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RESEARCH ARTICLE

Persistence of circannual rhythms under constant periodic and aperiodic light conditions: sex differences and relationship with the external environment

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

[1]DST IRHPA Center for Excellence in Biological Rhythm Research at Department of Zoology, University of Lucknow, Lucknow 226 007, India and [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)

SUMMARY

The timing and duration of gonadal phases in the year indicates that breeding cycles are regulated by endogenous mechanisms. The present study on tropical spotted munia (*Lonchura punctulata*) investigates whether such mechanisms are based on circannual rhythms, and whether circannual rhythms between sexes differ in their relationship with the light environment. Birds were subjected to 12 h light per day (12L:12D), alternate days of light and darkness (24L:24D, LL/DD) and continuous light (LL), with L=22 lx and D≤1 lx, for 28 months at constant temperature (18±1°C). Groups kept on natural day lengths (NDL) served as controls. Measurement of body mass, gonads and molts of the primary wing feathers and body plumage at regular intervals showed that birds underwent repeated cycles in gonads and molt, but not in body mass. Under NDL, gonadal phases in both sexes cycled with 12 month periods. Under other conditions, males cycled with similar periods of ~11 months, but females cycled with relatively large period variations, ~10–13 months. Gonadal recrudescence–regression phase was longer in males than in females and, in both sexes, longer in the second year compared with the first year. The molt of wing primaries was more closely coupled to gonadal maturation in groups on NDL and 12L:12D than in groups on LL and LL/DD, but this relationship drifted apart in the second year. Body plumage molts were relatively more highly variable in both frequency and pattern in females than in males. It is suggested that annual breeding cycle in spotted munia is regulated by the self-sustained circannual rhythms, which probably interact with the annual photoperiodic cycle to synchronize breeding cycles to calendar year. Both sexes seem to have independent timing strategies, but females appear to share a greater role in defining the reproductive season in relation with the environment.

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Key words: circannual rhythm, molt, testes, ovary, spotted munia.

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INTRODUCTION

Animals time their annual (seasonal) activities such as reproduction to the time in the year when conditions in the environment best favor the survival of their offspring. They accurately anticipate a favorable season and physiologically prepare themselves well in advance, as mistiming will have severe fitness consequences (Helm et al., 2009). Annual life-history stages begin and end at optimal times, and do not last shorter or longer than the optimal durations (Gwinner, 1996a; Gwinner, 1996b; Dawson, 2008; Budki et al., 2009).

Daily and seasonal rhythms are based on intrinsic timing mechanisms that integrate with predictive temporal cues such as day length, while buffering organisms from an acute change in the environment, such as a spell of high rise in temperature (Gwinner, 1986; Prendergast et al., 2002; Bradshaw and Holzapfel, 2007). These rhythms are not reactions to the light–dark (LD) cycle (Aschoff, 1981). This is evident from sustained rhythmic expressions of physiological and behavioral functions with periods close to 24 h (circadian rhythms; *circa*=about, *dien*=day) or 12 months (circannual rhythms; *circa*=about, *annum*=year), when individuals are exposed to constant conditions.

Circadian rhythms have been well studied. A temperature compensated circadian clock operating at the molecular level has been demonstrated in the suprachiasmatic nucleus as well as in other tissues in several vertebrate groups (Ruby et al., 1999; Reppert and Weaver, 2002; Yasuo et al., 2003; Kumar et al., 2004; Tosini et al., 2006; Reyes et al., 2008). A similar circannual clock is unknown, but various lines of evidence rule out the involvement of circadian clocks in generation or expression of the circannual rhythms (Dark et al., 1985; Pant and Chandola-Saklani, 1993; Kumar et al., 2004; Rani et al., 2006).

Circannual rhythms have been found to persist for several cycles in both birds and mammals, suggesting that a circannual clock can function throughout the life of an individual (Pengelley and Asmundson, 1969; Pengelley and Asmundson, 1974; Richter, 1978; Gwinner, 1986). The most compelling evidence for circannual rhythms comes from experiments in which captive birds kept on constant and 'neutral' photoperiods (e.g. 12 h or near 12 h light per day) exhibit repeated circannual cycles in reproduction (e.g. gonadal maturation and regression) and other phenotypic traits (e.g. body mass, feather molt, etc.) (Schwab, 1971; Gwinner, 1981; Gwinner, 1986; Gwinner, 2003; Gwinner and Dittami, 1990; Cadee et al., 1996; Dawson, 1997; Piersma, 2002; Piersma et al., 2008; Wikelski et al., 2008; Helm et al., 2009). It has been argued, however, that birds held under such

constant LD cycles are not completely devoid of the temporal information (see Dawson, 2007; Kumar et al., 2010).

A large body of evidence suggests that day length regulates annual reproductive cycle in birds (Kumar, 1997; Dawson et al., 2001). Also, Dawson (Dawson, 2007), from his studies on European starlings (*Sturnus vulgaris*), which do show repeated testicular cycles under 12 h:12 h light:dark regimes (Gwinner, 1981), suggests that annual gonadal cycle in starlings is the direct response to the prevailing photoperiod in nature. However, several photoperiodic species undergo spontaneous gonadal regression when kept on stimulatory day lengths (Nicholls et al., 1988; Kumar, 1997; Dawson et al., 2001), and show changes in responsiveness to long day lengths when kept on non-stimulatory day lengths (Misra et al., 2004). Therefore, the mechanisms of photoperiodism and circannual rhythm generation appear mutually inclusive, and possibly interact in the regulation of annual cycles in photoperiodic species (see Misra et al., 2004). In fact, the persistence of circannual rhythms in gonadal cycles has been shown in the photoperiodic, migratory junco (*Junco hyemalis*) (Holberton and Able, 1992).

In seasonal breeders, the annual breeding cycle can be described by four gonadal phases: recrudescence, breeding, regression and gonadal inactivity. The relative length of each phase in the year is species specific, but recrudescence and regression are the longest phases. Rigidly linked with gonadal phases, birds also exhibit seasonal cycles in phenotypic traits such as body mass and molt. Hence, the measurements of annual gonadal cycles and associated phenotypic traits under constant conditions have been found to be reliable markers of circannual rhythms.

The goal of the present study was to investigate the involvement of circannual rhythms in the regulation of annual gonadal cycle and associated phenotypic traits in the tropical spotted munia (*Lonchura punctulata*). They are photosensitive, but are not categorized as a typical photoperiodic species because they can respond to very short photoperiods as well, e.g. 1 or 3 h light per day (Chandola et al., 1975). Spotted munia have also been reported to show circannual cycles in food intake, body mass and testicular activity under constant light conditions (Chandola et al., 1982; Bhatt and Chandola, 1985). In this study, we investigated: (1) the persistence of circannual rhythms in gonadal phases and phenotypic traits under constant LD and continuous light (LL) conditions; (2) the differences in the circannual rhythm characteristics between males and females, because sexes can have independent timing strategies; and (3) the relationship of circannual rhythms to the external environment, because sexes can differ in how they use temporal cues to synchronize their circannual rhythms (Ball and Ketterson, 2008). We made a few general predictions in the present study. First, if spotted munia directly respond to light, then exposure to LD or LL at the end of breeding seasons will alter the course of gonadal regression. Alternatively, if spotted munia do not respond directly to light, then birds will continue to show repeated gonadal and molt cycles regardless of the external conditions. Second, if the total amount (hours) of light (or dark) received in the season/year influences the seasonal (annual) timing, spotted munia presented with light regimes providing identical amounts of light and dark periods in the year will exhibit similar circannual cycles. Third, male and female birds will show differences in circannual rhythm characteristics under given light environments if spotted munia evolved with a sex-specific timing strategy. We measured changes in body mass, gonadal recrudescence and regression, and feather molts in both sexes of spotted munia kept for a period of more than 2 years in an outdoor aviary providing natural conditions and in indoor aviaries providing controlled light (~22 lx) and temperature (18±1°C) conditions. Light at ~22 lx intensity was considerably low compared with that applied in several other avian circannual studies [100–300 lx or higher (e.g. Bhatt and Chandola, 1985; Gwinner et al., 1995; Gwinner, 1996a; Gwinner, 1996b)]. We proposed that a weak light environment would probably taper the direct effects, if any, of light on the gonadal recrudescence–regression cycle and, in turn, facilitate the expression of circannual rhythms in breeding cycle and associated phenotypic traits in the spotted munia. We also analyzed whether annual life-history traits in spotted munia are independent 'phenotype cycles' (Wingfield, 2005), but occur in a close temporal phase relationship in the natural environment.

