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RESEARCH ARTICLE

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

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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 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 with controls under 12 h light:12 h darkness (12 h:12 h LD), both P and F1 pairs showed a compromised reproductive success, as evidenced by fewer fledglings and fewer viable offspring with longer fledging durations, and increased offspring mortality with three successive clutches under LL. The overall negative effect of the no-night environment was increased in the F1 generation. As compared with P pairs, F1 pairs 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 hormone (testosterone in males and oestradiol 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 insight into the possible impact on physiology of animals whose surrounding environment is consistently losing the darkness of night.

KEY WORDS: Constant light, Biological clock, Reproduction, Song, *Taeniopygia guttata*

INTRODUCTION

Synchronization of the endogenous biological clock with the 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 is active at night and sleeps (or is inactive) during the day.

Disruption in the cyclic feature of the LD environment, for example via artificial light at night (ALAN) can have disruptive effects on 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 secretion, enzyme activity, metabolism), and higher brain functions such as 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 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 light-polluted sky (Falchi et al., 2016).

In birds, ALAN has been shown to affect the circadian time keeping. This is particularly evidenced by ALAN-induced effects on mRNA oscillations of genes forming the core transcription–translation 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 sleep–wake cycle, activity, foraging and singing activity 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; Kupprat et al., 2020). ALAN also affects metabolism (Batra et al., 2020), immune functions (Saini et al., 2019; Ziegler et al., 2021), mood states, and personality and cognitive performance, induces depressive-like symptoms (Taufique and Kumar, 2016; Jha and Kumar, 2017), and impacts reproduction (Kempenaers et al., 2010; Dominoni et al., 2013b, 2020). The effects can be long lasting, and may even show up in the subsequent generation (Jha and Kumar, 2017).

The question that we addressed is does exposure to LL (a no-night environment) have negative effects on reproduction of the adult parent, and of offspring 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 is a natural non-model experimental system to assess the consequences of chronic effects of perturbation of the light environment on reproduction and on fitness and survival of parents and offspring (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 long-term LL exposure on reproduction-associated responses between three successive clutches of parent (P) pairs, and changes since birth in F1 and F2 progeny. In particular, we measured the LL-induced effect on reproduction-associated changes and reproductive success of adults, and morphometric changes in their offspring. For example, we considered changes in phenotype and body size indicative of overall body condition, and

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the plasma levels of testosterone and cheek patch ratio in males and oestradiol levels in females 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 regions of the developing brain that are susceptible to an external influence. Additionally, 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 a measure of stress under LL.

MATERIALS AND METHODS

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, *Taeniopygia guttata* (Vieillot 1817), from our colony-bred stock maintained under 12 h light:12 h darkness (hereafter LD) and 24±2°C temperature. The experiments used 4 month 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, 42×30×54 cm) and kept them in separate rooms so that there was no visual or acoustic contact between birds of the opposite sex. This acclimated birds to a caged condition, and broke any pair (male–female) bonds that they might have established in the 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 gonadal state; gonad size 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 egg white was given as a protein supplement; both food and water were replenished only during daylight hours.

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 two perches and a rectangular nest box (9×6×11 cm) with a front opening (5×5 cm, 2 cm above floor). Then, they were exposed to either LL (i.e. no-night; L=150±5 lx; N=16) or remained on LD (lights on 06:00 h, lights off 18:00 h; N=16; L=150±5 lx), as before and served as a control. 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. The male remained alone in the cage for a week, and we then introduced a female, which had been housed in another room under LD and identical temperature conditions. We allowed 1 week acclimation of the male to its cage and nest box, and this in our experience was a sufficient duration for zebra finches to enthusiastically receive their mate. Housing a male first and introducing a potential female mate later is also consistent with the idea that social isolation followed by pairing increases courtship; isolated male zebra finches exposed to a new female demonstrate 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 a nest for the duration required for a pair to produce three clutches or for 24 weeks (Fig. 1, top).

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 the same light treatment (LL or LD) as their parents. Although the fledglings would hop in and out of the nest box, they spent a considerable amount of time on perches inside the cage. Our visual observation during the daytime suggested that fledged offspring rarely occupied the nest box; notably, the fledglings were never in complete darkness as even the rear end of the next box had shaded illumination. When the F1 progeny reached adulthood, they were again randomly paired (1 male and 1 female) and kept in separate breeding cages in the respective light conditions in which they were born (N=8 pairs each in LD or LL). Here, they lived together until they produced and nurtured a clutch of F2 adults or for 15 weeks, after which the experiment was terminated even if a few pairs had failed to produce an egg (Fig. 1, top).

Thus, we had three (P, F1 and F2) generations of birds at the end of the experiment. Various parameters associated with the 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 male zebra finches of all three generations.

Reproductive performance

Reproductive success was monitored until all 16 P pairs had produced three clutches or had been housed together for 24 weeks, whichever was earlier, as not all pairs laid three clutches; for example, a few LL females did not lay eggs at all. Likewise, we monitored the reproductive success of eight F1 pairs until they had one clutch or had been housed together for 15 weeks, whichever was earlier. Under LL, several F1 pairs did not engage in reproduction, as suggested by no eggs being laid by some females during 15 weeks of exposure. For monitoring egg laying, both cage and nest box were inspected daily between hour 3 and 5 of the lights-on period (09:00 h–11:00 h).

Reproductive performance was recorded pairwise, as follows. The laying of the first egg marked the initiation of breeding, and 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 also recorded the number attempts they made to build their nest. The length and breadth of each egg were measured to the nearest 0.1 mm using a Vernier calliper. The egg clutch was considered complete if no new eggs were added for 3 consecutive days. Whereas P pairs were allowed to breed until they laid three clutches, F1 pairs were only allowed to breed 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 number of eggs laid, clutch size, the number of hatchlings, fledglings and viable offspring, and mortality. We also recorded the number of days that a hatchling took to fledge (fledging duration) (Fig. 1, bottom). From these parameters, we calculated reproductive success (total

