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2 **Prenatal maternal effects appear to be insensitive to experimental
or natural environmental variation**

Caroline E. Thomson

4 Department of Zoology, Edward Grey Institute, University of Oxford, Oxford, OX1 3PS,
United Kingdom

6 `caroline.thomson@zoo.ox.ac.uk`

Jarrold D. Hadfield

8 Institute of Evolutionary Biology, University of Edinburgh, UK

`j.hadfield@ed.ac.uk`

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Correspondence: Caroline Thomson

Abstract

12 1. In many birds, hatching asynchrony is a common phenomenon, primarily driven
by patterns of incubation behaviour. However, experimental results in blue tits (*Cyanistes*
14 *caeruleus*) have shown that asynchrony is reduced by intrinsic properties of later eggs that
accelerate pre-natal development.

16 2. These intrinsic differences between early and late eggs could be driven by changes in
resource availability to females, which are then passively passed onto the egg. Alternatively,
18 it may be due to an anticipatory maternal effect, wherein some signal or resource is actively
placed within the egg, which is beneficial to those eggs laid late within the clutch.

20 3. In order to distinguish between these hypotheses we designed a supplementary
feeding experiment, wherein females were provided with food at certain times during
22 the laying phase. This had no discernible effect on development rate, or other egg
characteristics, consistent with anticipatory maternal effects.

24 4. Using a larger data set we also tested whether natural environmental variation
(weather) during egg formation affected maternal investment in eggs. Similarly, egg
26 characteristics were found to be relatively insensitive to the environmental variation,
supporting the experimental results.

28 **Keywords:** anticipatory maternal effect, *Cyanistes caeruleus*, development, eggs, food
supplementation, passive effects, weather

Introduction

30

Parents influence the phenotypes of their offspring, both through the genes they pass
32 on, and by directly modulating the environment offspring experience. These parental

effects have been shown to be present in many systems, and can have a major causal
34 contribution to an individual’s phenotype (Roach & Wulff 1987; Mousseau & Dingle 1991;
Mousseau & Fox 1998; Badyaev & Uller 2009; Wolf & Wade 2009). The effects are often
36 context-dependent – varying with the environment that the parents themselves experience
(Rossiter 1996, 1998). This could occur through the direct response of a parent to changes
38 in their own environment, which are then passed to offspring, regardless of the phenotypic
or fitness consequences of these effects to the offspring. Alternatively, parents may use
40 changes in their own environment to anticipate that which their offspring will experience,
and actively modulate their offsprings’ phenotypes accordingly (Marshall & Uller 2007).
42 Although the context-dependency of parental effects is unequivocal, whether anticipatory
responses are a common feature of context-dependency remains contentious (Uller *et al.*
44 2013; Burgess & Marshall 2014). Anticipatory parental effects (Marshall & Uller 2007)
are expected to occur when the environment experienced by parents is a predictor of the
46 environment that will be experienced by the offspring (Burgess & Marshall 2014), and
selection therefore favours parents producing offspring of an appropriate phenotype for
48 that environment. Whilst there has been some convincing evidence for such effects, such
as adaptation to maternal light environments (Galloway & Etterson 2007; Galloway 2005),
50 and transgenerational induction of defences in plants and animals (Agrawal *et al.* 1999), a
recent meta-analysis concluded that anticipatory effects were not widespread (Uller *et al.*
52 2013): In experiments that subjected parents and their offspring to two environments
in a fully factorial design, there was only weak evidence that offspring do better when
54 environments are matched rather than mismatched (Uller *et al.* 2013). Although at face
value this suggests that evidence for anticipatory parental effects is limited, studies such as
56 those included in the meta-analysis are open to the criticism that the environment parents
are subject to is not necessarily a good predictor of the environment offspring would have
58 experienced under natural conditions (Uller *et al.* 2013; Burgess & Marshall 2014)

In species that experience age-related sibling competition, anticipatory parental effects
60 are hypothesised to evolve in order to mediate these age-related effects, and may be related
to changes in the size and composition of eggs. Unlike those studies reviewed in Uller *et al.*
62 (2013), parents are assumed to know (rather than predict) where offspring from specific
eggs will be placed in any age hierarchy, and thus the competitive environment they may
64 experience. As a consequence, anticipatory parental effects may more easily evolve. For
example, Plaistow *et al.* (2007) found that female spider mites increase the size of their eggs
66 as they age, and attributed this to female anticipation of the level of sibling competition
later laid eggs will encounter. In birds, egg size (Slagsvold *et al.* 1984) and many egg
68 constituents have been shown to vary, both between females and across the laying sequence
of individuals (Williams 2012). These include carotenoids (Royle *et al.* 2001; Blount *et al.*
70 2002; Török *et al.* 2007; Saino *et al.* 2002), vitamin E (Royle *et al.* 2001) and hormones
(Gil 2008). Whilst egg size has downstream consequences on juvenile traits (Krist 2011),
72 hormonal changes in particular have been invoked as mediators of anticipatory parental
effects (e.g. Schwabl 1993; Schwabl *et al.* 1997; Lipar *et al.* 1999; Muller & Groothuis 2013),
74 and have also been shown to have downstream effects on multiple aspects of offspring
competitiveness (Schwabl 1993, 1996; Gorman & Williams 2005; Groothuis *et al.* 2005;
76 Smiseth *et al.* 2011; Williams 2012).

There is an alternative explanation, however, that changes in egg components across
78 the laying sequence may be the passive outcome of the response of females to their own
changing environments, rather than acting in anticipation of the environment offspring
80 will experience. For example, if dietary carotenoids increase over the season whilst eggs
are being laid, then the change in eggs may simply reflect the direct effect of carotenoid
82 availability (Török *et al.* 2007). Similarly, changes in female hormones may be seen in
preparation for and upon the onset of incubation, leading to different hormonal exposure
84 of eggs (Goldsmith & Williams 1980; Sockman & Schwabl 1999, 2000), reflecting changes

in cumulative exposure to the female’s endocrine state. Thus, care is needed to distinguish
86 between anticipatory and passive parental effects.

Previously, we found that prenatal development in blue tits (*Cyanistes caeruleus*) is
88 faster for eggs laid later in the laying sequence, and showed experimentally that this was in
part caused by something intrinsic to the eggs at the time of laying (Hadfield *et al.* 2013b).
90 Although consistent with an anticipatory parental effect that acts to reduce the extent of
hatching asynchrony, we offered the alternative explanation that it may simply be a result
92 of females being better able to provision later eggs as the amount of resource available to
them increases as spring progresses (Hadfield *et al.* 2013b), as there is a rapid change in
94 food availability in this time period (e.g. Dixon 1976). Here, we aim to test this hypothesis
by manipulating a female’s resource availability during the laying period to see if this
96 accelerates the prenatal development of her offspring. To ensure that any effects detected
are the result of changes to the size or composition of the eggs and not due to any effect
98 of the treatment on post-laying behaviour, we cross-fostered half the eggs of each nest on
the day they were laid. We are primarily interested in any effects of this treatment on the
100 hatching time of eggs, but also model whether it has any effect on egg weight or hatching
success.

