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RESEARCH ARTICLE

Circadian rhythms are not involved in the regulation of circannual reproductive cycles in a sub-tropical bird, the spotted munia

Puja Budki[1], Shalie Malik[1], Sangeeta Rani[1] and Vinod Kumar[1,2,*]

ABSTRACT

Circannual rhythms regulate seasonal reproduction in many vertebrates. The present study investigated whether circannual reproductive phenotypes (rhythms in growth of gonads and molt) were generated independently of the circadian clocks in the subtropical non-photoperiodic spotted munia (*Lonchura punctulata*). Birds were subjected to light:dark (LD) cycles with identical light but varying dark hours, such that the period of LD cycle (T) equaled 16 h (T16; 12 h L:4 h D), 21 h (T21; 12 h L:9 h D), 24 h (T24; 12 h L:12 h D) and 27 h (T27; 12 h L:15 h D), or to continuous light (LL, 24 h L:0 h D) at ~18°C. During the ~21 month exposure, munia underwent at least two cycles of gonadal development and molt; changes in body mass were not rhythmic. This was similar to the occurrence of annual cycles in reproduction and molt observed in wild birds. A greater asynchrony between circannual cycles of gonad development and molt indicated their independent regulation. Females showed reproductive rhythms with similar circannual periods, whilst in males, circannual periods measured between peak gonadal size were longer in T21 and T24 than in T16 or T27. This suggested a sex-dependent timing of annual reproduction in the spotted munia. Also, food availability periods may not influence the circannual timing of reproduction, as shown by the results on the rhythm in gonadal growth and regression in munia under T-photocycles and LL that provided differential light (feeding) hours. Further, a short-term experiment revealed that activity–rest patterns in munia were synchronized with T-photocycles, but were arrhythmic under LL. We conclude that circadian rhythms are not involved in the timing of the annual reproductive cycle in the spotted munia.

KEY WORDS: Body mass, Circadian rhythm, Circannual rhythm, Gonad, Molt, *Lonchura punctulata*, T-photocycle

INTRODUCTION

In seasonally breeding vertebrates, the annual reproductive cycle is recognized in gonadal recrudescence, breeding, gonadal regression and gonad-inactive phases. Rigidly linked with gonadal phases are the changes in food intake, body mass and molt. Each phase lasts for a definite period, but the gonadal recrudescence phase, i.e. the interval between the beginning of the initiation of growth and regression of the gonads, lasts for the longest period. In birds, as in several other vertebrates, circannual (circa=about, annum=year) rhythms are involved in the regulation of the timing and duration of yearly gonadal phases and associated phenotypic traits like body mass (Gwinner, 1986). Several bird species show repeated circannual cycles in food intake, body mass and reproductive phenotype (gonadal maturation, post-nuptial molt and feather regeneration) under constant 12 h photoperiods (Gwinner, 1981; Gwinner, 1986; Gwinner and Dittami, 1990; Cadee et al., 1996; Piersma et al., 2008; Wikelski et al., 2008; Helm et al., 2009) and constant bright [90 lx (Bhatt and Chandola, 1985)] or dim [1–3 lx (Holberton and Able, 1992), ~22 lx (Budki et al., 2012)] light. In the wild, circannual reproductive rhythms are synchronized with annual photoperiodic (zeitgeber: from the German zeit=time, geber=giver) cycle and exhibited as an annual reproductive cycle (Gwinner, 1981; Gwinner, 1986).

A number of studies further show that day length regulates the annual reproductive cycle in birds (Kumar, 1997; Dawson et al., 2001). Many, if not all, photoperiodic species undergo spontaneous gonadal regression if they are kept on stimulatory photoperiods for a longer duration (Kumar, 1997; Dawson et al., 2001). Interestingly, photoperiodic blackheaded buntings (*Emberiza melanocephala*) kept on a non-stimulatory short photoperiod [8 h light (L):16 h dark (D)] show a seasonal change in the amplitude of their testicular response to stimulatory long days (Misra et al., 2004). Also, the annual reproductive cycle in European starling (*Sturnus vulgaris*), which shows repeated testicular cycles under a 12 h light period (Gwinner, 1981), is suggested to be under strict photoperiodic control in nature (Dawson, 2007).

It is widely accepted that a photoperiodic species distinguishes between non-stimulatory and stimulatory day lengths by using its circadian clock. This is expressed as a circadian rhythm of photoinducibility, which daily passes through a period of inducibility by the external light environment (Kumar et al., 2010). Several studies have shown the involvement of circadian rhythms in the initiation and termination of the seasonal gonadal response in photoperiodic birds and mammals (Kumar, 1997). Unlike the circadian clock, a circannual clock has not yet been described at the anatomical, physiological or molecular level. Therefore, it may be argued that the circadian rhythms form the basis of the generation and/or expression of the circannual reproductive rhythms. However, evidence supporting this idea is wanting. In fact, in golden-mantled ground squirrels (*Callospermophilus lateralis*), the ablation of the suprachiasmatic nuclei (SCN) eliminated the circadian rhythms in activity, but not the circannual cycles in body mass and reproductive phenotypes (Dark et al., 1985). Also, circannual testicular cycles have been shown to persist in the pinealectomized spotted munia (*Lonchura punctulata*), although they express arrhythmicity in their perch-hopping activity (Pant and Chandola-Saklani, 1992).

Nevertheless, the circannual and circadian rhythms share many, if not all, common characteristics (Gwinner, 1986; Rani and Kumar, 2013). This gives credence to the idea that circadian cycles may generate circannual cycles by transformation into a lower frequency rhythm, in accordance with the frequency demultiplication hypothesis, FDH (Gwinner, 1973; Gwinner, 1981; Gwinner, 1986).

[1]DST IRHPA Center for Excellence in Biological Rhythm Research at Department of Zoology, University of Lucknow, Lucknow 226 007, India. [2]DST IRHPA Center for Excellence in Biological Rhythm Research at Department of Zoology, University of Delhi, Delhi 110 007, India.

*Author for correspondence (drvkumar11@yahoo.com)

Received 1 December 2013; Accepted 22 April 2014

The Journal of Experimental Biology (2014) doi:10.1242/jeb.100651

It postulates an intimate relationship between the circadian and circannual cycle periods (Gwinner, 1973; Gwinner, 1981) – the synchronized circadian rhythms determine the cycle length of the circannual phenotypes. A T21 or T27 day, falling within the range of the circadian entrainment (T=24±3 h), is expected to synchronize the circadian oscillator, as does a T24 day (Hut and Beersma, 2011). Thus, compared with a T24 day, which results in a 365 day annual cycle, the T21 and T27 days should result in the faster (short) and slower (long) circannual cycles, respectively.

An alternative idea, called the energy turnover hypothesis (ETH), has also been invoked for the circannual rhythm generation. As a mechanistic explanation, the ETH posits that the energy turnover during the metabolic processes underlying annual life history stages tracks the passage of time (Mrosovsky, 1980; Gwinner, 1986). Each life history stage has an average energy allocation to complete the underlying metabolic processes at an optimal level. Thus, during *ad libitum* resource availability in natural day–night conditions, animals exhibit a seasonal response at more or less similar times each year (Lincoln et al., 2006). However, a reduced food supply can enforce overall reduced energy expenditure and slow down the rates of metabolic processes, resulting in a longer circannual rhythm.

A number of lighting protocols have been developed to test the FDH and ETH in circannual rhythm generation. For example, subjecting a 'circannual' species to light:dark (LD) cycles that will synchronize the circadian rhythms tests the FDH. This involves exposure to LD cycles with periods (T) falling within the range of circadian rhythm entrainment; such a T-photocycle may be called a circadian day. It is predicted that animals under such circadian days will show synchronized circadian and circannual rhythms, resulting in corresponding frequencies (e.g. 365 circadian cycles=one circannual cycle), if their generation were interlinked. A mismatch between frequencies will strongly argue for separate mechanisms underlying their generation. A previous study showed that golden-mantled ground squirrels exposed for 17 months to T=23, 24 or 25 h cycles had synchronized circadian activity rhythms, but exhibited free-running circannual body mass and estrous cycles (Carmichael and Zucker, 1986).

