

Environmental heterogeneity affects seasonal variation in thyroid hormone physiology of free-living arctic ground squirrels (*Urocitellus parryii*)

Cory T. Williams, Helen E. Chmura, Victor Zhang, Danielle Dillon, Kathryn Wilsterman, Brian M. Barnes, and C. Loren Buck

Abstract: Thyroid hormones (TH) are key regulators of metabolism that could play an important role in altering physiology and energy allocation across life-history stages. Here, we examine seasonal TH dynamics from 345 plasma samples collected from 134 free-living arctic ground squirrels (*Urocitellus parryii* (Richardson, 1825)) across three consecutive years (2014–2016). We also examine whether unbound levels of triiodothyronine (free T_3) in plasma are correlated with total T_3 levels and total thyroxine (T_4) levels, and whether fecal T_3 metabolite levels correlate with plasma TH levels. We found significant differences in plasma TH levels across stages of the annual cycle, but these differences were highly inconsistent across years. However, within a given time period, pregnant females had lower free T_3 levels compared with lactating females. We also found that although free T_3 was correlated with both total T_3 and total T_4 in plasma, fecal T_3 was not positively correlated with plasma TH levels. Given the lack of consistent differences across life-history stages, we suggest environmental heterogeneity may be the primary driver of TH dynamics, masking any potential endogenous seasonal rhythms in TH secretion. We urge caution in the use of fecal T_3 metabolites as a proxy for circulating levels and encourage further research to understand the observed discrepancy.

Key words: *Urocitellus parryii*, arctic ground squirrel, fecal hormones, reproductive state, thyroid, thyroxine, triiodothyronine.

Résumé : Les hormones thyroïdiennes (HT) sont des régulateurs clés du métabolisme qui pourraient jouer un important rôle dans la modulation de la physiologie et de l'allocation d'énergie durant les différentes étapes du cycle de vie. Nous examinons la dynamique saisonnière des HT de 345 échantillons de plasma prélevés de 134 spermophiles arctiques (*Urocitellus parryii* (Richardson, 1825)) vivant en liberté sur trois années consécutives (2014–2016). Nous tentons aussi de déterminer si les concentrations de triiodothyronine libre (T_3 libre) dans le plasma sont corrélées aux concentrations totales de T_3 et aux concentrations totales de thyroxine (T_4), et si les concentrations fécales de métabolites de T_3 sont corrélées aux concentrations plasmiqes des HT. Nous relevons des différences significatives des concentrations plasmiqes des HT entre les différentes étapes du cycle annuel, mais ces différences sont fortement incohérentes d'une année à l'autre. Cependant, sur une période de temps donnée, les femelles gestantes présentent des concentrations de T_3 libre plus faibles que les femelles en lactation. Nous constatons également que, si la T_3 libre est corrélée à la T_3 totale et à la T_4 totale dans le plasma, la T_3 fécale n'est pas positivement corrélée aux concentrations plasmiqes des HT. Au vu de l'absence de différence cohérente entre les différentes étapes du cycle biologique, nous proposons que l'hétérogénéité du milieu pourrait être le principal facteur de modulation de la dynamique des HT, masquant tout rythme saisonnier endogène éventuel de la sécrétion des HT. Nous prônons la prudence dans l'utilisation de métabolites fécaux de la T_3 comme variable substitutive des concentrations en circulation et encourageons la réalisation de travaux visant à expliquer l'incohérence observée. [Traduit par la Rédaction]

Mots-clés : *Urocitellus parryii*, spermophile arctique, hormones fécales, état reproducteur, thyroïde, thyroxine, triiodothyronine.

Introduction

Physiological and behavioral flexibility allows free-living vertebrates to cope with environmental heterogeneity, including predictable seasonal changes and unpredictable challenges. Adjustments of physiology and behavior to environmental conditions are often mediated by hormonal mechanisms (Busch and Hayward 2009; McEwen and Wingfield 2010; Dantzer and Swanson 2012; Angelier et al. 2016). As fundamental regulators of metabolism, development, and thermogenesis, thyroid hormones (TH) are likely to play an important role in vertebrate responses to chronic environmental stressors (e.g., Thompson et al. 2017). However,

metabolism and TH physiology are also potentially modulated to meet the requirements dictated by annually recurring life-cycle events (life-history stages). Disentangling seasonal and endogenously generated life-history trends in hormone levels from those driven by changes in environmental conditions requires the collection of multiyear data sets.

There is increasing evidence that metabolic rate (MR), including basal MR (BMR), is a plastic trait with TH acting as a modulator of metabolic flexibility in endotherms (Williams and Tieleman 2000; Chastel et al. 2003; Mullur et al. 2014). Levels of triiodothyronine (T_3 ; the active TH) and thyroxine (T_4 ; the inactive precursor for T_3) have been found to fluctuate across time (e.g., Thompson et al.

