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1 Experimental increase in temperature affects eggshell thickness, and  
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3 *(Parus major)*

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18 **Running title:** egg feature, laying order and temperature

19

20 **Key words:** eggshell coloration, global warming, lysozymes, maternal effects, testosterone

21 **Abstract**

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

42

## 43 **Introduction**

44 Global warming has already caused phenotypic changes in many species from all taxa, with,  
45 for example, reports of decreasing body sizes, advancing phenology, or changing dispersal  
46 behaviours (Merilä and Hendry, 2014). There is evidence that these phenotypic changes are  
47 driven by genetic changes and plasticity (Charmantier and Gienapp, 2014; Merilä and  
48 Hendry, 2014; Visser, 2008). In this context, it seems promising to investigate the role of  
49 transgenerational plasticity in response to global warming (Meylan et al., 2012; Räsänen and  
50 Kruuk, 2007; Salinas and Munch, 2012; Shama et al., 2014). Transgenerational plasticity, also  
51 called parental effects or maternal effects, refers to any modification of the phenotype of  
52 the offspring due to the phenotype of its parents or to the environment experienced by its  
53 parents (Mousseau and Fox, 1998). Maternal effects can represent constraints, but  
54 interestingly, can also represent adaptive strategies if they pre-adapt the offspring to the  
55 environment they might encounter (predictive adaptive response) (Gluckman et al., 2005;  
56 Mousseau and Fox, 1998).

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

66 but they may also covary with temperature, the correlations being often positive (Christians,  
67 2002; Cucco et al., 2009; Nager and van Noordwijk, 1992; Saino et al., 2004) but not always  
68 (Christians, 2002; Schaper and Visser, 2013). Also, thinner eggshells may reduce hatching  
69 success because of desiccation during incubation or breaking of the shell (Drent and  
70 Woldendorp, 1989; Graveland, 1996). Eggshell mass and thickness are dependent on calcium  
71 availability and females forage actively on calcium-rich food sources during egg-laying  
72 (Wilkin et al., 2008). Temperature may affect female behaviour or female body condition  
73 and thus eggshell characteristics.

74 Regarding egg composition, it is known that hormones, and in particular androgens,  
75 transferred by females to egg yolk are major modulators of offspring phenotype (Gil, 2008,  
76 2003; Groothuis et al., 2005; Podmokła et al., 2018; Schwabl, 1993). Yolk androgens, and  
77 more precisely testosterone (T), androstenedione (A4) and dihydrotestosterone (DHT),  
78 affect offspring development by increasing chicks' growth rates, begging behaviour and  
79 survival. However, they are also associated with an altered immune response in the chicks of  
80 most species (Gil, 2008; von Engelhardt and Groothuis, 2011). Androgen deposition in the  
81 eggs depends on laying order and also on environmental factors such as food availability,  
82 mate quality or breeding density (Gil, 2008; von Engelhardt and Groothuis, 2011). However,  
83 the influence of temperature on yolk androgens has been less investigated, and the results  
84 are so far inconsistent. In two seabirds, yolk T and A4 levels did not differ between two years  
85 that were characterized by contrasted temperatures (Addison et al., 2008). In great tits, a  
86 study showed an interactive effect of temperature and laying sequence on yolk A4  
87 concentrations (Ruuskanen et al., 2016b). More specifically, at lower than average  
88 temperatures, there was an increase of yolk A4 concentrations with laying sequence, but no

89 pattern at higher than average temperatures (Ruuskanen et al., 2016b). They also showed  
90 that yolk T was positively correlated with temperature during yolk formation (Ruuskanen et  
91 al., 2016b). Yet, in two other studies in great tits, no correlation was found between ambient  
92 temperature and yolk T or A4 levels (Lessells et al., 2016; Remeš, 2011). Finally, Lessells *et al.*  
93 (2016) found a negative correlation between yolk DHT levels and temperature during yolk  
94 formation. Importantly, eggs also contain antimicrobial compounds in the albumen, called  
95 lysozymes. Egg lysozymes are major maternal component of innate immunity. Furthermore,  
96 they are known to enhance hatching success and they may facilitate offspring survival (Saino  
97 et al., 2002). In the barn swallow, egg lysozyme covary positively with temperature  
98 preceding laying (Saino et al., 2004). In the pied flycatcher there was no geographic trend in  
99 lysozyme activity (comparisons of 16 populations) (Ruuskanen et al., 2011). Yet, studies  
100 focusing on the effects of temperature, within and among populations, on lysozyme  
101 concentration are lacking.

102 In great tits and other tits, eggs are white with brown spots caused by protoporphyrin  
103 (Gosler et al., 2005), a pigment which is known to be pro-oxidant (Moreno and Osorno,  
104 2003). This oxidative property has led to opposite hypotheses. On the one hand, it has been  
105 suggested that increased pigmentation could be a signal of oxidative tolerance of females  
106 and thus a signal of good condition (Moreno and Osorno, 2003). On the other hand, it could  
107 indicate that females suffer from physiological stress, as suggested in blue tits by Martínez-  
108 de la Puente *et al.* (2007). Finally, it has also been shown that eggshell coloration is  
109 negatively correlated with eggshell thickness and calcium availability (Gosler et al., 2005;  
110 Sanz and García-Navas, 2009), thus supporting a structural function of pigmentation (Cherry  
111 and Gosler, 2010). More studies on eggshell spottiness are necessary to better understand

112 the correlations between eggshell characteristics and female condition, which may be  
113 affected by temperatures during egg formation.

