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*]

[1,2]DST IRHPA Center for Excellence in Biological Rhythm Research

[1]Department of Zoology, University of Lucknow, Lucknow 226 007

[2]Department of Zoology, University of Delhi, Delhi 110 007

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*Corresponding author

Email: 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 lux and D = <1 lux, for 28 months (mo) 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 the body mass. In NDL, gonadal phases in both sexes cycled with 12 mo periods. In other conditions, males cycled with similar periods of about 11 mo, but females cycled with relatively large period variations, about 10 to 13 mo. Gonadal recrudescence – regression phase was longer in males than in females and, in both sexes, in the second year as compared to 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 the frequency and pattern. 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.

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

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, since mistiming will have severe fitness consequences (Helm et al., 2009). Annual life-history stages begin and end at optimal times, and do not last for shorter or longer than the optimal durations (Gwinner, 1996a,b; Dawson, 2008; Budki et al., 2009).

Daily and seasonal rhythms are based on intrinsic timing mechanisms which integrate with predictive temporal cues like the 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 closely matching to 24 h (circadian rhythms; *circa* = about, *dien* = day) or 12 mo (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 (SCN) 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, 1992; Kumar et al., 2004; Rani et al., 2006).

Circannual rhythms have been found persisting 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, 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, 1986; 2003; Gwinner and Dittami, 1990; Cad’ee 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 (e.g., 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 (2007) from his studies on European starlings *(Sturnus vulgaris)*, which do show repeated testicular cycles under 12L:12D (Gwinner, 1981), suggest that annual gonadal cycle in starlings is the direct response to the prevailing photoperiod in the nature. However, several photoperiodic species when kept on stimulatory daylengths undergo spontaneous gonadal regression (Nicholls et al., 1988; Kumar, 1997; Dawson et al., 2001), and when kept on non-stimulatory daylengths show changes in responsiveness to long day lengths (Misra et al., 2004). Therefore, the mechanisms of photoperiodism and circannual rhythm generation appear mutually inclusive, and possibly interacting in 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 Juncos (*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 like body mass and molt. Hence, the measurements of annual gonadal cycles and associated phenotypic traits under constant conditions have been found as reliable markers of the circannual rhythms.

The goal of the current study was to investigate the involvement of circannual rhythms in 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 since they can respond to very short photoperiods as well, e.g. 1 or 3 h light per day (Chandola et al. 1975). Also, spotted munia are reported showing 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 in particular investigated (i) the persistence of circannual rhythms in gonadal phases and phenotypic traits under constant LD and light conditions, (ii) differences in the circannual rhythm characteristics between males and females, since sexes can have independent timing strategies, and (iii) the relationship of circannual rhythms to external environment, since

sexes can differ in using a temporal cue in synchronizing their circannual rhythms (Ball and Ketterson, 2008). We made few general predictions in the present study. First, if spotted munia directly responds to light, then exposure to LD or LL at the end of breeding seasons will alter the course of gonadal regression. Alternatively, birds will continue showing repeated gonadal and molt cycles regardless of the external conditions. Secondly, if the total amount (hours) of light (or dark) received in the season/ year influenced 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. Finally, male and female birds will show differences in circannual rhythm characteristics under given lighting environments, if spotted munia were 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 two years in an outdoor aviary providing natural conditions and in indoor aviaries providing controlled light (~22 lux) and temperature (18 ± 1^0C) conditions. Light at ~22 lux intensity was considerably low, as compared to that (100 to 300 lux or higher) applied in several other avian circannual studies (e.g. Bhatt and Chandola, 1985; Gwinner et al., 1995; Gwinner, 1996 a, b). We proposed that a weak light environment will probably taper the direct effects of light, if any, on 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 if 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

This study was done on adult spotted munia (*Lonchura punctulata*), a passerine finch (family: Estrildidae) measuring about 11 cm in length. Munia are widely distributed throughout the Indian subcontinent. They are a seasonal breeder with a long breeding season, extending in between June and October (Ali and Ripley, 1974; Thapliyal, 1981). Juveniles can easily be distinguished from adults (suppl. Fig. 1)

Experiment

It began in November 2007, when most birds had begun gonadal regression and the postnuptial molt. Wild caught birds were initially kept in an outdoor aviary (size = 2.95 x 1.73 x 2.21 m) for one week where they received natural light and temperature conditions (NDL). At this time, daylight and mid day temperature in Lucknow, India (26^0.55´N; 80^0.59´E) were about 10.9 h and 30^0C, respectively (suppl. Fig. 2). Acclimatized birds were brought indoors and provided with controlled light and temperature conditions in chronocubicles (size =2.2 x 1.8 x 2.8 m) located in the basement experimental facility. The experimental chronocubicles located completely underground greatly reduced the possibility of the effects of extraneous factors, if any, on the expression of annual cycles.

Birds were divided in 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 (suppl. Fig. 3). 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 watt fluorescent tube at an intensity of ~ 22 lux, obtained by covering the fluorescent tube with narrow black sheet strips. We proposed that a light intensity of ~22 lux, which was neither dim nor too bright, in constant illumination will disorganize circadian rhythms (Budki et al., unpublished obs.), but not the circannual rhythms.

Group 1 was exposed to 12 hours light: 12 hours darkness (12L:12D; L = 22 lux; D = <1 lux). 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 lux; D = <1 lux). 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 neither a constant light or dark environment (LL or DD), nor corresponded to a known natural light environment. We proposed that groups 1 and 2 will exhibit similar circannual cycles if the amount of light or dark influenced the annual timing in spotted munia. Alternatively, group 2 subjected to repeated alternating days of light and darkness (LL/DD) will exhibit responses significantly different from the group 1. Group 3 was exposed to constant light (L = 22 lux). In addition, beginning in March 2008, we kept groups of male and female birds (n = 15 each) to natural light and temperature conditions (suppl. Fig. 2) in the outdoor aviary (size = 2.95 x 1.7 x 2.2 m). At this time, birds were reproductively quiescent, and daylight and mid day temperature were about 11.6 h and 31 ^{0}C, respectively (suppl. Fig. 2). 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 multivitamin (Vimeral containing vitamin A, D3, E, and B12, marketed by Virbac Animal Health India Pvt. Ltd, Mumbai), was also given on alternate days (Singh et al., 2010). Birds also received an antibiotic (Tetracycline hydrochloride, Hoechst Roussel Vet Pvt. Ltd.) for five consecutive days every month. A few birds died during the experiment, and the mortality was especially high in LL/DD condition. 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 two or four 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 since changes in body mass over the experimental period in experimental groups did not show a regular cycle (suppl. Fig. 4).

