REVIEW

Limitations and requirements for measuring metabolic rates: a mini review

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Metabolic measurement of humans and model animals is an important aspect of biomedicine. Particularly, in the case of model animals, the limitations of currently widely used metabolic measurement methods are not widely understood. In this mini-review, I explain the theoretical underpinnings of flow-through respirometry as a linear time-invariant system, and the (usually serious) distortions of metabolic data caused by the interaction of chamber volume and flow rate. These can be ameliorated by increasing the flow rate through the chamber, though this is at the expense of the magnitude of the O_2 depletion and CO_2 enhancement signals from which metabolic rates are calculated. If achieved, however, the improvement in temporal response that follows higher flow rates can be marked, and allows confident and accurate measurement of resting and active energy expenditure. Applications of this approach in multiplexing gas signals from multiple cages, and in human room calorimetry, are also discussed.

INTRODUCTION

Living organisms must obtain, store and utilize energy to grow, reproduce, interact with their environment and stem the steady march of entropy. The energy expenditure (EE) of a model organism, or its 'fire of life' as Hans Kleiber[1] so memorably described it, is therefore a pivotally important parameter in biomedical science. Subtle or gross mismatches between energy acquisition, storage and utilization lie close to, or are at the very root of, a myriad of pathologies. It stands to reason, then, that accurate measurement of EE is a fundamentally important methodology.

There is, however, a fundamental paradox in the field of metabolic measurement. It is possible to obtain accurate EE measurements that span long intervals of time, and depending on research focus, such integrative measurements of EE may be sufficient. Examples may include overall energy balance investigations using human room calorimetry[2–4] and work on model animals such as mice and rats, where overall, 24 h or scotophase vs photophase EEs are of interest. Other investigators who are interested in fleeting phenomena, such as the EE corresponding to certain activities, or in direct measurements of resting EE (REE), may require higher temporal resolution. These are by no means as straightforward to obtain, especially if the experimental animal is housed in a relatively large and enriched 'home cage' environment to minimize stress artifacts.

It is the intent of this mini review to explain the methodological trade-offs involved in metabolic measurement, particularly concentrating on EE measurements in small experimental animals. The intent is not to survey the literature, but to introduce readers to quantitative aspects of metabolic measurement that are all, but unknown to researchers who may depend on them. Most researchers will agree that EE measurements should ideally reflect the moment-by-moment EE of the animal being measured, without delay or distortion. This is very far from being the case in real-world EE measurements, and it is hoped that this mini review of the methodology will explain why, and point the reader to possible solutions.

FLOW-THROUGH RESPIROMETRY

Virtually all the metabolic measurements on humans and experimental animals taking place in the world today are conducted using flow-through respirometry. Taking a mouse as an example, the animal is contained in a chamber through which a known flow rate of air is maintained. By comparing incurrent and excurrent gas concentrations, and thus measuring the reduction in oxygen concentration and increase in CO_2 concentration caused by the animal, it is possible to derive accurate measurements of the animal's O_2 consumption rate (VO_2) and CO_2 production rate (VCO_2), from which its respiratory quotient ($RQ = VCO_2/VO_2$) and EE can be calculated.

This is a gross oversimplification (for those interested, a fully detailed treatment is available[5]). For example, the air flow mentioned above can be maintained by pushing air into a sealed cage, or by pulling air from a cage that, if the air flow is carefully designed, does not necessarily have to be tightly sealed.[5] And, the equations utilized to derive VO_2 and VCO_2 are heavily dependent on the flow methodology, in addition to which both the incurrent and excurrent airstreams contain water vapor that dilutes the respiratory gas concentrations (*ibid*.). Unless the water vapor is removed or measured and accounted for, its presence will cause significant errors. However, these are methodological matters that are a question of system design choice and have relatively straightforward solutions.

TEMPORAL RESOLUTION LIMITATIONS

Two more subtle and interrelated problems combine to severely limit the temporal resolution of most metabolic measurement

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systems, whether for large (human) or small animals. We will treat these problems in turn.