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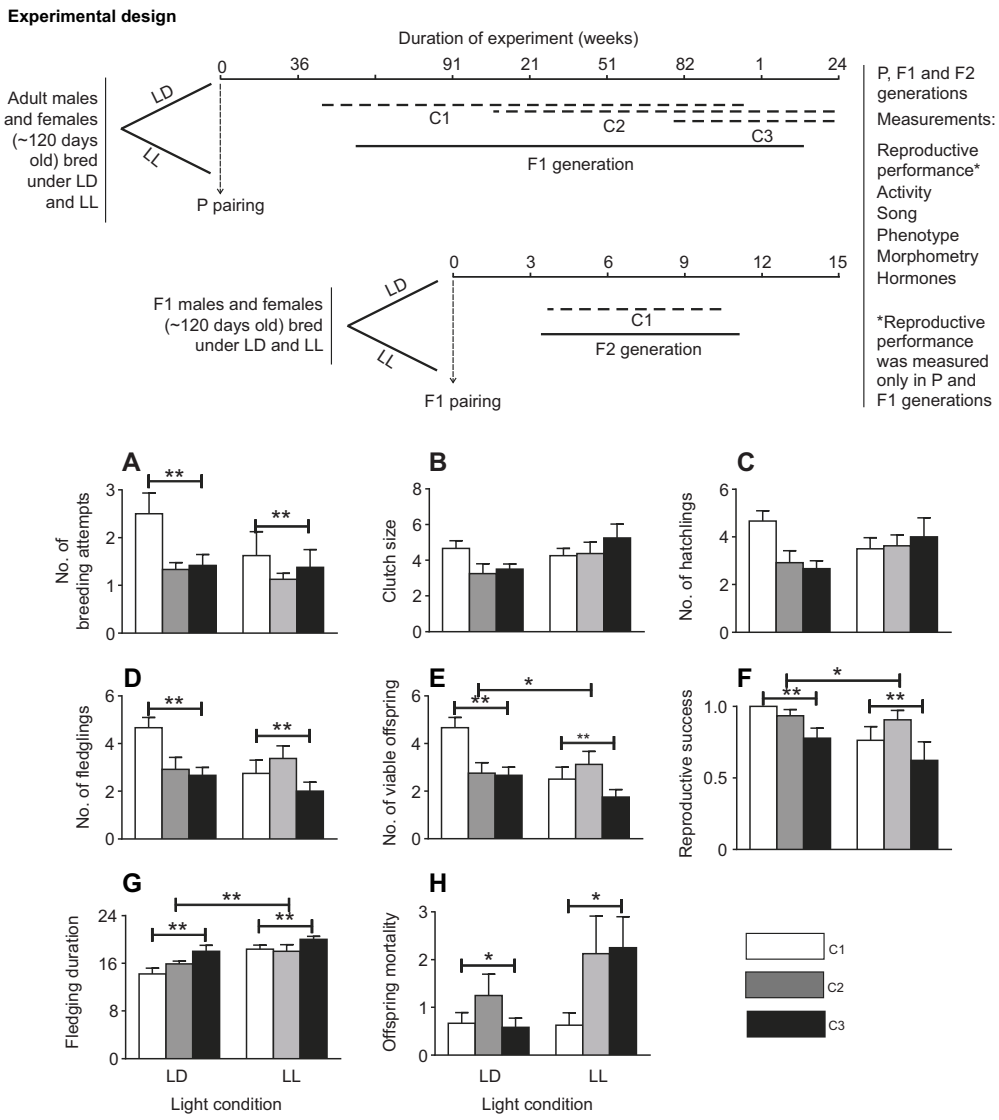

Fig. 1. Experimental design and measurement of reproductive performance of adult zebra finches. Top: adult male and female zebra finches (*Taeniopygia guttata*) that were housed in same-sex cages under a 12 h light:12 h dark (LD) photoperiod at 24±2° C were randomly paired and subjected to constant light (LL) or remained under LD, as before (controls; n=16 pairs per light condition), until they produced and raised three clutches (C1, C2 and C3) or for 24 weeks, whichever was earlier. Similarly, F1 progeny, when adult, were paired and exposed to LL or LD (n=8 pairs per light condition), as before, until they had produced and raised the first clutch (C1) or for 15 weeks, whichever was earlier. The dashed line underneath the clutch number indicates the time range (days) when pairs laid eggs that formed a clutch. The 24 h activity–rest pattern, song characteristics, male phenotype, morphometry and hormone levels of parent (P) generation birds were measured at the end of the breeding protocol, and of F1 and F2 progeny 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. Bottom: the reproductive performance of three successive clutches (C1–3) of adult zebra finches (P generation). Data are plotted as means±s.e.m. for number of breeding attempts (A), clutch size (B), number of hatchlings (C), number of fledglings (D), number of viable offspring (E), overall reproductive success (F), fledging duration (G) and offspring mortality (H). Asterisks indicate a significant difference (*P<0.05, **P<0.01), as revealed by the linear mixed effect model (LMEM) test.

number of fledglings) of each pair, and assessed and compared the reproductive performance of P pairs that raised all the three clutches. For the P generation, the overall reproductive success was the sum total number of fledglings of C1, C2 and C3. For the F1 generation, it was the total number of fledglings of C1 as they raised only one clutch.

Phenotypic and morphometric measurements

Adult physiology and morphology

The morphometric measures of P generation male and female zebra finches were taken at the time of their recruitment for 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. Body fat was recorded using a subjective criterion with a score of 0–3 (0=no subcutaneous fat, 1=light fat deposits underlying musculature, 2=heavier fat deposits underlying musculature, 3=copious fat deposits over several regions). The size of the testes and ovarian follicle was assessed by laparotomy, as routinely carried out 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 mass). The gonads were located by making a small incision between the last two ribs on the left flank of the bird. The dimensions 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 was quickly completed, with minimum pain or suffering (if any), and the bird was returned to its cage on a warm pad, which was removed after the bird recovered, after about 30 min. These birds were fully active and began perch hops in about 1 h. A veterinarian checked these birds the following day. We did not have mortality due to the laparotomy procedure in this experiment.

We also measured the length of the wing, tarsus, beak (bill) and tail of both male and female birds to the nearest 0.1 mm by a Vernier calliper (Sutherland et al., 2004). Wing length was the distance from

the carpal joint to the tip of the longest primary feather in a closed wing. Tarsal length was strictly the length of the tarsometatarsus, and was measured such that the tibiotarsus and tarsometatarsus bones formed an acute angle. Similarly, the length of the beak was measured from its base in the skull to the tip, and tail length was measured from the tip of the tail to the base of the feathers.

Adult male cheek patch

As a measure of sexually selected 'ornaments' in male birds, we quantified male cheek patch size and its coloration. For this, male birds (aged 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 Adobe Photoshop that allows for the selection of consistently coloured area. The 'histogram' function was used to analyse the number of pixels within the marked area. For this study, we calculated cheek patch area relative to the beak of the bird; for each bird, we made three independent measurements for each side of the head, which we averaged to get the final cheek patch ratio. We report cheek patch ratio instead of absolute cheek patch size as, despite our best efforts, the photographs may have differed slightly in distance from the subject because of 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).

