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# Circadian synchronization determines critical day length for seasonal responses

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## HIGHLIGHTS

- T-photocycles entrain circadian rhythm of photoinducibility (CRP) in buntings.
- Entrainment to T-cycles shifts photoinducible phase and alters critical day length.
- CRP-entrainment based critical day length (CD) involves changes at genetic levels.
- *EYA3* is involved in the photoperiod perception, rather than in the induction.
- Photoperiod effects on CD involve thyroid hormone responsive genes.

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## ABSTRACT

A photoperiodic species initiates fat deposition (in migrants) and gonadal recrudescence in response to a specific duration of natural daylight, called critical day length (CD), when light extends in the inductive phase of the endogenous circadian rhythm of photoinducibility (CRP). The molecular basis of species-specificCD, determined by the entrainment of the CRP, has been poorly understood. To investigate this, we measured expression levels of genes implicated in the photoperiod-induced changes in reproduction (*EYA3*, *TSH beta*, *DIO2*, *DIO3*, *GNRH* and *GNIH*) and metabolism (*SIRT1*, *HMGCR*, *FASN* and *PPAR alpha*) in photosensitive redheaded buntings subjected to light–dark cycles of varying period lengths (T-photocycles). Buntings were exposed to six T22, T24 or T26 photocycles, with 1 h additional light at night falling at different phases of the entrained CRP ($T22_{11L}$ = 6L:4D:1L:11D; $T24_{11L}$ = 6L:4D:1L:13D,$T24_{12L}$ = 6L:5D:1L:12D, $T24_{13L}$ = 6L:6D:1L:11D; $T26_{12L}$ = 6L:5D:1L:14D). Photoinduction at genetic and phenotypic levels in $T24_{12L}$ and $T24_{13L}$, not $T24_{11L}$, groups confirmed CD being close to 12 h in buntings under T24. Compared to T24, exposure to T22 advanced CD by 1 h, as evidenced by photoinduction in the $T22_{11L}$, not $T22_{6L}$, group. Similarly, CD appeared to be delayed under T26, with no photoinduction in the $T26_{12L}$ group. Further, to show that induction of response under a T-photocycle was because of the interaction of inductive phase of the CRP with 1 h during the dark period in each cycle, not with the 6 h main light periods falling 2 h earlier each successive 24 h day in a T22 paradigm, a group of buntings was exposed to 6L:16D ($T22_{6L}$), to which they did not respond. The mRNA expression of genes, particularly *TSH beta*, *DIO2*, *DIO3* and *PPAR alpha*, was significantly correlated with changes in reproductive and metabolic phenotypes. These results suggest CRP-entrainment based genetic regulation of the CD, and extend the idea that synchronization with environment is a critical measure in a seasonal species for its temporal adaptation in the wild.

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## 1. Introduction

Day length (photoperiod) is used to time reproduction and associated events to the best time of year in many vertebrates, including birds. To avoid resource competition, many species time gonadal recrudescence in response to a specific duration of the natural photoperiod. As a measure of this, the minimum light period in a 24 h environment that will induce a response in half of the test population has been defined as the critical day length, CD, which can be both species- and response-specific[1]. For example, CD for the induction of gonadal growth is close to 11.5 h in Japanese quail, *Coturnix coturnix japonica* [2], 12 h in blackheaded bunting, *Emberiza melanocephala*[3] and redheaded bunting, *Emberizabruniceps* [4] and 12.5 h in the golden hamster, *Mesocritecus auratus*[5]. Also, 11.5 h and 11.75 h photoperiods induce half-maximal testis growth and full, albeit at a slower rate, fat

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deposition and weight gain in the blackheaded bunting and redheaded bunting, respectively [3,4]. A photoperiod longer than CD acts as a long day; buntings fully fatten, gain weight and reproductively mature their testes in about 4-week exposure to a 13 h photoperiod [3,4].