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2017; Rimbach et al. 2017) and experimental manipulations of food availability affect both resting metabolic rate (RMR) and TH levels (Boelen et al. 2008). In birds, BMR correlates with circulating levels of T_3 , consistent with their role as potent modulators of metabolism (Chastel et al. 2003; Elliott et al. 2013; Welcker et al. 2013). For mammals, this relationship is less clear, although T_3 and BMR are both depressed in free-living animals that undertake natural fasts and captive animals that are food deprived (reviewed in Behringer et al. 2018). Additionally, T_3 has been shown to increase body temperature, heart rate, RMR, and activity levels in mice (Johansson and Thoren 1997; Jakobsons et al. 1999). Associations between TH and metabolism are evident in mammals (reviewed in Mullur et al. 2014), but how these relationships change with reproductive status remains unclear.

Studies of captive rodents indicate dynamic changes in TH physiology can occur across pregnancy and lactation, though there appear to be substantial differences across species (Castro et al. 1986; Calvo et al. 1990; Yokus et al. 2006). In pregnant Wistar rats, for example, plasma levels of total T_4 , free T_4 , and, to a lesser extent, total T_3 progressively decrease across pregnancy (Calvo et al. 1990). This depression in plasma TH levels persists throughout lactation, with levels returning to pre-reproductive levels after weaning, although the removal of pups during early lactation will cause a much earlier reversion to pre-reproductive levels (Fukuda et al. 1980). However, these changes in TH physiology do not appear universal across mammals, as Castro et al. (1986) found no differences in plasma total or free TH among male, non-reproductive female, and pregnant female guinea pigs (*Cavia porcellus* (Linnaeus, 1758)). This presents a challenge for studies of free-living vertebrates because it is difficult to assess whether observed changes in TH levels reflect transitions between life-cycle events, predictable seasonal changes in response to changing environmental conditions (e.g., resource availability), or responses to stochastic environmental variation.

In the present study, we investigate seasonal changes in total and free TH across the active season of free-living arctic ground squirrels (*Urocitellus parryi* (Richardson, 1825)). We measure TH during five sampling intervals: (I) the mating interval shortly after emergence, (II) late pregnancy to early lactation, (III) late lactation, (IV) post lactation, and (V) female fattening. Arctic ground squirrels occupy an extreme and highly seasonal environment (Williams et al. 2017); they avoid the long period of low resource availability by spending 6–8 months of the year hibernating, which leaves them with a relatively short interval to reproduce before they must fatten again to survive the next bout of hibernation (Buck and Barnes 1999). In our previous 1-year study of TH dynamics during the active season of free-living adult female arctic ground squirrels, we found that although T_4 levels decreased during pregnancy and lactation, relative to levels post hibernation, T_3 exhibited the opposite pattern, increasing during pregnancy and peaking during late lactation (Wilsterman et al. 2015). We attributed this increase in total T_3 to the higher metabolism that female ground squirrels need to sustain lactation as they forage intensively to meet their energy demands and those of their growing pups. Lactation is the most energetically demanding time of the season for female ground squirrels, with daily activity and energy expenditure peaking shortly before weaning commences (Kenagy et al. 1989; Williams et al. 2016a). The seasonal changes in T_3 that we observed in arctic ground squirrels correlated with seasonal variability in mean body temperature (Williams et al. 2016b), which is consistent with previous research showing BMR scales with body temperature (Clarke et al. 2010). However, given that animals were also exposed to predictable seasonal changes in weather or resource availability and unpredictable environmental variation, it remains unclear if plasma TH levels reflect endogenously driven changes in physiology, or simply reflect a response to dynamic environmental conditions.

Here, we build upon our previous work with two additional years of data, including measurement of TH in males, to disentangle these potential drivers of TH levels. We predicted that seasonal changes in TH levels would be consistent across years, but TH dynamics would differ between the sexes, mirroring the sexual dimorphism in the timing of energy allocation towards reproduction. Specifically, we predicted females would have elevated T_3 during late lactation, whereas males would have elevated T_3 during the mating season, when they exhibit higher levels of movement (C.T. Williams, B.M. Barnes, V. Zhang, and C.L. Buck, unpublished data) and thermoregulatory costs are high.

We also expand on our previous work by exploring how levels of unbound plasma T_3 (free T_3) and fecal T_3 correlate with total plasma TH concentrations. Feces can be collected easily and non-invasively, thereby allowing frequent sampling, even over long time periods (Wasser et al. 2010). However, before fecal TH analyses can be used widely as a proxy for energy balance (e.g., Schaebs et al. 2016), it is important to determine whether fecal TH are reflective of circulating levels, as the clearance of hormones from the blood stream and bacterial modification in the gut can generate systematic noise that distorts the “signal” (reviewed in Goymann 2012).

Materials and methods

Study sites and species

We conducted this study at two sites in northern Alaska, USA, that are ~20 km apart: Toolik Lake (68°38'N, 149°38'W; elevation 719 m) and Atigun River (68°27'N, 149°21'W; elevation 812 m). Population density is higher at Atigun, owing, in part, to the sandy substrate that is well drained and suited to burrowing; suitable substrate for burrowing is more dispersed at Toolik. Earlier loss of snow cover at Atigun also typically results in earlier timing of spring emergence from hibernation and reproduction, relative to Toolik (Sheriff et al. 2011). We collected environmental data (incident solar radiation, ambient temperature, and rainfall) at the Atigun River site using a Hobo U30-NRC weather station (Onset Computer Corporation, Bourne, Massachusetts, USA). We use only Atigun weather data in our analyses because some data from Toolik are missing due to sensor failure and environmental conditions in the summer months at Toolik are highly correlated with those at Atigun (Williams et al. 2016a).

