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Indirect calorimetry in laboratory mice and rats: principles, practical considerations, interpretation and perspectives

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Even PC, Nadkarni NA. Indirect calorimetry in laboratory mice and rats: principles, practical considerations, interpretation and perspectives. Am J Physiol Regul Integr Comp Physiol 303: R459 –R476, 2012. First published June 20, 2012; doi:10.1152/ajpregu.00137.2012.—In this article, we review some fundamentals of indirect calorimetry in mice and rats, and open the discussion on several debated aspects of the configuration and tuning of indirect calorimeters. On the particularly contested issue of adjustment of energy expenditure values for body size and body composition, we discuss several of the most used methods and their results when tested on a previously published set of data. We conclude that neither body weight (BW), exponents of BW, nor lean body mass (LBM) are sufficient. The best method involves fitting both LBM and fat mass (FM) as independent variables; for low sample sizes, the model $LBM^{0.2}/FM$ can be very effective. We also question the common calorimetry design that consists of measuring respiratory exchanges under free-feeding conditions in several cages simultaneously. This imposes large intervals between measures, and generally limits data analysis to mean 24 h or day-night values of energy expenditure. These are then generally compared with energy intake. However, we consider that, among other limitations, the measurements of $V_{O_2}$, $V_{CO_2}$, and food intake are not precise enough to allow calculation of energy balance in the small 2–5% range that can induce significant long-term alterations of energy balance. In contrast, we suggest that it is necessary to work under conditions in which temperature is set at thermoneutrality, food intake totally controlled, activity precisely measured, and data acquisition performed at very high frequency to give access to the part of the respiratory exchanges that are due to activity. In these conditions, it is possible to quantify basal energy expenditure, energy expenditure associated with muscular work, and response to feeding or to any other metabolic challenge. This reveals defects in the control of energy metabolism that cannot be observed from measurements of total energy expenditure in free feeding individuals.

indirect calorimetry; mouse; rat; energy metabolism; energy expenditure; locomotor activity; respiratory quotient; body size; body composition

BUILDING ON THE DISCOVERY of oxygen by Joseph Priestley, Antoine Lavoisier discovered that life is combustion—the chemical energy contained in nutrients is extracted by a process of oxidation that consumes oxygen and releases carbon dioxide and water (28). This energy is converted into energy-dense molecules, mainly ATP and creatine. These are then used when endergonic reactions are required, for example, synthesis processes and muscular work. Part of the reaction energy is lost as heat (~40%) which in homeotherms is used (and at thermoneutrality is sufficient) to maintain body temperature at ~37–39°C.

Comparison of heat production (measured by direct calorimetry) with $V_{O_2}$ and $V_{CO_2}$ showed that tight relationships exist between them. From 1890 to 1930, this line of work derived precise equations (well described in Ref. 28). Thus, measurement of respiratory exchanges has become a means to measure energy expenditure, bypassing the complex, delicate, and costly technique of direct measurement of heat production (indirect vs. direct calorimetry).

Indirect calorimetry (IC) is based on very simple principles, but, in practice, has long been a very delicate technique, since
the measurement of small changes in the oxygen content of air is prone to many errors due to changes in temperature, pressure, humidity, and so on. In the past, skilled technicians were allocated to tuning and running these devices—not only human respiration chambers, but also animal devices. Recently, largely in response to the need to phenotype large numbers of transgenic mice, user-friendly IC systems have multiplied. The pitfall of this development is that many investigators rely uncritically on the manufacturers’ unrealistic assertion that their systems require little calibration and intervention. However, by understanding some simple basic principles, it is possible to avoid misinterpretation.

The main goal of this paper is to describe in simple terms how IC operates, what IC can do, and what its limitations are. We shall also discuss how the data provided by IC can be corrected and analyzed.

Principles of Indirect Calorimetry

What units to use? Historically, the unit in nutrition is the calorie (cal). However, the joule (J) is the SI unit for energy, and therefore, the joule and the watt (W; J/s) rather than cal and cal/min should be used to express energy expenditure (EE) and metabolic rate. International agencies, such as the Food and Agriculture Organization and the European Food Safety Authority, now publish energy requirements in kilojoules and megajoules.

Glucose, lipid, and protein oxidation are usually expressed in grams or milligrams per minute. Here, we suggest that to further standardize the expression of EE and metabolic rate and better comprehend the relative participation of carbohydrates, proteins, and lipids in overall energy production, milligrams and milligram per minute should also be replaced by joules and Watts.

Table 1 summarizes the relationship between the usual formulations, and the one we suggest for a typical mouse and rat fed a standard diet in which lipids provide 10% of energy, protein provides 15% of energy, and carbohydrates provide 75% of energy.

Table 1. Example of conversion between kcal, cal/min, and mg/min to kJ and W in a mouse and a rat

<table>
<thead>
<tr>
<th>Calorie and Gram-Based Units</th>
<th>Conversion Factor</th>
<th>Joule-Based Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily EE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse: 13 kcal</td>
<td>4.185</td>
<td>54.5 kJ</td>
</tr>
<tr>
<td>Rat: 65 kcal</td>
<td></td>
<td>272 kJ</td>
</tr>
<tr>
<td>Metabolic rate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse: 9.03 kcal/min</td>
<td>(4.185 × 60) = 0.0698</td>
<td>0.630 W</td>
</tr>
<tr>
<td>Rat: 45.1 kcal/min</td>
<td></td>
<td>3.15 W</td>
</tr>
<tr>
<td>Carbohydrate oxidation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.69 mg/min</td>
<td>(4.0 × 4.185 ÷ 60) = 0.279</td>
<td>0.472 W</td>
</tr>
<tr>
<td>8.46 mg/min</td>
<td></td>
<td>2.36 W</td>
</tr>
<tr>
<td>Protein oxidation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.338 mg/mg/min</td>
<td>(4.0 × 4.185 ÷ 60) = 0.279</td>
<td>0.094 W</td>
</tr>
<tr>
<td>1.68 mg/mg/min</td>
<td></td>
<td>0.472 W</td>
</tr>
<tr>
<td>Lipid oxidation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.100 mg/mg/min</td>
<td>(9.0 × 4.185 ÷ 60) = 0.628</td>
<td>0.063 W</td>
</tr>
<tr>
<td>0.501 mg/mg/min</td>
<td></td>
<td>0.315 W</td>
</tr>
</tbody>
</table>

4.185 is the conversion factor from calories to joules. EE, energy expenditure. Energy content in carbohydrate, protein, and fat are assumed to be 4, 4, and 9 cal/g, respectively.

Relationship of Glucose, Lipid, and Protein Oxidation to \(\dot{V}O_2\) and \(\dot{V}CO_2\)

Carbohydrate, lipid, and protein are the three main energetic substrates oxidized to produce energy. The ratios of oxygen consumed (\(\dot{V}O_2\)) to carbon dioxide produced (\(\dot{V}CO_2\)), as well as the amount of energy released per gram is not the same for the three substrates. For a detailed description, see Refs. 27 and 57.

For carbohydrates, the ratio of \(\dot{V}CO_2\) to \(\dot{V}O_2\) is 1.0; 47.5 ml of O2 are consumed per kilojoule produced and 15.7 kJ are produced per gram of glucose oxidized [a little more 16.7 kJ/g for glycogen due to the additional energy available from the \(\alpha(1\rightarrow6)\)-linked branches]. The general equation is

\[
1 \text{ g glucose} + 746 \text{ ml O}_2 \rightarrow 746 \text{ ml CO}_2 + 0.6 \text{ g H}_2\text{O} + 15.7 \text{ kJ} \quad (1)
\]

For lipids, the ratio of \(\dot{V}CO_2\) to \(\dot{V}O_2\) is 0.705; 53.8 ml of O2 are consumed per kilojoule produced and 37.7 kJ are produced per gram of lipid oxidized. The general equation is

\[
1 \text{ g palmitate} + 2.029 \text{ ml O}_2 \rightarrow 1.430 \text{ ml CO}_2 + 0.45 \text{ g H}_2\text{O} + 37.7 \text{ kJ} \quad (2)
\]

For protein, despite some variability in the amino acid composition of proteins, a general equation can be used to approximate oxidation of a standard protein in which the ratio of \(\dot{V}CO_2\) to \(\dot{V}O_2\) is 0.81; 57.8 ml of O2 are consumed per kilojoule produced and 16.7 kJ are produced per gram of protein oxidized. The general equation assuming urea is the main waste product is

\[
1 \text{ g protein} + 966 \text{ ml O}_2 \rightarrow 782 \text{ ml CO}_2 + 0.45 \text{ g H}_2\text{O} + 0.50 \text{ g Urea} + 16.7 \text{ kJ} \quad (3)
\]

It appears from these equations that the ratio of \(\dot{V}CO_2:\dot{V}O_2\) (the respiratory quotient, RQ) is different for glucose, fatty acids, and proteins. Thus, RQ is typical of the substrate oxidized and can be used in IC to determine the relative participation of glucose, lipids, and proteins in energy production. RQ is different between carbohydrate, lipid, and protein because the C to O ratio is higher in proteins and lipids than in glucose. Consequently, more oxygen is needed to oxidize completely proteins and lipids than glucose, but more energy can be released (22 and 39 vs. 16 kJ/g, respectively). However, oxidation of glucose and lipids is complete (end products are CO2 and H2O), while oxidation of protein is not, since nitrogen has to be excreted as a waste product, mainly in the form of urea [CO(NH2)2] in mammals. The energy lost in this process reduces the metabolizable energy of protein down to 16.7 kJ/g, similar to that of carbohydrate. It should also be borne in mind that when special foods are consumed, the energy content and quotient of oxidation of triglycerides depend on their length and degree of saturation (46).

