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Urinary C-peptide of insulin as a non-invasive marker of energy balance in wild orangutans

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Abstract

Assessment of energetic condition is a critical tool for behavioral and reproductive ecologists. However, accurate quantification of energy intake and expenditure is labor-intensive, and it can be problematic for field scientists to obtain regular data on individual animals. C-peptide, a polypeptide segment of the proinsulin molecule that is secreted along with insulin in an equimolar relationship, can be measured in urine, and thus offers a potential means for the non-invasive assessment of energy balance in wild animals. Here, we validate C-peptide for the quantification of energetic condition, with specific application to wild orangutans (*Pongo pygmaeus*). We determined that application of urine to filter paper results in significantly lower C-peptide recoveries versus fresh samples. However, concentrations in filter paper samples were significantly correlated with fresh urine and were stable over various storage conditions and durations. We compared the C-peptide concentrations from wild orangutan urine samples with three independent measures of energetic condition: ketone bodies (urinalysis), caloric intake (nutritional biochemistry), and food availability (phenology). As expected, C-peptide concentrations were significantly lower in samples that tested positive for ketones in the field. Monthly average C-peptide concentrations of both male and female orangutans were significantly correlated with monthly determinations of energy intake and food availability. Therefore, we conclude that the collection and preservation of urine samples for C-peptide analysis are feasible under most field conditions and, in this species, presents a useful tool for assessing changes in energy balance.

Keywords: Energy intake; Food availability; Reproductive ecology; Energy metabolism; Urinary hormones; Insulin; Apes

Introduction

Energy balance has a critical influence on the behavior, physiology, and life history of primates and other animals. Temporal variation in the availability and intake of food resources impacts the timing of reproductive events, and thus the patterning of mating and competitive interactions (pinnipeds: Boyd, 2000; birds: Drent and Daan, 1980; ungulates: Sinclair et al., 2000; primates: Ganzhorn et al., 2003; Knott, 1999, 2001; Lindburg, 1987). Interpopulation and interindividual variation in resource access can influence both female reproductive rates and infant survival, ultimately making a significant impact on reproductive success (pinnipeds: Pitcher et al., 1998; birds: Martin, 1987; non-human primates: Altmann and Alberts, 2003a,b; Bercovitch, 1987; Emery Thompson et al., 2007, Emery Thompson and Wrangham, 2008; Knott, 2001; humans: Ellison, 1993, 2003). Among primates, increased food availability influences dietary selectivity (Conklin-Brittain et al., 1998; Doran, 1997; Leighton and Leighton, 1983; Stanford and Nkurunungi, 2003; Wrangham et al., 1998), association patterns (Chapman et al., 1994, 1995; Sakura, 1994; Sugardjito et al., 1987), ranging behavior (Altmann and Muruthi, 1988; Doran, 1997; Olupot et al., 1997), and the frequency of energy-intensive activities such as hunting (Gilby et al., 2006; Gilby and Wrangham, 2007) or extractive foraging (Nishida, 1972).

Given this wide-ranging influence, accurate methods for assessment of energetic condition are crucial tools for field biologists. It would be particularly valuable to be able to quantify the energetic condition of individual animals on a regular basis. For many species it is difficult to obtain weights of individuals regularly, and this typically requires either immobilization or

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provisioning that inherently changes the energetic condition of the subjects. Thus, field studies often rely on complex calculations of energy intake, energy expenditure, and energy balance (intake minus expenditure) estimated through a combination of intensive methods of data collection (e.g., nutritional biochemistry, food intake rates and processing times, time budgets, and travel distances) (Coelho et al., 1977, 1979; Altmann and Samuels, 1992; Knott, 1998, 1999).

Such data are problematic for several reasons. First, the intensive effort involved in collecting accurate data on energy intake and expenditure frequently precludes the collection of other behavioral data or limits the number of individuals that can be adequately sampled. Second, even after data collection, quantification of these behavioral variables and determination of the caloric values of foods requires even more intensive post-field data and sample processing. Third, as detailed as these methods are, they are subject to error due to factors such as the costs of food processing and digestion, interindividual and temporal variation in intake rates, and variation in metabolic rates in individuals of varying body size or reproductive state.

Given these problems, Sherry and Ellison (2007) proposed the use of urinary C-peptide of insulin for assessment of energy balance in wild primates. C-peptide ("connecting" peptide) is a protein released during the conversion of proinsulin to insulin by pancreatic beta cells. It is therefore produced on an equimolar basis with insulin, a hormone critical for compartmentalizing and storing energy (Melani et al., 1970; Rubenstein et al., 1969; Steiner and Oyer, 1967; Steiner et al., 1967). Serum insulin measures have proved effective in discriminating calorically-restricted, supplemented, or obese subjects from controls in humans (Polonsky et al., 1988; Doucet et al., 2000) and other primates (*Papio* spp.: Kemnitz et al., 2002; *Macaca mulatta*: Gresl et al., 2003; Kemnitz et al., 1994; Lane et al., 2000). However, while insulin itself undergoes significant breakdown and clearance by the liver, C-peptide does not and therefore represents a more accurate measure of pre-hepatic insulin production (Polonsky et al., 1986a). The metabolic clearance rate of C-peptide is not concentration-dependent (Meistas et al., 1982; Polonsky et al., 1986b; Polonsky and Rubenstein, 1986), nor is it significantly affected by factors such as age, sex, obesity or diabetic status (Faber et al., 1978; van Cauter et al., 1992), indicating that interindividual comparisons of C-peptide validly represent individual variation in energetic condition.

