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Seasonal timing in a warming world

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CHAPTER 6

MANIPULATION OF THE HYPOTHALAMIC PHOTOPERIODIC PERCEPTION AFFECTS GONADAL GROWTH BUT DOES NOT ADVANCE TIMING OF EGG-LAYING IN THE GREAT TIT

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Submitted

Abstract

Increasing spring temperatures have advanced phenology at different rates for species at different trophic levels, causing a phenological mismatch in many food chains. In the great tit (*Parus major*) the phenological match between the highest food demand of the offspring and the peak in caterpillars' biomass has been disrupted and as a consequence there is directional selection for earlier timing of reproduction. A response to this selection may be hampered by the elevated costs of earlier breeding. To test this hypothesis, wild birds need to be manipulated to start reproduction earlier in the season to assess the fitness consequences of earlier egg-laying. We attempted to advance timing of reproduction by modifying the photoperiodic perception in a free-living population of great tits in three consecutive years (2012-2014). As photoperiod, perceived through deep brain photoreceptors, is an important cue for initiating reproduction, we increased the light intensity by clipping head feathers. As a result of the manipulation, at sunrise and sunset, clipped birds received higher light intensities than control birds thereby perceiving a longer day length. The manipulated birds are expected to develop their gonads faster and lay their eggs earlier. We monitored gonadal size in the laboratory from March to May and timing of egg-laying in birds in the wild. The feather clipping treatment increased the intensity of light passing through the skull and gonadal size was indeed larger in the clipped birds in May. Egg-laying date in the wild was however not affected by treatment in any of the three experimental years. These results suggest that manipulating photoperiodic perception changes physiological mechanisms underlying reproduction, such as gonadal growth, but these changes do not directly translate into an earlier egg-laying date in the wild.

Introduction

Timing of various biological processes such as reproduction, migration or hibernation is crucial to the survival and reproductive success of an individual. Nowadays, particularly in temperate regions, rapid large-scale climate change (IPCC 2013) is altering phenologies of many organisms (Cotton 2003; Parmesan *et al.* 2003). Trends of phenological changes vary markedly among taxonomic groups. Secondary consumers have advanced their phenology less than primary producers over the past few decades (Thackeray *et al.* 2010). As a consequence, phenological mismatches between species at different trophic levels have occurred in many ecosystems (Visser *et al.* 2005). These developing asynchronies have been linked to reductions in individual fitness and declines in the population size (Both et al. 2006; Møller et al. 2008). When organisms become mismatched to their food supply, evolutionary theory predicts that natural selection will act on adjusting their timing in order to adapt to the new environmental conditions.

Seasonal timing of biological events, such as reproduction or migration, requires physiological and developmental preparations to be made well in advance of the actual event. To anticipate the favourable (and unfavourable) conditions organisms need to use information from the environment. In birds, photoperiod is the best-known cue used to initiate the physiological and behavioural cascade that leads to reproduction (Farner 1964; Bentley *et al.* 1998; Dawson *et al.* 2001; Bradshaw *et al.* 2007) . The duration of daylight provides indication of the time of the year. In spring the increase of day length leads to development of gonads (which are usually regressed outside the breeding period), hormone changes, initiation of courtship behaviour, nest building and ultimately egg-laying (Lofts *et al.* 1968; Murton *et al.* 1977; Wingfield *et al.* 1991; Silverin 1994; Reparaz *et al.* 2014).

Differently from mammals, in birds the photoperiodic information is not only perceived by the eyes but there are also photoreceptors located in the deep brain. In 1935 Benoit reported the first evidence of a deep brain photoreceptor to regulate seasonal reproduction in ducks. Blinded ducks developed their gonads, while ducks covered with black caps on their head did not (Benoit 1935). Extra evidence for a decisive role of extra-retinal photoreceptors was collected by Menaker and colleagues (Menaker 1968; Menaker *et al.* 1968; Underwood *et al.* 1970). For instance, sparrows with head feathers plucked grew their gonads whereas birds that were subcutaneously injected with India ink under their scalp did not show any reproductive photoresponse (Menaker *et al.* 1970). In Japanese quails, both pinealectomized and blinded individuals showed reproductive response to photic stimuli (Sayler *et al.* 1968; Siopes *et al.* 1974). Finally, recent studies have experimentally demonstrated the hypothalamic photoreceptors to be photosensitive (Nakane *et al.* 2014). The photoperiodic photoreception pathway in the avian brain has been localized to the cerebro-spinal fluid touching neurons located lateral to the hypothalamic third ventricle in the paraventricular organ. These neurons show a single outersegment which contain neuropsin (OPN5) as the functional photopigment (Nakane *et al.* 2014). Neuropsin has been reported to be bistable (Yamashita *et al.* 2010; Kojima *et al.* 2011) and action spectra were measured (or predicted) peaking at 380nm for mouse (Kojima *et al.* 2011), 380nm for human (Kojima *et al.* 2011), 415nm for chicken (Yamashita *et al.* 2010) and 419nm for quail (Nakane *et al.* 2010).