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 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 a caliper with reference to accurate scales plotted on a graph sheet. The procedure was quickly over, and the incision was sutured by the surgical thread. An antibacterial skin ointment (Soframycin skin cream, Aventis Pharma Ltd.) 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

The wing primary feathers were scored in a range of 0-5, as described by Trivedi et al. (2006). Briefly, this was done 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-third growth, 4=newly grown feather, but still incomplete, 5= fully grown feather. Thus, each primary could have a score of 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 (2004).

The body plumage was recorded by dividing whole bird's body into twelve 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, plumage body molt score ranged in between 0 and 12 (Trivedi et al., 2006).

Data presentation and analysis

Data on testes and ovarian follicle 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 the 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 primaries molt, which indicated 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 interval on the scale of -12 to +12, respectively, until values reached minima at both the ends. The timing of the onset of molt was plotted accordingly on the axis of the time scale (-12 to +12). This represented overall distribution of the molt pattern in the year in relation to gonadal growth- regression curve in the experiment.

We analyzed data using appropriate statistics that included t-test (both unpaired and paired t-tests), one-way and two-way analysis of variances (ANOVA), and post-hoc tests. Student's paired and unpaired t-tests were used to show differences between two values as the function of time (e.g. day and night), respectively, in the same and different light conditions. Similarly, one-way analysis of variance (1-way ANOVA) followed by 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 analysis of variance compared responses of two sexes in different experiments (factor 1: sex; factor 2: light condition). Significance was taken at $p<0.05$. The statistics was done using GraphPad Prism software, version 5.0, from San Diego, CA.

RESULTS

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

(i) Testicular and molt cycles

In all experiments, males underwent two cycles of gonadal growth and regression (Fig. 1, upper panels; 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 mo in the first year and 11.1±0.3 mo in the second year. Between two successive years, there was a significant difference in the duration of recrudescence-regression phase (p= 0.0418, paired t-test), but not in the amplitude (p= 0.6541, paired t-test), of the testicular cycle. The circannual period of the testicular cycle, the interval between successive testicular peaks, was 12.3 ± 0.2 mo (Fig.5G). Along with the testicular cycle, males underwent molts in wing primary feathers and body plumage (Fig. 1, upper panels). However, molt frequencies varied from two to three cycles in wing primary feathers and from two to four cycles in the body plumage (Fig. 1, upper panels).

(ii) Ovarian and molt cycles

Females in all groups also exhibited two cycles in growth and regression of the ovarian follicles (Fig. 1; lower panel, and 5A). The follicular growth was initiated in May/June 2008. The follicles grew largest by August/September 2008 and then regressed by the following January (3/9 birds) or March (6/9 birds). A second cycle followed similar pattern in timing, duration and the amplitude (Figs. 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 mo in the second year. There was no significant difference in the duration of growth-regression phase (p = 0.1108, paired t-test) or in the amplitude (p = 0.3400, paired t-test) 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 ± 0.3mo (Fig. 5H). Similar to males, females underwent molt in their primary wing feathers and body plumage with frequency of one to three cycles in primaries and two to four cycles in body plumage (Fig. 1; lower panels).

Cycles in gonadal phases and phenotypic traits in artificial light (12L:12D, LL/DD and LL) and temperature (18±1^{0}C) conditions

(i) 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 mo in group 1 on 12L:12D, 3.8 ± 0.3 mo in group 2 on 24L:24D, and 3.4 ± 0.2 mo in group 3 on LL. The next growth phase was initiated after 2.1 ± 0.3 mo, 1.3 ± 0.3 mo and 1.3 ± 0.3 mo in groups 1, 2 and 3, respectively. Irrespective of the lighting conditions, all groups underwent two testicular cycles (Figs. 2-4, upper panels; Figs. 5 B-D). However, the duration of testicular growth-regression phase in groups on 12L:12D and 24L:24D was significantly shorter ($p < 0.001$, paired t-test) in the first year than in the second year (first year: 12L:12D - 9.0 ± 0.4 mo, 24L:24D - 9.0 ± 0.8 mo; second year: 12L:12D - 12.4 ± 0.4 mo, 24L:24D - 13.3 ± 0.5 mo). In group 3 on LL, however, the duration of testicular growth-regression phase did not significantly differ ($p = 0.2085$, paired t-test) between the first (11.2 ± 0.3 mo) and second years (11.9 ± 0.5 mo). A similar difference was found in the testicular growth maxima between the first and second years in groups on 12L:12D ($p = 0.0004$, paired t-test) and 24L:24D ($p = 0.0117$, paired t-test), but not in the group on LL ($p = 0.7538$, t-test) (Fig. 5E).

Among three experimental groups, a significant difference occurred in both cycles in peak testicular response (Fig. 5E; first year: $F_{2,29} = 3.425$, $p = 0.0540$, second year: $F_{2,29} = 3.979$, $p = 0.0297$; 1-way ANOVA), but not in the circannual periods (Fig. 5G; cf. $F_{2,29} = 0.0057$, $p = 0.9943$, 1-way ANOVA). The latter, measured as intervals between two successive peaks were (Fig. 5G): 11.1±0.3 mo (group 1), 11.1 ± 0.5 mo (group 2) and 11.1 ± 0.4 mo (group 3). Interestingly, the circannual period of all three experimental groups was significantly different from the "annual" period of the group on NDL (NDL vs 12L:12D: $p = 0.0135$; NDL vs 24L:24D: $p = 0.0233$ and NDL vs LL: $p = 0.0261$, unpaired t-test).

All groups irrespective of the light conditions underwent molt cycles in wing primary feathers but with frequency varying from one to four cycles (Figs. 2-4, upper panel). Some birds in each group were molting at the beginning of the experiment, and hence three treatment groups did not differ from each other in the onset of molt ($F_{2,17} = 0.7008$, $p = 0.5100$, 1-way ANOVA). For example, in group 1, 6/14 birds were molting at the beginning and eight birds began molting after 4.0 ± 0.6 mo. Overall, there was no difference in the frequency ($F_{2,29} = 2.891$, $p = 0.0716$; 1-way ANOVA) or period ($F_{2,28} = 2.765$, $p = 0.0802$, 1-way ANOVA) of wing primary molts among three experimental groups. But, group comparisons revealed a significant increase in molt cycle in the group on LL than in the