Some background: to a very close approximation, flow-through metabolic measurement systems are linear and time invariant. Linearity, here, means that the input and output of such a system are mapped in a linear manner; if an input changes the output of the system, and a different input also changes the output, then the system output is the scaled and summed response to the two inputs. It can be shown that any linear time-invariant system is completely characterized by its reaction to a suddenly changing input signal, often referred to as an impulse or step response depending on its duration,[6] to which the output of the system will respond with the convolution of the input impulse or step.[7]

The time constant of the chambers

The above description is rather abstract. How do these concepts apply to real-world metabolic measurement? If a flow-through metabolic measurement system is a rigidly deterministic first-order time-invariant system, its output (which is to say, its excurrent respiratory gas concentrations as a function of time) may bear only a passing resemblance to its input, which is the actual animal EE within the chamber that we need to measure. The chief reason for this disparity is the time constant (τ) of the system, which is equal to the chamber volume divided by the flow rate through the chamber. It has units of time. In response to a step change in an animal's EE, the system (chamber plus animal) will exhibit an exponential approach to the final value of that step change. If $V(t)$ is the value of the system output at time t, and if V_0 is its value at time zero, the system output will change per standard first-order kinetics, proportionately to $V_o e^{-t/\tau}$. Over a time span equal to τ, the output of such a system will reach only $\sim$63% $(1-1/e)$ of its final value, whereas intuitively it might be expected to track changes in the animal's EE far more closely. It can easily be shown that to settle acceptably closely ($\sim$1%) to the final value of a step change, it is necessary to wait approximately five time constants.

Is the chamber time constant relevant? I do not wish to single out particular manufacturers of model animal metabolic measurement systems, but will take as typical of those in wide and prolonged use in the biomedical field, a flow rate of $\sim$400–450 ml/min and a 'home cage' volume of $\sim$9 liter (pers. comm. Karl Kaiyala, University of Washington; Louise Lantier, Vanderbilt). This equates to a time constant of $\sim$20–25 min. Thus, if a mouse suddenly changes its metabolic rate to a different, stable value, for example, during a brief bout of consistent activity, such a metabolic measurement system will take $\sim$100 min to reflect the new EE accurately. As anyone familiar with mouse behavior will attest, the mouse's behavior will have changed multiple times during that time. A far faster time constant will be required to reflect the dynamic, rapidly changing EE of a mouse. That said, if only broadly averaged means are of interest, such a system will produce acceptable results.

Even if only broad means are required, however, it is still frequently necessary to obtain accurate estimates of REE. Yet this is difficult or even impossible to do if the EE data are severely distorted by the slow time constants characteristic of most metabolic phenotyping systems. Many mathematical techniques of varying sophistication, such as linear regression analysis, Kalman filtration and penalized spline regression (ably summarized by Van Klinken et al.[8]) have been employed in an attempt to estimate REE from such records, with varying and controversial degrees of accuracy. And of course, the fleeting metabolic signatures of activity bouts are lost in the low-pass filtration of the system in the time domain, effectively smearing and blurring the original, fine-structured EE signal generated by the experimental animal.
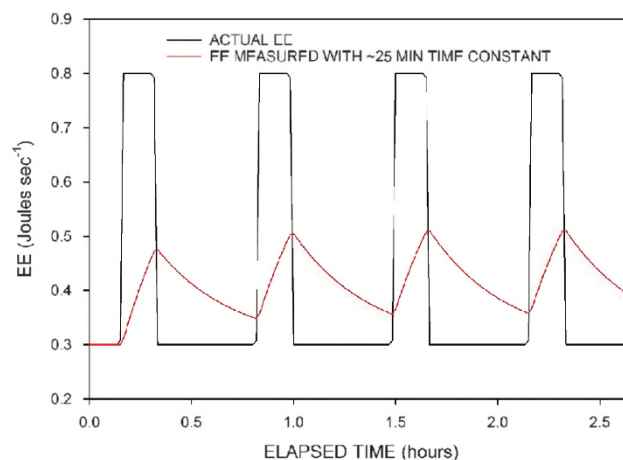

Figure 1. Mathematical model of the response of a flow-through metabolic measurement system with a time constant of ~25 min, to abrupt, repeated changes in the metabolic rate of an animal in the system. Black trace, underlying metabolic data; red trace, metabolic data distorted by the time constant of the system. See text for more details.

This can be illustrated by modeling such a system mathematically (Figure 1), assuming a time constant of 25 min. In this case, the model postulates a mouse with a REE of 0.3 J/s. Every 0.5 h, the mouse abruptly increases its EE for 10 min to 0.8 J/s. This is a factorial increase of 2.7, well within the range of a sudden, brief episode of activity in a small mammal.[5] The measured EE (red line) is grossly distorted relative to the actual EE of the animal in the chamber (black line). Neither REE nor active EE's can be ascertained with confidence from the data. Yet, this is entirely typical of >90% of the model animal EE data in the literature (again, I believe that citing and shaming typical investigations is counterproductive).