102 The results of our experiment showed little to no effect of the additional resources
on prenatal development and other egg characteristics. An alternative explanation is
104 that seasonal changes in the environment are causing laying-order effects on pre-natal
development, but in a way that was not be recapitulated in the feeding treatment. Thus,
106 using a larger data set we performed a more general (but correlative) test of whether the
rate of pre-natal development varied systematically with three weather variables, which
108 could be drivers of seasonal changes.

Materials and Methods

110 The experiment was carried out during the springs of 2012 and 2013 on a nest box
population of blue tits (*Cyanistes caeruleus*) on the Dalmeny estate, Edinburgh, UK. Nest
112 boxes were placed approximately 30m apart, on two study sites – 180 boxes on Cragie Hill
(grid reference NT 156 766) and 45 boxes along the Almond River (NT 179 758). From
114 early in the spring, nest boxes were checked systematically to detect nesting and onset of
laying. When nest boxes were empty, or had a very small amount of nesting material within
116 them, they were checked every 4 days. When ≥ 15 mm of nesting material was present, boxes
were checked every 2 days, and once the nest was lined it was checked every day. This
118 method meant that in all but very few cases, nests were checked on the day in which laying
began, and thus a single egg was found.

Cross-foster Design

120 The study was carried out within an ongoing partial cross-foster design used in this
population (for full details see Hadfield *et al.* 2013a). On the day of clutch initiation, nests
122 were randomly allocated into groups of three where possible (or two, four, or five if not),
between which eggs were switched. First eggs were then moved within triads, such that the
124 egg from nest A was placed in nest B, that from nest B in nest C, and that from C placed in
A. One egg is laid per day, so on alternate days, eggs were either moved between nests, or
126 remained within their own nests, and every egg was weighed and marked. Crossing ceased
when one or more nests had a laying pause and resumed when all nests recommenced
128 laying. Daily checks ceased when the female was incubating for the second day in a row, or
was found incubating after laying had ceased. The crossing of eggs within a triad stopped
130 when one nest in a given triad finished laying.

132

Feeding experiment

134 Within this cross foster design, a feeding experiment was also carried out in order
to determine how food availability to parents affects the hatching time of eggs. We used
those nests that were in crosses of 3 or more, and each group was assigned randomly to
136 either a control group or one of two feeding treatments. When a group was assigned a
feeding treatment, two nests within that group were fed, and the remaining nest (or nests
138 if the group was larger than 3) remained as a control. Nests in the feeding treatments
were provided with twenty wax worms (the larvae of the wax moth, *Gralleria mellonella*)
140 per day, which were pinned to narrow tree branches close to the nest box (j5m) early each
morning. On the following day, the number of wax worms consumed was recorded, and any
142 uneaten wax worms were removed. We set up video cameras at some nests to check that
the resident birds were taking the food. In some cases other birds were observed taking the
144 wax worms, but this was relatively rare. Blue tits carry out high levels of courtship feeding
during the egg laying period (Royama 1966; Krebs 1970; Cramp & Perrins 1993), so we
146 hope that resources from any wax worms taken by the male are passed to feed the female,
either directly or indirectly. Nests in the feeding treatments were split into an early group,
148 which were provided with wax worms on days 1 to 4 of the laying sequence, and a late
group that received wax worms on days 5 to 8. Both fed nests within a cross-fostering group
150 were given the same treatment. If development of the embryo is resource limited we expect
chicks from eggs laid in the treated nests to have more rapid prenatal development if the
152 food provided lifts this constraint. We predict that this effect should be more pronounced
in chicks from eggs in the early treatment if resource limitation is more acute at the onset
154 of laying than later in the sequence. Development of eggs takes around 4 days from the
onset of rapid yolk development (Haywood 1993), and so eggs 5-8 are expected to be the
156 most affected in the early treatment, and 9 to 12 in the late treatment. During 2012 there
were 20 nests in each of the early and late treatment, and 18 in each treatment in 2013, so

158 there were equal numbers in each treatment overall. There were 63 control nests in 2012,
and 60 in 2013. In addition, those nests that were not in cross-fostering groups of three
160 or more were never included within the feeding treatment, and so are classed as having no
treatment, as they were not true controls. There were 29 such nests in 2012, and 17 in
162 2013. The mean number of wax worms eaten per day in the early treatment was 15.8, and
17.7 per day in the late treatment.

164 The clutch sizes of the nests included in each treatment are shown in Figure 1, along
with the distribution of eggs within the laying sequences from each of these treatments. It
166 is worth noting that the clutch size and maximum egg number (a measure for each egg of
the days since the first egg was laid in a nest, with the first egg being numbered one) of a
168 nest do not necessarily match due to interruptions in the laying sequence. For example,
a female may lay a total of 9 eggs, but may pause in laying, such that eggs were not laid
170 on day 3 and 4 (i.e. there are no eggs three or four), and the maximum egg number is 11
rather than 9. Thus although mean clutch size is 8.26, many nests have several eggs with
172 egg numbers substantially greater than this, even if the total number of eggs in that nest is
less than this mean.

174

Figure 1 here

Hatching times

176 Nests were checked daily for hatching from 11 days after clutches had been completed.
As nests were checked daily, we found chicks within twenty-four hours of the first individual
178 hatching. On this occasion (day 0), the identities of any unhatched eggs were recorded, and

the same was done on the following two visits (day 1 and day 3). No eggs hatched after this
180 point.

Weather

182 Hourly temperature ($^{\circ}\text{C}$), rain (ml), and wind speed (knots) measurements were
obtained from the Met Office (UK Meteorological Office 1853-Current). Wind and
184 temperature data were from Turnhouse weather station (NT15988 73905, 2.72km from
Craigie Hill, and 2.69km from Almond River), whereas rain data were from Gogar Bank
186 (NT17088 71601, 5.21km from Craigie Hill, and 4.27km from Almond River). Hourly data
were used to calculate daily mean temperature, daily mean wind speed, and daily total
188 rainfall.

Analysis

190 Analyses were carried out in R (R Development Core Team 2012), using the package
MCMCglmm (Hadfield 2010) to fit Bayesian generalized linear mixed models. For those
192 models which had a binary or ordinal response, residual variances were fixed at 1, as these
could not be estimated from the data. For other models a flat improper prior for residual
194 variances was used. For the random effects, parameter expansion was used resulting in
scaled $F_{1,1}$ priors on the variances with scale 1000. Fixed effects had independent normal
196 priors with zero mean and large variances (10^8) except in binary/ordinal models. Here, a
prior correlation matrix was defined as one in which the fixed effects are identically and
198 independently distributed had the covariates been subject to Gelman’s (2008) scaling and
centering. The prior correlation matrix was then scaled by six (which in all models was
200 approximately the sum of the variance components) plus $\pi^2/3$ or 1, depending on whether

the logit or probit link was used. Feeding models were run for 130000 iterations, with
202 a burn in of 30000 and a thinning interval of 50, whereas weather models were run for
1300000 iterations, with a burn in of 300000 and a thinning interval of 500 as the chains
204 within these models took much longer to mix well.

