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REVIEW

Some mathematical and technical issues in the measurement and interpretation of open-circuit indirect calorimetry in small animals

JRS Arch[1], D Hislop[1], SJY Wang[1] and JR Speakman[2]

[1]*Clore Laboratory, University of Buckingham, Buckingham, UK and* [2]*Aberdeen Centre for Energy Regulation and Obesity, School of Biological Sciences, University of Aberdeen, Aberdeen, UK*

Indirect calorimetry is increasingly used to investigate why compounds or genetic manipulations affect body weight or composition in small animals. This review introduces the principles of indirect (primarily open-circuit) calorimetry and explains some common misunderstandings. It is not widely understood that in open-circuit systems in which carbon dioxide (CO_2) is not removed from the air leaving the respiratory chamber, measurement of airflow out of the chamber and its oxygen (O_2) content paradoxically allows a more reliable estimate of energy expenditure (EE) than of O_2 consumption. If the CO_2 content of the exiting air is also measured, both O_2 consumption and CO_2 production, and hence respiratory quotient (RQ), can be calculated. Respiratory quotient coupled with nitrogen excretion allows the calculation of the relative combustion of the macronutrients only if measurements are over a period where interconversions of macronutrients that alter their pool sizes can be ignored. Changes in rates of O_2 consumption and CO_2 production are not instantly reflected in changes in the concentrations of O_2 and CO_2 in the air leaving the respiratory chamber. Consequently, unless air-flow is high and chamber size is small, or rates of change of O_2 and CO_2 concentrations are included in the calculations, maxima and minima are underestimated and will appear later than their real times. It is widely appreciated that bigger animals with more body tissue will expend more energy than smaller animals. A major issue is how to compare animals correcting for such differences in body size. Comparison of the EE or O_2 consumption per gram body weight of lean and obese animals is misleading because tissues vary in their energy requirements or in how they influence EE in other ways. Moreover, the contribution of fat to EE is lower than that of lean tissue. Use of metabolic mass for normalisation, based on interspecific scaling exponents (0.75 or 0.66), is similarly flawed. It is best to use analysis of covariance to determine the relationship of EE to body mass or fat-free mass within each group, and then test whether this relationship differs between groups.
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Introduction

In direct calorimetry, the heat produced by a living organism is measured directly. Indirect calorimetry, by contrast, estimates heat production from the organism's oxygen (O_2) consumption and carbon dioxide (CO_2) production. Not only is indirect calorimetry easier to perform than direct calorimetry, but also, if both gases are monitored (and preferably if nitrogen excretion is measured as well), it is possible to calculate the use of fat and carbohydrate fuels, on the assumption that there is no net interconversion of fuels within the organism.

This article is not an attempt to provide a comprehensive review of the methods, mathematics and interpretation of open-circuit indirect calorimetry. There are some excellent articles that provide such information[1–6] and there would be little point in repeating them. There does, however, appear to be a need for an article that introduces those who work with animal models of obesity, and perhaps others, to the mathematics of indirect calorimetry and the limitations of its interpretation. Our own experience is mainly of indirect calorimetry studies on small animals like rodents, and this is our focus, although the arguments are equally applicable to studies in other animals and humans. Studies in rodents are frequently used to investigate whether a natural molecule, drug candidate or genetic modification alters

Correspondence: Professor JRS Arch, Clore Laboratory, University of Buckingham, Hunter Street, Buckingham, MK18 1EG, UK.
E-mail: jon.arch@buckingham.ac.uk
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energy expenditure (EE). It is apparent that authors often present their results as O_2 consumption, when in fact they may have a more accurate measure of EE, and they fail to realise that their treatment of data may not be sophisticated enough to show instantaneous EE. In addition, they frequently ignore the complexities of interpreting respiratory quotient (RQ), or of comparing the EE of animals that differ in body size or composition.

These are the issues that we shall discuss. We shall assume that the respiratory exchange ratio – the ratio of CO_2 expired to O_2 inspired – is the same as the RQ of the tissues, although it actually takes a finite time for the latter to be reflected in the former; we shall not complicate our equation by showing the corrections needed to adjust volumes to standard temperature (0°C) and pressure (760 mm mercury); and we hope that it helps the non-mathematician that we have assumed that normal air contains 21% O_2 and 79% N_2 plus inert gases, rather than using mathematical symbols for these parameters. Precise, correct values should be substituted if necessary. These are 20.93% for O_2 and 79.04% for N_2 plus inert gases in ambient air,[7] but they may be a little different in a calorimetry room.

Measurement of energy expenditure, oxygen consumption and respiratory quotient

We shall assume for now that a step change in the proportion of gases in the expired air of the animal is instantly reflected in the proportion of gases leaving the calorimetry chamber. This issue is addressed later. It is also assumed that there is no delay between the time when air exits the chamber and the measurement of its composition.

