Regulation of vernal migration in Gambel's white-crowned sparrows: role of thyroxine and triiodothyronine.

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21 **Abstract**

22 Appropriate timing of migratory behavior is critical for migrant species. For many temperate
23 zone birds in the spring, lengthening photoperiod is the initial cue leading to morphological,
24 physiological and behavior changes that are necessary for vernal migration and breeding. Strong
25 evidence has emerged in recent years linking thyroid hormone signaling to the photoinduction of
26 breeding in birds while more limited information suggest a potential role in the regulation of
27 vernal migration in photoperiodic songbirds. Here we investigate the development and
28 expression of the vernal migratory life history stage in captive Gambel's white-crowned
29 sparrows (*Zonotrichia leucophrys gambelii*) in a hypothyroidic state, induced by chemical
30 inhibition of thyroid hormone production. To explore possible variations in the effects of the two
31 thyroid hormones, triiodothyronine and thyroxine, we subsequently performed a thyroid
32 inhibition coupled with replacement therapy. We found that chemical inhibition of thyroid
33 hormones resulted in complete abolishment of mass gain, fattening, and muscle hypertrophy
34 associated with migratory preparation as well as resulting in failure to display nocturnal
35 restlessness behavior. Replacement of thyroxine rescued all of these elements to near control
36 levels while triiodothyronine replacement displayed partial or delayed rescue. Our findings
37 support thyroid hormones as being necessary for the expression of changes in morphology and
38 physiology associated with migration as well as migratory behavior itself.

39 **Introduction**

40 Increasing photoperiod in spring triggers a cascade of events leading to the expression of the
41 vernal life history stages that include prenuptial molt, migration, and breeding in migratory
42 songbirds such as the white-crowned sparrow (Ramenofsky and Wingfield, 2006; Wingfield et

al., 1990). Since Rowan's (1925) pioneering discovery of photoinduction of migratory behavior in captive dark-eyed juncos (*Junco hyemalis*) our understanding of the suite of vernal events triggered by photoperiod has increased greatly. However, the neural and hormonal mechanisms regulating the development of migratory readiness and expression of migratory behavior remain incomplete. Of the numerous endocrine candidates, thyroid hormones offer great promise although their role is poorly studied. Early investigations showed histological increases in thyroid gland activity in white-crowned sparrows with season and temperature (Oakeson and Lilley, 1960; Wilson and Farner, 1960). Building on these observations, increases in plasma levels of thyroid hormones were found in Canada geese (*Branta canadensis*; John & George 1978), Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*; Smith 1982), and rosy pastor (*Sturnus roseus*; Pathak and Chandola 1984) during the period preceding vernal migration. Furthermore, Wingfield et al. (1996) found significant increases in thyroid hormones from wintering levels 10 to 30 days following photostimulation with 20L:4D (Light:Dark). However, work on red-headed buntings (*Emberiza bruniceps*) showed direct involvement of thyroid hormones via thyroidectomy, which suppressed mass gain and expression of nocturnal restlessness (Pant and Chandola-Saklani, 1993). Furthermore, they demonstrated that administration of thyroid hormones partially reinstated both mass gain and nocturnal restlessness (Nair et al., 1994). Taken together these studies provide evidence that thyroid hormones may have a critical in role vernal migration.

More recently, the hypothalamic-pituitary-thyroid axis has been strongly implicated to regulate the photoinduction of breeding (reviewed in: Nakane and Yoshimura, 2010). Thyroid hormones acting within the brain have been identified as a key component in the pathway leading from transduction of photoperiodic information into the gonadotropin-releasing hormone (GnRH)

66 cascade ultimately responsible for the onset of breeding (Nakane and Yoshimura, 2010; Nakao et
67 al., 2008a; Nakao et al., 2008b). Recent work by Wang et al. (2013) showed that Gambel's
68 white-crowned sparrows exposed to long photoperiods under low-intensity light (green light 510
69 nm), displayed normal patterns of migratory fattening and nocturnal restlessness, previously
70 characterized for the species (Agatsuma and Ramenofsky, 2006), but no gonadal growth. These
71 findings suggest independent neural regulation of migration from breeding as noted previously
72 for this species (Ramenofsky, 2011; Stetson and Erickson, 1972; Yokoyama, 1976) and may
73 indicate involvement of thyroid hormones in the transduction of photoperiodic information in the
74 regulation of multiple pathways.

