Temperature-induced variation in yolk androgen and thyroid hormone levels in avian eggs

Suvi Ruuskanen, Ton G.G. Groothuis, Sonja V. Schaper, Veerle M. Darras, Bonnie de Vries, Marcel E. Visser

A R T I C L E  I N F O

Article history:
Received 11 December 2015
Revised 20 May 2016
Accepted 29 May 2016
Available online 30 May 2016

Keywords:
Testosterone
Thyroxine
Maternal effects
Plasticity
Global warming

A B S T R A C T

Global warming has substantially changed the environment, but the mechanisms to cope with these changes in animals, including the role of maternal effects, are poorly understood. Maternal effects via hormones deposited in eggs, have important environment-dependent effects on offspring development and fitness: thus females are expected to adjust these hormones to the environment, such as the ambient temperature. Longer-term temperature variation could function as a cue, predicting chick rearing conditions to which yolk hormone levels are adjusted, while short-term temperature variation during egg formation may causally affect hormone transfer to eggs. We studied the effects of ambient temperature on yolk androgens (testosterone and androstenedione) and thyroid hormones (thyroxine and triiodothyronine) in great tits (Parus major) using data from unmanipulated clutches from a wild population and from aviary birds (ad libitum food) exposed to different experimental temperature treatments during five years. Both in the wild and in captivity, longer-term pre-laying ambient temperature was not associated with clutch mean yolk hormone levels, while the way androstenedione and thyroxine levels varied across the laying sequence did associate with pre-laying temperature in the wild. Yolk testosterone levels were positively correlated with short-term temperature (during yolk formation) changes within clutches in both wild and captivity. We also report, for the first time in a wild bird, that yolk thyroxine levels correlated with a key environmental factor: thyroxine levels were negatively correlated with ambient temperature during egg formation. Thus, yolk hormone levels, especially testosterone, seem to be causally affected by ambient temperature. These short-term effects might reflect physiological changes in females with changes in ambient temperature. The adaptive value of the variation with ambient temperatures pre-laying or during egg formation should be studied with hormone manipulations in different thermal environments.

1. Introduction

Global warming has induced substantial changes in the environment, but the mechanisms in different organisms to cope with these changes are not well understood. One potential, rarely studied, physiological mechanism is via maternal effects (Galloway, 2005; Visser, 2008; Meylan et al., 2012; Salinas and Munch, 2012; Shama et al., 2014). Maternal effects occur when the maternal phenotype/environment affects offspring phenotype, for example via different developmental signals or care for the offspring (Mousseau and Fox, 1998). Maternal effects generate phenotypic variation which could either facilitate or hamper adaptation to changing conditions (Råsänen and Kruuk, 2007; Uller, 2008; Marshall et al., 2008; Meylan et al., 2012). To truly assess whether adaptive allocation occurs we need to quantify variation in the mediators of maternal effects in relation to influential environmental factors.

Oviparous species, such as birds, are a good study system for investigating climate-driven variation in prenatal maternal effects as their eggs develop outside the mother’s body, facilitating the measurement of maternal resources and signals. Steroid hormones...
in egg yolk, such as testosterone (T) and androstenedione (A4), are widely studied and vary substantially among clutches due to social and environmental factors (reviewed by Groothuis et al., 2005; Ruuskanen, 2015). They can affect both early and later offspring phenotype and survival (increasing growth, oxidative stress and metabolism; reducing immune response, Gil, 2008; von Engelhardt and Groothuis, 2011). However, other hormones, such as the thyroid hormones, thyroxine (T4) and triiodothyronine (T3), are present in egg yolk (McNabb and Wilson, 1997), but have rarely been studied in an ecological context. Nevertheless, maternal thyroid hormones (THs) are indispensable for normal development in other vertebrates (Power et al., 2001; Patel et al., 2011; Campinho et al., 2014) and have been shown to increase growth also in birds (Wilson and McNabb, 1997; Ruuskanen et al., 2016a).

For insectivorous passerine birds, such as our study species, the great tit (Parus major), temperature-related changes in the environment are important because their breeding success is heavily affected by timing of breeding in relation to the ambient temperature-dependent peak of insect food availability (Lack, 1950; Martin, 1987; Verhulst et al., 1995; Visser et al., 2006; Reed et al., 2013). Importantly, temperature patterns may be used as cues for timing of breeding (Visser et al., 2009, 2011; Schaper et al., 2012; Williams, 2012), making temperature a good candidate for explaining variation in maternal allocation. Furthermore, great tits are currently mismatched in egg laying in relation to the time of maximal prey availability; this is because the phenology of their prey advances faster in response to increased spring temperatures than the breeding time of great tits (e.g. Visser et al., 1998). However, the mechanisms (i.e. plasticity, microevolution) to keep up with this change are not fully understood and (hormone-mediated) maternal effects could play a role.

