Technical Note: Variation in Muscle Mass in Wild Chimpanzees: Application of a Modified Urinary Creatinine Method

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KEY WORDS body composition; muscle mass; creatinine; weight; apes

ABSTRACT Individual body size and composition are important variables for a variety of questions about the behavioral ecology and life histories of non-human primates. Standard methodologies for obtaining body mass involve either capture, which poses risks to the subject, or provisioning, which can disrupt the processes being studied. There are no methods currently available to assess body composition from living animals in the wild. Because of its derivation in muscle, the amount of creatinine that an individual excretes in 24 hours is a reliable and frequently used indicator of relative muscle mass in humans and laboratory animals. Although it is not feasible to collect 24-hour urine samples from wild primates, we apply here a simple method to approximate muscle mass variation from collections of spot urine samples. Specific gravity (SG), an alternative method for assessing urinary water content, is both highly correlated to creatinine and free of mass-dependent effects. Individuals with greater muscle mass should excrete more creatinine for a given SG. We examine this relationship in a dataset of 12,598 urine samples from wild chimpanzees in the Kibale National Park, Uganda. As expected from known differences in body composition, the slope of the relationship between SG and creatinine is significantly greater in adult males than adult females and in adults versus immature individuals. Growth curves generated through this method closely approximate published weight curves for wild chimpanzees. Consistent with the role of testosterone in muscle anabolism, urinary testosterone predicted relative creatinine excretion among adult male chimpanzees. Am J Phys Anthropol 149:622–627, 2012.

Physical condition is a critical variable for naturalistic studies because it conveys the somatic impacts of food availability, illness, aging, physical competition, reproduction, and other events. Obtaining regular and reliable measures of condition is a considerable challenge for field biologists, particularly when it is not feasible to capture or weigh subjects, as is the case for many primates. Although recent methods have allowed for non-invasive assessment of fluctuations in energetic condition via insulin production (Sherry and Ellison, 2007; Deschner et al., 2008; Emery Thompson and Knott, 2008; Emery Thompson et al., 2009) and protein insufficiency (Vogel et al., 2012), neither of these methods is informative about body mass. Investment in muscle anabolism, in particular, is an important component of strategic energy allocations for males, because it is energetically costly yet is predicted to contribute to male competitive success (Zurlo et al., 1990; Bribiescas, 1996; Mitani et al., 1996; Lassek and Gaulin, 2009).

A common approach for measuring lean body mass in humans is the measurement of creatinine excreted into urine over a 24-hour period. Creatinine is a breakdown product of two nitrogenous organic compounds, creatine and phosphocreatine, found chiefly (98% in humans) in muscle tissue, where they form a key component of cellular metabolic pathways. These compounds dehydrate into creatinine at a low and reasonably constant rate, and the creatinine is subsequently excreted into the urine (Heymsfield et al., 1983). Individuals with a greater quantity of muscle tissue, thus more creatine, excrete more creatinine per unit time. Urinary 24-hour creatinine has a high correlation with muscle mass estimated through other means, such as potassium-40 counts (Forbes and Bruining, 1976), bioelectrical impedance (mass–fat mass) (Baxmann et al., 2008), computed axial tomography (Wang et al., 1996), and dual-energy X-ray absorptiometry (Proctor et al., 1999), and has been used to track degeneration of muscle mass with age and degenerative illness in humans (Frontera et al., 1991; Mancini et al., 1992). This relationship is also consistent in animal models where 24-hour creatinine correlates with muscle mass estimation determined through carcass dissection (sheep, van Niekerk et al., 1963; rats, Chinn, 1966; bulls, De Campeneere et al., 2000).

Although creatinine levels are frequently obtained in primate studies as a method to adjust hormone levels for urinary water content, 24-hour collections are obviously infeasible in the wild. However, it is possible to obtain estimates of relative creatinine excretion rate by comparison to an alternative index of water content, specific

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gravity (SG), which is not affected by muscle mass. Individuals with more muscle tissue should secrete more creatinine at a given urine density, as measured by SG. Here, we test this premise in a large collection of urine specimens obtained from wild chimpanzees (*Pan troglodytes schweinfurthii*). We examine the relationship between relative creatinine excretion and known variation in body size between age-sex classes. Mature individuals are expected to excrete more creatinine than young individuals, and adult males should excrete more than adult females, consistent with the approximately 25% sexual dimorphism in body weight (Uehara and Nishida, 1987; Pusey et al., 2005). Because testosterone contributes to muscle anabolism (Griggs et al., 1989; Herbst and Bhasin, 2004), adult males with higher testosterone are also predicted to excrete relatively more creatinine.

