



Experimental increase in temperature affects eggshell thickness, and not egg mass, eggshell spottiness or egg composition in the great tit (*Parus major*)

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ABSTRACT

Phenotypic effects of global warming have been documented in many different taxa. However, the importance of transgenerational phenotypic plasticity in these adaptations are seldom studied. In birds, temperature could affect egg characteristics. Higher temperatures during egg-laying may reduce maintenance costs for females and allow a higher investment in reproduction. Yet, females may also use temperatures as a cue for the risk of mismatch later in the season. Thus, higher temperatures may be correlated to an acceleration of embryonic development (e.g. via hormonal manipulation). We performed an experiment in which night-time temperature was increased in the nestbox by approximately 1 °C throughout the entire laying period in great tits (*Parus major*). We collected one pre-treatment egg (beginning of the laying sequence) and one post-treatment egg (end of the laying sequence). Egg content (yolk androgens and lysozymes in the albumen), eggshell coloration, eggshell mass, egg mass, and shape were not affected by the treatment. However, last-laid eggs in clutches from control nestboxes had a thicker eggshell than last-laid eggs from heated nestboxes, suggesting a putative slight decrease of maternal investment with the experimental increase of temperature. We also observed effects of the laying sequence on egg characteristics. Eggs that were laid late in the laying sequence were heavier, larger, had larger spots and higher yolk androgens than eggs laid earlier. Lysozyme concentration decrease with the laying sequence in late clutches only. Thus, effects of temperature may also change with the laying sequence and it would be interesting in the future to test the effects on first-laid eggs.

1. Introduction

Global warming has already caused phenotypic changes in many species from all taxa, with, for example, reports of decreasing body sizes, advancing phenology, or changing dispersal behaviours (Merilä and Hendry, 2014). There is evidence that these phenotypic changes are driven by genetic changes and plasticity (Charmantier and Gienapp, 2014; Merilä and Hendry, 2014; Visser, 2008). In this context, it seems promising to investigate the role of transgenerational plasticity in response to global warming (Meylan et al., 2012; Räsänen and Kruuk, 2007; Salinas and Munch, 2012; Shama et al., 2014). Transgenerational plasticity, also called parental effects or maternal effects, refers to any modification of the phenotype of the offspring due to the phenotype of its parents or to the environment experienced by its parents (Mousseau and Fox, 1998). Maternal effects can represent constraints, but

interestingly, can also represent adaptive strategies if they pre-adapt the offspring to the environment they might encounter (predictive adaptive response) (Gluckman et al., 2005; Mousseau and Fox, 1998).

In small passerine birds, it has been shown for example that laying date can be advanced in response to warmer spring, reducing the mismatch between hatching and the peak of food availability (Charmantier et al., 2008; Visser et al., 2009). Yet, studies on other prenatal maternal effects are still scarce in the context of climate change (Bleu et al., 2017; Ruuskanen et al., 2016b; Vaugoyeau et al., 2017). Of particular interest in birds are prenatal maternal effects linked to egg morphological characteristics and egg content. For example, egg mass can be an important fitness determinant because it represents the total amount of energy invested in the egg, and therefore, is often correlated to egg hatchability (e.g. Perrins, 1996; Saino et al., 2004). Egg mass and size are highly heritable (Christians, 2002) but they may also covary

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with temperature, the correlations being often positive (Christians, 2002; Cucco et al., 2009; Nager and van Noordwijk, 1992; Saino et al., 2004) but not always (Christians, 2002; Schaper and Visser, 2013). Also, thinner eggshells may reduce hatching success because of desiccation during incubation or breaking of the shell (Drent and Woldendorp, 1989; Graveland, 1996). Eggshell mass and thickness are dependent on calcium availability and females forage actively on calcium-rich food sources during egg-laying (Wilkin et al., 2008). Temperature may affect female behaviour or female body condition and thus eggshell characteristics.

Regarding egg composition, it is known that hormones, and in particular androgens, transferred by females to egg yolk are major modulators of offspring phenotype (Gil, 2003, 2008; Groothuis et al., 2005; Podmokła et al., 2018; Schwabl, 1993). Yolk androgens, and more precisely testosterone (T), androstenedione (A4) and dihydrotestosterone (DHT), affect offspring development by increasing chicks' growth rates, begging behaviour and survival. However, they are also associated with an altered immune response in the chicks of most species (Gil, 2008; von Engelhardt and Groothuis, 2011). Androgen deposition in the eggs depends on laying order and also on environmental factors such as food availability, mate quality or breeding density (Gil, 2008; von Engelhardt and Groothuis, 2011). However, the influence of temperature on yolk androgens has been less investigated, and the results are so far inconsistent. In two seabirds, yolk T and A4 levels did not differ between two years that were characterized by contrasted temperatures (Addison et al., 2008). In great tits, a study showed an interactive effect of temperature and laying sequence on yolk A4 concentrations (Ruuskanen et al., 2016b). More specifically, at lower than average temperatures, there was an increase of yolk A4 concentrations with laying sequence, but no pattern at higher than average temperatures (Ruuskanen et al., 2016b). They also showed that yolk T was positively correlated with temperature during yolk formation (Ruuskanen et al., 2016b). Yet, in two other studies in great tits, no correlation was found between ambient temperature and yolk T or A4 levels (Lessells et al., 2016; Remes, 2011). Finally, Lessells et al. (2016) found a negative correlation between yolk DHT levels and temperature during yolk formation. Importantly, eggs also contain antimicrobial compounds in the albumen, called lysozymes. Egg lysozymes are major maternal component of innate immunity. Furthermore, they are known to enhance hatching success and they may facilitate offspring survival (Saino et al., 2002). In the barn swallow, egg lysozymes covary positively with temperature preceding laying (Saino et al., 2004). In the pied flycatcher there was no geographic trend in lysozyme activity (comparisons of 16 populations) (Ruuskanen et al., 2011). Yet, studies focusing on the effects of temperature, within and among populations, on lysozyme concentrations are lacking.

