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RESEARCH ARTICLE

Circannual testis and moult cycles persist under photoperiods that disrupt circadian activity and clock gene cycles in spotted munia

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ABSTRACT

We investigated whether circannual rhythms underlying annual testis maturation and moult cycles are independent of duration and frequency of the light period and circadian clock control in non-photoperiodic spotted munia. Birds were subjected to an aberrant light–dark (LD) cycle (3.5 h L:3.5 h D; T7, where T is the period length of the LD cycle) and continuous light (LL, 24 h L:0 h D), with controls on 12 h L:12 h D (T24, 24 h LD cycle). We measured the behavioural activity pattern of the birds and 24 h mRNA oscillations of circadian clock genes (*bmal1*, *clock*, *per2*, *cry1*, *cry2*) in the hypothalamus, the putative site of seasonal timing. Diurnal munia were rhythmic in behaviour with the period of the activity–rest cycle matched to T7 and T24, and became behaviourally arrhythmic with activity scattered throughout 24 h under LL. Similarly, exposure to 3.5 h L:3.5 h D and LL caused arrhythmicity in 24 h clock gene expression, suggesting disruption of internal circadian timing at the transcriptional level; a significant rhythm was found under 12 h L:12 h D. During an exposure of 80 weeks, munia showed two to three cycles of testis maturation and wing primaries moult under all photoperiods, although with a longer period under 12L:12D. Thus, the frequency of light period under 3.5 h L:3.5 h D or LL disrupted circadian clock gene cycles, but did not affect the generation of circannual testis and moult cycles. We conclude that the prevailing light environment and hypothalamic circadian gene cycles do not exert direct control on the timing of the annual reproductive cycle in spotted munia, suggesting independent generation of the circadian and circannual rhythms in seasonally breeding species.

KEY WORDS: Circadian rhythm, Circannual rhythm, Light–dark cycle, Moult, Spotted munia, *Lonchura punctulata*, Testicular cycle

INTRODUCTION

Most bird species reproduce at the best-suited time of the year, since mistiming will have severe fitness consequences for both parents and offspring (Helm et al., 2009; Helm and Lincoln, 2017). Birds show seasonal gonadal growth–regression cycles in response to the external environment, prevalently to the annual photoperiod changes (Kumar et al., 2010). Thus, annual time-keeping appears to operate at two levels. A wealth of accumulated evidence suggests the involvement of circannual clocks in the timing of changes in physiology and behaviour within each year. Captive birds show repeated cycles of gonadal maturation and moult with a cycle length of about 1 year under constant 'neutral' photoperiods [e.g. 12 h light:12 h darkness (12L:12D) or 12.25L:11.75D; Gwinner, 1986, 1996; Budki et al., 2012, 2014] or continuous light (LL; Holberton and Able, 1992; Budki et al., 2012, 2014). Also, birds show an annual gonadal cycle under the prevailing natural photoperiods, i.e. the annual photoperiod cycle is used as a calendar to time the gonadal maturation–regression cycle (Gwinner, 1986; Dawson et al., 2001; Kumar et al., 2010). This underscores that autonomous molecular switches respond to the photoperiod change and control the seasonal physiological states that make up an annual cycle.

Circannual and photoperiodic timing may not be mutually exclusive. For example, annual photoperiod variations can entrain circannual gonadal cycles to periods as short as 4 months and as long as 2 years (Gwinner, 1986). Contrary to this, many low-latitude and equatorial seasonally breeding birds show persistent circannual cycles in gonadal maturation–regression, and fail to show a typical short- or long-day response (Chandola et al., 1975; Thapliyal, 1981; Gwinner, 1996). Furthermore, following gonadal maturation, several photoperiodic seasonally breeding birds undergo regression and exhibit photorefractoriness, and they continue to remain gonadally regressed as long as held under stimulatory long days (Sansum and King, 1976; Malik et al., 2014).

As yet, less is understood about how a circannual timer operates at the cellular or molecular level (Kumar et al., 2010). A few recent studies advocate the pars tuberalis (PT) of the pituitary gland as the site of the 'circannual timer', based on the role of PT-derived thyroid-stimulating hormone (TSH) in driving the expression of genes encoding type 2 and 3 deiodinases (*dio2*, *dio3*) in the ependymal tanycytes (Hazlerigg and Loudon, 2008; Shinomiya et al., 2014). Intriguingly, much of the evidence for PT TSH-induced deiodinase-dependent control of gonadotrophin-releasing hormone (GnRH) release, and consequently the initiation–termination–reinitiation of the gonad development cycle, comes mainly from the photoperiodic species, irrespective of whether they breed in the summer or winter. Therefore, PT TSH could be a regulatory output, and is not necessarily an integral component of the circannual time generator. Clock genes are, by contrast, an integral component of circadian timing.