114 A change in temperature may affect egg characteristics through direct or indirect  
115 mechanisms. First, we can expect direct effects of temperature on breeding females. Higher  
116 ambient temperature provides an environment that is less energetically demanding for  
117 females (reduction of maintenance costs). Females could thus invest more in foraging and  
118 reproduction. Also, another direct effect of temperature could be due to the fact that  
119 temperature may be used as a cue of breeding season advancement. Under the predictive  
120 adaptive response hypothesis, we would expect egg characteristics to be adjusted in order  
121 to accelerate chick development and reduce the risk of mismatch. Second, the effects of  
122 temperature may be indirect as temperature can be correlated with other variables such as  
123 resource availability or habitat quality. In correlative studies, it is not possible to disentangle  
124 direct from indirect effects. Experimental studies are therefore necessary to highlight direct  
125 effects of temperature and to show causal relationship.

126 In this study, we investigated the direct effect of temperature on egg characteristics in a  
127 small passerine bird, the great tit (*Parus major*). We increased night-time temperature in the  
128 nestbox when females were laying eggs and we measured the consequences of this  
129 manipulation on egg characteristics, taking advantage that in this species laying females  
130 roost in their nestbox overnight. This experimental increase in nest temperature overnight  
131 was aimed at mimicking an increase in ambient temperature for females. We already  
132 reported its effects on phenology, chick development and female characteristics (Bleu et al.,  
133 2017). We found a negative effect of the treatment on clutch size in late-laying females  
134 experiencing an increase in nest temperature at night. In addition, the seasonal decline in

135 nestling health found in controls was not detected in nests from heated nestboxes, as  
136 measured by blood sedimentation rate (Bleu et al., 2017). These results suggest that females  
137 used temperature as a cue of seasonal advancement to adjust breeding phenology, and do  
138 not support the reduction of maintenance costs hypothesis (Bleu et al., 2017). The aim of  
139 the current paper is to analyse the characteristics of the eggs collected during this same  
140 experiment. We collected two eggs per clutch: one pre-treatment egg (beginning of the  
141 laying sequence; the treatment started on the day the first egg was laid), and one post-  
142 treatment egg (end of the laying sequence). Control nests monitored any changes of egg  
143 characteristics that may occur with the laying order. More precisely, we investigated egg  
144 morphological characteristics (mass, length, width, eggshell mass, eggshell thickness),  
145 coloration (eggshell pigmentation), and composition (yolk androgen concentration, albumen  
146 lysozyme concentration).

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

## 157 **Material and Methods**



158 Model species and field site

159 During spring 2015, we monitored a population of great tit (*Parus major*, Linnaeus, 1758)  
160 nesting in artificial nestboxes (Schwegler wood concrete nest-boxes 2M, Valliance, Saint  
161 Pierre La Palud, France) near the CEREEP field station (CEREEP-Ecotron Ile-de-France, UMS  
162 3194, École Normale Supérieure, St-Pierre-lès-Nemours). Nestboxes are evenly distributed  
163 within two sites in the Commanderie forest (48°17'N 2°41'E, site 1: 117 nestboxes, site 2:  
164 118 nestboxes, mean distance between the sites = 2 km), and used by great tits and blue tits  
165 (*Cyanistes caeruleus*). For this experiment, we used 82 nestboxes occupied by great tits (58  
166 in site 1 and 24 in site 2) (see also Bleu et al., 2017). In 2015, the earliest laying date for the  
167 first clutch was the 1<sup>st</sup> of April and the last one was the 20<sup>th</sup> of April.

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

174 Experimental treatment

175 The experimental heating treatment is described in details in a different publication focusing  
176 on the effects on phenology and reproductive success (Bleu et al., 2017). In brief, we had 41  
177 control nestboxes and 41 heated nestboxes. Starting on the day the first egg of a clutch was  
178 laid, we placed one hand warmer at the top inside the nestbox every evening, allowing us to  
179 heat the air in the nestbox during the night when females were present, without directly  
180 heating the eggs. Control nestboxes were equipped with used hand warmers that did not

181 produce heat anymore in order to expose females to the same level of disturbance. We used  
182 hand warmers releasing heat when in contact with oxygen during at least 7h (ref. HWES,  
183 Grabber 7+ Hour Hand Warmers). The heating treatment resulted in an increase of 1.1°C  
184 during the night (18h-05h) in heated as compared to control nestboxes (mixed-model with  
185 nestbox as a random effect:  $F_{1,6}=12.70$ ,  $P=0.012$ ), as measured in 8 empty nestboxes (4  
186 controls and 4 heated nestboxes) with iButton temperature loggers (DS1922L, Maxim  
187 integrated ; Bleu et al., 2017).

