Plasma Thyroid Hormone Concentrations in a Wintering Passerine Bird: Their Relationship to Geographic Variation, Environmental Factors, Metabolic Rate, and Body Fat

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ABSTRACT

Winter acclimatization among passerine birds involves metabolic adjustments that allow for high rates of thermogenesis. In previous studies, we observed geographic variation in the basal metabolic rate (BMR) of overwintering cardinals along a latitudinal gradient at two different longitudinal transects. Because thyroid hormones (THs) are important for metabolic adjustments in endotherms, we determined whether geographic variation in BMR can be explained by variation in thyroid status. We measured total plasma TH (thyroxine [T_4] and 3,5,3′-triiodothyronine [T_3]) concentrations by radioimmunoassay in birds from two latitudinal transects extending from approximately 31° to 42°. Birds from both transects had higher plasma THs in the late afternoon than in the early morning. Plasma T_3 increased with latitude, while plasma T_4 varied such that the southernmost birds and the northernmost birds had higher hormone concentrations than birds at the intermediate latitude. There was no correlation between plasma TH concentrations and BMR. To test whether thyroid status influences metabolic parameters in winter-acclimatized captive cardinals, we fed cardinals diets supplemented with T_4 (5 μg T_4 g^{-1} food), the goitrogen methimazole (1 mg g^{-1} food), or both. Plasma T_4 concentrations were altered by most of the treatments, but we observed no significant effects on any metabolic parameter. We conclude, therefore, that there is latitudinal variation in metabolic parameters in cardinals but that this variation is not explained by variation in plasma TH concentrations.

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Introduction

Winter acclimatization among passerine birds is primarily a metabolic process characterized by increased ability to sustain high rates of thermogenesis (Dawson et al. 1983*b*; Swanson 1990; O'Connor 1995*a*; Dawson and O'Connor 1996). By using endogenous stores of lipid to support shivering, birds in temperate climates can maintain energetic homeostasis during challenging winter conditions such as long, cold nights and temporary interruptions to foraging (Carey et al. 1978; Dawson et al. 1983*a*). Winter-acclimatized birds not only store lipid in larger quantities than nonacclimatized birds but also possess an enhanced ability to mobilize it as an energy substrate (e.g., O'Connor 1995*b*). The activities of certain metabolic enzymes appear to play a central role in this substrate mobilization (Dawson et al. 1983*b*; Carey et al. 1989; Marsh and Dawson 1989). These metabolic characteristics differ both seasonally and geographically: winter birds have greater cold tolerance than summer birds, and during winter, birds from colder climates have greater cold tolerance than birds from warmer climates (Dawson et al. 1983*a*; Swanson 1990; O'Connor 1996). Basal metabolic rate (BMR), which can also vary both seasonally and geographically (Root et al. 1991; Swanson 1991; Cooper and Swanson 1994; O'Connor 1996), may reflect functionally important adjustments to energetic considerations (Daan et al. 1990; Dawson and O'Connor 1996). However, because not all birds exhibit seasonal variation in BMR, it may not be an essential part of seasonal acclimatization (Dawson and Marsh 1989). Seasonal changes in BMR often, but not always, accompany changes in thermogenic capacity or endurance, but the nature of the relationship between them is not understood (Dawson and O'Connor 1996).

Although our understanding of the various adjustments that characterize winter acclimatization is improving, how these adjustments are regulated, both seasonally and geographically, remains largely unexplained. The metabolic nature of these adjustments, as summarized above, suggests that several hormones are probably involved, with likely candidates including the thyroid hormones (THs), among others (Dawson et al. 1992). THs thyroxine (T_4) and 3,5,3′-triiodothyronine (T_3) play important roles in substrate metabolism (Mariash and Oppenheimer 1983;

Heimberg et al. 1985); thermogenesis (Klandorf et al. 1981; Guernsey and Edelman 1983; Danforth and Burger 1984; Lam and Harvey 1990); and seasonal coordination of migration, premigratory fattening, and reproduction (Pathak and Chandola 1982; Smith 1982; Pant and Chandola 1995). This makes them prime candidates for involvement in the coordination of winter acclimatization in passerines.

THs are both hyperglycemic and lipolytic and are known to increase gluconeogenesis, glycogenolysis, glucose metabolism, and the release of fatty acids (see McNabb 1992). Changes in the activity of the thyroid system in response to environmental stimuli are complex and probably involve changes in thyroid gland output, peripheral conversion of T_4 to T_3, degradation of T_3, modulation of TH by plasma binding proteins, and regulation of cellular TH receptors (Rudas and Pethes 1984, 1986; McNabb 1992; Darras et al. 1995). Natural rhythms in plasma concentrations of THs occur seasonally and daily, probably in response to cold exposure and food intake (Kühn and Nouwen 1978; May 1978; Chandola and Pathak 1980; Klandorf et al. 1981; Sharp et al. 1984; May and Reece 1986; Stokkan et al. 1985; Cogburn and Freeman 1987). Food restriction and nutritional status also affect circulating concentrations of TH (Harvey et al. 1981; Klandorf and Harvey 1985; Eales 1988). Dawson et al. (1992) reported seasonal variation in TH of American goldfinches (*Carduelis tristis*), a species central to the characterization of winter acclimatization in passerines, and interpreted those patterns as supporting the hypothesis that THs may be involved in seasonal acclimatization. Goldfinches living in Michigan had higher plasma concentrations of T_4 in the summer than in the winter and higher concentrations of T_3 in the winter than in the summer.

In this study, we conducted both field sampling and captive experimental manipulations to determine whether previously observed latitudinal variation in metabolic parameters (Burger 1998) in a common, nonmigratory passerine bird, the northern cardinal (*Cardinalis cardinalis*), might be explained by variation in activity of the thyroid system. In the field, we collected blood samples and made geographic comparisons of plasma T_3 and T_4 during winter. Wintering cardinals fit the profile of acclimatized passerines in that they appear to rely primarily on lipid to meet nocturnal energy requirements: birds caught in the morning have lower fat stores than birds caught in the evening at the same locations (Burger 1998). Moreover, differences between morning and evening body reserves are greater at colder, northern sites than at milder, southern sites, which suggests that energy requirements vary geographically with ambient temperature (Burger 1998). Presumably in response to those energy requirements, cardinals exhibit geographic variation in both lipid stores and BMR (Burger 1998); however, to date, thermogenic capacity and enzyme activities have not been measured in this species. In addition to assessing how TH concentrations vary throughout the range of this species, we also examined how they may correlate with ambient temperature, body fat, and BMR. The experimental portion of this study involved captive cardinals. We manipulated plasma TH concentrations by providing captive birds with food supplemented with T_4 or the goitrogen methimazole in order to determine whether the induction of a hyper- or hypothyroid state affects BMR, body temperature, and body fat stores.