METHODS

Sample collection

Creatinine and SG were assessed in 12,598 urine samples collected between 1998 and 2010 from 70 wild, unprovisioned chimpanzees in the Kanyawara community of Kibale National Park, Uganda. Urine collections were made opportunistically during the course of daily chimpanzee follows either by capturing samples on plastic sheeting or by pipetting from vegetation. Contamination by soil and feces was avoided, and any particulates in the sample were pipetted out after settling and before freezing. Samples were frozen within 0–14 hours of collection and transported on ice to the Hominoid Reproductive Ecology Laboratory at the University of New Mexico for analysis.

Age estimation

Because many of the sampled chimpanzees were born before the onset of research at Kanyawara or are immigrants, ages are estimated. Age estimates at first encounter for individuals up to 15 years of age, including subadult males, nulliparous females, and juveniles, should have high reliability due to observable morphological, social, and reproductive differences. All older chimpanzees in the community were first individually indentified between 1983 and 1989. Thus, at the onset of sample collection in 1998, we would have high certainty in estimates for those individuals minimally aged 24 years (i.e., who were subadult when first identified). For many females, older age estimates were generated based on the presence and size of one or more dependent offspring. Chimpanzees exhibit age-related morphological changes (e.g., graying and balding of the back, wrinkling, protruding bones, worn teeth), which were used to assign relative ages to animals who were fully adult at the onset of research (Hill et al., 2001). The results of this study are not predicated on accurate discrimination of ages between relatively old individuals.

Urine analysis

SG was assessed on 100–300 μl aliquots of previously frozen, room-temperature urine samples (N = 12,598) using a handheld refractometer (Atago PAL-10S). The prism was wiped thoroughly between samples and rechecked against water every 10–12 samples. SG values, which represent the density of urine relative to water, varied between 1.000 and 1.060 with a resolution of 0.001. Creatinine was assessed on the first thawing of the sample using the colorimetric Jaffe reaction (Taussky, 1954). A recent critique suggested that creatinine might not be stable in field storage conditions, though no direct evidence was provided for this claim (Anestis et al., 2008). To examine the possibility of sample degradation, we re-assayed 193 samples, each of which had been originally assayed within 1 year of sample collection and subsequently frozen for between 3 and 11 years. The average coefficient of variation (CV) between original and repeat determinations was 1.77 $\pm$ 3.65% (N = 193). Chimpanzee creatinine values ranged from 0.0 to 3.5 mg/ml. Samples were assayed in triplicate or quadruplicate with intra-assay CV averaging 1%.

We additionally assayed a subset of 3,414 urine samples from adult males for immunoreactive testosterone. Because androgens are excreted into urine primarily as glucuronide metabolites (Hauser et al., 2008), we deconjugated samples via hydrolysis with beta-glucuronidase (*Helix pomatia*, Calbiochem, <2% aryl sulfatase activity), following the procedure of Muller and Wrangham (2004a). Extraction with an alternative beta-glucuronidase preparation (*Escherichia coli* VII-A, Sigma-Aldrich) produced significantly correlated testosterone results (Spearman's r = 0.92, N = 35, $P < 0.001$). Immunoreactive testosterone was determined via enzyme immunoassay with reagents and protocols obtained from Coralie Munro at the Clinical Endocrinology Laboratory at University of California at Davis (Munro and Lasley, 1988; deCatanzaro et al., 2003), with an approximate sensitivity of 16 pg/ml. In addition to testosterone itself, the antibody (R156/7) has high cross-reactivity (57%) only with one exclusive metabolite of testosterone, 5alpha-dihydrotestosterone, and low cross-reactivity with the adrenal androgen androstenedione (0.3%). Cross-reactivities with other steroids are minimal ($\leq$0.04). Intra-assay CV averaged 6.3%, and inter-assay CVs were 12.3% for a high sample and 15.7% for a low sample. Testosterone values were standardized for SG (T_{SG}) using the formula $T_{SG} = T_{pg/ml}*(SG_{pop}-1)/(SG-1)$, where 1.016 is the mean SG value for the population (SG_{pop}).

Data analysis

Linear and quadratic regression models for the relationship between SG and creatinine produced equivalent R squares. To determine relative creatinine excretion rate, and thus an estimate of relative muscle mass, we calculated the slope of the linear regression of creatinine (mg/ml) against SG (minus 1) for each sample subset of interest. Because the values of both are 0 for water (or a highly dilute urine sample), regressions were calculated through the origin. The resulting slopes ("SG-CR Slopes") were used in subsequent statistical analyses.