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

205 Egg characteristics

206 After collection, eggs were immediately weighted on a precision scale ( $\pm 0.001$  g) and stored  
207 at  $-20^{\circ}\text{C}$  until analyses. During summer 2015, eggs were photographed with a calibrated  
208 camera (see Mallard et al., 2013). We took 3 pictures for each egg: a lateral view, a view  
209 from above the pointed end of the egg (i.e. 'foot' sensu Gosler et al., 2005), and a view from  
210 above the flattened area (i.e. 'crown' sensu Gosler et al., 2005). The pictures were analysed  
211 with ImageJ (version 1.48, <http://imagej.nih.gov/ij/>). We determined for each picture, the  
212 total area of the egg, the number of spots on the eggshell and the total area of the eggshell  
213 covered with spots. We then deduced the proportion of the egg covered with spots for each  
214 picture (total area of spots / total area of the egg). For each egg, we thus have 9 measures of  
215 coloration: number, area and proportion of spots for each of the three pictures. After being  
216 photographed, eggs were measured (width and length) with a caliper ( $\pm 0.1$  mm). Then we  
217 opened the egg and separated the yolk and the albumen before full unfreezing, and stored  
218 them back at  $-20^{\circ}\text{C}$ . For the measures of androgens and lysozymes, we discarded eggs for  
219 which we suspected incubation started before collection (i.e. signs that the perivitelline  
220 membrane started to melt, albumen and yolk blending with each other (Biard, pers. obs.); 21  
221 eggs removed). We measured the thickness of unpigmented eggshell at the 'crown' where it  
222 is the thickest (Gosler et al., 2005)) with a micrometre (Mitutoyo, ref. 7-313,  $\pm 0.01$  mm). For  
223 each sample, we took 3 measures and used the mean of these 3 measures for the statistical  
224 analyses (coefficient of variation is 1.5%). Finally, total eggshell was dried at  $60^{\circ}\text{C}$  during 16h  
225 and then eggshell was weighted ( $\pm 0.0001$  g). From the 128 eggs collected, we could  
226 photograph and analyse eggshell spottiness for 126 eggs (60 eggs from control nestboxes  
227 and 66 eggs from heated nestboxes). For the analysis on egg morphology, we kept only the

228 118 eggs with no missing values for egg mass, egg length, egg width, eggshell mass and  
229 eggshell thickness (55 eggs from control nestboxes and 63 eggs from heated nestboxes).

#### 230 Egg content: lysozymes

231 Lysozyme concentration (107 eggs analysed including 51 control and 56 heated eggs) was  
232 measured using a micro-plate modification of the turbidimetric assay (Morosinotto et al.,  
233 2013; Ruuskanen et al., 2011). Shortly, 9,5 µL of albumen was distributed in the wells of a  
234 micro-plate. Each sample was run at least in duplicates (some samples were also repeated  
235 between different micro-plates). We added 9,5 µL of phosphate buffer (Sigma L6876, pH 6.3,  
236 9 g/L) in each well. We then suspended 20 mg of a dried strain of *Micrococcus lysodeikticus*  
237 (Sigma M3770) in 30 mL of phosphate buffer. The amount of *Micrococcus* was adjusted in  
238 order to obtain a solution with an absorbance of 1 at 450 nm (250µL were used to check the  
239 absorbance). Lysozymes from the albumen samples degrade bacterial cell walls, which can  
240 be measured from the absorbance with a micro-plate reader (Bio-rad 680). 250 µl of the  
241 *Micrococcus* solution were added to the wells on the plate and the absorbance was  
242 measured at 450 nm at 26°C after 10 min. We prepared a standard lysozyme solution by  
243 diluting crystalline hen egg white lysozyme (Sigma L6876) in phosphate buffer, which was  
244 serially diluted to obtain a standard curve (12.5 to 200 µg/ml). Two standard curves were  
245 added to the top and bottom row of each plate. Inter-assay variation was 7.7% and intra-  
246 assay variation was 6.1%.

#### 247 Egg content: Androgens

248 We could not retrieve the yolk for 2 eggs, thus we analysed 105 eggs for yolk androgen  
249 concentrations (50 control and 55 heated eggs). Yolk concentrations of T and A4 were  
250 determined by radio-immunoassay (RIA) at the CEBC as previously described (Paquet et al.,

251 2013). Each sample was run in duplicates. Briefly, 100 mg of each sample were homogenized  
252 in 1 mL of distilled water. Steroids were then extracted by adding 3 mL of diethyl-ether to  
253 300  $\mu$ L of the homogenized yolk, by vortexing, and by centrifuging (5 mins, 2000 rpm). The  
254 diethyl-ether phase containing steroids was decanted and separated after snap freezing the  
255 tube with an alcohol bath. The dried extracts were then dissolved in 900  $\mu$ L of phosphate  
256 buffer and each hormone was assayed in duplicate. 100  $\mu$ L of extract were incubated  
257 overnight with 4000 cpm of the appropriate  $^3$ H-steroid (Perkin Elmer, US) and polyclonal  
258 rabbit antiserum. The bound fraction was then separated from free fraction by addition of  
259 dextran-coated charcoal. Finally, activity was counted on a tri-carb 2810 TR scintillation  
260 counter (Perkin Elmer, US). Inter- and intra-assay variations were respectively 7.16% and  
261 7.67% for T, 10.38% and 7.04% for A4. T, A4 lowest detectable concentrations were  
262 respectively 1.26 pg/mg, and 1.06 pg/mg. T and A4 concentrations were highly correlated  
263 (Pearson's product-moment correlation:  $r=0.79$ ,  $t=13.29$ ,  $df=103$ ,  $p<0.001$ ).

