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A Noninvasive Method for Estimating Nitrogen Balance in Free-Ranging Primates

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Abstract The vital role of body protein as an energy reserve has received little focus in studies of wild primates. Owing to the relatively low protein content of fruit, some frugivorous primates could face a protein deficit if body protein is catabolized for energy during periods of low fruit availability. Such an imbalance can be detected if fatty acids, amino acids, and nitrogen (N) catabolites are reincorporated or recycled back to tissues. Here we describe a method to quantify protein recycling by measuring standardized urea concen-

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tration and N isotope signatures from urine samples collected from wild Bornean orangutans (*Pongo pygmaeus wurmbii*). Our overall goal was to explore if concentrations of urea and $\delta^{15}N$ values could be used as indicators of the amount of protein consumed and the degree of protein recycling, respectively, in wild, free-ranging primates. We examine the relationships between urea concentration, $\delta^{15}N$ values, protein intake, and fruit availability. Urea concentration increased with fruit availability, reflecting a slight increase in protein consumption when fruit was abundant. However, we found no relationship between $\delta^{15}N$ values and fruit availability, suggesting that orangutans avert a negative protein balance during periods of low fruit availability. These noninvasive methods complement recent advances in primate energy balance research and will contribute to our understanding of adaptations of primates during periods of fruit shortage.

Keywords Creatinine · $\delta^{15}N$ · Orangutans · *Pongo pygmaeus wurmbii* · Protein · Urea

Introduction

All animals depend on protein for growth, cell maintenance, homeostasis, immune function, and reproduction (Lee *et al.* 2008; Leonard 2000; Sare *et al.* 2005). Accordingly, the causes and effects of protein deficiency have been investigated intensively among human populations (Anonymous 1977; Edozien *et al.* 1976; Heiman *et al.* 1998; Jackson *et al.* 1993; Lechtig *et al.* 1981; Meling and Nylen 1996; Millward 1999; Wagenmakers 1998). Such studies have influenced the view that protein-impoverished habitats suppress the reproductive fitness of wild primates (Glander 1981; McKey *et al.* 1981; Milton 1979, 1981; White 1998). Yet testing the concept of protein limitation has been based largely on inductive reasoning. The tendency of primates to target proteinaceous foods (Conklin-Brittain *et al.* 1998; Felton *et al.* 2009b; Oftedal 1991; Rothman *et al.* 2008)—the accessibility of which correlates positively with primate biomass at large spatial scales (Chapman *et al.* 2004; Ganzhorn 1992; Ganzhorn *et al.* 2009; Oates *et al.* 1990)—suggests that primate reproductive success may be influenced by protein availability. As a result, it is important to consider the ways in which primates utilize protein by adapting methods from ecological studies (Barboza and Parker 2006; Goeyens *et al.* 1998; Mulvenna and Savidge 1992; Parker *et al.* 2005).

Tsuji *et al.* (2008) reported seasonal variation in protein balance among Japanese macaques. However, this study compared estimated protein intake relative to estimated requirements, and did not take protein assimilation into account. Other studies of wild primates have found that total ingested protein remains relatively stable compared to total ingested energy across periods of high and low fruit availability (Conklin-Brittain *et al.* 1998; Felton *et al.* 2009a; N'Guessan *et al.* 2009). Such findings suggest that cases of protein deficiency may be rare among primates (Oftedal 1991); however, the investigation of whether cases of actual imbalances occur among individuals has been challenging.

Background on Protein Balance Theory

The logic behind protein balance can be explained with a model of N balance in vertebrates (Martínez del Rio and Wolf 2005). Proteins are composed of amino acids, which are an important dietary source of N. Protein from wild animal plant foods can contain anywhere from 16% to 20% N (Ortmann *et al.* 2006). When dietary N intake exceeds N lost through urinary and fecal loss, an individual is in positive N balance, and endogenous levels of N remain stable (Fig. 1a). In a state of positive N balance, an individual excretes more concentrated urea because excess N is excreted in urine (Lechtig *et al.* 1976; Simmons 1972).

As N intake declines, an individual can maintain a neutral N balance state, so long as N intake does not fall below the amount necessary for cell maintenance (Fig. 1b). When energy intake declines below energetic needs, an individual will begin to rely heavily on lipid stores to meet energetic requirements and thus shift into negative energy balance (Fig. 1b, c). An individual can fall into a negative energy balance while still maintaining a neutral N balance, as long as adequate protein is consumed. If, however, N intake declines below endogenous rates of N loss in urine and feces but the individual obtains adequate energy from lipid stores, it can maintain a steady-state negative N balance, wherein N intake is less than N loss in urine and feces but body N is not utilized for energy (Fig. 1c). The duration of this steady-state negative

