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RESEARCH ARTICLE

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Developmental and environmental modulation of fecal thyroid hormone levels in wild Assamese macaques (*Macaca assamensis*)

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Abstract

Thyroid hormones are key modulators of development, as well as mediators of environmental conditions, by regulating developmental processes and metabolism in primates. Hormone measurement in noninvasively collected samples, that is, feces and urine, is a valuable tool for studying the endocrine function of wildlife, and recent studies have demonstrated the feasibility of measuring thyroid hormones in fecal samples of zoo-housed and wild nonhuman primates. Our study aimed to (i) validate the measurement of immunoreactive fecal total triiodothyronine (IF-T3) in wild Assamese macaques (*Macaca assamensis*) and (ii) to investigate its developmental changes and its response to environmental changes, including stress responses, in immature individuals. Fecal samples and environmental parameters were collected from individuals of three social groups of wild Assamese macaques living at Phu Khieo Wildlife Sanctuary, Northeastern Thailand. Our study confirmed the methodological feasibility and biological validity of measuring IF-T3 in this population. Specifically, the biological validation demonstrated higher IF-T3 levels in immatures compared to adults, and higher levels in females during late gestation compared to the preconception stage. Our analysis of IF-T3 levels in developing immature macaques revealed a significant increase with age. Furthermore, we found a positive association between IF-T3 and immunoreactive fecal glucocorticoid levels, an indicator of the physiological stress response. Neither minimum temperature nor fruit abundance predicted variation in IF-T3 levels in the immatures. Our findings indicate the possibility for differing effects of climatic factors and food availability on thyroid hormone level changes in immature versus adult animals and in wild compared to experimental conditions. Overall, our study provides the basis for

Abbreviations: HPA, hypothalamic-pituitary-adrenal; HPT, hypothalamus-pituitary-thyroid; IF-T3, immunoreactive fecal total T3; T3, triiodothyronine; T4, thyroxine; total T3, sum of free and bound T3; TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone.

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further investigations into the role of thyroid hormones in shaping species-specific traits, growth, and overall primate development.

KEYWORDS
fecal thyroid hormones, noninvasive measurement, primate development, triiodothyronine, wildlife endocrinology

1 | INTRODUCTION

The thyroid gland produces thyroid hormones, primarily thyroxine (T4) and triiodothyronine (T3). T3 is considered the more biologically active thyroid hormone as it directly affects target cells and tissues (Hulbert, 2000; Köhrle, 1999). The biological activity of T3 depends on its free fraction; unbound T3 (free T3) is biologically active and can enter target cells and tissues, whereas bound T3 (bound to plasma proteins such as T4-binding globulin, transthyretin, and albumin) is biologically inactive. The free T3 fraction accounts for less than 0.3% of total T3 in the bloodstream (Hulbert, 2000; Köhrle, 1999). T3 levels in the body are regulated by a feedback loop involving the hypothalamus-pituitary-thyroid (HPT) axis. In brief, the hypothalamus releases thyrotropin-releasing hormone (TRH), which stimulates the pituitary gland to secrete thyroid-stimulating hormone (TSH). TSH in turn stimulates the thyroid gland to produce and secrete T3 and T4. The HPT axis continuously monitors T3 and T4 levels in the blood and adjusts TRH and TSH secretion as needed (Fliers et al., 2014; Ortiga-Carvalho et al., 2016).

The main function of T3 is to increase the cellular metabolic rate, leading to higher energy expenditure and heat production (Goglia et al., 1999; Kim, 2008). This increase in metabolic rate impacts the overall energy expenditure of the body, thereby helping to maintain energy balance (Alvarez-Crespo et al., 2016; López et al., 2013). In addition, T3 is involved in the regulation of energy homeostasis by increasing the metabolic rate, thereby increasing the rate at which energy is utilized, and by modulating the expression of genes involved in glucose and lipid metabolism (Dev et al., 2016; McAninch & Bianco, 2014). In addition, T3 plays a role in tissue growth and development during the fetal and neonatal periods (Dev et al., 2016; Forhead & Fowden, 2014; Zoeller et al., 2002), as well as in oxygen consumption (Dev et al., 2016; Pascual & Aranda, 2013). In post-natal life, T3 continues to regulate the metabolism of cells, tissues, and organs, for example, brain and heart (Bernal, 2005; Dev et al., 2016). T3 also cross-talks with other systems, for example, with the immune system by modulating the expression of genes involved in the immune response, enhancing the proliferation and activation of immune cells, and affecting cytokine production (Jara et al., 2017; Wenzek et al., 2022). Furthermore, there is evidence for a cross-talk between the HPT and hypothalamic-pituitary-adrenal (HPA) axes. Cortisol, the primary hormone released by the HPA axis, can have an inhibitory effect on the HPT axis, leading to a decrease in thyroid hormone production. This may occur through the inhibition of TRH and TSH release from the hypothalamus and pituitary gland, respectively (Anifantaki et al., 2021; Douyon & Schteingart, 2002; Tsigos & Chrousos, 2002).

Since T3 plays such a crucial role in the postnatal development of mammals, immature individuals in mammalian species, including humans, have higher levels of thyroid hormones compared to adults. This pattern has been documented in several taxa in the wild and captivity including humans, goats, buffaloes, seals, whales, elephants, chimpanzees, and bonobos (reviewed in Behringer et al., 2018). As individuals grow and develop, they face challenges and stressors that can alter T3 levels such as changes in diet, physical activity, exposure to toxins, and infections (Hackney et al., 2003). Therefore, changes in T3 levels—being a key hormone in the regulation of metabolism and growth—can impact development, and therefore affect life history.

