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1 **Spring phenology does not affect timing of reproduction**
2 **in the great tit (*Parus major*)**

3
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14
15 **SUMMARY**

16 Many seasonal breeders adjust the timing of reproduction in response to year-to-year
17 variations in supplementary environmental cues, amongst which ambient temperature
18 is thought to be most influential. However, it is possible that for species such as the
19 great tit (*Parus major* L.), phenological cues from sprouting vegetation and the
20 consequent abundance of invertebrate prey, although dependent on temperature, may
21 provide supplementary environmental cues *per se*. This hypothesis was investigated in
22 breeding pairs of great tits kept in outdoor aviaries. In spring, experimental pairs were
23 provided with access to leafing birch branches and caterpillars as a visual food cue,
24 while control pairs were provided with non-leafing branches. Observations were made
25 on the onset of laying, and on concentrations of plasma luteinizing hormone (LH) at
26 regular intervals to monitor changes in reproductive function. The onset of egg laying
27 was not advanced by the presence of leafing branches and caterpillars. LH
28 concentrations increased during the course of the study, but phenological cues did not
29 affect plasma LH levels in both females and males. Early spring vegetation, such as
30 the leafing of birch branches and the appearance of caterpillar prey, do not appear to
31 play a significant role in fine-tuning the onset of egg-laying in great tits.

32
33 **Key words:** seasonal timing, laying date, *Parus major*, phenology, supplementary
34 cues, luteinizing hormone

35
36 **Short title:** Spring phenology and timing of laying

37

38

INTRODUCTION

39 Birds adapt their life histories to fluctuating environmental conditions, with energy-
40 demanding activities, such as reproduction and moult, occurring at a time of the year
41 that offers sufficient energetic resources. Matching the rearing of nestlings with the
42 seasonal food peak has large fitness consequences (Charmantier et al., 2008; Perrins,
43 1965; Sheldon et al., 2003; Thomas et al., 2001; van Noordwijk et al., 1995), and
44 there is strong selection pressure on mechanisms that enable females to predict future
45 food availability from proximate environmental cues (Visser and Lambrechts, 1999;
46 Visser et al., 2010).

47

48 In non-equatorial birds, lengthening photoperiod in spring provides the principal cue
49 for the timing of seasonal breeding (Dawson et al., 2001; Farner, 1985; Follett et al.,
50 1985; Sharp, 2005; Silverin et al., 1993), but supplementary cues derived from
51 rainfall, ambient temperature and phenology are available to increase the precision of
52 a timing decision (Dawson, 2008; Hau et al., 2004; Meijer et al., 1999; Perfito et al.,
53 2005; Salvante et al., 2007; Schaper et al., 2011; Small et al., 2008; Visser et al.,
54 2009; Wingfield et al., 1992; but see Visser et al., 2011). The aims of this paper are
55 firstly, to re-evaluate evidence for the involvement of phenological cues in the onset
56 of egg laying in opportunistic and strictly seasonally breeding birds, and secondly, to
57 assess experimentally whether there is a causal relationship between phenological
58 cues provided by leafing birch and caterpillars and the onset of reproduction in a
59 seasonal breeder, the great tit (*Parus major* L.).

60

61 Opportunistic breeding birds live in environments in which the distribution of their
62 food supply fluctuates erratically, and thus they require great sensitivity to
63 environmental cues predictive of increased food supply to stimulate reproductive
64 activity (Hahn, 1998). Most studies have been done on birds living in arid regions
65 with unpredictable rainfall (Table 1), and because of its importance for primary
66 productivity, rainfall is still the climatic parameter most frequently analysed with
67 regard to the onset of breeding (Barrientos et al., 2007). Rainfall stimulates the growth
68 of vegetation resulting in the production of leaves, flowers and seeds, and these not
69 only provide plant and associated invertebrate food to feed nestlings, but may also act
70 as phenological cues for the initiation of breeding (Hahn et al., 2008). For example, in

71 the granivorous zebra finch (*Taeniopygia guttata*) in central Australia, hatching
72 coincides with the availability of grass seeds to feed nestlings after the onset of rain,
73 with heavier rainfall resulting in longer breeding episodes, and repeated rainfall
74 stimulating repeated breeding (Zann et al., 1995). A similar phenomenon is observed
75 in Darwin's ground finches (*Geospiza spec.*) on the Galapagos Islands where hatching
76 coincides with flushes of insect availability occurring after semi-seasonal rains (Hahn
77 et al., 2008; Hau et al., 2004). However, in male Darwin finches, rainfall or even
78 rainfall-related noise stimulates singing (Grant, 1999) and may therefore act directly
79 as a proximate cue for reproduction.

80

81 Direct evidence that vegetation phenology is likely to provide an environmental cue
82 for breeding activity comes from red crossbills (*Loxia curvirostra*) that appear to
83 breed in response to the changing food availability of western hemlock (Hahn et al.,
84 2008), and Pinon jays (*Gymnorhinus cyanocephalus*) that breed in late summer only if
85 green cones of Pinon pines (*Pinus monophylla*) are abundant (Ligon, 1974; Ligon,
86 1978). The possibility that food acts as a phenological cue for breeding is
87 demonstrated in a study using captive male spotted antbirds (*Hylophylax n.*
88 *naevioides*) in Panama, in which gonadal growth and singing is stimulated by the
89 addition of live crickets to their diets, while singing is even induced when crickets are
90 only presented visually (Wikelski et al., 2000). In addition, Perfito et al. (2008)
91 showed in captive Lesser Sundas zebra finches (*Taeniopygia g. guttata*) that food
92 availability, in the form of seeds, is a more potent stimulus than increasing day length
93 in regulating testicular development. It thus appears that opportunistic breeders use
94 phenological cues alone or in combination with rainfall and/or temperature cues to
95 time the onset of breeding.