#### 264 Statistical analyses

265 We used R version 3.4.2 (R Core Team, 2017). The correlations between the variables  
266 describing egg morphological characteristics and eggshell spottiness are shown in Tables  
267 ESM1 and ESM2 respectively. Because of the high number of variables and of their  
268 correlations, we performed two separate principal component analyses (PCA) with package  
269 "ade4" version 1.7-8 (Dray and Dufour, 2007). For both analyses, variables were first  
270 standardized and we kept the two principal components that explained more than 70% of  
271 the total variation. Then, we analysed the first two principal components (PC1 and PC2) in  
272 separate mixed models (lmer procedure from package "lme4" version 1.1-14 with REML  
273 estimation) (Bates et al., 2015). Nestbox was defined as a random effect to account for the  
274 non-independence of the eggs laid by the same female. We tested the effect of treatment

275 and included the following covariates in all models: study site, laying order, clutch size,  
276 laying date. Laying order was a categorical binary variable, defining eggs as either pre-  
277 treatment eggs (position in the laying sequence 1 to 2.5) or post-treatment eggs (position in  
278 the laying sequence 7 to 11, Table 1). We included clutch size in order to control for the fact  
279 that post-treatment eggs do not have the same position in the laying sequence in all  
280 clutches. We tested the interaction between treatment and laying order, because we expect  
281 an effect of the treatment only on post-treatment eggs. We also tested the three-way  
282 interaction between treatment, laying order and laying date because we showed previously  
283 differential seasonal effects of the treatment (Bleu et al., 2017). Since we tested the three-  
284 way interaction, we also included the two-way interactions between laying order and laying  
285 date, and treatment and laying date. We removed the non-significant interactions step by  
286 step in all the models (all  $P > 0.10$ ). When analysing variation in PC1 for eggshell spottiness,  
287 the model was also run without one outlier (very small value, see Figure ESM2). This did not  
288 change the results (see Table ESM3).

289 To investigate variation in albumen lysozyme and yolk androgens concentrations, we  
290 performed similar mixed models with mean values (samples were run in duplicates). For  
291 lysozymes, 3 outliers were removed (small values) to conform to normality assumptions. The  
292 results are qualitatively similar if we keep all the data (see Table ESM4). For yolk androgens,  
293 data were first log-transformed to conform to normality and homoscedasticity assumptions.  
294 We present type III analysis of variance tables with Satterthwaite approximation for degrees  
295 of freedom (package "lmerTest" version 2.0-33). The estimates are presented as mean  $\pm$   
296 standard error. The assumptions of normality and homoscedasticity of residuals were  
297 fulfilled for all models, except for the model investigating variation in PC1 for eggshell  
298 spottiness. However, the residuals of the model followed a normal distribution when we ran

299 the model without the outlier. Post-hoc tests were computed as differences of least squares  
300 means between the factors (pairwise comparisons).

## 301 **Results**

### 302 Egg morphology

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

### 314 Eggshell spottiness

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

## 322 Lysozyme in the albumen and yolk androgens

323 Lysozyme concentration in the albumen was explained by an interaction between laying  
324 date and laying order (Table 4). This effect was not dependent on the experimental  
325 treatment. Lysozyme concentration decreased with laying date for post-treatment eggs ( $t=-$   
326  $2.41$ ,  $P=0.018$ ,  $df=97$ ) and not for pre-treatment eggs ( $t=0.89$ ,  $P=0.38$ ,  $df=97$ ). In late  
327 clutches, post-treatment eggs have less lysozymes than pre-treatment eggs for a given laying  
328 date (Figure 3A). Similarly, T and A4 concentrations were not affected by the treatment  
329 (Tables 5A and 5B) but there was an effect of laying order and laying date in interaction  
330 (Tables 5A and 5B, Figures 3B and 3C). T concentrations increase with laying date for pre-  
331 treatment eggs ( $t=2.36$ ,  $P=0.020$ ,  $df=96.80$ ) and not for post-treatment eggs ( $t=-0.46$ ,  $P=0.65$ ,  
332  $df=81.61$ ). For A4, the trend is similar but the slopes are not significantly different from zero  
333 (for pre-treatment eggs:  $t=1.37$ ,  $P=0.17$ ,  $df=96.5$  ; for post-treatment eggs:  $t=-0.73$ ,  $P=0.47$ ,  
334  $df=80.06$ ). However, the two slopes differ from each other ( $t=-2.08$ ,  $P=0.044$ ,  $df=41.21$ ). In  
335 both cases, post-treatment eggs have more androgens than pre-treatment eggs for a given  
336 laying date (Figure 3B and C).

## 337 **Discussion**

338 In this study, we increased night-time temperature in the nestbox by ca.  $1^{\circ}\text{C}$  during the egg-  
339 laying period. We measured the effect of this treatment on egg characteristics. The  
340 treatment only affected eggshell thickness. Post-treatment eggs from heated females had a  
341 thinner eggshell than post-treatment eggs from control females. Moreover, we highlighted  
342 effects of the laying order on egg shape, egg mass, eggshell coloration and differential  
343 effects of laying order with advancing season on egg content (yolk androgen concentrations  
344 and lysozyme concentration in the albumen).



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

361           The other variables describing egg characteristics were not affected by the heating  
362 treatment. In particular there was no effect on yolk androgen concentrations. In previous  
363 correlative studies (see introduction), the effects of temperature on androgen hormones  
364 were not clear. Our experimental study suggests that such effects may not be driven by  
365 temperature *per se*, which may explain the discrepancy between studies. But, we cannot  
366 exclude that our increase in temperature was not sufficiently high to trigger an effect.  
367 Interestingly, another experimental study (testing several manipulations of temperature,  
368 with some groups having temperatures that differed of 4 or 5°C) showed no effect of

369 temperature during yolk formation (4 days) or of mean temperature during the whole egg-  
370 laying period on yolk androgens (T and A4) concentrations in great tits (Ruuskanen et al.,  
371 2016b). However, they highlighted a positive effect of temperature within a clutch (effect of  
372 temperature deviation). The effect was positive, meaning that yolk T and A4 concentrations  
373 were higher when temperatures during formation of a particular egg were higher than on  
374 average during egg-laying of the entire clutch (Ruuskanen et al., 2016b). Concerning egg  
375 mass, we also expected a positive effect of the treatment and we did not find any. This lack  
376 of effect corroborates results from a previous experiment in blue tits (Yom-Tov and Wright,  
377 1993, experimental increase of temperature of 6°C). The relationship between egg mass and  
378 temperature found in some correlative studies (e.g. Ojanen, 1983, see also introduction)  
379 may be more likely linked to food availability (Ardia et al., 2006; Ruuskanen et al., 2016a).