A photoperiodic response is triggered when light period extends in the photoinducible phase (ϕi) lying in the second half of the endogenous circadian rhythm of photoinducibility (CRP; [6,7]).In a synchronized 24 h light–dark (LD) environment, the ϕi begins around midpoint of the daily cycle, i.e. about 12 h after lights on. This explains why a species with a CD of ~12 interprets 11- and 12 h light per day as non-inductive and inductive photoperiods, respectively.

Thus, synchronization of CRP determines the position of ϕi in an LD cycle [6,7,8]. This can be tested by exposure to LD cycles with varying period lengths (T) falling within the range of circadian entrainment (e.g. T = 21–27 h; T-photocycle). In this model, the entrainment of CRP under T-photocycles correspondingly alters the position of ϕi, hence the duration of CD for the photoperiodic induction. This has been demonstrated in a previous study on blackheaded buntings [9]. Buntings interpreted 11 h light as inductive and non-inductive photoperiods in T22 and T24 photocycles, respectively. Similarly, buntings interpreted 12 h light as inductive and non-inductive photoperiods in T24 and T26 photocycles, respectively; a 13 h photoperiod was inductive in a T26 photocycle [9]. Using a skeleton paradigm, in which a short 1 h light period was introduced in the dark period of non-stimulatoryT-photocycles (T22, 6L:16D; T24, 6L:18D; T26, 6L:20D), the ϕi was shown to begin 10-, 11- and 12 h after the onset of main 6 h light period under T22, T24 and T26 photocycles, respectively [9].

It is poorly understood how the entrainment of CRP affects molecular events underlying induction of seasonal responses. Identification of molecules in photoperiodic induction has now provided an opportunity to investigate this. Under the control of eye absent 3 (*EYA3*) and thyroid stimulating hormone-beta subunit (*TSH beta*) from the pars tuberalis (PT), there is a rapid switching between type 2 and 3 iodothyronine deiodinase (*DIO2* and *DIO3*) transcription in the tanycytes (ependymal cells) lining the third ventricle [10,11]. This occurs at the transcriptional levels as early as hour 14 (*EYA3* and *TSH beta*; 1st wave of induction genes) and hour 18 (*DIO2* and *DIO3*; 2nd wave of induction genes) of the first long day itself; i.e. when light impinges on to the ϕi [11,12,13]. As a result, T4 is converted into active T3 and in turn, GnRH (gonadotropin releasing hormone) released from the hypothalamus initiates gonadotropin secretion by the pars distalis [11,12,13,14]. In recent years, another hypothalamic dodecapeptide, GnIH (gonadotropin inhibiting hormone) has been shown regulating the synthesis and release of GnRH in response to change in the photoperiod environment [15]. Also, photoperiod-induced change in the mRNA expression of *EYA3*, *TSH beta*, *DIO2* and *DIO3* genes has been found around hours 15 and 19 after exposure to a single 13 or 16 h light period in night-migratory redheaded buntings [16].

A link between genes involved in the circadian timing and photoperiodic induction has been suggested. For example, *BMAL1*/*CLOCK* constituting positive element of the circadian clock feedback loop induces *TSH beta* mRNA expression, and *PERIOD1*, a core component of the negative element, reduces *BMAL1*/*CLOCK*-induced *TSH beta* levels in mice [17]. The presence of three E-box elements in *EYA3* promoter sensitive to *BMAL1*/*CLOCK* further supports association between circadian clock and photoperiodism. In mammals, circadian system mediated effects on photoperiod-induced gene expressions involve melatonin, a circadian clock driven output from the pineal gland [12,17]. Melatonin phase synchronizes gene expression and regulates *EYA3* expression in the PT[11]. Intriguingly, however, circadian clock governed melatonin secretion is not involved in the regulation of gonadal cycles in photoperiodic birds [18].

