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A Comparison of LED with Fluorescent Lighting on the Stress, Behavior, and Reproductive Success of Laboratory Zebra Finches (*Taeniopygia guttata*)

Alanna G Backx, DVM, MSc,[1] April Wu,[1] Alyx Tanner, BA,[2] and Niora J Fabian, DVM, MS, DACLAM[1,*]

There are limited evidence-based husbandry recommendations for laboratory zebra finches (*Taeniopygia guttata*), including appropriate light sources. Light-emitting diode (LED) technology has been shown to improve circadian regulation and reduce stress in some laboratory animal species, such as mice and rats, when compared with cool-white fluorescent (CWF) lighting, but the effects of LED lighting on zebra finches have not been published. We compared the effects of broad-spectrum, blue-enriched (6,500 Kelvin) CWF and flicker-free LED lighting on the behavior, stress, and reproductive outcomes of indoor-housed zebra finches. Using breeding pairs housed in cubicles illuminated with either CWF or LED lighting, we compared the reproductive output as determined by clutch size, hatching rate, and hatchling survival rate. We also compared the behavior of group-housed adult males, first housed under CWF followed by LED lighting, using video recordings and an ethogram. Fecal samples were collected from these males at the end of each recording period, and basal fecal corticosterone metabolite (FCM) levels were compared. A FCM assay for adult male zebra finches was validated for efficacy and accuracy using a capture-restraint acute stress response and parallelism analysis, respectively. The breeding pairs had no significant difference in the clutch size or percent hatching rate, but percent hatchling survival improved under LED with an increased proportion achieving 100% survival. There was no significant difference in FCM between the lighting treatments. However, the activity budgets of the birds were altered, with a reduction in flighted movement and an increase in enrichment manipulation under LED. Overall, these results support the use of blue-enriched, broad-spectrum flicker-free LED as a safe alternative to CWF lighting for breeding and nonbreeding indoor-housed zebra finches.

Abbreviations and Acronyms: CCT, correlated color temperature; CORT, corticosterone; CRI, color rendering index; CWF, cool white fluorescent; dph, days post-hatch; FCM, fecal corticosterone metabolites; K, kelvin; MANOVA, multivariate analysis of variance

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Introduction

The zebra finch (*Taeniopygia guttata*), an oscine passerine, is a well-established model of vocal learning and neurobiology.[54,60] The ease of breeding in captivity along with methods for transgenesis have further increased the popularity of zebra finches in various other fields such as genomics and developmental biology.[5,35,56,63,66,69,89] Despite their extensive use, there is a lack of evidence-based husbandry recommendations for zebra finches, potentially impacting their welfare and utility as a research model. In particular, the lighting regime is a fundamental environmental factor in avian husbandry. Light plays a critical role in synchronizing the avian neuroendocrine circadian rhythm and has direct impacts on reproductive physiology, metabolism, and behavior.[5,22,27,69] However, lighting recommendations for laboratory birds are scarce, rarely species-specific, and often empirically based and vague. Generally, it is recommended that lighting should mimic natural daylight and have high flicker frequencies.[5,63,69] The 8th edition of the *Guide for the Care and Use of Laboratory Animals* states that inappropriate photointensity, photoperiod, and spectral quality of light are potential photostressors for all animals; regarding birds, the *Guide* only mentions that chickens (*Gallus domesticus*) will not eat in low light or darkness.[47]

Zebra finches are endemic to arid regions of Australia with bright desert sunlight.[14,66,89] Thus, the low photointensity in facilities designed for rodents (that is, 325 lx) is thought to be insufficient for zebra finches.[73] Photoperiods are also important, and continuous light exposure has been associated with increased mortality in this species.[75] Furthermore, lights that provide full spectrum wavelengths (that is, from UV to infrared) have been suggested,[46,73] although specific wavelength ranges have not been experimentally validated in zebra finches. Light flicker, a temporal lighting artifact that is determined by the change in lighting output over time (measured in flicker percent or flicker index) and the frequency of that fluctuation (measured in Hz), is another important parameter for birds as they have higher spatial resolution than humans.[53,55,57,65,73] Some laboratory animal husbandry resources recommend cool white fluorescent (CWF) tube lighting for zebra finches.[5,63,69] However, one major drawback is that CWF and other readily available

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[1]*Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, Massachusetts; and* [2]*Department of Brain and Cognitive Sciences, McGovern Institute for Brain Research, Massachusetts Institute of Technology, Cambridge, Massachusetts*
**Corresponding author. Email: nfabian@mit.edu*

fluorescent tubes are low frequency (100 to 120 Hz) and can emit flicker frequencies that are detectable to the avian (but not human) eye, which has been found to adversely affect behavior and increase basal corticosterone (CORT) levels in other captive passerines.[27,55] In addition, fluorescent tubes contain toxic mercury vapors that can pose risks to animals, humans, and the environment if the tubes break.[32]

LED technology is being adopted as a replacement for CWF tubes in many facilities due to its superior energy efficiency and lifespan, recent reductions in up-front purchasing costs, and nonhazardous components.[18] Also, LED technology, compared with CWF, has superior color balance with fewer extreme spikes in the green and amber wavelength regions and does not produce flicker when housed in ballasts with direct current power. In laboratory rodents, LED has been shown to improve circadian regulation and downstream physiologic parameters when compared with CWF.[18] Therefore, LED may be a suitable alternative lighting source for zebra finches housed indoors. At present, there are no published reports that evaluate LED as a replacement for CWF lighting in zebra finches or other indoor-housed passerines. Our goal was to determine whether LEDs are a safe alternative to CWF lights and whether they offer any reproductive or welfare benefits to indoor-housed zebra finches.

In this study, we compared the effects of LED with CWF lighting on various parameters of behavior, physiologic stress, and reproduction in a large ($n > 200$) colony of indoor-housed zebra finches at the Massachusetts Institute of Technology. Our first aim was to compare the effects of LED and CWF lighting on reproductive outcomes of zebra finch breeding pairs as determined by egg production, hatch rate, and hatchling survival rate. Our second aim was to evaluate components of behavior and stress in adult male zebra finches housed under CWF followed by LED lighting using an ethogram-based behavioral assay and fecal CORT metabolite (FCM) measurements. We hypothesized that LED lighting would be associated with equivalent or improved breeding outcomes, behaviors (for example, decreased aggression), and stress (that is, decreased FCM levels) compared with CWF lighting.

Materials and Methods

Animals and husbandry. Healthy, adult (age greater than or equal to 3 mo), wild type, experimentally naïve zebra finches were maintained in an AAALAC-accredited indoor facility. Cubicle room level records indicate that the ambient temperature was maintained between 22 and 23 C° (72 and 74 °F) and the relative humidity was between 37% and 46% throughout the study period; these values were consistent across all 26 wk of the study. A millet–canary grass seed–oat mix fortified with amino acids and vitamins (Supreme Fortified Daily Diet: Finch, Kaytee Products, Chilton, WI) and pellets (Roudybush Breeder Nibles, Woodland, CA) constituted the main diet, with a high-protein supplement (High Potency Mash, Harrison's Bird Foods, HBD International, Brentwood, TN, moistened and mixed with minced hard-boiled eggs) fed several times each week. All birds received supplemental calcium in the form of cuttlebone. Fresh water was provided without restriction, with additional bathing bowls offered at least twice weekly. Finches were group housed with same-sex conspecifics in flight cages (maximum, 16 birds) or in breeding cages (male-female pairs with their offspring). All flight cages had a minimum of 8 perches (6 dowel and 2 swinging perches). All breeding cages had 2 perches (one dowel and one natural manzanita branch) and a nest box (Nest Leonardo, SKU: N011, 12 × 11 × 13.5 cm from S.T.A Soluzioni) that was attached to the outside of the cage. All cages received nesting material enrichment 3 times weekly in the form of strands of coconut fiber, jute fiber, and white linen cotton, in addition to cotton nestlets in the flight cages only. New pairs were provided with a nest box that contained layers of the nesting materials, except the cotton nestlets. The nesting materials within the nest box were in addition to the enrichment provided throughout each week. Before the onset of this study, a proportion of pairs were found to place whole, unshredded cotton nestlets in the center of their nest covering any preexisting eggs. To reduce the accumulation of unviable eggs and stress of egg production on the adults, nestlets were not provided to breeding pairs. Semiannual health monitoring was performed to screen for ectoparasites such as feather mites (for example, *Neocheyletiella* spp.), endoparasites such as coccidia, and bacterial pathogens such as *Salmonella* spp., along with regular (every 3 to 6 mo) postmortem surveillance for *Macrorhabdus ornithogaster* and mycobacterial organisms. All animal work was approved by the Massachusetts Institute of Technology's IACUC.

