# Title: Why early birds sing early: rebound singing from nighttime suppression can explain dawn chorus

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**Abstract:**

Many birds sing intensively at dawn, a phenomenon known as the "dawn chorus," yet its cause remains unclear. This is largely due to prior research being limited primarily to ecological studies of wild birds. We investigated the dawn chorus under controlled laboratory conditions using an animal model, the zebra finch. We found that birds became hormonally aroused in the dark long before initiating dawn singing, and that suppression of spontaneous singing by darkness enhanced their intrinsic motivation to sing. This elevated motivation subsequently triggered a rebound of intensive singing at dawn. Moreover, dawn singing accelerated refinement of song structure, supporting its role as intensive vocal exercise to optimize song performance after overnight silence. These findings provide mechanistic and functional explanations for the dawn chorus.

**Main Text:**

The dawn chorus (DC), the intensive singing in many bird species early in the morning, has been recognized as one of the most prominent biological indicators of daybreak since ancient times. The DC is observed daily, mostly during the breeding season and in most breeding habitats around the world(*1*, *2*), fascinating the general public as well as birders and researchers. Despite the ubiquity and prominence of the DC, however, its functions and underlying mechanisms remain surprisingly poorly understood. To date, at least 10 different hypotheses have been proposed to explain why birds produce the DC, but no consensus has yet been reached. Although there is some evidence for each of these hypotheses, it is mostly indirect. This is largely because most studies of the DC are conducted in wild birds, where precise controls and systematic manipulations of experimental conditions are difficult. Additionally, most research has focused on the adaptive functions of the DC (i.e. ultimate causes), leaving the behavioral and physiological mechanisms (i.e. proximate causes) of the DC largely unexplored. To address these issues, we conducted experiments on laboratory-bred zebra finches, a model organism widely used in life science research(*3*). Unlike previous studies of the DC, we controlled temperature, food availability, social environment, and age, and systematically manipulated lighting conditions in the morning to investigate the effects of morning light on the DC.

## Results

### *Captive zebra finches exhibit DC-like intensive singing when morning lights-on time is delayed.*

Male zebra finches housed alone in sound recording chambers spontaneously produce hundreds of songs per day (referred to as “undirected singing”) under light conditions, but virtually no songs under complete darkness(*4*). A previous study demonstrated that temporarily suppressing undirected singing by turning off the lights during the daytime induces intensive singing immediately after the lights are turned on and the suppression is lifted(*5*). Since this suppression-driven intensive singing resembles a typical DC, we hypothesized that this suppression-driven mechanism may underlie the DC. As the first step to test this hypothesis, we examined whether suppression-driven intensive singing occurs even in the morning following the dark period at night in captive birds. In particular, given that the magnitude of suppression-driven singing strongly depends on the duration of suppression(*5*), we predicted that extending or shortening the night period by delaying or advancing the morning lights-on time (LT) relative to the regular LT would enhance or attenuate morning singing activity, respectively (Fig. 1A). Although birds exhibited slight increases in singing rate relative to baseline even after the regular LT (0h LT in Fig. 1A-D & S1), these increases were markedly enhanced when the LT was delayed by 3 hours (+3 h LT in Fig. 1A–D & S1, Movie S1), resulting in intensive singing resembling the DC. In contrast, almost no transient increases in singing rate were observed when the LT was advanced by 3 hours (−3 h LT in Fig. 1A–D, Movie S2). Moreover, birds began singing sooner after the delayed LT than after the advanced LT (Fig. 1E). These results

demonstrate that DC-like intensive singing can be induced in socially-isolated songbirds simply by delaying the morning LT.

***Birds begin to sing in dimmer light conditions when LT is delayed.***

In addition to intensive singing in the morning, the early onset of singing under dim light at dawn is another key feature of the DC in wild birds and has given rise to various hypotheses regarding its underlying causes(*1*, *2*). We found that such early singing onset under dim light conditions can also be induced by delaying the morning LT. We modified the morning lighting paradigm to mimic natural dawn conditions: Instead of the abrupt lighting used in the previous experiment, the light level was gradually increased over a 2-h period (see Methods), and this "gradual lighting period" was either delayed or advanced by 3 h relative to the regular LT (Fig. 2A-B). When the gradual lighting period was delayed by 3 h (+3h LT), birds began singing earlier within the gradual lighting period compared to when the gradual lighting period was advanced by 3 h (-3h LT) (Fig. 2B-D), indicating that singing initiation occurred under lower light levels when the morning LT was delayed. Taken together, these results suggest that delaying the LT can induce both singing onset under dim light conditions and intensive singing, which are two major features of the DC.

***Birds wake up long before the delayed LT and their motivation to sing increases while spontaneous singing is being suppressed by darkness.***

Why do birds exhibit DC-like singing when the LT is delayed? Given that a similar pattern of intensive singing occurs following temporary singing suppression by lights-out during the daytime(*5*), we hypothesized that birds are mostly awake during the dark period before the delayed LT and and that their motivation to sing increases while singing is being suppressed by darkness, subsequently producing intensive singing as a 'rebound' from the suppression. To test this idea, we monitored the birds' movements in darkness before the LT (with the abrupt lighting paradigm described in Fig. 1A) using infrared cameras mounted above individual cages (Fig. 3A-B). We found that birds moved actively during the 3-h period prior to the delayed LT (+3h LT in Fig. 3C-E, Movie S3), but exhibited little movement before the advanced LT (-3h LT in Fig. 3C-E, Movie S4), providing evidence that birds are mostly awake during the dark period before the delayed LT but not before the advanced LT.

Although these results suggest higher arousal levels of the birds before the delayed LT, it does not necessarily indicate that they are highly motivated to sing at that time. To assess the singing motivation in the dark period before the LT more directly, we trained the birds to press a lever that triggered a 10-sec period of lighting (Fig. 3F, Movie S5). Since zebra finches sing almost exclusively under light conditions and not in darkness(*4*), the frequency of lever presses are likely to reflect, at least to some extent, the levels of singing motivation as well as the arousal levels. When the LT was delayed or advanced by 3 h, birds frequently pressed the lever and sang before the +3h LT, but only rarely before the -3h LT (Fig. 3G-I). These results support the idea that birds are highly motivated to sing in the dark before the delayed LT but not before the advanced LT. Given that suppression of undirected singing by darkness during the daytime

increases intrinsic singing motivation and results in intensive rebound singing immediately after the suppression is lifted(*5*), these results provide evidence that DC-like singing is driven by a rebound mechanism from dark-induced singing suppression before the delayed LT.

We also investigated the hormonal mechanisms underlying early awakening before the delayed LT. In zebra finches, plasma concentrations of melatonin, a hormone that regulates the sleep-wake cycle in vertebrates(*6*), are relatively high during the night and drop sharply to daytime levels approximately 0.5–2 hours before the regular LT(*7*). This pre-dawn drop in melatonin levels roughly coincides with the early awakening of birds before the delayed LT. Based on these findings, we hypothesized that birds wake up long before the delayed LT due to hormonal regulation including the early melatonin drop but are suppressed from singing by darkness, leading to an increase in singing motivation and eventually DC-like singing immediately after the LT. To test this hypothesis, we systemically administered the selective melatonin receptor antagonist luzindole 5 h before the regular LT and assessed whether this would induce DC-like singing. We found that birds began singing earlier after luzindole administration compared to saline administration (paired *t*-test: $t(7) = 2.79$, $p = 0.0269$; Fig. 3J), although the initial singing rates following the LT did not significantly differ between the two treatments ($t(7) = 1.162$, $p = 0.284$). These results suggest a key role of the early drop in plasma melatonin levels prior to the LT in inducing DC-like singing, thus supporting our hypothesis that the early morning awakening that is responsible for DC-like singing is caused by melatonin-related hormonal regulation.