The other important point that results from the differences in the chemical composition of carbohydrate, lipid, and protein, is that the oxygen cost of ATP production differs between the three substrates (3.72, 3.93, and 4.96 l/mol, respectively). In particular, \(\sim 30\%\) more oxygen is required when ATP is generated from amino acid than glycogen. This may explain why amino acids are so reluctantly mobilized when energy requirements are acutely increased, such as during muscular effort and why
glucose oxidation is favored at the expense of lipid as the intensity of exercise increases.

Equations to compute energy expenditure. Because the energy equivalent of oxygen depends on the substrate oxidized, \( \text{VO}_2 \), alone cannot be used to precisely compute metabolic rate. As shown in Fig. 1, the energy equivalent of oxygen increases from 80.8 kJ/l to 87.0 kJ/l when \( \text{RQ} \) increases from 0.7 to 1.0. Both \( \text{VO}_2 \) and \( \text{VCO}_2 \) are, thus, included in most equations developed to compute metabolic rate from respiratory exchanges. Figure 1 shows that four of the most popular equations (7, 15, 48, 66) never diverge by more than 1% and, thus, can be considered as equally valid. However, the Weir equation is, by far, the most popular. Protein oxidation is not taken into account in these equations because, in practice, it only marginally affects the energy equivalent of \( \text{VO}_2 \) and \( \text{VCO}_2 \) (see Fig. 2).

Equations to compute glucose, lipid, and protein oxidation.

The main equations to describe the oxidation of standard carbohydrate (glucose), lipid (palmitate), and protein (casein) are explained below (for details, see Refs. 27 and 57).

Protein oxidation (Pox) cannot be directly assessed from respiratory exchanges. The only way is to collect urinary nitrogen excretion. Approximately 80% of nitrogen is eliminated as urea in mammals, other sources being \( \text{NH}_4 \) nitrogen excretion. Approximately 80% of nitrogen is eliminated as urea in mammals, other sources being \( \text{NH}_4 \) nitrogen excretion. Approximately 80% of nitrogen is eliminated as urea in mammals, other sources being \( \text{NH}_4 \) nitrogen excretion. The formulas generally used derive from the ones described by Ferrannini (27). They can be converted from milligrams per minute to Watts, according to Table 1. (Units for Gox, Lox, and N are given in milligrams, while \( \text{VO}_2 \) and \( \text{VCO}_2 \) are given in milliliters.)

\[
\text{Gox} = (4.55 \times \text{VO}_2) - (3.21 \times \text{VCO}_2) \quad (5a)
\]

or taking N into account:

\[
\text{Gox} = (4.55 \times \text{VO}_2) - (3.21 \times \text{VCO}_2) - (2.87 \times N) \quad (5b)
\]

\[
\text{Lox} = (1.67 \times \text{VO}_2) - (1.67 \times \text{VCO}_2) \quad (6a)
\]

or taking N into account:

\[
\text{Lox} = (1.67 \times \text{VO}_2) - (1.67 \times \text{VCO}_2) - (1.92 \times N) \quad (6b)
\]

These equations, in contrast to the ones derived to compute EE, are sensitive to interconversion of substrates, and, in particular, to errors induced by de novo lipogenesis and gluconeogenesis. Possible adjustments when such processes occur are described by Elia and Livesey (16) and Ferrannini (27).

Taking into account protein oxidation. When Pox is not taken into account (Eqs. 5a and 6a), the conversion of \( \text{VO}_2 \) and \( \text{VCO}_2 \) into EE is not much affected but both Gox and Lox are overestimated. However, since the RQ of Pox (0.81) is closer to that of Lox (0.71) than of Gox (1.0), more of the error is reported on Lox than on Gox, and thus, Lox is overestimated relative to Gox (Fig. 2). There is no definitive solution to this problem, but it is generally suggested that when proteins account for more than 10–15% of the energy in the diet, the rate of protein oxidation should be taken into account for the calculation of Gox and Lox. Figure 2 shows how an increase from 0 to 50% of the contribution of Pox to energy production affects Gox, Lox, and the metabolic rate. It is apparent that assuming Pox is null only very marginally overestimates metabolic rate, but significantly overestimates Gox and particularly Lox. This example (mouse data) shows that at a level of

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**Fig. 1. Evolution of the energy equivalent of 1 liter of oxygen in relation to respiratory quotient (RQ).** According to the Brouwer, Lusk, Weir, and Elia formulas. Lusk: 15.79 \( \text{VO}_2 \) + 5.09 \( \text{VCO}_2 \), Weir: 16.3 \( \text{VO}_2 \) + 4.57 \( \text{VCO}_2 \), Elia: 15.8 \( \text{VO}_2 \) + 5.18 \( \text{VCO}_2 \), and Brouwer: 16.07 \( \text{VO}_2 \) + 4.69 \( \text{VCO}_2 \) (7). Inset: differences in % between Lusk, Elia, and Brouwer vs. Weir.
V_{O2}) milliliters per minute. Tissues do not produce the same energy per unit weight (14, 25, 65). Some organs are very active (liver, heart, kidney, brain), others are intermediate (resting muscles), and still others have a low or very low metabolic rate (bones, skin, and adipose tissue). Ideally, the exact weight of the various organs and tissues should be known, and whole body metabolic rate predicted from the exact body composition and specific metabolic rate of the various organs and tissues. This is, of course, not possible in most studies, and thus, normalization of EE relies on body weight (BW), allometric functions of BW, lean body mass (LBM) and fat mass (FM). However, if an increase in BW is not paralleled by an increase of the various tissues and organs of the body in proportion to BW, the difference in EE is not a linear function of the change in body size. In particular, in response to genetic manipulation or dietary manipulations, the size of a specific tissue (most often adipose tissue but sometimes LBM or muscle mass) can change relatively more than the size of the other organs and tissues. Not accounting for this can lead to an incorrect assignment of the relative effects of body composition and cellular metabolism on EE.

BW or exponents of BW. Correction of EE by BW is the easiest, most widely used and simplest method. It may be the least prone to generate significant bias when used to adjust EE between individuals that do not diverge much in body composition and in particular in which adiposity is comparable. This can be the case for lean mice between 15 and 25 g or lean rats between 250 and 500 – 600 g during their spontaneous growth, in which adipose tissue amounts to a fairly stable proportion of BW (15–20%). Thus, for the same strain raised in the same conditions, a group of rats should have the same per weight BEE across this limited weight range. In our hands, this is the case, except for a slight underestimation of BEE in larger animals (1.8 kJ per 100 g) (Fig. 3). Thus, BW may be a reasonable solution for adjusting EE between adult lean rats and mice of various weights when the differences in BW are not too large. However, when differences in adiposity are suspected or measured, adjusting EE to BW will induce an underestimation of the EE of the fatter animals because a larger BW (15–20% level of protein oxidation above which Pox should not be neglected. Not taking into account Pox induces a 12% overestimation of Gox and a 26.6% overestimation of Lox: Gox = [(4.57 × V_{CO2}) − (5.23 × V_{O2}) − (2.6 × N)] × (4 × 4.185 ÷ 60), Lox = [(1.69 × V_{O2}) − (1.69 × V_{CO2}) − (2.03 × N)] × (9 × 4.185 ÷ 60), Pox = N × 6.25 × 4 × 4.185 ÷ 60, and Pox, Gox, and Lox in W, N in milligrams per minute, V_{O2} and V_{CO2} in milliliters per minute.

15%, not taking account of Pox leads to an overestimation of Gox by 12% and of Lox by 27%.

Adjusting Energy Expenditure/Metabolic Rate for Body Weight and Body Composition

Even when raised under circumstances that are close to identical, laboratory mammals differ in size, and it is necessary to account for this when analyzing calorimetry data. However, there is a more complex and important issue—the various organs and tissues of the body have diverse metabolic rates (14), and their relative proportions vary depending upon circumstances (25). This means that if a manipulation has an effect on per-weight EE, it could come from two sources. The first, and usually considered less interesting, is changes in body composition—for example, increased food intake leading to proportionately increased adipose mass, a tissue with a relatively low metabolic rate. The more sought-after effect is changes in cellular metabolism, such as via altered motility or thermogenesis. To dissect how a manipulation affects EE, it is vital to know which source contributes, or in what proportion they both contribute—so it is vital to quantify and then normalize data to body composition, the one of the two sources that is measureable. How this should be done is an unresolved issue.

Normalization of EE should be performed on basal EE (BEE), that is to say on EE measured in standardized conditions (post-absorptive state, at rest and thermoneutrality) that reduce variability and best permit comparison between individuals. Alternatively, total EE (TEE) or resting EE (REE) measured in standardized conditions can be used. BEE and to a lesser extent TEE and REE directly depend on the sum of the energy expended by the various tissues of the body, and these tissues do not produce the same energy per unit weight (14, 25, 65). Some organs are very active (liver, heart, kidney, brain), others are intermediate (resting muscles), and still others have a low or very low metabolic rate (bones, skin, and adipose tissue). Ideally, the exact weight of the various organs and tissues should be known, and whole body metabolic rate predicted from the exact body composition and specific metabolic rate of the various organs and tissues. This is, of course, not possible in most studies, and thus, normalization of EE relies on body weight (BW), allometric functions of BW, lean body mass (LBM) and fat mass (FM). However, if an increase in BW is not paralleled by an increase of the various tissues and organs of the body in proportion to BW, the difference in EE is not a linear function of the change in body size. In particular, in response to genetic manipulation or dietary manipulations, the size of a specific tissue (most often adipose tissue but sometimes LBM or muscle mass) can change relatively more than the size of the other organs and tissues. Not accounting for this can lead to an incorrect assignment of the relative effects of body composition and cellular metabolism on EE.