Further, PT TSH expression is dictated by circadian clock-controlled night melatonin secretion in mammals (Dardente et al., 2010), and by direct light input from the hypothalamic photoreceptors in birds (Nakane et al., 2010). Avian hypothalamic photoreceptors may also be involved in the measurement of photoperiod length, as shown by changes in the expression of neuropsin and rhodopsin photopigments between short and long photoperiods (Majumdar et al., 2015; see also Nakane et al., 2010; Stevenson and Ball, 2012). Thus, we could envisage a role of the circadian pacemaker system (CPS) in the annual timing of seasonal events. In birds, CPS is composed of circadian clocks in the hypothalamus, pineal gland and retina (Cassone and Menaker, 1984; Kumar et al., 2004; Cassone and Yoshimura, 2015). Near-24 h timing in these clocks is generated by an autoregulatory

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Journal of Experimental Biology

Journal of Experimental Biology (2017) 220, 4162-4168 doi:10.1242/jeb.167809

mechanism involving a transcription–translation feedback loop comprising transcriptional activator (brain and muscle arnt-like protein, *bmal1*; circadian locomotor output cycles kaput, *clock*) and repressor (periods, e.g. *per2*; cryptochromes, *cry1* and *cry2*) genes (Bell-Pedersen et al., 2005; Kumar and Singh, 2006). There are differences in clock gene oscillations between short and long days (Yasuo et al., 2003) as well as between the photoperiod-induced seasonal states (Singh et al., 2015). Hypothalamic mRNA oscillations of core clock genes, and of genes involved in the photoperiodic transduction and neurosteroid-dependent processes in the hypothalamus show alterations in expression level, 24 h expression patterns, and 24 h rhythm waveforms (phase, amplitude) in parallel with seasonal changes in physiology and behaviour (e.g. reproduction, migration) of blackheaded buntings (*Emberiza melanocephala*; Singh et al., 2015; Mishra et al., 2017).

The role of light and/or of clock gene transcriptional oscillations in annual timing has not been investigated in a non-photoperiodic species that exhibits a strong circannual reproductive rhythm. Pinealectomized and enucleated birds continue to show a photoperiod-induced gonad development cycle (Menaker et al., 1970; Wilson, 1991; Kumar et al., 2002) or circannual gonadal cycle (Pant and Chandola-Saklani, 1992) and so, in all probability, the principal site of the light and circadian clock effects lies in the hypothalamus. We hypothesized that frequent light periods under an aberrant light–dark (LD, 3.5L:3.5D) cycle or under continuous light (LL) would induce perturbations in the hypothalamic circadian clockwork, and consequently that this might affect circannual reproductive rhythm. In the present study, we tested this proposal in the subtropical spotted munia (*Lonchura punctulata*), in which circannual cycles of gonadal maturation and moult persist under a constant 'neutral' photoperiod (i.e. 12L:12D), LL, and LD cycles with period length (T) shorter or longer than 24 h (e.g. T16, 12L:4D; T21, 12L:9D; T27, 12L:15D; Budki et al., 2012, 2014). Pinealectomized munia show abolition of circadian rhythm in perch-hopping activity, but continue to show circannual rhythm in the gonad development cycle under LL (Pant and Chandola-Saklani, 1992). Here, we monitored the activity pattern, which is a reliable assay of internal circadian timing, measured the 24 h mRNA expression pattern of core clock genes (*bmal1*, *clock*, *per2*, *cry1* and *cry2*) in the hypothalamus, and recorded testicular growth–regression and moult cycles in spotted munia exposed to 3.5L:3.5D (T7) and LL, with controls on 12L:12D (T24). Although an aberrant T7 LD cycle may not affect circadian timing, as shown by body temperature and corticosterone rhythms in mice (LeGates et al., 2012), LL at bright intensity has been shown previously to affect the circadian timing system, as shown by disrupted daily activity rhythms in both birds and mammals (Aschoff, 1981; Singh et al., 2012).

MATERIALS AND METHODS

Animals and experimental design

This study was performed on spotted munia, *Lonchura punctulata* (Linnaeus 1758), at the Department of Zoology, University of Lucknow, Lucknow, India, in accordance with guidelines of the Institutional Animal Ethical Committee (IAEC). Spotted munia is an autumn-breeding songbird, and belongs to family: Estrildidae; order: Passeriformes. Adult birds were captured in February from the wild around Lucknow (26°55′N, 80°59′E), and acclimated to captive conditions for a week in an outdoor aviary (2.95×1.73×2.21 m) under natural light–dark and temperature conditions (NDL). Using an identical experimental design, we performed the following experiments, beginning in March when the birds had small, reproductively inactive testes.