Fixed effects are considered significant if the 95% credible intervals did not overlap
206 zero, and pMCMC (twice the posterior probability that the estimate is either negative or
positive, depending on which is the smaller probability) is less than 0.05. For groups of
208 fixed effects (e.g. year, treatment) their significance was tested using omnibus Wald tests.

Feeding Experiment: egg-level effects

210 A series of models were developed to look at the effect of the treatment on individual
egg characteristics. The main focus was on hatching time, although we also analysed
212 hatching success, egg weight, and pausing in laying. In all models, clutch size, year, day
of clutch initiation (from 1st April) and whether the egg was laid after a pause in the
214 laying sequence or not were fitted as fixed effects. Where necessary, we distinguish between
variables measured in the nest-of-origin and nest-of-rearing using the subscripts o and r
216 respectively. The rank $_r$ of the egg (the number of days between it and the final egg in
the nest-of-rearing being laid), was fitted as a spline to capture any non-linearity in the
218 relationship induced by incubation behaviour (Hadfield *et al.* 2013b). In all models we
excluded eggs that had not been found on the day they were laid, and those from clutches
220 smaller than three eggs (due to early desertion of the clutch). We also excluded three
eggs that weighed less than 0.6g, as these were abnormally small (below half the mean egg
222 weight), and do not develop.

For each egg we also included the treatment group of its nest-of-origin (treatment $_o$) and

224 its nest-of-rearing (treatment_r) as a fixed factor. Nests that were neither fed nor assigned
as controls were included in the analysis as a fourth level in treatment group (coded as
226 ‘none’). Generally, these were nests that initiated laying early or late in the season when
few nests initiated, so that they could not be assigned to groups of three or more.

228 Eggs within nests of a given feeding treatment vary in when and how many wax worms
were consumed by their mother during their development. For example, the fifth egg in
230 an early-treated nest could be affected by up to 80 wax worms provided over the four
consecutive days prior to it being laid, whereas the second egg could only be influenced by
232 up to 20 wax worms on the day prior to it being laid. Blue tits are income breeders, and
eggs develop over a period of four days (Haywood 1993), and thus the main eggs expected
234 to be affected by the feeding are those laid around four days after feeding occurs (e.g. egg
5 for extra food given on day 1) if supplementary feeding has a direct effect on eggs. To
236 accommodate this, we included the number of wax worms that were consumed on each day
prior to the laying of an egg as multiple predictors. Their effects were modelled using a
238 varying coefficient model (Wood 2006; Hastie & Tibshirani 1993): a type of spline that in
this context allows changes in the effect of the predictor (number of wax worms eaten) to
240 be a smooth function of some other covariate (the interval of time between the wax worms
being eaten and the egg itself being laid). These models have recently been used in an
242 ecological context (Roberts *et al.* 2015) and in our context can capture any physiological
lags that exist between the consumption of food and any effect of that food on the egg.
244 Separate varying coefficient splines were fitted for the early and late groups.

The degree to which the treatment splines improved model performance was assessed
246 using 20-fold cross-validation (Stone 1974, 1977), where models were rerun using data with
the k^{th} subset removed. The posterior mean predicted values for the omitted data were
248 obtained with the random nest-of-origin and nest-of rearing effects marginalised using

posterior predictive simulation. Where responses were discrete, the correct classification
250 of the response variable was used as a measure of predictive ability. For continuous
responses the coefficient of determination (R^2) was used. Differences between models in
252 their predictive ability were often small, and so we tested whether an increase in predictive
ability relative to that of the null model (i.e. one without treatment splines) was greater
254 than expected from Monte Carlo error (i.e. differences were not solely due to random
allocation of observations to subsets). This was achieved using repeat-measure ANOVA
256 with subset as a random effect.

Hatching time was analysed in two ways. Firstly, hatching asynchrony was fitted as
258 an ordinal response, which considers the hatching interval of an egg within a clutch (i.e.
the hatching day, relative to the first egg in the clutch to hatch, which could be 0, 1 or
260 2-3). Secondly, as a censored Gaussian response, which fits the time from the final egg in a
nest being layed to the hatching of each egg with the censoring points being the day/time
262 of nest visits between which an egg hatched. Eggs were assumed to be laid at 6am, as
the exact laying time was not known, but eggs had always been found after this time
264 (Pullen 1946; Perrins 1979). This measure captured differences between nests in incubation
behaviour of females (time to commence incubation, and duration of incubation), and
266 within-nests it captures intrinsic differences in hatching time between eggs. Hatching
success was analysed as a binary response (hatching/not-hatching) and all abandoned nests
268 were excluded. Egg weight was analysed as a Gaussian response, and the probability of an
egg being laid as a binary response (where an egg being laid was given the value 1). These
270 two phenotypes are not dependent on post-laying (and therefore post-crossfostering) effects
and so nest-of-rearing terms (both fixed and random) were dropped from the model. In
272 addition, clutch size_o was dropped from the model of pausing, as the two are confounded.

Feeding Experiment: nest-level effects

274 A second set of models investigated the nest-level effects of the feeding experiment,
specifically how it affected clutch size and the onset of incubation. The onset of incubation
276 is taken as the number of days before (negative value) or after (positive value) the last
egg was laid that the female was found incubating, or the eggs were found to be warm.
278 Both traits were treated as Gaussian and the models had the same form as above, although
treatment was included only as a fixed effect, without spline terms, and egg rank was not
280 included in the model. In addition, only nest-of-origin level terms were retained.

Weather

282 In addition to the main analyses, we ran the same models as those described above,
but also included terms to model how weather (a measure of external conditions) affects
284 egg- and nest-level characteristics. In these analyses we used comparable data collected in
the previous two years (Hadfield *et al.* 2013b,a) although egg weight data were unavailable
286 for 2010. Treated nests from 2012-13 were excluded, as were those eggs included in an
experiment performed in 2010-2011 (Hadfield *et al.* 2013b). Consequently any terms
288 associated with treatments were dropped from the model. The effect of weather variables
(daily mean temperature, daily mean wind speed, and total daily rainfall) were modelled
290 using varying coefficient splines, as was done for the effect of wax worm consumption. Thus
each model had three varying coefficient splines within it. Daily weather 50 days prior to,
292 and 40 days after, laying of a given egg was used in these splines, so that both long- and
short-term effects of weather might be captured. The sample sizes for each model, along
294 with those eggs and nests that have been excluded are shown in Table 1.

