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1 **Nest box temperature affects clutch size, incubation initiation and nestling**
2 **health in great tits**

3 Josefa Bleu*^{1,2}, Simon Agostini³, Clotilde Biard¹

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5 ¹ Sorbonne Universités, UPMC, CNRS, INRA, IRD, Université Paris Diderot, Université
6 Paris-Est Créteil, UMR 7618, Institute of Ecology and Environmental Sciences, 75005 Paris,
7 France

8 ² Université de Strasbourg, CNRS, IPHC UMR 7178, F-67000 Strasbourg, France

9 ³ CEREEP-Ecotron Ile-de-France, UMS 3194, École Normale Supérieure, St-Pierre-les-
10 Nemours, France

11
12 *Corresponding author: josefa.bleu@iphc.cnrs.fr

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14
15 **Abbreviated title:** Nest box temperature during egg laying in great tits

16
17 **Lay summary:** Seasonal organisms use temperature as a cue in reproductive decisions, but
18 the role of climate change is not yet clear. Here, an experimental increase in temperature
19 during laying in a small passerine bird reveals that heated females adjusted their incubation
20 behavior and the number of eggs laid, and produced nestlings of equal condition over the
21 season, unlike control females. A small temperature increase may therefore influence
22 breeding strategy and offspring phenotype.

24 **Abstract:** Pre-natal maternal effects can be a source of phenotypic plasticity and may play a
25 role in adaptation to climate change. However, we do not know how far temperature could
26 influence such effects, if at all. We studied the influence of temperature during egg laying on
27 maternal reproductive investment, and on the phenotype of adult females, adult males, and
28 nestlings. We expected temperature to have an effect, as it influences maintenance costs for
29 females, who can also use it as a cue of the advancement of the breeding season. We
30 experimentally increased night-time nest box temperatures by approximately 1°C throughout
31 the entire laying period in great tits (*Parus major*). Clutch size was negatively correlated with
32 laying date in heated females. Heated females did not delay incubation after clutch
33 completion as frequently as control females did. Finally, blood sedimentation rate, which is an
34 indicator of acute infections and inflammatory diseases, was positively correlated with
35 hatching date in control broods. This suggests that nestlings were of lower quality in late-
36 hatched broods than in early-hatched broods. This seasonal effect was not detected in heated
37 nests. Our results show that a small increase in temperature during laying can influence
38 breeding strategy and nestling characteristics. These results suggest that birds used
39 temperature as a cue of seasonal advancement to adjust breeding phenology, with beneficial
40 effects on nestling health. To better understand the consequences of maternal adjustments
41 during egg laying, it would be interesting to combine studies with heating treatment during
42 different periods of the breeding cycle.

43

44 **Key words:** Global warming, Phenological mismatch, Phenotypic plasticity

45 **Introduction**

46 Over the past century, the Earth's climate has warmed and has already caused great ecological
47 changes, including shifts in species ranges and phenology such as earlier timing of flowering
48 and breeding (Walther et al. 2002; Parmesan 2006; Ovaskainen et al. 2013). Micro-evolution
49 and phenotypic plasticity are two mechanisms that can explain population responses to
50 climate change (Visser 2008). One particular form of phenotypic plasticity is the occurrence
51 of maternal effects, whereby the phenotype of a mother or the environment in which she is
52 located influence the phenotype of her offspring (Mousseau and Fox 1998). Maternal effects
53 have been widely studied because they affect the morphology, physiology and behavior of the
54 offspring and thus influence not only offspring fitness but also population dynamics
55 (Mousseau et al. 2009). Maternal effects may therefore be important mechanisms underlying
56 evolution on ecological timescales (Räsänen and Kruuk 2007), yet they have not been studied
57 in detail in the context of adaptation to climate change.

58 Previous studies have shown that for insectivorous passerine birds, climate change can lead to
59 a mismatch between the peak in food availability (caterpillar abundance) and the peak in
60 energetic needs during reproduction, namely for chick feeding (Visser et al. 1998; Visser et al.
61 2004; Visser et al. 2006). This mismatch is due to phenology advancement in response to
62 climate change at different rates for birds and their preys. However, studies have shown that
63 this pattern of response can differ between populations. In fact, the sensitivity of small
64 passerines to the effects of climate change on resource availability for chick feeding is
65 correlated with their ability to fine-tune the start of egg laying and the length of the incubation
66 period (Cresswell and McCleery 2003; Nussey et al. 2005; Charmantier et al. 2008). Maternal
67 effects in response to climate change thus have a considerable impact on the development and
68 fitness of offspring. Still, although numerous studies have described the effect of climate
69 change on phenology, little is known about how climate change affects other prenatal

70 maternal effects. Nevertheless, nest temperature has been shown to be an important factor for
71 reproductive success in several bird species. For example, fledging success in tree swallows is
72 positively correlated to overnight nest box temperature and negatively correlated to daytime
73 temperatures (Ardia 2013), whilst in northern flickers, clutch size, but not fledging success, is
74 positively correlated to mean cavity temperature (Wiebe 2001). Other (mostly correlative)
75 studies reveal that egg size and mass may positively covary with temperature (Christians
76 2002; Saino et al. 2004; Cucco et al. 2009). Also, females and/or nestlings were found to be in
77 better condition (immunity or body condition) in warmer nests in different species such as
78 tree swallows (Pérez et al. 2008; Ardia et al. 2010) or blue tits (Nilsson et al. 2008).