Experiment 1

This experiment examined activity–rest pattern and the 24 h mRNA oscillation in core clock genes (*bmal1*, *clock*, *per2*, *cry1* and *cry2*) in male spotted munia exposed to an aberrant LD cycle (3.5L:3.5D; T7) and continuous light (LL), with controls exposed to 24 h LD cycle (12L:12D; T24). Acclimated birds (n=16 per group) were brought indoors and singly housed in activity cages (60×45×35 cm) placed in light-tight boxes at 20±1°C, and exposed to T24 (group 1), T7 (group 2) or LL (group 3) for 5 weeks at 90±5 lx light intensity (the dark phase in the T7 and T24 LD cycle was ∼0.1 lx). Each activity cage had two perches and was mounted with a passive infrared motion sensor (Haustier PIR-Melder; Intellisense XJ-413T, C & K Systems, Conrad Electronic, Hirschau, Germany) that continuously detected general movement of a bird in its cage. Chronobiology Kit software (Stanford Software Systems, Stanford, CA, USA) was used to collect and collate activity in 5 min bins, and to analyze the activity–rest pattern by a computerized data-logging system (Malik et al., 2004; Singh et al., 2015). At the end of the experiment, and when light-on [zeitgeber time (ZT) 0] of T7 coincided with that of T24 (which occurred at weekly intervals), we recorded body mass and measured testis size (see below), and sacrificed four birds at 6 h intervals, beginning 2 h after light-on at 07:00 h (i.e. ZT2, 8, 14 and 20) by decapitation, which is a quick and unanticipated procedure and hence avoids trauma to the bird and possible anaesthesia effects on gene expression (Pekny et al., 2014; Staib-Lasarzik et al., 2014). The hypothalamus was dissected from quickly excised brains and stored in Trizol (15596026, Invitrogen, Carlsbad, CA, USA) at −80°C until analysis for gene expression.

Experiment 2

This experiment examined the persistence of testicular growth–regression and moult cycles in spotted munia exposed to identical light conditions to those in experiment 1 except in the duration of exposure. Acclimated birds under NDL were brought indoors into a chronocubicle (2.2×1.8×2.8 m) at 20±1°C, and exposed to T24 (12L:12D, group 1), T7 (3.5L:3.5D, group 2) or LL (24L:0D) at 90±5 lx light intensity (the dark period in T24 and T7 was not illuminated) for 80 weeks so that the onset of a subsequent testicular cycle could be discerned. To provide conditions in captivity as close as possible to the free-living condition, we included equal numbers of females in each group, housed birds in chronocubicles that allowed much freer movement, and physically enriched chronocubicles with artificial plants and leafy twigs and perches that were periodically refreshed.

We measured changes in body mass and testis size, and wing primaries moult at 4 week intervals, as per methods regularly used in our laboratory and as published for this species (Budki et al., 2012, 2014). Briefly, birds were weighed on a top-pan balance providing an accuracy of 0.1 g. The size of the left testis was measured by unilateral laparotomy. The procedure involves a small incision made between the last two ribs on the left flank of the bird under anaesthesia (ketamine–xylazine solution administered at 0.003 ml g^{-1} body mass; Surbhi et al., 2015). The left testis was located, and its dimensions (length and width) measured to an accuracy of 0.5 mm. The incision was then immediately sutured, and an antibacterial skin ointment (Soframycin skin cream, Aventis Pharma, Goa, India) was applied. The bird was returned to its cage on a warm pad, which was removed after it had recovered in about 30 min. The testis volume was calculated using the formula $4/3\pi ab^2$, where a and b denote half the length and width of the testis, respectively. For moult, we examined primary wing feathers and scored them in a range of 0 to 5: score 0, worn or old feather;

Journal of Experimental Biology (2017) 220, 4162-4168 doi:10.1242/jeb.167809

1, missing feather (i.e. just dropped); 2, from the stage of emergence to one-third growth of a new feather papilla; 3, a new feather papilla with two-thirds growth; 4, newly grown feather, but still incomplete; 5, fully grown feather, as published earlier (Budki et al., 2012, 2014). Thus, an individual primary feather could have a score of 0 to 5, and a wing primary (left or right) could have a total score of 0 to 45. From this score, a linear increase in new feather mass was calculated as described (Dawson and Newton, 2004).

Food (seeds of *Setaria italica* and *Oryza sativa*) and water were provided *ad libitum*. A supplementary food rich in protein and vitamins, which was prepared by mixing bread crumbs, boiled eggs, crushed egg shells, cottage cheese and multi-vitamins (Vimeral, containing vitamins A, D3, E and B12, Virbac Animal Health India, Mumbai, India), was also given on alternate days.

Measurement of gene expression

We measured mRNA expression of *bmal1*, *clock*, *per2*, *cry1* and *cry2* in the hypothalamus collected at 6 h intervals. We cloned partial sequences of the genes of interest, as per protocols standardized and used in our laboratory for songbirds (Singh et al., 2013). Briefly, 2 µg total RNA extracted by Trizol, as per manufacturer's protocol, was first treated with RQ1 RNase-free DNase (M6101, Promega, Madison, WI, USA) to remove genomic DNA contamination, and then reverse transcribed to synthesize cDNA using the Revert-Aid first-strand cDNA synthesis kit (K1622, Thermo Scientific, Vilnius, Lithuania). Then, the gene of interest was amplified from hypothalamic cDNA using degenerate primers designed from the conserved region of gene sequence (Table S1A). Amplified products were resolved through a 1.5% agarose gel (0710, Ameresco, Solon, OH, USA), and PCR amplicons were excised and eluted using a gel extraction kit (28704, Qiagen, Hilden, Germany). The purified DNA product was ligated into a pGEM-T vector (pGemT-easy, Promega cat. 1360), competent *E. coli* DH5α cells transformed and plasmids isolated using the QIAprep Spin Miniprep Kit (27104, Qiagen). Positive clones were sequenced (MWG, Bangalore, India) and gene identity was determined by nucleotide BLAST (NCBI).