380         Finally, we also report interesting patterns of change in egg characteristics with laying  
381 order. Within-clutch changes in egg characteristics could be adaptive and have been  
382 classically explained by two distinct strategies: brood reduction and brood survival strategies  
383 (Lack, 1947; Slagsvold et al., 1984). In the first case, the investment is skewed towards first  
384 laid eggs to facilitate the death of late offspring in case of adverse conditions. In the second  
385 case, the investment increases with the laying sequence to compensate for the negative  
386 effects of hatching asynchrony. We found that post-treatment eggs were heavier (total egg  
387 mass and eggshell mass) and had higher concentration of yolk androgens (especially for early  
388 clutches), which support the brood survival hypothesis. The increase of androgens with the  
389 laying sequence had already been described in great tits (Tschirren et al., 2004), but not  
390 always (Ruuskanen et al., 2016a). For example, a decrease of yolk T was observed in another  
391 wild population of great tits (Ruuskanen et al., 2016a, 2016b). Also, the effect of laying order  
392 can be affected by other variables, such as temperature (e.g. Ruuskanen et al., 2016b for

393 yolk A4), personality (Groothuis et al., 2008 for yolk T) or laying date (this study). It should be  
394 noted that the range of laying dates in our study site is short (20 days). This effect may be  
395 even more pronounced in study population with a larger range of lay dates. The increase in  
396 eggshell mass may indeed represent a change of allocation with the laying sequence (brood  
397 survival hypothesis). However, it is also known that calcium is a limited resource for breeding  
398 passerines (Gosler et al., 2005; Graveland, 1996). Thus, the increase in eggshell mass may  
399 also result from a change of foraging-mediated calcium acquisition (Gosler et al., 2005).  
400 There is no effect of laying date on this pattern so we do not think that calcium-rich prey  
401 availability changes over the short time-scale of the laying sequence. Nevertheless, the  
402 ability of females to forage on calcium-rich preys may improve and this may result in females  
403 producing heavier eggshell at the end of the laying period.

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

415 Egg coloration also changed with laying order. Post-treatment eggs had larger spots  
416 and more proportion of the eggshell covered with spots than pre-treatment eggs, as also

417 reported by others (Gosler et al., 2005). First, we should note that the heating treatment did  
418 not affect this pattern. Thus, within the limits of our manipulation, female condition does  
419 not seem to influence eggshell coloration, contrary to the predictions of the hypotheses that  
420 pigmentation signals oxidative tolerance or physiological stress (Martínez-de la Puente et al.,  
421 2007; Moreno and Osorno, 2003). Also, the experimental treatment did not affect calcium  
422 availability for the females (no effect of the treatment on eggshell mass), thus we cannot  
423 test the structural hypothesis (Gosler et al., 2005). This hypothesis posits that a change of  
424 pigmentation may be due to the use of protoporphyrin in replacement to calcium. It may  
425 seem surprising that eggshell thickness and eggshell coloration were not correlated contrary  
426 to a previous study (Sanz and García-Navas, 2009). However, we did not measure eggshell  
427 thickness at the same position on the egg, and eggshell thickness may be variable within an  
428 egg (Gosler et al., 2005).

429         The results of this study do not support the hypothesis that a slight increase of  
430 temperature during laying allows the females to invest more in their eggs because of a  
431 reduction of their maintenance costs. Females can also use temperature as a cue of the  
432 advancement of the breeding season, and thus accelerate their reproduction in response to  
433 higher temperature to reduce the risk of mismatch. However, yolk androgens, which  
434 accelerate chick development, were not affected by the treatment. We observed a negative  
435 effect of the treatment on eggshell thickness. This would suggest a strategy of brood  
436 reduction, which may fit the hypothesis of temperature being used as a cue of the  
437 advancement of the breeding season (but all the other parameters of maternal investment  
438 were not affected by the treatment). We have also more support for the “cue hypothesis”  
439 than the reduction of maintenance costs hypothesis concerning phenology (see the effects  
440 on clutch size and incubation behaviour in Bleu et al., 2017). However, this reduction in

441 eggshell thickness in heated nestboxes should be interpreted with caution because there is  
442 no difference in eggshell mass (thus the investment in total calcium may not differ). Finally,  
443 we have clear evidence that females adopt a strategy of brood survival, independent of the  
444 heating treatment: the effect of laying order is positive on yolk androgens (and more  
445 pronounced in early clutches), egg mass and eggshell mass. Since laying order affects several  
446 characteristics of eggs, it could be interesting also to test the effects of temperature on first-  
447 laid eggs. More studies are needed to understand sources of variation in pre-natal maternal  
448 effects between populations and environments (Ruuskanen et al., 2011), in particular in the  
449 context of adaptation to rapidly changing environments, in which they may play a crucial  
450 role. The effects of temperature may be difficult to predict because temperature may have  
451 direct physiological consequences but may also be used as a seasonal cue by the organisms,  
452 and trigger differential reproductive strategies.

453 **Data accessibility** Data available from the Dryad Digital Repository (data will be deposited  
454 when the manuscript is accepted for publication).

455 **Conflict of interest** The authors declare that they have no conflict of interest.

