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Urinary C-peptide as a method for monitoring body mass changes in captive bonobos (*Pan paniscus*)

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ABSTRACT

In recent years methodological improvements have allowed for more precise estimates of nutrient intake in wild primates. However, estimates of energetic condition have remained relatively imprecise due to the difficulties of estimating digestive efficiency and energy expenditure in these animals. In the absence of a reliable intake-expenditure calculation, a method is needed that directly links changes in energetic condition, such as body mass, to physiological changes that can be detected via markers in body excretions such as urine or feces. One promising marker is C-peptide, a metabolic byproduct of insulin synthesis. Here we present the results of a food restriction experiment carried out in a group of captive bonobos (*Pan paniscus*). We measured changes in food availability and body mass and determined urinary C-peptide levels with the help of a time-resolved fluoroimmunoassay routinely used for measuring C-peptide in human blood. Urinary C-peptide levels decreased during a period of food restriction and increased again when food availability was continuously increased. During this refeeding phase an increase in body mass was significantly correlated with an increase in urinary C-peptide levels. Our results suggest that urinary C-peptide levels are an accurate indicator of individual energy balance. In conclusion, measuring C-peptide in urine is a promising method to quantify the energetic condition of wild apes.

Introduction

A major task in the daily life of wild animals is to consume enough food to obtain the energy that is needed to maintain basal body function and meet travel costs and thermal requirements. Energy availability influences growth rate, age of maturity, body size and mass, probability of survival, and the number and physical condition of offspring (Lindström, 1999; Schneider and Wade, 2000). By estimating and measuring search effort, investment for food preparation, food quality, digestive efficiency and metabolic rate, it is possible to calculate the optimal values for diet composition and energy supply (MacArthur and Pianka, 1966). However, predicted values are often not met, and animals may grow and reproduce even if conditions are chronically suboptimal (Altmann, 1998). Although seasonal changes in food availability might affect all individuals within a population in a similar way, dominance rank differences in group-living animals can lead to within-group variance in resource acquisition potential (Janson and Vogel, 2006; Rubenstein, 1986). Although there is large consensus that nutritional status is a key parameter contributing to individual fitness (Koenig et al., 1997), accurate monitoring of energetic condition in wild living large-bodied animals is a significant methodological challenge.

Various methods are used to measure the nutritional status of wild animals. In animals hunted for game, nutritional status can be estimated by determining subcutaneous, omental, renal, pericardial and marrow fat stores (Brooks et al., 1977; Dauphiné, 1971; Harris, 1945). In captive animals, techniques to assess nutritional status include measurements of body mass and dual-energy X-ray absortiometry (Blanc et al., 2005). Live traps have been used to capture wild animals. This invasive method can be used to measure body mass (Richard and Dewar, 1991), body temperature, and oxygen consumption (Schmid and Speakman, 2000), and to take blood to assess serum insulin, and cholesterol levels (Kemnitz et al., 2002). If animals can be held temporarily in captivity, doubly labeled water analysis allows for the determination of energy balance (Lifson and McClintock, 1966). In some cases, observers have used baits to attract wild animals to step on balances (Goodall, 1986; Hofer and East, 2003; Pusey et al., 2005; Uehara and Nishida, 1987). It is more common to use indirect methods that rely on visible changes of subcutaneous tissue that are related to temporal variations in energy supply, metabolism or both (Koenig et al., 1997; Mendoza et al., 1978).

Changes in body fat can be monitored by measuring ketones in urine samples. Ketones are produced when an animal is metabolizing its own body fat reserves to meet its energetic needs (Robinson, 1980).

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This technique has been used successfully in wild orangutans living in a habitat that is characterized by an alternation of mast fruiting events and periods of extreme food scarcity (Knott, 1998). Fluctuations in food availability led to significant changes in energy supply and adult males underwent dramatic changes in body mass (Conklin-Brittain et al., 2006). During periods of mast fruiting, urine samples were free of ketones whereas in the period of severe fruit shortage, ketones were found in a large proportion of the samples. Attempts to apply the same method to samples from other hominoids did not succeed or produced conflicting results (Kaur and Huffman, 2004; Knott, 2005a, b; Krief et al., 2005). It is unclear if the changes in energetic condition in these latter studies were too small to induce a ketone signal or if other factors interfered with the analysis.