Table 1 here

296

Results

Feeding Experiment: egg-level effects

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Overall, we found little support for any effects of the feeding treatment on hatching time and other egg characteristics. This is seen particularly in the fitting of treatment splines – in all models the treatment splines did not improve the predictive ability of the model, as evaluated using cross-validation (Table 2). Consequently, we report the results of these models with the treatment splines removed in the text and tables. However, in Figure 2, and Figure S2 in the Supporting Information, we present the predictions based on the full model.

Figure 2 here

306

Table 2 here

308

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316

The fixed effects for the best model for hatching asynchrony are shown in Table 3. The 95% credible intervals of all fixed treatment effects overlapped zero, and thus there was no significant effect of treatment on asynchrony. There were significant positive effects of clutch size_r, and the day of clutch initiation, showing nests are more asynchronous when clutches are larger and laid later in the season. The spline of egg rank (Figure S1a) shows that lower rank eggs hatch later (i.e. late laid eggs hatch late), although this ceases to be the case for eggs of rank > 3 which tend to have equivalent hatching times. Nest-of-origin explained a small amount of variance in hatching asynchrony (0.068 [0.018 - 0.149]), whereas the nest-of-rearing explained a greater proportion of variation in the spread of hatching, 0.562 [0.446 - 0.613].

Table 3 here

318 The fixed effects from the censored Gaussian model of hatching time (from the laying
of the last egg in a nest) are summarised in Table 4, again this is the best model (without
320 the treatment splines). Overall, the results are broadly similar to the hatching asynchrony
model with all fixed treatment effects overlapping zero and therefore non-significant. There
322 was a significant negative effect of clutch size_r, with eggs hatching 0.287 days ([0.127 -
0.445], P<0.001) faster for each additional egg in a nest, and eggs in later clutches hatched
324 faster by 0.106 days ([0.074 - 0.137], P<0.001) for each day later in the season. In addition
the rank spline (Figure S1b) shows that there is a decrease in time from laying to hatching
326 with increasing rank, although this change is most prominent for those eggs of low rank.
The variance explained by the nest-of-origin was small, with an intraclass correlation of
328 0.002 [0.000 - 0.007], but the variance explained by the nest-of-rearing was much larger
(0.972 [0.962 - 0.976]) as the censored Gaussian model also includes variation due to
330 differences in incubation behaviour between females. However, the nest-of-origin effect
within nest-of-rearing explained more variance (0.079 [0.000 - 0.202]).

332 *Table 4 here*

For the best model of hatching success, the fixed effects are summarised in Table 5 (no
334 treatment splines were in the best model). No significant differences between treatment
groups were found, although eggs in none-fed none-control nests were significantly less
336 likely to hatch (-0.863 logits [-1.684 - -0.100] P=0.040). Eggs laid after a pause show a
significantly lower probability of hatching than those not laid after a pause. The rank spline
338 (Figure S1c) shows that later laid eggs in a clutch (low rank) are more likely to hatch than

earlier ones, and Figure 2c suggests this is mainly due to first eggs having lower hatching
340 probability. Both the intraclass correlation for nest-of-origin (0.153 [0.000 - 0.264]), and
nest-of-rearing (0.167 [0.055 - 0.287]) were reasonably small and of similar magnitude.

342

Table 5 here

The fixed effects from the best model of egg weight are summarised in Table 6; this
344 model did not include any treatment splines. The 95% credible intervals of all treatment
effects overlapped zero, thus treatment did not have an effect on egg weight. Eggs were
346 significantly smaller (-0.080 g [-0.111 - -0.049] $P < 0.001$) in 2013 than 2012 and egg weight
increased by 0.002 g per day ([0.000 - 0.003], $P = 0.028$) as clutch initiation day increased.
348 There was also a significant effect of being laid after a pause, with eggs laid after an
interruption in laying being 0.051 g ([0.043 - 0.059], $P < 0.001$) heavier. The rank spline
350 (Figure S1d) shows that there is a general decrease of egg weight with increasing rank,
implying that late laid eggs tend to be heavier than earlier ones. A large proportion of the
352 variance in egg weight was explained by nest-of-origin (0.800 [0.768 - 0.832]).

Table 6 here

354 The fixed effects from the best model of the probability of an egg being laid are
summarised in Table 7 (treatment splines did not improve the model). These results
356 suggest that feeding had no significant effect on the probability that an egg is laid. There
is a significant negative effect of the day of clutch initiation, such that pausing occurs more
358 frequently later in the season. The rank spline, Figure S1e, shows a negative effect of egg
rank on the probability that an egg is laid, such that interruptions in the laying sequence

360 are more likely to happen early in a clutch. The intraclass correlation for nest-of-origin was
moderate: 0.179 [0.088 - 0.312].

362

Table 7 here

Feeding Experiment: nest-level effects

364 The fixed effects from the model of clutch size are summarised in Table 8. Neither
treatment had a significant effect on clutch size – early treatment resulted in 0.413 more eggs
366 ([-0.130 - 0.964], P=0.136) than control nests; late treatment resulted in 0.386 more eggs
([-0.197 - 0.947], P=0.170). The difference between early and late treatments overlapped
368 zero (0.026 [-0.640 - 0.676] P=0.917), and the omnibus test showed there was no overall
significant effect from the treatments (Wald test $\chi^2=3.172$, P=0.205). There is a significant
370 negative effect of the date on which the clutch started, with females laying 0.073 fewer eggs
([0.049 - 0.095], P<0.001) with each additional day after April 1st that the clutch started.

372

Table 8 here

The fixed effects from the model of incubation onset are summarised in Table 9. There
374 was a marginal effect of early treatments on the onset of incubation, with incubation
commencing 0.639 days later ([-0.119 - 1.309], P=0.090) than in controls. Although there
376 was no effect of the late treatment, the difference between early and late treatments
overlapped zero (0.703 [-0.164 - 1.609] P=0.107). As with clutch size, there was a significant
378 negative effect on the incubation onset of the clutch initiation day – incubation began 0.080

days ([0.048 - 0.114], $P < 0.001$) earlier, relative to the final egg, with each day after 1st of
380 April that the female commenced laying. Additionally, there is a significant negative effect
of clutch size, such that incubation onset advanced by 0.261 days ([0.087 - 0.417], $P = 0.003$)
382 for each additional egg in a clutch.

Table 9 here

384

Weather

Generally, we found little support for weather having effects on hatching time, and
386 other egg characteristics. In all models, except the censored gaussian model of hatching
time and the model of clutch size, the weather splines did not improve the predictive
388 ability of the model as evaluated using cross-validation (Table 10). The results of the null
models were qualitatively similar to the feeding treatment models and are presented in the
390 supplementary information. The main difference was support for inter-annual differences
in all models, even for those egg characteristics and nest effects for which no significant
392 differences between 2012 and 2013 were found. In addition, the significant effect of eggs
laid after a pause having a lower probability of hatching (-0.842 logits [-1.520 - -0.126]
394 $P = 0.028$), were not replicated in the larger data set (-0.306 logits [-1.016 - 0.370] $P = 0.377$).
The splines for each weather variable are shown in Figures S3, S4 and S5.