Material and Methods

Geographic Comparison Field Sites and Methods

To analyze geographic variation in plasma TH concentrations, we captured northern cardinals ($N = 418$) in mist nets at six locations (Fig. 1) between January 1 and February 21 during the winters of 1992–93 and 1993–94 (hereafter 1993 and 1994, respectively). These locations included private and University of Michigan properties near Ann Arbor, Michigan (42°17′, 83°44′ W); Paynetown State Recreation Area, Monroe Reservoir property, and Indiana University properties near Bloomington, Indiana (39°13′ N, 86°35′ W); Eufaula National Wildlife Refuge near Eufaula, Alabama (31°53′ N, 85°09′ W); Ledges State Park and U.S. Army Corps of Engineers Saylorville Lake property near Boone, Iowa (42°04′ N, 93°53′ W); Fountain Grove Wildlife Area near Chilicothe, Missouri (39°47′ N, 93°33′ W); and U.S. Army Corps of Engineers Wallace Lake Reservoir property and Bodcau Wildlife Area near Shreveport, Louisiana (32°28′ N, 93°46′ W). From north to south, the Michigan, Indiana, and Alabama sites comprise the eastern transect; the Iowa, Missouri, and Louisiana sites comprise the western transect.

All cardinals captured in 1993 ($N = 161$) were caught within the first 2 h of civil sunrise (morning); between 22 and 33 cardinals were captured at each location. In 1994, a total of 257 cardinals were captured. Of these, 124 were captured in the morning and 133 were captured in the evening (within 2 h of civil sunset); at each time (morning and evening), between 15 and 28 cardinals were captured at each location.

We weighed all captured birds. From most ($N = 243$), we removed a small blood sample (100–200 μL) via the brachial vein. These birds we then released. From the rest ($N = 175$, between eight and 11 adult males per location per time), we removed blood samples via cardiac puncture. We then killed these birds via thoracic compression for use in body composition analyses. For each bird, the times when it was first caught in a net and when its blood sampling was completed were recorded so that we could determine whether the time that elapsed between the two events had an effect on plasma hormone concentrations. Whenever possible, this time interval was minimized.

We immediately placed the carcasses of the birds we killed into individual plastic bags and put them on ice; within 4 h, the bags were placed in a freezer or on dry ice and frozen. We collected all blood with EDTA-treated equipment and stored it on ice for up to 2 h before centrifuging it in the field. Immediately after centrifuging the blood, we pipetted the plasma

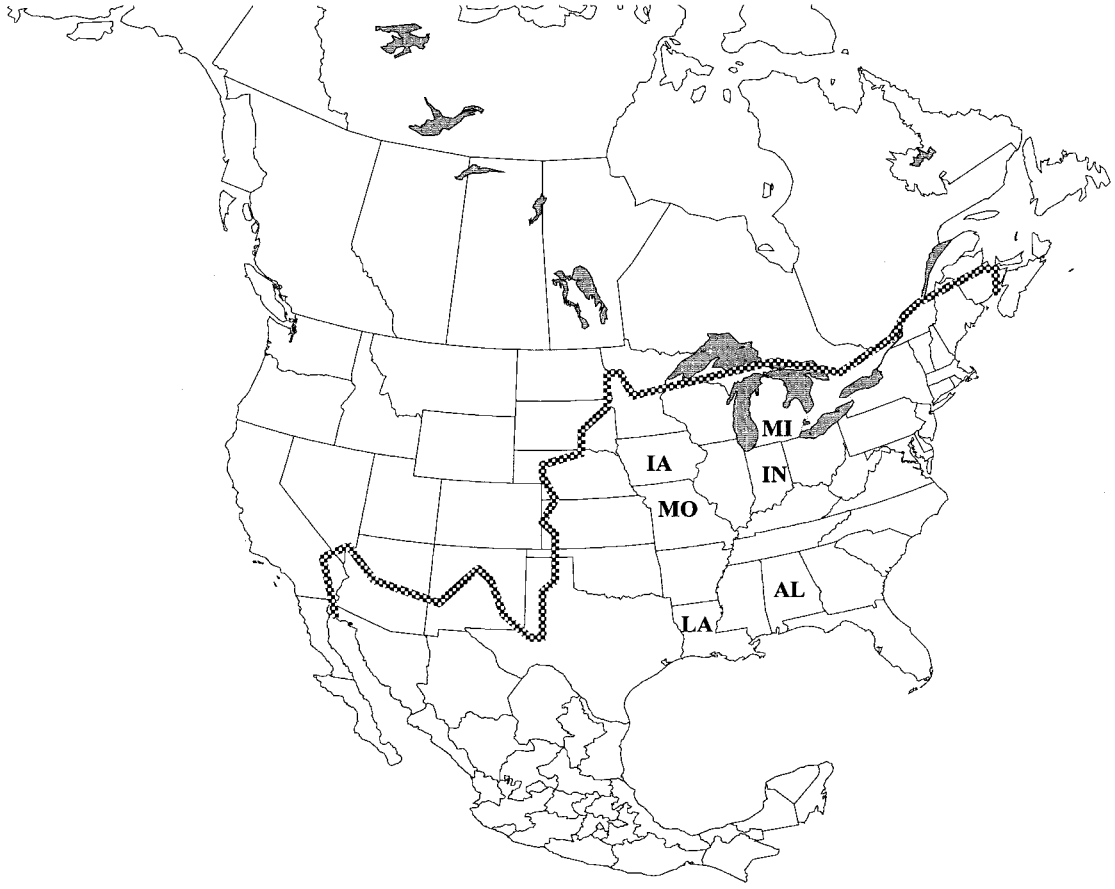

Figure 1. Location of field sites along a latitudinal gradient at two longitudinal transects. The heavy line running east to west represents the northern limit to the cardinal's range (see Root 1991).

into separate EDTA-treated cryotubes, froze it in liquid nitrogen, and then stored it at −70°C until radioimmunoassay (RIA) for T_3 and T_4 (see "Plasma Hormone Concentrations").

Captive Experimental Methods

In the second part of this study, we captured 29 cardinals between January 6 and February 28, 1997, and held them in outdoor aviaries at the Michigan site. We did this to determine whether experimental manipulation of plasma TH concentrations affects BMR or body composition. We captured birds for this part of the study in the evening, weighed them, and then removed a small blood sample from them via their brachial vein. We handled the blood in the manner described above. We then transported the birds to the laboratory (we arrived just after civil sunset), where we measured their BMRs in the manner described below (see "Basal Metabolism").