75 Given the effects of thyroidectomy on migratory behavior and its role in the induction of the
76 hypothalamic-pituitary-gonadal axis, we hypothesized that thyroid hormones may play a similar
77 role in mediating the response to photoperiod that triggers the onset of the vernal migratory life
78 history stage. If this is the case, it is possible that local thyroid signaling, analogous to that
79 involved in breeding, is responsible for the photoperiodic response of the vernal migration life
80 history stage. Here we present our findings from two experiments designed to address the role
81 thyroid hormones in vernal migration in a seasonally breeding songbird.

82 First we sought to establish the validity of chemical inhibition of thyroid hormone production as
83 a reversible alternative to surgical or radiation based methods of thyroidectomy in wild birds as
84 well as appropriate dosages (Weng et al., 2007). We further sought to confirm abolishment of
85 major vernal events following chemical inhibition of thyroid hormones. To investigate the
86 observed effects of the thyroid inhibition were due to the absence of circulating thyroid
87 hormones and not pharmacological effects of the anti-thyroid agent methimazole, we conducted
88 a thyroid replacement experiment. The replacement experiment allowed for disassociation

between effects of 3,5,3'-triiodothyronine (T3), classically considered the active form of the hormone (higher receptor affinity), and thyroxine (T4) the primary product of the thyroid gland. We present two predictions: first, that inhibition of thyroid hormone production would abolish pre-migratory fattening, mass gain, increases in muscle size and the onset of nocturnal restlessness. Second, that thyroid replacement would restore all changes in morphology/physiology and behaviors to levels observed in control birds.

Methods

Study Species

The Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelii*) is a seasonally breeding, long distance, nocturnal migrant. In preparation for the onset of migration, birds undergo a pre-migratory development phase (Ramenofsky and Wingfield, 2007; Wingfield and Farner, 1978a). During this phase both wild and captive birds exhibit hyperphagia, deposition of subcutaneous fat stores, hypertrophy of pectoralis flight muscles, and changes in metabolic enzymes (Bairlein and Simons, 1995; Bauchinger and Biebach, 2005; Price and Guglielmo, 2010; Price et al., 2010; Ramenofsky, 2011). These changes serve to support endurance flight during migration (Ramenofsky, 2011; Ramenofsky et al., 2012). The transition between the development of migratory capability and expression of migration is poorly characterized in the wild, while in captivity, the behavior of migratory passerines has been well documented (Agatsuma and Ramenofsky, 2006). Captive white-crowned sparrows show a characteristic pattern of twilight quiescence followed by nocturnal restlessness, nighttime movements including wing flapping and bill up behaviors; these are considered to be an active expression of the urge to migrate and are typically seen during the first portion of the night (Agatsuma and

Ramenofsky, 2006; Coverdill et al., 2008; Coverdill et al., 2006). All research was conducted in compliance with Institutional Animal Care and Use Committee guidelines under protocol #17812, the *Guidelines to the Use of Wild Birds in Research*, and National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experiment 1: Chemical Thyroid Inhibition

Eleven male Gambel's white-crowned sparrows were caught in the vicinity of the University of California, Davis, California (N 38.554, W 121.738) in the late autumn of 2012 and brought into captivity. Sex determination was conducted in the field using a wing length cut off of ≥ 75 mm to identify males (Fugle and Rothstein, 1985) and confirmed via laparotomies at the end of the experiment. Birds were acclimated to captivity in flight aviaries for two weeks prior to the start of the experiment, at which point they were moved to individual registration cages. Each cage was equipped with an infrared photodetection system to record locomotor activity and transfer the output to a Mini Mitter Acquisition System – Vital View (Sunriver, OR) per the methods of Ramenofsky and Németh (2014). This system monitors breaks of an infrared beam crossing the perch and has been successfully used to tie raw activity to nocturnal restlessness behavior (Agatsuma and Ramenofsky, 2006; Ramenofsky and Németh, 2014). Birds were held on natural photoperiod ($\sim 38.5^\circ$ N), at 21-24°C, and given water and food ad libitum.