In great tits and other tits, eggs are white with brown spots caused by protoporphyrin (Gosler et al., 2005), a pigment which is known to be pro-oxidant (Moreno and Osorno, 2003). This oxidative property has led to opposite hypotheses. On the one hand, it has been suggested that increased pigmentation could be a signal of oxidative tolerance of females and thus a signal of good condition (Moreno and Osorno, 2003). On the other hand, it could indicate that females suffer from physiological stress, as suggested in blue tits by Martínez-de la Puente et al. (2007). Finally, it has also been shown that eggshell coloration is negatively correlated with eggshell thickness and calcium availability (Gosler et al., 2005; Sanz and García-Navas, 2009), thus supporting a structural function of pigmentation (Cherry and Gosler, 2010). More studies on eggshell spottiness are necessary to better understand the correlations between eggshell characteristics and female condition, which may be affected by temperatures during egg formation.

A change in temperature may affect egg characteristics through direct or indirect mechanisms. First, we can expect direct effects of temperature on breeding females. Higher ambient temperature provides an environment that is less energetically demanding for females (reduction of maintenance costs). Females could thus invest more in

foraging and reproduction. Also, another direct effect of temperature could be due to the fact that temperature may be used as a cue of breeding season advancement. Under the predictive adaptive response hypothesis, we would expect egg characteristics to be adjusted in order to accelerate chick development and reduce the risk of mismatch. Second, the effects of temperature may be indirect as temperature can be correlated with other variables such as resource availability or habitat quality. In correlative studies, it is not possible to disentangle direct from indirect effects. Experimental studies are therefore necessary to highlight direct effects of temperature and to show causal relationship.

In this study, we investigated the direct effect of temperature on egg characteristics in a small passerine bird, the great tit (*Parus major*). We increased night-time temperature in the nestbox when females were laying eggs and we measured the consequences of this manipulation on egg characteristics, taking advantage that in this species laying females roost in their nestbox overnight. This experimental increase in nest temperature overnight was aimed at mimicking an increase in ambient temperature for females. We already reported its effects on phenology, chick development and female characteristics (Bleu et al., 2017). We found a negative effect of the treatment on clutch size in late-laying females experiencing an increase in nest temperature at night. In addition, the seasonal decline in nestling health found in controls was not detected in nests from heated nestboxes, as measured by blood sedimentation rate (Bleu et al., 2017). These results suggest that females used temperature as a cue of seasonal advancement to adjust breeding phenology, and do not support the reduction of maintenance costs hypothesis (Bleu et al., 2017). The aim of the current paper is to analyse the characteristics of the eggs collected during this same experiment. We collected two eggs per clutch: one pre-treatment egg (beginning of the laying sequence; the treatment started on the day the first egg was laid), and one post-treatment egg (end of the laying sequence). Control nests monitored any changes of egg characteristics that may occur with the laying order. More precisely, we investigated egg morphological characteristics (mass, length, width, eggshell mass, eggshell thickness), coloration (eggshell pigmentation), and composition (yolk androgen concentration, albumen lysozyme concentration).

Under the hypothesis of reduction of maintenance costs, we predict a positive effect of temperature on egg morphological characteristics and on lysozyme concentration. A positive effect is expected on egg coloration if it is an indicator of female health. On the contrary, if it is an indicator of eggshell structure and if females with reduced maintenance costs can spend more time foraging on calcium-rich preys, we expect a negative effect of the heating treatment on egg coloration. Under the hypothesis that temperature is used as a cue of the advancement of the breeding season and the predictive adaptive response hypothesis, we predict a positive effect on yolk androgen concentration that are known to accelerate embryonic and chick development. Our experimental design allows us also to investigate the change of egg characteristics with the laying order.

2. Material and methods

2.1. Model species and field site

During spring 2015, we monitored a population of great tit (*Parus major*, Linnaeus, 1758) nesting in artificial nestboxes (Schwegler wood concrete nest-boxes 2 M, Valliance, Saint Pierre La Palud, France) near the CEREEP field station (CEREEP-Ecotron Ile-de-France, UMS 3194, École Normale Supérieure, St-Pierre-lès-Nemours). Nestboxes are evenly distributed within two sites in the Commanderie forest (48°17'N 2°41'E, site 1: 117 nestboxes, site 2: 118 nestboxes, mean distance between the sites = 2 km), and used by great tits and blue tits (*Cyanistes caeruleus*). For this experiment, we used 82 nestboxes occupied by great tits (58 in site 1 and 24 in site 2) (see also Bleu et al., 2017). In 2015, the earliest laying date for the first clutch was the 1st of April and the

last one was the 20th of April.

Great tits are insectivorous small passerine birds. They produce one or two clutches per year and females usually lay one egg per day and start full incubation around clutch completion. During egg-laying, females typically roost in their nestbox at night (Gosler, 1993) and may start some nocturnal incubation (Vedder, 2012). Only females incubate, and part of their nutritional requirements during incubation is provided by the male who feeds her in the nest (Gosler, 1993).