Based on this knowledge, here we experimentally manipulated the hypothalamic photoperiodic perception in great tits in order to advance reproduction and to measure fitness consequences of earlier egg-laying in the wild. We experimentally increased the intensity of the light reaching the hypothalamic photoreceptors. That way, we aimed to advance and delay the time of the day at which birds would start and stop perceiving light, respectively (Figure 1). As a result, we expected that clipping head feathers would expose birds to a higher light intensity both at sunrise and sunset, thereby elongating the perceived photoperiod, which in turn would advance gonadal growth and the onset of egg-laying. In captive great tits kept under natural photoperiod and temperature conditions, we compared gonadal growth of females that had their head-feathers clipped with gonadal growth of control females. In parallel, we monitored egg-laying dates in wild great tits that were subjected to the same experimental manipulations.

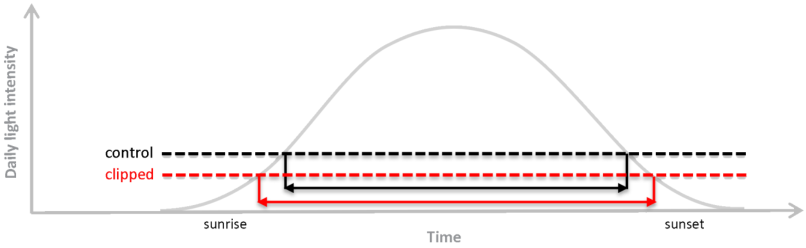

Figure 1 Daily variation in light intensity and hypothetical durations of the photoperiodic perception in clipped and control birds. We assumed that the perceived day length in clipped birds is longer because at sunrise and sunset light intensity levels are higher earlier (or later) in the day.

Materials and methods

Field study

The field experiment was performed in a mixed deciduous woodland area (Oosterhout, Nijmegen, The Netherlands) in three consecutive years (2012-2014) in the period between late February and July. The study site comprises a long-term study site where the breeding biology of the great tit has been recorded since 1956 (Van Balen 1973). The tree species composition of the experimental area consists mainly of oaks, where about 150 nest-boxes for passerines are available. Each experimental year female great tits were caught in late February or early March while sleeping in nest boxes at night. Immediately after catching, each bird was aged and ringed following the standardized procedures, and randomly allocated to one of the experimental treatments. After the approximately 15 minutes treatment the bird was released in the same nest box at which it was caught. During the breeding season (April-July) egg-laying date and the identity of the females breeding in the area were recorded.

Experimental treatments

The experimental groups consisted of a focal group and two control groups: a true control and a behavioural control group (Figure 2). Birds belonging to the focal group had their head feathers clipped (“clipped group”), in order to enhance the amount of light that penetrates through the skull. Feathers were clipped and not plucked to prevent the growth of new feathers before the end of the breeding season. In the true control group (hereafter just referred to as “control group”) the plumage was left intact and birds were only handled for the same amount of time as the focal animals. The second control treatment (“behavioural control”) was established to rule out the effect of clipping on the behaviour of the bird. Birds belonging to the behavioural control group had their head feathers clipped and ink was injected subcutaneously between the skin and the skull, to restore the light filtering properties of the intact feathered skull. This latter group was used to exclude any potential effect of the feather clipping independent of the modification of light perception. In 2013 and 2014 we also injected ink subcutaneously on the back in the clipped group. We made this modification based on the results of the first experimental year (2012) to completely exclude the possibility that the ink injection had negative effects on the bird’s performance. Moreover in 2014, we did not include a behavioural control group as we had sufficient data for this group, and our main interest was in comparing the clipped and the control groups. The sample size of each experimental group in the three different years is shown in Table 1.