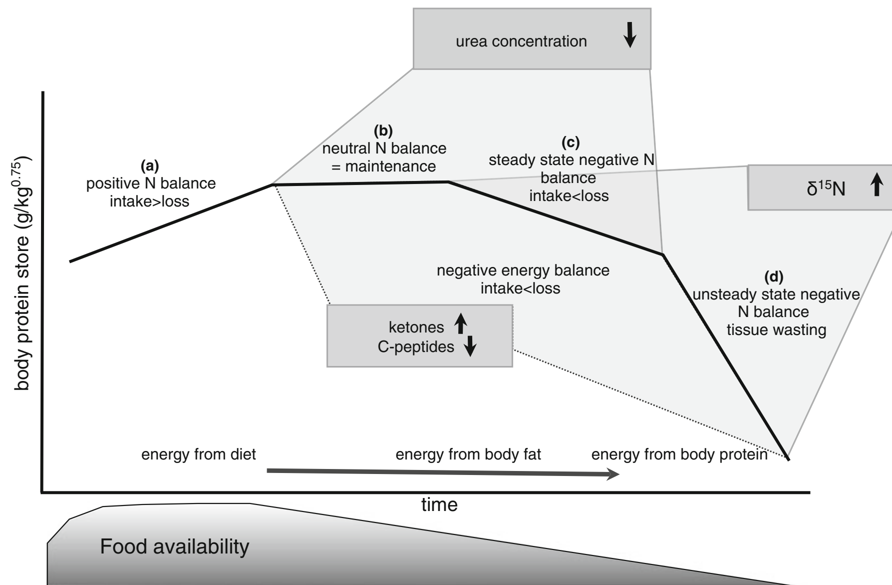

Fig. 1 Theoretical protein balance model (modified from Barboza and Parker 2006). An individual is in a positive N balance when N intake exceeds N loss **(a)**. As food availability declines, N intake equals loss and an individual transitions into a neutral N balance **(b)**. When N intake is less than N loss, but other body reserves such as fat are used for energy, an individual transitions into a steady state negative N balance **(c)**. Once those alternative reserves are expended, an individual transitions into an unsteady negative N balance or tissue wasting state **(d)**. The dotted lines surrounding the shaded area represent hypothetically where indicators of negative energy balance may be detected along the protein balance continuum.

N balance will depend on the body fat stores and activity levels of the individual (Barboza and Parker 2006).

If dietary protein intake continues to be lower than N loss in the body, urine, and feces for a prolonged period, body fat stores used for energy become depleted. An individual then enters an unsteady state of negative N balance (Fig. 1d), during which body protein stores are used to meet energy requirements. This results in the depletion of lean mass, or tissue wasting (Barboza and Parker 2006; Koch 2007). This unsteady state of negative N balance, which we will refer to as tissue wasting, cannot be maintained for long and may result in death.

Urinary urea concentrations can be used to track this transition from a positive to negative protein balance. For example, an animal consuming a high-protein diet will experience a proportionally large loss of N as urea, while one consuming a low-protein diet will have lower urea concentrations in their urine and feces because they utilize most of their dietary N to build body protein. Indeed, dietary protein intake and urea concentrations correlate positively in humans and the former is commonly used as a diagnostic tool for studying protein deficiency in humans (Lechtig *et al.* 1976, 1981; Simmons 1972). A study on Papua New Guinea highlanders demonstrated that urea N is utilized in the synthesis of body protein in human subjects with low protein intake (Miyoshi *et al.* 1986). Thus, it is likely that nonhuman primates utilize similar metabolic pathways to avoid tissue wasting.

Two methods, urea concentration, standardized by creatinine concentration, and stable isotope biogeochemistry, have been combined to detect the transition from positive protein balance to tissue wasting in a variety of vertebrates including birds (Kempster *et al.* 2007), rats (Sick *et al.* 1997), ungulates (Barboza and Parker 2006; Parker *et al.* 2005; Sponheimer *et al.* 2003), and reptiles (McCue and Pollock 2008). If dietary N intake is held constant, then any change in the ratio of urea/creatinine should reflect increased oxidation of body protein. If dietary protein increases, the ratio of urea/creatinine should also increase, provided protein intake exceeds protein expenditure (Barboza and Parker 2006; Parker *et al.* 2005; Simmons 1972). However, the ratio of urea/creatinine cannot distinguish whether excreted protein originates from the breakdown of body protein or excess dietary protein.

Fortunately, stable N isotope values in urine can be used to distinguish between the excretion of dietary and body-derived N. N isotope values are reported using a standardized "δ" notation wherein $\delta^{15}N=(R_{sample}/R_{standard}) - 1) * 1000$, $R={}^{15}N/{}^{14}N$ and the standard is "Air." Stable N isotope values in animal tissues reflect their diet with some enrichment. N isotope values for animal tissues are several permil higher than for diet (DeNiro and Epstein 1981; Koch 2007; Steele and Daniel 1978). Urinary N waste comes from deaminated amino N liberated during protein catabolosis. The $\delta^{15}N$ values in urine should, therefore, indicate if an animal is predominantly using N derived from dietary protein or tissue protein (Martínez del Rio and Wolf 2005).

When an animal transitions from positive or neutral N balance to negative N balance, it will start using tissue stores to meet metabolic requirements. In a neutral N balance (Fig. 1b), $\delta^{15}N$ values in urine will continue to closely track $\delta^{15}N$ values in the diet (Steele and Daniel 1978). If the animal enters steady-state negative N balance, there may be no change in urinary isotope values because the animal can use lipid stores to meet metabolic requirements and avoid tissue wasting (Fig. 1c). If,

however, the animal enters an unsteady negative N balance state and tissue wasting commences (Fig. 1d), body-derived N will be excreted and urinary δ^{15}N values should increase (Barboza and Parker 2006; Hobson *et al.* 1993). Urinary δ^{15}N values will then continue to increase until the animal starts consuming adequate amounts of protein, or dies.

Although the protein balance model has not been tested in wild primates, the theoretical relationship between dietary protein and body protein has been demonstrated in semicaptive and provisioned caribou (*Rangifer tarandus*) from harsh arctic environments (Barboza and Parker 2006, 2008; Parker *et al.* 2005). These studies used stable isotope values combined with concentrations of N metabolites in urine and blood to examine protein dynamics in reindeer. Elevated δ^{15}N values during the winter indicated that the focal individuals recycled tissue-derived amino-N. In addition, both urinary urea-N concentration and δ^{15}N values tracked dietary protein intake throughout the winter (Parker *et al.* 2005).

A recent study on a group of captive bonobos (*Pan paniscus*) used an experimental approach to examine N balance (Deschner *et al.* 2010). In agreement with the N-balance model, these authors found that during a 30% reduction of daily energy intake, urinary δ^{15}N values increased, while during periods of food abundance they decreased (Deschner *et al.* 2010). These results concur with the idea that the bonobos broke down body protein when faced with energy deficits. However, the authors did not observe a difference in protein consumption during the 2 food abundance periods because they replaced energy-rich food items with energy-poor food items that were higher in protein but had lower dry weights, e.g., lettuce (Deschner *pers. comm*. 2011). They concluded that the measurement of urinary stable isotope ratios of wild apes could be a promising method to identify variation in individual energy status during periods of fluctuating food availability.

