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Are thyroid hormones mediators of incubation temperature-induced phenotypes in birds?

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Incubation temperature influences a suite of traits in avian offspring. However, the mechanisms underlying expression of these phenotypes are unknown. Given the importance of thyroid hormones in orchestrating developmental processes, we hypothesized that they may act as an upstream mechanism mediating the effects of temperature on hatchling phenotypic traits such as growth and thermoregulation. We found that plasma T_3, but not T_4 concentrations, differed among newly hatched wood ducks (*Aix sponsa*) from different embryonic incubation temperatures. T_4 at hatching correlated with time spent hatching, and T_3 correlated with hatchling body condition, tarsus length, time spent hatching and incubation period. In addition, the $T_3 : T_4$ ratio differed among incubation temperatures at hatch. Our findings are consistent with the hypothesis that incubation temperature modulates plasma thyroid hormones which in turn influences multiple aspects of duckling phenotype.

1. Introduction

The early developmental environment is important in shaping offspring phenotype, and parents have tremendous influence over this environment. In birds, incubation temperature, which is largely influenced by parental behaviour, is an important determinant of phenotype in precocial [1–8] and altricial [9] species, affecting many avian hatchling characteristics that have implications for the offspring's ability to survive and reproduce. For example, in wood ducks (*Aix sponsa*) artificial incubation at variable, naturally occurring temperatures influenced plasma corticosterone concentration, locomotor performance, thermoregulation, energy expenditure, immune function, growth and survival of ducklings [1–4,7]. In general, performance is lower in ducklings incubated at low temperatures. However, a mechanism responsible for temperature-dependent effects on avian offspring phenotype has not been determined.

One potential mechanism behind the temperature-dependent effects on offspring phenotype is altered thyroid hormone production and/or function. Thyroid hormones are critical for early development in vertebrates [10]. Thyroxine (T_4), the primary secretory product of the thyroid gland, is converted to 3,5,3'-triiodothyronine (T_3) by 5'-monodeiodinases in target tissues; T_3 has 10–15 times the biological activity of T_4 [10,11]. In precocial birds, plasma thyroid hormone concentrations increase dramatically during late stages of incubation and are thought to be important for embryonic growth, tissue growth and differentiation, organ maturation and hatching [10,12]. Thyroid hormones continue to facilitate development post-hatch, influencing the critical transition to homeothermy, and stimulating immune cell proliferation, feeding

and metabolism [10,11]. As thyroid hormones are integral to the above processes, many of which are influenced by incubation temperature [1–4,8], we hypothesized that the phenotypic effects of embryonic incubation temperature are mediated in part by thyroid hormones. Thus, we predicted that (i) ecologically relevant variation in incubation temperature would positively correlate with thyroid hormone concentrations of nestlings and (ii) thyroid hormones would positively correlate with nestling mass, body condition and incubation period, but negatively correlate with tarsus length because of protracted development [2,4]. To test our hypothesis, we incubated wood duck eggs at three, ecologically relevant temperatures, which have been shown to produce different phenotypic outcomes in this species [3]. We then measured hatchling body size and circulating plasma concentrations of T_3 and T_4 on days 0, 4 and 10 post-hatch.

2. Material and methods

(a) Study species

Wood ducks are an abundant dabbling duck that readily use nest-boxes [13]. Their egg-laying season begins in February and continues until mid-July. Average clutch size is 12 eggs [13]. Minimum, maximum and average temperatures of naturally incubated wood duck nests at our study site are 34.98°C, 38.70°C and 36.79°C, respectively (G. R. Hepp 2008, unpublished data).

(b) Egg collection and incubation

We monitored 92 nest-boxes on 12 isolated wetlands in SC, USA. We collected freshly laid eggs daily and incubated them at mean temperatures of 35.0°C, 35.9°C or 37.0°C in incubators (Grumbach, Germany) with two decreases in temperature per day to mimic females' recesses for foraging [6]. These three temperatures produced incubation periods of 37, 35 and 31 days, respectively [4]. Eggs were randomly assigned to an incubation temperature; for more details on IACUC-approved egg collection and incubation protocols, see the electronic supplementary materials and [1,2,4].

