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Clock gene is associated with individual variation in the activation of reproductive endocrine and behavior of Asian short toed lark

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Within year individual variation in the timing of seasonal reproduction within wild bird populations in highly seasonal environments can be pronounced, but the molecular and physiological mechanisms responsible for this variation are unclear. We investigated the relationship between *Clock* gene poly-Q length, activation of the HPG endocrine axis, and the timing of breeding behavior, in a wild population of the Asian short-toed lark (*Calandrella cheleensis*) in Inner Mongolia, China. Six variants of *Clock* gene poly-Q alleles were identified in this population. *Clock* poly-Q mean allele length was positively correlated with the mean peak date deviation of individual birds. The shorter an individual's *Clock* poly-Q mean allele length, the earlier its plasma LH, T and E2 values peaked. Mean *Clock* poly-Q allele length of nestlings in the same nest were positively correlated with the standardized laying date of the first egg in that nest. These results suggest that the *Clock* gene influences the reproductive timing of birds through its effect on the HPG endocrine axis, and that individual variation in the timing of reproduction may have a genetic basis.

Seasonal timing of breeding is a nearly ubiquitous feature of birds in mid to high latitude areas. Proper timing of breeding is a key component of fitness because birds must synchronize hatching within a narrow time window of maximal food abundance to enhance their reproductive success. However, within year individual variation in the timing of reproduction within populations inhabiting highly seasonal environments can be considerable^{1–5}. Although some quantitative genetic analyses have demonstrated that part of this variation has a genetic basis^{6–10}, the biological mechanism of such individual variation is still not very clear. Recent studies have highlighted some candidate genes in which STR length polymorphism is associated with phenological traits^{11–15}. The most commonly studied gene so far is the *Circadian Locomotor Output Cycles Kaput* (CLOCK) gene, a highly conserved transcription factor central to the rhythmicity of the circadian oscillator¹⁶. The CLOCK possesses a poly-glutamine (poly-Q) binding region that is polymorphic in length (the number of Q repeats), which affects its binding affinity to its transcription factor¹⁷. The *Clock* gene was first identified in mice and has subsequently been characterized in a large number of animals, including several bird species^{18,19}. The *Clock* gene poly-Q region has been found to be polymorphic in 65 of 69 passerine populations of 12 species^{18,20–25}. At the individual level, length polymorphism in the *Clock* gene poly-Q region has been found to be positively correlated with laying date, hatching date, and incubation duration, in blue tits (*Cyanistes caeruleus*)²⁶, and with laying date in barn swallows (*Hirundo rustica*)²³. Therefore, it seems quite likely that genetic variation in the *Clock* gene is involved in the within year individual variation of seasonal breeding rhythm within bird populations.

Birds in mid to high latitude areas use photoperiod as a reliable cue to activate hypothalamus–pituitary–gonad (HPG) axis endocrine to adjust the timing of their breeding behavior^{27–30}. In spring, an increase in day length leads to elevated secretion of gonadotropin releasing hormone (GnRH) from the median eminence at the base of the hypothalamus³¹. This stimulates the synthesis and release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) in the pituitary³². These two hormones are secreted into the blood and induce gonadal maturation,

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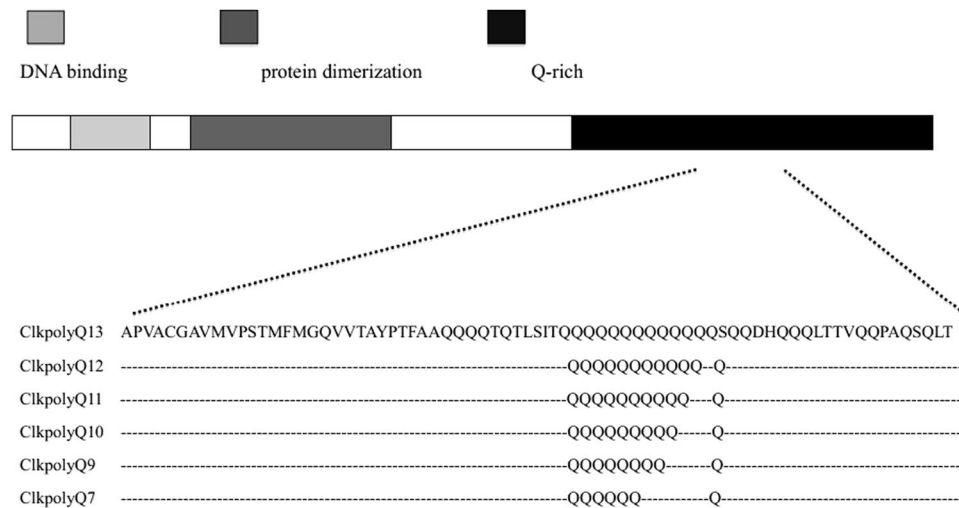


