




Thyroid hormone fluctuations indicate a thermoregulatory function in both a tropical (*Alouatta palliata*) and seasonally cold-habitat (*Macaca fuscata*) primate

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Thyroid hormones boost animals' basal metabolic rate and represent an important thermoregulatory pathway for mammals that face cold temperatures. Whereas the cold thermal pressures experienced by primates in seasonal habitats at high latitudes and elevations are often apparent, tropical habitats also display distinct wet and dry seasons with modest changes in thermal environment. We assessed seasonal and temperature-related changes in thyroid hormone levels for two primate species in disparate thermal environments, tropical mantled howlers (*Alouatta palliata*), and seasonally cold-habitat Japanese macaques (*Macaca fuscata*). We collected urine and feces from animals and used ELISA to quantify levels of the thyroid hormone triiodothyronine (fT₃). For both species, fT₃ levels were significantly higher during the cooler season (wet/winter), consistent with a thermoregulatory role. Likewise, both species displayed greater temperature deficits (i.e., the degree to which animals warm their body temperature relative to ambient) during the cooler season, indicating greater thermoregulatory pressures during this time. Independently of season, Japanese macaques displayed increasing fT₃ levels with decreasing recently experienced maximum temperatures, but no relationship between fT₃ and recently experienced minimum temperatures. Howlers increased fT₃ levels as recently experienced minimum temperatures decreased, although demonstrated the opposite relationship with maximum temperatures. This may reflect natural thermal variation in howlers' habitat: wet seasons had cooler minimum and mean temperatures than the dry season, but similar maximum temperatures. Overall, our findings support the hypothesis that both tropical howlers and seasonally cold-habitat Japanese macaques utilize thyroid hormones as a mechanism to boost metabolism in response to thermoregulatory pressures. This implies that cool thermal pressures faced by tropical primates are sufficient to invoke an energetically costly and relatively longer-term thermoregulatory pathway. The well-established relationship

between thyroid hormones and energetics suggests that the seasonal hormonal changes we observed could influence many commonly studied behaviors including food choice, range use, and activity patterns.

KEYWORDS

energetics, temperature, thermal environment, thermoregulation, thyroid hormone

1 | INTRODUCTION

The thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) play a multifaceted role in the mammalian body, influencing a diverse array of processes including growth, reproduction, metabolism, and thermoregulation (Yen, 2001). Thyroid hormone levels impact an animal's energetic balance by increasing ATP turnover, reducing the efficiency of ATP synthesis, and as a result boosting basal metabolic rate (BMR) (for greater detail see Silva, 2003). This heightened cellular metabolism leads to both increased energy expenditure and heat production (Freake & Oppenheimer, 1995; Silva, 2003, 2006). As such, these hormones represent an important, and metabolically costly, thermoregulatory strategy in mammals (Jessen, 2001; Silva, 2006).

Exposure to cold temperatures stimulates the production of thyroid hormone (Silva, 2003, 2006; Tomasi, 1991). Under experimental conditions, sudden drops in temperature can lead to rapid increases in T_3 . However the physiological effects, including mitochondrial activity and increased metabolism, take from 2 to 10 days to fully manifest (Goglia, Liverini, De Leo, & Barletta, 1983; Oppenheimer & Samuels, 1983; Roodyn, Freeman, & Tata, 1965; Silva, 2006; Tata et al., 1963). For example, a single T_3 administration to rats (*Rattus norvegicus*) led to increases in BMR after a 1 day delay, with the effect peaking after 3 days and lingering up to 8 days (Tata et al., 1963). These well-documented lags indicate that thyroid hormones are a relatively longer-term (i.e., over days) strategy to cope with cold temperatures, in comparison to relatively shorter-term thermoregulatory responses such as shivering, vasomotion, or microhabitat selection that can occur within minutes. Thyroid hormone levels also decrease as environmental temperatures increase, illustrating that this is a plastic hormonal pathway that responds to changing conditions over time (Goglia et al., 1983; Tomasi, 1991).

The relationship between thyroid hormones and metabolism also extends beyond temperature regulation. Since these hormones increase animals' energetic requirements, elevated thyroid hormone levels are associated with increased caloric consumption (Pijl et al., 2001; Silva, 2003; Silvestri, Schiavo, Lombardi, & Goglia, 2005). Likewise, under caloric restriction animals decrease thyroid hormone levels as a strategy to lower BMR and conserve energy (Danforth & Burger, 1989; Eales, 1988; Fontana, Klein, Holloszy, & Premachandra, 2006). Given that free-ranging animals can experience changes in food availability across a year, thyroid hormone levels may change in response to severe seasonal food shortages.

Thyroid hormone levels also fluctuate in association with seasonal reproduction, although the exact nature of their role is not entirely clear (Barrett et al., 2007; Nakao, Ono, & Yoshimura, 2008). Thyroid hormones have been implicated as both an initiator of pathways leading to seasonal breeding, as well as a mechanism that halts reproduction (Nakao et al., 2008). For primates, a few seasonally breeding species show increases in thyroid hormone levels during or prior to the mating season, particularly for males (Cristóbal-Azkarate, Maréchal, Semple, Majolo, & MacLarnon, 2016; Kaack, Walker, & Walker, 1980; Petter-Rousseaux, 1984). This is consistent with the role thyroid hormones play in mammalian spermatogenesis (Wagner, Wajner, & Maia, 2009). As such, a combination of factors could lead to seasonal fluctuations in thyroid hormone levels under natural conditions. Our project will focus on the thermoregulatory aspects of thyroid hormone function in primates.

