE_{O_2} (which, as well as v_g, are STPD).

However, repeated-measurement fluorometry differs in that the respirometry chamber remains sealed, and the P_{O_2} is measured *in situ*. This has the advantage of allowing repeated measurements of chamber P_{O_2}, but has the disadvantage that any change in P_b within the chamber (e.g. resulting from fluctuating temperature, CO_2 accumulation and evaporation or condensation) will proportionally influence the P_{O_2}. As far as we can tell, such changing partial pressures between successive measurements are not currently accounted for using commercially available automated fluorescence analysers; indeed, the descriptions regarding the temperature corrections described in the Q_2 user manual (Anonymous, 2014) refer to analysis corrections made in response to the fluorometer sensor temperature, not *in situ* correction.

Changes in water vapour pressure inside the chamber by evaporation or condensation also alter the P_{O_2}. In the worst case, an initially dry chamber atmosphere will almost certainly reach H_2O vapour saturation throughout a metabolic trial because of evaporation from the organism(s), which would increase internal chamber P_b. This is an important consideration for calculating v_g of the chamber prior to sealing it. As such, we advocate allowing the respirometry chamber to reach saturation water vapour pressure prior to sealing, to avoid any effects of subsequent evaporation on barometric pressure in the chamber (hence, measured P_{O_2}). The potential errors associated with these approaches are discussed below. Once the respirometry chamber is sealed, however, and assuming the chamber is rigid, changing water vapour pressure affects P_{O_2} and O_2 mole fraction in opposite directions. Effectively, changes in P_{O_2} and mole fraction of O_2 balance each other out, which is the previously unreported basis of how the Q_2 fluorescence analyser measures P_{O_2} independent of chamber humidity (Bello and Bradford, 2016; Bradford et al., 2013; Van Asbrouck and Taridno, 2009).

Accounting for resampling effects

The Q_2 fluorescence analyser attempts to account for resampling effects (sensor drift and *in situ* physical changes in P_{O_2}) by standardising measurements of P_{O_2} against a known zero (0% O_2=0.0 FS_{O_2}) and atmospheric air (20.95% O_2=1.0 FS_{O_2}) for every series of repeated measurements. These standards are maintained in the same incubator as the experimental chambers, and are therefore subject to the same ambient temperature fluctuations as the experimental chambers, but these fluctuations are not recorded. The environment within the respirometry chambers can differ in terms of the standards as a result of experimental treatment, and also the time between measurement of the standards and measurement of the experimental chambers. We found it necessary to further correct the measured P_{O_2} of respirometry chambers by incorporating a series of saturated [100% relative humidity (RH)] 'blank' chambers (Szela and Marsh, 2005). Since a blank chamber should always return an FS_{O_2} of 1.00, being identical to the upper standard, this can be used as a further control to correct for any fluctuations in the data output:

$$FS_{O_2} = \frac{FS_{O_2,\text{sample}}}{FS_{O_2,\text{blank}}}, \tag{4}$$

where $FS_{O_2,\text{sample}}$ denotes the raw standardised value and $FS_{O_2,\text{blank}}$ denotes the raw standardised value measured for the 'blank' chamber.

For the purposes of applying the calculations derived from Vleck (1987), the initial fractional O_2 value (FI_{O_2}) and a subsequent fractional O_2 value (FE_{O_2}) can be calculated by the multiplication of the standardised output derived from Eqn 4 by 0.2095, assuming atmospheric air (FI_{O_2}) is 0.2095 (dry, CO_2-free air):

$$FI_{O_2}(t_0) = FS_{O_2}(t_0) \times 0.2095, \tag{5}$$

$$FE_{O_2}(t_n) = FS_{O_2}(t_n) \times 0.2095, \tag{6}$$

where FS_{O_2} denotes the raw standardised value, t_0 denotes the initial measurement and t_n denotes the measurement after n periods.

