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Estimation of energetic condition in wild baboons using fecal thyroid hormone determination

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Abstract

Understanding how environmental and social factors affect reproduction through variation in energetic condition remains understudied in wild animals, in large part because accurately and repeatedly measuring energetic condition in the wild is a challenge. Thyroid hormones (THs), such as triiodothyronine (T3) and thyroxine (T4), have a key role in mitigating metabolic responses to energy intake and expenditure, and therefore are considered important biomarkers of an animal's energetic condition. Recent method development has shown that T3 and T4 metabolites can be measured in feces, but studies measuring THs in wild populations remain rare. Here we measured fecal T3 metabolites (mT3) in baboons, and tested whether the conditions of collection and storage used for steroid hormones could also be used for mT3; we focused on mT3 as it is the biologically active form of TH and because fecal T4 metabolites (mT4) were below detection levels in our samples. We also tested if mT3 could be determined in freeze-dried samples stored for long periods of time, and if these concentrations reflected expected biological variations across seasons and reproductive states. Our results show that mT3 can be measured with accuracy and precision in baboon feces. The conditions of collection and storage we use for steroid hormones are appropriate for mT3 determination. In addition, mT3 concentrations can be determined in samples stored at -20°C for up to 9 years, and are not predicted by the amount of time in storage. As expected, wild female baboons have lower mT3 concentrations during the dry season. Interestingly, mT3 concentrations are lower in pregnant and lactating females, possibly reflecting an energy sparing mechanism. Retroactive determination of mT3 concentration in stored, freeze-dried feces opens the door to novel studies on the role of energetic condition on fitness in wild animals.

Keywords

fecal; thyroid hormone; baboon; method validation; season; reproductive status

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1. Introduction

The energetic condition of an animal depends not only on the amount of energy it has stored, but also on its energy balance, i.e., on the difference between its energy intake and energy expenditure (Ellison, 2001). An animal will be in positive energy balance and will gain weight when its energy intake surpasses its energy expenditure, and conversely will be in negative energy balance when the opposite applies. An animal's energy balance in natural environments will be influenced by the physical environment and, in social species, also by the social environment. For instance, seasonal variation in rainfall and temperature can lead to pronounced variation in energy balance over the course of the year because rainfall and temperature affect food availability; seasonal food shortages may affect not only energy intake but also energy expenditure if animals need to travel farther to encounter food (Alberts et al., 2005; Bronson, 1995; Dunbar, 1992; Johnson et al., 2015; van Schaik and Pfannes, 2005). The impact of food shortage on energy balance may be further exacerbated by adverse ambient temperatures due to the additional costs of thermoregulation (Bronson, 1995). In social species, several aspects of the social environment can impact an animal's energy balance. For instance, the social status of an animal within its group can mediate energy balance if high-ranking animals have priority of access to food and experience fewer feeding interruptions than lower-ranking animals, leading to higher foraging efficiency (Deag, 1977; Ellis, 1995; Holekamp et al., 1996; Koenig, 2002; Saito, 1996; Vogel, 2005). The size of an animal's social group will also influence its energy intake. Individuals in larger social groups are usually at an energetic disadvantage compared to individuals in smaller groups, because of more intense intra-group competition, but this disadvantage may be alleviated if larger groups can monopolize better patches of food than smaller groups (Markham et al., 2012; Markham and Gesquiere, 2017; Potts et al., 2015; Wrangham et al., 1993).

Available energy is allocated between several competing processes such as self-maintenance, growth and reproduction. In the wild, most animals have a finite amount of energy available, and trade-offs between survival and reproduction will be necessary if the available energy is insufficient to meet the costs of all these processes (Bronson, 1985; Charnov, 1997; Stearns, 1989). Some species have adapted to periods of low food availability by reproducing seasonally when environmental conditions are favorable (Bronson, 1985). Other species are able to reproduce all year-long, relying on their energy reserves, and on their capacity to increase metabolic efficiency and decrease energy expenditure in order to cover the high costs of pregnancy and lactation (Alam et al., 2003; Butte and King, 2005; reviewed in Bronson, 1995). Understanding how environmental and social factors regulate an individual's energetic condition in nonseasonal breeders and how, in turn, an individual's energetic condition mediates the tradeoff between reproduction and survival is a topic of considerable interest that has remained understudied in wild animal populations. Indeed, in the wild it is challenging to accurately and repeatedly measure an animal's energy status and energy balance. Factors contributing to this challenge include repeatedly trapping the animal to obtain its weight, as well as recording the amount and energetic value of the food it consumed, and the animal's energetic expenditures, including the energy it spent finding and processing food, mating, caring for its offspring, and maintaining its body temperature.