To test this promising method in wild primates, we analyzed the ratio of urea/creatinine and δ^{15}N values in urine samples collected from wild orangutans (*Pongo pygmaeus wurmbii*) during periods of varying fruit availability. The forests of Southeast Asia, where orangutans occur, are characterized by unpredictable periods of fruit abundance, known as mastings, that are commonly followed by long periods of fruit scarcity (Cannon *et al.* 2007a, b; Curran and Leighton 2000; Knott 1998; Leighton and Wirawan 1986; van Schaik *et al.* 1993; Wich and van Schaik 2000). Orangutans prefer to feed on ripe, and in some cases unripe, fruit when it is available, but they often increase the percentage of inner-bark/cambium, leaves, flowers, and other vegetative plant parts in their diets when fruit is scarce (Bastian *et al.* 2010; Harrison *et al.* 2010; Knott 1998; Morrogh-Bernard *et al.* 2009; Vogel *et al.* 2008; 2009; Wich *et al.* 2006).

Orangutans: A Model System

The past decade has witnessed new techniques for collecting urine samples from free-ranging arboreal primates in tropical settings, and the effects of long-term storage in varying conditions have been tested with orangutan samples (Emery Thompson and Knott 2008; Knott 1997, 1998, 2005a, b). These studies verified that urine samples dried and stored on filter paper remain relatively stable over time. Using such collection and storage methods, these authors tested ketone levels in the

field using fresh urine (Knott 1998) and they examined C-peptide levels as a proxy for energy balance (Emery Thompson and Knott 2008) in wild orangutans. Results suggest that when fruit is scarce orangutans: 1) consume significantly fewer total kilocalories per day (Emery Thompson and Knott 2008; Harrison *et al.* 2010; Knott 1998); 2) are in a negative energy balance, i.e., energy intake < energy loss, as evidenced by low C-peptide levels (Emery Thompson and Knott 2008); and 3) metabolize excess body fat, as evidenced by the presence of ketones in their urine (Harrison *et al.* 2010; Knott 1998). Such findings suggest that orangutans could also be susceptible to a negative protein balance; however, nothing is known about protein cycling in these populations or how it might be related to periodic energy deficits.

Based on the aforementioned ecological and dietary characteristics, combined with the fact that orangutans regularly excrete large quantities of urine, orangutans serve as a good focal species to investigate the use of these noninvasive methods to examine protein balance in a wild, primate species. Although the percentage of protein in the diets of orangutans at Gunung Palung was relatively stable across months (mean 9.7%) and less variable than other macronutrients (range 5.3–16%), total grams of protein intake showed significant differences across the year and total intake was higher during fruit rich periods vs. fruit poor periods (Knott 1999). This variation in protein differs from spider monkeys that have been found to maintain a relatively constant amount of protein in their diets (Felton *et al.* 2009a).

Knott (1998) found that when fruit availability was low, total energy intake (kcal/d) was significantly lower for this population of orangutans, and that these orangutans were also energetically stressed (Emery Thompson and Knott 2008; Knott 1998). We predicted that if orangutans experience lower levels of dietary protein intake during periods of fruit scarcity, they should also have lower corrected urea concentration (μM urea/μM creatinine) during these intervals. We focused on fruit in this study, as previous studies have documented energy deficits when fruit was scarce in this population (Emery Thompson and Knott 2008; Knott 1998, 2005a). Despite energy deficits, it is possible that orangutans obtain sufficient protein from nonfruit items when fruit is scarce. These items, such as leaves and bark, are available year round and thus should not vary in availability. In this case, we may observe no relationship between corrected urinary urea concentration and fruit availability. We also predicted that if the orangutans are protein limited during periods of fruit scarcity and enter into a tissue wasting state, δ^{15}N values should increase.

Methods

Study Site and Subjects

We collected urine samples and behavioral data from 23 wild adult orangutans (*Pongo pygmaeus wurmbii*) in the area surrounding the Cabang Panti Research Station, Gunung Palung National Park in Borneo, West Kalimantan, Indonesia (1° 13′S, 110°7′E), from October 1994 to April 1999 (Knott 1998). Our sample included 12 females and 11 males (Table I; ESM Table SI). Detailed descriptions of the

Table I Description of urine samples analyzed in this study

Sex	Age class	Reproductive state	Number of urine samples
F	Adult	Lactating	11
F	Adult	Not pregnant	21
F	Adult	Pregnant	7
F	Independent subadult	Not pregnant	6
M	Adult flanged	N/A	29
M	Adult unflanged	N/A	7
M	Independent subadult	N/A	3

F females, *M* male. Males were either with facial disks present (flanged) or absent (unflanged)

research site are provided elsewhere (Cannon *et al.* 2007b; Cannon and Leighton 2004; Knott 1998; Knott *et al.* 2010; Marshall 2009).

Fruit Availability

We quantified monthly fruit availability as the percentage of fruiting trees that are known orangutan food species, commonly referred to as the fruit availability index (FAI) (Conklin-Brittain *et al.* 2006; Emery Thompson and Knott 2008; Knott 1998). Briefly stated, we determined fruit availability by monitoring 1368 orangutan fruit trees distributed along 16 phenological routes, spread across the habitat zones found at Cabang Panti Research Station. We divided FAI into high and low abundance categories. We determined periods of high fruit abundance by calculating *z*-scores, with months above 0% labeled as high fruit periods and below 0% as low fruit periods. This resulted in months with >15% of the trees fruiting labeled as high fruit periods.

Protein Intake

We estimated protein intake from feeding data collected using full-day, continuous focal animal sampling (Altmann 1974). Once we encountered the focal subjects, we followed them until they made a night nest and then we returned to the nest before dawn the next morning to continue following the same individual. We followed subjects continuously until they were lost or left the study area with an average follow of 3.16 d. If focal individuals were in a feeding tree but were out of view, we assumed they were eating unless we did not hear food remains dropping to the ground.

