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

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SUMMARY

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

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

Short title: Spring phenology and timing of laying

INTRODUCTION

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

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

Opportunistic breeding birds live in environments in which the distribution of their food supply fluctuates erratically, and thus they require great sensitivity to environmental cues predictive of increased food supply to stimulate reproductive activity (Hahn, 1998). Most studies have been done on birds living in arid regions with unpredictable rainfall (Table 1), and because of its importance for primary productivity, rainfall is still the climatic parameter most frequently analysed with regard to the onset of breeding (Barrientos et al., 2007). Rainfall stimulates the growth of vegetation resulting in the production of leaves, flowers and seeds, and these not only provide plant and associated invertebrate food to feed nestlings, but may also act as phenological cues for the initiation of breeding (Hahn et al., 2008). For example, in the granivorous zebra finch (*Taeniopygia guttata*) in central Australia, hatching coincides with the availability of grass seeds to feed nestlings after the onset of rain, with heavier rainfall resulting in longer breeding episodes, and repeated rainfall stimulating repeated breeding (Zann et al., 1995). A similar phenomenon is observed in Darwin's ground finches (*Geospiza spec*.) on the Galapagos Islands where hatching coincides with flushes of insect availability occurring after semi-seasonal rains (Hahn et al., 2008; Hau et al., 2004). However, in male Darwin finches, rainfall or even rainfall-related noise stimulates singing (Grant, 1999) and may therefore act directly as a proximate cue for reproduction.

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

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

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

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

A close relationship between spring phenology and laying date has also been observed in some non-Parids. In song sparrows (*Melospiza melodia morphna*), in the Northwest USA, differences in the timing of reproductive development between coastal and montane populations can be best explained by an integration of temperature cues and vegetation cues in the form of fresh shoots (Perfito et al., 2004). In Danish barn swallows (*Hirundo rustica)*, the onset of laying is also closely correlated with the phenology of local vegetation, such as broad-leaved elm (*Ulmus glabra*) and snowdrop (*Galanthus nivalis*), which is related to increased temperature (Møller, 2008). In contrast with these studies, in the pied flycatcher, a migratory insectivorous passerine, seasonal vegetation phenology does not provide a strong cue for the timing of breeding (Slagsvold, 1976, Table 1).

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

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

In order to test the hypothesis that vegetation and invertebrate phenology might advance the onset of laying by providing a visual stimulus, it is first necessary to identify a suitable temperature-dependent cue and an appropriate measure of reproductive neuroendocrine response. If birds have evolved to adjust their reproductive timing to vegetational cues, these cues should reliably provide information on the future timing of an invertebrate food peak. After dormancy release, deciduous trees of mature forests, e.g. oaks, respond to increased photoperiod in spring, which is modulated by temperature. In contrast, many short-lived, early successional trees, e.g. birches, are primarily temperature-sensitive (Körner and Basler, 2010). The leafing of these trees marks the onset of spring in temperate zones (Chmielewski and Rotzer, 2001), and is thus available to insectivorous birds as a cue integrating past temperature patterns and predicting the temperature-dependent hatching of lepidoptera caterpillars.

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

MATERIALS AND METHODS

Experimental birds & housing

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

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

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

Treatments

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

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

Measurements

Nest boxes were checked daily for eggs. The day that the first egg was found is referred to as the laying date. Blood samples of 100 μl were taken from the jugular vein every two weeks for luteinizing hormone (LH) analysis. Additionally, an initial sample was taken a week prior to the provisioning of phenological cues. Plasma was separated from red blood cells and stored at -80°C. Plasma LH concentrations were determined using a chicken LH radioimmunoassay (Sharp et al., 1987) validated for use in blue tits (Caro et al., 2006). The assay reaction volume was 60 μl comprising 20 μl plasma sample or standard, 20 μl primary antibody (rabbit anti-chicken LH), and 20 μl of I^{125}-labeled chicken LH. The primary antibody was precipitated to separate free and bound I^{125} label using 20 μl of donkey anti-rabbit precipitating serum and 20 μl of non-immune rabbit serum. The samples were measured in a single assay, in duplicate. The intra-assay coefficient of variation was 6.4% for a high value plasma pool and 8.1% for a low value plasma pool, and the minimum detectable dose 0.15 ng/ml.