Birds were randomly assigned to two groups: control implant (n=4) and treatment (n=7). All birds in the study received two 14 mm silastic implants, sealed with silicone sealant at both ends, inserted subcutaneously beginning on February 21, 2013 and replaced every two weeks until the termination of the study. The implants for the treatment groups were packed with the anti-thyroid agent methimazole (M8506, Sigma-Aldrich, St. Louis MO) and the vehicle controls were

left empty. Methimazole acts to block the production of thyroid hormones within the thyroid gland, targeting the enzyme thyroid peroxidase. In addition to administration of silastic implants, treatment birds were also given filter sterilized methimazole orally (500mg/L; per Weng et al. 2007) in their drinking water. The combination of implants and oral administration was chosen to ensure sufficient dosage of methimazole to achieve inhibition of thyroid hormone, as implants alone were determined to be insufficient (Pérez unpublished data).

Experiment 2: Thyroid Replacement

Thirty-two adult white-crowned sparrows were caught near the campus of the University of California, Davis in early winter 2014. Sex was again confirmed at the end of the experimental period by necropsy; 29 males and 3 females had been included. Inclusion of the females did not affect the results of analyses for all variables examined thus they were not removed from final analyses presented here. Birds were housed and monitored as described previously for experiment 1.

Birds were randomly assigned to four treatment groups (n=8): control, methimazole treated (M), methimazole + T3 (MT3), and methimazole + T4 (MT4). Two 14mm silastic implants packed with Methimazole, along with oral administration (500mg/ml) were begun on Feb. 20, 2014. Additionally, the MT3 birds received a single 10 mm subcutaneous silastic implant packed with T3 (T2877, Sigma Aldrich, St. Louis MO) while the MT4 birds received a 12 mm silastic implant packed with T4 (T2376, Sigma Aldrich, St. Louis MO) inserted on March 6th or 7th, 2014. T3 and T4 implants were punctured twice with a needle to enhance diffusion. Emptying rates for both T3 and T4 implants had been previously determined to be slow enough that

implants were only replaced once halfway through the experimental period to ensure continuous delivery, as thyroid hormone remained in all implants when removed.

Measurements & Sampling

Bi-monthly morphometrics of subcutaneous fat were scored on a scale from 0 (no fat) to 5 (greatly bulging with fat) in both the furcular and abdominal regions (Wingfield and Farner, 1978b), and pectoralis muscle size were visually scored (Bairlein and Simons, 1995; Salewski et al., 2009). Mass was measured by Pesola spring scale to the nearest 0.1 g. Blood samples for hormonal analysis were collected monthly. Briefly, the alar vein in the wing was punctured with a 26-gauge needle and surface blood collected into heparinized microcapillary tubes. Blood samples were centrifuged at 10,000 rpm for 5 minutes to separate plasma from formed elements. Plasma was aspirated via Hamilton syringe and stored in a microcentrifuge tube at -30°C until assaying.

Thyroxine Assay

Thyroxine levels were quantified via radioimmunoassay. Briefly, a standard curve was created by serial dilution of a thyroxine standard from 50 ng to 0.195 ng. Samples were run in duplicate using 20 µl of plasma. Water blanks, low (6.25 ng) and high standards (25 ng) were run to internally validate the assay. 20 µl of charcoal stripped white-crowned sparrow plasma was added to non-specific binding, total binding, standard curve and standard tubes as validation testing indicated this addition decreased non-specific binding and improved parallelism over buffer alone. Rabbit polyclonal anti-thyroxine antibody diluted 1:500 was used as the primary antibody (GWB-7B9782 Genwaybio, San Diego CA). Following primary antibody addition 250 µl of barbital buffer with bovine gamma globulins and 8-anilio-1naphthalenesulfonic acid was

added to all samples. Subsequently, 20 μ l (~10000 cpm) of iodinated T4 tracer (NEX111X PerkinElmer, Waltham MA) was added to all tubes. Samples were then incubated for 90 minutes in 37°C water bath. Following the incubation, a 1:4 dilution of goat anti-rabbit gamma globulins (Antibodies, Inc. Davis CA) was added to precipitate bound hormone. Samples were then incubated overnight at 4°C. On the second day, 250 μ l of 5% polyethelene glycol (Sigma Aldrich, St Louis MO) was added to enhance pellet formation and the samples were centrifuged at ~3000 rpm for 30 minutes at 0°C. Supernatant was aspirated from tubes and pellets were counted using a Cobra II Auto-Gamma counter (Packard, Meriden CT). All samples from Experiment 1 were assayed in a single assay, while Experiment 2 were done in two assays. Average inter-assay variation for the assay was 13.6% and intra-assay variation was 6.98%. The limit of detection was 8.44 pg of hormone per assay tube.