Until now, changes in glucocorticoid (GCs) levels have often been used as a measure of energetic condition in studying wild animals. GCs are a family of steroid hormones secreted by the adrenal glands in periods of energy deficiency; they are responsible for an increase in feeding behavior and up-regulation of glucose metabolism to provide the energy necessary to restore homeostasis (Landys et al., 2006; Sapolsky et al., 2000; Wingfield et al., 1998). However, GC secretion is also stimulated by psychological stressors, making it challenging to interpret the source of elevated GC concentrations (Sapolsky, 1994). Therefore, obtaining a more direct and fine-grained measure of energetic condition is essential to shed more light on the role of an animal's energetic condition in the regulation of reproduction by environmental and social factors.

Recent advances in non-invasive assessment of thyroid hormones (THs) in feces create a novel opportunity to achieve the goal of a more accurate measure of energetic condition. Two major THs are secreted by the thyroid gland: triiodothyronine (T3) and its prohormone, thyroxine (T4). T4 is the major form in the blood, but T3 is the biologically active form. T3 and T4 are largely transported bound to proteins, such as thyroglobulin, and less than one percent is found in a free form in the blood (Robbins, 1992). THs have key roles in growth and development, in the regulation of the basal metabolic rate (BMR), and in the metabolism of proteins, lipids and carbohydrates. When food is limited, and energy demands exceed energy intake, T4 conversion into T3 is substantially reduced, so that free and total T3 concentrations decrease (Eales, 1988; see review by Chatzitomatis et al., 2017). This down-regulation of T3 allows animals to conserve their energy reserves and thereby increase the chance that they can meet energy demands. By contrast, when food is not limited, and energy intake exceeds energy expenditure, total T3 concentrations are up-regulated in response to increased energy demands — i.e., during molting (Gobush et al., 2014), somatic growth (Behringer et al., 2014), testicular development (Wagner et al., 2008), mating (Cristobal-Azkarate et al., 2016), pregnancy (Chatzitomatis et al., 2017; Glinioer, 1997), and exposure to low temperatures (Cristobal-Azkarate et al., 2016).

Traditionally, TH concentrations have been determined in blood or urine (Behringer et al., 2014; Genin and Perret, 2000; Ortiz et al., 2010; Wingfield et al., 2003), but collecting blood or urine in wild animals is not always feasible. Because THs are excreted in the bile of birds and mammals (DiStefano, 1988; DiStefano and Sapin, 1987; Taurog et al., 1951), their concentrations can be determined in feces, providing a valuable non-invasive tool to measure an animal's energetic condition. Recent studies have validated and applied fecal determination of T3 metabolites in several species including killer whales (*Orcinus orca*), monk seals (*Monachus schauinslandi*), caribou (*Rangifer tarandus*), and northern spotted owls (*Strix occidentalis caurina*) (Ayres et al., 2012; Gobush et al., 2014; Hayward et al., 2011; Joly et al., 2015). Four studies have measured T3 metabolites in primate feces: two have been conducted in wild populations and two in captivity (wild barbary macaques, *Macaca sylvanus*, Cristobal-Azkarate et al., 2016; wild howler monkeys, *Alouatta palliata*, Dias et al., 2017; captive howler monkeys, *Alouatta palliata*, Wasser et al., 2010; captive yellow breasted capuchins, *Sapajus xanthosternus*, Schaebs et al., 2016).

Here we validate the measurement of T3 metabolites in the feces of wild savannah baboons (hereafter “baboons”). Baboons are large semi-terrestrial monkeys that are wide spread

across sub-Saharan Africa (Henzi and Barrett, 2003; Jolly, 1993). They are eclectic omnivores with a diverse diet that encompasses grass, fruits, flowers, shrub leaves, tree gum, and insects (Alberts et al., 2005; Altmann, 1998; Altmann, 2009; Norton et al., 1987). Baboons are considered nonseasonal breeders, as they breed throughout the year, but nevertheless exhibit slight seasonality in their reproduction that is a function of food availability and their energetic reserve (Alberts et al., 2005; Altmann, 1980; Bentley-Condit and Smith, 1997; Bercovitch and Harding, 1993; Janson and Verdolin, 2005; Melnick and Pearl, 1987). Pregnancy and lactation are highly energetically demanding states in human and non-human primates (Altmann, 1980; Prentice et al., 1996; Thompson, 2013). During pregnancy, extra energy is needed to cover the costs of the development and maintenance of the fetus and associated tissues (i.e. placenta, enlargement of blood volume, enlargement of the uterus), as well as the costs associated with the increase in BMR (Butte and King, 2005; Cikrikci et al., 1999). During lactation, extra energy is needed for milk production as well as infant carrying (Altmann, 1980; Barrett et al., 2006; Butte and King, 2005). A corollary to these costs of reproduction in humans and other nonseasonal primates is that females transition from amenorrhea to cycling, and from cycling to pregnancy, only when they have a surplus of energy (Ellison, 2003; Rosetta et al., 2010; Thompson et al., 2012; Valeggia and Ellison, 2004; 2009).