96

97 Strictly seasonal breeders may also use phenological cues, such as bud burst and
98 associated appearance of invertebrate prey, to fine-tune the timing of breeding to local
99 conditions, superimposed on an underlying seasonal reproductive pattern (Hahn,
100 1998; Hahn et al., 2008), particularly if the seasonality of their environment has an
101 unpredictable component (Wingfield et al., 1992). These cues may be dependent on
102 changes in ambient temperature, for example in insectivorous seasonal breeders
103 relying on a food peak in spring to rear their young (Both et al., 2004; Cresswell and
104 McCleery, 2003; Crick et al., 1997; Dhondt and Eyckerman, 1979; Kluyver, 1952;

105 Perrins, 1965; Perrins and McCleery, 1989; Schmidt, 1984; Sokolov, 2000; van
106 Balen, 1973; Visser et al., 1998; Visser et al., 2003). It is thus difficult to distinguish
107 between direct effects of increasing temperature (Schaper et al., 2011) and
108 phenological phenomena cues for timing the onset of breeding.

109

110 Possible phenological cues used by insectivorous seasonal breeders for reproductive
111 timing have been most extensively studied in great tits (*Parus major*) and blue tits
112 (*Cyanistes caeruleus*), which appear to respond to the leafing of trees and appearance
113 of caterpillars (Table 1). Egg-laying of great tits in Oxford is associated with oak bud
114 burst and the first appearance of caterpillars (Jones, 1972; Perrins, 1965; van
115 Noordwijk et al., 1995), while in some other European populations, it coincides with
116 the timing of birch (*Betula pubescens*) leafing (Slagsvold, 1976). In Switzerland,
117 laying dates of great tits correlate with the appearance of caterpillars in mixed forests
118 of conifer, beech (*Fagus sylvatica*), oaks (*Quercus spp.*) and hornbeam (*Carpinus*
119 *betulus*, Nager and van Noordwijk, 1995). In Swedish coastal and inland habitats
120 variation in laying dates of great and blue tits correlate with leafing phenology of oak
121 (*Q. robur*) and birch (*B. pendula*, Nilsson and Källander, 2006). In blue tit
122 populations in Corsica, living in broad-leaved deciduous downy oak (*Q. pubescens*) or
123 in evergreen Holm oak forests (*Q. ilex*), egg laying occurs at different times
124 depending on forest type, but regardless of forest type, there is a close correlation
125 between bud burst date and laying dates (Blondel et al., 1993; Bourgault et al., 2010).
126 In contrast to these studies, Visser et al. (2002) observed in great tits, in a ‘natural
127 experiment’ in the Netherlands, that the bud burst of pedunculate oaks (*Q. robur*) did
128 not correlate with the onset of egg laying, when it was delayed in 1992 by unseasonal
129 frost in the previous year.

130

131 A close relationship between spring phenology and laying date has also been observed
132 in some non-Parids. In song sparrows (*Melospiza melodia morphna*), in the Northwest
133 USA, differences in the timing of reproductive development between coastal and
134 montane populations can be best explained by an integration of temperature cues and
135 vegetation cues in the form of fresh shoots (Perfito et al., 2004). In Danish barn
136 swallows (*Hirundo rustica*), the onset of laying is also closely correlated with the
137 phenology of local vegetation, such as broad-leaved elm (*Ulmus glabra*) and
138 snowdrop (*Galanthus nivalis*), which is related to increased temperature (Møller,

139 2008). In contrast with these studies, in the pied flycatcher, a migratory insectivorous
140 passerine, seasonal vegetation phenology does not provide a strong cue for the timing
141 of breeding (Slagsvold, 1976, Table 1).

142

143 Only experiments under controlled conditions can answer questions about the causal
144 effects of supplementary phenological cues on the timing of seasonal avian
145 reproduction, and observations reported to date are conflicting. The timing of the
146 onset of laying in captive great or blue tits kept in outdoor aviaries has been compared
147 in a multi-site experiment carried out in the Netherlands, Sweden and Corsica (Visser
148 et al., 2002). In the Netherlands, the onset of breeding in pairs of great tits given
149 leafing pedunculate oak branches was not affected by the stage of development of
150 leaves (Visser et al., 2002). In Sweden, gonadal growth and concentrations of plasma
151 testosterone were the same in male great tits in the presence or absence of leafing
152 branches of birch (*B. pubescens*), although an increase in plasma luteinizing hormone
153 (LH) was advanced in the presence of branches (Visser et al, 2002). In contrast, in a
154 study of captive blue tits from two populations in Corsica, provided with phenological
155 cues from branches of downy oak (*Q. pubescens*), the most common tree in the
156 habitat of one population, or of evergreen Holm oak (*Q. ilex*), the most common tree
157 in the habitat of the other population, the laying dates were advanced in both
158 populations when provided with leafing evergreen oak (Visser et al., 2002). In a study
159 on song sparrows from the Northwest USA, differences in the timing of the onset of
160 laying in free living birds observed at different altitudinal temperatures were not
161 replicated in a laboratory study in which the birds were exposed to the same
162 temperatures, but not provided with phenological cues (Perfito et al., 2005). White-
163 crowned sparrows (*Zonotrichia leucophrys gambelii*) receiving green leaves of wheat
164 sprouts as a food additive for 20 days showed a significant increase in ovarian weight
165 compared to controls, even though their body weights, as well as testicular weights,
166 were not affected (Ettinger and King, 1981). Finally, in a study on wild island
167 canaries (*Serinus canaria*) held under short day conditions, the onset of breeding was
168 advanced after exposure to green grass (*Poa pratensis*), bamboo (*Phyllostachys*
169 *aureosulcata*) and white spruce (*Picea glauca conica*), but not after exposure to
170 simulated rainfall (Voigt et al., 2007).