For each feeding bout, we calculated both the dry weight (grams) consumed and the percentage of each of the macronutrients (protein, lipid, carbohydrate, Neutral Detergent Fiber (NDF)) for each food item. Using this information, we then calculated the total protein consumed for the bout. Methods and procedures for nutritional analyses are documented in detail elsewhere (Conklin-Brittain *et al.* 2006; Knott 1998). Briefly stated, we used crude protein (CP), which was determined using the Kjeldahl procedure for total N and multiplying by 6.25 (Pierce and Haenisch 1947). Some researchers have argued that the 6.25 conversion factor typically used to estimate the protein content of vegetation may be inappropriate for

tropical plant parts consumed by primates and that a conversion factor of 4.3–4.4 may provide a more accurate estimate of total protein in such samples (Conklin-Brittain *et al.* 1999; Milton and Dintzis 1981). However, a new conversion factor for protein has not been adopted in primate studies. We calculated total grams consumed per feeding bout by multiplying dry weight for each food item by the mean percentage of protein in a given food item by the number of food items eaten per bout:

$$\text{Grams dry weight/food item} \times \%\,\text{protein/food item} \times \text{no. of food items eaten/bout}$$

If we did not have nutritional data for a given species, we used the mean value for the food item. We then summed all bouts from each day to calculate the total grams of protein consumed per day for each individual. We analyzed a total of 604 h of focal feeding data to calculate protein intake rates.

Urine Sample Collection and Preservation

In total, we analyzed 90 urine samples collected by C. D. Knott and her field staff from October 1994 to April 1999. We collected all urine samples opportunistically, in most cases from first-morning voids during focal follows. For female samples, C. D. Knott determined the reproductive status, e.g., pregnant, lactating, or not pregnant, through observation and the use of radioimmunossay of urine samples (Knott 1997). We dried and stored all urine samples on filter paper, using previously documented and validated methods (Emery Thompson and Knott 2008; Knott 1997, 1998, 2005b). Briefly stated, we aliquoted 200 μl of urine onto uniform squares of sterile, high-absorbency filter paper. At the time of the study, we used Schlicker and Schloom (no. 11610) filter papers, but this product is now unavailable. We currently use Whatman 903 protein saver cards (no. 10534612), which can store *ca.* 300 μl of urine per card. If the urine sample was large enough, we made replicates of each urine sample on additional pieces of filter paper. Once aliquoted, we placed the samples into an airtight container with desiccant (silica gel beads), until completely dry (generally 12–24 h). We then placed the dried samples into plastic slide sheets and stored them in a different airtight container with desiccant until we could transport them to the Primate Reproductive Ecology Laboratory at Harvard University.

We froze the samples at −20°C on arrival at the laboratory. Prior validation tests of the filter paper storage method for C-peptide and creatinine recovery determined that although samples stored on filter paper had a lower recovery than samples immediately frozen at −20°C, the rate of degradation was uniform across specimens (Emery Thompson and Knott 2008; Knott 2005b; Sherry and Ellison 2007). Similar results have been detected with urea concentration in frozen and nonfrozen samples (Saude and Sykes 2007). In addition, no differences in C-peptide or creatinine recovery from filter paper stored under varying temperatures or lengths of time have been found (Emery Thompson and Knott 2008; Knott 2005b). Thus, although the absolute values in the orangutan data presented here may be underestimates of the urinary urea and creatinine concentrations at the time of collection, the values should be comparable across samples stored in a similar manner (Emery Thompson and Knott 2008; Saude and Sykes 2007; Sherry and Ellison 2007).

Urea and Creatinine Concentrations

In the laboratory at UC Santa Cruz, we eluted the urine from filter paper using methanol. We placed each sample into a glass test tube using sterile forceps, and then filled each tube with 5 ml of methanol. We covered and incubated the samples in a refrigerator for 24 h. After 24 h, we removed the filter paper and transferred half of each eluted sample to a separate tube for stable isotope analysis. We did not split samples with duplicate filter papers. We dried down all samples under a N drying block. Once samples were dry, we added 2 ml of Tris assay buffer (0.1 M Tris, 0.9% NaCl, 0.1% NaN_3 (sodium azide), 0.1% gelatin) to each sample destined for urea and creatinine analysis.

Because our initial urine samples were small (200 μl), and in some cases very dilute, standard urea concentration test kits for humans did not work. Instead, we determined urea concentration by modifying a diacetylmonoxime reaction developed for determining urea concentrations in seawater (Goeyens *et al.* 1998; Mulvenna and Savidge 1992; Price and Harrison 1987). This method is extremely sensitive, with a calculated detection limit of 0.14 μM (Goeyens *et al.* 1998). Because this method was developed for detecting urea concentrations in seawater, we had to dilute our urine samples to be within the correct detection range. We determined the most appropriate dilution factor for urea to register well on the standard dose–response curve. We created 3 dilutions for each sample: full strength, 1:10, and 1:100 using the Tris assay buffer. For most samples, a dilution factor of 1:100 provided a result within the detectable limits of the standard curve.

We modified the preparation of reagents from the original procedure (Mulvenna and Savidge 1992). The protocol calls for two reagents, A and B. Reagent A consisted of 8.5 g of diacetylmonoxime added to 250 ml distilled water (DIW) and 10 ml of thiosemicarbazide solution (0.95 g of thiosemicarbazide added to 100 ml of DIW). We made reagent B by slowly adding 535 ml of DIW to 300 ml of concentrated sulfuric acid, resulting in an exothermic reaction. Once reagent B cooled, we added 0.5 ml of a ferric chloride solution (0.15 g of $FeCl_3$ in 10 ml of DIW). We modified the protocol for the urea analysis from the Goeyens method (Goeyens *et al.* 1998). We dispensed 5 ml of each sample, at full strength and the 2 dilutions (1:10 and 1:100), into sterile Coring 50 ml centrifuge tubes. We then added 1.25 ml of reagent A and 4.0 ml of reagent B to each tube, mixing thoroughly between each addition. We created reagent blanks by adding reagents A and B to Tris assay buffer. We made a stock 500 μM urea standard by adding 0.75 g of urea to 250 ml of DIW. We then removed aliquots to make a serial dilution standard curve with Tris assay buffer in concentrations of 0.5, 1, 10, 30, and 50 μM.