Baboons, like humans and other nonseasonally breeding primates, show evidence that their reproduction is energy limited. Female baboons are more likely to start cycling (onset of puberty) or to resume cycling following post-partum amenorrhea, in periods of food abundance, after they have regained a positive energy balance (Alberts et al., 2005; Gesquiere et al., submitted). The probability of conception may also vary with rainfall and body condition (Beehner et al., 2006b; Bercovitch, 1987; but see Gesquiere et al., submitted). Social factors such as dominance rank and group size also affect a baboon's access to food, their energy balance, and consequently their fitness. For example, high-ranking female baboons mature earlier, have shorter inter-birth intervals (IBIs) between successive live births, and show higher offspring survival than low-ranking females (Altmann and Alberts, 2003), advantages that seem to result from their priority of access to food resources and their higher foraging efficiency (Barton, 1993; Post et al., 1980). By contrast, female baboons in large groups spend more time foraging (likely a consequence of within-group competition for food resources), mature later, and have longer IBIs than those in smaller groups (Altmann and Alberts, 2003; Bulger and Hamilton, 1987; Wasser and Starling, 1988). These data all point to energetic condition as being key for understanding how reproduction is regulated by environmental and social factors in baboons.

To estimate a baboon female's energetic condition in this study, we measured concentrations of total fecal T3 metabolites (hereafter "mT3"). Our focus was on mT3 because this is the biologically active TH, and is excreted in feces in proportion to its synthesis (Wasser et al., 2010). We also measured total fecal T4 metabolites (mT4), but they were below detection levels in our samples (see Methods). We first investigated whether the commercially available radioimmunoassay (RIA) for total T3 previously used for measuring mT3 in fecal samples of other mammal species (Wasser et al., 2010) was well suited to measure mT3 in baboon feces. We conducted a validation of the T3 antibody by checking for parallelism, accuracy, and intra- and inter- coefficient of variation (CV) for fecal pools of captive and

wild baboons. Secondly, using feces from captive baboons, we assessed experimentally whether conditions of collection and storage used for steroid hormones in the wild are appropriate for mT3 measurements. Finally, we determined the biological relevance of mT3 concentrations, using left-over freeze-dried fecal powders from our steroid hormone analyses that were stored at -20°C for periods of time between 1 to 9 years after collection.

We next assessed the effect of season and reproductive status on mT3 concentrations in wild female baboons, taking into account the effect of time in storage. TH secretion is up-regulated in response to short-term energy demands, but will decrease under conditions of chronic energetic stress, leading to the following predictions. We predicted that mT3 concentrations should be lower during the dry season when food availability was reduced, as has been shown by other studies (Ayres et al., 2012; Cristobal-Azkarate et al., 2016; Joly et al., 2015; Schaebs et al., 2016; Wasser et al., 2010; see also review by Chatzitomaris et al., 2017). We also predicted that because of the high energetic costs of pregnancy and lactation, mT3 concentrations should be higher in pregnant and lactating females than in cycling females, as shown for human and non-human primates (Chatzitomaris et al., 2017; Dias et al., 2017; Glinioer, 1997, but see also Iwatani et al., 1987; Yamamoto et al., 1979 for an opposite pattern during lactation in humans).

2. Methods

2.1. Subjects

The fecal samples collected for the validation of the T3 assay came from adult captive and wild baboons. The captive baboons (*Papio ssp.*) were housed at Six Flags Safari Park, Jackson, NJ (4 males and 3 females). The wild baboons were females belonging to a well-studied population of individually-identified baboons in the Amboseli basin in Kenya; this population consists of yellow baboons (*P. cynocephalus*) that experience some admixture with neighboring populations of olive baboons (*P. anubis*) (Alberts and Altmann, 2001; Charpentier et al., 2012; Tung et al., 2008). Near-daily data on the demography, behavior and reproduction of the population have been collected over 45 years by the Amboseli Baboon Research Project (ABRP) (e.g. Alberts and Altmann, 2012; Gesquiere et al., 2007; see also www.amboselibaboons.nd.edu). Since December 1999, fecal samples have also been collected and used to determine steroid hormone concentration (e.g. Gesquiere et al., 2008; Khan et al., 2002). All data collection procedures were non-invasive, adhered to the laws and guidelines of Kenya (Research Permit NACOSTI/P/15/1973/7878), and were approved by the Animal Care and Use Committee at Princeton University (IACUC 1821), and at Duke University (IACUC A028-12-02).