A small but consistent fraction of C-peptide is excreted into the urine, leading to a strong correlation between C-peptide secretion by pancreatic beta cells and urinary C-peptide excretion, thus validating the use of urinary C-peptide for human clinical applications traditionally assayed with serum (Meistas et al., 1982; Gjessing et al., 1989; Kruszynska et al., 1987). While 24-hour urine collections have been used in the clinical assessment of C-peptide, it is feasible that opportunistic urine sampling, particularly when replicated, can also be an effective marker of energy balance. This supposition is supported by the use of urine samples for monitoring the energetics of lactational amenorrhea in humans (Ellison and Valeggia, 2003) and by the correlation of serum and urinary C-peptide levels from captive chimpanzees (Sherry and Ellison, 2007).

Recent pilot studies demonstrated that C-peptide could be recovered in urine samples collected from wild orangutans and chimpanzees, and recoveries were higher in samples collected during periods of highest versus lowest fruit availability (Sherry and Ellison, 2007). While this examination was promising, it was based on a very small number of urine samples (43 chimpanzee samples, 6 orangutan samples) and was used to distinguish only very broad contrasts in fruit availability. Thus, as suggested by Sherry and Ellison (2007), we present here an analysis of C-peptide measurement using a larger sample size, a longer study period, and more detailed comparative data on energetics.

Knott (1998, 1999) conducted a field study of the wild orangutans in which urine samples were collected in conjunction with detailed data on forest phenology and individual energy intake. This dataset thus provides a context for evaluating the efficacy of C-peptide measurement as a means to assess energetic condition. Our study included three components. First, because orangutan samples were stored on filter paper (due to lack of facilities for freezing), we assessed the reliability of filter paper as a short- and long-term means to preserve urine samples for C-peptide measurement. Second, we compared C-peptide concentrations with an independent, but less sensitive, physiological marker of energetic condition, the presence of ketone bodies in urine. We compared C-peptide concentrations from orangutan urine samples testing positive for ketone bodies, indicative of highly negative energy balance, with those testing ketone-negative. Third, we compared monthly mean C-peptide concentrations for orangutans with detailed estimates of food availability and energy intake calculated for the same individuals over the same period.

Methods

Study site

Data were collected from orangutans (*Pongo pygmaeus wurmbii*) in Gunung Palung National Park in Borneo, West Kalimantan, Indonesia, from October 1994 to September 2001 (Knott, 1998, 1999). Gunung Palung National Park consists of 90,000 hectares of mostly primary rainforest. Our core research area at the Cabang Panti research site comprised approximately 2100 hectares, including lowland and montane rainforests in addition to peat swamp, freshwater swamp, and alluvial bench forests at elevations of 1–1000 m (Curran and Leighton, 2000).

The study population consisted of a core group of 27 individuals, including 12 adult females, 8 flanged adult males, and 6 adult or subadult unflanged males. Urine samples from 22 individuals are represented in this study (Table 1). In our analysis of C-peptide levels in relation to energy intake and energy balance, we focus on 11 mature individuals encountered and sampled most consistently between October 1994 and December 1996, when detailed observations on energy intake were also conducted. This sample consists of 5 flanged males (2 prime males and 3 past-prime) and 6 adult (parous) females.

Collection and preservation of wild orangutan urine

Urine samples were collected by CDK or field staff opportunistically, particularly during first-morning voids and focal follows (Knott, 1997). When possible, plastic sheeting was used to catch samples, though some samples were collected by pipetting directly from vegetation. Samples contaminated by feces were discarded. Remaining samples were transferred to polystyrene tubes and labeled with the subject's identity and the date and time of urination.