Lighting regimens and spectral of transmittance measurement. Adjacent windowless cubicles with opaque walls ($n = 3$) receiving 12:12-h diurnal artificial lighting (lights on, 0700) were used to deliver different lighting treatments. Each cubicle contained a hardwired standard 48-in. T8-sized standard electric ballast (E113705, Cooper Lighting, Americus, GA) in addition to supplemental lighting at the cage level. Supplemental lighting consisted of a single high-output, 48-in. T5-sized light fixture that spanned each row of either 3 standard breeding cages (dimensions: 17′′ L × 16′′ H × 14′′ D each; Figure 1A) or one flight cage (dimensions: 60′′ L × 20′′ H × 24′′ D each). The supplemental light on:off schedule was staggered by approximately 10 min with the standard overhead lights by digital timers, such that the lights would turn on at approximately 0710 and turn off at approximately 1850. The supplemental light sources were chosen to mimic natural daylight (broad-spectrum, correlated color temperature [CCT] of 6,500 Kelvin [K], color rendering index [CRI] of 100) as closely as possible to deliver light resembling daylight, and the ceiling lights complemented the supplemental lighting. Specifically, the CWF (control; current practice) cubicles had fluorescent Sylvania Octron 800 F032/835/ECO T8 ceiling lights (3,500 K, 32 W, and 85 CRI) and Sunlite F28T5/865 T5 supplemental cage lights (6,500 K, 28 W, and 82 CRI) in standard plug-in electric ballasts. The LED (experimental treatment) cubicles had Waveform Lighting Centric Daylight T8 ceiling lights (6,500 K, 18 W, and 95+ CRI) and Waveform Lighting Centric Daylight T5 supplemental cage lights in plug-in direct current flicker-free ballasts (6,500 K, 18 W, and 95+ CRI).

Throughout the study period, each cubicle was monitored weekly for ambient lighting characteristics at the cage level in the upper third of the cage using a hand-held spectrometer with a built-in sensor (UPRtek MK350N Premium, Taiwan) held parallel to the cage walls. For the flight cages ($n = 3$), measurements were taken from the middle of the most central perch from the level of the perch. For breeding cages, one cage on each level on all racks (3 levels, 2 racks; $n = 6$ cages per cubicle) was measured at approximately 10 cm from the opening of the nest box at the level of the adjacent perch (Figure 1B). Flicker percent and index were calculated automatically by the spectrometer software according to the Illuminating Engineering Society definitions.

Reproductive success. With the use of 2 adjacent cubicles dedicated to breeding pairs and fashioned with 18 breeding cages each, cubicle 1 was equipped with CWF and cubicle 2 with LED lighting. Over 29 wk, starting in the fall of 2022, 89

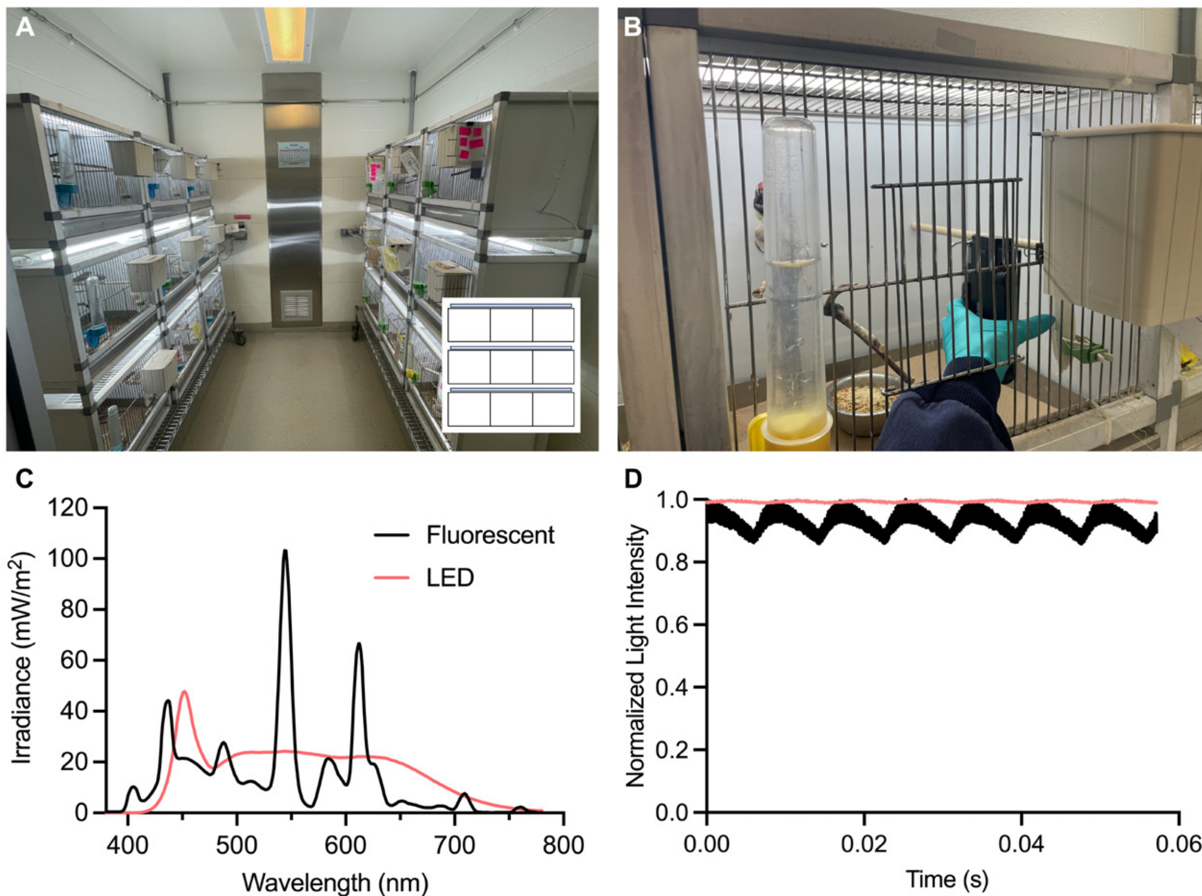

Figure 1. Lighting setup and measurements. Each cubicle (n = 3 total) consisted of 1 to 2 racks that were included in the study. Each rack had 3 rows of breeding cages or a single flight cage. (A) Each cubicle contained a hard-wired 4-ft T8-sized fixture in the ceiling and a single 4-ft T5-sized light across each row of breeding cages or each flight cage; inset is a graphic of rack setup. (B) Radiospectrometry measurements were recorded at the cage level from 6 cages in each breeding cubicle and from each flight cage approximately once weekly throughout the study. A representative image of the recording location in the breeding cages is shown. Representative (C) spectral irradiance distributions and (D) normalized temporal flicker or power modulation of the LED (red line) and fluorescent (black line) lights as measured by a handheld spectrometer at the cage level.

male-female pairs (n = 44 to 45 pairs per cubicle) were randomly assigned to breeding cages in either cubicles 1 or 2 and were monitored for an average of 40 consecutive days per pair. Up to 3 breeding pairs were randomly assigned to each cubicle weekly. All pairs were assessed at least twice weekly for nest building, egg laying, hatching, and hatchling development until 11 d post-hatch (dph), when hatchlings were relocated with the adult female by the research laboratory staff for sound isolation (Figure 2A). Bonding time (time from pairing to first egg laid), clutch size (total number of eggs laid), hatch rate (number of eggs hatched per clutch size), and hatchling survival rate (total number of hatchlings surviving to 11 dph divided by the original number of hatchlings born per clutch) were calculated for each pair and compared between treatments. Birds that failed to produce offspring after 2 or more consecutive pairings were excluded from the study.