Male arctic ground squirrels terminate hibernation, on average, 25–36 days before females, as they spend, on average, 16–25 days below ground at euthermic body temperatures consuming a food cache while undergoing seasonal gonadal recrudescence and spermatogenesis (Barnes 1996; Sheriff et al. 2011). This belowground interval also allows them to recuperate their body condition such that they emerge to the surface ready to establish territories and compete with other males for access to females, which emerge ~10–14 days later (Sheriff et al. 2011; Richter et al. 2017). Female arctic ground squirrels emerge from their hibernacula in late April to early May at their lowest annual body mass and become pregnant, on average, within 6 days of ending hibernation (Buck and Barnes 1999; Williams et al. 2011). Gestation lasts approximately 26 days; lactation lasts an additional 4–5 weeks. Shortly after weaning their young, females undergo an interval of hyperphagia as they store energy endogenously as fat in preparation for hibernation (Buck and Barnes 1999; Sheriff et al. 2011). Females enter hibernation in early to mid-August, whereas males do not begin hibernating until early to mid-October, after they have accumulated their food hoards for the subsequent spring (Buck and Barnes 1999; Sheriff et al. 2011).

Animal capture and blood sampling

All procedures were approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee (IACUC No. 340270 and No. 864841) and followed the guidelines estab-

Table 1. Range of dates that arctic ground squirrels (*Urocitellus parryii*) were sampled within each sampling interval across the 3-year study.

Year	I. Mating	II. Late gestation to early lactation	III. Late lactation	IV. Post lactation	V. Female fattening
2014	19 April–1 May	NA	2–13 June	30 June–7 July	9–14 August
2015	17–27 April	13–19 May	3–17 June	9–11 July	1–6 August
2016	14–25 April	20–24 May	30 May–6 June	2–7 July	30 July–3 August

Note: No animals (NA) were sampled during interval II in 2014.

lished by the American Society of Mammalogists (Sikes et al. 2016). We captured arctic ground squirrels using Tomahawk live traps (Tomahawk Trap Co., Hazelhurst, Wisconsin, USA) baited with carrot. Animals were given unique ear tags and pit tags for individual identification. Immediately prior to sampling, we anesthetized squirrels by briefly exposing them to isoflurane vapors and then weighed and assessed individuals for reproductive status (females: pregnant (assessed via palpation), lactating, post lactation; males: non-scrotal, scrotal). We collected blood samples for hormone analysis via cardiac puncture using 26 gauge needles in 3 mL syringes. No more than 1.5 mL was collected at a single capture (<1% body mass for all animals). Blood was placed on ice in EDTA-coated tubes in the field. Samples were centrifuged in the laboratory for 10 min at 6500 r/min within 7 h of collection to separate plasma. Plasma was stored in microcentrifuge tubes at -80°C until analysis. Fecal samples were collected when the animal was first trapped and stored on ice until transfer to a freezer. In the mating season, animals from both sites were transported to the laboratory at Toolik Field Station prior to sampling and subsequently released at their site of capture the following day. At all other times of the year, squirrels were transported to and processed at a vehicle adjacent to the site and returned to their burrow of capture ~ 15 min after sampling. Animals were not processed at the vehicle during the mating season because of the colder conditions.

Animals were trapped during four (2015) or five (2016 and 2017) sampling intervals across the active season, though there was slight year to year variation in the timing of trapping owing to logistical constraints. We obtained samples from animals shortly after emergence (i.e., during the mating interval, sampling interval I), during late pregnancy to early lactation (2016 and 2017 only; interval II), late lactation (interval III), post lactation (interval IV), and during female fattening (interval V); dates of sampling intervals in each year for these intervals are shown in Table 1. Although females undergo pre-hibernation fattening in August, males do not complete fattening until sometime in September (Buck and Barnes 1999). Because reproduction is not synchronized among all animals, some females sampled during interval II had recently given birth and were lactating, whereas others were still gestating (pregnancy determined by palpation). This provided the opportunity to test whether plasma TH levels were correlated with differences in reproductive state within a sampling interval.