At the end of the day, 200 μl aliquots of each sample were transferred to multiple replicate 2.5×2.5 cm squares of absorbent filter paper (Schleicher and

Table 1
Orangutan subject and sampling information

Subject	Sex	Age category	Urine samples	Observation days (hours)[a] Oct 94–Dec 96
JM	Male	Flanged, prime	59	3 (32.1)
RM	Male	Flanged, prime	240	108 (973.9)
BB	Male	Flanged, prime	16	n/a[b]
BF	Male	Flanged, past-prime	19	29 (334.4)
FK	Male	Flanged, past-prime	15	33 (355.8)
TB	Male	Flanged, past-prime	10	17 (163.8)
BT	Female	Parous adult	80	90 (997.6)
EZ	Female	Parous adult	75	60 (607.5)
KT	Female	Parous adult	58	9 (73.3)
KR	Female	Parous adult	67	20 (193.7)
MR	Female	Parous adult	520	152 (1659.7)
SR	Female	Parous adult	12	n/a[b]
EM	Female	Immature	19	n/a[b]
KL	Female	Immature	48	n/a[b]
MS	Female	Immature	143	n/a[b]

[a] Indicates number of follows of ≥3 h, from which energy intake estimates were calculated, and total number of observation hours during those follows.
[b] Some individuals were only sampled after 1996; their data and 8 additional samples from miscellaneous individuals are used in the analyses of ketones but not in analyses of energy intake.

Schuell 903 grade) according to methods described previously (Knott, 1997, 2005a). Samples were stored in a plastic container containing silica gel for an initial drying period of at least 12 h and were subsequently transferred to plastic slide sheets, also stored with color-indicating silica gel dessicant (Shideler et al., 1995). Samples were sequestered from light and heat (Campbell, 1994), and silica gel was replaced as needed to avoid contamination of samples by moisture and mold.

After transport to the Primate Reproductive Ecology Laboratory at Harvard University, filter paper samples were placed in the freezer at −20 °C until analysis and for long-term storage. We analyzed a total of 1389 urine samples for this study.

Urinalysis and C-peptide measurement

Urine samples were tested for the presence of ketone bodies (Chemstrips, Roche Diagnostics) either at the time of collection or at the end of the study day (Knott, 1998). Ketones build up in the blood as a result of the metabolism of fat, and when levels exceed the body's removal mechanisms, are excreted in the urine (Robinson, 1980). Clinical detection of urinary ketone bodies, as with Chemstrips, is indicative of carbohydrate shortage and/or impaired ability to metabolize carbohydrates. Wild orangutans do appear to dip into sufficiently low energy balance that ketone bodies are detectable in the field (Knott, 1998; but see Wich et al., 2006). We compared C-peptide measurements in those samples which tested negative for ketones to those with trace or positive detection of ketones, with the expectation that ketone-positive samples should have lower C-peptide concentrations than ketone-negative samples.

C-peptide of insulin was assayed with a commercially-available competitive radioimmunoassay kit designed for use with urine (Diagnostic Systems Laboratories, Webster, TX). One day prior to the assay, samples were prepared by punching with a standard hole punch 6 circles of each filter paper sample into 0.3 ml C-peptide zero standard, creating an approximate 1:2.5 dilution. After soaking overnight at 4 °C, 50 μl duplicates of each eluted sample were assayed as per kit instructions (overnight assay procedure). Samples with C-peptide levels lower than detectable limits were assigned values just below the minimum sensitivity of the assay. We applied creatinine corrections to adjust for urinary concentration (Taussky, 1954). Samples with creatinine levels <0.10 mg/ml were discarded due to the potential to overinflate hormonal estimates. Intra-assay coefficients of variations (CVs) were 11.8 and 6.9% for low and high controls, respectively, and inter-assay CVs were 14.8 and 12.2% for low and high controls (N=28 assays). The sensitivity of the assay was 0.01 ng/ml.

Validation of filter paper storage

Primate field studies in remote locations may have limited or unreliable access to refrigeration. When freezing of urine samples is not feasible, storage of urine specimens on filter paper provides a reliable means of preserving some analytes, such as steroid hormones (Knott, 1997, 2005a). In their initial validation of 12 samples, Sherry and Ellison (2007) determined that filter paper storage did not significantly impact C-peptide recoveries. Here, we repeat this validation to test for an initial degradation of C-peptide during the application and elution from filter paper. In addition, we tested the recovery of C-peptide stored under various long-term conditions mimicking potential field storage techniques.

Using procedures identical to those used in the field with orangutan samples, we created 22 filter papers from fresh frozen human urine samples. Note that in both field and laboratory contexts, filter paper was handled with gloves and sterilized forceps before and after urine application. Using the above protocol, we then compared the C-peptide concentrations of urine extracted from filter paper with C-peptide in the original thawed urine sample.

For examination of long-term storage effects on C-peptide recovery, we prepared multiple filter papers from human urine samples. Replicates of each of three urine samples were stored (1) at −20 °C; (2) at 4–8 °C; and (3) at room temperature. We then assayed each of these 9 replicates after 2 weeks, after 1 month, and after 1 year.

Fruit availability and energy intake

Detailed methods for our assessment of fruit availability and energy intake have previously been published (Conklin-Brittain et al., 2006; Knott 1998). Briefly, fruit availability was assessed by monthly surveys of 558 reproductively-mature orangutan food trees and lianas on 12 census routes. These surveys represented all major habitat types and 93% of all fruit genera eaten by orangutans at Gunung Palung. Interobserver bias was reduced through three means: (a) observers were rotated each month between different transects, (b) transects were periodically checked by a second observer, and (c) samples of reproductive parts from each censused tree were returned to camp for independent confirmation of observer assessment. Crop sizes of mature or ripening fruit were assessed, and fruit availability was converted to kilocalories (kcal) per hectare (ha) based on the average weight and nutritional biochemistry of individual fruit species (Knott, 2005b).