The majority of primates live in tropical habitats, although several species occupy higher latitudes and elevations with seasonally cold thermal pressures (Hanya, 2010; Hanya, Tsuji, & Grueter, 2013). Tropical environments do not have the large seasonal swings in thermal conditions seen at high latitudes. However, they do display distinct wet and dry seasons with more modest thermal changes. In comparison to the dry season, wet seasons are typically characterized by lower temperatures, reduced solar radiation, and higher precipitation (van Schaik & Pfannes, 2005; Wright & van Schaik, 1994). These thermal variables can also work in conjunction to create an even cooler perceived thermal environment (e.g., animals are "wet and cold") (Hill, Weingrill, Barrett, & Henzi, 2004). Although the majority of thermoregulation research has focused on heat stress in tropical mammals (e.g., Lovegrove et al., 2014; Tewksbury, Huey, & Deutsch, 2008) or cold thermal pressures in highly seasonal habitats (e.g., Lovegrove, 2005; Nowack, Wippich, Mzilikazi, & Dausmann, 2013), there is a growing body of evidence that mammals in milder tropical climates still experience cold thermal pressures (Dausmann, Glos, Ganzhorn, & Heldmaier, 2004; Takemoto, 2004; Thompson, Williams, Glander, Teaford, & Vinyard, 2014; Thompson, Williams, Glander, & Vinyard, 2016; Turbill, Law, & Geiser, 2003).

We investigated patterns of thyroid hormone variation in two primates. If fluctuations in thyroid hormone levels are related to their thermoregulatory function (via increased metabolism), then we predict: 1) there will be seasonal differences in thyroid hormone levels, with animals displaying higher values in the cooler season; 2)

animals will display a greater temperature deficit (the extent to which they warm their body temperature relative to ambient temperatures) during the cooler season; and 3) there will be a negative relationship between thyroid hormone levels and the temperature experienced by animals. We tested these predictions in two species: tropical-dwelling mantled howlers (*Alouatta palliata*) and seasonally cold-habitat Japanese macaques (*Macaca fuscata*). Despite howlers' tropical habitat, previous work has demonstrated that they experience seasonally cool thermoregulatory pressures (Thompson et al., 2014, 2016). Japanese macaques face warm temperatures and high humidity in the summer, and cold, snowy winters in their natural habitat (e.g., Hanya, 2010; Hanya, Kiyono, & Hayaishi, 2007). It is known that Japanese macaques' energy requirements increase with decreasing ambient temperatures (Agetsuma, 2000) and that they acclimatize to seasonally cold conditions through increasing pelage insulation (Hori, Nakayama, Tokura, Hara, & Suzuki, 1977), accumulating fat (Hanya, 2010; Hanya et al., 2013; Kurita, Shinomura, & Fujita, 2002; Muroyama, Kanamori, & Kitahara, 2006), and behavioral modifications (Hanya et al., 2007). This between-species comparison will allow us to investigate the extent to which primates in disparate thermal habitats display seasonal and temperature-related changes in thyroid hormone levels, which may ultimately impact metabolism and thermoregulation.

2 | METHODS

2.1 | Data collection

2.1.1 | Mantled howlers

Thyroid hormone levels were measured using urine samples collected from wild howlers at Hacienda La Pacifica, Costa Rica (10°28'N, 85°07'W). La Pacifica is a tropical lowland and seasonally dry deciduous forest with howlers inhabiting riparian forests and more seasonal dry, non-river forest patches (Glander, 1975). Based on weather monitoring from 1971 to 2006 (collected with a Davis Weatherlink Station), daily temperatures during the dry season (November–April) had an absolute range from 22.5 to 34.1°C (\bar{x} = 28.1°C) while wet season (May–October) temperatures had an absolute range from 21.8 to 33.8°C (\bar{x} = 26.1°C) (Glander, unpublished data; Daubenmire, 1972). Relative humidity in the dry season averages 42% compared to 94% in the wet season (Daubenmire, 1972). Mean annual precipitation is 1,368 mm (range: 831–2,701 mm), with heavy rains during the wet season and relatively little precipitation during the dry season (Daubenmire, 1972). More details of the thermal environment are provided in Thompson et al. (2016).

Data collection occurred in 2013 and 2014, representing the dry season (2/26–3/16/14), and wet season (5/22–5/31/13 and 7/11–7/23/14). Urine was collected by placing a plastic sheet underneath resting animals and pipetting samples immediately after voiding was observed. We collected a total of 49 samples across both seasons (dry: N = 30; wet: N = 19). Samples were most often collected in the morning (76% of samples before 12:00). Using regression curve estimation,

there was no significant relationship found between collection time and fT_3 levels. Animals were sampled opportunistically, based on the feasibility of capturing urine from their position in the canopy. We obtained samples from 11 different identified adult animals (\bar{x} = 4 samples/animal; range = 1–10), including N = 7 females and N = 4 males. Howlers in the study group were identified by distinctive collars or anklets placed during long-term work at the site (Glander, 2006).

We focused hormonal assays on freely circulating triiodothyronine (fT_3). fT_3 is the more bioactive hormone (Silvestri et al., 2005) and initial fT_4 assays yielded lower, nearly undetectable values, consistent with data from other mammals (Wasser et al., 2010). fT_3 levels were evaluated using ELISA kits (Item Code: 1650, Alpha Diagnostics Inc., San Antonio, TX). Intra-assay coefficient of variation (CV) was $4.26 \pm 4.7\%$; inter-assay CV was 8.71% over five assays. There was not a significant difference in slope between serial dilutions of samples and the standard curve (t = 0.65, p = 0.549), indicating parallelism. Spike recovery analyses were performed following Brown, Walker, and Steinman (2004). A single spike recovery test was performed in duplicate, using 16 pg/ml standard for the spikes. For howlers this yielded 100.2% recovery with a mean CV of 8.2% between duplicates. Previous studies established a relationship between serum and urinary levels of thyroid hormones for humans (Burke & Shakespear, 1976), but this relationship has not been examined for howlers. We measured samples' specific gravity with a handheld refractometer (PAL-10S, ATAGO Co., Tokyo, Japan) at collection (Anestis, Breakey, Beuerlein, & Bribiescas, 2009). Specific gravity measures urine density and serves as a control for urine dilution, which can lead to variation in measured hormone concentration. These types of corrections are standard in endocrine analysis (Anestis et al., 2009; Hodges & Heistermann, 2011; White et al., 2010).