TH assays

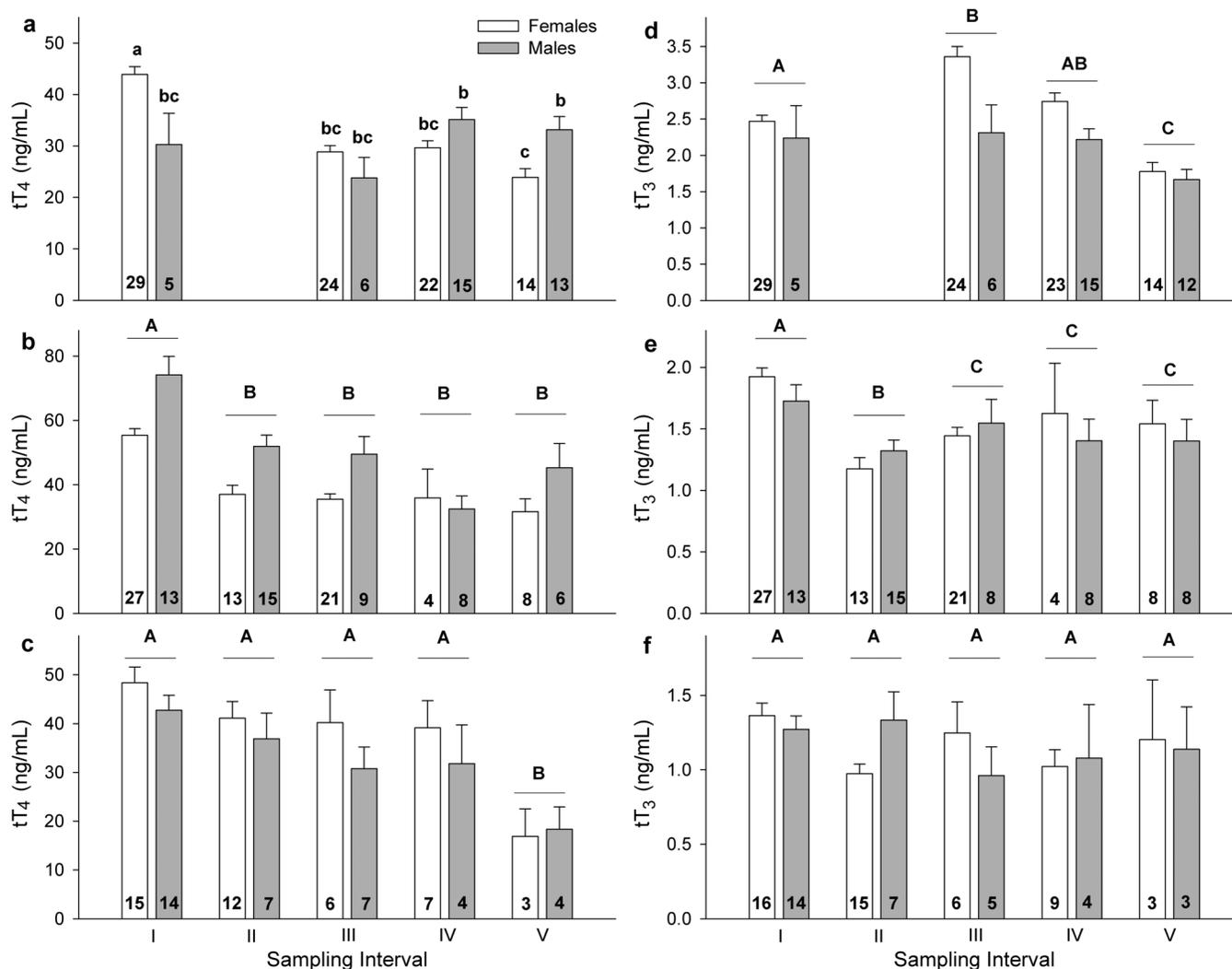
We measured total T_4 of plasma samples using a commercially available total T_4 enzyme immunoassay (EIA) for human serum (07BC1007; MP Biomedicals, Solon, Ohio, USA). For measures of total T_3 , we extracted plasma samples using a standard barbital buffer protocol (Crane et al. 2004) prior to using a commercially available total T_3 radio-immunoassay (RIA) for serum (06B254215; MP Biomedicals). Assays for total T_4 and total T_3 were validated for this species using the standard addition method and tests of parallelism according to the manufacture specifications. Data for females in 2014 were previously reported in Wilsterman et al. (2015). We also measured plasma free T_3 from 2015 and 2016 plasma samples using a commercially available RIA (06B258709; MP Biomedicals); we validated this assay kit using tests of parallelism. Only a single free T_3 plate was run in 2016. For most assays, we ran

samples in triplicate, but occasionally samples were run in duplicate or singlet if we had insufficient plasma. Different years were run in separate assays. For 2015 and 2016, we used a common pool to assess interassay variation. Within a year, samples were randomized across the plate and across assays. The mean intra- and inter-assay coefficients of variation were 4.1% and 24.8% for total T_4 , 2.9% and 12.4% for total T_3 , and 4.6% and 14.4% for free T_3 , respectively. Finally, in 2015, we used the same total T_3 kit as used for plasma to measure T_3 in 51 fecal samples collected at the time of capture for blood sampling; fecal samples were freeze dried, powered using a mortar and pestle, and 0.2 g was extracted twice in 5 mL of 70% ethanol following Wasser et al. (2010). We validated the fecal assay for arctic ground squirrel feces using standard addition and tests of parallelism; this kit (06B254215; MP Biomedical) has also previously been validated for fecal analyses in a variety of other mammals (Wasser et al. 2010). Intra- and inter-assay variations for fecal T_3 were 3.9% and 9.1%, respectively.