79 We can expect to observe a direct positive effect of an increase in nest temperature on
80 reproductive investment by the female because nests with higher temperatures provide an
81 environment that is less energetically demanding (reduction of maintenance costs). Moreover,
82 temperature may also be used as a cue of breeding season advancement. In this case, we
83 would expect reproductive adjustments that allow hatching to be brought forward in order to
84 reduce the risk of mismatch. This could be achieved by a reduction in clutch size or by an
85 increase in incubation behavior, for example. These hypotheses have mainly been tested with
86 experimental manipulations of nest temperature during incubation (e.g. Nilsson et al. 2008;
87 Pérez et al. 2008; Ardia et al. 2009; Ardia et al. 2010; Vaugoyeau et al. 2016), and
88 experimental studies during the egg-laying period are scarcer (but see Nager and van
89 Noordwijk 1992; Yom-Tov and Wright 1993; Vedder 2012).

90 This study focuses on the effects of temperature on great tits (*Parus major*) during the egg-
91 laying period. Our aim was to investigate the effects of temperature on breeding adults in the
92 light of the hypotheses presented above (maintenance cost, risk of mismatch) by measuring
93 reproductive behavior and adult phenotype. We also investigated the consequences in terms of
94 fitness by measuring reproductive success and the phenotype and growth of nestlings. More

95 precisely, a change of temperature during egg laying can have consequences for the offspring
96 when carry-over effects occur. Indeed, the temperature experienced by the female during egg
97 laying may affect her investment in the egg, potentially leading to changes in egg quality (see
98 Saino et al. 2004) or have carry-over effects on her behavior during the rest of the
99 reproductive season, with consequences such as changes in incubation or parental care
100 behaviors (Vedder 2012). Nest boxes temperature during laying could thus indirectly affect
101 offspring development and characteristics.

102 We conducted an experimental study in order to control for confounding effects (for example,
103 global warming can be associated with a change in food availability). Control nest boxes were
104 left unheated, and the remaining nest boxes were heated during the night because egg-laying
105 great tit females roost in their nest box at night (e.g. Nager and van Noordwijk 1992; personal
106 observation). The temperature treatment started when the first egg was laid to avoid
107 influencing nest box selection or nest building (Nager and van Noordwijk 1992). We
108 compared the phenology and breeding behavior (laying gaps, onset of incubation, incubation
109 date, hatching date) and reproductive success (clutch size, hatching success, fledging success)
110 between heated and control nests. Moreover, we assessed the consequences of an increase in
111 temperature during egg laying on adult phenotype during the nestling feeding phase, shortly
112 before fledging. Finally, we investigated the potential carry-over effects on offspring, namely
113 on growth patterns and nestling phenotype before fledging.

114 We measured morphological and physiological indices reflecting the general condition and
115 health status of the individuals. First, blood sedimentation rate, which indicates the levels of
116 circulating immunoglobulins and fibrinogens, was used as an indicator of acute infections and
117 inflammatory diseases (see Sturkie 1986; Biard et al. 2006). We then measured hematocrit
118 levels, which not only reflect health status (Svensson and Merilä 1996; Hōrak et al. 1998; Fair
119 et al. 2007) but may also be an indicator of water balance, with increased hematocrit levels

120 indicating an increase in evaporative heat loss (Ardia 2013). Third, we measured the
121 proportion of leukocytes in total blood volume, which increases in case of stress or infection
122 (Sturkie 1986). Finally, in adults, we used blood smears to assess the heterophil/lymphocyte
123 (H/L) ratio, which is proportional to the level of glucocorticoid released and can thus be used
124 to estimate stress (Davis et al. 2008). The haematozoan parasite counts resulting from these
125 blood smears were used to estimate infection (Hörak et al. 1998; Bentz et al. 2006). In great
126 tits, a better immunological state and a lower stress is correlated to a higher survival
127 probability (Kilgas et al. 2006).

128 This study tests the following hypotheses: first, according to the hypothesis that the thermal
129 treatment reduced maintenance costs for females, we predicted better physical and
130 physiological conditions for the heated females than control females (with less infection, a
131 lower H/L ratio, a smaller leukocyte buffy coat, a lower sedimentation rate, a higher
132 hematocrit levels and a greater body mass), a greater clutch size, higher reproductive success
133 and nestlings that are in better condition. It should also be noted that higher hematocrit levels
134 may also reflect a lower plasma volume due to evaporative heat loss. If an increase in clutch
135 size occurred, we predicted indirect effects of the thermal treatment on adult males, which
136 attend the female during incubation and participate in chick feeding, and should therefore be
137 in worse physical condition in the heated group than in the control group. Secondly, given the
138 hypothesis that females use temperature as a cue of the advancement of the breeding season to
139 reduce the possible risk of mismatch, we predicted a change in reproductive investment
140 and/or breeding behavior. This change should allow the females to bring the hatching date
141 forward, for example through a reduction in clutch size, and/or an earlier or more efficient
142 incubation through increased nest attentiveness. In the case of a reduction in clutch size, we
143 predicted a better physical condition for both adult males and females due to the lower
144 energetic costs of incubation and chick feeding.

145 **Materials and Methods**

146 Model species and field site

147 During spring 2015, we monitored a population of great tits (*Parus major*) nesting in nest
148 boxes (Schwegler wood concrete nest-boxes 2M, Valliance, Saint Pierre La Palud, France)
149 located near the CEREEP field station (CEREEP-Ecotron Ile-de-France, UMS 3194, École
150 Normale Supérieure, St-Pierre-lès-Nemours). Nest boxes were evenly distributed within two
151 sites in the Commanderie forest (48°17'N 2°41'E, site 1: 117 nest boxes, site 2: 118 nest
152 boxes, mean distance between the sites = 2 km), and were used by great tits and blue tits
153 (*Cyanistes caeruleus*). For this experiment, we used 82 nest boxes occupied by great tits (58
154 on site 1 and 24 on site 2).