Behavioral recordings. Adult nonbreeding males were randomly distributed into 3 flight cages (n = 45 birds total, 11 to 16 birds per cage) in cubicle 3 (separate from breeding cubicles). Ceiling and supplemental cage lighting first consisted of CWF, followed by LED. Flight cages were remotely recorded using Wi-Fi-enabled HD video cameras (ReoLink E1-3MP Indoor Wifi Camera) mounted across from each cage before the first acclimation period. Recordings occurred 3 times per week for 4 wk, following a 3-wk acclimation;[27] a morning (0700 to 1000) and afternoon (1500 to 1800) recording on Sundays (day with least disturbance by researchers and care staff) and a recording on Tuesday morning (0900 to 1200) after twice-weekly water bath dishes were provided by care staff (Figure 3A). Time-matched (across cage replicates), representative 15-min clips from each set of recordings were randomly selected for evaluation by a blinded observer using an ethogram (Table 1);[15] behaviors of all birds were counted every 15 s per recording. This approach was used to quantify the proportion of birds performing each type of behavior at predetermined time points to create activity budgets.

Fecal sampling, storage, and extraction. For each light treatment, at the end of the 4-wk behavioral observation (videorecording) period, pooled fecal samples were collected at 1020, 1140, 1240, 1340, and 1610 to evaluate basal levels of FCM and any associated diurnal changes during the sampling period (Figure 3A). Samples were collected from each cage on a Sunday when birds were least likely to be disturbed by veterinary and research staff and were collected directly from freshly placed paper cage liner. To limit disturbances created by serial sampling the appropriate number of sheets was placed prior to the sampling period. Fresh droppings were collected with the handle end of wood-based sterile cotton-tipped applicators and transferred to clear polyethylene resealable bags. Attempts were made to exclude large amounts of urates, seeds, seed hulls, and pellets. Each sample consisted of approximately

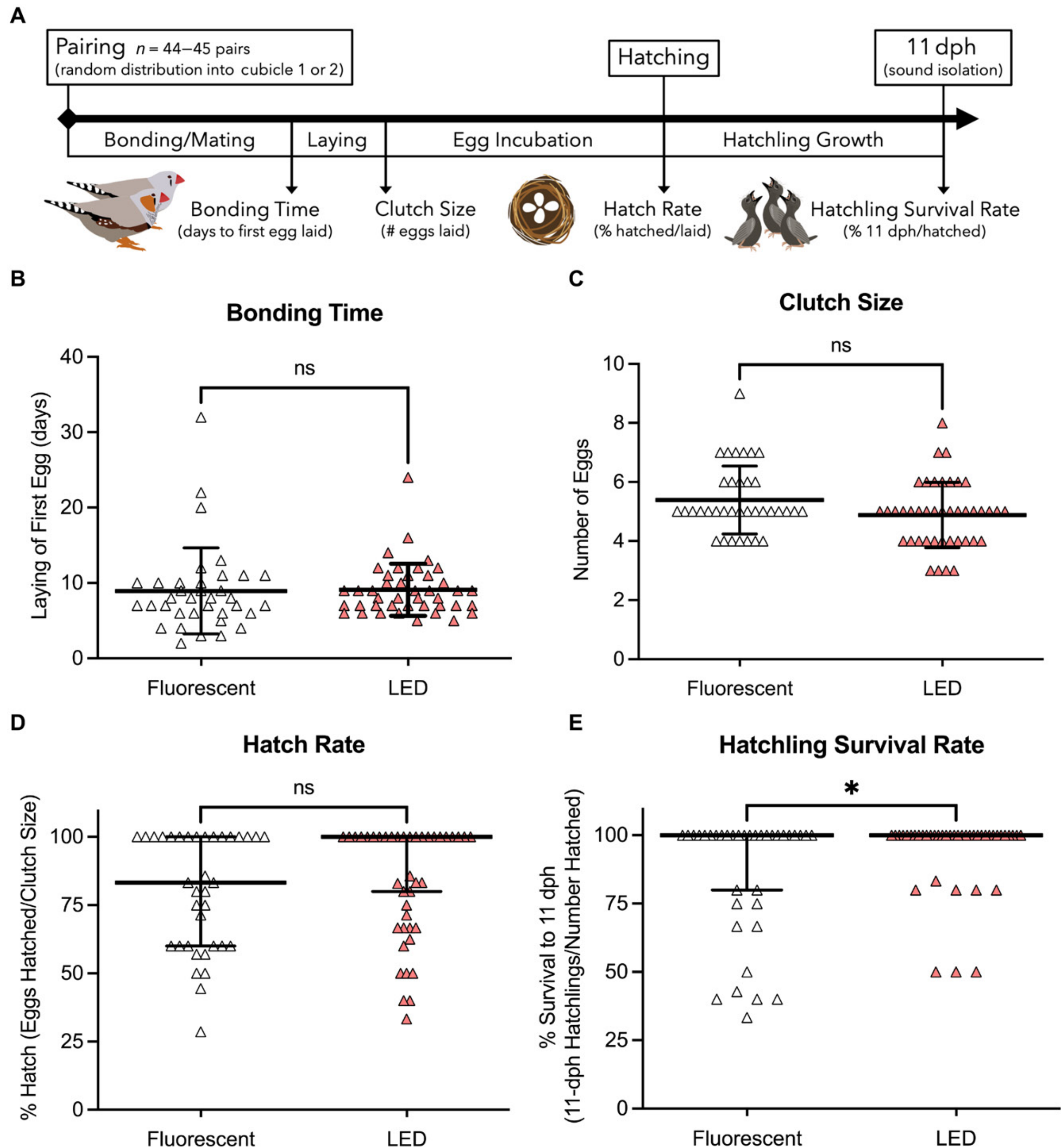

Figure 2. Summary of experiment comparing reproductive success of newly formed male-female zebra finch breeding pairs under fluorescent or LED lighting systems in different, adjacent cubicles. The study timeline for each reproductive pair is demonstrated (A). Pairs were monitored at least twice weekly for an average of 40 d from pairing through 11 d post-hatch (11 dph). Reproductive parameters of breeding pairs housed either under fluorescent lighting (control; white) or LED lighting (experimental treatment; red) are summarized. There was no difference between the 2 treatment groups for bonding time (B) and clutch size (C). Results displayed as scatter plots with the means ± SD. The hatch rate did not differ between the treatments (D), but the hatchling survival rate was significantly higher for the LED-treated pairs (E). Results displayed as scatter plots with the median ± 95% confidence interval. (*, $P \leq 0.05$; ns, not significant).

30 pooled droppings from each cage at each specified time point (approximately 0.2 g of lyophilized feces).[83] Samples were stored at −80 °C (–112 °F) until lyophilization and extraction. Frozen samples were lyophilized for 24 h, weighted, transferred to 2 mL cryovials, and extracted in 7.5 mL of 70% ethanol per gram of dried feces. Samples were broken up and shaken for 30 min before centrifugation for 15 min at 4 °C (39 °F) and 5,000 rpm.[8,78] The supernatant was transferred to a new 2 mL cryovial, dried to remove all alcohol (Eppendorf 5301 Vacufuge Concentrator), and reconstituted in 0.5 mL of room temperature assay buffer (kit diluent). Samples were stored at −20 °C (–4 °F) until being assayed.