Statistical analyses

We performed all statistical analyses in R version 3.4.4 (R Core Team 2016) using the packages lme4 (version 1.1-15) and lmerTest (version 2.0-36). Sample sizes for analyses are shown in Figs. 1a–3c. We used a natural log transformation for all hormone data to ensure normality of model residuals. We assessed the normality of residuals using quantile–quantile plots and by plotting residuals against fixed effects. In 2016, log transformation did not fully normalize model residuals for total T_4 , so data were also analyzed using a rank transformation. As modeling results were equivalent regardless of the transformation used, we report only log-transformed results. Data for each year were analyzed separately because hormone samples collected each year were run in separate assays and because all life-history stages were not sampled every year. For each year, we fitted a linear mixed-effects model using restricted maximum likelihood (REML) with fixed effects for sex, sampling interval, and the interaction between sex and sampling interval. A random effect of individual identification, nested within a random site effect, was used to account for repeated measures within years. We calculated p values using a Satterthwaite approximation to estimate denominator degrees of freedom. We do not include reproductive state as a factor in our models because reproduction was relatively synchronous among animals such that reproductive stage corresponds to sampling interval. However, in both 2015 and 2016, a sampling interval (II) occurred in which some females were in late gestation, whereas others had already given birth and were in early lactation; we used linear mixed-effects models with a random individual identification effect to compare hormone levels between lactating and gestating females within this window. We used Pearson's product moment correlation analyses to determine whether free T_3 , total T_4 , and free T_3 were correlated with one another and to test whether fecal T_3 metabolites were correlated with plasma total T_3 , free T_3 , or total T_4 . Finally, we tested for annual differences in environmental conditions (mean daily ambient temperature, mean daily solar radiation, and daily rainfall (as a categorical "yes" or "no")) within each sampling interval using generalized linear mixed models (logit link for rainfall).

Fig. 1. Plasma levels (mean \pm SE) of (a–c) total thyroxine (tT_4) and (d–f) total triiodothyronine (tT_3) during five sampling intervals across 3 years in free-living arctic ground squirrels (*Urocitellus parryii*). Numbers on bars indicate sample size. A significant interaction between sex and sampling interval was only found for tT_4 in 2014, where different lowercase letters indicate significant differences among groups ($p < 0.05$). For all other panels, different uppercase letters indicate significant differences between sampling intervals; males also had higher tT_4 than females in 2015. Sampling intervals correspond to stages of the annual cycle as follows — interval I: post hibernation, including mating season; interval II: late gestation to early lactation; interval III: late lactation; interval IV: post lactation; interval V: female fattening. Animals were not sampled during interval II in 2014.



Results

Stage-dependent variability in TH

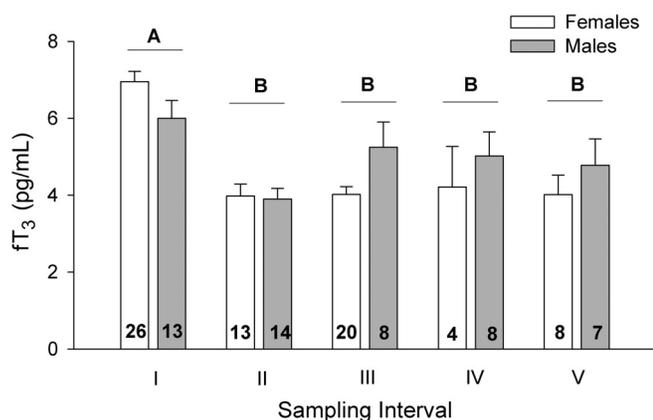
A summary of all statistics and p values are shown in Supplementary Table S1.¹ In 2014, total T_4 levels in plasma were affected by an interaction between sex and sampling interval. In females, total T_4 was higher during the mating season compared with all other sampling windows (Fig. 1a). Males also had lower total T_4 than females during the mating season and there were no significant differences in male total T_4 levels across the season. In contrast, total T_3 levels differed across sampling intervals and were consistently higher in females than in males; the interaction between sex and sampling interval was not significant. Plasma total T_3 levels were highest during late lactation, intermediate during mating and post lactation, and lowest during the interval of female fattening (Fig. 1d). Animals were not sampled during late

gestation to early lactation (interval II) in 2014 and assays for free T_3 were not performed on any 2014 samples.

In 2015, total T_4 was affected by sampling interval and was higher in males compared with females, but the interaction between sex and sampling interval was not significant. Plasma total T_4 was higher during mating compared with all other sampling intervals (Fig. 1b). Plasma total T_3 levels in 2015 were affected by sampling interval, but the effect of sex and the interaction between sex and sampling interval were not significant. In 2015, plasma total T_3 levels were highest during mating and lowest during late gestation to early lactation (Fig. 1e). The results for free T_3 in 2015 mirrored the findings for total T_3 with an effect of sampling interval, but with no significant effect of sex and no significant interaction between sex and sampling interval. Plasma free T_3 levels were higher during mating compared with all other

¹Supplementary table and figure are available with the article through the journal Web site at <http://nrcresearchpress.com/doi/suppl/10.1139/cjz-2018-0302>.