In nonhuman primates, thyroid hormones have been studied also outside of the clinical context. These studies have provided insights into differences in thyroid hormone metabolism, TSH levels, and thyroid hormone-binding protein concentrations in nonhuman primates (Aliesky et al., 2013; Arbelle et al., 1994; Gagneux et al., 2001; Kaack et al., 1979; Seo et al., 1989; Whittow et al., 1979). Those studies were mainly conducted in blood samples. However, collecting blood samples can affect an animal's physiology and subsequently alter T3 levels, and moreover, is not feasible in many wild living mammals. With advancements of noninvasive sampling methods (Wasser et al., 2010), thyroid hormone measurement has gained popularity, for example, through the measurement of immunoreactive total T3 levels in nonhuman primate urine (e.g., Behringer et al., 2014; Sadoughi et al., 2021; Touitou et al., 2021b) and feces (e.g., Cristóbal-Azkarate et al., 2016; Schaebs et al., 2016; Thompson et al., 2017). These noninvasive methods have become particularly valuable for animal studies, specifically in wild animals (e.g., Lemos et al., 2020; Shi et al., 2021; Szott et al., 2020), as it allows for the repeated measurement of total T3 levels without the need for frequent blood draws or other invasive procedures.

In numerous mammalian species, fecal samples have been demonstrated to be suitable for investigating the broader patterns of thyroid hormone dynamics, encompassing factors like reproductive status or food availability (e.g., Gesquiere et al., 2018; Gobush et al., 2014; Kozlowski et al., 2020; Lemos et al., 2020; Liu et al., 2022; Mondol et al., 2020). Although, to our knowledge, serum and fecal T3 have not been measured together in any species, both measures demonstrate consistent biological patterns over the long term (reviewed in Behringer et al., 2018). Studies in rats and humans have consistently shown that a substantial proportion, ranging from 20% to 30% of the daily produced or administered

thyroid hormone is excreted in feces (DiStefano & Sapin, 1987; Fenneman et al., 2023; Shakespear & Burke, 1976). Additionally, a radiometabolism study in dogs revealed a substantial fecal excretion of T3, with a time lag of 24–48 h between T3 administration and peak excretion in feces. This time lag aligns with the 24 h peak clearance observed for fecal T3 in humans (Chopra et al., 1975; Wasser et al., 2010). These findings support existing evidence that fecal thyroid hormone measurements serve as a valuable noninvasive tool for assessing thyroid hormone dynamics. By providing an integrated measure over time, fecal measurements offer distinct advantages over blood measurements, as they are less susceptible to minor fluctuations, such as diurnal changes (Behringer et al., 2018).

Noninvasive measurements of thyroid hormone levels have provided valuable insights into various aspects of the biology of primates. For example, studies have investigated the thyroid hormonal regulation of individual adaptation to the environment (*Macaca sylvanus* Cristóbal-Azkarate et al., 2016; *Saimiri sciureus* Kaack et al., 1980; *Macaca mulatta* Liu et al., 2022; *Macaca fuscata*, *Alouatta palliata* Thompson et al., 2017). Changes in thyroid hormone levels have also been linked to energetic changes (*Pan paniscus* Deschner et al., 2020; *A. palliata* Dias et al., 2017; *Papio spec.* Gesquiere et al., 2018; *M. mulatta*, *Macaca fasciciularis* Sadoughi et al., 2021; *Sapajus xanthosternos* Schaebs et al., 2016), as well as thermoregulation (*Rhinopithecus roxellana* Chen et al., 2021; *M. sylvanus* Cristóbal-Azkarate et al., 2016; *Macaca assamensis* Touitou et al., 2021b) and reproductive status (*A. palliata* Rangel Negrín et al., 2021; *M. assamensis* Touitou et al., 2021b). Additionally, concentrations of thyroid hormones have been found to decline during development (*P. paniscus*, *Pan troglodytes* Behringer et al., 2014). Therefore, changes in urinary or fecal immunoreactive total T3 levels provide a valuable tool for investigating development, metabolism, and reproductive function in nonhuman primates. However, studies investigating these aspects are still rare in primates, particularly in the wild, and to our knowledge, the influence of environmental and demographic factors on thyroid hormone dynamics in immature individuals living in the wild has not been investigated yet.

Against this background, we conducted a study to establish baseline information on the effects of environmental conditions and developmental changes on T3 changes in immature Assamese macaques (*M. assamensis*) in their natural habitat. Assamese macaques are mainly frugivorous primates that live in large multi-male multi-female groups. They are forest-dwelling and predominantly arboreal with 90% of their activity time spent off the ground (Schülke et al., 2011). Females are philopatric, while males leave their natal group at 4 years of age on average (Anzà et al., 2022; Berghänel et al., 2015). Females of the study population at Phu Khieo Wildlife Sanctuary, Northeastern Thailand, breed seasonally with a distinct October to February mating season and a March to August birth season (Fürtbauer et al., 2010). The vast majority of births (79%) are confined to a 3-month period from April to June (Anzà et al., 2022; Fürtbauer et al., 2010; Touitou et al., 2021a). Females reach adult body size at 5–6 years of age, while males attain full size at 9–10 years of age (Anzà et al., 2022; Fürtbauer et al., 2010). The weaning age for this species is around 1 year (Berghänel et al., 2016; Ostner et al., 2013), and females typically give birth for the first time between the ages of 5–7 years (Anzà et al., 2022).