We measured near-animal ambient temperatures for long-term periods prior to urine sample collection (2010–2012), as well as during urine sample collection (wet and dry seasons of 2014; but not 2013) by attaching an automated temperature logger (iButton: Maxim Integrated Products Inc., San Jose, CA) to an anklet worn by animals (2014: N = 6; total: N = 15) (Thompson et al., 2014, 2016). This placement has the advantage of measuring temperatures at the animal's exact location in the forest canopy, rather than at a stationary site. We also recorded subcutaneous temperatures (during 2014, but not 2013) by implanting a sterilized iButton directly under the skin (immediately deep to the hypodermis) between the shoulder blades while animals were under general anesthesia (Table 1) (Lovegrove, 2009; Thompson et al., 2014). While subcutaneous temperatures are more variable than core body temperatures, layers of the skin are known to act as thermosensors responsible for initiating thermoregulatory responses (Bratincsák & Palkovits, 2005; Romanovsky, 2014). Subcutaneous temperature loggers were implanted and extracted at the same time that near-animal loggers were fitted and retrieved during captures. We waited >14 hr postcapture to collect subcutaneous temperatures to avoid thermal effects of anesthesia on primates (Lopez, Gibbs, & Reed, 2002). Loggers were certified as accurate to $\pm 0.5^\circ\text{C}$ by the manufacturer with post-production between-logger precision reported as $\pm 0.4^\circ\text{C}$ (Johnson et al., 2005). Testing of our own loggers

TABLE 1 Summary of seasonal and daily minimum, maximum, and mean subcutaneous and ambient temperatures of mantled howlers at La Pacifica, Costa Rica, and Japanese macaques at Inuyama, Japan

	Subcutaneous (°C)					Ambient (°C)				
	Seasonal		\bar{x} daily (\pm SEM)			Seasonal		\bar{x} daily (\pm SEM)		
	Min	Max	Min	Max	\bar{x}	Min	Max	Min	Max	\bar{x}
Mantled howlers ^a										
Dry	35.2	40.9	36.0 (0.19)	39.1 (0.36)	37.3 (0.20)	24.9	44.9	27.0 (0.27)	36.4 (0.41)	31.2 (0.30)
Wet	31.0	42.6	35.4 (0.12)	39.6 (0.12)	37.1 (0.08)	22.3	42.6	25.9 (0.18)	36.0 (0.19)	30.1 (0.18)
Japanese macaques ^b										
Summer	32.9	41.4	34.5 (0.07)	39.9 (0.08)	37.2 (0.04)	12.4	39.6	21.0 (0.42)	30.0 (0.57)	25.0 (0.46)
Winter	27.6	42.1	31.0 (0.22)	39.9 (0.11)	35.7 (0.06)	-3.6	15.8	0.7 (0.27)	9.4 (0.31)	4.7 (0.24)

^aLong-term near-animal ambient temperatures collected during dry (February–March) and wet (July–August) seasons from 2010 to 2012 and 2014. Sample hours: dry: near-animal ambient = 827.0 animal hrs, subcutaneous = 190.5 animal hrs; wet: near-animal ambient = 2468.2 animal hrs, subcutaneous = 2432.2 animal hrs.

^bAmbient temperatures collected from the on-site weather station from December 2014 to February 2015 (Winter) and June–August 2015 (Summer). Sample hours: summer: ambient = 1605.0 hrs, subcutaneous = 7370.5 animal hrs; winter: ambient = 2155.5 hrs, subcutaneous (July–August only) = 5784.5 animal hrs.

after data collection revealed a mean pairwise difference between loggers (in the same location) of 0.02°C (SD = 0.68; $N = 129$ measurements from three loggers at 20.3–34.3°C). Loggers recorded at a resolution of 0.0625°C every 10 min.

We also sought to measure temperatures experienced by animals immediately prior to sample collection. However, due to the relatively short period of data collection, ambient temperatures from the above loggers were not available for urine samples obtained at the beginning of the data collection period (i.e., only samples collected on the fifth day of temperature collection would have four prior days of temperature data), leading to small sample sizes. As an alternative, we accessed public temperature data from the National Oceanic and Atmospheric Administration's (NOAA) National Climatic Data Center. These data were obtained from the nearest temperature monitoring station to the field site, collected in Liberia, Costa Rica, located 38.6 km away (straight line distance). This distance is a drawback, as these temperatures reflect general climatic patterns, rather than temperatures directly experienced by the animal (Thompson et al., 2016). However, utilizing this data set yields a sufficiently robust sample size to perform analyses, and provides temperature estimates for the 2013 wet season sample, during which on-site temperatures were not recorded. Only daily minimum and maximum temperatures were available from the NOAA location. With these data, we determined the minimum and maximum absolute temperatures that animals experienced 4 days prior to urine sample collection.

2.1.2 | Japanese macaques

Data were collected on semi-free ranging Japanese macaques housed at the Kyoto University Primate Research Institute (KUPRI) in Inuyama, Japan (35°23'N, 136°57'E). Animals were housed in an outdoor enclosure (~900 m²) exposed to natural thermal conditions. Winters (December–February) at KUPRI have mean daily temperatures of

5.7°C with mean daily lows of -1.9°C and mean daily highs of 15.6°C. The summer season (June–August) has mean daily temperatures of 27.3°C, with mean daily lows of 19.2°C and mean daily highs of 35.6°C (NOAA, 2016). Inuyama has a humid warm-temperate climate with an average precipitation of 407.2 mm in the winter and 496.4 mm in summer (NOAA, 2016). Provisioned feedings of monkey biscuits and other foods (e.g., sweet potato, carrots, etc.) occurred at roughly the same time each day (9:00 and 16:00 hr on weekdays; 13:00 on weekends) while water and ground foliage were available ad libitum.

Sample collection occurred during winter 2014 (12/11–12/23) and summer 2015 (7/14–7/26). As urine was comparatively more difficult to obtain for semi-terrestrial Japanese macaques than howlers, we collected fecal samples by observing defecation, identifying the animal, gathering samples, and freezing at -80°C until analysis. A total of 63 samples were collected across both seasons (winter: $N = 26$; summer: $N = 37$). Samples were most often collected in the morning (91% of samples before 12:00). Using regression curve estimation, there was no significant relationship found between collection time and fT_3 levels. We obtained samples from 24 animals ($\bar{x} = 3$ samples/animal; range = 1–9) identified via unique facial tattoos. This included $N = 15$ females and $N = 9$ males, aged 1–17 ($\bar{x} = 7.0$ yrs).