This method is a colorimetric determination. Thiosemicarbazide is photosensitive and light can interfere with color development; therefore, after adding reagents A and B, we covered the samples with aluminum foil and stored them at room temperature in the dark for 72 h. After 72 h, we read the absorbance for each sample in duplicate at 520 nm on a SpectraMax 340PC384 Absorbance Microplatereader. Precision for duplicate samples was 0.0–0.03 μM. The diacetylmonoxime method has been documented to react with very few organic and inorganic N compounds other than urea, and thus is insensitive, in terms of reacting, to the blanks or reagents used (Price and Harrison 1987). Nevertheless, we subtracted the average of duplicate blank readings from both urea and creatinine samples in our calculations.

We applied creatinine corrections to adjust for urinary concentration (Taussky 1954). Urea concentrations are commonly considered in relation to creatinine, facilitating comparisons that are not associated with water turnover and dilution. Creatinine was measured based on colorimetric determination by the Jaffe reaction (Taussky 1954). We used a BioAssay Systems QuantiChrom™ Creatinine Assay Kit (DICT-500) designed for use with urine or blood samples. The detection limit of the creatinine assay was 0.10 mg dl^{-1} (8 μM) creatinine. Of 90 samples, only 4 were below this limit and thus were excluded from data analysis owing to their potential to overinflate urea estimates. For the remainder of this study, we refer to the urea concentration (μM) divided by creatinine concentration (μM) as the corrected urea concentration (μM urea/μM creatinine).

Most studies examining protein balance in mammals have examined the ratio of urea N/creatinine (Barboza and Parker 2006, 2008; Parker *et al.* 2005). We did not isolate N from urea because our initial volume of urine samples, 200 μl, was low and isolating N from urea results in only *ca.* 68% recovery of N from the urea (Parker *et al.* 2005). Instead, we examined the ratio of whole urea/creatinine (μM/μM), as urea is the main nitrogenous component of the urine in mammals and other organisms (Withers 1998). Studies on humans have historically examined the whole urea/creatinine ratio as a valid method for estimating protein intake both in populations and in individuals (Bergstrom *et al.* 1993; Lechtig *et al.* 1976, 1981). Because we collected and preserved all samples using the same exact methods, any N loss due to ammonia volatilization should be consistent across samples (Saude and Sykes 2007).

Preparation of Samples for Isotope Analysis

We re-eluted dried urine samples by adding 1 ml of methanol to vials containing samples that were split for urea concentration and isotope analysis, and 2 ml of methanol to each vial containing a full duplicate urine sample. We then added eluted samples directly into 5×7 mm tin weighing boats (Costech no. 041077). To maximize sample retention, we first arranged the tin boats into a cup-and-saucer configuration by unfolding and flattening one tin boat (the saucer), and placing a second tin boat on top of this flattened boat (the cup). Using a microbalance, we recorded the cumulative mass of each cup and saucer pair, and placed each pair into a clean plastic weighing dish. We then added eluted samples in 100-μl aliquots via pipette into the preweighed tin "cups," and any urine–methanol solution that leaked from the cup was collected on the "saucer." After allowing each aliquot to dry thoroughly, we recorded the cumulative mass of each sample, cup, and saucer. We then determined the mass of each dried urine sample by subtracting the total mass from the initial mass of each cup and saucer. We continued to add and dry 100-μl aliquots of urine–methanol solution until dried urine samples weighed 550–1000 μg. Very dilute urine samples rarely yielded >150 μg dried urine. We ran these smaller samples separately and in some cases excluded them from statistical analyses.

Once dried urine samples were within the appropriate mass range, e.g., 50–200 μg or 550–1000 μg, we rolled each tin cup and saucer pair into a single compact ball, being careful to minimize any loss of dried urine. We then reweighed each sample to verify sample mass. Urine samples were combusted on a Finnigan

ThermoElectron Delta+XP continuous flow system (Bremen, Germany) connected to a Carlo Erba Elemental Analyzer (Milan, Italy) at the UC Santa Cruz Stable Isotope Laboratory. We measured both $\delta^{15}N$ and weight percent N (hereafter referred to as %N). We measured N isotopes relative to air (0‰). To account for minor instrument error, we normalized data analyzed on different days using tin boat blanks and standard $\delta^{15}N$ and %N values for both IAEA Acetanilide and Pugel, a UCSC standard. The analytical precision (±1 SD) for %N and $\delta^{15}N$ was 0.6% and 0.3‰, respectively, based on 7 IAEA Acetanilide replicates, and 0.8% and 0.1‰, respectively, based on 38 Pugel replicates. The average difference in %N and $\delta^{15}N$ values between replicate urine samples was 0.9% and 0.2‰, for duplicates (N=12), and 0.8% and 0.06‰ for triplicates (N=2).

Statistical Analyses

We fit linear mixed models (LMM) to the data using maximum likelihood estimation to predict 4 outcome measures. These outcome measures included natural log of corrected urea concentration (μM/μM creatinine), $\delta^{15}N$ (‰), weight %N, and total protein (g) consumed/d. We estimated fixed effect coefficients for predictors fruit abundance index (FAI), sex (female vs. male), and female reproductive status (pregnant or lactating vs. not pregnant and not lactating). We estimated random effect coefficients for subject ID. We selected variables for inclusion in the model using the AIC criterion. We considered interactions among fixed effect factors and among fixed and random effect factors, but they were not selected using the AIC criterion. In general, the best-fit model for most tests only included FAI and did not include sex or reproductive state (ESM Tables SII–SVI). We fit all models with the lme (linear mixed-effect) function from the nlme package in R (version 2.10.1; Pinheiro *et al.* 2011). All variance component estimates that were produced using this function were positive, even those close to 0. All probability levels are 2-tailed, and we set the significance for all tests at $\alpha < 0.05$.

