Module 2: Introduction to Analysis of Covariance to Adjust Energy Expenditure for Body Size

Module 1 explained why traditional ratio normalization is invalid. Module 2 provides a concise explanation of analysis of covariance (ANCOVA), a preferred method for adjusting EE for differences in body mass / composition when certain assumptions are met. ANCOVA is a well-established tool with a long history of use in many scientific fields, including basic animal energetics research and human obesity / diabetes research.

The simple elegance of ANCOVA is often obscured in a fog of mathematical notation. This module provides a picture-based explanation of how ANCOVA works. We use synthetic data configured to highlight key features of the method. Module 3 demonstrates the use of the MMPC website to apply ANCOVA to the real data of Module 1.

How ANCOVA works We consider an experiment in which EE and total body mass are measured in two groups (Figure 1): experimental (EXP) and control (CON). Average total body mass is significantly greater in the EXP group than the CON group. Without adjusting for differences in total body mass, EE is higher in the EXP group than in the control group. Because this difference in EE is confounded by the difference in total body mass, we need to statistically control for the difference in EE that depends only on the difference in total body mass. In other words, the primary goal is to estimate the group difference in EE (± SE) that would pertain, if the groups had the same mean body mass. Similarly, we often want to estimate the mean EE (± SE) that would pertain for each group, if the groups had the same mean body mass.

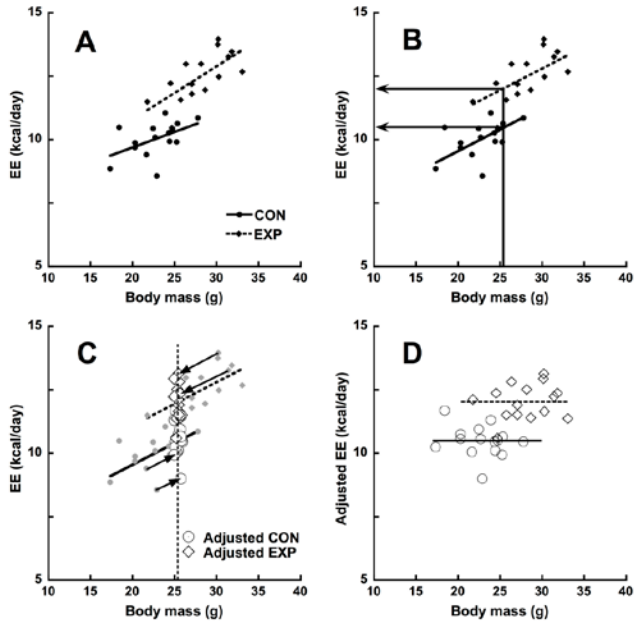


Figure 1. **A**: The individual regression slopes do not differ significantly ($p > 0.05$), so ANCOVA uses a weighted average of the two slope estimates to characterize the dependence of EE on body mass. **B**: The ANCOVA model fit depicting a single "pooled" slope estimate for the two groups. The arrows point to the adjusted group mean EE values at a given body mass; the difference in elevations is the adjusted group difference in EE. **C**: ANCOVA mathematically "slides" each EE point parallel to its regression line (per arrows) until directly over mean body mass to obtain an adjusted individual EE value. These adjusted EE values are the basis for the adjusted group EE means. **D**: Sliding the adjusted EE values horizontally until they are directly over the body mass values reveals that the adjusted values are uncorrelated with body mass.

ANCOVA is the appropriate model whenever you can assume that slope of the relationship between EE and body mass is the same in both groups (In stat-speak, this is the "homogeneity of regression slopes" assumption. The other assumptions of ANCOVA are detailed below). Figure 1A depicts separate regression fits for each group. Note that the steepness (i.e. slope) of the regressions of EE on body mass look similar for the two groups, suggesting that the equal slopes assumption is reasonable. Stated differently, it appears reasonable to assume that the two samples reflect two populations that share the same slope of EE on body mass. Accordingly, for now we will assume the slopes are the same for both groups, although this assumption can be tested as detailed below. (If we have to reject the assumption of equal slopes, there is an alternative statistical approach that will be explored in the last section of this module).