The Amboseli basin in Kenya ($2^{\circ}40'S$, $37^{\circ}15'E$, 1100m altitude) is a semi-arid short-grass savannah ecosystem, characterized by a predictable five-month-long dry season (June–October) and a seven-month wetter period (November–May). The availability of food and drinking water progressively declines throughout the dry season, which is devoid of rain. The seven-month wetter period exhibits highly variable and unpredictable rainfall from month to month. The result of these rainfall patterns is highly seasonal variation in food availability for baboons, with periodic droughts when the rains fail. Some food sources are available all year (i.e. corms, tree gum, grass blade base, insects), while other are highly

seasonal (i.e. fruits, flowers, acacia seeds, grass blades) (Alberts et al., 2005; Altmann, 1998; Norton et al., 1987).

For the wild females, we assigned reproductive state based on records of female sexual swelling state (turgescence or deturgescence) and size, presence of menstrual blood, and the color of the paracallosal skin (Altmann, 1973; Gesquiere et al., 2007; see also the ABRP Monitoring Guide at www.amboselibaboons.nd.edu for details on data collection protocols). Menstrual cycles in female baboon are easy to identify by the successive turgescence (follicular phase) and deturgescence (luteal phase) of the sexual skin. Failure to cycle after 40 days and the absence of menstrual blood usually indicates that the female is pregnant (Beehner et al., 2006a). Pregnancy is then confirmed by the change in color of the paracallosal skin from black to pink, approximately two months after conception (Altmann, 1973). The average gestation length for female baboons is 177 days (Altmann, 1980). After birth, the female remains in a state of post-partum amenorrhea for an average of one year unless her infant dies, in which case the female usually resumes cycling about three weeks after the infant's death (Altmann et al., 1978).

2.2. Fecal collection and extraction

2.2.1. Fecal collection and storage: wild and captive baboons—For experimental testing of the collection and storage effects, a fecal sample was collected immediately upon defecation for 7 captive baboons from Six Flags Safari Park. Each sample was homogenized and divided into four aliquots: one was stored in an empty tube and 3 were stored in 95% ethanol (See Fig. 1 and section 2.4. for further explanation of these treatments). All aliquots were kept on ice, for less than 4 hours before they were frozen at -20°C . The samples were then shipped on dry ice to our lab at Duke University and kept at -20°C until processed as indicated in section 2.3.1. Processed samples were freeze dried and sifted to a fecal powder (Gesquiere et al., 2008; Khan et al., 2002).

The fecal samples from wild baboons were collected and stored according to our protocol used to determine steroid hormone concentration (e.g. Gesquiere et al., 2008; Khan et al., 2002; see also Gesquiere et al., 2017 for the details on the hormone lab protocols). In brief, samples from known individuals were collected immediately after defecation, mixed and placed in 95% ethanol (2.5 to 1 ratio of ethanol to feces), and kept at ambient temperature until return to our camp site (within 8 hours). Samples were then stored at 4°C for no longer than two weeks before being shipped to University of Nairobi where the samples were freeze dried and sifted to remove the vegetative matter, and stored as a fecal powder at -20°C . Here we analyzed mT3 concentrations in 377 fecal samples from 61 adult females collected between January 2008 and October 2015.

2.2.2. Fecal TH extraction—For each sample from both captive and wild baboons, we extracted 0.05 g of fecal powder into 5 ml of 70% ethanol. We then mixed the samples on a multipulse vortexer for 30 min and centrifuged the samples for 20 min at 3200 rpm. The supernatant was collected and the procedure was repeated once, and the second supernatant combined with the first one. We pipetted 2.5 ml of the ethanol extract, evaporated it and

added 250 µl of buffer (T3 standard '0' from the RIA kit), thereby concentrating the samples 10-fold as done by others (Wasser et al., 2010).

We assessed extraction recovery by adding 12,000 cpm of ^{125}I -T3 to 6 tubes containing 0.05 g of fecal powder from the wild baboons and incubating the mixture at ambient temperature for 1 hr prior to ethanol extraction. The radioactivity was measured in the ethanol extracts and we calculated the T3 extraction recovery.