To investigate the impact of environmental conditions and development on changes in immunoreactive fecal total T3 (IF-T3), our first aim was to assess the analytical and biological validity of a commercial ELISA designed for the measurement of total T3 (the sum of free and bound T3) in human blood samples to reliably quantify T3 in fecal samples of Assamese macaques. The assay has already been applied for the measurement of total T3 in the urine and feces of other nonhuman primate species (e.g., Cristóbal-Azkarate et al., 2016; Deschner et al., 2020; Schaebs et al., 2016). To examine analytical validity, in a first step, we tested whether fecal alcoholic extracts generated from field extractions could be measured without matrix interference effects in the selected ELISA. In a second step, we examined whether the IF-T3 measurements would provide biologically valid results using a dual approach. First, based on findings in humans (Soldin et al., 2004), we compared IF-T3 levels during the cycling stage and late-gestation within individual females, expecting IF-T3 levels to be significantly elevated at the end of gestation compared to nonpregnancy levels. Second, we compared IF-T3 levels of adult individuals with those of immatures, predicting IF-T3 levels in immatures to be significantly higher than those in adult individuals, as shown for many other mammalian species (reviewed in Behringer et al., 2018). Finally, following the analytical and biological validation of IF-T3 measurements, we investigated the effects of demographic and environmental factors potentially affecting IF-T3 levels in immature Assamese macaques. If environmental factors affect IF-T3 levels of immatures and adults in a similar way, although the immatures are still growing, we expected to see a negative association between total T3 levels and minimum ambient temperature in immature individuals, as well as a positive association with fruit availability, based on patterns observed in adult nonhuman primates (e.g., Chen et al., 2021; Cristóbal-Azkarate et al., 2016; Gesquiere et al., 2018) and other adult mammals (reviewed in Behringer et al., 2018). Since stressors affect T3 secretion (reviewed in Behringer et al., 2018; Charmandari et al., 2005; Chrousos, 1992), we expected a negative association of fecal glucocorticoids (an indicator of the physiological stress response) and IF-T3 (Hackney & Dobridge, 2009).

2 | MATERIALS AND METHODS

2.1 | Study site and subjects

The study was carried out on a population of Assamese macaques that has been studied since 2005 at Phu Khieo Wildlife Sanctuary, Northeastern Thailand, located at 16°05–35′N and 101°20–55′E. The sanctuary is part of the >6500 km^2 connected and protected forest of the Western Isaan Forest Complex. At the study site Huai Mai Sot Yai (16°27′N, 101°38′E, 600–800 m a.s.l.) the pristine forest

comprises mainly hill and dry evergreen forest with patches of bamboo and dry dipterocarp forest (Borries et al., 2002; Koenig et al., 2004; Kumsuk et al., 1999). The climate is characterized by a cold dry season from November through mid-March with minimum temperatures in January just above freezing sometimes and a warm rainy season (1374 mm/year) with peak precipitation in May and September (Richter et al., 2016).

Between November 2017 and June 2019, fecal samples were collected from three study groups. Group composition in April 2019 were for the first group 14 adult males, 20 adult females, and 52 immatures, for the second group 7 adult males, 14 adult females, and 26 immatures, and for the third group 8 adult males, 10 adult females, and 23 immatures. Immatures included in this study were of two different developmental stages, infant and juvenile. The infants were immatures born between April–June 2018 and between 6 and 13 months old at the beginning of immature sample collection. The juveniles were immatures born between March–July 2014, and consequently 4.5–5.1 years of age at the time of sampling. Females in this population are considered adult from their first conception onwards and males when they reached adult body length.

As environmental conditions, such as temperature were found to affect thyroid hormone levels, and climatic factors further affect food and fruit availability, we collected data on daily minimum ambient temperature with an automated HOBO data logger (ranging from 10°C to 22.5°C during the period of sample collection of immature individuals). At the middle of each month, we assessed fruit availability based on phenological scores from samples of more than 650 food trees and tree species abundance scores from 21 ha of botanical plots and calculated a monthly fruit availability index according to methods described previously (Heesen et al., 2013). Using a linear relationship of fruit availability between 2 consecutive months, we were able to estimate a daily value of fruit availability (see Touitou et al., 2021a, for details). We also collected data on precipitation with a data-logging rain gauge (HOBO), however, due to strong correlation of this factor with the other environmental factors (variance inflation factor > 41), we were not able to include it directly into the statistical model (see below).

2.2 | Fecal sample collection, extraction, and analysis

We collected 16 samples from eight adult females for validation purposes (A): Comparing IF-T3 levels in late gestation with pregestation levels within a female; see aims. (B) 247 fecal samples were collected from 28 immatures (11 females, 17 males), and 21 samples from five adult males and five lactating females for comparing IF-T3 levels in immatures versus adults; see aims. Samples, collected immediately after defecation and only when uncontaminated with urine, were homogenized manually before approximately 1 g was transferred into a 15 mL PPT tube containing 5 mL 90% ethanol. The samples were shaken by hand for 1-min to create a fecal suspension and kept in the dark until extraction on the same day.

Sample extraction was performed in the field camp following the protocol described in Shutt et al. (2012). In brief, we manually shook the fecal-ethanol suspension horizontally for 5 min, and then centrifuged it at 5000 rpm. Afterward, we pipetted 1 mL of the supernatant into a 2 mL PPT tube and stored it at −20°C until shipment to the endocrinology laboratory of the German Primate Center, Göttingen, Germany, for hormone analyses.

Before IF-T3 measurement, 130 µL of fecal extract was transferred to a glass tube, dried down under an air stream at 35°C, and resuspended in 130 µL of sample diluent (standard 0 of the total T3 ELISA kit, as recommended by the assay supplier and used in other studies [Cristóbal-Azkarate et al., 2016; Schaebs et al., 2016]).