Having assumed that the slopes are equal in both groups, we use ANCOVA to fit a single "pooled" slope to each group (Figure 1B; details on obtaining the pooled slope estimate are presented in a separate module titled Statistical Formula). In Figure 1B you can see that now the slope of both lines are identical. The only difference is that the y-intercepts (and hence the line elevations as depicted in Figure 1B) are now different to allow the regression line to go through the data points for each group.

Each line elevation at a given body mass in Figure 1B is an estimate of a group mean EE at that body mass. The difference in line elevations (i.e. y-intercept) is the estimate of the mean difference in EE between groups. Typically, EE is adjusted to the grand mean of whatever mass compartment is being adjusted for (illustrated in Figure 1 at 25.4 g, the grand mean for body mass for all animals). Each ANCOVA-adjusted EE value corresponds to "sliding" each point parallel to the group-specific slope for that subject until it is directly aligned with the grand mean of body mass (Figure 1C). For example, if a particular mouse weighed 30 g and had an EE of 12 kcal/day and ANCOVA indicated that EE changed by 0.2 kcal/day per g change in body mass (i.e. slope), then the adjusted EE for that mouse would be approximately 11 kcal/day (this can be calculated follows:

$$12\ \text{kcal/d} - (30g - 25.4g) \times 0.2\text{kcal/d per g change in body mass} = 11.08\ \text{kcal/d}.$$

The MMPC web program automatically adjusts each animal's raw data using this approach, allowing you to present your work in terms of the adjusted EE group difference (±SE), p-value, and EE group means.

The formula for the SEs of adjusted EE means and the SE of the difference in the two slope elevations (i.e. y-intercept) are beyond the scope of this module (interested readers may refer to the module titled Statistical Formula). It is worth noting that these uncertainty estimates depend on the variance in EE that is not accounted for by the ANCOVA model (i.e. on the degree to which the individual EE data do not fall on the regression lines in Figure 1B).

In Module 1, we stated that a normalization method is valid if the adjusted EE values are minimally or un-correlated with the relevant body mass compartment. ANCOVA satisfies this criterion. ANCOVA statistically removes from every EE value the part of EE that is explained by individual differences in body mass and by group membership. It does so not by dividing by body weight (i.e. ratio normalization) but by simply adjusting the EE based on the slope of the relationship between EE and body weight (see Fig.1C). If we plot the adjusted EE for each animal against their original body weight (Fig 1D) one can see in contrast to what we saw with ratio normalization (Module 1 Figure 1B) the ANCOVA adjusted EE values are completely uncorrelated (slope =zero) with body mass (Figure 1D).

Unequal slopes The situation above describes the standard ANCOVA model that fits to both groups a single pooled slope estimate for the dependence of EE on body mass. This parsimonious model (i.e. uses only a few parameters to predict a relationship) is appropriate (and has optimal statistical power) unless there is good reason to decide that the slopes actually do differ between groups. The easiest way to determine whether the slopes of EE on body (or fat free) mass differ between groups is to evaluate a regression model that includes as predictor (independent) variables *group, body mass, and the group by body mass interaction term* (the web tool does this automatically as described below) The interaction term represents the extent to which the slopes differ between groups. If the group by body mass interaction term is not significant (customarily using $p < 0.05$), then the slopes of EE on body mass do not differ significantly between groups and the interaction term is eliminated from the final regression model. Alternatively, one can determine whether the slopes differ by doing a Student's t test on the

slope estimates as shown in the module titled Statistical Formula (although this is cumbersome). This option and the test for significance of the group by body mass interaction term described yield identical t statistics and p-values.

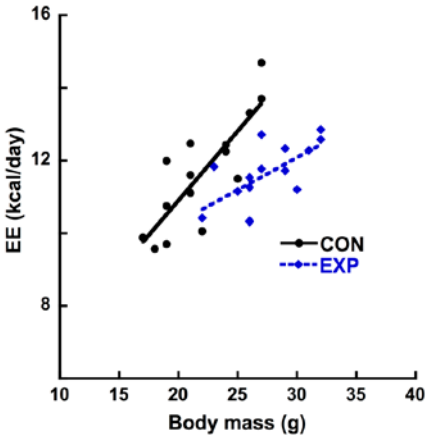


Figure 2. An example in which the slopes of EE on body mass differ significantly between groups (p=0.04). The Johnson-Neyman technique indicates that the groups have significantly different EE above a body mass of approximately 22 g.