Closed-circuit systems

The simplest system is a closed-circuit, indirect calorimeter (Figure 1a). In this system, all the CO_2 produced by the occupant(s) of the chamber is absorbed. This causes the pressure in the chamber to fall, which activates a valve permitting the entry of O_2 and the return of the pressure within the chamber to its initial level. The O_2 does not simply replace the CO_2 absorbed – if it did, the volume of O_2 supplied would provide a measure of CO_2 production: it also replaces any O_2 that the occupants have used but did not replace with an equivalent volume of CO_2, as happens when the RQ is less than 1. Closed-circuit, indirect calorimetry therefore provides a measure of O_2 consumption. It is potentially very sensitive, but it is also sensitive to changes in ambient temperature and pressure. Moreover, O_2 consumption does not always provide an accurate measure of EE, although if the CO_2 absorbed is measured gravimetrically, both RQ and a more accurate measurement of EE can be obtained. This is explained below. One other problem is that ammonia builds up in the chamber, so that closed-circuit systems are probably best used for short-term measurements.

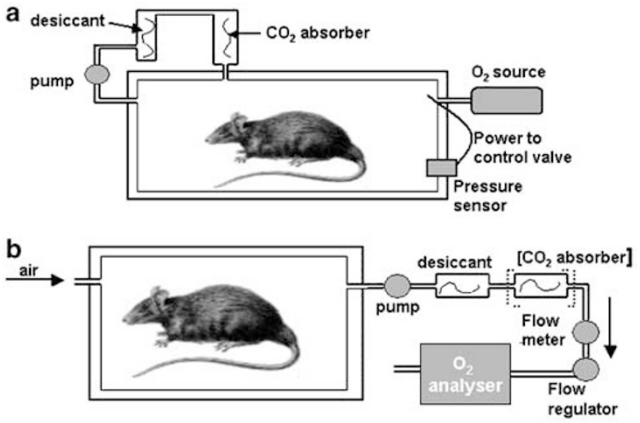

Figure 1 Organisation of (**a**) closed-circuit and (**b**) open-circuit indirect calorimetry systems. In the open-circuit system, CO_2 leaving the respiratory chamber can be absorbed, but usually it is not. The air usually passes to both O_2 and CO_2 analysers. The schematics are provided primarily so that the mathematics can be better understood. In practice, there may be multiple respiratory chambers, and the system may include equipment to sample air from each chamber in turn, pump it to the analysers and regulate flow to the analysers.

Open circuit with carbon dioxide removal

In open-circuit systems, it is air rather than O_2 that enters the chamber. O_2 consumption can be calculated from the O_2 content only and the volume of the air leaving the chamber, but as the CO_2 content is not known, this can be carried out accurately only if CO_2 is absorbed before the exiting air reaches the O_2 analyser (Figure 1b). The reason becomes clear when we look at the mathematics. The O_2 consumed (VO_2) is the difference between the amount of O_2 that entered and the amount that left the chamber. Thus,

$$VO_2 = 0.21V^{i} - (0.21 - x)V^{o} \quad (1)$$

where V^i is the volume of dry air at standard temperature and pressure that entered and V^o the volume that exited the chamber; 0.21 is assumed to be the fraction of O_2 in the dry air entering the chamber; x is the difference in the fraction between air entering and leaving the chamber – in other words, the difference that the O_2 analyser measures.

The problem is that the flow meter in the configuration shown in Figure 1b measures V^o but not V^i. There could be a second flow meter that measures V^i, but it would have to be very accurately calibrated relative to the flow meter on the exit side of the chamber to avoid errors, because x is usually kept below 0.01 to ensure that respiratory rate does not increase.[8] Consequently, a 1% error in the calibration of one of the flow meters relative to the other would result in an error of at least 21% in the calculated value of VO_2.

If the objective is to measure VO_2, rather than measuring V^i as well as V^o, it is better to absorb the CO_2 produced by the

occupant of the chamber before measuring the O_2 content of the air that has left the chamber. If no CO_2 enters the chamber and none exits it, the difference between the volume of dry air that entered and the volume that exited the chamber is the O_2 consumption, that is,

$$VO_2 = V^i - V^o$$

so

$$V^i = VO_2 - V^o \quad (2)$$

Replacing V^i in (1) with (2) gives

$$VO_2 = \frac{xV^o}{0.79} \quad (3)$$

If the CO_2 content of the air entering the chamber is that normally found in atmospheric air (0.03%), it is unnecessary to remove it or to adjust the equation, which changes only marginally to

$$VO_2 = \frac{xV^o}{0.789937} + 0.00008\,V^o$$

It may be a different matter if the CO_2 content of the room in which the chamber is located rises, emphasising the importance of having a supply of fresh air.

(It is also possible to measure flow into, rather than out of, the chamber and substitute equation (1) with the term $V^o = VO_2 - V^i$.)

Open circuit without carbon dioxide removal

Most workers do not remove the CO_2 in the air leaving the chamber (Figure 1b). It is then possible to measure RQ by using a CO_2 as well as an O_2 analyser. This makes the calculation of V^i more complicated, however.

If is often assumed that $V^i = V^o$ and therefore $VO_2 = xV^o$, but this is only true when RQ = 1, that is, the fuel is carbohydrate. Many workers who make this assumption to calculate VO_2 then discuss VO_2 as if it were the same as EE. Their final conclusions are then valid, as the rest of this section explains. If the fuel is fat, RQ = 0.718.[1] Then,

$$V^i = V^o + (1 - 0.718)\,VO_2 \quad (4)$$

In other words, V^o is reduced by a value equal to 28.2% of the O_2 consumption – the proportion of the O_2 consumed that is not replaced by CO_2.

