Received: 20 June 2023 | Revised: 18 September 2023 | Accepted: 1 October 2023

DOI: 10.1002/ajp.23563

RESEARCH ARTICLE

AMERICAN JOURNAL OF PRIMATOLOGY WILEY

Urinary C-peptide and total triiodothyronine as energetic biomarkers for studies of lemurs

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Funding information
Duke Lemur Center; The Graduate Center of the City University of New York

Abstract

Measuring energy balance and energy metabolism can provide crucial information for understanding the ecological and behavioral drivers of an animal's energetic and physiological condition. Both urinary C-peptide (uCP) of insulin and urinary total triiodothyronine (uTT3) have been validated as noninvasive biomarkers of energy balance and metabolic activity in haplorrhine primates. This study attempts to validate uCP and uTT3 measures in strepsirrhines, a phylogenetically distinct primate clade, using the ruffed lemur (genus *Varecia*) as a model. We experimentally manipulated the diet of captive black-and-white (*Varecia variegata*) and red (*Varecia rubra*) ruffed lemurs at Duke Lemur Center across a 4-week period. We collected urine samples from subjects ($n = 5$) each day during 1 week of control diet, 2 weeks of calorie-restricted diet and 1 week of refeeding, designed to temporarily reduce energy balance and metabolism. We also tested the outcome of filter paper as a storage method by comparing to controls (frozen at −20°C) to assess its suitability for studies of wild populations. We successfully measured uCP and uTT3 levels in frozen urine samples using commercial enzyme immunoassay kits and found that both biomarkers were excreted at lower concentrations (C-peptide: 1.35 ng/mL, 54% reduction; TT3: 1.5 ng/mL, 37.5% reduction) during calorie-restricted periods compared to normal diet periods. Filter paper recovery for uCP was 19%, though values were significantly positively correlated with frozen control samples. uTT3 could not be recovered at measurable concentrations using filter paper. These methods enable noninvasive measurement of energetic conditions in wild strepsirrhines and subsequent assessment of relationships between energy balance and numerous socioecological drivers in primate populations.

KEYWORDS

energy balance, food restriction, insulin, metabolism, primates, thyroid hormone

1 | INTRODUCTION

Energy is a key resource throughout an individual's life and is among the primary drivers of behavior, physiology, and life history (Emery Thompson, 2017). Wild animals experience a variety of environmental and social stressors across life history stages that challenge their ability to maximize energy acquisition while both maintaining metabolic function and maximizing reproduction. Energy is finite and thus must be allocated between competing functions, which has led to diverse strategies between and within species to survive and reproduce.

Abbreviations: uCP, urinary C-peptide; uTT3, urinary total triiodothyronine.

Energy balance—or the balance between energy intake and energy expenditure (Andersson et al., 1991)—is one common measure used to indicate an animal's energetic condition. Insulin, secreted by the pancreas in response to food ingestion, is integral to the maintenance of energetic homeostasis (Rubenstein et al., 1969). Insulin plays a vital role in the storage and utilization of energy during feeding and fasting states, by controlling the synthesis and degradation of glucose and glycogen (Magkos et al., 2010; Qaid & Abdelrahman, 2016). In response to energy intake, concentrations of serum insulin increase to uptake and store carbohydrates (Bergman et al., 1970; Trenkle, 1970). Conversely, in response to energy deficits, serum insulin decreases, and carbohydrate stores are utilized to produce glycogen (Bergman et al., 1968; Shoemaker et al., 1963). C-peptide (CP) is an equimolar by-product of insulin biosynthesis, separated from proinsulin when it is cleaved into A and B chains (Steiner et al., 1967). However, CP has limited biological activity and is excreted largely intact in urine (Hoekstra et al., 1982; Zavaroni et al., 1987). When measured against baseline insulin levels, urinary C-peptide (uCP) can indicate shifts in energy balance between states of energy storage (positive) and utilization (negative) (Andersson et al., 1991).

Metabolic activity is a second important component of energetics. Basal metabolic activity comprises >50% of daily energy expenditure (Silva, 1995), and interindividual differences in metabolic regulation have fitness consequences (Burton et al., 2011). Basal metabolic rate is controlled by the hypothalamus–pituitary–thyroid axis (Douyon & Schteingart, 2002; Klieverik et al., 2009; Welcker et al., 2013), and thus, thyroid hormones can provide vital information about the regulation of energy metabolism and allocation. The thyroid hormone, thyroxine, is converted into triiodothyronine (T3), its biologically active form (Chatzitomaris et al., 2017). These thyroid hormones play a vital role in regulating the body's metabolic rate. In response to surplus energy, concentrations of serum T3 increase and act to decrease the efficiency of energy formation (Dauncey, 1990; Silva, 2006). Conversely, when energy is in deficit (e.g., during fasting or starvation), concentrations of serum T3 decrease (Azizi, 1978; Kozlowska & Rosolowska-Huszcz, 2004; Roth et al., 2002), increasing the efficiency of energy formation, thereby lowering metabolic activity (Eales, 1988; Forsum et al., 1981). T3 and its metabolites are excreted by the kidneys into the urine (Burke & Shakespear, 1976; Burke et al., 1972), resulting in a positive correlation between urine and serum T3 concentrations (Burke & Eastman, 1974; Gaitan et al., 1975; Yoshida et al., 1980)—akin to the relationship between insulin and CP production.