Energy intake was estimated by combining systematic observations of intake rates during focal follows with the results of nutritional biochemistry analyses on foods consumed (Conklin-Brittain et al., 2006; Knott, 1998). Five or more representative samples (i.e., fruits similar in size and appearance to those eaten by the orangutans) were collected of each fruit type or species and dried and weighed in the field to obtain mean weights. Approximately 100 g of fresh weight of each food were collected and processed in bulk for nutrient analysis. Crude protein (Pierce and Haenisch, 1947), lipid (AOAC, 1984), and total nonstructural carbohydrate (TNC, digestible carbohydrates) composition were determined and total caloric content calculated by assuming values of 9 kcal/g lipid, 4 kcal/g protein, and 4 kcal/g carbohydrate (Conklin-Brittain et al., 2006; National Research Council, 1980). In addition, we calculated the potentially available energy in neutral detergent fiber (NDF, Robertson and van Soest, 1980). Assuming a fiber digestion coefficient approximately similar to chimpanzees (54.3%, Milton and Demment, 1988), a fiber fuel value of 1.6 kcal/g was used following Knott (1998) and Conklin-Brittain et al. (2006). Energy intake during each feeding bout was assessed by multiplying the kilocalories of metabolizable energy per gram by the grams ingested per bout. For each study month, we calculated the rate of kilocalorie consumption per hour for adult flanged males and adult females separately based on dietary observations during focal follows of 3 h or longer.

Results

Reliability of filter paper storage

Estimates of C-peptide concentration (ng/ml) were significantly lower in urine samples preserved on filter paper than in

those stored by freezing (76.3%±9.5 SE, Wilcoxon matched-pairs test $z=-3.036$, $n_1=n_2=22$, $p_2=0.002$). However, there was a strong correlation between the C-peptide values from filter paper and frozen samples ($r_s=0.902$, $n=22$, $p_1<0.0001$, Fig. 1a), indicating that the rate of degradation was uniform across specimens. Creatinine values were also significantly correlated between the two storage methods ($r_s=0.664$, $n=22$, $p_1<0.001$). Thus overall corrected C-peptide concentrations (ng/mg creatinine) from filter paper were significantly correlated with those derived from frozen urine ($r_s=0.844$, $n=22$, $p_1<0.0001$, Fig. 1b).

Despite the initial degradation of C-peptide detected from the transfer of urine to filter paper, we did not find significant variation in C-peptide recoveries from filter papers stored under various storage conditions or for various lengths of time prior to extraction and analysis (Fig. 2, Two-factor ANOVA with replication. Effect of duration: $F=1.061$, $df=2$, $p=0.37$; effect of temperature: $F=0.084$, $df=2$, $p=0.92$; interaction: $F=1.011$, $df=4$, $p=0.43$).

C-peptide concentrations given in the remainder of the results were derived from filter-paper stored samples. Our experiments on filter paper storage methods indicate that the absolute values presented in the orangutan data are underestimates of true urinary C-peptide concentrations yet should validly represent relative C-peptide levels among samples stored in an equivalent manner.

Comparison with urinalysis of ketones

As expected, samples which tested positive for ketone bodies had significantly lower C-peptide concentrations (mean±standard error: 1385.7±177.6, $N=103$) than samples which tested ketone-