MATERIALS AND METHODS

Animals

This study was performed on adult spotted munia [*Lonchura punctulata* (Linnaeus 1758)], a passerine finch (family: Estrildidae) measuring ~11 cm in length. Munia are widely distributed throughout the Indian subcontinent. They are a seasonal breeder with a long breeding season, extending between June and October (Ali and Ripley, 1974; Thapliyal, 1981). Juveniles can easily be distinguished from adults (supplementary material Fig. S1). The study was carried out at the Department of Zoology, University of Lucknow, Lucknow, India, as per approval of the Institutional Ethics Committee.

Experiment

The experiment began in November 2007, when most birds had begun gonadal regression and the post-nuptial molt. Wild-caught birds were initially kept in an outdoor aviary (2.95×1.73×2.21 m) for 1 week where they were maintained under natural light and temperature conditions (NDL). At this time, daylight and mid-day temperature in Lucknow, India (26°55′N, 80°59′E) were approximately 10.9 h and 30°C, respectively (supplementary material Fig. S2). Acclimatized birds were brought indoors and maintained under controlled light and temperature conditions in chronocubicles (2.2×1.8×2.8 m) located in the basement experimental facility. The underground location of the experimental chronocubicles greatly reduced the possibility of the effects, if any, of extraneous factors on the expression of annual cycles.

Birds were divided into three groups (groups 1 to 3) each of males and females (N=14–16 per group), and housed for 28 months in one of the three cubicles on programmed light but identical temperature conditions. Experimental cubicles were enriched by several perches, artificial creepers and regularly replenished fresh twigs of green plants (supplementary material Fig. S3). Birds were un-caged, and so they freely moved in their experimental cubicles. Both males and females were kept in the same cubicles. Artificial lighting was provided by a Phillips 40 W fluorescent tube at an intensity of ~22 lx, obtained by covering the fluorescent tube with narrow black strips of paper. We proposed that a light intensity of ~22 lx, which was neither dim nor too bright, in constant illumination would disorganize circadian rhythms (P.B., unpublished), but not circannual rhythms.

Group 1 was exposed to a 12 h:12 h light:dark regime (12L:12D; L=22 lx, D≤1 lx). This equinox light environment in square wave form with identical photorefraction did not provide temporal information to birds about the seasonal environment. Group 2 was similarly exposed to 24L:24D (L=22 lx, D≤1 lx). In spite of being identical to 12L:12D in the total amount of light and dark periods that birds received during the entire duration of the experiment, 24L:24D was not a constant light or dark environment (LL or DD), nor did it correspond to a known natural light environment. We

proposed that groups 1 and 2 would exhibit similar circannual cycles if the amount of light or dark influenced the annual timing in spotted munia. Alternatively, we proposed that group 2, subjected to repeated alternating days of light and darkness (LL/DD), would exhibit responses significantly different from group 1. Group 3 was exposed to constant light (L=22 lx). In addition, beginning in March 2008, we kept groups of male and female birds (N=15 each) exposee to natural light and temperature conditions (supplementary material Fig. S2) in the outdoor aviary (2.95×1.7×2.2 m). At this time, birds were reproductively quiescent, and daylight and mid-day temperature were approximately 11.6 h and 31°C, respectively (supplementary material Fig. S2). All experiments ended in March 2010.

Food (seeds of *Setaria italica* and *Oryza sativa*) and water were freely available and replenished at intervals during the light phase. A supplement food, rich in protein and vitamins and prepared by mixing bread crumbs, boiled eggs, crushed egg shells, cottage cheese and multivitamins (Vimeral, containing vitamins A, D3, E and B12, Virbac Animal Health India, Mumbai, India), was also given on alternate days (Singh et al., 2010). Birds also received an antibiotic (tetracycline hydrochloride, Hoechst Roussel Vet, Mumbai, India) for five consecutive days every month. A few birds died during the experiment, and the mortality was especially high under LL/DD conditions. Group size at the end of the study was as follows: NDL (male, N=10; female, N=9), 12L:12D (male, N=14; female N=12), 24L:24D (LL/DD: male, N=7; female, N=6) and LL (male, N=11; female, N=13).

Data recording

Observations on body mass, testes and ovary, and molt of wing primary feathers and body plumage were taken at regular intervals of 2 or 4 weeks throughout the experiment. The data from birds that died during the period of study were excluded from the presentation and analysis. Also, we have not presented data on body mass because changes in body mass over the experimental period in experimental groups did not show a regular cycle (supplementary material Fig. S4).

Testis size and follicle size

The testicular and ovarian responses were measured as testis volume (mm^3) and diameter of the largest follicle (mm), respectively. For this, birds were laparotomized at monthly intervals under local anesthesia, as described in an earlier studies (Kumar et al., 2001). Briefly, gonads were located in the abdominal cavity through a small incision in between the last two ribs on the left flank, and the size of the left testis or the largest ovarian follicle was measured using calipers with reference to accurate scales plotted on a graph sheet. The procedure was quickly over, and the incision was sutured by surgical thread. An antibacterial skin ointment (Soframycin skin cream, Aventis Pharma, Goa, India) was applied to the wound. Healing was rapid; post-operative infections were generally absent. Testis volume was calculated using formula $4/3\pi ab^2$, where a and b denote half of the long (length) and short (width) axes, respectively.

Molt: wing primary feathers and body plumage

Wing primary feathers were scored on a scale from 0 to 5, as described by Trivedi et al. (Trivedi et al., 2006). Briefly, scale was 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 from 0 to 5, and a primary wing feather could have total score of 0 to 45. From this, a linear increase in new feather mass was calculated, as per Dawson and Newton (Dawson and Newton, 2004).

Body plumage was recorded by dividing the bird's body into 12 different regions as follows: 1=head, 2=neck, 3=shoulder, 4=back, 5=pelvic, 6=caudal, 7=throat, 8=chest, 9=abdomen, 10=flank, 11=shank, and 12=sub-caudal. Any region could have a score of 0 (no molt; i.e. the region has fully grown or old feathers) or 1 (molt; the region has no feathers or new feathers emerging). Thus, the plumage body molt score ranged from 0 to 12 (Trivedi et al., 2006).

Data presentation and analysis

Data on testes and ovarian follicles were plotted against the time axis, which, when connected by a line, revealed gonadal phases during the period of the experiment. To better illustrate the growth–regression response curve for individuals and for each group, we also calculated moving averages for the entire data set by averaging three consecutive values for each time point. The maximum value of testis size or follicle diameter attained in each cycle was considered as the amplitude (peak) of the circannual cycle, and intervals between two successive peaks gave the period of circannual rhythms.

The gonadal recrudescence–regression curve was also plotted along with wing primary molt, which indicated the temporal relationship between gonadal cycle and an associated phenotypic trait. For this, the peak gonadal response in an individual was given 0 on the time scale, and successive values preceding and following this maximum were plotted at monthly intervals on a scale of –12 to +12, respectively, until values reached minima at both ends. The timing of molt onset was plotted accordingly on the time scale (–12 to +12). This represented the overall annual distribution of the molt pattern in relation to the gonadal growth–regression curve in the experiment.

We analyzed data using appropriate statistics that included t-tests (both unpaired and paired), one-way and two-way ANOVAs, and *post hoc* tests. Student's paired and unpaired t-tests were used to show differences between two values as a function of time (e.g. day and night) in the same and different light conditions, respectively. Similarly, one-way ANOVA followed by a Newman–Keuls multiple comparison *post hoc* test was used to determine significant changes in a measurement among different groups in the experiment (e.g. significant difference in circannual period or peak testicular response among the experimental groups at one time point). A two-way ANOVA compared responses of two sexes in different experiments (factor 1: sex; factor 2: light condition). Significance was taken at $P<0.05$. Statistical analysis was carried out using GraphPad Prism software (version 5.0, La Jolla, CA, USA).

RESULTS

Cycles in gonadal phases and phenotypic traits under natural conditions (NDL)

Testicular and molt cycles

In all experiments, males underwent two cycles of gonadal growth and regression (Fig. 1, Fig. 5A). Testes in all groups started recrudescing in April/May, attained maximal growth by July/August, and regressed by the following January. The second testicular cycle followed a similar pattern (Fig. 5A). The recrudescence–regression phase lasted for 10.1±0.4 months in the first year and 11.1±0.3 months in the second year. Between two successive years, there was a significant difference in the duration of the recrudescence–regression phase (paired t-test, P=0.0418), but not in the amplitude (paired t-test, P=0.6541), of the testicular cycle.