In the morning, we transported birds to a quiet, remote campus location and placed them individually into aviaries (see description below). We randomly assigned the birds to one of four treatment groups: control, T_4 supplement, methimazole, and both T_4 and methimazole. Methimazole is a goitrogen that specifically inhibits thyroid peroxidase-catalyzed iodination of thyroglobulin within the thyroid gland (see Taurog 1996). We administered treatments via food provided ad lib. Thyroxine (sodium salt), methimazole, both, or neither were dissolved in a small amount (between 0.3 and 0.5 mL) of 0.1 M NaOH. Distilled water was then added to adjust the concentrations of these solutions, which were sprayed onto food (equal parts shelled sunflower seeds and cracked corn) and allowed to dry. Food for the control group was sprayed with the vehicle only (NaOH and ddH_2O), while food for the other groups was treated as follows: T_4-supplement group food was 5 μg T_4 g^{-1} food, methimazole group food was 1 mg methimazole g^{-1} food, and both T_4 and methimazole group food were both 5 μg T_4 g^{-1} food and 1 mg methimazole g^{-1} food. Dosages were chosen (based on results of a pilot study in 1995) to affect changes in T_3 and T_4 within the physiological ranges of these hormones

found in free-living cardinals. Treated food was stored in airtight containers shielded from sunlight. All other aspects of handling and living conditions were similar for all birds (see below).

Each wire aviary measured 0.61 m wide × 1.52 m deep × 1.83 m high and had a plywood roof over the back half (away from the door) of the top and a plywood wall covering the top third of the back. Burlap covered the rest of the back and both sides. A small cut conifer tree (commercially available Christmas tree of the same size and species) was placed upright in the back of each aviary to provide roosts similar to those used by free-living cardinals (M. F. Burger and R. J. Denver, personal observation). Small branches from deciduous trees were placed in each aviary so that birds could perch in or out of any sunlight. Water (in a 25-cm-diameter plastic dish with an electric bird bath heater) was available at all times in each aviary and refreshed daily. The 10 aviaries were placed side by side and oriented with their doors facing south.

At dusk, on days 5, 9, and 13 of captivity, we retrieved birds from their aviaries, weighed them, and took another blood sample via the brachial vein. We then transported them to the laboratory for BMR measurement. In the mornings after BMR measurements on the fifth and ninth days of captivity, we returned birds to their aviaries, where they remained with minimal disturbance until the next time we measured them. At the end of BMR measurements on the thirteenth day, we killed the birds via thoracic compression, placed them into plastic bags, and froze them. Sample sizes were not always equal due to losses attributable to severe weather or equipment malfunction; however, sample sizes were sufficient in each treatment group.

Plasma Hormone Concentrations

Total T_3 and T_4 concentrations were measured in the plasma of free-living and captive cardinals by RIA (see Dawson et al. 1992). Both T_3 and T_4 RIAs were carried out in barbital buffer (0.09 M, pH = 8.6) that contained 8-anilino-1-napthalene sulfonic acid (ANS; 0.1 g L^{-1}; Kodak) and 1 mg mL^{-1} bovine gamma-globulins (Cohn fraction II; Sigma). Primary antiserums (rabbit) were from Endocrine Sciences, and high specific activity [^{125}I]-T_3 or [^{125}I]-T_4 were from New England Nuclear. Duplicate 25- and 50-μL plasma samples were run for each bird in the T_3 and T_4 RIAs, respectively. Standards for both assays were made in hormone-stripped chicken plasma. We separated bound hormone from free hormone by adding 1 mL of secondary antiserum (sheep antirabbit gamma globulins; Antibodies) and carrier proteins (rabbit immunoglobulins; Antibodies) dissolved in ice-cold 5% polyethylene glycol (PEG 8000; Sigma) to the plasma samples and by incubating the samples at 4°C for 60 min before centrifuging them at 2,000 *g* for 30 min.

We accomplished quality control and standardization among RIAs by including high, medium, and low immunoassay controls (Bio-Rad) in each assay. We included approximately 30 samples (in duplicate) in each T_3 RIA: six T_3 RIAs for 1993 samples, 11 RIAs for 1994 samples, and one RIA for captive samples. Intraassay variation ranged from 0.1% to 10.8%; interassay variation was 15.8%. However, there were no differences in control concentrations between the 1993 assays (1.80 ± SD of 0.21 ng mL^{-1}) and 1994 assays (1.79 ± SD of 0.34 ng mL^{-1}; $T = 0.06$; $P = 0.953$). We included between 60 and 120 samples (in duplicate) in each T_4 RIA: two T_4 RIAs for 1993 samples, two RIAs for 1994 samples, and one RIA for captive samples. For the T_4 RIA, intraassay variation ranged from 3.2% to 7.2%; interassay variation was 8.5%. For each year, samples from the different latitudes, transects, and times of day were randomly distributed among the different RIAs.

We had two methods for validating T_3 and T_4 RIAs. First, we verified parallelism between the standards and dilutions of cardinal plasma. Second, we conducted analyses of recoveries of unlabeled hormone from a pool of cardinal plasma; that is, we determined the quantities of hormone in a sample of cardinal plasma and an aliquot of diluted, dissolved, unlabeled hormone via RIA and found them to sum with the quantity of hormone in a sample that we created by combining an identical aliquot of unlabeled hormone with an identical sample of cardinal plasma. Controls in all assays fell within expected ranges (Bio-Rad).

Body Composition

We thawed and dissected birds in the laboratory. We removed ingesta from the entire length of the alimentary canal, wrapped the remaining carcass (including feathers) in filter paper, freeze-dried it, and weighed it daily until the change in mass from one day to the next was 0.05 g or less. Total freeze-drying time usually was 4 or 5 d. This mass, which we obtained after subtracting the mass of filter paper, comprised the dry mass of the bird. We broke each dry carcass (in filter paper) into pieces <1 cm^3 and placed the pieces into a cellulose thimble. We then placed the thimble in a Soxhlet apparatus and added petroleum ether for 24 h to extract the nonpolar lipids. After extraction, we dried the thimble and its contents in a fume hood for 1 h, oven-dried them at 90°C for 40 min, cooled them in a desiccator at room temperature for 20 min, and then weighed them. We repeated oven-drying, cooling, and weighing until the mass of the thimble and its contents changed by no more than 0.05 g. We stored filter papers and cellulose thimbles in desiccators, and their dry masses were determined in advance. Subtracting the sum of the dry masses of the filter paper and thimble from the dry mass of the thimble and its contents after extraction gives the lean dry mass of the bird, and subtracting lean dry mass from dry mass yields lipid mass (i.e., depot fat). Dry, lean dry, and lipid masses were determined to the nearest 0.01 g.

Basal Metabolism

BMRs of cardinals were measured as oxygen consumption in a portable flow-through respirometry system. Birds were weighed to the nearest 0.1 g immediately before being placed into metabolic chambers (1,800 mL, painted flat black) at approximately civil sunset. To provide a surface to grip and to prevent birds from touching the bottoms of the chambers, we placed wire-mesh platforms in each chamber; the chambers were then immersed in a water bath maintained at 25°C. Dry air was supplied to each chamber at a regulated flow rate of 900 mL min^{-1}, and the birds were left undisturbed (i.e., in dark and quiet) for at least 4 h. At the end of this time, while the birds were still undisturbed, the oxygen content of the dry, CO_2-free excurrent air from the chambers was measured for at least 1 h with paramagnetic oxygen analyzers (Servomex model 570A). The oxygen content of ambient air was measured for 5–10 min at the beginning and end of each measurement period to establish a baseline value for incurrent air. At the end of the measurement period, birds were weighed again to the nearest 0.1 g. We calibrated flowmeters and oxygen analyzers before making measurements. We determined BMRs (mL O_2 h^{-1}) by finding the minimum average oxygen consumption of any 10-min period during the hour, which we calculated with the methods described in Withers (1977). We measured the body temperature of the birds immediately after their removal from the metabolic chambers by inserting a thermocouple approximately 1 cm into their cloaca.