Figure 1. CLOCK poly-Q types identified in an Asian short-toed lark population in Inner Mongolia, China.

which, in turn, secrete testosterone (T) and estradiol (E_2) to initiate reproductive behavior^{33,34}. Although some studies imply that within year individual variation in the timing of breeding within populations could be due to genetic variation^{23,26}, the relationship between the *Clock* gene and activation of the hypothalamus–pituitary–gonad (HPG) axis remains unclear. Research on rats (*Rattus rattus*)³⁵ suggests that this relationship is the key to explaining the mechanism by which the *Clock* gene controls the timing of the breeding behavior. The results of this study suggested that the *Clock* gene influences the timing of reproduction in rats by binding to E-box elements in the promoter of the GnRH receptor, a key reproductive gene. This suggests that individual differences in the timing of breeding behavior could be because individuals have different versions of the *Clock* gene and consequently different HPG axis activation rates. Determining the relationship between *Clock* gene poly-Q region length and activation of the HPG axis in birds is an important test of the generality of this hypothesis, and for understanding the underlying physiological mechanism responsible for individual variation in the timing of breeding behavior.

In this paper we present data on the relationship between *Clock* gene poly-Q (hereafter ClkpolyQ) length, the activation of HPG endocrine axis, and the timing of breeding behavior in a wild population of the Asian short-toed lark (*Calandrella cheleensis*, Passeriformes, Alaudidae) in Inner Mongolia, China, a population that displays significant within year individual variation in the timing of breeding behavior³⁶. Our objective was to investigate the physiological mechanism through which the *Clock* gene influences the timing of breeding of individuals in this population. Previous research conducted by us on this population indicates that it only breeds once a year, and that there is considerable variation between pairs in the dates on which the first eggs are laid and the first chicks are hatched; the earliest and latest laying and hatching dates are about 15 days apart³⁷. Therefore, this species is very suitable for exploring the genetic basis of individual variation in the timing of breeding behavior. Based on evidence from blue tits and barn swallows^{23,26}, we hypothesized that individuals with longer *Clock* gene alleles initiate the HPG endocrine axis later, and consequently breed later than those with shorter *Clock* gene alleles. To test this hypothesis, we investigated the relationship between the secretion rate of plasma LH, T (males only), E_2 (females only) and *Clock* gene poly-Q region length, in wild-caught birds housed in field aviaries at the study site in the spring of each year between 2014 and 2016. We also investigated the relationship between laying dates and nestling ClkpolyQ length in free-living birds at the study site. In this part of the study we inferred mean ClkpolyQ allele length of two parents from mean allele length of all their nestlings because breeding adults were difficult to catch, and because the mean allele length of parents would be expected to be equal to the mean allele length of all their offspring.

Results

We successfully amplified, sequenced, and genotyped, the ClkpolyQ variable length region of all birds we collected blood samples from. Six length variants of ClkpolyQ alleles were identified: ClkpolyQ_{7, 9, 10, 11, 12, 13} (the subscript indicates the number of poly-Q repeats) (Fig. 1). ClkpolyQ₉ and ClkpolyQ₁₁ were the two most common alleles (Table 1). *Clock* Poly-Q allele frequencies did not deviate significantly from the Hardy–Weinberg equilibrium (HWE). Observed overall heterozygosity is shown in Table 1.