Fig. 2. Plasma levels (mean \pm SE) of free triiodothyronine (fT₃) across five sampling intervals in 2015 in free-living arctic ground squirrels (*Urocitellus parryii*). Different letters indicate significant differences ($p < 0.05$) between sampling intervals; the effects of sex and the interaction between sex and sampling interval were not significant. Sampling intervals correspond with stages of the annual cycle as follows — interval I: post hibernation, including mating season; interval II: late gestation to early lactation; interval III: late lactation; interval IV: post lactation; interval V: female fattening.



sampling intervals and lower in late gestation to early lactation than all sampling intervals with the exception of the period of female fattening (Fig. 2).

In 2016, total T₄ levels were affected by sampling interval, but the effect of sex and the interaction between sex and sampling interval were not significant. The seasonal pattern for total T₄ in 2016 was similar to 2014 and 2015, although plasma levels were lower during female fattening compared with all other intervals (Fig. 1c). Similar to prior years, plasma total T₄ levels were highest during mating, although this difference was not significant, except compared with the time period of female fattening. Plasma total T₃ in 2016 was not significantly affected by sampling interval, sex, or the interaction between sampling interval and sex (Fig. 1f). Similarly, free T₃ was not significantly affected by sampling interval or by sex; we were unable to test for the effects of an interaction between sampling interval and sex on free T₃ in 2016 because the model was rank deficient due to low sample size.

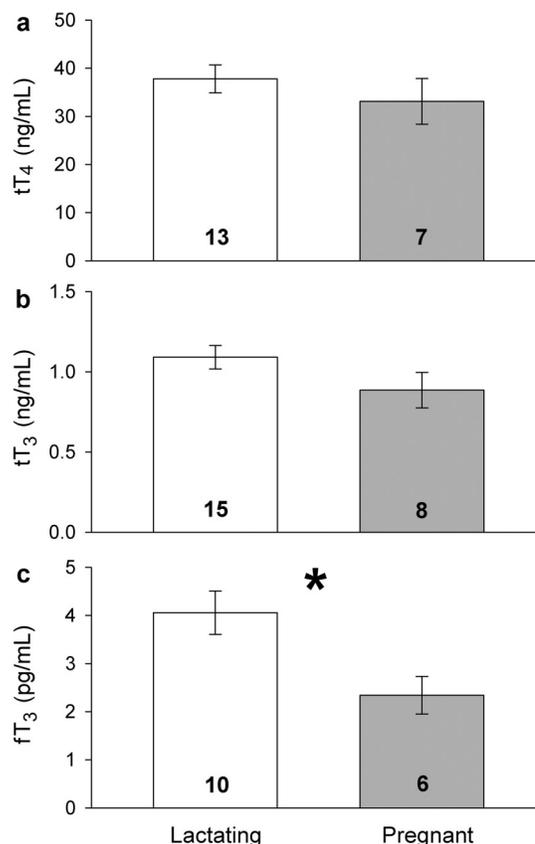
During the second sampling interval (2015 and 2016), although the majority of females were lactating ($n = 15$), some were in the late stages of pregnancy ($n = 8$), whereas others were non-reproductive ($n = 3$) or had lost their litters ($n = 2$). Compared with pregnant females, lactating females did not differ in total T₄ ($p = 0.79$) or total T₃ ($p = 0.07$), but had higher levels of free T₃ ($p < 0.0001$; Figs. 3a–3c).

Correlations between plasma free TH, plasma total TH, and fecal T₃ levels

In 2015, plasma free T₃ was positively correlated with total T₃ ($r = 0.63$, $n = 118$, $p < 0.0001$; Fig. 4a) and with total T₄ ($r = 0.45$, $n = 119$, $p < 0.0001$; Fig. 4b). In 2016, when variability in plasma total T₃ and total T₄ levels was lower and sample size was smaller, free T₃ was not correlated with either total T₃ ($r = 0.20$, $n = 28$, $p = 0.30$) or total T₄ ($r = 0.31$, $n = 29$, $p = 0.09$). Plasma total T₃ was also positively correlated with total T₄ in 2015 ($r = 0.36$, $n = 122$, $p < 0.001$), but the correlation between total T₃ and total T₄ was also weaker in 2016, and only approached significance ($r = 0.21$, $n = 75$, $p = 0.07$; Fig. 4c).

Measures of fecal T₃ (i.e., T₃ metabolites) ranged between 17 and 55 ng/g but were not correlated with plasma total T₃ ($r = -0.06$, $n = 47$, $p = 0.69$) or with plasma total T₄ ($r = -0.19$, $n = 51$, $p = 0.19$). There was, however, a negative relationship between fecal T₃ and free T₃ ($r = -0.55$, $n = 18$, $p = 0.01$; one outlier removed).