IF-T3 was measured with a commercial competitive T3 Total ELISA (EIA-4569 DRG Instruments GmbH; the assay is identical with Ref. RE55251, IBL International GmbH) which has already been successfully used to measure T3 in feces and urine of other macaque species (Cristóbal-Azkarate et al., 2016; Sadoughi et al., 2021), and in urine of our study species (Touitou et al., 2021b). The assay was performed according to the manufacturer's instructions, and each sample was measured in duplicate. Interassay coefficients of variation (CV) calculated from the repeated measurement of high and low-value quality controls provided by the supplier were 3.6% and 9.7%, respectively ($N = 7$ plates). The intra-assay CV, calculated as the average value of CVs from sample duplicates was <10%. IF-T3 concentrations were expressed as nanogram hormone per gram feces.

2.3 | Analytical validation of IF-T3 measurements

To examine the analytical validity of the human total T3 ELISA for the measurement of IF-T3 in alcoholic fecal extracts of Assamese macaques, and specifically to test whether the fecal sample matrix would show potential interferences with the assay, we conducted a linearity/parallelism test. For this, we used fecal extract pools prepared from individuals of different age/sex categories, namely adult males, nonpregnant females, pregnant females, infant females, infant males, and male/female juveniles. Since previous studies in other nonhuman primates, macaques and capuchins, found IF-T3 levels to be generally low (Schaebs et al., 2016; Wasser et al., 2010), for the parallelism test we initially concentrated fecal pool samples by a factor of 10. We then assessed the degree of parallelism in these samples by comparing serial 1:2 dilutions from the concentrated pools to serial dilutions of the total T3 standard, and tested the dilution and standard curves for differences between slopes (Cohen et al., 2013; Soper, 2006).

2.4 | Fecal glucocorticoid metabolite (fGCM) measurement

To assess the effect of HPA axis activity on IF-T3 levels, we used fGCM levels generated from the same alcoholic extracts as used for

IF-T3 analysis. The fGCM measurements were performed before the IF-T3 analysis as part of a separate study (Anzà et al., 2021) using a validated enzyme immunoassay for immunoreactive 11ß-hydroxyetiocholanolone (Ganswindt et al., 2003), a major metabolite of cortisol found in the feces of numerous primate species (Heistermann et al., 2006), including Assamese macaques (Ostner et al., 2008). The assay was performed as described by Heistermann et al. (2004) using duplicate measurements of each sample and sample dilutions of 1:200 to 1:4000 depending on concentration. Intra- and inter-assay CVs, calculated from the repeated measurement of high- and low-value quality controls, were <10% and <15%, respectively. fGCM results are expressed as nanogram hormone per gram feces.

2.5 | Statistical analysis

We compared IF-T3 levels within a female (pregestation vs. late gestation state) using a two-tailed paired *t*-test.

To examine age-related differences in IF-T3 concentrations as part of our biological validation (B) and to investigate the influence of demographic (age and sex), metabolic (fGCM levels) and environmental parameters (fruit availability, minimum temperature, daytime) on IF-T3 levels in immatures, we ran two linear mixed models (LMM) (Baayen, 2008). The models were fitted in R 4.1.0 (R Development Core Team, 2022) using the R-package lme4, function "lmer" (Bates et al., 2015).

In our first model, we compared IF-T3 levels of immatures ($N = 210$ samples) with those of adults ($N = 37$ samples). We fitted log-transformed IF-T3 levels as response variable and controlled for sex (female and male), time of day at fecal collection (z-transformed), and the day of sample collection, age category was added as a predictor with two levels (immature or adult). Individual identity was included as random effects to control for repeated sampling. We compared the full model with the null model to investigate the significance of our fixed effect age category using a likelihood ratio test (Dobson & Barnett, 2008). In the null model only age-class category was excluded, all other variables were retained.

We ran a second LMM to explore demographic (age and sex), metabolic (fGCM levels) and environmental factors (fruit availability, minimum temperature, and daytime) explaining variation in IF-T3 levels in immature individuals. The full model included as the response variable log-transformed IF-T3 levels. We included in the model as predictor variables fGCM concentrations (measured in a previous study, Anzà et al., 2021), individual's age at the time of sampling (continuously, z-transformed to achieve comparable estimates, Schielzeth, 2010), sex (female and male), a daily fruit abundance index (details see above), and minimum ambient temperature (at each day). We also included the time of day of sample collection to control for potential circadian variation on IF-T3 levels. To limit type I error rates to a nominal level of 5% (Barr et al., 2013), we included individual and group as random effects, with random slopes for age at sample collection. We compared the full model with the null model to investigate the significance of each fixed effect using a likelihood ratio test (Dobson & Barnett, 2008). The null model excluded the predictor variables while retaining the random effects and the random slopes.

In both models, we assessed the required homogeneity of residuals and normal distribution by visual inspection of the q–q plot of the residuals and by plotting residuals against fitted values. These tests revealed no deviation from the assumption. We also evaluated collinearity by determining variance inflation factors (VIF) (Field, 2009) using the function "vif" from the R-package "car" (Fox, 2011), which revealed that collinearity was not an issue (model 1 maximum VIF: 2.3; model 2 maximum VIF: 2.9). We further examined model stability by excluding levels of the random effects one at a time from the model and comparing the resulting model estimates for these data with those of the full data set. The results revealed no indication of any influential levels of random effects to exist. Significance for all tests was set to $p = 0.05$. We used ggplot from the packages "ggplot2" to plot results of the model.

3 | RESULTS

3.1 | Analytical validation of IF-T3 measurements

Serial dilutions of concentrated fecal extracts from samples of all age classes and sexes gave displacement curves that run parallel to the total T3 standard curve with no differences in slopes between displacement curves and standard curve ($t = 0.0554–1.3411$, all $p > 0.2$) (Figure 1).