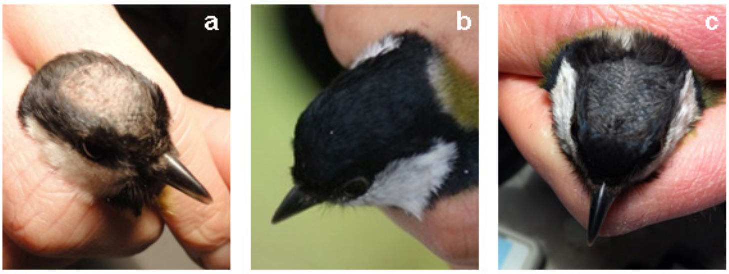

Figure 2 Experimental treatments: a) clipped; b) control and c) behavioural control. In clipped birds the head feathers were clipped; in the control group birds were only handled and in the behavioural control group head feathers were clipped and ink was injected subcutaneously.

Table 1 Sample sizes of each experimental group in the field studies in 2012, 2013 and 2014. Number and percentage of birds in the experiment that were found back breeding later in the season are also indicated.

	Treatment	Sample size	No of birds breeding	% of birds breeding
2012	clipped	11	9	84%
	control	10	10	
	behavioural control	10	7	
2013	clipped	12	6	54%
	control	12	5	
	behavioural control	11	8	
2014	clipped	19	14	76%
	control	15	12	
	Total	100	71	71%

Light measurements

To verify that clipping the head feathers increased the light intensity reaching the brain we measured the light transmission through the skull in naturally deceased birds. After surgical removal of the brain, the diffusor of the photospectrometer (JETI specbos 1211 with cosine diffusor mounted fibreoptic, Spectrapartners, Haarlem, the Netherlands) was placed under the head of the bird and the light intensity transmitted was measured. The spectrum measured ranged from 380 to 780 nm. All measurements were done outdoor under clear sky natural light conditions around noon. Each bird (n=3) was measured three times: i) with head feathers intact (control); ii) with the feathers clipped (clipped) and iii) with feathers clipped and ink injected subcutaneously on the skull (behavioural control). Each measurement in the three different conditions was replicated three times and averaged.

Captive study and gonadal measurements

The captive study was performed in 2012 on birds caught in a mixed deciduous forest (Bennekom, Ede, The Netherlands). In early March, 17 female great tits were captured at night while sleeping in nest boxes and brought to the laboratory where they were temporarily housed in individual cages. The next morning, they were unilaterally laparotomized under isoflurane anaesthesia by making a small incision between the last two ribs on the left side of the bird. Diameter of the largest ovarian follicle was measured (to the nearest 0.1 mm), using a scale engraved in the ocular of a binocular microscope. Follicle volume was calculated as: $V=4/3\pi a^3$, where *a* is diameter/2. After the laparotomy, birds were returned to their individual cages for one more day to facilitate the recovering process. After full recovery, they were randomly allocated to the clipped or to the control group as described in the previous section. Each bird was also randomly allocated to one of two outdoor aviaries, where they were exposed to natural photoperiod and temperature conditions. Food and water were provided *ad libitum*. In early April and early May, the diameter of the largest follicle was again measured, following the same procedures as in the first laparotomy. A few days after the third laparotomy, all birds were released in the wild at the place of capture.

Statistical analysis

To analyse the differences in light intensity transmitted through the skull of birds belonging to the three treatments we used a linear mixed model. Treatment, wavelength and their interaction (treatment*wavelength) were fit as fixed effects and bird identity was fit as random effect. To analyse the effect of the clipping treatment on gonadal size in the captive study we used linear mixed models. The model included treatment, sampling date and their interaction (treatment*date) as fixed effects and bird identity as random effect. Date was fit as continuous variable in a first analysis and then, it was fit as factor to analyse the effect of treatment in each month. All data on gonadal size were log transformed to account for exponential growth.

On the data obtained in the field study, to describe the effect of treatment on egg-laying date we performed two analyses. In the first analysis, data from the three years were pooled and we performed a linear mixed-effects model analysis followed by a Kenward-Roger approximation (*KRmodcomp* function in the R package *pbkrtest* (Halekoh *et al.* 2014)) for model selection. The full model included treatment, experimental year and their interaction (treatment*experimental year) as fixed effect and bird identity as random effect. Bird identity was included in the model as some of the birds used in the previous year(s) as control were re-captured and used again in the following year(s). To perform this analysis we only considered data relative to the clipped and the control group as the data relative to the behavioural control group were available for two out of the three experimental years. In the second analysis, we used linear models with treatment as fixed effect on three separated data-sets, one per each experimental year (2012, 2013 and 2014). In this analysis, all three treatments were considered. All statistical analyses were performed with the R software (version. 3.0.2).