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part of their BW is composed of tissue that has a low metabolic activity. Not taking this into account, despite extensive warnings, has often led to the incorrect conclusion that EE is decreased in obese mice/rats and increased in lean ones (6, 8, 9, 36, 40).

Many studies account for differences in body composition related to body size by adjusting EE, according to the Kleiber allometric law of BW raised to the power 0.75 (44). Despite the fact that it was recently suggested that this adjustment was valid in rats (42), we, as well as many others, consider the 0.75 Kleiber exponent can be valid only to describe interspecies differences in EE. It accounts, among other still poorly understood mechanisms (31), for the fact that in larger animals the quantity of tissue with a medium and low metabolic rate is relatively large compared with that of tissues with a high metabolic rate. For example, Wang et al. (64) showed that the four organs with the highest metabolic rate (liver, brain, kidney, and heart) account for 68% of REE in a 100-g mammal and for only 34% in a 100-kg mammal. Rats and mice grow throughout their life, but the relative size of the main metabolically active tissues and organs (apart from the brain) remains fairly constant when they remain lean. In contrast, in obese subjects, adipose tissue size increases much more than other tissues. Therefore, differences in BW due to either age or adiposity cannot be adjusted by the Kleiber exponent. In our hands, in lean Wistar rats, correcting BEE for BW0.75 strongly overestimates it in larger animals (25 kJ/100 g) (Fig. 3).

Adjustment to LBM and fat mass. Adjusting to LBM may be considered a solution to correct for large differences in BW and body adiposity because LBM is the “metabolically active” part of BW. This is unfortunately also incorrect since the metabolic activity of adipose tissue is not null. Therefore, adjustment of EE to LBM will tend to underestimate EE in fatter subjects (since adipose tissue activity gets incorporated into LBM, inflating it) in the same way that adjustment to BW underestimates it (1, 2, 39, 60).

Integration at the whole body level by regression analysis has also led to the estimation that the specific metabolic activity of adipose tissue is 15–25% of LBM in mice (discussed in Ref. 2). Therefore, if it can be considered that this 15–25% ratio between the specific EE of adipose tissue and LBM is the norm (let us say 20% for now), it is possible to compute from LBM and FM a “metabolically adjusted” weight as the sum of LBM + (0.2 FM). We have not yet used this method of correction in our previous publications and are not aware of other groups that have used it. However, on a theoretical basis, it should be more accurate than the use of BW only or LBM only (see below). At the present stage, however, because of uncertainties about the precise specific per-unit mass metabolic activity of adipose relative to LBM (see for example, Refs. 39 and 40) and possible variations of it with total adiposity (5), this approach needs to be tested.

Analysis of covariance of any known composition parameters. The use of analysis of covariance (ANCOVA) has been strongly advocated as the best and simplest statistical approach that has the main advantage of making no prior assumptions about the scaling of the relationship between different body compartments and EE (detailed and defended in Refs. 2 and 60). Accordingly, utilization of this method to compare BEE and REE in growth hormone-deficient mice suggests that it does, indeed, have a great potential to adjust EE between individuals with large differences in body weight and body adiposity (49). However, as noted in Ref. 2, one main caveat in using ANCOVA is that this approach does not work well when the within-group variances are low compared with the between-group effects on both mass and EE. Another limitation is that, because more parameters are necessarily included in the analysis, the power of the ANCOVA to reveal a difference is reduced as the number of body compartments used is increased. Where large numbers of animals are used, this will not pose a problem; however, for smaller-scale studies, it can have a substantial effect (8), though the importance of this has been questioned (see supplemental note 5 in Ref. 60). Our view is that a basic fact in statistics—the greater the number of parameters estimated, the greater the sample size required to observe effects—has to be taken into account. Therefore, although ANCOVA can be a valuable method to take into account LBM and fat mass, it may become less efficient if three or more body compartments (for example, liver, brain, kidney, muscle, and fat mass) are used, unless a large number of animals were studied.

Organ-tissue-based model. In humans, equations to predict EE from the weight and the specific metabolic activity of various organs and tissues have been developed from the measure of body composition by MRI and prediction of the specific metabolic activity of the various tissues, according to the historical report of Elia (14) recently validated by Wang (65). We also reported that in rats, organ-specific metabolic rates were 4.9 times higher than in humans, but their relative contribution to BEE was the same (25). Therefore, as detailed analysis of body composition by imaging techniques develops, we should start considering the possibility of fixing predictive equations for EE in rats and mice, and using these equations to estimate the “normality” of EE in our studies. The results we reported for rats in 2001, based on carcass analysis by dissection, strongly suggest that it is, indeed, possible to fit accurate predictive estimations of BEE, according to organ and tissue masses and from there, to study whether experimental groups are hypermetabolic or hypometabolic.

Example comparison of the various adjustment methods in a group of experimental subjects: lean rats (Wistar, Sprague-Dawley, and Zucker Fa?) vs. genetically obese (Zucker fa). In this section, we have reused the data of our 2001 publication (25) to compare the effect of the various adjustment methods on the comparison of BEE between control lean WT rats [a mix of Wistar, Sprague-Dawley, and lean Zucker Fa?] rats previously shown to constitute a homogenous group (25)] and genetically obese Zucker fatty (fa) rats (69). Statistics were calculated using R (53). At first glance, it appears that both BW and fa explain a large part of the variance in BEE (Fig. 4).

To make the comparison more about adjustment methods than statistical tests, all of them have been brought into the general linear model (GLM) framework. Our model is BEE = intercept + x1(adjustment) + x2(fa) + x3(adjustment × fa), where adjustment is a known continuous independent variable (such as BW), fa is a known discrete independent variable with a value of 0 or 1 (presence or absence of the allele, respectively), and the magnitude and significance of the intercept and the variable coefficients x1 (for adjustment), x2 (for fa), and x3 (for the interaction between adjustment and fa) are evaluated. If the adjustment involves more than just one factor (such as
using both LBM and FM), the equation necessarily becomes more complex with extra additive and interactive terms.

To take the example of adjustment by BW correction, rather than dividing all the BEE values by the corresponding BW then doing a t-test between WT and fa rats, a linear model is constructed with BEE as the dependent variable and fa and BW as independent variables. Very crudely, the GLM evaluation of the significance of x2 in the equation above becomes equivalent to the t-test, with x1 acting as the division that removes the effect of BW. The adjustment × fa interaction x3 does not have to be included in the model, but it is standard practice to start with the maximal model and then delete unimportant parameters. The interaction would mean that the effect of fa changed in magnitude with a rise in BW (and/or vice versa) rather than just being a constant to be added. This is biologically plausible, since changes in adipose volume could affect adipocyte lipid storage efficiency and basal metabolism (25), and via release of leptin and adipokinin have a hormonal effect on LBM metabolic activity (2, 39).

A very important aspect of these tests is to determine whether fa and/or its interaction(s) are useful explanatory variables. It is possible that the adjustment method removes all difference between WT and fa rats. In this case, an effect of fa on basal cellular metabolism can be ruled out in favor of a simpler situation where fa merely affects body weight and/or composition.

Table 2 outlines results from the five simplest corrections discussed above, with and without the interaction term x3. The results of the two more complex ones (LBM and FM, and multiorgan) do not lend themselves to convenient inclusion in the table and so are discussed later.

In all models, the intercept should not be substantially different from 0, since if there is no BW, LBM, or FM, there can be no BEE. The first base model (per rat) involves no correction. This model has almost no explanatory power (adjusted $R^2$ of 0.0000). The intercept is effectively the mean BEE of the lean group, with the fa effect being what has to be added to get the mean of the obese group. Because of high intragroup variance, the difference of 0.1141 ± 0.1139 is not significant ($P = 0.322$).

Regarding interactive models, these all have high explanatory power (adjusted $R^2$ of 0.77–0.79). However, there are differences between them. BW and BW$^{0.75}$ both have significant interaction terms ($-0.002 ± 0.0007, P = 0.005$ and $-0.011 ± 0.0040, P = 0.011$, respectively), while the LBM and LBM + 0.2 FM models do not have any significant terms for fa. BW$^{0.75}$ is the only one with a significant intercept ($-0.570 ± 0.2592, P = 0.0342$).

If the interaction is removed and an additive model used, in the cases of BW, BW$^{0.75}$ and LBM, the fa term becomes significant ($P = 0.000$ for all three). While BW$^{0.75}$ no longer has a significant intercept term, BW does ($0.5149 ± 0.1375, P = 0.0000$). Explanatory powers for all remain similar (0.75–0.78).

Since fa was not a significant term for LBM + 0.2 FM with or without an interaction, a final model was performed in which BEE was just explained by LBM + 0.2 FM. This was very powerful, with an adjusted $R^2$ of 0.787, insignificant intercept, and significant correction term ($0.0061 ± 0.0005, P = 0.0000$).

