Born without night: The consequence of the no-night environment on reproductive performance in diurnal zebra finches

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Summary statement

Loss of night negatively affects reproductive performance of adults born and raised in normal day-night, with a more severe effect when adult in animals born in such environment since birth.

Abstract

We investigated the consequence of no night environment (constant light, LL) on reproductive performance in zebra finches in the parent (P) and subsequent F1 generation. As a measure of the overall effects on the metabolic reproductive health, we monitored daily activity behaviour, recorded song and cheek patch size in males, and measured body size and hormone levels. As compared to controls under 12 h light: 12 h darkness (12:12 h LD), both P and F1 pairs showed a compromised reproductive success, as evidenced by fewer fledglings and viable offsprings with longer fledging durations, and increased offspring mortality with successive three clutches under LL. The overall negative effect of the no-night environment was increased in the F1generation. As compared to P pairs, F1pairs had more failed nesting and breeding attempts, took longer to initiate reproduction, incubated fewer eggs, produced fewer viable offspring with longer fledging duration, and showed increased

offspring mortality. Consistent with negative reproductive effects, P males showed significant changes in the motif duration and other spectral features of song, and both F1 and F2 males copied poorly the song of their parent under LL. Plasma corticosterone and sex hormones (testosterone in males and estradiol in females) levels were significantly lower under LL. Daily plasma melatonin rhythm persisted but with a reduced amplitude under LL. These results demonstrate the importance of night in reproduction in a continuously breeding diurnal species, and give insights into the possible impact on physiology of animals whose surrounding environment is consistently losing the darkness of night.

1. Introduction

Synchronization of the endogenous biological clock with cyclic 24-h light/dark (LD) environment is adaptive. The developing biological clock becomes responsive at an early stage in life, and a cyclic LD environment shapes its functions (Ohta et al., 2006). This is exhibited in many temporally compartmentalized behavioural and physiological activities into the day or night. For example, a diurnal species is active during the day and sleeps (or is inactive) at night; conversely, a nocturnal species in active at night and sleeps (or is inactive) during the day.

Disruption in the cyclic feature of LD environment, for example via artificial light at night (ALAN) can have disruptive effects on the clock-controlled functions. In fact, ALAN has been to shown to have negative effects on daily behaviour (activity-rest, sleep-wake and feeding-no feeding) and physiology (hormone secretions, enzyme activities, metabolism), and higher brain functions like the cognitive performance and personality (Benca et al., 2009; Rotics et al., 2011; Dominoni et al., 2016; Attia et al., 2019). A complete absence of the night (i.e. exposure to constant light, LL) also causes both acute and long-term disruptive effects on the developing biological clock of mice (Ohta et al., 2006). The negative effects are so widespread that ALAN is referred to as the light pollution (Kurvers and Hoelker, 2015; Dominoni and Nelson, 2018; Falcón et al., 2020). It is alarming that a large proportion of the world's population lives under the light polluted sky (Falchi et al. 2016).

Among birds, ALAN has been shown to affect the circadian timekeeping. This is particularly evidenced by ALAN-induced effects on mRNA oscillations of genes forming the core transcriptional-translational feedback loop that generates circadian time (Batra et al., 2019, 2020; Renthlei and Trivedi, 2019; Prabhat et al., 2020), as well as on downstream

clock-controlled functions such as the daily cycle of sleep-wake, activity, foraging and singing activities and melatonin secretion (Miller, 2006; Dominoni et al., 2013a; Da Silva et al., 2014; Dominoni and Partecke, 2015;Raap et al., 2015; Silva et al., 2017; Ulgezen et al., 2019; Batra et al., 2020; Kuppart et al., 2020). ALAN also affects the metabolism (Batra et al.2020), immune functions (Saini et al. 2019; Ziegler et al. 2021), mood states, personality and cognitive performance, induces depressive like symptoms (Taufique and Kumar,2016; Jha and Kumar, 2017), and affects reproduction (Kempenaers et al., 2010; Dominoni et al., 2013b; Dominoni et al., 2020). The effects can be long-lasting, and may even show up in the subsequent generation (Jha and Kumar, 2017).

The question that we have addressed is whether the exposure LL (a no-night environment) will have negative effects on reproduction of the adult parent, and of offsprings born and raised under LL. We investigated this using zebra finches (*Taeniopygia guttata*), a diurnal songbird that breeds successfully year-round in captivity, and responds to changes in both the light and food environment (Jha and Kumar, 2017; Mishra and Kumar, 2019). This species can be a natural non-model experimental system to assess the consequence of chronic effects of the perturbation in light environment on reproduction and on fitness and survival of parents and offsprings (Naguib and Gil, 2005; Naguib et al., 2006; Tschirren et al., 2009; Griffith and Buchanan, 2010; Brust et al., 2014; Gaston et al., 2015; Farine et al., 2015; Jha and Kumar, 2017; Moaraf et al., 2020). Here, we assessed the effects of a long-term LL exposure on reproduction associated responses between three successive clutches of the parent (P) pairs, and changes since birth in F1 and F2 progenies. In particular, we measured the LL-induced effect on the reproduction associated changes and reproductive success of adult, and morphometric changes in the offspring. For example, we considered changes in the phenotype and body size indicative of the overall body condition, and the plasma levels of testosterone and male cheek patch ratio in male and estradiol in levels as indices of the reproductive health of the animal (Williams, 1999; Crino et al., 2014; Mishra et al., 2020). We also examined the characteristics of male song in both F1 and F2 progenies to assess the LL-induced effects on a reproduction-linked trait, controlled by the hippocampus (Clayton and Krebs, 1994) and song-control nuclei (Buchanan et al., 2003), which are susceptible regions of the developing brain to an external influence. Along with, we monitored daily activity pattern and plasma melatonin levels to assess both direct and circadian clock effects, and measured plasma corticosterone levels in the morning and evening as measure of stress under LL.

2. Methods

2.1. Birds and experimental setup

All the procedures strictly adhered to the guidelines for the use of animals where the research was conducted, and were approved by the Institutional Animal Ethics Committee (IAEC) of the Department of Zoology, University of Delhi, India (Protocol No.: DU/ ZOOL/IAEC-R/2015/01). The study was done on zebra finches from our colony-bred stock maintained under 12 h light: 12 h darkness (12:12 h LD) and 24 ± 2 ^{o}C temperature. The experiments used 4 months old zebra finches, which are considered sexually adult (Roper and Zann, 2006). We housed male and female birds in same sex cages (N = 5 per cage, size = 42 x 30 x 54 cm) and kept them in separate rooms so that there was no visual and acoustic contact between birds of the opposite sex. This acclimated birds to a caged condition, and broke pair (male-female) bond that they might have established in breeding aviary. Females that laid eggs during the 2-week acclimation period were excluded from the experiment. Both, the light and temperature conditions were kept identical to those maintained in the breeding aviary.

All birds were weighed on a top pan balance to an accuracy of 0.1 g, and the size of gonads was measured by unilateral laparotomy (see below) to assess the gonadal state; the size of gonad reflects the summation of the gametogenic activity over a period of time (Lofts, 1975). Birds were fed *ad libitum* on *Setaria italica* seeds (main food), and the egg white was given as the protein supplement; both food and water were replenished only during the light hours.

2.2. Manipulation of the light condition

At the beginning of the experiment, all birds had similar body mass (12.7 ± 0.8 g, N = 64) and were in similar gonadal state (testis volume = 6.2 ± 0.4 mm^3; ovarian follicle diameter = 1.3 ± 0.2 mm, N = 32 each; testis volume was calculated using the formula $4/3\pi ab^2$, where 'a' and 'b' denote half of the length and width of the testis, respectively).

To begin with, male zebra finches were singly housed in cages equipped with 2 perches and a rectangular nest-box (size = 9 x 6 x 11 cm) with a front opening (size = 5 x 5 cm, 2 cm above floor). Then, they were exposed to either LL (hence no-night; L = 150 ± 5 lux; N = 16) or remained on 12:12 h LD (light on 0600 h, light off 1800 h; N = 16; L = 150 ± 5 lux), as before and served as control. The regular visual observation and images captured showed that birds were always in bright illumination, except when they went inside the nest-box whose extreme rear end had shaded illumination. After a week of male occupying it, we

introduced in the cage a female that was housed in another room under 12:12 h LD and identical temperature condition. We considered that a week of prior stay period led the acclimation of the male to its cage and nest box, and this in our experience was a sufficient time duration for zebra finches to enthusiastically receive its mate. Housing a male first and introducing a potential female mate later is also consistent with the idea that the social isolation followed by pairing increases courtship; isolated male zebra finches exposed to a new female demonstrates increased directed singing (Shukla and Sadananda, 2021).Thus, there were 16 male-female P generation pairs who lived together under LL or LD, with regular replenishment of food and water, and materials (grass, cotton) to build the nest for the duration a pair produced three clutches or for 24 weeks (Fig. 1; Upper panel).

The offspring (F1 progeny) lived with their parents inside the cage until they were 90-100 days old, when they were considered adult (Roper and Zann, 2006). The eggs were laid in the nest-box and the hatchlings flew out of the nest-box once they fledged. So, all the offspring received same light treatment (LL or LD) as their parents did. Although the fledglings would hop-in and hop-out of the nest-box, they spent considerable amount of time on perches in the cage. Our visual observation during daytime suggested that fledged offspring rarely occupied the nest box; notably, the fledglings were never in completely darkness since even the very rear end of next box had a shaded illumination. The F1 progenies when adult were again randomly paired (1 male and 1 female) and kept in separate breeding cages in respective light conditions in which they were born (N = 8 pairs each in 12:12 h LD or LL). Here, they lived together and bred until the time they produced and nurtured a clutch of F2 adults or for 15 weeks after which the experiment was terminated even if few pairs failed to produce an egg (Fig. 1).

Thus, we had three (P, F1 and F2) generations of birds at the end of the experiment. Various parameters associated with health and reproductive performance of P and F1 generations were assessed, as described below. It may be noted that an earlier publication by Jha and Kumar (2017) has reported the results on 24-h activity and singing patterns, cognitive performance and personality traits of zebra finches of all the three generations.