2.2. Experimental treatment

The experimental heating treatment is described in details in a different publication focusing on the effects on phenology and reproductive success (Bleu et al., 2017). In brief, we had 41 control nestboxes and 41 heated nestboxes. Starting on the day the first egg of a clutch was laid, we placed one hand warmer at the top inside the nestbox every evening, allowing us to heat the air in the nestbox during the night when females were present, without directly heating the eggs. Control nestboxes were equipped with used hand warmers that did not produce heat anymore in order to expose females to the same level of disturbance. We used hand warmers releasing heat when in contact with oxygen during at least 7 h (ref. HWES, Grabber 7 + Hour Hand Warmers). The heating treatment resulted in an increase of 1.1 °C during the night (18 h–05 h) in heated as compared to control nestboxes (mixed-model with nestbox as a random effect: $F_{1,6} = 12.70$, $P = 0.012$), as measured in 8 empty nestboxes (4 controls and 4 heated nestboxes) with iButton temperature loggers (DS1922L, Maxim integrated; Bleu et al., 2017).

During the course of the heating treatment, every evening after having replaced hand warmers, any new egg laid was numbered with a pencil, and the temperature of the eggs checked. This allowed us to establish the laying sequence and the start of incubation (i.e. when eggs were warm). The heating treatment was stopped when incubation started. We collected two eggs per clutch at (or near) clutch completion. We aimed to collect the first laid-egg (considered as a pre-treatment egg) and the last laid-egg (considered as a post-treatment egg) but it happened that the female laid a new egg after collection of the eggs (Table 1). When the laying sequence was not known for sure (e.g. we forgot to mark one egg and thus found two unmarked eggs the following day) we calculated a mean laying sequence (e.g. laying sequence 1.5 is for the eggs that were laid as first or second eggs). We did not collect eggs from nests abandoned before incubation ($n = 5$), and from nests with small clutch sizes ($n = 5$). Also we did not reduce clutch size by more than two eggs. This means that we did not collect eggs or collected only one egg when some eggs were found broken in the nest ($n = 2$) or were broken during manipulation ($n = 7$). This means that from the 82 nestboxes at the start of the experiment, we collected and analysed 128 eggs from 69 nestboxes. We had 90 eggs from site 1 and 38 from site 2, and we had 65 pre-treatment eggs and 63 post-treatment eggs (Table 1).

Table 1
Sample sizes and laying order.

	Pre-treatment eggs				Post-treatment eggs**								
Laying sequence	1	1.5*	2	2.5*	7	7.5*	8	8.5*	9	9.5*	10	11	12
Number of eggs	53	8	3	1	4	2	22	1	18	1	9	5	1

*When the laying sequence was not known for sure (e.g. we forgot to mark one egg and thus found two unmarked eggs the following day) we calculated a mean laying sequence (i.e. laying sequence 1.5 is for the eggs that were laid as first or second eggs).

**The post-treatment eggs are not always the last-laid eggs as some females laid one or two eggs after the start of incubation/after egg collection.

2.3. Egg characteristics

After collection, eggs were immediately weighted on a precision scale (± 0.001 g) and stored at -20 °C until analyses. During summer 2015, eggs were photographed with a calibrated camera (see Mallard et al., 2013). We took 3 pictures for each egg: a lateral view, a view from above the pointed end of the egg (i.e. ‘foot’ sensu Gosler et al., 2005), and a view from above the flattened area (i.e. ‘crown’ sensu Gosler et al., 2005). The pictures were analysed with ImageJ (version 1.48, <http://imagej.nih.gov/ij/>). We determined for each picture, the total area of the egg, the number of spots on the eggshell and the total area of the eggshell covered with spots. We then deduced the proportion of the egg covered with spots for each picture (total area of spots/total area of the egg). For each egg, we thus have 9 measures of coloration: number, area and proportion of spots for each of the three pictures. After being photographed, eggs were measured (width and length) with a caliper (± 0.1 mm). Then we opened the egg and separated the yolk and the albumen before full unfreezing, and stored them back at -20 °C. For the measures of androgens and lysozymes, we discarded eggs for which we suspected incubation started before collection (i.e. signs that the perivitelline membrane started to melt, albumen and yolk blending with each other (Biard, pers. obs.); 21 eggs removed). We measured the thickness of unpigmented eggshell at the ‘crown’ where it is the thickest (Gosler et al., 2005) with a micrometer (Mitutoyo, ref. 7-313, ± 0.01 mm). For each sample, we took 3 measures and used the mean of these 3 measures for the statistical analyses (coefficient of variation is 1.5%). Finally, total eggshell was dried at 60 °C during 16 h and then eggshell was weighted (± 0.0001 g). From the 128 eggs collected, we could photograph and analyse eggshell spottiness for 126 eggs (60 eggs from control nestboxes and 66 eggs from heated nestboxes). For the analysis on egg morphology, we kept only the 118 eggs with no missing values for egg mass, egg length, egg width, eggshell mass and eggshell thickness (55 eggs from control nestboxes and 63 eggs from heated nestboxes).