171

172 Spring phenology could influence the onset of laying in several ways. First, if the
173 onset of reproduction is energy-limited, the increase in prey abundance and diversity
174 might provide energy and nutrient resources to build up reproductive tissues, thus
175 facilitating an early onset of laying. The effect of food availability on the onset of
176 breeding may be dependent on the appropriate ecological conditions (Bourgault et al.,
177 2009). Pre-breeding food supplementation experiments in single brooded passerines
178 have produced ambiguous results, either showing no effect, or advancing the onset of
179 laying by no more than one week (Harrison et al., 2010; Meijer and Drent, 1999; but
180 see e.g. Scheuerlein and Gwinner, 2002). Secondly, spring phenology could influence
181 the onset of laying through changes in the composition of the bird's diet, adding
182 chemical compounds that speed up reproductive development. This possibility is
183 suggested by an observation in montane voles (*Microtus montanus*), in which
184 testicular development and mating is stimulated by 6-methoxybenzoxazolinone (6-
185 MBOA), a substance found in growing seedlings eaten by the animals in spring
186 (Berger et al., 1981; Berger et al., 1987). It is possible that birds ingest similar
187 secondary chemical compounds while feeding on buds of deciduous trees in early
188 spring (Betts, 1955), which might affect their reproductive system in a comparable
189 way. However, Bourgault et al. (2006) investigated the amount of oak bud scale
190 remains in blue tit gizzards and concluded that a consumption of large amounts of
191 buds does not occur before egg laying. Lastly, temperature-dependent vegetation and
192 invertebrate phenology might accelerate the onset of laying by providing a visual
193 stimulus that is translated into a reproductive neuroendocrine response, comparable to
194 the effect of photostimulation in early spring (Ball and Ketterson, 2008; Hahn et al.,
195 1997; Moore et al., 2006; Stevenson et al., 2008). It thus seems likely that
196 phenological cues providing a visual stimulus may be used to fine-tune the onset of
197 reproduction in a photoperiodic seasonal breeder, such as the great tit (*Parus major*).

198

199 In order to test the hypothesis that vegetation and invertebrate phenology might
200 advance the onset of laying by providing a visual stimulus, it is first necessary to
201 identify a suitable temperature-dependent cue and an appropriate measure of
202 reproductive neuroendocrine response. If birds have evolved to adjust their
203 reproductive timing to vegetational cues, these cues should reliably provide
204 information on the future timing of an invertebrate food peak. After dormancy release,
205 deciduous trees of mature forests, e.g. oaks, respond to increased photoperiod in

206 spring, which is modulated by temperature. In contrast, many short-lived, early
207 successional trees, e.g. birches, are primarily temperature-sensitive (Körner and
208 Basler, 2010). The leafing of these trees marks the onset of spring in temperate zones
209 (Chmielewski and Rotzer, 2001), and is thus available to insectivorous birds as a cue
210 integrating past temperature patterns and predicting the temperature-dependent
211 hatching of lepidoptera caterpillars.

212

213 We experimentally investigated whether great tits make use of phenological cues from
214 birch, since the leafing of birch branches coincides with the beginning of egg-laying
215 of great tits in the natural population used in our study (see Fig. 1 for details). The
216 development of vegetation in early spring promotes an increase in invertebrate food
217 sources, especially caterpillars feeding on developing leaves (Buse and Good, 1996;
218 van Dongen et al., 1997). Therefore, caterpillars were also presented as a visual
219 phenological cue. It was predicted that reproductive development and onset of laying
220 of breeding pairs provided with these supplementary cues would advance relative to
221 control pairs. The causal reproductive neuroendocrine response was assessed in both
222 sexes by measuring changes in the concentrations of plasma luteinizing hormone,
223 which correlate with increasing gonadal activity and the onset of breeding in blue tits
224 exposed to natural lighting (Caro et al., 2006).

225

226

227

MATERIALS AND METHODS

228

229

Experimental birds & housing

230 Eighty great tits from a long-term study population at the Hoge Veluwe (the
231 Netherlands) were taken into captivity as nestlings in 2008. Broods were selected
232 from early- or late-laying maternal lines (Schaper et al., 2011). All chicks were blood
233 sampled, sexed (Griffiths et al., 1998), and extra-pair offspring identified (Saladin et
234 al., 2003) prior to brood-choice. On day 10 post-hatching, chicks were taken to the
235 Netherlands Institute of Ecology (Heteren) for hand-raising (Drent et al., 2003). After
236 independence they were kept in single-sex groups in open outdoor aviaries (2 x 4 x
237 2.5 m). The birds were fed *ad libitum* with a constant daily amount of food, consisting
238 of a mixture of minced beef, proteins and vitamins, complemented by sunflower
239 seeds, fat balls, a mix of dried insects (Carnizoo, Kiezebrink International, Putten, the

240 Netherlands), proteins, vitamin and mineral supplements (Nekton S and Nekton Bio,
241 NEKTON GmbH, Pforzheim, the Netherlands), calcium and water for drinking and
242 bathing. In December 2008, 36 breeding pairs were transferred to climate controlled
243 aviaries. During the 2009 breeding season the birds were kept under naturally
244 increasing photoperiod and either on an average temperature of 14°C or 8°C, which
245 did not affect the onset of laying (Schaper et al., 2011). The birds were moved back to
246 outdoor aviaries in December 2009, kept in single-sex groups over winter and sixteen
247 pairs were reformed again in spring for their second breeding season in 2010. These
248 pairs had bred together in 2009, except in two cases where the females were paired
249 with a new mate as their original mates had died. Two pairs did not lay eggs in 2009,
250 but bred successfully in the experiment reported here.