Measurement of plasma hormone levels

We measured plasma levels of testosterone, oestradiol and corticosterone in blood samples collected early in the day (hour 0–0.5, where hour 0=lights on) from birds of all three (P, F1 and F2) generations. In each bleed, we collected 50–100 µl blood into a heparinized capillary tube by puncturing the wing vein, 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 (see Mishra and Kumar, 2019). Each hormone assay was carried out as briefly described below.

Testosterone

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

Oestradiol

Oestradiol concentration was measured in plasma of female birds using an enzyme immunoassay kit (Estradiol EIA Kit, Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions (Ubuka et al., 2014). Briefly, 100 or 50 µl of EIA buffer was added to NSB and Bo wells in a 96-well plate, respectively; 50 µl of standards and samples was added to respective wells. Then, 50 µl of oestradiol-acetylcholinesterase tracer was added to all wells, except TA and blank wells. Next, 50 µl of oestradiol antiserum was added to each well, except the TA, NSB and blank wells. The plates was incubated at room temperature for 1 h on an orbital shaker (200 rpm), then washed 5 times with washing buffer. Thereafter, 200 µl of Ellman's reagent was added. The plate was incubated at room temperature in the dark on an orbital shaker (200 rpm). The assay developed optimal colour (i.e. Bo wells ≥0.3 OD) in 1 h. OD was read at 405 nm with a SpectraMax M2e microplate reader (Molecular Devices LLC). The intra-assay variability was 5.2%.

Corticosterone

An enzyme immunoassay kit (ADI-901-097, Enzo Life Sciences) was used to measure plasma corticosterone levels in both male and female birds. In addition, we measured plasma corticosterone levels in F1 birds before and after lights on and off; for this, blood samples were collected 2 h before and after lights on as well as 4 h before and after lights off. The assay was run in 96-well plates, as per the manufacturer's protocol (Wada et al., 2008). Assays were run in duplicate with 10 µl of plasma samples at 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 was added to the standard and sample wells, respectively, and 50 µl of assay buffer was added to the NSB wells to make up the volume, then 50 µl blue conjugate (alkaline phosphate conjugated with corticosterone) added to each standard and sample well. Then, 50 µl of antibody was added to each well, except the blank, TA and NSB wells, and incubated for 2 h at room temperature on a shaker at 500 rpm. Post-incubation, the plate was washed 3 times with 400 µl of wash buffer. A 5 µl sample of conjugate was added to TA wells and 200 µl pNpp substrate solution was pipetted into each well. The plate was again incubated for 1 h at room temperature without shaking. The reaction was stopped by adding 50 µl of stop solution, and OD was read at 405 nm with a SpectraMax M2e microplate reader (Molecular Devices LLC). The corticosterone concentration of experimental samples was calculated with reference to values in the standard curve drawn from the OD of standard samples. The intra-assay variability was 9.5%.

Melatonin

Plasma melatonin concentration (pg ml^{-1}) was measured by ELISA using a specific melatonin kit (RE54021, IBL International GmbH, Hamburg, Germany). Melatonin levels were assayed in blood samples taken from F1 birds at 6 h time intervals, beginning from lights on in LD (Zeitgeber time, ZT: 0, 6, 12 and 18). Melatonin was extracted and assayed from plasma, as per the manufacturer's protocol and instructions, using a procedure standardized and

routinely used in our laboratory (see Singh et al., 2012; Taufique et al., 2018). The dried pellet obtained from extraction was reconstituted in 0.15 ml of bi-distilled water and vortexed for 1 min, and used immediately in the assay. First, 50 µl of sample with 50 µl of melatonin antiserum (rabbit, polyclonal) was incubated in a 96-well plate for 20 h at 4°C. Then, 150 µl of freshly prepared enzyme conjugate was added and incubated for 2 h at 25°C with shaking (500 rpm). Thereafter, 200 µl of freshly prepared 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. OD was read at 405 nm with a SpectraMax M2e microplate reader (Molecular Devices LLC), using 650 nm wavelength as a reference. The melatonin concentration of experimental samples was calculated with reference to values in the standard curve drawn from the OD of standard samples. The intra-assay variability was 5.8%.

Monitoring of 24 h activity

As a behavioural marker of the LL effects, the 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 a 10 day period at the end of the experiment for the P generation, and after the F1 and F2 generations had reached sexual maturity, as per the 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, DSC, Canada) mounted on the cage continuously monitored the birds' 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, CT, USA). For a better visual resolution, we obtained a double-plotted activity record (actogram), wherein activity on successive days 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 report data on activity–rest for F1 generation females, as data for male birds have already been published (see Jha and Kumar, 2017).

Assay of male song characteristics

We assessed song features 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 a period of about 48 h. Sound was recorded at a rate of 16 bits and 44.1 kHz sampling frequency and sound files were saved as digital audio files in avi format. The song of P males was recorded before and after the experiment, whereas that of F1 and F2 males was recorded when they reached adulthood (>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 analysed song similarity between generations (P versus F1; F1 versus 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 parents and offspring were also calculated from the similarity feature module.

Statistics

Statistical analyses were performed using IBM SPSSv20, GraphPad Prism version 5.0 (GraphPad, La Jolla, CA, USA) and GraphPad Prism version 5.0 320, 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 LD and LL across the three clutches (C1, C2 and C3) or across P and F1 generations. Further, a linear mixed effect model (LMEM) compared the effect of light condition (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 an interaction factor, and individual as the random factor. Here, we compared only those P pairs that raised all three clutches, i.e. 12 pairs in LD and 8 pairs in LL. In cases where factor 2 (clutch sequence) had a significant effect on reproductive variables, we used Bonferroni adjustment to determine whether groups differed significantly from each other. We used Mann–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 analyse the effect of light condition (LD and LL; factor 1) and generation (P and F1; factor 2) on reproductive performance of zebra finches across generations. For this analysis, we used data from the first clutch (C1) only as F1 pairs had completed only the first clutch.

GLM was also used to analyse the effects of light condition (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 (oestradiol, testosterone and corticosterone), and vocal imitation of a 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 analysed the effect of light condition (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 $y=A+B\times\cos(2\pi(x-C)/24)$, where 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, R^2 values and number of predictors (mesor, amplitude and phase: https://www.danielsoper.com/statcalc/calculator.aspx?id=15). For statistical significance, alpha was set at 0.05.