Statistical Analyses

All statistical analyses were performed in R version 3.1.1-3.2.4 (Team, 2014a) with the lme4 (Bates et al., 2014), lmerTest (Kunznetsova et al., 2014), glmmADMB (Fournier et al., 2012; Skaug et al., 2016), rstan (Team, 2014b, c), and rethinking (McElreath, 2015) packages. All analyses were conducted independently for the two experiments.

Both plasma thyroxine concentrations and body mass were analyzed via linear mixed effects models using the lme4 package. For both chemical thyroid inhibition and the thyroid replacement experiments, thyroxine concentrations and body mass were modeled using the main effects of sampling date, treatment (categorical), and their interaction. Bird ID was included in all models as a random effect to account for the repeated sampling of individuals. Within

sampling time point comparisons were done via ANOVA and between group post hoc comparisons by Tukey's HSD.

Total fat and pectoralis muscle size were modeled by ordinal logistic regression using a ordered categorical log-odds probability density link function in the rethinking package coupled with the rstan package (McElreath, 2015). In all cases the outcome variable was modeled using random effects for individual to account for repeated sampling. We *a priori* chose to use a model including a universal intercept and treatment by Julian date interactions as fixed effects. Appropriate mixing of the Markov chains was confirmed by visual inspection of plot traces.

The presence of nocturnal restlessness was assessed by comparison of nighttime activity levels between treatment groups. Activity was monitored as the number of infrared beam breaks produced within a fifteen minute interval by each bird, with higher counts indicating more activity. Data were restricted to the period of lights out (scotophase) and averaged by week prior to analysis. Previous work in this system has shown that birds display continuous low levels of activity at night, however, these levels do not represent nocturnal restlessness but rather generalized activity, rather onset of nocturnal restlessness is shown by a further elevation of activity levels (Agatsuma and Ramenofsky, 2006; Ramenofsky and Németh, 2014). Nocturnal restlessness was modeled by generalized linear mixed effects models with random intercepts for each individual to control for repeated sampling and fixed effects of Treatment and week including their interaction. Prior to analysis activity was averaged for each week by individual to reduce noise. For experiment 1 a negative binomial (nbinom1 option in glmmADMB package) distribution was used to handle over dispersion present in the data, while for experiment 2 a zero inflate Poisson distribution was used.

Cohen's d effect sizes were calculated for all ANOVAs and paired tests aside from the lmerTest package's built in t-tests for fixed effects using an online calculator (<http://www.campbellcollaboration.org/escalc/html/EffectSizeCalculator>). Effect sizes for linear mixed effects models were approximated using Ω_2 , which provides a value similar to R_2 (Xu, 2003)

Results

Experiment 1: Chemical Thyroid Inhibition

Comparison of circulating levels of plasma thyroxine in Experiment 1 indicated successful suppression of thyroxine levels by methimazole treatment as reflected by the significant interaction of methimazole treatment and Julian date in the model ($t_{51} = 2.76$, $p = 0.008$; Fig. 1A). Post hoc comparisons indicated that thyroxine levels were the same across groups at the first sampling point of the experiment ($F_{1,9} = 1.30$, $p = 0.283$, $d = 0.72$). However, methimazole treated birds had lower thyroxine levels at the termination (final sampling point) of the experiment ($F_{1,9} = 65.79$, $p < 0.001$, $d = 5.08$).

Initial mass was no different between the two groups at the onset (Julian Day 18) of the experiment ($t_7 = 1.50$, $p = 0.178$, $d = 0.94$). The model ($\Omega_2 = 0.84$) found that mass increased with Julian date ($t_{75} = 7.25$, $p < 0.001$; Fig. 2A). Methimazole treatment suppressed vernal mass gain, resulting in a significant interaction between Julian date and Methimazole treatment ($t_{75} = -4.341$, $p < 0.001$). There was no main effect of Methimazole treatment ($t_{13} = -0.54$, $p = 0.596$). Control birds were estimated to increase mass at a rate of 0.059 g/day while methimazole treated birds were estimated to increase by only 0.015 g/day.

Composite fat scores for control birds increased over the experimental period while methimazole treated birds showed a decline in composite fat scores (Fig. 2B). Modeling of composite fat scores over Julian date for the inhibition of thyroid hormone confirmed visual observation of data. Cumulative probability estimates for composite fat score classes drawn from the posterior distribution of the model indicate that control birds showed an increased probability of having high fat scores over the course of the experiment while methimazole treated birds show a decline in likelihood of high fat scores (Supplemental Fig. 1).

