Module 1: Why is it Invalid to Normalize Energy Expenditure by Dividing by Total or Lean Body Mass?

Introduction Energy expenditure (EE) phenotyping requires methods to account for the influence of body size on EE. The importance of this requirement is difficult to overstate as an observed difference in body weight is an important clue that a drug, diet or genetic manipulation may have altered EE. Thus in many cases investigators have to compare EE in two groups of animals of differing size and/or body composition. Many investigators attempt to normalize EE (which is usually expressed as kcal or kJ per unit time) by dividing EE by total body weight (TBM) or by lean body weight (LBM). This method is called **ratio normalization**. For example, imagine that you have two groups of mice with one group (A) having an average weight of 20 g and an average EE of 10 kcal/day and the other group (B) having average TBM of 25 grams and an average EE of 11 kcal/day. If you divide the EE by TBM, the average EE would be approximately 0.5kcal/d per g TBM for group A *vs.* $0.45 \text{ kcal/d per g}$ for group B (we say approximately because the ratio of means is not exactly equal to the mean of ratios). You might be tempted to conclude that one reason animals in group B are larger is that their energy expenditure is lower, but the basis for this conclusion is flawed (see [1-10]). When you divide by TBM or LBM you implicitly assume that the relationship between body size and EE is both linear *and* that when you extrapolate your body size covariate to zero, then EE will also be zero (i.e. the y-intercept is zero). While for many data sets the relationship between EE and TBM (or LBM) is linear, the y-intercept is rarely zero. Non-zero y-intercepts invalidate ratio normalization as a means to control for the effect of body size on EE [1-10].

The purpose of this module is to illustrate the problem with using traditional ratio normalization to correct EE for differences in body size. Subsequent modules present valid statistical approaches of varying complexity to evaluate the EE data. Here we use a data set comparing wild type and myostatin knockout mice to illustrate the inherent problem of the ratio normalization approach.

About the dataset The data in MyostatinKOdata.csv were measured in the Nutrition and Obesity Research Center's Energy Balance Core at the University of Washington and represent a subset of published data [11]. Groups are MKO, denoting myostatin knockout mice (n=13) and CON, denoting control mice (n=31). CON mice include wildtype mice and mice that were heterozygous for the myostatin mutation. EE is in units of kcal/d and mass variables are in g. Abbreviations are: TBM, total body mass; LBM, lean body mass; FM, fat mass; EEkcal.d, average 24h EE in kcal/d; EE.lbm, ratio of EE to LBM in units of kcal/d per g; EE.tbm, ratio of EE to TBM in kcal/d per g. EE normalized both for TBM and LBM are included in the file if you wish to analyze these variables for yourself.

What we are trying to accomplish The primary goal of adjusting EE for body size is to estimate the group difference in EE (± SE) that would pertain if the groups had the same mean TBM or LBM. Similarly, we often want to estimate the mean EE (± SE) for each group, if the groups had the same mean TBM or LBM.

Traditional ratio normalization is simple, and seems intuitively valid because it yields 'EE per unit mass' but it is easy to demonstrate why this method is incorrect. Using the myostatin data [11] we plotted EE against body mass (Figure 1A). MKO mice are more muscular and have higher LBM than control mice. As expected there is a positive relationship between LBM and EE (i.e. larger animals have higher EE). If we combine WT and MKO we can use regression analysis to calculate the dependency of EE on LBM for the entire data set. The regression line in Figure 1A gives the estimated mean EE given

a particular value of LBM. This is illustrated by right angle line with arrow showing that the estimated mean EE at 18.5 g is 9.45 kcal/d. (Note: the mean LBM if you combine WT and MKO data is 18.5 g). The slope parameter (Δ EE / Δ LBM) of the regression equation (0.21) tells us that for every 1.0 g change in LBM, the estimated mean EE changes by 0.21 kcal/d. It is important to recognize that the regression line models the EE component that simply reflects the average influence of LBM on EE for the entire sample. Separate regression lines for MKO and controls can be fit and may in fact differ in an informative way as described in a subsequent module. The existence of the positive relationship between EE and LBM is precisely what we are trying to control for in comparing the two groups.