A similar lighting protocol to test the ETH involves manipulation of the food availability periods by restricting the daily light hours to a diurnal species. Wikelski and colleagues (Wikelski et al., 2008) measured the circannual cycles in temperate house sparrows (*Passer domesticus*) subjected for 15 months to T21, T24 and T27 photocycles, and to a skeleton photoperiod that had an additional resting phase, which enforced a reduced food availability period. Contrary to the FDH predictions, sparrows exhibited similar circannual period lengths regardless of the T-photocycles. However, in keeping with the ETH, sparrows on the skeleton photoperiod exhibited a longer circannual reproductive rhythm (Wikelski et al., 2008).

The spotted munia, *L. punctulata* (Linnaeus 1758) is an estrilid seasonal breeder with a reproductive season extending from June to October (Ali and Ripley, 1974). It is not a typical photoperiodic species in the sense that on prolonged exposure it shows gonadal growth regardless of the duration of the light period or the absence of a light period altogether, i.e. continuous darkness (Chandola et al., 1975). However, it may still use light in the synchronization of its annual gonadal cycle, as shown by the effects of small increments of 1.5–2 min daily light periods during the time around the vernal equinox (Chandola-Saklani et al., 2004). Spotted munia have been reported to show both circadian and circannual rhythms (Bhatt and Chandola, 1985; Pant and Chandola-Saklani, 1992; Budki et al., 2012) (P.D., S.M., S.R. and V.K., unpublished observations).

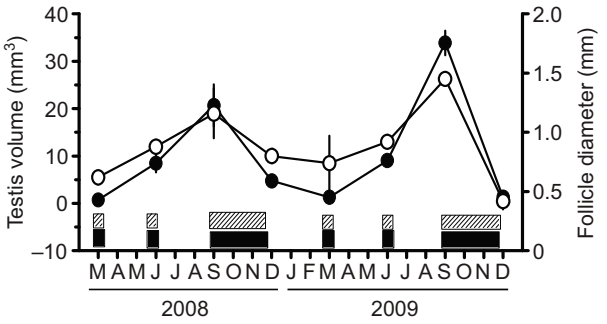

Fig. 1. Annual gonadal and molt cycles in spotted munia. Mean (±s.e.m.) changes in gonadal size in male and female spotted munia that were captured from the wild in the middle of March, June, September and December over 2 years (filled circles, males; open circles, females). The bars at the bottom of graph show the time of molt in both sexes (hatched bars, females; solid bars, males).

Removal of the pineal gland, a circadian oscillator in songbirds (Kumar et al., 2004), impairs circadian but not circannual rhythmicity (Pant and Chandola-Saklani, 1992) in the spotted munia under constant light. Using T-photocycles as the experimental paradigm, the goal of this study was to investigate whether circannual reproductive phenotypes (rhythms in gonad growth and molt) are generated independently of the circadian clocks in the spotted munia. Also, we analyzed whether the circannual reproductive phenotypes exhibit independent cycles in a year, and whether male and female munia use independent strategies in timing their annual reproductive cycles.

RESULTS

Results from birds from the wild are shown in Fig. 1. All birds exhibited yearly cycles in the growth of gonads, with minimum and maximum gonadal size in March and September, respectively. Whereas birds began to show gonadal recrudescence in June, they had regressed gonads in December. Thus, gonads underwent significant changes in their size during the year (one-way ANOVA, male: $F_{7,32}$=32.16, P<0.0001; female: $F_{7,32}$=7.035, P<0.0001; Fig. 1). Although birds showed a short pre-nuptial molt period, the post-nuptial molt period was relatively complete and occurred in all birds (Fig. 1).

Circannual cycles in males

Spotted munia underwent significant changes in body mass in all light conditions, with large intra-group individual variations and a lack of distinct cyclicity (data not shown). However, birds exhibited testis growth–regression cycles in all light conditions (Fig. 2A–E), but with significantly different circannual periods (one-way ANOVA, $F_{4,44}$=5.945, P=0.0006; Fig. 2A–E and Fig. 3C; supplementary material Figs S1–S5). The circannual periods under T21 (12 h L:9 h D) and T24 (12 h L:12 h D) were similar to each other, but were significantly longer (Newman–Keuls test, P<0.05) than those under T16 (12 h L:4 h D), T27 (12 h L:15 h D) and LL (24 h L:0 h D) (Fig. 3C). The duration of the recrudescence phase (breeding window, Table 1) also differed between groups in the first but not in the second cycle (one-way ANOVA, first cycle: $F_{4,44}$=4.643, P=0.0033; second cycle: $F_{4,44}$=1.617, P=0.1868). The responses to T-photocycles in the first cycle, measured as the time taken to attain peak testis maturation, were similar among all T-photocycles, but slower in LL (one-way ANOVA, $F_{4,44}$=11.19, P<0.0001; Newman–Keuls test, P<0.05; Table 2). However, the

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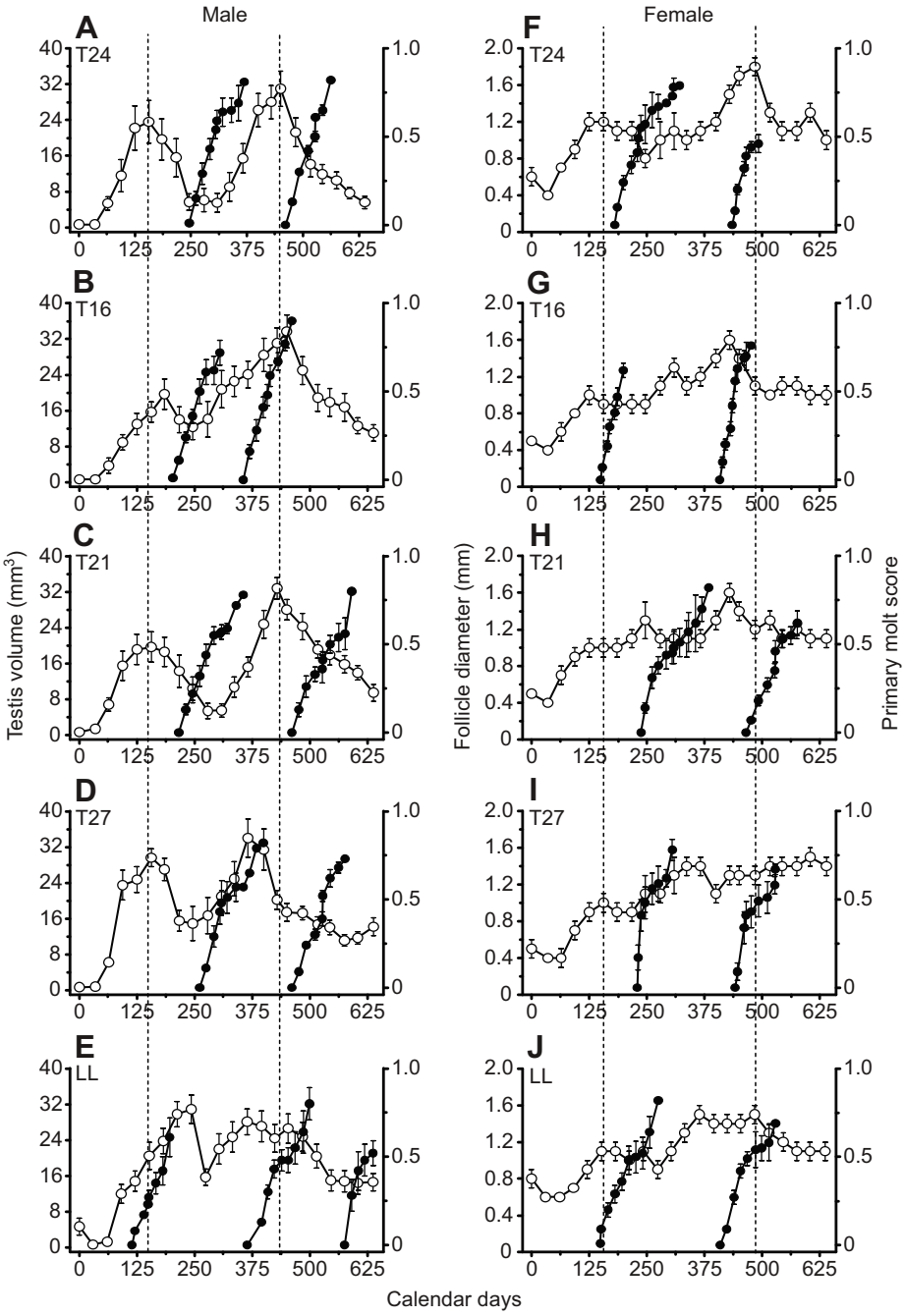