Results

Variation in Urea Concentration and N Isotope Values

Corrected urea concentration (μM/μM creatinine) ranged from 0.42 to 147.19 μM (Table II). During the high fruit period, the corrected urea concentration was 2.6 times higher when compared to the lower fruit period. Considerably less variation was observed for %N and $\delta^{15}N$: %N ranged from 2.2 to 14.6 while $\delta^{15}N$ ranged from −1.6 to 3.0‰. We found little difference between high and low fruit periods for %N, whereas for $\delta^{15}N$ we observed slightly more positive values during periods of fruit scarcity (Table II).

Testing Our Predictions and the Protein Balance Model

In support of our prediction that if orangutans consumed lower levels of dietary protein during periods of fruit scarcity, they would have lower corrected urea

Table II Summary statistics for urea concentration, %N, and $\delta^{15}N$

	Urea concentration (μM/μM creatinine)	%N	$\delta^{15}N$ (‰)
Mean±SE (*n*=69)	20.60±3.29	6.36±0.30	−0.14±0.12
High fruit period (*n*=28)	32.67±6.83	6.37±0.52	−0.16±0.20
Low fruit period (*n*=41)	12.36±2.30	6.35±0.33	−0.12±0.14
Range (min–max)	0.42–147.19	2.2 – 14.6	−1.6 to 3.0
High fruit period	1.07–147.19	3.5–14.6	−1.6 to 3.0
Low fruit period	0.42–63.16	2.2–12.1	−1.8 to 1.5

concentrations, we found a significant positive relationship between fruit availability and corrected urea concentration (LMM: t=3.06, df=50, p=0.0035; ESM Table SII; Fig. 2). In addition, we found a positive relationship between protein intake (g/d) and fruit availability (LMM: t=2.01, df=22, p=0.05; ESM Table SIII). During high fruit periods, protein intake (g/d) was 1.4 times greater compared to the low fruit periods (high: 73.0±33.9 (g/d); low: 54.0±33.6 (g/d)). Perhaps surprisingly, our estimates of daily protein intake were unrelated to urea concentration (LMM: t=−0.28, df=20, p=0.78; ESM Table SIV). Given that orangutans had lower N intake and output during periods of fruit scarcity, we expected they may have entered a negative steady state of N balance. We found no evidence for tissue wasting when examining the relationship between FAI and either $\delta^{15}N$ (‰) or weight %N (LMM: t=−0.86, df=44, p=0.39; LMM: t=−0.05, df=44, p=0.96; ESM Tables SV and VI; Figs. 3 and 4).

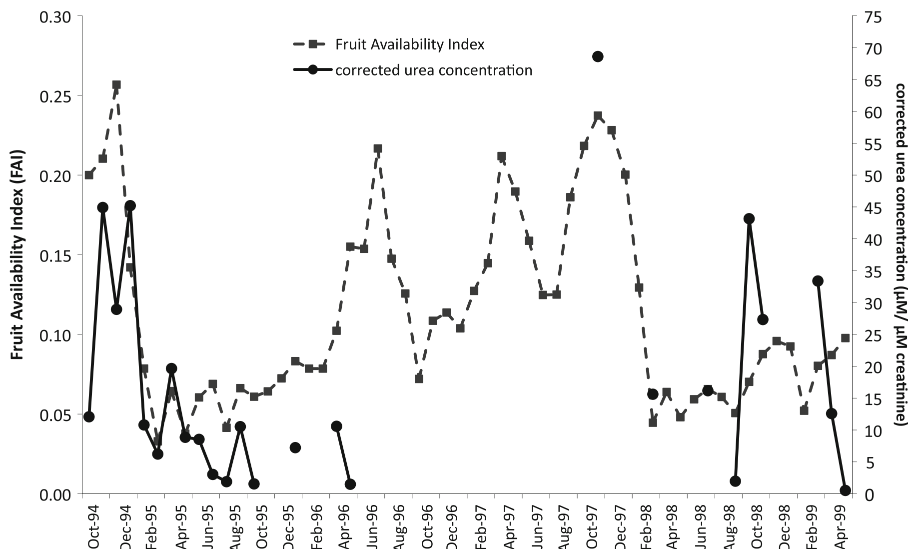

Fig. 2 Relationship between corrected urea concentration (μM/μM creatinine) and the fruit availability index (LMM: t=3.06, df=50, p=0.0035).

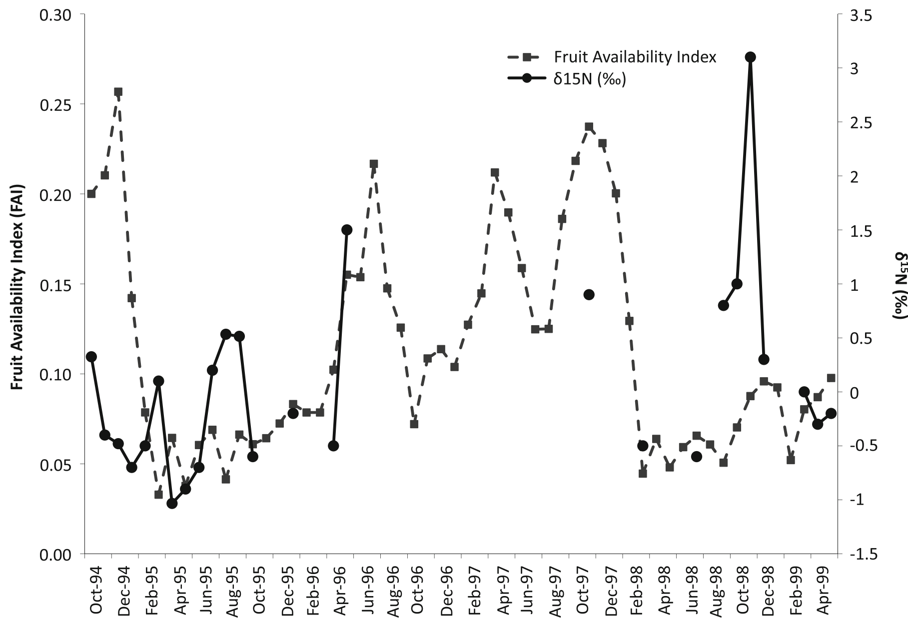

Fig. 3 Relationship between urinary δ^{15}N and the fruit availability index (LMM: t=−0.86, df=44, p=0.40).