Using gene-specific primers designed from cloned cDNA sequences (Table S1B), the mRNA expression of a candidate gene was measured by RT-PCR on a ViiA7 thermal cycler (Applied Biosystems, Foster City, CA, USA) using Power SYBR Green PCR MasterMix (4387669, Applied Biosystems), as described in previous publications from our laboratory (Singh et al., 2013; Trivedi et al., 2014). Both the sample and reference (*β-actin*) were run in duplicate, and the fold change in mRNA levels was calculated as $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). Briefly, we first calculated

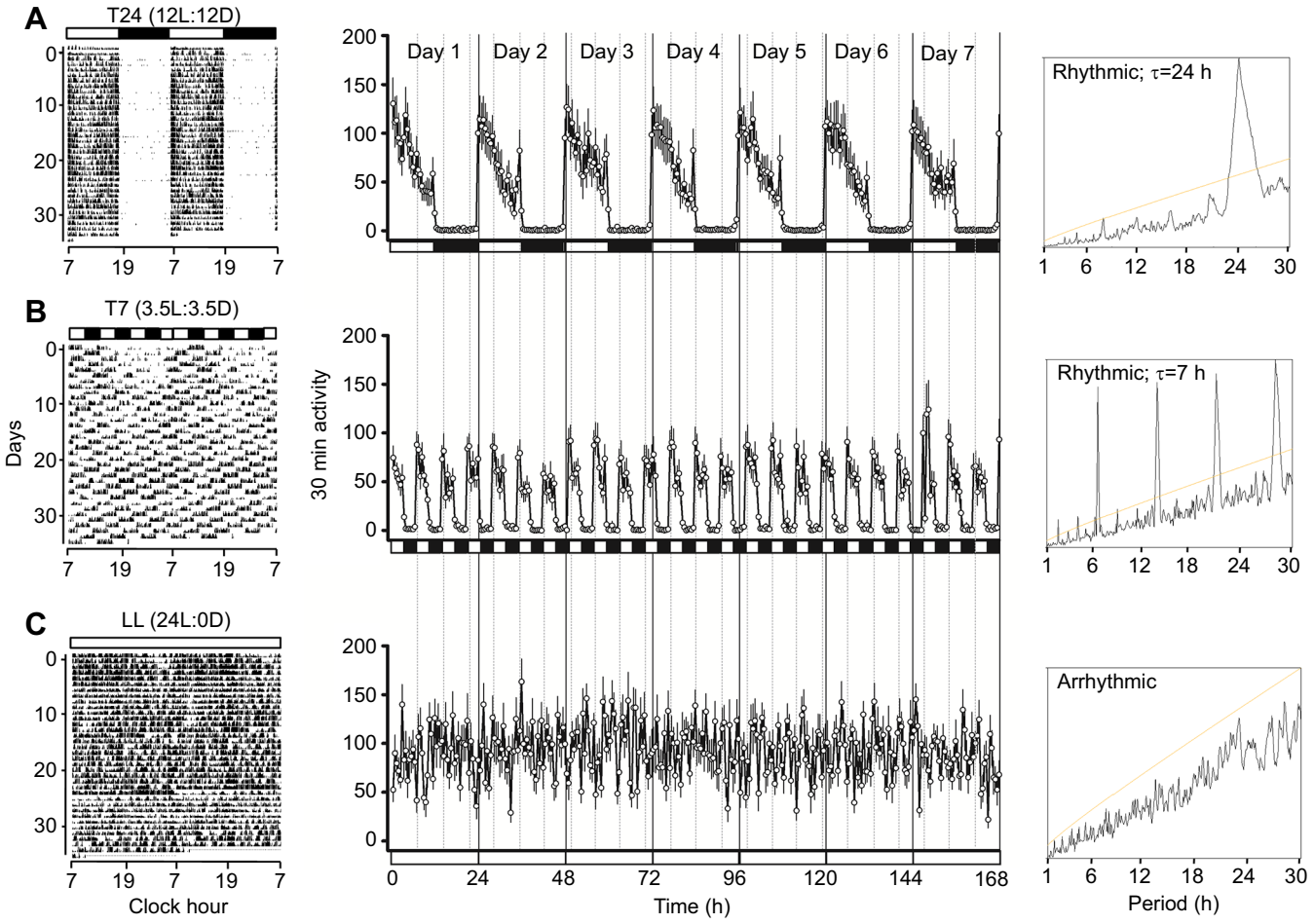

Fig. 1. Activity–rest pattern of spotted munia under T24 and T7 light-dark cycles and constant light. (Left) Double-plotted representative actograms of spotted munia exposed to (A) T24, (B) T7 or (C) LL light conditions for 5 weeks. (Middle) Mean (±s.e.m.) 30 min activity profile (n=16 birds per group) over seven consecutive days (separated by solid lines); dotted lines indicate the T7 LD. Note that the activity and rest patterns are synchronized with the light and dark periods, respectively, under T24 and T7, whereas the activity is scattered throughout the 24 h under LL. (Right) χ^2 periodogram analysis showing the period of activity rhythm. Horizontal bars (left and middle) represent light (white) and darkness (black).