251

252 The breeding pairs were housed in two rows of outdoor aviaries from January 2010
253 onwards. One side of the aviary complex opened to a grass field, while the birds from
254 the other aviary row could see a hedge, mainly consisting of elder (*Sambucus nigra*)
255 and hawthorn (*Crataegus sp.*) at about 15 m distance. In 2010, all birds were kept
256 under natural temperature and day light conditions. Lighting was supplemented by
257 two 1 tubular lights which were on for two hours after sunrise to compensate the
258 shading effect of the aviary roof. The aviaries offered a choice of four nest boxes.
259 Moss as nesting material was provided from mid-February onwards.

260

261

Treatments

262 Pairs of birds were randomly and equally assigned to a control and a treatment group
263 in the two rows of aviaries. To simulate an early onset of spring, the birds were
264 provided with leafing birch (*B. pendula*) branches and caterpillars to simulate the
265 availability of prey in the environment. Phenological cues were added from March 9th
266 until the end of May. During this period day length increased from 11 h 18 min to 16
267 h 21 min. The cues consisted of branches, which had been kept at room temperature
268 for one week until an advanced bud burst occurred. Five branches about 1.5 m long
269 with just unfolding leaves were provided for each breeding pair and replaced twice
270 weekly. In addition, a covered transparent 20 cm Petri dish was placed on a feeding
271 table in the centre of each aviary, containing about 20 caterpillars of the great cabbage
272 white (*Pieris brassicae*) at larval instars 2-3 on a cabbage leaf. The larvae were
273 replaced weekly, after they developed into instars 3-4.

274

275 Birds from the control group received undeveloped birch branches with tightly closed
276 buds, and for an equivalent cage enrichment, paper ‘leaves’ were added consisting of
277 4x4 cm red and blue cardboard squares slid over the branches. Control branches were
278 rotated twice a week to simulate branch replacement and torn ‘leaves’ were replaced.
279 As an equivalent to the presentation of caterpillars, small twigs, which could freely
280 roll around, were placed in the Petri dishes. The birds made extensive use of both the
281 birch branches with young leaves and the control branches with paper ‘leaves’ by
282 climbing in them, and pecking and destroying buds and leaves. It is likely that birds
283 from the treatment group regularly consumed buds and leaves. The birds were also
284 attracted to the caterpillars and in few cases succeeded in opening the Petri dishes to
285 eat them. It was therefore concluded that the caterpillars provided a satisfactory food
286 cue.

287

288

Measurements

289 Nest boxes were checked daily for eggs. The day that the first egg was found is
290 referred to as the laying date. Blood samples of 100 µl were taken from the jugular
291 vein every two weeks for luteinizing hormone (LH) analysis. Additionally, an initial
292 sample was taken a week prior to the provisioning of phenological cues. Plasma was
293 separated from red blood cells and stored at -80°C. Plasma LH concentrations were
294 determined using a chicken LH radioimmunoassay (Sharp et al., 1987) validated for
295 use in blue tits (Caro et al., 2006). The assay reaction volume was 60 µl comprising
296 20 µl plasma sample or standard, 20 µl primary antibody (rabbit anti-chicken LH),
297 and 20 µl of I¹²⁵-labeled chicken LH. The primary antibody was precipitated to
298 separate free and bound I¹²⁵ label using 20 µl of donkey anti-rabbit precipitating
299 serum and 20 µl of non-immune rabbit serum. The samples were measured in a single
300 assay, in duplicate. The intra-assay coefficient of variation was 6.4% for a high value
301 plasma pool and 8.1% for a low value plasma pool, and the minimum detectable dose
302 0.15 ng/ml.

303

304

Statistics

305 Laying dates in 2010 were analysed with linear models in R 2.10.0 (R Development
306 Core Team, 2009), including phenology treatment, as well as laying dates of the
307 female’s and male’s mother in the wild (a measure of genetic disposition for early or

308 late laying) and laying date of the pair in the previous year as covariates. LH data
309 were log-transformed to achieve normality and analysed in general linear models for
310 females and males separately. First, we tested whether initial LH concentrations
311 differed between treatment groups. Second, we tested if plasma LH concentrations
312 increased over time. Third, we tested if the seasonal change in LH following the
313 addition of phenological cues differed between the groups in a mixed model with bird
314 identity as a random factor (procedure lmer, package lme4). Fourth, we tested in a
315 general linear model whether the rise in plasma LH two weeks after addition of
316 phenological cues was different between treatment groups, as plasma LH
317 concentrations can increase within days of exposure to a stimulatory cue (Meddle and
318 Follett, 1995; Wingfield et al., 1997). Explanatory variables were week of
319 measurement (as a factor), phenology treatment, as well as the interaction between the
320 two. Fifth, we tested in a linear model whether LH concentrations at the end of April
321 were related to laying dates.

322

323

324

RESULTS

325 Initial LH concentrations did not differ between phenology treatment and control
326 groups at the start of the experiment (females: $t_{1,16}=0.15$, $P=0.88$, males: $t_{1,16}=1.23$,
327 $P=0.24$, Fig. 2 A,B). In both treatment and control groups, plasma LH increased with
328 time (females: sampling week: $\chi^2_1=27.5$, $P<0.001$; males: sampling week: $\chi^2_1=12.8$
329 $P<0.001$). Two weeks after the start of the experiment, compared to initial values, the
330 difference in LH concentrations was not affected by the addition of phenological cues,
331 (females: $t_{1,16}=1.45$, $P=0.17$, males: $t_{1,16}=1.33$, $P=0.21$, Fig. 2 A,B). However, while in
332 females there was no interaction between treatment and sampling date on the increase
333 in plasma LH (treatment*sampling week: $\chi^2_3=4.61$, $P=0.20$, treatment: $\chi^2_1=1.48$,
334 $P=0.22$, sampling week: $\chi^2_3=22.3$, $P<0.001$, Fig. 2 A), in males there was a significant
335 interaction (treatment*sampling week: $\chi^2_3=11.29$, $P=0.010$). In males exposed to
336 phenological cues, LH concentrations were already near their maximum in early
337 spring, just after the addition of phenological cues, while concentrations in control
338 males increased more slowly, with the steepest rise in late April (Fig. 2 B). Females
339 with higher LH concentrations at the end of April tended to lay earlier ($t_{1,14}=-2.06$,
340 $P=0.062$, Fig. 3).