Given these physiological relationships, in addition to the ethical and practical issues associated with invasive sample collection in large mammals, researchers have developed noninvasive techniques to facilitate the investigation of these patterns in the wild. Biological validation studies have confirmed the utility of using uCP as a biomarker of energetic status in several animals, with uCP concentrations being higher when more food and/or calories are consumed (e.g., Emery Thompson & Knott, 2008; Emery Thompson et al., 2009; Girard-Buttoz et al., 2011). Likewise, thyroid hormones have been increasingly used as noninvasive markers of energy metabolism and allocation. Fecal T3 metabolites show higher concentrations when more calories are consumed (e.g., Ayres et al., 2012; Cristóbal-Azkarate et al., 2016; Dias et al., 2017; Schaebs et al., 2016), and lower concentrations during the seasons when food availability and energy intake are usually more limited (Gesquiere et al., 2018). Much of this research has focused on nonhuman primate energetics. Because most primates live in tropical and subtropical environments, they therefore experience seasonal variation in the availability, distribution, and quality of food resources (Van Schaik & Brockman, 2005; Van Schaik et al., 1993). These selection pressures have led primates to develop diverse behavioral and physiological strategies to meet their energetic needs. Positive correlations between energetic condition and uCP concentration have been found in haplorrhine primates (chimpanzees: Emery Thompson et al., 2009; Sherry & Ellison, 2007; bonobos: Deschner et al., 2008; colobus monkeys: Harris et al., 2010; macaques: Girard-Buttoz et al., 2011; Higham, Girard-Buttoz, et al., 2011; gorillas: Grueter et al., 2014; blue monkeys: Thompson et al., 2020; capuchins: Bergstrom et al., 2020; Sacco et al., 2021). Likewise, relationships between energetics and fecal T3 have also been examined in this clade (Chen et al., 2021; Cristóbal-Azkarate et al., 2016; Gesquiere et al., 2018; Schaebs et al., 2016). Correlates of urinary T3 in wild primates are somewhat scarce (e.g., Behringer et al., 2022; Thompson, 2017), although research has identified a positive correlation between urinary total triiodothyronine (uTT3) and caloric intake in captive bonobos (Deschner et al., 2020) and macaques (Sadoughi et al., 2021). However, to date, uCP and uTT3 validations have not been published for the other major primate lineage, the strepsirrhines. Though insulin structure and function are strongly conserved in mammals (Snell & Smyth, 1975; Wallis, 2009), strepsirrhine insulin gene sequences have not been compared to those of other primates and may have altered structure and/or function. Similarly, strepsirrhine thyroid hormone genes and T3 structure and function have received little attention to date.

In this study, we aimed to validate uCP and uTT3 as biomarkers of energy balance and energy metabolism in lemurids, one of five major strepsirrhine clades, using the ruffed lemur (genus: *Varecia*) as a model. Lemurs have evolved a unique combination of energy-saving traits characterized as an adaptive response to coping with Madagascar's harsh and often unpredictable environment (Wright, 1999). While many studies have examined this "energy frugality hypothesis" through a behavioral lens, most have not directly measured energetics (Simmen & Rasamimanana, 2018). Moreover, what little we do know about lemur energetic strategies primarily stems from nocturnal species that undergo torpor (e.g., cheirogaleids: Blanco et al., 2018). However, changes to climatic conditions and food availability likely present significant energetic challenges for diurnal lemurs, as well. Ruffed lemurs are endemic to the highly seasonal eastern rainforests of Madagascar (Morelli et al., 2020; Vasey et al., 2022; Wright, 1999). Ruffed lemurs are anatomical fruit specialists, with simple digestive tracts and short gut transit times suited to readily digestible energy and nonfibrous foods (Campbell et al., 2000; Edwards, 1995; Edwards & Ullrey, 1999). Several studies have shown the behavioral impacts of seasonal resource availability on these lemurs (Balko, 1998; Balko & Brian Underwood, 2005; Beeby & Baden, 2021; Britt, 2000; Ratsimbazafy, 2002, 2006; Rigamonti, 1993); fewer have investigated the physiological impacts of this seasonality (Morland, 1993; Vasey, 2004, 2005). Due to their adaptations to frugivory, in combination with declines in calorie intakes during fruit-lean seasons (Beeby