3.2 | Biological validation of IF-T3 measurements

3.2.1 | IF-T3 increases from preconception to late gestation

Figure 2 shows the IF-T3 levels recorded for the preconception and late gestation stage in eight female Assamese macaques. Although IF-T3 levels varied among females, in all individuals, levels were significant higher during the late gestation stage compared to the preconception stage ($t = -5.344$, $N = 8$, $p = 0.00107$). On average, IF-T3 levels increased by 48.7% (range: 9.9%–128.2%). The percentage of increase in IF-T3 levels was not related to the preconception level.

3.2.2 | IF-T3 levels of adults are lower than those of immatures

In immatures, IF-T3 levels were significantly higher compared to adults ($\chi^2 = 5.13$, $df = 1$, $p = 0.02347$), despite the fact that samples of adults included gestating females (Figure 3). Although in both age classes, IF-T3 levels showed a large variation and values overlapped (Table 1). IF-T3 levels in immatures were on average about two-fold higher than those of adults (Table 1).

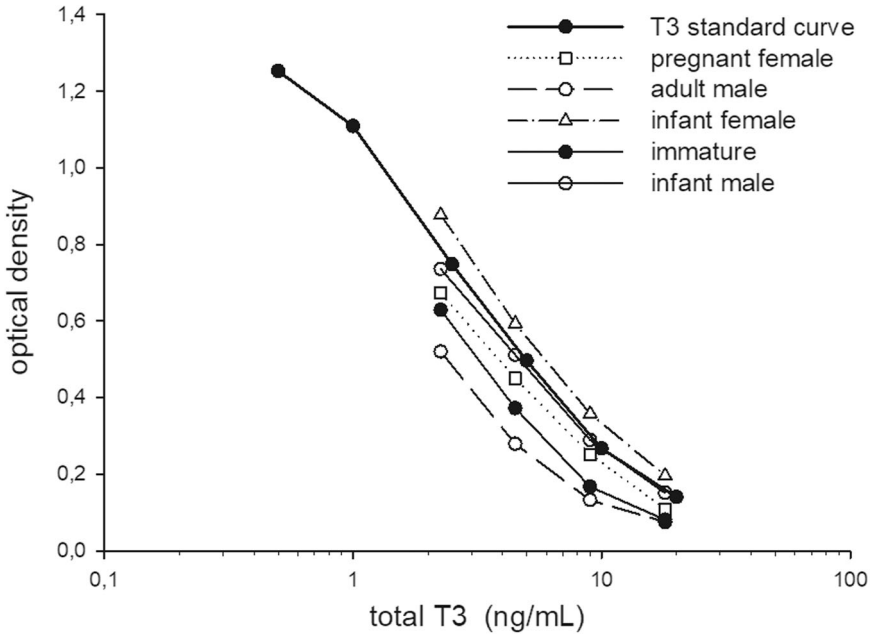

FIGURE 1 Parallel displacement curves of serial dilutions of concentrated fecal extract pools generated from various age/sex categories of wild Assamese macaques (see Section 2) in the total triiodothyronine (T3) ELISA. *x*-axis is log-transformed.

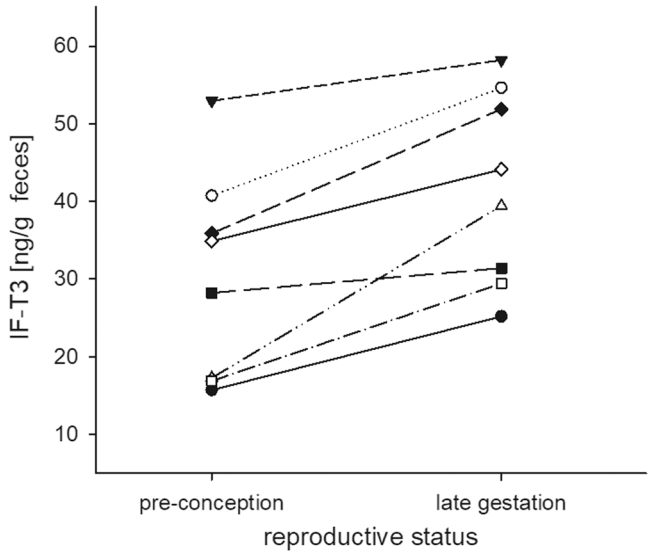

FIGURE 2 Biological validation (A): Immunoreactive fecal total triiodothyronine (IF-T3) levels of eight adult female Assamese macaques during the preconception and late gestation stage. IF-T3 levels were significantly increased at the end of gestation compared to preconception.

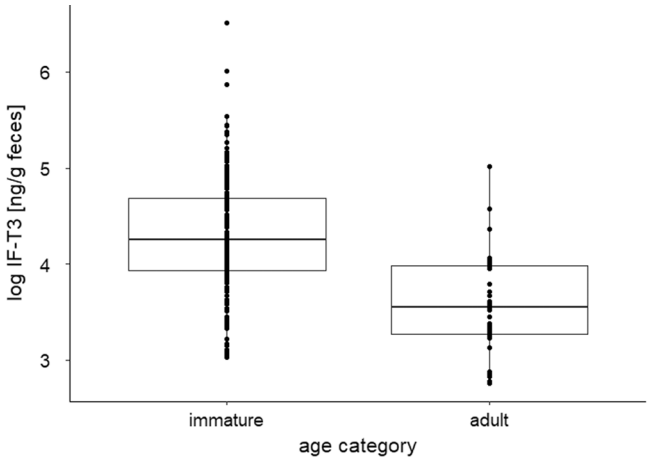

FIGURE 3 Biological validation (B): Log transformed immunoreactive fecal total triiodothyronine (IF-T3) levels of immature ($N = 210$ samples) and adult ($N = 37$ samples) Assamese macaques. IF-T3 levels were significantly higher in immature compared to adult individuals. Boxes indicate quartiles (25% and 75%), and vertical lines represent quantiles (2.5% and 97.5%).