To determine the minimum sample size needed to generate valid slopes, we performed a randomization procedure in which data obtained from a well-sampled chimpanzee in one calendar year were subsampled and slopes recalculated at intervals of five from 5 to 100 samples. This procedure was repeated six times each for one adult female and one male (Fig. 1). CV across the six trials fell to 5% when at least 25 samples were included. We therefore used 25 samples as a minimum criterion for statistical comparisons of individuals.

To determine whether this method could discriminate known differences in body size between age-sex classes,

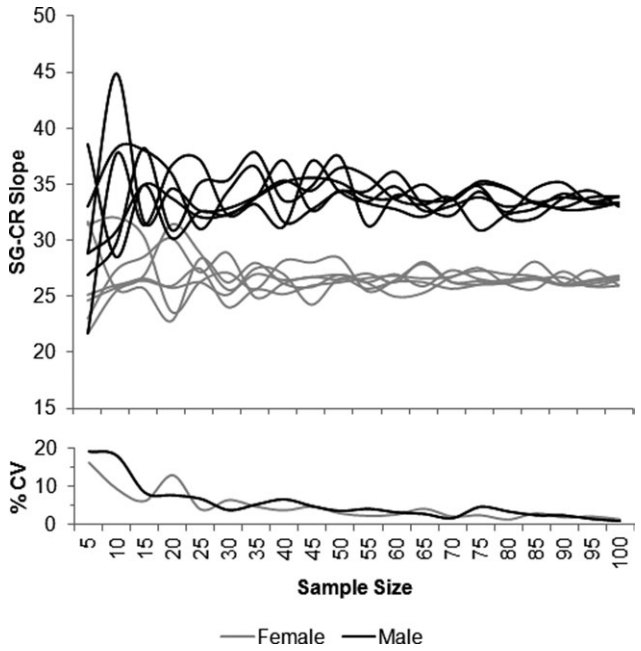

Fig. 1. Results of random sampling simulation for male (dark lines) and female (gray lines). Variation in SG-CR slope estimates is reduced when regressions are calculated with at least 25 samples.

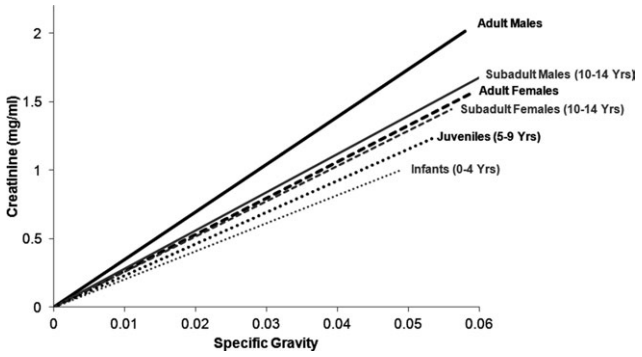

Fig. 2. Linear regression lines describing the relationship between SG and creatinine of urine samples within age-sex classes: adult males (N = 5,975 samples, slope = 34.7), subadult males (N = 1,177, slope = 28.0), adult females (N = 3,387, slope = 26.5), subadult females (N = 621, 25.9), juveniles (N = 1,197, slope = 23.1), and infants (N = 241, slope = 20.4).

we first performed the regression analysis separately for each age-sex class, combining samples for all individuals in the class: adult males (15+ years, N = 5,975), adult females (15+ years, N = 3,387), subadult males (10–14 years, N = 1,177), subadult females (10–14 years, N = 621), juveniles (5–9 years, males and females combined, N = 1,197), and infants (0–4 years, N = 241). Groups were compared statistically by conducting t-tests using slopes for individual chimpanzees sampled 25 or more times. To investigate the finer-scale relationship between age and creatinine excretion and evaluate patterns of growth in chimpanzees, we calculated SG-CR slopes separately for each chimpanzee-year. Because individual immature chimpanzees were often not sampled 25 times, we included all slopes that were associated with a significant ($P < 0.05$) relationship between creatinine and SG. The tradeoff was that this allowance made it possible to construct age curves based on a larger set of individual slopes at each time point. Finally, to evaluate the relationship between testosterone and muscle mass estimated through creatinine excretion, we obtained annual testosterone averages (log-transformed) and SG-CR slopes for each male and calculated Pearson's rank correlations between them, including only males whose slopes derived from 25 or more samples.