Statistics

Laying dates in 2010 were analysed with linear models in R 2.10.0 (R Development Core Team, 2009), including phenology treatment, as well as laying dates of the female’s and male’s mother in the wild (a measure of genetic disposition for early or late laying) and laying date of the pair in the previous year as covariates. LH data were log-transformed to achieve normality and analysed in general linear models for females and males separately. First, we tested whether initial LH concentrations differed between treatment groups. Second, we tested if plasma LH concentrations increased over time. Third, we tested if the seasonal change in LH following the addition of phenological cues differed between the groups in a mixed model with bird identity as a random factor (procedure lmer, package lme4). Fourth, we tested in a general linear model whether the rise in plasma LH two weeks after addition of phenological cues was different between treatment groups, as plasma LH concentrations can increase within days of exposure to a stimulatory cue (Meddle and Follett, 1995; Wingfield et al., 1997). Explanatory variables were week of measurement (as a factor), phenology treatment, as well as the interaction between the two. Fifth, we tested in a linear model whether LH concentrations at the end of April were related to laying dates.

RESULTS

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

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

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

DISCUSSION

Vegetation phenology and food abundance have often been suggested as proximate supplementary cues in avian timing of reproduction, but there is little evidence for causality, especially in seasonal breeders. In the current experiment great tits were exposed to phenological cues that are naturally present in their environment at the time of egg laying and are strongly affected by temperature. Contrary to prediction, exposure to leafing birch branches and caterpillars did not advance the onset of laying in great tits housed in outdoor aviaries exposed to natural light and temperature where birds had access to *ad libitum* food. This observation is consistent with an earlier study showing no effect of developing oak and birch branches on the timing of reproduction in captive great and blue tits (Visser et al., 2002). The lack of an effect of phenological cues in these earlier studies is therefore not a consequence of inhibitory cues associated with, for example, indoor caging. The failure to demonstrate an effect of phenological cues on the onset of laying is in contrast with many observations in free living bird populations, which imply, or suggest, that the correlation between either bud burst or food phenology and the onset of laying or reproductive activity is causal (see Introduction for references). The interpretation of earlier studies now requires critical re-assessment bearing in mind the following.

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

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

Thirdly, the correlation between temperature, tree phenology and insect abundance excludes any inference of the causal relationship between any one of these cues and the timing of reproduction under natural conditions. Even though many studies report on relationships between phenological cues and laying dates (see Introduction), there is little experimental evidence for a causality, which should be a focus of future efforts.

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

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

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

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

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

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

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

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

LIST OF ABBREVIATIONS

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

Main text:	LH	= luteinizing hormone
	FSH	= follicle-stimulating hormone
Table 1:	gran.	= granivorous
	insect.	= insectivorous
	obs.	= observational study
	exp.	= experimental study

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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 ± 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 ± 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