2.4. Egg content: lysozymes and androgens

Lysozyme concentration (107 eggs analysed including 51 control and 56 heated eggs) was measured using a micro-plate modification of the turbidimetric assay (Morosinotto et al., 2013; Ruuskanen et al., 2011). Shortly, 9.5 μ L of albumen was distributed in the wells of a micro-plate. Each sample was run at least in duplicates (some samples were also repeated between different micro-plates). We added 9.5 μ L of phosphate buffer (Sigma L6876, pH 6.3, 9 g/L) in each well. We then suspended 20 mg of a dried strain of *Micrococcus lysodeikticus* (Sigma M3770) in 30 mL of phosphate buffer. The amount of *Micrococcus* was adjusted in order to obtain a solution with an absorbance of 1 at 450 nm (250 μ L were used to check the absorbance). Lysozymes from the albumen samples degrade bacterial cell walls, which can be measured from the absorbance with a micro-plate reader (Bio-rad 680). 250 μ L of the *Micrococcus* solution were added to the wells on the plate and the absorbance was measured at 450 nm at 26 °C after 10 min. We prepared a standard lysozyme solution by diluting crystalline hen egg white

lysozyme (Sigma L6876) in phosphate buffer, which was serially diluted to obtain a standard curve (12.5–200 µg/ml). Two standard curves were added to the top and bottom row of each plate. Inter-assay variation was 7.7% and intra-assay variation was 6.1%.

We could not retrieve the yolk for 2 eggs, thus we analysed 105 eggs for yolk androgen concentrations (50 control and 55 heated eggs). Yolk concentrations of T and A4 were determined by radio-immunoassay (RIA) at the CEBC as previously described (Paquet et al., 2013). Each sample was run in duplicates. Briefly, 100 mg of each sample were homogenized in 1 mL of distilled water. Steroids were then extracted by adding 3 mL of diethyl-ether to 300 µL of the homogenized yolk, by vortexing, and by centrifuging (5 mins, 2000 rpm). The diethyl-ether phase containing steroids was decanted and separated after snap freezing the tube with an alcohol bath. The dried extracts were then dissolved in 900 µL of phosphate buffer and each hormone was assayed in duplicate. 100 µL of extract were incubated overnight with 4000 cpm of the appropriate ³H-steroid (Perkin Elmer, US) and polyclonal rabbit antiserum. The bound fraction was then separated from free fraction by addition of dextran-coated charcoal. Finally, activity was counted on a tri-carb 2810 TR scintillation counter (Perkin Elmer, US). Inter- and intra-assay variations were respectively 7.16% and 7.67% for T, 10.38% and 7.04% for A4. T, A4 lowest detectable concentrations were respectively 1.26 pg/mg, and 1.06 pg/mg. T and A4 concentrations were highly correlated (Pearson's product-moment correlation: $r = 0.79$, $t = 13.29$, $df = 103$, $p < 0.001$).

2.5. Statistical analyses

We used R version 3.4.2 (R Core Team, 2017). The correlations between the variables describing egg morphological characteristics and eggshell spottiness are shown in Tables ESM1 and ESM2 respectively. Because of the high number of variables and of their correlations, we performed two separate principal component analyses (PCA) with package “ade4” version 1.7-8 (Dray and Dufour, 2007). For both analyses, variables were first standardized and we kept the two principal components that explained more than 70% of the total variation. Then, we analysed the first two principal components (PC1 and PC2) in separate mixed models (lmer procedure from package “lme4” version 1.1-14 with REML estimation) (Bates et al., 2015). Nestbox was defined as a random effect to account for the non-independence of the eggs laid by the same female. We tested the effect of treatment and included the following covariates in all models: study site, laying order, clutch size, laying date. Laying order was a categorical binary variable, defining eggs as either pre-treatment eggs (position in the laying sequence 1–2.5) or post-treatment eggs (position in the laying sequence 7–11, Table 1). We included clutch size in order to control for the fact that post-treatment eggs do not have the same position in the laying sequence in all clutches. We tested the interaction between treatment and laying order, because we expect an effect of the treatment only on post-treatment eggs. We also tested the three-way interaction between treatment, laying order and laying date because we showed previously differential seasonal effects of the treatment (Bleu et al., 2017). Since we tested the three-way interaction, we also included the two-way interactions between laying order and laying date, and treatment and laying date. We removed the non-significant interactions step by step in all the models (all $P > 0.10$). When analysing variation in PC1 for eggshell spottiness, the model was also run without one outlier (very small value, see Fig. ESM2). This did not change the results (see Table ESM3).

To investigate variation in albumen lysozyme and yolk androgens concentrations, we performed similar mixed models with mean values (samples were run in duplicates). For lysozymes, 3 outliers were removed (small values) to conform to normality assumptions. The results are qualitatively similar if we keep all the data (see Table ESM4). For yolk androgens, data were first log-transformed to conform to normality and homoscedasticity assumptions.

We present type III analysis of variance tables with Satterthwaite approximation for degrees of freedom (package “lmerTest” version 2.0-33). The estimates are presented as mean ± standard error. The assumptions of normality and homoscedasticity of residuals were fulfilled for all models, except for the model investigating variation in PC1 for eggshell spottiness. However, the residuals of the model followed a normal distribution when we ran the model without the outlier. Post-hoc tests were computed as differences of least squares means between the factors (pairwise comparisons).

3. Results

3.1. Egg morphology

The first principal component (PC1) expresses 52.1% of the total variation and is mainly explained by the mass and shape of the egg (Fig. ESM1). The second principal component (PC2) expresses a further 21.1% of the total variation and is mainly explained by eggshell thickness (Fig. ESM1). The experimental increase in ambient temperature in the nestbox during laying had no effect on egg mass and shape (PC1) but post-treatment eggs and eggs in site 2 were heavier and larger than pre-treatment eggs (Table 2A and Fig. 1A) and eggs in site 1 (Table 2A). On the contrary, for eggshell thickness (PC2), there is a significant interaction of treatment and laying order (Table 2B). Post-hoc comparisons show that post-treatment eggs in the control group have a thicker eggshell than post-treatment eggs in the heated groups ($t = 2.65$, $P = 0.01$, $df = 96.6$) but that there is no difference for pre-treatment eggs ($t = 0.099$, $P = 0.92$, $df = 99.5$) (Fig. 1B).