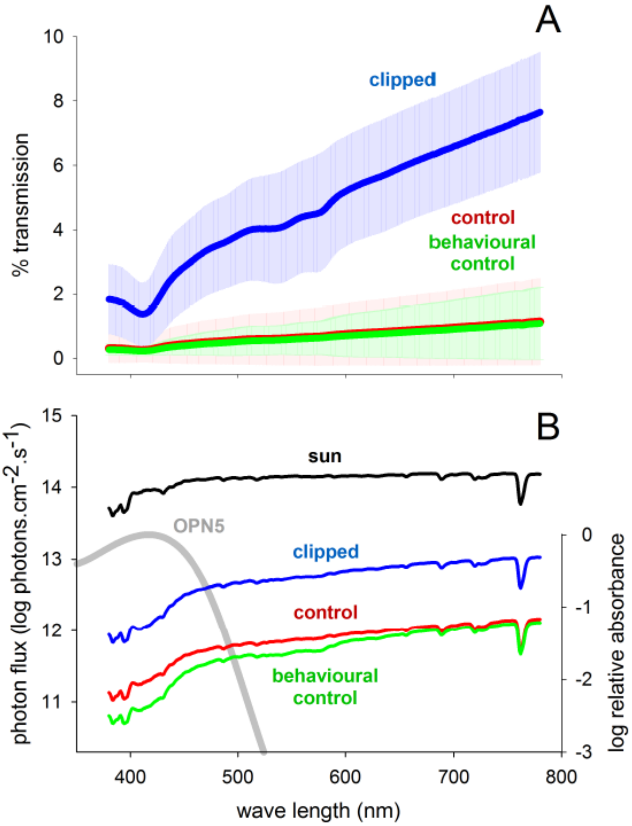

Figure 3 Light transmissions and penetration curves for birds in the three treatments. a) Light transmission in percentage of light intensity measured through the skull with feathers (control, red), no feathers (clipped, blue) or no feathers + inked (behavioural control, green). All three measures were collected subsequently on the same bird skull in three different bird skulls. b) Solar photon flux spectrum with resultant photon flux through the skull in the three conditions (clipped, control, behavioural control). For comparison the relative absorbance curve of the putative avian photoperiodic photopigment neuropsin (OPN5) is also presented, with peak absorbance of 417nm, as measured in quail and chicken (Nakane *et al.* 2010; Yamashita *et al.* 2010), using the opsin nomogram equations by Stavenga (Stavenga *et al.* 2000).

Results

The measurements of the light transmitted through the skull showed that the clipping treatment significantly increased the light intensity (lux) throughout the spectrum measured (380-780 nm) (Figure 3). The amount of light transmitted through the skull of clipped birds was significantly higher than the intensity in birds of the two control groups (Figure 3, $p<0.001$). Moreover, the light intensity transmitted through the skull did not differ between birds of the control and behavioural control groups (Figure 3, $p=0.66$).

In captivity, treatment affected gonadal size as the interaction treatment*date was significant ($p=0.016$). While follicle sizes did not differ between the groups in March (i.e. before the treatments started, $p=0.22$) and in April ($p=0.22$), clipped females had larger follicles than control females in May (Figure 4; $p=0.015$).