155 Great tits are small, insectivorous passerine birds. They produce one or two clutches per year
156 and females usually lay one egg per day, starting full incubation around the time of clutch
157 completion. During egg laying, females typically roost in their nest box at night (Gosler 1993)
158 and may start occasional nocturnal incubation (Vedder 2012). Only females incubate the eggs,
159 and males provide part of their nutritional requirements during incubation by feeding them in
160 the nest (Gosler 1993). In our population, clutches typically hatch within 24h (C.B., personal
161 observation over 6 years).

162 Experimental treatment

163 Nest boxes were checked every day to record the laying date of the first egg. We started the
164 treatment on the day the first egg was laid in each nest box occupied by great tits. The species
165 was identified according to egg mass: eggs weighing more than 1.3 g were assigned to great
166 tits based on data collected during previous reproductive seasons (2010 to 2014) in the same
167 population (mean \pm S.D. egg mass was 1.59 ± 0.12 g and 1.14 ± 0.10 g for great tits and blue
168 tits, $n=205$ and $n=183$ eggs, respectively). Identification of the species was confirmed during

169 incubation and only one blue tit egg had been erroneously identified as that of a great tit. This
170 nest box was initially assigned to the heated group, but was removed from the experiment
171 when the species had been correctly identified. Data from this nest box was excluded from all
172 data analysis. Nest boxes were randomly assigned to the heated or control group (41 nest
173 boxes in each group).

174 The nest boxes were heated using hand warmers that release heat when in contact with
175 oxygen for a minimum of 7h (ref. HWES, Grabber 7+ Hour Hand Warmers). The hand
176 warmer was attached to the ceiling of the nest box, allowing us to heat the air of the nest box
177 during the night when females were present, without directly heating the eggs. Control nest
178 boxes were equipped with used hand warmers that no longer produced heat in order to expose
179 females to the same level of disturbance. Every evening all hand warmers were replaced
180 (between 17.00 and 20.00, with one replacement session lasting until 21.00). The effect of
181 the heating treatment was monitored by recording the temperature every 10 min with iButton
182 temperature loggers (DS1922L, Maxim integrated) positioned at an intermediate height in 8
183 empty nest boxes (4 controls and 4 heated nest boxes). We could not record temperature in
184 occupied nest boxes; the females removed the iButton wherever it was placed, unless it was
185 hidden inside or under the nest, which would not have indicated the air temperature in the nest
186 box. During heating treatment, heated nest boxes temperatures were 1.1°C higher during the
187 night (18.00-05.00) than those recorded in control nest boxes (mixed-model with nest box as a
188 random effect: $F_{1,6}=12.70$, $P=0.012$, see Figure S1). The maximum difference in temperature
189 between heated and control nest boxes did not exceed 3.5°C. During the day (06.00-17.00),
190 no difference was observed in temperature between treatments ($F_{1,6}=2.19$, $P=0.19$).

191 Every evening throughout the period of heating treatment, any new egg laid was numbered
192 with a pencil and the temperature of the eggs was checked after the replacement of the hand
193 warmers. This allowed us to identify the laying sequence and the start of incubation (i.e. when

194 eggs were warm). The heating treatment was stopped when incubation started. We also
195 collected two eggs per clutch at (or near) clutch completion for other analyses that are not
196 presented here.

197 Offspring and adult phenotype

198 For logistical reasons, we monitored offspring body mass growth on site 1 only. Hatchlings
199 were individually marked one day after hatching by the selective clipping of some or all of the
200 down feathers from the 6 feather tracts on the head. Then, every other day until the age of 13
201 days, each nestling was weighed to the nearest 0.10 g (when body mass was below 10 g) or
202 0.25 g with a Pesola spring balance. When nestlings were 7 days old, they were marked with
203 numbered aluminum rings. Nestlings were finally captured in the nest a few days before
204 fledging, at the age of 14 or 15 days (except for 13-day-old birds in one nest box), to obtain
205 morphological measures and blood samples. Adults were trapped while feeding nestlings aged
206 5 days old or more to limit the risk of nest desertion, and they were identified with a
207 numbered aluminum ring, measured and their blood was sampled.

208 For both adults and fledglings, we measured tarsus length to the nearest 0.1 mm with a
209 caliper, and body mass to the nearest 0.25 g with a Pesola spring balance. A blood sample
210 (50–100 μ L) was taken from the brachial vein in heparinized micro-hematocrit tubes. Blood
211 samples were stored in a cooling bag in the field until arrival at the lab, where they were
212 stored at 4°C in an upright position to measure sedimentation after 8 h. All blood samples
213 were then centrifuged for 8 min at 10000 rpm. The lengths of the plasma layer and the red
214 blood cell layer were measured to the nearest 0.5 mm and length of the ‘buffy coat’ layer was
215 measured to the nearest 0.01 mm with a graduated magnifying glass. Sedimentation rate was
216 measured as plasma volume (length of the plasma layer) divided by total blood volume (total
217 length of the tube filled with blood) and hematocrit was measured as the red blood cell
218 volume divided by total blood volume (Svensson and Merilä 1996). In the same way, the

219 proportion of leukocytes in total blood volume was measured as the ratio of the ‘buffy coat’
220 layer to total blood volume (Gustafsson et al. 1994).