group on 12L:12D (p = 0.05, unpaired t-test), and a shorter molt duration in group on LL/DD than in the group on LL (p = 0.05, unpaired t-test). Eight, five and one birds underwent molt in primaries for two (8.1 ± 1.1 mo intervals), three (8.0 ± 0.5 mo intervals) and four (6.0 ± 0.6 mo intervals) times, respectively. The pattern also varied from partial to complete molt among individuals of the group 1. In group 2, 2/7 birds were in molt at the beginning of the experiment and remaining five individuals underwent first molt after 5.4 ±1.4 mo. In total, 6/7 birds had three molts, with second and third molts at the intervals of 7.8 ± 1.1 mo and 7.0 ± 1.0 mo, respectively. The remaining one individual showed molt after seven months. Group 3 of eleven birds had two (n = 2), three (n = 5) and four (n = 4) molt cycles. Four birds were already in molt phase when the experiment began. Remaining seven individuals began first molt after 3.7 ± 1.1 mo. The subsequent molt occurred after 10.5 ± 0.5 mo (n =2), 12.3 ± 0.41mo (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, upper panels; Figs. 5F-H). In all groups, all, but one, birds were in molt at the beginning of experiment; one individual started molting after four (group 1), ten (group 2) or two months (group 3). There was no significant difference among three groups in the number of body molt cycles ($F_{2,29}$ = 1.701, p = 0.2003, 1-way ANOVA) or the duration of molt cycle ($F_{2,29}$ = 2.698, p = 0.0842, 1-way ANOVA). In group 1 on 12L:12D, nine, three and two individuals underwent three (11.1 ± 0.8 mo intervals), four (8.7 ± 0.5 mo intervals) and five (6.1 ± 0.4 mo intervals) body plumage molts, respectively. Similarly, there were two (n = 1, 8.0 mo intervals), three (n = 2; 7.5 ± 1.0 mo intervals), four (n = 3; 7.7 ± 0.2 mo intervals) and five (n = 1; 6.8 ± 0.3 mo intervals) body plumage molts in group 2 on 24L:24D. Group 3 birds also exhibited three (n = 4; 9.4 ± 0.9 mo), four (n = 3; 7.9 ± 0.6 mo), five (n = 2; 6.4 ± 0.6 mo) and six (n = 2; 5.7 ± 0.3 mo) molts during their exposure to LL. The intervals in body molts differed among three groups ($F_{2,29}$ = 3.944, p < 0.05, 1-way ANOVA). The interval period in the body molt in group on 12:12D was significantly longer than in the group on 24L:24D (LL/DD) (p <0.05, t-test), but not in the group on LL.

(ii) Ovarian and molt cycles

Similar to males, females in all experimental groups had fully regressed ovary by the end of 5.0 ±0.4 mo (group1, 12L:12D), 2.3 ± 0.3 mo (group 2, 24L:24D) or 2.7 ± 0.4 mo (group 3, LL). A subsequent follicular growth phase was initiated in these groups after 3.6 ±

0.4 mo of 12L:12D (group 1), 5.8 ± 0.8 mo of 24L:24D (group 2) or 4.5 ± 0.4 mo of LL (group 3). During the experiment, all groups exhibited two ovarian cycles with follicular growth - regression phase of 6.3 ± 0.4 mo (group 1), 6.5 ± 0.6 mo (group 2) or 8.5 ± 0.6 mo (group 3) in the first year, and 10.8 ± 0.6 mo (group 1), 12.5 ± 0.9 mo (group 2) or 9.1 ± 0.8 mo (group 3) in the second year (Figs. 2-4 lower panel; Figs. 5 B-D). Thus, growth - regression phase was significantly longer ($p<0.01$, paired t-test) in the second cycle in groups 1 and 2, but not in the group 3 ($p = 0.5896$, paired t-test). When compared to controls (NDL), the growth-regression phase was significantly shorter in the first cycle in groups 1 and 2, but not in the group 3 ($F_{3,36}=10.31$, $p<0.0001$, 1-way ANOVA). Interestingly, in the second cycle, the growth-regression phase was significantly longer in group 2 as compared to other groups including the NDL ($F_{3,36} = 3.186$, $p = 0.0353$, 1-way ANOVA). A similar difference was found in the follicular growth maxima among three experimental groups with significantly smaller ($p< 0.01$, paired t-test, Fig. 5F) follicle in the first cycle as compared to the second cycle. When compared with response in NDL, there was a significantly smaller follicular growth among experimental groups in the first cycle ($F_{3,36} = 9.219$, $p<0.0001$, 1-way ANOVA) but not in the second cycle ($F_{3,36} = 2.401$, $p=0.0837$, 1-way ANOVA). Further, the periods of cyclicity, measured as the interval between two peaks, were 11.8 ± 0.4 mo (group 1, 12L:12D), 9.8 ± 0.6 mo (group 2, 24L:24D) and 13.5 ± 0.8 mo (group 3, LL) (Fig. 5H). Thus, the circannual periods of groups 2 and 3, but not group1, were significantly different from that of the NDL group ($F_{3,36} = 4.705$ $p = 0.0072$, 1-way ANOVA; Fig. 5H).

Like males, females showed no difference among the three treatment groups in the onset of the wing primary molts ($F_{2,21} = 2.178$, $p = 0.1381$, 1-way ANOVA). However, group on 12L:12D molted slowly than the group on 24L:24D (LL/DD; $p = 0.05$, unpaired t-test). All birds underwent molts in wing primaries irrespective of the light condition (Figs. 2-4, lower panels), although with varying pattern (partial to complete) and frequency (1 – 4 cycles; Figs. 2-4 lower panels). The number of wing primary molts over the duration of study did significantly differ among the treatment groups ($F_{2,28} = 2.030$, $p = 0.1502$, 1-way ANOVA). A few birds in each group (8/12 in group 1, 3/6 in group 2 and 7/13 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 mo of exposure to 12L:12D. The interval between two molts was 11.6 ± 1.0 mo. Similarly, group 2 molted once (n =1), twice (n = 2), thrice (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 mo of exposure to

24L:24D. The subsequent molt occurred after 10.0 ± 2.0 mo, 9.0 ± 0.5 mo and 6.7 ± 0.9 mo in birds showing two, three and four molts, respectively. Group 3 also molted once (n =3), twice (n = 4), thrice (n = 2) or four times (n = 4). Birds that were not in molt at the beginning, initiated molt after 5.8 ± 1.6 mo of LL exposure. Subsequent molt occurred after 9.5 ± 1.2 mo, 7.0 ± 0.5 mo and 8.5 ± 0.3 mo 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 ($F_{2,22}$ = 4.341, p = 0.0258, 1-way ANOVA). In particular, group on LL had significantly shorter period than the group on 12L:12D (p <0.05, t-test).