C-peptide is another potential biomarker of variation in body mass, nutritional status, and energetic condition (Hoogwerf and Goetz 1983). This is a small polypeptide of 31 amino acids with a molecular mass of 3020Da. It is produced during insulin synthesis when the primary product of transcription, proinsulin, is enzymatically cleaved in the β-cells of the pancreas into active insulin and C-peptide. Insulin and C-peptide are secreted in equimolar amounts into the blood-stream, but because C-peptide is broken down at a much slower rate in the body than insulin (Horwitz et al., 1975), it has become a useful marker for monitoring insulin production in medical studies (Hoffman, 2004; Tsai et al., 2006).

C-peptide levels in non-diabetic patients correlate positively with body mass index (French et al., 1992; Polonsky et al., 1988). Studies of anorexic patients have also shown that C-peptide levels rise with increasing body mass after the start of a therapy (Yoshida et al., 2006). Therefore, C-peptide is a plausible indicator for both between- and within-individual variation in energetic condition.

While most clinical studies have focused on serum and plasma as a source for C-peptide analyses, the use of urine has been successfully applied in a number of studies (Goetz et al., 2002; Murata et al., 2000; Suzuki et al., 2004).The results indicate that insulin secretion is highly correlated with urinary C-peptide excretion (Meistas et al., 1981, 1982). Furthermore, in captive chimpanzees there was a significant correlation in C-peptide levels between matched serum and urine samples (Sherry and Ellison, 2007). Urine can therefore be used for measurement of C-peptide with the same assay methods, incorporating a change in the dilution.

Few studies have investigated the usefulness of measuring urinary C-peptide levels for detecting variation in energetic condition. Results from a study of captive chimpanzees showed that captive animals assumed to be better fed than wild ones, had significantly higher C-peptide levels than wild animals (Sherry and Ellison, 2007). Furthermore, urinary C-peptide levels of adult male chimpanzees varied with food availability, suggesting that individual C-peptide levels corresponded with differences in energy supply. In wild orangutans, urinary C-peptide levels correlate with presence of urinary ketone bodies, fruit availability and energy intake estimates (Emery Thompson and Knott, 2008).

It is currently unclear how sensitive and responsive urinary C-peptide measurements are to individual differences and within individual changes in energetic condition. Urinary C-peptide levels could be an indicator of energy status indicating the amount of energy reserves (fat tissue) in an animal. Alternatively, C-peptide levels could be a better indicator of energy balance, — i.e. recent changes in energy intake and expenditure. To resolve this question, direct investigations of body mass changes in relation to C-peptide levels are needed. We therefore implemented a study in which we modified the energy content of food given to a group of captive bonobos (*Pan paniscus*) while simultaneously measuring changes in body mass and urinary C-peptide levels. Energy content was gradually reduced for 2weeks and then slowly increased over two additional weeks until the animals received food with considerably more energy than the normal diet. This period of increasing energy availability was followed by another week during which food composition returned to normal levels. We also conducted experiments on the stability of urinary C-peptide to evaluate degradation problems that might exist under the difficult collection conditions typical of field work. The data collected during this experimental study addressed the following questions:

- Is there a predictable relationship between absolute body mass and urinary C-peptide levels?
- Do changes in dietary energy content lead to corresponding changes in urinary C-peptide levels?
- Do changes in body mass correlate with changes in urinary C-peptide levels?
- How stable is urinary C-peptide at room temperature and when exposed to repeated freezing and thawing?