Substituting the term for V^i from equation (4) into equal 1 gives

$$VO_2 = \frac{xV^o}{0.94}$$

Thus, when fat is the fuel, the calculated value of VO_2 is 6% too low if the equation used is $VO_2 = xV^o$

Some workers regard a maximum error of 6% as acceptable, given the other errors involved in the experiments. However, this error can be almost entirely avoided simply by recognising that the measurement of x and V^o gives an accurate estimate of EE, rather than O_2 consumption. In many cases, unless the aim is to measure RQ, EE is a more useful value than O_2 consumption. Moreover, it can be measured without the need for a CO_2 analyser. How is this possible?

Table 1 Energy released when macronutrients are combusted with 1 l of O_2

Macronutrient	*RQ*	*Energy released (kcal)*
Carbohydrate	1.0	5.047
Fat	0.718	4.735
Protein	0.802	4.463

Abbreviations: O_2, oxygen; RQ, respiratory quotient. Values are taken from Weir.[1]

The classic paper of Weir[1] (and more recent papers by Koteja[9] and Speakman[10]) explains why it is relatively easy to measure and calculate EE. Weir pointed out that for the same volume of O_2 consumed, 6% more energy is obtained from carbohydrate than from fat (Table 1). Consequently, when fat is the fuel, if one multiplies a value of VO_2 that is 6% too low by a value of EE per litre of O_2 that is 6% too high, the errors are cancelled out. In fact, the error in the calculation of EE is less than one in 600 when fat is the fuel, and approaches zero as the proportion of carbohydrate increases. A key point is that one must use the energy equivalence per litre of O_2 consumed that is appropriate for carbohydrate (5.04 kcal/l), whatever fuel the animal may be using. Using a value appropriate for an average RQ of 0.75 or 0.8, as some workers have, introduces more error than assuming that all the fuel is carbohydrate.

Weir showed that for 1 l of expired air

$$\mathrm{EE\,(kcal)} = \frac{0.0504x^{\%}}{1 + 0.082P} \quad (5)$$

Here $x^{\%}$ is the difference in percentage O_2 between inspired and expired air. (The fact that most of the air that passes through the chamber is not inspired makes no difference to this equation.) P is the fraction of calories from combustion of protein. Typically, in humans $P = 0.125$, in which case

$$\mathrm{EE} = 0.050x^{\%}\ \mathrm{kcal} \quad (6A)$$

a neat equation, or

$$\mathrm{EE} = 0.2086x^{\%}\ \mathrm{kJ} \quad (6B)$$

The simple message is 'if you do not use a CO_2 analyser, calculate EE, not VO_2, and make sure that you use an EE equivalence appropriate for carbohydrate'.

Measurement of respiratory quotient

If the aim is to measure RQ (the volume of CO_2 produced (VCO_2) divided by the volume of O_2 used (VO_2)), then one has to use a CO_2 analyser – or measure CO_2 production gravimetrically. In theory, one could calculate VCO_2 by measuring the difference between the volume of air entering and the volume leaving the chamber, but it is almost impossible to do this with the required accuracy. Calculation

of VCO_2 is easy:

$$VCO_2 = V^{o}y \quad (7)$$

where y is the fraction of CO_2 in the air exiting the chamber minus the fraction in the air entering the chamber. Because the fraction of CO_2 in the air entering the chamber is usually very small, it makes almost no difference to this calculation that the precise value of V^{i} is not known. For 0.03% CO_2 in the entering air, VCO_2 is underestimated by 0.01%.

To calculate the VO_2, an approach called 'The Haldane Correction' is used. This is based on the fact that the volume of N_2 (and inert gases) entering the chamber is the same as that leaving it. For simplicity, we shall say that 0.79 of the entering air is composed of N_2 and inert gases. This fraction increases by the value of x and decreases by the value of y in the exiting gases. Thus,

$$0.79\,V^{i} = V^{o}[1 - (0.21 - x) - y] \quad (8)$$

Strictly, the last term should be y–0.0003 to allow for the 0.03% CO_2 in the entering air. If $x = 0.008$ (i.e. $x^{\%} = 0.8$), this simplification results in VO_2 being underestimated by 1%.

The value of V^{i} from equation (8) can be substituted into equation (1) to give

$$VO_2 = V^{o}(1.266x - 0.266y) \quad (9)$$

(Even *et al*.[2] provide an equivalent equation – equation (7) of their paper. There is, however, an error in this equation: F^{in} CO_2 should be replaced by F^{out} O_2, as correctly used in their equation (6).)

When the fuel is carbohydrate, the values of x and y are equal and

$$VO_2 = xV_o$$

which is, of course, what one gets if $V^{i} = V^{o}$ (carbohydrate combustion). However, when the fuel is not pure carbohydrate, $V^{i} \neq V^{o}$ and $x \neq y$, and the correct equation is equation (9). VCO_2 from equation (7), divided by VO_2 from equation (9) gives RQ.