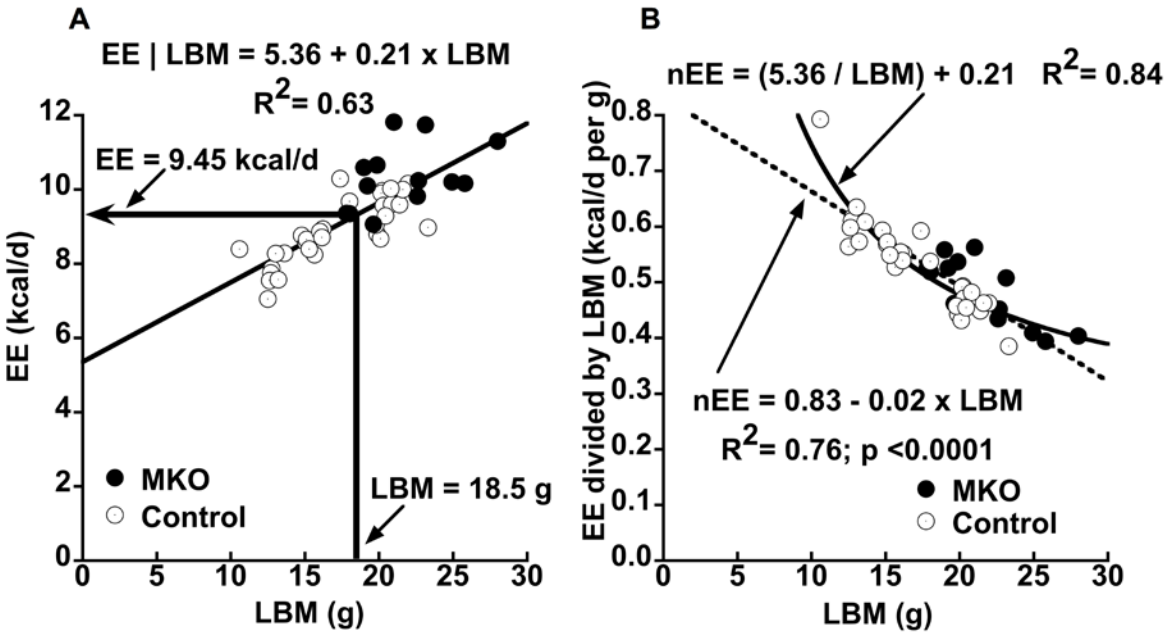


Figure 1. **A**: The linear regression fit line predicts mean EE given a particular value of body size (note: "EE | LBM" means "EE given a particular value of LBM"). Note the presence of a positive y-intercept. **B**: The positive y-intercept forces ratio normalized EE to be negatively correlated with LBM.

Non-zero y-intercepts invalidate ratio normalization Based on the example shown in Figure 1A, you could ask, "Why not just divide EE by LBM?" The answer lies in the fact that the y-intercept for the regression line in Figure 1A is not zero. Non-zero (usually positive) y-intercepts are common in regressions of resting/basal or 24h average EE on TBM or LBM. The data in Figure 1A conform to this expectation because when LBM equals zero g, EE equals 5.36 kcal/h based on the regression equation. Because this positive y-intercept is outside the range of observed data **and is a physiological impossibility**, this value is simply a mathematical feature of fitting a straight line to the data (see [10] for additional details). Although a straight line is a reasonable model for the data in the example, where LBM ranges from 10-30 g, we cannot assume that this linear relationship holds outside of the observed LBM range. In fact, the actual relationship between EE and LBM across a much larger range of LBM is not linear but it does have a true zero intercept; this is called allometric scaling [10]. The common application of linear regression in EE analysis is possible because the range in body mass typically observed in a typical study is rather narrow; it is essentially linear (if you are a purist you could call it "local linearity") [10].

Importantly, the resulting positive (or negative) y-intercept in linear EE analysis invalidates traditional ratio normalization whenever the goal is to answer the main questions noted above. If we just divide EE by LBM and plot the ratio-normalized EE against LBM, we observe a non-linear relationship (depicted

in Figure 1B). If the ratio normalization procedure were correct, the slope of the relationship between ratio-normalized EE and body weight in Figure 1B would be zero or close to zero. Instead, we see that ratio-normalized EE is actually correlated more strongly with LBM than is the case for the original relationship in Figure 1A (see comment below). Mathematically, ratio normalization is equivalent to dividing the regression equation in Figure 1A by LBM such that $\text{EE in kcal/d per g} = \frac{5.36}{LBM} + 0.21$ (Figure 1B). Obviously, the result is not a constant term (0.21), rather we have created function that sharply decreases with increasing LBM due to the $\frac{5.36}{LBM}$ term. Therein lies a serious mathematical artifact that **forces** heavier animals to have a lower normalized metabolic rate. Not surprisingly, therefore, a Student's independent groups t-test on ratio-normalized EE indicates that MKO mice have significantly lower normalized EE than control mice (difference = -0.05 ± 0.022 kcal/d; p = 0.035). Prior to the publication of [11], researchers were baffled by why a *hyper*muscular phenotype would be associated with a *hypo*metabolic phenotype. The reason turns out to be that MKO mice suffer from a serious statistical artifact when EE is normalized using the traditional ratio method. When the data are analyzed appropriately (using analysis of covariance, ANCOVA), MKO exhibit a *hyper*metabolic phenotype [11]. ANCOVA is developed in greater detail in subsequent modules.

Key point: The whole point of adjusting EE for LBM is to remove the influence that individual and group differences in LBM have on the EE values. To succeed in controlling for the influence of a measure of body size on EE, the normalized EE of the subjects must be minimally or un-correlated (i.e. slope=zero) with body size. This rule is critically important. If after normalization EE is significantly correlated with body size, the normalization method is invalid! Ratio normalization creates a systematic bias; Mice with greater LBM tend to have lower ratio-normalized EE. By contrast, ANCOVA is an unbiased statistical approach that can be used to compare groups of differing sizes and body composition.

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