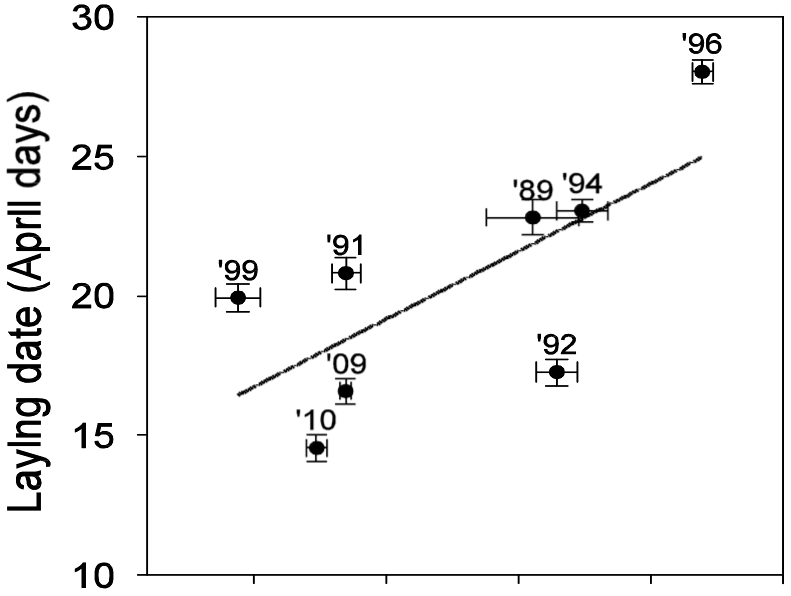
Laying dates per pair of great tits breeding in climate-controlled aviaries in 2009 and in outdoor aviaries in 2010. Females with access to leafing birch branches and visual cues of caterpillars are given as closed diamonds, those with access to undeveloped branches and visual cues of pieces of twigs 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 (*Gymnorhinus cyanocephalus*)	gran.	green cones of Pinon pines	breeding commences in summer when cones are present	obs.	Ligon 1978
zebra finches (*Taeniopygia guttata*)	gran.	rainfall	hatching coincides with ripening of grass seeds after rain	obs.	Zann et al. 1995
red crossbills (*Loxia curvirostra*)	gran.	cones of hemlock	breeding commences when cones are present	obs.	Hahn 1998
Darwin's ground finches (*Geospiza spec*.)	insect.	rainfall	breeding commences after rainfall	obs.	Hau et al. 2004
Darwin's ground finches (*Geospiza spec*.)	insect.	rainfall	rainfall-related noise stimulates singing	exp.	Grant 1999
spotted antbirds (*Hylophylax n. naevioides*)	insect.	addition of live crickets to diet	faster testis growth when live crickets present	exp.	Wikelski et al. 2000
spotted antbirds (*Hylophylax n. naevioides*)	insect.	visual cues of live crickets	increased song rates when crickets visible	exp.	Wikelski et al. 2000
zebra finches (*Taeniopygia g. guttata*)	gran.	food availability, day length	faster gonadal growth when unrestricted food present	exp.	Perfito et al. 2008
b) strictly seasonal breeders					
great tits (*Parus major*)	insect.	oak and caterpillar phenology	breeding coincides with oak and caterpillar phenology	obs.	Jones 1972
great tits (*Parus major*)	insect.	oak and caterpillar phenology	breeding coincides with oak and caterpillar phenology	obs.	Perrins 1965
great tits (*Parus major*)	insect.	tree phenology	breeding coincides with birch leafing	obs.	Slagsvold 1976
pied flycatchers (*Ficedula hypoleuca*)	insect.	tree phenology	breeding correlates only weakly with vegetation phenology	obs.	Slagsvold 1976
great tits (*Parus major*)	insect.	caterpillar phenology	breeding coincides with caterpillar phenology	obs.	Nager and van Noordwijk 199
great tits (*Parus major*)	insect.	tree and caterpillar phenology	breeding coincides with oak and caterpillar phenology	obs.	van Noordwijk et al. 1995
great tits (*Parus major*)	insect.	oak phenology	no correlation between delayed oak bud burst and breeding	obs.	Visser et al. 2002
song sparrows (*Melospiza melodia morphna*)	insect.	temperature, emergence of shoots	testis growth coincides with presence of vegetational cues	obs.	Perfito et al. 2004
great tits (*Parus major*)	insect.	oak and birch phenology	breeding coincides with leafing phenology of oak	obs.	Nilsson and Källander 2006
blue tits (*Cyanistes caeruleus*)	insect.	oak and birch phenology	breeding coincides with leafing phenology of birch	obs.	Nilsson and Källander 2006
barn swallows (*Hirundo rustica*)	insect.	plant phenology	breeding coincides with leafing of elm and flowering of snowdrop	obs.	Møller 2008
blue tits (*Cyanistes caeruleus*)	insect.	oak bud burst	breeding coincides with oak bud burst	obs.	Bourgault et al. 2010
white-crowned sparrows (*Zonotrichia leucophrys*)	gran.	sprouted wheat leaves	ovary, but not testis development advanced by food supplement	exp.	Ettinger and King 1981
great tits (*Parus major*)	insect.	branches of pedunculate oaks	no correlation between development of branches and breeding	exp.	Visser et al. 2002