FCM ELISA. FCM measurements were made using the DetectX Corticosterone Enzyme Immunoassay Kit (Arbor Assays, Ann Arbor, MI; K014-H5). Samples and standards were treated according to the manufacturer's instructions. Each sample and

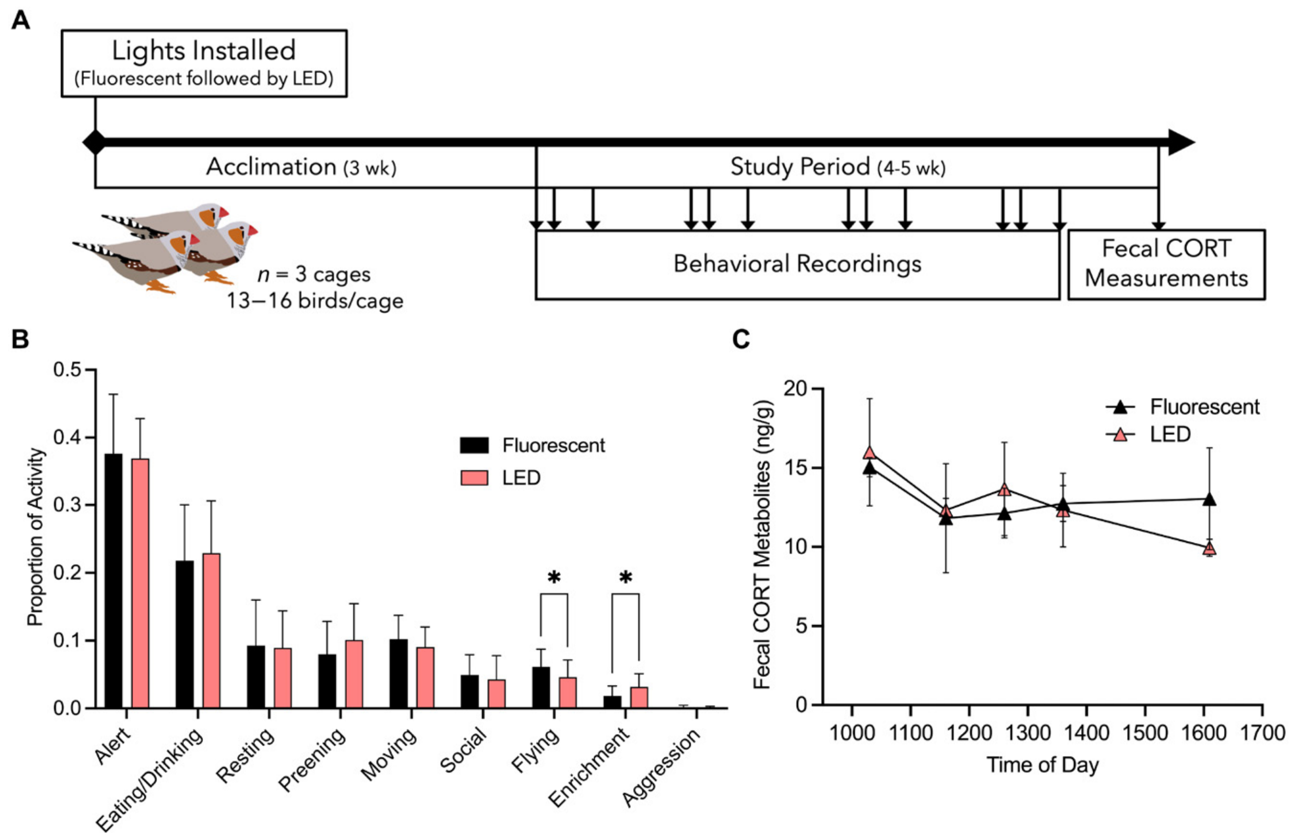

Figure 3. Summary of experiments evaluating welfare in nonbreeding adult males. (A) Three cage replicates were used, with each cage containing 11 to 16 birds. A cross-over design was used with the males housed under fluorescent lighting and then under LED. Video recordings were made 3 times a week for 4 wk, after a 3-wk acclimation period for each lighting type. Fecal samples were collected at the end of the recording periods at 5 standardized time points in a single day. (B) Behavior time budgets of adult males were created for each lighting treatment type based on an ethogram. Proportions of total activity for each treatment group are displayed as means ± SD to demonstrate relative changes in activity budgets. Pair-wise comparisons were made with Student *t* tests and Holm-Šídák correction. (C) Diurnal pattern of fecal corticosterone (CORT) in male zebra finches housed under fluorescent or LED lights. Means ± SD are displayed. Fecal CORT did not differ between the 2 treatments and did not significantly fluctuate during the sampling period (2-way ANOVA, time × treatment, $P > 0.05$; ng/g = ng CORT per g of dried feces; *, $P \leq 0.05$).

Table 1. Ethogram describing video assessment behavioral coding

Behavior	Description
Moving	Locomotor activity without use of wings from perch to perch, perch to floor, moving sideways on a perch, swinging (balancing) on plastic clothes hanger, or hopping on floor.
Flying	Locomotor activity with the use of wings from perch to perch, or perch to floor.
Alert	Frequent head movements and an absence of locomotor activity, not performing any of the other defined behaviors.
Resting	Absence of head/wing movement or locomotor activity.
Feeding/drinking	Drinking water, eating seeds, supplemental mash, or cuttlefish bone; if the bird pauses the behavior for a maximum of 3 s and then returns to it, then the whole duration of that activity is considered as the same “bout” of foraging.
Interacting with enrichment	Pecking at or holding nesting material (for example, sisal, hay, nestlet); if the bird pauses the behavior for a maximum of 3 s and then returns to it, then the whole duration of that activity is considered as the same “bout” of enrichment interaction.
Preening	Grooming feathers, bathing (standing or hopping in, preening or flapping wings in water bath); if the bird pauses the behavior for a maximum of 3 s and then returns to it, then the whole duration is considered as the same “bout” of preening.
Social behavior	Allopreening (bird grooms conspecific or is groomed by conspecific).
Aggression	Biting/pecking at, chasing another bird.

Table was adapted from Reference 15.

standard were measured in duplicate. The following control values were determined: nonspecific binding and maximum binding/zero standard. A standard curve was created for every analysis. All samples had CORT levels that were above the manufacturer’s reported limit of detection for this assay (26.99 pg/mL). Sensitivity of the assay for CORT at 50% binding was approximately 0.8 ng/mL. Measurements were adjusted for the mass of the fecal sample and concentrations are expressed as

nanograms/gram of lyophilized fecal matter. The mean intra- and interassay coefficients of variation (n = 3 plates) were 8.9% (n = 134) and 5.69% (n = 19), respectively. Cross-reactivity for the CORT antibody, according to the manufacturer was as follows: corticosterone (100%), 1-dehydrocorticosterone (18.90%), desoxycorticosterone (12.30%), 1α-hydroxycorticosterone (3.3%), 11-dehydrocorticosterone (2.44%), tetrahydrocorticosterone (0.76%), aldosterone (0.62%), cortisol (0.38%), progesterone (0.24%), dexamethasone (0.12%), testosterone (0.03%), corticosterone-21-hemisuccinate (<0.1%), cortisone (<0.08%), estradiol (<0.08%), 17-hydroxyprogesterone (<0.01%), allopregnanolone (<0.01%), dehydroepiandrosterone sulfate (<0.01%), estrone-3-glucuronide (<0.01%), and estrone-3-sulfate (<0.01%).