At this point, it is worth taking a brief segue into another methodological aspect of high-throughput metabolic phenotyping. It is customary to share a gas analysis chain (typically O_2 and CO_2) between several chambers, sampling from each in succession. This is referred to as multiplexing. The time taken to obtain a new sample from a given cage is referred to as the cycle time of the system.[5,9] This can vary from $\sim$2 min for a highly optimized, modern system to >45 min for an older system. 'Continuous' gas analysis is often proposed as a solution to the poor temporal resolution of multiplexed metabolic phenotyping systems, because each cage utilizes a dedicated gas analysis chain that is sampled much more frequently, as often as 1 Hz in some systems. Inspection of the red line in Figure 1 shows, however, that the amount of new information obtained with each sample drops rapidly as soon as the sampling interval drops below the time constant of the system's chambers. By the time the sampling interval or cycle time falls below approximately half the system's time constant, very little additional information is captured or benefit derived if the sampling interval or cycle time decreases further. In practice, this means that a metabolic phenotyping system with a long chamber time constant will benefit marginally, if at all, from so-called 'continuous' measurement. I will formalize this relation in due course.

The solution to the unacceptably long time constant of most metabolic phenotyping systems (and with that, the indirect and admittedly inaccurate methods required to estimate temporally sensitive information such as REE from their data) is obviously to reduce the time constant.

The resolution of gas analyzers

At first sight, reducing the time constant of the chamber would appear to be trivial. If the volume cannot be reduced (doing so would create a cramped and high-stress environment), the flow rate through the chamber must increase.

The concentration changes of O_2 and CO_2 between the incurrent and excurrent airstreams are customarily expressed in fractional concentration terms (= percent/100 which is what the respirometric equations expect), and are generally referred to as ΔO_2 and ΔCO_2. To convert these dimensionless fractional concentrations into VO_2 and VCO_2 in volume units per unit time, ΔO_2 and ΔCO_2 are multiplied by the flow rate through the chambers. VO_2 and VCO_2 then inherit the dimensions of the flow rate, for example, ml/min. (The full story is more complex and includes compensation for interacting dilution and concentration effects, among others.[5])

From this, it follows that if VO_2 and VCO_2 remain constant, ΔO_2 and ΔCO_2 must fall in inverse proportion to flow rate. A simple thought experiment will convince you that as flow rate through a chamber containing an animal approaches infinity, ΔO_2 and ΔCO_2 must approach zero (in the spirit of physics examples, the hypothetical animal is not affected by the flow rate). It is generally accepted that O_2 and CO_2 analyzer resolution in the range of 0.01% is adequate for modern metabolic phenotyping systems.[10] In other words, with such analyzers, a ΔO_2 or ΔCO_2 of 0.01% would be indistinguishable from the background noise and drift that the analyzers normally produce. It is rational, given this resolution, to aim for a ΔO_2 and ΔCO_2 in the range of 0.5%, in which case the noise contribution falls to ~ 2% of the signal (for equations relating flow rates to analyzer noise figures, see Lighton and Halsey[9]). That optimal flow rate is ~ 400–450 ml/min, which happens to be the consensus flow rate that has been utilized for many years in metabolic phenotyping systems—for good reason, as can be seen.

With appropriate design care and choice of suitable sensor technologies, it is, however, possible to push both O_2 and CO_2 analyzer resolution down to a $1 - \sigma$ value of 0.0001% (1 p.p.m.) or below. For example, a commercially available O_2 analyzer has been found able, with care, to resolve 0.00003% (0.3 p.p.m.[11]).
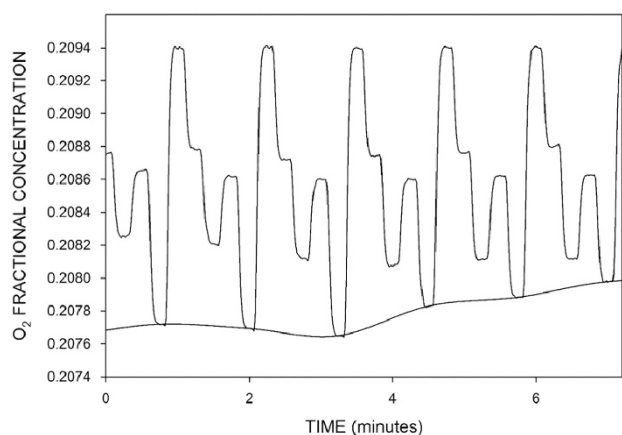