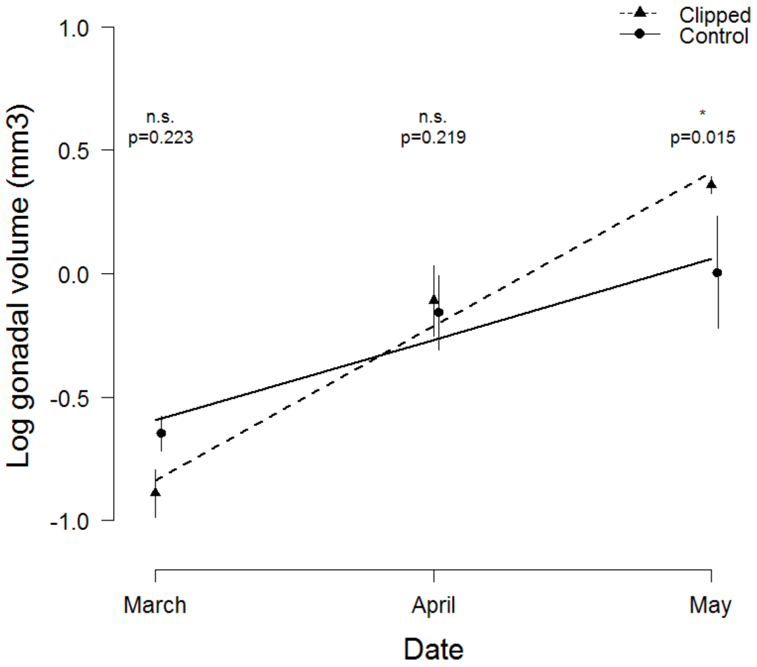

Figure 4 Mean (±SE) gonadal volume of clipped and control birds in the captive study in 2012. Gonadal volume was measured prior (March) and after (April and May) treatment was applied. Gonads were larger in clipped birds in May. There were no differences between treatments in the previous months. Symbols represent group means and lines are the linear mixed model estimates. Sample size of both groups was 8 birds at all sampling events.

In the field, females from the clipped group did not breed earlier than control females (Figure 5; Table 2; Kenward-Roger approximation test, *p*=0.44). Overall, egg-laying dates significantly differed only across experimental years (Table 2; *p*<0.001). When years are analysed separately, and thus when all three treatments are considered, we found no statistical differences between treatments, except in 2012, where females from the behavioural control group laid significantly later than the two other groups (Table 3; year 2012, *p*=0.03). However, this effect was not found in the following year (Table 3; year 2013, *p*=0.24). Overall, the egg-laying dates of clipped birds were never earlier than the control group (Table 3).

Table 2 Statistical analysis of the effect of the experimental treatments on egg-laying date in the field in 2012, 2013 and 2014. Results of the Kenward-Roger approximation used for model selection are reported. F values (F), numerator of degrees of freedom (ndf), denominator degree of freedom (ddf), and *p* values are indicated. The data-set used here only includes the clipped and the control groups.

Explanatory variable	**F value**	**ndf**	**ddf**	***p***
Treatment*year	0.58	2	49.0	0.57
Treatment	0.61	1	51.1	0.44
Year	22.96	2	1	**<0.0001**

Table 3 Model results for the analysis of the effect of treatment on egg-laying date in the field in 2012, 2013 and 2014. The same analysis was performed on each experimental year separately. Egg-laying date indicate the day that the first egg of the clutch was laid in the wild. All estimates are relative to the control group. Treatment did not have any effect on egg-laying date with exception of year 2012 when the egg-laying date in the behavioural control group was significantly later than the control group.

Linear model: laying date ~ treatment

	Treatment	**Estimate$^{(*)}$**	**SE**	**t**	***p***
2012	Clipped	-1.40	2.63	-0.53	0.59
	Behavioural control	6.46	2.82	2.29	**0.03**
2013	Clipped	3.50	2.18	1.60	0.13
	Behavioural control	2.50	2.05	1.22	0.24
2014	Clipped	-3.4	4.15	-0.83	0.41