Methods

Animals

We conducted the experiment on one group of captive bonobos at the Frankfurt Zoo, Germany, consisting of 1 adult male, 6 adult females, 2 juveniles and 1 infant (Table 1). The group had access to two indoor and one outdoor enclosure, and all individuals remained in social groups throughout the day and night. Bonobos are a great ape species, closely related to chimpanzees, and their distribution range is restricted to the tropical lowland rainforest in the Democratic Republic of Congo, south of the Congo river (Coolidge, 1933).

Feeding experiment

Energy content of the bonobos' diet was modified by changing the composition of the provisioned food. For the first 2weeks, we replaced caloric-rich food items (e.g., cereals, meat, and fruit and vegetables rich in sugar and lipids) with low-caloric food items such as salads, leaves, and tree branches. Thereafter, dietary energy content was gradually increased over 2weeks until it surpassed the usual amount of food that the group normally received. Samples from all food items were analyzed for content of macronutrients, fibers, and energy content (Hohmann et al., in prep.) Throughout the experiment the animals were not separated but remained in social contact. The experiment was approved by the Regierungspräsidium Darmstadt, Hessen, Germany and adhered to all animal care, legal and ethical requirements of Germany.

Table 1
Age, sex, reproductive status, body mass and average C-peptide levels during a period of normal food provisioning of study subjects

Animal	Sex	Age (years)	Rep. condition	Average bodyweight (kg)	Average C-peptide levels (nmol/g Crea)[a]
Ludwig	Male	23	–	48.6	0.96 (+/−0.44, *N*=9)
Margrit	Female	56[b]	Post reproductive	29.9	1.21 (+/−0.56, *N*=7)
Natalie[c]	Female	43[b]	Pregnant	31.8	5.08 (+/−1.99, *N*=5)
Ukela	Female	22	Cycling	25.3	0.85 (+/−0.85, *N* =5)
Kamiti	Female	20	Cycling	32.0	0.61 (+/−0.38, *N*=9)
Zomi	Female	9	Cycling	25.6	0.76 (+/−0.45, *N*=5)
Kutu[c]	Female	9	Pregnant	30.2	0.83 (+/−0.38, *N*=5)
Heri[d]	Male	6	–	15.0	–
Haiba[d]	Female	6	–	13.2	–
Kelele[d]	Male	3	–	8.5	–

[a] Collected during period of normal food provisioning after the refeeding period.
[b] Age estimated because animal was wild caught.
[c] Individual was pregnant.
[d] Less than five samples available during the period of normal food provisioning.

Sampling protocol

Data were collected from January 20 to February 18, 2007 and included body mass measurements and urine sampling. To determine body mass, subjects had to enter a compartment, within an indoor enclosure, that was connected to a balance (EZI, range: 0–500kg, resolution: 0.5kg). Since moving on the scale and measuring body mass were part of the routine training program for these apes, most individuals performed this task easily. However, efforts to take body mass on a daily basis deviates from the normal routine, and individuals did not always cooperate. Accordingly, weights are not available for every day of the feeding experiment and the number of records per individual ranges from 22 to 32 with an average of 28 measures. One female, Ukela, could only be measured 3 times and was therefore excluded from analyses involving body mass. Before the onset of the experiment, subjects were trained to deliver urine into containers held by zoo personnel. Urination was enhanced by providing nettle tea 20min prior to the collection time. Urine samples were collected twice each day. Morning samples were taken between 8:00 and 8:30 before the bonobos received their first meal. A second sample was taken in the afternoon around 14:00, about 4h after they had received their main meal consisting of fruit and vegetables. Samples were collected directly into plastic cups or taken off the ground with disposable plastic pipettes. Samples were stored at − 20°C and transported on dry ice to the laboratory. As with body mass measurements, urine sample collection varied according to the cooperation of the animals.

Sample preparation and C-peptide assay

We measured C-peptide with AutoDELFIA® C-peptide, a commercially available time-resolved fluoroimmunoassay kit from PerkinElmer, designed to measure C-peptide in human serum and plasma. Serial dilutions of urine samples gave displacement curves parallel to those obtained with the appropriate standard (C-peptide Fragment 3–33, human, Sigma C 8662). Intra- and interassay coefficients of variation calculated from replicate determinations of samples and quality controls were 2.3% and 11%. The sensitivity of the assay is typically better than 4.97pmol/l.