Repeated sampling complicates the simple calculation of MR by Vleck (1987) using FI_{O_2} and a single FE_{O_2}, and we suggest for repeated measurements that the previous O_2 measurement be assigned as FI_{O_2} and the next as FE_{O_2} to calculate MR over the elapsed interval between the two measurements. This introduces substantial MR variation as the signal to noise ratio between consecutive measurements of F_{O_2} made over a short period of time is quite low, so we advocate a moving window average (Kenney and Keeping, 1962) or linear regression (Crispin and White, 2013) to account for this variation. This effectively applies a statistical smoothing across the data to further minimise any changes due to noise between consecutive measurements, such as negative MRs, that result from temperature and pressure oscillations in the respirometry chambers, and signal noise. Data averaging may be less necessary for organisms with higher MR.

Biological effects on chamber atmosphere

Over time, the changing partial pressure of CO_2 by metabolism in the chamber needs to be accounted for. This can be done by estimating a respiratory exchange ratio [RER; the ratio of CO_2 excreted by the organism(s) relative to the O_2 consumed] to eliminate the effect of CO_2 on FE_{O_2}:

$$\dot{V}_{O_2} = \frac{v_g(\text{STPD}) \times (FI_{O_2} - FE_{O_2})}{1 - FE_{O_2}(1 - \text{RER})}. \tag{7}$$

Following Vleck (1987), RER can be assumed to range between 0.7 and 1; the maximum error associated with assumption of 0.7 or 1.0 is 6.4% and the maximum potential error is 3.2% for an assumed RER of 0.85 (Vleck, 1987).

In summary, there are a number of points where repeated-measures closed-system fluorometric respirometry differs from standard closed-system respirometry. In the latter, CO_2 and H_2O can be removed prior to measurement of the P_{O_2} (Lighton, 2008; Vleck, 1987), but this is not possible for fluorometric measurements because P_{O_2} is measured inside the sealed respirometry chamber. Rather, any evaporation after sealing the respirometry chamber needs to be accounted for to calculate v_g(STPD), changes in P_{O_2} over time by changes in ambient temperature and sensor drift need to be accounted for, and the contribution of CO_2 production must be accounted for by assuming an RER. If done so

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appropriately, in the manner that we have developed here, then the equation for calculation of $\dot{V}_{O_2}$ for repeated-measures fluorometric aerial closed-system respirometry reduces to the same as for conventional closed-system respirometry (Eqn 7).

Measuring RMR in plant seeds

We have used seeds as model organisms to demonstrate the calculation of MR from P_{O_2} measurement by repeated-measures fluorometric closed-system respirometry. We measured seed RMR, which has rarely been done by fluorometric respirometry, but has been done using other methodologies (Dalziell and Tomlinson, 2017; Garwood and Lighton, 1990). However, there are several complications when measuring RMR of seeds that must be considered.

Firstly, metabolic processes in dry seeds are limited by solute mobility, and MR is not readily detectable in seeds with moisture contents in equilibrium with an RH below 90% (Bewley et al., 2013; Walters et al., 2005). Consequently, seeds need to be hydrated sufficiently for metabolic processes to be initiated prior to respiratory measurements being made. Secondly, seeds must not be fully imbibed during measurements of respiration, as germination (a metabolically active process that produces much higher MR compared with quiescent seeds) may occur during the trial. Therefore, in our metabolic trials, to ensure germination would not occur, seeds were pre-equilibrated to 95% RH prior to respirometry measurements. The signal to noise ratio is significantly reduced in quiescent seeds compared with germinating seeds, and to compensate for this we have measured more than one individual seed per chamber. This may offset some of the intrinsic utility suggested for repeated-measures fluorometric respirometry in making single seed measurements (as per Bello and Bradford, 2016), but this is countered somewhat by our ability to measure many replicate chambers at once. Finally, the interactions between seed dormancy status and MR is not well understood (Dalziell and Tomlinson, 2017), and the relationship between seed dormancy and germination/respiration is unclear (Bello and Bradford, 2016; Booth and Sowa, 2001; Bradford et al., 2007; Footitt and Cohn, 1995). As such, we measured RMR of dormant and non-dormant seeds (see methods below).