The University of Michigan committee on the use and care of animals in research approved our housing, handling, and experimental techniques for both free-living and captive birds.

Plasma T_4 Binding

We attempted to assess an additional parameter of thyroid status, plasma T_4 binding activity, in an attempt to determine whether plasma T_4 binding proteins might vary geographically (and perhaps alter T_4 availability to tissues). We assessed plasma T_4 binding activity in two ways: (1) polyacrylamide gel electrophoresis (PAGE) followed by autoradiography to examine the distribution of [125I]T_4 binding among plasma proteins and (2) minicolumns of Sephadex G-25 to attempt to quantify the high-affinity binding activity present in cardinal plasma. The latter method is based on the ability of plasma proteins with high affinity for T_4 to remove [125I]T_4 bound to the columns. In developing the two methods, a pool of cardinal plasma was used, and chicken and sheep plasma were also run for comparative purposes. Methods used in this study were exactly the same as those described by Licht et al. (1990). For PAGE, 5 μL of each type of plasma was fractionated in a 7% native gel. For Sephadex G-25 analysis, plasma was diluted in phosphate-buffered saline (PBS; 50 mM sodium phosphate, 100 mM NaCl, pH 7.4) before being applied to the columns. The dilutions of plasma in PBS were 1 : 5.3 for cardinals, 1 : 25 for chickens, and 1 : 100 for sheep.

Environmental Variables

Three temperature variables (TEMP1, TEMP7, TEMPLT in °C) were used in this analysis: TEMP1 is the midpoint between the minimum and maximum temperatures of the day of capture, TEMP7 is the mean of the midpoints between minimum and maximum temperatures for 7 d before capture and including the day of capture, and TEMPLT is the midpoint between long-term average minimum and maximum temperatures in January and February at the site of capture.

The data from which we calculated TEMP1 and TEMP7 were taken from "Record of River and Climatological Observations" forms (obtained from the National Climatic Data Center [NCDC], Asheville, N.C.) for the weather station nearest to each field site. Field sites included the University of Michigan, Ann Arbor; Indiana University, Bloomington; the Eufaula Wildlife Refuge, Alabama; Boone, Iowa; Chillicothe, Missouri; and the Red River Research Station, Bossier County, Louisiana. Long-term average temperatures (TEMPLT) were calculated from data (including a minimum of 47 winters; see below) taken from the NCDC Summary of the Day (EarthInfo 1997) for a period beginning as early as possible (with the establishment of the weather station) and ending in December 1996. Weather stations near two sites had been discontinued and replaced by new nearby stations at some point during this period. The Eufaula station covered the period from January 1930 to February 1967, when it was replaced by the Eufaula Wildlife Refuge station (in March 1967), and the Chillicothe Radio KCHI station covered the period from January 1918 to September 1980, when it was replaced by the Chillicothe station (in October 1980). Because the temperature differences between the old and new stations are small compared to those among field sites, we combined records of the two stations with averages weighted for the number of years covered by each station. For the remaining field sites, the periods of record began in the following months: January 1897 in Michigan, March 1901 in Indiana, April 1948 in Iowa, and January 1930 in Louisiana (Shreveport AP station).

Statistics

Plasma concentrations of T_3 and T_4 were compared among locations (latitudes and transects) and times (time of day, year) with ANOVA. When ANOVA indicated a significant main effect of latitude but not a significant interaction involving latitude, we made pairwise comparisons among latitudes with Fisher's least significant difference approach. We examined relationships between plasma hormone concentrations and environmental variables with correlation analysis. We used ANCOVA to determine

whether environmental variables explained additional variation in THs after we accounted for the geographic variation.

We analyzed captive study results—including plasma TH concentrations, body mass, body temperature, and BMR—with repeated measures ANOVA (or ANCOVA) that allow for missing data. We analyzed data to detect differences among times (sample periods) within each treatment group and differences among treatment groups within sample periods. If at least one significant difference existed among groups within these analyses, we made pairwise multiple comparisons among groups and controlled α (0.05) by dividing it by the number of comparisons within each family of comparisons (Sokal and Rohlf 1994). We used ANOVA and ANCOVA to determine whether body composition of captive birds differed among treatment groups.

Unless otherwise noted, we accepted significance at $\alpha = 0.05$. There were no serious deviations from the assumptions of normality or equal variances for any of the analyses. We performed all statistical tests with SYSTAT 5.2 (ANOVA, ANCOVA, correlation) or SAS (repeated measures ANOVA and ANCOVA).

Results

Geographic Variation in THs

Preliminary analyses showed that total plasma T_3 but not T_4 concentration was influenced by the length of time that had elapsed between capture in the mist net and blood sampling. Regression analysis indicated that plasma T_3 was negatively related to sample time ($P = 0.002$), while there was no relationship between T_4 and sample time ($P = 0.900$). Plasma T_3 concentration declined abruptly when the time elapsed since capture had reached approximately 85 min. A *t*-test verified that plasma T_3 was significantly greater when sample time was <85 min (0.602 ± 0.014 ng mL^{-1}) than when it was >85 min (0.334 ± 0.034 ng mL^{-1}; $P < 0.001$). Therefore, all T_3 data derived from blood samples taken after an elapsed time of 85 min or more were removed from further analysis. No differences in total plasma T_3 or T_4 were found between male and female cardinals nor were there significant interactions between sex and transect, latitude, time of day, or year ($P > 0.05$); therefore, data for both sexes were pooled for all subsequent analyses.

Both total plasma T_3 and T_4 exhibited significant temporal as well as geographic variation. Because birds were not sampled in the evening in 1993, two separate ANOVAs were conducted for each hormone in order to fully analyze for temporal and geographic patterns. One analysis was restricted to birds sampled in 1994 to determine whether there are significant differences between morning and evening samples as well as geographic differences (i.e., main effects in the model are time of day, transect, and latitude). The other analysis was restricted to birds caught in the morning in both 1993 and 1994 to determine whether there are significant differences between years as well as geographic differences (i.e., main effects in the model are year, transect, and latitude).

In general, plasma T_3 (Fig. 2) tended to increase from south to north (with exceptions), was greater in the evening than in the morning, and was greater in 1994 than in 1993. In the ANOVA of 1994 birds, T_3 was significantly greater in the evening than in the morning ($F_{1,238} = 45.74$, $P < 0.001$) and differed among latitudes ($F_{2,238} = 8.77$, $P < 0.001$) but not between transects ($F_{1,238} = 0.84$, $P = 0.360$; Fig. 2). There were no significant interactions between time of day and transect and latitude. Post hoc tests showed that, in 1994, plasma T_3 concentrations of southern birds were lower than those of midlatitude and northern birds ($P = 0.005$ and $P < 0.001$, respectively), whose plasma T_3 levels did not differ ($P = 0.175$).