221 Thin blood smears were made using a drop of blood obtained from each adult bird after blood
222 sampling. Slides were air-dried and fixed in absolute methanol for 1 min, left to air-dry and
223 then stained with a Giemsa solution (Sigma GS128) for 45 minutes. Blood smears were
224 examined at x 1000 with oil immersion. Parasites were determined on the basis of an
225 examination of 10 000 erythrocytes per smear, and identified to the genus level
226 (Haemoproteus, Leucocytozoon and Plasmodium; Valkiūnas 2004). Due to the low number of
227 blood parasites, we only compared the presence/absence of any of these three genera in the
228 analyses. After scanning for blood parasites, the number of lymphocytes and heterophils were
229 counted on the basis of an examination of a total of 100 leukocytes (heterophils, lymphocytes,
230 eosinophils, basophils, monocytes), and used to calculate the ratio of heterophils to
231 lymphocytes (H/L ratio) (Davis et al. 2008).

232 Statistical analyses

233 A total of 82 nest boxes were observed in the experiment, of which 5 nests were abandoned
234 before incubation, 2 before hatching and 3 before fledging. There is no hatching date for two
235 nests. We captured 51 females and 34 males, and monitored the body mass growth of 296
236 nestlings (8 of which died within 13 days of hatching) from 48 nest boxes on site 1. At
237 fledging, we captured 436 nestlings from 71 nest boxes on both sites. There were no
238 differences in laying date ($F_{1,79} = 0.55$, $P = 0.46$, model including site as a covariate), female
239 morphology (tarsus length: $F_{1,49} = 0.06$, $P = 0.80$, wing length: $F_{1,49} = 0.51$, $P = 0.48$) or
240 male morphology (tarsus length: $F_{1,32} = 0.58$, $P = 0.45$, wing length: $F_{1,32} = 0.61$, $P = 0.44$)
241 between the two groups.

242 We used R version 3.2.3 (R Core Team 2015) for statistical analyses. First, we analyzed the
243 phenology, using linear models (lm procedure) for incubation and hatching dates. Treatment,

244 clutch size (or number of eggs incubated), the interaction between these covariates, and site
245 were used as explanatory variables. We also analyzed binary variables to estimate the
246 probability that females would (i) interrupt the laying sequence, (ii) start incubating before, or
247 (iii) delay the start of incubation after clutch completion (1 or several days of time lag). A
248 generalized linear model (GLM) with a binomial distribution was used for (i) and (ii), and a
249 quasi-binomial for (iii) with treatment, clutch size, site, and laying date as explanatory
250 variables.

251 In a second step, we analyzed components of reproductive success. Clutch size (total number
252 of eggs laid) was modelled with a generalized linear model (GLM procedure) using a quasi-
253 Poisson distribution to account for overdispersion. Hatching success was defined as the
254 number of hatchlings divided by the number of eggs incubated (clutch size - eggs collected),
255 and fledging success by the number of fledglings divided by the number of hatchlings. We
256 used a GLM with a quasi-binomial or binomial distribution, respectively. For these three
257 models, treatment, laying date, the interaction between these covariates, and site were used as
258 explanatory variables.

259 We then compared adult phenotypes (females and males separately) in the two experimental
260 groups. We used a linear model for body mass, sedimentation rate, hematocrit, H/L ratio and
261 leukocytes. Prior to the analyses one outlier was removed from the male dataset for
262 hematocrit, and arcsine square root transformation was applied to ensure that the H/L ratio
263 and leukocyte buffy coat conformed to normality assumption. For H/L ratio in males, as
264 variances were not similar between treatments, we implemented a model with different
265 variances per treatment (option `varIdent`, see Zuur et al. 2009). The presence/absence of blood
266 parasites was modelled using a GLM with binomial (for females) or quasi-binomial (for
267 males) distribution. All of these models tested the effects of treatment, hatching date, brood
268 size, the interactions of the aforementioned variables with treatment (not for males due to

269 small sample sizes), nestling age at capture, site, tarsus length and hematocrit (solely for the
270 sedimentation rate model).

271 Finally, offspring phenotypes were compared between the two experimental groups. We used
272 a non-linear mixed model for offspring body mass growth (NLME procedure and function
273 SSlogis from “nlme” package). Growth was best modelled with a logistic function expressed
274 as: $y_t = A/(1+\exp((I-t)*K))$ where y_t = mass at time t (g), A = asymptotic mass (g), K =
275 growth rate constant (1/day), I = the inflection point of the growth curve (days) and t =
276 nestling age in days (Aldredge 2016). We used random effects to control for the non-
277 independence of nestlings within the same nest box and repeated measurements of individual
278 nestlings at different ages. More precisely, models included nestling identity nested within the
279 nest box as a random intercept. To decide which combination of growth parameters should
280 also be included as random effects, we compared the AIC of the models (Table S1) (see
281 Burnham et al. 2011). The best model included random effects for A and I (Table S1). For the
282 fixed effects, the effect of the treatment was tested on all three parameters of the logistic
283 function. Phenotype at fledging was then analyzed. This was achieved using a linear mixed
284 model (LME procedure from the “nlme” package) for body mass (1 outlier removed), tarsus
285 length (2 outliers removed), wing length, sedimentation rate, hematocrit and leukocyte buffy
286 coat (arcsine square root transformed). The nest box was entered as a random effect to
287 account for similarities between nestlings sharing the same nest. For all those models, we
288 tested the effects of treatment, hatching date, brood size, the interactions of the
289 aforementioned variables with treatment, the site, the age of the nestlings at capture, the tarsus
290 length (when relevant) and hematocrit levels (for the model of sedimentation rate only).

291 We present the full models in the results section. We have not applied a model selection
292 procedure in order to avoid cryptic multiple hypothesis testing and inflation of type I error
293 (Forstmeier and Schielzeth 2011). The estimates of the models are presented with t -statistics

294 and REML estimation for mixed models, t-statistics for linear models or GLM models with
295 overdispersion and z-statistics for GLM models (Zuur et al. 2009). Estimates are given with
296 standard errors, and we used the control group and site 2 as references. The assumptions of
297 normality and homogeneity of variances were fulfilled. The inclusion of the outliers in the
298 analyses did not change the results qualitatively (data not shown).