It is widely known that egg size, i.e. general resource allocation to eggs, decreases with ambient temperature in wild populations (Nager and Zandt, 1994; Christians, 2002; Lessells et al., 2002), but opposite, potentially adaptive patterns can arise where food is not limiting (Schaper and Visser, 2013). However associations between ambient temperature and egg composition, such as yolk hormones, have rarely been studied (but see Remes, 2011).

An ultimate, causal explanation of the variation in yolk hormones with ambient temperature is that temperatures before egg-laying may be used as cues for timing of breeding and/or the quality of the season (see above) and thus the conditions for off-spring rearing; Yolk hormones are then adjusted to the different predicted conditions (predictive adaptive response; PAR, Nager et al., 1997; Gluckman et al., 2008; Groothuis and Schwabl, 2008). Indeed the effects of yolk hormones on e.g. growth may be dependent on environmental conditions (Boncoraglio et al., 2011; Kankova et al., 2014; Muriel et al., 2015). We hypothesize that as yolk androgen and thyroid hormones decrease developmental time and increase growth (McNabb and Wilson, 1997; Groothuis et al., 2005; in great tits Tschirren et al., 2005; Ruuskanen et al., 2016a) individuals starting to breed in warmer ambient temperatures, interpreted as cues for being closer to the food peak, should show higher clutch mean hormone levels to speed up the embryonic development of the chicks.

In addition to clutch mean hormone levels, such adaptive allocation may also concern variation in hormone levels across the laying sequence within a clutch. In great tits, androgen and thyroid hormone levels increase across the laying sequence but within-clutch patterns may also vary in relation to environmental conditions (Tschirren et al., 2004; Tobler et al., 2007; Groothuis et al., 2008; Heylen et al., 2012; Ruuskanen et al., 2016b). Such patterns in other species have been interpreted as a way to facilitate brood survival or brood reduction, in relation to hatching asynchrony (HA), or a bet-hedging strategy to increase offspring diversity (Schwabl et al., 1997; Laaksonen, 2004; Groothuis et al., 2005). Interestingly, HA increases in higher ambient temperatures and later clutches in tits (Verhulst et al., 1995; Vedder, 2012; Alvarez and Barba, 2014). Thus, we hypothesize that the increase (or decrease) of yolk hormones over the laying sequence may be more pronounced in warmer temperatures, either to provide more diversity in offspring phenotype or to account for greater HA.

Furthermore, there could be a causal effect of short-term variation in ambient temperature during yolk formation on yolk hormone levels, mediated through variation in circulating hormone levels in the female. For thyroid hormones (THs) this is likely, as plasma TH levels in birds is affected by ambient temperature (Kuhn and Nouwen, 1978; e.g. Cogburn and Freeman, 1987; reviewed by McNabb, 2007). Yolk THs originate from plasma and seem to correlate with circulating plasma thyroid levels (McNabb and Wilson, 1997; McNabb, 2007; with potential scope for independent regulation, Van Herck et al., 2013). For yolk androgens, there is inconsistent data for the correlation between circulating and yolk hormones, with some evidence for independent regulation (steroids being locally produced in the ovaries, e.g. Groothuis and Schwabl, 2008; Okuliarova et al., 2011).

Finally, the observed temperature effects could be mediated indirectly via food availability: insect food availability is strongly affected by ambient temperature, and temperature may further affect foraging behavior and food intake (e.g. Perrins, 1991; Winkler et al., 2013). However, studies with experimentally altered food supply show contradictory effects on yolk hormone levels (e.g. Benowitz-Fredericks et al., 2013 and ref therein). Thus, to separate the effects of food and temperature, controlled experiments are needed.

We studied the effect of variation in ambient temperature on the levels of two influential yolk hormone classes, androgens and thyroid hormones in great tits. We used data from unmanipulated clutches of wild birds to describe patterns in relation to ambient temperature. However, patterns associated with temperature may be mediated via food availability; thus, to reveal direct causal effects of temperature on yolk hormone levels, we analyzed the eggs of captive birds with ad libitum food, exposed to different experimental temperature treatments. This temperature-controlled data from a wild species is a truly unique dataset. We analyzed the effect of pre-laying ambient temperatures on both clutch mean hormone concentration and patterns across the laying sequence. We further analyzed the effect of temperatures during yolk formation. As captive birds are not energetically limited, we predict weak association with ambient temperature in captivity if temperature-related variation in yolk hormone levels is mediated indirectly via food availability.