(c) Duckling hatching and husbandry

We monitored eggs for pipping and hatching approximately hourly between 06.00 and 21.00 h, to calculate the amount of time needed for a duckling to complete the hatching process (i.e. hatch duration), a vulnerable developmental window that could be influenced by thyroid hormones. Only ducklings of known incubation length were included in this study. At hatching, we measured duckling body weight (g), tarsus length (mm) and culmen length (mm), and calculated body condition of ducklings as the residual of body weight regressed against tarsus. Ducklings were housed in groups of two to three according to previous methods ([1,2,4]; and see the electronic supplementary material).

(d) Blood collection

We collected blood samples following previous methods [1] from each duckling to quantify plasma T_3 and T_4 concentration at 0, 4 and 10 days post-hatch. Sampling on day 0 occurred within 0.25 and 4.70 h of hatching. Sampling on days 4 and 10 occurred between 13.00 and 18.00 h.

(e) Analysis of plasma T_3 and T_4 by radioimmunoassay

Plasma concentrations of total T_3 and T_4 were measured by radioimmunoassays (RIAs) following previous methods [14]

with minor modifications. Primary antiserums were purchased from AbCam (anti-T_3, ab30803; anti-T_4, ab30833-200) and high specific activity tracers from Perkin-Elmer ([125]T_3 NEX110X; [125]T_4, NEX111X). All samples were run in single RIAs for each hormone, and intraassay variation was 3.6% for T_3 and 5.9% for T_4. Analysis of three plasma pools demonstrated that wood duck plasma produced dilution curves that were parallel to the standards. Plasma samples were run in duplicate at two dilutions in each assay.

(f) Statistics

To avoid pseudoreplication and account for parental effects, we only included one randomly selected duckling per female per incubation temperature ($n = 14$–20 ducklings/incubation temperature/age); the remaining ducklings were used in other experiments. All statistical analyses were run in SAS v.9.1 (SAS Institute Inc., Cary, NC, USA) or Microsoft EXCEL and statistical significance was recognized at $\alpha < 0.05$.

We tested for differences in mass, tarsus length and body condition (residuals of mass versus tarsus) at hatching among incubation temperatures using three ANCOVAs (egg mass and lay sequence were covariates). Models contained incubation temperature, hatchling sex and egg storage time (i.e. prior to incubation) as main effects. We also ran simplified models only containing incubation temperature (main effect) and egg mass (covariate). We examined the influence of incubation temperature on plasma T_3, T_4 and the $T_3 : T_4$ ratio (all $\log_{10}$-transformed) of ducklings using three repeated measures ANOVAs (SAS proc mixed; repeated factor = age). Satterthwaite's approximation was used in order to calculate degrees of freedom in the T_3 and T_4 models to account for mild violations of homoscedasticity. We ran models including duckling sex (main effect), age (repeated factor), lay sequence (covariate), cage mate (random effect), time from hatching to sampling on Day 0 (covariate) and egg storage time (main effect) in the models, as well as simplified models only containing age and incubation temperature. We also regressed plasma T_3, T_4 and $T_3 : T_4$ measured on day 0 with measures of body size (body weight and tarsus length), body condition at hatching, hatch duration, incubation period and the time after hatching that blood samples were collected. For details on hatching success and incubation period for eggs incubated in 2011, see [4].