2.3. T3 and T4 radioimmunoassays

Total mT3 concentrations were determined using the T3 ^{125}I kit (catalog # 06B254216, MP Biomedicals, Costa Mesa, CA), following the supplier's instructions. Per the manufacturer, the primary antibody cross-reacts 100% with L-triiodothyronine (T3), 0.18% with L-thyroxine (T4), 0.44% with 3,5-diiodothyronine, 0.01% with 3,3'-L-triiodothyronine (r-T3), and <0.01% for 3,5-diiodotyrosine, phenylbutazone, sodium salicylate, diphenylhydantoin and dicumerol. We validated the mT3 radioimmunoassay for use with both the captive and wild baboon fecal samples. We first assessed parallelism by running increasing dilutions of two fecal pools (one for captive, one for wild baboons) and by comparing the slope of the fecal pool dilutions to the slope of the standard curve. For the captive baboons, the fecal pool mT3 concentration was 408 ng/dl, and we used the following dilutions: 1:1, 3:4, 1:2, 3:8, 1:4, 3:16, 1:8, 1:16. For the wild baboons, mT3 concentration of the pool was only about one fifth of the captive pool (82 ng/dl), probably a consequence of the lower food intake in wild populations. In order to have a comparable number of dilutions that fall within the standards range (50–800 ng/dl), we spiked the wild fecal pool with a T3 standard to increase the initial T3 concentration to 882 ng/dl and used the following dilutions 1:2, 1:3, 1:4, 1:6, 1:8, 1:12, 1:16. The assay accuracy was assessed by spiking the T3 standards with either a fecal pool from captive or wild baboons (200 ng/dl and 50 ng/dl, respectively). The intra-assay coefficient of variation (CV) was evaluated by running in the same assay a captive (408 ng/dl) and wild fecal pool (28 ng/dl) multiple times (N=13 for captive, and N=8 for wild). The inter-assay CV was assessed by running in each assay three T3 controls (50, 100 and 200 ng/dl) and a captive fecal pool (115 ng/dl).

THs are preferentially excreted in feces as mT3, but mT4 can also be detected in some animal species (see Wasser et al. 2010), therefore we initially attempted to measure both mT3 and mT4 in the wild baboon feces. Total mT4 concentrations were determined using the T4 MAb ^{125}I kit (catalog # 06B254011, MP Biomedicals, Costa Mesa, CA, standards range: 2–20 µg/dl, assay sensitivity 0.76 µg/dl) following the supplier's instructions. However, even after concentrating the samples 20 times, mT4 concentrations were below detection level in our wild baboon feces (see Wasser et al., 2010, for comparable results in howler monkeys and other herbivores). We thus focused our subsequent analyses on mT3, which are excreted in feces in proportion to its production, and by consequence are a better estimate of TH function than mT4 (Wasser et al., 2010).

2.4. Effect of fecal collection and storage on mT3 concentrations

To avoid hormone degradation by bacteria, fecal samples are typically frozen at -20°C immediately following collection (Groh et al., 1993; Taylor, 1971). However, in field

conditions, access to a freezer is usually not an option, and samples are commonly stored at ambient temperature for several hours in 95% ethanol to prevent bacterial degradation of the hormones (Khan et al., 2002; Lynch et al., 2003; Whitten et al., 1998). Here we experimentally tested how collection and storage conditions encountered when working with wild populations affected mT3 concentrations (Fig. 1). Specifically, using the aliquots collected from the captive baboons, we treated the four aliquots as follows. The aliquot without ethanol (aliquot 1) and one of the aliquots stored in 95% ethanol (aliquot 2) were kept at -20°C ; the other two aliquots stored in 95% ethanol were kept for 8 hours at 4°C (aliquot 3) or at 25°C (aliquot 4) before being stored for 2 weeks at 4°C (Fig. 1). Aliquot 1 represented optimal conditions of collection and storage in a lab setting. Aliquots 2–4 tested aspects of field conditions for collection and storage. Specifically, aliquot 2 tested the effect of simple ethanol storage on mT3 concentrations. Aliquot 3 and 4 tested field conditions in which samples were kept in a cooler containing ice packs until return to camp site (aliquot 3) or when samples were kept at ambient temperature until return to camp site (aliquot 4).

2.5. Biological validation

We assessed whether seasonal variation in rainfall and female reproductive status affected mT3 concentrations in wild baboons by using 377 fecal samples from 61 wild adult females. These samples were the leftover freeze-dried fecal powder from samples collected between January 2008 and October 2015 for steroid hormone analyses. The freeze-dried fecal powder had been stored at -20°C for period of time from 1.3 years (for the samples collected in October 2015) to 9.1 years (for the samples collected in January 2008).