299 **Results**

300 Phenology, clutch size, incubation behavior and reproductive success

301 The incubation date depended on an interaction between treatment and clutch size (Table 1,
302 Figure 1). Incubation date was positively correlated to clutch size in control females but not in
303 heated females (Table 1, Figure 1). Hatching date did not significantly differ among treatment
304 groups (Table 1). Clutch size was negatively correlated to laying date in heated females but
305 not in control females (interaction between treatment and laying date, Table 2, Figure 2), i.e.,
306 females heated at night stopped laying with smaller clutches compared to control females
307 when the season advanced. Increasing the temperature in the nest box at night did not affect
308 the probability that the female would interrupt the laying sequence (Table 3). Although
309 temperature treatment did not significantly affect the probability of a female starting a full
310 incubation before clutch completion (Table 3), it did affect the probability of a female
311 delaying the onset of full incubation after clutch completion (Table 3). Control females
312 delayed the onset of incubation more often than heated females (Table 3, 23% of control
313 females and 11% of heated females). Hatching and fledging success did not significantly
314 differ among treatment groups (Table 2). Mean \pm s.e. hatching and fledging success were 0.89
315 \pm 0.02 and 0.96 \pm 0.02 for heated females, and 0.88 \pm 0.03 and 0.96 \pm 0.01 for control
316 females, respectively.

317 We also highlighted the effects of some covariates. There were significant differences
318 between the two sites for clutch size, incubation date and hatching date (Tables 1 and 2).
319 Incubation and hatching started earlier (Table 1) and females laid smaller clutches (9.82 ± 0.24
320 vs. 8.98 ± 0.18 eggs) in the study site 1 compared to the study site 2. The probability of an
321 interruption in the laying sequence decreased with clutch size (Table 3). The probability of
322 starting a full incubation before clutch completion increased with clutch size and laying date,
323 whilst the contrary was true for the probability of delaying the onset of incubation (Table 3).
324 Finally, hatching success and fledging success were not influenced by any of the covariates
325 (Table 2).

326 Adult phenotype

327 None of the variables used to describe adult body condition and health were affected by the
328 treatment (Tables S2a and S2b). In females, body mass was positively correlated with tarsus
329 length and negatively correlated with the age of nestlings at capture (Table S2a).
330 Sedimentation rate was negatively correlated to tarsus length and to hematocrit levels (Table
331 S2a). Finally, the presence of blood parasites, hematocrit, leukocytes and H/L ratio were not
332 correlated to any of the tested variables (Table S2a). In males, the presence of blood parasites,
333 sedimentation rate, H/L ratio and hematocrit were not correlated to any of the tested variables
334 (Table S2b). Body mass tended to be positively correlated with tarsus length. There was also a
335 positive trend between leukocytes and brood size (Table S2b).

336 Nestling growth and phenotype

337 Concerning body mass growth, there was no significant effect of the treatment on the
338 parameters of the logistic growth curve (parameter A: $t = 0.39$, $P = 0.70$; parameter K: $t = -$
339 0.66 , $P = 0.51$; parameter I: $t = 0.90$, $P = 0.37$). Nestlings of the same age had similar body
340 mass in both treatments (Figure S2).

341 Shortly before fledging, nestlings from the two treatment groups did not significantly differ in
342 body size (tarsus and wing length) or mass, nor did they differ for hematocrit values or
343 relative proportion of circulating leukocytes (Table S2c). However, the temperature treatment
344 during egg laying had an effect on the sedimentation rate of nestlings. Indeed, the variation in
345 sedimentation rate was explained by an interaction between hatching date and treatment
346 (Table 4). There was a positive correlation between hatching date and sedimentation for
347 control nestlings, whereas no correlation was found for heated nestlings (Table 4 and Figure
348 3). Sedimentation rate was also significantly and negatively related to hematocrit levels and
349 was marginally negatively related to tarsus length (Table 4). Moreover, at fledging, tarsus
350 length, wing length and hematocrit were negatively correlated to hatching date (Table S2c).
351 Wing length was positively correlated to the age at capture of the nestlings (Table S2c). Body
352 mass was negatively correlated to brood size, and site 2 nestlings were heavier than those
353 from site 1 (Table S2c). Site 2 nestlings had a greater proportion of circulating leukocytes
354 than those from site 1 (Table S2c).

355

356 **Discussion**

357 In this study of great tits, we analyzed the effects of an increase of the nest box temperature
358 during egg laying on reproductive phenology and success and on the characteristics of
359 nestlings and adults at the end of the breeding season. We experimentally increased the
360 temperature by approximately 1°C during the night over the egg-laying period. The main
361 differences between heated and control nests were a change in clutch size and in the
362 sedimentation rate of nestlings, both according to the timing in the season, and also a change
363 in the incubation behavior of the female.

364 Breeding success and phenology

365 We observed a negative effect of increased temperature during laying on the clutch size of
366 great tits that started laying eggs late in the season. This effect is in contradiction with the
367 hypothesis of a positive effect of the heating treatment on the energetic budget of the female
368 (reduction of maintenance cost). Indeed, we expected the better thermal properties of the
369 heated nest boxes to allow the females to save energy (O'Connor 1978), leading females to
370 invest more in reproduction, and particularly in clutch size (Yom-Tov and Hilborn 1981). Our
371 results do not support this hypothesis. Moreover, previous experimental studies did not find
372 any effect of heating during laying on clutch size in great tits and blue tits (Nager and van
373 Noordwijk 1992; Yom-Tov and Wright 1993; Vedder 2012).