A similar pattern and frequency was found in the body plumage molt (Fig. 2-4, lower panel). Like wing primaries, many birds (10/12 birds in group 1, 5/6 birds in group 2, 7/13 birds in group 3) were in molt of their body plumage at the beginning of the experiment. The onset of the first body molt appeared to vary significantly ($F_{2,28}$ = 4.345, p = 0.0227, 1-way ANOVA). In particular, group on LL molted faster than the group on 12:12D (p <0.05, unpaired t-test) Birds that were not in molt, initiated molt after two months (groups 1 and 2) or 3.2 ± 0.4 mo (group 3). Also, most birds in all groups had partial body plumage molt with 2-7 cycles, as indicated above (Figs. 2-4, lower panels). In 12L:12D, birds molted twice (n = 1), thrice (n = 4), four (n = 5) or five (n = 2) times. The subsequent molts occurred after 9.0 ± 0.0 mo, 10.8± 0.3 mo, 7.6 ± 0.2 mo and 6.0± 0.3 mo in birds molting for two, three, four and five times, respectively, during the experiment. Similarly, in 24L:24D, birds molted twice (n = 2), four (n = 2), five (n =1) and six (n = 1) times at intervals of 7.5 ± 0.5 mo, 7.7± 0.0 mo, 5.8 ±1.0 mo and 5.0 ± 0.7 mo, respectively. In group on LL also, birds molted twice (n = 2), thrice (n = 1), four (n = 1), five (n = 5), six (n = 3) and seven (n = 1) times at intervals of 8.0 ± 0.0 mo, 10.8 ± 0.3 mo, 9.0 ± 2.9 mo, and 5.7 ± 0.2 mo, 5.1 ± 0.2 mo and 5.0 ± 0.5 mo, respectively. In spite of individual differences, the frequency of body molt was significantly different among three groups ($F_{2,28}$ = 2.563, p = 0.0950, 1-way ANOVA) with a particular difference between the groups on 12L:12D and LL (p <0.05, unpaired t-test); the latter groups had more molts. The period of molt cycles also differed significantly in group 3 than in group 1 ($F_{2,28}$ = 3.627, p = 0.0398, 1-way ANOVA) indicating that birds molted faster and at shorter intervals than other groups.

Among experimental groups, in spite of the similar gonadal cycle, both sexes differed in the duration of growth-regression phase, amplitude of cycle defined by testicular and follicular maxima, and in the periods over two cycles. Two way ANOVA revealed a significant effect of sex (factor 1) and light condition (factor 2) as well as interaction between

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 1x2- $F_{2,57} = 0.3499$, $p = 0.7062$; second cycle: factor 1- $F_{1,57} = 7.670$, $p = 0.0076$, factor 2- $F_{2,57} = 8.222$, $p = 0.0007$; factors 1x2- $F_{2,57} = 0.4401$, $p = 0.6461$) and circannual period (factor 1- $F_{1,57} = 1.478$, $p = 0.2291$; factor 2- $F_{2,57} = 4.123$, $p = 0.0210$; factors 1x2- $F_{2,57} = 4.405$, $p = 0.0166$)..

Two way ANOVA analyzed the effects of sex and light condition. The frequency of wing primary molt was significantly influenced by the sex ($F_{1,57} = 5.377$, $p = 0.0240$), light condition ($F_{2,57} = 4.449$, $p = 0.0160$) as well as by interaction between them ($F_{2,57} = 0.3663$, $p = 0.6949$). In general, females on LL underwent molt more frequently than the males on LL. Further, photoperiod ($F_{2,50} = 4.542$, $p = 0.0154$), but not the sex ($F_{1,50} = 0.05126$, $p = 0.8218$) had significant effect on the period of 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- $F_{2,57} = 6.627$) of body molts in this study.

Relationship between breeding and wing primaries molt cycles

Figure 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 mo (NDL), 9.9 ± 0.2 mo (12L:12D), 10.8 ± 0.6 mo (24L:24D) or 10.9 ± 0.5 mo (LL). Subsequent breeding season in these groups lasted for 12.0 ± 0.3 mo (NDL), 12.0 ± 0.4 mo (12L:12D), 11.4 ± 0.6 mo (24L:24D) or 11.5 ± 0.5 mo (LL). Similarly, breeding periods in females lasted for 9.9 ± 0.2 mo (NDL), 6.8 ± 0.4 mo (12L:12D), 6.5 ± 0.6 mo (24L:24D) and 8.2 ± 0.5 mo (LL) in the first cycle, and 12.0 ± 0.2 mo (NDL), 9.7 ± 0.5 mo (12L:12D), 11.8 ± 0.5 mo (24L:24D) and 9.7 ± 0.5 mo (LL) in the second cycle. Overall, the breeding seasons appeared to range from 6 to 12 months, with second gonadal growth – regression phase being longer than the first one.

Also, plotted in Figure 6 is the distribution of wing primaries molts in different groups in relation to gonadal cycle. In NDL, wing primaries molt coincided with gonadal regression (Figs. 6A, E). This pattern was nearly maintained in 12L:12D, but got increasingly lost in 24L:24D and LL (Figs. 6B, F). When compared, the molt patterns were scattered and

430 more dissociated in females than in males, and in the second cycle as compared to the first
431 cycle (Fig. 6).

432

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 the consequence of the prevailing photoperiods, since 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 (cf. Figs. 1-5). These results are in agreement with previously reported circannual rhythm in testicular cycle, but not in body mass, in the spotted munia (see Bhatt and Chandola, 1985). Unlike those reported by 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 underwent seasonal cycle in body mass (suppl. Fig. 4).

The present study differs from previous studies on circannual rhythm in songbirds (Gwinner, 1986), including the spotted munia (Chandola et al., 1982; Bhatt and Chandola, 1985). In particular, we included both sexes in the same sex ratio (1:1), whereas the study of Bhatt and Chandola (1985) was done only on males. Also, we simultaneously measured circannual cycles in groups kept on both constant periodic (12L:12D, 24L:24D) and aperiodic (LL) light conditions, which addressed on the causal effect of the photoperiod, if any, on circannual rhythm generation. Further, we housed un-caged birds in chronocubicles (size = 2.2 m x 1.8 m 2.8 m); so, each individual shared approximately 65 folds more space in volume as compared to that they had in the study of Bhatt and Chandola (1985), in which birds were housed in groups of 5 or 6 birds per cage (size = 25 x 48 x 28 cm). This could be an important factor since social crowding is known to affect reproduction at various levels, including the development of reproductive endocrine organs (Rahe et al., 1986). The onset of breeding was delayed among crowded ptarmigans *(Lagopus lagopus)* subjected to stimulatory increasing daylengths (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 lux light intensity differed in the reproductive performance, but not in the duration of reproductive phases or circannual periods (Gwinner et al., 1995).