Muscle size of control birds increased over the course of the experiment while methimazole birds remained unchanged (Fig. 2C). Model estimates generated from the posterior distribution of an ordinal logistic regression support these observations (Supplemental Fig. 2). Estimates for control birds indicated an increased probability of high muscle scores (larger muscle) with increasing Julian date, while methimazole birds showed a decrease in the probability of large muscle size over time.

Methimazole treated birds showed no increase in nighttime activity levels, while controls increased activity towards the end of the experimental period, coinciding with the time free-living birds were commencing spring migration from our field sites (Fig. 3A). There was a significant effect of week and a week by Methimazole treatment interaction (Table 1).

Experiment 2: Thyroid Replacement

In the Experiment 2 we detected no main effects of treatment for circulating T4 levels but found significant Julian date by Treatment interactions for all treatments (Table 2). Comparison of initial T4 levels between treatment groups found no differences ($F_{3, 27} = 0.55$, $p = 0.651$, $d = -0.23$)

in thyroxine levels. Beginning on Julian date 106 there were significant differences in thyroxine levels between treatment groups ($F_{3,6}=10.01$, $p<0.001$, $d= -1.91$) that persisted through the final bleed on day 125 ($F_{3,27}=13.18$, $p<0.001$, $d= -2.27$). On day 106 Methimazole (diff= -6.12, $p<0.001$) and MT3 (diff=-6.41, $p<0.001$) birds had lower thyroxine levels than controls while MT4 birds displayed successful rescue (diff=-2.19, $p=0.458$). On day 125 Methimazole (diff=-7.20, $p<0.001$) and MT3 (diff=-7.43, $p<0.001$) birds again showed significantly lower levels than control birds while MT4 birds showed no difference (diff=-3.61, $p=0.07$).

Initial mass between treatment groups were not different ($F_{3,28}=0.14$, $p=0.938$, $d= -0.02$). Our model detected several significant effects ($\Omega_2=0.67$). There was a significant main effect of Julian date ($t_{218}=9.66$, $p<0.001$; Fig.2D), with mass increasing over time. We also detected a significant main effect of methimazole treatment as compared to the control group ($t_{79}=3.38$, $p=0.001$). There were significant interactions of Methimazole and Julian date ($t_{219}=-6.98$, $p<0.001$) and MT3 and Julian date ($t_{219}=-2.32$, $p=0.021$), with both groups showing reduced mass gain compared to controls. There was no interaction between MT4 and Julian date ($t_{219}=-1.35$, $p=0.178$).

Control, MT4 and MT3 birds increased composite fat scores over the experimental period (Fig. 2E). Modeling of composite fat scores predicted increasing probability of high fat scores with Julian date for control birds (Supp. Fig. 3). There was a marginal increase in fat scores for methimazole birds, though the increase was below that observed in all other groups. Model estimates for MT4 birds were nearly identical to those of control birds, while MT3 birds were estimated to fatten intermediately between methimazole and control birds.

Control, MT4, and MT3 birds all showed increased muscle size over the experimental period, while methimazole birds showed decreases in average size (Fig. 2F). Estimates from ordinal logistic models of muscle size predicted an increased probability of high muscle scores with increasing Julian date for control, MT4 and MT3 birds, but decreased probability of high scores in methimazole birds (Supp. Fig. 4).

Control and MT4 birds displayed increased nocturnal activity towards the end of the experimental period, while MT3 birds showed minimal increases (Fig. 3B). Activity levels in Methimazole treated birds remained roughly constant though elevated with the exception of a spike in activity around day 65 that quickly returned to previous levels. The spike however may have represent an artifact of birds entering the initial stages of hypothyroidism as Methimazole treatment led to depletion of thyroid hormone stores. Manual inspection of Methimazole treated birds daily activity profiles indicates that observed activity appears to be confined to the early morning hours prior to lights on, thus inconsistent with classic nocturnal restlessness, which begins shortly after lights out and tapers off towards lights on (Agatsuma and Ramenofsky, 2006). However, such patterns are consistent with nighttime activity ween in birds known to be in the wintering life history stage in captivity (Ramenofsky and Pérez pers. observation). The main effect of week as well as all three week by treatment interaction terms were significant (Table 3).