374 In wild birds, and in particular in great tits, a decline in clutch size as the season progresses is
375 common (Perrins and McCleery 1989; Garant et al. 2007). The absence of a significant
376 correlation between laying date and clutch size in control females may therefore seem
377 surprising. However, it has also been shown that laying date does not always affect clutch size
378 (Wawrzyniak et al. 2015), and also that the laying date effect can be dependent on where the
379 population is located (Winkler et al. 2014), or the breeding year (Gladalski et al. 2015). This
380 experiment was performed in spring 2015, which was not a typical breeding season in our
381 population. The breeding season started later than usual and was very intense: almost all the
382 females started laying within a 2-week interval, and only 5 second clutches were observed.
383 This could explain the absence of a laying date effect on clutch size for control females.

384 Nevertheless, we observed a negative effect of laying date on clutch size for heated females.
385 In great tits, there is a positive selection for both early laying and increased clutch size
386 (Garant et al. 2007). In fact, there is evidence that environment is more favorable early in the
387 season and that late-laying birds are of lower quality than early-laying birds (Verhulst and
388 Nilsson 2008). We can thus hypothesize that the treatment only had an effect late in the
389 season because low quality birds may be more sensitive to a change in environmental

390 conditions than high quality birds. Moreover, birds use temperature (or an increase therein) as
391 a cue to adjust their laying date to achieve better synchrony with peak food abundance (e.g.
392 Charmantier et al. 2008; Visser et al. 2009; Schaper et al. 2012; Caro et al. 2013). There is
393 also evidence that birds can continue this adjustment throughout the laying and incubation
394 periods (Cresswell and McCleery 2003; Vedder 2012). This could imply that late-laying
395 heated birds may have adopted a strategy of clutch size reduction to limit the putative
396 mismatch between food requirements during chick feeding and food abundance. This result
397 supports the hypothesis of a reduction of the risk of mismatch.

398 As adjustments may not be limited to clutch size, we also compared the laying and incubation
399 behavior of heated and control females. The onset of daily continuous incubation by the
400 females was only dependent on clutch size in the control group. This result is a logical
401 consequence of the treatment effect on clutch size. Also, gaps in the laying sequence were
402 rare and were not affected by the treatment. This was reported by Vedder (2012), but not by
403 Yom-Tov and Wright (1993) or Matthysen et al. (2011) (correlational study). We did not
404 observe any difference in the probability that females would begin full incubation before
405 clutch completion in females in either group, thus we do not expect to see greater hatching
406 asynchrony in our heated group contrary to that observed in another study in blue tits (Vedder
407 2012), (but see also Podlas and Richner 2013). However, control females delayed full
408 incubation for one or several days after clutch completion more frequently than heated
409 females did. This result is in line with the hypothesis that heated females used temperature as
410 a cue of breeding season advancement. Finally, the heating treatment did not directly
411 influence hatching success and fledging success. It would have been interesting to know
412 whether the unhatched eggs were more often the first-laid or last-laid eggs, and if the effect of
413 laying order on egg hatchability was linked to the temperature treatment. However, this
414 statistical comparison was not possible given the high hatching success.

415 Nestling and adult phenotypes

416 Contrary to the results of similar studies during incubation (see introduction), the
417 morphological and physiological characteristics of the adults were not affected by the
418 treatment during egg laying in our study. Again this result does not support the hypothesis
419 that heating reduced maintenance costs for females, and suggests that reproductive
420 adjustments (change in clutch size, incubation behavior) were not costly for the adults. This
421 result may also indicate that the adults did not significantly change their parental care
422 behavior. Indeed, we did not measure directly parental care but rather measured adult
423 physiological and morphological characteristics that could reflect their investment, namely
424 indicators of energetic expenditure (body mass), health status (immunological indices and
425 blood parasites load) and stress (H/L ratio). No inter-group differences were observed in these
426 characteristics. As far as females are concerned, it should however be noted that any change
427 during the egg-laying period could have been compensated for during the incubation or chick-
428 rearing period, thus making them undetectable at the time of capture.

429 The morphological characteristics of nestlings were not affected by the treatment, but certain
430 physiological characteristics differed between heated and control group nestlings. We could
431 expect the thermal treatment during laying to affect nestling phenotype and physiological
432 status if it affected (i) egg quality, and/or (ii) female behavior during incubation and parental
433 care, and/or (iii) the environment of the nest box.

434 First, we will consider a potential effect on egg quality. Egg formation lasts approximately 4
435 days (Perrins 1996). We could therefore expect the treatment to have an effect on the eggs
436 laid at the end of the laying sequence and on the nestlings resulting from these eggs. This
437 hypothesis is confirmed by one of the rare previous experimental studies on great tits, which
438 showed that temperature can affect egg volume (Nager and van Noordwijk 1992), which can
439 in turn affect the body mass or size of the nestlings (Williams 1994). Hatching was highly

440 synchronous, making it impossible to determine which nestling hatched from which egg. The
441 subsequent comparison of nestling characteristics according to laying order was therefore
442 impossible. This may partly explain why so few detectable differences were observed
443 between nestlings from control and heated nests. However, one such difference was observed
444 in nestlings from broods hatching late in the season: the sedimentation rate of nestlings was
445 higher in late-hatched than in early-hatched broods of control nests, indicating that nestling
446 physiological status decreased as the season progressed. No such correlation was found for
447 nestlings in heated nests. We may therefore hypothesize that the reduction in clutch size by
448 heated females late in the season allowed the heated females to raise healthier nestlings than
449 those of control females.