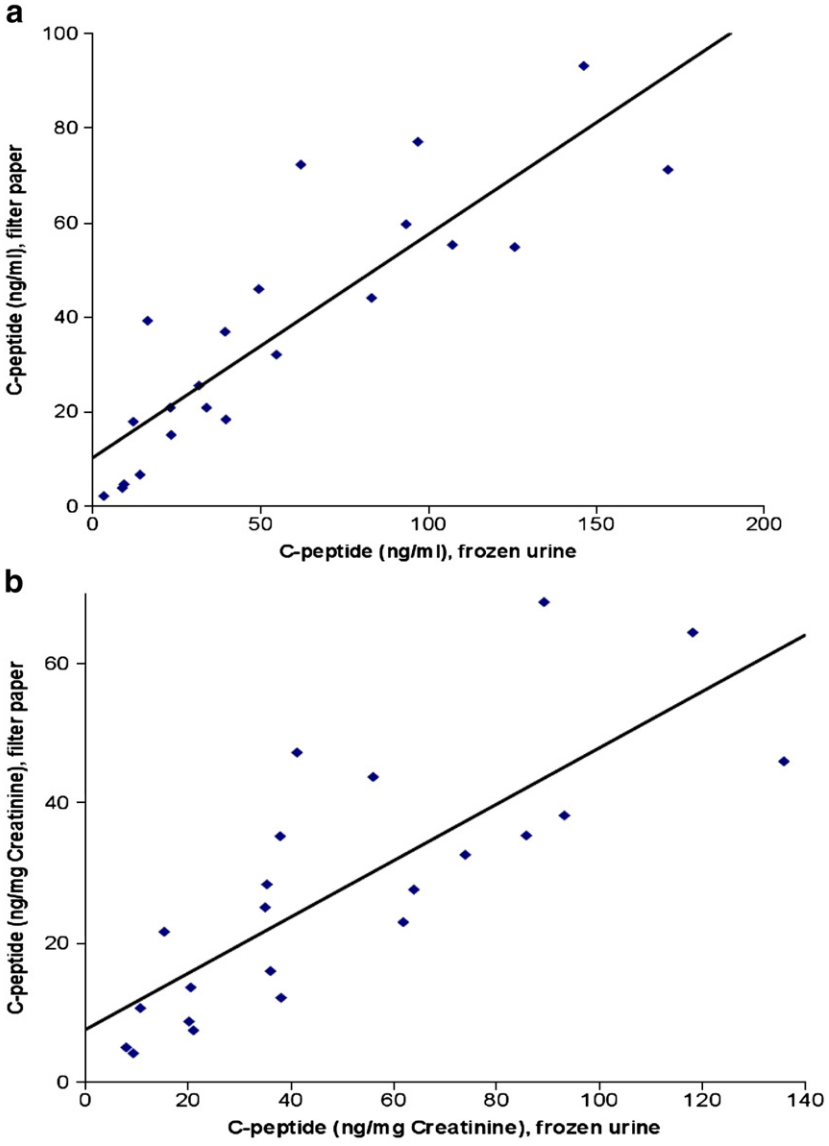

Fig. 1. Comparison of urinary C-peptide concentrations in matched frozen and filter paper-preserved samples: (a) ng/ml, $r_s=0.902$, $n=22$, $p<0.0001$; and (b) ng/mg creatinine, $r_s=0.844$, $n=22$, $p<0.0001$.

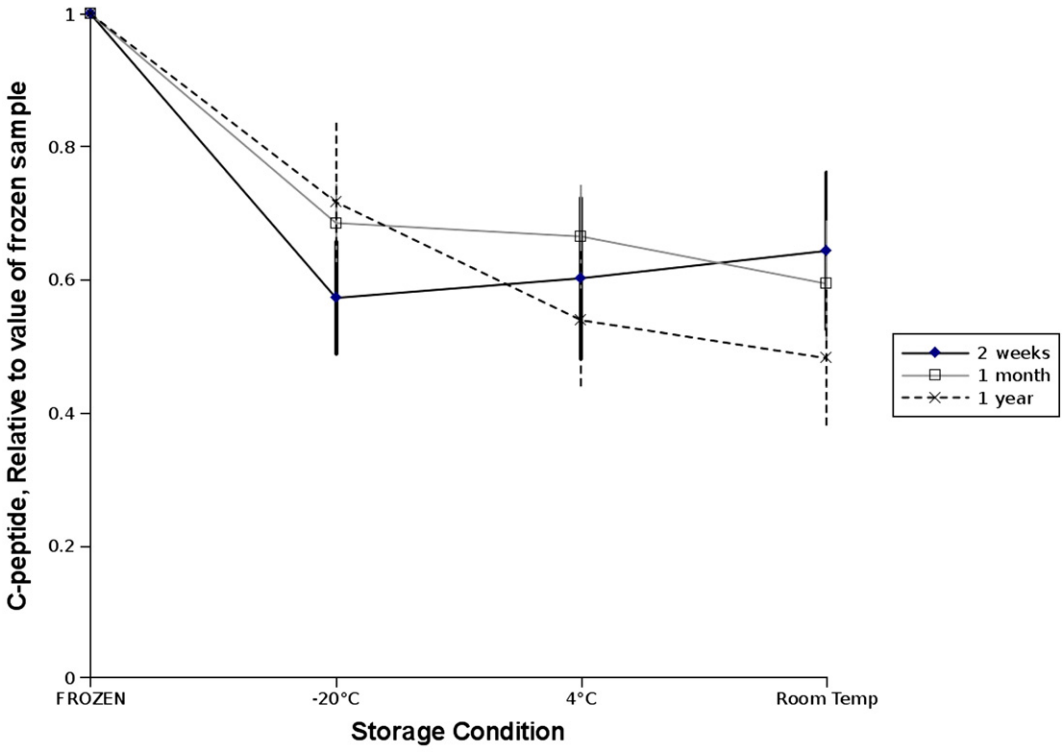

Fig. 2. C-peptide concentrations derived from 3 urine samples transferred to filter paper and stored at different temperatures (−20 °C, 4 °C, room temperature) and for different storage durations (2 weeks, 1 month, 1 year). Values on the *y*-axis are given relative to the C-peptide concentration derived from assay of the same urine sample stored by freezing.

negative (2159.5±125.2, $N=1230$; Mann–Whitney U test, $z=-3.552$, $p_1=0.0002$, Fig. 3).