There was considerable individual variation in the dates on which plasma LH, T and E_2 concentrations peaked in all three years of the study (Fig. 2). The results of LMMs indicate that of the explanatory variables ClkpolyQ length, body mass, sex and year, only ClkpolyQ length had a significant effect on plasma LH, T and E_2 Peak date Deviation (PD; see Methods for definition) values (Table 2). Individual mean ClkpolyQ allele length (see Methods for definition) was positively correlated with the individual LH, T and E_2 PD of adult birds (LH: $n = 257$, $r = 0.541$, $P < 0.001$; T: $n = 171$, $r = 0.484$; $P < 0.001$; E_2 : $n = 86$, $r = 0.523$, $P = 0.001$). The mean plasma LH, T and E_2 PD of individuals with shorter ClkpolyQ genotypes were smaller (Fig. 3) and individuals with lower PD values had shorter mean allele lengths (Fig. 4). The mean ClkpolyQ allele length of nestlings in one nest was positively correlated with the first egg laying date for that nest ($n = 240$; $r = 0.559$; $P < 0.001$) (Fig. 5). Generally, the shorter a nestling's mean ClkpolyQ allele length, the earlier the eggs were laid.

	n	Q7	Q9	Q10	Q11	Q12	Q13	H
Total	257	0.004	0.438	0.050	0.434	0.041	0.033	0.585
Males (2014 + 2015 + 2016)	171	0.000	0.456	0.044	0.450	0.031	0.019	0.540
Females (2014 + 2015 + 2016)	86	0.012	0.402	0.061	0.402	0.061	0.061	0.674

Table 1. *Clock* gene poly-Q allele frequencies and observed heterozygosity (*H*) of adult Asian short-toed larks in Inner Mongolia, China.

Discussion

Our results show that there is considerable within year individual variation in ClkpolyQ length in the Asian short-toed lark population, and that the ClkpolyQ length of individual birds is associated with temporal variation in the activation of reproductive hormone secretion and laying date. This supports our hypothesis that individuals with longer ClkpolyQ lengths activate the HPG axis and initiate breeding activity relatively late. Taken together, these results suggest that within year individual variation in the activation of the HPG axis and timing of breeding behavior in the Asian short-toed lark may have a genetic basis.

Although several studies have found evidence that the *Clock* gene influences the timing of laying in passerine birds^{23,26}, the physiological mechanism responsible for this has not been identified. Our results imply that the *Clock* gene influences the timing of laying via the HPG axis. The discovery that the *Clock* gene may influence the timing of reproduction by binding to E-box elements in the GnRH receptor gene promoter of rats suggests that it regulates reproductive gene expression in the upper stream of the HPG axis³⁵. Vitellogenesis and oocyte growth in birds are characterized by the estrogen-dependent hepatic synthesis of two main yolk precursors: VTG and yolk-targeted very low-density lipoprotein (VLDL_y). These are the primary sources of yolk protein and lipid, respectively, providing all the nutrients and energy required by the developing embryo^{38–40}. Estrogens initiate gene transcription and synthesis of VTG and apo-VLDL-II protein, and also reprogramme hepatic lipid and protein synthesis to support the transition from generic VLDL to VLDL_y synthesis. Therefore, females in which E2 levels increase earlier could complete vitellogenesis sooner, and consequently lay earlier. Further research on the relationship between the *Clock* gene and the HPG axis in birds is required to clarify the physiological mechanism through which *Clock* affects the timing of breeding.

The majority of studies suggest that the timing of seasonal reproduction of birds is influenced by environmental cues and there is considerable knowledge of the potential mechanisms through which day length, ambient temperature and food availability affect the timing of reproduction^{41–44}. However the potential mechanisms underlying individual variation in the timing of reproduction remain unclear. Our results reveal that such individual variation could have a genetic basis such that individuals with different *Clock* genotypes activate the HPG axis, and consequently breed, at different times. This suggests that the timing of reproduction in birds is not only influenced by environmental factors, but also by endogenous factors.