RESULTS

LL negatively affects reproductive performance across generations

There was an overall reduced reproductive performance in both P and F1 generation pairs under LL, compared with the LD condition. The number of P pairs engaged in raising three clutches was significantly reduced in LL, compared with LD controls (Chi-square test: $\chi^2_3=47.52$, $P<0.001$). Over a 24 week period, the reproductive performance of 16 P pairs was as follows: LD 12 pairs produced 3 clutches, 2 pairs produced 2 clutches and 2 pairs produced 1 clutch; LL 8 pairs produced 3 clutches, 3 pairs produced

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2 clutches, 2 pairs produced 1 clutch, and 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: χ^2_1=36.40, P<0.001). Out of 8 F1 pairs that were recruited, all reproduced and raised their first clutch under LD, whereas only 3 out of 8 pairs reproduced and raised their first clutch under LL.

The overall reproductive success of LL parents was lower than that of LD parents (Mann–Whitney U test; P=0.05). We then evaluated the long-term effect of the 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 fewer viable offspring raised, lower reproductive success, and longer fledging duration in LL than in LD (P≤0.05, LMEM and Bonferroni adjustment for multiple comparisons; Fig. 1, Table 1). The effects were increased with progressive clutches, as assessed by the number of breeding attempts, fledglings and viable offspring, fledging duration and offspring mortality. The number of breeding attempts of pairs by the time they had a clutch was in the following order: C1>C2>C3; however, this was significantly different between C1 and 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 number of fledglings and viable offspring raised from C3 was significantly lower than in 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 the interaction of light condition and clutch sequence had a significant effect on clutch size, number of hatchlings and number of viable offspring (P≤0.05, LMEM and Bonferroni adjustment for multiple comparisons; Fig. 1, Table 1). Although there was an overall effect of clutch sequence on offspring mortality, the three clutches did not significantly differ from each other (C1 versus C2: P=0.058; C1 versus C3: P=1.14; C2 versus C3: P=1.00). Whereas the fledging duration of C3 was significantly longer than that of C1 (P=0.004) and C2 (P=0.002), values for C1 and C2 were not significantly different from each other (P=1.00, Bonferroni adjustment for multiple comparisons).

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

Table 1. Linear mixed effect model (LMEM) output showing the effect of light condition and clutch number (C1, C2 and C3) on reproductive performance of parent (P) generation zebra finches (*Taeniopygia guttata*) across the three clutches

Parameters	Factors	*F*	d.f.	*P*
Breeding attempt	Light	1.00	1.18	0.330
	Clutch number	6.25	2.18	0.009**
	Light×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×clutch number	3.86	2.18	0.040*
No. of hatchlings	Light	0.53	1.18	0.477
	Clutch number	2.91	2.18	0.080
	Light×clutch number	6.14	2.18	0.009**
No. of fledglings	Light	3.39	1.18	0.08
	Clutch number	7.88	2.18	0.003**
	Light×clutch number	3.36	2.18	0.058
No. of viable offspring	Light	5.62	1.18	0.029*
	Clutch number	8.27	2.18	0.003**
	Light×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×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×clutch number	2.84	2.18	0.085

Zebra finches were exposed to control (12 h:12 h light:dark, LD) or constant light (LL) conditions. Asterisks indicate a significant difference (*P<0.05, **P<0.01).

LL affects phenotype and morphometric measurements

GLM univariate analysis revealed that there was no effect of the light condition, generation and their interaction (light condition×generation) on fat score (but females showed copious fat deposits under LL), body mass and gonad size (testicular volume and follicular diameter) of zebra finches (Table 3, 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. 3F, Table 3). The LD birds had a higher cheek patch ratio than the LL birds; likewise, F2 birds had a higher cheek patch ratio than F1 birds. The tarsus was smaller in length in F2 than in 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 length (significantly shorter tarsus and beak in F2 than in P generation) of females; however, there was an interaction effect of the light condition × generation on tarsus length of both male and female birds (P≤0.05, GLM univariate analysis; Table 3).

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

Daily activity behaviour 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

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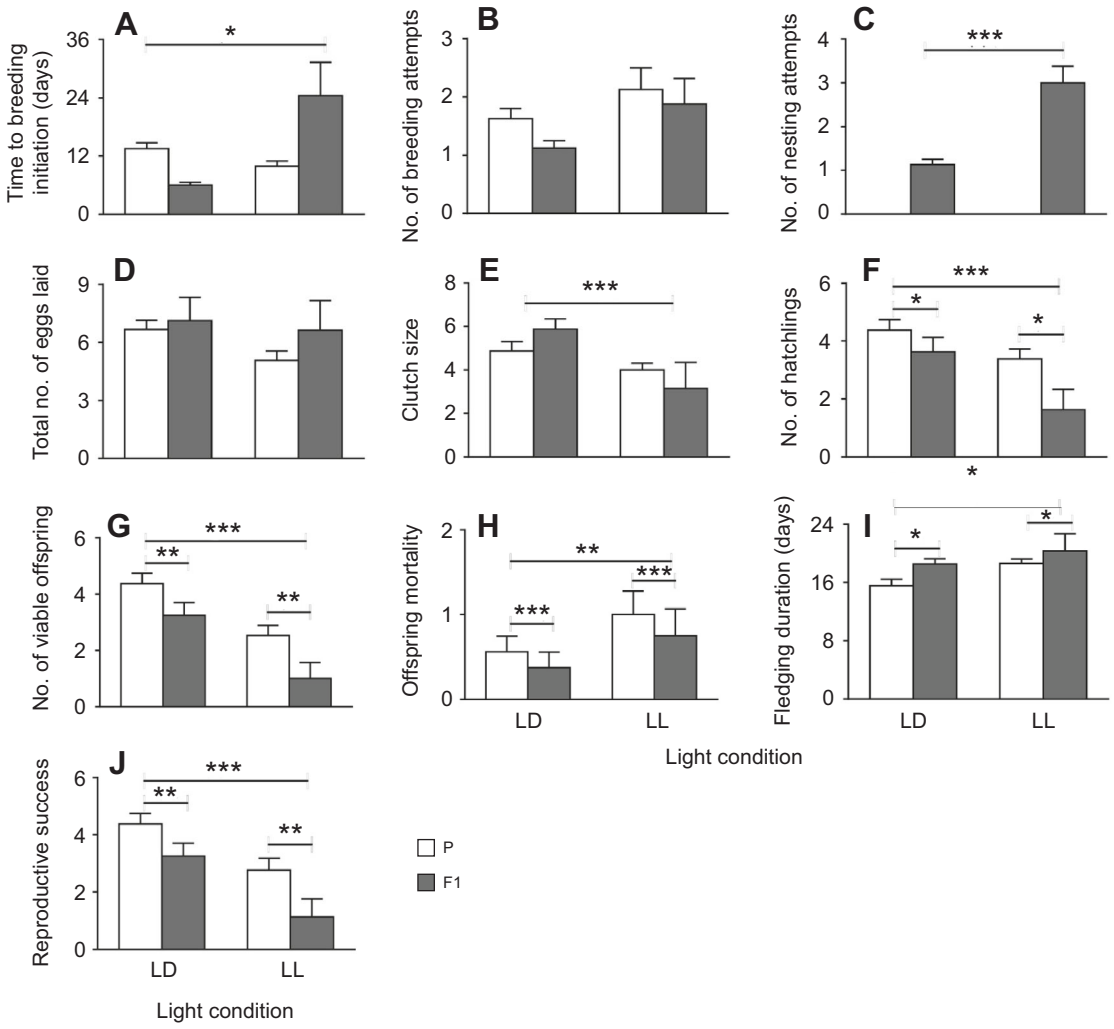