Exclusion based on C-peptide recovery

In the initial assays, more than 10% of all urine samples proved to have C-peptide levels lower than the sensitivity level of the assay, and these levels remained low even after spiking these samples with C-peptide standard (C-peptide Fragment 3–33 human, C8662, Sigma). Since addition of protease inhibitor (complete mini, EDTA free, Roche) did not improve recovery, we concluded that this problem was not caused by direct proteolytic degradation of C-peptide. To address this problem, we measured all samples twice, once as pure urine and once as urine spiked with C-peptide standard. We excluded all samples from the analysis in which the recovery rate of the spiked standard was lower than 50%. For the remaining samples, we corrected the determined C-peptide level with the respective recovery rate for the spiked pendant of this sample. For ten samples (3.4% of the assayed samples) C-peptide levels were below the sensitivity level of the assay although their recovery rate was above 50%. Five of the samples were from the infant and juveniles and five were from adult females. All samples were from periods when food provisioning was low, either during the second week of the diet ($N = 7$) or the first week of refeeding ($N = 3$). In these cases, we assigned them the C-peptide concentration of the sensitivity level of the assay (4.97pmol/l) and retained these data in the analysis so as not to artificially eliminate samples with low C-peptide levels from the study. However exclusion of these data does not change the qualitative outcome of statistical analyses (data not shown).

To compensate for the variation in the volume and concentration of the voided urine, we measured creatinine (Cr) concentrations in each urine sample (Bahr et al., 2000) and expressed all C-peptide values as nmol/g creatinine. Since very low creatinine concentrations lead to an overestimation of C-peptide, we excluded all urine samples with creatinine < 0.1mg/ml from the analyses ($n = 28$, 9.6% of all assayed samples).

Stability experiments

To test the stability of C-peptide in urine samples stored in less than optimal conditions, we conducted two experiments with a subset of six samples that were divided into several portions each. In the first experiment, we thawed and froze portions of the same 6 samples for 1 to 5 times. In the second experiment, we left portions of the same samples for 1, 4, 18, 24 and 45h at room temperature.

Statistics

We used a paired sample *t*-test to explore differences in C-peptide levels in morning and afternoon samples. The data met the assumption of normally distributed differences. Differences in the proportion of samples with low recovery between morning and afternoon samples were tested with a chi-square test.

A Pearson correlation was used to investigate the association between average female C-peptide levels and average female body mass during normal feeding.

We investigated the association between body mass change and C-peptide levels by calculating the Spearman correlation for each individual and phase and then testing with a one-sample *t*-test if this association across subjects was significantly different from 0.

Results

Body mass dynamics

During the energy restriction period (diet phase), the animals lost on average 4.5% of their body mass (Table 2). The adult male had the highest loss of absolute body mass (3.7kg). The two pregnant females and the infant lost almost no weight during the diet phase and were therefore excluded from all analyses related to changes in C-peptide levels. However, the decrease in body mass over time across all group members was significant (mean correlation: $r = -0.51$, one-sample *t*-test, $t_8 = -3.922$, $p = 0.004$).

During the refeeding period, when the availability of energy-rich food items was continuously increased, the animals gained on average 11.5% of their body mass relative to the start of this period. During this phase, the adult male gained 7.2kg, the greatest gain in terms of

Table 2
Absolute and relative weight loss and gain of the study subjects during the feeding experiment