396

Table 10 here

The best supported model for the censored Gaussian model of hatching time was that
398 with temperature and rain splines included, however the full model had a very similar

classification rate so there was little difference between models with and without wind
400 included (correct classification rate of best model 0.2190; Full model 0.2189; Null model
0.2181). The fixed effects from the best model are shown in Table S3, whilst those for the
402 full and null model are shown in Table S2. The weather spline in Figure S4b shows that
there was a positive effect of temperature on hatching time (days from last egg laid in a
404 nest to hatching), up to three weeks prior to the egg being laid, implying that eggs that
are developing in the female when conditions are warmer then hatch later. However, the
406 converse appears to be the case post-laying, where there is a negative effect of temperature
on hatching time, such that warm conditions after an egg has been laid leads to an
408 acceleration in hatching time. Both rain (Figure S5b) and wind (Figure S3b) prior to laying
appear to have little effect on hatching time in the full model, although the effect of rain
410 is supported by cross validation. Year has a significant effect on hatching time in the best
model (Wald test on year $\chi^2=13.651$, $P=0.003$), with the biggest estimated difference of
412 an increase of 1.746 days ([0.721 - 2.675], $P<0.001$) to hatching between 2011 and 2012.
However, year effects were larger in the null model with an increase of 2.429 days ([2.045 -
414 2.847], $P<0.001$) to hatching between 2011 and 2012 (Wald test on year $\chi^2=153.13$, $P<0.001$),
implying that year and weather effects may be confounded. Similarly, laydate was not
416 significant in the best model, although it was in the null model. In contrast to the feeding
model, clutch size_r did not have a significant effect in the full model, but did in the null
418 model, whereas clutch size_o had a significant positive effect on hatching time in both the
full and null model.

420 Inclusion of splines of temperature and wind improved the predictive ability of the
model of clutch size ($R^2=0.308$; Null model $R^2=0.259$). The weather splines for wind and
422 temperature are seen in Figures S3f and S4f respectively. Wind appears to have a negative
effect on clutch size, such that females lay fewer eggs as mean daily wind speed increases.
424 Similarly, temperature negatively affects clutch size, such that smaller clutches are laid

as daily mean temperature increases. Year has a non-significant effect (Wald test on year
426 $\chi^2=0.305$, $P=0.883$), although this then appears significant in the null model. Laydate did
not have an effect on clutch size, although this was significant and negative in the null
428 model, suggesting that seasonal effect as well as year are confounded with weather in this
model.

430

Discussion

The experiment described above was designed to test whether the increased rate of
432 prenatal development across the laying sequence of blue tits could be directly caused by an
increase in the resource availability to the mother at the time of laying. We found little
434 support for supplementary feeding accelerating prenatal development; If anything, there
was a positive effect of food consumption on hatching time, i.e. hatching occurred later
436 due to supplementary feeding. Thus, we conclude that the changes in resource availability
are not likely to be responsible for the intrinsic acceleration of hatching of late laid eggs
438 observed in Hadfield *et al.* (2013b). We do not see any significant effect of treatment on
female behaviour, although the effect size for the fixed effect of treatment on clutch size
440 is reasonable large (increased clutch size by half an egg), suggesting that we may lack the
power to estimate this effect.

442 Our results suggest that differences between eggs across the laying sequence are the
result of changes in the female that are independent of either the nutritive or climatic
444 environment. Consequently, these results are consistent with the idea that females are
manipulating the developmental rate and consequent hatching time of their offspring
446 independently of their own state. The mechanism by which they achieve this could be
through increasing the provision of resources to eggs laid later. Consistent with this idea
448 is the fact that egg size increases over the laying sequence, and larger eggs hatch faster

(Rubolini *et al.* 2005; Hadfield *et al.* 2013b). This is a clear extension of previous work
450 that suggested that larger size of late-laid eggs should benefit late-hatched chicks through
the effect on the size at hatching of those chicks (Howe 1976; Clark & Wilson 1981), and
452 thus reducing the effect of hatching asynchrony. Where size also affects hatching time,
then size differences may affect the extent, as well as the effect, of hatching asynchrony. In
454 addition, Ferrari *et al.* (2006) and Alquati *et al.* (2007) found that experimental removal
of albumen delayed hatching, thus the relative amount of albumen and yolk within an egg
456 may be important in influencing prenatal development. Maternally deposited hormones are
a more commonly invoked candidate as a mediator of anticipatory maternal effects. More
458 specifically, androgens generally act to accelerate pre- and post-hatching development (e.g.
Lipar & Ketterson 2000; Eising *et al.* 2001), reviewed in von Engelhardt & Groothuis (2011),
460 although contrary results have been found (Sockman & Schwabl 2000; Von Engelhardt
et al. 2006). Previous work on blue tits, however, suggests there is little variation across
462 the clutch in androgen concentration (Kingma *et al.* 2009); a pattern predicted from
interspecific comparisons of initial size differences between first and last hatching chicks
464 and yolk testosterone compensation (Muller & Groothuis 2013). Thus, it seems unlikely
that androgens, are driving the differences in prenatal development in this particular
466 case, although other hormones may be at play. Nonetheless, our experiment is unable to
differentiate between a passive effect of changes in the females endocrine state and an active
468 anticipatory role if hormones are mediating such effects. It is not known whether females
are able to independently control blood plasma and egg hormone concentrations, but most
470 hormones in egg yolks are derived from cells in the developing follicle (Huang *et al.* 1979;
Hackl *et al.* 2003; Williams *et al.* 2004; Gil 2008), and so the possibility of independent
472 control exists (Groothuis & Schwabl 2008).

It is also possible that laying order effects are a direct maternal response to changing
474 nutritive conditions, but such an effect might not be seen if providing wax worms failed

to recapitulate such conditions. However, the wax worms provided to the birds should
476 contain sufficient calorific and protein content to lift seasonal resource constraints; twenty
wax worms constitute 12kcal and 0.67g protein (Sauter *et al.* 2006), whereas blue tits
478 require 13.8kcal per day (Gibb 1957, winter conditions) and each egg contains about 0.12g
of protein (Bourgault *et al.* 2007; Murphy 1994, given a mean egg weight of 1.18g) . Thus,
480 although there is evidence that responses to feeding experiments depend upon the protein
content of the food provided (Nager *et al.* 1997; Ramsay & Houston 1997, 1998), we
482 believe our supplementary feeding would lift any constraints imposed by limited protein
availability. Furthermore, if changing nutritive conditions were generating changes in
484 hatching time, this would be detected in the models using climatic variables as predictors,
which was not seen. In addition to macronutrients, it is possible that birds become less
486 constrained by micronutrients as laying progresses. In particular, blue tits do not use
skeletal calcium for egg production, so all calcium must be obtained in the diet (Woodburn
488 & Perrins 1997) mainly through consumption of snail shells, bones and grit (Betts 1955;
Graveland & Berends 1997; Reynolds & Perrins 2010). Twenty wax worms contains just
490 0.230mg calcium, much less than the content of an eggshell (58mg; Graveland & Berends
1997). However, although the wax worms may be limiting in certain resources, the energy
492 they provide may still allow greater time and resources to be spent in obtaining those
micronutrients.