3.2. Eggshell spottiness

In the PCA, PC1 expresses 54% of the total variation and is mainly explained by the size of the spots (total area covered with spots and proportion of the egg covered with spots) (Fig. ESM2). PC2 expresses a further 23% of the total variation and is mainly explained by the number of spots (Fig. ESM2). The area of the eggshell covered with spots is larger in post-treatment eggs compared to pre-treatment eggs (Table 3A, Fig. 2A), but the number of spots is smaller (Table 3B, Fig. 2B). In both cases, there is no effect of the treatment on these changes (Table 3, Fig. 2A and B).

Table 2

Analysis of variance for egg morphological characteristics. The principal components (PC) are described in the text and in the Fig. 1 and ESM1. The among-nestboxes variance of the intercept is 1.17 and 0.51 and the residual variance is 1.24 and 0.54 (for PC1 and PC2 respectively). The full models included the interactions: laying order*laying date*treatment, laying order*laying date, laying date*treatment, laying order*treatment. Non-significant interactions were removed. Significant effects are in bold ($P < 0.05$).

	DF	F-value	P-value
A) Egg mass and shape (PC1)			
Laying date	1,61.642	0.19	0.66
Clutch size	1,64.124	1.88	0.17
Study Site	1,63.74	5.40	0.023
Treatment	1,62.062	0.0096	0.92
Laying order	1,56.914	11.74	0.0011
B) Eggshell thickness (PC2)			
Laying date	1,59.559	0.14	0.71
Clutch size	1,62.053	0.028	0.87
Study Site	1,61.670	0.16	0.69
Treatment	1,59.989	2.60	0.11
Laying order	1,54.802	1.14	0.29
Treatment × Laying order	1,54.881	5.46	0.023

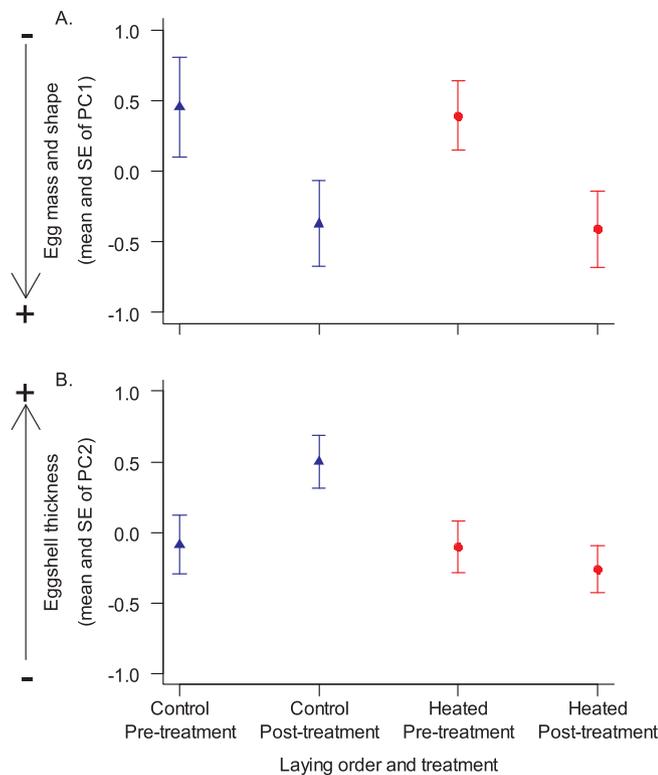


Fig. 1. Effect of laying order and experimental heating during egg-laying on egg morphological characteristics. To describe egg morphology we measured 5 variables: egg mass, egg length, egg width, eggshell thickness and eggshell mass. The 2 main principal components (weighted linear combinations of all these factors), PC1 and PC2 (see Fig. ESM1), were used as dependent variables in linear mixed models (see statistics in Table 2 and in the main text). Laying order affected egg characteristics described by PC1 (egg mass and shape, panel A). Treatment in interaction with laying order affected the egg characteristics described by PC2 (eggshell thickness, panel B). Eggs from heated females are the red circles and eggs from the control females are the blue triangles.

Table 3

Analysis of variance for eggshell spottiness. The principal components (PC) are described in Fig. 2 and ESM2. The among-nestboxes variance of the intercept is 0.81 and 0.90 and the residual variance is 3.16 and 0.99 (for PC1 and PC2 respectively). The full models included the interactions: laying order*laying date*treatment, laying order*laying date, laying date*treatment, laying order*treatment. Non-significant interactions were removed. Significant effects are in bold ($P < 0.05$).

	DF	F-value	P-value
A) Size of the spots (PC1)			
Laying date	1,61.566	0.39	0.53
Clutch size	1,64.346	2.61	0.11
Study Site	1,64.456	0.0027	0.96
Treatment	1,62.769	0.19	0.67
Laying order	1,64.213	37.88	< 0.0001
B) Number of spots (PC2)			
Laying date	1,62.175	0.52	0.47
Clutch size	1,64.279	0.0001	0.99
Study Site	1,64.490	1.37	0.25
Treatment	1,63.077	2.11	0.15
Laying order	1,61.406	19.27	< 0.0001

3.3. Lysozyme in the albumen and yolk androgens

Lysozyme concentration in the albumen was explained by an interaction between laying date and laying order (Table 4). This effect