Subject	Mean weight at the beginning of diet (kg)	Mean weight at the end of diet (kg)	Relative weight loss (% bodyweight)	Mean weight at the end of transition (kg)	Relative weight gain (% bodyweight)
Ludwig	50.5	46.8	−7.3	54.0	15.3
Margrit	29.2	29.0	−0.6	31.7	9.2
Natalie[a]	32.2	32.5	1.0	32.5	0.0
Ukela[b]					
Kamiti	33.0	31.0	−6.1	34.0	9.7
Kutu[a]	30.0	29.7	−1.1	31.8	7.3
Zomi	25.8	23.7	−8.4	29.2	23.2
Heri	15.7	14.5	−7.4	15.0	3.4
Haiba	12.8	12.0	−6.5	14.0	16.7
Kelele	8.3	8.0	−4.0	9.5	18.8
Average			−4.5		11.5

[a] Subject was pregnant.
[b] Subject refused to be weighed.

absolute body mass. The increase in body mass over time across group members was significant (mean correlation: $r = 0.66$, one-sample t-test, $t_8 = 6.368$, $p < 0.001$).

C-peptide levels in morning and afternoon samples

Morning and afternoon samples did not differ in their C-peptide levels (paired sample t-test, $t_{16} = -1.158$, $p = 0.264$). Furthermore, there was a good correlation between samples collected in the morning and samples collected in the afternoon (Pearson correlation, $r = 0.73$, $p = 0.001$). However, whereas only 14.3% of morning samples showed an unacceptably low recovery rate, this rate was significantly higher for the afternoon samples (38.5%, $p = 0.001$, $chi^2 = 14.32$, df = 1). We therefore restricted our analysis of the effect of food availability and body mass changes on C-peptide levels to samples collected in the morning.

Individual C-peptide levels during normal feeding and association with body mass

Mean individual C-peptide levels for adult subjects during normal feeding ranged from 0.61nmol/g Crea (Kamiti) to 5.08nmol/g Crea (Natalie) (mean ± SD = 1.48 ± 0.58, $N = 7$; Table 1). There was no significant correlation between individual female's mean C-peptide level and individual female's mean body mass (Pearson correlation, $r = 0.433$, $N = 6$, $p = 0.392$). This result did not change when excluding the two pregnant females (Pearson correlation, $r = -0.019$, $N = 4$, $p = 0.981$).

C-peptide level changes in relation to dietary regime

Individual C-peptide decreased with ongoing food restriction (Fig. 1: profile for the adult male). The decrease in C-peptide level during the diet phase across individuals over time was significant (mean correlation: $r = -0.40$, one-sample t-test, $t_6 = -4.792$, $p = 0.003$) (Fig. 2). When food availability was continuously increased during the refeeding phase, C-peptide levels increased significantly across individuals over time (mean correlation: $r = 0.54$, one-sample t-test, $t_6 = 3.267$, $p = 0.017$).