Fig. 2. Reproductive performance of parent (P) and F1 generation zebra finches exposed to LL versus LD. Data are plotted as means±s.e.m. for time to initiation of breeding (A), number of breeding attempts (B), number of nesting attempts (C), total number of eggs laid (D), clutch size (E), number of hatchlings (F), number of viable offspring (G), offspring mortality (H), fledging duration (I) and reproductive success (J). Asterisks indicate a significant difference (*P<0.05, **P<0.01, ***P<0.001), as revealed by the general linear model (GLM) test.

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 LD and LL (Jha and Kumar, 2017). The daily activity pattern of both male and female birds under LD and LL differed 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 oestradiol in females showed a significant effect of the light condition, with levels significantly lower in LL than in the LD condition (P≤0.05, GLM univariate analysis; Fig. 3D,E, Table 3); however, we found no effect of generation or its interaction with light condition. Light condition, generation and their interaction did not affect corticosterone levels in females. Further, in F1 birds, plasma corticosterone levels measured 2 h and 4 h before and after the lights on and lights off, respectively, were not significantly different between LD and LL. The cosinor analysis also revealed a significant daily rhythm of melatonin in both LD and LL conditions, albeit with a reduced amplitude under LL (Fig. S2).

LL affects song characteristics

Song structure and spectral features of male parent songs

There was no significant difference between LD and LL in song structure, i.e. song bout duration and number of motifs per bout both at the beginning and end of the experiment (LMEM; Table 4). 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 4). 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, e.g. mean frequency, FM, entropy, and variance of the amplitude, FM, AM and entropy (LMEM; see Table 4).

Vocal imitation of the parent's song

The vocal imitation of the 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 percentage similarity (P=0.02), percentage accuracy (P=0.021), pitch (P=0.014) and AM (P=0.03), and of generation on pitch (P=0.002) and AM (P<0.001). The percentage similarity and accuracy of the parent and progeny

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Table 2. Model output from general linear model (GLM) univariate analysis showing the effects of light condition (LD and LL) and generation (P and F1) on reproductive performance of zebra finches (*Taeniopygia guttata*)

Parameters	Factors	*F*	d.f.	*P*
Time to initiation of breeding	Light	6.79	1.44	0.013*
	Generation	1.11	1.44	0.298
	Light×generation	16.16	1.44	<0.001***
No. of breeding attempts	Light	3.44	1.47	0.070
	Generation	1.24	1.47	0.272
	Light×generation	0.14	1.47	0.712
No. of nesting attempts	Light	22.98	1.15	<0.001***
Total no. of eggs laid	Light	1.61	1.44	0.211
	Generation	1.43	1.44	0.239
	Light×generation	0.45	1.44	0.505
Clutch size	Light	11.69	1.44	0.001***
	Generation	0.05	1.44	0.832
	Light×generation	3.70	1.44	0.061
No. of hatchlings	Light	10.17	1.44	0.0003**
	Generation	7.16	1.44	0.011*
	Light×generation	1.19	1.44	0.288
No. of viable offspring	Light	21.85	1.44	<0.001***
	Generation	9.28	1.44	0.004**
	Light×generation	0.22	1.44	0.639
Reproductive success	Light	15.85	1.44	<0.001***
	Generation	8.74	1.44	0.005**
	Light×generation	0.31	1.44	0.582
Offspring mortality	Light	11.47	1.44	0.002**
	Generation	28.32	1.44	<0.001***
	Light×generation	0.29	1.44	0.587
Fledging duration	Light	5.27	1.44	0.029*
	Generation	4.70	1.44	0.038*
	Light×generation	0.35	1.44	0.561

Asterisks indicate a significant difference (*P<0.05, **P<0.01, *** P<0.001).

song was lower in LL males. Likewise, under LL, there was a significant increase in pitch difference and decreased AM in progeny, as compared with the parents ($P \leq 0.05$, GLM; Fig. 4 and Table 5).

DISCUSSION

We demonstrate a long-term effect of the no-night environment on reproductive behaviour and associated physiology of diurnal zebra finches. Two conclusions can be made from the present results.

First, the absence of a night component from the day–night environment negatively impacted the reproductive performance of P generation birds that were born under LD and exposed to LL when adult. This was evident by reduced efforts in raising the offspring and enhanced negative impact with the three successive clutches: compared with C1, C3 pairs made fewer breeding attempts, produced fewer fledglings and viable offspring, and took more time to fledge under LL, compared with LD controls. Differential reproductive success also seemed to be related to differences in structure and spectral features of the song, which is a secondary sexual trait, supporting 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 such as mean frequency, FM and entropy. However, notwithstanding with the overall reduced reproductive success, P pairs were found to engage in reproduction activity quicker under LL than under LD, and this we attribute to an accelerated activation of the reproductive axis in response to the acute LL exposure (Sharp, 1993).