An obvious advantage of the circannual rhythms in breeding cycles is to prepare two sexes for synchronized reproductive processes. However, there can be sex 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 lacked a circannual rhythm in breeding cycle, then the reported circannual rhythm in testicular cycle (Bhatt and Chandola, 1985) may have much less of the consequence unless it is assumed that males directly drive the female sexual state. This could be a possibility for final stages of ovum maturation (called exponential growth phase), in order to maximise 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 is unclear at the present, we have observed repeated gonadal cycles in spotted munia with circannual periods within the narrow range of ± 3.0 mo among individuals of both male and female groups held under constant photoperiods (T=24, 12L:12D; T=48, 24L:24D) and LL (Fig. 5). The circannual periods in testicular and ovarian cycle were close to 11 mo. and 10 -13 mo, respectively (Fig. 5). A 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 has been reported in few other bird species (Schwab, 1971; Schwab and Lott, 1971; Gwinner, 1975, 1981, 1986; Gwinner and Dittami, 1990; Cad'ee 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 the 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 (*Saxicola torquata;* Helm et al., 2009). When *S. t. 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 most likely situation 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 (cf. Figs. 1-4). It is not surprising since 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). The 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, the 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 some could argue that our data set represent n= 1 for a condition, we would nevertheless like state that our results indeed showed difference between two sexes in gonadal recrudescence-regression cycles (cf. Figs. 1-5). In all three experimental groups, mean circannual testicular rhythm measured close to 11.1 mo, while mean circannual ovarian cycles significantly varied among these groups (p<0.05, Fig. 5). Further, there were sex differences in the circannual periods between groups on 24L:24D and LL, but not 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 on 12L:12D (Fig. 5). The difference in circannual rhythms between males and females in present experiments could be taken to suggest that spotted munia is 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 were kept separately and perhaps individuals were isolated.

Further, the amount of light does not seem to affect the circannual programs in spotted munia, since 12L:12D (T=24) and 24L:24D (T=48) conditions had identical total hours of light and dark periods per year. However, we do not rule out if 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. (2008). Could it be 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 circannual periods under 12L:12D almost averaged to that under LL/DD and LL. If that

were true, it could be argued that changing daily light and dark periods in the year impose opposing effects, and thereby synchronize circannual rhythms to a period of 12 mo.

Linked with gonadal phases, spotted munia exhibited distinct cycles in phenotypic traits, as measured in molts of the wing primaries (Fig. 6). Overall, wing primaries 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 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 as compared to 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 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 mo period (cf. Fig. 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 the wing primaries molts (cf. 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 (cf. Figs. 1-4; Fig. 6). It appears that the experimental conditions affected body plumage molts more readily than they affected the wing primaries 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 since birds require responding to demands of their surroundings, and one of the mechanisms they might employ for metabolic adjustments is by changing their body feathers.

It is suggested that annual breeding cycles in spotted munia is regulated by self-sustained circannual components, which is not the part of the circadian timing system, since birds continued showing circannual cycles in 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 lux; both treatments abolished circadian activity rhythms (Pant and

565 Chandola-Saklani, 1992). Circannual rhythms also persist in SCN-lesioned ground squirrels
566 (Zucker et al., 1983; Dark et al., 1985). We would suggest that circannual rhythms are
567 intimately involved in temporal organization of the annual cycles in vertebrates, and provide
568 substrate for interaction with the environment. However, there can be species and sex
569 differences in the circannual rhythms, as well as in the response of circannual rhythms to
570 environmental regulating seasonal cycles (Ball and Ketterson, 2008). Possibly, circannual
571 rhythms in spotted munia are synchronized by social interactions among the members of the
572 groups, and this needs to be investigated in a future study.

573

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757

758

759 FIGURE LEGENDS

760 Fig. 1. Changes in testis volume and diameter of the largest follicle (open circles, left axis)
761 and the occurrence of molt in wing primaries (solid circles, right axis)and body plumage
762 (solid horizontal bars along the x-axis) in spotted munia (n = 10 males, top two panels; 9
763 females, bottom two panels) kept in an outdoor aviary at Lucknow, India (26^0,55'N;
764 80^0.59'E) for 25 months, March 2008 to March 2010. All the birds receiving natural day
765 length and temperature cycles showed repeated cycles of gonadal recrudescence – regression
766 and molts. Asterisks on gonadal cycle indicate the times of peak gonadal growth in an
767 individual bird.

768 Fig. 2. Changes in testis volume and diameter of the largest follicle (open circles, left axis)
769 and the occurrence of molt in wing primaries (solid circles, right axis) and body plumage
770 (solid horizontal bars along the x-axis). Fourteen males (top two panels) and twelve females
771 (bottom two panels) spotted munia were moved indoors in experimental rooms, located
772 underground) to a photoperiodic regime of 12 h light: 12 h darkness (12L:12D; L = 22±1 lux;
773 D = <1 lux) at 18±1^{0}C temperature. Data for only ten individuals of each sex are shown;
774 remaining birds responded similarly. All the birds showed repeated cycles of gonadal
775 recrudescence – regression and molts during 28 months of exposure to 12L:12D, beginning
776 from November 2007. Asterisks on gonadal cycle indicate the times of peak gonadal growth
777 in an individual bird.

778 Fig. 3. Changes in testis volume and diameter of the largest follicle (open circles, left axis)
779 and the occurrence of molt in wing primaries (solid circles, right axis) and body plumage
780 (solid horizontal bars along the x-axis). Seven males (top two panels) and six females
781 (bottom two panels) spotted munia were moved indoors in experimental rooms, located
782 underground) to a photoperiodic regime of 24 h light: 24 h darkness (24L:24D; L = 22±1 lux;
783 D = <1 lux) at 18±1^{0}C temperature. Thus, these birds received alternating days of light and
784 darkness. All the birds showed repeated cycles of gonadal recrudescence – regression and
785 molts during 28 months of exposure to 24L:24D, beginning from November 2007, albeit with
786 an attenuated amplitude, especially in females. Asterisks on gonadal cycle indicate the peak
787 gonadal growth in an individual bird.

Fig. 4. Changes in testis volume and diameter of the largest follicle (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 males (top two panels) and thirteen females (bottom two panels) spotted munia were moved indoors in experimental rooms, located underground, to a constant light regime (LL, L = 22±1 lux) at 18±1^{0}C temperature. Data for only ten individuals of each sex are shown; remaining birds responded similarly. All the birds showed repeated cycles of gonadal recrudescence – regression and molts during 28 months of exposure to LL, beginning from 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 on gonadal cycle indicate the peak gonadal growth in an individual bird.

Fig. 5. The recrudescence - regression cycle in testis (solid circles, left axis) and ovary (open circles, right axis) in spotted munia subjected to natural conditions (NDL, A) and photoperiodic regimes (12L:12D, B; 24L:24D, C; LL, D) for the period of 25 (NDL) or 28 months, ; group 3) from November 2007 to February 2010. The data plotted in this figure is the moving average of the group. The peak response for both cycles has been plotted as Ist and IInd peaks in the figure 5E (testis volume) and 5F (follicle diameter). The intervals between two peaks gave the period of annual/ circannual cycle, plotted as mean ± SE in 5G (male) and 5H (female). The annual/ circannual periods are as follows: NDL - 12.3±0.2 mo (males), 12.4±0.36 mo (females); 12L:12D - 11.14±0.33 mo (males), 11.8±0.4 mo (females); 24L:24D - 11.1±0.46 mo (males), 9.83±0.6 mo (females); LL - was 11.1±0.4 mo (males), 13.5±0.8 mo (females). Bars with identical alphabets - no difference; bars with different alphabets – $p< 0.05$. Asterisks indicate significance of difference between two peaks ($p< 0.05$)

Fig. 6. The relationship between gonadal cycles and wing primaries 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 successive twelve months before and after the time of peak responses were accorded values from -12 to +12, respectively. The frequency of the onset of wing primaries molts in individual birdswas plotted on the timescale by different open symbols (first molt, inverted triangle; second molt, diamond; third molt, triangle; fourth molt, circle). The number of each symbol denotes the

820 number of birds in molt, but at several places these symbols overlap which is indicated by a
821 number.