et al., 2023), ruffed lemurs may be at high risk of experiencing negative energy balance (expending more energy than they consume) during sustained periods of fruit scarcity. Moreover, the ability to maintain energetic conditions throughout lean seasons may be a driver of certain ruffed lemur life history traits, such as their "boom-bust" reproduction (Ratsimbazafy, 2002) and communal nesting (Baden, 2011; Baden et al., 2013). Measures of energy balance and energy metabolism in this clade will allow us to better understand the diversity and flexibility of lemur energetic strategies and how species are able to survive in unpredictable and increasingly disturbed habitats. To this end, we measured uCP and uTT3 levels using commercially available enzyme immunoassays to examine the viability of these biomarkers for quantifying energetics in lemurs. Additionally, we examined the outcome of filter paper as a storage method by comparing to controls (frozen at −20°C) to optimize later studies on wild populations.

2 | METHODS

2.1 | Data collection

Study subjects: We collected samples from two groups of captive ruffed lemurs (genus: *Varecia*) at Duke Lemur Center (DLC). Individuals comprised three *Varecia variegata* (two female and one male) and two *Varecia rubra* (one female and one male).

Dietary manipulation: We conducted feeding experiments over a 4-week period during March 2022. Study animals were subjected to a manipulated diet during two of the four study weeks and all animals were fed separately to ensure controlled dietary intakes. Diets comprised water-soaked primate biscuits with varying mixtures of fruits and vegetables. The dietary restriction comprised a 30% reduction in total dietary calories, designed to induce short-term negative energy balance. This was achieved through a reduction in the quantity of primate biscuits provided, in addition to the replacement of specific fruits and vegetables with less calorie-dense alternatives. This was deemed by DLC veterinary staff to be a sufficient reduction to induce negative energy balance while maintaining ethically responsible practice (IACUC #A012-22-01). Moreover, study animals were closely monitored to ensure no more than 15% mean body weight was lost during the restricted diet weeks. The feeding schedule was as follows: Week 1—Control, Week 2—Diet 1 (calorie restriction), Week 3—Diet 2 (calorie restriction), Week 4—Refeeding. The purpose of two consecutive weeks of dietary restriction was to decouple the effects of short-term reduced dietary energy intake and sustained negative energy balance. Refeeding indicates a return to the control diet.

Sample collection and storage: We collected one urine sample from each individual each weekday, with a minimum of 0.5 mL per sample and three samples per individual per week. The total number of samples collected was $n = 99$. Urine samples were collected using sterile, disposable transfer pipettes from trays placed on the ground beneath animals. Urine samples contaminated with feces or dirt were not collected. All samples were collected between 9 a.m. and 12 noon to control for potential circadian patterns and were processed and stored within 2 h of collection. Each sample was measured for specific gravity using a handheld refractometer (Atago 4410 PAL-10S urine specific gravity [SPG] refractometer) to correct for urine concentration during analyses (Anestis et al., 2009). Samples were then aliquoted into two subsamples: (1) microcentrifuge tubes stored at −20°C and (2) 200 µL dried onto Cytiva Whatman™ Grade 5 Circles filter paper and stored in aluminum foil in an airtight container, to compare storage methods for optimization of later field studies (99 samples × 2 aliquots = 198 samples). Samples were stored for 12 months before analyses.