We measured seeds of five species of Fabaceae native to Western Australia. Using seeds from a single family (i.e. a phylogenetically constrained group) limited the potential influences of embryo type and size, as well as seed dormancy, on seed MR (Dalziell and Tomlinson, 2017). Seeds were collected from wild populations in the Pilbara region of Western Australia between 2010 and 2013 and stored in a controlled environment room at 15°C and 15% RH prior to experiments. Prior to respirometry measurements, all seeds were X-rayed (Faxitron Specimen Radiography System MX-20 Cabinet, Tucson, AZ, USA) to determine seed fill (Erickson et al., 2016). Seeds that did not contain an intact embryo or showed any internal damage were discarded. Seeds were immersed in hot water (95°C) for 2 min to alleviate physical dormancy (Erickson et al., 2016), then blotted dry with paper towel. To reduce the metabolic effect of microbial contamination, all seeds (both dormant and non-dormant) were sealed in porous nylon-mesh bags and soaked in a biocide [2% solution of Plant Preservative Mixture (Plant Cell Technology, Washington, DC, USA), with two drops of the surfactant Tween 20 (Sigma-Aldrich, Castle Hill, NSW, Australia)] for 30 min, after which seeds were rinsed in autoclave-sterilized, reverse-osmosis purified water. All seeds were then placed in respirometry chambers (14 replicate 1800 µl chambers containing seven seeds for each dormant and non-dormant treatment), and placed inside an air-tight electrical enclosure box (NHP Fibox, NHP Electrical Engineering Products, Sydney, Australia) containing a non-saturated solution of LiCl (~4.8 g per 100 ml H_2O; Hay et al., 2008) to allow seeds to equilibrate to 95% RH. The box was placed in an incubator at 20°C for 10 days. To ensure that they had reached 95% RH at the end of the incubation period, non-dormant seeds were tested with a hygrometer (HygroPalm, Rotronic, Crawley, UK). Chambers were then removed from the RH box, capped and measured at 25°C (regulated using an in-built temperature controller) at 30 min intervals over a period of 2 days, using the standard data acquisition software. These data were further analysed according to Eqns 1–7 using the accompanying R script (Script 1). To calculate the initial v_g, we used the wet mass of seeds at 95% RH, multiplied by the number of seeds in each respirometry chamber, and P_b was measured using a portable weather station (TechBrands XC0394, Electus Distributions, Rydalmere, NSW, Australia). Subsequent to measurement, to ensure successful dormancy break and seed viability, all seeds were removed from the chambers and plated in Petri dishes containing water agar (0.7% w/v), placed in an incubator at 25°C, and assessed for germination over a period of 28 days.

Table 1. Patterns of metabolic rate for seeds of Australian Fabaceae calculated from Q_2 O_2 consumption measurements, showing that dormant seeds have lower metabolic rates than non-dormant seeds

	Dry mass (mg)						
	Total seed	Embryo	Embryo: seed (%)	$\dot{V}_{O_2}$ (µl seed^{-1} h^{-1})	$\dot{V}_{O_2}$ (µl mg^{-1} seed h^{-1})	$\dot{V}_{O_2}$ (µl mg^{-1} embryo h^{-1})	Dormant/ non-dormant
Senna notabilis							
Dormant	15.1±0.25	4.1±0.12	27.5±0.67	0.16±0.060	0.011±0.004	0.039±0.014	0.62
Non-dormant				0.26±0.062	0.017±0.004	0.063±0.015	
Acacia ancistrocarpa							
Dormant	31.2±2.31	19.3±1.53	61.7±0.42	1.47±0.167	0.047±0.005	0.076±0.009	0.74
Non-dormant				2.01±0.715	0.064±0.023	0.104±0.037	
Acacia wanyu							
Dormant	70.1±3.09	23.2±0.56	33.0±0.71	0.21±0.093	0.003±0.001	0.009±0.004	0.27
Non-dormant				0.77±0.118	0.011±0.002	0.033±0.005	
Acacia inaequilatera							
Dormant	43.3±1.75	24.7±1.61	57.1±1.74	0.61±0.240	0.014±0.006	0.025±0.010	0.45
Non-dormant				1.39±0.656	0.032±0.015	0.056±0.027	
Acacia tumida var. *pilbarensis*							
Dormant	64.3±1.55	32.7±1.11	50.8±0.54	0.20±0.148	0.003±0.002	0.006±0.005	0.28
Non-dormant				0.69±0.113	0.011±0.002	0.021±0.003	

Data are presented as means±1 s.e.m., n=7.