***Captive zebra finches housed under semi-natural conditions exhibit DC singing.***

Although the suppression-driven rebound singing observed after the delayed LT in socially isolated zebra finches housed under artificial light conditions resembles the DC that is generally observed in many bird species in the wild, it is unclear whether these two phenomena are based on the same mechanisms. While zebra finches have been extensively studied in laboratory settings for life science research, to our knowledge, no studies have reported the DC in wild zebra finches.

To investigate whether the DC-like singing that we observed is closely related to the DC that is generally observed in the wild, we recorded the vocal activity of captive zebra finches housed with conspecifics in an aviary exposed to natural light through large windows over a 20-day period from mid-September to early December in 2022. In these birds kept under social and natural light conditions (with almost constant temperature and humidity), the onset of their morning vocal activity appeared to vary depending on sunrise time and weather conditions (Fig. 4A), suggesting that light levels in the aviary critically influence morning vocal behavior. To determine whether these birds exhibited DC-like vocal activity, we accounted for variations in sunrise time and weather conditions by aligning the vocal activity data to a "dawn reference time (DRT)," defined as the time when light levels in the aviary surpassed a predetermined threshold (see Methods). This analysis revealed a marked increase in vocal activity around the DRT, followed by a gradual decline (Fig. 4B–C), resembling the typical DC observed in wild birds. Furthermore, detailed analysis of this morning vocal activity showed a relationship between

dawn timing and the onset of morning vocalizations consistent with our findings in socially isolated birds under artificial lighting (Fig. 1 & 2). Specifically, during the first 10-day recording period, in which both DRT and sunrise occurred relatively early, the average vocal activity tended to have a peak that was later than that during the subsequent 10-day period (Fig. 4B-C). Also, when the DRT occurred later due to seasonal changes and weather conditions, vocal activity around the DRT tended to be greater (Fig. 4D) and to start earlier (Fig. 4E). These findings in the birds under social and natural light conditions are consistent with the earlier onset and greater amplitude of morning singing after delayed LT observed in the birds under socially isolated and artificial light conditions (Fig. 1 & 2), suggesting similar mechanisms of DC vocal activity in both conditions. Given these results, we suggest that zebra finches in the wild may produce the DC through the suppression-driven rebound mechanisms identified in captive birds.

**DC singing accelerates morning changes in song structure.**

Our findings in captive zebra finches revealed the behavioral and hormonal mechanisms (i.e., the proximate cause) underlying DC singing: In the birds that woke up long before LT, suppression of spontaneous singing by pre-dawn darkness increases singing motivation, leading to intensive rebound singing shortly after the LT. We also investigated why the pre-dawn singing suppression increases singing motivation and induces rebound singing, which is the adaptive function (i.e., the ultimate cause) of DC singing. Previous studies in captive adult finches have demonstrated that daily singing serves as vocal exercise (or practice) to optimize and maintain song performance. In particular, recent studies have shown that song performance, which is mostly stable throughout life, substantially changes after long-term (1–2 weeks) suppression of daily singing and recovers with subsequent singing(*8*, *9*). Based on these findings, we hypothesize that DC singing serves as an intensive vocal exercise to compensate for the lack of singing at night, thereby accelerating the optimization of song performance and ultimately increasing reproductive success. Importantly, this hypothesis explaining the function of DC singing in captive zebra finches is virtually identical to the “warm-up hypothesis” previously proposed to explain the function of the DC in wild birds(*10–12*). To test this warm-up hypothesis in captive zebra finches, we analyzed possible changes in song structure during morning singing in the songs recorded under the shifted LT conditions shown in Fig. 1, and examined whether morning song changes are accelerated by DC singing.

We examined potential changes in morning song structure by comparing each song produced during the first hour after LT with all songs produced during the second hour in each LT condition (see Methods). Comparisons were made for each song syllable (n = 18 syllable types from 6 birds) by calculating mean acoustic distances in acoustic feature space (cosine distance, see Methods). In both the -3h LT and +3h LT conditions, many syllables showed gradual decreases in acoustic distance during the first hour (i.e., negative slopes of the acoustic distance trajectories; Fig. 5A-B), indicating substantial changes in acoustic structure during morning singing. If these song changes were caused by singing behavior rather than simply by the passage of time, the speed of the song changes should depend on the singing rate during that period. Consistent with this prediction, we found that the slopes of song changes over the first 30

min were significantly steeper in the +3h LT condition, where DC singing occurred, than in the -3h LT condition (Fig. 5A-B and Table S1). Moreover, the slopes were significantly correlated with normalized singing rate during the same period (the number of syllable renditions in the first 30-min period normalized to that in the first 2-h period) (Fig. 5C and S2). These results suggest that morning song structure changed as a function of recent singing activity and that such changes were accelerated by DC singing. This conclusion was further supported by the findings that, when acoustic distances were plotted as a function of syllable order instead of time, there was no significant difference in the slopes between the -3h and +3h LT conditions (Fig. 5D and Table S2).

**Discussion**

Our results offer a comprehensive explanation for DC singing in captive zebra finches, covering both its underlying mechanisms and functional significance. Before exhibiting DC singing, birds wake up in the dark long before dawn, likely through the hormonal mechanisms associated with melatonin, and their intrinsic motivation to sing increases while spontaneous singing is being suppressed by the darkness. When dawn arrives and ambient light levels increase, the singing suppression is lifted, resulting in rebound singing characterized by an early singing onset and a high singing rate, the two main features of the DC in general. Moreover, DC singing accelerates changes in song structure that naturally occur in the morning, suggesting that DC singing may serve to compensate for the loss of vocal exercise during the night and to rapidly re-optimize the vocal system and song performance to increase reproductive success. Since a vocal exercise function has been proposed for the DC in wild songbirds as well(*10–13*), we propose that these mechanisms and function of DC singing may be applicable, at least in part, to the DC generally observed in wild birds.

A key aspect of the mechanisms underlying DC singing that we revealed is the time lag between when birds wake up and when light intensity rises (the LT). During this time lag, birds are awake but suppressed from singing by darkness and their intrinsic singing motivation appears to increase depending on the duration of the time lag, just as in birds that are suppressed from singing by lights-out during daytime(*5*). This suppression-dependent increase in singing motivation is reminiscent of and consistent with the so-called “Lorentz’s psychohydraulic model”, in which a reservoir of motivational impulse builds up and is ultimately released by appropriate environmental stimuli(*14*). Although mechanisms underlying this motivational increase remain largely unclear, a brain region and neuromodulators related to singing motivation have recently been reported(*5*, *15*, *16*). On the other hand, the mechanisms by which birds awaken before the LT likely involve hormonal regulation including a drop in plasma melatonin levels from early night levels(*7*), which is caused by a circadian clock even in the absence of light inputs(*17*, *18*). A similar trend of decreased melatonin levels during the late night phase has been reported in songbirds other than zebra finches, although the timing of those changes is unclear(*19–22*). Also, in wild songbirds, nighttime melatonin concentrations are positively associated with the onset time of early morning behaviors including singing(*21*, *23*). Thus, it is reasonable to assume that the mechanisms underlying DC singing in captive zebra

finches are applicable to the DC in other songbird species. Whereas the circadian secretion of melatonin has been hypothesized to regulate the timing of DC(*1*, *2*), a number of studies have suggested that the DC timing is strongly influenced by light inputs to the birds, such as intensity of morning light and the size of birds' eyes(*24–27*). Our findings reconcile these seemingly contradictory ideas and provide a clear explanation of the mechanisms by which the circadian secretion of melatonin and light inputs interact to determine the timing of the DC as well as its magnitude.