RESULTS

Over the entire dataset, creatinine levels of urine samples were significantly predicted by SG (R^2 = 0.84, N = 12,598, $P < 0.001$). This relationship was significant within all age-sex classes ($R^2 > 0.80$, $P < 0.001$). However, as predicted, the slope of the relationship was greater in larger-bodied age-sex classes, varying from 20.4 in infants to 34.7 in adult males (Fig. 2). To test the statistical significance of these differences, we calculated SG-CR slopes for all individuals with at least 25 urine samples (Table 1). As expected, subadults of both sexes had significantly higher SG-CR slopes than juveniles (subadult females vs. juveniles: t = 2.75, N_{sf} = 7, N_{jv} = 11, P = 0.005; subadult males vs. juveniles: t = 3.33, N_{sm} = 8, N_{jv} =11, P = 0.011). Adult males had higher slopes than either subadult males (t = 4.25, N_{am} = 15, N_{sm} = 8, P = 0.002) or adult females (t = 8.93, N_{am} = 15, N_{af} = 15, $P < 0.001$). There were no significant differences between adult and subadult females (t = -0.001, N_{af} = 15, N_{sf} = 7, P = 0.26) or between subadult males and subadult females (t = 1.19, N_{sf} = 7, N_{sm} = 8, P = 0.26), classes containing individuals of similar size. We did not have enough samples from individual infants to test for significant differences, but the mean slope for 3–4-year-old infants was considerably lower than that of juveniles, consistent with the size difference between them. Infants younger than 3 years had unusually high slopes, comparable to large males in the community.

TABLE 1. SG-CR slopes for chimpanzees according to age-sex class

Age-sex class	N	Mean	Std dev	Minimum	Maximum
Adult male	15	35.8	2.4	32.0	41.7
Adult female	15	27.0	3.0	22.3	32.6
Subadult male (10–14)	8	29.1	4.1	23.8	36.0
Subadult female (10–14)	7	27.0	2.5	24.8	32.4
Juvenile (5–9)	11	23.0	3.7	18.4	32.0
Infant (3–4)[a]	9	19.1	3.0	15.0	22.5
Infant (0–2)[a]	8	34.5	8.6	16.0	43.3

[a] Whereas other ages classes include only individuals with ≥25 samples, no infants were sampled that frequently. We report all infants with ≥5 samples from whom statistically significant regressions were obtained.

We next examined whether SG-CR slopes could capture more moderate differences in body size, such as those occurring during growth. Individual slopes plotted by age (Fig. 3) produced realistic growth curves. Female muscle mass estimated by SG-CR slopes increased linearly until the age of 15 years and remained stable through most of adulthood. Male muscle mass estimated by this method also increased linearly until the age of 15 years, surpassing females by the age of 10 years. Con-

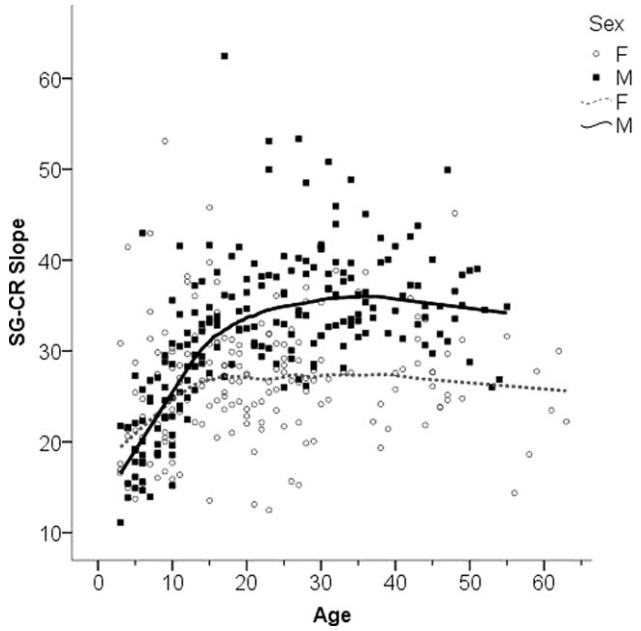

Fig. 3. Slopes from regression of creatinine on SG calculated for each chimpanzee and year, fitted with loess curve. Data limited to individuals aged ≥3 years and slopes based on statistically significant ($P < 0.05$) regressions.