Discussion

We found strong support for our hypothesis that thyroid hormone signaling is necessary for the expression of vernal migration. Chemical thyroid inhibition completely abolished both the

development and expression of the vernal migratory life history stage in white-crowned sparrows. While, continuous administration of exogenous T4 restored all migratory traits; exogenous T3 only partially restored mass gain, fattening, and muscle hypertrophy, but failed to restore nocturnal restlessness. Though ties between thyroid hormones and expression of vernal migration have frequently been suggested (reviewed in Ramenofsky, 2011) this is only the second study to show conclusive involvement of thyroid hormones in avian migration (Jenni-Eiermann et al., 2002; Nair et al., 1994; Pant and Chandola-Saklani, 1993).

Though our results are broadly consistent with previous findings in red-headed buntings, which showed abolishment of nocturnal restlessness and mass gain in response to surgical thyroidectomy with both T3 and T4 replacement rescuing these traits, they diverge in two key ways. Firstly, ours is the first study to show abolishment of hypertrophy of the pectoralis muscles (by visual scale) and subsequent rescue by T4 administration. Our results show abolishment of all major behavioral and morphological changes associated with vernal migration that were measured, in response to chemical thyroid inhibition, suggesting thyroid action at the level of the brain. Secondly, in contrast to red-headed buntings we found limited efficacy for T3 replacement treatments across all variables measured. T3 replacement appears to be either absent or delayed when compared to MT4 and control birds. Similar differences in efficacy between T4 and T3 have been previously noted in regards to the effects of peripheral thyroid hormone administration on gonadal growth in quail (Follett et al., 1988). However, these T3 results still need to be interpreted with caution.

Several possible explanations for reduced efficacy of T3 as compared to T4 treatment exist. Firstly, it may be possible that migratory processes rely directly on T4 signaling and not T3 in our species. Second, thyroid hormone transporters, that control hormone entry in to the cells,

328 may have higher affinity for T4 and thus little or no T3 is able to reach target cells. This
329 explanation would be consistent with the discrepancies observed between thyroid hormone
330 administered peripherally (T4 more effective) and centrally (T3 more effective) in quail with
331 regards to gonadal growth (Follett and Nicholls, 1988; Follett et al., 1988; Yoshimura et al.,
332 2003). At present several different thyroid transporters have been identified (Arjona et al., 2011;
333 Sharlin et al., 2011; Visser et al., 2011a; Visser et al., 2011b). However, differences in
334 transporter affinities for T4 versus T3 are not well characterized. In addition transporters have
335 been shown to vary in their distribution within the brain (Alkemade et al., 2011). We note that
336 their distribution within the brains of white-crowned sparrows is completely unknown.
337 Complicating the picture further, multiple isoforms of nuclear thyroid hormone receptors exist
338 with varying transactivation efficiency and distributions (Cheng et al., 2010). Furthermore,
339 putative non-genomic receptors have also been identified which are more responsive to T4 than
340 T3 (Davis et al., 2005; Davis et al., 2008). Any one or more of these elements acting alone or in
341 concert with others may explain the lack of response to T3 replacement.

342 Alternatively, T3 has been shown in some cases to display effects opposite to their normal
343 function when circulating levels exceed certain thresholds (Cooke et al., 2004; Holsberger and
344 Cooke, 2005; Holsberger et al., 2005; Yoshimura et al., 2003). Given the imprecise nature of
345 implant administration and lack of an assay for plasma T3 we are unable to rule out
346 pharmacological elevation of circulating T3 leading to a suppressive effect. The final explanation
347 is that T3 implants did not provide adequate rescue of circulating T3 levels. Though this is a
348 possibility the presence of holes in the walls of the implants to aid diffusion make this
349 explanation unlikely.

Though we have shown here that development of migratory condition and expression of migratory behavior is dependent upon thyroid hormones our study is limited in its ability to address mechanistic pathways. Hormonal actions are immensely complicated; depending not only on changes in circulating levels of hormone, but also on carrier proteins, regulatory enzyme systems, membrane transporters (particularly in the case of thyroid hormones) and receptors interacting with their co-regulatory protein complexes (Wingfield, 2013). Ultimately it is a combination of these components and circulating hormones that will be responsible for the observed effects of thyroid hormones on migration. A review of the current literature reveals little is known about changes in distribution or abundance of deiodinase enzymes or thyroid hormone receptors with regards to vernal migration. In fact there is, to the best of our knowledge, no distributional study of thyroid hormone receptors within the adult avian brain. Future work will need to focus on understanding the seasonal changes in the neural components of thyroid signaling if we are to understand the mechanisms by which thyroid hormones act in migration.