(*) contrast to the control group

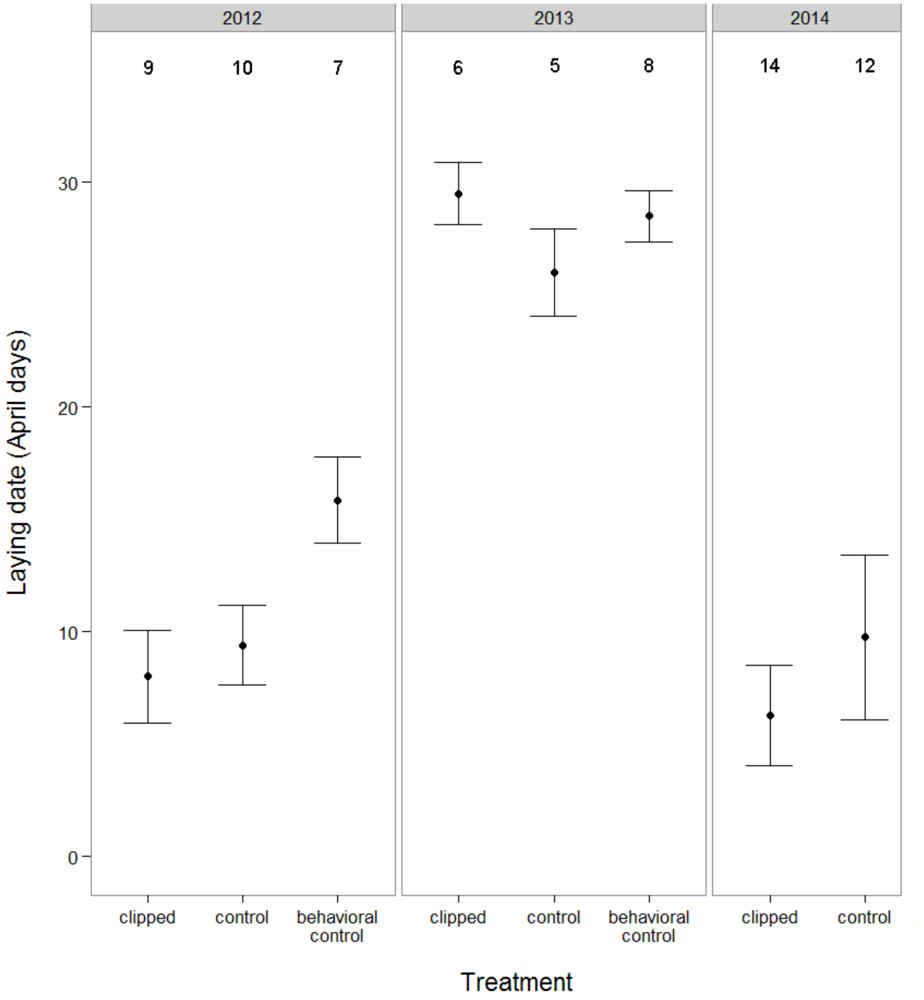

Figure 5 Mean (±SE) egg-laying date of clipped and control birds in the field study performed in 2012, 2013 and 2014. In 2012 the egg-laying date of the behavioural control group was significantly later than the control group. In the two other years, egg-laying dates did not differ among groups. Sample size of each group is indicated above.

Discussion

In this study we manipulated the perceived photoperiod in great tit females by clipping their head feathers and we monitored gonadal growth and timing of egg-laying. This manipulation significantly increased the light intensity transmitted through the skull and affected gonadal growth but it did not advance egg-laying. Overall these findings suggest that the clipping treatment altered the physiological mechanisms underlying reproduction (i.e. gonadal development) but it did not translate in earlier egg-laying decisions. These results are consistent with other studies on the same species in captivity where there was also no correlation between gonadal size and egg-laying date (Schaper et al. 2012a).

Birds use the increasing photoperiod as major cue to initiate gonadal growth and breeding (Lambrechts *et al.* 1997; Dawson *et al.* 2001). The physiological response to the photoperiodic manipulation (i.e. development of larger gonads) and, at the same time, the lack of the behavioural response (advanced onset of egg-laying) suggests that other cues, rather than photoperiod, must be involved in egg-laying decision. Increasing temperatures have been shown, in studies in captivity, to fine-tune the onset of egg-laying (Meijer et al. 1999; Visser et al. 2009; Schaper et al. 2012b; Caro et al. 2013a). Few other experimental studies have succeeded to advance egg-laying date via food availability (Nilsson 1994; Gienapp *et al.* 2006b). In blue tits food provisioning resulted in earlier egg laying, however mortality of manipulated earlier female was higher, supporting the hypothesis that cost of reproduction earlier in the season might be too high (Nilsson 1994). Similarly, female great tits in the Hoge Veluwe that had experimentally increased food availability in the previous year, laid their eggs earlier in the next year (Gienapp *et al.* 2006b).

Similar experimental studies using only light cues in field conditions did not succeed to advance egg-laying date. In te Marvelde et al. (te Marvelde et al. 2012) great tits exposed to a long day in winter develop their gonads earlier than the control birds whereas timing of egg-laying date did not differ. Both the findings of this latter study and our study could be due to too high fitness costs of egg-laying and/or incubation earlier in the season. The costs of egg-laying at cold temperatures may be larger than the fitness benefits gained via increased offspring's fitness.

Finally, our current understanding on how phenology of egg-laying is regulated in the field is based mainly on correlative studies. Although already much work has been carried out, more experimental approaches focused on the exploration of the proximate causes of egg-laying decisions in the wild are needed for the understanding of fitness consequences of avian timing of reproduction in the wild.

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Ethical statement

All the experiments performed in this study were approved by the Animal Experimentation Committee of the Royal Dutch Academy of Sciences (DEC-KNAW; protocol number NIOO12.05). All surgery was performed under Isoflurane anesthesia, and all efforts were made to minimize discomfort.