Fig. 3. Plasma levels (mean \pm SE) of (a) total thyroxine (tT₄), (b) total triiodothyronine (tT₃), and (c) free T₃ (fT₃) in female arctic ground squirrels (*Urocitellus parryii*) that have recently given birth to offspring and females that are late in gestation but have not yet given birth. The asterisk indicates a significant difference ($p < 0.05$). Data shown are from 2015 and 2016 combined; year was included as a random effect in mixed models.



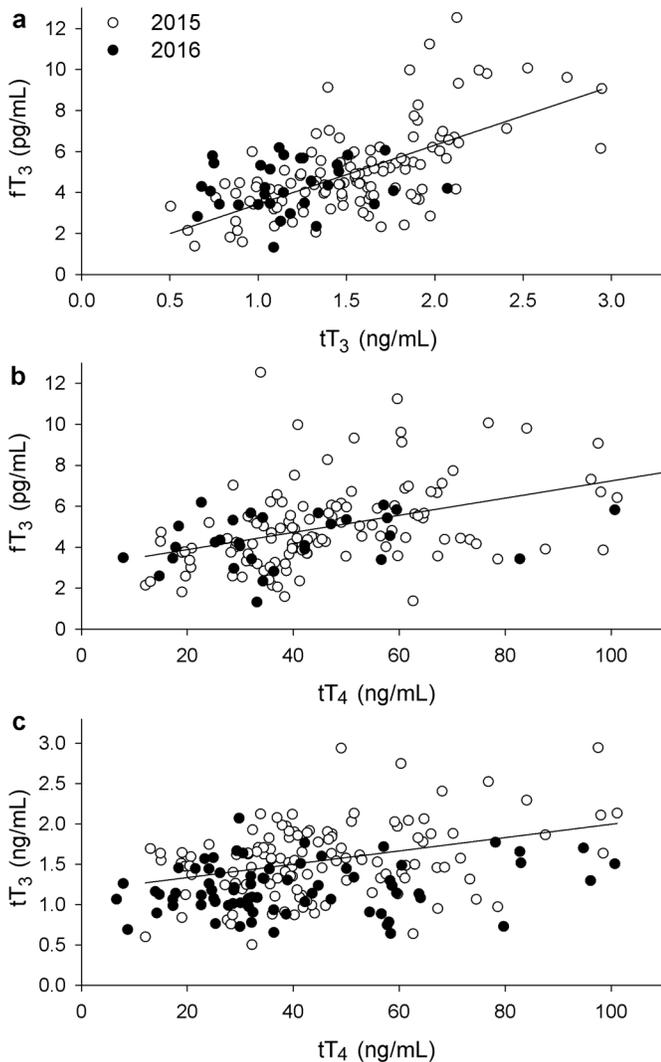
Weather

Environmental conditions during the active season of arctic ground squirrels across the 3 years are shown in Supplementary Fig. S1.¹ Overall, day-to-day weather was highly variable and total levels of precipitation across the active season were higher in 2014 (156 mm) and 2016 (183 mm) relative to 2015 (93 mm). However, within the animal sampling intervals, we found no significant effects of year on solar radiation ($p > 0.26$ for all intervals) or on the frequency of rainy days ($p > 0.07$ for all intervals). However, the sampling interval that included late gestation to early lactation was, on average, 9.1 °C (95% CI: 5.3 °C, 12.9 °C) cooler in 2014 compared with 2015 and 4.3 °C (95% CI: 0.68 °C, 8.1 °C) cooler in 2014 compared with 2016. No other intervals differed across years ($p > 0.32$ for all).

Discussion

TH play an important role in modulating metabolism (Mullur et al. 2014), stimulating thermogenesis (Silva 2006), and promoting reproduction through both central and peripheral mechanisms (Cooke et al. 2004). For these reasons, we predicted plasma TH levels may exhibit consistent sex-dependent seasonal patterns as arctic ground squirrels transition across reproductive stages during their active season. However, the effects of sex and season on plasma TH levels were relatively inconsistent across years. In 2014, total T₃ levels were highest during lactation, a result that is opposite to what has been observed in some studies of captive rodents (Fukuda et al. 1980; Calvo et al. 1990). We previously in-

Fig. 4. The correlations between (a) free triiodothyronine (fT_3) and total T_3 (tT_3), (b) fT_3 and total thyroxine (tT_4), and (c) tT_3 and tT_4 in 2015 (open circles) and 2016 (solid circles) in free-living arctic ground squirrels (*Urocitellus parryii*). Trend lines indicate significant relationships in 2015.



interpreted high total T_3 during lactation as likely being associated with the high MR associated with acquiring food and lactating (Wilsterman et al. 2015). However, during the present study, we found the opposite pattern in 2015, with arctic ground squirrels exhibiting lower total T_3 and free T_3 during gestation and lactation. We also found that sex differences in TH dynamics were inconsistent across years. Thus, sex differences in when energy is allocated towards reproductive effort do not appear to be strongly associated with seasonal TH dynamics.