Calculation of energy expenditure from VO_2 *and* VCO_2

Weir,[1] in his equation (9), states that when 12.3% of EE is produced by protein oxidation

$$EE(kcal) = 3.9\,VO_2 + 1.1\,VCO_2 \quad (10)$$

So it is possible to use values of VO_2 and VCO_2 to calculate EE. It should make little difference whether equation (10) or equations (6A) or (6B), which do not require the analysis of CO_2, are used. Equation (10) introduces slightly less error if variations in protein oxidation are not accounted for, however (see below).

Protein oxidation

The percentage of calories obtained from combustion of protein may vary widely in some rodent studies, but nevertheless introduce only a small error into the measurement of EE. For example, some studies compare energy balance in animals fed on diets that contain widely differing protein contents.[11,12] If differences in dietary protein content of 8 and 20% by energy are fully reflected in the percentage of calories obtained from protein, then values of EE from equation (5) would differ by 1.1% for the same value of $x^{\%}$. If equation (10) is used, which contains both VO_2 and VCO_2 terms, this error is reduced by about 40% because variations in protein oxidation are reflected in RQ.

Interpretation of respiratory quotient

When measuring VO_2 and VCO_2, we assume that the O_2 and CO_2 pools in the body remain stable. The CO_2 pool is not stable, however, and this limits our ability to interpret short-term changes in RQ. It is similarly important to recognise that interconversions of macronutrients that result in variations in their pool sizes will affect RQ.

Pool sizes are not normally stable *during* the course of 24 h, nor during rapid weight gain or loss where these are associated with interconversion of fuel types. On the other hand, it is reasonable to expect that pool sizes will change little over a 24 h period when animals are in or near energy balance. Figure 2, based on Ferrannini,[4] shows RQ values for gluconeogenesis, lipogenesis and ketogenesis. For example, the RQ for the conversion of glucose to fat is 5.55. The RQ for oxidation of fat is 0.7. As the volumes of O_2 and CO_2 involved in the oxidation of the fat are greater than those involved in its production from glucose, the overall RQ for the conversion of glucose to fat followed by its oxidation is 1.0, exactly as if the glucose was oxidised directly. Clearly, therefore, short-term measurements of RQ can by themselves provide little quantitative information on fuel oxidation; they are better interpreted as measures of the net rate of fuel disappearance. These issues are discussed in more detail by others.[3,4,6]

Protein oxidation has far more influence on the calculation of RQ than of EE (see above).[6] Therefore, protein oxidation should be measured if it might vary significantly between experimental groups, and RQ is to be calculated.

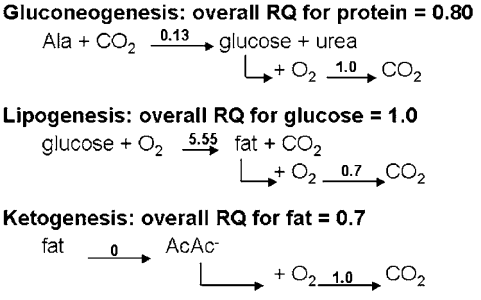

Figure 2 Respiratory quotient (RQ) values for gluconeogenesis from alanine, lipogenesis and ketogenesis.[4] If the glucose, fat or acetoacetate ($AcAc^{-}$) produced is subsequently oxidised, the overall weighted average of the RQ is that of the original substrate (alanine, glucose or fat, respectively).

If protein oxidation is measured from nitrogen excretion, it is possible to calculate the nonprotein RQ with the knowledge that for each gram of urinary nitrogen 5.941 of O_2 is consumed and 4.761 of CO_2 is produced. The proportions of carbohydrate and fat disappearing can then be read from tables.[7] An alternative mathematical approach is to calculate glucose, fat and protein disappearance from equations based on VO_2, VCO_2 and urinary nitrogen.[6]

The simple message is 'without information on fuel interconversions, it may be difficult to interpret RQ'.

'Instant' measurements

Up to now, we have assumed that a step change in the proportion of gases in the air that an animal expires is instantly reflected in a step change in the proportion of gases leaving the chamber. This is obviously not true. If the occupants of the chamber were removed, it would take a finite time for the air in the chamber to reach the composition of the entering air. This is illustrated in Figure 3. In fact,

$$VO_2 = VO_2^{i} - VO_2^{o} - \Delta O_2 \text{ in chamber} \qquad (11)$$

ΔO_2 is negative and $-\Delta O_2$ is therefore positive when the amount of O_2 in the chamber has fallen.

If the ΔO_2 term is not used, the calculated rate of change of VO_2 or EE is 'smoothed out'. This may not affect the interpretation of data if control and experimental data are subject to the same smoothing. If so, it may be best to work with smoothed data because correcting it may introduce noise into the calculations. Smoothed data may not be acceptable, however, if VO_2 or EE is compared to instantaneous measurements, such as locomotor activity, feeding behaviour, body temperature or heart rate. Nor are smoothed data appropriate if accurate maximum or minimum values are required, for example because they are to be interpreted in relation to absolute organ weights, rather than compared between treatment groups.