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The majority of seed RMR should result from the combined activity of the cotyledons, plumule and radicle, which we broadly considered as the 'embryo'. To compare the RMR of seeds between species, and to understand the interspecific effects of seed mass and embryo mass, dry masses of whole seeds and excised embryos were determined. To determine embryo masses, a subsample of 30 non-dormant seeds of each species were imbibed for 24 h, and the embryos were excised using a pair of fine forceps and a scalpel under a binocular microscope. The embryos and the seed coats were separated, and split into three replicates (i.e. 10 seeds per replicate) and placed in individual metal pans and dried for 48 h at 105°C. Average dry masses of the 30 embryos and remaining seed contents were measured using a five-point balance (Mettler Toledo, Switzerland).

RESULTS AND DISCUSSION

RMR of angiosperm seeds

Oxygen depletion was linear for all species and resulted in constant RMR following our calculations. RMR ranged between 0.26±0.062 and 2.01±0.71 μl O_2 h^{-1} for non-dormant seeds. Dormant seeds had RMR approximately 53% lower than this (Table 1). These values are within the range reported for aerobic metabolism of seeds, showing similar metabolic depression in dormant seeds (Dalziell and Tomlinson, 2017; Garwood and Lighton, 1990).

Biological variation in RMR

The biological sources of error described below contribute to both interspecific and intraspecific variation in MR. Although these form the basis of the biological patterns that most ecologists and evolutionary biologists are interested in, they also contribute potential errors to the measurement of MR, and need to be appropriately addressed (Felsenstein, 1985).

Mass is the most pervasive influence on MR of organisms, overpowering the combined influences of ecology and evolution (Darveau et al., 2002; Gillooly et al., 2001). Previous repeated-measures fluorometric respirometry studies (Bello and Bradford, 2016; Bradford et al., 2013) have not considered the influence of seed mass, which has substantial implications for the calculation of initial gas volume and the expression of MR corrected for metabolically active mass. Calculation of RMR in absolute terms (i.e. millilitres O_2 consumed per hour) does not account for the potentially complex allometric effects of mass that have a pervasive influence on RMR (Dalziell and Tomlinson, 2017). RMR of seeds (Table 1) shows that, even in closely related taxa, mass correction and embryo mass correction markedly influence the species comparisons. We have avoided phylogenetic influence to a large degree by measuring closely related species, but phylogenetically independent contrast analyses (Felsenstein, 1985) should be considered for broader interspecific comparisons, especially because the phylogenetic diversity of plant seeds is greater than that of any animal group studied to date. Finally, we specifically avoided using seeds with complex dormancy types, as there is a limited understanding about how these kinds of dormancy may affect RMR, and populations of seeds with physical dormancy are simple to manipulate into non-dormant fractions. Nevertheless, differences in RMR between dormant and non-dormant seeds (Table 1) are interspecifically variable, even in closely related species, and patterns are variable depending on different mass corrections. Investigating these patterns was not our aim here, but the data that we have presented indicate that a substantial amount of research is required to do so.

Methodological errors can contribute biological errors, e.g. some seeds became contaminated with fungal and/or bacterial infections.