The suppression-driven rebound singing that underlies DC singing in captive zebra finches is remarkably consistent with the recent report of DC-like vocal behavior observed in many species of wild birds after the daytime darkness caused by a solar eclipse(*28*). Both behaviors are caused solely by changes in ambient light levels and are likely independent of other factors previously hypothesized to contribute to the DC, such as non-light environmental conditions, social interactions with conspecifics, predation risk, and food availability(*1*, *2*). This consistency between the two studies strongly supports the idea that rebound singing is the fundamental mechanism underlying the DC observed across many bird species. Although the solar eclipse produced relatively short darkness (totality lasted for about 4 minutes) compared to the darkness that induces rebound singing in captive zebra finches(*5*), this difference is likely attributable to interspecies differences in baseline singing motivation and its enhancement by darkness, as evidenced by the varying effects of solar eclipses on vocalization rates among different species(*28*).

Our hypothesis that DC serves as an intensive vocal exercise that rapidly optimizes song performance is virtually identical to the the warm-up hypothesis established in previous studies using wild and wild-caught birds(*10–12*). Those studies defined high-performance songs as those approaching physiological limits and demonstrated that song performance improves rapidly during the DC(*10–12*). Although our results of rapid changes in syllable structure during DC singing do not directly indicate improvement in song performance, several lines of evidence suggest that captive zebra finches indeed improve song performance through morning singing. In juvenile zebra finches in the process of song development, song structure deteriorates overnight and then dramatically improves through morning singing(*29*). Also, in adult zebra finches, which normally produce songs with stable overall structure, song performance defined as the degree of female's preference to that song significantly decreases by prolonged suppression of daily singing and gradually recovers after singing is resumed(*8*, *9*). Thus, it is likely that song changes that were accelerated by DC singing in our data represent improvement of song performance. Given that socially-isolated adult zebra finches produce undirected songs daily throughout the day likely for vocal exercise to maintain song structure(*8*, *9*, *13*, *30–35*), DC singing presumably serves to compensate for the loss of vocal practice at night and to regain peak song performance. Because peak song performance is operationally defined by female conspecific preference(*9*) and thus has adaptive benefit, our results strongly support the warm-up hypothesis, which explains the adaptive function of the DC. Moreover, based on the previous studies examining mechanisms of adult song improvement following suppression-induced deterioration(*8*, *9*), it is likely that song improvement during the DC is caused largely by vocal exercise and resulting physiological

changes in the muscles of the vocal system, rather than hearing-dependent adjustment of song motor circuits. Therefore, the mechanisms and function that we proposed to explain the DC are applicable not only to oscine birds, which develop their songs through audition-dependent learning, but also to suboscine birds and other avian species that produce a DC with innate vocalizations.

While our rebound singing hypothesis offers a novel perspective on the mechanisms and functions of DC, it does not necessarily contradict the existing hypotheses about DC. In fact, our hypothesis can explain many DC-related findings that support the existing hypotheses. For example, previous studies have reported that the extent of DC (e.g., singing duration and timing of the first song) is associated with specific reproductive stages such as the female fertility period and also correlated positively with reproductive success (see (*1*, *2*) for review). Based on these findings, it has been proposed that the DC represents an honest signal of male quality(*36–38*) and directly regulates female reproductive behavior around dawn(*39*, *40*). Our rebound singing hypothesis states that the DC functions to rapidly optimize the quality of song performance, which is critical for mate attraction when produced in courtship context. Thus, it is possible that birds exhibit greater DC at the female fertility period to optimize song performance as early as possible in the morning and to maximize their mating success. The rapid song optimization by the DC could also be advantageous in territory defense for many territorial species. Although they need to defend their territories throughout the day, they may rapidly optimize their song performance through the DC early in the morning to prepare for the territory invasion by other birds. This view is consistent with a previous study that the extent of the DC is affected by territorial intruders(*41*) and also explains the fact that many songbird species show the DC even after the female fertility period. Additionally, because birds exhibiting more intense DC are likely to have higher motivation, not only for singing, but also for other reproductive behaviors, such higher motivation for non-singing behaviors could also contribute to higher reproductive success. Moreover, our rebound singing hypothesis aligns with the unpredictable overnight-condition hypothesis as well, which proposes that birds sing intensively at dawn to expend the surplus of energy stored in preparation for unpredictable overnight conditions(*42*). This hypothesis is supported by findings that greater food availability leads to a more pronounced DC(*43–45*). Since food availability positively affects birds' nutritional state and overall singing motivation, as shown in captive zebra finches(*15*), sufficient food availability could amplify the DC by elevating overall singing motivation and enhancing suppression-driven rebound singing.

Given that our experiments were conducted under laboratory conditions and considering the unique characteristics of the zebra finch as a non-territorial and opportunistic breeder, further research is needed to determine whether our rebound-singing hypothesis also applies to the DC in other songbird species. In particular, although territorial birds may also produce the DC through rebound-singing mechanisms similar to those in zebra finches, its function may involve re-establishing territory boundaries and social relationships with neighbors(*46*), rather than, or in addition to, the rapid optimization of song performance. Because it is often difficult to determine in the wild whether birds are singing for vocal exercise or for territory defense and social interaction, the functional roles of the DC may not be easily clarified. In contrast, the behavioral

mechanisms underlying the DC, including rebound singing, can be examined more readily. Even in field settings, it should be possible to test the relationship between the intensity of the DC and the timing of the increase in ambient light at dawn, as we did in birds housed under natural light conditions. It would also be important to determine when birds wake up before dawn and whether this timing correlates with the intensity of the DC. Such studies would help elucidate the general mechanisms driving the DC and potentially reveal broader principles of avian biology related to singing behavior.

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Visualization: SK, EBS, CM, YK, JW

Funding acquisition: SK, CM

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Supervision: SK

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**Supplementary Materials**

Materials and Methods

Figs. S1 to S2

Tables S1 to S2

References (*50–53*)