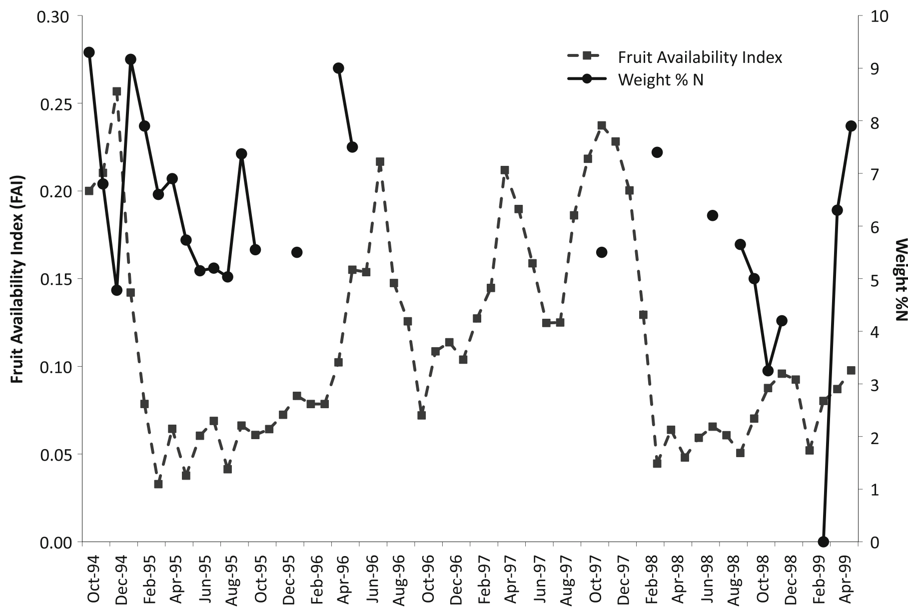

Fig. 4 Relationship between urinary weight %N and the fruit availability index (LMM: t=−0.05, df=44, p=0.96).

Discussion

Our findings, combined with previously published studies on energy balance (Emery Thompson and Knott 2008; Knott 1998), indicate that Bornean orangutans were most likely in a neutral N balance and negative energy balance during periods of fruit scarcity, as evidenced by lower concentrations of urea and the presence of ketones in the urine (Knott 1998). Although we could not directly quantify declines in body fat through the low fruit periods, the presence of ketones and lower C-peptide concentrations in urine (collected during the same study period) provides evidence of fat catabolism during these periods (Emery Thompson and Knott 2008; Knott 1998). During high fruiting periods, corrected urea concentrations were 2.6 times greater than during lower fruiting periods, suggesting that urea excretion was minimized when fruit availability was lower. Thus, it is likely that orangutans recycled excess urea to increase N retention when protein intake was lowest. Our findings on wild orangutans support a previous captive study on primates that found higher levels of serum urea when 2 species of lemurs (*Propithecus verreauxi* and *P. tattersalli*) were fed a diet higher in protein (Campbell *et al.* 1999).

Surprisingly, we did not find a significant correlation between our measure of urea cycling, corrected urea concentration, and the protein intake of individuals. This unexpected result, which we attribute to a type II error, is a testament to the challenge of estimating the rate and mass of foods ingested by wild arboreal animals (Schuelke *et al.* 2006). One possible source of error is that we used protein intake data for the day the urine sample was collected instead of using data from the day before the urine sample collection. This was necessary because for several focal follows we did not have a full day of behavioral data for the day before sample collection and this would have greatly reduced our sample size. Similar to energy intake (Knott 1999), it is unlikely that protein intake varies dramatically between consecutive days within a given focal follow, although this is worth investigating further.

Our method of estimating protein content in the food items may have inflated the amount of protein in the samples. There is often error in estimating the amount of protein in the food items, as values may include fiber-bound N or nonprotein N that is not digested by the individual (Conklin-Brittain *et al.* 1999; Izhaki 1993; Milton and Dintzis 1981; Rothman *et al.* 2008). Indeed, young leaves and other immature vegetation contain higher amounts of nonprotein N vs. mature plant parts such as mature leaves and fruit (Milton and Dintzis 1981). Because orangutans increase the percentage of young leaves in their diets when fruit is scarce (Knott 1998, 1999), our estimates of protein intake during these periods may overestimate the amount of available protein to the focal individuals.

Further, estimating daily protein intake in the wild can be difficult and current methods can produce rather imprecise measurements. Most researchers estimate the amount of protein consumed by taking the product of the averages of number of items ingested per minute, the amount of protein per item as estimated in the laboratory, and the length of the feeding bout (Conklin-Brittain *et al.* 1998; Rothman *et al.* 2008). However, along with there often being measurement error involved in estimating the number of food items ingested by primates in the field (Schuelke *et al.* 2006), there is variation in the chemical composition of food items collected from

different feeding trees (Rothman *et al*. this issue; Rothman *et al.* 2008; 2009; Worman and Chapman 2005), and it is reasonable to assume that feeding rates do not remain constant throughout a feeding bout (Charnov 1976). However, the effect of such bias would be expected to be similar across samples days.