2.2 | Data analysis

Laboratory analyses: All urine samples were shipped to New York University for analysis. CP analyses were first conducted using a commercial human CP enzyme-linked immunosorbent assay (ELISA) from IBL International GmbH (RE53011; Tecan), which has been validated for use in multiple other primates, including macaques, baboons, and capuchins (Fürtbauer et al., 2020; Girard-Buttoz et al., 2011; Higham, Girard-Buttoz, et al., 2011; Higham, Heistermann, et al., 2011; Sacco et al., 2021). TT3 analyses were conducted using a commercial human TT3 ELISA from DRG Instruments GmbH (EIA-4569R) which has been validated for use in multiple other primates, including macaques, bonobos, and capuchins (Deschner et al., 2020; Girard-Buttoz et al., 2011; Sadoughi et al., 2021; Touitou et al., 2021a). Subsequent CP analyses were then conducted using a rodent CP ELISA from RayBiotech (EIAM-CPE/EIAR-CPE). These rodent kits were chosen as they were designed to measure nonprimate mammalian CP, and both have low cross-reactivity with human and macaque CP, meaning these kits detect differently structured CP molecules and therefore may have better cross-reactivity with lemur CP. We measured urine SPG for selected samples ($n = 20$) a second time, to assess potential evaporation and subsequent concentration of samples and all the samples had the same SPG after freezing as they did during initial collection. For all other samples, we used SPG values from initial collection. We then used ELISA kits to detect metabolite levels above assay sensitivity (CP: 0.6 ng/mL; TT3: 0.1 ng/mL) in *Varecia* urine by running serial dilutions using appropriate assay buffers and comparing these to standard curves for each kit. We ran all samples at appropriate dilutions (between 1:1 and 1:16) to measure both uCP and TT3 concentrations. In some cases ($n = 18$), we concentrated samples to achieve minimum assay sensitivity for CP. We concentrated these samples 3X by lyophilizing 600 µL aliquots and reconstituting them in 300 µL of deionized water, which brought concentrations to within the linear part of the standard curve. For CP, interassay variation, determined by the coefficients of variation (CVs) calculated from high- and low-value quality controls were 2.5% and 2%, respectively. Intra-assay variation was 5.2%. For T3, interassay variation, determined by the CVs calculated from high- and low-value quality controls were 6% and 5.2%, respectively. Intra-assay variation was 15.9%.

Finally, to assess the viability of filter paper as a sample collection and storage method, we reconstituted urine samples from filter paper and measured metabolite concentrations. We used a standard 6 mm hole punch, sterilized with ethanol between samples, to collect 10X punches per sample and eluted these in 1 mL of 80% methanol overnight. We then dried off the methanol in a dry bath incubator, and resuspended extracted metabolites with 150 µL of assay buffer provided in the ELISA kits.

Statistical analyses: To determine if samples diluted in parallel to the standards we built linear models using dilution factor as a predictor of optical density and examined regression coefficients of samples versus standards. To examine whether metabolite levels change as biologically expected (with changes in calorie intake), we used analysis of variance (ANOVAs) to compare values across each of the diet types (weeks). Additionally, we used post hoc Tukey–Kramer tests to identify where any statistical differences between diet types were found. To avoid losing statistical power, we did not use more sophisticated methods for biological validation due to the limited sample size. To test the outcomes of the two different urine storage methods, we performed a linear regression across match-paired frozen and filter paper samples to examine the correlation coefficient of storage methods. All analyses were performed in RStudio ver. 2023.03.0.

3 | RESULTS

3.1 | Effects of calorie restriction on weight

We observed a trend of decreasing weight from control through Diet 2 (mean reduction: 140 g, 3.75%), which reflected the 30% calorie reduction during Diet 1 and Diet 2 weeks. These results suggest the calorie reduction was sufficient to impact energetics in these lemurs. No individuals lost more than 6% of their original weight, even during Week 3. Weights of all study subjects then increased from Diet 2 to refeeding (mean increase: 136 g, 3.72%).

3.2 | uCP and TT3

Initially, we used Human CP ELISA kits from Tecan (RE53011), as this has been validated for use in several non-human primates (Deschner et al., 2020; Girard-Buttoz et al., 2011; Sadoughi et al., 2021; Touitou et al., 2021b). However, we could not reliably detect CP in lemur urine using this kit. CP concentrations were low, almost entirely falling outside of the linear range of the assay. Moreover, serial dilutions of urine (neat, 1:2, 1:4, 1:8, 1:16) did not show changes in CP concentration, nor any consistent patterns. This suggests minimal displacement resulting from low cross-reactivity between the anti-mouse antibodies and the CP antigens in lemur urine (Figure 1). Further examination of a subset of samples expected to have the highest concentrations—those with high SPG from normal-diet weeks—showed similar results. We found that uCP concentrations can, however, be reliably measured using mouse/rat CP ELISA kits from RayBiotech (EIAM-CPE/EIAR-CPE). Parallelism of serial dilutions indicates precise detection of uCP concentrations using this method, with no significant differences in regression slopes between the samples and the standard curve (Supporting Information: Figure S1; samples coefficient = 0.027, standards coefficient = 0.010, $R^2_{adj.} = 0.93$, $p = 0.08$). Moreover, uCP concentrations decreased a mean of 1.35 ng/mL (54% reduction) from Week 1 to Weeks 2 and 3, when calorie intake was restricted by 30% (ANOVA: $f = 4.255$, $df = 1$, $p = 0.043$), supporting that variation in the measurements indicate biologically meaningful variation in energetic status. However, uCP concentrations did not increase upon refeeding during Week 4, which likely contributed to the relatively low statistical power of this result. Post hoc Tukey–Kramer tests revealed significant differences between the control and all other diet types (control-Diet 1: $p_{adj} = 0.02$, control-Diet 2: $p_{adj} = 0.02$, control-refeeding: $p_{adj} = <0.01$). All other comparisons were nonsignificant (Diet 1–Diet 2: $p_{adj} = 0.098$, Diet 1-refeeding: $p_{adj} = 0.99$, Diet 2-refeeding: $p_{adj} = 0.95$).