Long-term studies of wild bird populations have shown that the timing of reproduction is often under strong selection within populations and that females that time reproduction to coincide with peaks of food availability produce a greater proportion of the next generation^{45,46}. It has been well established that the phenology of many species has been affected by global warming, which threatens the survival of some species^{47,48}. A trophic mismatch between birds and insects has been described in several European birds^{49–52}. This is thought to be caused by the timing of reproduction in birds and the emergence of insects (the main food of nestlings) being initiated by different environmental cues^{53,56}. Individual phenotypic polymorphism exists within populations, and if heritable, may allow populations to adapt to environmental change through microevolution. Physiologists are in general less interested in variation among individuals than in inter-species or inter-population variation^{53–55}. Our results show that within year individual variation in the timing of reproductive hormone secretion and breeding behavior could have a genetic basis, and that this could be an important component of the microevolutionary adaptive potential of Asian short-toed lark populations. Asian short toed larks live on the grasslands of northeast China where the main food of nestlings (grasshoppers) peak at different times in different years due to variation in local ambient temperature³⁶. As the timing of these annual food peaks in our study area varied significantly, polymorphism in the *Clock* gene could allow individual Asian short toed larks to breed successfully despite this marked annual fluctuation in peak nestling food abundance³⁶. Further work is required to investigate how the Asian short-toed lark *Clock* gene might evolve in response to climate change.

Methods

Study site and species. The study site was located in the Dalai National Nature Reserve (47°45′50″N – 49°20′20″N; 116°50′10″E – 118°10′10″E) situated in the northeastern part of the Inner Mongolian Autonomous Region, China. This reserve is a semiarid, steppe region where the mean annual temperature, precipitation, and potential evaporation are –0.6 °C, 283 mm, and 1754 mm, respectively. The dominant plant species are *Stipa krylovii*, *Leymus chinensis*, and *Cleistogenes squarrosa*. Winter is longer than summer and the average maximum daytime temperatures in January and July are –20.02 °C and –22.72 °C, respectively. The Asian short-toed lark is the most common lark species on the grasslands of northeastern Inner Mongolia. This species begins nesting in the middle of April. Nest-building takes 7–10 days, the average clutch size is 3.05 ± 0.51, and the incubation period is 10–12 days³⁶.

Experiment design and data collection. Adult Asian short-toed larks were captured in mist-nets from March 10 to March 15 in 2014, 2015 and 2016 in the study site (sample sizes in each year are shown in Table 1)

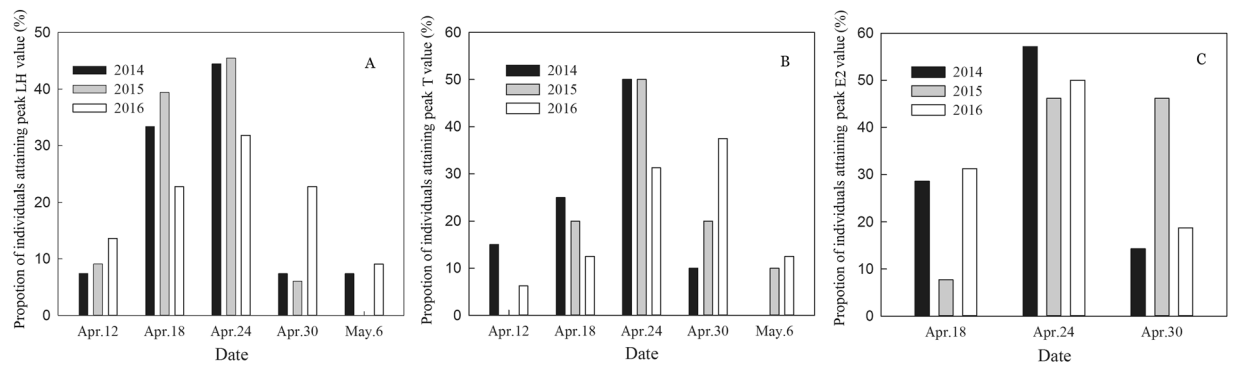


Figure 2. Variation in the dates on which (a) plasma LH, (b) T, and (c) E_2 , concentrations of individuals attaining the peak levels within a population of Asian short-toed larks in Inner Mongolia, China.