To assess fT_3 levels from fecal samples of Japanese macaques, we followed established procedures for thyroid hormone extraction (Hodges & Heistermann, 2011; Wasser et al., 2010). Wet samples were first homogenized and lyophilized to standardize hormone level by dry weight, then sifted through a 40 mesh sieve to remove plant matter (Hodges & Heistermann, 2011; Khan, Altmann, Isani, & Yu, 2002). We then added 0.1 gm fecal powder to 15 ml of 70% ethanol, vortexed for 30 min, and centrifuged at 2200 rpm for 20 min. The supernatant was decanted and the original pellet re-extracted; extracts were combined and stored at -20°C until assayed (Wasser et al., 2010). Similar to howlers, fT_3 levels were evaluated using ELISA kits (Item Code:1650, Alpha Diagnostics Inc.). The intra-assay CV was

$5.7 \pm 0.45\%$; inter-assay CV was 7.1% over five assays. There was not a significant difference in slope between serial dilutions of samples and the standard curve ($t = -0.53$, $p = 0.624$), indicating parallelism. Our spike recovery test (using the same procedure described for howlers) yielded 108.2% recovery with a mean CV of 5.9% between duplicates. Previous work has found that feces are a reliable and accurate medium for measuring thyroid hormone in a range of mammals (including howler monkeys) (Wasser et al., 2010), although this has not been verified for Japanese macaques specifically.

Ambient temperatures at KUPRI were recorded using an on-site weather station (HOBO U30, Onset Computer Corporation, Bourne, MA) located near the animals' enclosure (Table 1). Temperature accuracy is stated by the manufacturer to be $\pm 0.2^\circ\text{C}$, measured at a resolution of 0.03°C . Subcutaneous temperatures were recorded on five adult macaques ($N = 2$ males; $N = 3$ females) following the procedure given above for howlers and in accordance with KUPRI standard operating procedures. Using these data, we determined the minimum and maximum absolute temperatures that occurred within 4 days prior to fecal sample collection. Animal procedures for howlers were approved by NEOMED and GVSU IACUCs; for Japanese macaques by the KUPRI Ethics Committee and GVSU IACUC. All methods complied with legal requirements of Costa Rica and Japan, as well as the American Society of Primatologists' Principles for the Ethical Treatment of Primates.

2.2 | Data analysis

2.2.1 | Factors impacting fT_3

We first examined within-individual changes in fT_3 levels between seasons. For howlers we created the variable "adjusted fT_3 " to account for variation in urine concentration by performing an ANCOVA on fT_3 levels with z-transformed specific gravity as a covariate and saving predicted fT_3 values. For individual howlers sampled in both seasons of the same year (2014: $N = 6$), we compared mean adjusted fT_3 levels between seasons via a paired t -test. Similarly, a paired t -test of mean fT_3 levels was conducted for individual Japanese macaques sampled in both seasons ($N = 11$). This test allows us to attribute potential seasonal differences to actual within-animal changes in hormone levels.

To more holistically assess the impact of season, recently experienced temperature, sex, and individual variation on fT_3 levels, we used linear mixed modeling. This procedure is designed to incorporate unbalanced designs, as the seasons in our study had unequal samples sizes (Cnaan, Laird, & Slasor, 1997; Verbeke & Molenberghs, 2000). A separate linear mixed model analysis was performed for each species. Given our relatively low sample size for this statistical procedure, limiting the number of predictor variables in our models was particularly important to avoid overfitting (Matuschek, Kliegl, Vasishth, Baayen, & Bates, 2017). As such, our approach was to 1) select pertinent, biologically relevant predictor variables that are either known to influence thyroid hormone levels or are expected to under our hypothesis, and 2) following well-established recommendations (e.g., Johnson & Omland, 2004; Matuschek et al., 2017), use

information criterion as a model selection procedure to objectively and systematically determine which predictor variables comprise the models that best fit our data. We used a combination of the Bayesian information criterion (BIC) and Akaike's information criterion for small sample sizes (AICc) to select models. When AICc and BIC yielded conflicting candidates for the top models, the model with the lower log-likelihood estimate was selected (Kuha, 2004). The analysis considered all possible combinations (including full inclusion and exclusion) of the following variables: 1) season; 2) absolute minimum ambient temperature experienced by animals during the 4 days prior to sample collection (i.e., the lowest temperature in those four days); 3) absolute maximum ambient temperature experienced by animals during the four days prior to sample collection; 4) individual identity of the animal; and 5) sex. Variables 1–3 were categorized as climate variables, included in the analysis as variables of interest for assessing the links between fT_3 levels and their thermoregulatory function. It should be noted that ambient temperatures, rather than measures of body temperature, serve as the most direct external signal that initiates thermoregulatory responses. Variables 4–5 were included as animal-based variables, with the goals of (a) generating a better explanatory model, as these factors are known to influence either thyroid hormone levels and/or thermoregulatory ability, and (b) characterizing variability in fT_3 for our study species. In particular, sex is known to impact thyroid hormone levels in various primates (Kaack, Walker, Brizzee, & Wolf, 1979; Wasser et al., 2010). There is also evidence that primate sexes differ in their thermoregulatory strategies (Lubbe et al., 2014; Terrien, Perret, & Aujard, 2010). We also included z-transformed specific gravity in all howler models to control for variable dilution of urine samples. This approach, compared to simply adjusting raw concentrations by a dilution factor, avoids generating erroneous relationships between hormone levels and predictor variables that are actually due to a relationship with the dilution factor, rather than the hormone (Barr et al., 2005). Specific gravity and individual animal identity were designated as random factors in models; all others were treated as fixed factors. Collinearity was assessed via variance inflation factors (VIF).