As will be described in Module 4, the MMPC web tool automatically determines the significance of the group by body mass interaction term (without requiring you to enter it into the model) and retains the interaction in the model if this term is significant (i.e. slopes differ). Furthermore, as the slopes are not equal the lines will intersect. So there may be ranges of body mass where the two groups will not be different and other ranges where they may be different. When the interaction is significant, the web tool automatically determines ranges of the body mass covariate for which there is a significant difference in EE between groups. This evaluation uses the Johnson-Neyman technique (for computational details and further references see [1]). The web tool also includes an option to determine the significance of the difference in EE at a specific value of the body mass covariate (e.g. at a specific body weight). Computational details are in the module titled Statistical Formula. Here we simply illustrate an example involving slopes that differ significantly between groups (Figure 2).

Causes of unequal slopes Why might the slopes of EE on body mass or fat free mass differ between groups? This issue is complex. Broadly speaking, the change in EE per unit change in body mass is expected to be greater in animals with lean body composition phenotypes compared to fat phenotypes (lean tissue has a higher metabolic rate per unit mass than fat tissue). Accordingly, in comparisons involving such animals, the slopes of EE on total body mass might differ whereas the slopes of EE on fat free or lean body mass (FFM, LBM) might be similar. However, the relationship between FFM and EE is complicated by the fact that FFM is a heterogeneous tissue compartment composed of organs and tissues with widely differing mass-specific (EE per kg) metabolic rates (reviewed in [2] and illustrated in [3]). Remarkably, in humans, liver, brain, kidneys and heart account for >60% of resting EE despite representing only ~6% of body mass [4] and other data suggest that those four organs might contribute even more to resting EE in mice [5,6]. By contrast, skeletal muscle and bone, much larger contributors to fat free mass, exhibit relatively low mass-specific metabolic rates (kcal/h per gram of tissue). Genetic and/or environmental factors may affect the mass and/or mass-specific metabolic rate of one or more organ-tissues that comprise FFM. Such factors may alter the manner in which individual organ-tissue EE scales with total or fat free body mass; this might favor unequal slopes of EE vs. body size. This topic is considered in greater detail in [3]. Therefore, the relationship between EE and body or fat free mass is complex and poorly understood.

Assumptions of ANCOVA

1. The regression slopes of EE on the body size covariate (e.g. body mass) are the same for the groups (homogeneity of regression slopes).
2. The residuals (difference between measured and predicted value) are normally distributed. This issue is usually evaluated by inspecting the residuals and that usually works well enough. Formal tests exist to evaluate the normality of residuals (e.g.,[7]).
3. The variances within the treatment groups should be the same.

4. The relationship between EE and the body mass covariate should be linear within the measured range of body mass.
5. The residuals (i.e. difference between what is measured for each animal and what the regression equation predicts) should be uncorrelated.

Some sources assert that EE and the body mass covariate should be normally distributed. However, these are not assumptions of ANCOVA [8].

An original and now 'relaxed' assumption of ANCOVA in EE analysis was that the mean and variance of the covariate should be similar between groups. Indeed, the major reason for the adoption of ANCOVA in EE analysis is to provide a method to adjust for group differences in a body size covariate. However, a large between-groups difference in the body size covariate can pose major problems to the validity of an ANCOVA. If you compare very obese mice to lean mice, there may be no overlap in body weight. We know that the relationship between EE and weight over a very large range of weights is not linear (i.e. slope is not constant). At a minimum, there should be some overlap in the ranges of the covariate (e.g. body weight). Investigators should design studies to minimize the group difference in body mass or FFM (e.g. look at young animals before the weight difference is as apparent). Pair feeding could be used to better match the groups. However pair feeding may or may not be appropriate as animals tend to change their metabolic rate in response to food restriction. Good research design is the key to good EE analysis.

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