341

342 One male of a pair given phenological cues died, and one female of a pair also given
343 phenological cues died after laying her first egg, The remaining male was transferred
344 to breed with the remaining female, which started laying 11 days later. Her laying
345 date was included in the analysis. However, one female of the control group was ill
346 and did not lay. Another female of the phenology group started laying extremely late
347 on June 11th, which was considered to be too abnormal to be a consequence of the
348 experimental design and was therefore excluded from subsequent analysis (Grubb's
349 test for outliers: $G=2.7$, $p=0.008$).

350

351 Laying commenced on May 1st, approximately eight weeks after the birds were
352 allocated to treatment or control groups. The onset of laying was not advanced by
353 exposure to leafing birch branches and caterpillars (treatment: $t_{1,14}=-0.40$, $P=0.71$, Fig.
354 3). Neither the genetic background of the female (laying date of female's mother:
355 $t_{1,14}=-1.38$, $P=0.20$) nor the male (laying date of male's mother: $t_{1,14}=1.68$, $P=0.13$)
356 influenced laying date. The onset of laying in 2010 in outdoor aviaries correlated with
357 onset of laying in 2009 in indoor climatized aviaries under standardized conditions
358 ($t_{1,12}=3.73$, $P=0.004$, Fig. 4), which means that individual females laid consistently
359 early or late in both years independent of supplementary cues.

360

361

362

DISCUSSION

363 Vegetation phenology and food abundance have often been suggested as proximate
364 supplementary cues in avian timing of reproduction, but there is little evidence for
365 causality, especially in seasonal breeders. In the current experiment great tits were
366 exposed to phenological cues that are naturally present in their environment at the
367 time of egg laying and are strongly affected by temperature. Contrary to prediction,
368 exposure to leafing birch branches and caterpillars did not advance the onset of laying
369 in great tits housed in outdoor aviaries exposed to natural light and temperature where
370 birds had access to *ad libitum* food. This observation is consistent with an earlier
371 study showing no effect of developing oak and birch branches on the timing of
372 reproduction in captive great and blue tits (Visser et al., 2002). The lack of an effect
373 of phenological cues in these earlier studies is therefore not a consequence of
374 inhibitory cues associated with, for example, indoor caging. The failure to

375 demonstrate an effect of phenological cues on the onset of laying is in contrast with
376 many observations in free living bird populations, which imply, or suggest, that the
377 correlation between either bud burst or food phenology and the onset of laying or
378 reproductive activity is causal (see Introduction for references). The interpretation of
379 earlier studies now requires critical re-assessment bearing in mind the following.

380

381 First, some experiments measure reproductive development without reporting laying
382 dates in response to environmental cues. These experiments do not take into account
383 the possibility that a given phenological cue may not affect ovarian development, but
384 instead, the laying decision itself. This decision is made by the female (Caro et al.,
385 2009), which may be responsive to supplementary cues that differ from those
386 recognized by males (Ball and Ketterson, 2008). In less favourable conditions than
387 used in the present study, captive females often do not lay while males tend to show
388 full gonadal maturation, which is why most experimental work has been restricted to
389 males. The observation that females may not show full gonadal development under
390 captive conditions indicates that cues additional to increasing photoperiod are
391 required for the initiation of egg laying, which might be phenological or social cues.
392 Researchers need to critically investigate if the choice of physiological measures used
393 to deduct changes in reproductive timing in response to a likely cue is appropriate.

394

395 Secondly, leafing date of, for example, the tree species hosting lepidoptera prey, or
396 caterpillar emergence itself, is a standard phenological measure used to predict the
397 timing of avian breeding (Table 1). Selection for synchrony with the food peak
398 facilitates this correlation, but the bud burst of e.g. oak trees often commences late in
399 spring, sometimes after the onset of egg laying and can therefore not be considered a
400 predictive cue (Visser et al., 2002). It thus requires careful observations of natural
401 systems to identify cues that are both relevant, in terms of predictability of future
402 events, and timed in advance of changes in the phenological trait under investigation.

403

404 Thirdly, the correlation between temperature, tree phenology and insect abundance
405 excludes any inference of the causal relationship between any one of these cues and
406 the timing of reproduction under natural conditions. Even though many studies report
407 on relationships between phenological cues and laying dates (see Introduction), there

408 is little experimental evidence for a causality, which should be a focus of future
409 efforts.

410

411 In females, phenological cues did not affect the photoperiodically-dependent seasonal
412 increase in luteinizing hormone (LH). In males receiving phenological cues, LH
413 concentrations were coincidentally high from the beginning onwards, but did not
414 increase much over time after the addition of cues. In contrast, control males showed
415 a rise to levels similar to males from the treatment group over two months time. One
416 can only speculate what would have happened if initial LH values in males from the
417 phenology treatment group would have been lower, but given the hormonal
418 development in females we would not expect a difference between experimental
419 groups.

420

421 Unfortunately, at the moment there is no assay for avian follicle-stimulating hormone
422 (FSH) available, the gonadotropin directly inducing follicle maturation, restricting
423 researchers to measure LH instead. It is therefore possible that FSH, and not LH,
424 could be the mediator for the integration of phenological cues, but as here we found
425 no effect of vegetation cues on the timing of laying itself, we would not expect
426 different results for FSH.