494 Previous feeding experiments lend support to the idea that supplementary feeding can
lift constraints associated with breeding, since supplemented birds often show advanced lay
496 dates and increased clutch size (see Christians 2002; Robb *et al.* 2008a). However, these
experiments usually commence feeding prior to laying, ranging from several months prior
498 to breeding (e.g. Robb *et al.* 2008b) to a few days (e.g. Ramsay & Houston 1997), and
the few that have looked at effects on prenatal development often attribute differences to
500 behavioural changes in the female rather than changes in egg characteristics (e.g. Wiebe &

Bortolotti 1994). In the experiment shown here we failed to find any effect of supplementary
502 food on female behaviour, either through clutch size or incubation. Nevertheless, there is
limited evidence that supplementary feeding can alter egg characteristics. Effects on egg
504 size in particular have been investigated, and some increases in response to supplementary
food have been found (Wiebe & Bortolotti 1995; Horsfall 1984; Ramsay & Houston 1997),
506 although the results are not always replicated (Nilsson & Svensson 1993; Nager *et al.* 1997),
and the majority of studies have failed to find any significant effect of feeding (reviewed
508 in Christians 2002). A recent paper from Ruuskanen *et al.* (2016) carried out a similar
experiment to the one shown here, and found increased egg mass in fed nests. However,
510 there appear to be differences between mass of eggs in control and fed treatments prior to
feeding, and thus the conclusions from this may be questionable.

512 In this study we fail to see any major effect of the environment on the prenatal
development of chicks, either through the provision of food or through correlations with
514 the weather at the time of laying. Thus it seems that egg characteristics and prenatal
development are insensitive to external conditions as experienced by the mother. Consistent
516 with this insensitivity is the high repeatability of egg size (Christians 2002; Williams 2012),
and other egg constituents (Postma *et al.* 2014), within females. This suggests that the
518 observed laying-order effects are regulated by the female without regard to her external
environment. If the laying-order effects are the result of differential resource provisioning
520 by the female, then we suggest that this is in anticipation of the level of sibling competition
the chicks will experience. However, if the laying-order effects are mediated by maternal
522 androgens our results are silent as to whether they are passive by-products of the females
endocrinological state or whether they constitute an anticipatory parental effect.

524 *Author Contributions*

CET designed the experiment, carried out field work and statistical analysis, and wrote

526 the manuscript. JDH helped in experimental design, carried out field work, designed the
statistical analysis, and contributed to writing the manuscript.

528

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Data Accessibility

536 Data and scripts used for this paper are available on Data Dryad DOI: [doi:10.5061/dryad.26h4q](https://doi.org/10.5061/dryad.26h4q).

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754 *SUPPORTING INFORMATION*

Additional supporting information may be found in the online version of this article.

756 Figure 1: Egg Rank Splines from the Feeding Analyses

Figure 2: Feeding Splines from the Feeding Analyses

758 Figure 3: Splines of the effect of Wind from the Weather Analyses

Figure 4: Splines of the effect of Temperature from the Weather Analyses

760 Figure 5: Splines of the effect of Rain from the Weather Analyses

762 Tables 1-9: Results from the models of the effects of Weather on Egg- and Nest
characteristics.

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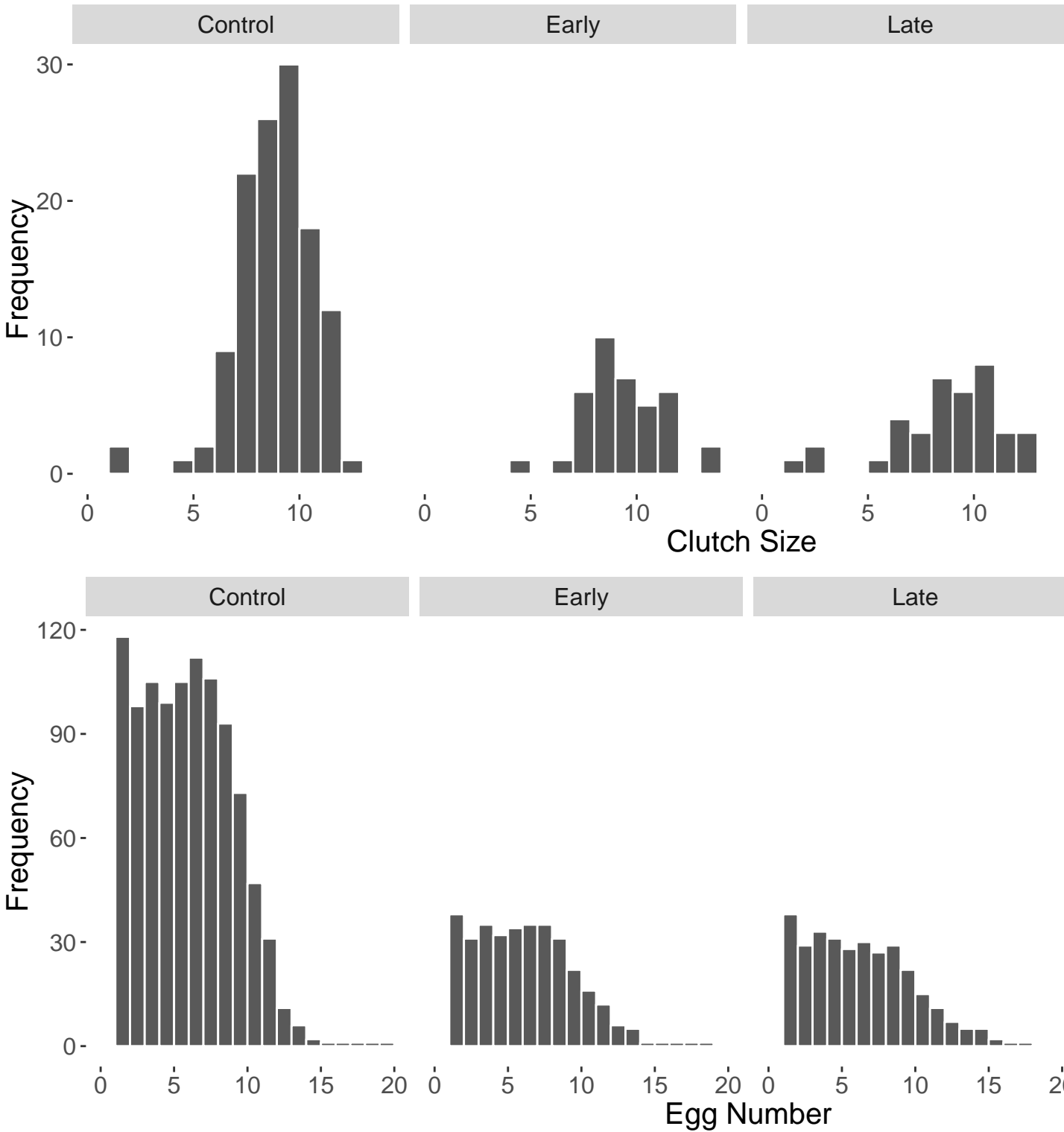
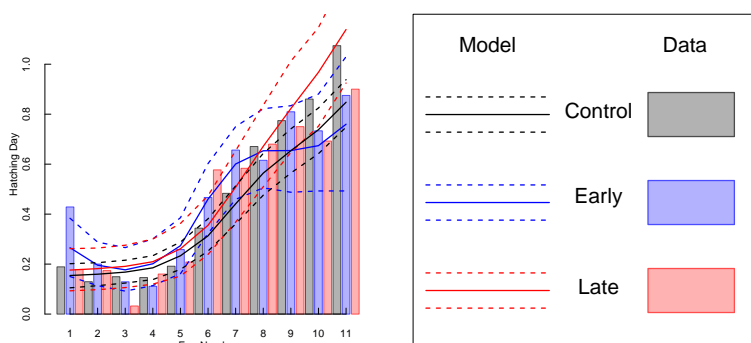
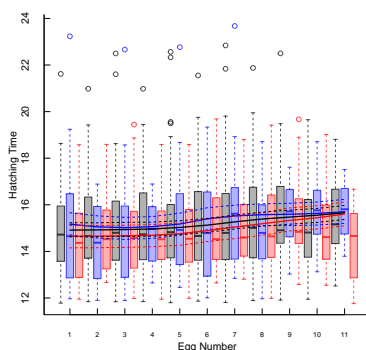