2.3. Reproductive performance

The reproductive success was monitored until the time all 16 P pairs had produced three clutches or were housed together for 24 weeks, whichever was earlier, since not all pairs laid 3clutches; for example, a few LL females did not lay eggs at all. Likewise, we monitored the reproductive success of eight F1 pairs until the time they had one clutch or were housed

together for 15 weeks whichever was earlier. Under LL, several F1 pairs did not engage in reproduction as suggested by no eggs laid by females during 15 weeks of the exposure. For monitoring egg laying, both cage and nest box were inspected daily between hour 3 and 5 of the light on period (0900 h - 1100 h).

The reproductive performance was recorded pairwise, and as follows. The laying of the first egg marked the initiation of breeding, for which we recorded the number of days taken to lay the first egg since pairing. For F1 pairs, which unlike P pairs, did not engage early in nesting, we recorded also the number attempts they made to build their nest. The length and breadth of each egg were measured to the nearest 0.1 mm by using a Vernier Caliper. The egg clutch was considered complete if no new egg was added for 3 consecutive days. Whereas P pairs were allowed to breed until they laid 3 clutches, F1 pairs were allowed only until the completion of the first clutch. The completion of a clutch and hatching of at least one of its eggs was counted as a successful breeding attempt. Each hatchling was tagged with a coloured and numbered leg ring. We thus recorded the eggs laid, clutch size, number of hatchlings, fledglings and viable offspring, and mortality. We also recorded the number of days that a hatchling took to fledge (fledging duration), and the time duration from the beginning of the experiment until the day a bird fledged. From these parameters, we calculated reproductive success (total number of fledglings) of each pair, and assessed and compared the reproductive performance of P pairs that raised all the three clutches. For P generation, the overall reproductive success was the sum total number of fledglings of C1, C2 and C3. For F1 generation, it was the total number of fledglings of C1 as they raised only one clutch.

2.4. Phenotypic and morphometric measurements

2.4.1. Adult physiology and morphology: The morphometric measures of P generation male and female zebra finches were taken at the time of their recruitment in the experiment. Similarly, the measurements of F1 and F2 male and female birds were taken when adult (120 ± 20 days). The birds were weighed on a top pan balance to an accuracy of 0.1 g. The body fat was recorded using a subjective criterion in a score of 0-3 (0 = no subcutaneous fat, 1= light fat depots underlying musculature, 2 = heavier fat depots underlying musculature, 3 = copious fat depots over several regions). The size of the testes and ovarian follicle was assessed by laparotomy, as we routinely carryout in our laboratory (for details, see Jha and Kumar, 2017). This procedure was done under general anaesthesia with a mixture of ketamine/ xylazine solution (67.5 mg ketamine + 7.5 mg xylazine per kg body weight). The

gonads were located by making a small incision between the last two ribs on the left flank of the bird. The dimension of the left testis and the diameter of the largest ovarian follicle were measured to an accuracy of 0.5 and 0.1 mm, respectively. The incision was immediately sutured by surgical thread, and an antibacterial skin ointment (Soframycin skin cream, Aventis Pharma Ltd.) was applied. The procedure with minimum pain or suffering, if any, was quickly completed, and the birds was returned to its cage on the warm pad, which was removed after the bird recovered in about 30 min. These birds were fully active and began perch hops in about 1 h time. The veterinarian checked these birds the following day. We did not have mortality due to laparotomy procedure in this experiment.

We also measured the length of wing, tarsus, beak (bill) and tail of both male and female birds to the nearest 0.1 mm by a Vernier caliper (Sutherland et al., 2004). The wing length indicated the distance from carpal joint to tip of the longest primary feather in a closed wing. The tarsal length meant strictly the length of tarsometatarsus, and was measured such that tibiotarsus and tarsometatarsus bones formed an acute angle. Similarly, the length of beak was measured from its base in skull to the tip, and tail length from the tip of the tail to feathers base.

2.4.2. Adult male cheek patch: As a measure of sexually selected 'ornaments' in male birds, we quantified male cheek patch size and its colouration. For this, male birds (age = 120 ± 20 days) were photographed against a black background by a digital camera (Sony Digicam 20 MP H200 Blk) from the same fixed distance. The photographed files were imported into Adobe Photoshop 7.0 (Adobe Systems Inc. San Jose, CA, USA), and pigmented cheek area and shape of the beak were determined by the "magic wand" tool function of the Adobe Photoshop that allows for the selection of consistently coloured area. The "histogram" function was used to analyze the number of pixels within the marked area. For this study, we calculated cheek patch area relative to beak of the bird; for each bird, we made three independent measurements for each side of the head, which we averaged to get finally the cheek patch ratio. We report cheek patch ratio instead of the absolute cheek patch size, since despite our best efforts the photographs may have differed slightly in distance from the subject because of a possible movement of the subject and/ or associated human error. This is also consistent with the method used in other studies in zebra finches (Leader and Nottebohm, 2006; Krause and Naguib, 2015).

2.5. Measurement of plasma hormone levels

We measured plasma levels of testosterone, estradiol and corticosterone in blood samples collected early in the day (hour0 - 0.5; hour 0 = light on) from birds of all the three (P, F1 and F2) generations. In each bleed, we collected 50 - 100 µl blood by puncturing the wing vein into a heparinized capillary tube, and the procedure was completed within 2 min to avoid stress-induced changes in hormone levels (Wada et al., 2008). The blood was centrifuged immediately at 845 g for 10 min, and plasma was harvested and stored at -20 °C until assayed for hormones. We performed hormone assays by using ELISA immunoassay kits and protocols that have been validated and used for zebra finches in our laboratory (for example, see Mishra and Kumar, 2019). Briefly, each hormone assay was done as follows.

2.5.1. Testosterone: Using testosterone (T) ELISA kit from Enzo Life science (Ann Arbor, MI; Catalog # ADI-900-065), plasma testosterone (T) levels were measured in 10µlduplicate samples from males (1:20 dilution: 10 µl plasma + 10 µl 1% steroid displacement buffer + 180 µl assay buffer, Lynn et al., 2015). 100 µl of standard diluent was pipetted out into the NSB (nonspecific binding) and Bo (maximum binding) wells, and 100 µl of standards and diluted samples were pipetted out in respective wells. Then, 50 µl of antibody was added to each well except for the blank, TA (total activity) and NSB wells. The 96 well-plate was incubated for 1 h at the room temperature on a plate shaker at 5000 rpm. The plate was again incubated after adding 50 µl of conjugate to each well except the blank and TA wells. Thereafter, the plate was washed 3 times, and then 200 µl of p-nitrophenyl phosphate in buffer (pNpp) substrate solution was added and the plate was again incubated for 1 h at the room temperature without shaking. After incubation, 50 µl of stop solution was added to stop the reaction and the SpectraMax M2e microplate reader (Molecular Devices LLC, USA) read the optical density at 405 nm. T concentrations of experimental samples were calculated with reference to values in the standard curve drawn from OD of standard samples. The sensitivity and intra-assay variability of the assay were 0.08 ng/ml and 6.7 %, respectively. The intra-assay variability was 6.7%.

2.5.2. Estradiol: Estradiol concentration was measured in plasma of female birds by using an enzyme immunoassay kit (Estradiol EIA Kit, Cayman Chemical Company, MI, USA) according to the manufacturer's instruction (Ubuka et al., 2014). Briefly, 100 µl and 50 µl of EIA buffer was added to NSB and Bo wells, respectively. 50 µl of standards and

samples were added to respective wells. After that 50 µl of estradiol- acetylcholinesterase tracer was added to all wells, except TA and blank wells. Then, 50 µl of estradiol antiserum was added to each well, except the TA, NSB and the blank wells. It was incubated at room temperature for 1 h on an orbital shaker (200 rpm). The plate was washed 5-times with washing buffer. Thereafter, 200 µl of Ellman's reagent was added. The plate was incubated at room temperature in dark on an orbital shaker (200 rpm). The assay developed optimal colour (i.e. Bo wells ≥ 0.3 OD) in 1 h. The plate was read at 405 nm by SpectraMax M2e microplate reader (Molecular Devices LLC, USA). The intraassay variability was 5.2 %.

2.5.3. Corticosterone:An enzyme Immunoassay kit from Enzo Life Science (Ann Arbor, MI; catalog # ADI-901-097) was used to measure the plasma corticosterone levels in both male and female birds. In addition, we measured plasma corticosterone levels in F1 birds before and after the time of lights on and off; for this, blood samples were collected 2 h before and after the light on as well as 4 h before and after the light off. The assay was run on 96 well plates, as per the manufacturer's protocol (Wada et al., 2008). 10 µl of plasma samples were run in duplicates with 1:40 dilution in 1 % steroid displacement buffer (10 µl plasma + 10 µl 1 % steroid displacement buffer + 380 µl assay buffer). 100 µl each of standards and samples were added to the standard and sample wells, respectively. 50 µl of assay buffer was again added to the non-specific binding (NSB) wells to make up the volume for 50 µl blue conjugate (alkaline phosphate conjugated with corticosterone) added to each standard and sample well. 50 µl of Antibody was added to each well, except the blank, TA and NSB wells and incubated for 2 hours at the room temperature on shaker at 500 rpm. Post-incubation, the plate was washed 3 times with 400 µl of wash buffer. A 5 µl of conjugate was added to TA wells and the 200 µl of p-nitrophenyl phosphate in buffer (pNpp) substrate solution was pipetted out in each well. The plate was again incubated for 1 h at the room temperature without shaking. The reaction was stopped by adding 50 µl of stop solution, and the SpectraMax M2e microplate reader (Molecular Devices LLC, USA) read the optical density at 405 nm. The corticosterone concentrations of experimental samples were calculated with reference to values in the standard curve drawn from OD of standard samples. The intra-assay variability was 9.5 %.