Comparison with fruit availability and energy intake

C-peptide concentrations of both male and female orangutans peaked during months of peak fruit availability and declined during low fruit availability (Fig. 4a). Linear regressions of monthly ripe fruit availability, assessed as kcal/ha, demonstrated a significant positive relationship with C-peptide concentrations of both flanged male ($R^2=0.488$, $N=17$, $p_2=0.0009$) and adult female orangutans ($R^2=0.269$, $N=18$, $p_2=0.023$).

In addition, estimates of caloric consumption during a given month significantly predicted C-peptide concentrations from urine samples collected in that month. This relationship was significant for both adult females ($R^2=0.790$, $df=18$, $p_2<$

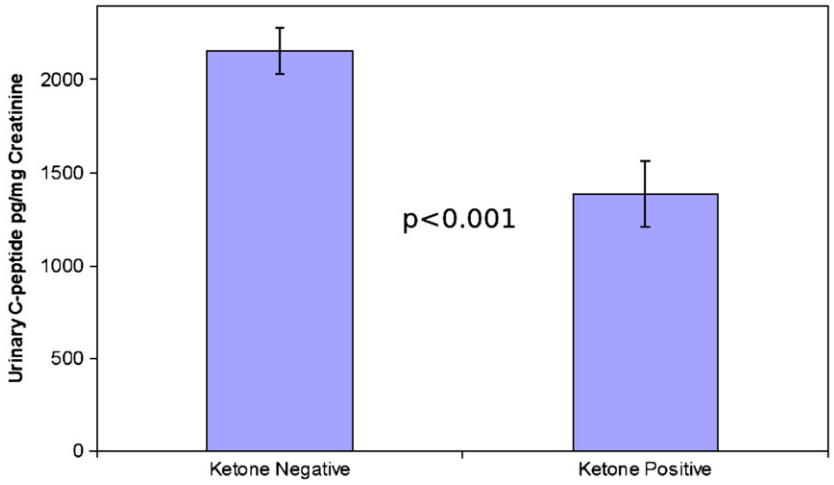

Fig. 3. C-peptide concentrations (mean±standard error) obtained from samples testing trace/positive versus negative for ketone bodies using Chemstrip urinalysis strips.