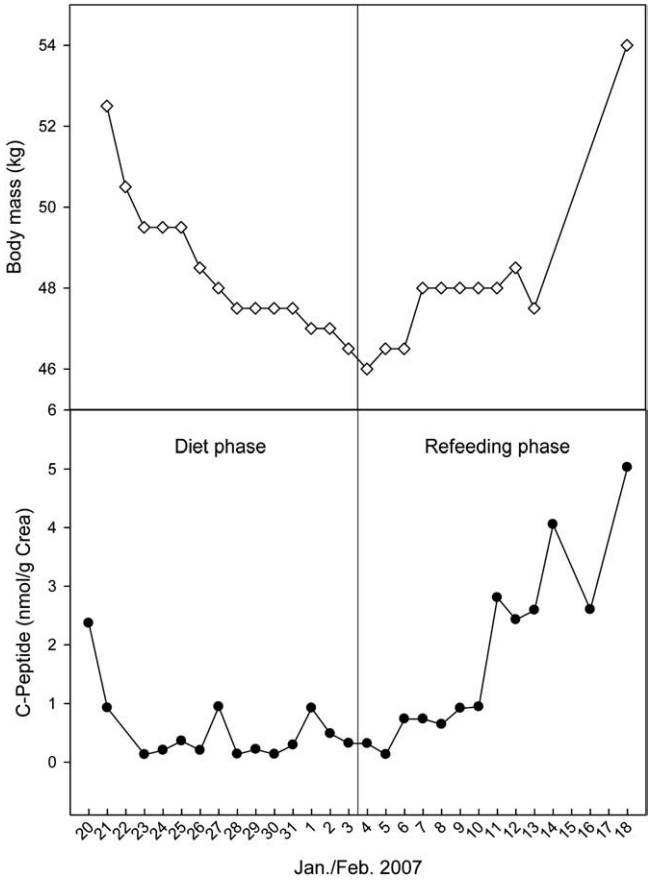

Fig. 1. Profile of body mass and urinary C-peptide throughout the diet and refeeding phase of the food restriction experiment for the adult bonobo male Ludwig.

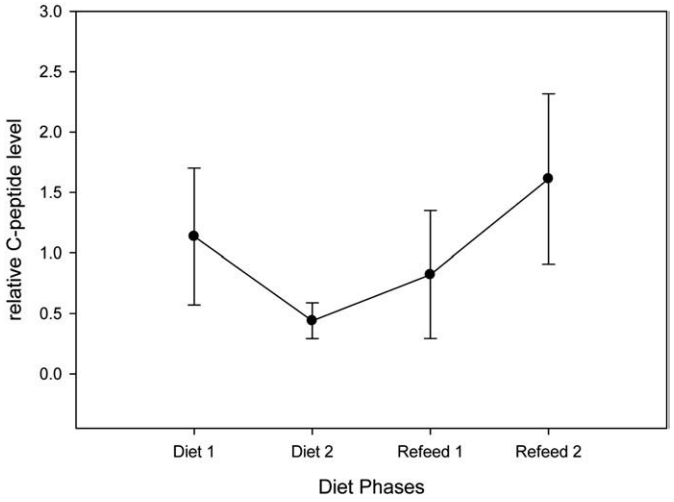

Fig. 2. Composite profile of average C-peptide levels during the 4 weeks of the feeding experiments. Levels are given relative to the overall average level of each individual (values for the two pregnant females and the infant were excluded). Error bars indicate the standard deviation calculated across individuals. Decrease during diet phase: mean correlation: $r = -0.40$, $p = 0.003$, increase during refeeding phase: mean correlation: $r = 0.54$, $p = 0.017$.

Body mass dynamics and C-peptide levels

During the diet phase, there was no significant association between body mass and C-peptide levels (mean correlation: $r = 0.31$, one-sample t-test, $t_4 = 1.559$, $p = 0.194$). However, the sample size was small since two individuals could not be weighed and the pregnant females and the infant showed no weight loss. During the refeeding phase, however, increase in body mass was significantly correlated with increase in C-peptide levels (mean correlation: $r = 0.54$, one-sample t-test, $t_5 = 4.02$, $p = 0.01$) (Fig. 3).

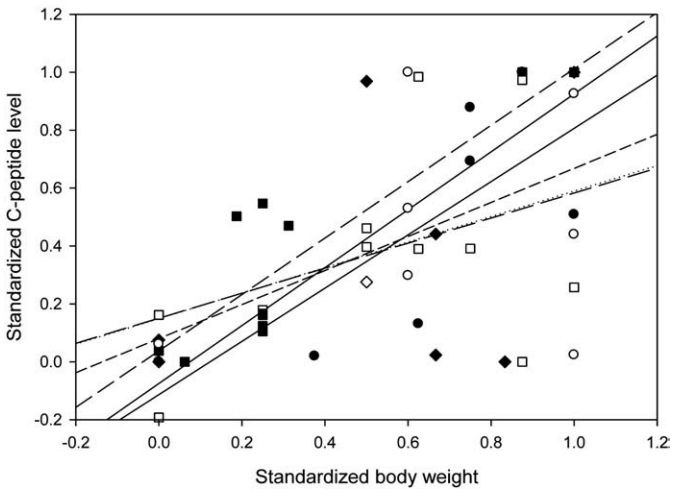

Fig. 3. Standardized daily C-peptide levels in relation to standardized body mass for six bonobos during the refeeding period (standardization: Std $x_i = (x_i - x_{min})/(x_{max} - x_{min})$. Mean correlation: $r = 0.54$, $p = 0.01$.