2.5.4. Melatonin: Plasma melatonin concentration (pg/ml) was measured by ELISA using a specific melatonin kit (product no. RE54021, IBL International GmbH, Hamburg, Germany. The melatonin levels were assayed in blood samples taken from F1 birds at 6 h

time intervals, beginning from the light on in LD (zeitgeber time, ZT, 0, 6, 12 and 18). The melatonin was extracted and assayed from plasma, as per the manufacturer's protocol and instructions, and procedure standardized and routinely used in our laboratory (see for example, Singh et al., 2012; Taufique et al., 2018). The dried pelletobtained from extraction was reconstituted in 0.15 ml of bidistilled water and vortexed for 1 min, and used immediately the assay. 50 µl of sample with 50 µl of melatonin anti-serum (rabbit, polyclonal) were incubated in 96-well plate for 20 h at 4 ºC. 150 µl of freshly prepared enzyme conjugate was then added and incubated for 2 h at 25 ºC with 500 rpm. Thereafter, 200 µl of freshly prepared p-nitophenyl phosphate (pNpp) substrate solution was added to each well and incubated for another 40 min. Addition of 50 µl of pNpp stop solution stopped the reaction. The optical density (OD) was measured at 405 nm wavelength, using 650 nm wavelength as reference, by SpectraMax M2e microplate reader (Molecular Devices LLC, USA). The melatonin concentrations of experimental samples were calculated with reference to values in the standard curve drawn from the OD of standard samples. The intra-assay variability was 5.8 %.

2.6. Monitoring of 24-h activity behavior

As a behavioural marker of the LL effects, 24-h activity-rest pattern was recorded for both male and female birds of all three generations. For this, the activity of each individual bird was continuously monitored over 10-day period at the end of the experiment for P generation, and after the sexual maturity for F1 and F2 generations, as per method described in several previous publications from our laboratory (e.g., Singh et al., 2010, Jha and Kumar, 2017). Briefly, a passive infrared sensor (digital PIR motion detector, LC-100-PI) mounted on the cage continuously monitored birds's general activity and transmitted it in 5-min bins to a designated channel of the computerized data-recording system. The collection, graphics and analysis of activity were done by 'The Chronobiology Kit' software program of Stanford Software Systems, Stanford, USA. For a better visual resolution, we obtained a double-plotted activity record (actogram), wherein successive days' activity was plotted sideways and underneath. Daily activity profile was further plotted in a graph format, for which the activity record over a 7-day segment was first averaged for each hour for every individual and then plotted over 24 h. We are reporting data on activity-rest for F1 generation females, as data for male birds have already been published (see Jha and Kumar, 2017).

2.6.1. Assay of male song characteristics: We assessed the features of the song as a marker of the secondary sexual characteristics of male birds. The song of each male was recorded by a Behringer C-2 Studio Condenser microphone fitted in each cage, using M-Audio Profire 2626 8-channel Sound Card and Nuendo application software (Steinberg Media Technologies GmbH, Hamburg, Germany) over about 48h period. The sound was recorded at the rate of 16 bit and 44.1 kHz sampling frequency and the sound files were saved as digital audio files in the avi format. The song of P male was recorded before and after the experiment, whereas that of F1 and F2 male was recorded when adult (> 120 days old).

Individual singing records were screened, and song motifs were identified and analysed. Various acoustic features of the song motif, namely the duration, amplitude, amplitude modulation (AM), pitch, frequency, frequency modulation (FM) and Wiener entropy were calculated. We analyzed song similarity between generations (P vs. F1; F1 vs. F2), by comparing 10 song motifs that were identified by visual inspection of the sonograms. This was essentially mapped by using the automated Similarity Feature of Sound Analysis Pro 2011 (SAP 2011) software, which detected sections of similarity across songs in terms of pitch, FM, Wiener entropy and spectral continuity. Differences in acoustic features of the song between parent and offsprings were also calculated from the similarity feature module.

2.7. Statistics

Statistical analyses were performed using IBM SPSSv20, GraphPad Prism version 5.0(GraphPad, La Jolla, CA, U.S.A) and Canoco version 4.5 software, as appropriate.Data were first checked for meeting the assumptions of parametric statistics. If assumptions were not met, data were log transformed for further statistical analyses. We used a chi-square test to compare the proportions of recruited birds engaged in raising successful clutches between 12:12 h LD and LL across the three clutches (C1, C2 and C3) or across P and F1 generations. Further, the linear mixed effect model (LMEM) compared the effect of light condition (12:12 h LD and LL; factor 1) and clutch sequence (C1, C2 and C3; factor 2) on reproductive measures of P birds, with factor 1 and factor 2 as fixed factors, factor1*factor 2 as interaction factor, and individuals as the random factor. Here, we compared only those P pairs that raised all three clutches, i.e. 12 pairs in 12:12 h LD and 8 pairs of LL. In case, factor 2 (clutch sequence) had a significant effect on reproductive variables, we used Bonferroni adjustment to determine if groups differed significantly from each other. We used Man-Whitney U test to compare the overall reproductive success of P pairs in LD and LL. We also used a generalized linear model (GLM

univariate analysis) to analysethe effect of the light condition (12:12 h LD and LL; factor 1) and generation (P and F1; factor 2) on reproductive performance of zebra finches across generations. For this analysis, we have used data from first clutch (C1) only since F1 pairs had completed only the first clutch.

GLM was also used to analysethe effects of light condition (12:12 h LD and LL; factor 1) and generation (P, F1 and F2; factor 2) on phenotypic and morphometric measures of male and female birds, plasma hormone levels (estradiol, testosterone and corticosterone), and vocal imitation of parent's song by its progeny. If factor 2 had a significant effect, we performed Tukey's post hoc test to show significant differences between groups. Similarly, LMEM analyzed the effect of light condition (12:12 h LD and LL; factor 1) and time/ duration of light exposure (before and after; factor 2) on song structure and spectral features of the P zebra finch song.

The cosinor analysis tested a 24-h rhythm in activity behaviour and melatonin levels based on unimodal cosinor regression ($y = A + B \times \cos(2\pi (x- C)/24]$; A, B and C are the mesor (mean value), the amplitude and the acrophase (the estimated time of peak) of the rhythm, respectively). The significance of the regression analysis was calculated by using the number of samples, R2 values and numbers of predictors (mesor, amplitude and phase: Soper, 2013; http://www.danielsoper.com/statcalc3/calc.aspx?id¼15). For statistical significance, alpha was set at 0.05.

3. Results

3.1. LL negatively affected the reproductive performance across generations

There was an overall reduced reproductive performance found in both P and F1 generation pairs under LL, compared to the LD condition. The number of P pairs engaged in raising three clutches was significantly reduced in LL, compared to 12:12 h LD controls (Chi-square test: $\chi^2_3 = 47.52$, $P<0.001$). Over 24-week period, the reproductive performance of 16 P pairs was as follows: LD = 12 pairs produced 3 clutches, 2 pairs 2 clutches and 2 pairs 1 clutch; LL= 8 pairs produced 3 clutches, 3 pairs 2 clutches, 2 pairs1 clutch each, 3 pairs laid no eggs. The overall participation or engagement of breeding pairs in raising successful clutches was affected by both, the light condition and generation; the LL pairs raised fewer clutches and the effects were more pronounced in the F1 generation (Chi-square test: $\chi^2_1 = 36.40$,

P<0.001). Out of 8 F1 pairs that were recruited, all reproduced and raised first clutch under LD, whereas only 3 out 8 pairs reproduced and raised first clutch under LL.

The overall reproductive success of LL parents was lower than that of LD parents (Man-Whitney U test; p = 0.05). We then evaluated the long-term effect of no night environment on reproductive performance, for which we assessed whether the reproductive effect was related to the duration of LL exposure, by comparing various reproductive parameters of three clutches obtained from P pairs within and between LD and LL. There was an overall significant effect of the light condition, with lesser number of viable offspring raised, lower reproductive success, and longer fledging duration in LL than the LD ($p \leq 0.05$, LMEM and Bonferroni adjustment for multiple comparisons; Fig. 1: Lower panel, Table 1). The effects were increased with progressive clutches, as assessed in the breeding attempt, number of fledglings and viable offspring, fledging duration and offspring mortality. The breeding attempt of pairs by the time they had a clutch was in the following order; C1 > C2 > C3; however, this was significantly different between C1and C3 (p = 0.007) or close to being significantly different between C1 and C2 (p = 0.055), but not between C2 and C3 (p = 1.000, Bonferroni adjustment for multiple comparisons). Similarly, the fledglings and viable offspring raised from C3 were significantly lower in number than those from the C1 (fledglings: p = 0.003; viable offspring: p = 0.002), but it was not different between those from C1 and C2 (fledglings: p = 0.856; viable offspring p = 0.546) or C2 and C3 (fledglings: p = 0.411; viable offspring p = 0.432). We found interaction of light condition and clutch sequence had a significant effect on clutch size, number of hatchlings and viable offspring ($p \leq 0.05$, LMEM and Bonferroni adjustment for multiple comparisons; Fig. 1: Lower panel, Table 1). Although there was an overall effect of the clutch sequence on offspring mortality, the three clutches did not significantly differ from each other (C1 vs C2: p = 0.058; C1 vs C3: p = 1.14; C2 vs C3: p = 1.00). Whereas, C3 had fledging duration significantly longer than the C1 (p = 0.004) and C2 (p = 0.002), theC1 and C2 were not significantly different from each other (p = 1.00, Bonferroni adjustment for multiple comparisons).

Further, we examined consequence of LL exposure on reproductive performance of F1 birds with respect to P birds. The reproductive performance was compromised under LL, with a significant effect on breeding initiation, nesting attempt, clutch size, number of hatchlings, viable offspring, offspring mortality, fledging duration and reproductive success ($p \leq 0.05$, GLM; Fig. 2, Table 2). There was a significant generation effect on the number of

hatchlings, viable offspring, mortality of offspring, fledging duration and reproductive success ($p \leq 0.05$, GLM univariate analysis; Fig. 2, Table 2). Under LL, birds took a longer time to initiate breeding, made several nesting attempts before laying an egg and successfully raising a clutch. Although LD controls and LL birds laid almost similar total number of eggs per pair, there was a significant difference between two generations in the number of eggs that they incubated (clutch size) and got hatched, viable offspring, offspring mortality, fledging duration, and the overall reproductive success. F1 birds had significantly fewer hatchlings, fledglings (also took much longer to fledge) and viable offspring under LL. There was interdependence of the light condition and generation on initiation to breed, as revealed by the significant effect of the light condition x generation interaction ($p \leq 0.05$, GLM univariate analysis; Fig. 2, Table 2).