We suggest that circulating levels of T_3 are likely being modulated in response to environmental conditions, although what is driving the interannual variability is unclear. Interestingly, total T_3 appeared to be correlated with time spent above ground in 2014 (Wilsterman et al. 2015), though whether total T_3 actually influences activity patterns or whether changes in activity and levels of TH are both simply responsive to weather conditions (or another unmeasured factor) remains unclear. The first year of this study was cooler during late lactation to early gestation than other years, which likely increased thermoregulatory costs. However, we could not detect significant effects of year on ambient conditions within the sampling intervals in which TH differed across

years. Additionally, differences in forage quality or availability between years seems likely because lactating females were significantly heavier in 2015 compared with 2014 (Williams et al. 2016a). Food deprivation in captivity and natural fasting are known to depress T_3 levels and BMR (reviewed in Behringer et al. 2018), but experimental manipulations of free-living individuals are needed to determine how fluctuating resource availability influences TH levels in natural populations.

TH metabolism

The hypothalamus–pituitary–thyroid (HPT) axis involves the production of thyrotropin-releasing hormone (TRH) in the paraventricular nucleus of the hypothalamus triggering the production and release of thyroid-stimulating hormone (TSH) from the anterior pituitary, which, in turn, stimulates the release of the two primary THs (T_4 and T_3) from the thyroid gland. Although T_3 is considered the biologically active form of TH, T_4 acts as a precursor for T_3 and occurs at much higher concentrations in plasma, both as total hormone and as free (unbound) hormone. Both T_3 and T_4 are transported in the plasma bound to thyroid-binding globulin (TBG) and albumin, as well as to a lesser degree to the thyroid hormone distributor protein transthyretin (Richardson et al. 2015). We found significant correlations between free T_3 and total T_3 , although the strength of these correlations was moderate. However, correlations between free T_3 and other hormones were not significant in 2016, possibly owing to the smaller sample sizes and decreased TH variability in that year. Interestingly, the seasonal patterns observed for free T_3 were similar to seasonal changes in total T_3 , despite our smaller sample sizes. Although the current study suggests similar seasonal patterns in total T_3 and free T_3 , more work is needed to understand when and how levels of binding globulins are modulated and the potential for the correlations between total T_3 and free T_3 to vary across space and time.

Although the molecular actions of steroids are directly related to the concentrations of free (unbound) hormone in plasma, TH actions in target tissues are modulated by the density and composition of transporters and intracellular deiodinases (i.e., DIO1, DIO2, and DIO3) in target tissues (Visser et al. 2011). Consequently, some actions of T_3 occur independently of plasma TH levels and are instead controlled almost entirely by variation in TH transporters and (or) by deiodinase-driven changes in T_3 concentrations within the target tissue (Bianco and Kim 2006; Dardente et al. 2014). Nevertheless, despite the variety of intracellular mechanisms that affect the bioavailability of T_3 in target tissues, BMR has been shown to be strongly correlated with both free T_3 and total T_3 concentrations in plasma or serum, particularly in birds (Sechman et al. 1988; Chastel et al. 2003; Elliott et al. 2013; Welcker et al. 2013). BMR can exhibit predictable changes across stages of the annual cycle (e.g., Piersma et al. 1995) and therefore we anticipated predictable and endogenously driven changes in circulating TH levels across the active season. Instead, we found that seasonal pattern of TH dynamics varied across years suggesting that environmental heterogeneity might be driving the observed changes in TH physiology and masking any endogenous seasonal rhythms, assuming such rhythms exist.

Fecal vs. plasma TH

Although TH concentrations are most often measured in blood, there is increasing interest in non-invasive assessment of TH status by measuring TH metabolites in feces (Behringer et al. 2018; Gesquiere et al. 2018). However, although fecal hormone analyses are becoming more popular in wildlife conservation and ecological research, it is important to recognize that caveats relating to sex, diet, MR, and individual differences in hormone metabolite formation can sometimes reduce the inferences that can be drawn from fecal hormone measurements (reviewed in Goymann 2012). We found no relationships between fecal T_3 and total T_3 or

between fecal T_3 and total T_4 . Our findings with respect to the relationships between fecal T_3 and free T_3 were surprising because animals with higher free T_3 had lower fecal T_3 , which is opposite to what we predicted. Given the absence of a positive relationship between fecal T_3 and circulating TH levels, we suggest that caution should be used in drawing conclusions from fecal T_3 in this species.

Conclusions

Metabolic flexibility is likely critical for the survival and reproduction of arctic ground squirrels in their natural environments, which are characterized by extreme seasonality with unpredictable inclement weather during short active seasons (Williams et al. 2017). Although plasma T_3 and T_4 exhibit predictable declines across gestation and lactation in some captive rodents (Fukuda et al. 1980; Calvo et al. 1990), we found that TH dynamics across the active season of arctic ground squirrels varied substantially from year to year. Our findings indicate that annual variation in environmental conditions may override or mask seasonal changes in plasma TH levels, assuming endogenous seasonal TH rhythms exist. Given the importance of TH as modulators of metabolism and thermoregulation, there is a need for more studies of TH dynamics in free-living systems. Specifically, we encourage more experimental manipulations designed to better understand the role of these hormones in coordinating physiological and behavioral responses to environmental variation.

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