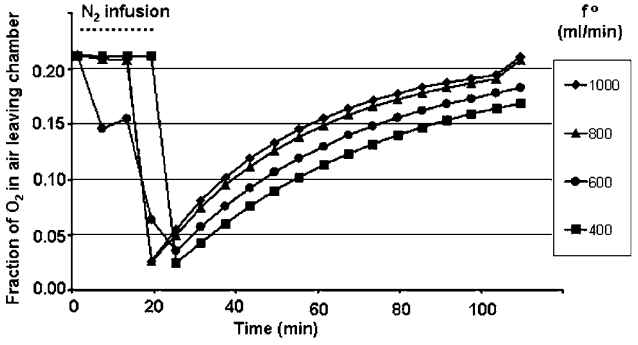

Figure 3 Return of composition of air leaving respiratory chamber to that of ambient air after nitrogen was infused into the chamber. The volume of the chamber was 23 l. The flow of air through the chamber after the nitrogen infusion was varied as shown. The figure illustrates in an exaggerated fashion how a change in gas exchange is not instantaneously fully reflected in the composition of the exiting gases. Data from this figure are analysed in Table 2.

To calculate instant VO_2, we need to differentiate equation (11) with respect to time

$$\frac{dVO_2}{dt} = fO_2^{i} - fO_2^{o} + C\,\frac{dx}{dt} \qquad (12)$$

where f is flow (of O_2 in or out of the chamber) and C is the volume of the chamber. x has a positive value, so that if the value of x is increasing, dVO_2/dt is larger than if this correction is not made.

From the equations of Weir,[1] we obtain a similar equation:

$$\frac{dEE}{dt} = \left[f^{o}x + C\,\frac{dx}{dt}\right]0.05 \qquad (13)$$

where f^{o} is the flow of air out of the chamber. So if we need to calculate 'instant' EE, we need to know the volume of the chamber and the rate of change of x. The latter might be obtained from a sophisticated curve-fitting programme, or it may be roughly determined from $\Delta x/\Delta dt$ for times either side of the time for which EE is being calculated.[5] In many set-ups for rodents, x is measured only once every 10–15 min, which makes accurate determination of dx/dt difficult. For really accurate measurement of EE, a continuous measurement of x is the best approach. Ravussin *et al*.[5] describe such a system for calorimetry in humans, and measure Δx over 2 min periods.

The term $C\,dx/dt$ in equations (12) and (13) is based on the assumption that the rate of change of x in air leaving the chamber is the same as that for air inside the chamber. This is only true when there is good mixing of expired air with the air in the chamber. If mixing is poor, the rate of change of x for the air in the chamber (an average for the whole chamber – x will vary within the chamber) may be greater (more positive or more negative) than that of the air reaching the O_2 analyser. This is because the air entering the chamber is, to some extent, flowing straight through without 'picking up' the expired air.

If the measured value of dx/dt is too small, then equations (12) and (13) will only be correct if the chamber volume (C) is replaced by a higher value, C_{apparent} (C_{app}). Fortunately, it is not difficult to determine C_{app} in the absence of chamber occupants, and so to determine the maximum flow rate that gives $C_{\text{app}} = C$. It may be best not to exceed this flow rate if the $C_{\text{app}}\,dx/dt$ term is used in the calculation of EE, because the presence of animals in the chamber and their movement in the chamber may improve mixing and reduce C_{app}.

C_{app} can be determined by making the O_2 content of the air in chamber different from that of ambient air, and then, with no occupants in the chamber, following the rate at which it returns to that of ambient air.

From equation (13), since $\frac{dE}{dt} = 0$

$$\frac{dx}{dt} = -\frac{f^{o}x}{C_{\text{app}}} \qquad (14)$$

This is the equation of a first-order decay. In other words, the rate of fall of x is proportional to x. The rate constant of this

first-order decay is

$$\frac{f^{o}}{C_{app}} \quad (15)$$

and the half-life ($t_{1/2}$) is

$$\frac{0.693 C_{app}}{f^{o}} \quad (16)$$

We have applied this equation to the data shown in Figure 3, which were obtained for a system that uses normal rodent cages with a measured volume of 23 l. Table 2 shows that when f^{o} for this system was 0.4 l/min, the measured value C_{app} was equal to the actual value, but as the flow rate increased, C_{app} increased.

The $t_{1/2}$ of this system when f^{o} was 0.4 l/min was 39.8 min. This is obviously not a good system for measuring rapid changes in EE, or short-lived maxima or minima of EE. The dx/dt term, which is the least accurate in equations (12) and (13), assumes greater importance for the calculation when C_{app} is high or f^{o} is low, the ratio C_{app}/f^{o} being proportional to $t_{1/2}$ (equation (16)). For measurements of rapid changes in EE, it is therefore best to keep the chamber volume (C) low and the airflow (f^{o}) high. The problem with using small chambers is that the animals may be restricted in their movement, and it may not be possible to keep them in small chambers for very long. The problem with f^{o} being high is that this reduces the size of x, and if x is too low, accuracy will be reduced. The system described with its large chambers is nevertheless suitable for measurement of EE over 24 h or perhaps 6 h periods. This is usually all that is needed when attempting to understand energy balance in a genetically modified animal, or in response to a potential anti-obesity drug. If, however, we wish to relate EE to instant measurements, it is important to use the C_{app} dx/dt correction factor and it is best to keep C/f^{o} as low as possible, given other experimental constraints.