3.2. LL affected the phenotype and morphometric measurements

GLM univariate analysis revealed that there was no effect of the light condition, generation and their interaction (light condition x generation) on fat score (but females showed copious fat deposits under LL), body mass and gonads (testicular volume and follicular diameter) of zebra finches (Table 3A; Fig. 3). A few morphological parameters also showed a significant effect of the light condition and/ or generation. For example, there was a significant effect of both light condition and generation, but not of their interaction, on cheek patch ratio, and of generation alone on wing and tarsus length of male birds ($p < 0.01$, GLM univariate analysis; Fig. 3f1, Table 3A). The LD birds had higher cheek patch ratio than the LL birds; likewise, F2 birds had higher cheek patch ratio than the F1 birds. The tarsus was smaller in length in F2 than P birds. Similarly, we found a significant effect of the light condition on the tail length (longer tail in LD) and of generation on tarsus and beak lengths (significantly shorter tarsus and beak in F2 than P generation) of females; however, there was an interaction effect ofthe light condition x generation on tarsus length of both male and female birds ($p \leq 0.05$, GLM univariate analysis Table 3A).

3.3. LL-induced changes in daily activity behaviour and plasma hormone levels

Daily activity behavior of female zebra finches also showed a significant 24-h rhythm in activity-rest pattern, as revealed by cosinor analysis, with activity consolidated during the light period under LD and subjective daytime under LL (Fig. S1). This is similar to the pattern and 24-h rhythm reported previously for both activity and singing behaviours of male zebra finches under 12:12 h LD and LL (Jha and Kumar 2017). The daily activity pattern of both male and female birds under LD and LL were different in terms of the synchronization (synchronized to light phase with 24 h period in LD, and rhythmic with a circadian period in LL), but not in daily total activity(Fig. S1).

Further, the plasma concentration of corticosterone and testosterone in males and estradiol in females showed a significant effect of the light condition, with levels significant lower in LL than the LD condition ($p \leq 0.05$, GLM univariate analysis; Fig. 3d1, e1 and e2, Table 3B); however, we found no effect of the generation or its interaction with light condition. Both light condition, generation or their interaction did not affect the corticosterone levels in females. Further in F1 birds, the plasma corticosterone levels measured 2 h as well as 4 h before and after the light on and light off, respectively, were not significantly different between LD and LL. The cosinor analysis also revealed a significant daily rhythm of melatonin in both 12:12 h LD and LL conditions, albeit with a reduced amplitude under LL (Fig. S2).

3.4. LL affected the song characteristic

Song structure and spectral features of male parent songs: There was no significant difference between LD and LL in the song structure, namely the song bout duration and motifs per bout both at the beginning and end of the experiment (LMEM; Table 4A). However, the spectral features of song did show an effect of the light condition. In particular, there was a significant shortening of the motif duration in LL males (LMEM; Fig. 4c, Table 4A). There was significant effect of the light condition on mean amplitude, variance peak frequency and entropy. In particular, the duration of exposure influenced light condition effects on several song features, for example mean frequency, frequency modulation (FM), entropy, and on variance of the amplitude, FM, amplitude modulation (AM) and entropy (LMEM; see Table 4A).

Vocal imitation of the parent's song: The vocal imitation of parent's song was measured in F1 and F2 males, with respect to their parents (P for F1, and F1 for F2). There was an effect of the light condition on percent similarity ($p = 0.02$), percent accuracy ($p = 0.021$), pitch ($p = 0.014$), AM ($p = 0.03$), and of the generation on pitch ($p = 0.002$) and AM ($p < 0.001$). The percent similarity and accuracy of the parent and progeny song was lower in LL males. Likewise, under LL, there was a significant increase in pitch difference and decreased AM in progenies, as compared to the parents ($p \leq 0.05$, GLM; Fig. 4 and Table 4B).

4. Discussion

We demonstrate long-term effect of the no-night environment on reproductive behavior and associated physiology of diurnal zebra finches. Two conclusions can be made from the present results. First, the absence of night component from day-night environment negatively impacted the reproductive performance of P generation birds that were born under 12:12 h LD and exposed to LL when adult. This was evident by reduced efforts in raising the offspring and enhanced negative impact with successive three clutches: compared to C1, C3 pairs made lesser breeding attempts, produced fewer fledglings and viable offsprings, and took more time to fledge under LL, compared to LD controls. Differential reproductive success seemed also related to differences in structure and spectral features of the song, which is a secondary sexual trait, support the reproductive effect of LL in zebra finches. In particular, by the time the experiment ended, LL males had shortened their song motifs, and showed alteration in song features like the mean frequency, FM and entropy. However, notwithstanding with the overall reduced reproductive success, P pairs were found engaged initially in reproduction activity quicker under LL than the LD, and this we attribute to an accelerated activation of the reproductive axis in response to the acute LL exposure (Sharp, 1993).

These results are thus in agreement with those suggesting a negative reproduction associated effects of ALAN in other songbirds. For example, female blue tits (*Cyanistes caeruleus*) laid eggs earlier and males obtained more extra pair mates under the streetlight influence (Kempenaers et al., 2010). This, as the authors argued, was because females targeted the earliest-singing males, considering that this was their dawn song, an indicator of a better-quality male. Perhaps, the street light ALAN led to a maladaptive mate choice decision of the female with respect to an extra-pair behavior and, consequently altered the selection pressures on the mating behavior. Black-tailed godwits (*Limosa limosa*) were also

found to have preference for the nesting away from the roadway lighting (Longcore and Rich, 2004).The nocturnal illumination also affected the fledgling mass, but not fledgling production, in great tits and pied flycatchers (de Jong et al., 2015; Dominoni et al., 2020).Likewise, there was an effect on the territorial singing behaviour in male mockingbirds (*Mimus polyglottos*; Derrickson, 1988) and American robins (*Turdos migratorius*), with birds starting to sing much before the dawn in areas that were lit by the artificial lighting (Miller, 2006). In similar lines, ALAN influenced the dawn singing, extra-pair siring success and laying dates in five forest-breeding birds, namely the chaffinches (*Fringilla coelebs*), blue tits, great tits (*Parus major*), blackbirds (*Turdus merula*) and robins (*Erithacus rubecula*) (Kempenaers et al., 2010). However, blackbirds developed their gonads during the first year, but not after the second year of the exposure to very dim (0.3 lux) light at night (Dominoni et al., 2013a).The ALAN influence on reproduction seems to be widespread among animal taxa, as shown by its reported effects on the mating behavior in moths (*Operophtera brumata*; van Geffen et al., 2015), mating calls in male frogs (*Physalaemus pustulosus*; Longcore and Rich, 2004), copulatory behaviour in sexually naïve adult male rats (Fantie et al., 1984), structural and functional changes in the pituitary-thyroid axis in female rats (Miler et al., 2014), andreduced reproductive efficiency, reproductive organ mass and sperm counts in male south Indian gerbils (*Tatera indica cuvieri*; Thomas and Oommen, 2001).

Second, F1 generation zebra finches that were born in the no-night environment had reduced reproductive success. Under LL, the lower plasma testosterone and estradiol levels in males and females, respectively, support this. A similarinhibition of the reproductive hormones was found in western scrub-jays (*Aphelocoma californica*; estradiol levels were low in both sexes and testosterone levels were low in females) exposed to ALAN (Schoech et al., 2013). In the current study, F1 pairs took longer time to initiate breeding, made more nesting and breeding attempts before they successfully laid and raised the first clutch, had fewer hatchlings and fledglings with increased fledging duration, and increased offspring mortality, less viable offspring and lower reproductive success under LL, compared to LD controls. Interestingly, the LL environment resulted in poor copying of the parent's song by both F1and F2 progenies. This is consistent with previously reported learning and memory deficits, evidenced by reduced participation and performance in the spatial and association task tests in F1 generation zebra finches (Jha and Kumar, 2017). Our results are consistent also with evidence that unpredictable LD cycles can impair learning performance with effects passed on to F1 generation in chicken (Lindquivst et al., 2007).

We found an overall low plasma level of corticosterone (in males, not females), testosterone in males and estradiol in females in LL, as compare to than in the LD condition. Sex differences in corticosterone levels, an indicator of stress response, is similar to that reported in chicken with males showing higher levels than females (Goerlich et al., 2012). Further, a relatively lower corticosterone level under LL is similar to those found in European starlings (*Sturnus vulgaris*), which showed decreased basal and stress-induced corticosterone levels in response to chronic stress (Rich and Romero, 2005), but inconsistent to that found in response to ALAN in free living great tits (Grunst et al., 2020). The present results may be considered with caution since we did not measure corticosterone levels after acute as well aslong-term exposure to LL. We would like to reason though that the LL environment was not stressful, since there were no overt signs of the physiological stress, such as the lack of feeding and loss of weight, and decreased mobility or activity under LL. Additionally, the corticosterone levels measured in F1 birds both before and after the time of light on as well as light off were not significantly different between the LD and LL (Figure S3).

Daily patterns of the activity (of females) and singing (of males) behaviour also reveal that these were consolidated and rhythmic rather than being scattered over 24 h period under LL, as also reported previously for male birds (see Jha and Kumar (2017). F1 female birds were found also rhythmic in their 24-h activity-rest pattern, as measured at the end of the experiment irrespective of the light condition there were exposed to, albeit with relatively a lower amplitude under LL (Fig. S1). Interestingly, these birds also showed a 24-h rhythm in plasma melatonin levels with a relatively lower peak under LL, consistent with persistence of daily melatonin rhythm in free-living willow warblers during the arctic summer (Steiger et al., 2013).