450 Parental care behavior may have been affected by the treatment. During incubation, thermal
451 treatment can have a direct effect on the duration and the regularity of female incubation (see
452 Ardia et al. 2009; Álvarez and Barba 2014). Moreover, a study by Vedder (2012) shows that
453 even if thermal treatment is carried out solely during the laying period, it can still influence
454 female incubation behavior, with the treatment resulting in an increase in nocturnal incubation
455 prior to clutch completion. It is also important to note that parental chick-feeding strategies
456 are flexible in many bird species, and male birds could have adjusted their investment in
457 response to female behavior (e.g. David et al. 2015). However, as discussed above, our
458 indirect measures of investment in parental care did not detect such effects in adults.

459 The quality of the environment provided by the nest box may have been modified by the
460 treatment. More specifically, warmer conditions may be more favorable to the development of
461 parasites such as biting flies (Martínez de la Puente et al. 2010). However, we do not have any
462 evidence that warmer nests would have been less suitable: immunological indices do not
463 suggest an increase in infection rates in nestlings or adult birds, and there was no difference in
464 the prevalence of ectoparasites between nests from the two treatment groups (data not shown).

465 In fact, we detected a positive effect for nestlings from heated nests (see above). Warmer
466 nests could also induce higher evaporative heat loss, possibly resulting in lower plasma
467 volume as suggested by Ardia (2013) or increased water loss in the eggs before the onset of
468 incubation (Booth and Rahn 1990). In our study, however, no increase was observed in
469 hematocrit in females, nor did we observe a lower hatching success for eggs in heated nests.
470 Thus, our results do not support the hypothesis that an increase in nest temperature during
471 laying could have negative effects on females or nestlings due to parasites or overheating.
472 Finally, the effects of increasing the temperature in the nest box at night during egg laying
473 probably vary according to the magnitude of the treatment, which was moderate in this study.
474 Effects may also depend on the magnitude of the difference between internal and external
475 temperatures, resulting in different effects in different breeding years, and potentially more
476 detectable effects during colder spring periods.

477 Conclusion and perspectives

478 Our results show that a small increase in ambient temperature during egg laying can influence
479 clutch size, incubation behavior and nestling phenotype. Clutch size decreased with laying
480 date in heated females, which does not support the hypothesis that reduced maintenance costs
481 enable females to lay more eggs. This result rather suggests that females use temperature as a
482 cue to assess the advancement of the breeding season not only before, but also during egg
483 laying. We detected some carry-over effects on nestling sedimentation rate, indicating that the
484 negative seasonal effect on nestling physiological status was alleviated by increasing nest
485 temperature during egg laying. These changes did not have any detectable costs or benefits for
486 adults, nor did they influence hatching or fledging success. It would now be interesting to
487 study the combined effects of temperature increase at different periods of the breeding season,
488 with a view to assess whether the responses measured are adaptive.

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502 The authors declare that there is no conflict of interest. This study was conducted in full
503 compliance with French laws and regulation, including recommendations for ethical treatment
504 of experimental animals.

505

506 **Data accessibility**

507 Analyses reported in this article can be reproduced using the data provided by Bleu et al.
508 (2017).

509

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- 641

642 **Figure legends**

643

644 **Figure 1. Incubation date as a function of clutch size and female heating treatment in**

645 **great tits.** Females from heated nest boxes are represented with red triangles and those from
646 control nest boxes with blue circles. Symbol size is proportional to sample size (from 1 to 4
647 for larger symbols). Slopes are estimates from the linear model presented in Table 1. For
648 heated females, there was no correlation between clutch size and laying date ($t_{1,72} = -0.45$,
649 $P = 0.66$), and a positive correlation was found for control females, ($t_{1,72} = 2.84$, $P = 0.0059$).
650 Dates are encoded as Julian dates, i.e. 100 = 10th of April.

651 **Figure 2. Clutch size as a function of laying date and female heating treatment in great**

652 **tits.** Heating treatment started on the day the first egg was laid (i.e. laying date). Females from
653 heated nest boxes are represented with red triangles and those from control nest boxes with
654 blue circles. Symbol size is proportional to sample size (from 1 to 3 for larger symbols).
655 Slopes are estimates from the generalized linear model presented in Table 2. For heated
656 females, there was a negative correlation between clutch size and laying date ($t_{1,72} = -2.55$,
657 $P = 0.013$), but not for control females ($t_{1,72} = 0.89$, $P = 0.38$). Results are qualitatively similar
658 when data for the female from the control nest box with an early laying date is excluded from
659 analysis (effect of the treatment: $t_{1,71} = 2.22$, $P = 0.029$; slope for heated group: $t_{1,71} = -2.53$,
660 $P = 0.014$; slope for control group: $t_{1,71} = 0.54$, $P = 0.59$). Dates are encoded as Julian dates,
661 i.e. 100 = 10th of April.

662 **Figure 3. Nestling red blood cell sedimentation rate as a function of hatching date and**

663 **female treatment.** Nestlings were blood sampled before fledging (at 14 or 15 days old), and
664 sedimentation rate was determined as the volume of plasma relative to total blood volume
665 approximately 8h after blood sampling. Nestlings from heated nest boxes are represented with
666 red triangles and those from control nest boxes with blue circles. The slopes are the estimates

667 from the statistical linear mixed model presented in Table 4 (with nest box as the random
668 effect). For heated nestlings, there is no significant correlation between the sedimentation rate
669 and the hatching date ($t_{1,60} = -0.62$, $P = 0.53$), but there is a positive correlation for control
670 nestlings ($t_{1,60} = 2.63$, $P = 0.011$). Results are qualitatively similar when data for the two
671 nestlings from the control nest box with an early hatching date are excluded from analysis
672 (effect of the treatment: $t = -2.06$, $P = 0.044$; slope for heated group: $t_{1,59} = -0.61$, $P = 0.54$; slope
673 for control group: $t_{1,59} = 2.36$, $P = 0.022$). Dates are encoded as Julian dates, i.e. 100 = 10th of
674 April.