Table 2 Apparent volume of respiratory chamber determined from rate at which composition of exiting air approaches that of ambient air

Flow rate (f^{o}; l/min)	*Rate constant (k; min^{-1})*	*Apparent chamber volume (C_{app}^{1}; l)*
0.4	0.0175	22.9
0.6	0.0210	28.5
0.8	0.0256	31.2
1.0	0.0283	34.7

Data are taken from Figure 3. Calculations (equations (14)–(16)) are described in the text. The measured chamber volume was 23 l.

Figure 4 illustrates the importance of keeping C/f^{o} low and of using the C_{app} dx/dt correction term to accurately measure transient changes in EE, both with respect to time and magnitude. In this system, a short-tailed field vole weighing approximately 25 g was placed in a chamber with a volume of 843 ml, and the value of f^{o} was 230 ml/min.[13] Thus, the calculated $t_{1/2}$ of the system was only 2.5 min. Nevertheless, the magnitude of the transient thermogenic response to noradrenaline was increased by 40% and the time from the noradrenaline injection to the peak response cut by more than half when the C_{app} dx/dt correction was made. In fact, with such a short-lived response, buffering of gas exchange within the vole's body fluids and airways may mean that the response was even greater and sooner than the value calculated using the C_{app} dx/dt term. By contrast, the baseline metabolic rate, which is relatively stable, was unaffected by the correction term. The example of Figure 4 can easily be extrapolated to other situations: for example, the point of injection of noradrenaline can be viewed as the start of a short bout of physical activity.

The simple message is 'know the half-life of your system and whether rate of change of x is included in the calculation of EE. Then, you can decide whether your values are 'instant enough' for your purposes'.

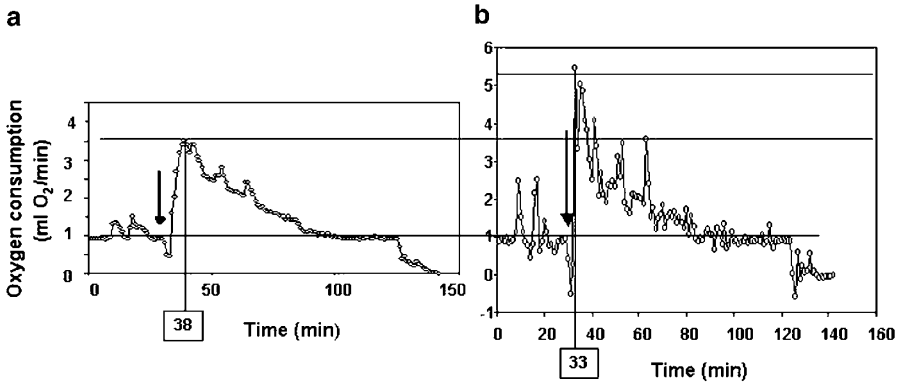

Figure 4 Effect of applying a C_{app} dx/dt term to correct for the fact that the composition of air exiting a respiratory chamber does not immediately respond fully to a change in the rate of gas exchange by the chamber's occupant(s). (**a**) Oxygen (O_2) consumption without correction, and (**b**) corrected O_2 consumption, for a short-tailed field vole injected with noradrenaline.[13] The system employed a much smaller chamber (843 ml) than the one used for the data shown in Figure 3 and Table 2. The flow of air out of the chamber was 230 ml/min. The horizontal lines show how the peak but not the basal O_2 consumption is underestimated when the C_{app} dx/dt term is not used in the calculation. The vertical lines show how the time of peak O_2 consumption appears too late if the C_{app} dx/dt term is not used in the calculations. The dip in the apparent O_2 consumption shortly before the peak was due to removal of the vole from the respiratory chamber so that it could be injected with noradrenaline at the point indicated by the arrows.

Comparison of energy expenditure in lean and obese animals

The dominant factor influencing levels of EE is body weight. This has been known since the seminal observations of energy demands using calorimetry that were made by Lavoisier and Laplace in the late 1700s. There is therefore a widespread understanding that to compare the levels of EE between animals that differ in their body sizes, it is necessary to somehow correct for the differences in body size. The aim of normalisation of EE is to obtain some understanding of the differences between animals or treatment groups, with the effects of differences in body size removed. A simple way that this might be achieved is to divide the estimated EE by body weight. Comparisons of different animal species made in this way by Rubner[14] demonstrated that dividing by body weight overcorrects the estimated metabolic rates of larger animals. Nevertheless, to this day, the metabolic rates of lean and obese rodents are often compared only after dividing by body weight.[15] Rubner instead argued that, because heat loss is a surface phenomenon, it would be more logical to divide the metabolic rates of the animals by their surface areas rather than their body weights. However, this causes a practical problem because body weights are easy to measure, whereas surface areas are not. Rubner devised an elegant solution to this problem by making arguments of dynamic similarity. When a simple object like a cube increases in size, but keeps the same shape, the relation between surface area and mass follows a simple dynamic rule. Because all animals are roughly of the same shape, he argued that, like objects that retain shape similarity, their surface area scales in relation to body weight raised to the power 0.66. Applying this to data from dog breeds, he judged that correcting metabolic rate estimates by expressing them in relation to $\text{Mass}^{0.66}$ adequately corrected for the differences in body size.[14]