ELISA validation. To validate a commercially available corticosterone ELISA kit, healthy, adult male zebra finches (n = 11 to 14 birds/cage) underwent hand capture and restraint as an acute stress challenge. Following this challenge, a time course of FCM measurements was recorded to identify acute changes in adrenal activity and subsequent return to basal levels. This is a previously validated method when challenging adrenocortical tissue and elevating endogenous CORT concentrations in small birds.[50,79,80,86,88] We used 2 variations of a "capture/restraint" stress method, either via an established protocol with an opaque cotton bag (method 1, n = 1 cage) or a novel protocol with an extended (60 s) hand capture and placement in a new home cage (method 2; n = 3 cages).[50,86] Fresh basal fecal samples were collected and then each bird was hand caught, examined, and restrained using one of the 2 methods. Post-stress fecal samples were then collected 1, 2, 3, and 5.5 h after restraint (Figure 4A). Timepoints were based on similar studies in other passerines.[78] Time-matched, unstressed controls were used for comparison. All samples were collected between 1000 and 1630 during the middle of the diurnal, lights-on period. To determine the assay validity across multiple concentrations, parallelism was compared between serially diluted (1:4 to 1:64, n = 4 samples) fecal extracts from pooled adult male zebra finch samples and kit-provided CORT standards.[8,78]

Statistical analysis. FCM levels were measured in nanograms of CORT metabolite per gram of dried feces. FCM ELISA validation and experimental basal stress repeated measured samples were analyzed using a 2-way ANOVA (time × stress method, or time × treatment), and Bonferroni post hoc tests were used to compare stressed and unstressed time-matched values. Parallelism was assessed by linear regression. Behaviors were summarized for each 15-min video as a percentage of behavior performed during that period and were compared across treatments, recording week (first through fourth week), and recording period (morning, evening, or with water bath) using a 3-way full-factorial multivariate analysis of variance (MANOVA). Paired t tests with a Holm-Šídák correction were performed for each behavioral category to further characterize the observed variance from the MANOVA. Bonding time and clutch size were compared with unpaired t tests, and hatch and survival rates were compared by nonparametric one-sided Mann–Whitney U tests. Values of $P \leq 0.05$ were considered

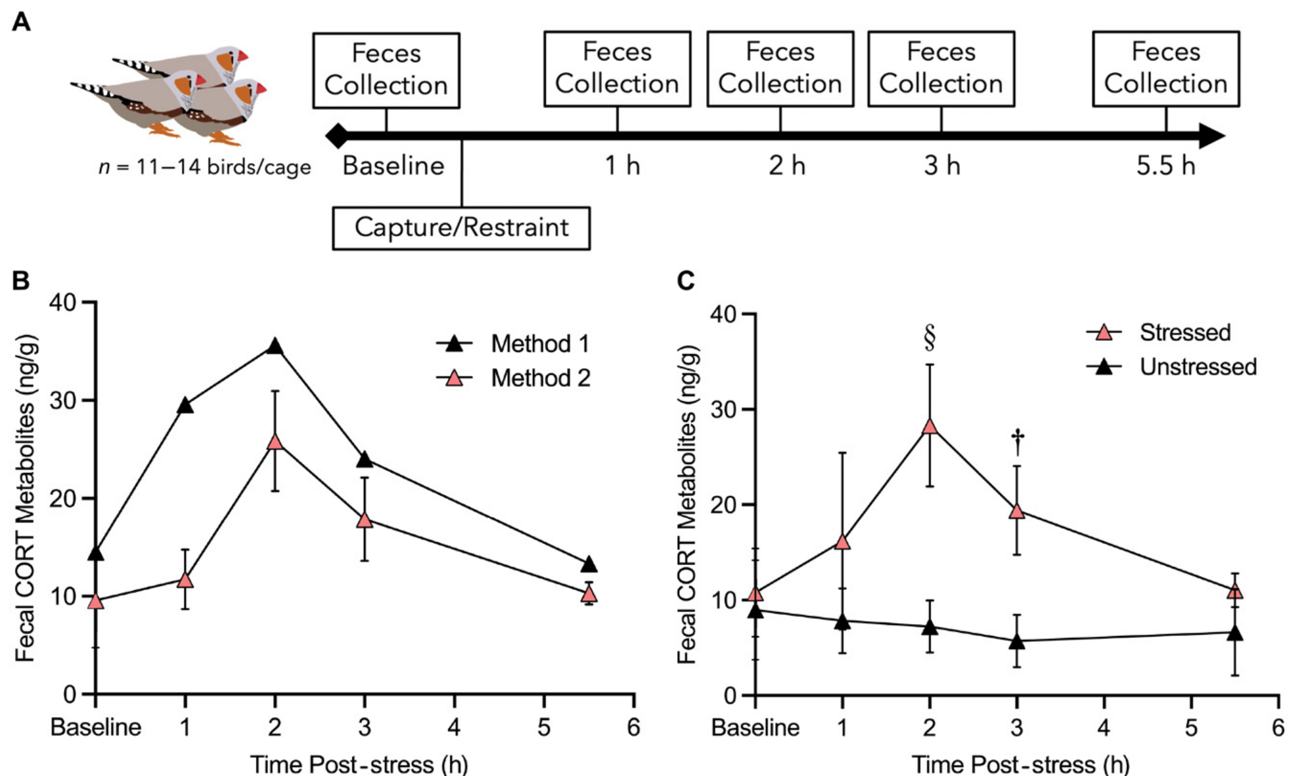

Figure 4. Fecal corticosterone (CORT) metabolite assay validation. (A) Schematic of capture/restraint physiologic stress test. Basal fecal samples were collected. Birds were then restrained to generate a stress-induced CORT release. Post-stress samples were collected at 1, 2, 3, and 5.5 h post-restraint. On a separate day from restraint, time-matched fecal samples were collected from unstressed birds from the same cages to serve as controls (n = 4 cages). (B) Stress was induced using either an established "capture/restraint" protocol with an opaque cotton bag (method 1) or a novel protocol with an extended hand capture and new home cage (method 2). Means ± SD over the time course are presented. There was no significant difference between the methods (2-way ANOVA time × stress method, $F_{4,8} = 1.442$, $P = 0.305$); the results were combined for comparison against unstressed controls. (C) Mean ± SD fecal CORT in stressed male finches compared with time-matched, unstressed controls. Restraint resulted in a significant elevation compared with controls at 2 and 3 h post-stress (2-way ANOVA, time × treatment, $P \leq 0.0001$), with a return to baseline by 5.5 h post-stress (Bonferroni post hoc test). (†, $P \leq 0.001$; §, $P \leq 0.0001$; ng/g = ng CORT per g of dried feces).

significant. All tests were performed using GraphPad PRISM 10.0.2 (GraphPad Software, Boston, MA) or SAS JMP Pro 16.2.0 (SAS Institute, Cary, NC). Sample sizes were based on a minimum of 80% power ($\beta = 0.2$) at a significance criterion of $\alpha = 0.05$ and determined using G*Power version 3.1.9.6.[29] FCM ELISA validation sample size was calculated based on similar tests in other passerine species with an estimated $\delta = 6$ and SD = 2 between baseline and peak elevation in CORT.[78] Minimum sample size based on a one-sided t test was $n = 3$. To determine whether LED would result in similar or improved reproductive outcomes compared with CWF treatment, sample size for the reproductive study was based on the one-sided Mann–Whitney U test with an estimated medium effect size of $d = 0.65$ (from an estimated $\theta_1 = 0.85$, $\theta_2 = 0.75$, SD = 0.15). Minimum sample size was estimated to be $n = 34$ per group.

Results

Lighting measurements. Cage-level illumination from T5 lights placed above each row of cages was measured from a standard location (displayed in Figure 1B) with the detector facing upward to measure ambient lighting within the breeding cages. Measurements ($n = 101$ total per cubicle) were similar to the manufacturer's specifications. Irradiance (and illuminance) values in cubicle 1 (CWF; control) were 7.91 ± 1.96 mW/m^2 ($2{,}555 \pm 644$ lx) with a CCT of $5{,}647 \pm 120$ K and a CRI of $83.7 \pm 0.56\%$. cubicle 2 (LED) cages had an irradiance (and illuminance) of 6.09 ± 1.27 mW/m^2 ($1{,}638 \pm 346$ lx) with a CCT of $6{,}128 \pm 178$ K and a CRI of $97.5 \pm 0.15\%$. Representative cage-level spectral power distributions from 380 to 780 nm are shown in Figure 1C. The CWF tubes emitted unnatural peaks in the green (approximately 545 nm) and amber (approximately 612 nm) regions, which was expected based on previous studies.[18,20] The LED light had an expected blue (approximately 448 nm) peak and a more sunlight-mimicking, natural distribution across the visual spectral output. Flicker percent and index were measured, and power modulation fluctuations are visually represented in Figure 1D. The frequency of flicker (or power modulation/fluctuation) cycled at 128 Hz for both lighting sources, as determined by the municipal power source. However, the change in output as determined by the flicker percent and flicker index was approximately 7.6% and 0.014 for the CWF lights and 0.63% and 0.0009 for the LED lights, respectively. The flight cage lighting measurements were similar to those from breeding cages reported above but were measured from the level of the most central perch.