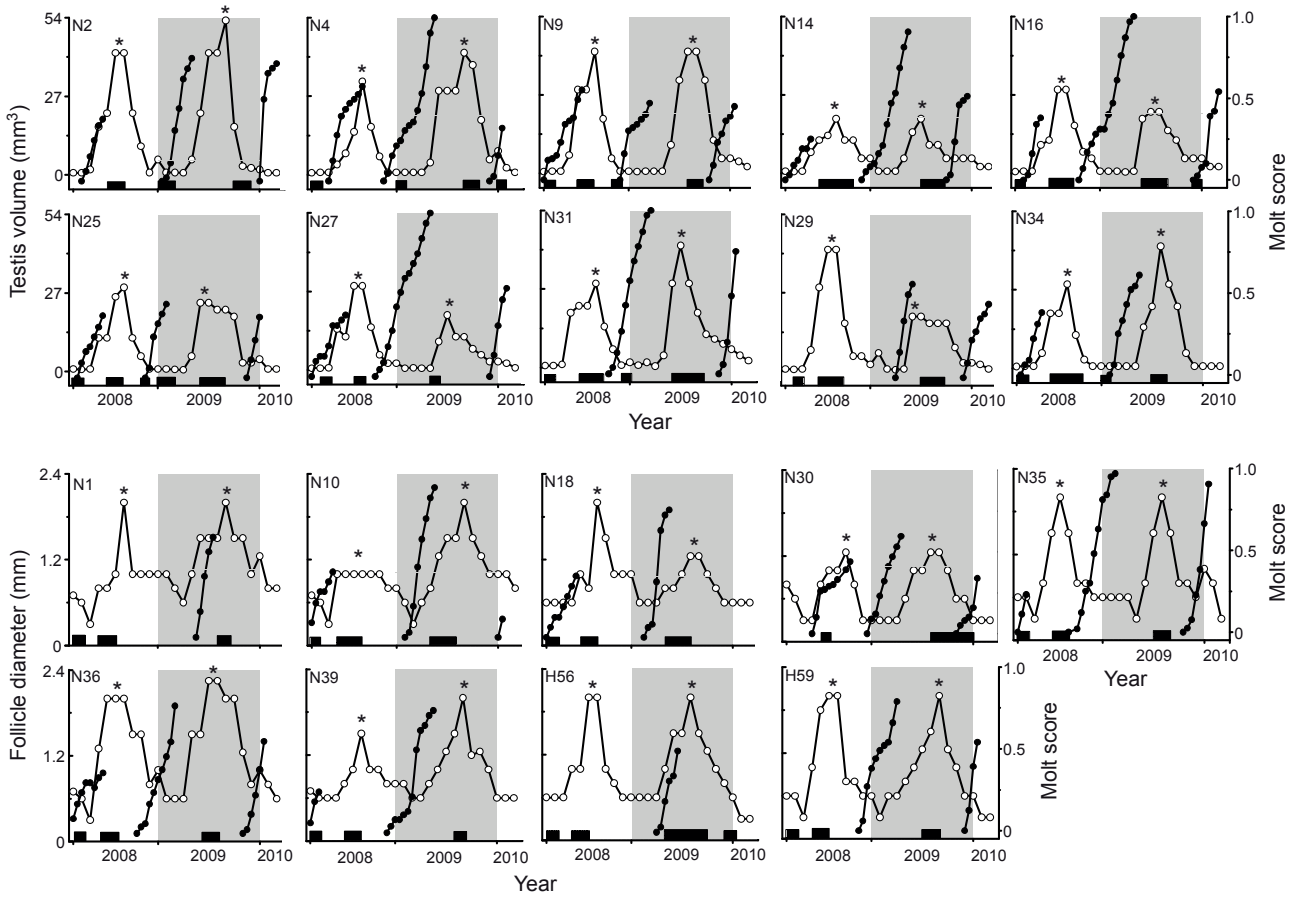

Fig. 1. Changes in testis volume (males) and diameter of the largest follicle (females) (open circles, left axis) and the occurrence of molt in wing primaries (solid circles, right axis) and body plumage (solid horizontal bars along the *x*-axis) in spotted munia (*N*=10 males, 9 females) kept in an outdoor aviary at Lucknow, India (26°55′N, 80°59′E) for 25 months, March 2008 to March 2010. All birds were maintained under natural day length and temperature cycles and showed repeated cycles of gonadal recrudescence–regression and molts. Asterisks indicate the times of peak gonadal growth in an individual bird.

The circannual period of the testicular cycle, the interval between successive testicular peaks, was 12.3±0.2 months (Fig. 5G). Along with the testicular cycle, males underwent molts in wing primary feathers and body plumage (Fig. 1). However, molt frequencies varied from two to three cycles in wing primary feathers and from two to four cycles in body plumage (Fig. 1).

Ovarian and molt cycles

Females in all groups also exhibited two cycles in growth and regression of the ovarian follicles (Fig. 1, Fig. 5A). Follicular growth was initiated in May/June 2008. The follicles grew largest by August/September 2008 and then regressed by the following January (three of nine birds) or March (six of nine birds). A second cycle followed pattern similar in timing, duration and amplitude (Fig. 5A,H). The growth–regression phase of the annual ovarian cycle lasted for 9.8±0.4 mo in the first year and 10.6±0.4 months in the second year. There was no significant difference in the duration (paired *t*-test, P=0.1108) or amplitude (paired *t*-test, P=0.3400) of the growth–regression phase of the follicular cycle between two successive years. The circannual period of ovarian cycle, the interval between two successive peaks in follicular growth, was 12.4±0.3 months (Fig. 5H). Similar to males, females underwent molt in their primary wing feathers and body plumage with a frequency of one to three cycles in primaries and two to four cycles in body plumage (Fig. 1).

Cycles in gonadal phases and phenotypic traits under artificial light (12L:12D, LL/DD and LL) and temperature (18±1°C) conditions

Testicular and molt cycles

In experiments, testes, which were still large at the beginning of the experiment, were fully regressed by the end of 4.2±0.3 months in group 1 on 12L:12D, 3.8±0.3 months in group 2 on 24L:24D, and 3.4±0.2 months in group 3 on LL. The next growth phase was initiated after 2.1±0.3, 1.3±0.3 and 1.3±0.3 months in groups 1, 2 and 3, respectively. Irrespective of the lighting conditions, all groups underwent two testicular cycles (Figs 2–4, Fig. 5B–D). However, the duration of testicular growth–regression phase in groups on 12L:12D and 24L:24D was significantly shorter (paired *t*-test, P<0.001) in the first year than in the second year (first year: 12L:12D, 9.0±0.4 months, 24L:24D, 9.0±0.8 months; second year: 12L:12D, 12.4±0.4 months, 24L:24D, 13.3±0.5 months). In group 3 on LL, however, the duration of the testicular growth–regression phase did not differ significantly between the first (11.2±0.3 months) and second years (11.9±0.5 months; paired *t*-test, P=0.2085). Similar differences were found in the testicular growth maxima between the first and second years in groups on 12L:12D (paired *t*-test, P=0.0004) and 24L:24D (paired *t*-test, P=0.0117), but not in the group on LL (*t*-test, P=0.7538; Fig. 5E).

Among three experimental groups, a significant difference occurred in both cycles in peak testicular response (one-way