As more data accumulated, the appropriateness of the 0.66 scaling exponent advocated by Rubner was questioned. It seemed to apply as much to ectotherms as to endotherms, undermining the theoretical basis of its derivation. By 1932, Kleiber[16] was already advocating an exponent of 0.72, which in the days before computers were widely available became 'rounded' to $\frac{3}{4}$ or 0.75. In recent years, a body of theoretical work has become established, based on fractal geometry of O_2 supply networks, explaining why 0.75 would be expected to be the exponent for the scaling of metabolic rate.[17] But this work largely ignores the fact that exponents of 0.75 have never been an established feature of the empirical database. Indeed, more recent empirical work has established that, once phylogenetic effects are accounted for (in other words, effects due to a shared evolutionary origin), the most parsimonious exponent is strongly dependent on the group being studied,[18–20] and there is no uniform scaling exponent – be it 0.66 or 0.75 – for either basal or maximal metabolic rate.[21] This widespread confusion over the most appropriate method for 'correcting' estimates of metabolism for body weight differences has led to a diversity of different approaches in the literature, which is replete with estimates of metabolism divided by body weight, or divided by 'metabolic weight', which is weight raised to 0.66 or weight raised to 0.75.

One issue of importance is whether corrections for body size differences that are derived from comparisons across different species of animal have any relevance anyway when making comparisons within a particular species. This is particularly germane to studies of obesity, where it is generally the case that one is interested in whether metabolic rate differences between lean and obese individuals of a single species (e.g. transgenically manipulated animals, or animals on different dietary or drug treatments) contribute to their differential storage of body fat. It is seldom the case that one might be interested in interspecific comparisons. Scaling exponents within species generally do not conform at all to scaling exponents between species. The reasons are obvious. When one compares a 20 g mouse to a 60 g mouse, the difference between the two is largely due to excess fat storage. In contrast, when one compares a 20 g mouse to a 60 g hamster, the body composition remains similar.

In the literature on humans, this problem has long been recognised and a 'solution' has been to divide the metabolic rate not by body weight, or weight raised to a scaling exponent, but rather by the lean body tissue weight or fat-free mass. The rationale for this approach is that fat tissue is largely inert[22] and contributes little to the metabolic rate, which is generated mostly by the activity of the lean body compartment. Animal studies have tended not to employ this approach largely because, until recently, accurately estimating the fat-free mass has been technically difficult, but some studies have started to employ such calculations, as the potential for non-invasive body composition measurements has improved.[23] It is worth noting, however, that dividing metabolic rate by lean body mass may not be an ideal solution to the problem of normalising metabolic rate data. There are two problems in ignoring adipose tissue. First, although the metabolic rate of fat tissue may be low, it is not zero. Secondly, adipose tissue releases adipokines, notably leptin and adiponectin, that affect EE in other tissues. When regression models are used, the contribution of fat mass appears to be around 0.15–0.2 of the contribution of the same mass of lean tissue.[24,25] Studies in humans show that in this species as well, fat mass makes a contribution to EE that is a 0.15–0.2,[5,26,27] or a greater[28] proportion of that of the equivalent lean mass.

Let us consider the example of a hypothetical lean mouse of 20 g, which consists of 18 g of lean tissue and 2 g of fat. (We will ignore the bones for simplicity.) If the resting metabolism of the lean tissue is say 0.1 W and the fat expends energy at 0.2 times the rate of the lean tissue, the total metabolic rate will be $(18 \times 0.1) + (2 \times 0.1 \times 0.2) = 1.84$ W. Expressed on a fat free mass basis, this would be 0.102 W/g. If the mouse gained 20 g of fat to become a 40 g

mouse, and (ignoring any influence of adipose on other tissues) the metabolism of its tissues was unchanged, the total metabolism would be $(18 \times 0.1) + (22 \times 0.1 \times 0.2) = 2.28$ W. Expressed per gram of fat-free mass, the metabolism would be 0.124 W/g. In other words, using fat-free mass as the comparison basis (as is often the case in studies on humans), one would conclude that the metabolic rate was increased by 22% in the *fat* mouse, when in fact the metabolic rates of fat and fat-free tissue were unchanged. Incidentally, dividing by total body weight in this situation results in a 64% higher estimated metabolism in the *lean* mouse, using a scaling exponent of 0.66 results in a 29% greater metabolic rate in the lean mouse and using a scaling exponent of 0.75 gives a 38% greater metabolic rate in the lean mouse, demonstrating that these approaches are also inadequate.

A further problem with using fat-free mass is that the lean tissue compartment is not homogeneous. As animals vary in their fat-free mass and total body weight, the contributions of different tissues change. Some tissues like the brain, with high rates of metabolism, change relatively little in absolute weight, but others such as skeletal muscle, with lower rates of metabolism, are subject to extensive remodelling. The result is that the intercept of the relation between fat-free mass and metabolism is generally not zero, even if the contribution and influence on other tissues of fat tissue remain constant.[29,30] Simply dividing by fat-free mass is therefore inappropriate, because the scaling is not isometric. These problems are as important in normalisation of metabolism data in humans as they are in animals.