2.6. Statistical Analysis

Parallelism between standard curves and serial dilutions of fecal pools were determined by a test of the equality of two slopes (Neter et al., 1990). To assess the effect of collection and storage in the field, we compare mT3 concentrations of aliquots that were collected in presence or absence of ethanol and stored under different conditions of temperatures and duration, using the non-parametric Wilcoxon Signed Ranks Test. The effect of season and reproductive status on mT3 concentrations in wild females was assessed by running a Linear Mixed Model (LMM), with season (wet or dry), female reproductive status (cycling, pregnant, or lactating) and time in storage (number of months) as predictor variables, and baboon identity, hydrological year (the sample was collected) and assay number as random factors. Significance levels for all the tests were set at $p < 0.05$.

3. Results

3.1. Extraction efficiency and mT3 assay validation: parallelism, accuracy, and precision

Ethanol extraction efficiency of a fecal pool from wild baboons was $85.5\% \pm 0.9\%$ (Mean $\pm$ SD, $N=6$). The slope of a serial dilution of fecal pools from captive and wild baboons showed strong parallelism with the slope of the standard curve (captive: $F_{1,8}=0.310$, $p=0.593$, $N=12$, Fig. 2A; wild: $F_{1,9}=0.361$, $p=0.563$, $N=13$, Fig. 2B). The assay accuracy, assessed by spiking each standard with an aliquot of the captive and wild fecal pools, were $92.6\% \pm 9.8$ (Mean $\pm$ SD, $N=7$) and $104.6\% \pm 6.7$ (Mean $\pm$ SD, $N=7$), respectively. The intra-assay coefficients of variation (CV) were 3.22% ($N=13$) for a 408 ng/dl fecal pool from

captive baboons and 7.9% (N=8) for a 28 ng/dl fecal pool from wild baboons. The inter-assay CVs were 11.77% (N=14), 6.42% (N=14) and 4.84% (N=13) respectively for a 50, 100 and 200 ng/dl T3 control and 10.85% for a 115 ng/dl fecal pool from captive baboons (N=9).

3.2. Field collection and storage

mT3 concentrations were not affected by the addition of 95% ethanol to the tube (aliquot 2 vs. aliquot 1: Wilcoxon Signed Ranks Test $Z=-0.169$, $p=0.866$, $N=7$). Storage of the samples for 8 hours at 4°C (aliquot 3) or at 25°C (aliquot 4) followed by 2 weeks at 4°C, did not significantly affect mT3 concentrations when compared to samples in ethanol that were immediately frozen (aliquot 3 vs. aliquot 2: $Z=-0.676$, $p=0.499$, $N=7$, aliquot 4 vs. aliquot 2: $Z=-0.338$, $p=0.735$, $N=7$).

3.3. Biological validation

mT3 concentrations in wild baboons ranged between 28–349 ng/g feces (Mean±SD: 141 ± 56 , $N=377$), while captive baboons had mT3 concentrations ranging between 276–704 ng/g feces (Mean±SD: 454 ± 143 , $N=7$). mT3 concentrations were not predicted by the number of months in storage ($F_{1,57}=3.017$, $p=0.088$, $b=0.139$; Fig. 3A). That is, even after periods of storage for up to 9 years, mT3 concentrations did not appear different from those measured after a year of storage. As predicted, wild female baboons had lower mT3 concentrations during the dry season, a period where food availability is reduced ($F_{1,371}=72.374$, $p<0.001$, $b=-21.894$; Fig. 3B). Wild females' mT3 concentrations varied with their reproductive condition ($F_{2,352}=4.112$, $p=0.017$); during pregnancy and lactation, concentrations were significantly lower than during sexual cycling, but not different from each other (pregnant vs. cycling: $b=-9.619$, $p=0.011$; lactating vs. cycling: $b=-7.532$, $p=0.018$; lactating vs. pregnant: $b=2.086$, $p=0.554$; Fig. 3C).

4. Discussion

Taken together, our results showed that mT3 concentrations can be accurately measured in baboon feces, are stable under storage conditions used for steroid hormone analysis, and reflect known biological variation. Furthermore, mT3 concentrations can be assayed in freeze-dried fecal samples that have been stored for up to 9 years at -20°C .