Figure 2. Rapid switching (multiplexing) of an O_2 gas analyzer between multiple chambers, $N = 4$, in a metabolic phenotyping system. The excurrent air from each chamber is sampled for 15 s, and interleaved by periodic sampling of incurrent O_2 concentrations. This allows the ΔO_2 caused by the animal to be measured with optimal accuracy. Note the rapid response of the O_2 analyzer to sample concentration changes, enabled by response correction based on the very low intrinsic noise level of the analyzer. The line near the bottom of the graph shows the extraction of interpolated O_2 data from cage 4. The chamber time constant of this system is ~ 4 min, so multiple samples are taken from each cage per time constant. Data from a Promethion metabolic phenotyping system (Sable Systems, Las Vegas, NV, USA).

On an autobiographical note, I designed that analyzer. I found it necessary to use or design such high-resolution analyzers to conduct my own research on the energetics and gas exchange kinetics of diverse insects,[12] foraging in ants,[13–15] flight energetics in Drosophila[16] and hummingbirds,[17] and so on. I have also found that knowledge of the mathematical behavior of respirometry systems (a field pioneered by my doctoral supervisor at UCLA, George Bartholomew[18,19]) is exceedingly useful in accelerating gas analyzer response times and permitting very rapid, sequential sampling in multi-chamber metabolic measurement systems (Figure 2).

REQUIREMENTS FOR HIGH TEMPORAL RESOLUTION

Use of very high-resolution O_2 and CO_2 analyzers permits a corresponding increase in chamber flow rate, and thus a proportional decrease in chamber time constant. Assuming a conservative 0.001% analyzer noise figure, a approximately five-fold increase in chamber flow rates still yields twice the signal-to-noise ratio of a conventional system, although decreasing its time constant and increasing its temporal resolution five-fold to ~ 4 min. This is done without sacrificing the interior volume and floor surface area required to house a model animal at minimal stress levels. In addition, the higher flow rate reduces the CO_2 concentration within the chamber from ~ 0.5 to ~ 0.1%, which is close to normocapnic levels and poses no risk of the cognitive impairment and behavioral changes reported at higher CO_2 concentrations (albeit in humans—no such work has been done in mice or rats to my knowledge).[20,21]

ROOM CALORIMETRY AND CONVECTIVE AIR MOVEMENT

With some care, it is possible to utilize knowledge of the kinetics of flow-through respirometry to reverse the distortions caused by chamber time constants. This is particularly relevant to human room calorimetry, where chamber (= room) volumes of 25 000+ liters are common, together with relatively low flow rates in the 30–80 l/min range.[4] This yields time constants of the order of several hours. Some degree of compensation for the distortion caused by the time constant of the room is obviously essential, and if transient EEs corresponding, for example, to treadmill running, are to be reliably detected within a short time span, that compensation must be extraordinarily fine-tuned via several ingenious methods.[22] Underlying our mathematical understanding of response distortions is the implicit assumption that perfect convective mixing occurs within the respirometry chamber. To utilize response correction on the chamber level, it is therefore necessary to actively mix the air within the chamber. In room calorimeters, this is generally accomplished by powerful air circulators.[4] Unfortunately, such vigorous circulation is difficult to achieve within typical model animal chambers, because it carries the risk of greatly accentuating convective heat loss, thus further distorting the metabolic signal. Thus most model animal chambers are not actively, convectively ventilated, and application of mathematical response correction to the resulting data is problematic because of imperfect mixing and slight stratification. It should also be noted that unless very low noise gas analyzers are used, application of mathematical response correction may be difficult or impossible because it relies on the highly amplified first derivative of the data. Consequently, increasing the chamber flow rate remains the best method for decreasing the chamber time constant although, as pointed out above, this approach makes considerable demands on the resolution of the gas analyzer chain.