Reproductive success. New pairs acclimated over a 10-d bonding/pairing period, approximating the time needed for successful pairing before mating.[89] They were then monitored from egg laying, through incubation, and finally until 11 dph. The number of days to produce the first egg of the clutch, maximum clutch size, percent hatching rate (eggs hatched per maximum clutch size), and percent hatchling survival to 11 dph (nestlings per maximum clutch size) were recorded. Eleven pairs ($n = 9$ CWF treated, 2 LED treated) were excluded due to aggression ($n = 1$, CWF treated) or unsuccessful pairing with no hatchling production ($n = 8$ CWF treated, 2 LED treated). Results ($n = 36$ CWF-treated pairs and 42 LED-treated pairs after exclusions) showed no statistically significant difference in the timing of the first egg produced ($t_{76} = 0.144$, $P = 0.886$; Figure 2B), clutch size ($t_{76} = 1.980$, $P = 0.051$; Figure 2C), or percent hatching rate ($U = 684$, $P = 0.223$; Figure 2D), but percent hatchling survival was higher in the LED group ($U = 623.5$, $P = 0.043$; Figure 2E).

Behavior. Using an ethogram, a blinded observer scored twelve 15-min videos for each cage of group-housed male birds. This was repeated under CWF and LED lighting. Behavioral time budgets were created for each treatment over 4-wk recording periods, with 3 time points each week for each cage, resulting in 36 budget summaries per treatment. The total proportion of each type of behavior for each time video was calculated. A 3-way full-factorial MANOVA (treatment × recording week × time of day) showed no significant interactions between independent variables (Wilk's $\lambda_{18,104} = 0.806$, $P = 0.845$). There was also no significant interaction between treatment and time of day of recording or treatment and week of recording (treatment × time of day, Wilk's $\lambda_{18,104} = 0.753$, $P = 0.606$; treatment × recording week, $F_{9.52} = 0.291$, $P = 0.117$). However, treatment alone did significantly influence the time budget profiles (one-way MANOVA, $F_{9,62} = 0.454$, $P = 0.0036$), so each behavior type was subsequently analyzed by individual paired Student t tests and Holm-Šídák correction for multiple comparisons for each behavior type to determine the source of variability detected by the MANOVA. Trends between the 2 treatments are summarized in Figure 3B. The "flying" and "enrichment interaction" terms were found to be significantly different between the treatment groups. Specifically, when treated with LED lights, there was a decrease in proportion of birds flying (4.6% compared with 6.1%, $t_{35} = 3.599$, $P = 0.009$) and an increase in proportion of birds interacting with enrichment (3.2% compared with 1.9%, $t_{35} = 3.051$, $P = 0.034$), compared with the CWF treatment.

Physiologic stress. Using 3 groups of adult male zebra finches randomly assigned to flight cages, we used a crossover design first using CWF lighting type (control; current practice), followed by LED (experimental). We used FCM as a measure of physiologic stress, as CORT is the major stress hormone in birds.[42] Pooled fecal samples collected at the end of each 4-wk behavioral observation period for each lighting treatment were analyzed for basal FCM levels using a 2-way ANOVA (time × treatment; Figure 3C). There was no difference in the FCM levels between the 2 treatments. In addition, the FCM levels did not fluctuate during the sampling period across all samples combined (effect of time; $F_{2.214,8.856} = 2.401$, $P = 0.1444$) or within each treatment group (Tukey's honestly significant difference, $P > 0.05$).

ELISA validation. There was no significant difference in the FCM elevation between the 2 stress methods (2-way ANOVA, time × stress method, $F_{4,8} = 01.442$, $P = 0.305$; Figure 4B). This result demonstrates the validity of the novel capture-restraint stress method with a 60-s extended manual restraint (method 2) as an alternative to the traditional method using an opaque cotton bag (method 1). The results obtained from both methods were combined and compared with unstressed, time-matched controls (Figure 4C). There was a significant increase in FCM from baseline at 2 and 3 h (2-way ANOVA, time × stress method, $F_{4,24} = 9.463$, $P \leq 0.0001$, Bonferroni post hoc test, $P \leq 0.0001$ and $P = 0.002$, respectively) and a return to baseline by 5.5 h, demonstrating that the ELISA can detect increased FCM resulting from physiologic stress in laboratory zebra finches.

We also assessed regression curves between CORT standards and a dilution series of pooled samples (1:4 through 1:64), which demonstrated parallelism with the kit-provided standard curve ($F_{1,24} = 0.4415$, $P = 0.5127$; standard slope = −2.262; sample slope = −2.210; Figure 5). All samples were within the log-logit linear range (13.9% to 92.2% binding) of the standard curve, demonstrating that this ELISA can robustly detect and quantify FCM within this range. These results demonstrate that FCM ELISA can be used as a reliable, noninvasive test to monitor stress in adult male zebra finches.

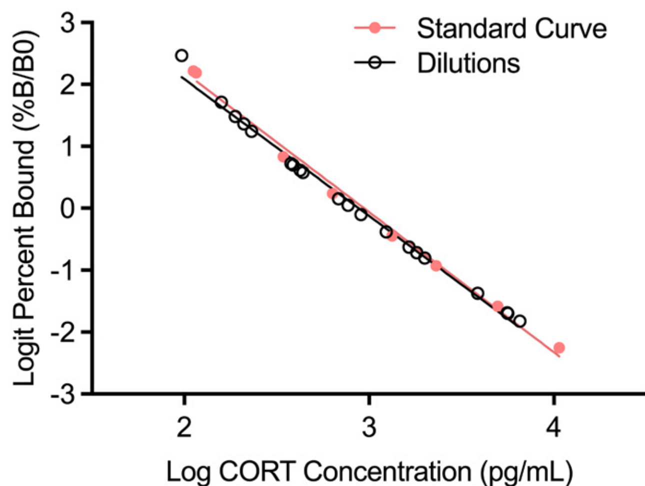

Figure 5. Parallelism between serially diluted standards and pooled fecal corticosterone (CORT) levels detected in feces using ELISA. A log–logit transformed standard curve ($Y = -2.210 \times X + 6.506$, $r^2 = 0.9940$, $P \leq 0.0001$) and log–logit transformed curve of serial dilutions of fecal extracts collected from adult male zebra finches ($Y = -2.262 \times X + 6.723$, $r^2 = 0.9925$, $P \leq 0.0001$). The standard curve is indicated by the red line and closed circles. The fecal dilutions are indicated by the black line and open circles.

Discussion

LED lighting is becoming more widely adopted and is expected to be used in the majority of animal facilities within the next decade.[16,20,40,45] This shift is largely due to the improved energy efficiency, reduced heat, and low vibration or noise from the technology compared with fluorescent and incandescent lights, compounded with the improved manufacturing costs and accessibility of the technology. Lighting quality and characteristics, including spectral irradiance and flicker, have been shown to influence avian physiology, behavior, and reproduction.[5,27,69] In addition, photoperiod is an important signal for migratory birds.[7,36] However, no prior study has specifically compared the effect of broad-spectrum LED and CWF technologies on the reproduction and welfare of indoor-housed passerines. This study compared the effects of LED with CWF lighting on the reproductive outcomes, behavior, and physiologic stress of laboratory zebra finches. We found that LED lighting resulted in an increase in the proportion of breeding pairs without any hatchling deaths within the first 11 dph and had no negative impact on zebra finch welfare in terms of fecal CORT metabolite measurements. We also found that LED lighting resulted in altered behavior of group-housed males, as evidenced by less flighted movement and more interaction with provided nesting material enrichment items. Overall, we concluded that LED lighting did not induce more stress or negative reproductive outcomes compared with the standard CWF lighting that is used in most facilities today, supporting the use of LED as a safe and viable lighting alternative for these birds.