Journal of Experimental Biology

Journal of Experimental Biology (2017) 220, 4162-4168 doi:10.1242/jeb.167809

ΔC_t [C_t(target gene)−C_t(reference gene)], and then normalized it against the ΔC_t value of the calibrator sample (lowest ΔC_t value at ZT2) to give $\Delta\Delta C_t$. Then, $2^{-\Delta\Delta Ct}$ was plotted as the fold change in relative mRNA expression level (Livak and Schmittgen, 2001).

Statistics

Unless specified otherwise, statistical analyses were performed using GraphPad Prism software, version 5.0 (GraphPad, La Jolla, CA, USA), and for statistical significance alpha was set at 0.05. In experiment 1, we calculated and presented activity in 30 min bins to better illustrate 24 h variation in the activity over 7 days, so that it covered the period when light period coincided between T7 and T24 LD cycles on every eighth day. We first averaged activity for each 30 min beginning from light-on in T24 or T7 over 7 days for each individual, and from this the mean (±s.e.m.) for the group was determined. The period of circadian rhythm (τ) in the activity pattern was calculated by χ^2 periodogram analysis using the Chronobiology Kit software program. Further, one-way ANOVA was used to determine 24 h variation in mRNA expression levels, and a unimodal cosinor regression analysis $\{y=A+[B\cdot\cos(2\pi(x-C)/24)]$, where A is the mean (mesor), B is the amplitude and C is the acrophase of the rhythm (Cuesta et al., 2009)} was used to assess a daily rhythm in mRNA expression levels over 24 h, as previously (e.g. Singh et al., 2013; Mishra et al., 2017). The significance of cosinor regression analysis was calculated using the number of samples, R^2 values, and numbers of predictors, i.e. mesor, amplitude and acrophase (Soper, 2013). The mesor, amplitude and acrophase values were used as markers of the baseline, maximum change in mRNA expression levels, and estimated time of peak mRNA expression, respectively.

In experiment 2, we calculated and presented mean (±s.e.m.) from six males that had complete data for the entire duration of the experiment, excluding data from individuals that died during the experiment (two males from each 12L:12D and LL, and one from T7) or data that were lost for technical reasons (one from T7). One-way ANOVA was used to determine overall differences between the time points or groups and, following significance, the Newman–Keuls *post hoc* test was used to compare time points or groups. Two-way ANOVA further tested the effects of the light condition (factor 1), exposure duration (factor 2), and factor 1×factor 2 interaction on changes in the size of testes over the period of exposure.

RESULTS

Experiment 1: 24 h variations in activity and mRNA expression patterns

This experiment tested the persistence of 24 h rhythms both in activity behaviour and clock gene expression in spotted munia under typical (T24) and aberrant (T7) LD cycles, and bright LL. Body mass and testis size did not show any differences during the 5-week exposure, irrespective of the photoperiod (data not shown). However, as expected, diurnal spotted munia were active during the daytime under T24 (12L:12D), and exhibited a synchronized 24 h activity pattern with a period equal to 24 h (τ=T=24 h, χ^2 periodogram; Fig. 1A). Also, birds showed activity only during the light period, and exhibited a rhythmic activity–rest pattern with a period equal to 7 h under 3.5L:3.5D (τ=T=7 h, χ^2 periodogram; Fig. 1B, Fig. S1). However, munia became gradually arrhythmic and exhibited activity scattered throughout 24 h under LL (Fig. 1C). The 30-min activity profile (mean±s.e.m.) calculated and plotted over seven calendar days, as light onsets of the T24 and T7 LD cycles coincided with each other every eighth day, further verified rhythmicity in activity behaviour under both T24 and T7 LD cycles, and arrhythmicity in activity behaviour under LL (Fig. 1).