2. Methods

2.1. Study species

The great tit is a model species in ecological and evolutionary research. Importantly, maternally derived androgen and thyroid hormones in eggs have been found to affect offspring growth and dispersal (Tschirren et al., 2005; Ruuskanen et al. 2016a; Tschirren et al., 2007; Tschirren and Richner, 2008; Podlas et al., 2013), and vary with multiple environmental factors (Tschirren et al., 2004; Groothuis et al., 2008; Remes, 2011; Ruuskanen et al., 2016b).

2.2. Data from a wild population

The correlative field data was collected in 2013 from a population with 125 nest-boxes in Bennekom, the Netherlands. Nest-building was monitored from end of March twice a week.
The eggs were collected on the day of laying until clutch completion, replaced with plastic dummy eggs, and frozen at −20 °C until dissection. Egg collection was conducted throughout the season (dates: 20.4.–14.5., see Online resource 1), but only including the first nest of a female in that year. Eggs were collected from every 3rd nest initiated in the population, a total of 23 full clutches. Temperature data was acquired from a Dutch meteorological station (De Bilt). We calculated the average of ambient temperatures during 14 and 7 days before laying the 1st egg as the pre-laying, longer-term temperature variables. We further calculated the average of ambient temperatures during 4 days before laying each egg (hereafter −4d) to represent the period during rapid yolk formation, which takes ca 3–4 days in small passerines (Badayev et al., 2005). We also analyzed other temperature periods (−1d and −7d before each egg), to ensure that a causal effect of temperature indeed overlaps with the yolkinc period (see statistics). We analyzed all eggs from all 23 clutches for yolk androgens; testosterone (T) and androstenedione (A4). For thyroid hormones (thyroxine, T4 and triiodothyronine, T3) we needed ca 400–500 mg of yolk for accurate measurements (based on pilot assays). As the average yolk mass is ca 250–350 mg, and part of the egg was used for androgen assays, we decided to pool pure yolks from eggs 1&2 (hereafter egg 1), eggs 5&6 (hereafter egg 5) and eggs 8&9 (hereafter egg 8) for thyroid hormone analysis. The mass taken from each of the two pooled yolks was kept as similar as possible (average 15% difference in mass from each of the two eggs). Androgen and thyroid hormones were analyzed with radioimmunoassays (see Online resource 2).

2.3. Data from captive birds used in temperature experiments

Temperature experiments have been described in detail elsewhere (Visser et al., 2011; Schaper et al., 2013; Schaper and Visser, 2013), see Online resource 3 for details on the treatments. The birds were the offspring of 50 known wild parents from a long-term monitored population at the Hoge Veluwe National Park (the Netherlands). On day 10 post-hatching, chicks were taken to captivity at the Netherlands Institute of Ecology (NIOO-KNAW) as complete broods for hand-feeding under standardized conditions (Drent et al., 2003). Each year, breeding pairs were formed in captivity avoiding sib-matings and pairs were kept in climate-controlled aviaries (one pair/aviary) with ad libitum food. See Visser et al. (2011), Schaper et al. (2013) and Schaper and Visser (2013) for aviary conditions.

During five years of experiments, birds were exposed to varying temperature regimes in individual aviaries per breeding pair, which were designed to test the effects of ambient temperature on the onset of reproduction (see Online resource 3 for full description of the treatments and realized temperatures). For a description of temperature effects on the onset of laying see (Visser et al., 2011 and Schaper et al., 2012). Briefly, in 2006 and 2007 birds were exposed to gradually increasing temperatures during egg-laying, with 4 °C absolute difference between the two treatments. In 2008 birds were laying in a constant ambient temperature (7 or 15 °C), but were exposed to 7 °C for a month in either February, March or April. In 2009 birds were also laying in constant ambient temperatures (9 vs 15 °C), and there was no seasonal temperature pattern, but the temperature fluctuated with either high or low day-night amplitude. In 2010 birds were again laying in constant ambient temperatures (15 vs 20 °C), but treatment groups experienced increases of temperatures at different periods in the spring prior to laying. Temperature treatments affected the onset of laying in 2008 and 2010, but not in other years. Realized temperatures in the aviaries were recorded every 10 min.