Fecal pools from wild and captive baboons showed parallelism, accuracy and precision when using the total T3 antibody from the MP Biomedicals RIA kit, as was shown for feces of several other mammal species (Ayes et al., 2012; Gobush et al., 2014; Hayward et al., 2011; Joly et al., 2015; Wasser et al., 2010). By contrast, mT4 concentrations were below detectable levels (see Wasser et al., 2010, for similar results in howler monkeys and other herbivores). mT3 were relatively stable when stored in ethanol, and were not degraded even if the samples were kept for up to 8 hours at 25°C followed by 2 weeks at 4°C, conditions that have been used for steroid hormone analysis (Gesquiere et al., 2017; Khan et al., 2002; Lynch et al., 2003). Therefore, both steroid hormones and mT3 concentrations can be determined from a single fecal sample, and the method of collection and storage already in

place for steroid hormone determination can be used without further modification for mT3 analyses.

A particularly important and unexpected result from our study was that mT3 concentrations could still be determined in freeze-dried fecal samples that were stored for up to 9 years at -20°C . We had expected that mT3 concentrations would be below detection levels in fecal powder stored for more than 3 years, assuming that mT3 degraded at a similar and consistent rate than that reported by Schaebs et al. (2016). Indeed, these authors showed that mT3 concentrations of fecal powders stored for 17 months at -20°C decreased by 42.8% relative to original concentrations. Being able to retroactively determine mT3 concentrations in fecal powders stored for several years will allow researchers to obtain mT3 concentrations that are complementary to already available steroid hormone concentrations. The finding that season strongly predicted mT3 in this relatively small dataset is very encouraging and suggests that important biological pattern can be detected even using fecal powder stored for variable lengths of time. The possible confounding effect of the length of time in storage was taken in account as a fixed effect in our linear mixed model (see Methods), but did not significantly predict variation in mT3 concentrations in our wild female baboons.

The lower mT3 concentrations during the dry season are consistent with data reported by several authors who showed that in periods of low food availability, mT3 concentration decreased, presumably reflecting a metabolic strategy of energy conservation (Ayres et al., 2012; Cristobal-Azkarate et al., 2016; Joly et al., 2015; Schaebs et al., 2016; Wasser et al., 2010; see also review by Chatzitomatis et al., 2017). Because of the negative correlation between the hypothalamic-pituitary-adrenal (HPA) axis and the thyroid axis (Charmandari et al., 2005), these lower mT3 concentrations during the dry season are in agreement with previous data from our lab showing that both male and female baboons have elevated fecal GC (fGC) concentrations during the dry season, as food availability declines (Gesquiere et al., 2008; 2011). Baboons are highly adaptable, and in response to food scarcity they switch their diet from green grass blades and fruits to grass corms (the underground stem bases of grass) (Alberts et al., 2005). However, grass corms require extensive harvesting time relative to the nutrients they provide, and therefore their consumption results in an increase in the time spent foraging (Alberts et al., 2005; Altmann, 2009). The presumed increase in energy expenditure combined with a decrease in energy intake when animals switch to dry season foods such as corms will likely lead to a state of negative energy balance, and will result in a decrease in animals' energy reserves. The decrease in mT3 documented in this study supports this scenario, as does the deterioration in animals' body condition that we observe during the dry season (ABRP unpublished data). The decrease in mT3 that we have documented, in combination with the increase in fGC (Gesquiere et al., 2008; 2011) suggest that baboons employ behavioral and physiological strategies during the dry season that help to restore their energy balance and improve their energy condition.

We also found that mT3 concentrations varied with reproductive state in female baboons. However, in contrast to our predictions, we found that pregnant and lactating females had lower mT3 concentrations than cycling females. Lower mT3 in lactating female baboons, while in disagreement with data in wild howler monkeys (Dias et al., 2017), agrees with previous reports in humans (Iwatani et al., 1987; Yamamoto et al., 1979). This decrease in

T3 levels is unexpected in the context of the high energetic demands of lactation, and may reflect energy conservation strategies on the part of the mother. Lactating mothers may be compensating by increasing the local production of T3 directly in the mammary tissues, where it will provide the necessary energy for milk production, as suggested in rats, without involving a systemic increase in T3 for the mother (Jack et al., 1994). These authors showed that 5'-deiodinase – an enzyme that converts T4 into T3 – is present in the mammary tissue of lactating rats, suggesting a local increase in the conversion of T4 into T3 during lactation. Because iodine is essential for the synthesis of TH, another possible explanation for the low levels of T3 during lactation is the iodine deficiency that is commonly observed during lactation, as iodine is secreted in milk (Brown-Grant, 1957).