When various observations are considered together, zebra finches appear to have adapted at least partially to LL, which during the long-term exposure was probably interpreted as a predictable environment; this needs to be tested further. There seems to be a tradeoff between the reproductive performance and non-reproductive aspects, viz. the activity and singing behaviour, the phenotype and morphometric changes. The overall results on LL-induced phenotypic and morphometric effects suggest that the chronic LL exposure was not as deleterious in its effects as one would have expected. Some of the LL-induced negative effects also showed sex differences; for example, female zebra finches but not males showed copious fattening under LL, as previously reported when they were subjected to an acute exposure to dim light at night (Batra et al., 2019). However, we found no difference in the body mass, consistent with no difference in total 24 h activity levels between LD and LL

conditions, indicating that the birds were otherwise in the healthy state. Further, LL males showed reduced cheek patch (a male sex trait), and both sexes showed negative effects in only few morphometric measurements, such as the tarsus or tail length. Increased offspring mortality further supports the harmful effect of the loss of night in zebra finch survival, as evidenced from ALAN-induced mortality in other birds (Jones and Francis, 2003; Rodríguez et al., 2012).

Although the present study was not designed to answer the mechanism by which LL affects the reproduction, we can make few postulations nonetheless. First, under LL, the disrupted daily circadian timekeeping and consequently mismatch of the biological processes led to the effects on reproduction-associated events in zebra finches. LL can induce disruption of the circadian clock gene oscillations (Prabhat et al., 2020) and overt circadian behaviours such as24-h cycle of activity-rest and singing patterns (males) of zebra finches (see also Jha and Kumar, 2017). It is widely established that in interaction with the prevailing photoperiod, the endogenous circadian clock regulates gonadal maturation of reproduction in seasonally breeding species (Kumar et al., 2010). Although zebra finch is not a seasonal breeder, there are indeed within year differences in its reproductive investment (Williamson et al., 2008), which do not preclude the circadian clock influence on the reproductive physiology of this species. There is evidence from the other taxa that the loss of circadian functions decreases reproductive fitness, e.g. in male *Drosophila melanogaster* (Beaver et al., 2002), and that the circadian clock gene (e.g. *period 2* and *brain muscle arnt like 1*) deficient mice exhibit irregular estrus cycle (Boden et al., 2010; Pilorz et al.,2009). Second, LL can affect the motivation to engage in reproduction, as also suggested by decreased participation and performance in cognitive test tasks, reduction in the exploratory behaviour, attenuated singing bouts in males, and probably reduced involvement in parental care, as reported in the previous publication (Jha and Kumar, 2017). Although it cannot be known from the current study, the pathways involved in these motivational behaviours may have similarly been affected. Consistent with this, Indian house crows showed increased errors and search-time during the learning-retrieval tasks, and attenuated the activity of the dopaminergic pathway after acute exposure to LL (Taufique and Kumar, 2016). As measured by immunohistochemistry, both ZENK (a protein marker of the neuronal activity) and tyrosine hydroxylase (the rate limiting enzyme of dopamine biosynthesis) show reduced expressions in mid brain area after 10 days of LL exposure (Taufique and Kumar, 2016).

Finally, one can ask about the relevance of the present study when in nature zebra finches or other species inhabiting 66.5 degrees north or south of the equator never experience a complete loss of the night (an LL environment) in the wild. To answer this, we will emphasize that the New World Artificial Night Sky Brightness atlas reveals that more than 80 % of the world is under light-polluted skies (Falchi et al., 2016). And, apart from such a general increase in ALAN, the special work environments like for those involved in night-shift work, intensive care units in hospitals, shopping malls, space flights and vigilance departments do experience at night the light environment that almost mimics the daytime in terms of the physiological effect. In several cases, the lighted environment is illuminated at a level higher than ~150 lux light intensity that was used in the present study. Exposure to light beyond normal lighting hours can have effects on physiology and behaviour at multiple levels. In this context, the present long-term study demonstrating the effects of the no-night environment on diurnal zebra finches is significant and lays ground for future researches with implications for the public health.

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Data availability: Data as appropriated will be made available from the first or corresponding author on a reasonable request.

Authors' contribution: VK: Conceptualization of the idea; NAJ and SKT: Experiment-monitoring behaviour and collecting samples and assays; NAJ and VK: Analyses and preparation of figures; VK and NAJ: Wrote the paper; VK and NAJ: Revision and production of the final version VK: funding, logistics and other resources. All authors gave final approval for publication.

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Figures

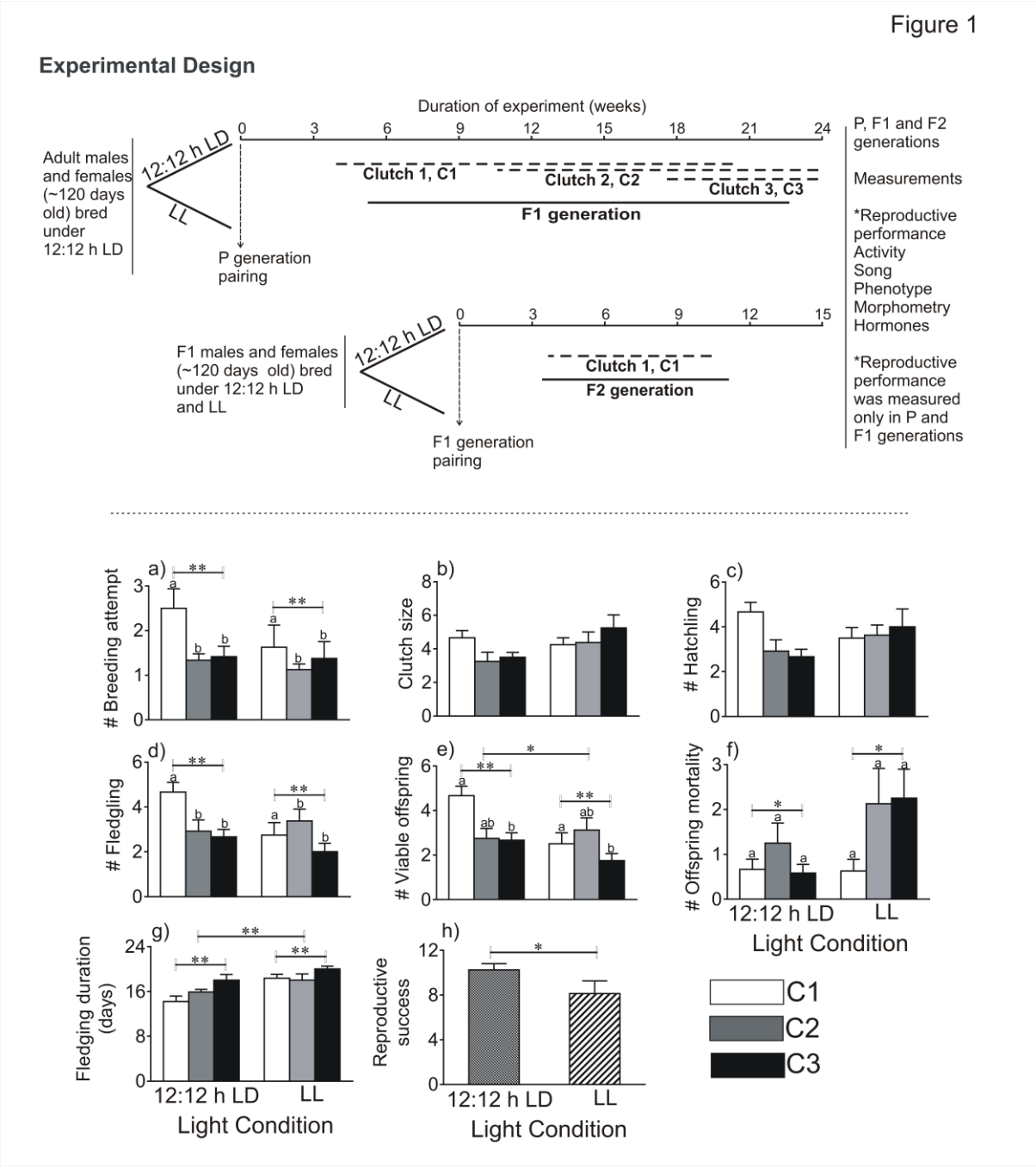


Figure 1. Upper panel: experimental design. Adult male and female zebra finches *(Taeniopygia guttata)* that were housed in same-sex cages under a 12 h light:12 h dark (12:12 h LD) photoperiod at 24±2°C were randomly paired and subjected to constant light (LL) or remained under 12:12 h LD, as before (controls; n = 16 pairs per light condition), for the time

until they produced and raised three clutches (C1, C2 and C3) or for 24 weeks, whichever was earlier. Similarly, F1 progenies when adult, were paired and exposed to LL or LD (n = 8 pairs per light condition), as before, for the time until they had produced and raised the first clutch (C1) or for 15 weeks, whichever was earlier. Broken line underneath the clutch number indicates the time range (days) when pairs laid egg that formed a clutch.24-h activity-rest pattern, song characteristics, male phenotype, morphometry and hormone levels of P generation birds were measured at the end of the breeding protocol, and of F1 and F2 progenies after they had reached sexual maturity, i.e.~120 days. The parameters that we used to define reproductive performance were measured in P and F1 generation pairs.

Lower panel represents the reproductive performance of three successive clutches of adult zebra finches (parent, P, generation). Data are plotted as mean ± SE for breeding attempt (a), clutch size (b), number of hatchlings (c), number of fledglings (d), number of viable offspring (e), offspring mortality (f), fledging duration (g), and overall reproductive success of P birds (h). The open, grey and black bars represent clutch 1 (C1), clutch 2 (C2) and clutch 3 (C3), respectively. Asterisk (*) indicates a significant difference at $p < 0.05$ level, as revealed by the linear mixed effect model (LMEM)test.