2.2.2 | Seasonal temperature differences

We assessed seasonal differences in temperatures experienced by howlers in two ways. First, for howlers with attached loggers (sampled in 2014), we calculated each individual's mean daily near-animal ambient minimum, maximum, and mean temperatures experienced during the study. We compared temperatures between wet and dry seasons via two sample t -tests. However, since the actual days of urine sample collection that overlapped with near-animal ambient temperature recording were relatively low (dry: $N = 14$; wet: $N = 10$), we also used long-term near-animal ambient data from 2010 to 2012 and 2014 to more broadly assess overall seasonal differences in temperatures. For these data, we compared average daily minimum, maximum, and mean temperatures between wet and dry seasons via a two-way ANOVA controlling for individual. This test was done for both near-animal ambient and subcutaneous temperatures. We also calculated

animals' temperature deficits as the difference between simultaneous subcutaneous and near-animal ambient temperatures. We conducted a two-way ANOVA controlling for individual to compare temperature deficits between wet and dry seasons.

For Japanese macaques, we compared seasonal differences in daily minimum, maximum, and mean ambient temperatures (collected at the on-site weather station) via two sample *t*-tests. To compare subcutaneous temperatures between seasons, we conducted a two-way ANOVA including season and animal identity. Temperature deficits were compared between seasons via two-way ANOVA, controlling for individual. All statistical procedures were performed in SPSS.

2.3 | Comparability of interspecies fT_3 values

For this study, absolute fT_3 values are not directly comparable between mantled howlers and Japanese macaques, as hormone levels were evaluated through different media (urine vs. feces). While both of these media are standards for primate hormone analysis (Hodges & Heistermann, 2011), there are several key reasons values cannot be directly compared. 1) Feces are standardized by dry weight through lyophilization, with fT_3 levels expressed as a portion of dry weight (ng/g); urine hormone concentrations are standardized via specific gravity and expressed as a portion of volume (pg/ml). 2) There are differences in how hormones are excreted into these two media and in the related lag times from circulating levels, which are commonly 4–8 hrs for urine and 36–48 hrs for feces. However, there are no data specifically quantifying this lag for thyroid hormone in our study species (Hodges & Heistermann, 2011). Nonetheless, this general difference should be noted when interpreting this study's results. 3) There are known differences in baseline thyroid hormone levels between primate species (Gagneux et al., 2001; Kaack et al., 1979). As such, the interspecies comparison made here should be based on qualitative patterns rather than quantitative measures of hormone levels. Given these factors, we assessed variation in fT_3 levels in relation to season and recently experienced temperature (without accounting for lag times).

3 | RESULTS

3.1 | Prediction 1: Seasonal differences in fT_3

Individual animals of both species showed a strong seasonal pattern with elevated levels of fT_3 in the cooler season. In comparing seasonal fT_3 changes of individual animals, six of seven howlers that were sampled in both seasons (including both sexes) displayed elevated levels of fT_3 in the cooler wet season (\bar{x} difference = 1.84 pg/ml, $t_5 = -3.14$, $p = 0.013$) (Figure 1a; Supporting Information Table S1). Similarly for Japanese macaques, 10 of 11 animals sampled in both seasons (also including both sexes) displayed elevated levels of fT_3 in the winter (\bar{x} difference = 1.02 ± 0.67 ng/g, $t_{10} = 5.05$, $p < 0.001$) (Figure 1b; Supporting Information S1). Overall, only one animal of each species deviated from the predicted seasonal pattern of fT_3 ; there was nothing noteworthy about these data points to indicate why they differed from the modal pattern.

Our linear mixed model confirmed a significant effect of season on fT_3 levels for both howlers and Japanese macaques. The variable season was included and statistically significant in all selected models for both species (Table 2). All models showed larger effect sizes (β) for the impact of season on fT_3 relative to other explanatory variables in the model.

3.2 | Prediction 2: Seasonal differences in temperature

Both species experienced seasonal differences in environmental temperature. For the exact dates of howler urine collection, near-animal ambient temperatures were lower for wet season daily temperature minimums (\bar{x} difference = -1.22°C , $t_4 = -2.22$, $p = 0.046$) but the two seasons did not show significant differences for daily mean (\bar{x} difference = 0.03, $t_4 = 0.05$, $p = 0.480$) or maximum near-animal ambient temperatures (\bar{x} difference = -0.36°C , $t_4 = -0.30$, $p = 0.388$). For long-term howler temperature data, wet seasons had significantly

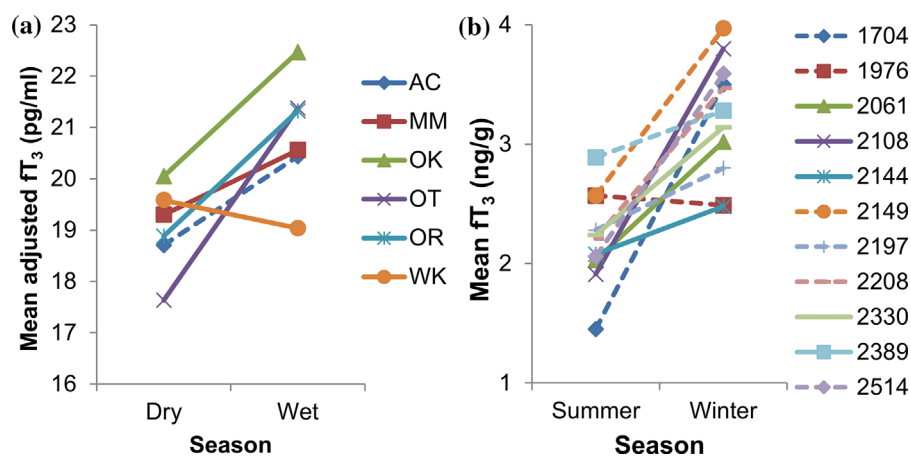


FIGURE 1 Seasonal differences in fT_3 levels for individual (a) mantled howlers and (b) Japanese macaques. Dashed lines = males; solid lines = females. The mean standard error of the mean for all points was 1.1 ± 0.20 pg/ml for howlers and 0.2 ± 0.03 ng/g for Japanese macaques. Data are only reported for animals that were sampled in both seasons. For a full list of animals' individual fT_3 levels, see Supporting Information S1

TABLE 2 Top three linear mixed models predicting fT_3 concentrations from mantled howler urine and Japanese macaque feces