822

Figure 1

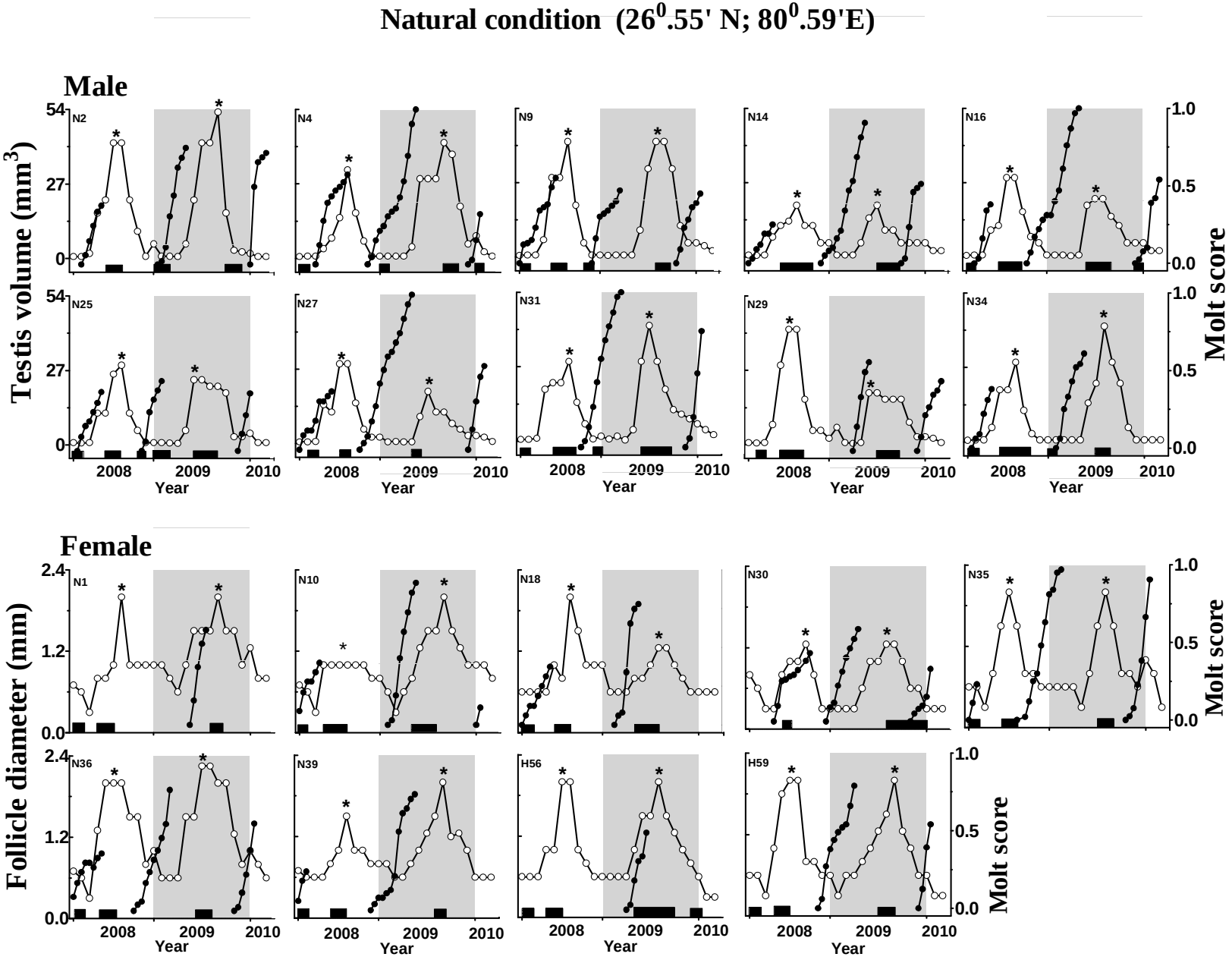

Figure 2

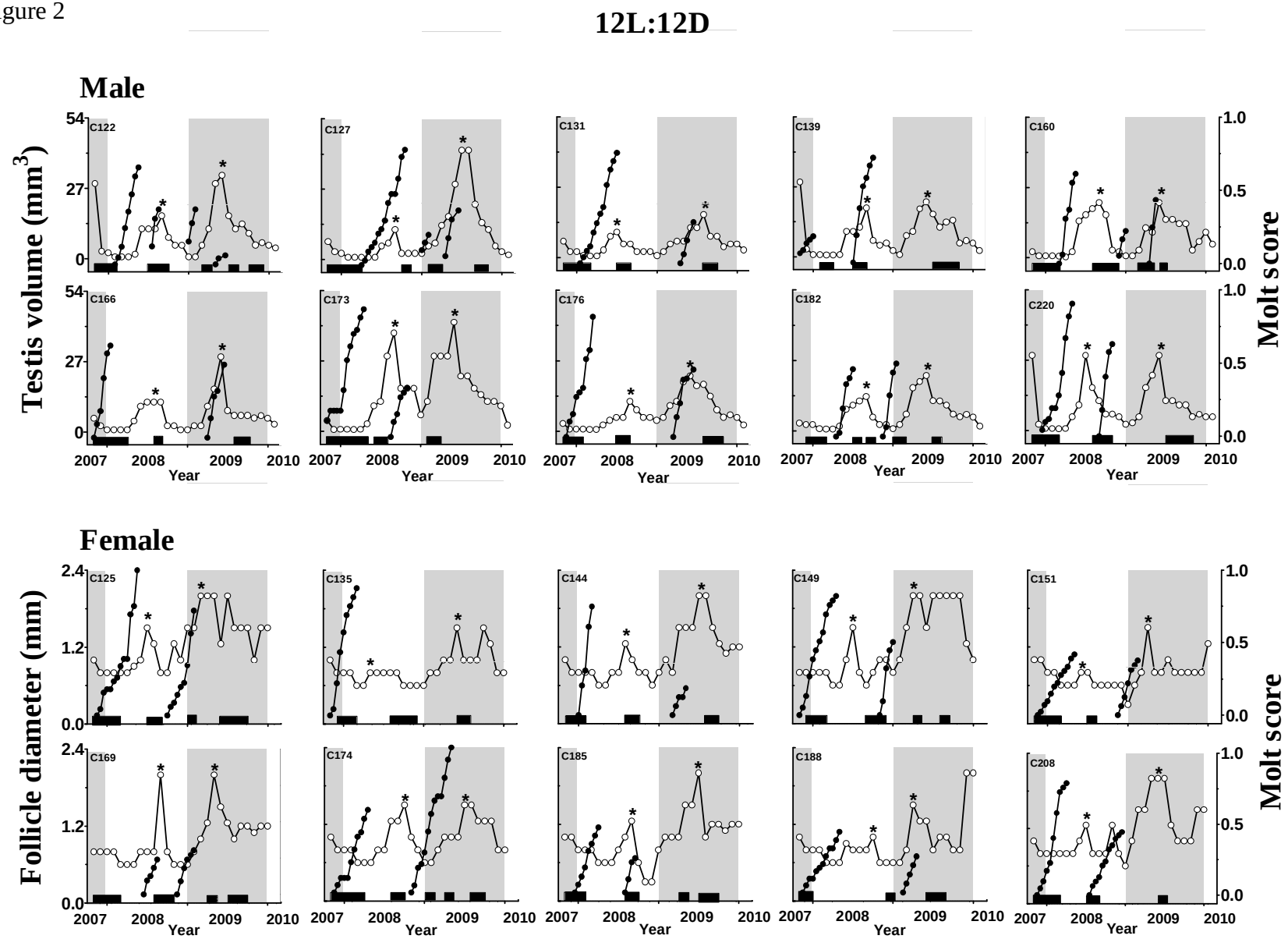