Birds have a wider visual spectrum than humans, from approximately 300 to 700 nm, and like reptiles and freshwater fish, most diurnal birds have 4 types of cones (tetrachromacy) involved in color vision, rather than 3, with the maximum sensitivities at 567 nm (red), 502 nm (green), 430 nm (blue), and 360 nm (UV).[10,38] These cones contain colored oil droplets that are thought to narrow cone spectral sensitivities and improve color discrimination,[81] which is more accurate at higher light intensities.[67,68] These are important considerations for 2 reasons. First, the established photometric measurements, including lux brightness estimates, are not directly applicable to birds and other tetrachromats.[17] Second, as diurnal birds perceive light and visual signals in a different manner, providing a species-specific full spectrum of light (that is, wavelengths including the UVA range) and ensuring the quality of the light is appropriate (for example, high CRI or related measurements of color quality, low flicker, balanced wavelengths) is arguably more important for avian species compared with rodents. The optimization of artificial lighting systems for indoor-housed zebra finches and other passerines should be based on well controlled studies, with careful consideration for species-specific anatomy and physiology.

Light flicker from artificial light sources is a temporal artifact that is caused by the inconsistent output of light over time due to oscillating power delivery. The level of flicker is a factor of both the frequency of the fluctuation and the difference between maximum and minimum output (peaks and troughs), which is described as flicker index or flicker percent. Low frequency and/or high flicker index or percent light sources are more likely to be perceived as flickering, but perception of this variation is also determined by an individual animal's temporal resolution. Animals with high temporal resolution such as flighted birds and insects are much more likely to perceive the flickering of a low-frequency or highly fluctuating light.[46] While a human may perceive flicker under 50 to 90 Hz lighting, rock pigeons (*Columba livia*) and other diurnal birds have been recorded as high as 100 to 143 Hz.[21,53,55,57,65] In our study, we determined that the flicker frequency was 128 Hz for both lighting sources, but both flicker index and flicker percent measured at the cage level were consistently higher in the CWF-illuminated cages. Specifically, the flicker percent and flicker index were approximately 7.6% and 0.014 for the CWF lights and 0.63% and 0.0009 for the LED lights, or approximately 1,200% higher under the CWF lights.

The perception of flicker has been shown to affect behavioral patterns and stress levels in several avian species.[26,70,84] In a series of studies with European starlings (*Sturnus vulgaris*), one study found that low-frequency (100 Hz) full-spectrum light was associated with altered behavior, including increased muscle spasms, as compared with high-frequency (>30,000 Hz) light.[27] The observed behavioral changes remained for 3 wk following the end of the low-frequency light exposure period. In addition, in a choice experiment, European starlings consistently spent more time under high-frequency lighting, suggesting that they found high-frequency lighting less aversive, although, like our findings, there was no change in basal CORT levels.[34] Lighting flicker has also been shown to affect European starling visual communication and reproductive behavior. High-frequency lighting was associated with more consistent mate choice by adult females, which was associated with feather type.[24] The LED near-zero flicker percent and flicker index in our study may have been perceived by the birds as less aversive and less stressful than the flicker produced by the CWF lighting, providing a more stable environment. This is one possible explanation for the reduced hatchling deaths and the altered behavior of males associated with LED lighting. Based on this key difference between the lighting types used, LED lights powered by high-quality flicker-free drivers should be used for all cage-level supplemental lighting or for overhead lighting where cage-level lights are not used.

Another important photometric parameter to consider when choosing an artificial light source is the CCT or color balance, which indicates the color tone of white light and is measured on the Kelvin scale. Lights with a warmer color have a lower

color temperature or CCT; for example, warm, red-toned late afternoon light is approximately 2,000 to 3,000 K, while cool, blue-toned midday sunlight is approximately 6,500 K. Diurnal animals in the wild are adapted to natural sunlight, and the visible spectrum of sunlight is best simulated by cool-toned or blue-enriched broad-spectrum lighting.[18] The CCT of white light and single-wavelength lights have both been shown to affect avian behavior and reproduction. Broiler chickens raised under cool-toned (5,000 K) white LED lights had reduced stress and fear responses compared with those raised under warm-toned (2,700 K) white LED lights.[4] In addition, broilers reared under blue LED lights had the highest reproductive performance, lowest CORT and behavioral stress scores, and highest melatonin levels compared with red, green, white, and mixed LED light.[1,31] However, the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* recommends between 3,000 and 3,500 K for stimulating egg production in layer poultry.[30] Similar lighting tone and color studies in budgerigars (*Melopsittacus undulatus*) found that blue LED light was associated with an increase in social behavior, but birds spent more time under yellow light compared with blue, red, or white LED light.[23] The lights used in our study were both cool-toned, broad-spectrum white lights, with a CCT over 5,500 K at the cage level (with higher levels reported by the manufacturers).

Importantly, cool-toned daylight-mimicking broad-spectrum lighting is optimized when delivered by LED technology due to the balance of irradiance across all wavelengths of light. Conversely, CWF lights of the same overall CCT will inherently emit unnatural spikes in the green and amber regions, as demonstrated in our cage-level measurements. Previous comparisons of these 2 lighting technologies on rodent physiology found that animals housed under LED lighting had a significant increase in melatonin release in the middark phase and an enhancement of the circadian regulation of multiple neuroendocrine, metabolic, and physiologic parameters.[3,19,20] For example, when comparing groups of rats exposed to either LED or CWF technology during the day, LED-exposed rats had similar circadian patterns of release, but overall lower plasma glucose, lactic acid, and CORT levels, which are associated with improved physiology and well-being.[20] Similarly, our study compared cool-toned broad-spectrum LED and CWF lighting. However, the effect of LED and CWF lights on physiologic changes in zebra finches beyond FCM were not compared in this study and would require further investigation.

Our study found an increased hatchling survival rate of 11 dph in the LED-treated male-female breeding pairs, with a greater proportion of pairs achieving 100% survival. Thus, while the hatch rate did not differ, LED lighting appeared to positively alter the post-hatching conditions and increase the chances of survival of the hatchlings. Therefore, the LED lighting had nuanced effects on different aspects of reproductive success in these birds. An improvement to the cage microenvironment due to reduced flicker or optimized spectral profile may have enhanced parental care, for example, through optimized feeding and brooding behaviors. However, this explanation for the improvement in hatchling survival under LED lighting is speculative, and further studies are needed to elucidate the specific mechanisms underlying this phenomenon.

Captive animals can be negatively impacted by the inability to express or perform natural behaviors.[58] To counter this, biologically relevant enrichment has been studied in many production, companion, exhibitory, and laboratory species. Avian studies have identified species-specific positive changes in behavior associated with the addition of environmental enrichment to baren or otherwise lightly furnished enclosures, including increased foraging and preening in chickens,[25] reduced fear responses in Amazonian parrots (*Amazona amazonica*),[59] reduced periods of inactivity in zebra finches and mute swans (*Cygnus olor*),[13,48] and increased vocalizations and singing in zebra finches.[47] However, a study with garden warblers (*Sylvia borin*) found no correlation between basal CORT levels with the addition of environmental enrichment.[28,61] In our study, the environmental enrichment offered during each lighting period did not change and included a variety of nesting fibers. Overall, the most common behaviors recorded did not differ between the lighting types, but the nonbreeding males spent more time manipulating the provided nesting fiber enrichment in the second half of the study when the lights were changed from CWF to LED. This manipulation included collecting, shredding, and piling. During this time, there was no change in ambient temperature or relative humidity. We consider this a positive behavioral shift as male zebra finches are the sex that collects and deposits nesting material, while the female will shape it into a dome nest, and nest building is associated with an increase in activity in the dopaminergic/reward circuits in the brains of zebra finches.[37] In addition, the nonbreeding males in this study spent less time in flighted locomotion ("flying") when lights were changed from CWF to LED. Although the welfare implications of this finding are less clear, the decreased locomotion could be associated with a positive affective state. For example, it has been reported in the literature that male zebra finches increase their "perch hopping" activity (similar to our "flying" term) after separation from their breeding partner, and ravens (*Corvus corax*) increase their locomotor activity when in a negative affective state.[2,71] Alternatively, the decreased flight could be a result of the increased time spent interacting with enrichment.