Due to differences in the technical efficiency of temperature regulation, slight discrepancies in mean ambient temperature existed among aviaries belonging to the same treatment group in a given year. Thus, mean realized, i.e. measured, temperatures per breeding pair were used in the analysis. Furthermore, in year 2008, there was day-to-day variation in the realized temperatures (see below). Similarly as for the wild population, we calculated average temperatures (midnight to midnight) during 14 and 7 days before laying the first egg (i.e. pre-laying temperature) and during 4 days before laying each egg (during yolk formation, i.e. short-term effect).

In captivity, eggs were collected on the day of laying until clutch completion, replaced with plastic dummy eggs, and frozen at −80 °C until hormone analyses in spring 2013/2014. We included in our dataset all first clutches of all years with clutch size between 4 and 15 eggs (yolk steroids; N = 98 clutches (410 eggs), yolk THs; N = 77 clutches (189 eggs), see Table 3a and b for sample sizes between treatments and years). Analysis was restricted to these clutch sizes as they are in the range of normal clutch sizes in Dutch wild populations. For A4 and T we analyzed all eggs in the laying sequence from 10 clutches (divided across years). From the rest (88 clutches), we analyzed egg 1, 3, every 3rd and always the last egg. For yolk T4 and T3, a complete yolk was needed for the analysis, thus we used egg 2, 4 (if available) and the penultimate egg. See details of the analysis methods in Supplementary methods.

2.4. Ethical note

All experimental procedures were approved by the Animal Experimentation Committee (DEC) of the Royal Netherlands Academy of Arts and Sciences (KNAW). All applicable institutional and/or national guidelines for the care and use of animals were followed.

3. Statistics

3.1. Correlative data from a wild population

The responses were yolk mass and concentrations (pg/mg) of yolk T, A4 and T4 and T3: all concentrations were log10 transformed to achieve normality. The hormone content showed similar results, and thus data from hormone concentrations only has been reported. Sample sizes were the following: yolk androgens N = 158 eggs (23 clutches), yolk THs: 45 eggs (21 clutches). The explanatory factors included egg order (proportional, i.e. scaled from 0 to 1 due to highly variable clutch sizes, range: 5–11 eggs) and proportional egg order squared (to model non-linear within-clutch patterns). We included multiple temperature variables in order to test our hypotheses. Each temperature variable was included separately in the model due to their correlatedness. The temperature variables are presented in Table 1. We analyzed longer-term pre-laying ambient temperature variation: #1a) Pre-laying temperature = Average ambient temperature during 14 and 7 days before laying the 1st egg of the clutch, and #1b) Interaction between pre-laying temperature and egg order, testing if patterns across the laying sequence differ in relation to pre-laying temperatures. To study short-term effects of ambient temperature we analyzed:

- #2) Absolute temperature −4d = Average of temperatures during 4 days before laying each egg, i.e. during yolk formation, separately for each egg. However, temperature can have an effect independently at two levels, both among clutches (i.e., mean temperatures on mean clutch values), and within clutches (i.e. an effect of temperature during formation of an egg on an individual egg within the clutch) – thus, using absolute temperatures as above, these cannot be fully separated (van de Pol and Wright, 2009; Dingemanse and Dochtermann, 2013). Thus we dissected this variation to the following among- and within-clutch temperature
variation parameters: #3) Clutch mean temperature = Average of ambient temperatures during the whole egg-laying-period period (average of #2, see above); #4) Temperature deviation from the clutch mean ambient temperature = individual ambient egg temperature value (see #2) – clutch mean ambient temperature (see #3). Positive values indicate that temperatures during formation of a particular egg were higher than on average during egg-laying. The average absolute temperature deviation from the clutch mean was $1.7 \pm 1.0 \text{(SD)} \, ^\circ \text{C}$. ClutchID was always included as a random factor to account for the non-independence of the eggs from the same clutch. For yolk THs, the random factors were ClutchID and analysis batch (due to differing recovery rates across hormone assay batches) and the quadratic term of egg order effect was not tested due to most clutches only having two eggs. Models with absolute egg order (instead of proportional egg order) produced similar results (results not shown). Models with ambient temperatures –1 or –7 days before laying each egg (results not shown), showed no correlation with yolk hormone levels, or less strong correlation than using –4d supporting the idea that ambient temperatures during yolk formation can have a causal effect.