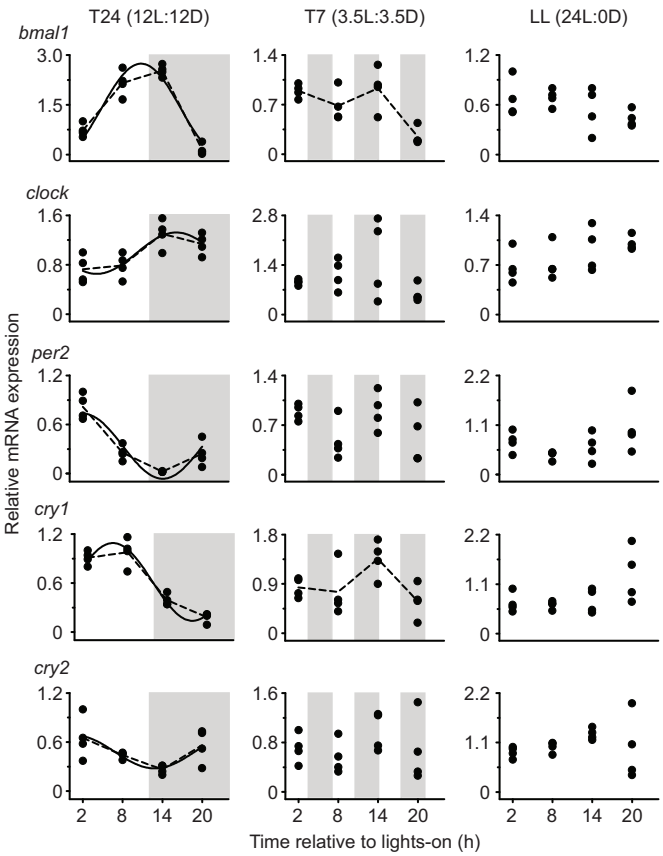

Fig. 2. Daily variation and cosinor waveform of 24 h mRNA expression pattern of core clock genes. The daily (24 h) mRNA expression levels of core circadian clock genes (*bmal1*, *clock*, *per2*, *cry1* and *cry2*) measured four times a day (n=4 per time point) in the hypothalamus of spotted munia under T24 (left), T7 (middle) and LL (right). Each data point represents the relative mRNA expression of a gene normalized to *β-actin*, which was used as a reference gene. A dashed line connecting mean values indicates a significant difference between 24 h mRNA levels, as determined by one-way ANOVA. The solid line, which is a regression curve drawn through the time points, represents a significant 24 h rhythm in mRNA expression levels, as determined by cosinor analysis. Shaded areas represent dark periods. For statistical significance, alpha was set at 0.05. Note that the scales on the *y*-axis differ.

The 24 h mRNA expression patterns of core clock genes showed differences between the three light conditions (Fig. 2). In birds under T24, both activator (*bmal1* and *clock*) and repressor (*per2*, *cry1* and *cry2*) genes showed significant variations in mRNA levels (*bmal1*, $F_{3,15}$=82.1, P<0.0001; *clock*, $F_{3,15}$=6.9, P=0.006; *per2*, $F_{3,15}$=32.8, P<0.0001; *cry1*, $F_{3,15}$=52.5, P<0.0001; *cry2*, $F_{3,15}$=3.8, P=0.040; one-way ANOVA; Fig. 2). Also, there was a significant 24 h (daily) rhythm in the mRNA expression patterns of all genes (P<0.05, cosinor analysis), although with gene-specific variation in the rhythm waveform (e.g. amplitude and acrophase; Table 1, Fig. 2). Whereas the acrophase of activator gene oscillations was later during the day (ZT11, *bmal1*) or early at night (ZT16, *clock*), that of the repressor gene oscillations was very early or in the middle of the day (ZT2, *per2*; ZT6, *cry1*; ZT1, *cry2*) (Table 1, Fig. 2). By contrast, mRNA expression levels of both activator and repressor genes neither varied over 24 h, except for *bmal1* ($F_{3,15}$=9.2, P=0.002) and *cry1* ($F_{3,15}$=3.6, P=0.041; one-way ANOVA) under T7, nor showed a significant 24 h rhythm under T7 and LL

Journal of Experimental Biology (2017) 220, 4162-4168 doi:10.1242/jeb.167809

Table 1. Rhythm waveform characteristics in the mRNA expression patterns of core clock genes of spotted munia under T24 (12L:12D) light conditions, as determined by cosinor analysis

Gene	Waveform characteristic	Mean±s.e.m.*
bmal1	Mesor	1.39±0.09
	Amplitude	1.35±0.13
	Acrophase	10.79±0.36
clock	Mesor	0.99±0.05
	Amplitude	0.33±0.07
	Acrophase	16.08±0.82
per2	Mesor	0.33±0.04
	Amplitude	0.40±0.05
	Acrophase	2.06±0.50
cry1	Mesor	0.61±0.03
	Amplitude	0.47±0.04
	Acrophase	5.80±0.31
cry2	Mesor	0.48±0.04
	Amplitude	0.20±0.06
	Acrophase	0.85±1.11

*Acrophase is in hours. There is an absence of rhythm in birds under the T7 (3.5L:3.5D) and LL (24L:0D) conditions (not shown).

conditions (Fig. 2). This suggested disruption of circadian rhythm generation at the transcriptional level under the aberrant T7 LD and LL conditions.