Figure 3

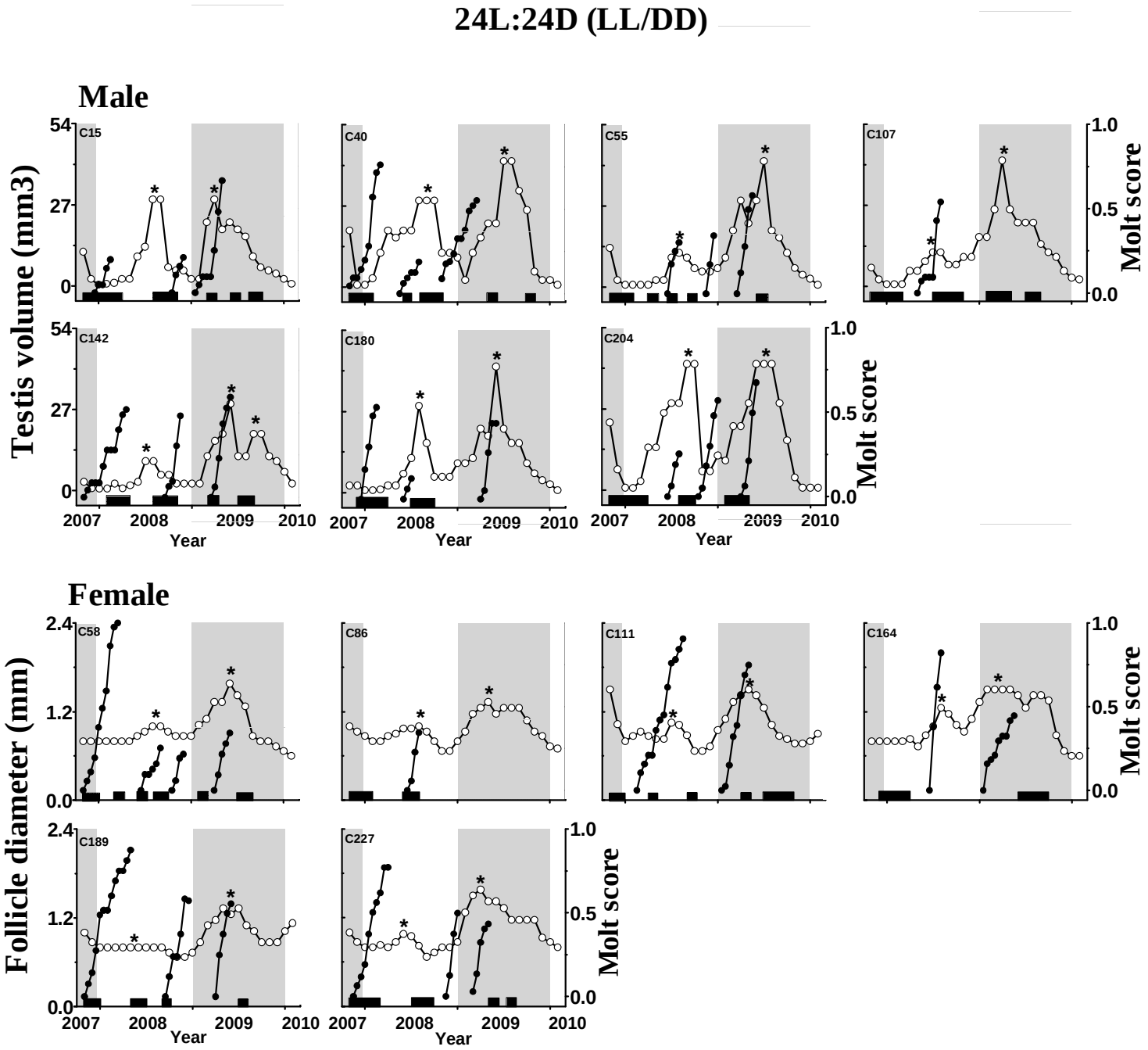

Figure 4