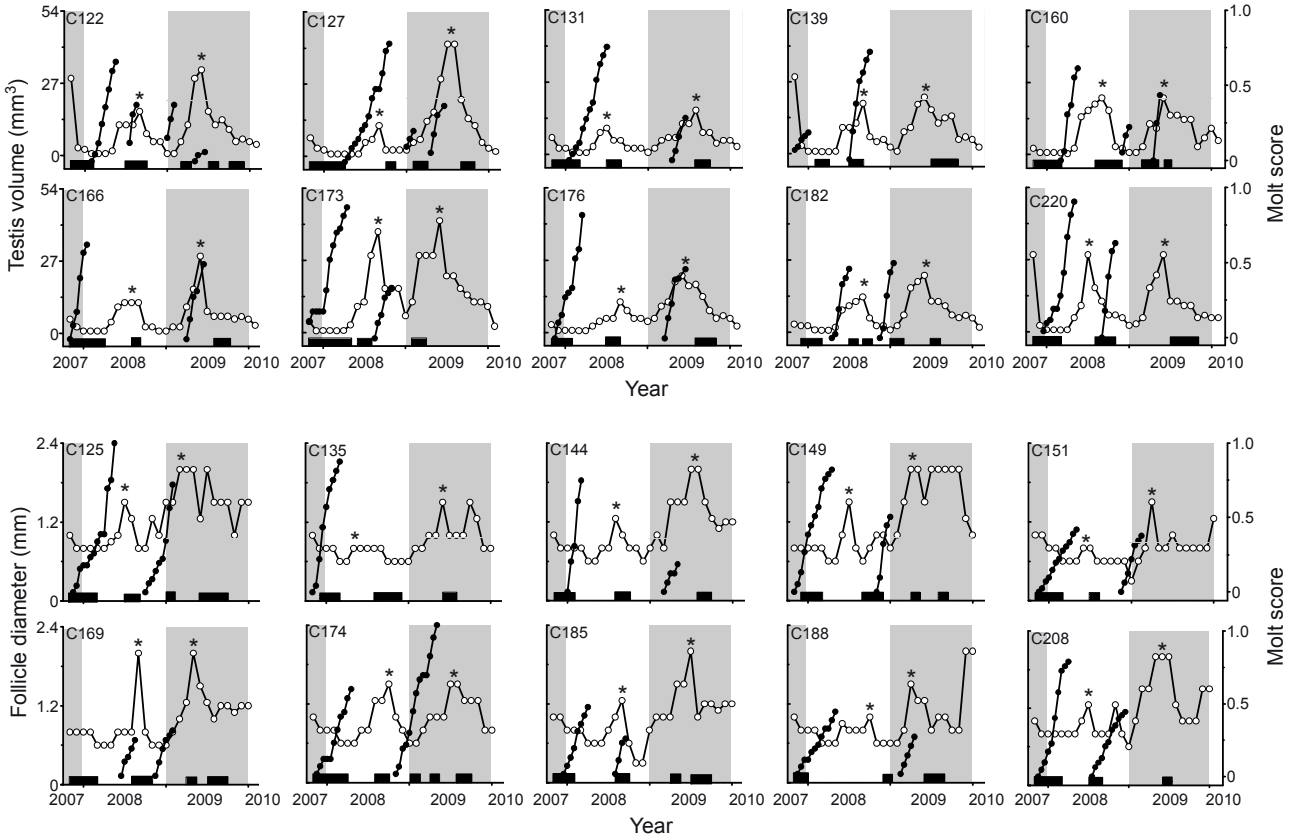

Fig. 2. Changes in testis volume (males) and diameter of the largest follicle (females) (open circles, left axis) and the occurrence of molt in wing primaries (solid circles, right axis) and body plumage (solid horizontal bars along the *x*-axis). Fourteen male and 12 female spotted munia were moved indoors in experimental rooms, located underground, and were subjected to a photoperiodic regime of 12 h:12 h light:dark (12L:12D; L=22±1 lx, D≤1 lx) at 18±1°C. Data for 10 individuals of each sex are shown; the remaining birds responded similarly. All birds showed repeated cycles of gonadal recrudescence–regression and molts during 28 months of exposure to 12L:12D, beginning in November 2007. Asterisks indicate the times of peak gonadal growth in an individual bird.

ANOVA, first year: $F_{2,29}$=3.425, P=0.0540; second year: $F_{2,29}$=3.979, P=0.0297; Fig. 5E), but not in the circannual periods (one-way ANOVA, $F_{2,29}$=0.0057, P=0.9943; Fig. 5G). The latter, measured as intervals between two successive peaks, were 11.1±0.3, 11.1±0.5 and 11.1±0.4 months for groups 1, 2 and 3, respectively (Fig. 5G). Interestingly, the circannual period of all three experimental groups was significantly different from the 'annual' period of the group on NDL (unpaired *t*-test, NDL *versus* 12L:12D, P=0.0135; NDL *versus* 24L:24D, P=0.0233; and NDL *versus* LL, P=0.0261).

All groups, irrespective of light condition, underwent molt cycles in wing primary feathers but with frequency varying from one to four cycles (Figs 2–4). Some birds in each group were molting at the beginning of the experiment, and hence the three treatment groups did not differ from each other in the onset of molt (one-way ANOVA, $F_{2,17}$=0.7008, P=0.5100). For example, in group 1, six of 14 birds were molting at the beginning of the experiment and eight birds began molting after 4.0±0.6 months. Overall, there was no difference in the frequency (one-way ANOVA, $F_{2,29}$=2.891, P=0.0716) or period (one-way ANOVA, $F_{2,28}$=2.765, P=0.0802) of wing primary molts among three experimental groups. However, group comparisons revealed a significant increase in molt cycle in the group on LL than in the group on 12L:12D (unpaired *t*-test, P=0.05), and a shorter molt duration in the group on LL/DD than in the group on LL (unpaired *t*-test, P=0.05). Eight, five and one bird underwent molt in primaries two (8.1±1.1 month intervals), three (8.0±0.5 month intervals) and four (6.0±0.6 month intervals) times, respectively. The pattern also varied from partial to complete molt among individuals of group 1. In group 2, two of seven birds were in molt at the beginning of the experiment and the remaining five individuals underwent their first molt after 5.4±1.4 months. In total, six of seven birds had three molts, with second and third molts at intervals of 7.8±1.1 and 7.0±1.0 months, respectively. The remaining individual molted after 7 months. The 11 birds in group 3 exhibited two (N=2), three (N=5) and four (N=4) molt cycles. Four birds were already in molt phase when the experiment began. The remaining seven individuals began their first molt after 3.7±1.1 months. The subsequent molt occurred after 10.5±0.5 (N=2), 12.3±0.41 (N=5) or 8.5±0.6 months (N=4).

Similar to wing primaries, body plumage molt varied in pattern (partial or complete) and frequency (Figs 2–4, Fig. 5F–H). In all groups, all but one of the birds were in molt at the beginning of experiment; the remaining individuals in each group started molting after 4 (group 1), 10 (group 2) and 2 months (group 3). There was no significant difference among three groups in the number of body molt cycles (one-way ANOVA, $F_{2,29}$=1.701, P=0.2003) or the duration of molt cycle (one-way ANOVA, $F_{2,29}$=2.698, P=0.0842). In group 1 on 12L:12D, nine, three and two individuals underwent three (11.1±0.8 month intervals), four (8.7±0.5 month intervals) and five (6.1±0.4 month intervals) body plumage molts, respectively. Similarly, there were two (N=1, 8.0 month intervals), three (N=2; 7.5±1.0 month intervals), four (N=3; 7.7±0.2 month intervals) and

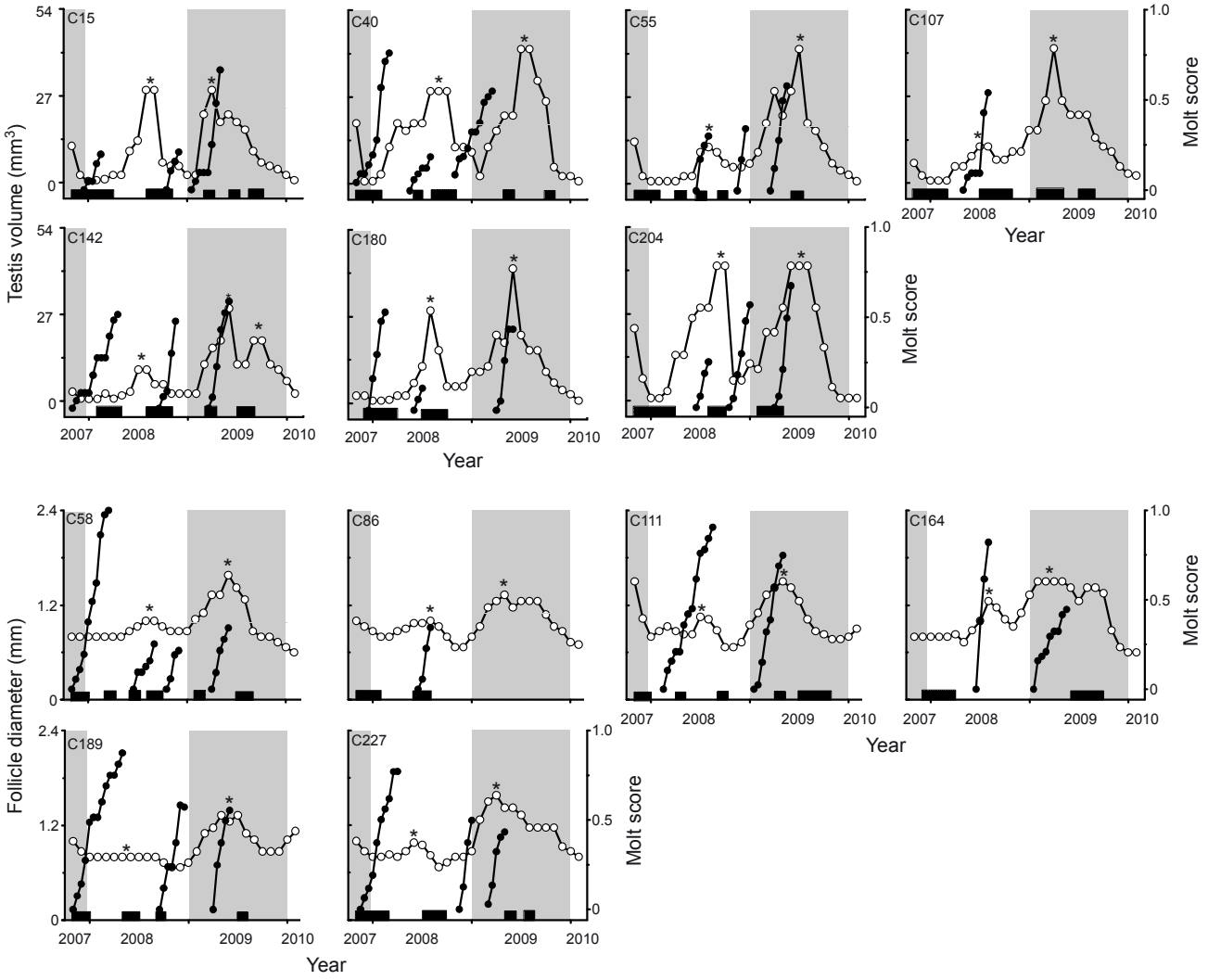

Fig. 3. Changes in testis volume (males) and diameter of the largest follicle (females) (open circles, left axis) and the occurrence of molt in wing primaries (solid circles, right axis) and body plumage (solid horizontal bars along the *x*-axis). Seven male and six female spotted munia were moved indoors in experimental rooms, located underground, and were subjected to a photoperiodic regime of 24 h:24 h light:dark (24L:24D; L=22±1 lx, D≤1 lx) at 18±1°C. Thus, these birds received alternating days of light and darkness. All birds showed repeated cycles of gonadal recrudescence–regression and molts during 28 months of exposure to 24L:24D, beginning in November 2007, albeit with an attenuated amplitude, especially in females. Asterisks indicate the peak gonadal growth in an individual bird.