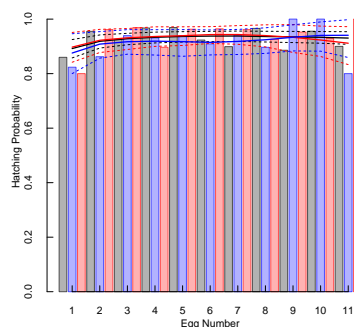
Fig. 1.— The clutch sizes (upper panel) and egg numbers included in the data for each of the treatments. An egg's number is the day on which it was laid (relative to the first egg in the clutch), such that egg 1 is the first egg, and egg 3 is laid 2 days after the first egg. Numbering is irrespective of whether a pause in the laying sequence has occurred, so does



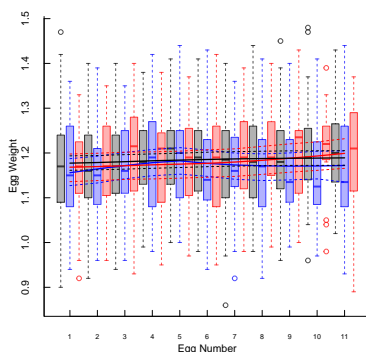
(a)



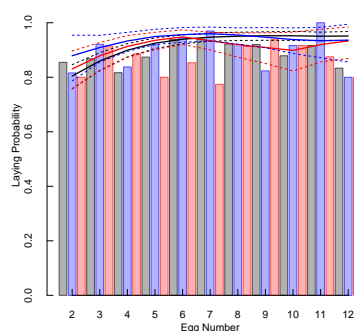
(b)



(c)



(d)



(e)

Fig. 2.— The cumulative effect of wax worms eaten on (a) hatching asynchrony within nest, (b) time from laying of the final egg in a nest to hatching, (c) probability of hatching, (d) weight of eggs, and (e) the probability that an egg is laid. Boxplots and barplots show the raw data, split by treatment, together with predictions (solid lines) and 95% credible intervals (dashed lines). Predictions were made holding all predictors at their mean value except rank and the number of wax worms eaten for which we use the mean for each egg-number/treatment combination.

Table 1: Sample sizes (n), the number of eggs that are excluded, and the number of nests that those eggs are found in, for each model

	Feeding				Weather			
	Included		Excluded		Included		Excluded	
	Eggs	Nests	Eggs	Nests	Eggs	Nests	Eggs	Nests
Ordinal Hatching	1649	214	67	3	3076	426	775	39
Censored Gaussian Hatching	1649	214	67	3	3076	426	775	39
Hatching Success	1825	215	49	2	3425	428	829	37
Weight	1979	235	51	12	2754	319	730	91
Pausing	1739	235	0	0	3259	431	794	93
Clutch Size		229		18		409		115
Onset of Incubation		210		24		391		104

Table 2: Mean predictive power from 20-fold cross validation of feeding experiment models, comparing the full model (all fixed and random effects included, along with splines of the effects of the feeding experiment) with those that drop the splines for each of the early and late treatments, and the null model in which both are dropped (but all other fixed and random effects are retained). Ordinal and Censored Gaussian are the two models of hatching time.. For weight, predictive power is measured as an R^2 value, and for other models it is the rate of correct classification. In all cases the predictions are marginal with respect to nest_o and nest_r (where appropriate). The final column is the probability that the estimated predictive power of the best model (in bold) exceeds that of the null model more than would be expected from Monte Carlo error alone.

	Full Model	Early Spline Dropped	Late Spline Dropped	Both Dropped	$\text{Pr}(iW)$
Ordinal	0.616	0.615	0.616	0.615	0.1240
Censored Gaussian	0.178	0.178	0.178	0.177	0.0830
Hatching Success	0.842	0.842	0.842	0.843	
Weight	0.094	0.095	0.096	0.097	
Laying Success	0.793	0.793	0.794	0.793	0.5090

Table 3: Summary of the fixed effects from an ordinal model of hatching asynchrony (day of hatching relative to the first day of hatching within the nest). These results are from a model without treatment splines since they did not significantly increase predictive ability (Table 2). P-values from wald tests on fixed effects are also presented. The mean is the posterior mean, l-95% and u-95% are the lower and upper 95% credible intervals.

	mean	l-95%	u-95%	pMCMC	Pr(j W)
Intercept	-6.067	-8.539	-3.654	0.001	
Treatment _r .None	0.288	-0.442	1.120	0.464	
Treatment _r .Early	-0.200	-1.150	0.675	0.646	0.223
Treatment _r .Late	0.731	-0.210	1.588	0.121	
Treatment _o .Early	0.585	-0.057	1.285	0.101	0.232
Treatment _o .Late	-0.082	-0.789	0.586	0.803	
Clutch size _r	0.284	0.105	0.495	0.002	
Clutch size _o	0.083	-0.048	0.212	0.221	
Year.2013	-0.389	-1.156	0.403	0.308	
Laydate _r	0.052	0.014	0.091	0.007	
After pause	0.365	-0.131	0.867	0.163	