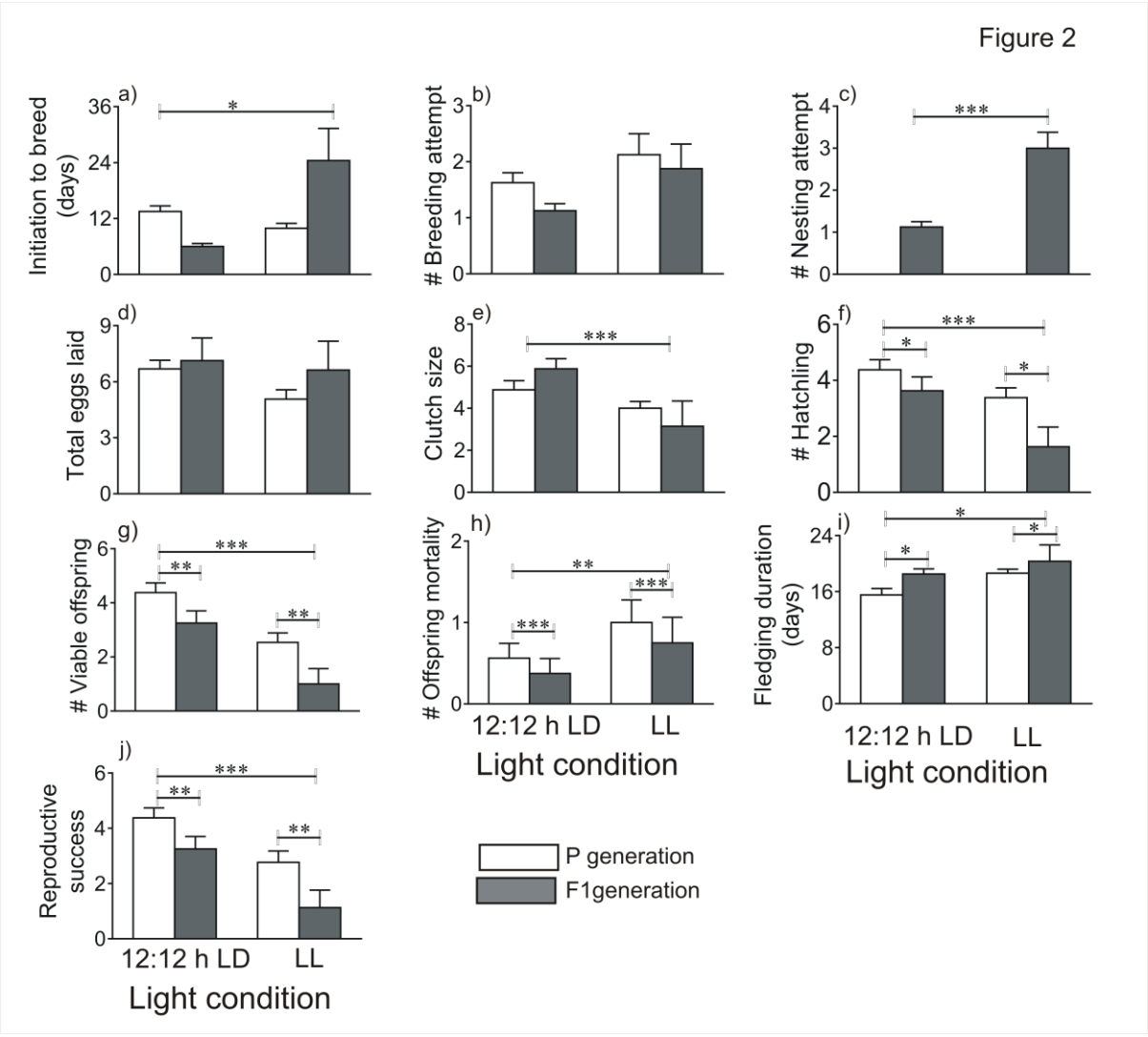
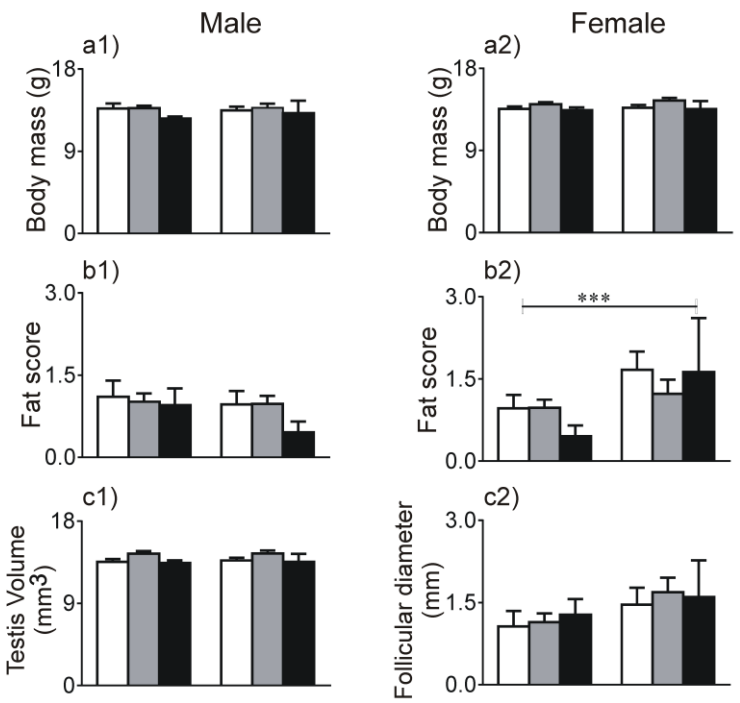


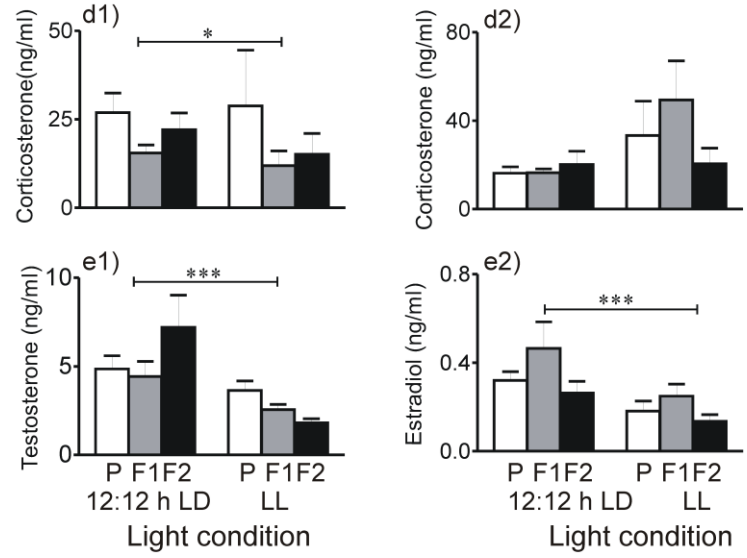
Figure 2. Reproductive performance of parent (P) and F1 generation zebra finches exposed to constant light (LL), with controls under 12 h day: 12 h night (12:12 h LD). Data are plotted as mean ± SE for initiation to breed (a), breeding attempt (b), nesting attempt (c), total eggs laid (d), clutch size (e), number of hatchling (f), number of viable offspring (g), offspring mortality (h), fledging duration (i), and reproductive success (j). The open, grey and black bars represent P and F generations, respectively. Asterisk (*) indicates a significant difference at $p < 0.05$ level, as revealed by general linear model (GLM) test.

Figure 3

Changes in body fattening, weight and gonad



Changes in hormones



Changes in secondary sexual characteristic

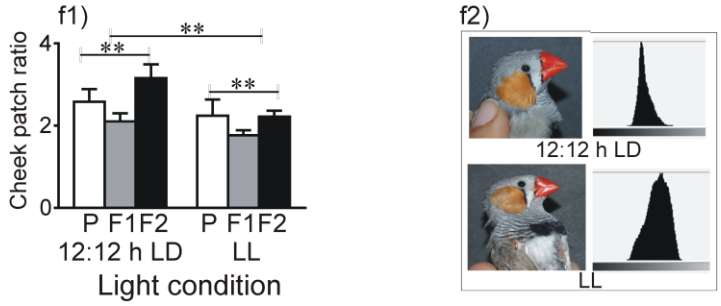


Figure 3. Upper panels: Changes (mean ± SE) in body mass, fat score and gonadal size of male (left) and female (right) zebra finches over three (P, F1 and F2) under constant light (LL) environment, with controls under 12 h day: 12 h night (12:12 h LD). Data are plotted for: body mass (a1: males; a2: females), fat score (b1: males; b2: females), testis volume in males (c1), and follicular diameter in females (c2). Middle panels: Changes (mean ± SE) in plasma levels of corticosterone (male and female; d1 and d2, respectively), testosterone (male; e1) and estradiol (female; e2) in three generations of zebra finches under LL and 12:12 h LD. Bottom panels: Cheek patch ratio of males (a male secondary sexual characteristics; f1) over three generations, and representative photograph and corresponding pixel-histogram showing differences under LD and LL conditions (f2). Asterisk (*) indicates a significant difference at $p < 0.05$ level, as revealed by general linear model (GLM) test.