675

676 **Tables**

677 **Table 1. Statistical models of breeding phenology.** Linear models were used, and all the
 678 tested variables are presented. Clutch size is the total number of eggs laid in the incubation
 679 date model, and the total number of eggs incubated in the hatching date model (see Methods
 680 section for more details). We used the control treatment (TRT) and site 2 as references for the
 681 estimations. Dates are encoded as Julian dates, i.e. 100 = 10th of April.

	Incubation date				Hatching date			
	Estimate	SE	t-value	<i>P</i>	Estimate	SE	t-value	<i>P</i>
Intercept	102.25	3.97	25.76	<0.0001 ***	117.81	3.91	30.16	<0.0001 ***
TRT:heated	12.54	4.97	2.52	0.014 *	6.73	4.76	1.41	0.16
Clutch size	1.14	0.40	2.84	0.0059 **	0.99	0.50	2.00	0.050 .
Site:1	-1.89	0.81	-2.32	0.023 *	-2.00	0.91	-2.21	0.030 *
TRT:heated x Clutch size	-1.31	0.53	-2.45	0.017 *	-0.87	0.65	-1.35	0.18

682 . P<0.10 * P<0.05 ** P < 0.01 *** P <0.001

683

684 **Table 2. Statistical models of reproductive success.** Clutch size is the total number of eggs laid, hatching success is the number of hatchlings
685 divided by the number of eggs incubated, and fledging success is the number of fledglings divided by the number of hatchlings. We used a
686 generalized linear model with a quasi-Poisson distribution (clutch size), a quasi-binomial (hatching success) or a binomial distribution (fledging
687 success). All the tested variables are presented in the table. We used the control treatment (TRT) and site 2 as references for the estimations.

688

	Clutch size				Hatching success				Fledging success			
	Estimate	SE	t-value	<i>P</i>	Estimate	SE	t-value	<i>P</i>	Estimate	SE	z-value	<i>P</i>
Intercept	1.81	0.56	3.22	0.0019 **	10.79	6.88	1.57	0.12	1.26	8.66	0.15	0.88
TRT:heated	2.13	0.85	2.52	0.014 *	-15.12	9.98	-1.52	0.13	17.56	14.74	1.19	0.23
Site:1	-0.10	0.035	-2.91	0.0048 **	-0.26	0.41	-0.62	0.54	0.42	0.50	0.84	0.40
Laying date	0.0048	0.0054	0.89	0.38	-0.083	0.065	-1.27	0.21	0.017	0.083	0.20	0.84
TRT:heated x Laying date	-0.021	0.0082	-2.56	0.013 *	0.15	0.096	1.53	0.13	-0.17	0.14	-1.20	0.23

689 . *P*<0.10 * *P*<0.05 ** *P* < 0.01 *** *P* <0.001

690 **Table 3. Statistical models of breeding behavior.** We modelled the probability that females would interrupt egg-laying, begin full (i.e. day and
691 night) incubation before clutch completion, or delay the onset of full incubation after clutch completion (1 or several days of time lag). We used
692 generalized linear models with a binomial distribution or a quasi-binomial distribution for the last model. We used the control treatment (TRT)
693 and site 2 as references for the estimations.

	Interruption in the laying sequence				Early start of incubation				Late start of incubation			
	Estimate	SE	z-value	<i>P</i>	Estimate	SE	z-value	<i>P</i>	Estimate	SE	t-value	<i>P</i>
Intercept	12.14	8.77	1.39	0.17	-48.41	12.88	-3.76	0.00017 ***	82.04	17.27	4.75	<0.0001 ***
TRT:heated	0.12	0.58	0.21	0.84	0.50	0.58	0.86	0.39	-1.72	0.71	-2.41	0.018 *
Clutch size	-0.45	0.25	-1.82	0.069 .	0.93	0.29	3.25	0.0012 **	-1.04	0.33	-3.13	0.0025 **
Site:1	0.17	0.77	0.21	0.83	0.59	0.67	0.88	0.38	0.66	1.05	0.63	0.53
Laying date	-0.092	0.076	-1.21	0.23	0.37	0.11	3.48	0.00051 ***	-0.73	0.15	-4.77	<0.0001 ***

694 . P<0.10 * P<0.05 ** P < 0.01 *** P <0.001

695

696 **Table 4. Statistical model of offspring blood sedimentation rate.** We used a linear mixed
 697 effect model (with nest box as the random effect). We used the control treatment (TRT) and
 698 site 2 as references for the estimations.

699

	Estimate	SE	t-value	P
Intercept	-0.20	0.41	-0.49	0.63
TRT:heated	0.97	0.45	2.14	0.036 *
Tarsus length	-0.012	0.007	-1.66	0.097 .
Age of nestlings	0.010	0.016	0.64	0.53
Site:1	0.011	0.014	0.79	0.43
Hatching date	0.0060	0.0023	2.63	0.011 *
Brood size	0.0062	0.0073	0.84	0.40
TRT:heated x Hatching date	-0.0079	0.0037	-2.14	0.036 *
TRT:heated x Brood size	0.00083	0.0093	0.089	0.93
Hematocrit	-0.39	0.083	-4.75	<0.0001 ***

700 . P<0.10 * P<0.05 ** P < 0.01 *** P <0.001