five (N=1; 6.8±0.3 month intervals) body plumage molts in group 2 on 24L:24D. Group 3 birds also exhibited three (N=4; 9.4±0.9 months), four (N=3; 7.9±0.6 months), five (N=2; 6.4±0.6 months) and six (N=2; 5.7±0.3 months) molts during their exposure to LL. The intervals in body molts differed among three groups (one-way ANOVA, $F_{2,29}$=3.944, P<0.05). The interval period in the body molt in the group on 12L:12D was significantly longer than in the group on 24L:24D (t-test, P<0.05), but not in the group on LL.

Ovarian and molt cycles

Similar to males, females in all experimental groups had fully regressed ovaries by the end of 5.0±0.4 (group 1, 12L:12D), 2.3±0.3 (group 2, 24L:24D) or 2.7±0.4 months (group 3, LL). A subsequent follicular growth phase was initiated in these groups after 3.6±0.4 months of 12L:12D (group 1), 5.8±0.8 months of 24L:24D (group 2) or 4.5±0.4 months of LL (group 3). During the experiment, all groups exhibited two ovarian cycles with a follicular growth–regression phase of 6.3±0.4 (group 1), 6.5±0.6 (group 2) or 8.5±0.6 months (group 3) in the first year, and 10.8±0.6 (group 1), 12.5±0.9 (group 2) or 9.1±0.8 months (group 3) in the second year (Figs 2–4, Fig. 5B–D). Thus, the growth–regression phase was significantly longer (paired t-test, P<0.01) in the second cycle in groups 1 and 2, but not in group 3 (paired t-test, P=0.5896). When compared with controls (NDL), the growth–regression phase was significantly shorter in the first cycle in groups 1 and 2, but not in group 3 (one-way ANOVA, $F_{3,36}$=10.31, P<0.0001). Interestingly, in the second cycle, the growth–regression phase was significantly longer in group 2 compared with the other groups, including NDL (one-way ANOVA, $F_{3,36}$=3.186, P=0.0353). A similar difference was found in the follicular growth maxima among three experimental groups, with significantly smaller follicles in the first cycle compared with the second cycle (paired t-test, P<0.01; Fig. 5F). When compared with responses in birds under NDL, there was a significantly smaller follicular growth among experimental groups in the first

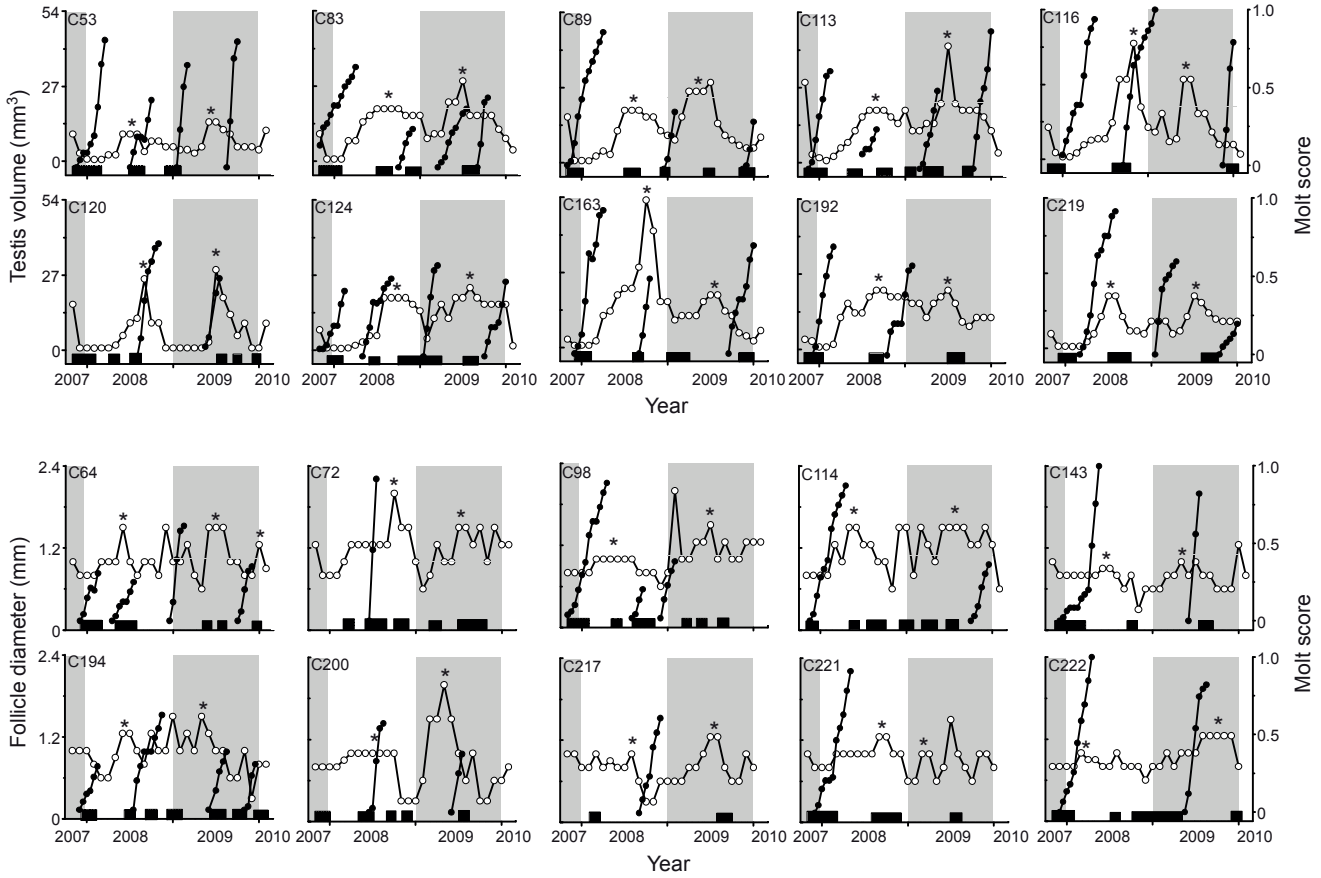

Fig. 4. Changes in testis volume (males) and diameter of the largest follicle (females) (open circles, left axis) and the occurrence of molt in wing primaries (solid circles, right axis) and body plumage (solid horizontal bars along the *x*-axis). Eleven male and 13 female spotted munia were moved indoors in experimental rooms, located underground, and subjected to a constant light regime (LL; L=22±1 lx) at 18±1°C. Data for 10 individuals of each sex are shown; the remaining birds responded similarly. All birds showed repeated cycles of gonadal recrudescence–regression and molts during 28 months of exposure to LL, beginning in November 2007, albeit with an attenuated amplitude. The cyclicity was relatively less pronounced in females, but still in most individuals the timing of peaks could be discerned. Asterisks indicate the peak gonadal growth in an individual bird.

cycle (one-way ANOVA, $F_{3,36}$=9.219, P<0.0001) but not in the second cycle (one-way ANOVA, $F_{3,36}$=2.401, P=0.0837). Further, the periods of cyclicity, measured as the interval between two peaks, were 11.8±0.4 (group 1, 12L:12D), 9.8±0.6 (group 2, 24L:24D) and 13.5±0.8 months (group 3, LL; Fig. 5H). Thus, the circannual periods of groups 2 and 3, but not group 1, were significantly different from that of the NDL group (one-way ANOVA, $F_{3,36}$=4.705, P=0.0072; Fig. 5H).