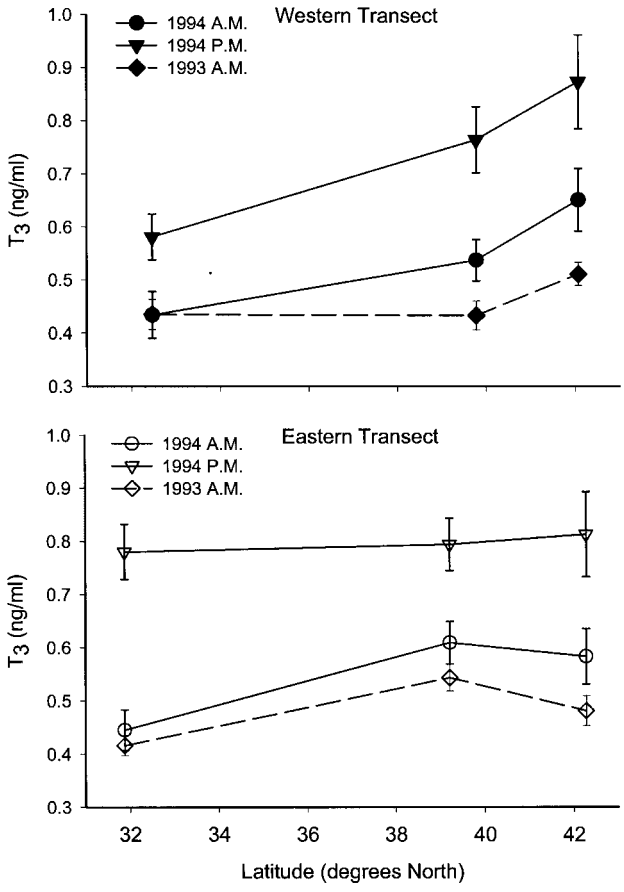

Figure 2. Total plasma 3,5,3′-triiodothyronine (T_3) of wintering northern cardinals measured on two latitudinal transects, eastern and western, during two seasons, 1993 and 1994, in the morning and the evening (for 1993, only morning samples were obtained). See the text for details of the statistical analysis. Blood samples were collected and analyzed by radioimmunoassay as described in "Material and Methods." Points represent the means; bars represent SE. Sample sizes ranged from 15 to 25.

In the ANOVA of morning birds (from both 1993 and 1994), T_3 was significantly lower in 1993 than in 1994 ($F_{1,260} = 12.97$, $P < 0.001$) and differed among latitudes ($F_{2,260} = 12.99$, $P < 0.001$) but not between transects ($F_{1,260} = 0.38$, $P = 0.538$; Fig. 2). Similar latitudinal patterns of morning T_3 concentrations were found both years on each transect (apparent in Fig. 2 and indicated by nonsignificant year × latitude and year × transect interactions, $P > 0.075$), but those patterns differed between the eastern and western transects (as indicated by a significant latitude × transect interaction in this model, $F_{2,260} = 4.33$, $P = 0.014$). In the east, plasma T_3 concentrations of southern birds were lower than those of midlatitude and northern birds ($P < 0.001$ and $P = 0.003$, respectively), whose plasma T_3 concentrations did not differ ($P = 0.186$). On the western transect, plasma T_3 concentrations of southern and midlatitude birds did not differ ($P = 0.205$) but were lower than those of northern birds ($P = 0.003$ and $P = 0.008$, respectively).

Plasma T_4 (Fig. 3), like T_3, was greater in the evening than in the morning, but unlike T_3, it did not differ between years and exhibited a very different geographic pattern of variation. In general, T_4 concentrations tended to decrease from southern to midlatitude sites and to increase from midlatitude to northern sites. In the ANOVA of 1994 samples, T_4 concentrations (Fig. 3) were significantly more elevated in birds caught in the evening than in those caught in the morning ($F_{1,183} = 16.72$, $P < 0.001$); they differed among latitudes ($F_{2,183} = 11.08$, $P < 0.001$) and did not differ between transects ($F_{1,183} = 1.31$, $P = 0.253$). Similar latitudinal patterns of T_4 were found in both morning and evening on each transect (apparent in Fig. 3 and indicated by nonsignificant interactions between time of day and latitude and transect), but those patterns differed between the eastern and western transects (as indicated by a significant latitude × transect interaction in this model, $F_{2,183} = 5.74$, $P = 0.004$). On the eastern transect, plasma T_4 concentrations were higher in southern birds than in midlatitude or northern birds ($P < 0.001$ for both), whose plasma T_4 concentrations did not differ ($P = 0.279$). On the western transect, plasma T_4 concentrations of southern birds did not differ from those of midlatitude or northern birds ($P = 0.116$ and $P = 0.368$, respectively), whose plasma T_4 concentrations did differ ($P = 0.015$).

In the ANOVA of morning-caught birds from 1993 and 1994, T_4 did not differ between years ($F_{1,228} < 0.01$, $P = 0.980$) or transects ($F_{1,228} = 1.88$, $P = 0.172$) but differed significantly among latitudes ($F_{2,228} = 9.42$, $P < 0.001$). The latitudinal patterns in morning T_4 concentrations were similar for both years (Fig. 3; as nonsignificant interactions between year and transect and latitude indicate; $P_s > 0.075$), but those patterns differed between the eastern and western transects (as indicated by a significant transect × latitude interaction effect; $F_{2,228} = 3.65$, $P = 0.027$). On the eastern transect, the plasma T_4 concentrations of southern birds were greater than those of midlatitude and northern birds ($P < 0.001$ and $P = 0.020$, respectively), whose plasma T_4 concentrations did not differ ($P = 0.089$). On the western transect, the plasma T_4 concentrations of southern birds did not differ from those of either midlatitude or northern birds ($P = 0.099$ and $P = 0.240$, respectively), whose plasma T_4 concentrations did differ ($P = 0.004$).