We detected TT3 in urine collected from ruffed lemurs using human TT3 ELISA kits (EIA-4569R). Parallelism of serial dilutions

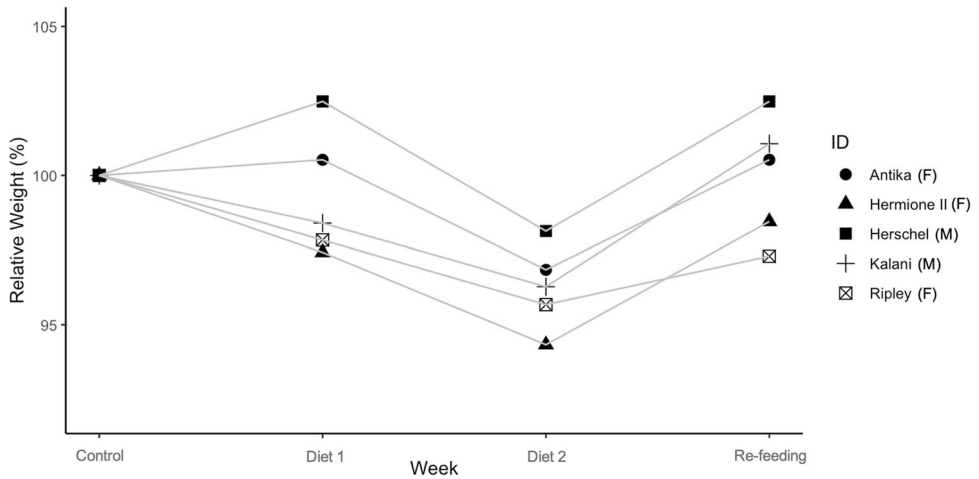

FIGURE 1 Relative change in weekly weight (%) of ruffed lemur individuals ($n = 5$) during the 4-week diet experiment. Percentages presented are relative to the original weight per individual. F, female; M, male.

indicates precise detection of uTT3 concentrations using this method, with no significant differences in regression slopes between samples and the standard curve (Supporting Information: Figure S2; samples coefficient: 0.083, standards coefficient: 0.094, $R^2_{adj} = 0.91$, $p = 0.66$). Moreover, uTT3 concentrations decreased a mean of 1.5 ng/mL (37.5% reduction) during weeks when calorie intake was restricted by 30% (ANOVA: $f = 3.076$, $df = 1$, $p = 0.008$), indicating that variation in the measurements indicates biologically meaningful variation in energetic status. Post hoc Tukey–Kramer tests revealed significant differences between the two diet weeks and refeeding (Diet 1-refeeding: $p_{adj} = <0.01$, Diet 2-refeeding: $p_{adj} = 0.02$). All other comparisons were nonsignificant (control-Diet 1: $p_{adj} = 0.43$, control-Diet 2: $p_{adj} = 0.68$, control-refeeding: $p_{adj} = 0.26$, Diet 1–Diet 2: $p_{adj} = 0.98$).

3.3 | Storage method

We found uCP concentrations from filter paper samples were 81% lower than uCP concentrations from the same sample stored at −20°C, however, there was a strong positive correlation between matched pairs of filter paper and frozen storage samples ($R = 0.7$, $n = 13$, $p = 0.0081$). We could not detect uTT3 concentrations above minimum assay sensitivity from filter paper, and therefore could not compare these to values measured in frozen urine samples.

4 | DISCUSSION

We found that uCP and uTT3 can be detected in lemur urine using commercial enzyme immunoassays and are both reliable indicators of lemur energetics. Both biomarkers are excreted at lower concentrations in urine during calorie-restricted periods than normal diet periods. Additionally, uCP can be reliably detected from samples stored on filter paper, whereas uTT3 cannot.

Commercial ELISA kits designed for human biological samples, which have been validated for use in other primate clades, are not suitable for reliably measuring uCP in lemurs. However, kits designed for rodent biological samples can reliably detect lemur uCP. As expected, we found that CP concentrations decreased from the control diet to calorie-restricted diet weeks. However, upon refeeding, CP concentrations did not increase back to baseline levels (Figure 2).