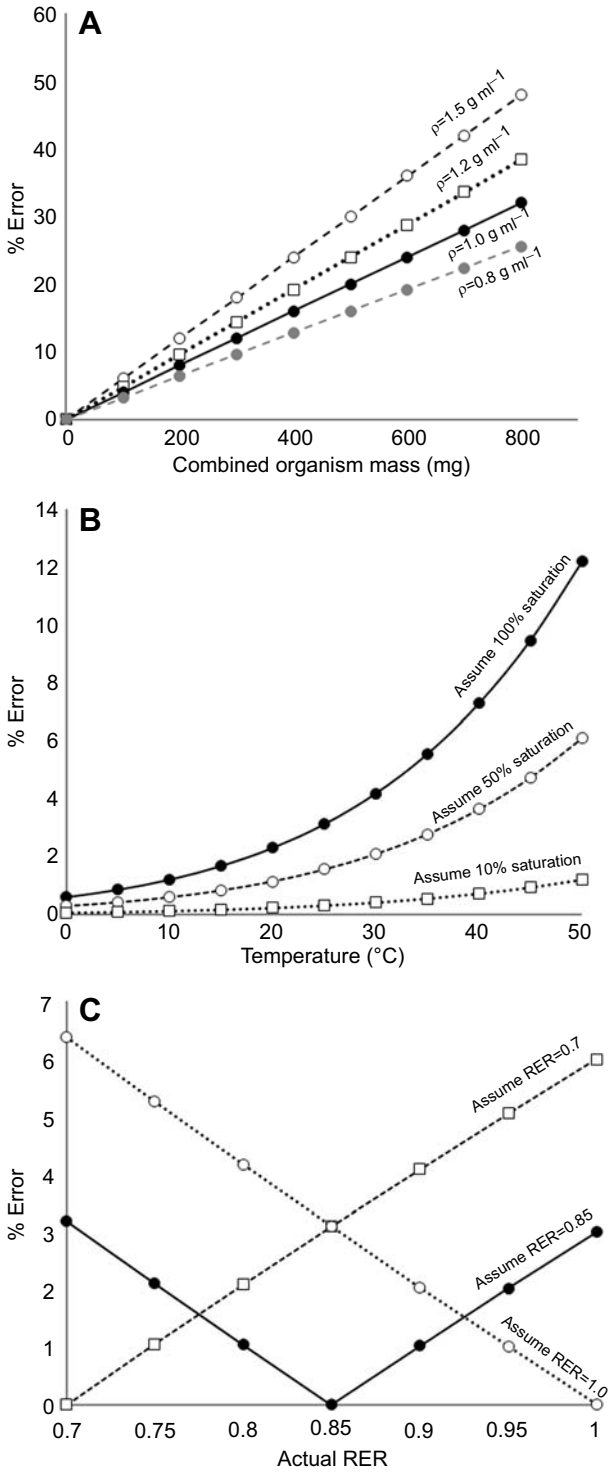

Fig. 1. Potential error in calculations of metabolic rate (MR) resulting from three major assumptions in our calculations. (A) Density of the study organism causes increasing rates of error as more individuals are added to the respirometry chamber, and rates roughly halve as density decreases from, for example, 1.5 g ml^{-1} (open circles) to 1.0 g ml^{-1} (black circles). (B) Water vapour pressure contributes different rates of error dependent on temperature, the worst-case scenario being the assumption of saturation when chamber atmosphere is initially dry (black circles) and decreasing when chamber atmosphere is at 50% (open circles) or 10% relative humidity (open squares), and there is no error for assuming 100% (*x*-axis). (C) The effect of the respiratory exchange ratio (RER) is minimal when RER is assumed to be 0.85 (black circles), and greatest where assumed to be 1.0, but actual RER is 0.7 (open circles), and vice versa (open squares).

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Alternatively, when dealing with multiple individuals in a single chamber, some replicates might include more-or-less metabolically inactive individuals. These errors result in much quicker or slower O_2 depletion rates, respectively. We have removed trials where O_2 depletion rates were greater than two standardised residuals different to average O_2 depletion rates (Dataset 1). We have not specifically included this process in the calculation algorithm (Script 1) because this is one of several statistical approaches to identify outliers.

Respirometry errors

As previously stated, although the biological variation described above contributes to patterns of interest to ecologists and evolutionary biologists, these patterns need to be appropriately accounted for so as to not influence the MR measured by repeated-measures fluorometric aerial closed-system respirometry.

Organism density and chamber gas volume

To account for organism volume in the respirometer (Vleck, 1987), mass is corrected to volume by using an estimate of organism density. Seed density typically ranges between 1.0 and 1.5 g cm^{-3} (Gupta and Das, 1997; Nelson, 2002). Resulting errors in gas volume by not correcting organismal volume can be considerable; for four 100 mg seeds in a 2200 µl respirometry chamber, error ranges from an ∼16% (ρ=1.0) to 24% (ρ=1.5) overestimate of $\dot{V}_{O_2}$ (Fig. 1A). As such, we advocate an appropriate organism mass density be accounted for and suggest assuming (if necessary) the median of published values of 1.2 g cm^{-3} for seeds. This results in a nominal error of approximately 1.25% if ρ is between 1.0 and 1.5 g cm^{-3}.