### 3.2. Temperature experiment data from the captive birds

Similarly as for the wild birds, the responses were yolk mass and concentrations (pg/mg) of yolk T, A4, T4 and T3, and all were log10 transformed to achieve normality. The hormone content showed similar results, and thus data from hormone concentrations only is reported. We first analyzed the effect of different pre-laying temperature patterns/treatments (four treatments/years, see Online resource 3 for description of the treatments) separately for each year, on clutch mean hormone levels and patterns across the laying sequence by including treatment and its interaction with proportional egg order as the fixed factors. Proportional egg order and its squared term were covariates as in previous models, and dropped if not significant.

Secondly, similarly as for data from the wild population, we analyzed the effect of realized (i.e. measured) ambient temperatures before egg-laying (#1, see Table 1) and during yolk formation (#2–4, see Table 1) on yolk hormones. We used data from all experimental years (2006–2010, see Online resource 3). Sample sizes: yolk steroids: $N = 410$ eggs (98 clutches), yolk THs: 189 eggs (77 clutches). The explanatory factors were proportional egg order and its squared term and the same temperature parameters as for the wild population (see above). The effect of within-clutch variation (#4) could only be analyzed in year 2008 where temperatures were stable on average (i.e. no seasonal increase), but with within-clutch variation in ambient temperatures, independent of laying order (average absolute temperature deviation from the clutch mean, $0.36 \text{^\circ C, max 1.5 ^\circ C}$). Sample sizes: yolk steroids, $N = 79$ eggs (18 clutches), yolk THs $N = 33$ eggs (13 clutches). We included ClutchID, year and female family (to account for relatedness and common early growth environment) as random effects. All analyses were conducted with SAS 9.4. PROC MIXED. Kenward-Roger method was used for estimating degrees of freedom. Homogeneity and normality of residuals was checked. Alpha level was 0.05.

### 4. Results

#### 4.1. Correlative data from a wild population

The results from wild birds are shown in Table 2A. Ambient temperature 14 or 7 days before egg-laying (code #1 in Table 1) was not associated with clutch mean levels of any of the hormones (14 day pre-laying temperature: Table 2A; 7d temperature: all F-values < 2.0, p-values > 0.14). However the pattern of A4 and T4 over the laying sequence was associated with average temperatures 14 days (but not 7 days) before egg-laying: a statistically significant interaction between egg order and pre-laying temperature (Table 2A, Online resource 4: Fig. 1a) suggested that at lower temperatures (lower than the average, $10.4 \text{^\circ C}$), there was a slight increase of A4 over the laying sequence (prop. egg order $\beta \pm SE: 0.055 \pm 0.026; F_{1,62} = 4.5, p = 0.036$), but no such pattern at higher than average temperatures ($>10.4 \text{^\circ C}$) (prop egg order $-0.037 \pm 0.027, F_{1,71} = 1.8, p = 0.18$). For T4 there was a similar interaction (Table 2A, Online resource 4: Fig. 1b), suggesting that at lower temperatures there was no pattern over the laying sequence in T4 (prop egg order $\beta \pm SE: 0.05 \pm 0.03, F_{1,8} = 3.1, p = 0.11$) but at higher temperatures there was an increase in T4 over the laying sequence (prop. egg order $\beta \pm SE: 0.16 \pm 0.03, F_{1,12} = 32.9, p = 0.001$). Finally, there was a trend for a similar interaction for yolk mass ($F_{1,134} = 3.4, p = 0.06$; Online resource 4, Fig. 1c, below $10.4 \text{^\circ C}$: $\beta \pm SE: 16.1 \pm 8.5, F_{1,62} = 2.78, p = 0.09$, above $10.4 \text{^\circ C}$: $-13.7 \pm 10.2; F_{1,71} = 1.8, p = 0.18$).

Air temperature during yolk formation of each egg (see code #2 in Table 1) was positively correlated with yolk T concentration and negatively correlated with yolk T4 concentration ($\beta \pm SE, T: 0.007 \pm 0.003; T4: -0.0095 \pm 0.004$, Table 2A): The correlation seemed to be predominantly at within-clutch but not at between-clutch level: we found a positive correlation between temperature deviation from the clutch mean (code #4) and yolk T concentration, and a negative correlation with T4 concentration (Table 2A, Fig. 1A, $\beta \pm SE, T: 0.0079 \pm 0.003; T4: -0.0095 \pm 0.0043$). This means that a positive deviation from clutch mean ambient temperature was associated with higher yolk T and lower yolk T4 concentrations. Yolk A4 or T3 concentrations did not show any association with short-term ambient temperature during egg-laying (Table 2A, Fig. 1A).