Performing ANCOVA with LBM and FM or with multiple organs is more complex, since there are many more options for whether to include/delete interactions, and in the case of multiple organs, which organs to include. The best ANCOVA

Table 2. Linear modeling of basal energy expenditure in lean wild-type (Wistar, Sprague-Dawley, and Zucker Fa/?; n = 26) and fatty fa (n = 16) rats adjusted by the 5 simplest methods, with and without interaction terms

<table>
<thead>
<tr>
<th>BEE adjustment</th>
<th>Intercept</th>
<th>x1 (Adjustment)</th>
<th>x2 (fa)</th>
<th>x3 (Adjustment × fa)</th>
<th>Adjusted $R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Per rat</td>
<td>1.955</td>
<td>0.0896</td>
<td>0.000</td>
<td>0.0050</td>
<td>0.0005</td>
</tr>
<tr>
<td>BW</td>
<td>0.0760</td>
<td>0.1933</td>
<td>0.6964</td>
<td>0.0297</td>
<td>0.0330</td>
</tr>
<tr>
<td>BW$^{0.75}$</td>
<td>-0.570</td>
<td>0.2592</td>
<td>0.0342</td>
<td>0.0072</td>
<td>0.0080</td>
</tr>
<tr>
<td>LBM</td>
<td>-0.003</td>
<td>0.2092</td>
<td>0.989</td>
<td>0.0671</td>
<td>0.0077</td>
</tr>
<tr>
<td>LBM + 0.2FM</td>
<td>0.0076</td>
<td>0.2000</td>
<td>0.570</td>
<td>0.0067</td>
<td>0.0007</td>
</tr>
<tr>
<td>BW</td>
<td>0.5149</td>
<td>0.1375</td>
<td>0.0000</td>
<td>0.0038</td>
<td>0.0044</td>
</tr>
<tr>
<td>BW$^{0.75}$</td>
<td>-0.050</td>
<td>0.1846</td>
<td>0.789</td>
<td>0.0236</td>
<td>0.0221</td>
</tr>
<tr>
<td>LBM</td>
<td>0.1039</td>
<td>0.1618</td>
<td>0.5247</td>
<td>0.0068</td>
<td>0.0006</td>
</tr>
<tr>
<td>LBM + 0.2FM</td>
<td>0.1909</td>
<td>0.1504</td>
<td>0.212</td>
<td>0.0060</td>
<td>0.0005</td>
</tr>
<tr>
<td>LBM + 0.2FM</td>
<td>0.1902</td>
<td>0.1486</td>
<td>0.208</td>
<td>0.0061</td>
<td>0.0005</td>
</tr>
</tbody>
</table>

According to the equation stated in the text, adjustment has coefficient $x_3$, fa has $x_2$, and interaction has $x_3$. BEE, basal energy expenditure; WT, wild-type; BW, body weight; LBM, lean body mass; FM, fat mass; Std. Err., standard error. Significant difference ($P < 0.05$) results are in bold.
of LBM and FM (derived by starting with a maximal model and deleting insignificant/unimportant terms), similar to LBM + 0.2 FM, involved no inclusion of a fa effect, just LBM and FM additively. LBM was evaluated to be 0.00599 ± 0.0006 (P = 0.0000) and FM to be 0.00128 ± 0.0003 (P = 0.0001), with an adjusted $R^2$ of 0.7819. The ratio of these LBM and FM coefficients (0.00128 + 0.00599 = 0.2130) is, interestingly, very similar to the 0.2 in the LBM + 0.2 FM model discussed earlier. The dropping of the discrete fa term means this is now, in effect, multiple regression.

Choosing what to include in the multiorgan model is yet more complicated since many of the components are correlated to each other, presumably because they are not fully independent. The best multiorgan model accounted for BEE using the kidneys (0.2000 ± 0.0956, P = 0.0431), liver (0.0366 ± 0.0063, P = 0.0000), and the nonkidney and nonliver part of LBM (0.0030 ± 0.0008, P = 0.0004), with adjusted $R^2$ of 0.8776. Many other models, including FM, or substituting kidney for heart (whose effects were very similar) could also produce $R^2$ much greater than 0.8. Again, fa was not a useful factor for explaining the variance in BEE.

Overall, in this situation, even just a simple BW correction seems to explain a great majority of the variance in BEE. However, it is still prone to claim an effect for fa that is not necessarily there. LBM is slightly better, but has the same problem. BW$^{0.75}$ does not seem particularly useful. The most widely recommended method, ANCOVA of LBM and FM, is able to explain the variance without a need to ascribe an importance to fa, though in the end its results are very similar to the LBM + 0.2 FM model. Unsurprisingly, organ-specific correction is able to explain the most variance, though model selection is complicated. This all implies that fa exerts its effects on BEE via body composition rather than cellular metabolism, information that is useful for ascertaining the mechanism of fa action on BEE.

Thus, it appears that in cases of very small sample sizes, LBM + 0.2 FM would be a perfectly adequate correction to make. Where larger sample sizes are available, ANCOVA of LBM and FM is recommended, and if both a large sample size and the relevant information are available, multiorgan fitting provides the highest explanatory power.

Avoiding the need for correction. Assuming that EE can be compared properly between animals of close size and body composition, one possible strategy is to compare subjects before differences in body weight and body adiposity have developed, or at a very early stage of difference. This also has the advantage of assuring that any metabolic differences seen are causes of future changes (such as adiposity gain), rather than being consequences of such changes that originally occurred for some nonmetabolic reason (13, 38, 41, 59).

How to Develop a Precise and Reliable Indirect Calorimetry System

IC is simple in theory (just record VO2 and VCO2) but difficult in practice since many factors coalesce to disturb these measurements. The experimenter must constantly pay a great deal of attention that no parameter or tuning has drifted. The main danger with the developing user-friendly push-button devices is the excessive degree of confidence that an inexperienced user can have in the “auto-xxx” processes. In the next section, we summarize the results of our practical and far from perfect experiences and suggest some basic strategies to get a well-calibrated system, and confirm that this is, indeed, the case. We are not, of course, pretending that what we present are the best and unique solutions. There is a lot of dependency on how the calorimeter is designed, and solutions can be different from one setup to the next. Figure 5 gives the general design of an IC system.

Measuring VO2 and VCO2. The changes in O2 and CO2 levels in the metabolic cage must not exceed 1% to consider that the environment of the animal is maintained in a state that does not affect respiration or metabolism. Considering that in rats and mice periods of spontaneous activity can increase VO2 and VCO2 2 to 3-fold, at rest, the difference between ambient and cage O2 and CO2 should not be more than 0.3–0.4%.

Reliably measuring an increase in CO2 of 0.3% relative to room air in which CO2 is ~0.003% can be easily performed with infrared gas analyzers (the level of CO2 to measure is 150–500 times larger than the ambient CO2). In addition, this methodology is little affected by pressure, temperature, humidity, and air flow, so accuracy can be maintained over the long term. Measuring O2 is much more challenging because 1) the main technologies used (paramagnetic and electrochemical) are more complex and are sensitive to numerous artifacts (including temperature, humidity, pressure, gas flow) and 2) the background oxygen content of ambient air from which changes in O2 must be measured is very high relative to the signal (for example, a 0.3% decrease in cage O2 is only 1.4% of the 20.95% background level). In this context, our opinion and experience are that the O2 and CO2 gas analyzers should be differential ones in which the assessment of cage air is continuously compared with that in the room. For O2, paramagnetic measurement should be preferred for long-term stability (unless it is very important that the response of the gas analyzer be extremely rapid, such as breath to breath). Differential gas analyzers can measure more precisely small changes vs. ambient air, which is particularly difficult for O2 and can correct for potential drift in the ambient O2 and CO2 air content. Modern devices include electronic regulation of temperature and pressure.

It must be clear, however, that even differential gas analyzers cannot properly correct for shorter-term changes in room O2 and CO2 related to the circulation of people and/or the presence of other animals. These variations can be taken into account by using an empty cage, “a blank”, that provides a periodical “reference zero” value for VO2 and VCO2 that can be subtracted from the VO2 and VCO2 values measured in other cages. We shall not enter into the detail of such a procedure, which has already been fully described previously (37). This method is well adapted to multiplexed systems in which VO2 and VCO2 are measured on each cage only periodically but can be difficult to apply when it is necessary that data be acquired at high frequency and without interruption during several hours (for example, measuring responses to food intake or glucose/insulin tolerance tests). In these conditions, it is necessary that the air supplying the cage(s) and the reference cells of the gas analyzers be protected from short-term changes in O2 and CO2. This can be done by sampling ambient air from the outside, the compressed air circuit, or a bottle previously filled with ambient air.
Measuring air-flow. A precise measurement of air flow is also an important point because computation of $\dot{V}O_2$ depends on the correct measurement of air flow, as much as on the correct measurement of $O_2$ level ($\dot{V}O_2 = [O_2] \times \text{flow}$). It is surprising that in most publications, it is well explained how the gas analyzers are calibrated, but very little information is available on how air-flow through the cage(s) is controlled, including frequency and method of meter calibration. For long-lasting stable recordings of air flow, electronic mass flow-controllers should be preferred because of their long-term stability and resistance to variations in temperature and pressure. However, despite their long-term stability, owing to the extreme precision required to make measurements of $\dot{V}O_2$ and $\dot{V}CO_2$, the flow controllers must be calibrated every 1–2 mo, ideally at the beginning and end of a cycle of measures. With rat and mouse setups, because air flow is below 2 l/min, it is easy to control the signal of the electronic flowmeters with bubble flowmeters corrected for standard temperature and pressure (1 atm and 20°C). Calibrating the flowmeters oneself is a delicate procedure, and it is not practical to send the flowmeters back to the manufacturer for calibration each time a small drift has been observed. A practical solution is, knowing from the calibration process what the air-flow error is, to correct it either online or postacquisition by adjusting the results in the data file. Figure 6 is a copy of a spreadsheet prepared to control the signal of a 0–2-liter mass flow-controller with a bubble flowmeter. It compares the airflow value given by the mass flowmeter to the air flow value measured with the bubble flowmeter (corrected for temperature and pressure) and generates an equation to adjust the response of the mass flow controller. A similar spreadsheet is used to control and adjust the response of a 0–100-ml flowmeter.