Table 3. Model output from GLM univariate analysis showing the effects of light condition (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*)

Parameters	Factors	*F*	d.f.	*P*
Males				
Body mass	Light	0.04	1.106	0.84
	Generation	1.60	2.106	0.21
	Light×generation	0.11	2.106	0.90
Fat score	Light	1.94	1.100	0.19
	Generation	0.72	2.100	0.86
	Light×generation	0.05	2.100	0.95
Testis volume	Light	2.70	1.100	0.10
	Generation	0.31	2.100	0.73
	Light×generation	0.02	2.100	0.98
Wing length	Light	0.01	1.106	0.92
	Generation	3.11	2.106	0.049*
	Light×generation	0.99	2.106	0.38
Tarsus length	Light	0.96	1.106	0.33
	Generation	3.41	2.106	0.037*
	Light×generation	3.71	2.106	0.028*
Tail	Light	0.24	1.106	0.62
	Generation	0.08	2.106	0.93
	Light×generation	0.72	2.106	0.44
Beak	Light	0.82	1.106	0.37
	Generation	0.36	2.106	0.70
	Light×generation	0.22	2.106	0.81
Cheek patch	Light	7.84	1.85	0.008**
	Generation	6.76	2.85	0.004**
	Light×generation	0.99	2.85	0.38
Testosterone	Light	20.48	1.69	<0.0001***
	Generation	1.45	2.69	0.241
	Light×generation	2.71	2.69	0.074
Corticosterone	Light	5.25	1.58	0.026*
	Generation	2.75	2.58	0.073
	Light×generation	0.13	2.58	0.880
Females				
Body mass	Light	0.73	1.98	0.393
	Generation	2.58	2.98	0.09
	Light×generation	0.89	2.98	0.41
Fat score	Light	7.52	1.95	0.007**
	Generation	0.38	2.95	0.68
	Light×generation	1.06	2.95	0.35
Follicular diameter	Light	0.48	1.98	0.57
	Generation	0.06	2.98	0.94
	Light×generation	0.13	2.98	0.88
Wing length	Light	0.01	1.98	0.93
	Generation	2.48	2.98	0.09
	Light×generation	0.08	2.98	0.93
Tarsus length	Light	1.97	1.98	0.16
	Generation	4.13	2.98	0.019*
	Light×generation	4.47	2.98	0.014*
Tail	Light	3.91	1.98	0.05*
	Generation	6.88	2.98	0.42
	Light×generation	1.21	2.98	0.30
Beak	Light	0.61	1.98	0.44
	Generation	3.47	2.98	0.035*
	Light×generation	1.43	2.98	0.24
Oestradiol	Light	9.49	1.49	0.004***
	Generation	1.27	2.49	0.291
	Light×generation	0.05	2.49	0.956
Corticosterone	Light	0.02	1.62	0.903
	Generation	0.38	2.62	0.687
	Light×generation	0.31	2.62	0.736

Asterisks indicate a significant difference (*P<0.05, **P<0.01, *** P<0.001).

These results are thus in agreement with those suggesting negative reproduction-associated effects of ALAN in other songbirds. For example, female blue tits (*Cyanistes caeruleus*)

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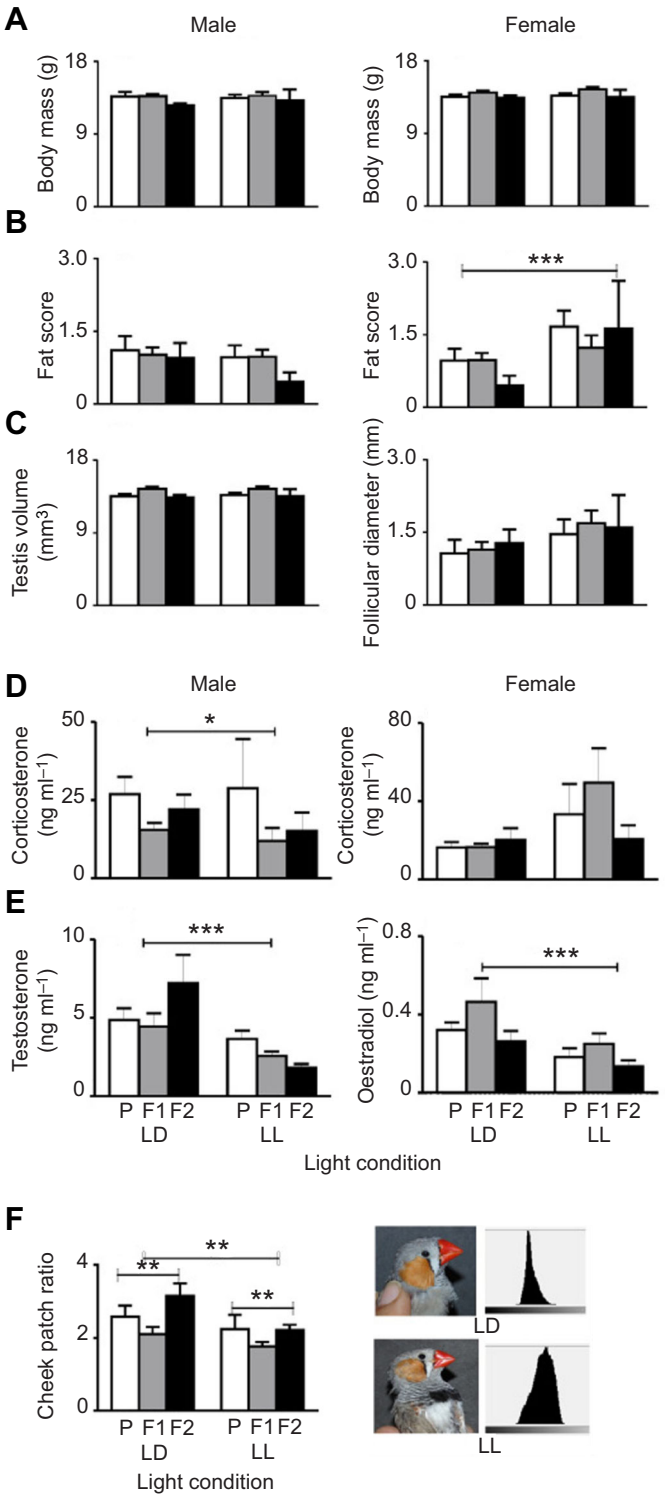

Fig. 3. Body metrics, hormone levels and cheek patch ratio of zebra finches exposed to LL versus LD. (A–C) Changes in body mass (A), fat score (B) and gonad size (testis volume or follicular diameter; C) of male (left) and female (right) zebra finches over three generations (P, F1 and F2) under LL and LD. (D,E) Changes in plasma levels of corticosterone (D) and sex hormones (testosterone or oestradiol; E) of male (left) and female (right) zebra finches over three generations (P, F1 and F2) under LL and LD. (F) Cheek patch ratio of males (a male secondary sexual characteristic; left) over three generations, and representative photograph and corresponding pixel-histogram (right) showing differences under LD and LL conditions. Data are means±s.e.m. Asterisks indicate a significant difference (*P<0.05, **P<0.01, ***P<0.001), as revealed by GLM test.