Experiment 2: circannual cycles in body mass, testis maturation and moult

This experiment examined the persistence of testis maturation–regression and 'post-nuptial' moult cycles over a 80 weeks under aberrant T7 LD cycle and bright LL, with controls on T24. Interestingly, contrary to the disruption of 24 h circadian gene cycles under T7 and LL, testes underwent maturation–regression cycles in all three photoperiods. Hence, we found a significant change in testis size over the period of the experiment (T24, $F_{20,125}$=10.7, P<0.0001; T7, $F_{20,125}$=7.6, P<0.0001; LL, $F_{20,125}$=7.9, P<0.0001; one-way RM ANOVA; Fig. 3A–C, Fig. S3), although with differences in period length (interval between successive testis size maxima) and waveform of the circannual testicular cycle between the light conditions ($F_{2,16}$=6.3, P=0.011; one-way ANOVA; Fig. 3E). Whereas the circannual testicular cycle freeran with similar period lengths under T7 (28.0±3.9 weeks) and LL (23.2±1.5 weeks), it was significantly longer under T24 (39.3±3.5 weeks; Newman–Keuls test, P<0.05; Fig. 3E). The peak testis size was not significantly different between cycles, both within a group and between groups (Fig. 3F). Also, with reference to day 0, the time taken to attain the second peak, not the first peak, in testicular growth was significantly different between the three light conditions ($F_{2,16}$=5.9, P=0.013; one-way ANOVA): it was shorter under T7 (54.0±5.3 weeks) and LL (44.8±1.5 weeks) than under T24 (64.0±3.1 weeks; Newman–Keuls test, P<0.05). Overall, we found that the effect of the light condition on the testicular cycle varied with the duration of exposure (light condition, $F_{2,315}$=18.6, P<0.0001; duration of exposure, $F_{20,315}$=14.5, P<0.0001; condition×duration interaction, $F_{40,315}$=2.3, P<0.0001; two-way ANOVA; Fig. 3A–C).

Similar to testes, the birds also showed cycles in the post-nuptial wing primaries moult, although with differences between individuals within and between light conditions. In general, birds underwent two to three moult cycles under T24 and T7, and two to four moult cycles under LL. Hence, the mean period length of moult cycles (interval between successive moults) was not significantly different between the light conditions (Fig. 3D).

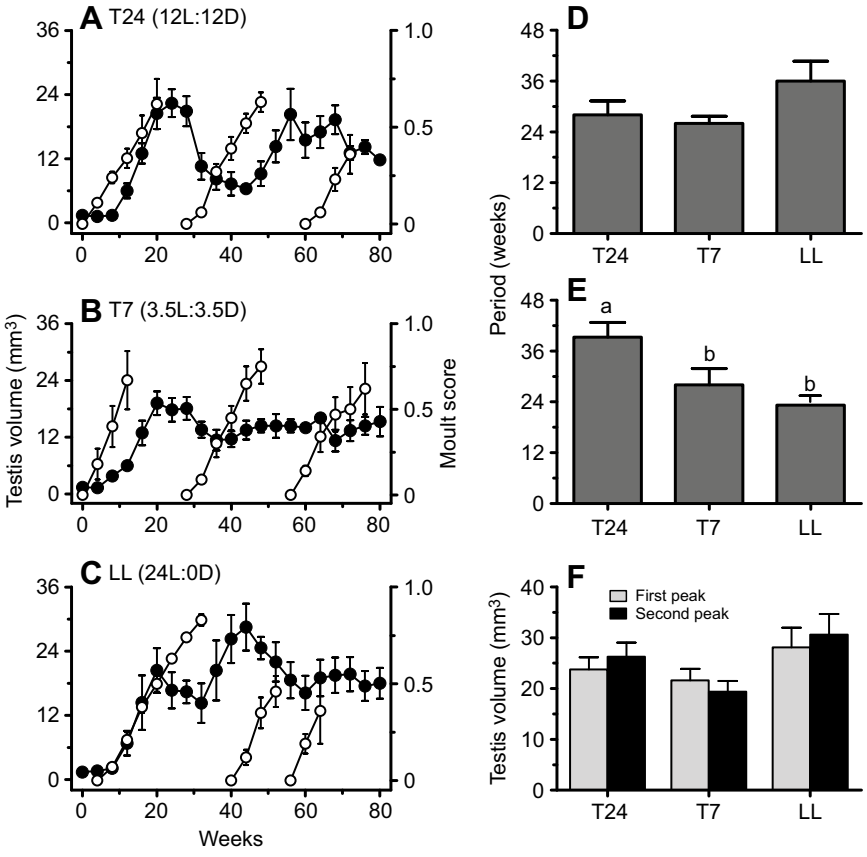

Fig. 3. Testicular and moult cycles under T24 and T7 light–dark cycles and constant light. (A–C) Mean (±s.e.m.) testis size (black circles) of spotted munia exposed to (A) T24, (B) T7 or (C) LL light conditions for a period of 80 weeks. Also shown is the moult pattern (mean±s.e.m.) of wing primaries (white circles). (D,E) Mean (±s.e.m.) period length of (D) wing primaries moult (the interval between successive initiations of the moult) and (E) the testicular cycle (interval between successive testis maxima). (F) Peak testis size attained (mean±s.e.m.) in the first and second testicular cycle. Each data point is for six birds. Different lowercase letters indicate a significant difference between groups (P<0.05, Newman–Keuls *post hoc* test).

Journal of Experimental Biology

Journal of Experimental Biology (2017) 220, 4162-4168 doi:10.1242/jeb.167809

Finally, there were significant differences in body mass during the period of the experiment under T24 and T7 (T24, $F_{20,125}$=6.1, P<0.0001; T7, $F_{20,125}$=3.6, P<0.0001; one-way RM ANOVA), but not under LL, although such variations in body mass were not consistent with a circannual rhythm (Fig. S2).