Fig. 2. Gonadal and molt cycles under different T-photocycles and constant light. (A–E) Mean (±s.e.m.) testicular cycles (testis volume) and (F–J) ovarian cycles (largest follicle diameter; open circles) in the spotted munia exposed to T24 (12 h L:12 h D; *N*=8 males, 11 females), T16 (12 h L:4 h D; *N*=10 males, *N*=10 females), T21 (12 h L:9 h D; *N*=11 males, *N*=11 females) and T27 (12 h L:15 h D; *N*=9 males, *N*=9 females) light:dark cycles and to LL (24 h L:0 h D; *N*=11 males and *N*=11 females) for about 21 months. Superimposed on each figure are the molt patterns of wing primaries (filled circles, means ± s.e.m.). Details of T-photocycles are given in Fig. 7. Vertical lines passing through each figure have been drawn with reference to the time of peak gonadal response under T24.

time taken to attain the second peak in testis maturation was significantly shorter in birds under T27 (one-way ANOVA, $F_{4,44}$=9.043, P<0.0001; Newman–Keuls test, P<0.05; Table 2). Between cycles, the peak testicular response was similar in all groups, except in the T16 group, in which birds had a significantly larger response in the second cycle (paired t-test, P=0.0315; Fig. 3A). Among groups, the LL group had larger testes than the T16 and T21 groups (unpaired t-test, LL versus T16: P=0.0098; LL versus T21: P=0.0201) in the first cycle, while all the five groups had similar peak response in the second cycle (one-way ANOVA, $F_{4,44}$=0.4930, P=0.7408; Fig. 3A).

Similar to the findings for testes, birds underwent two molt cycles for the wing primaries under the T-photocycles, but three cycles under the LL. Each group, however, had large individual variations. In the first year, the timing of the molt initiation was similar in all the T-photocycles, but in the second year the molt was initiated earlier in the T16 group than in the T21, T24 and T27 groups (one-way ANOVA, first year: $F_{3,34}$=1.218, P=0.3182; second year: $F_{3,34}$=4.406, P=0.0105). The LL group initiated molt earlier than the T-photocycle groups in both years (one-way ANOVA, first year: $F_{4,44}$=3.907, P=0.0084; second year: $F_{4,42}$=2.463, P=0.0598; Fig. 3E). The circannual period of molt was significantly longer in the LL group than in the T16, T24 and T27 groups, but not T21 group (Newman–Keuls test, P<0.05; Fig. 3D).

Circannual cycles in females

Females also underwent significant changes in body mass, with large intragroup individual variations and a lack of defined rhythmicity (data not shown). Similar to males, however, most exhibited follicular growth–regression cycles with similar circannual

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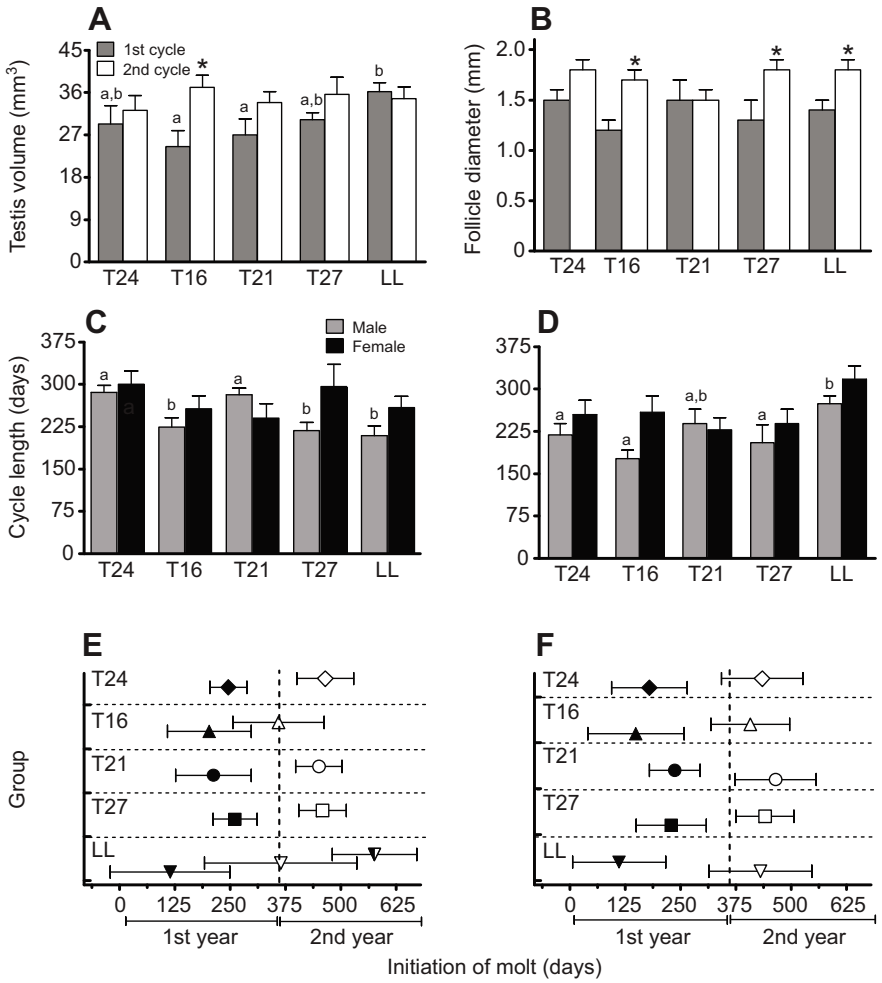

Fig. 3. Peak gonadal response, circannual period of gonad development cycle and distribution of time of molt initiation in spotted munia. (A,B) Peak gonadal response (means and s.e.m.) in male (A) and female (B) spotted munia in the first and second circannual cycle under T-photocycles and LL. (C,D) Cycle length (means and s.e.m.) of circannual rhythms in gonad development (C, the interval between successive gonadal peaks) and wing primary molt (D, the interval between successive times of molt initiation). (E,F) Distribution (means ± s.d.) of the time of molt initiation in the first and second cycles shown separately for males (E) and females (F) in relation to the peak gonadal response, in the first (filled symbols) and second (open symbols) year. The half-filled triangle in the second year for the LL group indicates the beginning of the third molt in these birds. Different letters indicate a significant difference between groups at $P<0.05$. Asterisks indicate a significant difference at $P<0.05$ between the first and second cycle values of the same group.

periods, measured between two peak follicular responses (Fig. 2F–J; supplementary material Figs S1–S5). The amplitude of the ovarian cycles was low, especially in the second cycle, mainly because ovaries did not fully regress. Using the criterion described in Materials and methods, most birds had a peak follicular response in each cycle, except 1/10 in the T16 group, 2/11 in the T21 group and 1/9 in the T27 group. Thus, the duration of the recrudescence phase based on birds in which it was calculated significantly varied among groups in both cycles (one-way ANOVA, first cycle: $F_{4,47}=7.054$, $P=0.0002$; second cycle: $F_{4,44}=3.299$, $P=0.0190$; Table 1). In the first cycle, birds in the T21 group had a longer recrudescence phase than those in the T16, T24, T27 or LL groups (Table 1 and Fig. 2F–J). Similarly, in the second cycle, birds in the T24 group had a longer recrudescence phase than those in the T21 and T27 groups, but not those in the T16 or LL groups. There was no difference in the peak follicular response in the first or second cycle among the groups (Fig. 3B). Between cycles, however, the T16, T27 and LL groups had a significantly larger response in the second than in the first cycle (paired *t*-test, $P<0.05$; Fig. 3B); in contrast, the T24 and T21 groups did not have a significant difference in the peak follicular size between the two cycles. Nevertheless, the time taken to attain peak follicular growth varied among the groups. It was significantly longer in the first cycle in birds in the T21 group than in those in the T24 and T27 groups, but was not different in the T16 or LL group (one-way ANOVA, $F_{4,47}=3.909$, $P=0.0080$; Table 2). The time taken to attain the second peak follicular growth was similar in all the groups (one-way ANOVA, $F_{4,47}=1.738$, $P=0.1575$; Table 2).