Movies S1 to S5

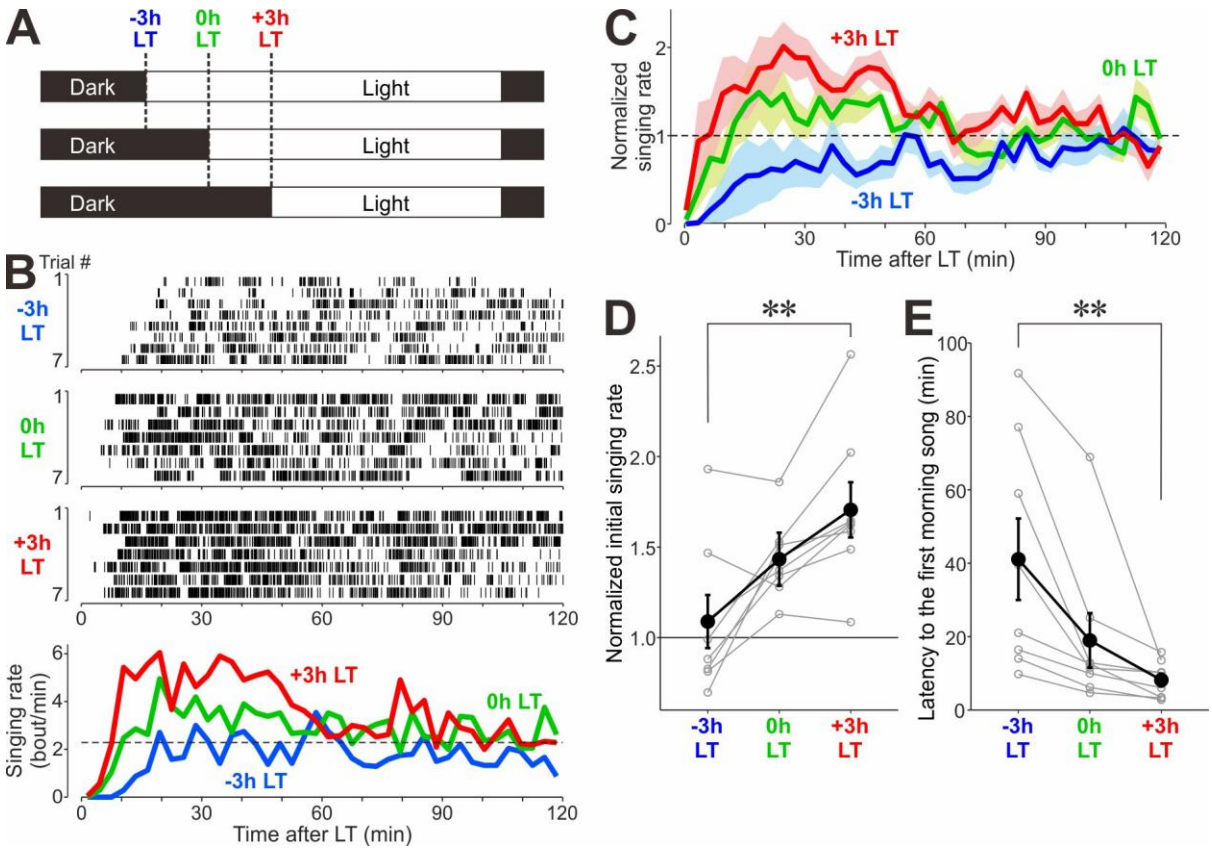


**Fig. 1. DC-like intensive singing can be induced in captive zebra finches by delaying the LT in the morning. (A)** Daily schedules of light and dark periods with variable LT. The 3 LT conditions were administered in a randomized order. **(B)** Raster plots of song bouts (*top*) produced after -3h LT, 0h LT, and +3h LT, and corresponding singing rate histograms (bin size, 3 min) (*bottom*) in a representative bird. **(C)** Time course of instantaneous singing rate in the three different LT conditions (normalized to the baseline rate, mean ± SEM, n = 8 birds). **(D)** Initial singing rate in -3h, 0h, and +3h LT conditions (normalized to baseline singing rate); gray circles with lines indicate the data of individual birds ($p = 0.005$ for 3 conditions, Friedman's test; $^{**}p = 0.005$ for -3h LT vs. +3h LT, Bonferroni corrected Durbin-Conover test). **(E)** Latency to the first morning song in 3 LT conditions ($p = 0.0003$ for 3 conditions; $^{**}p = 0.005$ for -3h LT vs. +3h LT).

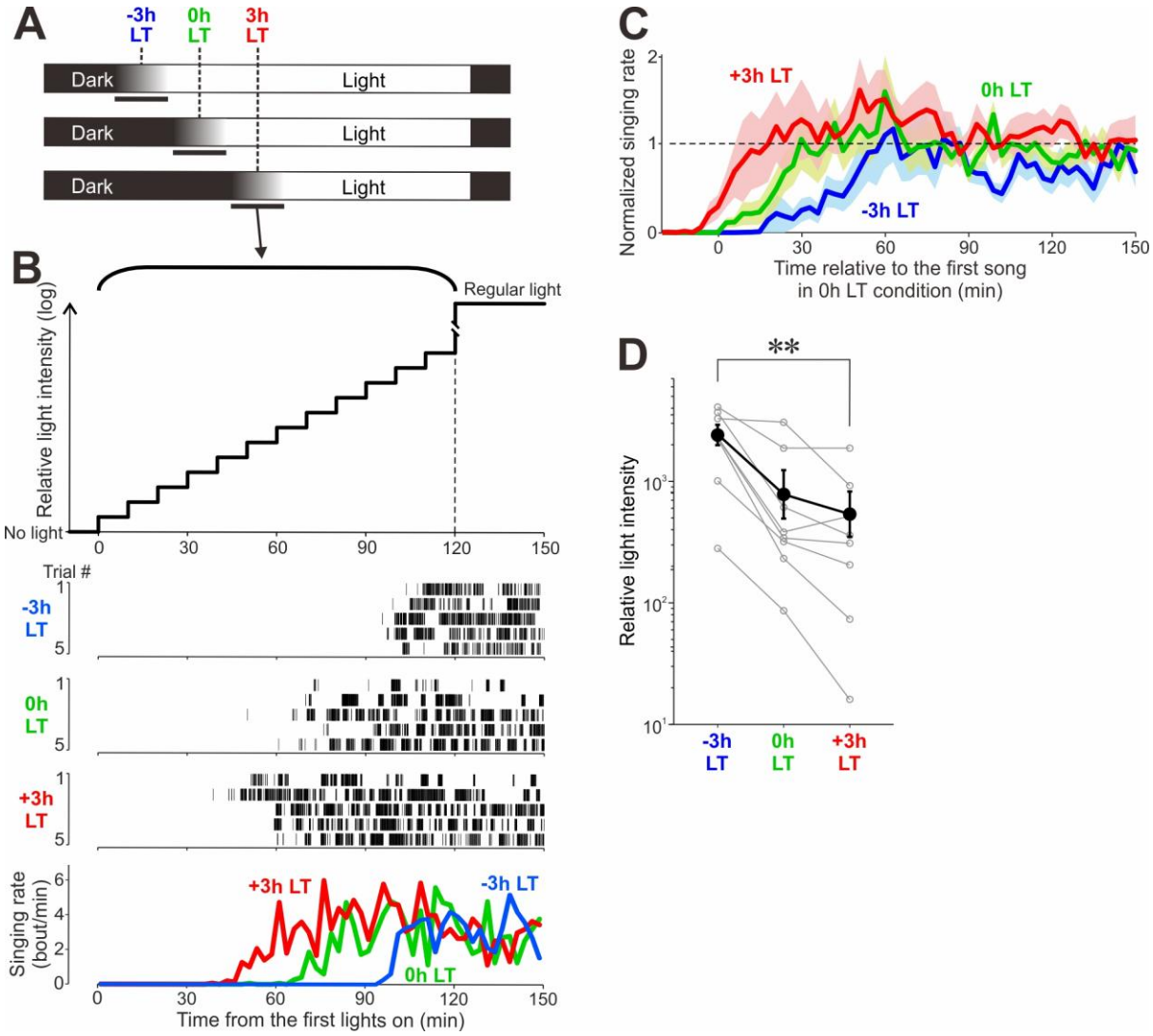


**Fig. 2. Birds begin to sing in dimmer light conditions when LT is delayed. (A)** Daily schedules of light and dark periods with gradual lighting. The 3 LT conditions were administered in a randomized order. **(B)** Time course of light level changes during a gradual lighting period (*top*), raster plots of song bouts during gradual lighting periods in -3h LT, 0h LT, and +3h LT conditions (*middle*), and corresponding singing rate histograms (*bottom*) in a representative bird. **(C)** Time course of instantaneous singing rate in the three different LT conditions (normalized to the baseline rate, mean ± SEM, n = 8 birds). **(D)** Relative light intensity of first song in -3h, 0h, and +3h LT conditions ($p$ = 0.0008 for 3 conditions; **$p$ = 0.008 for -3h LT vs. +3h).