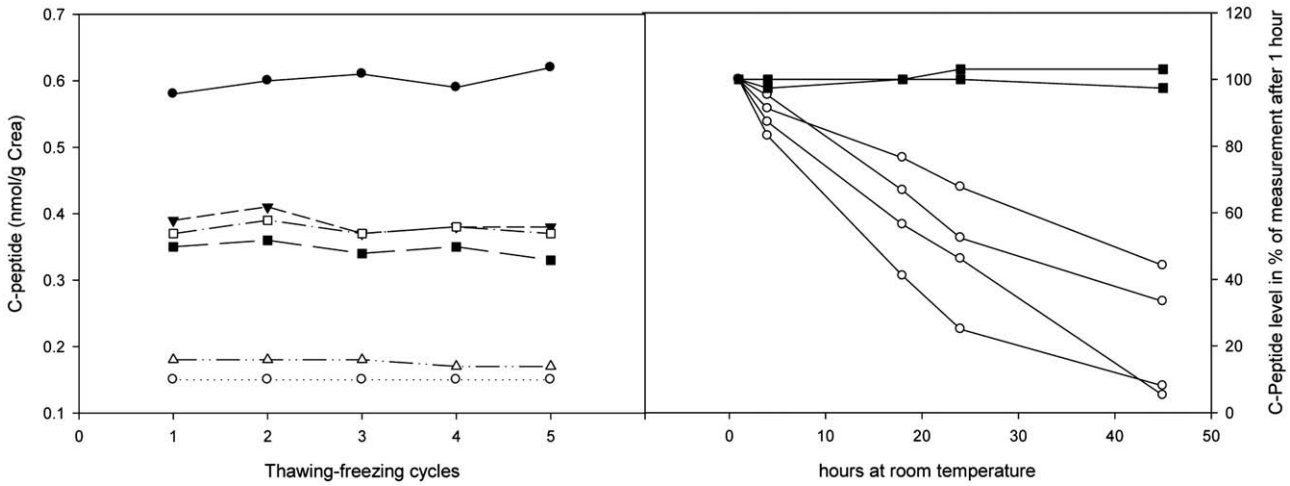

Fig. 4. (a) C-peptide levels in six urine samples during five freezing and thawing cycles and (b) decrease in relative C-peptide levels of urinary samples left at room temperature. Black squares indicate male samples, white circles female samples.

Urinary C-peptide stability

Freezing and thawing

Repeated thawing up to 5 times did not have a detrimental effect on C-peptide levels. Samples that were thawed for five times still had on average 98.8% (range: 94.3 to 106.9%) of their original C-peptide level (Fig. 4a).

Degeneration at room temperature

C-peptide levels of urine samples left at room temperature showed diverging degrees of degradation. Whereas in two samples from the adult male virtually no degradation could be detected after 45 h, C-peptide levels in four female urine samples dropped continuously until, after 45 h, only 22.6% of the original C-peptide level could be detected (range: 5.1 to 45.0%) (Fig. 4b).

Discussion

By experimentally restricting diets and monitoring changes in body mass and changes in urinary C-peptide level changes we were able to show that measuring C-peptide levels in bonobo urine is a useful physiological marker reflecting changes in energetic condition in this species. We furthermore showed that degradation caused by unidentified substances could cause a problem for the interpretation of urinary C-peptide levels. At the same time, we applied a means (spiking samples) to control for this problem.