The wing primaries had two molt cycles in all the light conditions, with significant variations among groups in the timing of the initiation of molt in the first but not the second year (one-way

Table 1. Duration of gonadal recrudescence phase in spotted munia exposed to different light conditions

	Male		Female	
Light condition	First cycle (days)	Second cycle (days)	First cycle (days)	Second cycle (days)
LL (24 h L:0 h D)	223.7±13.1[a]	280.7±21.9	138.1±10.4[a,*]	236.5±25.1[a]
T16 (12 h L:4 h D)	173.5±20.7[a,*]	344.1±16.1	194.0±31.6[a,*]	236.9±28.0[a]
T21 (12 h L:9 h D)	227.5±9.3[b,*]	300.4±10.8	305.5±36.0[b]	162.1±15.5[b]
T24 (12 h L:12 h D)	194.0±12.6*	307.5±13.6	168.6±19.1[a]	254.4±28.0[a]
T27 (12 h L:15 h D)	159.2±12.2[a,*]	318.2±27.6	152.6±22.9[a]	145.1±23.0[b]

Different superscript letters indicate a significant difference among groups (column wise). Asterisks indicate a significant difference within the group (first versus second cycle). Level of significance: $P<0.05$; Newman–Keuls *post hoc* test.

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Table 2. Time to attain the peak gonadal response in the first and second cycles in spotted munia under different light conditions

	Male				Female			
	First cycle		Second cycle		First cycle		Second cycle	
Light condition	Calendar days	Circadian (T) days	Calendar days	Circadian (T) days	Calendar days	Circadian (T) days	Calendar days	Circadian (T) days
LL (24 h L:0 h D)	231.7±12.3[b]	–	440.4±13.6[a]	–	181.9±18.2[a]	–	440.4±21.2	–
T16 (12 h L:4 h D)	185.8±14.4[a]	279.0±21.7	410.5±13.1[a]	615±19.6	191.9±31.7[a]	288.0±47.6	407.3±18.5	652.4±27.2
T21 (12 h L:9 h D)	149.9±11.5[a]	171.2±13.2	431.4±5.9[a]	492.7±6.7	252.4±30.9[b]	288.4±35.3	492.2±25.5	562.5±29.2
T24 (12 h L:12 h D)	144.1±8.0[a]	144.1±8.0	492.6±7.3[a]	492.6±7.3	130.5±8.0[c]	130.5±8.0	430.5±18.5	430.5±18.5
T27 (12 h L:15 h D)	142.0±9.0[a]	126.3±8.0	360.1±7.4[b]	320.0±7.4	168.9±16.0[d]	150.2±14.3	464.9±39.5	413.3±35.1

Time has been calculated in terms of both calendar (24 h) and circadian (period, T, of the light:dark cycle) days. For details please refer to Fig. 2.
Different superscript letters indicate a significant difference among groups (column wise). Level of significance, $P<0.05$; Newman-Keuls *post hoc* test.

ANOVA, first year: $F_{4,47}=3.747$, $P=0.0100$; second year: $F_{4,47}=0.5305$, $P=0.7139$; Fig. 3F). Birds in the LL group began molting earlier than those on the T21 and T27 photocycles in the first cycle (Newman–Keuls test, $P<0.05$). The interval in the initiation of wing primary molts between the first and second cycle was not different among groups (one-way ANOVA, $F_{4,43}=1.645$, $P=0.1805$; Fig. 3D).

Time-dependent effect of T-cycles

Comparing all T-cycles for the effect of factor 1 (treatment or light condition) and factor 2 (time), we found that different T-cycle regimes had a significant effect on gonadal growth and development in both male and female birds (two-way ANOVA, male – factor 1: $F_{3,748}=9.051$, $P<0.0001$; factor 2: $F_{21,748}=32.13$, $P<0.0001$; female – factor 1: $F_{3,814}=3.315$, $P=0.0195$; factor 2: $F_{21,814}=33.71$, $P<0.0001$; Table 1 and Fig. 2). When all T-cycle groups along with the LL group were compared for the effect of time and light condition in both male and female birds the difference was still significant in these treatments (two-way ANOVA, male – factor 1: $F_{4,968}=6.817$, $P<0.0001$; factor 2: $F_{21,968}=37.21$, $P<0.0001$; female – factor 1: $F_{4,1034}=2.447$, $P=0.0448$; factor 2: $F_{21,1034}=39.02$, $P<0.0001$; Table 1 and Fig. 2).

Circannual phenotypes: sex dependence

A two-way ANOVA (factor 1: light condition, factor 2: sex) was used to determine the sex-dependent effects of the light conditions on circannual gonadal and molt cycles (Fig. 2). Although there was no difference in the circannual gonadal cycle between males and females in the first cycle (factor 1: $F_{4,91}=8.362$, $P<0.0001$; factor 2: $F_{1,91}=0.08153$, $P=0.7759$; Table 1 and Fig. 2), the two sexes showed significant difference in the second cycle (factor 1: $F_{4,88}=2.720$, $P=0.0346$; factor 2: $F_{1,88}=54.25$, $P<0.0001$; Table 1 and Fig. 2). The two sexes also differed in the time taken to attain the peak gonadal response in the second year (factor 1: $F_{4,91}=2.518$, $P=0.0466$; factor 2: $F_{1,91}=6.938$, $P=0.0099$; Table 2 and Fig. 3) but not in the first year (factor 1: $F_{4,91}=5.156$, $P=0.0009$; factor 2: $F_{1,91}=1.493$, $P=0.2249$; Table 2 and Fig. 3). Under T27, the peak response occurred earlier in males than in females (Bonferroni test, $P<0.05$; Table 2 and Fig. 3). The time of the initiation of molt, however, was not different between male and female birds in both cycles (first cycle – factor 1: $F_{4,91}=6.915$, $P<0.0001$; factor 2: $F_{1,91}=2.133$, $P=0.1476$; second cycle – factor 1: $F_{4,86}=2.427$, $P=0.0539$; factor 2: $F_{1,86}=0.5987$, $P=0.4412$; Fig. 3E,F). Similarly, the circannual period of the gonadal cycle was different among light conditions but not between sexes (factor 1: $F_{4,91}=3.216$, $P=0.0162$; factor 2: $F_{1,91}=1.763$, $P=0.1876$; Fig. 3C). However, the two sexes differed in the circannual molt cycles: males had a shorter circannual period than females (factor 1: $F_{4,91}=2.299$, $P=0.0654$; factor 2: $F_{1,91}=7.229$, $P=0.0086$; Fig. 3D).

Relationship between gonadal and molt cycles

Fig. 4 shows the distribution of molt in the first and second cycle in relation to the peak gonadal response in both males and females. In males, molt in the first cycle coincided with the testicular regression phase, and the two phenotypes were more tightly coupled in the T24 and T27 groups than in the T21 and T16 groups (Fig. 4A). In the second cycle, the gonad and molt phenotypes remained tightly coupled in birds in the T24 and T27 groups, but they were increasingly uncoupled in birds on T21 and T16. Under LL, the molt was distributed on both sides of the peak testis response. Between

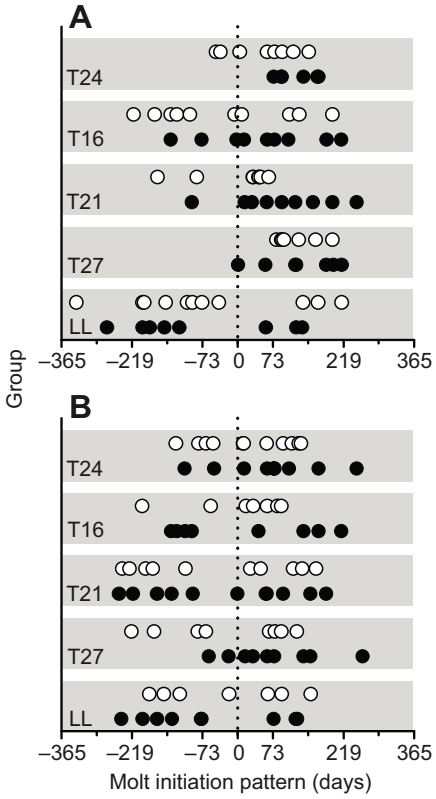

Fig. 4. Relationship between the timing of gonadal peak and initiation of wing primary molt in spotted munia under T-photocycles and LL. Data are shown for male (A) and female (B) spotted munia. The peak gonadal size of each individual in both cycles was given a value of zero on the time scale (*x*-axis, midline), and the time of molt of each bird in the first (filled circles) and second (open circles) cycles was plotted in each condition with respect to this zero value, on a scale from −365 to +365 days; minus (–) and plus (+) signs indicate before and after the time of peak gonadal response (time 0), respectively.