427

428 From an ecological point of view, the functional significance of higher LH plasma
429 concentrations in males exposed to predictive environmental cues in early spring is
430 uncertain. As the development of the male reproductive system is preceding the one
431 of the female, it is less likely for males to show an adaptive response to phenological
432 cues to fine-tune gonadal development. Yet, in an opportunistic breeder, the rufous-
433 winged sparrow (*Aimophila carpalis*), environmental factors associated with summer
434 rains stimulated both GnRH synthesis and LH secretion in males, which was,
435 however, unrelated to gonadal growth earlier in the season (Small et al., 2008).
436 Similarly, the higher LH concentrations reported in Visser et al. (2002) did not induce
437 a greater increase in testis size, and also in the present experiment there was only a
438 weak correlation between female LH concentrations and the actual laying date. These
439 findings demonstrate that different components of the hypothalamo-pituitary-gonadal
440 axis might be influenced by various supplementary cues in different species. In

441 addition, measuring the actual laying decision of the female is crucial to drawing
442 conclusions about timing of breeding.

443

444 As there was no effect of spring vegetational cues on the timing of reproduction in
445 great tit females, it seems that the between-year variation in laying dates is triggered
446 directly by temperature, which thus causes the correlation between birch bud burst
447 and the onset of laying in the wild population (Fig. 1). In recent years, warmer springs
448 advanced both the leafing of birches, as well as the egg laying in great tits. The results
449 of this experiment support a study by Schaper et al. (2011), who recently showed that
450 different patterns of increasing spring temperatures, rather than mean temperature
451 itself, affected the onset of egg laying differently for early- and late-laying female
452 great tits from the same population used in this setup, implying genetic differences in
453 sensitivity to temperature cues. The current experiment thus indicates that sensitivity
454 to early spring vegetation, or food cues, plays only a minor role in fine-tuning the
455 onset of egg-laying.

456

457 Besides influencing the decision when to lay, temperature can also affect the
458 photoinduced timing of gonadal growth, which has been shown for white-crowned
459 sparrows (*Zonotrichia leucophrys*, Wingfield et al., 2003; Wingfield et al., 1997). To
460 date, possible pathways that can accommodate this temperature effect, which might
461 act on a physiological level or as a proximate cue, remain to be discovered. Low
462 temperatures might also limit the speed of gonadal maturation by increasing the daily
463 energy expenditure under natural conditions where food is scarce (Perrins, 1970;
464 Stevenson and Bryant, 2000). In captive great tits, however, we did not observe an
465 effect of ambient temperature regulating gonadal growth (Schaper et al. 2011).

466

467 The high repeatability in the timing of laying between 2009 and 2010 in individual
468 pairs, irrespective of whether they were early or late layers, supports findings by
469 Visser et al. (2009) that laying dates of great tits in climate controlled aviaries are
470 closely correlated with laying dates of the same females under natural conditions. This
471 consistency again stresses a genetic component in the mechanisms underlying the
472 timing of reproduction, which could well be sensitivity to environmental cues, such as
473 photoperiod or temperature (Visser et al., 2011), but is apparently not related to
474 phenological cues.

475

476 In 2009, the birds in this study bred in climate controlled aviaries (Schaper et al.,
477 2011) and in 2010 bred again exposed to more natural conditions in open aviaries.
478 Against expectations, egg laying commenced later in 2010 than in 2009, even though
479 second-year breeders normally lay earlier than first-year breeders and additional
480 environmental information, also in form of vegetational growth, was available to the
481 birds in outdoor aviaries. Part of this effect could be attributed to the lower light levels
482 caused by the roofing in the outdoor aviaries, as the increase in day length is the
483 primary cue for timing of reproduction. However, this is unlikely, as supplementary
484 light was provided in outdoor aviaries. A different explanation could be that birds
485 experienced colder night conditions in 2010 than in climate-controlled aviaries in
486 2009, which delayed the onset of laying relative to the previous year.

487

488 In conclusion, both from previous work and from the experimental observations
489 presented here, there is little direct evidence for effects of tree phenology or presence
490 of lepidopteran prey on the onset of reproduction in great tits. Nonetheless several
491 studies reported close correlations between tree phenology and laying dates of both
492 opportunists and seasonal breeders in the field. Experimental work on a range of
493 species is needed to further investigate if those potential proximate cues assumed to
494 advance, or even induce breeding are really causal for the timing of reproductive
495 development. This is one of few studies examining direct effects of phenological cues
496 on both male and female reproductive development, as well as egg laying under
497 controlled conditions. More thorough physiological work concentrated on the
498 reproductive development and behavioural decisions of the female is needed to
499 investigate in how far seasonal breeders make use of phenological cues. It is likely
500 that, at least in great tits, the correlation between spring phenology and onset of laying
501 is mediated by other proximate factors, such as direct temperature cues stimulating
502 both vegetation growth and avian breeding.

503

LIST OF ABBREVIATIONS

504

505

506 Note: All abbreviations have been fully explained in the text/head of the table.

507

508 Main text: LH = luteinizing hormone

509 FSH = follicle-stimulating hormone

510 Table 1: gran. = granivorous

511 insect. = insectivorous

512 obs. = observational study

513 exp. = experimental study

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516

517

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Figure legends

Fig. 1: Laying dates of great tits in the wild in relation to birch bud burst

Laying dates of great tits of the Hoge Veluwe population in relation to birch (*Betula pendula*) bud burst dates, defined as the stage when green tips of leaves are unfolding. The analysis was restricted to years (see labels in the graph) in which at least ten birches from long-term monitoring sites were scored twice a week (range 10-51 trees). The exact laying dates based on daily nest visits only encompass first clutches. If including the exceptionally late year 1992 (see the discussion of Visser et al. 2002 in the Introduction), the relationship is marginally non-significant (linear model, $t_{1,7} = 2.34$, $P = 0.058$), while without 1992 bud burst is predicting laying dates well (linear model, $t_{1,6} = 3.32$, $P = 0.021$). Means \pm standard errors are given. Note the advancement of laying in recent years 2009 and 2010 relative to the birch bud burst.