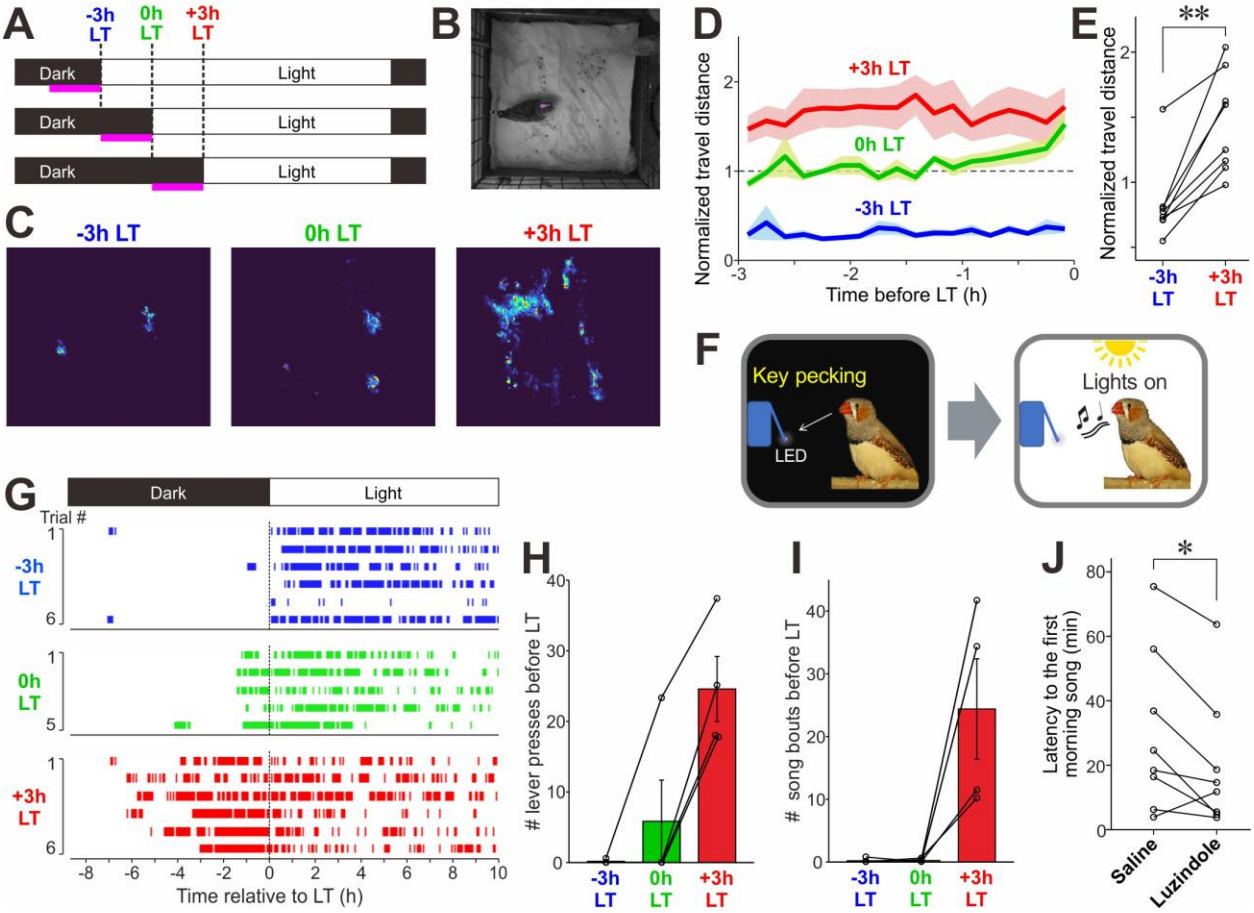


**Fig. 3. Birds are awake before the delayed LT and their intrinsic singing motivation increases while singing is being suppressed by darkness. (A)** Daily schedule of light/dark periods and the 3-h periods of IR video recording (magenta lines). **(B)** Example IR video image of a bird in the dark. Magenta dot on the bird head indicates the point used to track bird's movement. **(C)** Representative images indicating the positions of a bird during the 3-h dark periods immediately before the -3h, 0h, and +3h LTs (magenta lines in *A*). **(D)** Travel distances over the pre-LT 3-h periods (mean ± SEM across birds, normalized to the mean of 0h LT data for each bird). **(E)** Mean travel distances during the pre-LT 3-h periods, normalized to the 0h LT data, in individual birds (**$p$ = 0.0078, Wilcoxon signed-rank test). **(F)** Schematics of operant conditioning paradigm to associate a lever press with a 10-sec period of lighting. **(G)** Representative plots of lever press activities (vertical bars) before and after the 3 different LTs. **(H & I)** The number of lever presses (H) and song bouts produced (I) before the 3 different LTs; lines represent the data of individual birds (averaged across trials) ($n$ = 4 birds, $p$ = 0.0324 and 0.038 for *H* and *I*, respectively, Friedman's test). **(J)** The effect of the selective melatonin receptor antagonist luzindole on first song latency. Note that birds started singing significantly earlier when injected with luzindole compared to saline (*$p$ = 0.027, paired $t$-test).

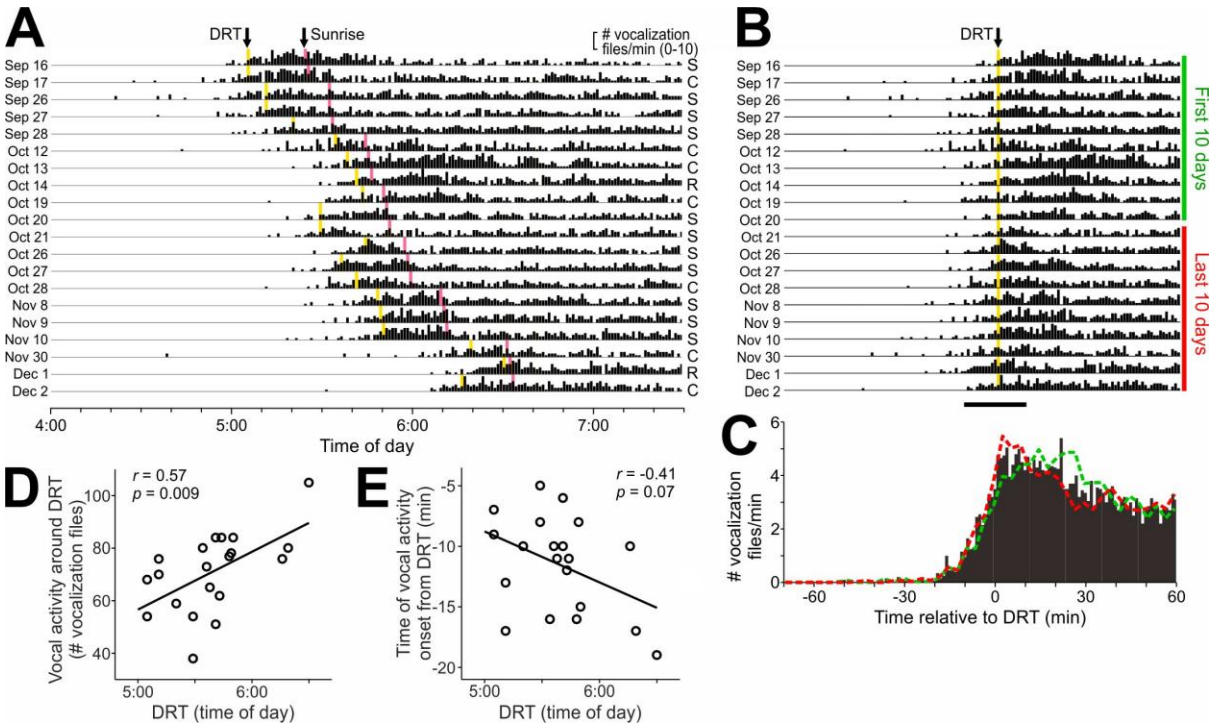


**Fig. 4. Captive zebra finches housed under social and natural light conditions exhibit DC singing. (A)** Vocal activity (number of vocalization files/min) recorded around dawn for 20 days from mid-September to early December. Yellow lines, dawn reference time (DRT); pink lines, sunrise time. Letters on the right side of the actogram indicate the weather on individual days: S, sunny; C, cloudy; R, rainy. **(B)** Vocal activity aligned to DRT; Green and red vertical lines indicate the first and last 10-day periods analyzed for the data in **(C)** Horizontal bar at the bottom indicates the period in which vocal activity on individual days were measured for the plot *D*. ***C.*** DRT-aligned vocal activity averaged over the all recording days (black histogram), the first 10-day period (green line) and the last 10-day period (red line). **(D)** Vocal activity during the 20-min period around DRT (horizontal bar in *B*) plotted against DRT; circles represent the data of individual days. Note that vocal activity around DRT was greater on days with later DRTs. **(E)** Time of vocal activity onset relative to DRT plotted against DRT. Note that vocal activity tended to start earlier relative to DRT on days with later DRTs.