	Model 1				Model 2				Model 3			
	β	SE	Test statistic ^a	<i>p</i> -value	β	SE	Test statistic ^a	<i>p</i> -value	β	SE	Test statistic ^a	<i>p</i> -value
Mantled howlers												
Season	-15.12	4.41	-3.43	0.001^d	-6.28	1.50	-4.20	<0.001	-14.84	4.40	-3.38	0.001^d
Minimum temperature ^b	-2.27	1.07	-2.12	0.039^d					-2.17	1.07	-2.02	0.049^d
Maximum temperature ^c	2.44	0.79	3.10	0.003	2.37	0.82	2.89	0.006	2.46	0.78	3.14	0.003
Individual												
Sex									1.12	1.33	0.84	0.405
Specific gravity	1.43	2.76	0.52	0.603	0.81	1.94	0.42	0.675	1.14	2.36	0.48	0.629
Japanese macaques												
Season	5.95	1.63	3.65	0.001^d	5.79	1.69	3.43	0.001^d	6.01	1.64	3.68	0.001^d
Minimum temperature ^b												
Maximum temperature ^c	-0.25	0.06	-4.12	<0.001^d	-0.26	0.06	-3.91	<0.001^d	-0.26	0.06	-4.15	<0.001^d
Individual									0.002	0.02	0.15	0.879
Sex	-0.24	-2.10	0.040						-0.24	0.12	-2.07	0.049

Model selection criteria (lower values indicate better model fit) for howlers: AICc: model 1 = 294.69, 2 = 296.26, 3 = 296.74; BIC: 1 = 303.86, 2 = 304.19, 3 = 307.04; Japanese macaques: AICc: 1 = 77.24, 2 = 79.12, 3 = 79.68; BIC: 1 = 86.70, 2 = 86.85, 3 = 90.79.

Test values with $p < 0.05$ in bold italics.

Empty spaces indicate that the variable was not included in the specified model.

^aFixed variables (season, minimum and maximum temperatures, and sex) were assessed with a *t* statistic; random variables (individual and specific gravity) were assessed with Wald's *Z*.

^bAbsolute minimum temperature experienced during the four days prior to sample collection.

^cAbsolute maximum temperature experienced during the four days prior to sample collection.

^dVIF > 10. Despite being correlated, we retained both season and temperature in models due to their potentially distinct effects on thyroid hormone (following O'Brien, 2007). Correlations between minimum and maximum recently experienced temperatures are presented in Supporting Information Table S2.

lower daily minimum and mean near-animal ambient temperatures (minimum: \bar{x} difference = -1.15°C , $F_{1,145} = 10.34$, $p = 0.002$; mean: \bar{x} difference = 0.97°C , $F_{1,145} = 7.18$, $p = 0.008$), but slightly higher, although not significantly different, daily maximums (\bar{x} difference = 0.54°C , $F_{1,145} = 1.25$, $p = 0.266$) (Table 1).

For howlers, temperature deficits were greater in the wet than dry season ($F_{1,9216} = 787.99$, $p < 0.001$), with animals experiencing ambient temperatures on average $6.90 \pm 0.04^\circ\text{C}$ below subcutaneous temperature in the wet season, compared to $3.52 \pm 0.12^\circ\text{C}$ below subcutaneous temperature in the dry season (Figure 2). Subcutaneous temperatures alone did not show any statistically significant differences between seasons (minimums: \bar{x} difference = -0.67°C , $F_{1,113} = 1.68$, $p = 0.198$; maximums: \bar{x} difference = 0.15°C , $F_{1,113} = 0.93$, $p = 0.337$; means: \bar{x} difference = -0.53°C , $F_{1,113} = 0.01$, $p = 0.921$) (Table 1).

Ambient temperatures for Japanese macaques were also significantly colder in winter than summer for daily minimums (\bar{x} difference = -23.26°C , $t_{23} = -32.94$, $p < 0.001$), means (\bar{x} difference = -23.70°C , $t_{23} = -29.07$, $p < 0.001$), and maximums (\bar{x} difference = -24.10°C , $t_{23} = -19.52$, $p < 0.001$) (Table 1). Subcutaneous temperatures also differed between seasons (\bar{x} difference = 0.74°C , $F_{1,5016} = 343.83$, $p < 0.001$), with warmer subcutaneous temperatures in the summer (Table 1). Temperature deficits were far greater in the winter than summer ($F_{1,4680} = 63605.48$, $p < 0.001$), with animals experiencing ambient temperatures on average $33.04 \pm 0.07^\circ\text{C}$ below subcutaneous temperature in the winter, compared to ambient temperatures

$10.05 \pm 0.05^\circ\text{C}$ below subcutaneous temperatures during summer (Figure 2).

3.3 | Prediction 3: fT_3 and recently experienced temperature

For howlers, all three best-fit linear mixed models demonstrated a relationship between recently experienced temperatures and fT_3 levels (Table 2). Minimum temperatures were included as a factor in two of the top three models, and in both showed a significant negative relationship with fT_3 levels, consistent with a thermoregulatory function. However maximum temperatures, which were included as a factor in all three top models, showed a significantly positive relationship with fT_3 , the opposite of that predicted from a thermoregulatory function.

For Japanese macaques, all selected models included maximum, but not minimum, temperatures as a factor impacting fT_3 . In all models, maximum temperatures had a significant negative relationship with fT_3 , consistent with a thermoregulatory function.

3.4 | Variation in fT_3 based on animal-effects

Individual animals' fT_3 levels are reported by sex and season in Supporting Information S1. For howlers, sex was included in one of the top linear mixed models, but was not a significant predictor of fT_3 and