Furthermore, yolk testosterone concentration decreased and yolk T4 concentration increased with laying sequence of the eggs within the clutch (proportional egg order, $\beta \pm SE, T: -0.076 \pm 0.019; T4: 0.10 \pm 0.02$), whereas A4 showed a non-linear pattern across the laying sequence, values being highest in the middle of the clutch ($\beta \pm SE$: proportional egg order 0.15 $\pm 0.067$; proportional order$^2$ $-0.14 \pm 0.065$, Table 2A). Yolk mass did not show any association with ambient temperature during egg-laying (all-p-values > 0.13).

#### 4.2. Experimental data from captive birds

Clutch means or within-clutch patterns over the laying sequence of any of the yolk hormones were not affected by the

| Table 1 Description of the different temperature variables and their calculations. |
|-----------------------------------|----------------------------------|
| **Coding** | **Temperature variable** | **Description** |
| #1 | Pre-laying temperature | Average ambient temperature during 14 or 7 days before laying the 1st egg of the clutch |
| #2 | Absolute temperature – 4d | Average of ambient temperatures during 4 days before laying each egg, i.e. during yolk formation, separately for each egg |
| #3 | Clutch mean temperature | Average of absolute – 4d ambient temperatures (#2) during the whole egg laying-period period |
| #4 | Temperature deviation | Absolute – 4d temperature value (#2) – clutch mean temperature (#3), i.e. within clutch temperature deviation. Positive values indicate that temperatures during formation of a particular egg were higher than on average during egg-laying |
temperature treatments (Table 3A, B). When analyzing the effect of realized (i.e. measured) ambient temperature (ambient temperature as a continuous variable, using data from all years together) we found that ambient temperature 14 or 7 days before egg-laying (code #1 in Table 1) was not associated with clutch mean hormone levels, similarly as for the field data. There was also no variation in the patterns across the laying sequence of any of the yolk hormones in relation to pre-laying temperature, which is in contrast to the field data (Table 2B; 7d pre-laying; all F-values < 0.72, p-values > 0.40).

There was no effect of absolute temperatures during yolk formation of each egg (code #2 in Table 1) on any of the yolk hormones, nor effects of clutch mean ambient temperature (code #3 in Table 1) during yolk formation (Table 2B). However, when analyzing associations with within-clutch temperature deviation (code #4 in Table 1, in 2008, the only experimental year where temperature variation within clutches (independent of egg order) exists) we found that ambient temperature 14 or 7 days before egg-laying were associated with patterns of yolk A4 over the laying sequence under war- ming temperatures. However the patterns were only found in the wild with a rather small sample size, and not in captive conditions. In wild and captive birds, clutch mean yolk androgen levels were not related to ambient long-term pre-laying temperatures. However, in the wild population (but not in captivity), the within-clutch patterns of A4, T4 and yolk mass over the laying sequence were associated with pre-laying (14 days) ambient temperatures.

5.1. Yolk androgen hormones in relation to ambient temperature

In neither wild nor captive birds, clutch mean yolk androgen levels were related to the ambient long-term mean temperatures or temperature patterns (treatments) prior to egg-laying; this contrasts our PAR hypothesis that clutch mean hormone levels are adjusted according to pre-laying temperature cues, to speed up offspring development to match the food peak. However, pre-laying ambient temperatures over 14 days (but not 7 days) before egg-laying were associated with patterns of yolk A4 over the laying sequence; with increases in A4 over the laying sequence under colder conditions and no pattern in warmer pre-laying temperatures. This contrasts our predictions of stronger increase (or decrease) of yolk hormones over the laying sequence under warmer temperatures. However the patterns were only found in the wild with a rather small sample size, and not in captive conditions nor in relation to experimental temperature treatments, thus should be interpreted with caution. We also found a tendency for similar within clutch variation in yolk mass (which is correlated with A4, r = 0.16, p = 0.06), which may partly explain the pattern in A4. If variation in the hormonal patterns over the lay sequence would represent a response to pre-laying temperature cues, it is unclear whether the average, or certain types of temperature patterns are the cue to respond. For example, the best predictor for the start of egg-laying in great tits was an increase in ambient temperatures in certain periods, not absolute temperatures (Visser et al., 2005; Schaper et al., 2012).