Like males, females showed no difference among the three treatment groups in the onset of the wing primary molts (one-way ANOVA, $F_{2,21}$=2.178, P=0.1381). However, the 12L:12D group molted more slowly than the 24L:24D group (unpaired *t*-test, P=0.05). All birds underwent molts in wing primaries irrespective of the light condition (Figs 2–4), although with varying pattern (partial to complete) and frequency (one to four cycles; Figs 2–4). The number of wing primary molts over the duration of the study did differ significantly among the treatment groups (one-way ANOVA, $F_{2,28}$=2.030, P=0.1502). A few birds in each group (eight of 12 in group 1, three of six in group 2 and seven of 13 in group 3) were in molt at the beginning of the experiment in November/December 2007. In total, group 1 molted once (N=2) or twice (N=10), beginning after 3.2±0.7 months of exposure to 12L:12D. The interval between the two molts was 11.6±1.0 months. Similarly, group 2 molted one (N=1), two (N=2), three (N=2) or four times (N=1). In birds that were not in molt at the beginning of the experiment, the first molt occurred after 6.3±1.2 months of exposure to 24L:24D. The subsequent molt occurred after 10.0±2.0, 9.0±0.5 and 6.7±0.9 months in birds showing two, three and four molts, respectively. Group 3 also molted one (N=3), two (N=4), three (N=2) or four times (N=4). Birds that were not in molt at the beginning of the experiment initiated molt after 5.8±1.6 months of LL exposure. Subsequent molts occurred after 9.5±1.2, 7.0±0.5 and 8.5±0.3 months in birds molting two, three and four times, respectively. Unlike frequency, however, the period of wing primary molts was significantly different among the three groups (one-way ANOVA, $F_{2,22}$=4.341, P=0.0258). In particular, the group on LL had a significantly shorter period than the group on 12L:12D (*t*-test, P<0.05).

A similar pattern and frequency was found in the body plumage molt (Figs 2–4). Like wing primaries, many birds (10 of 12 birds in group 1, five of six birds in group 2 and seven of 13 birds in group 3) were molting their body plumage at the beginning of the experiment. The onset of the first body molt appeared to vary significantly (one-way ANOVA, $F_{2,28}$=4.345, P=0.0227). In particular, the group on LL molted faster than the group on 12L:12D (unpaired *t*-test, P<0.05). Birds that were not in molt at the beginning of the experiment initiated molt after 2 (groups

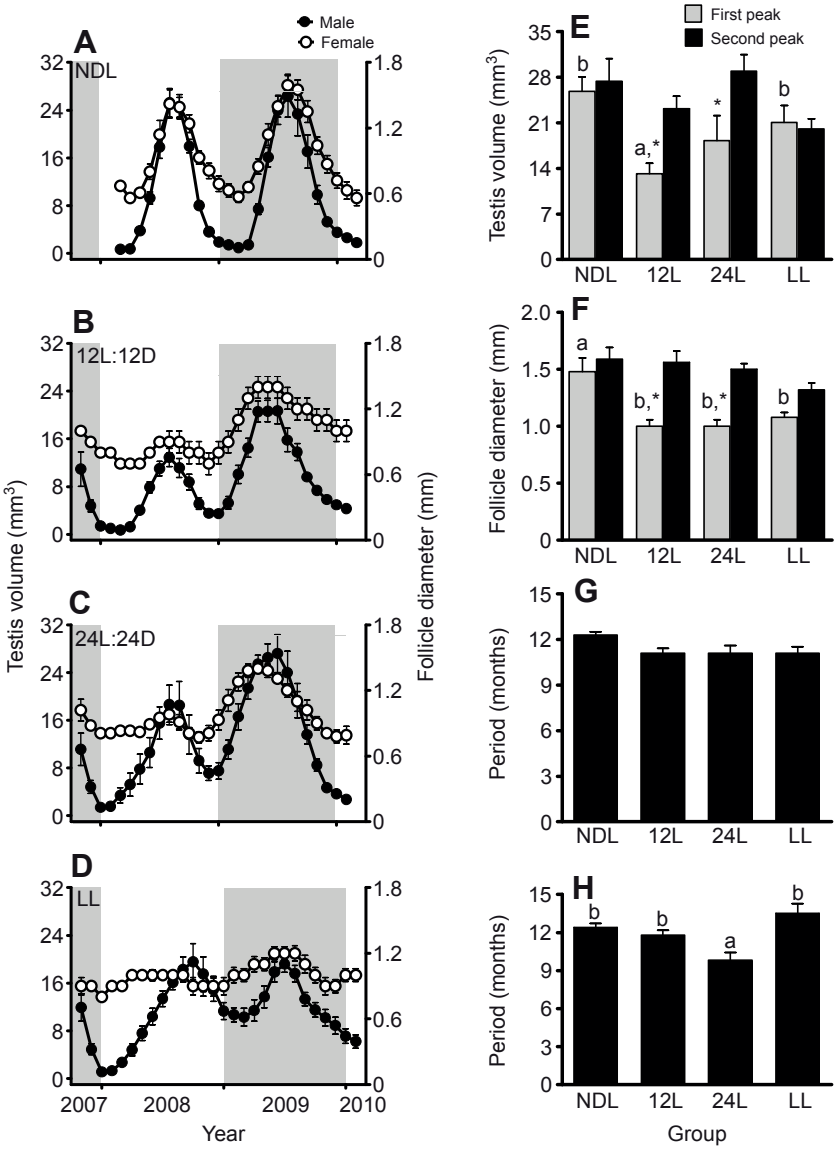

Fig. 5. The recrudescence–regression cycle in testis (solid circles, left axis) and ovary (open circles, right axis) in spotted munia subjected to (A) natural conditions (NDL) and the photoperiodic regimes (B) 12L:12D, (C) 24L:24D and (D) LL for a period of 25 (NDL) or 28 months from November 2007 to February 2010. The data plotted in this figure are the moving averages of the groups. The peak responses for both cycles are plotted for (E) testis volume and (F) follicle diameter. The intervals between two peaks gave the period of annual/circannual cycle, plotted as means ± s.e.m. for (G) males and (H) females. The annual/circannual periods are as follows: NDL, 12.3±0.2 months (males), 12.4±0.36 months (females); 12L:12D, 11.14±0.33 months (males), 11.8±0.4 months (females); 24L:24D, 11.1±0.46 months (males), 9.83±0.6 months (females); LL, 11.1±0.4 months (males), 13.5±0.8 months (females). Different letters indicate significant differences between treatment groups (P<0.05). Asterisks indicate significant differences between the two peaks (P<0.05).

1 and 2) or 3.2±0.4 months (group 3). Also, most birds in all groups had partial body plumage molt with two to seven cycles, as indicated above (Figs 2–4). Under 12L:12D conditions, birds molted two (N=1), three (N=4), four (N=5) or five (N=2) times. The subsequent molts occurred after 9.0±0.0, 10.8±0.3, 7.6±0.2 and 6.0±0.3 months in birds molting for two, three, four and five times, respectively, during the experiment. Similarly, under 24L:24D conditions, birds molted two (N=2), four (N=2), five (N=1) and six (N=1) times at intervals of 7.5±0.5, 7.7±0.0, 5.8±1.0 and 5.0±0.7 months, respectively. In the group on LL, birds molted two (N=2), three (N=1), four (N=1), five (N=5), six (N=3) and seven (N=1) times at intervals of 8.0±0.0, 10.8±0.3, 9.0±2.9, 5.7±0.2, 5.1±0.2 and 5.0±0.5 months, respectively. In spite of individual differences, the frequency of body molt was significantly different among three groups (one-way ANOVA, $F_{2,28}$=2.563, P=0.0950), with a particular difference between the groups on 12L:12D and LL (unpaired t-test, P<0.05); the latter groups had more molts. The period of molt cycles also differed significantly between groups 1 and 3 (one-way ANOVA, $F_{2,28}$=3.627, P=0.0398), indicating that birds molted faster and at shorter intervals in group 3.

Effects of sex and light condition

Among experimental groups, in spite of the similar gonadal cycle, both sexes differed in the duration of the growth–regression phase, the amplitude of the cycle defined by testicular and follicular maxima, and the periods over two cycles. Two-way ANOVA revealed a significant effect of sex (factor 1) and light condition (factor 2) as well as a significant interaction between the two factors (only in the first cycle) on duration of gonadal growth–regression phase (first cycle: factor 1, $F_{1,57}$=41.91, P<0.0001; factor 2, $F_{2,57}$=17.23, P<0.0001; factors 1×2, $F_{2,57}$=0.3499, P=0.7062; second cycle: factor 1, $F_{1,57}$=7.670, P=0.0076; factor 2, $F_{2,57}$=8.222, P=0.0007; factors 1×2, $F_{2,57}$=0.4401, P=0.6461) and circannual period (factor 1, $F_{1,57}$=1.478, P=0.2291; factor 2, $F_{2,57}$=4.123, P=0.0210; factors 1×2, $F_{2,57}$=4.405, P=0.0166).