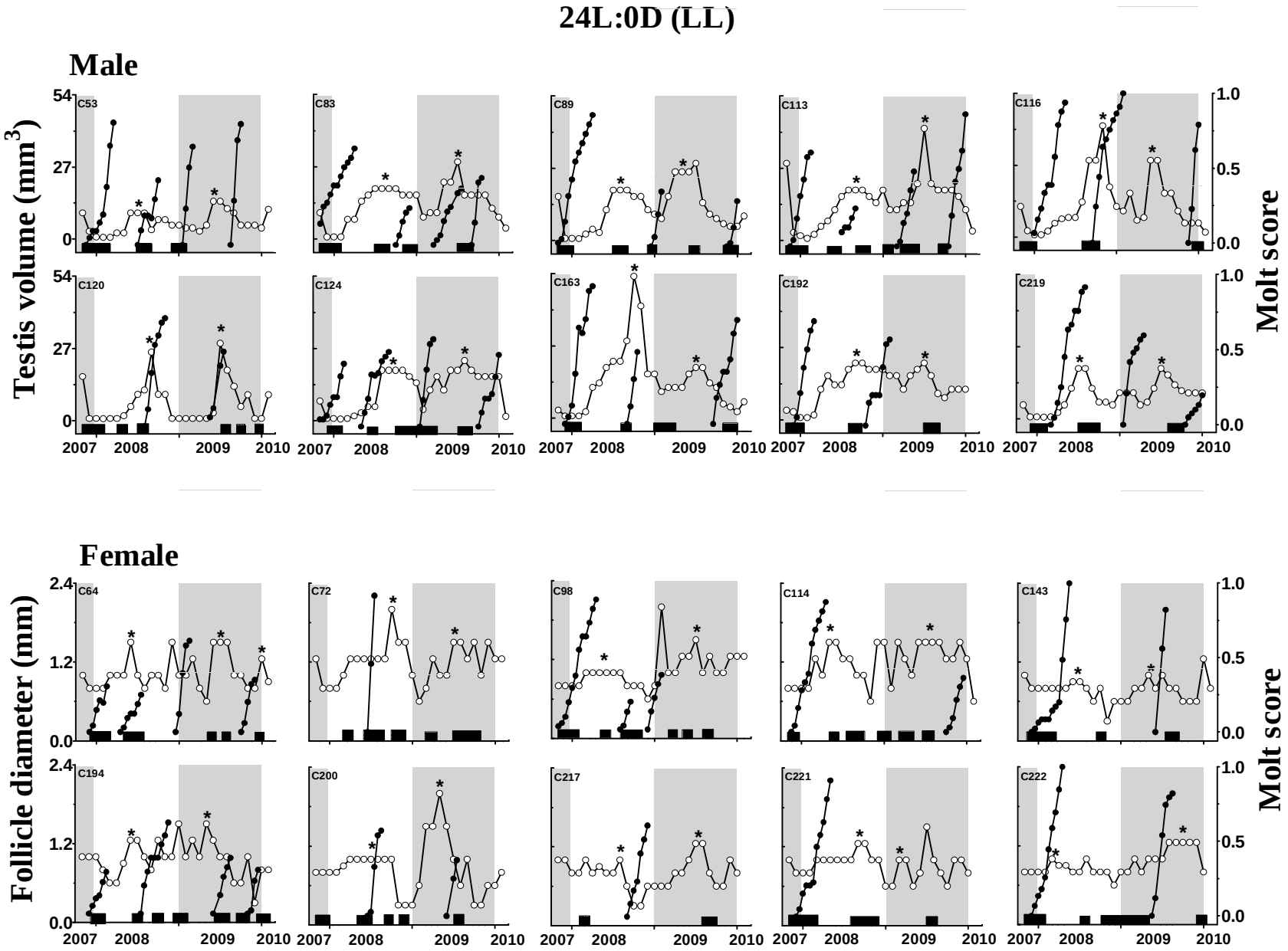

Figure 5

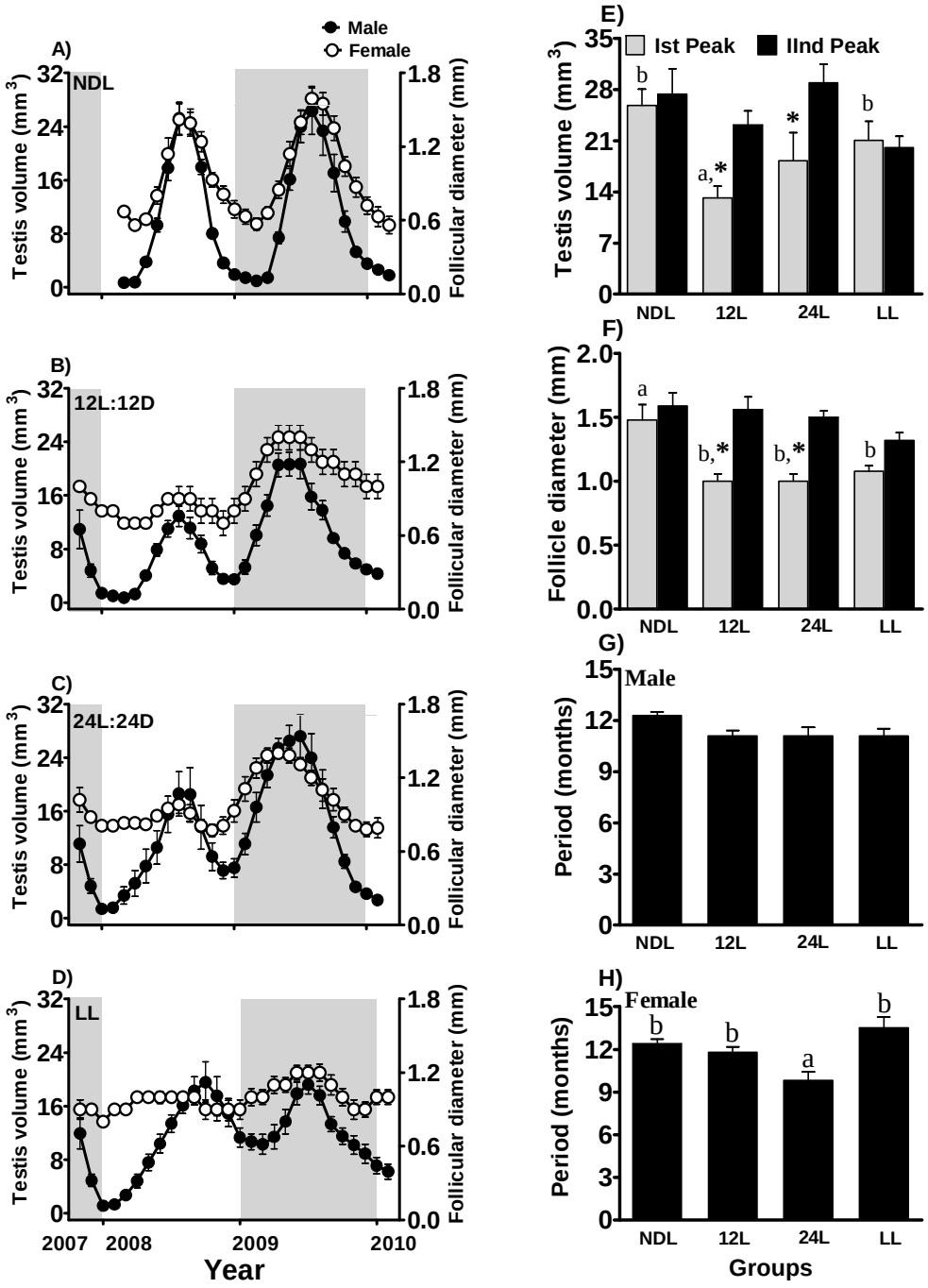

Figure 6