Response variable	Explanatory variable	F	P
PD of LH	ClkpolyQ length	15.907	<0.001
	body mass	0.509	0.826
	sex	0.888	0.348
	year	0.535	0.846
	ClkpolyQ length \times body mass	0.535	0.846
	ClkpolyQ length \times sex	1.819	0.167
	ClkpolyQ length \times year	1.648	0.154
PD of T	ClkpolyQ length	3.333	0.042
	body mass	1.942	0.079
	year	0.982	0.473
	ClkpolyQ length \times body mass	1.562	0.132
	ClkpolyQ length \times year	0.971	0.516
PD of E_2	ClkpolyQ length	4.482	0.021
	body mass	0.332	0.914
	year	1.025	0.431
	ClkpolyQ length \times body mass	1.459	0.263
	ClkpolyQ length \times year	1.042	0.348

Table 2. Results of linear mixed models (LMMs) on the effects of ClkpolyQ length, body mass, sex, and year, on peak date deviation (PD) of plasma luteinizing hormone (LH), and on the PDs of plasma testosterone (T) and estradiol (E_2), of wild-caught, captive, Asian short-toed larks in Inner Mongolia, China.

and were individually identified by leg-bands when first captured to avoid repeated sampling of the same individuals in different years. All captured birds were housed in out-door aviaries (100 \times 100 \times 70 cm) (six birds per aviary), fed mixed seeds, boiled eggs, and mealworms, and provided with water. From 29 March to 6 May, at least 100 μ l of whole blood was collected from each bird between 12:00 and 12:30 every 5 days for LH, T, E_2 , and *Clock* gene, assays. Blood was collected into heparinized microcapillary tubes within 1–3 min of capture by puncturing a brachial wing vein with a disinfected 23 G needle. The skin around the puncture site was disinfected with medical alcohol before, and after, puncturing. Pressure was applied to the puncture site for 1 min with an alcohol-soaked cotton wool swab to stop bleeding. Blood samples were stored at 4 $^{\circ}$ C for up to 8 h until centrifuged at 3000 rpm for 10 min. The resultant blood plasma and cells were separated into different micro-centrifuge tubes and then kept frozen until assayed. Blood plasma was used for the hormone assays and the blood cells were used as a source of DNA to analyze *Clock* gene polymorphism. All caged birds were released at the end of the experiment. Experimental procedures comply with the ARRIVE guidelines and were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978), and approved by the Animal Research Ethics Committee of Hainan Provincial Education Centre for Ecology and Environment, Hainan Normal University (permit no. HNECEE-2013–002).

We started monitoring laying behavior of the free living birds on April 20 each year. Eighty, 82 and 78 nests were monitored from the first egg to fledging in 2014, 2015 and 2016, respectively. Laying dates of each egg were recorded for each nest. At least 20 μ l of whole blood was collected from each six day old nestling for the *Clock* gene assay. The blood sampling and storage protocols for nestlings were the same as those for adults.

Hormone assay. Plasma LH, T and E_2 levels were measured using chicken enzyme immunoassay kits from MyBioSource (cat #MBS165746), Enzo (cat #ADI-901–065 and –174), respectively, which had been previously validated by our laboratory for use on Tree sparrows (*Passer montanus*)⁵⁶. In order to confirm that the kits would