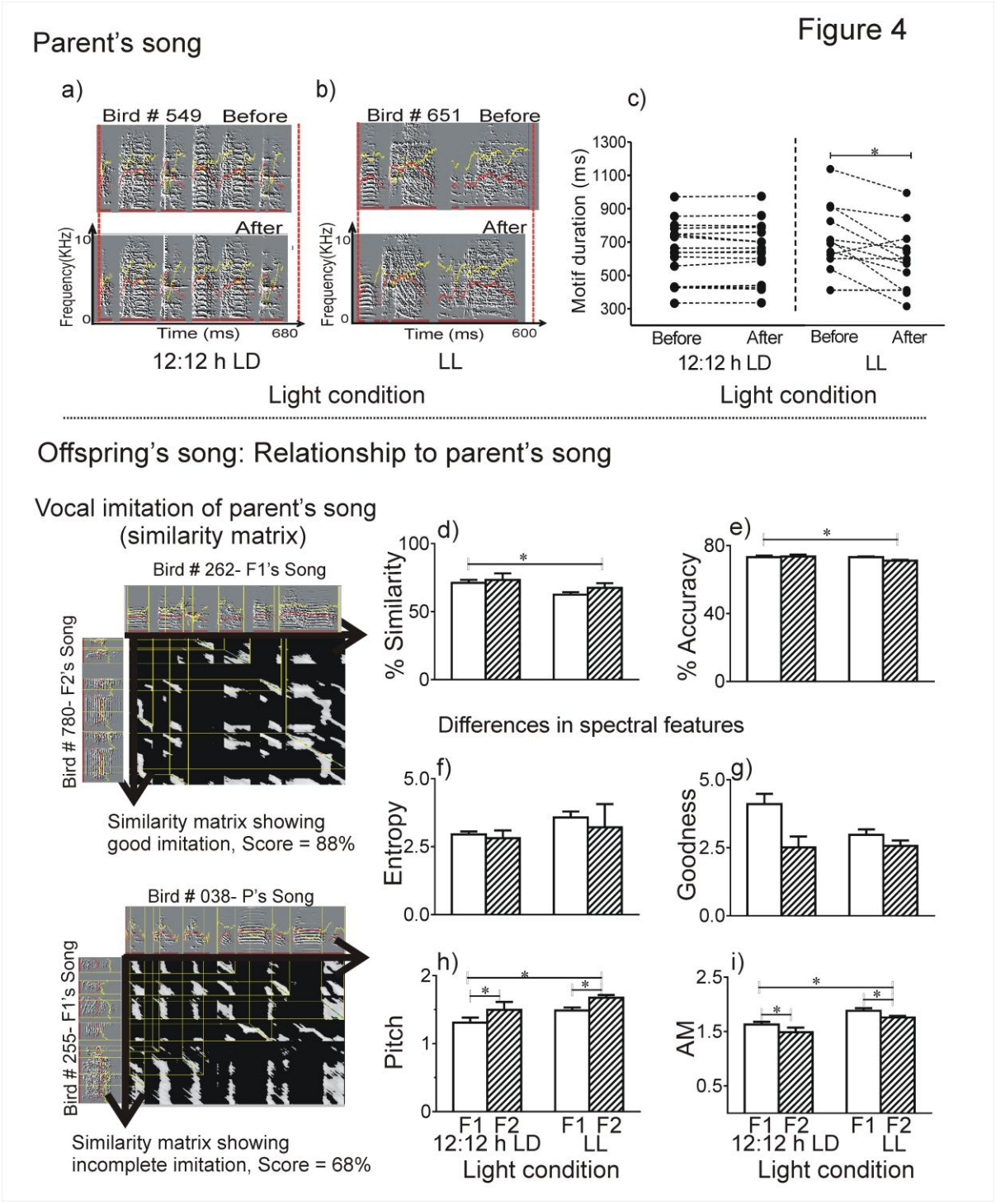


Figure 4. The song features of parent and offspring zebra finches (exposed to a no-night (constant light, LL) environment, with controls under 12 h day: 12 h night (12:12 h LD). Upper panels: Representative song motif P generation males before and after the long-term LD (a) and LL (b) exposures; c) shows mean (± SE) song motif duration. Lower panels show similarities between parent and offspring's song. The left panel shows similarity matrices

with representative copying of the parent's song: F1 copying P song, and F2 copying F1 song. Note higher similarity matrix score indicating good copying by F2 of F1 song, and lower similarity matrix score indicating poor copying by F1 of P song. Right panels: Mean (± SE) % similarity (d), % accuracy (e) and spectral features viz. entropy, goodness, pitch, AM (f-i) of F1 and F2 n generations under two light conditions. Asterisk (*) indicates a significant difference at $p < 0.05$ level, as revealed by linear mixed effect model (LMEM) and general linear model (GLM) tests.

Table 1: Linear Mixed Effect Model (LMEM) output showing the effect of light condition (12:12 h LD and LL) and clutch number (C1, C2 and C3) on reproductive performance of parent (P) generation zebra finches (*Taeniopygia guttata*) across the 3 clutches. An asterisk indicates a significant ($p < 0.05$) factor.

Parameters	Factors	F	df	p
Breeding attempt	Light	1.00	1,18	0.330
	Clutch number	6.25	2,18	0.009**
	Light x clutch number	3.14	2,18	0.068
Clutch size	Light	2.64	1,18	0.122
	Clutch number	0.89	2,18	0.429
	Light x clutch number	3.86	2,18	0.040*
Hatchlings	Light	0.53	1,18	0.477
	Clutch number	2.91	2,18	0.080
	Light x clutch number	6.14	2,18	0.009**
Fledglings	Light	3.39	1,18	0.08
	Clutch number	7.88	2,18	0.003**
	Light x clutch number	3.36	2,18	0.058
Viable offsprings	Light	5.62	1,18	0.029*
	Clutch number	8.27	2,18	0.003**
	Light x clutch number	4.24	2,18	0.031*
Fledging duration	Light	9.34	1,18	0.008**
	Clutch number	8.76	2,18	0.003**
	Light x clutch number	1.53	2,18	0.248
Offspring mortality	Light	3.94	1,18	0.063
	Clutch number	4.04	2,18	0.036*
	Light x clutch number	2.84	2,18	0.085

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Table 2: Model output from GLM univariate analysis showing the effects of light condition (12:12 h LD and LL) and generation (P and F1) on reproductive performance of zebra finches (*Taeniopygia guttata*). An asterisk indicates a significant ($p < 0.05$) factor.

Parameters	**Factors**	**F**	**df**	**p**
Initiation to breed	Light	6.79	1,44	0.013*
	Generation	1.11	1,44	0.298
	Light x generation	16.16	1,44	<0.001***
Breeding attempt	Light	3.44	1,47	0.070
	Generation	1.24	1,47	0.272
	Light x generation	0.14	1,47	0.712
Nesting attempt	Light	22.98	1,15	<0.001***
Total eggs laid	Light	1.61	1,44	0.211
	Generation	1.43	1,44	0.239
	Light x generation	0.45	1,44	0.505
Clutch size	Light	11.69	1,44	0.001***
	Generation	0.05	1,44	0.832
	Light x generation	3.70	1,44	0.061
Hatchlings	Light	10.17	1,44	0.0003**
	Generation	7.16	1,44	0.011*
	Light x generation	1.19	1,44	0.288
Viable offsprings	Light	21.85	1,44	<0.001***
	Generation	9.28	1,44	0.004**
	Light x generation	0.22	1,44	0.639
Reproductive success	Light	15.85	1,44	<0.001***
	Generation	8.74	1,44	0.005**
	Light x generation	0.31	1,44	0.582
Offspring mortality	Light	11.47	1,44	0.002**
	Generation	28.32	1,44	<0.001***
	Light x generation	0.29	1,44	0.587
Fledging duration	Light	5.27	1,44	0.029*
	Generation	4.70	1,44	0.038*
	Light x generation	0.35	1,44	0.561

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Table 3: Model output from GLM univariate analysis showing the effects of light condition (12:12 h LD and LL) and generation (P, F1 and F2) on phenotypic and morphometric measures, and hormone levels of male and female zebra finches (*Taeniopygia guttata*). An asterisk indicates a significant ($p < 0.05$) factor.

Table 3A: Body mass, fat score, gonad size and morphometric measures

Parameters	Factors	F	df	p
Male				
Body mass	Light	0.04	1,106	0.84
	Generation	1.60	2,106	0.21
	Light x generation	0.11	2,106	0.90
Fat Score	Light	1.94	1,100	0.19
	Generation	0.72	2,100	0.86
	Light x generation	0.05	2,100	0.95
Testis volume	Light	2.70	1,100	0.10
	Generation	0.31	2,100	0.73
	Light x generation	0.02	2,100	0.98
Wing length	Light	0.01	1,106	0.92
	Generation	3.11	2,106	0.049*
	Light x generation	0.99	2,106	0.38
Tarsus length	Light	0.96	1,106	0.33
	Generation	3.41	2,106	0.037*
	Light x generation	3.71	2,106	0.028*
Tail	Light	0.24	1,106	0.62
	Generation	0.08	2,106	0.93
	Light x generation	0.72	2,106	0.44
Beak	Light	0.82	1,106	0.37
	Generation	0.36	2,106	0.70
	Light x generation	0.22	2,106	0.81
Cheek patch	Light	7.84	1,85	0.008**
	Generation	6.76	2,85	0.004**
	Light x generation	0.99	2,85	0.38
Female				
Body mass	Light	0.73	1,98	0.393
	Generation	2.58	2,98	0.09
	Light x generation	0.89	2,98	0.41
Fat Score	Light	7.52	1,95	0.007**
	Generation	0.38	2,95	0.68
	Light x generation	1.06	2,95	0.35
Follicular diameter	Light	0.48	1,98	0.57
	Generation	0.06	2,98	0.94
	Light x generation	0.13	2,98	0.88
Wing length	Light	0.01	1,98	0.93
	Generation	2.48	2,98	0.09
	Light x generation	0.08	2,98	0.93
Tarsus length	Light	1.97	1,98	0.16
	Generation	4.13	2,98	0.019*
	Light x generation	4.47	2,98	0.014*
Tail	Light	3.91	1,98	0.05*
	Generation	6.88	2,98	0.42
	Light x generation	1.21	2,98	0.30

Beak	Light	0.61	1,98	0.44
	Generation	3.47	2,98	0.035*
	Light x generation	1.43	2,98	0.24

* p < 0.05; ** p < 0.01; *** p < 0.001

Parameters	Factors	F	df	p
Estradiol	Light	9.49	1,49	0.004***
	Generation	1.27	2;49	0.291
	Light x generation	0.05	2,49	0.956
Testosterone	Light	20.48	1,69	<0.0001***
	Generation	1.45	2;69	0.241
	Light x generation	2.71	2,69	0.074
Male corticosterone	Light	5.25	1,58	0.026*
	Generation	2.75	2;58	0.073
	Light x generation	0.13	2,58	0.880
Female corticosterone	Light	0.02	1,62	0.903
	Generation	0.38	2;62	0.687
	Light x generation	0.31	2,62	0.736

Table 3B: Plasma levels of estradiol, testosterone and corticosterone hormones

* p < 0.05; ** p < 0.01; *** p < 0.001

Table 4A: Linear Mixed Effect Model (LMEM) output showing the effect of light condition (12:12 h LD and LL) and time (before and after the light exposure) on song structure and mean and variance values of spectral features of song motif of male P birds. An asterisk indicates a significant ($p < 0.05$) factor.