Air drying. Measurements of $CO_2$ and particularly $O_2$ are sensitive to humidity. Thus, it is necessary to completely dry the inflow before it reaches the gas analyzers. In our experience, dryers like silica gel that can be regenerated by heating are not very efficient and should be avoided. Air drying by cooling is more efficient but not complete, so we consider that addition of a chemical dryer after cooling is a good precaution. This ensures that the water vapor level in the air is constant (and null). We use anhydrous calcium chloride in small pellets (1–3 mm). These have a strong absorptive power but come with the big pitfall that they cannot be regenerated. Because mice and rats do not produce much water vapor, in our setups, no predrying by cooling is performed, and the gas from the cages is directly pushed to the dryers. Two 100-ml tubes in series are usually sufficient for mice, assuming air flow is 0.3 to 0.6 l/min (the first is changed daily, the second serves as a
buffer and is changed weekly). For a rat, assuming an air-flow of 1.3 to 2 l/min, the volume of the first dryer should be at least 500 ml. Having two dryers in series, the second remaining in place during the whole week, also prevents vapor entering the gas analyzers when the dryers are changed. For the reference cells, a 500-ml dryer is sufficient for 1 wk. The buffer dryers on the measuring lines and the dryers on the reference lines are changed on Friday evening or early on Monday morning because we observed that full reequilibration of the analyzers after water vapor has entered the reference cells can take a long time.

**Synchronization of the response of the gas analyzers.** Differences in the delay (when positioned serially) and the response profiles of the gas analyzers must be minimized or corrected to reduce noise in the calculation of RQ during rapid changes in \( VO_2 \) and \( VCO_2 \). When air flow is above 1 l/min (configuration with rats), the \( O_2 \) and \( CO_2 \) analyzers can be affixed separately (in parallel) for the best synchronization of their responses. Figure 7 shows the effects on the calculation of RQ of a slight advance in the response of the \( CO_2 \) analyzer.

**Calibration of the gas analyzers.** It is obvious that a proper calibration of the gas analyzers is essential to get correct data. The unfortunate fact is that it is rather difficult to get them to remain stable over the long term.

Calibration of the gas analyzers is generally performed by using room air, or better, a span gas bottle filled with room air for the “zero” point, and a span gas bottle containing \( \approx 20\% \) oxygen and 0.8 to 1% carbon dioxide in nitrogen for the “span” point. This typically provides a two-point calibration curve. According to our experience, this quasi-universal method of calibration has several limitations:

- **The accuracy of the calibration strongly relies on the homogeneity of the \( O_2-CO_2-N_2 \) mix in the span gas bottle (assuming room air is always taken in proper conditions). This mixing is a ternary mixing, which is expensive and difficult to perform with a great accuracy.**
- **It is not possible to perform a multipoint calibration unless several span gas bottles are used, and, therefore, not possible to control that the response of the gas analyzers remains perfectly linear.**
- **The span gas must be injected directly into the analyzers; thus, calibration consumes a lot of gas each time. As a consequence, the bottle has to be changed frequently, with uncertainties related to the accuracy of the new gas mixture vs. the previous one.**

To avoid these limitations, we use a span gas bottle with only two gases, 80% \( N_2 \) and 20% \( CO_2 \). This gas is mixed with room air using two mass flow controllers. Typically, room air is passed at a constant flow of 1 l/min regulated by the first flowmeter (span 0 –2 l/min) and the span gas mixed (after room-air flow has been measured) at various rates (0, 10, 25, 50 . . . ml/min) that are controlled with the second flowmeter (span 0–100 ml/min) (see Fig. 5). The resulting \( O_2 \) and \( CO_2 \) content of the mixed air is (using 20.95 as the %\( O_2 \) content of room air):

\[
\begin{align*}
\text{\% } O_2 & = 20.95 \\
\text{\% } CO_2 & = 20 + \text{mass flow controller setting} \\
\text{\% } N_2 & = 100 - \text{mass flow controller setting}
\end{align*}
\]

For example, if the span gas has 5% \( CO_2 \), the %\( O_2 \) and %\( N_2 \) are 85.95 and 14.05, respectively.
Indeed, since the O2 content of air is 20.95, injecting into and this RQ should be chosen to be close to the mean RQ of room air a gas without O2 decreases O2 by a value proportional to gas flow.

%O2 = \frac{20.95 \times \text{air flow}}{(1,000 + \text{gas flow})} \quad (7a)

%CO2 = \frac{20.00 \times \text{gas flow}}{(1,000 + \text{gas flow})} \quad (8a)

For example, if room-air flow is 1,000 ml/min, span-gas flow is 25 ml/min.

%O2 = \frac{20.95 \times 1,000}{1,000 + 25} = 20.43\% \quad (7b)

%CO2 = \frac{20 \times 25}{1,000 + 25} = 0.488\% \quad (8b)

The choice of a CO2 content of 20% in the span gas bottle is important because it controls the “RQ” of the span gas bottle, and this RQ should be chosen to be close to the mean RQ measured in practice, meaning it should be between 0.85 and 1.0. Indeed, since the O2 content of air is 20.95, injecting into room air a gas without O2 decreases O2 by a value proportional to gas flow \times 20.95, and increases CO2 by a value proportional to gas flow \times CO2% in gas. The \frac{\Delta CO2}{\Delta O2} in the mixed air will be \%CO2 in gas \div 20.95. Thus \%CO2 must be chosen in order that this ratio remains within physiological limits—around 0.9 to 1.0. In the example given above, the ratio is 20 \div 20.95 = 0.954. Accordingly, if we compare the results of Eqs. 7b and 8b, the ratio of the \%CO2 to \%O2 changes is 0.488 \div 0.511 = 0.954.

This technique requires that both flowmeters be very precisely calibrated, but it has many advantages.

- Because it is a simple two-gas mixture, it is generally more precisely measured by the manufacturer (and less expensive). A possible error of, for example, 0.02% on the measured CO2 content of the bottle is a much smaller relative error when the CO2 content is 20% than when it is 0.8%.

- Oxygen in the span gas bottle is always 0%; thus, O2 calibration is not affected when the bottle is changed.

- The CO2 content of a new bottle can be measured easily by comparing the bottle quotients of the old and new bottles.

- With this method, it is possible to generate multiple calibration points which permit %O2 and %CO2 derivation from the slope of a regression line generated by the calibration procedure. This allows for correction of potential small errors at individual points (thus reduction of calibration errors) and permits control and possible adjustment of the linearity of the responses of the gas analyzers.

- The volume of the gas bottle required for calibration is ~40 times lower than the volume required when the span gas is injected directly into the gas analyzers; thus, the bottle lasts much longer (typically more than 1 yr).

- When injected into the cage at various flows to mimic various levels of EE, this span gas can be used to control for potential leaks and can be used as an artificial rat or mouse to test the response of the system in experimental conditions. In practice, we routinely start and finish all measurements with 5 min on room air (zero), then 5 min at each of 3 levels of injection of the span gas to control that zero and RQ values are correct at the onset of the experiment and are still correct at the end of the measuring period. If this is not the case, comparison of the initial and final response of the O2 and CO2 gas analyzers often allows correction for the drifts.

- Finally, it is possible to study the delay and distortions between the signals generated in the cage and the responses of the gas analyzers to develop precise correction procedures when required.

**Multiplexed vs. continuous data acquisition.** Many papers have been published on good practice in IC, and many practical details can be obtained from these articles. Commercially available multiplexed systems usually follow the principles described by Jensen et al. (37), in which potential drifts of the gas analyzers and/or variations in the room air O2 and CO2 content are periodically corrected by using a blank cage that serves as a floating reference. Our best experience is with uninterrupted data acquisition at high frequency on a system that monitors VO2 and VCO2 in one single cage. This kind of setup must be able to remain stable during 24 h, with incoming air having very stable O2 and CO2 levels. In the next sections, we shall explain why we consider that uninterrupted high-frequency measurements (every 2 to 10 s) of VO2 and VCO2 on one single animal to analyze short-term changes in the various components of energy metabolism should be preferred to periodical (every 10–30 min) measurements on several (5–10) animals simultaneously to attempt energy balance (EB) studies.

**Limitations of Indirect Calorimetry to Perform EB Studies**

Most of the published studies that involve IC are devoted to measuring EB by comparison of EE with caloric intake (CI). The usual goal is to get a demonstration that the increase(s)/decrease(s) in BW and FM that are observed between groups over the long-term originate from an increase/decrease in EE relative to CI. There are three main pitfalls with this approach.
First, it is not necessary to use expensive devices and time-consuming procedures to demonstrate that if BW or FM have increased/decreased in one group more than another, it is because more/less energy has been ingested than expended (first law of thermodynamics, principle of conservation of energy, nothing is gained, nothing is lost).