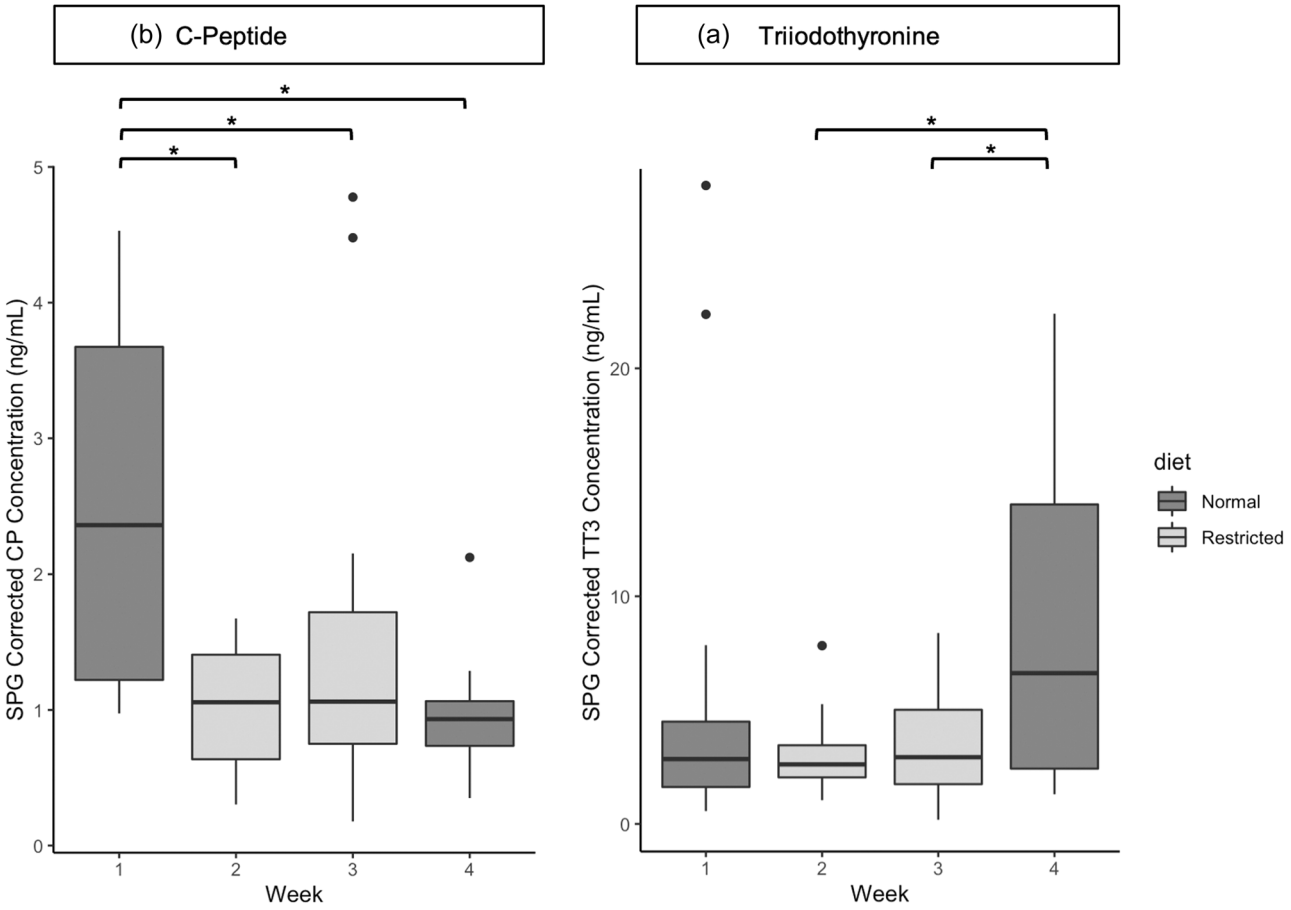

FIGURE 2 Urinary biomarker concentrations in captive ruffed lemurs across a 4-week experimental dietary manipulation with weeks representing: (1) control, (2) Diet 1, (3) Diet 2, (4) refeeding: (a) SPG corrected uCP concentrations, (b) SPG corrected uTT3 concentrations. *Significant pairwise comparisons are shown based on post hoc Tukey–Kramer tests. SPG, specific gravity; uCP, urinary C-peptide; uTT3, urinary total triiodothyronine.

Though we expected to see increases during refeeding, this result may reflect the limited collection period of only 1 week after refeeding, and CP concentrations likely would increase shortly after this time. Sustained low uCP concentrations upon refeeding may indicate a metabolic response to food insecurity, such as increased energy efficiency and/or shifts from glucose and glycogen metabolism to lipid metabolism, resulting in altered insulin production (Bateson et al., 2021; Secor & Carey, 2011). Despite the lack of observed increase in CP upon refeeding (Figure 2a), the significant decrease in CP concentrations during calorie restriction indicates that uCP is a reliable indicator of energy balance in lemurs. CP concentrations in urine should decrease when calorie consumption decreases such that individuals are expending more calories than they are consuming, thus falling into more negative energy balance. This state of negative energy balance appears to extend for several days after refeeding, but we expect that individuals would return to a more positive energy balance after a few more days.