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sexes, the molt pattern in females was more scattered in both the cycles in all light conditions, which indicated an uncoupling between the ovarian and molt cycles (Fig. 4B).

Effect of T-photocycles on activity rhythms, food intake and body mass

Synchronization of activity rhythms

Results from a short-term experiment that examined the effects of T-photocycles on circadian entrainment are presented in Fig. 5. Birds exhibited activity during the light period, and were synchronized with the LD cycle regardless of the period of T-photocycle (Fig. 5Ai–Di and 5Aii–Dii). The activity rhythm period under LD conditions closely matched the period of the T-photocycle (Fig. 5Aiii–Diii), which confirmed that birds were entrained to T-photocycles. Under LL, however, the activity rhythm was gradually disrupted and birds became arrhythmic or showed short activity bouts scattered all over the day, indicating multiple rhythmicity (Fig. 5Ei, Eii, Eiii). When released into dim constant light (LL_{dim}) for 10 days, all birds had a circadian period of >24 h, regardless of the T-photocycle to which they were previously exposed (cf. Fig. 5Aiii–Diii and Aiv–Div). Interestingly, birds that were in LL showed a variable response under LL_{dim}; half of them became rhythmic (Fig. 5Eii), whereas half were still arrhythmic (Fig. 5Eiii).

Changes in food intake and body mass

After 30 days of exposure, body mass was significantly increased (paired *t*-test, $P<0.05$) in the T16 and T27 groups, but not in the T21, T24 and LL groups (Fig. 6A). Two-way RM ANOVA of all five groups over 30 days revealed that there was a significant effect of the duration of exposure (factor 2) to T-photocycles but not of light condition (factor 1) or the interaction between them (factor 1: $F_{4,22}=1.446$, $P=0.2523$; factor 2: $F_{1,22}=23.71$, $P<0.0001$; interaction factor 1 × factor 2: $F_{4,22}=2.462$, $P=0.0752$; Fig. 6A). Also, under

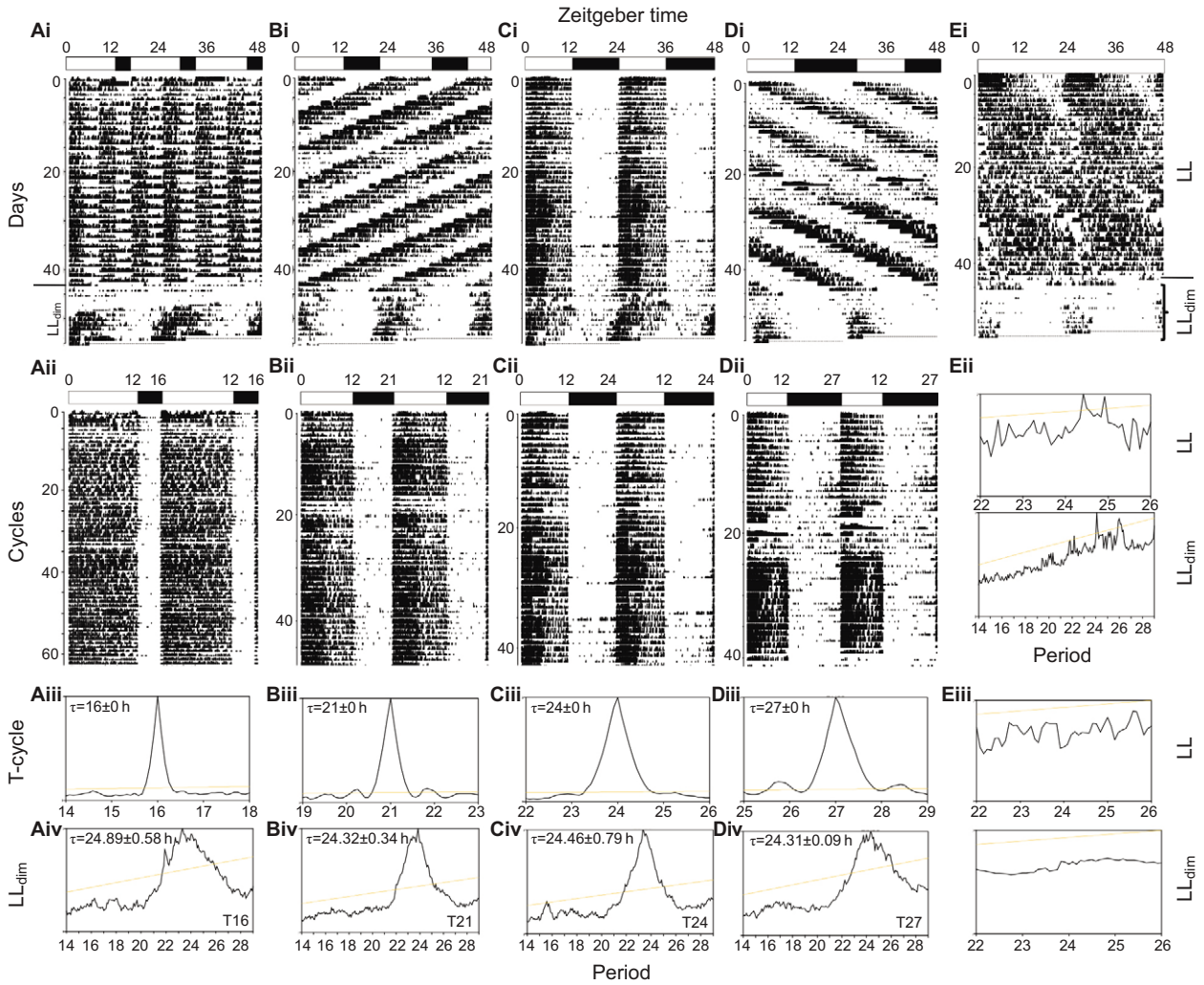

Fig. 5. Representative actograms of spotted munia exposed to different T-photocycles or LL for 40 days and then released into dim constant light (LL_{dim}) to examine the phase of circadian activity rhythm. (Ai–Ei) Activity records plotted in 24 h day format. (Aii–Dii) Activity records plotted in modulo tau (as per cycle length) for T-cycle groups. The period of activity rhythm, determined by chi-square periodogram analysis using a segment of activity recorded under LD or LL, and LL_{dim} (Ai–Di) are plotted in Aiii–Diii and Aiv–Div, respectively. The upper and lower panels of Eii and Eiii show similar plots under LL and LL_{dim} of birds in the Ei group that were circadianly rhythmic and arrhythmic, respectively. Horizontal bars above Ai–Ei represent light and dark periods over two calendar days (48 h), while those above Aii–Dii represent light and dark periods over two T-photocycles. Note the synchronization of activity rhythm under T-photocycles, the relative disorganization of activity rhythm under LL, and circadian rhythm under LL_{dim}. Yellow lines in period plots show the definition of circadian rhythmicity. Individuals showing values crossing vertical lines are rhythmic, whereas values remaining below the line indicate arrhythmicity.