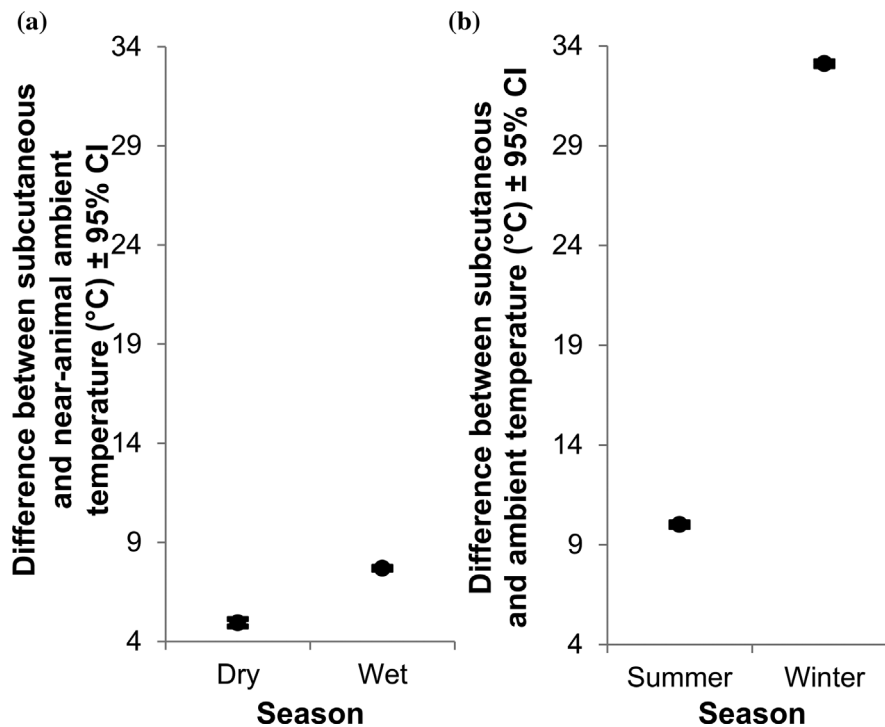


FIGURE 2 Differences between simultaneously collected subcutaneous and ambient temperatures for (a) howlers in the wet ($N = 8,376$) and dry ($N = 1,140$) seasons, and (b) Japanese macaques in the winter ($N = 1,585$) and summer ($N = 3,098$). Howler ambient temperatures were recorded from on-animal loggers; Japanese macaque ambient temperatures were from the on-site weather station

displayed lower effect sizes than other variables included in the model (Table 2). For Japanese macaques, sex was included in two of the three top models and was a significant predictor of fT_3 levels in both (Table 2), with females having lower fT_3 levels than males (Supporting Information S1).

4 | DISCUSSION

4.1 | Thermoregulation and fT_3

We assessed whether seasonal variation in thyroid hormone levels is consistent with a thermoregulatory function for both tropical mantled howlers and seasonally cold-habitat Japanese macaques. Our main findings (Table 3) were: 1) thyroid hormone levels differed between seasons, with both mantled howlers and Japanese macaques exhibiting higher fT_3 during the cooler season. Moreover, these differences were attributable to within-animal changes in hormone levels. This pattern is consistent with (but not necessarily exclusive to) a thermoregulatory function of fT_3 . 2) Both tropical howlers and Japanese macaques showed a seasonal temperature deficit, with significantly greater differences between subcutaneous and ambient temperatures during the cooler season. This indicates that animals of both species warmed their bodies more during the cooler season, suggesting greater thermoregulatory pressures during this time. 3) Recently experienced temperatures were related to fT_3 levels, although not in a straightforward manner. For howlers, thyroid hormones increased with decreasing recently experienced minimum

temperatures (when controlling for season), which is consistent with a thermoregulatory function. However, recently experienced maximum temperatures showed a positive relationship with fT_3 , the opposite of the expected relationship for a thermoregulatory function. For Japanese macaques, model selection indicated that only maximum, rather than minimum, temperatures played a role in predicting fT_3 ; recently experienced maximum temperatures consistently showed the predicted negative relationship with fT_3 . In interpreting these results, the longer lag time from circulating hormone levels to feces for Japanese macaques, relative to urine from howlers, should be noted, although minimum ambient temperatures were persistently well below subcutaneous temperatures for Japanese macaques in the winter. Overall, our findings are consistent with a thermoregulatory function of thyroid hormone for Japanese macaques, with animals raising fT_3 levels during the cooler season and showing a negative relationship between temperature and thyroid hormone, although only for recently experienced maximum temperatures. However, howlers only showed mixed support for a thermoregulatory pattern (Table 3). Consistent with this pattern, howlers displayed greater temperatures deficits and higher thyroid hormone levels during the cooler wet season, as well as higher fT_3 levels when recently experienced minimum temperatures were low. Yet contrary to expectations, howlers had elevated fT_3 levels when recently experienced maximum temperatures were high.

The difference in results between minimum and maximum temperatures for howlers could relate to the nature of temperature variation at the field site. Our long-term temperature data at La

TABLE 3 Support for the hypothesis that thyroid hormone has a thermoregulatory function in mantled howlers and Japanese macaques

Prediction	Supported for mantled howlers?	Supported for Japanese macaques?
1) fT_3 levels should be higher in the colder season	Yes	Yes
2) Animals have greater temperature deficits in the colder season	Yes	Yes
3) Ambient temperatures display a negative relationship with fT_3	Minimums: Yes Maximums: Positive relationship	Minimums: No relationship Maximums: Yes

Pacifica found that while wet seasons exhibited significantly lower minimum and mean temperatures, maximum temperatures were actually slightly higher (albeit non-significantly) in the wet season. In short, wet season temperature peaks are just as warm, or warmer, than dry season peaks, even though animals still experience cooler temperature minimums and means. In turn, increasing fT_3 levels at cooler minimums may be linked to the opposite, epiphenomenal relationship with maximum temperatures (Supporting Information S2). This demonstrates the idea that tropical environments do not necessarily reflect the same seasonal patterns of thermal variation as habitats at higher latitudes, in which minimum, maximum, and mean temperatures are consistently lower in the winter. Not surprisingly, Japanese macaques in our study did experience larger magnitude differences in seasonal temperatures and temperature deficits than howlers. Nonetheless, the magnitude of temperature deficits displayed by howlers in this study (wet: $\bar{x} = 6.90^\circ\text{C}$, dry: $\bar{x} = 3.52^\circ\text{C}$, Figure 2) appear to be biologically meaningful. As a comparison, thermoneutral zones of primate species range from 3 to 10°C (although have not been evaluated for our study species) (Thompson et al., 2016). Likewise, using standardized measures of hormone variance (the range of hormone values observed, expressed as a percent of the minimum value: MacLarnon et al., 2015), Japanese macaque fT_3 varied 173.5% over our data set, compared to 55.3% for howlers. This suggests that fT_3 does vary more in Japanese macaques than howlers, likely in response to the larger magnitude of seasonal thermal swings in their habitat.