Water vapour pressure

The next potential error in the calculation of $\dot{V}_{O_2}$ also relates to v_g, on the basis of initial water vapour density, to avoid the influences of initial and changing water vapour pressure on v_g. Because saturation water vapour pressure is temperature dependent (Parrish and Putnam, 1977), the water present in saturated air changes with temperature, and hence error is also temperature dependent. Exploring the worst-case scenario where chamber atmosphere was initially dry (0% RH) but we assume saturation, the v_g error at 10°C is approximately 1% underestimated (Fig. 1B). As temperature increases to 40°C, this increases to approximately 7%. In a more realistic scenario, where chamber humidity is actually 50%, the error is approximately 2.7% at 10°C, and increases to 13% at 40°C. Although these errors are small at biologically appropriate temperatures, they need to be minimised to standardise MR measurements, especially under experimental treatments where a broader range of temperatures may be applied.

Respiratory exchange ratio and O_2 limitation

We have made several assumptions relating to P_{CO_2}, and how this influences the changing conditions in the respirometry chamber and the subsequent effects on quantifying MR. The first effect, that

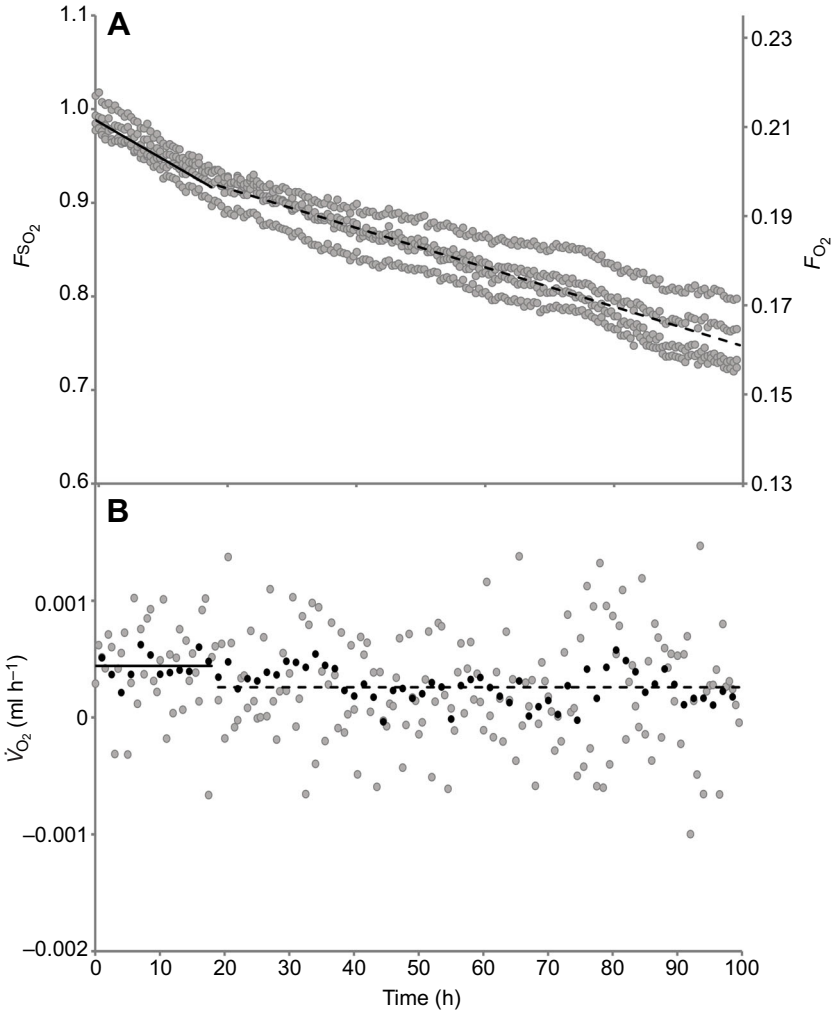

Fig. 2. The effects of measurement duration and oxygen limitation on respirometry measurements. (A) Patterns of O_2 consumption showing Q_2 standardised O_2 values (F_{SO_2}; 0–1) and their equivalent fractional atmospheric O_2 (F_{O_2}; 0–0.025). At ∼19 h, the slope decreases in a manner suggestive of O_2 limitation. (B) Conversion of O_2 partial pressure (P_{O_2}) to absolute MR ($\dot{V}_{O_2}$; grey circles) with a five-point moving average (black circles) to minimise noise. Solid lines represent the average calculated over the first 19 h prior to O_2 limitation and dashed lines indicate the subsequent average.