3.2.3 | Variation in fecal T3 among and within immatures

In the 180 fecal samples of immatures, the comparison of the full model to the null model was significant ($\chi^2 = 35.005$, $df = 6$, $p < 0.001$). IF-T3 levels were positively associated with the age of immatures at sampling time ($p = 0.002$, Figure 4, Table 2).

IF-T3 levels were also positively associated with fGCM levels (fGCM; $p < 0.0001$) (Table 2, Figure 5).

All other predictors, daily fruit availability index, sex, minimum ambient temperature, and daytime at sample collection did not explain variation in immature IF-T3 levels (Table 2).

4 | DISCUSSION

Our study had two aims, the first was to validate a commercial ELISA for measuring total T3 in fecal samples of wild Assamese macaques, and the second was to investigate demographic (age and sex), metabolic (fGCM levels) and environmental factors (fruit availability,

TABLE 1 Immunoreactive fecal triiodothyronine (T3) levels in adult and immature Assamese macaques.

Age class	Sample no.	Mean	Median	Std. Dev.	Std. Error	Max.	Min.
Adult	37	41.2	34.9	25.8	4.2	150.0	15.7
Immature	210	98.5	74.1	80.7	5.6	668.7	16.7

Note: Values are given in ng/g feces

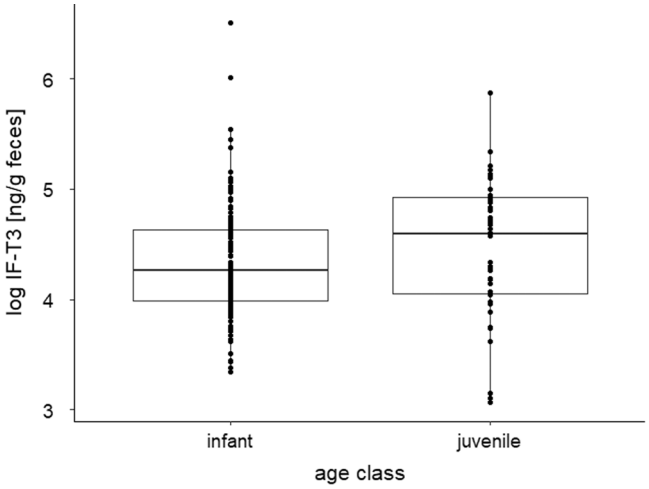

FIGURE 4 Log transformed immunoreactive fecal T3 (IF-T3) levels in infant (0.5–1 year of age) and juvenile (4.5–5 years of age) Assamese macaques ($N = 180$ samples of 27 immatures; 11 females, 16 males). Boxes indicate quartiles (25% and 75%), and vertical lines represent quantiles (2.5% and 97.5%).

minimum temperature, and daytime) that may explain variation in IF-T3 levels in immature individuals of a wild-living population of this species. First, the validation part of our study demonstrated the analytical and biological validity of measuring IF-T3 levels in wild Assamese macaques. In the second part, which focused on IF-T3 levels in developing immatures, our results revealed a positive association of IF-T3 levels with immature age, with infants having lower levels than juveniles. Additionally, IF-T3 levels of immatures were positively associated with fGCM levels. Overall, IF-T3 levels in immatures showed a large variation, but this variation was not explained by sex, fruit availability or minimum ambient temperature.

Validation of a method in a given species, such as measuring thyroid hormones in wild Assamese macaques, is crucial for establishing reproducibility and confidence in study results (Behringer & Deschner, 2017; Buchanan & Goldsmith, 2004; Goymann, 2005; Higham, 2016; Palme, 2005). Our validation results, including testing for potential fecal matrix effects and two biological validations, indicated and provide assurance that the selected ELISA designed for total T3 analysis in human blood samples is also valid for the noninvasive assessment of T3 in fecal samples of wild Assamese macaques. Thus, our data confirm findings from other studies, in which this particular ELISA has been used successfully to monitor T3 levels in noninvasively collected samples in other macaque species (Cristóbal-Azkarate et al., 2016; Sadoughi et al., 2021). The general validity of the established IF-T3 assay is also confirmed indirectly by the finding that the IF-T3 levels (range: 15–700 ng/g feces) measured in our Assamese macaque samples are consistent with those recorded in previous studies (range: 4–450 ng/g feces) in nonhuman primate feces using various assays (Chen et al., 2021; Cristóbal-Azkarate et al., 2016; Dias et al., 2017; Gesquiere et al., 2018; Liu et al., 2022; Schaebs et al., 2016; Thompson et al., 2017; Wasser et al., 2010).

The finding of higher levels of IF-T3 in immatures compared to adults in our study is consistent with previous mammalian studies, which have shown that immature individuals generally have higher levels than adults (reviewed in Behringer et al., 2018; Brown et al., 2007; Flower et al., 2015; Nilssen et al., 1985). Similar patterns have been observed in humans (Gunapalasingham et al., 2019; Verburg et al., 2011; Walsh, 2022) and other nonhuman primate species (Behringer et al., 2014). The variation of IF-T3 levels among immature individuals compared to adults was found to be large in our study. Similarly, in humans, the T3 reference range is also typically wider in children than in adults (Walsh, 2022). Based on all these findings, we conclude that the commercial total T3 ELISA used and the general methods described here are suitable for the determination of T3 levels excreted into the feces of wild Assamese macaques.