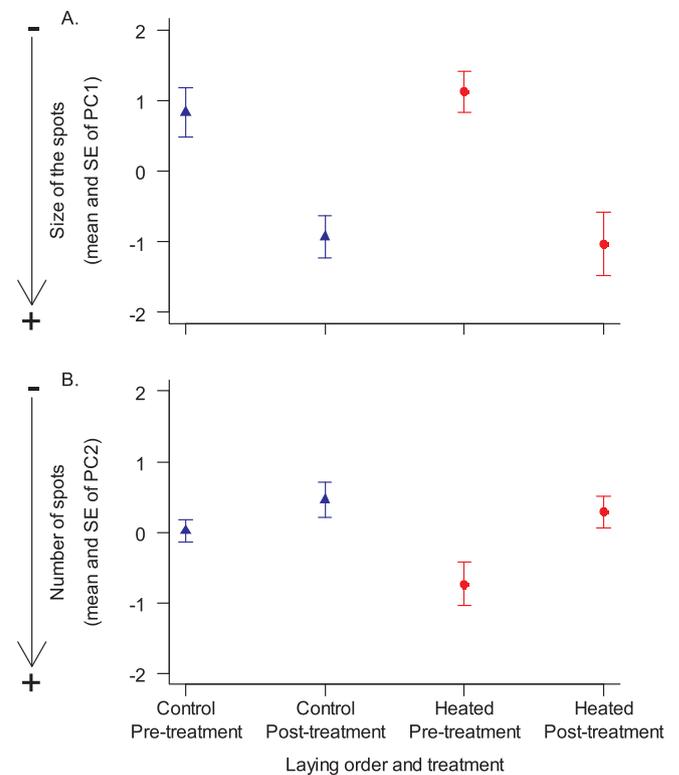


Fig. 2. Effect of laying order on eggshell spottiness. To describe eggshell spottiness we measured 3 parameters from 3 different pictures (lateral view, crown region and foot region) of each egg: proportion of the egg covered with spots, total area of the egg covered with spots, and the number of spots. The 2 main principal components (PC1 and PC2, see Fig. ESM2) were used as dependent variables in linear mixed models (see statistics in Table 3). Laying order affected the egg characteristics described by PC1 (size of the spots, panel A) and PC2 (number of spots, panel B). Eggs from heated females are the red circles and eggs from the control females are the blue triangles.

Table 4

Analysis of variance for lysozyme concentration in the albumen. The among-nestboxes variance of the intercept is 0.0 and the residual variance is 224.7. The full models included the interactions: laying order*laying date*treatment, laying order*laying date, laying date*treatment, laying order*treatment. Non-significant interactions were removed. Significant effects are in bold ($P < 0.05$).

	DF	F-value	P-value
Laying date	1,97	0.60	0.44
Clutch size	1,97	0.33	0.57
Study Site	1,97	2.89	0.093
Treatment	1,97	0.013	0.91
Laying order	1,97	4.72	0.032
Laying date × Laying order	1,97	4.96	0.028

was not dependent on the experimental treatment. Lysozyme concentration decreased with laying date for post-treatment eggs ($t = -2.41$, $P = 0.018$, $df = 97$) and not for pre-treatment eggs ($t = 0.89$, $P = 0.38$, $df = 97$). In late clutches, post-treatment eggs have less lysozymes than pre-treatment eggs for a given laying date (Fig. 3A). Similarly, T and A4 concentrations were not affected by the treatment (Tables 5A and B) but there was an effect of laying order and laying date in interaction (Tables 5A and B, Fig. 3B and C). T concentrations increase with laying date for pre-treatment eggs ($t = 2.36$, $P = 0.020$, $df = 96.80$) and not for post-treatment eggs ($t = -0.46$, $P = 0.65$, $df = 81.61$). For A4, the trend is similar but the slopes are not significantly different from zero (for pre-treatment eggs: $t = 1.37$, $P = 0.17$, $df = 96.5$; for post-treatment eggs: $t = -0.73$,