The present study asked a specific question, as to whether synchronization of the CRP to external photoperiod environment would determine CD by corresponding changes in the timing of transcription of genes in systems associated with the reproduction and metabolism. If yes, then the exposure to shorter and longer T cycles would advance and delay, respectively, the timing of the transcriptional activation of photoperiod response genes, hence CD, compared to that in the T24 cycle. We investigated this, by examining gene expression in strongly circadian photosensitive redheaded buntings entrained to shorter and longer T-photocycles in a skeleton paradigm. Buntings were exposed to T22, T24 and T26 cycles, with a 6 h main photophase and 1 h additional light introduced during the darkness such that it lasts before or into the ϕi. Hypothalamic expression of six genes measured in the system associated with reproduction included *EYA3* and *TSH beta* – 1st wave of induction genes, *DIO2* and *DIO3* – 2nd wave of induction genes, and G*NRH* and *GNIH* – downstream response genes [11]. As a measure of metabolic states, mRNA expression of genes involved in the glucose (*SIRT1*, *silent mating type information regulation 2 homologue type 1*) and lipid (*3-hydroxy-3-methyl-glutaryl-CoA reductase*, *HMGCR or HMG-COA*, *fatty acid synthase*, *FASN*, *peroxisome proliferator-activated receptor alpha*, *PPAR alpha*) metabolism was measured in the hypothalamus and liver. The prediction was that a 1 h light pulse would trigger a transcriptional activation response only if it interacted with the ϕi, beginning around midpoint in an entrained CRP.

## 2. Material and methods

The redheaded bunting is a Palearctic-Indian latitudinal night-migratory songbird, which overwinters in India (~25°N). It arrives in India in late September/October and begins to return to its breeding grounds in west Asia and east Europe (~40°N; north-west spring migration) generally in early April [19]. The present study was done on male buntings procured from the overwintering flocks in the first week of March 2013, at least three weeks before they generally initiate spring migration. After acclimation to natural day length and temperature conditions (NDL, sunrise to sunset = ~11.5 h) in an outdoor aviary for a week, birds were maintained indoors on a short photoperiod (8 h light:16 h darkness, 8L:16D) and constant temperature (24 ± 2 °C) conditions for two weeks, until the beginning of the experiment. The experiment was performed and the procedures including the ones for the sampling of tissues for mRNA expression measurements, described below, were adopted as per the approval of the Institutional Animal Ethics Committee at the University of Delhi, India.

### 2.1. Experiment

We used an experimental design that was tested in a previous study on blackheaded buntings (*E. melanocephala*) that measured fat deposition and body mass gain, and testis recrudescence as a result of exposure to T-photocycles[9]. A total of 28 birds were used in this study. They were first exposed to 6L:18D (L = ~250 lx; D = 0 lx) for two weeks, and then housed in activity cages (*n* = 2 or 3) placed individually in photoperiodic chambers providing 6L:18D (L = ~250 lx; D = 0 lx) and constant temperature (24 ± 2 °C). After a week, they were randomly distributed in six groups, each of 4 or 5 birds, and exposed for 6 cycles of light–dark cycles (days) with varying period lengths (T), as schematically represented in Fig. 1. Groups 1 and 2 were exposed to a 22 h day (T = 22 or T22) with a 6 h light period (6L:16D, $T22_{6L}$), but group 2 received an additional 1 h light period interposed at zeitgeber time, ZT, 10 (ZT 0 = light onset) in the 16 h dark period (6L:4D:1L:11D, $T22_{11L}$). Likewise, groups 3–5 were exposed to a 24 h day (6L:18D, T24) with an additional 1 h light period interposed at ZT10 (6L:4D:1L:13D, $T24_{11L}$), ZT11(6L:5D:1L:12D, $T24_{12L}$) or ZT12 (6L:6D:1L:11D, $T24_{13L}$). A sixth group was exposed to a 26 h day (T26) with 1 h light pulse given at ZT11 (6L:5D:1L:14D, $T26_{12L}$). Thus, we did not compensate light illumination period for the difference in 'T', given a short period of exposure (6 cycles) and longer light pulse duration (1 h). Here, a 6L:16D ($T22_{6L}$) group was included to confirm that

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