In the second part of our study, which focused on T3 variation in immature individuals, we found lower levels of IF-T3 in infants (0.5–1 year of age) compared to 4-to-5-year-old juveniles. This difference in IF-T3 levels between our younger and older immature subjects may be related to higher metabolic rate in the older juveniles, as the stage of adolescence is an important time of growth and development in which thyroid hormones play a key role (Walsh, 2022). The potentially higher metabolic rate of older immatures may also be related to increased physical activity and independence from their mothers, which is in contrast to infants who rely on their mothers for movement and sleep in close contact with her (Pereira & Fairbanks, 1993). Additionally, body mass growth in congeneric species such as rhesus (*M. mulatta*, Vančata et al., 2000) and toque macaques (*M. sinica*, Cheverud et al., 1992) is significantly higher at the age of four compared to 1-year-old, and it is conceivable that this increased growth velocity may be linked to an overall increased metabolism modulated by elevated thyroid hormone levels. Overall, our findings suggest that thyroid hormones play an important role in the growth and development of immature Assamese macaques. Future studies should investigate the relationship between thyroid hormone levels, metabolic rate, and physical activity in different age groups of macaques to further improve our understanding of the role of thyroid hormones and the physiological mechanisms underlying growth and development.

TABLE 2 Linear mixed model results testing the influence of age at sampling time, sex, daily fruit abundance index, minimum ambient temperature, fecal glucocorticoid metabolites (fGCM), and daytime at collection in immature wild Assamese macaques on IF-T3 levels (ng/g of fecal T3).

Term	Estimates	SE	CI lower	CI upper	χ^2	*p*-value
Intercept	2.045	0.435	1.206	2.922		
Age	0.195	0.054	0.085	0.308	10.007	**0.002**
Sex (male)	0.010	0.083	−0.157	0.162	−0.025	0.982
Daily fruit index	0.003	0.004	−0.006	0.011	0.410	0.522
Minimum temperature	0.013	0.020	−0.027	0.051	0.417	0.519
fGCM	0.398	0.076	0.248	0.542	24.837	**<0.001**
Daytime	0.024	0.038	−0.050	0.094	0.414	0.520

Note: Estimates, standard error (SE), as well as lower (CI lower) and upper (CI upper) confidence intervals are included. Statistically significant effects are indicated in bold.

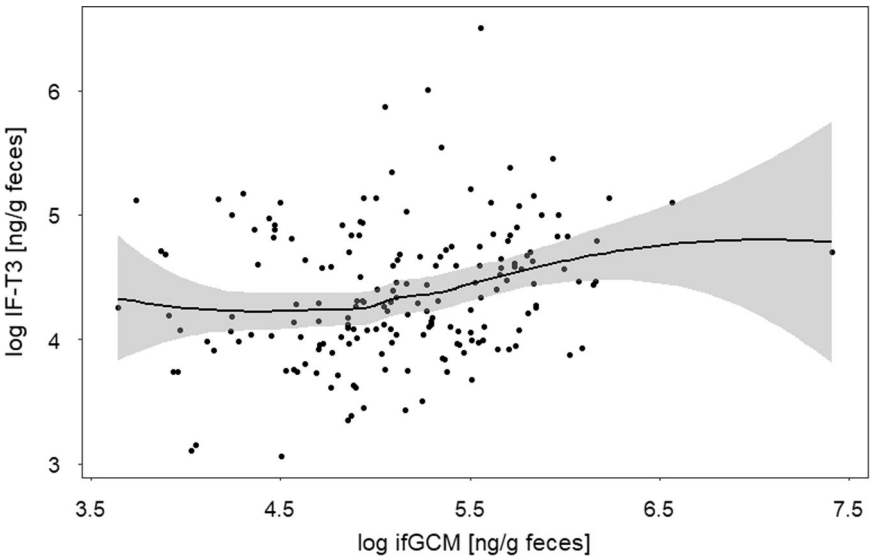

FIGURE 5 Relationship between log-transformed immunoreactive fecal T3 (IF-T3) levels and log-transformed fecal glucocorticoid metabolite (fGCM) levels in immature Assamese macaques ($N = 180$ samples of 27 immatures; 11 females, 16 males). Data points represent individual hormonal measures with 95% confidence intervals shown in gray. A smooth trend line was used to fit the relationship.

Previous studies have explored the relationship between glucocorticoids and IF-T3 levels in mammals across different contexts, including thermoregulation and feeding strategies (e.g., Nilssen et al., 1985; Sadoughi et al., 2021; Touitou et al., 2021a). Positive and negative associations have been reported. Some studies found a negative association between the two hormones (Hackney & Dobridge, 2009), others have found a positive relationship, for example, in Hawaiian monk seals (*Monachus schauinslandi* Gobush et al., 2014), in two cetacean species (*Delphinapterus leucas* and *Balaena mysticetus* Hudson, 2019), and in free-ranging moose (*Alces alces* Jesmer et al., 2017), consistent with our findings. However, it's important to note that many of these studies were conducted in captive environments (du Dot et al., 2009; summarized in Jesmer et al., 2017; Sadoughi et al., 2021), and the associations between the hormones may differ when measured in wild animals.

In contrast to our results in immature individuals, no relationship was found between glucocorticoid levels and T3 levels measured in urine samples from adult females of the same study population (Touitou et al., 2021b). This discrepancy may be due to differences in sample matrix, sex, and age of subjects. The earlier study focused on adult females and found associations with rank and reproductive status (Touitou et al., 2021b), factors associated with T3 levels also observed in wild-mantled howler monkeys (Dias et al., 2017). Since our study focused on immature individuals who are not yet reproductively active and have not entered the adult dominance hierarchy, these factors may not have impacted T3 levels in our sample. This aligns with the state-dependent hypothesis, suggesting that animals adjust their behavior based on their current condition, which can vary between life stages (Jesmer et al., 2017). Furthermore, factors such as developmental stage, growth requirements, immune function, and the interplay between the immune system and the hypothalamic-pituitary-thyroid axis can influence thyroid hormonal responses differently in immatures compared to adults (Bernal, 2005; Dev et al., 2016; Jara et al., 2017; Wenzek et al., 2022). Additionally, immatures engage more in play behavior than adults (Berghänel et al., 2015), and exercise in humans has been shown to increase both cortisol and thyroid hormones (Hackney & Dobridge, 2009; Moore et al., 2005).