Fig. 2: Luteinizing hormone development

Luteinizing hormone (LH) concentrations measured in female (A) and male (B) great tits either with access to leafing birch branches and visual cues of caterpillars (closed diamonds, straight line) or with access to undeveloped branches and visual cues of pieces of twigs (open dots, broken line). Arrows indicate the addition of cues. Means \pm standard errors are given.

Fig. 3: Luteinizing hormone concentration and laying date

Relationship between female luteinizing hormone (LH) concentrations at the end of April (21.4.) and laying date. Females with access to leafing birch branches and visual cues of caterpillars are given as closed diamonds, those with access to undeveloped branches and visual cues of pieces of twigs as open dots. Laying dates are given as April days, where 1 = 1st of April.

Fig. 4: Relationship between laying date 2009 and 2010

Laying dates per pair of great tits breeding in climate-controlled aviaries in 2009 and in outdoor aviaries in 2010. Females with access to leafing birch branches and visual cues of caterpillars are given as closed diamonds, those with access to undeveloped branches and visual cues of pieces of twigs as open dots. Laying dates are given as April days, where 1 = 1st of April.

Table 1: Review of selected publications reporting effects of spring phenology on the seasonal timing of reproductive development and egg laying in both

a) seasonal opportunists and b) strictly seasonal breeders. gran.=granivorous, insect.=insectivorous, obs.=observational study, exp.=experimental study.

bird species	food	cue	behavioural or physiological measure	study	reference
a) seasonal opportunists					
Pinon jays (<i>Gymnorhinus cyanocephalus</i>)	gran.	green cones of Pinon pines	breeding commences in summer when cones are present	obs.	Ligon 1978
zebra finches (<i>Taeniopygia guttata</i>)	gran.	rainfall	hatching coincides with ripening of grass seeds after rain	obs.	Zann et al. 1995
red crossbills (<i>Loxia curvirostra</i>)	gran.	cones of hemlock	breeding commences when cones are present	obs.	Hahn 1998
Darwin's ground finches (<i>Geospiza spec.</i>)	insect.	rainfall	breeding commences after rainfall	obs.	Hau et al. 2004
Darwin's ground finches (<i>Geospiza spec.</i>)	insect.	rainfall	rainfall-related noise stimulates singing	exp.	Grant 1999
spotted antbirds (<i>Hylophylax n. naevioides</i>)	insect.	addition of live crickets to diet	faster testis growth when live crickets present	exp.	Wikelski et al. 2000
spotted antbirds (<i>Hylophylax n. naevioides</i>)	insect.	visual cues of live crickets	increased song rates when crickets visible	exp.	Wikelski et al. 2000
zebra finches (<i>Taeniopygia g. guttata</i>)	gran.	food availability, day length	faster gonadal growth when unrestricted food present	exp.	Perfito et al. 2008
b) strictly seasonal breeders					
great tits (<i>Parus major</i>)	insect.	oak and caterpillar phenology	breeding coincides with oak and caterpillar phenology	obs.	Jones 1972
great tits (<i>Parus major</i>)	insect.	oak and caterpillar phenology	breeding coincides with oak and caterpillar phenology	obs.	Perrins 1965
great tits (<i>Parus major</i>)	insect.	tree phenology	breeding coincides with birch leafing	obs.	Slagsvold 1976
pied flycatchers (<i>Ficedula hypoleuca</i>)	insect.	tree phenology	breeding correlates only weakly with vegetation phenology	obs.	Slagsvold 1976
great tits (<i>Parus major</i>)	insect.	caterpillar phenology	breeding coincides with caterpillar phenology	obs.	Nager and van Noordwijk 1999
great tits (<i>Parus major</i>)	insect.	tree and caterpillar phenology	breeding coincides with oak and caterpillar phenology	obs.	van Noordwijk et al. 1995
great tits (<i>Parus major</i>)	insect.	oak phenology	no correlation between delayed oak bud burst and breeding	obs.	Visser et al. 2002
song sparrows (<i>Melospiza melodia morphna</i>)	insect.	temperature, emergence of shoots	testis growth coincides with presence of vegetational cues	obs.	Perfito et al. 2004
great tits (<i>Parus major</i>)	insect.	oak and birch phenology	breeding coincides with leafing phenology of oak	obs.	Nilsson and Källander 2006
blue tits (<i>Cyanistes caeruleus</i>)	insect.	oak and birch phenology	breeding coincides with leafing phenology of birch	obs.	Nilsson and Källander 2006
barn swallows (<i>Hirundo rustica</i>)	insect.	plant phenology	breeding coincides with leafing of elm and flowering of snowdrop	obs.	Møller 2008
blue tits (<i>Cyanistes caeruleus</i>)	insect.	oak bud burst	breeding coincides with oak bud burst	obs.	Bourgault et al. 2010
white-crowned sparrows (<i>Zonotrichia leucophrys</i>)	gran.	sprouted wheat leaves	ovary, but not testis development advanced by food supplement	exp.	Ettinger and King 1981
great tits (<i>Parus major</i>)	insect.	branches of pedunculate oaks	no correlation between development of branches and breeding	exp.	Visser et al. 2002

great tits (<i>Parus major</i>)	insect.	branches of downy birch	luteinizing hormone rise accelerated in presence of branches	exp.	Visser et al. 2002
blue tits (<i>Cyanistes caeruleus</i>)	insect.	branches of downy or evergreen oak	breeding advanced in presence of evergreen oak	exp.	Visser et al. 2002
island canaries (<i>Serinus canaria</i>)	gran.	simulated rainfall or vegetation	rainfall induces rise in testosterone and advances breeding	exp.	Voigt et al. 2007