Second, the daily error made by the regulatory network controlling EB in the rat or the mouse (or in humans) is usually very small. Consider, for example, studying EE in a mouse model sensitive to obesity because BW gain increased 10 g more during a period of 25 wk, accounting for 7 g more fat and 3 g more LBM [a strong model of obesity (67)]. Assuming that 37.7 kJ are stored per gram of fat and 4.2 kJ per gram of LBM, a mouse retains $(7 \times 37.7) + (3 \times 4.2) = 276.5$ kJ over 175 days, giving 1.58 kJ per day. Assuming that the average EE of a mouse is $\approx 55$ kJ per day, this means that the imbalance (CI-EE) amounted to $\approx 3\%$ of EE. This imbalance is near impossible to reveal considering the limits in the accuracy of the measurements of CI ($\approx 3\%$) and EE ($\approx 2\%$) (see also Refs. 37, 60, and supplementary notes 3 and 5 in Ref. 60). In addition to the lack of sufficient accuracy in the raw data, this approach is impaired by the fact that in the rat and mouse, as in humans, daily CI does not match daily EE. CI adjusted to EE over a much longer timescale, more like week-to-week than day-to-day (3, 4, 55). Therefore, EE and CI should be continuously monitored during at least 1 wk, which is very rarely the case.

Finally, and this is not the smallest problem, the usual behavior of the mice or rats housed in calorimetry cages is modified because numerous environmental parameters are changed (temperature, single housing, accessibility to food, and so on), such that BW gain during the calorimetry studies is often less that in usual conditions, and therefore, EB is not the same as the usual EB. This is such a common observation that Longo et al. (47) devoted a specific study to correct EB for changes in BW during calorimetry recordings, although the methodology they proposed did not convince all (32). Some devices now make it possible to move either the rat or mouse and its home cage directly into the IC system, considerably reducing changes in the environment of the animals. Such an approach should be generalized as a first step to improve the capacity of IC to assess EB. One important check to do when EB values have been obtained in such studies is to assess their “realism” by projecting their long-term consequences on the evolution of BW and FM, and seeing if this fits with observations.

To perform precise EB studies, measuring EE with IC and CI from food intake over 2–5 days is not the correct method. The correct technique is much more difficult and time consuming. It consists of initial measurements of BW and body energy content with bomb calorimetry from initial carcasses in a reference group, then precise measurement over the long term (several weeks, still using bomb calorimetry) of CI and energy losses (urines, feces, spilled food collected in the tray), and again final analysis of BW and body energy content. With these parameters, it is possible to measure with $\approx 2\%$ accuracy the CI, and energy retained in the body, and thus to compute whether the differences in energy retention were due to increased CI, reduced EE, or any combination of changes in CI and EE (3). This is a difficult method that requires a specific environment, skilled technicians, substantial time, and death of numerous animals.

Suggested Procedure to Analyze Differences in the Components of EE

As discussed above, we consider that using IC for EB studies is not the correct approach. Additionally, this kind of study exploits only a tiny fraction of the information contained in IC records. Now that automated data acquisition and computer-assisted data processing and analysis are universal, the possibility exists to delve much deeper into the study of the relationships between respiratory exchanges, spontaneous activity, and food intake.

Total EE (TEE) results from the energy expended in four main compartments: BEE, energy expended with motor activity (AEE), thermic effect of food (TEF), and thermoregulation: $\text{TEE} = \text{BEE} + \text{AEE} + \text{TEF} + \text{Thermoregulation}$.

AEE itself results from the amount of activity (act) and the energy cost of activity (cost). TEF is a percentage of CI that is different depending on the relative proportion of the macronutrients making up these calories and expressed as a percentage of them: $\text{TEE} = \text{BEE} + (\text{act} \times \text{cost}) + (\% \text{TEF} \times \text{CI}) + \text{Thermoregulation}$.

If resistance or sensitivity to obesity is related to a defective control of energy metabolism, it should appear in BEE, cost of activity, or $\% \text{TEF}$. If none of them is affected, the phenotype is not the result of a defective energy metabolism, but rather of a modification in the central mechanisms controlling CI (55). Therefore, the procedure used with IC should allow access to all of the subcomponents of TEE. This can be done by measuring TEE and activity in standardized conditions where CI is controlled and thermoregulation EE is reduced to zero by housing the animals at thermoneutrality. If defects in thermoregulatory processes are suspected, specific experiments should be conducted separately (9, 49).

Importance of eliminating cost of thermoregulation when measuring EE in rats and mice. Rats and mice are small, and at standard laboratory facility temperatures (20–22°C), they have to produce heat above that released by the resting metabolism to maintain body temperature. When the long-term evolution of their phenotype is studied, rats and mice usually benefit from having isolative bedding, being housed in groups, and being able to engage in behavioral strategies, such as clustering when sleeping to limit heat losses. In this favorable context, the EE (measured from CI) is about 60% higher than at thermoneutrality (10). When singly housed in calorimetry cages, sometimes with wire mesh floors to allow a better recovery of food spillage or urine/feces collection, they have to produce more heat to maintain body temperature than in their usual living conditions. REE in a mouse housed singly at 20°C is two to three times the REE measured when housed at 30°C (8, 43, 49, 56). This is an increase comparable to that measured in a lightly dressed human moved from 28°C to 10°C (33). In the rat, EE is increased $\sim 50\%$ between 30°C and 20°C (estimated from Ref. 17).

In addition to the fact that the cost of thermoregulation by nonshivering thermogenesis (NST) may be very different between normal living conditions and the ones that prevail during measurements of EE, the importance of the thermoregulatory processes raises several concerns about the proper measure-
ment of EE and its components. Below thermoneutrality, since body temperature is maintained by the sum of the heat produced by BEE + (act × cost) + TEF + NST, increases in BEE, act, cost, and TEF reduce NST because more heat is produced by these processes. Thus alterations in metabolic response measured below thermoneutrality may reveal thermoregulatory defects and/or hide alterations in intrinsic metabolic rate (see Ref. 9). This has important consequences; for example, changes in TEF and cost of activity may be hidden by a compensatory decrease in NST. On the other hand, working with models bearing a mutation of UCP1 can affect the efficiency of heat production and the preferential use of lipids to fuel brown adipose tissue (BAT) thermogenesis (49). Therefore, if these mutations affect EB, they will involve metabolic processes usually inactive in humans. Thus observing that a mouse can become obese when fed a high-fat diet at 20–22°C may rather reveal a defective utilization of lipids by the BAT, while this same mouse may have resisted DIO at thermoneutrality. The reverse is true; a mouse resistant to obesity at 20°C thanks to an extensive amount of energy expended in relation to thermoregulation may have become obese at 30°C (see example of the UCP1 KO mice as described by Feldmann et al. (26)).

Mutations can also alter the thermoneutral zone by affecting fur density or skin thermoconductivity [(54, 68), and for a more complete review, see Ref. 9, p. 251]. Thermoregulation may be an important component of EB in many mouse models of sensitivity/resistance to obesity and where possible should be studied specifically [see Ref. 8 for an extensive discussion of this question].

Access to the various components of EE. In the next section, it is assumed that rats or mice are housed at thermoneutrality under a standard 12:12-h light-dark cycle (lights on 0800 to 2000).

Basal energy expenditure. BEE is the major component of TEE in humans and rodent models, accounting for 60 to 70% of TEE. Therefore, even a small decrease in BEE, if not compensated for by a similar reduction in CI, can significantly affect EB. BEE must be measured at thermoneutrality and in the postabsorptive state, that is, when the TEF of previous meals has vanished. TEF is a long-lasting process, and a whole night of food deprivation is necessary to completely extinguish it. Therefore, in practical terms, BEE should only be measured after an overnight fast. On the other hand, great care must be taken to ensure that true BEE values, not artificially increased by VO2 and VCO2 residuals of previous activity periods, are considered. The increases in VO2 and VCO2 induced by an activity period can last long after the end of activity since there is an increase of %O2 and %CO2 in the cage. This must be washed out after the end of the activity period before BEE can be measured. A rule of thumb is that the time required by a system to respond to 95% of the change in %O2 and %CO2 is equal to three times the cage volume divided by the flow rate through the chamber (typically 15–30 min; compare activity and changes in %O2 and %CO2 in Fig. 7). This phenomenon will be referred to later in the text as the dampening effect of the cage volume. In practice the “cage volume” is best estimated empirically and includes the dampening effects of the tubing and time constants of the gas analyzers.

Thermic effect of food (and responses to other metabolic challenges). TEF can provide information on the cost of digesting, absorbing and metabolizing the nutrients, as well as on the changes in RQ, glucose, and lipid oxidation induced by various types of meals (such as low-fat vs. high-fat meals, response by obese vs. lean subjects and so on). Differences revealed in the intensity of TEF or in the RQ response to the meal can be suspected to alter the components of EE and substrate utilization under usual free feeding conditions. Although these differences may be extremely small and difficult to reveal when spread over many meals throughout 24 h, they can be more easily revealed by looking at the acute response to a standardized meal in individuals previously normalized in the basal state. Thus, measuring TEF can be done after BEE has been measured in overnight fasted subjects, that is, by giving overnight fasted mice or rats a calibrated test meal. Giving the meal between 0900 and 1000 leaves 6–7 h to follow most of the thermogenic response. Instead of a meal, to interfere more specifically with some aspects of energy metabolism, various metabolic tests can be performed, such as glucose or insulin tolerance tests, injection of various molecules, or changes in cage temperature (see Refs. 18 and 20). One main advantage of these tests is that the response to most of them depends on their intensity but not on the size of the animal. Thus, the results can be compared from noncorrected VO2 and VCO2 changes (for example, the TEF of a 1-g meal is the same, 10–15% of the energy content of the pellet, whether ingested by a small or a large mouse).