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

Figure 1



Laying date (April days)
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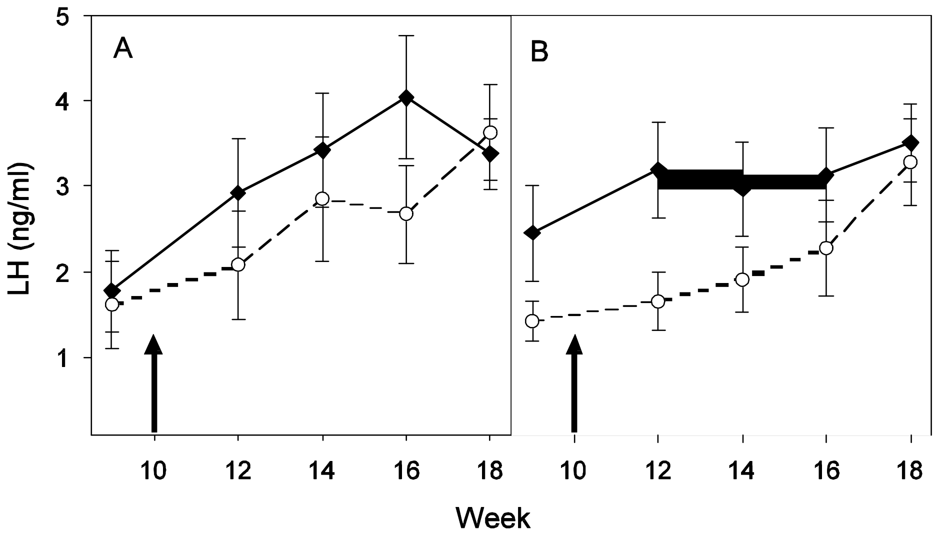

LH (ng/ml)
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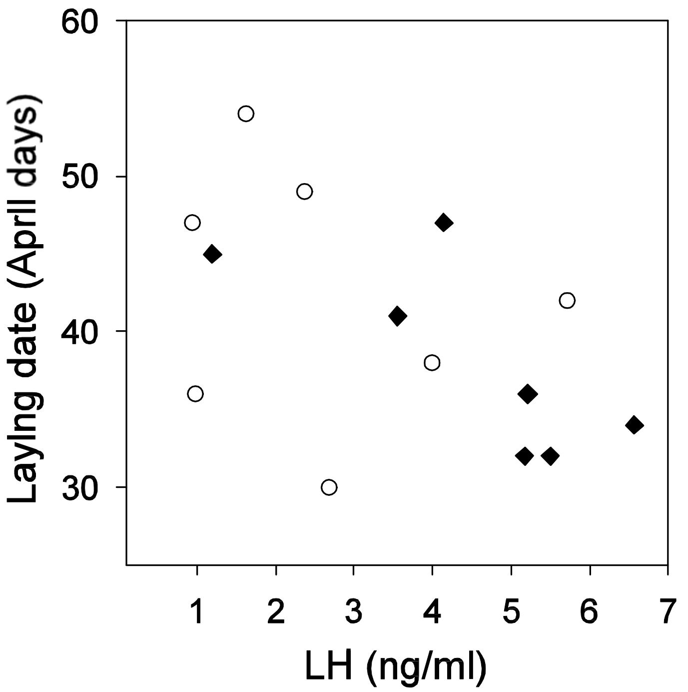

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Laying date (April days)
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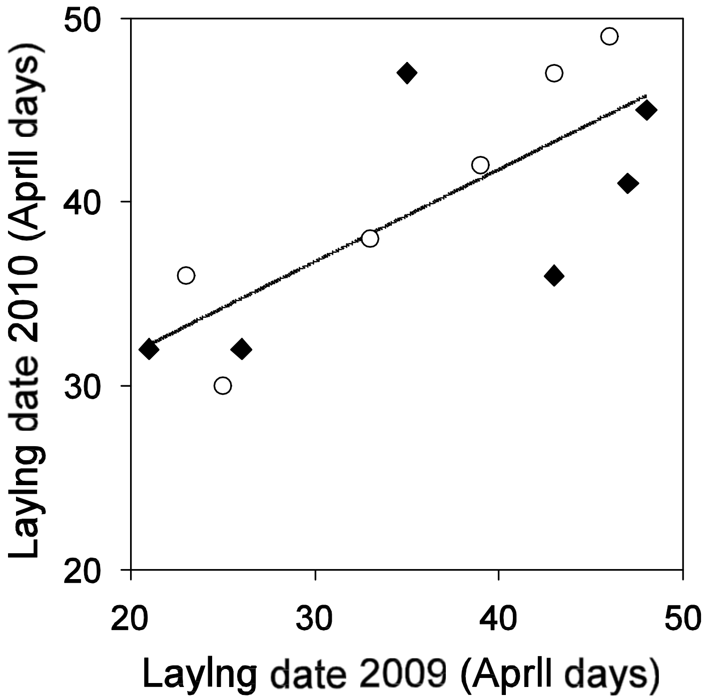
7
LH (ng/ml)



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Laying date 2010 (April days)
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Laying date 2009 (April days)