Two-way ANOVA analyzed the effects of sex and light condition. The frequency of wing primary molt was significantly influenced by sex ($F_{1,57}$=5.377, P=0.0240) and light condition ($F_{2,57}$=4.449, P=0.0160), as well their interaction ($F_{2,57}$=0.3663, P=0.6949). In general, females on LL underwent molt more frequently than males on LL. Further, photoperiod ($F_{2,50}$=4.542, P=0.0154), but not sex ($F_{1,50}$=0.05126, P=0.8218), had significant effect on the period of

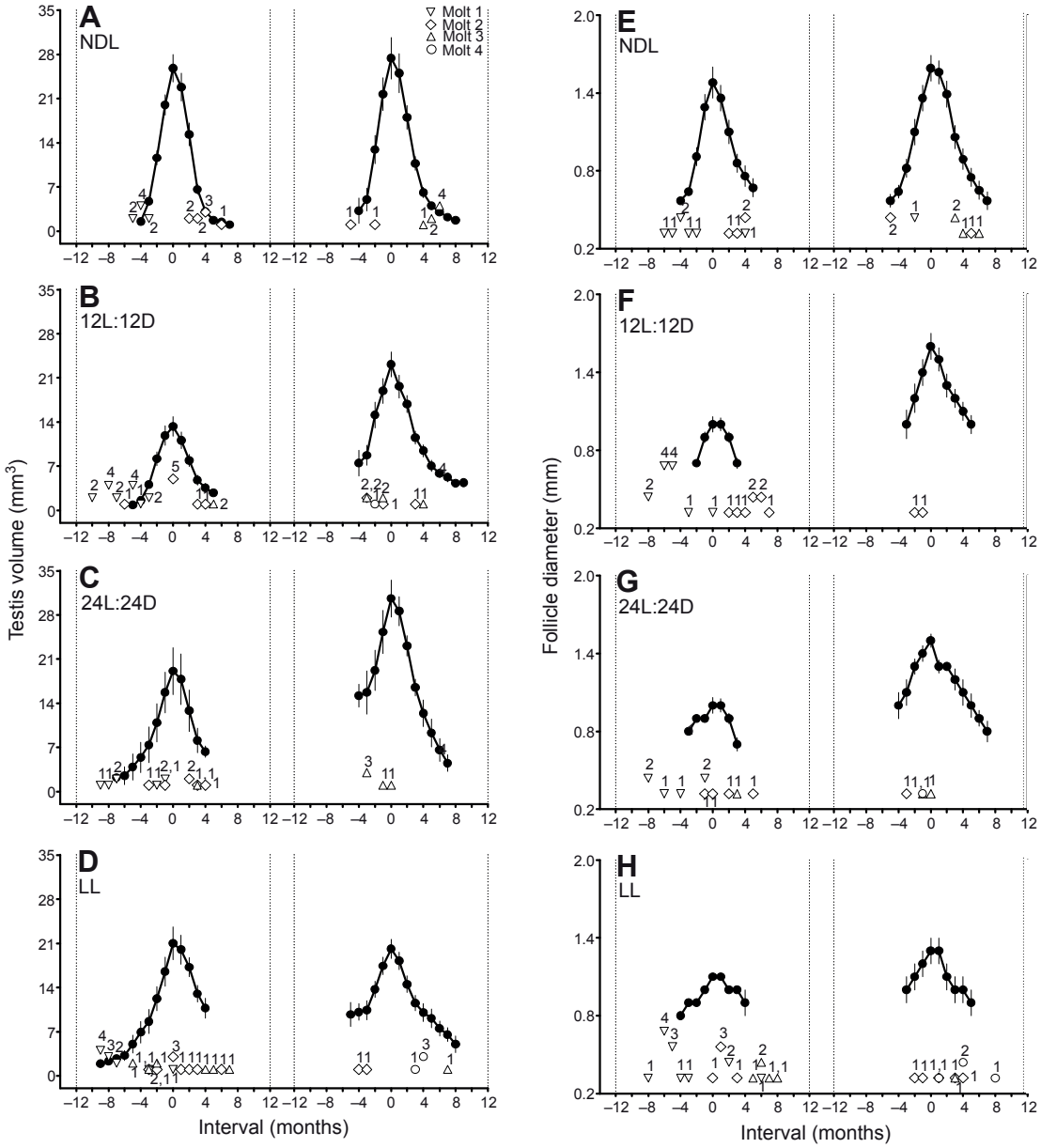

Fig. 6. The relationship between gonadal cycles and wing primary molt in male (left panels) and female spotted munia under natural (NDL) and artificial lighting conditions (12L:12D, 24L:24D and LL). The peak gonadal response in an individual during the first and second cycle was given the value zero on the time scale (*x*-axis), and the 12 months before and after the time of peak responses were accorded values from –12 to +12, respectively. The frequency of the onset of wing primary molts in individual birds was plotted on the time scale (first molt, inverted triangle; second molt, diamond; third molt, triangle; fourth molt, circle). The number of each symbol denotes the number of birds in molt. Data are means ± s.e.m.

wing primary molt. Similarly, there was a significant effect of sex (factor 1) and light condition (factor 2) on the frequency (factor 1, $F_{1,57}$=11.16, P=0.0015; factor 2, $F_{2,57}$=5.474, P=0.0067) and period (factor 1, $F_{1,57}$=4.049, P=0.0489; factor 2, $F_{2,57}$=6.627, P=0.0026) of body molts in this study.

Relationship between breeding and wing primary molt cycles

Fig. 6 shows two successive breeding cycles, plotted with reference to peak gonadal growth. The first breeding period reflected by gonadal growth–regression in males lasted for 10.7±0.3 (NDL), 9.9±0.2 (12L:12D), 10.8±0.6 (24L:24D) or 10.9±0.5 months (LL). The subsequent breeding season in these groups lasted for 12.0±0.3 (NDL), 12.0±0.4 (12L:12D), 11.4±0.6 (24L:24D) or 11.5±0.5 months (LL). Similarly, breeding periods in females lasted for 9.9±0.2 (NDL), 6.8±0.4 (12L:12D), 6.5±0.6 (24L:24D) and 8.2±0.5 months (LL) in the first cycle, and 12.0±0.2 (NDL), 9.7±0.5 (12L:12D), 11.8±0.5 (24L:24D) and 9.7±0.5 months (LL) in the second cycle. Overall, the breeding seasons appeared to range from 6 to 12 months, with the second gonadal growth–regression phase being longer than the first one.

Also plotted in Fig. 6 is the distribution of wing primary molts in different groups in relation to gonadal cycle. In NDL, wing primary molt coincided with gonadal regression (Fig. 6A,E). This pattern was nearly maintained under 12L:12D conditions, but became increasingly lost in 24L:24D and LL (Fig. 6B,F). When compared, the molt patterns were scattered and more dissociated in females than in males, and in the second cycle compared with the first cycle (Fig. 6).

DISCUSSION

The present study demonstrates circannual rhythms in gonadal cycle in the tropical spotted munia (Figs 1–5). Clearly, repeated cycles in gonadal phases (Figs 1–5) are not a consequence of the prevailing photoperiods, because exposure of birds to different light conditions did not alter the course of gonadal regression. Birds exhibited two consecutive gonadal cycles with circannual periods of 10–13 months under 12L:12D, 24L:24D and LL, and with gonadal phases comparable to those in the control group on NDL (see Figs 1–5). These results are in agreement with previously reported circannual rhythms in testicular cycle, but not in body mass, in the spotted munia (see Bhatt and Chandola, 1985). Unlike Bhatt and Chandola (Bhatt and Chandola, 1985) we did not find a circannual (or seasonal) rhythm in body mass in experimental groups in the present study, although birds on NDL underwenta seasonal cycle in body mass (supplementary material Fig. S4).

The present study differs from previous studies on circannual rhythms in songbirds (Gwinner, 1986), including the spotted munia (Chandola et al., 1982; Bhatt and Chandola, 1985). In particular, we included both sexes in an equal sex ratio (1:1), whereas the study of Bhatt and Chandola (Bhatt and Chandola, 1985) was performed only on males. Also, we simultaneously measured circannual cycles in groups kept on both constant periodic (12L:12D and 24L:24D) and aperiodic (LL) light conditions, which addressed on the causal effect, if any, of the photoperiod on circannual rhythm generation. Further, we housed un-caged birds in chronocubicles ($2.2\times1.8\times2.8$ m), so each individual shared approximately 65-fold more space in volume than the birds in the study of Bhatt and Chandola (Bhatt and Chandola, 1985), in which birds were housed in groups of five or six in much smaller cages ($25\times48\times28$ cm). This could be an important factor because social crowding is known to affect reproduction at various levels, including the development of reproductive endocrine organs (Rahe et al., 1986). For example, the onset of breeding was delayed among crowded ptarmigans (*Lagopus lagopus*) subjected to stimulatory increasing day lengths (Sharp and Moss, 1981). Some temperate birds remain reproductively active during the second cycle when housed in pairs, but not when singly housed (Schwab and Lott, 1971; Wingfield and Farner, 1979). Crowding extended the duration of breeding in African village weaverbirds (*Ploceus cucullatus*) (Collias et al., 1971). Also, paired and unpaired African stonechats (*Saxicola torquata axillaris*) held for 29 months under 12.25 h light per day at 300 lx differed in terms of reproductive performance, but not in the duration of reproductive phases or circannual periods (Gwinner et al., 1995).