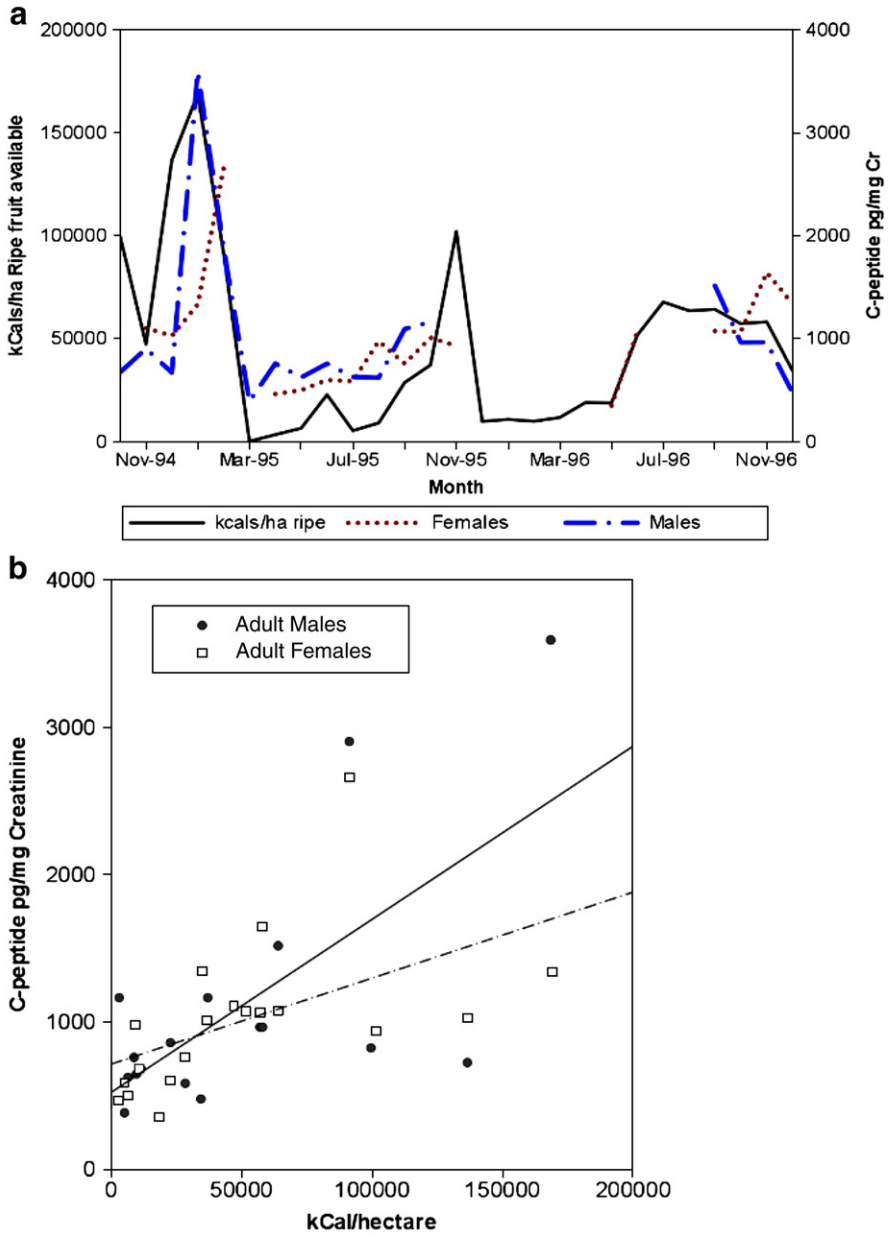

Fig. 4. Correlation of ripe fruit availability (kcal/ha) with C-peptide levels of adult males and females: (a) monthly changes in fruit availability (black line), mean female C-peptide levels (dotted line), and mean male C-peptide levels (broken line); and (b) scatterplot of fruit availability versus mean male (open squares) and female (closed circles) C-peptide levels.

0.00001, Fig. 5a) and for adult males (R^2=0.525, df=18, p_2= 0.0007, Fig. 5b).

Discussion

Collection of urine samples has allowed for non-invasive assessment of general health status (e.g., Kelly et al., 2004; Knott, 1997; Krief et al., 2005), stress physiology (e.g., Muller and Wrangham, 2004; van Schaik et al., 1991), and reproductive function (e.g., Andelman et al., 1985; Deschner et al., 2004; Emery Thompson, 2005a; Emery Thompson et al., 2006, 2007; Emery Thompson and Wrangham, 2008; Knott, 1999; Marshall and Hohmann, 2005) in wild primates. In this paper, we present a feasibility study of urinary methods for assessment of energetic condition, a potentially valuable tool for behavioral ecologists in the field. While C-peptide levels most specifically reflect the dynamic process of insulin response to caloric intake, mean urinary C-peptide levels were predicted to be higher when individuals were experiencing periods of relatively high energy intake. In support of this, our results demonstrate significant relationships between C-peptide concentrations and behavioral measures of energy intake, phenological measures of food availability, and the presence or absence of urinary ketone bodies.

Our experiments with filter paper storage methods indicated strongly correlated but moderately lower recoveries of C-peptide