Temperature is often considered to be the main climatic variable that differs between seasons. However temperatures in tropical habitats can overlap between the warm (dry) and cool (wet) seasons (Table 1). Instead, tropical seasons show greater differences in solar radiation, humidity, and rain (van Schaik & Pfannes, 2005; Wright & van Schaik, 1994), which are known to impact mammalian thermoregulation and perceived thermal environment (Hill et al., 2004; Höpfe, 1999). It is worth noting that our limited time frame of sample collection provides only a discrete comparison of these seasonal differences that does not reflect the actual, gradual change animals

experience as seasons transition. As such, the relationship between fT_3 and recently experienced temperature may reflect minor adjustments to hormone levels compared to larger, but more gradual, changes in animals' physiology that occur between seasons. Long-term longitudinal studies will be able to more finely assess how thermoregulatory strategies transition over the long (seasonal) versus short (daily) term.

4.2 | Non-thermoregulatory factors impacting fT_3

The seasonal patterns of fT_3 we found in howlers and Japanese macaques are consistent with, but not necessarily exclusive to, a thermoregulatory role. Non-thermoregulatory factors such as changes in reproductive status and nutritional intake could also generate seasonal fluctuations in thyroid hormone which could in turn impact BMR. While this study did not formally assess these factors, we can gain some insight by comparing our results to expected patterns from the literature.

First, in regard to seasonal reproduction, howlers breed year-round (Glander, 1980). As such, the reproductive role of thyroid hormones cannot account for our observed seasonal pattern in this species. Japanese macaques are seasonal breeders, with mating taking place during the winter (Watanabe, Mori, & Kawai, 1992). For males of three other seasonally breeding primates, squirrel monkeys (*Saimiri sciureus*), mouse lemurs (*Microcebus murinus*), and Barbary macaques (*Macaca sylvanus*), the mating season coincides with an increase in thyroid hormone levels (Cristóbal-Azkarate et al., 2016; Kaack et al., 1980; Petter-Rousseaux, 1984), similar to the pattern we observed. There are currently no published accounts specifically demonstrating that seasonally breeding female primates show increased thyroid hormone levels in the mating season. Instead Kaack et al. (1979) reported similar T_3 levels for both pregnant and non-pregnant rhesus macaques (*Macaca mulatta*). In our study, both sexes of Japanese macaques showed seasonal increases in fT_3 . Based on these reports, reproductive processes could contribute to seasonal differences in thyroid hormone for male Japanese macaques, but there is not currently enough information to assess reproduction-based changes for females. A possible discrepancy between thyroid hormone's reproductive role between male and female Japanese macaques may be linked to the significant sex difference found in this study, with males having elevated fT_3 relative to females (Table 2; Supporting Information S1). However, the effect of season was still significant when controlling for sex (Table 2). Likewise, the consistent negative relationship between maximum temperatures and fT_3 suggests that a thermoregulatory role is operating in conjunction with any possible reproductive effects.

Seasonal changes in diet and nutritional status are a second potential factor impacting thyroid hormone levels. Previous studies report that feeding time and body weight remained constant between seasons for howlers at our field site (Glander, 1981, 2006). This suggests that animals are not experiencing seasonal caloric deficits, however, we have no direct evidence to fully exclude an impact of diet on thyroid hormone levels. Under seasonal caloric restriction, Japanese macaque fT_3 should be lower during the leaner winter

season; our study found the opposite pattern. The Japanese macaques in our study were also provisioned and hence not subject to natural variation in food availability. As such, dietary changes do not adequately explain the seasonal fT_3 patterns for Japanese macaques in this study.

4.3 | Implications for primate behavioral ecology

We documented seasonal differences in fT_3 levels for mantled howlers and Japanese macaques. Regardless of whether this pattern was driven by thermoregulatory or other processes, these changes have important implications for primates' biology. Since increased levels of thyroid hormone are linked with increased cellular metabolism and BMR (Silva, 2003, 2006), fluctuations in this hormone influence energy expenditure, and consequently a broad array of associated variables including foraging decisions, activity patterns, and range use. Given these potential impacts, our finding that thyroid hormones seasonally differ in both a tropical and a seasonally cold-habitat primate has important implications for interpreting commonly collected variables in primate field studies.

Food consumption patterns provide an illustrative example of the potentially overlooked consequences of seasonal differences in thyroid hormone levels. Seasonal changes in food consumption have been documented for many primates, including tropical ones (e.g., Hemingway & Bynum, 2005; Wallace, 2005), with these shifts often attributed to seasonal differences in food availability (Dasilva, 1994; Poulsen, Clark, & Smith, 2001). Yet, there is strong evidence that altering the levels of circulating thyroid hormone changes animals' feeding habits, increasing overall caloric consumption and altering macronutrient preferences toward a more carbohydrate-rich diet (Pijl et al., 2001; Silva, 2003; Silvestri et al., 2005). This indicates that seasonal shifts in macronutrient consumption, caloric intake, and feeding behavior may be responses to thyroid hormone fluctuations. From the current study population, howlers spend similar amounts of time feeding in both seasons, but do consume more energy-rich fruit during the wet season, as fruit availability also increases during this time (Glander, 1981). These findings suggest that patterns previously attributed to external, environmental factors (e.g., fruit availability) could actually be driven by aspects of animals' internal physiology such as hormone levels (which may themselves be responding to other external factors).

5 | CONCLUSIONS

Our findings lend support to the hypothesis that thyroid hormone fluctuations are consistent with a thermoregulatory function in both tropical howlers and seasonally cold-habitat Japanese macaques. This physiological link corroborates a growing body of behavioral evidence suggesting that tropical primates face greater cool thermoregulatory pressures than previously thought (Gestich, Caselli, & Setz, 2014; Takemoto, 2004; Thompson et al., 2014, 2016). The finding that thyroid hormone increases during the cooler season and decreases in the warmer season for both mantled howlers and Japanese macaques

suggests that animals seasonally adjust energy expenditure, which could have wide-ranging implications for how we interpret commonly collected data such as food choice and activity patterns.

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