Commercial ELISA kits originally designed for human biological samples are suitable for reliably measuring urinary TT3 in lemurs. We found that TT3 concentrations decreased a small amount from the control diet to calorie-restricted diet weeks. However, upon refeeding, TT3 concentrations increased considerably (Figure 2b). This indicates that uTT3 is a reliable indicator of metabolic activity in lemurs, as TT3 concentrations in urine should decrease as metabolic activity is reduced to conserve energy during times of limited calorie intake and increase as metabolic activity is upregulated to store energy during times of surplus calorie intake.

Contrary to what has been found in haplorrhine primates (see Girard-Buttoz et al., 2011), both uCP and uTT3 levels remained relatively constant across the restricted diet period. The initial decline in both metabolites correlates well with the decrease in food supply (and therefore energy intake). However, body mass continued to decline throughout the calorie-restricted period, despite metabolite levels remaining relatively constant. This suggests that uCP and uTT3 excretion are sensitive to caloric intake but less so to body mass in this taxon. However, based on our assessment, it appears that the assay antibody is cross-reacting well with the antigens of interest in both kits. To identify exactly which metabolites are being excreted as breakdown products of each hormone, a radiometabolite study could be conducted (e.g., Möhle et al., 2002). However, such studies, comprise substantial ethical concerns and are extremely difficult to undertake, with institutes now requesting animals to be purchased and euthanized after the study, which we do not believe can be ethically justified for the present study. The differences we see between the two metabolite profiles in response to calorie restriction are likely due to compensatory physiological mechanisms, such as altered CP and T3 function, to avoid energy scarcity. However, the precise nature of this relationship is difficult to interpret without further investigation.

Filter paper appears to be a viable storage method for CP in lemur urine. As expected, samples stored using this method did not reflect absolute CP values in the urine, due to degradation of the metabolite upon drying. CP concentrations from filter paper samples were 81% lower than CP concentrations from the same sample stored at −20°C, however, there was a strong positive correlation between matched pairs of filter paper and frozen storage samples (Figure 3). This is a lower percentage recovery than other validations of filter paper to store primate urine (see Emery Thompson & Knott, 2008; Higham, Heistermann, et al., 2011), but nonetheless, the correlation was significant. The sample size for the storage method

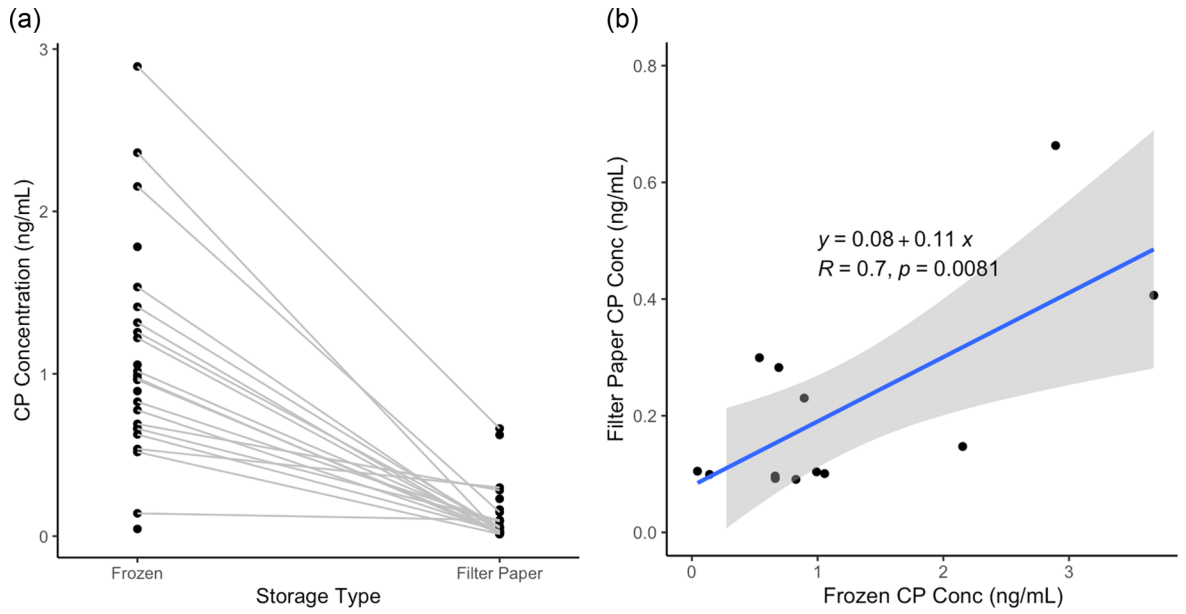

FIGURE 3 Comparison of urinary C-peptide concentrations (ng/mL) of frozen and filter paper preserved samples from ruffed lemurs: (a) recovery concentrations for filter paper compared to match-paired frozen samples; filter paper samples were on average 19% of the value of frozen counterparts (range: 14%–30%), (b) correlation between frozen and filter-paper match-paired samples; $R = 0.7$, $n = 13$, $p = 0.0081$.