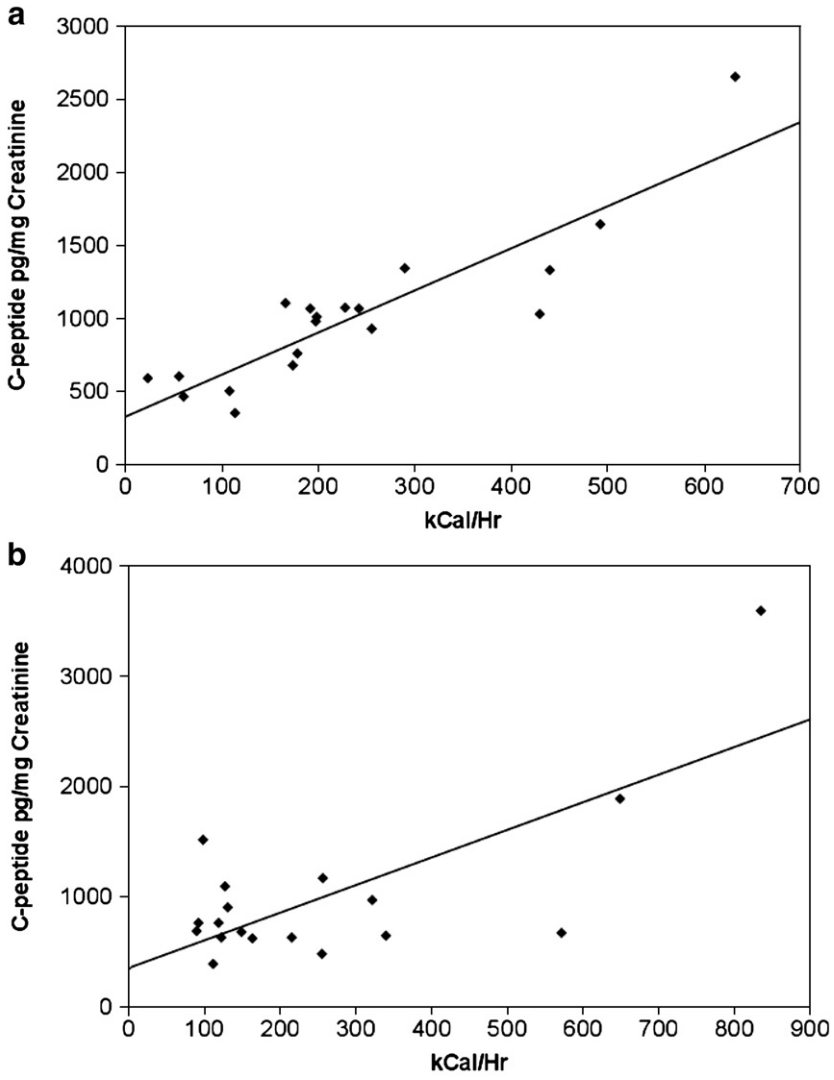

Fig. 5. Scatterplots of monthly energy intake estimates (kcal/hr) versus C-peptide concentrations for (a) adult female and (b) adult male orangutans.

in filter paper versus frozen urine samples. It is unclear whether this may have resulted from degradation of the hormone during the drying process, interference by other media such as the silica gel used to facilitate drying, or incomplete extraction of C-peptide from the filter papers during analysis. We suspect that the loss of C-peptide occurred during the initial drying process rather than during extraction because creatinine recoveries were not significantly different from frozen samples (see also Knott, 2005a,b). While it was not possible to replicate the long storage times of our orangutan filter paper samples, comparisons of recoveries after 2 weeks, 1 month, and 1 year of storage suggest that C-peptide molecules are relatively stable once fully dried onto filter paper.

In our analysis C-peptide correlated well with measures of food availability and caloric intake of both male and female orangutans. However, energy expenditure is also an important variable contributing to the organism's energy balance. In addition, variables such as developmental status, reproductive state, and aging may also impact the physiology of energy storage. This may explain some of the scatter we see in the data.

Based on our examinations, urinary C-peptide appears to be superior to other means available for the non-invasive assessment of energetic condition in primates. Cortisol, a key metabolic hormone, can be readily measured in urine. However, the use of urinary cortisol as a measure of energetic stress in individual animals is complicated by cortisol's additional responsiveness to psychosocial stress, particularly in highly social species (Sapolsky, 1992). Urinary ketone assessment, via urinalysis strips, can diagnose severe carbohydrate shortage (Knott, 1998), but this method is not sensitive enough to detect the more moderate variation in energy balance experienced by many primates (Emery Thompson, 2005b; Kaur and Huffman, 2004; Kelly and Huffman, 2004; Krief et al., 2005; Muller and Wrangham, personal communication).

A key strength of C-peptide is that it offers a potentially integrated measure of energy balance, incorporating variation in metabolic costs of individuals (e.g., due to lactation and pregnancy) or different food items (e.g., fiber content and digestibility). Alternatively, the more labor-intensive methods for calculating energy intake and energy expenditure are still

necessary for studies wishing to understand variation in energy intake independent of these factors.

While C-peptide has a slower clearance time than insulin (Katz et al., 1975; Kjems et al., 2000; Kuzuya and Matsuda, 1976; Oyama et al., 1975), it is still likely to be subject to diurnal fluctuations as the animal changes its feeding behavior. Unlike hormones such as cortisol and testosterone, this variation may not be strictly based on a predictable circadian pattern. Thus, as with behavioral data, it is necessary to obtain multiple urine samples to adequately characterize the C-peptide levels of an individual for the time period being studied.

As with other hormones (e.g., Whitten et al., 1998), properties of urinary clearance of C-peptide, its adequacy for measuring energy intake, and the sensitivity and applicability of commercial C-peptide assays (designed for application to humans) will vary by study species. In particular, amino acid sequences of C-peptide are relatively more variable among mammals than insulin itself (Henry et al., 1993; Oyer et al., 1971). Thus, until wider validations are available, the use of urinary C-peptide in other species should first be validated against independent measures of energetic condition.

Potential applications of C-peptide are wide ranging. In humans, urinary C-peptide has been used to understand the relationship between changing metabolic load and the duration of lactational amenorrhea (Ellison and Valeggia, 2003). C-peptide is a particularly valuable tool for applications in reproductive ecology because the gonadotropic actions of insulin suggest a direct means by which energetic condition may influence ovarian function (Poretsky and Kalin, 1987). We therefore envision further wide-ranging applications for understanding the influence of energy balance on reproductive function and how this may differ in various primate species. A more sensitive quantification of energy-rich versus energy-poor seasons will assist in understanding behavioral responses to energetic stress. Quantification of the energy balance of individuals may also help us to better understand the consequences of behavioral variation, such as the effects of social status or different levels of resource competition. Finally, a physiological indicator of energy balance improves the validity of comparing energetic condition across populations when differing habitat and food types preclude the direct comparison of energy availability.

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