CONCLUSION

I have shown that metabolic measurement conducted using flow-through respirometry suffers from serious but generally unrecognized
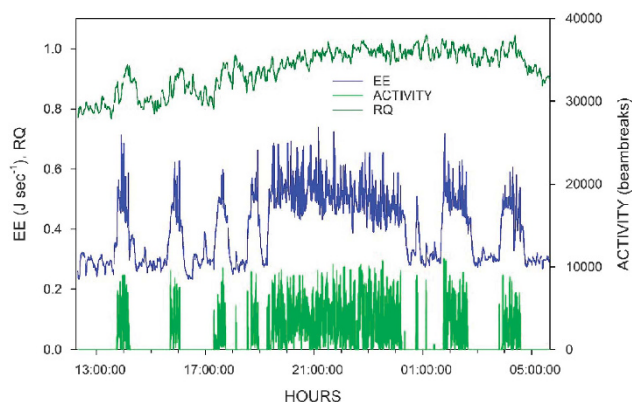
Figure 3. Activity (lower, light green trace), EE (blue trace) and RQ (respiratory quotient, an indicator of metabolic substrate; upper, dark green trace) of a C57BL/6 J mouse, body mass 25.8 g, 23 °C. A metabolic phenotyping system with a chamber time constant of ~ 4 min and a cycle time of ~ 2.5 min was used (Promethion data). See text for more details.
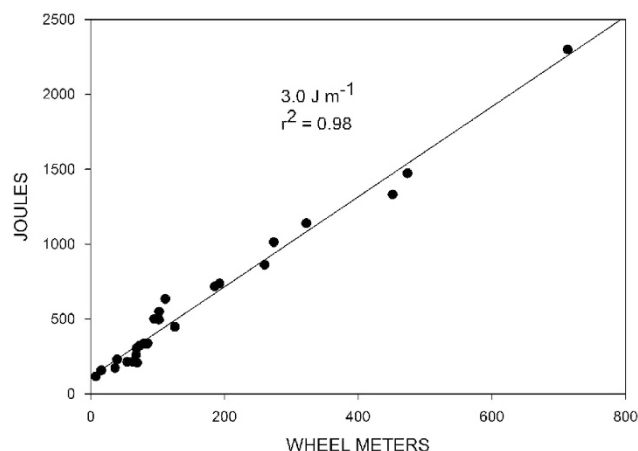

Figure 4. Integrated EE (y axis) versus meters traveled in pedestrian locomotion on a running wheel (x axis) for several bouts of wheel running for a C57BL/6 J mouse, mass 23.1 g. The slope of the line corresponds to the cost of transport of the mouse in units of J/m. Note that the mouse was unrestrained and running voluntarily; see text for details.

limitations in temporal resolution, and I propose that temporal resolution can be improved several fold by increasing chamber flow rates, and at the same time decreasing the relative contribution of analyzer noise to the metabolic signal, provided appropriate gas analyzers, signal handling and data acquisition technologies are utilized to their best effect.

In the space available, the synergistic effects of this new approach to metabolic phenotyping are best shown graphically. (Again, I do not reproduce data from conventional systems here; readers are no doubt familiar with many examples from the literature.) In Figure 3, a clear contrast between REE and active EE can be seen, together with tight correlation between bouts of activity (lower green trace) and EE (blue trace). There is no need to estimate REE using indirect methods; it can be directly measured without contamination from activity.

It will be noted from Figure 3 that each bout of activity, particularly in the photophase, yields a cleanly separated peak of EE. By analyzing data from a similar recording with equivalent temporal resolution from a mouse housed in a chamber with a running wheel, it is possible to separate each bout of wheel running and its corresponding EE signature. By integrating the

running speed in m/s against time in seconds to yield meters run for each bout, and by integrating the resulting EE in J/s against time in seconds to yield J, performing a linear regression of EE and distance run yields the cost of transport of the mouse as the slope of this relation (Figure 4). These data were generated voluntarily by an unrestrained, unshocked mouse without requiring a treadmill, and are in excellent accord with literature values (Figure 2 in Vance Tucker's classic cost of transport review[23]). This approach requires an ambiguous separation of EE events corresponding to each locomotion bout, and is thus not feasible with traditional metabolic phenotyping approaches.

Many other applications for high temporal resolution in metabolic phenotyping exist, including detailed investigation of non-activity thermogenesis, the kinetics of drug reactions, quantification of the thermic effect of food and 'luxus consumption', the role of activity in overall EE, and a host of others. This mini review of metabolic measurement methodology hardly scratches the surface of this complex and controversial field. If it raises some awareness of the limitations inherent in metabolic measurement, and points the way to resolving some of these limitations, it will have achieved its goal.

CONFLICT OF INTEREST

JRBL, a recovering academic, is president and CTO of Sable Systems International, a company that designs and produces metabolic measurement and metabolic phenotyping systems.

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