Figure 1

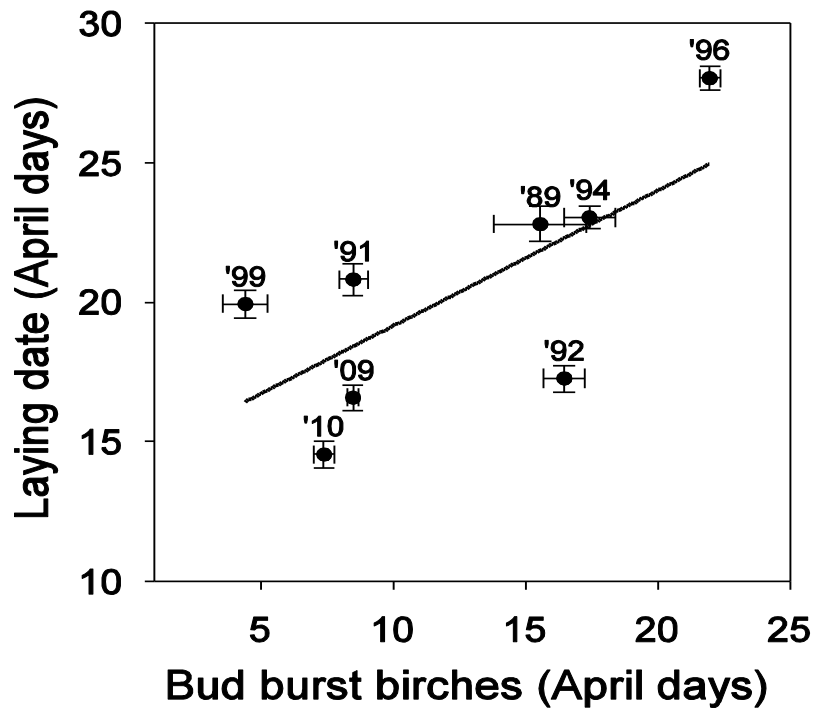


Figure 2

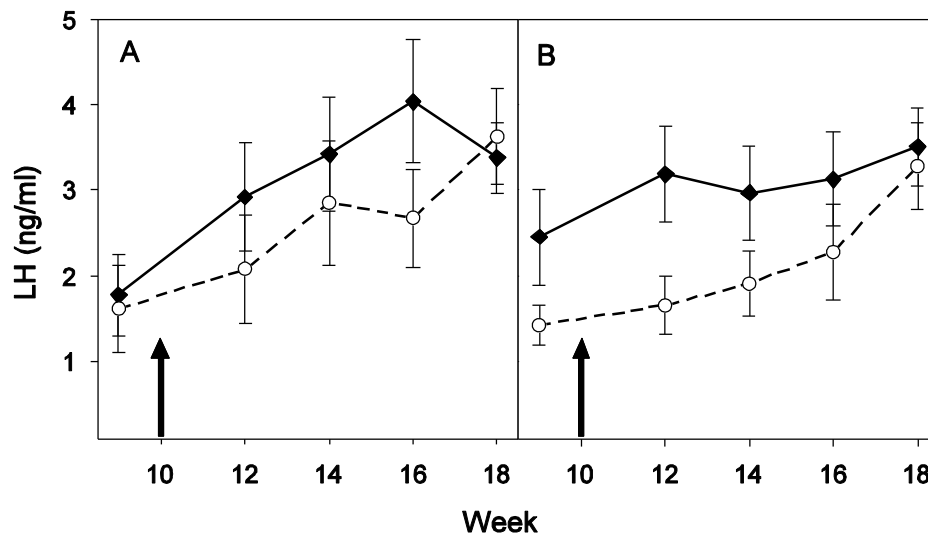


Figure 3

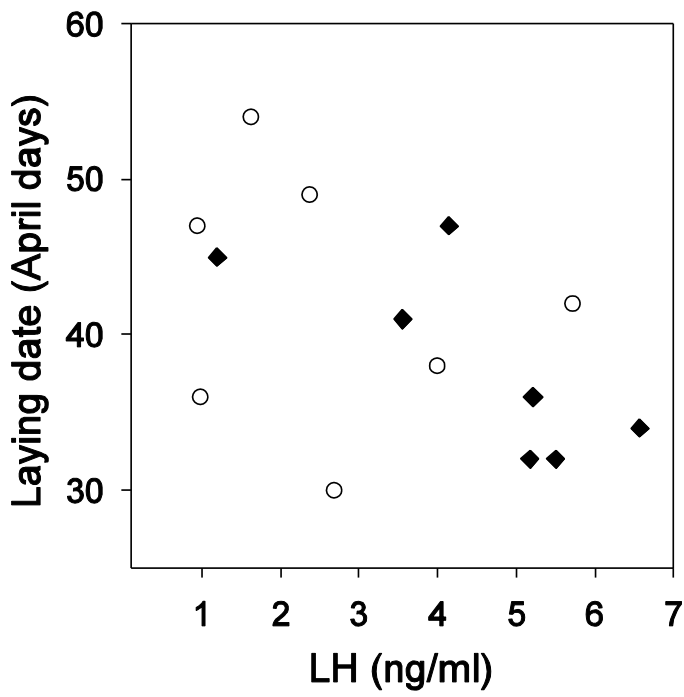


Figure 4