Despite changes in the concentration of urinary urea, we observed no significant differences in urinary $\delta^{15}N$ or weight %N with varying fruit availability. Similar urinary $\delta^{15}N$ and weight %N values during periods of varying fruit availability suggest that orangutans are in a positive, neutral, or the early stages of a steady-state negative N balance, even when fruit availability is low. Combined with the urea concentration data and the presence of ketones in urine samples, it is more likely that individuals were in neutral N or in the early stages of a steady-state negative balance during these periods of fruit scarcity and, therefore, were not experiencing tissue wasting. Instead, orangutans were able to rely on fat reserves for energy during low fruit periods to avoid tissue wasting.

For bonobos in a controlled experiment, a 30% reduction in total energy intake resulted in significantly higher $\delta^{15}N$ values. We observed a 26% reduction in protein intake from high to low fruit periods, yet did not detect differences in urinary $\delta^{15}N$ values. Although there were some days in our study when the orangutans consumed as little as 12.6 g/d of protein, during low fruit periods they still maintained an average of 54.0±33.6 g/d. In a study on Papua New Guinea highlanders who have a low-protein diet, average protein intake was 32.2±8.6 g/d (Rikimaru *et al.* 1985). Thus, perhaps the orangutan's protein intake in our study never fell below critical values necessary to enter into a negative N balance state. Future research should explore variation in the use of lipid reserves among wild great apes, as orangutans appear to be unique in the extent to which they can store excess calories as fat reserves when fruit is abundant (Wheatley 1987).

Our conclusion that orangutans are, on average, not protein limited requires 2 important assumptions: 1) N isotope ratios are similar among orangutan food items, and 2) urinary $\delta^{15}N$ values reflect body $\delta^{15}N$ values. Both of these assumptions can be supported. First, although we were not able to measure directly $\delta^{15}N$ values for orangutan food items, it is unlikely that they vary dramatically. Previous work has demonstrated that on average, bark tissues have slightly lower $\delta^{15}N$ values than fruit, and leaves have slightly higher $\delta^{15}N$ values than fruit. Nevertheless, these isotopic differences are small, and they can differ among species (Codron *et al.* 2005; Hyodo *et al.* 2010). Mean insect $\delta^{15}N$ values were likely only slightly higher (*ca.* 1–2‰) than those in plants (Hyodo *et al.* 2010). The small positive shift in urinary $\delta^{15}N$ values during periods of fruit scarcity could, therefore, reflect small increases in insect or leaf consumption. Although several studies have documented a direct link between dietary $\delta^{15}N$ values and $\delta^{15}N$ values in serum, feces, and urine (Barboza and Parker 2006, 2008; Parker *et al.* 2005; Sponheimer *et al.* 2003), the majority of these studies used controlled high- and low-protein diets with notable variation. The relative constancy of urinary $\delta^{15}N$ values in our sample despite substantial variation in dietary items consumed supports our assumption that N isotopes are similar among orangutan foods.

Second, although we were not able to measure the N isotope values of orangutan tissues, we anticipate that urinary $\delta^{15}N$ values should reflect those in body tissues. Differences in the N pool size and protein turnover among tissues can lead to small

differences in $\delta^{15}N$ values between urine and body N as well as among tissues (Barboza and Parker 2006; Parker *et al.* 2005). Nevertheless, maintaining a balance between N fluxes necessitates that urine and body $\delta^{15}N$ values are closely linked (Martínez del Rio and Wolf 2005). If anything, urinary $\delta^{15}N$ values should be more sensitive to changes in N balance than body $\delta^{15}N$ values. During protein catabolosis, body protein is broken down into amino acids, and the amino acids are then deaminated or transported to other organs. Excreta such as urine represent the immediate byproducts of this deamination and transamination, and therefore should be sensitive to any changes in the percentage of body protein recycled for protein synthesis (Martínez del Rio *et al.* 2009).

Lastly, we measured whole urine $\delta^{15}N$ values rather than urea-specific isotope ratios. Whereas urea $\delta^{15}N$ values can shift with changes in N balance, $\delta^{15}N$ values for other urinary components, e.g., creatinine, are less affected (Barboza and Parker 2006). It is, therefore, possible that small isotopic shifts in the urea were masked by small declines in the relative proportion of urea in the urine samples (Barboza and Parker 2006). We believe that this possibility is unlikely for 2 reasons. First, similar $\delta^{15}N$ values have been found in whole urine and urea, despite substantial fluctuations in the relative proportion of urea in total urinary N (30–77%) (Parker *et al.* 2005). Second, we observe the largest changes in $\delta^{15}N$ between June and October, 1995, which was a period marked by relatively small changes in corrected urea concentration (Figs. 2 and 3).

The new methods we have presented here, combined with previous methods to detect energy balance in free-ranging primates (Emery Thompson and Knott 2008; Knott 1998), provide us with a unique set of tools to explore the importance of protein in the diets of wild primates. Collecting large quantities of urine can be challenging when studying free-ranging primates. The urea detection method described here was modified from a method for detecting urea concentration in ocean water and as a result it has the advantage that it can accommodate small quantities of urine (200 μl/sample) that can have very low urea concentrations. Indeed, all of our samples required further dilution. As protein has been suggested to be a major limiting factor for primate body size, diet, and behavior (Chapman *et al.* 2004; Ganzhorn *et al.* 2009; Glander 1981; McKey *et al.* 1981; Milton 1979, 1981; White 1998), developing techniques to better understand protein limitations and cycling in free-ranging primates is critical to exploring the role that protein plays in shaping primate communities. Further, our methods could be used as a conservation tool to assess the health and viability of orangutans or other primates living in more degraded or marginal habitats. Although orangutans living in this relatively undisturbed primary rain forest were able to avoid protein limitation, individuals in more compromised habitats may not be able to avoid it. We anticipate that our new urea methods for estimating protein balance state combined with methods for estimating protein intake (Conklin-Brittain *et al.* 1998; 1999; Felton *et al.* 2009b; Oftedal 1991; Rothman *et al.* 2008) will be useful for determining the importance of protein acquisition for nonhuman primates.

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