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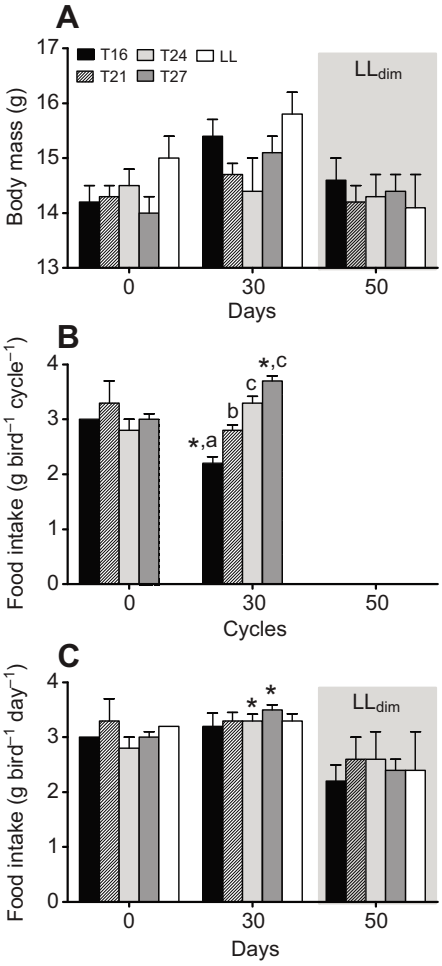

Fig. 6. Body mass and food intake over a period of exposure to T-photocycles and constant light. Mean (and s.e.m.) body mass (A), food intake per bird per cycle (B) and food intake per bird per day (24 h, C) in spotted munia (N=4–6) exposed first to T-photocycles and LL (T24, T16, T21, T27) or constant light (LL) for about 40 days and then released into dim constant light (LL_{dim}) for 10 days. Asterisks indicate a significant difference (P<0.05, paired t-test) within the group as function of number of cycles (B) or days (C).

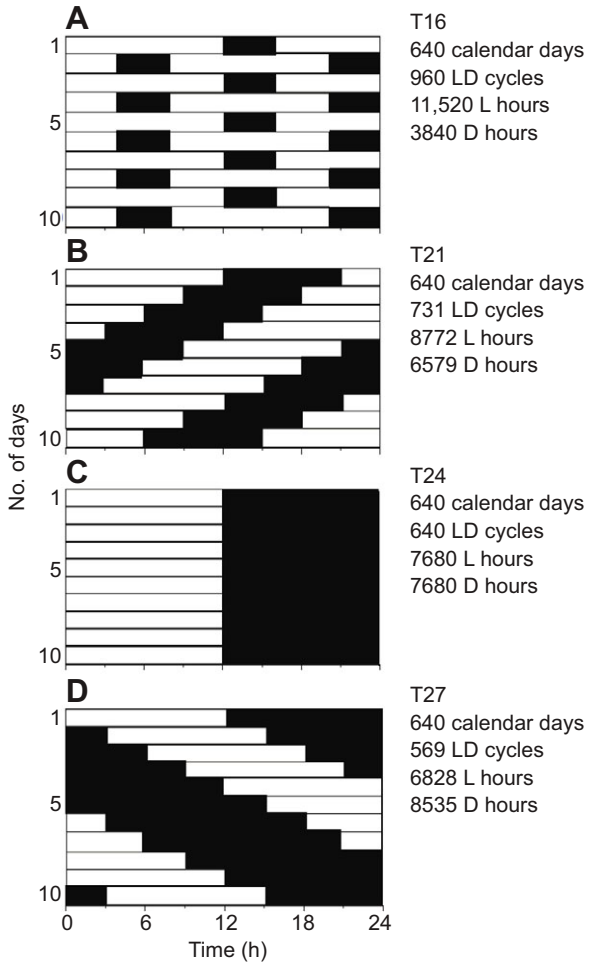

Fig. 7. Schematic presentation of the T-photocycles, to which spotted munia were exposed in the experiment. (A) T16, 12 h light (L):4 h dark (D); (B) T21, 12 h L:9 h D; (C) T24, 12 h L:12 h D; and (D) T27, 12 h L:15 h D. The total number of calendar days, light:dark (LD) cycles and total light and dark hours that birds received during the experiment are shown on the right-hand side of the figure. Note differences in the light and dark phases among the T-photocycles.

LL_{dim}, body mass did not show a difference among the groups (one-way ANOVA, $F_{4,22}$=0.3137, P=0.8657).

Food intake was measured in terms of exposure both for 30 cycles and for 30 calendar days. Per cycle, it was significantly decreased and increased in T16 and T27 groups, respectively (paired t-test, P<0.05); in the other groups, food intake did not differ over 30 cycles of exposure (Fig. 6B). Two-way RM ANOVA of data from only T-photocycle groups revealed the effect of light condition (factor 1) and the interaction between light condition and the number of cycles, but not of the number of cycles alone (factor 2) (light condition: $F_{3,19}$=8.702, P=0.0008; cycles: $F_{1,19}$=0.04959, P=0.8262; interaction: $F_{3,19}$=20.34, P<0.0001). However, per calendar day (24 h), food intake was significantly increased (paired t-test, P<0.05) after 30 days in T24 and T27, but not in the other groups (Fig. 6C). Two-way RM ANOVA showed a significant effect of the duration of exposure (factor 2), but not of light condition (factor 1) or the interaction between light condition and duration of exposure (light condition: $F_{3,19}$=0.834, P=0.4895; days: $F_{1,19}$=7.977, P=0.0108; interaction: $F_{3,19}$=1.205, P=0.3350; cf. Fig. 6B,C). A similar result was obtained when all groups including LL were analyzed together by two-way RM ANOVA. Further group comparison among the T-photocycles showed that birds on the T16 photocycle had a significantly lower (Bonferroni test, P<0.05) food intake than those on other T-photocycles. Similarly, birds on T21 had a lower food intake than those on T24 and T27, but those on T24 and T27 did not differ in their food intake (Bonferroni test, P<0.05). Under LL_{dim}, all groups had a similar food intake (one-way ANOVA, $F_{4,22}$=0.4838, P=0.7474).

DISCUSSION

Fig. 2 shows that spotted munia underwent two circannual gonadal and molt cycles, regardless of the external light conditions (see Fig. 7), similar to the yearly reproductive cycle in the wild (Fig. 1). This confirms the previous results on the involvement of circannual rhythms in the regulation of seasonal responses in the spotted munia (Bhatt and Chandola, 1985; Budki et al., 2012) and supports the idea that seasonal physiology is not a direct response to the prevailing photoperiod, as has been argued at times (Dawson, 1997; Kumar et al., 2010). Further, the light intensity does not influence circannual reproductive rhythms in the spotted munia: circannual gonadal

cycles under LL at ~90 lx (Fig. 2) are similar to those reported under LL at ~22 lx (Budki et al., 2012), except that in the former the higher light intensity reduced variations and increased synchrony in the gonadal and molt cycles among individuals of the group. This is consistent with a much more synchronous circannual testicular cycle reported in the spotted munia exposed to LL at 150 lx (Bhatt and Chandola, 1985). An increased synchrony in the free-running circannual testicular and molt cycles has also been reported in African stonechats (*Saxicola torquata axillaris*) under 300 lx light intensity (Gwinner and Scheuerlein, 1998).

The FDH postulates an intimate relationship between circadian and circannual cycle periods (Gwinner, 1973; Gwinner, 1981). Under this hypothesis, the synchronized circadian oscillations determine the cycle length of the reproductive phenotypes in the year. A T21 or T27 day, falling within the range of the circadian entrainment (T=24±3 h), is expected to synchronize the circadian oscillator, as in the T24 day in which the period of the entrained circadian oscillator determines the timing of an event in a 24 h day (Hut and Beersma, 2011) and hence in the 365 day year. Thus, compared with the T24 day, which results in a 365 day circannual cycle, the T21 and T27 days should result in faster (short) and slower (long) circannual cycles, respectively. The results from the activity experiment (Fig. 5) do show that birds were entrained to T-photocycles, had disrupted activity under LL, and free-ran under LL_{dim}. Birds had activity rhythms matched with the period of the external T-photocycles. This suggests that the circadian system in munia possibly has a larger range of circadian entrainment, from T16 to T27. Further support for this comes from the observation when released into LL_{dim}, all birds had a free-running circadian period (>24 h). All this appears to be consistent with the idea that birds living in tropical environments have a flexible timing system compared with that in birds living in temperate environments (Wingfield, 2008).