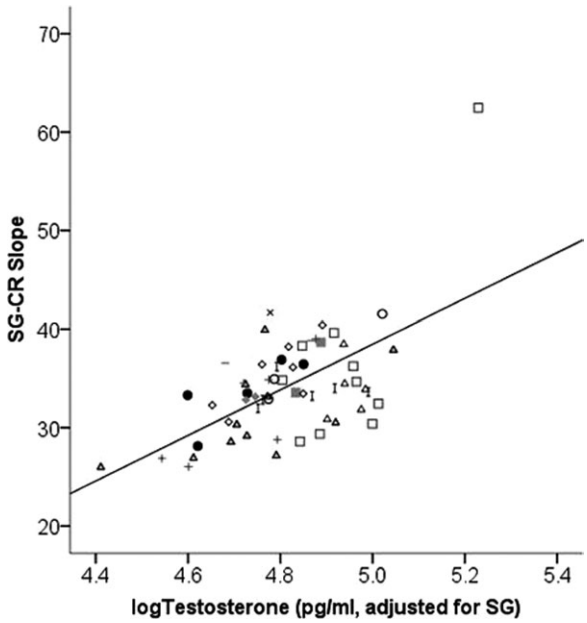

Fig. 4. Urinary testosterone predicts the slope of the regression of creatinine on SG. Each point represents one adult male for one calendar year, with symbols designating individual males.

sistent with a cessation of skeletal growth but continued investment in muscle mass, male slopes continued to increase in the early 20s. Adult SG-CR slopes were highly variable.

Consistent with the role of testosterone in muscle anabolism, mean annual testosterone levels of individual males predicted their individual SG-CR slopes (Pearson's $r = 0.590$, $N = 60$ male-years, $P < 0.001$, Fig. 4). There was a positive relationship between annual testosterone and SG-CR slope for each of the seven males sampled in 5 or more years, though this relationship was only significant for four of them ($0.60 < r < 0.81$, $P \leq 0.05$).

DISCUSSION

We tested the premise that a modification of the urinary creatinine method used to quantify muscle mass in humans could be used without obtaining 24-hour specimens. We proposed an alternative method in which the slope of the relationship between creatinine excretion and SG is calculated using multiple spot urine samples. Using a large sample of urine specimens from wild chimpanzees, we found that this method reliably discriminated age-sex classes with known differences in size. Furthermore, testosterone levels of adult males significantly predicted their muscle mass, as estimated through the modified creatinine method.

The most promising result of our study was the production of a realistic growth curve for muscle mass in wild chimpanzees. These curves closely approximated those obtained for body mass in Gombe chimpanzees using baited scales (Pusey, 1990; Pusey et al., 2005). For example, an important shared feature was that in both studies, the size of males exceeded females by approximately the age of 10 years. Males at this age begin to try to dominate adult females and begin to be targeted for aggression by adult males (Pusey, 1990). Growth of both males and females slowed in the early to mid teens, at approximately the time when both sexes are considered fully mature (Goodall, 1986). Among immature individuals, relative creatinine excretion most likely reflects the proportional increase in musculature with body size. However, once skeletal growth is complete, individuals are expected to differ in their relative investment in muscle tissue, and this investment may also vary over time. In our study, SG-CR slopes of adults were considerably more variable than those of juveniles. After adulthood, males but not females exhibited continued increases in muscle mass, peaking around an age of 30 years.

Many of the infants below the age of 3 years were outliers in that they exhibited SG-CR slopes more like large adults. Some of this may be due to the relatively poor sample size for these infants. However, it is notable that humans also exhibit elevated urinary creatinine in early infancy (Finney et al., 2000). This has been attributed to low glomerular filtration rate during maturation of the kidney, as well as the elevated presence of cross-reacting chromagens, such as bilirubin (Stonestreet et al., 1979). The duration of elevated creatinine in chimpanzees exceeded that observed in human infants (~4–12 months, Finney et al., 2000; Price and Finney, 2000). Generally, it should be noted that injury or disease to the kidney may alter serum and urinary creatinine excretion (Barr et al., 2005).