comparison was smaller than for the biological validation due to many filter paper sample measures falling well below the linear part of the standard curve, and in many cases below minimum assay sensitivity. Therefore, we included in our analyses only those paired samples for which both frozen and filter paper samples were above accurately detectable assay sensitivity. It is likely that samples stored on filter paper for shorter periods of time (<12 months) would have better recoveries.

Filter paper was not, however, a viable storage option for T3, due to the substantial degradation of the metabolite. It is possible that filter paper could be used for short-term storage, but our samples, which were stored for 12 months after collection, did not contain any measurable T3 above minimum assay sensitivity. Further studies should examine the timescale of T3 degradation by analyzing filter paper samples stored for short- to medium-term periods (<12 months).

Direct, noninvasive measurement of energy balance and energetic condition made possible by these methods will enable greater insight into lemur energetics. For decades, the unusual suite of lemur demographic, behavioral, and life history traits have been explained as energy-saving adaptations (Wright, 1999). This "energy frugality" is typically considered to be an adaptive strategy to conserve energy in Madagascar's harsh and unpredictable environment. However, few studies have looked beyond lemur behavioral syndromes, and fewer still have directly measured energetics (but see Simmen & Rasamimanana, 2018). Now validated, uCP and uTT3 will be highly useful biomarkers for noninvasively quantifying energetic conditions in wild lemurs and directly assessing relationships between energetic conditions and numerous socioecological drivers in these primates. Future research should also consider comparing lemur proinsulin gene sequences to those of other primate taxa. The low antibody–antigen cross-reactivity we found when using CP plates validated for humans and other non-human primates may indicate structural and/or functional differences in lemur insulin amino acid sequences. This may be relevant to further understanding the unusual energetic strategies we see in this primate clade.

AUTHOR CONTRIBUTIONS

Nina Beeby: Conceptualization (lead); data curation (lead); formal analysis (lead); funding acquisition (lead); methodology (equal); writing—original draft (lead); writing—review and editing (equal). **Andrea L. Baden**: Conceptualization (lead); data curation (supporting); supervision (equal); writing—original draft (supporting); writing—review and editing (equal). **James P. Higham**: Conceptualization (supporting); formal analysis (supporting); methodology (equal); resources (lead); software (lead); supervision (equal); writing—original draft (supporting); writing—review and editing (equal).

ACKNOWLEDGMENTS

The authors would like to thank the CUNY Graduate Center Early Research Initiative and the Duke Lemur Center Director's Fund (DLC publication number #1579) for funding for this research. The authors thank Stacey Tecot, Mitchell Irwin, April Binder, and Melissa Emery Thompson for advice on project feasibility as well as the discussion of urine collection, storage, and analysis methods. Thank you to all Duke Lemur Center staff, especially Erin Ehmke, Kay Welser, Julie McKinney, and Gabbi Hirschkorn for their invaluable training and assistance during data collection. James P. Higham thanks Michael Heistermann for discussion during the troubleshooting phases. Nina Beeby thanks Connor Whalen for discussions during laboratory analyses. Finally, thank you to the reviewer and editor who provided constructive feedback on our manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data are available upon reasonable request.

ETHICS STATEMENT

The protocol for this study at the Duke Lemur Center (DLC) was approved by the Institutional Animal Care and Use Committee at Duke University (protocol number: A012-22-01). All research undertaken adhered to all animal care, legal and ethical requirements of the United States and the American Society of Primatologists "Principles for the Ethical Treatment of Non-Human Primates." All DLC animals are housed either as pairs or small groups, allowing for social interactions. Animals were housed indoors during sample collection hours but were provided with outdoor access before and after sample collection each day (temperature permitting). All samples were collected non-invasively, and animals were only handled during weight checks once per week by trained DLC staff.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Beeby, N., Baden, A. L., & Higham, J. P. (2023). Urinary C-peptide and total triiodothyronine as energetic biomarkers for studies of lemurs. *American Journal of Primatology*, e23563. https://doi.org/10.1002/ajp.23563