The choice of sample matrix may contribute to the different association patterns observed between glucocorticoids and T3 levels. Fecal hormonal excretion has a longer and more variable time-lag compared to urinary excretion patterns (Anestis, 2010; Behringer &

Deschner, 2017; Palme, 2005). As a result, the association pattern between hormones within a sample may differ depending on the sample matrix. Additionally, the duration of glucocorticoid release can influence the effect on thyroid hormone secretion, with acute increases in glucocorticoids inhibiting thyroid function but this effect disappearing after a few days (Nicoloff et al., 1970).

In conclusion, investigating the relationship between glucocorticoids and T3 levels requires consideration of various factors, including the individual's environment, demographics, and duration of stressor exposure. Thyroid function can be influenced by multiple stimuli, including negative feedback from thyroid hormones, temperature, and stressors such as food scarcity. As a result, the relationship between glucocorticoids and thyroid hormones may not be consistent in all situations.

In the current study, IF-T3 levels of immature individuals were independent of minimum ambient temperature. Several studies on mammals have reported seasonal changes in thyroid hormone levels, which were associated with changes in climatic factors such as temperature or food availability (e.g., Flower et al., 2015; Gabrielsen et al., 2015; Kozlowski et al., 2020; Mayahi et al., 2014). In primates, however, fecal T3 measurements yielded mixed results. For example, fecal T3 levels were negatively associated with temperature and rainfall in some wild-living species (Cristóbal-Azkarate et al., 2016; Gesquiere et al., 2018; Thompson et al., 2017), whereas in others for example, wild rhesus monkeys, fecal T3 levels were independent of ecological factors such as rainfall, sunlight, humidity, or day length (Liu et al., 2022). These differences could be the result of various factors, such as the degree and frequency of temperature fluctuations, as well as the coping mechanisms of the primates in response to temperature variation. Additionally, temperatures not dropping drastically below the thermoneutral zone of the species, differences in temperature associated with other environmental factors such as rainfall, and/or the fact that climatic changes often occur in combination with changes in food availability could also play a role. For instance, in our population of Assamese macaques both climatic conditions and fruit availability are highly seasonal (Fürtbauer et al., 2010; Richter et al., 2016). Assamese macaques are highly frugivorous (Heesen et al., 2013; Schülke et al., 2011), and high fruit availability is often associated with the rainy season (Heesen et al., 2013). If lower fruit availability results in lower energy intake, this will result in decreased thyroid hormone levels to lower metabolic rate and conserve energy (reviewed Behringer et al., 2018; Deschner et al., 2020; Sadoughi et al., 2021). Similarly, in another Assamese macaque population, living in Limestone Forest of Southwest Guangxi, China, the coldest month also coincided with minimum rainfall, but also with a peak in resting behavior (Li et al., 2020). Increased resting may be a behavioral adaptation to conserve energy (and would not lead to an increase in T3 levels during the cold). We have not measured behavioral responses to cold in our immature subjects, but similar behavioral strategies may be at play.

While our study provides initial insights into the developmental modulation of fecal thyroid hormone levels in wild immature Assamese macaques, there are several limitations to consider. First, our study was limited to a 6-month period, which may have missed some seasonal and environmental differences that could have been present during other parts of the year. Additionally, our study included only a few samples per individual, which limits our ability to examine individual-level variation and may have influenced the accuracy of our results. Finally, our sample size was relatively small, and our findings may not be generalizable to other primate species or to different populations of Assamese macaques. Despite these limitations, our study provides an important foundation for further investigations of thyroid hormone regulation in wild primates and the factors that shape their development and physiology.

5 | CONCLUSION

We validated the measurement of immunoreactive total T3 in feces of wild Assamese macaques. Furthermore, we found that glucocorticoid metabolite levels and age influenced total T3 levels in immature individuals. These findings provide the basis to investigate mechanisms of primate development and the role of hormones in shaping species-specific traits.

AUTHOR CONTRIBUTIONS

Verena Behringer: Conceptualization (equal); formal analysis (lead); investigation (equal); methodology (equal); validation (lead); visualization (equal); writing—original draft (lead); writing—review and editing (equal). **Michael Heistermann**: Methodology (equal); resources (equal); writing—review and editing (equal). **Suchinda Malaivijitnond**: Methodology (equal); project administration (equal); writing—review and editing (equal). **Oliver Schülke**: Conceptualization (equal); funding acquisition (equal); methodology (equal); project administration (equal); resources (equal); writing—original draft (supporting); writing—review and editing (equal). **Julia Ostner**: Conceptualization (equal); funding acquisition (equal); investigation (equal); methodology (equal); project administration (equal); resources (equal); writing—original draft (equal); writing—review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

We adhered to the American Society of Primatologist Principles for Ethical Treatment of Nonhuman Primates and we followed the American Society of Primatologists Code of Best Practices for Field Primatology. We further conform to Directive 2010/63/EU, and obtained permission for all aspects of our study from the National Research Council of Thailand and the Department of National Parks, Wildlife and Plant Conservation of Thailand (permits 0002/4139 June 9, 2017, 0002/2747 May 4, 2018, 0402/2798 October 4, 2019, 402/4707 October 2, 2020; 401/11121 August 26, 2021).

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