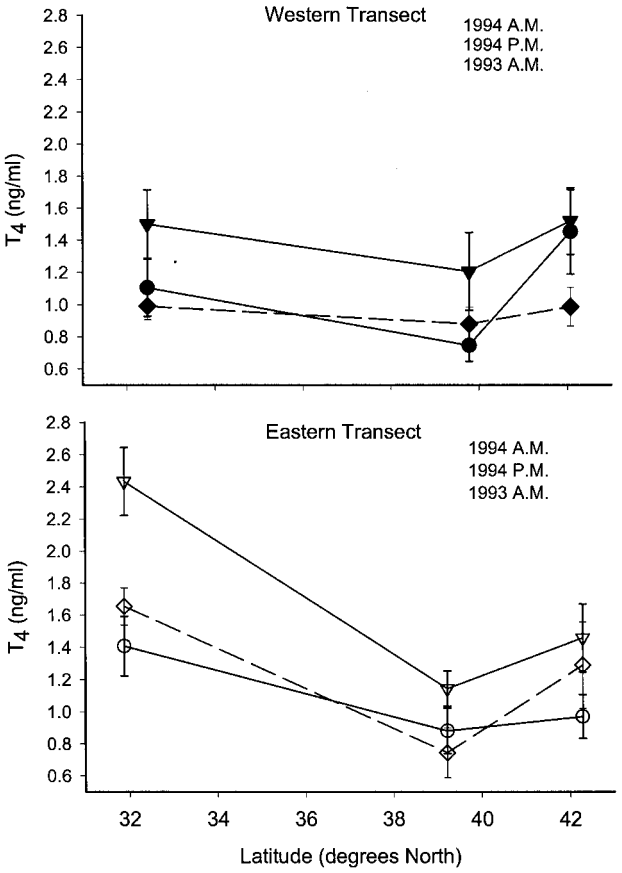

Figure 3. Total plasma thyroxine (T_4) of wintering northern cardinals measured on two latitudinal transects, eastern and western, during two seasons, 1993 and 1994, in the morning and the evening (for 1993, only morning samples were obtained). See the text for details of the statistical analysis. Blood samples were collected and analyzed by radioimmunoassay as described in "Material and Methods." Points represent the means; bars represent SE. Sample sizes ranged from 10 to 25.

Plasma T_4 Binding

Plasma T_4 binding activity of cardinal plasma was limited to low affinity binding apparently associated with albumin. PAGE/autoradiographic analysis indicated much greater [125I]T_4 binding to plasma proteins (thyroxine binding globulin [TBG] in the sheep, transthyretin [TTR] in sheep and chicken) in the same volume (5 μL) of sheep and chicken plasma than in cardinal plasma (data not shown). Similarly, we detected no

high-affinity binding of [125I]T_4 by cardinal plasma by Sephadex G-25 analysis, even when we used plasma at five and 19 times the amount that was required to demonstrate high-affinity binding in the chicken and sheep, respectively (data not shown).

Environmental Correlates of Plasma TH Concentrations

The differences in morning TH concentrations between years were not explained by temperature variables; thus 1993 and 1994 data were analyzed separately. Also, because TH concentrations differed between morning and evening in 1994, samples from these times were analyzed separately. Plasma T_3 was significantly, negatively correlated with all three temperature variables in all three data subsets (Table 1); that is, higher temperatures are associated with lower plasma T_3 concentrations. By contrast, plasma T_4 concentration was positively correlated with temperature variables in most cases; the relationships were weak and mostly nonsignificant for T_4 of birds caught in the morning and stronger for T_4 of birds caught in the evening (Table 1).

In no case did a temperature variable explain significant variation in a TH in addition to that explained by geography; the effects of the temperature covariables were not significant in ANCOVA models that included latitude, transect, and their interactions (all $P > 0.05$).

Table 1: Correlation of plasma THs and environmental variables for three data subsets (1993 morning, 1994 morning, 1994 evening) of wintering cardinals

Environmental Variable[a]	T_3		T_4	
	R	*N*	*r*	*N*
1993 morning:				
TEMP1	−.307***	155	.109	136
TEMP7	−.285***	155	.180*	136
TEMPLT	−.235**	155	.220*	136
1994 morning:				
TEMP1	−.288**	117	.003	104
TEMP7	−.352***	117	.023	104
TEMPLT	−.335***	117	.113	104
1994 evening:				
TEMP1	−.199*	133	.280**	91
TEMP7	−.173*	133	.302**	91
TEMPLT	−.205*	133	.344***	91

[a] Environmental variables defined in the text.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

THs, BMR, and Body Fat of Free-Living Cardinals

We did not measure THs and BMR in the same individual, free-living cardinals during the winters of 1993 and 1994; therefore, to determine a relationship between them we limited our analysis to the mean values of birds from the same locations and times. Geographic variation in BMR is reported elsewhere (Burger 1998); in summary, BMR tended to increase with latitude but peak in Indiana during both years on the eastern transect and did not differ among latitudes in 1994 on the western transect. Correlation analysis of mean values of THs and BMR per site (each year) revealed that neither morning nor evening values of plasma T_3 or T_4 were significantly correlated with BMR (morning T_3: $r = 0.416$, $P = 0.266$, $N = 9$; morning T_4: $r = -0.268$, $P = 0.485$, $N = 9$; evening T_3: $r = 0.299$, $P = 0.565$, $N = 6$; evening T_4: $r = -0.438$, $P = 0.385$, $N = 6$).

Both body fat and THs were measured in 175 free-living cardinals from all locations during 1993 and 1994. Hormone concentrations did not completely explain differences in body fat between years or times of day; therefore, three data subsets were analyzed separately to determine the relationships between body fat and THs. For morning-caught birds from 1993, neither T_3 nor T_4 was significantly correlated with body fat ($r = 0.214$, $P = 0.124$, $N = 53$; $r = -0.217$, $P = 0.126$, $N = 51$, respectively). Neither T_3 nor T_4 was significantly correlated with body fat of either morning-caught birds from 1994 ($r = 0.273$, $P = 0.053$, $N = 51$; $r = -0.093$, $P = 0.513$, $N = 52$, respectively) or evening-caught birds from 1994 ($r = 0.207$, $P = 0.133$, $N = 54$; $r = -0.215$, $P = 0.138$, $N = 49$, respectively). When we included T_3 or T_4 as covariables in ANCOVA of body fat, the results indicated that neither hormone explained a significant amount of the variation in fat (all $P > 0.200$) after our accounting for of geographic variation (i.e., the model includes the main effects of latitude and transect and their interaction).

Captive Study Results

While treatments with T_4 or methimazole resulted in significant, although inconsistent, alterations in thyroid status, we did not observe any effects of these manipulations on body weight, BMR, body temperature, or body lipid mass. The results of the experimental treatments are given in Table 2. Plasma T_4 tended to increase over time in control birds, but plasma T_3 was unaffected. Methimazole treatment did not significantly alter plasma T_4 concentrations over the course of the experiment, and plasma T_4 concentrations were not statistically different from control T_4 concentrations within any sample period. Methimazole treatment did lower plasma T_3 during the experiment, although methimazole birds had statistically lower T_3 than control birds only in the final sample period (day 13). Both plasma T_4 and T_3 of thyroxine- and thyroxine and methimazole–treated birds were the same during each sample period. Plasma T_4 concentrations of birds in these two treatment groups increased approximately

Table 2: Effects of manipulations of thyroid status of captive cardinals on plasma TH concentrations, basal metabolic rate, body mass, and body temperature