Parameters	Factors	F	df	p
Bout duration	Light	0.93	1,26	0.346
	Time	0.02	1,26	0.969
	Light x Time	0.23	1,26	0.635
Motifs per bout	Light	0.34	1,26	0.563
	Time	0.57	1,26	0.459
	Light x Time	0.01	1,26	0.936
Mean motif duration	Light	1.34	1,26	0.257
	Time	15.86	1,26	<0.001***
	Light x Time	14.92	1,26	0.001***
Mean amplitude	Light	5.88	1,26	0.023*
	Time	0.90	1,26	0.352
	Light x Time	1.20	1,26	0.284
Mean pitch	Light	0.73	1,26	0.401
	Time	0.39	1,26	0.537
	Light x Time	1.01	1,26	0.325
Mean mean frequency	Light	0.34	1,26	0.563
	Time	4.54	1,26	0.043*
	Light x Time	5.83	1,26	0.023*
Mean peak frequency	Light	0.19	1,26	0.666
	Time	4.08	1,26	0.054
	Light x Time	5.83	1,26	0.023*
Mean goodness	Light	1.09	1,26	0.305
	Time	2.15	1,26	0.154
	Light x Time	1.81	1,26	0.190
Mean FM	Light	0.04	1,26	0.847
	Time	15.67	1,26	0.001***
	Light x Time	17.83	1,26	<0.001***
Mean AM	Light	2.02	1,26	0.167
	Time	3.39	1,26	0.077
	Light x Time	1.37	1,26	0.252
Mean entropy	Light	0.182	1,26	0.673
	Time	8.201	1,26	0.008**
	Light x Time	8.080	1,26	0.009**
Variance amplitude	Light	1.42	1,26	0.710

	Time	33.34	1,26	<0.001***
	Light x Time	31.85	1,26	<0.001***
Variance pitch	Light	0.11	1,26	0.744
	Time	0.02	1,26	0.965
	Light x Time	0.09	1,26	0.764
Variance mean frequency	Light	0.09	1,26	0.766
	Time	0.92	1,26	0.763
	Light x Time	0.97	1,26	0.575
Variance peak frequency	Light	5.51	1,26	0.027*
	Time	1.31	1,26	0.262
	Light x Time	1.45	1,26	0.239
Variance goodness	Light	0.76	1,26	0.390
	Time	3.53	1,26	0.072
	Light x Time	2.31	1,26	0.141
Variance FM	Light	1.69	1,26	0.205
	Time	6.02	1,26	0.021*
	Light x Time	2.16	1,26	0.154
Variance AM	Light	0.63	1,26	0.436
	Time	10.91	1,26	0.003**
	Light x Time	16.21	1,26	<0.001***
Variance Entropy	Light	23.83	1,26	<0.001***
	Time	39.41	1,26	<0.001***
	Light x Time	42.76	2,18	<0.001***

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Table 4B: Model output from GLM univariate analysis of vocal imitation of male zebra finch (*Taeniopygia guttata*) song by their progenies; vocal imitation of P birds by F1 progeny and vocal imitation of F1 birds by F2 progenies under two light conditions (12:12 h LD and LL). An asterisk indicates a significant factor effect ($p < 0.05$).

Parameters	Factors	F	Df	p value
% Similarity	Light	5.44	1,44	<0.02*
	Generation	1.31	2,44	0.26
	Light x generation	0.26	2,44	0.65
% Accuracy	Light	5.49	1,44	0.021*
	Generation	2.00	2,44	0.14
	Light x generation	2.36	2,44	0.09
Entropy difference	Light	0.67	1,44	0.42
	Generation	2.70	2,44	0.11
	Light x generation	0.11	2,44	0.72
Goodness difference	Light	3.00	1,44	0.09
	Generation	1.32	2,44	0.26
	Light x generation	2.94	2,44	0.09
Pitch difference	Light	2.28	1,44	0.014*
	Generation	8.54	2,44	0.002**
	Light x generation	0.06	2,44	0.94
AM difference				
	Light	4.03	1,44	0.03*
	Generation	15.23	2,44	<0.001***
	Light x generation	0.02	2,44	0.88

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Figure S1

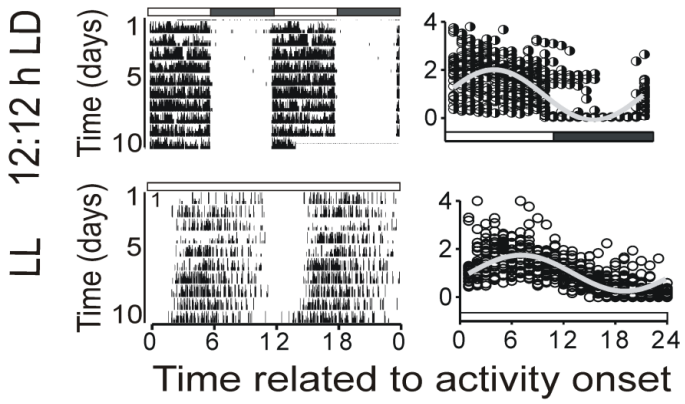


Table: Mean ± SE cosine waveform parameters of female activity rhythms of zebra finches (*Taeniopygia guttata*) under 12:12 h light:dark (LD) and constant light (LL) environment.

Parameter	Light condition	Mesor	Amplitude	Acrophase
Female activity	12:12 h LD	0.96 ± 0.027	1.22 ± 0.05	5.28 ± 0.39
	Constant LL	0.96 ± 0.027	0.95 ± 0.06	7.34 ± 0.30

Mesor = mean value; amplitude = maximum extent of peak; acrophase = time of estimated peak

Fig. S1. 24-hour activity-rest pattern of adult F1 female zebra finches (*Taeniopygia guttata*) under the no-night (constant light, LL) environment, with controls under 12 h day: 12 h night (12:12 h LD). The curves passing through individual data points indicate a significant 24-h rhythm, as determined by cosinor rhythm analysis.

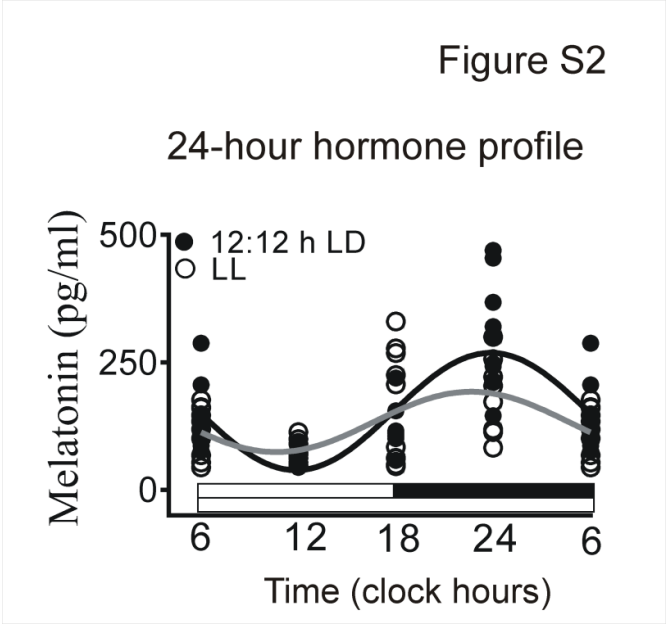


Table: Mean ± SE cosine waveform parameters of melatonin rhythm of zebra finches (*Taeniopygia guttata*) under 12:12 h light:dark (LD) and constant light (LL) environment.

Parameter	Light condition	Mesor	Amplitude	Acrophase
Melatonin	12:12 h LD	153.70 ± 10.85	115.00 ± 15.88	17.85 ± 0.47
	Constant LL	133.4 ± 9.53	59.15 ± 14.31	16.65 ± 0.81

Mesor = mean value; amplitude = maximum extent of peak; acrophase = time of estimated peak

Fig. S2. 24-hour pattern of plasma melatonin levels adult F1 zebra finches (*Taeniopygia guttata*) under the no-night (constant light, LL) environment, with controls under 12 h day: 12 h night (12:12 h LD). The curves passing through individual data points indicate a significant 24-h rhythm, as determined by the Cosinor rhythm analysis.

Figure S3

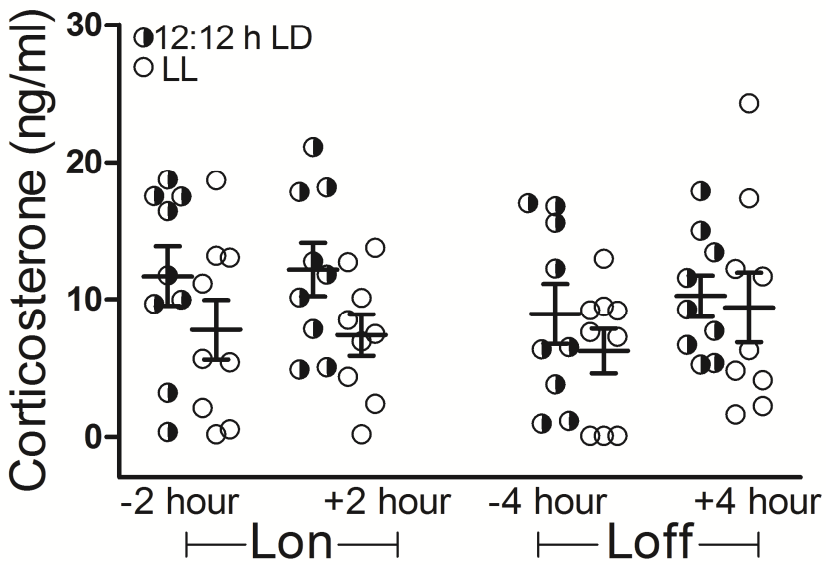


Fig. S3. Plasma corticosterone levels (ng/ml) in F1 zebra finches (*Taeniopygia guttata*) under constant light (LL) environment, with controls under 12 h day: 12 h night (12:12 h LD), in blood samples taken 2 h before and after the light on (Lon), and4 h before and after the light off (Loff).