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475

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637

638 **Tables**

639

640 **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	

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

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

646

647

648 **Table 2. Analysis of variance for egg morphological characteristics.** The principal  
649 components (PC) are described in the text and in the figures 1 and ESM1. The among-  
650 nestboxes variance of the intercept is 1.17 and 0.51 and the residual variance is 1.24 and  
651 0.54 (for PC1 and PC2 respectively). The full models included the interactions: laying  
652 order\*laying date\*treatment, laying order\*laying date, laying date\*treatment, laying  
653 order\*treatment. Non-significant interactions were removed.

654

A) Egg mass and shape (PC1)			
	DF	F-value	P-value
Laying date	1,61.642	0.19	0.66
Clutch size	1,64.124	1.88	0.17
<b>Study Site</b>	<b>1,63.74</b>	<b>5.40</b>	<b>0.023</b>
Treatment	1,62.062	0.0096	0.92
<b>Laying order</b>	<b>1,56.914</b>	<b>11.74</b>	<b>0.0011</b>
B) Eggshell thickness (PC2)			
	DF	F-value	P-value
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
<b>Treatment x Laying order</b>	<b>1,54.881</b>	<b>5.46</b>	<b>0.023</b>

655

656

657 **Table 3. Analysis of variance for eggshell spottiness.** The principal components (PC) are  
658 described in figures 2 and ESM2. The among-nestboxes variance of the intercept is 0.81 and  
659 0.90 and the residual variance is 3.16 and 0.99 (for PC1 and PC2 respectively). The full  
660 models included the interactions: laying order\*laying date\*treatment, laying order\*laying  
661 date, laying date\*treatment, laying order\*treatment. Non-significant interactions were  
662 removed.

A) Size of the spots (PC1)			
	DF	F-value	P-value
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
<b>Laying order</b>	<b>1,64.213</b>	<b>37.88</b>	<b>&lt;0.0001</b>
B) Number of spots (PC2)			
	DF	F-value	P-value
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
<b>Laying order</b>	<b>1,61.406</b>	<b>19.27</b>	<b>&lt;0.0001</b>

663

664

665 **Table 4. Analysis of variance for lysozyme concentration in the albumen.** The among-  
666 nestboxes variance of the intercept is 0.0 and the residual variance is 224.7. The full models  
667 included the interactions: laying order\*laying date\*treatment, laying order\*laying date,  
668 laying date\*treatment, laying order\*treatment. Non-significant interactions were removed.

	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
<b>Laying order</b>	<b>1,97</b>	<b>4.72</b>	<b>0.032</b>
<b>Laying date x Laying order</b>	<b>1,97</b>	<b>4.96</b>	<b>0.028</b>

669

670

671 **Table 5. Analysis of variance for yolk androgen concentration.** The among-nestboxes  
672 variance of the intercept is 0.043 and 0.060 and the residual variance is 0.039 and 0.050 (for  
673 testosterone and delta4 androstenedione respectively). The full models included the  
674 interactions: laying order\*laying date\*treatment, laying order\*laying date, laying  
675 date\*treatment, laying order\*treatment. Non-significant interactions were removed.

A) log(Testosterone)			
	DF	F-value	P-value
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
<b>Laying order</b>	<b>1,42.892</b>	<b>9.57</b>	<b>0.0035</b>
<b>Laying date x Laying order</b>	<b>1,42.758</b>	<b>7.98</b>	<b>0.0072</b>
B) log(Delta 4 androstenedione)			
	DF	F-value	P-value
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
<b>Laying order</b>	<b>1,41.341</b>	<b>5.66</b>	<b>0.022</b>
<b>Laying date x Laying order</b>	<b>1,41.214</b>	<b>4.34</b>	<b>0.044</b>