laid eggs earlier and males obtained more extra pair mates under the influence of street light (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 behaviour and consequently altered the selection pressures on the mating behaviour. Black-tailed godwits (*Limosa limosa*) were also found to have a preference for nesting away from the roadway lighting (Longcore and Rich, 2004). The nocturnal illumination also affected 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 territorial singing behaviour in male mockingbirds (*Mimus polyglottos*; Derrickson, 1988) and American robins (*Turdos migratorius*), with birds starting to sing long before the dawn in areas that were lit by artificial lighting (Miller, 2006). On a similar line, ALAN influenced the dawn singing, extra-pair siring success and laying date in five forest-breeding birds, namely 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 exposure to very dim (0.3 lx) 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 mating behaviour in moths (*Operophtera brumata*; van Geffen et al., 2015), mating calls in male frogs (*Physalaemus pustulosus*; Longcore and Rich, 2004), copulatory behaviour in sexually naive adult male rats (Fantie et al., 1984), structural and functional changes in the pituitary–thyroid axis in female rats (Miler et al., 2014), and reduced reproductive efficiency, reproductive organ mass and sperm count 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 oestradiol levels in males and females, respectively, support this. A similar inhibition of the reproductive hormones was found in western scrub-jays (*Aphelocoma californica*; oestradiol 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 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 showed increased offspring mortality, fewer viable offspring and lower reproductive success under LL, compared with LD controls. Interestingly, the LL environment resulted in poor copying of the parent's song by both F1 and F2 progeny. 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 also consistent with evidence that unpredictable LD cycles can impair learning performance with effects passed on to the F1 generation in chickens (Lindqvist et al., 2007).

We found an overall low plasma level of corticosterone in males (but not females), testosterone in males and oestradiol in females in LL, as compared with that in the LD condition. Sex differences in corticosterone levels, an indicator of the stress response, are similar to those reported in chickens, with males showing higher levels than females (Goerlich et al., 2012). Further, a relatively lower corticosterone level under LL is similar to findings in European starlings (*Sturnus vulgaris*), which showed decreased basal and

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Table 4. LMEM output showing the effect of light condition (LD and LL) and time (before and after light exposure) on song structure and mean and variance values of spectral features of song motif of male P birds

Parameters	Factors	*F*	d.f.	*P*
Bout duration	Light	0.93	1.26	0.346
	Time	0.02	1.26	0.969
	Light×Time	0.23	1.26	0.635
No. of motifs per bout	Light	0.34	1.26	0.563
	Time	0.57	1.26	0.459
	Light×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×Time	14.92	1.26	0.001***
Mean amplitude	Light	5.88	1.26	0.023*
	Time	0.90	1.26	0.352
	Light×Time	1.20	1.26	0.284
Mean pitch	Light	0.73	1.26	0.401
	Time	0.39	1.26	0.537
	Light×Time	1.01	1.26	0.325
Mean frequency	Light	0.34	1.26	0.563
	Time	4.54	1.26	0.043*
	Light×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×Time	5.83	1.26	0.023*
Mean goodness	Light	1.09	1.26	0.305
	Time	2.15	1.26	0.154
	Light×Time	1.81	1.26	0.190
Mean FM	Light	0.04	1.26	0.847
	Time	15.67	1.26	0.001***
	Light×Time	17.83	1.26	<0.001***
Mean AM	Light	2.02	1.26	0.167
	Time	3.39	1.26	0.077
	Light×Time	1.37	1.26	0.252
Mean entropy	Light	0.182	1.26	0.673
	Time	8.201	1.26	0.008**
	Light×Time	8.080	1.26	0.009**
Variance amplitude	Light	1.42	1.26	0.710
	Time	33.34	1.26	<0.001***
	Light×Time	31.85	1.26	<0.001***
Variance pitch	Light	0.11	1.26	0.744
	Time	0.02	1.26	0.965
	Light×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×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×Time	1.45	1.26	0.239
Variance goodness	Light	0.76	1.26	0.390
	Time	3.53	1.26	0.072
	Light×Time	2.31	1.26	0.141
Variance FM	Light	1.69	1.26	0.205
	Time	6.02	1.26	0.021*
	Light×Time	2.16	1.26	0.154
Variance AM	Light	0.63	1.26	0.436
	Time	10.91	1.26	0.003**
	Light×Time	16.21	1.26	<0.001***
Variance entropy	Light	23.83	1.26	<0.001***
	Time	39.41	1.26	<0.001***
	Light×Time	42.76	2.18	<0.001***

Asterisks indicate a significant difference (*P<0.05, **P<0.01, *** P<0.001).

stress-induced corticosterone levels in response to chronic stress (Rich and Romero, 2005), but is inconsistent with that found in response to ALAN in free-living great tits (Grunst et al., 2020). The present results should be considered with caution as we did not measure corticosterone levels after acute and long-term exposure to LL. We reason, though, that the LL environment was not stressful, as there were no overt signs of physiological stress, such as lack of feeding and loss of mass, and decreased mobility or activity under LL. Additionally, the corticosterone levels measured in F1 birds both before and after the time of lights on and lights off were not significantly different between the LD and LL birds (Fig. S3).

Daily patterns of activity (of females) and singing (of males) behaviour also reveal that these were consolidated and rhythmic rather than being scattered over the 24 h period under LL, as also reported previously for male birds (see Jha and Kumar, 2017). F1 female birds were also found to be rhythmic in their 24 h activity–rest pattern, as measured at the end of the experiment irrespective of the light condition they were exposed to, albeit with a relatively 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 the 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 trade off between reproductive performance and non-reproductive aspects; namely, activity and singing behaviour, phenotype and morphometric changes. The overall results on LL-induced phenotypic and morphometric effects suggest that 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 body mass, consistent with no difference in total 24 h activity levels between LD and LL conditions, indicating that the birds were otherwise in a 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 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 investigate the mechanism by which LL affects reproduction, we can make a few postulations nonetheless. First, under LL, the disrupted daily circadian time keeping and consequent mismatch of biological processes led to effects on reproduction-associated events in zebra finches. LL can induce disruption of circadian clock gene oscillations (Prabhat et al., 2020) and overt circadian behaviours such as the 24 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 the 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 influence of the circadian clock 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 circadian clock gene-(e.g. *period 2* and *brain muscle arnt like 1*) deficient mice exhibit irregular oestrus cycles (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