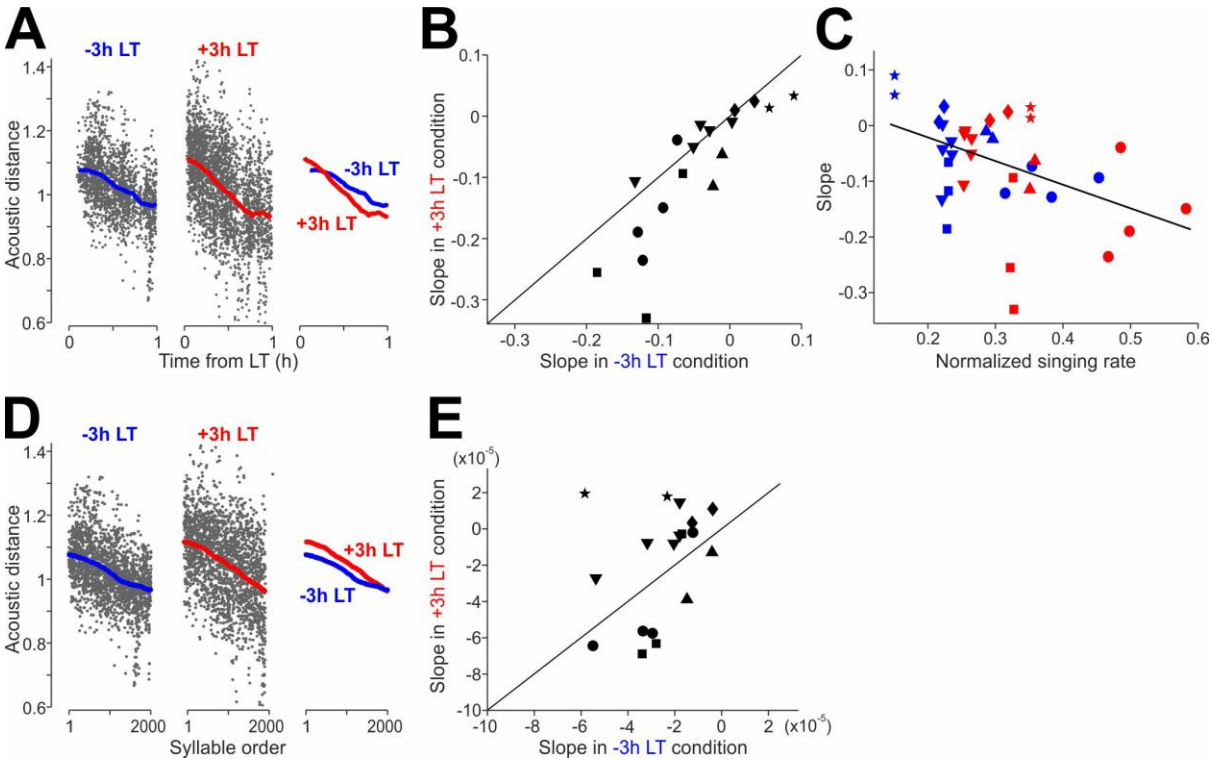


**Fig. 5. DC singing accelerates morning changes in song syllable structure. (A)** Changes in acoustic distances of syllable structure in the renditions during the first hour after LT relative to the renditions during the second hour in -3h and +3h LT conditions for an example syllable. Gray dots and thick lines indicate the data of individual syllable renditions and their local mean trajectories, respectively; the local mean trajectories in the 2 conditions are overlayed in the rightmost plot. **(B)** Comparisons of the slopes of acoustic distance changes over the first 30 min between -3h and +3h LT conditions (n = 18 syllable types from 6 birds, t = 2.74, $p$ = 0.0140, a linear mixed-effects model including random intercepts for bird identity and syllable; see Table S1). Each data point indicates a data from individual syllable type; different symbols represent different birds; Diagonal line indicates unity. **(C)** The same slope data in the 2 LT conditions plotted against the singing rate during the the first 30-min period (the singing rate was normalized to that in the first 2-h period). Blue and red symbols indicate data in -3h and +3h LT conditions, respectively; symbols are as in *B* (n = 36, pairwise linear correlation, ρ = -0.4445, $p$ = 0.0066). **(D)** Acoustic distances of the same syllable as shown in A, plotted as a function of syllable order instead of time. Conversions are identical to those in A. **(E)** Comparisons of the slopes over the first 1000 syllable renditions between -3h and +3h conditions (t = 0.35, $p$ = 0.3509, linear mixed-effects model with random intercepts for bird identity and syllable; see Table S2).

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# Supplementary Materials for

## Why early birds sing early: rebound singing from nighttime suppression can explain dawn chorus

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**The PDF file includes:**

Materials and Methods
Figs. S1 to S2
Tables S1 to S2
References (*50–53*)

**Other Supplementary Materials for this manuscript include the following:**

Movies S1 to S5

## Materials and Methods

Subjects

The subjects used for song recording under socially isolated and artificial light conditions were adult male zebra finches (*Taeniopygia guttata*, 87–260 dph) bred in the songbird facility at the Korea Brain Research Institute. They were raised with their parents and siblings until ~60 dph and then housed with their siblings and/or other males conspecifics until the experiments started. Their care and treatment were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the Korea Brain Research Institute. The subjects used for recording vocal activity under social and natural light conditions were adult male and female zebra finches bred in the songbird facility at Teikyo University. Their care and treatment were reviewed and approved by the Animal Care and Use Committee at Teikyo University. All experimental procedures were carried out in compliance with the ARRIVE guidelines(*50*).

Song recording

For the experiments with song recording under socially isolated and artificial light conditions, each bird was housed in a sound-attenuating and light-proof chamber (MC-050, Muromachi Kikai, or custom-made chambers) throughout the experiment. Songs were recorded using a microphone (PRO35, Audio-Technica) positioned above the cage and a custom-written song recording program. Output from the microphone was amplified by a mixer (402-VLZ4, Mackie) and digitized via an audio interface (Octa-Capture UA-1010, Roland) at 44.1-kHz (16-bit). Recorded signals were down-sampled to a sampling rate of 32-kHz. Recording was triggered if the program detected 3 or 4 consecutive sound notes, each of which was defined based on sound amplitude, duration, and intervening gap duration; those parameters for triggering sound recording were optimized for each bird so as to record all songs produced. Each recording ended if a silent period lasted longer than 0.5 sec (i.e. each song file contains a single "song bout" that is separated from other bouts by >0.5-sec silent periods). Songs were recorded throughout the day, and all song recordings were of undirected song (i.e. no female was present). Birds with sufficient singing rates (>300 song bouts per day) were used for the experiments. The procedures to record vocal activity of the birds housed under social and natural light conditions and to analyze the recorded vocal activity are described below.