initial F_{CO_2} is zero, is minor if the initial F_{CO_2} is actually at atmospheric levels, resulting in <0.04% error in v_g (current atmospheric F_{ICO_2} is 0.000406; https://www.esrl.noaa.gov/gmd/ccgg/trends/). The second effect, where CO_2 accumulates throughout the measurement period, is substantial, if accumulated metabolic CO_2 production is not accounted for. RER is usually between 0.7 and 1.0, and the maximum possible errors in calculated $\dot{V}_{O_2}$ will occur when estimated RER=0.7 when actually RER=1, or vice versa (Fig. 1C) (Vleck, 1987). Overestimates of $\dot{V}_{O_2}$ result if actual RER is less than assumed RER, and underestimates result if the reverse is true, with a maximum error of approximately 6.5% (Vleck, 1987). On this basis, minimal error is obtained by assuming RER=0.85, restricting the maximum possible error to ±3.2% (Vleck, 1987). Prolonged closure of the metabolic system can cause more substantial errors of approximately 10–13% because of the greater storage capacity of organismal water for CO_2 than for O_2 (Malte et al., 2016).

Hypoxia has a potential effect on MR per se. A rule of thumb for terrestrial animals suggests that O_2 levels below 12% (atmospheric) begin to affect MR (Bradford and Seymour, 1988; Seymour et al., 1995; Wheatly, 1981); 12% O_2 is equivalent to approximately 58% of initial O_2 using the standardised Q_2 scale. As O_2 becomes limiting in the chamber, it decreases MR over time (Fig. 2A), resulting in an asymptotic O_2 depletion curve rather than the expected straight line. This can be corrected by analysing data prior to the time at which hypoxia may have occurred. However, during dynamic state applications, such as germination, O_2 limitation generates unpredictable outcomes. Peak MR, such as that estimated at the midpoint of O_2 depletion curves during germination (R_{50}; Bello and Bradford, 2016; Bradford et al., 2013) are potentially underestimated because they have been measured in hypoxic atmospheres and this potentially reduces the MR of the seeds.

Conclusions and broader applicability

The approaches to utilising repeated-measures fluorometric aerial closed-system respirometry explored here potentially expand the comparative approach to seed biology, enabling quantitative evaluation of metabolic adaptations to a range of environmental and biological factors, as explored in animals (Bartholomew, 1964; Cloudsley-Thompson, 1991; Degen, 1997). Although evolutionary patterns in the MR of animals are well documented and continue to be debated (Uyeda et al., 2017), they have yet to be explored for plant seeds. Although our study reveals the complexity of calculating accurate RMR from repeated-measures fluorometric respirometry, even greater and less predictable complexity is entailed in making dynamic state calculations, and these will require further exploration. Nevertheless, repeated-measurement fluorescence sensors extend the capabilities of closed-system respirometry in ways that potentially revolutionise the study of MR for small taxa. Although measurement of $\dot{V}_{CO_2}$ using infra-red gas analysers has been accomplished for quite small taxa (Ayton et al., 2016; Lighton and Fielden, 1995; Terblanche et al., 2004), fluorometric closed-system respirometry has the capacity to extend this lower size limit substantially, including different life history stages such as eggs and larvae (Köster et al., 2008; Szela and Marsh, 2005). Measurements of the MR of such organisms, particularly as a response of thermal performance, are often crucial to understanding ecophysiological responses to changing environments (Kearney et al., 2010; Tomlinson et al., 2018). As energetics is the basis of many processes structuring ecosystems (McNab, 2002; Tomlinson et al., 2014), we expect great potential insights to be gained from measurement of MR using repeated-measurement fluorometric respirometers for small organisms.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: S.T., E.L.D., D.J.M.; Methodology: S.T., E.L.D., P.C.W., W.L., D.J.M.; Software: S.T., W.L.; Investigation: S.T., E.L.D., P.C.W., W.L.; Resources: D.J.M.; Writing - original draft: S.T.; Writing - review & editing: S.T., E.L.D., P.C.W., W.L., K.W.D., D.J.M.; Funding acquisition: S.T., E.L.D., P.C.W., K.W.D., D.J.M.

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Supplementary information
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