We also found that relative increases in ambient temperatures during yolk formation within clutches were associated with higher...
Fig. 1. Association between yolk hormones and temperature deviation from the clutch mean ambient temperature (as °C, #4, Table 1); Yolk testosterone (T), yolk androstenedione (A4), yolk thyroxine (T4) and yolk triiodothyronine (T3) concentration (pg/mg). Panel A) data from a wild population; panel B) data from temperature experiments in captivity (year 2008, see Section 2). Yolk T4 and T3 are standardized according the analysis batch to account for variation across analysis batches.
yolk androgen concentrations, both in the wild and in captivity, suggesting a short-term causal effect of temperature on yolk androgen levels. In the wild, this kind of temperature effect could also be caused via correlation with insect food availability. However, as the same within-clutch temperature effect on yolk T was also found in captivity with ad libitum food, it is likely not mediated only via food availability. Interestingly, variation in yolk androgens in relation to ambient temperature during yolk formation was not found among clutches, although absolute temperature variation among clutches was larger than within clutches. Detection of subtle effects using a within-clutch comparison may be possible as it controls for genetic and other parental effects and thus removes other sources of variation, whereas the among-clutch comparison does not. We can speculate that the proximate mechanism to explain these changes in yolk hormone levels from egg to egg within clutches could reflect short-term changes in female physiology (e.g., metabolism) or temperature-related behavioral changes, potentially associated with short-term increases in circulating androgens and correlated changes in yolk hormone levels. Such variation can be adaptive, maladaptive or neutral. Unfortunately we do not have data on circulating androgen levels in relation to temperature in the study species, and in general, data such data within natural range is scarce and ambiguous (Frigerio et al., 2004; Dorn et al., 2014). Previous studies show a negative correlation between short-term temperature variation within clutches and dihydrotosterone (but not T or A4, Lessells et al., 2016) and no correlation between clutch means of ambient temperature and androgen concentration (Remes, 2011). Thus the exact mechanism behind the association between temperature and yolk steroids should be further investigated, including multiple species and temperature ranges.

Finally, it is important to compare the temperature effects to other sources of variation in yolk hormone levels to understand their relative magnitudes and the potential for this environmental variation really affecting offspring traits. In this study, the mean increase in yolk T concentration across the whole temperature range was 17% (from, on average, 64 pg/mg to 76 pg/mg, Fig. 1A) in the wild population. This is in similar range as the observed decrease in yolk T over the laying sequence (17%, from, on average, 75 pg/mg in the 1st eggs to 63 pg/mg in the last eggs). The change in A4 with ambient temperature in captivity was even larger, 41% over the temperature range (77–109 pg/mg). This shows that the effect of temperature on yolk hormones can be in the same range as other environmental/individual variables, such as laying order, age (e.g., Laaksonen et al., 2011; 20% difference in relation to male age) or social environment (Hargitai et al., 2009; 20% difference in respect to social stimuli).

5.2. Yolk thyroid hormones in relation to ambient temperature

Similarly as for yolk androgens, clutch mean T4 or T3 concentration was not associated with ambient pre-laying temperatures (#1) in neither wild nor captive populations, which is in contrast to our PAR hypothesis. However, long-term pre-laying ambient temperatures (14 days before egg-laying) were related to the within-clutch patterns of yolk T4 over the laying sequence in the wild population, but not in captivity. Similarly as for A4 (see above), variation in these patterns over the laying sequence may relate to associated changes in yolk mass. The functional significance of these patterns is not yet understood. To our knowledge, this is the first time that variation in yolk THs in relation to environmental factors has been shown.

Interestingly, yolk T4 concentration was negatively correlated with short-term temperature changes during yolk formation within clutches (code #4, Table 1) in the wild population. THs regulate metabolic and thermoregulatory processes, circulating TH levels increasing in colder ambient temperatures (Kuhn and Nouwen, 1978; e.g., Cogburn and Freeman, 1987; reviewed by McNabb, 2007; Kamely et al., 2015). As yolk THs are correlated with plasma circulating levels (McNabb and Wilson, 1997), a proximate mechanism for the associations with temperature during egg formation might simply be a correlation with changes in circulating TH levels in response to short-term temperature variation. It needs to be noted that most work on temperature-TH associations