Manipulation of light-dark schedule and illumination level

Birds for the experiments under socially isolated and artificial light conditions (Figs 1-3) were bred and kept on a constant 14:10hr light:dark (LD) cycle (with abrupt light-dark transitions) in the bird colony and after being transferred to the sound-attenuating chambers for > 5 days until the experiments began. During the experiments, we used two different lighting protocols. For the "abrupt lighting" experiment, the time of abrupt dark-light transition (the lights-on time, LT) was shifted daily ±3-h from the original 14:10hr LD cycle; birds underwent the -3hr, 0hr, and +3h LT conditions in a randomized order (neither the -3h LT nor the +3h LT condition was administered on consecutive days to prevent the birds from adapting to the LT

condition). Each condition was administered multiple times (ranging from 4 to 8 trials). For the "gradual lighting" experiment, the illumination level was exponentially increased with 12 steps over 2 hr (10-min period for each step) using LED lights with an Arduino-based control unit; the illumination level of the 12$^{th}$ step was approx. 350 lux, and it was followed by a constant illumination level equivalent to the one used for regular light periods (approx. 500 lux). This 2-hr gradual lighting period was shifted daily ±3-h in a randomized order just as for the abrupt lighting experiment. Illuminance was measured at the center of the experimental cages using a light meter (Tenmars, TM-205).

Song analysis

To analyze singing activity recorded in sound-attenuating chambers, we screened all sound files recorded during the time periods of interest to exclude non-song files using a semi-automated method previously described(*5*). Briefly, we sorted song files (sound files that include at least one full motif of song) and non-song files by focusing on temporal trajectories of sound amplitude (amplitude envelopes) and those of Weiner entropy, both of which are highly stereotyped across motif renditions and clearly distinct from other sounds including non-song vocalizations. We calculated the maximum correlation coefficients (mCCs) of amplitude envelopes and that of entropy trajectories between a canonical song motif and all sound files recorded, and excluded files that had mCCs for both amplitude envelopes and entropy trajectories below certain thresholds as non-song files. We then visually inspected spectrograms of those excluded files to ensure that no song files were excluded from the song dataset to be analyzed.

To quantify the magnitude of DC-like singing activity, we used two measures, the initial singing rate and first song latency(*5*). The initial singing rate was measured as the mean singing rate over a 1-hr period starting at the onset of the first song produced in the morning for each trial and averaged across trials with the same LT. The initial singing rate was then normalized to the baseline singing rate, which was cross-trial and cross-LT average of the mean singing rate over the 30-min period starting at 90 min after the morning LT. We also plotted time courses of singing activity by measuring instantaneous singing rates using 3-min bins and averaged across trials with the same LT. The average instantaneous singing rates were then normalized by the baseline singing rates. The first song latency was measured in the abrupt lighting experiments as the time interval from the LT to the onset of the first song recorded in the morning. The latencies were then averaged across trials with the same LT.

To investigate morning changes in song structure, songs recorded in the -3h LT and +3h LT conditions were segmented into syllables using a custom program on Matlab based on the following parameters: amplitude, minimum and maximum syllable duration, and minimum and maximum frequency. These parameters were manually adjusted for each bird. From individual syllables, 8 time-varying acoustic features (centroid, spread, skewness, kurtosis, flatness, slope, pitch, goodness of pitch) and 2 other features (amplitude and duration) were extracted and projected onto a two-dimensional space by dimensionality reduction algorithm t-SNE (t-

distributed Stochastic Neighbor Embedding). Syllables were then manually labelled based on clusters formed on the two-dimensional space. All labeled syllables were manually checked and incorrect labels were corrected. In the syllables that had stereotyped temporal structure across renditions, morning changes in acoustic structure were examined in each LT condition by comparing each syllable rendition produced during the first hour after LT with all syllable renditions produced during the second hour (the reference period) and by averaging their acoustic distances. The acoustic distance was quantified as cosine distance computed using the within-syllable mean, standard deviation, and mean absolute derivative of the 8 acoustic features and amplitude described above. The acoustic distances of individual syllable renditions were plotted as a function of time after LT and the local means over a sliding window of ±10 min durations were computed with 1-min increments. Slopes of the local mean changes over the first 30-min period starting at the first rendition were compared between the -3h and +3h LT conditions. The same acoustic distance data were also plotted as a function of syllable order instead of time, and slopes of the local mean changes were compared between the 2 conditions. Additionally, the slopes of acoustic distances were plotted against the number of syllable renditions recorded during the first 30-min period normalized to the number of renditions recorded during the first 2-h period to examine correlations between them.

Recording and analysis of bird movements

In the birds that underwent the ±3h LT conditions with the abrupt lighting paradigm, their movements in the dark before the LT were monitored using infrared cameras (SQ12, Mrs Win, and UI-3040CP Rev. 2, iDS imaging) positioned on the top of the individual cages. Infrared LED lights were also positioned ~10 cm above the cages and they were on during the night dark period. Each recording session spanned 3 hours, finishing at the LT (-3h, 0h, or +3h LT). To increase the precision of movement tracking, a small fluorescent sticker was affixed on the top of each bird's head. Movements of the birds' heads were tracked using the machine learning software DeepLabCut(*51*), and their travel distances were calculated over 10-min time bins. Mean travel distances over the 3-h period were calculated for each LT condition and compared across different LT conditions.

Operant lever-pressing task for lighting

To examine the level of motivation in the dark to seek light conditions that allow birds to sing, we trained birds to press a lever that is associated with the activation of ambient light. Birds were kept individually in an experimental cage that was placed in a sound-attenuating and light-proof chamber throughout the experiments. On the ceiling of the chamber, white LED lights were installed in addition to the built-in lights of the chamber. In the cage, a lever switch (Omron Electronics, SS-01GL111-E) was placed on the rear wall near a perch, and a small red LED was also placed on the rear wall above the lever. Throughout the experiment, including day and night, pressing the lever immediately activated the white LED lights for 10 seconds, and the timing of lever pressing by the bird was recorded; the small red LED was always lit so that the lever

location could be identified in the dark. Undirected songs were also recorded throughout the experiment using a microphone positioned above the cage. Birds were initially housed in the experimental cage with the lever operational on a regular 14/10 light-dark (LD) cycle for more than 7 days for the purpose of habituating the bird to the experimental apparatus and having them to learn the association between lever pressing and activation of white LED lights. Subsequently, we introduced the the behavioral paradigm of shifting the morning LT described above (the "abrupt lighting experiment"): birds underwent -3hr, 0hr, and +3h LT in a randomized order. Each condition was administered multiple times (ranging from 4 to 7 trials). The frequencies of lever pressing and of song bouts over the 3-h period before the LT were averaged across trials and compared across different LT conditions.

Luzindole injections

A potential role of melatonin in morning singing was examined using luzindole (Tocris, 0877), a competitive melatonin MT1/MT2 receptor antagonist(*52*). Luzindole was stored as stock solution in DMSO (40 mg/ml) at −20 °C and diluted in PBS before use (2 mg/ml). Birds were kept on a regular 14/10 LD cycle with abrupt light-dark transitions, and injected subcutaneously with luzindole (5.0 mg/kg) or PBS 5 h before the LT. Each bird received 4 injections for both luzindole and PBS in a randomized order (a single injection per day). Undirected songs were recorded throughout the experiment, and morning singing was analyzed by focusing on the first song latency and initial singing rate. Results were averaged across trials for each bird.