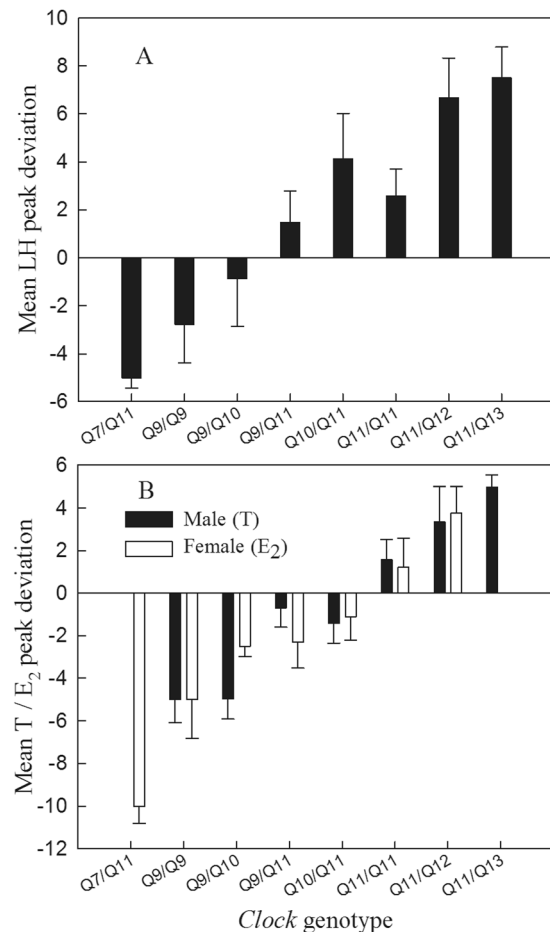


Figure 3. The LH, T and E₂ mean peak date deviation of all *Clock* poly-Q genotypes. The mean LH, T and E₂ peak date of the population in each year was scored as 0. The peak deviation of an individual bird was defined as the deviation days from the population peak date.

work on Asian short-toed lark plasma, plasma samples from 20 Asian short-toed larks were pooled and diluted by 1, 1/2, 1/4, 1/8, 1/16 and 1/32, according to the methods used by Chastel *et al.*⁵⁷. We then used the kits to analyze the concentration-dependent binding dynamics of the diluted samples. The resultant LH, T and E₂ dilution curves were parallel to the standard ELISA Kit curves, confirming that the kits could reliably assess levels of LH, T and E₂ in Asian short-toed lark plasma. The respective inter- and intra-plate coefficients of variation for LH, T and E₂ were, 7.06% and 6.5%, 9.1% and 7.7%, and 8.5% and 2.4%, respectively.

Sequencing of the *Clock* gene. We extracted total DNA from blood samples for PCR reactions⁵⁸. PCR primers (forward primer: 5'-TTTTCTCAAGGTCAGCAGCTTGT-3' and reverse primer: 5'-CTGTAGGAAGTGTG (C/T) GG (G/T) TGCTG-3') were designed to amplify an approximately 250–290 bp region of the *Clock* gene for fragment-length analysis. The *Clock* gene was amplified using touchdown PCR in 25 μ L reactions (12.5 μ L Premix (rTaq), 9.5 μ L dd H₂O, 1 μ L of each primer (20 pmol/ μ L), and 1 μ L template DNA (approximately 50–100 ng/ μ L)) under the following conditions: 94 °C/2 min; 94 °C/30 s, 58 °C/30 s, 72 °C/60 s, 10 cycles; 94 °C/30 s, 62 °C/30 s, 72 °C/60 s increasing 5 s/cycle, 30 cycles; 72 °C/7 min, hold at 4 °C, catalyzed using Taq DNA polymerase. Amplified PCR products were prepared for sequencing using QIAGEN MinElute PCR Purification Kits. Nucleotide sequences of purified PCR fragments were determined with the BigDye Terminator version 3.1 cycle sequencing ready reaction (Applied Biosystems) under standard sequencing conditions according to the manufacturer's protocol. The reaction products were detected on an ABI PRISM genetic analyser 3730xl (Applied Biosystems), and analyzed with the GeneMapper 4.0 software package. We succeeded in genotyping 99.6% of adult samples and 96% of nestling samples. After sequencing, we translated *Clock* gene sequences into *Clock* amino acid sequences with DNAMAN software, and counted the number of the Q in the poly-Q regions.

Data analysis. We used “peak date deviation (PD)” to measure the secretion rate of LH, T and E₂. Firstly we calculated the mean LH, T and E₂ values of all the birds on each sample day in each year. The dates on which the peak mean LH, T and E₂ values occurred were defined as the peak dates for each hormone of the population in each year. The peak date of the population in each year was scored as 0. If the peak LH, T and E₂ values of an individual bird occurred n days earlier than the population peak date, in which case the PD of the individual was