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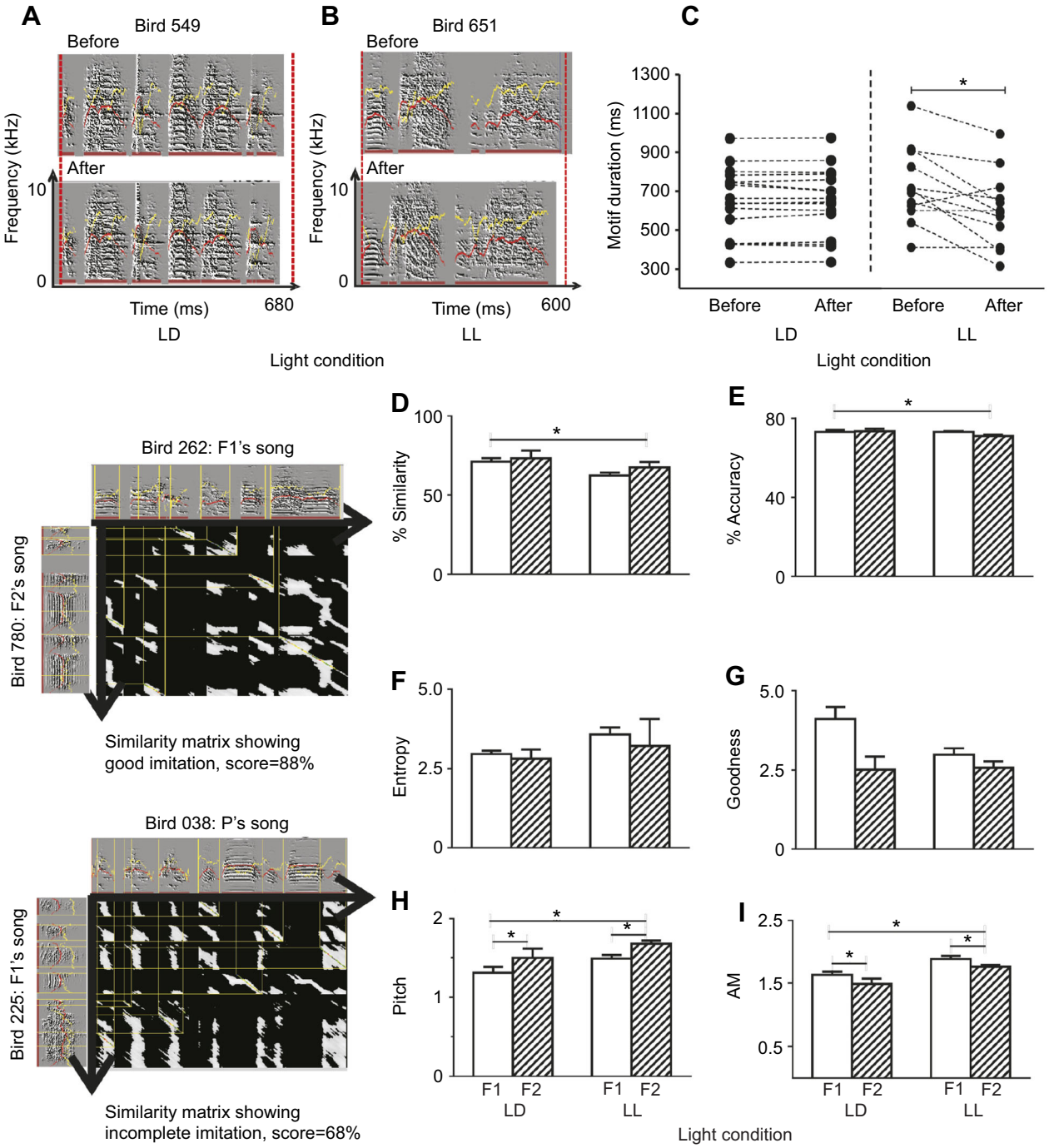

Fig. 4. Song features of parent and offspring zebra finches exposed to LL versus LD. (A,B) Representative song motif of P generation males before and after long-term LD (A) and LL (B) exposure. (C) Mean±s.e.m. song motif duration. (D–I) Similarity between parent and offspring song: mean±s.e.m. percentage similarity (D), percentage accuracy (E) and spectral features, i.e. entropy (F), goodness (G), pitch (H) and amplitude modulation (AM; I) of F1 and F2 generations under the two light conditions. Asterisks indicate a significant difference (*P<0.05) as revealed by LMEM and GLM tests. Similarity matrices with representative copying of the parent's song are shown on the left: F2 copying F1 song (top) and F1 copying P song (bottom). Note the 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.

cognitive test tasks, reduction in exploratory behaviour, attenuated singing bouts in males, and probably reduced involvement in parental care, as reported in our 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 neuronal activity) and tyrosine hydroxylase (the rate limiting enzyme of dopamine biosynthesis) show reduced expression in the 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 the wild zebra finches and other species living 66.5 deg north or

Table 5. Model output from GLM univariate analysis of vocal imitation of male zebra finch (*Taeniopygia guttata*) song by their progeny: vocal imitation of P birds by F1 progeny and vocal imitation of F1 birds by F2 progeny under two light conditions (LD and LL)

Parameters	Factors	*F*	d.f.	*P*
% Similarity	Light	5.44	1.44	<0.02*
	Generation	1.31	2.44	0.26
	Light×Generation	0.26	2.44	0.65
% Accuracy	Light	5.49	1.44	0.021*
	Generation	2.00	2.44	0.14
	Light×Generation	2.36	2.44	0.09
Entropy difference	Light	0.67	1.44	0.42
	Generation	2.70	2.44	0.11
	Light×Generation	0.11	2.44	0.72
Goodness difference	Light	3.00	1.44	0.09
	Generation	1.32	2.44	0.26
	Light×Generation	2.94	2.44	0.09
Pitch difference	Light	2.28	1.44	0.014*
	Generation	8.54	2.44	0.002**
	Light×Generation	0.06	2.44	0.94
AM difference	Light	4.03	1.44	0.03*
	Generation	15.23	2.44	<0.001***
	Light×Generation	0.02	2.44	0.88

Asterisks indicate a significant difference (*P<0.05, **P<0.01, *** P<0.001).

south of the equator never experience a complete loss of night (an LL environment). 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, people exposed to special work environments such as those for night-shifts, intensive care units in hospitals, shopping malls, space flights and vigilance departments experience at night a light environment that almost mimics the daytime in terms of its physiological effects. In several cases, the lighted environment is illuminated at a level higher than the ∼150 lx 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 the ground for future research with implications for the public health.

Competing interests

The authors declare no competing or financial interests.

Author contributions

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

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