Contrary to the FDH, circannual cycles in spotted munia were not circadian day dependent; rather, they free-ran (Fig. 2 and Fig. 3C). Among T-photocycles, circannual periods were similar in females and were significantly longer in males under T21 and T24 than under T27 (Newman–Keuls test, $P<0.05$; Fig. 2 and Fig. 3C), regardless of the number of circadian days that spotted munia experienced under T21 (731 days), T24 (640 days) and T27 (569 days; Fig. 7) photocycles. A few other studies have reported similar circannual periods among animals on T21, T24 and T27 circadian days (Gwinner, 1973; Kenagy, 1980; Gwinner, 1981; Wikelski et al., 2008). Further, a T16 day did not result in the corresponding circannual response in the spotted munia (Fig. 2 and Fig. 3C). Interestingly, the T16 day, generally considered to lie outside the range of the circadian entrainment, overlapped with the T24 day on alternate calendar days (days 1, 3, 5 and so on, 3×T16=2×T24). Therefore, the response induced by T16 could be analyzed with reference to a T24 cycle. Our results showed that this was not the case; instead, circannual periods in males were significantly longer under the T24 than under the T16 photocycle (Newman–Keuls test, $P<0.05$; Fig. 2 and Fig. 3C).

Spotted munia on T-photocycles received variable daily (per cycle) as well as yearly (per 365 cycles) dark (rest) hours, whilst those on LL did not have a dark-induced rest phase. Further, in spite of all the T-photocycles containing equal (12 h) light (feeding) hours per cycle, they provided hugely different total light (feeding) and dark (no-feeding) hours during the period of the experiment. For example, the T16 cycle provided 1.5 times more light (feeding) hours than the T24 cycle (T16=11,520 h versus T24=7680 h; Fig. 7). Similarly, munia on T21 and T27 received 1.14 and 0.89 times more and fewer light (feeding) hours, respectively (T21=8772 h and T27=6828 h versus T24=7680 h; Fig. 7). The reverse was true of the dark (no-feeding) hours (Fig. 7). In the absence of the measurement of a metabolic marker in this study, we are unable to conclude that the energy turnover in birds was different among these T-photocycles that provided different dark-induced rest hours. However, we have some indications from the results on food intake from the short-term activity experiment. Per cycle, food intake in different groups was as follows: T16<T21<T24<T27 (Fig. 6B). Also, there was an effect of the light condition and an interaction between the light condition and number of cycles, but not of the number of cycles alone (see Results). However, food intake was not different over 30 days among the groups when it was measured per day (24 h) (Fig. 6C). Could this suggest that the length of no-feeding (dark) hours affected the food intake? Or that feeding rhythms, unlike activity rhythms (Fig. 5Aii–Dii), were not synchronized with the T-photocycles? In either case, the present results seem not to conform to the ETH.

A comparison of the gonadal and molt cycles shows differences between the two sexes in their response to external light conditions. Fig. 4 compares the relationship between the rhythms in gonadal maturation and molt in both the male and female munia. Overall, the onset of molt coincided with the time of gonadal regression on natural day lengths, but this relationship was lost in birds kept on constant LD cycles (Budki et al., 2012). There was a loose coupling between the cycles in the gonad development and wing primary molt (Fig. 4), as also reported in our earlier study (Budki et al., 2012). This could mean that reproduction and molt cycles are independent circannual phenotypes, although they probably share a common regulatory mechanism. By comparison, however, African stonechats showed the persistence of testicular and molt cycles with a much tighter coupling over 12 years (Gwinner, 2003). Further, our results (Fig. 4) show that the gonadal and molt cycles become more asynchronous in females than in males, in LL than in T-photocycles and, among the T-photocycles, in T16, T21 or T27 than in the T24 cycle. Between the sexes, molt in relation to gonadal maturation was more dispersed in females than in the males. A greater variation in the circannual periodicity among females may suggest that they share a greater role in defining the reproductive season in relation to the environment (Budki et al., 2012). Thus, the two sexes appear to have evolved with an independent circannual timing strategy for controlling their annual reproductive cycle in interaction with the environment (Zucker et al., 1983). This makes sense as the timing of the gonadal cycle in one sex cannot be subservient to the other sex. In any case, it cannot be assumed that males drive the female sexual state, or vice versa. Therefore, an obvious advantage that circannual rhythms confer to reproduction is that they prepare the male and female for synchronized reproductive processes. This synchrony of testicular and ovarian cycles and associated phenotypes can be much closer, even overlapping, in the wild when the two sexes live together.

In summary, annual reproductive phenotypes of spotted munia are independently regulated. The FDH is not a possible explanation for the regulatory mechanism underlying seasonal reproduction as the circannual rhythms in the growth of gonads and molt observed in this study are inconsistent with the prediction derived from the theory of the circadian clock entrainment with the T-photocycles. Thus, circadian rhythms may not be the basis for annual reproduction in the spotted munia. However, a link between the circadian and circannual rhythms is not ruled out at present as the circannual system may influence circadian rhythms (Ball and Ketterson, 2008; Mrosovsky et al., 1976). Although food availability can influence gonadal growth

in seasonally breeding songbirds (Kumar et al., 2001; Budki et al., 2009), we would like to speculate based on the present results that energy turnover, enforced by the seasonality in the abundance of food resources, is not controlling the circannual rhythm underlying seasonal reproduction in the spotted munia.

MATERIALS AND METHODS

Animals

The experiments were carried out on spotted munia (*L. punctulata*) at Lucknow, India (26°55′N, 80°59′E), in accordance with the guidelines of the Institutional Ethics Committee. Adult male and female birds were captured around the vernal equinox period (mid-March to mid-April) in 2008 when they had small reproductively inactive gonads. Birds were kept in an outdoor aviary (2.95×1.75×2.20 m) in natural daylight and temperature conditions. At this time, daylight and midday temperature were about 12 h and 30°C, respectively.

Experiment

Acclimated birds in four groups of males and females (N=11 or 12 pairs per group) were brought indoors in separate chronocubicles (1.37×0.79×2.74 m) and exposed to programmed light:dark (LD) cycles, as follows: T16 (12 h L:4 h D; Fig. 7A), T21 (12 h L:9 h D; Fig. 7B), T24 (12 h L:12 h D; Fig. 7C), and T27 (12 h L:15 h D; Fig. 7D). These LD cycles were called T-photocycles, as they varied in their period by having different durations of dark hours per cycle. Although all the T-photocycles ran for a total of 640 calendar days, they presented very different LD cycles in terms of the number of circadian days as well as the total number of light and dark hours. During the experiment, T16, T21, T24 and T27 cycles presented the total number of LD cycles (circadian days) and total light hours in decreasing order, and total dark hours in increasing order (Fig. 7). One group each of male and female munia (N=14 each) was kept in the continuous illumination (LL, 24 h L:0 h D), and considered as controls, as spotted munia exhibit circannual testicular cycles under LL (Bhatt and Chandola, 1985; Budki et al., 2012). Observations were also recorded for a group of male and female spotted munia (N=5 each) that were freshly captured at four different times during the year, corresponding with the times of equinox (March, September), and short (December) and long (June) day lengths.

To further examine the circadian rhythm synchronization with T-photocycles in munia, we monitored the activity–rest pattern in individually housed birds in activity cages. Birds (N=4–6) were first exposed to T16, T21, T24, T27 and LL (as above) for 40 days and then to dim constant light (LL_{dim}, <1.0 lx, illumination intensity at night during the T-photocycles) for 10 days. Activity of all birds was recorded continuously. We also measured food intake and body mass at the beginning (day 0) and end (day 30) of the LD cycle, and after 10 days of LL_{dim} exposure. Additionally, we measured food intake in all birds at cycle 30 of each T-photocycle, so that birds in all groups had been exposed to identical light hours.

Each chronocubicle was lit with compact fluorescent lamps (15 W, Phillips India) at about 90.0±5.0 lx intensity at floor level. Here, we used a higher light intensity than that (~22 lx) used in our previous study (Budki et al., 2012) to ensure that light intensity was sufficient enough to entrain the circadian rhythms to T-photocycles. This ‘unnaturalness’ of reduced light intensity provided in the laboratory by cool white fluorescent lamps may correspond to that underneath shades in the wild. Interestingly, for most of the day, birds preferably inhabit shade areas (e.g. forest canopies, shaded bush areas or under trees) where they receive natural light at intensities reduced many fold on a log-scale (Corth, 1980). Also, circadian testicular cycles have been shown to persist in the spotted munia under 90 lx LL (Bhatt and Chandola, 1985). Temperature was maintained at around 20°C (±1.0°C). Food and water were freely available. In addition to seeds of *Setaria italica* provided *ad libitum*, a supplementary food prepared from breadcrumbs, boiled eggs, crushed egg shells, cottage cheese and multivitamin (Vimeral containing vitamins A, D3, E and B12; Virbac Animal Health India Pvt. Ltd, Mumbai, India) was given on alternate days (Singh et al., 2010). Physical enrichment via the periodic addition of fresh foliate twigs and perches to the aviary environment also occurred.