3. Results

Incubation temperature, sex, lay sequence and egg storage time did not affect duckling body mass at hatching (in all cases $p \geq 0.38$). However, both tarsus length and body condition at hatching varied with incubation temperature in the directions we predicted (tarsus (mm): $F_{2,42} = 8.3$, $p < 0.001$; LSMeans: 35.0°C = 18.9, 35.9°C = 18.8, 37.0°C = 17.9; body condition: $F_{2,42} = 4.1$, $p = 0.024$; LSMeans: 35.0°C = -0.87, 35.9°C = -0.01, 37.0°C = 1.09), but were not influenced by any of the other variables (in all cases $p \geq 0.44$). As egg mass increased so did duckling body mass ($F_{2,42} = 125$, $p < 0.001$), tarsus length ($F_{2,42} = 33.2$, $p < 0.001$) and body condition ($F_{2,42} = 37.5$, $p < 0.001$) at hatching. For mass, tarsus and body condition, the simple models only containing incubation temperature and egg mass produced similar statistical outcomes.

Mean plasma T_4 concentration decreased with age for all incubation temperature treatments (figure 1a; age: $F_{2,73.4} = 25.12$, $p < 0.001$). However, there was no influence of incubation temperature on plasma T_4 concentration at any age (incubation temperature: $F_{2,52.3} = 0.96$, $p = 0.39$;
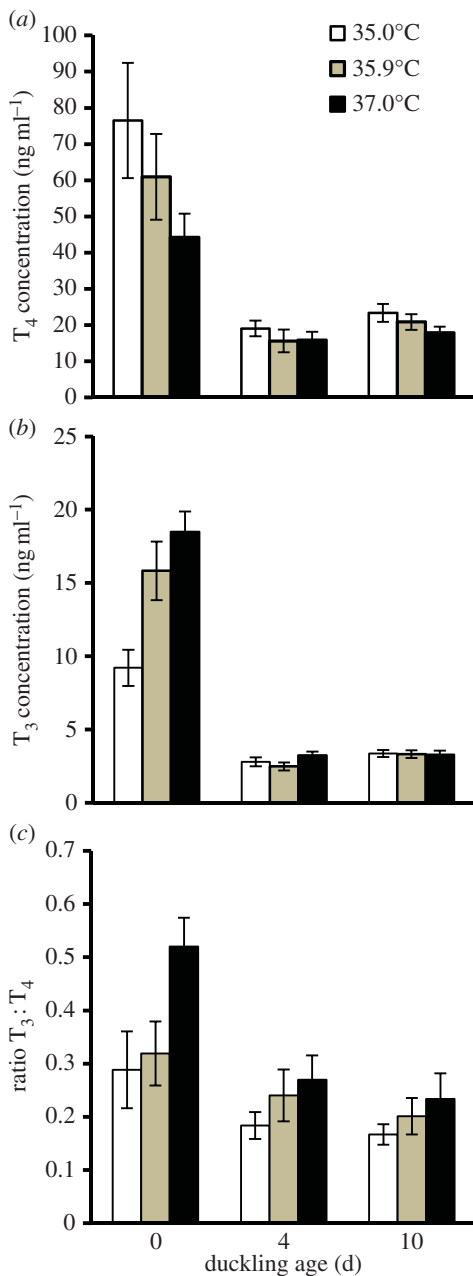
Figure 1. The relationship between incubation period and (*a*) plasma T_4 concentration, (*b*) plasma T_3 concentration and (*c*) plasma $T_3 : T_4$ ratio of ducklings sampled on days 0, 4 and 10 post-hatch. Eggs were incubated at either 35.0°C, 35.9°C or 37.0°C. $n = 14 – 20$ ducklings/age/incubation temperature. Error bars are ± 1 s.e.m.