In the rat and mouse (contrary to humans in which subjects are simply asked to stay still and relax), measuring TEF or the response to any metabolic test is challenging because the changes in VO2 and VCO2 measured after the test(s) involve two components that must be separated: the effect on spontaneous activity and, thus, on the energy expended with activity, and the effect on REE (the parameter we are most often interested to measure). This is obviously difficult and time consuming if no specific process of data analysis has been developed to take into account the noise induced by the variability in spontaneous activity. At the very least, data acquisition should be performed at a high rate (every 10 s at least), and corrections for the dampening effect of the cage volume should be performed to get more frequent access to REE values (2). Better still, specific procedures should be developed to precisely measure spontaneous activity and how it affects TEE. The next section deals with this specific question.

Cost of activity, RQ of activity, and continuous access to REE in freely moving rats and mice. Proper measurements of REE, TEF, and responses to various metabolic challenges can be impaired by variations in the energy expended with spontaneous activity, which makes precise measurement of spontaneous activity and analysis of its effects on VO2 and VCO2 of critical importance. In addition to favoring more reliable measurements of the responses to metabolic challenges, a precise analysis of the relationships between VO2, VCO2, and activity can provide information on the control of muscle energy metabolism in vivo, a component of TEE that is more and more suspected to play a key role in the regulation of body energy homeostasis (34, 52, 61).

Detailed analysis of the relationship between spontaneous activity and VO2 and VCO2 also gives access to the cost of activity on VO2 (ΔVO2/ΔAct) and VCO2 (ΔVCO2/ΔAct) and to...
decreased V̇O₂ specifically associated with muscular work \( \Delta V̇O₂(\text{Act})/\Delta V̇O₂(\text{Act}) \) = Activity-RQ (Fig. 8). By difference with whole body V̇O₂ and V̇CO₂, V̇O₂, and V̇CO₂ changes free from activity, meaning resting V̇O₂ and V̇CO₂ and, thus, resting RQ, can be computed. The cost of activity can be affected by mechanical efficiency of the movement between animals with a large difference in adiposity, but most often by the biological efficiency of the coupling between V̇O₂ and ATP synthesis in working muscles. Therefore, the cost of activity can give in vivo an indication of the level of the uncoupling processes in muscles. Separate analysis of resting-RQ and activity-RQ and of their changes in relation to the fasted-fed state or in response to various manipulations has the potential to provide a deep insight into the way metabolic fluxes are controlled into the body.

Example Analysis of a File Created by High-Speed Uninterrupted Data Acquisition of V̇O₂, V̇CO₂, and Spontaneous Activity in the Rat

This section describes a “standardized” 23-h recording session performed in a rat. The goal of the study was to compare the TEF of a low-fat meal between young obesity prone (OP) and resistant (OR) rats. The light-dark cycle was 12:12 h, lights off at 2000. The rat was housed in the metabolic cage at around 1800 with water but without food. Temperature in the cage was measured and regulated at 28°C to switch off thermoregulation. The next day at 1000, a calibrated test meal (48 kJ) was given, and the TEF was measured until 1700. The 1700–1800 time window is used to clean the cage, calibrate the cage was measured and regulated at 28°C to switch off the lights off at 2000. The rat was housed in the metabolic cage at around 1800 with water but without food. Temperature in the cage was measured and regulated at 28°C to switch off thermoregulation. The next day at 1000, a calibrated test meal (48 kJ) was given, and the TEF was measured until 1700. The 1700–1800 time window is used to clean the cage, calibrate the analyzers, and restart an experiment. Data acquisition was performed at 2-s intervals on O₂, CO₂, air flow, and motor activity. Figure 9A shows the recorded V̇O₂, V̇CO₂, RQ, and activity values. The activity signal is characterized by periodical bursts, though in between there are frequent events of shorter duration and intensity. V̇O₂ and V̇CO₂ exhibit short-term changes related to activity and long-term changes related to feeding. RQ is largely insensitive to the intensity of activity but strongly affected by the fed, fasted, or refed status.

**Recording and processing the activity signal.** The most common method to measure spontaneous activity is the use of beams located around the metabolic cage that measure the \( x-y \) or \( x-y-z \) displacements of the animal. We have never used this technique and, thus, cannot comment on its efficiency at producing an activity signal proportional to the work performed by the animal. That said, we suspect it ignores a lot of the activity that occurs without significant displacement, in particular, in small metabolic cages. We chose to use force transducers rather than beams from the very beginning of our studies (22, 23), a method that has also been implemented for large animals (63). Force transducers located below the floor of the cage should produce a signal directly proportional to the work released on the floor of the cage. This includes when no \( x-y \) displacements are involved. We (23) and others (63) have observed that force transducers generate a signal that is tightly correlated to changes measured in V̇O₂ and V̇CO₂, and thus can be used to study the energy cost of activity.

The signal provided by the force transducers resembles an EEG signal and must be acquired at high frequency (typically 100 Hz) before being filtered (window 0.1 Hz-30 Hz), rectified, averaged, and stored in 1- to 10-s bins, according to the periodicity selected for V̇O₂ and V̇CO₂ acquisition (Fig. 10).

**Decomposition of TEE and RQ into their subcomponents.** It is possible from the raw V̇O₂ and V̇CO₂ values to access basal metabolism, as this animal was relatively inactive between 0830 and 1000 (Fig. 9A). However, this may not always be the case. Considering the large activity that developed after presentation of the meal (given at 1000), it is clearly impossible to properly measure the TEF response to the meal that is mixed with the activity response. To faithfully measure TEF, it is thus necessary that resting V̇O₂ and V̇CO₂ be computed. This is done from analysis of the relationship between changes in V̇O₂, V̇CO₂, and activity, according to a process that we have described in detail elsewhere (21, 23). However, a summary of this processing is laid out below.

A model is formulated with a response delay and damping coefficient that describe the distortion of the V̇O₂ response due to the cumulative effects of the volume of the cage, but also the tubing and the electronic filtering of the gas analyzers (determined empirically by injecting the CO₂/N₂ mixture of the span gas bottle in the cage). In our experimental configuration, the delay was 8 s, and the dampening of the response of the system equivalent to the one induced by a cage volume of 10.5 liters. With these parameters, the model predicts the expected changes in V̇O₂ from the activity signal assuming that Total V̇O₂ = Resting V̇O₂ + [Activity × Cost(ox)]. The prediction is compared with the actual Total V̇O₂ value by an adjustment algorithm (Kalman filtering). This is tuned to iteratively adjust after each acquisition the values of resting V̇O₂ and/or cost of activity predicted by the model to maintain the predictions in close agreement with the actual measurements. Intuitively, one can understand that this process requires that data acquisition be uninterrupted and that the frequency of data acquisition be high to feed the filtering process with many temporally close data points to permit repeated small adjustments of resting V̇O₂ and Cost(ox). The process is applied in parallel to the V̇CO₂ signal using the same distortion model. It is, thus, necessary that the responses of the analyzers be perfectly synchronized. Figure 8 graphically details the results obtained during a rest-activity-rest period. Nowadays, modeling tools and statistical software probably offer many other possibilities to solve this problem.
Equation [D]. The separation is particularly useful, since it allows calculation of specific movement-related RQ effects, which is the final parameter of activity RQ, shown in Eq. [E]. RQ is linked to the nutrient mix fuelling a metabolic process, and the (Kalman filtering) is able to separate out nonresting (in other words activity) V˙CO2 and V˙O2 from whole body V˙CO2 and V˙O2. The activity RQ parameter that we are subsequently able to derive, therefore, effectively represents the nutrient mix specifically fuelling movement, in other words, fuelling skeletal muscle work. Note that activity RQ flatlines outside the movement period because the input data become too small to allow it to be calculated.

Separate analysis of RQs computed from total V˙O2 and V˙CO2 (total RQ), resting V˙O2 and V˙CO2 (resting RQ), and RQ computed from activity V˙O2 and V˙CO2 (activity-RQ). Three different RQs are now available, RQ computed from total V˙O2 and V˙CO2 (total RQ), RQ computed from resting V˙O2 and V˙CO2 (resting RQ), and RQ computed from activity-V˙O2 and V˙CO2, all shown in Fig. 9C. In this rat, it can be observed that activity-RQ closely follows the changes in resting RQ, indicating that in this animal, the substrate mix used by the working muscle to fuel muscular contraction remains consistently close to the mix used by the resting tissues. In particular, working muscles switched to lipids in the fasted state and rapidly returned to glucose utilization when the animal was reared with about the same kinetics as resting-RQ. The activity-RQ signal appears shakier than resting-RQ because activity-RQ can be fully adjusted only when the activity signal is strong enough to generate significant level of Act-V˙O2 and Act-V˙CO2. Thus adjustment of activity-RQ values cannot be as continuous as it is for resting-RQ.