To overcome these problems, the best and simplest statistical approach is to use analysis of covariance (ANCOVA).[31,32] This approach is useful because it makes no prior assumptions about the nature of the scaling relationships between different body compartments and metabolism, but rather derives these empirically using the actual data. The generalised linear model for ANCOVA includes the assumption that the effects of body mass or fat-free mass on metabolic rate are linear and the traits are normally distributed. It is best to check these assumptions first by plotting the data for individual groups (say different treatment groups or genotypes on different diets) and using a standard normality test such as the Anderson–Darling or Kolmogorov–Smirnov tests. If the data are nonlinear or not normally distributed, they can generally be corrected by using a normalisation procedure, such as a standard logarithmic transformation, or the Box–Cox procedure. Having convinced oneself that the data conform to the test assumptions, the analysis assesses the effects of body weight, the effects of the group allocation and the interaction of these effects.

The interaction evaluates whether the gradients of the effects of the independent variable (weight or fat-free mass) on metabolism are different between treatment groups. If they are, then a formal comparison of a group effect is not possible. This is because at some point two non-parallel lines (i.e. that differ in their gradients) must cross. However, in practical terms, one is not interested in whether the lines may eventually cross, but whether they differ in the region where one has data. The problem of an interaction can be overcome by using the regression line for each group to predict what the EE of each animal in that group would have been, if it had the average body weight for the two groups combined. The normalised data may then be compared using a standard *t*-test. Alternatively, more sophisticated approaches such as the Johnson–Neyman technique may be applied,[33] which defines the region of body weights over which no significant differences in metabolism are detectable.

If, on the other hand, the interaction is not significant, this means that the gradients of the effects of body weight (or fat-free mass) are not different in the two groups (i.e., they are parallel). Re-running the ANCOVA excluding the interaction term from the model can then formally test for a group effect.

The strength of this approach is that it makes no *a priori* assumptions about scaling relationships or differences in body composition, and can be run using either body weight or fat-free mass as the independent variable. Indeed, if fat-free and fat mass are known, they can both be included in the analysis as two independent covariable factors. There may, however, be interventions that are better interpreted using only fat-free mass as the covariate, so that effects of fat mass on EE in other tissues are not factored out. An example is where enhanced metabolic efficiency after weight loss was investigated in obesity-prone rats.[34] Other examples of the use of ANCOVA are a recent study of mice with a missense mutation in growth hormone,[35] and a study on EE in type 2 diabetes.[28]

Nevertheless, few studies on genetically modified mice have used ANCOVA to interpret alterations in energy balance. It must seem strange, even to those who are not familiar with ANCOVA, that fundamentally similar data have been expressed relative to body weight to argue that genetically modified animals are obese or lean because they have a reduced or raised EE respectively, but expressed per individual to argue that obese humans are obese (or sustain their obesity) because they overeat.[26,36,37] It is particularly illogical to attempt to interpret energy balance data when food intake is expressed on a whole animal basis, but EE is expressed relative to body weight.[15] At least if both energy intake and expenditure are expressed in the same way, some attempt can be made to determine whether fat accretion or resistance to diet-induced obesity is due to altered intake or expenditure. However, it is important to recognise that in humans and to a lesser extent rodents, severe obesity can develop as a result of very small mismatches of energy intake and expenditure. There are a number of examples of genetically modified mice where energy intake and expenditure are both disturbed in the same direction, where hyperphagia develops after obesity has developed, or where it has not been possible to

determine why obesity has developed by measuring intake and expenditure.[38]

One final caveat in using ANCOVA to analyse the effects of treatments on EE corrected for body mass is that this approach does not work well when the within-group variances are very low compared to the between-group effects on both mass and EE. Normally, this is not an issue, as individual variations in response are sufficient to generate a large enough within-group variation in mass/body composition. In those cases where there is exceptionally low within-group variance relative to the treatment effect, ANCOVA will generally not detect an independent treatment effect, but assign all the EE effect to the covariate differences (mass or body composition). In these unusual cases, one is reduced back to using normalisation processes based on division by mass or lean body mass. We suggest using lean body mass by preference over mass or metabolic mass for the reasons given above.

The simple message is 'use ANCOVA to analyse studies of treatment effects on EE. In those cases where ANCOVA is not appropriate (low within-group variance relative to treatment effect on mass and EE) at the very least give EE per animal as well as transformed data (preferably divided by lean body mass and not divided by whole body mass or metabolic mass). Always make comparisons between intake and expenditure using equivalent units (mass corrected or absolute)'.

Conclusion

The use of indirect calorimetry in small rodents has increased in the obesity field in recent years owing to the need to discover why many genetically modified mice are obese, lean or resistant to obesity. Moreover, thermogenic responses to compounds aimed at the many new targets suggested by genetically modifed mice have been investigated. Some workers have reported O_2 consumption despite using an O_2 but not a CO_2 analyser, not realising that paradoxically their data can give a more reliable estimate of EE; the calculated EE or O_2 consumption is sometimes assumed to be precise and instantaneous, when the equation used in its calculation results in it being smoothed over time; and it is important when interpreting differences in EE between lean and obese animals to appreciate that different tissues have different energy requirements and that of adipose tissue is low relative to its weight. Analysis of covariance offers the best approach for the comparison of EE data between lean and obese animals.

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