incubation temperature × age: $F_{4,75.4} = 0.61$, $p = 0.66$). Similar to T_4, plasma T_3 concentrations decreased with age in all treatments (figure 1*b*: age: $F_{2,75.5} = 163.52$, $p < 0.001$). In addition, there was a significant age by incubation temperature interaction ($F_{4,78.4} = 5.43$, $p < 0.001$), in which ducklings from the lowest incubation temperature had significantly lower plasma T_3 concentration on day 0 than ducklings from the higher incubation temperatures (*post hoc* slice by age: day 0, $p < 0.001$; day 4, $p = 0.08$; day 10, $p = 0.94$). However, by day 4, plasma T_3 concentrations were similar among incubation temperatures. There was no effect of lay sequence, sex or egg storage time on T_4 concentrations (in all cases $p \geq 0.58$) or T_3 (in all cases $p \geq 0.09$), and models not containing these factors or cage mate yielded similar results (T_4: age, $p < 0.001$; incubation temperature, $p = 0.40$; incubation temperature × age, $p = 0.63$; T_3: age, $p < 0.001$; incubation temperature, $p = 0.004$; age × incubation temperature, $p < 0.001$).
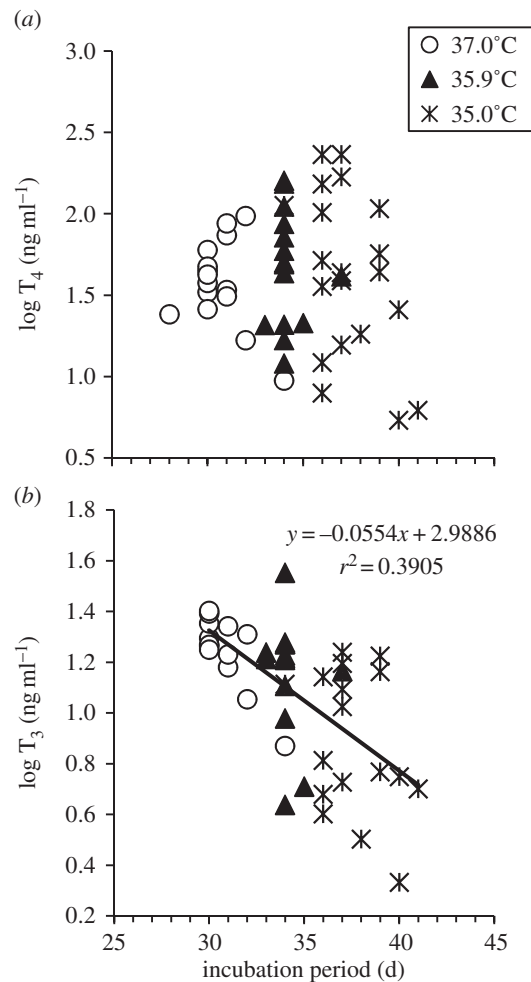

Figure 2. The relationship between incubation period and (*a*) plasma T_4 concentration and (*b*) plasma T_3 concentration of ducklings sampled within $0.25 – 4.70$ h of hatching. Eggs were incubated at either 35.0°C, 35.9°C or 37.0°C. $n = 14 – 20$ ducklings/incubation temperature.

For all incubation temperatures, the $T_3 : T_4$ ratio was the highest at hatching and then decreased approximately 25–55% by days 4 and 10 (age: $F_{2,68.3} = 8.81$, $p < 0.001$). Ducklings from the lowest incubation temperature had 46% lower ratios than ducklings from the high incubation temperature, but ratios were similar by day 4 (incubation temperature: $F_{2,44.9} = 5.71$, $p = 0.006$; incubation temperature × age $F_{4,69.9} = 0.83$, $p = 0.51$; *post hoc* slice by age: day 0, $p = 0.004$; day 4, $p = 0.18$; day 10, $p = 0.55$). There was no effect of lay sequence, sex or egg storage time on ratios (in all cases $p \geq 0.35$), and models not containing these factors or cage mate yielded similar results (age, $p < 0.001$; incubation temperature, $p = 0.004$; incubation temperature × age, $p = 0.67$).