676

677 **Figure legends**

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

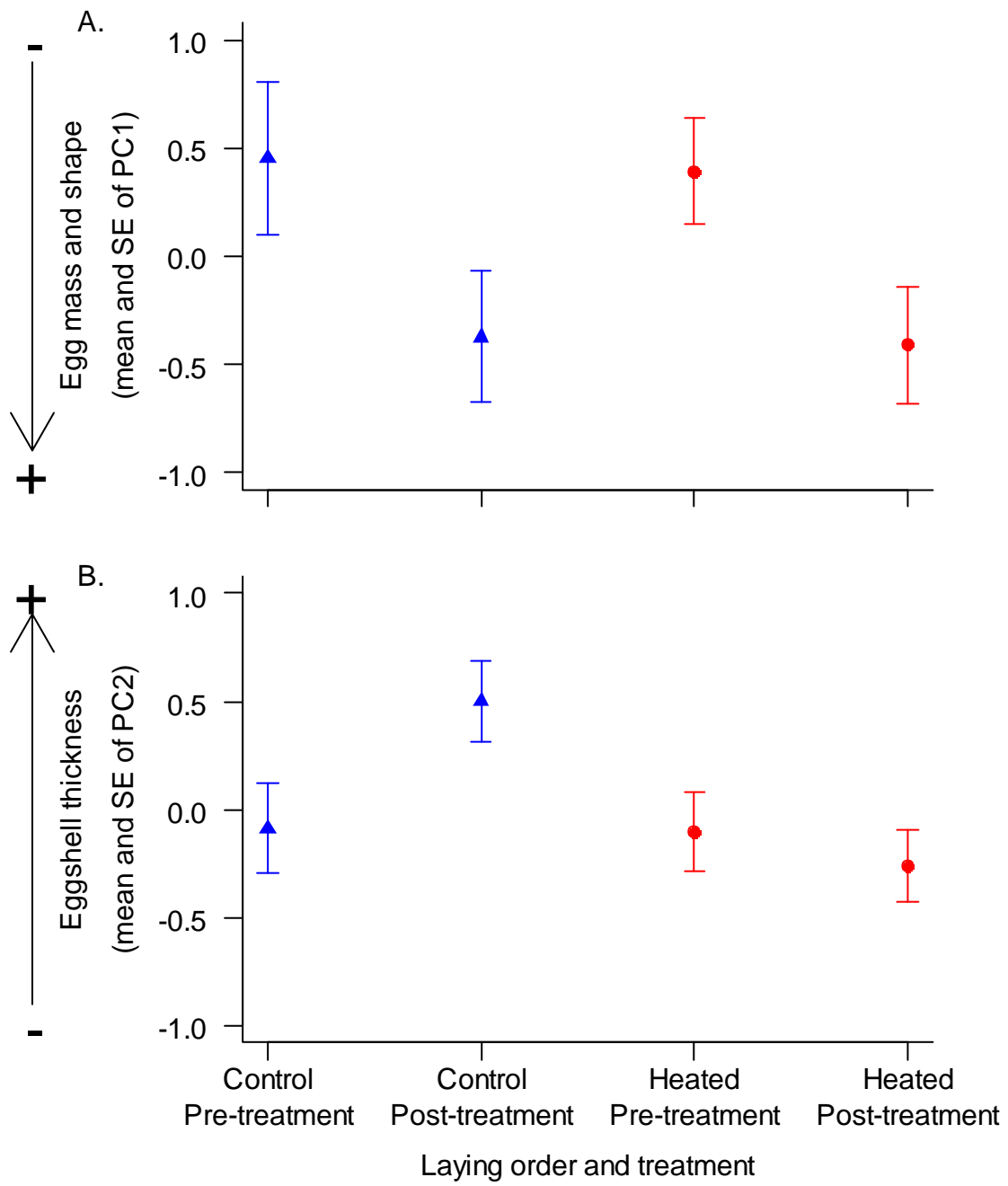
687 **Figure 2 Effect of laying order on eggshell spottiness.** To describe eggshell spottiness we  
688 measured 3 parameters from 3 different pictures (lateral view, crown region and foot  
689 region) of each egg: proportion of the egg covered with spots, total area of the egg covered  
690 with spots,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

695 **Figure 3. Interactive effect of laying order and laying date on lysozyme and yolk androgen**  
696 **concentrations.** We measured lysozyme in the albumen ( $\mu\text{g}/\text{ml}$ , panel A), testosterone (T,  
697 panel B) and delta 4 androstenedione (A4, panel C) in egg yolk ( $\text{pg}/\text{mg}$ ). Lysozyme  
698 concentration in the albumen decreased with laying date for post-treatment eggs only  
699 (slopes are estimates from the model in Table 4, see also the main text for statistics). Yolk  
700 concentration of both androgens increased with laying date for pre-treatment eggs only

701 (slopes are estimates from the models in Table 5, see also the main text for statistics). Dates  
702 are encoded as Julian dates, that is, 100 = 10th of April. Post-treatment eggs are the green  
703 stars (green regression line), pre-treatment eggs are the squares (dashed regression line).