Conclusions

The presence of circulating thyroid hormones is necessary for the development of migratory readiness and expression of captive nocturnal restlessness. Definitive roles for T3 versus T4 and their relative importance to constituent processes of the migratory life history stage remain unclear, although at least T4 administration can fully rescue the effects of inhibited thyroid hormone synthesis. Further work at the molecular level within the brain will be necessary to elucidate mechanisms of thyroid signaling in this system.

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Figure 1. Circulating thyroxine levels as measured by radioimmunoassay for Experiment 1: chemical inhibition of thyroid hormone (A) and for experiment 2: thyroid replacement (B). Data are presented for control ("C"), methimazole treated ("M"), methimazole+T3 ("MT3") and methimazole+T4 ("MT4") birds. Start of methimazole treatment is indicated by "↓" and onset of replacement treatments by "‡". All values are presented as means $\pm$ sem.

Figure 2. Changes in morphometric variables in response to chemical thyroid inhibition (A,B,C) and replacement experiments (D,E,F). Changes in mass under chemical inhibition (A) and replacement regimes (D). Composite fat score (B) and pectoralis muscle profile scores (C) in response to chemical inhibition of thyroid hormone production and in response to thyroid replacement treatment (E and F respectively). Treatment categories include control ("C"), methimazole ("M"), methimazole+T3 ("MT3") and methimazole+T4 ("MT4"). Start of methimazole treatment is indicated by ↓, onset of replacement treatments are indicated by ‡. All values are presented as means $\pm$ sem.

Variable	Estimate	Std Error	Z value	p-value
Intercept	4.15	0.39	10.67	<0.001*
Meth Treatment	0.145	0.49	0.30	0.765
Week	0.09	0.02	4.13	<0.001*
Meth X Week	-0.09	0.03	-3.01	0.003*

Table 1. Fixed effect for negative binomial generalized linear mixed effects model of nighttime activity in experiment 1: Chemical Inhibition of Thyroid Hormone. Intercept represents model intercept for control birds.

Variable	Estimate	Std Error	df	t	p-value
Intercept	-1.94	0.97	139	-2.00	0.048
Julian Date	0.08	0.01	117	8.25	<0.001*
Meth Treatment	3.16	1.38	140	2.28	0.24*
MT3 Treatment	3.26	1.34	138	2.43	0.017*
MT4 Treatment	0.83	1.41	139	0.59	0.56
Julian X Meth	-0.09	0.02	117	-5.85	<0.001*
Julian X MT3	-0.09	0.1	117	-6.16	<0.001*
Julian X MT4	-0.3	0.2	117	-2.29	0.024*

Table 2. Effects of Julian date, treatment and their interaction on circulating T4 levels during Experiment 2: Thyroid Replacement using linear mixed effects model. Main effects of treatment for Methimazole (Meth Treatment), MT3 and MT4 are compared to control birds (Intercept) which serve as the baseline of all comparisons.

Figure 3. Nighttime activity expressed as mean number of beam breaks/15minute interval for each week for control (“C”), Methimazole (“M”), Methimazole+T3 (“MT3”), and Methimazole+T4 (“MT4”) birds. Experiment 1: chemical inhibition of thyroid hormone (A) and experiment 2: thyroid replacement (B). Start of methimazole treatment is indicated by ↓, onset of replacement treatments are indicated by ‡. All values presented as means ± sem.

Variable	Estimate	Std Error	Z value	p-value
Intercept	3.17	0.56	5.72	<0.001*
Meth Treatment	0.85	0.73	1.17	0.24
MT3 Treatment	0.43	0.75	0.57	0.57
MT4 Treatment	-0.03	0.77	-0.04	0.97
Week	0.6	0.004	15.55	<0.001*
Meth X Week	-0.11	0.006	-19.86	<0.001*
MT3 X Week	-0.11	0.006	-19.43	<0.001*
MT4 X Week	0.04	0.002	25.36	<0.001*

Table 3. Fixed effects for zero inflate poisson generalized linear mixed effects model of nighttime activity in Experiment 2: thyroid replacement. Main effects of treatment for Methimazole (Meth Treatment), MT3 and MT4 are compared to control (Intercept) which serve as the baseline.