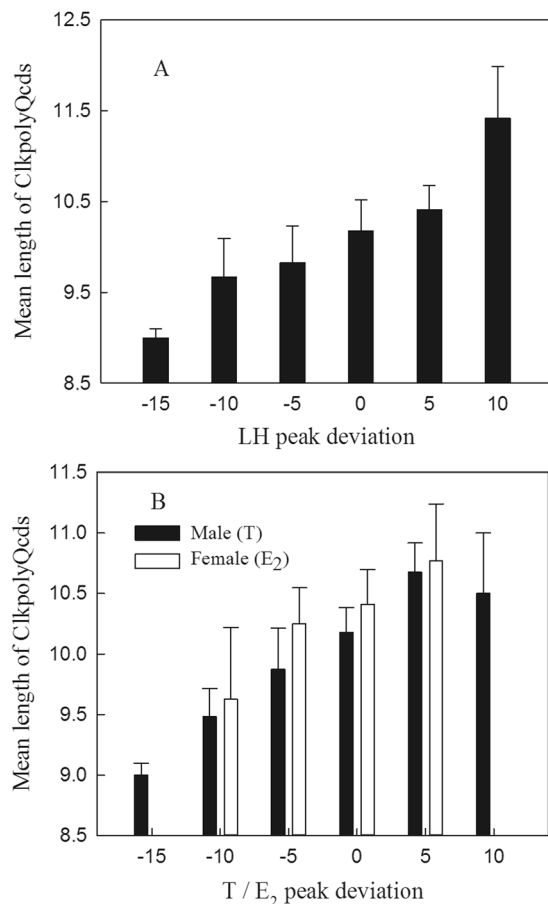


Figure 4. The mean lengths of *Clock* poly-Q allele length of the birds with different LH, T and E₂ peak date deviation.

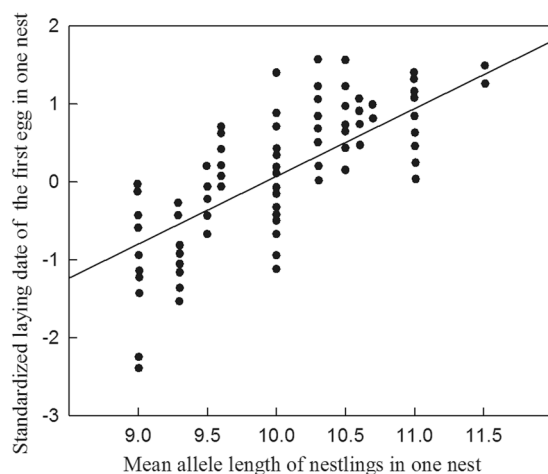


Figure 5. The correlation between mean *Clock* poly-Q allele length of the nestlings in one nest and the standardized laying date of the first egg in one nest ($n = 240$; $r = 0.559$; $P < 0.001$).

recorded as $-n$. However, if the peak LH, T and E₂ dates of an individual bird was n days later than the mean peak date, the PD of the individual was recorded as n .

Timing of laying of the free living birds were standardized for each year using the method described by Liedvogel *et al.*²⁶. The laying date (LD) is defined as the number of days that the actual LD was from January 1st. Standardized laying date (LDstand) was defined as follows: $LDstand = (LD - LD(\text{average per year})) / SD$ (where SD is the standard deviation of LD per year). Because a clutch can be laid within two days, we recorded the laying date of each clutch as the date on which the first egg was laid.

We defined *Clock* poly-Q (ClkpolyQ) genotype of an individual as the mean *Clock* poly-Q allele length, which allows the combined effect of both alleles on phenotype to be assessed. The mean poly-Q allele length of all nestlings in one nest was used to reflect the mean allele length of their parents.

The effects of ClkpolyQ length, body mass, adult sex, and sample year, on plasma LH PD were analyzed with a Linear Mixed Model (LMM) in which individual was a random factor. The effects of all factors, except sex, on the plasma T and E₂ PD of adults were assessed with a LMM with individual as a random factor. We use Pearson correlation analysis to analyze the correlation between individual ClkpolyQ length and the PD of three hormones and the correlation between the mean ClkpolyQ length of the nestlings in one nest and the LD_{stand}. Statistical analyses were performed in SPSS 18.0; $\alpha = 0.05$ in all tests.

Data availability. All data generated or analyzed during this study are included in this published article

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Author Contributions

S. Z. and W. L. conceived and designed the experiment. S.Z. wrote the paper. X.X. analyzed the data. X.X., W.W. and W.Y. collected the data.

Additional Information

Competing Interests: The authors declare that they have no competing interests.

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