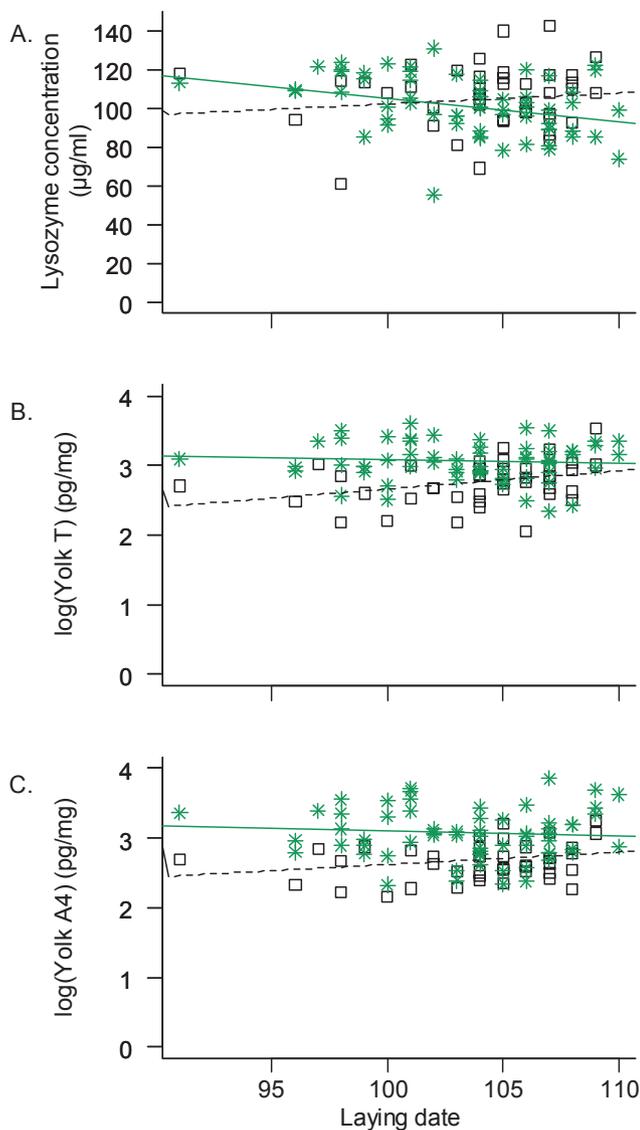


Fig. 3. Interactive effect of laying order and laying date on lysozyme and yolk androgen concentrations. We measured lysozyme in the albumen ($\mu\text{g/ml}$, panel A), testosterone (T, panel B) and androstenedione (A4, panel C) in egg yolk (pg/mg). Lysozyme concentration in the albumen decreased with laying date for post-treatment eggs only (slopes are estimates from the model in Table 4, see also the main text for statistics). Yolk concentration of both androgens increased with laying date for pre-treatment eggs only (slopes are estimates from the models in Table 5, see also the main text for statistics). Dates are encoded as Julian dates, that is, 100 = 10th of April. Post-treatment eggs are the green stars (green regression line), pre-treatment eggs are the squares (dashed regression line).

$P = 0.47$, $df = 80.06$). However, the two slopes differ from each other ($t = -2.08$, $P = 0.044$, $df = 41.21$). In both cases, post-treatment eggs have more androgens than pre-treatment eggs for a given laying date (Fig. 3B and C).

4. Discussion

In this study, we increased night-time temperature in the nestbox by ca. 1°C during the egg-laying period. We measured the effect of this treatment on egg characteristics. The treatment only affected eggshell thickness. Post-treatment eggs from heated females had a thinner eggshell than post-treatment eggs from control females. Moreover, we highlighted effects of the laying order on egg shape, egg mass, eggshell coloration and differential effects of laying order with advancing season

Table 5

Analysis of variance for yolk androgen concentration. The among-nestboxes variance of the intercept is 0.043 and 0.060 and the residual variance is 0.039 and 0.050 (for testosterone and androstenedione respectively). The full models included the interactions: laying order*laying date*treatment, laying order*laying date, laying date*treatment, laying order*treatment. Non-significant interactions were removed. Significant effects are in bold ($P < 0.05$).

	DF	F-value	P-value
A) log(Testosterone)			
Laying date	1,61.020	1.56	0.22
Clutch size	1,62.000	0.04	0.84
Study Site	1,63.248	0.73	0.40
Treatment	1,60.195	1.63	0.21
Laying order	1,42.892	9.57	0.0035
Laying date \times Laying order	1,42.758	7.98	0.0072
B) log(Androstenedione)			
Laying date	1,60.182	0.22	0.64
Clutch size	1,61.004	1.89	0.17
Study Site	1,62.221	1.32	0.25
Treatment	1,59.250	0.68	0.41
Laying order	1,41.341	5.66	0.022
Laying date \times Laying order	1,41.214	4.34	0.044

on egg content (yolk androgen concentrations and lysozyme concentration in the albumen).

Contrary to our predictions, the increase of temperature during egg-laying did not seem to increase maternal investment in their eggs (hypothesis of reduction of maintenance costs) or to increase androgen concentration (predictive adaptive response hypothesis, see introduction). We however observed a negative effect of the treatment, resulting in eggs with thinner eggshells. It is known in poultry that heat stress results in a decrease in eggshell quality (decrease in eggshell thickness, higher probability of egg breakage) (Lin et al., 2004). This may be due to the decrease in the amount of calcium in plasma because of respiratory alkalosis during heat stress (Mahmoud et al., 1996). However, in our experiment females were not in a context of heat stress because overnight temperature was only slightly increased (1°C). The heating treatment was moderate and only during night-time, with no detrimental effects on female health (Bleu et al., 2017). Moreover, there is no effect of the treatment on eggshell mass, suggesting that the total amount of calcium invested in eggshell is not different between the groups (Gosler et al., 2005). However, the distribution of eggshell mass may differ between the groups (Gosler et al., 2005). We measured eggshell thickness at the crown and thus we cannot test this hypothesis. This should be investigated in more details in future studies.

The other variables describing egg characteristics were not affected by the heating treatment. In particular there was no effect on yolk androgen concentrations. In previous correlative studies (see introduction), the effects of temperature on androgen hormones were not clear. Our experimental study suggests that such effects may not be driven by temperature *per se*, which may explain the discrepancy between studies. But, we cannot exclude that our increase in temperature was not sufficiently high to trigger an effect. Interestingly, another experimental study (testing several manipulations of temperature, with some groups having temperatures that differed of 4 or 5°C) showed no effect of temperature during yolk formation (4 days) or of mean temperature during the whole egg-laying period on yolk androgens (T and A4) concentrations in great tits (Ruuskanen et al., 2016b). However, they highlighted a positive effect of temperature within a clutch (effect of temperature deviation). The effect was positive, meaning that yolk T and A4 concentrations were higher when temperatures during formation of a particular egg were higher than on average during egg-laying of the entire clutch (Ruuskanen et al., 2016b). Concerning egg mass, we also expected a positive effect of the treatment and we did not find any.

This lack of effect corroborates results from a previous experiment in blue tits (Yom-Tov and Wright, 1993, experimental increase of temperature of 6 °C). The relationship between egg mass and temperature found in some correlative studies (e.g. Ojanen, 1983, see also introduction) may be more likely linked to food availability (Ardia et al., 2006; Ruuskanen et al., 2016a).