When dietary energy content was restricted, individuals lost on average 4.5% of body mass over a period of 15 days. Only pregnant females were able to maintain their body mass. Whether this was due to their ability to monopolize food or to changes in metabolic rates is unclear. Under conditions of dietary restrictions, metabolic rate has been observed to decrease in pregnant women, thereby sparing energy for fetal development (Poppitt et al 1993). Both explanations are possible since our experimental design did not allow us to provision food to individual animals. Body mass across all individuals increased significantly with the increase in dietary energy content. These changes in body mass were correlated with changes in C-peptide levels, strongly suggesting a link between dietary energy content, changes in body mass and urinary C-peptide levels. The ability to estimate the effect of moderate changes in body mass on urinary C-peptide levels is especially important for a species such as bonobos, since their natural habitat is thought to be relatively stable in terms of food availability and nutrient content. Therefore temporal changes in energy supply are expected to be moderate (Chapman et al., 1994; Hohmann et al., 2006; Wrangham, 1986).

Decreased urinary C-peptide levels during the dietary restriction phase might be an outcome of two different causes. Either they are directly linked to the reduced availability of energy-rich macronutrients such as lipids and carbohydrates, or they correspond to decreased body mass. In humans, C-peptide levels correlate with body mass index (Polonsky et al., 1988). In this study, urinary C-peptide levels did not differ between morning and afternoon samples, where the morning samples were collected before the animals had received any food, while the afternoon samples were collected 4 h after one of the two main food provisionings of the day. The results therefore indicate that the observed pattern relates at least partly to changes in body mass rather than to transient changes due to differences in nutrient supply.

While our results show that urinary C-peptide is a useful marker for changes in body mass and therefore in energy balance, it remains unclear if it can also reliably reflect individual differences in energy status. We did not detect any correlation between average urinary C-peptide levels during the standard food provisioning period and individual body mass, as has been described for humans (Polonsky et al., 1988). Our finding might be due to several factors. First, body mass index may be a better predictor of C-peptide levels than simply body mass because BMI accounts for individual differences in size and is a good measure of the amount of individual fat tissue. Secondly, our sample size was small and we could not control for female reproductive status, therefore any existing effect might have been too small to be detected. This finding is of importance in the application of this method to field studies. To relate average C-peptide levels to social parameters such as rank, especially in females, individual parameters such as reproductive state must be taken into consideration, which suggests studies will require larger sample sizes than are typical in field studies. More detailed experimental studies with accurate information on individual energy intake are needed to resolve the question of whether individual differences in energy status can be monitored by measurements of urinary C-peptide levels.

Repeated freezing and thawing of samples did not affect urinary C-peptide levels. This finding is especially important for handling samples in the lab. However, in some cases, degradation at room temperature took place. While we could not detect any degradation in male samples after 45 h, in female urine samples C-peptide levels dropped to an average of 22.6% after this time. Male samples were collected principally by directly holding a plastic cup into the stream, but female samples were collected with pipettes from the ground. We infer that contamination was responsible for the degradation pattern. The contamination was still active when we spiked urine samples, and

degradation could not be stopped by addition of a protease inhibitor. This could mean that some non-enzymatic component of the cleaning water was responsible for this effect. This inference is supported by the finding that suppression of spiked C-peptide levels occurred significantly more often in the afternoon samples, after the floor had been wiped with a commercial detergent, than in morning samples which were taken before cleaning. Contamination in a field situation can be avoided by capturing and pipetting urine samples off clean plastic bags instead of pipetting from vegetation. Alternatively, the effect of possible contamination from vegetation can be validated by controlled experiments as has been done for steroid hormones and creatinine (Knott, 2005a,b, Muller and Wrangham 2004).

Furthermore, these results show how important spiking of samples can be to detect measurement problems. We therefore recommend spiking a certain percentage of collected samples to detect if any problems with degradation exist. If not, then measurements can be performed without a spiked sample in parallel. If, however, some samples show degradation, every sample should be spiked in parallel to determine the reliability of the measured value.

In summary, we have shown that measuring urinary C-peptide levels in bonobos is a useful tool for estimating energy balance and changes in body composition. This method will allow researchers to answer important questions about seasonal and populational variation in different great ape species in relation to food availability, nutritional quality and feeding.

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