An obvious advantage of circannual rhythms in breeding cycles is that they prepare two sexes for synchronized reproductive processes. However, there can be sex-specific differences in the existence of the circannual rhythms. In humans, for example, a circannual rhythm of prolactin secretion is found in females, but not in males (Touitou et al., 1983). If female spotted munia lack a circannual rhythm in breeding cycle, then the reported circannual rhythm in testicular cycle (Bhatt and Chandola, 1985) may have much less of a consequence unless it is assumed that males directly drive the female sexual state. This could be a possibility for the final stages of ovum maturation (the exponential growth phase), in order to maximize the chances of successful reproduction at the most favourable time, but not for the timing of the initiation of development of ovarian cycle. In all probability, it seems much less likely that the timing of ovarian cycle is subservient to the male state. Regardless of the underlying reasons, which are unclear at present, we have observed repeated gonadal cycles in spotted munia with circannual periods within the narrow range of ±3.0 months among individuals of both male and female groups held under constant photoperiods (T=24, 12L:12D; T=48, 24L:24D, where T is the period of zeitgeber) and LL (Fig. 5). The circannual periods in testicular and ovarian cycle were close to 11 and 10–13 months, respectively (Fig. 5). Similar repeated circannual cycles in reproductive functions (e.g. gonadal maturation and regression) and other phenotypic traits (body mass, molt and plumage) under constant photoperiods have been reported in few other bird species (Schwab, 1971; Schwab and Lott, 1971; Gwinner, 1975; Gwinner, 1981; Gwinner, 1986; Gwinner and Dittami, 1990; Cadee et al., 1996; Dawson, 1997; Piersma, 2002; Piersma et al., 2008). Also, reproductive asynchrony and population divergence between two equatorial populations of rufous-collared sparrows (*Zonotrichia capensis*) has been found to be associated with local weather and not photoperiod (Moore et al., 2005). It may be noted that circannual rhythms can be very specific, as revealed by a study on closely related subspecies of stonechats: when *Saxicola torquata maurus* and *S. t. torquata*, which breed at similar latitudes, were exposed to same annual photoperiodic cycle, the two subspecies exhibited responses corresponding to their different natural breeding schedules (Helm et al., 2009).

Our experiments allowed birds freer movement and close interactions within and between sexes, a situation thought to mimic the natural environment. If social interactions between sexes affected the duration of gonadal recrudescence–regression phase, then the consequence would be reflected on the entire breeding cycle, and hence on the circannual reproductive rhythms. The present results do not exclude such a possibility. Birds (especially males) on 24L:24D and LL underwent cycles with similar periods, and with some synchrony among individuals of each group (see Figs 1–4). This is not surprising because the effects of social interactions between sexes on reproduction are well known across vertebrate taxa. In tungara frogs (*Physalaemus pustulosus*), the interaction with males modulates reproductive hormone levels in females (Lynch and Wilczynski, 2006). In addition, male presence is required for the progression of reproductive processes in female blood pythons (*Python curtus*) (DeNardo and Autumn, 2001) and female ring doves (*Streptopelia risoria*) (Freidman, 1977). Similarly, female presence affects testosterone levels and male sexual behaviour in songbirds (Moore, 1982; Wingfield and Monk, 1994; Pinxten et al., 2003). Perhaps male and female effects are the components of a self-reinforcing cycle that results in the synchronized reproductive activity (Walkden-Brown et al., 1999). We cannot eliminate the possible impact of living together on gonadal cycles, and although some may argue that our data set represents N=1 for a condition, we would nevertheless like state that our results indeed showed sex-specific differences in gonadal recrudescence–regression cycles (see Figs 1–5). In all three experimental groups, mean circannual testicular rhythm measured close to 11.1 months, while mean circannual ovarian cycles significantly varied among these groups (P<0.05; Fig. 5). Further, there were sex-specific differences in the circannual periods between groups on 24L:24D and LL, but not between these groups and the group on 12L:12D (Fig. 5). Females exhibited greater variations in the period and frequency of the seasonal cycles. Circannual periods in females on 24L:24D and LL were significantly shorter and longer, respectively, than in females on 12L:12D (Fig. 5). The difference in circannual rhythms between males and females in the present experiments could be taken to suggest that the spotted munia evolved with a sex-specific annual timing strategy, with females possibly sharing a greater role in defining the reproductive season in relation to the environment.

However, the present conclusion on sex differences needs to be validated by experiments where two sexes are kept separately and perhaps individuals are isolated.

Further, the amount of light does not seem to affect the circannual programs in spotted munia, as 12L:12D (T=24) and 24L:24D (T=48) conditions had identical total hours of light and dark periods per year. However, we cannot rule out the possibility that the T=24 and T=48 light regimes had differential energy turnovers, and affected the circannual timing by modulating the speed of the circannual clocks, as proposed by Wikelski et al. (Wikelski et al., 2008). It is possible that in T=48 light regimes, the alternate days of constant light and dark periods (LL/DD) had opposing effects: LL lengthened and DD shortened the circannual period. (This might explain why the average circannual periods under 12L:12D were similar to those under LL/DD and LL.) If that were true, it could be argued that changing daily light and dark periods during the year imposes opposing effects, and thereby synchronizes circannual rhythms to a period of 12 months.

Linked with gonadal phases, spotted munia exhibited distinct cycles in phenotypic traits, as measured in molts of the wing primaries (Fig. 6). Overall, wing primary molts were relatively synchronized with gonadal cycle during the first year, but were scattered during the second year (Fig. 6). The gonadal and molt cycles drifted apart both under LL/DD and LL (Fig. 6). Thus, the phase relationship between gonad and molt cycles in resident spotted munia was not as close as in migratory stonechats, in which two cycles remained almost coupled for more than 12 years (Gwinner, 2003). Could it be that the timing of annual cycles is under the control of a more rigid circannual clock in migrants than in resident species? Also, the possibility that such differences reflect species-specific adaptation cannot be ruled out. For example, white-crowned sparrows (*Zonotrichia leucophrys*) held under 12L:12D have been shown to exhibit circannual rhythms in testicular size, but not in the post-nuptial molt of wing primaries (see Farner et al., 1983; Donham et al., 1983).

Cycle lengths of body plumage molts in spotted munia were highly variable as reflected in molt frequencies during the 28 month period (see Figs 1–4). Overall, molt had more individual variations than the gonad development, and of the two molts, body plumage molts were more frequent and variable than wing primary molts (see Figs 1–4). Furthermore, the molt patterns were more variable in females than in males, in LL than in other light conditions, and in the second cycle than in the first cycle (see Figs 1–4, 6). It appears that the experimental conditions affected body plumage molts more readily than they affected the wing primary molts. It is known that photoperiod, food and environmental stressors including captivity influence the timing, rate, frequency and extent of molt in birds (Swennen, 1977; Thompson, 1999; Thompson and Kitaysky, 2004). This seems adaptive because birds respond to the demands of their surroundings, and one of the mechanisms they might employ for metabolic adjustments changing their body feathers.

It is suggested that annual breeding cycles in spotted munia are regulated by self-sustained circannual components, which is not part of the circadian timing system, as birds continued to show circannual cycles under 24L:24D and LL that might disrupt circadian rhythmicity. This is consistent with evidence for the independence of circannual rhythm generation from circadian rhythms in several species including the spotted munia. In spotted munia, circannual rhythms in testicular size persisted in birds that were pinealectomized or held under LL at 300 lx; both treatments abolished circadian activity rhythms (Pant and Chandola-Saklani, 1993). Circannual rhythms also persist in suprachiasmatic-nucleus-lesioned ground squirrels (Zucker et al., 1983; Dark et al., 1985). We suggest that circannual rhythms are intimately involved in temporal organization of the annual cycles in vertebrates, and provide substrate for interaction with the environment. However, there can be species- and sex-specific differences in the circannual rhythms, as well as in the response of circannual rhythms to environmental regulating seasonal cycles (Ball and Ketterson, 2008). Circannual rhythms in spotted munia may be synchronized by social interactions among the members of the groups, and this needs to be investigated in a future study.

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