Observations and data recording

Body mass, gonad size and molt

In birds exposed to T-photocycles and LL, the body mass and primary wing feather molt were measured every 15 days, and gonadal size every 30 days. Observations on body mass and gonadal size at four times in the year in groups of freshly captured birds were recorded in the middle of the month. Similar observations on body mass and gonadal size in birds used in the activity experiment were taken on days 0, 30 and 50 of the experiment. All measurements were made in the middle of the day. Birds were weighed on a top-pan balance providing an accuracy of 0.1 g. Gonadal size was measured as testis volume (mm^3) in males and diameter (mm) of the largest follicle in females, as has been described in our previous publications (Kumar et al., 2001; Budki et al., 2012). Briefly, birds under local anesthesia were laparotomized and the size of the left testis and ovarian follicle was measured. Such a procedure has no effect on activity rhythms or gonad development cycle in a number of species that we study in our laboratory (Rani et al., 2005; Rani et al., 2007; Singh et al., 2012). Testis volume was calculated using the formula $4/3\pi\ ab^2$, where a and b denote half of the long (length) and short (width) axes, respectively. At each observation, the primary wing feathers (wing primaries) were scored in a range of 0 to 5, as per earlier publications (Newton, 1966; Trivedi et al., 2006, Budki et al., 2012) as follows: 0, worn or old feather; 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; and 5, fully grown feather. Thus, each primary could have a score of 0 to 5, and a wing primary could have total score of 0 to 45. From this, a linear increase in new feather mass was calculated, as per the Dawson and Newton protocol (Dawson and Newton, 2004). Data from birds that died during the experiment have been excluded from the presentation and analysis.

Food intake

Food intake was measured in singly housed birds in activity cages in the second short-term experiment only. For this, the floor of the cage was covered with a paper sheet such that it extended onto the side wall of the cage. A weighed quantity of food was dispensed in the food cup and was removed after a calendar day (24 h) or after a LD cycle. The difference between food supplied and recovered in a specified duration gave the amount of food consumed (food intake) by an individual bird, and from this the mean (±s.e.m.) food intake (g $bird^{-1}$ day^{-1}) was calculated. Food intake was measured at the beginning and after 30 days/cycles under T-photocycles and LL, and on day 50 under LL_{dim}. When measured over a calendar day, food was given at a particular time of day and was removed at the same time the next day, i.e. after 24 h. However, when food intake was measured over a cycle, it was given at the time of lights on and removed 3–5 min before the next lights on. This was repeated consecutively over 2 days/cycles, and mean food intake of an individual was used for further calculations.

Activity recordings

The general movement of a bird in its cage was monitored as a measure of the response of the circadian system. This is the easiest and most reliable marker of the circadian system (Daan and Aschoff, 1975). A bird was kept in a specially designed activity cage (60×45×35 cm) that was furnished with two perches and mounted with a passive infrared motion sensor. Each sensor was connected to separate channels of a computerized data recording system, and the bird’s movement was recorded and plotted as a double plot using The Chronobiology Kit program from the Stanford Software System, USA, as described in previous publications (Malik et al., 2004; Singh et al., 2012).

Data presentation and analysis

Data from birds that died during the experimental period were excluded from the analysis and presentation. These included two birds of each sex under T16, one bird of each sex under T21, three males and one female under T24, two birds of each sex under T27 and three birds of each sex under LL. The results on body mass, gonadal size and molt are plotted as means (±s.e.m.). A line connecting observations plotted against time (calendar days) indicated the course of changes in these parameters during

the experiment. The day that molt of a wing primary began was recorded for individual birds, and from this the mean (±s.e.m.) was calculated for the group. The interval between the initiation of molt in the first and second cycles was calculated as the circannual period of molt. Also, the data on wing primary molt in the first and second cycles were plotted along with the gonadal growth–regression cycle to show the relationship between two seasonal phenotypes. Further, the time of attainment of peak testis or follicle size of individual birds in each cycle was marked, and the duration between successive peaks gave the circannual period of the gonadal cycle. Thus, circannual period was calculated for birds that showed two cycles during the experiment. In general, females exhibited cyclicity with less precision. They had a low amplitude ovarian cycle because ovaries did not fully regress, particularly in the second cycle. In fact, many times the follicles regressed to only about 0.6 mm in diameter compared with the 0.3 mm value we usually assign for a completely regressed ovary. Therefore, we subjectively evolved a criterion for designating a peak ovarian response, which was when the diameter of the largest follicle was ≥1 mm. By this criterion, most birds with at least a 1.5-fold difference between regressed and recrudesced follicles had shown a peak follicular response in each cycle, except 1/10 in the T16 group, 2/11 in the T21 group and 1/9 birds in the T27 group.

In most, if not all, birds, wing primary molt immediately followed the breeding season (Dawson, 2004; Neto and Gosler, 2006). Spotted munia also showed such a phase relationship between the rhythm in the growth of gonads and the rhythm of molt. Initiation of molt in munia coincides with the termination of the breeding season, indicated by gonadal regression in laboratory conditions (Budki et al., 2012). Therefore, we have plotted wing primary molt with reference to the peak gonadal response, with the latter being indicated as the zero line in the middle of the *x*-axis (see Fig. 4). A molt initiated before the peak gonadal response was allocated a minus sign (–) and plotted on the left side of the zero line. Similarly, a molt initiated after the peak gonadal response was allocated a plus sign (+) and plotted on the right side of the zero line. This gave a plot of the molt pattern in the range between −365 and +365 days, and enabled us to compare the molt patterns between the first and second cycles. We also calculated mean (±s.e.m.) the time taken from small reproductively inactive gonads to attain the full maturation (peak gonadal size; Table 2) as well as the duration of the recrudescence phase [reproductive window, the time between initiation of gonadal recrudescence (the time of the first significant increase in gonad size) and regression (the time after which no further decrease in gonad size occurred); Table 1].

The activity data recordings, called actograms, are shown as double plots of representative birds. The period of the activity rhythm under LD, LL and LL_{dim} conditions was calculated from a segment of the activity records by chi-square periodogram analysis using the Chronobiology Kit software program. Food intake for each group was calculated as means (±s.e.m.) and is presented as per cycle and per day (24 h).

Groups of birds exposed to different light conditions were randomly selected from a batch of birds that were procured from the wild. We tested data first for homogeneity of variance, and then analyzed these using parametric tests. The circadian system remained synchronized with the T-photocycles, except under the LL condition in which we had speculated its possible disruption during very long exposures. Statistical analysis was done using GraphPad Prism software (version 5.0, La Jolla, CA, USA). One-way ANOVA with or without repeated measures was used to compare time-dependent effect of different light conditions within the group and multiple groups at one time point. Two-way ANOVA was used to compare the effect of light conditions (treatment, factor 1) and sex (factor 2; interaction factor 1 × factor 2) or to compare the effect of treatment (factor 1) over time (factor 2). Newman–Keuls (following one-way ANOVA) and Bonferroni (following two-way ANOVA) *post hoc* tests were used for group comparisons, if ANOVA indicated a significant difference. Two data points from the same and different groups were compared by Student's paired and unpaired *t*-tests, respectively. Significance was taken at $P<0.05$.

Acknowledgements
We thank anonymous reviewers for helpful comments which improved the manuscript. Dr Sudhi Singh helped in the analysis and presentation of the data.

Competing interests
The authors declare no competing financial interests.

Author contributions
V.K. conceived the idea; V.K., S.R. and P.B. designed the experiments; P.B., S.M. and S.R. carried out the experiments and analyzed the results; V.K., P.B. and S.R. wrote the manuscript.

Funding
This work was supported by a generous research grant from the Department of Science and Technology, New Delhi, India, through Intensive Research in High Priority Areas (IRHPA) research funding (IR/SO/LU-02/2005).

Supplementary material
Supplementary material available online at
http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.100651/-/DC1

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