In summary, this data processing gives access to TMR, RMR, activity, cost of activity, whole body RQ, resting RQ, activity-RQ, and their evolution during transition from the fed to the fasted state. From this point, basal metabolism can be measured from RMR measured in the post-absorptive state and TEF can be computed as the increase in RMR after ingestion of the meal. It is not the place here to give experimental results, but, as an example, the resting and activity RQ responses of these two groups of rats in response to ingestion of the low-fat test meal are presented in Fig. 11. It shows that the evolution of the cost of activity and activity-RQ are the same in both groups, suggesting that there is no difference in the control of energy metabolism in muscles. RMR in the OP rats is significantly higher than in OR rats, but the difference is erased after correction for differences in either BW or LBM (data not shown). The meal-induced increase in RMR is identical in OR and OP animals, indicating that there is no defective meal-induced thermogenesis in the OP rats. Premeal and postmeal activity were also similar, suggesting that OP rats were not less active. The only difference between the OP and OR rats was observed at the level of activity-RQ responses of these two groups of rats in response to ingestion of the low-fat test meal are presented in Fig. 11. It shows that the evolution of the cost of activity and activity-RQ are the same in both groups, suggesting that there is no difference in the control of energy metabolism in muscles. RMR in the OP rats is significantly higher than in OR rats, but the difference is erased after correction for differences in either BW or LBM (data not shown). The meal-induced increase in RMR is identical in OR and OP animals, indicating that there is no defective meal-induced thermogenesis in the OP rats. Premeal and postmeal activity were also similar, suggesting that OP rats were not less active. The only difference between the OP and OR rats was observed at the level of the resting-RQ response to feeding. This increased more and above FQ in OP rats, suggesting that, since TEF was the same, glucose oxidation increased more and lipid oxidation decreased more in the OP than in the OR group. It is very probable that the small difference in the RQ response to feeding was relaxed here thanks to the acute transition from the fasted to the fed state induced by the test meal would not have been visible under free-feeding conditions. We hope that this example can convince some readers of the potential of IC protocols that give access to the subcomponents of TEE and analyze their
evolution in response to various metabolic challenges. The example given here is the response to a meal, but as quoted above, many other metabolic tests can be considered. Fixing the process to determine the cost of activity may initially be time-consuming, but it is worth the investment. Once done, each experiment gives access to measures of TMR, RMR, resting-RQ, activity-RQ, intensity of activity, and cost of activity, precise noise-reduced measurements of basal metab-

Fig. 9. A: changes recorded in $\dot{V}O_2$, $\dot{V}CO_2$ (ml/min), RQ, and spontaneous activity (arbitrary units derived from integration of the force transducer signal) in response to overnight food restriction (18:47 to 10:00) and refeeding (10:00 to 18:45). A 48-kJ test meal was given at 1000. Data acquisition was frozen ~10 min before 10:00 to remove the perturbations due to the opening of the cage when the meal was introduced. B: changes recorded in total metabolic rate (TMR) and resting metabolic rate (RMR) (in Watts), cost of activity (W/AU), and spontaneous activity (AU) in response to overnight food restriction (18:47 to 10:00) and refeeding (10:00 to 18:45). A 48-kJ test meal was introduced at 1000. Data acquisition was frozen ~10 min before to remove perturbations caused by opening the cage to introduce the meal. C: changes recorded on total RQ and resting RQ activity RQ and spontaneous activity (AU) in response to overnight food restriction (18:47 to 10:00) and refeeding (10:00 to 18:45). A 48-kJ test meal was introduced in the cage at 10:00. Data acquisition was frozen ~10 min before 10:00 to remove the perturbations due to the opening of the cage when the meal was introduced.

Recording of activity with force transducers

Fig. 10. Activity recorded by force transducers. In this example, the signals of three piezoelectric force transducers are integrated and acquired at 100 Hz, and average intensity was stored every 2 s.
olism and TEF. This gives much more opportunity to observe differences between groups than from only mean 24 h TMR and RQ values.

Discussion

In this article, we have tried to give a simple and practical description of the basic concept of IC and answer specific questions related to data recording and analysis. Several important reviews have been published recently, in particular, while we were working on this paper, one by Tschop et al. (60), which included among the authors several essential contributors to the domain. Tschop and colleagues extensively discussed the experimental conditions and statistical methods that they consider should be performed. This article and, in particular, the extensive supplement attached provide a lot of detailed information and clear discussion of the various aspects of IC (specifically, in mice). However, we are cautious about several of their conclusions, in particular, the optimism on the possibility of obtaining sufficiently accurate measurements of EE and CI to assess EB. On the other hand, they have also recognized that measuring EE in mice far below thermoneutrality is a real problem. However, we are cautious about several of their conclusions, in particular, the optimism on the possibility of obtaining sufficiently accurate measurements of EE and CI to assess EB. On the other hand, they have also recognized that measuring EE in mice far below thermoneutrality is a real problem. However, they also concluded that because it is under usual housing conditions that phenotypes develop, it is also better to perform IC measurements below thermoneutrality. Although we agree that the argument is logical, we insist, here, that it is very problematic to use animals that can spend more than 50% of their daily EE just on thermoregulatory processes as models of EE and BW regulation in humans. Thus, it is really important to put into consideration the question of whether a mouse housed at 20–22°C can be used as a model for human energy metabolism. Considering the money invested in developing mutant mice and in treating the pandemic of obesity, a definitive and motivated answer to this question should be given. In this field, see Refs. 8, 9, and 45 for review.

The authors also pointed to limitations imposed by multiplexed multicage systems but did not challenge this method by proposing an alternative. It was one main goal of this review to discuss various practical details important for obtaining reliable data and bringing the reader to our opinion that multiplexed multicage systems cannot obtain the goal for which they have been developed—comparing EE and energy intake to measure EB. As detailed above, we consider that “dissecting” the components of EE in well-controlled metabolic conditions may bring much more useful information than EB studies. Simultaneous analysis of BEE, EE of activity, resting, and activity RQs can give a more detailed outline of how energy metabolism is regulated and increase the chances of finding a metabolic defect. Inclusion of metabolic challenges, such as meals of various compositions, injections of various drugs, hormones, antimetabolites, variations in ambient temperature and so on target various components of energy metabolism possibly involved in defective EB. Conditions are, of course, not normal, but since differences are usually too small to be revealed in normal conditions, experimental protocols in which energy metabolism is challenged and the various components of TEE simultaneously studied may be more efficient.

This approach may not appeal to many because it requires measurement of spontaneous activity and correct computation of its short-term effects on respiratory exchanges. However, we think that, contrary to the situation in 1984 when we first developed this method (19, 51), this problem can today be easily solved. An article published while we were correcting the proofs of this article confirms this (62). Those performing IC and/or companies developing IC systems should implement such processing.

A major question in analyzing EE in rats and mice is of normalization to BW and body composition. Several solutions have been proposed, although no single one objectively stands out as overwhelmingly better than all of the others; not the LBM + 0.2 FM that worked well with our set of data (though its simplicity

Fig. 11. RMR, cost of activity, resting-RQ, and activity-RQ measured in response to ingestion of a test meal given at time 0. Values are expressed as means ± SE plotted at 15-min intervals; −600 to 0 before a meal denotes clock time from 00:00 to 10:00, during which the rat is fasted. 0 to 420 denotes clock time from 10:00 to 17:00, during which the response to ingestion of the test meal is measured. Note that ~10 h are required to measure the whole of TEF. For obesity-resistant (OR) rats, n = 12; for obesity-prone (OP) rats, n = 12.
makes it a very attractive option for low sample sizes), nor ANCOVA that appears for now the most robust method (but requires that within-group variance in BW, FM, or LBM be much larger than within-group variance in REE—not often the case since, generally, groups are constituted to be homogenous). Researchers working in humans often compare EE measured in their subjects to the results of predictive equations established in control subjects. These equations can be very simple (just based on weight or height and weight) or much more complex (including age, sex, ethnicity, and so on) (35). For several years, equations have also been developed that use detailed analysis of body composition by MRI (29, 30). These organ-based equations appear very efficient at predicting BEE and to correct for age, sex, ethnicity, and adiposity. We suggest that this approach should be developed for mice and rats given that it has already been shown to be reliable. We have already demonstrated that BEE can be predicted in several rat strains (Wistar, Sprague-Dawley, and Zucker lean) using the same organ-based prediction as in humans after correction by a single exponent factor accounting for allometric scaling (25). Because methods of analysis of body composition in vivo, in particular MRI, are developing rapidly, it should now become possible to work on a more systematic way to develop organ-based equations to predict BEE in mice and rats.

**Perspectives and Significance**

After a long period during which integrative physiology was an understudied subject, the necessity to make measurements at the whole body level on freely moving live animals to study the consequences of and possible adaptations to various metabolic and genetic challenges has become urgent. In this context, indirect calorimetry has benefited from an important renewal of interest. Reacting to this, several companies have developed user-friendly devices for nonexpert researchers. This has multiplied the number of devices available. It is a challenge for the new generation of users to invest time to take full control of their devices and obtain more parameters of interest than they might realize is possible. The current wider availability of computing hardware and software enables acquisition, exploitation, and analysis of much more than just whole body energy expenditure, and may be a fruitful area for young scientists. To give a well-timed example, while we were writing the proofs of this article, Van Klinken et al. (62) published a paper in which they challenge the method of Kalman filtering to compute the cost of activity. They suggest that penalized spline regression should be preferred as it is more resistant to default measurements of the activity signal. There is much to discuss about their results and analysis, but it is encouraging that another strategy has finally been proposed that, because it can be used on data acquired at a low frequency, will also possibly challenge our claim that there is little interesting to be exploited from multiplexed systems.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

Author contributions: P.C.E. conception and design of research; P.C.E. performed experiments; P.C.E. and N.A.N. analyzed data; P.C.E. and N.A.N. interpreted results of experiments; P.C.E. and N.A.N. prepared figures; P.C.E. and N.A.N. drafted manuscript; P.C.E. and N.A.N. edited and revised manuscript; P.C.E. and N.A.N. approved final version of manuscript.

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