At hatching, we did not detect a relationship between incubation period and plasma T_4 concentration (figure 2*a*; $r^2 = 0.01$, $p = 0.46$), but we did detect a strong negative correlation between incubation period and plasma T_3 concentration (figure 2*b*: $r^2 = 0.39$, $y = -0.055x + 3.00$, $p < 0.001$) and a mild negative correlation with $T_3 : T_4$ ($r^2 = 0.10$, $y = -4.162x + 35.8$, $p = 0.056$). $T_3 : T_4$ ratios did not correlate with any other variable (for all variables $r^2 \leq 0.04$, $p \geq 0.21$). The only significant correlation detected with T_4 at hatching was a negative relationship with hatch duration ($r^2 = 0.14$, $y = -0.007x + 1.98$, $p = 0.007$; for all other variables $r^2 \leq 0.06$, $p \geq 0.10$). By contrast, plasma T_3 at hatching negatively correlated with hatch duration ($r^2 = 0.14$, $y = -0.006x +$

1.32, $p = 0.012$) and tarsus length ($r^2 = 0.11$, $y = -0.091x + 2.78$, $p = 0.031$), and positively correlated with body condition ($r^2 = 0.13$, $y = 0.044x + 1.07$, $p = 0.017$). We found no relationship between plasma T_3 and duckling body weight (in both cases $r^2 \leq 0.03$, $p \geq 0.24$).

4. Discussion

We demonstrated that biologically relevant differences in incubation temperature affect plasma thyroid hormone concentrations of bird hatchlings from a natural population. Plasma thyroid hormone concentrations in ducklings from all incubation temperatures were within the concentrations measured for other precocial bird hatchlings [9,10,15]. We found that ducklings from the lowest incubation temperature had reduced plasma T_3 concentrations and lower $T_3 : T_4$ ratios at day 0 compared with ducklings from the higher incubation temperatures (figure 1). In addition, plasma T_3 at hatching was negatively correlated with incubation period (figure 2b), which is strongly influenced by incubation temperature [4]. As the bird thyroid gland secretes very little T_3 [11,16], our findings suggest that the cool incubation temperatures could be reducing deiodination of T_4 into T_3, perhaps through reduced expression of liver deiodinases which increase rapidly around the time of hatching [11]. Additional possibilities for altered T_3 may include differences in hormone clearance or degradation, or differential abundance of T_3 binding proteins (e.g. transthyretin) that influence T_3 degradation [11].

Reductions in T_3, the more biologically active thyroid hormone, could be influencing many other phenotypic traits of ducklings from the low incubation temperature which might explain their reduced physiological performance (e.g. slower growth and poor thermoregulatory ability) relative to ducklings from higher incubation temperatures. Consistent with this hypothesis, ducklings with low T_3 hatched later with larger structural size, but in lower body condition, than their counterparts from the high incubation temperature. This suite of effects is attributable to the more protracted growth period associated with slower development at low temperatures [2,4]. In addition, some of the effects of incubation temperature on phenotype appear to attenuate with age [1,7], which could correspond with the convergence of duckling plasma T_3 concentration among incubation temperatures by day 4. Other studies also support the importance of incubation temperature for thyroid expression, although the direction of effects can differ across species [9,17]. For example, lower incubation temperatures caused an increase in plasma T_4 of hatchling snapping turtles incubated at different temperatures [17]. Studies in poultry reveal that exposing embryos to higher incubation temperatures on specific days during incubation when the thyroid and adrenal axes are developing reduced plasma T_3 and T_4 of hatchlings (e.g. [9]). Although not measured in this study, covariations in other hormones (e.g. testosterone) could also influence embryonic development and hatchling phenotype (e.g. [18]).

Our findings suggest that changes in plasma thyroid hormone concentration may serve as an upstream mechanism for the effects of incubation temperature on traits like thermoregulation, metabolism, growth and immune responses. Ultimately, incubation temperature may modulate gene expression during development of the thyroid axis, including deiodinase expression or enzyme activity. Future research on gene expression, administration of exogenous thyroid hormones, and/or blocking of thyroid hormone production will help elucidate potential causal relationships between incubation temperature, thyroid hormones and expression of variable phenotypes in birds.

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