<table>
<thead>
<tr>
<th>a. b. Experimental data from captive great tits (Parus major): The effect of pre-laying temperature treatments on yolk hormone concentrations using data from experimental years 2006 + 2007, 2008, 2009 and 2010 separately (see Section 2 and Online resource 3 for treatments) for a) yolk testosterone (T) and androstenedione (A4); b) for thyroxine (T4) and triiodothyronine (T3). GLMM with normal error distribution. Random factor: female identity. Proportional egg number and its square were included if significant (not shown). The factors below the dashed line have been included in the model after removing the initial temperature parameters. In 2009 we further analyzed treatment as a 2-class variable: high vs low daily variation and in 2010 we analyzed treatment as a 2-class variable: early or late temperature increase (see Online resource 3 for details of the treatments). In year 2010, no all treatment effects could be tested due to small sample sizes (NA).</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>3a</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>Treatment*prop. egg order</td>
</tr>
<tr>
<td>Year 2009/2010: treatment</td>
</tr>
<tr>
<td>Year 2009/2010 treatment</td>
</tr>
<tr>
<td>2-class*egg order</td>
</tr>
<tr>
<td><strong>3b</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>Year 2009/2010: treatment</td>
</tr>
<tr>
<td>2-class*egg order</td>
</tr>
</tbody>
</table>
originates from poultry studies with rather extreme experimental cool or heat conditions, thus the response to small-scale variation in ambient temperatures is not well understood. Via changes in peripheral deiodination, circulating TH levels can, however, respond very rapidly (<1 h) to changes in ambient temperature (e.g. Klandorf and Harvey, 1985; Darras et al., 1995; Reyns et al., 2002). In captivity (ad libitum food) no effect of temperature during yolk formation (nor pre-laying temperature) was found on T4, which suggests that the effect found in the wild population could be mediated mostly via temperature-related changes in food availability. However the small sample size in captivity and limited temperature variation needs to be taken into account in the interpretation. Interestingly, T3 concentration was not associated with environmental variation either pre-laying or during yolk formation. We may speculate that as T3 is the biologically active form, multiple mechanisms may have evolved to regulate its levels in the egg, independent of variation in circulating T3 levels (Van Herck et al., 2013).

5.3. Patterns across the laying sequence

In addition to the variation in A4 and T4 in response to pre-laying temperatures, we found general patterns over the laying sequence for most of the hormones. T levels decreased across the laying sequence in the wild population and a showed curvilinear pattern in captivity (with highest values in the middle of the clutch). The decreasing pattern is contrary to the increasing pattern previously reported for great tits (Tschirren et al., 2004; but see Groothuis and Schwabl, 2008; Heylen et al., 2012) and many other passerines (Gil, 2008). It may be explained by the unusual study year which was extremely late (14 days later than average) and temperatures several degrees colder than the average (M. Visser, unpubl. data). Interestingly, we also found, for the first time in a wild altricial bird (see Ruuskkanen et al. 2016b), that yolk T4 concentration varied systematically within the laying sequence, increasing with egg order. We can only speculate if within-clutch patterns would have similar function in yolk THs as in steroid hormones (brood survival, reduction or bet-hedging, see Section 1) or whether this is an epiphenomenon reflecting changes in female circulating hormone levels. Certainly more data is needed from other species and environmental conditions to reveal the generality of these patterns and their functional significance.

5.4. Conclusions

Yolk hormone levels (especially testosterone) can be causally affected by ambient temperature during egg-formation within clutches. As these are short-term effects, this variation may reflect female physiological changes and constraints but the exact mechanism and adaptive value is not known. What are the consequences of such temperature-dependent variation in yolk androgens and THs within clutches? Potentially even these small differences in yolk androgen and TH levels within the clutch may affect growth and sibling competition (Tschirren et al., 2005; Ruuskkanen et al., 2016a; Podlas et al., 2013), and thus have consequences for offspring and maternal fitness. We also found some indication for variation in the patterns over the laying sequence within clutches depending on the longer-term (14 day) pre-laying temperature. The adaptive value of both the longer-term and short-term variation should be studied using yolk hormone manipulations at different thermal environments. In the global change scenario with changing spring temperatures, adjustment of hormone levels to ambient temperature cues could function as a mechanism in modulating phenotypes in a context-dependent way. However, if the effects of ambient temperature on yolk hormones are constrained via female physiology, more variable temperature profiles could lead to more variable yolk hormone levels within clutches. For THs, it is immature to make any further conclusions as their functional significance is not well understood.

Acknowledgments

SR was financially supported by Academy of Finland (grant: 258419). MEV was supported by a NWO-VICI grant. We thank all field and laboratory assistants and animal caretakers, especially Anouk de Plaa and Lut Noterdaeme for their great effort. Data is available at Dryad. We have no conflict of interest to declare.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2016.05.026.

References