DISCUSSION

The persistence of 24 h rhythm in activity behaviour and in the hypothalamic expression of core clock genes under 12L:12D suggests that the circadian clock operates at both behavioural and molecular levels in spotted munia. Consistent with 24 h mRNA oscillations demonstrated in other bird species [Japanese quail (Yasuo et al., 2003); chicken (Karaganis et al., 2009); house sparrow (Helfer et al., 2006); redheaded bunting (Singh et al., 2013)], the acrophases of transcriptional activator gene oscillations (late in the day for *bmal1*, and early in the night for *clock* expression) were in antiphase with those of the repressor gene oscillations (early in the day; *per2*, *cry2*) (Fig. 2). By contrast, there was decay of rhythms in both activity behaviour (under LL only) and clock genes under T7 and LL; under T7, the activity–rest pattern perhaps showed a masked (direct) effect of the LD cycle. This suggests an effect of the prevailing light environment on the endogenous circadian system regulating changes in physiology and behaviour within each day in spotted munia. Animals received light throughout 24 h under LL, whereas they received equal weekly amounts (84 h) of light or darkness but at different frequencies under the T7 and T24 LD cycles. Per calendar week, T7 and T24 received 24 and 7 periods of light (and dark), respectively (Fig. 1).

In a similar exposure to the aberrant 3.5L:3.5D photoperiod, mice freeran with a circadian period and exhibited a 24 h rhythm in suprachiasmatic nucleus (SCN) PER2 and liver *Per2* expression (LeGates et al., 2012). However, another study found abolition of circadian rhythms in core body temperature, corticosterone and clock gene expression in peripheral tissues, although feeding and drinking rhythms persisted, in mice under 3L:3D (T6; Oishi et al., 2015). Also, LL has been shown to induce arrhythmicity in the activity pattern and in PER2 and *Rev-erbα* (thyroid hormone receptor alpha) and *Bmal1* expression in the SCN (Beaulé et al., 2003; Nováková et al., 2011) and of circadian clock and clock-controlled genes in peripheral tissues (colon and liver) (Polidarová et al., 2011) in rats. Although it is unclear how arrhythmicity in activity behaviour and clock gene expression occur in spotted munia, we speculate, based on evidence from mammals (Ohta et al., 2005), that T7 and LL induce desynchronization in phases of individual rhythms within CPS neurons.

Irrespective of the synchronized (under T24) or disrupted (under T7 and LL) molecular circadian clockwork, spotted munia exhibited circannual cycles in testicular maturation and post-nuptial moult. This suggested that seasonal (annual) timing was not a direct response to the prevailing photoperiods, as has been argued (Dawson, 1997; Kumar et al., 2010). We would not conclude, though, that the annual reproductive cycle in spotted munia is completely independent of the external light environment, which might still have a modulating effect on the circannual reproductive rhythm. There was indeed a difference in the period and waveform of the testicular cycles between the three light conditions: mean circannual period was significantly longer and the overall amplitude of the growth–regression curve was higher under T24 than under T7 or LL (Fig. 3). However, differences in the timing and pattern of the testicular and moult cycles between light conditions also suggest their independent regulation, although both probably share a common functional basis, i.e. circannual clock control.

Clearly, hypothalamic circadian gene oscillations do not appear to be involved in circannual rhythm generation, with the caveat that a direct connection between the two timing mechanisms could not be shown by the present study. Hence, we would not completely rule out the possibility of a linkage between the circannual and circadian rhythms. This is because (1) the circannual system might influence circadian rhythms (Mrosovsky et al., 1976; Ball and Ketterson, 2008) and photoperiod-induced seasonal states (Misra et al., 2004); and (2) we have not identified, as yet, the hypothalamic site(s) of circannual rhythm generation. Notably, however, PT is suggested to contain the circannual timer (Wood and Loudon, 2017), based on evidence that PT-derived TSH controls reciprocal switching of *dio2* and *dio3* in ependymal tanycytes and, in turn, triiodothyronine (T3)-controlled GnRH release and the gonadal growth–regression cycle in both seasonally breeding birds and mammals (Hazlerigg and Loudon, 2008; Shinomiya et al., 2014). Intriguingly, the TSH-induced increase and decrease of *dio2* and *dio3* expression, respectively, has been shown in photoperiodic species in response to long days only, irrespective of whether they breed in the summer or winter. Therefore, it is not unlikely that PT-dictated changes in deiodinase expression constitute the regulatory output, and are not necessarily part of the circannual time generator across seasonally breeding species.

To our knowledge, this is the first demonstration of light-induced concurrent effects on activity behaviour, hypothalamic expression of circadian clock genes, and testis maturation and moult cycles in a subtropical species that shows a robust self-sustained circannual reproductive cycle. In particular, we show that annual reproductive phenotypes are independent of direct control by light and the circadian clock system in spotted munia.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: V.K.; Methodology: S.R., V.K.; Formal analysis: N.A., I.M., V.K.; Investigation: N.A., I.M., R.K., S.R.; Resources: S.R., V.K.; Data curation: N.A., I.M., R.K.; Writing - original draft: N.A., I.M., V.K.; Writing - review & editing: V.K.; Supervision: S.R., V.K.; Project administration: S.R., V.K.; Funding acquisition: S.R., V.K.

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Supplementary information

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