Recording and analysis of vocal activity of the birds under social and natural light conditions

To examine morning vocal activity under social and natural light conditions, audio and video recordings were made in the zebra finch aviary at Teikyo University, which has large glass windows that let in natural light from outdoor, for 20 days intermittently from September 16th to December 2nd, 2022. In the aviary, ~90 zebra finches were housed in 18 cages (8 cages for breeding pairs and 10 cages for adult birds with the same sex) that were placed in a metal rack (4 cages x 3 tiers) near the windows. Audio recordings were made using a microphone (PRO35, Audio-Technica) placed in the middle of the cage rack and connected to an audio interface (Octo-Capture UA-1010, Roland) for digitization at 44.1 kHz (16 bit). To capture vocal sounds, we used a custom-made recording program (tRec, R. O. Tachibana, 2020), which started sound recording when it detected 5 successive sound notes, each of which reached predefined thresholds of amplitude and duration, and ended when it detected a silent period lasted >0.8 sec. This recording system captured mostly vocal sounds (i.e., songs and calls), but non-vocal sounds such as cage noises and wing-flapping sounds were occasionally recorded. To measure vocal activity around dawn, we visually sorted the files that contain vocalizations using Avisoft Saslab (Avisoft Bioacoustics), and calculated the number of the files recorded during 1-min time bins over the time period from 4:00 to 7:30 am. The daily vocal activity data were then aligned relative to the "dawn reference time (DRT)", which was defined as follows: During the periods

of audio recordings, we simultaneously videotaped all cages and windows in the aviary using a wide-angle web camera (BSW200MGK, Buffalo) placed 80 cm away from the cage rack (frame rate, 16 Hz). On the recorded videos, the minute-by-minute changes in luminance for four window locations (10 x 10 pixels) were measured using Fiji (ImageJ, version 1.54f(*53*)). For each day, the time point when the luminance reached 200 was defined as the DRT, and the vocal activity data were aligned to the DRT. The aligned data were then averaged across days to obtain the average vocal activity patterns. We obtained sunrise times and whether conditions for individual experimental days at the website of the National Astronomical Observatory of Japan (https://eco.mtk.nao.ac.jp/cgi-bin/koyomi/koyomix_en.cgi) and that of the Japan Weather Association (https://tenki.jp/past/2022/09/weather/).

Statistics

Statistical tests were performed using MATLAB (MathWorks) or R (R Core Team and R Foundation for Statistical Computing). To the differences in singing and lever-press activity across different LT conditions, we used a Friedman's test followed by Bonferroni corrected Durbin-Conover test. To examine the effects of different LT conditions on birds' travel distances and the effects of luzindole administration on morning singing activity, we used a Wilcoxon signed-rank test and a paired t-test, respectively. To assess the effects of different LT conditions on the slopes of morning song changes, we fitted a linear mixed-effects model predicting each syllable's slope from LT condition (–3h LT vs +3h LT), while accounting for two levels of nesting: each bird has its own baseline (random intercept) and, within each bird, each syllable has its own baseline (another random intercept). A p-value of $<0.05$ was used as the criterion for statistical significance.

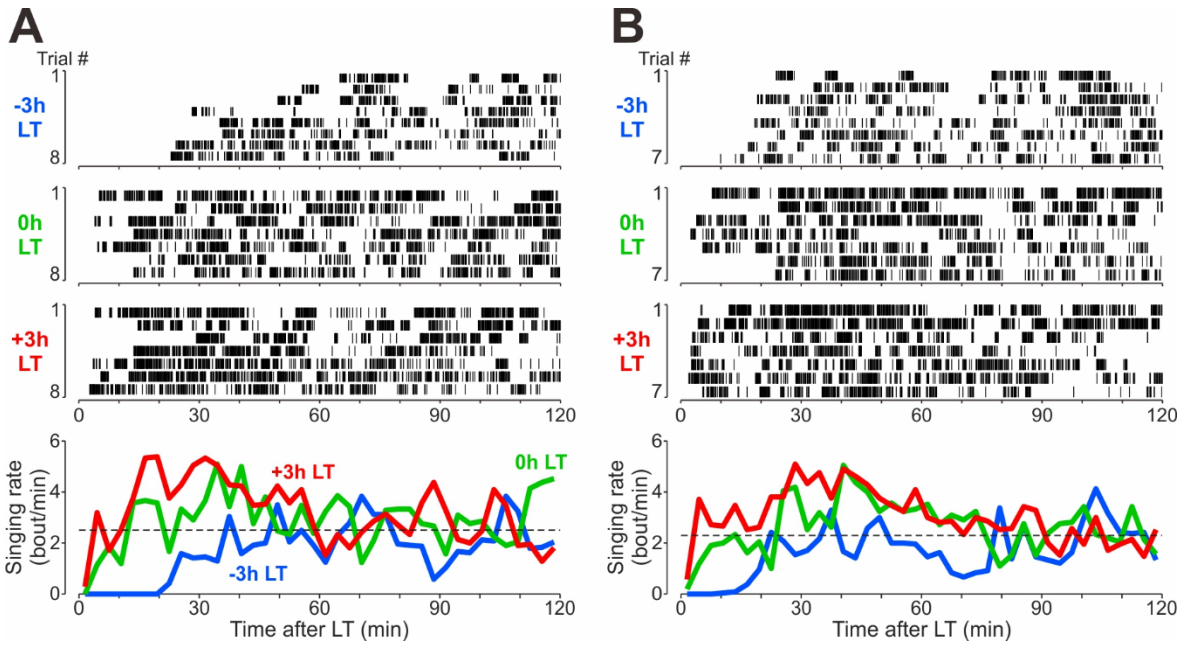


**Fig. S1.** LT-dependent DC-like singing in two addition birds. Conventions are as in Fig. 1B.

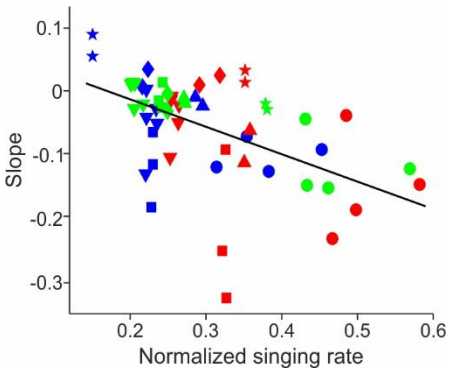


**Fig. S2.** The slope data in -3h (blue) and +3h (red) LT conditions shown in Fig. 5C are replotted together with the data in 0h LT condition (green) ($n = 54$, pairwise linear correlation, $\rho = -0.5045$, $p < 0.001$).

| effect | group | term | estimate | std error | statistic | df | p-value |
|---|---|---|---|---|---|---|---|
| fixed | NA | (Intercept) | -0.03843 | 0.03509 | -1.095 | 5.33 | 0.32 |
| fixed | NA | Lighting condition | -0.03899 | 0.01424 | -2.738 | 17 | 0.014* |
| random | Bird | (Intercept) | 0.07812 | NA | NA | NA | NA |
| random | Bird:Syllable | (Intercept) | 0.04136 | NA | NA | NA | NA |
| random | Residual | Observation | 0.04272 | NA | NA | NA | NA |

| effect | group | term | estimate | std error | statistic | df | p-value |
|---|---|---|---|---|---|---|---|
| fixed | NA | (Intercept) | −2.495e-05 | 6.903e-06 | −3.610 | 8.22 | 0.00653 |
| fixed | NA | Lighting condition | 6.737e-06 | 7.009e-06 | 0.96 | 17 | 0.34993 |
| random | Bird | (Intercept) | 1.023e-10 | NA | NA | NA | NA |
| random | Bird:Syllable | (Intercept) | 8.673e-11 | NA | NA | NA | NA |
| random | Residual | Observation | 4.422e-10 | NA | NA | NA | NA |

**Table S1 & S2.** A linear mixed effects model was run to evaluate whether the LT conditions (−3h LT vs +3h LT) influence the slope of acoustic-distance change in syllable structure during morning singing. Table S1 shows the results of the slopes over the first 30-min period following LT. Table S2 shows the results of the slopes for the first 1000 syllable renditions following LT. In both tables, the outcome variable and the fixed effect were slope and LT condition, respectively. Random effects included random intercepts for bird, and for syllable nested within bird, to account for hierarchical structure and non-independence of renditions.

**Movie S1.** An example of DC-like intensive singing following +3h LT.

**Movie S2.** An example of DC-like intensive singing following -3h LT in the same bird as in Movie S1.

**Movie S3.** Infrared recording of bird movement in the dark before +3h LT, displayed at 16x speed.

**Movie S4.** Infrared recording of bird movement in the dark before +3h LT (the same bird as in Movie S3).

**Movie S5.** Operant conditioning to associate a lever press with lighting.