Dependent Variable and Treatment Group	Day 1 of Captivity	Day 5 of Captivity	Day 9 of Captivity	Day 13 of Captivity
Plasma T_4 (ng mL^{-1}):				
Control	1.34 ± .28^{A,1} (8)	1.10 ± .31^{A,1} (5)	2.56 ± .16^{A,2} (5)	2.12 ± .78^{A,1,2} (3)
Methimazole	1.23 ± .29^{A,1} (6)	1.63 ± .60^{A,1} (6)	1.38 ± .69^{A,1} (6)	.39 ± .33^{A,1} (5)
Thyroxine	1.24 ± .38^{A,1} (7)	13.79 ± 2.97^{B,2} (6)	14.20 ± 2.50^{B,2} (5)	11.58 ± 4.32^{B,2} (3)
T_4 + methimazole	1.12 ± .35^{A,1} (5)	15.80 ± 2.64^{B,2} (5)	18.50 ± 2.50^{B,2} (5)	21.33 ± 5.03^{B,2} (4)
Plasma T_3 (ng mL^{-1}):				
Control	.87 ± .04^{A,1} (7)	.86 ± .13^{A,B,1} (5)	.65 ± .12^{A,B,1} (5)	.94 ± .14^{B,1} (3)
Methimazole	.71 ± .08^{A,1} (5)	.56 ± .04^{A,1,2} (6)	.48 ± .06^{A,2,3} (6)	.31 ± .04^{A,3} (4)
Thyroxine	.77 ± .07^{A,1} (6)	1.08 ± .22^{B,1} (6)	1.09 ± .12^{B,C,1} (5)	.66 ± .02^{A,B,1} (2)
T_4 + methimazole	.82 ± .10^{A,1} (4)	1.08 ± .13^{B,1} (5)	1.26 ± .23^{C,1} (5)	1.23 ± .25^{B,1} (4)
BMR[a] (mL O_2 h^{-1}):				
Control	142.9 ± 8.0^{A,1} (7)	151.4 ± 9.0^{A,1} (5)	156.5 ± 9.9^{A,1} (4)	152.2 ± 11.2^{A,1} (3)
Methimazole	159.1 ± 9.1^{A,1} (5)	145.3 ± 8.3^{A,1} (6)	145.3 ± 8.3^{A,1} (6)	151.3 ± 8.9^{A,1} (5)
Thyroxine	158.1 ± 9.2^{A,1} (5)	135.8 ± 8.6^{A,1} (6)	157.3 ± 10.3^{A,1} (4)	153.7 ± 11.4^{A,1} (3)
T_4 + methimazole	142.7 ± 9.5^{A,1} (5)	148.9 ± 9.2^{A,1} (5)	150.3 ± 9.8^{A,1} (4)	149.6 ± 9.9^{A,1} (4)
Body mass (g):				
Control	49.9 ± 1.4^{A,2} (9)	44.8 ± .9^{A,B,1} (5)	44.2 ± 1.0^{A,1} (5)	44.9 ± 1.5^{A,1} (3)
Methimazole	48.8 ± 1.2^{A,2} (6)	45.9 ± 1.1^{A,B,1} (6)	46.5 ± 1.5^{A,1,2} (6)	46.0 ± 1.1^{A,1,2} (5)
Thyroxine	48.7 ± 1.4^{A,2} (7)	44.1 ± 1.3^{A,1} (6)	44.4 ± 1.8^{A,1} (5)	44.2 ± 3.3^{A,1} (3)
T_4 + methimazole	50.0 ± 1.2^{A,2} (6)	49.6 ± .9^{B,C,1,2} (5)	47.0 ± 1.2^{A,1} (5)	48.3 ± 1.2^{A,1,2} (4)
Body temperature (°C):				
Control	40.6 ± .3^{A,1} (9)	41.0 ± .5^{A,1} (5)	41.0 ± .6^{A,1} (4)	41.1 ± .5^{A,1} (3)
Methimazole	40.5 ± .4^{A,1} (4)	40.7 ± .3^{A,1} (5)	40.8 ± .3^{A,1} (6)	40.4 ± .7^{A,1} (5)
Thyroxine	40.8 ± .2^{A,1} (7)	40.8 ± .4^{A,1} (6)	40.9 ± .3^{A,1} (5)	40.8 ± .5^{A,1} (3)
T_4 + methimazole	40.1 ± .4^{A,1} (5)	40.4 ± .3^{A,1} (5)	40.5 ± .2^{A,1} (4)	40.8 ± .1^{A,1} (4)

Note. Mean ± SE, sample size in parentheses. Letters indicate significance among treatments within times, and numbers indicate significance among times within treatments ($\alpha = 0.008$). For each dependent variable, groups with the same letters did not differ within that sample period (i.e., day); groups with the same numbers did not differ within that treatment.

[a] BMR is adjusted for the significant effect of body mass ($P = 0.05$) via ANCOVA.

10–20 times over initial concentrations and were five to 15 times greater than control concentrations within sample periods on days 5, 9, and 13. Plasma T_3 concentrations of birds in these treatment groups did not change significantly over time, and only on day 9 did concentrations in one of the treatment groups (thyroxine and methimazole) differ significantly from those of the control group for same sample period. On days 5, 9, and 13, however, T_3 concentrations of birds from both the thyroxine- and thyroxine and methimazlole–treated groups were greater than T_3 concentrations of the methimazole group (except for the thyroxine group on day 13).

Body mass of birds in all treatment groups decreased initially and then stabilized, but there was only one difference (and no consistent trends were evident) among treatments within a sample period (Table 2). Neither BMR nor body temperature differed over time or among treatments within sample periods (Table 2). Body lipid mass of captive birds treated for at least 1 wk (3.36 ± 0.20 g) did not differ between sexes ($F_{1,13} = 0.29$, $P = 0.598$) or treatment groups ($F_{3,13} = 1.40$, $P = 0.288$), nor was the sex × treatment interaction significant ($F_{3,13} = 0.27$, $P = 0.846$). Inclusion of lean dry mass as a covariate in the analysis of body lipid was not significant ($F_{1,13} = 1.39$, $P = 0.261$) and did not change any of the conclusions.

Discussion

To our knowledge, this is the first report of its kind of geographic variation in circulating TH concentrations in a free-living avian species. Indeed, few reports on TH concentrations of free-living birds are available, but those that are often compare hormone concentrations seasonally and not geographically. In brief, Dawson et al. (1992) reported finding T_4 concentrations higher in summer than in winter and T_3 concentrations higher in winter than in summer in American goldfinches. Similarly, Silverin et al. (1989) did not assay T_3 but found higher concentrations of T_4 of great tits (*Parus major*) and willow tits (*Parus montanus*)

during warm periods of the year than during winter and spring. In contrast, Smith (1982) did not find significant variation in either T_3 or T_4 of house sparrows (*Passer domesticus*) or in T_3 of migratory white-crowned sparrows (*Zonotrichia leucophrys gambelii*). Plasma T_4 of white-crowned sparrows varied seasonally but not in a manner correlated with day length or ambient temperature. In Smith's study, house sparrows were sampled only from late fall to early spring and breeding and nonbreeding white-crowned sparrows were from separate populations sampled in separate locations. Stokkan et al. (1985) found that T_4 of svalbard ptarmigan (*Lagopus mutus hyperboreus*) was constant throughout the year but that T_3 was elevated during the period of high food intake and fat deposition (i.e., August).