Finally, we also report interesting patterns of change in egg characteristics with laying order. Within-clutch changes in egg characteristics could be adaptive and have been classically explained by two distinct strategies: brood reduction and brood survival strategies (Lack, 1947; Slagsvold et al., 1984). In the first case, the investment is skewed towards first laid eggs to facilitate the death of late offspring in case of adverse conditions. In the second case, the investment increases with the laying sequence to compensate for the negative effects of hatching asynchrony. We found that post-treatment eggs were heavier (total egg mass and eggshell mass) and had higher concentration of yolk androgens (especially for early clutches), which support the brood survival hypothesis. The increase of androgens with the laying sequence had already been described in great tits (Tschirren et al., 2004), but not always (Ruuskanen et al., 2016a). For example, a decrease of yolk T was observed in another wild population of great tits (Ruuskanen et al., 2016a,b). Also, the effect of laying order can be affected by other variables, such as temperature (e.g. Ruuskanen et al., 2016b for yolk A4), personality (Groothuis et al., 2008 for yolk T) or laying date (this study). It should be noted that the range of laying dates in our study site is short (20 days). This effect may be even more pronounced in study population with a larger range of lay dates. The increase in eggshell mass may indeed represent a change of allocation with the laying sequence (brood survival hypothesis). However, it is also known that calcium is a limited resource for breeding passerines (Gosler et al., 2005; Graveland, 1996). Thus, the increase in eggshell mass may also result from a change of foraging-mediated calcium acquisition (Gosler et al., 2005). There is no effect of laying date on this pattern so we do not think that calcium-rich prey availability changes over the short time-scale of the laying sequence. Nevertheless, the ability of females to forage on calcium-rich preys may improve and this may result in females producing heavier eggshell at the end of the laying period.

Lysozymes concentration in the albumen decreased with laying order, but only for late clutches. In barn swallows, Saino et al. (2002) also found a negative relationship between laying order and egg lysozyme concentration. They suggested that this pattern may be adaptive because first-laid eggs are at greater risk for infection than last-laid eggs and because lysozyme production is costly for females. This would suggest that females breeding early in the season are in better condition than late breeding females since they can invest comparable amount of lysozyme to all their eggs. Interestingly, for several other bird species, no pattern of decreasing lysozyme concentration with laying sequence was found (D'Alba et al., 2010; Shawkey et al., 2008; for studies on blue tits and 8 other bird species). Such a pattern might actually be overlooked if other variables (such as laying date) affecting female reproductive strategies are not taken into account.

Egg coloration also changed with laying order. Post-treatment eggs had larger spots and more proportion of the eggshell covered with spots than pre-treatment eggs, as also reported by others (Gosler et al., 2005). First, we should note that the heating treatment did not affect this pattern. Thus, within the limits of our manipulation, female condition does not seem to influence eggshell coloration, contrary to the predictions of the hypotheses that pigmentation signals oxidative tolerance or physiological stress (Martínez-de la Puente et al., 2007; Moreno and Osorno, 2003). Also, the experimental treatment did not affect calcium availability for the females (no effect of the treatment on eggshell mass), thus we cannot test the structural hypothesis (Gosler et al., 2005). This hypothesis posits that a change of pigmentation may be due to the use of protoporphyrin in replacement to calcium. It may seem surprising that eggshell thickness and eggshell coloration were not correlated contrary to a previous study (Sanz and García-Navas, 2009).

However, we did not measure eggshell thickness at the same position on the egg, and eggshell thickness may be variable within an egg (Gosler et al., 2005).

The results of this study do not support the hypothesis that a slight increase of temperature during laying allows the females to invest more in their eggs because of a reduction of their maintenance costs. Females can also use temperature as a cue of the advancement of the breeding season, and thus accelerate their reproduction in response to higher temperature to reduce the risk of mismatch. However, yolk androgens, which accelerate chick development, were not affected by the treatment. We observed a negative effect of the treatment on eggshell thickness. This would suggest a strategy of brood reduction, which may fit the hypothesis of temperature being used as a cue of the advancement of the breeding season (but all the other parameters of maternal investment were not affected by the treatment). We have also more support for the “cue hypothesis” than the reduction of maintenance costs hypothesis concerning phenology (see the effects on clutch size and incubation behaviour in Bleu et al., 2017). However, this reduction in eggshell thickness in heated nestboxes should be interpreted with caution because there is no difference in eggshell mass (thus the investment in total calcium may not differ). Finally, we have clear evidence that females adopt a strategy of brood survival, independent of the heating treatment: the effect of laying order is positive on yolk androgens (and more pronounced in early clutches), egg mass and eggshell mass. Since laying order affects several characteristics of eggs, it could be interesting also to test the effects of temperature on first-laid eggs. More studies are needed to understand sources of variation in prenatal maternal effects between populations and environments (Ruuskanen et al., 2011), in particular in the context of adaptation to rapidly changing environments, in which they may play a crucial role. The effects of temperature may be difficult to predict because temperature may have direct physiological consequences but may also be used as a seasonal cue by the organisms, and trigger differential reproductive strategies.

5. Data accessibility

Data available from the Dryad Digital Repository ([doi:10.5061/dryad.nh3ss87](https://doi.org/10.5061/dryad.nh3ss87)).

Conflict of interest

The authors declare that they have no conflict of interest.

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All applicable institutional and national guidelines for the care and use of animals were followed.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ygcen.2019.02.004>.

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