The factors that affect optimal parental care and reproductive outcomes in birds are complex and multifactorial, including experience or parity, overall health, genetics, and age.[85] Other considerations more specific to diurnal lighting quality include the interactions between melatonin and other hormones in avian parental care physiology. For example, in groups of rats housed under natural light (sunlight) or CWF lights, natural light was associated with greater melatonin peaks during the dark cycle and a stronger circadian pattern of prolactin release.[51] Therefore, while melatonin is involved in regulating sleep-wake cycles in most vertebrate species, changes in melatonin patterns can have cascading effects on other hormones. In zebra finches, prolactin plays a critical role in maternal and paternal behaviors, influencing the time and effort invested in nurturing offspring, and plasma prolactin is highest in the post-hatching period in this species.[11,74] While it is unclear how zebra finch melatonin and prolactin levels vary under different diurnal lighting conditions, it is possible that the LED lighting resulted in higher hormone levels in both nonbreeder and breeder birds, possibly through changes in melatonin release. If this were the case, changes in prolactin levels may have altered the allocation of time and resources toward nesting material manipulation due to a perceived need for nest building and parental care. Thus, it might explain both the behavioral change in the nonbreeding males and the improved hatchling survival rate associated with LED lighting. Although beyond the scope of this study, investigating how LED lighting influences nighttime melatonin secretion, prolactin plasma levels, and daytime behavior in zebra finch breeding pairs could provide a better understanding of the influence of lighting on hormone modulation and breeding success.

FCM measurements have been used to noninvasively assess stress in a variety of mammalian and bird species, but validation had not been performed in zebra finches.[64,83] We validated a FCM ELISA and demonstrated its utility as a low-stress alternative to blood sampling to assess physiologic stress in adult male zebra finches. This method was preferred over plasma measurements, as the handling and restraint required for blood sampling have been shown to increase CORT levels within a few minutes, requiring investigators using this method to collect blood within the first 2 min after capture.[33,39,62] To demonstrate that the assay accurately detected changes in CORT release, we used 2 variations of a capture-restraint stress method to cause a physiologic increase in CORT. The opaque cotton bag method (method 1) has been used extensively in field studies that require short-term capture of wild birds[43,50,77,86] or in captive avian physiology studies to induce an acute stress response.[49] We compared this traditional method to a novel extended (60 s) hand-restraint method (referred to as method 2). The elevation in FCM from baseline was equivalent between the 2 methods, with an approximately 2-fold increase by 2 h post-restraint and a return to baseline by 5.5 h post-restraint. As the 2 methods resulted in comparable elevations in FCM, extended manual restraint may be a safe, easily applied alternative for the experimental induction of stress in indoor-housed domesticated birds. These results also highlight the importance of considering any study effects or variables secondary to handling and restraint and whether an acclimation or a recovery period should be standardized in studies using zebra finches. In addition, we found a high level of parallelism between the kit-provided standard curve and serial dilutions of 4 concentrated samples, demonstrating validity of experimental samples across a wide range of concentrations with little interference by contaminants, including dyes used in commercial seed and pelleted diets. This assay is most suitable for comparisons within a single population; between 2 or more treatment groups or over a time course. Additional validation should be completed before using this assay to assess FCM in juveniles, adult female zebra finches, or wild zebra finch populations, as physiologic variation from the adult male population in our study can result in altered metabolism of CORT excreted in the feces. Finally, as there were no significant variations in FCM seen within each group between 1020 and 1610, smaller groups of birds may be analyzed by pooling samples collected during a predetermined timeframe (for example, between 1100 and 1600).

There was no change in the basal FCM of the nonbreeding male finch population after 7 wk of exposure to each lighting type. However, this result may not fully capture the complex dynamics of the avian neuroendocrine response to chronic environmental stressors. Some avian studies have indicated that responses to chronic stressors can be highly adaptive and involve various physiologic and behavioral adjustments.[87] Thus, the impact of the lighting conditions on CORT excretion may present as a short-term change that is modulated over time. Therefore, transient deviations in basal CORT may have been missed. As an alternative approach, previous avian studies have suggested that the magnitude of an acute stress response more reliably reveals the overall stress state compared with relying solely on basal CORT levels.[12,49,82] This approach is grounded in the idea that acute stress responses are often more pronounced and consistent, and the magnitude of change can be influenced by chronic stress states.[72] For example, capture restraint or exposure to novel environments can induce rapid elevations in CORT levels, which can reflect both the habituation to the event and the underlying physiologic state of the individual or population. In the context of our study, considering the acute stress response of the adult nonbreeding males, in addition to early changes in basal FCM levels, may have provided a more comprehensive understanding of how the different lighting conditions influenced their physiologic stress levels.

Our study did not consider UV wavelengths, and both cage-level light sources emitted a negligible amount of UVA (315 to 400 nm; less than or equal to 1% of maximum irradiance output) and UVB (280 to 315 nm; less than 1% of maximum irradiance output), as stated by the manufacturers and verified by a spectrometer (SpectraPro 2300i, Acton Research Corporation; data not shown). However, birds do see into the UVA range, and these wavelengths are an inclusive portion of the full avian visual spectrum of light. As such, UVA is important for visual communication cues in birds, and female zebra finches will alter their mate selection behavior when visualizing males under a full avian spectrum of light.[6,44] Thus, artificial lighting excluding UVA wavelengths can withhold important visual cues in the form of UV plumage reflectance and can influence mate choice. In addition, UVB is important for vitamin D production and calcium absorption, although an appropriate diet can generally overcome the absence of UVB.[52,76] Conversely, inappropriate exposure to UV wavelengths can have negative consequences for birds (for example, retinal damage) and for vivarium care staff and research personnel, so its inclusion in the overall lighting regimen in a facility should be carefully considered, and decisions for implementation should include input from occupational health and safety specialists.[9,41]

Our study has shown that lighting spectral quality appears to affect zebra finch reproductive success, which we suspect is due to a change in reproductive behavior and investment; however, the physiologic cause is not known. In addition, these lighting factors will influence the behavior of group-housed nonbreeding adult males, with an increase in manipulation of nesting materials under LED light. While the link between increased nest material manipulation by nonbreeding males and enhanced nestling survival of breeding pair offspring under LED lighting remains speculative, it is plausible that improved nest construction, mate attraction, and parental care may collectively contribute to the observed outcomes. Changes to the cage microenvironment due to lighting spectral color balance and flicker may have altered photoperiodic cues, potentially affecting circadian hormone regulation and stress responses and ultimately impacting nest-building behaviors and parental care. Further inquiry into the underlying mechanisms and behavioral processes is needed to elucidate the complex relationship between lighting conditions, nest building, and reproductive success in zebra finches.

The findings of this study support the use of broad-spectrum LED as an alternative to CWF lighting for indoor-housed zebra finch colonies without compromising key aspects of their reproductive behavior.[89] Specifically, we recommend tubes or strips housed in a frosted casing and powered by an LED-specific high quality flicker-free ballast. The CCT should be blue-enriched (6,500 K) with a balanced irradiance and have a CRI of 95% or higher. Cage-level supplemental lighting should also provide enough illuminance to mimic the light levels these birds are adapted to, and we recommend 1,000 lx or higher at the cage level. Future studies on the effects of both dawn-dusk color-shifting technology and UV supplementation on physiology and well-being are recommended to provide additional evidence-based lighting recommendations for zebra finches. Continued optimization and standardization of the husbandry practices for zebra finches will improve their welfare and the use of this species as a research model.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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Author Contributions

AGB and NJF designed the research study; AGB, AT, and NJF cared for the animals in the study and created breeding pairs; AGB, AW, and NJF performed the laboratory assays; AGB, AW, and NJF analyzed the data; AW created original images for the figures; AGB and NJF wrote the first draft of the paper. All authors reviewed and critically edited the manuscript.

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