Table 4: Summary of the fixed effects from a censored Gaussian model on the time (in days) from laying to hatching of eggs. These results are from a model without treatment splines since they did not significantly increase predictive ability (Table 2). P-values from wald tests on fixed effects are also presented. The mean is the posterior mean, l-95% and u-95% are the lower and upper 95% credible intervals.

	mean	l-95%	u-95%	pMCMC	Pr(χ^2)
Intercept	21.347	19.327	23.197	0.001	
Treatment _r .None	-0.430	-1.106	0.189	0.193	
Treatment _r .Early	0.079	-0.675	0.711	0.822	0.663
Treatment _r .Late	-0.277	-0.978	0.377	0.438	
Treatment _o .Early	0.101	-0.018	0.219	0.107	0.264
Treatment _o .Late	0.015	-0.118	0.136	0.818	
Clutch size _r	-0.287	-0.445	-0.127	0.001	
Clutch size _o	0.018	-0.005	0.040	0.111	
Year.2013	-0.358	-0.972	0.337	0.295	
Laydate _r	-0.106	-0.137	-0.074	0.001	
After pause	0.049	-0.038	0.146	0.322	

Table 5: Summary of the fixed effects from a model of hatching success of eggs. These results are from a model without treatment splines since they did not significantly increase predictive ability (Table 2). P-values from wald tests on fixed effects are also presented. The mean is the posterior mean, l-95% and u-95% are the lower and upper 95% credible intervals.

	mean	l-95%	u-95%	pMCMC	Pr(j W)
Intercept	0.331	-2.086	3.032	0.809	
Treatment _r .None	-0.863	-1.684	-0.100	0.040	
Treatment _r .Early	-0.490	-1.557	0.499	0.349	0.56
Treatment _r .Late	0.258	-0.794	1.319	0.627	
Treatment _o .Early	0.149	-0.830	1.195	0.778	0.918
Treatment _o .Late	-0.155	-1.162	0.899	0.784	
Clutch size _r	0.150	-0.050	0.365	0.160	
Clutch size _o	0.133	-0.024	0.318	0.116	
Year.2013	-0.630	-1.499	0.128	0.125	
Laydate _r	0.034	-0.003	0.076	0.089	
After pause	-0.842	-1.520	-0.126	0.028	

Table 6: Summary of the fixed effects from a model of egg weight. These results are from a model without treatment splines since they did not significantly increase predictive ability (Table 2). P-values from wald tests on fixed effects are also presented. The mean is the posterior mean, l-95% and u-95% are the lower and upper 95% credible intervals.

	mean	l-95%	u-95%	pMCMC	Pr(j W)
Intercept	1.160	1.059	1.251	j0.001	
Treatment _o .None	-0.005	-0.035	0.027	0.728	
Treatment _o .Early	-0.012	-0.045	0.020	0.467	0.766
Treatment _o .Late	-0.004	-0.037	0.031	0.771	
Clutch size _o	-0.001	-0.009	0.007	0.896	
Year.2013	-0.080	-0.111	-0.049	j0.001	
Laydate	0.002	0.000	0.003	0.028	
After pause	0.051	0.043	0.059	j0.001	

Table 7: Summary of the fixed effects from a model of the probability that an egg is laid. These results are from a model without treatment splines since they did not significantly increase predictive ability (Table 2). P-values from wald tests on fixed effects are also presented. The mean is the posterior mean, l-95% and u-95% are the lower and upper 95% credible intervals.

	mean	l-95%	u-95%	pMCMC	Pr($j W$)
Intercept	4.469	3.466	5.530	0.001	
Treatment _o .None	-0.530	-1.232	0.082	0.094	
Treatment _o .Early	0.394	-0.330	1.116	0.286	0.504
Treatment _o .Late	-0.091	-0.782	0.603	0.820	
Year.2013	-0.283	-0.987	0.379	0.399	
Laydate	-0.039	-0.069	-0.004	0.024	

Table 8: Summary of the fixed effects from a gaussian model of clutch size. P-values from wald tests on fixed effects are also presented. The mean is the posterior mean, l-95% and u-95% are the lower and upper 95% credible intervals.

	mean	l-95%	u-95%	pMCMC	Pr($j W$)
Intercept	10.828	10.092	11.564	j0.001	
Treatment _o .None	-0.112	-0.620	0.467	0.689	
Treatment _o .Early	0.413	-0.130	0.964	0.136	0.205
Treatment _o .Late	0.386	-0.197	0.947	0.170	
Laydate	-0.073	-0.095	-0.049	j0.001	
Year.2013	0.232	-0.312	0.692	0.402	

Table 9: Summary of the fixed effects from a gaussian model of the onset of incubation relative to the date on which the last egg in a nest was laid. P-values from wald tests on fixed effects are also presented. The mean is the posterior mean, l-95% and u-95% are the lower and upper 95% credible intervals.

	mean	l-95%	u-95%	pMCMC	Pr($j W$)
Intercept	6.185	4.018	8.138	j0.001	
Treatment _o .None	-0.154	-0.820	0.519	0.687	
Treatment _o .Early	0.639	-0.119	1.309	0.090	0.187
Treatment _o .Late	-0.064	-0.731	0.715	0.838	
Laydate	-0.080	-0.114	-0.048	j0.001	
Year.2013	-0.371	-1.002	0.311	0.298	
Clutch size _o	-0.261	-0.417	-0.087	0.003	

Table 10: Mean predictive power from 20-fold cross validation of weather models, comparing the full model (all fixed and random effects included, and splines of the effects of temperature, rain and wind) with those with each of the individual weather splines dropped, and the null model in which all three weather splines are dropped (but all other fixed and random effects are retained). Ordinal and Censored Gaussian are the two models of hatching time. For weight, predictive power is measured as an R^2 value, and for other models it is the rate of correct classification. All are run with $nest_o$ and $nest_r$ (where appropriate) marginalised. The final column is the probability that the predictive power of the best model (in bold) exceeds that of the null model more than would be expected from Monte Carlo error alone.

	Full Model	Wind Spline Dropped	Temperature Spline Dropped	Rain Spline Dropped	All Dropped	$\Pr(j>W)$
Ordinal	0.574	0.592	0.578	0.569	0.593	
Censored Gaussian	0.219	0.219	0.219	0.218	0.218	0.0180
Hatching Success	0.831	0.831	0.831	0.831	0.830	0.0730
Weight	0.066	0.062	0.063	0.064	0.063	0.4000
Pausing	0.868	0.869	0.868	0.868	0.868	0.1990
Clutch Size	0.303	0.305	0.271	0.308	0.259	0.0020
Incubation	0.243	0.247	0.248	0.249	0.256	