The results of Dawson et al. (1992) and Stokkan et al. (1985), while seasonally dissimilar, both indicate the potential involvement of T_3 during periods when food intake and lipid metabolism are high. Similarly, Pathak and Chandola (1982) suggested a role for T_3 in premigratory fattening of redheaded buntings (*Emberiza bruniceps*). Chandola and Pathak (1980) reported that, in the annual cycle of the spotted munia (*Lonchura punctulata*), restricted food intake in late summer and early autumn precedes the decline in thyroid activity at that season and that increased food intake in response to cold temperature in the winter is likely a cause of increased thyroid activity at that time. Thus, food intake is perhaps the driving force behind variation in THs. The relationship between food intake and plasma T_3 concentration is now well established in many vertebrates (e.g., Almeida and Thomas 1981; Sharp and Klandorf 1985; May and Reece 1986; Eales 1988; Darras et al. 1995; Bruggeman et al. 1997) and may represent a unifying theme for the role of this hormone, that is, in signaling nutritional status in order to coordinate the flow of energy through the system (Eales 1988).

Geographic variation in T_3 of wintering cardinals in this study is also in support of that idea. Energy requirements (and, thus, also daily food intake) of wintering cardinals increase with latitude, likely in response to ambient temperature (Burger 1998). Not surprisingly, plasma T_3 of these birds also increased with latitude and was negatively correlated with ambient temperature variables. However, ambient temperature did not explain the significant variation in T_3 in addition to that explained by geography, even though, because birds were collected on more than one day at each site, it was possible for temperature to have explained more variation in hormone concentrations than geography.

In spite of general geographic agreement between T_3 concentrations and energy requirements, evidence that the THs are closely related to characteristics associated with winter acclimatization among birds (e.g., body fat and BMR) is lacking. Neither body fat nor BMR of free-living cardinals was significantly correlated with plasma concentrations of T_3 or T_4. Our results with manipulation of thyroid status of captive cardinals also fail to support a causal relationship between plasma concentrations of T_3 or T_4 and body fat or BMR. Studies of day-old chickens suggest that THs affect body temperature and oxygen consumption (Abdel-Fattah et al. 1990; Lam and Harvey 1990). However, effects of reduced plasma THs on heat production of 25-wk-old chickens was minimal, restricted to the period of darkness, and unlikely alone to account for adjustments in energy expenditure observed during food deprivation (Mitchell et al. 1986). In this study, captive cardinals held in outdoor aviaries exposed to cold ambient temperatures and natural day lengths and provided with food ad lib. showed no response in BMR, body temperature, or body fat to differences in THs. Even though THs did not directly affect body fat or BMR in this study, future research will be necessary to determine whether they affect other characteristics of winter acclimatization of passerines, such as metabolic enzyme activities or thermogenic capacity and endurance.

The overall geographic variation in plasma T_4 of wintering cardinals in this study was consistent among years and across transects. Birds caught in Alabama during both years had relatively high plasma concentrations of T_4. These high concentrations in birds caught in Alabama appear to be primarily responsible for the positive correlations between T_4 and ambient temperature variables; plasma T_4 concentration in birds caught in Alabama were most elevated in evening-caught birds from 1994, and consequently, this group had the strongest correlation between T_4 and ambient temperature. During both years, on both the eastern and western transects, and during both times of day in 1994, latitudinal trends in T_4 concentrations showed an inflection point at the midlatitude sites (Indiana and Missouri; Fig. 3). In contrast to the pattern we found in plasma T_4 concentrations, most environmental conditions (e.g., temperature and day length) vary in a uniform manner with latitude, which suggests that regulation of the plasma T_4 concentration of wintering cardinals is not a simple response to environmental conditions. The consistent inflection of this pattern at midlatitude sites to higher concentrations at northern sites is especially intriguing in light of latitudinal patterns of variation in breast musculature and heart mass. Pectoralis muscle and heart masses of wintering cardinals increase with latitude but peak at midlatitude sites in a manner suggestive of food limitation at the northern sites (see Burger 1998). Darras et al. (1995) and Bruggeman et al. (1997) reported elevated T_4 concentrations in chickens subjected to long-term partial food restriction. Like geographic variation in body composition, geographic variation in plasma T_4 of cardinals may indicate relative variation in the energetic conditions of the sites (i.e., food limitation at northern sites), but evaluating such an association requires additional research.

Sampling free-living cardinals at the beginning and end of the photoperiod allows some determination of circadian rhythms of THs. Plasma T_3 concentrations were lower in the morning than in the evening, which is in agreement with the results of laboratory studies of chickens (Klandorf et al. 1978;

Kühn and Nouwen 1978; Sharp et al. 1984; May and Reece 1986; Cogburn and Freeman 1987) and Japanese quail (*Coturnix coturnix japonica*; Almeida and Thomas 1981) that showed that feeding (usually during the photoperiod) causes an increase in plasma T_3. However, the results of this study contradict those of the laboratory studies regarding the daily variation of T_4. In the laboratory studies of chickens and quail, T_4 tended to rise during the scotophase and to decline during the photophase in an apparent reciprocal effect, which was likely a result of hepatic conversion of T_4 to T_3. However, in cardinals at all locations, T_4, like T_3, was found at higher concentrations in evening-caught birds than in morning-caught birds, which suggests that thyroid output of T_4 is increased at the same time that peripheral conversion to T_3 is increased, that is, during the photophase.

Interpretation of total plasma TH concentrations, as reported here, can be complicated by variation in plasma binding proteins (e.g., McNabb and Hughes 1983) that can influence the concentration of free hormone available to bind to receptors in target tissues. However, PAGE and Sephadex G-25 minicolumn analyses detected no high-affinity binding in cardinal plasma; cardinal plasma has very low capacity for binding thyroxine relative to the plasma of sheep and chickens. The small amount of binding that was detected in cardinal plasma appeared to be associated with albumin, although PAGE may not have resolved prealbumin and albumin. Consequently, because the total capacity for binding thyroxine of cardinal plasma is very low, total plasma hormone concentrations reported here probably correspond closely with free plasma hormone concentrations.

Conclusions

Plasma T_3 concentrations of wintering, free-living northern cardinals increased with latitude in accordance with decreasing ambient temperature and increasing energy requirements, but they were not significantly related to body fat or BMR in either free-living or captive cardinals. Latitudinal patterns of plasma T_4 concentrations consistently dipped at midlatitudes and were not strongly correlated with ambient temperature. Additional research is needed to determine whether THs affect thermogenic capacity or indicate relative nutritional status of free-living birds.

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