A limitation of our study is that we were unable to validate our creatinine-based muscle mass estimates against alternative measures of muscle mass. Valid measures of muscle mass are notoriously difficult to obtain, even among humans in a clinical setting (Heymsfield et al., 1983). Although the creatinine method could be compared to body weights of captive animals with the assumption that variation in muscle mass correlates with variation in total body mass, sample sizes would be limited. With an existing sample of over 12,000 urine specimens, we had a unique opportunity to study creati-

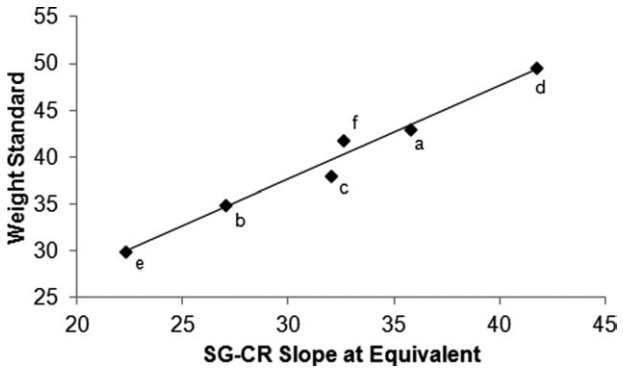

Fig. 5. Comparison of known body weights for adult *Pan troglodytes schweinfurthii* with SG-CR slopes for equivalent individuals in this study. **a,b**: mean beta for adult males and females in this study matched to average body weights of published non-Gombe adult male and female specimens (Rahm, 1967; Uehara and Nishida, 1987; Carter et al., 2008; Emery Thompson and Wrangham, in press). **c-f**: minimum and maximum betas for adult males (c,d) and adult females (**e**,f) in this study matched to minimum and maximum adult body weights of Mahale chimpanzees (Uehara and Nishida, 1987); excluded weight of one Mahale male noted to be unusually small. These preliminary data indicate a creatinine equivalent for adult chimpanzees of weight in kg = 1.0 × Beta + 7.7 (R^2 = 0.98, $P < 0.005$).

nine excretion in a large number of individuals over a 13-year study period, thus offering a better sample in which to examine age-related variation. The similarity of our findings to those of a body mass study from wild chimpanzees at Gombe suggest that our method is indeed capturing important variation in muscle mass, as strongly implied by the known relationship of muscle to creatinine excretion.

Studies using the 24-hour creatinine method in humans have frequently attempted to establish a creatinine equivalence by which to convert units of creatinine to increments of muscle mass or total body mass. For the reasons above, we can at this point make only a tentative estimation of creatinine equivalence for chimpanzees by comparing our mean, minimum, and maximum SG-CR slopes for adult males and females to published body mass estimates for *Pan troglodytes schweinfurthii*. Unfortunately, Gombe chimpanzees, for which the best weight data were available, are approximately 10% lighter than other chimpanzees of the subspecies (Uehara and Nishida, 1987). We instead used mean body masses from small samples in Tanzania, the Congo, and Uganda (Kibale) and minimum and maximum individual body weights of adults at Mahale (Rahm, 1967; Uehara and Nishida, 1987; Carter et al., 2008; Emery Thompson and Wrangham, in press). The relationship of these weights to our corresponding SG-CR slopes (Fig. 5), leads to an initial estimate that 1.0 increment in SG-CR slope is approximately equivalent to 1 kg increase in adult body mass in chimpanzees.

An important conclusion of this study is that, when using creatinine to standardize urinary hormone levels, as is common practice, it is necessary to consider that body composition may bias or obscure variation in the target hormone. Studies that wish to compare urinary hormone levels across age-sex classes should use specific-gravity correction if at all possible. Within age-sex classes, the effects should be moderate. Based on the average SG of urine samples obtained in this study, differences in creatinine excretion would contribute an average error of 9% for adult females and 5% for adult males. In most cases of published research on chimpanzees, the direction of significant effects cannot be readily explained by individual differences in creatinine excretion. For example, high-ranking male chimpanzees have been reported to excrete higher levels testosterone and cortisol, after correction for creatinine (Muller and Wrangham, 2004a,b). If rank were systematically related to muscle mass (which is not necessarily the case), it should be in the direction of higher muscle in dominant individuals, which would produce a systematic underestimation of steroid levels in high-ranking males. In other words, muscle mass should create a bias against the result obtained, not for it.

This method offers considerable potential for studying growth, as well as how muscle mass varies in relation to factors such as seasonality, age, dominance rank, and mating success. This is likely most applicable to studies of large, arboreal primates, from which urine can be reliably collected and for which darting and/or weighing are not feasible methodologies for obtaining relative body size estimates. In fact, no method currently available allows for discrimination of body composition for live animals in the wild. Although studies will require an intense effort to obtain a suitable number of samples on which to base muscle mass estimates, creatinine and SG values can be derived with a minimum of expense and expertise.

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