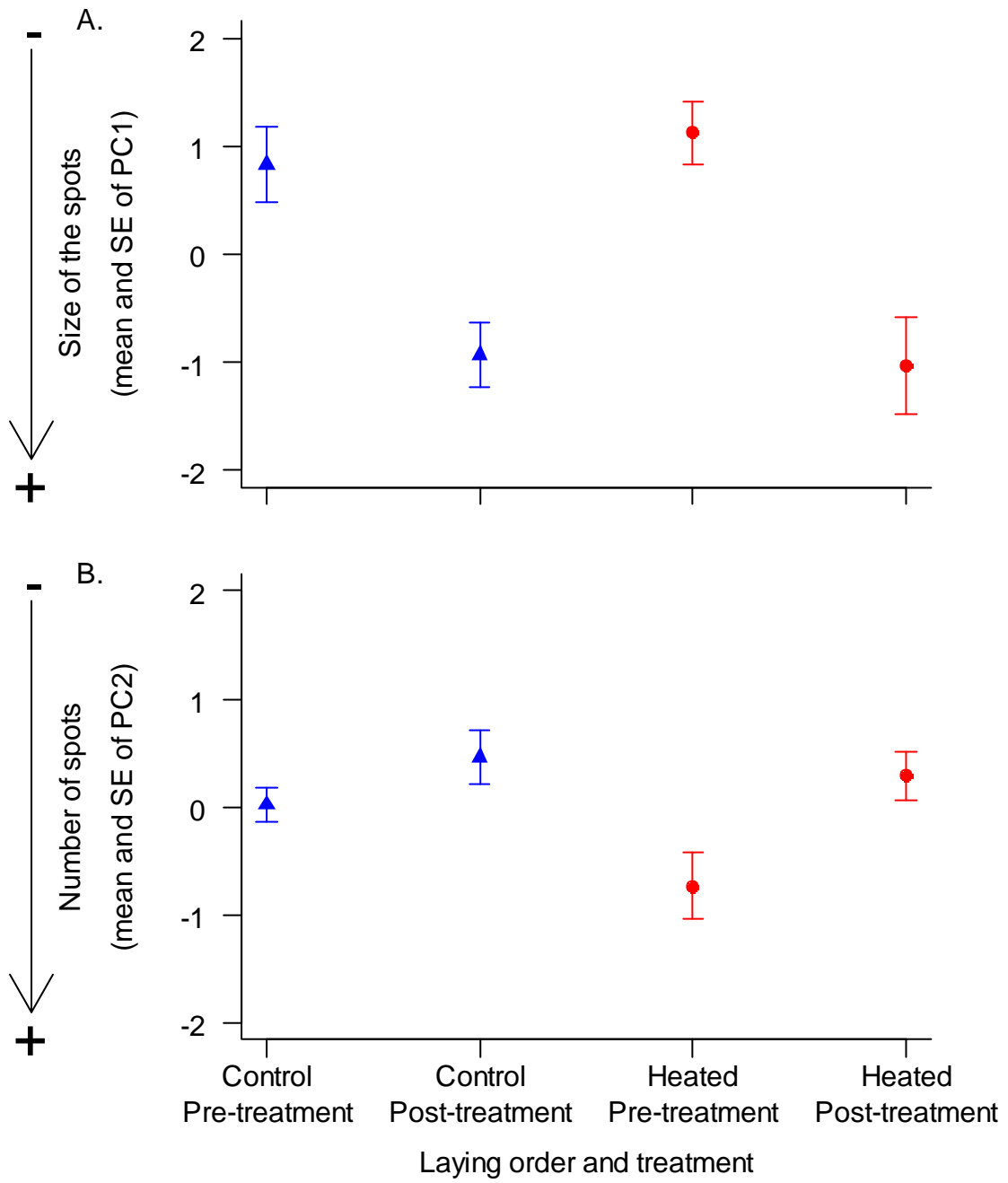


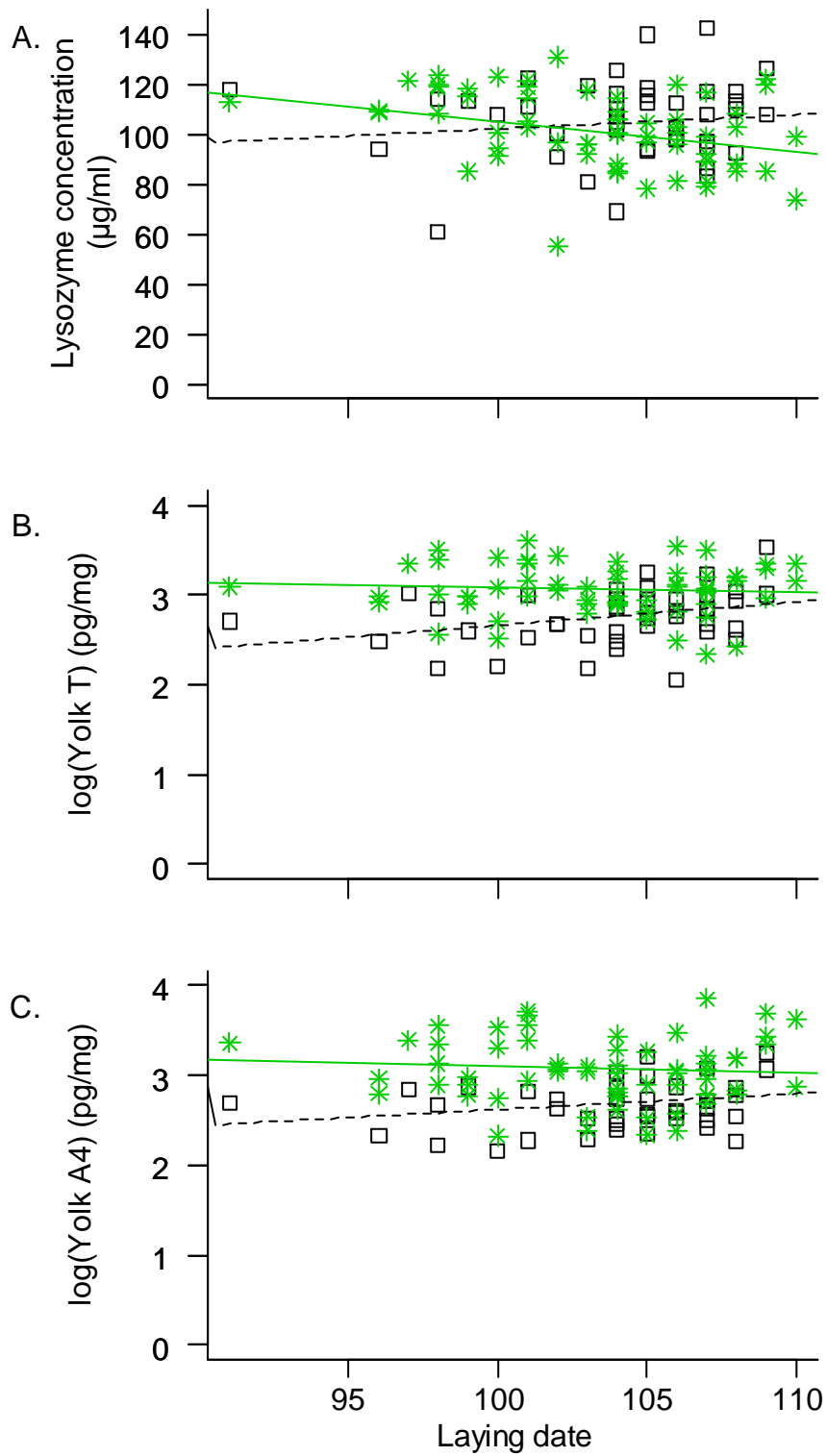
704 **Figure 1**



705

706





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711