The low concentrations of mT3 in pregnant female baboons are also unexpected, as both human and non-human primate studies have reported elevated total T3 and T4 concentrations during pregnancy (Chatzitomaridis et al., 2017; Dias et al., 2017; Glinioer, 1997). So why would female baboons have low mT3 concentrations in an energetically demanding period such as pregnancy? First, it is possible that blood concentrations of T3 and fecal concentrations of mT3 are not tightly positively correlated in pregnant females. For example, maternal TH production increases to provide adequate TH for the fetus, resulting in an increase in circulating TH, while hepatic clearance and excretion of TH in feces may be reduced (DiStefano, 1988). However, findings from Dias et al. (2017) do not support this hypothesis, as they found an increase in fecal mT3 in pregnant howler monkeys. Another possibility is that species differences in TH metabolism occur, and the reduced concentrations of mT3 in pregnant baboons may reflect a particularity in TH metabolism in that species. For example, if pregnant baboons had less of 5' deiodinase – an enzyme that convert T4 into T3 – and more of 5 deiodinase – an enzyme that converts T4 to reverse T3 (rT3) and T3 into diiodothyronine (T2) – this could produce lower concentrations of both circulating T3 and fecal mT3. Finally, in Amboseli food is often limited, and it is possible that low levels of T3 in pregnant baboons are a consequence of iodine deficiency, or chronically low energy intake, or both. TH concentrations are highly influenced by food availability (Eales, 1988; see review by Chatzitomaridis et al., 2017). Reports of elevated TH during pregnancy have been documented in well-nourished women, and results may be different in undernourished populations. Unfortunately, to our knowledge no studies have measured TH in pregnant undernourished women. However, TH concentrations are tightly linked to changes in basal metabolic rate (BMR), and studies measuring BMR during pregnancy have been conducted in well-nourished as well as undernourished populations (Butte and King, 2005; Cikrikci et al., 1999). While in well-nourished women from England a steady increase in BMR was reported during pregnancy, in undernourished women from Gambia a decrease in BMR was seen in the first two trimesters of pregnancy followed by a small increase in the last few weeks of pregnancy. It is therefore possible that pregnant females from our wild baboon population exhibit energy sparing metabolic responses similar to that of undernourished women, with relatively low BMR and low mT3 concentrations. While pregnant and lactating female baboons in our population have lower mT3 concentrations than cycling females, these concentrations appear sufficient not to cause any significant health impairment of the infants, in contrast to the report of mental retardation

and impaired motor function in a child of mother with hypothyroidism (Smallridge and Ladenson, 2001).

In conclusion, we have established that mT3 can be reliably and accurately determined in baboon feces, providing a valuable tool to study energy condition in wild populations for which urine and blood collection are not possible. Because mT3 can provide a more direct and fine-grained measure of energetic condition than fGC concentrations (Wasser et al., 2010), researchers will now have a unique opportunity to examine both mT3 and fGC, potentially allowing the respective roles of energetic and psychosocial stressors to be disentangled, an approach that is not possible with fGC measure alone. The simultaneous measurement of mT3 and reproductive hormones (such as estrogen or testosterone) will also provide critical complementary information that will help shed light on the role of energetic factors on reproduction.

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- mT3 can be reliably and accurately determined in baboon feces using a commercial RIA
- Collection and storage used for steroid hormones can be used for thyroid hormones
- mT3 in freeze dried samples were not affected by storage at -20°C for up to 9 years
- Female baboons had lower mT3 in the dry season, and during pregnancy and lactation
- mT3 measurement in feces is a valuable tool to study energy balance in wild animals

Figure 1. Experimental design using feces collected from captive baboons to test the effect on mT3 concentrations of fecal collection and storage in the wild

Aliquot 1 represents optimal conditions of collection and storage in a lab setting. Aliquot 2 tested the effect of storage in ethanol on mT3 concentrations. Aliquots 3 and 4 tested field conditions in which samples were kept in a cooler containing ice packs until return to camp site (aliquot 3) or when samples were kept at ambient temperature until return to camp site (aliquot 4).

Figure 2. Comparison of a standard curve using the standards from the T3 MP Biomedical kit with a serial dilution of a fecal pool plotted on logit-log scale
 (A) fecal pool from wild baboons, standards: $y = -0.978x + 2.121$, $R^2 = 0.983$, fecal pool wild: $y = -1.043x + 2.256$, $R^2 = 0.997$ and (B) fecal pool from captive baboons, standards: $y = -0.914x + 1.924$, $R^2 = 1.000$, fecal pool wild: $y = -0.886x + 1.948$, $R^2 = 0.992$.

Figure 3. Wild baboon residual mT3 concentrations obtained from the LMM assessing the effect of (A) time in storage, (B) season and (C) female reproductive state

Female identity, year of sample collection and RIA assay number were entered as random factors. The residual values were calculated by included the predictors and random factor listed above minus the variable of interest (see Methods).