Artificial light at night does not affect telomere shortening in a developing free-living songbird: A field experiment
Artificial light at night and telomere dynamics

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HIGHLIGHTS

- Artificial light at night (ALAN) might induce developmental stress.
- We experimentally exposed great tit nestlings to ALAN in nest boxes.
- Nestlings exposed to ALAN gained less body condition.
- Nevertheless, ALAN did not affect telomere shortening.
- Body condition was positively related to telomere length and fledging success.

GRAPHICAL ABSTRACT
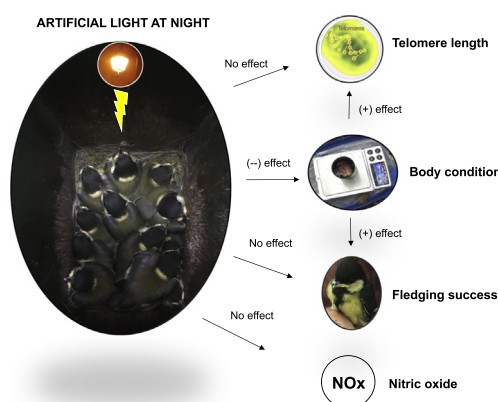
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ABSTRACT

Artificial light at night (ALAN) is an increasingly pervasive anthropogenic disturbance factor. ALAN can seriously disrupt physiological systems that follow circadian rhythms, and may be particularly influential early in life, when developmental trajectories are sensitive to stressful conditions. Using great tits (*Parus major*) as a model species, we experimentally examined how ALAN affects physiological stress in developing nestlings. We used a repeated-measure design to assess effects of ALAN on telomere shortening, body mass, tarsus length and body condition. Telomeres are repetitive nucleotide sequences that protect chromosomes from damage and malfunction. Early-life telomere shortening can be accelerated by environmental stressors, and has been linked to later-life declines in survival and reproduction. We also assayed nitric oxide, as an additional metric of physiological stress, and determined fledging success. Change in body condition between day 8 and 15 differed according to treatment. Nestlings exposed to ALAN displayed a trend towards a decline in condition, whereas control nestlings displayed a trend towards increased condition. This pattern was driven by a greater increase in tarsus length relative to mass in nestlings exposed to ALAN. Nestlings in poorer condition and nestlings that were smaller than their nest mates had shorter telomeres. However, exposure to ALAN was unrelated to telomere shortening, and also had no effect on nitric oxide concentrations or fledging success. Thus, exposure to ALAN may not have led to sufficient stress to induce telomere shortening. Indeed, plasticity in other physiological systems could

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allow nestlings to maintain telomere length despite moderate stress. Alternatively, the cascade of physiological and behavioral responses associated with light exposure may have no net effect on telomere dynamics.

1. Introduction

Anthropogenic environments expose organisms to novel stressors that have not been experienced over the course of evolutionary history, including light, chemical and noise pollution (Gaston et al., 2013; Swaddle et al., 2015; Bauerová et al., 2017). These stressors have the potential to overwhelm biological coping mechanisms, resulting in physiological stress, decreased performance and fitness declines. Exposure to artificial light at night (ALAN), or light pollution, may have particularly potent effects on physiology and behavior (Hölker et al., 2010; Gaston et al., 2013). Organisms have evolved with the periodicity of light-dark cycles, such that light is an important *Zeitgeber*, mediating adaptive daily and seasonal adjustments in organismal phenotypes (Gwinner and Brandstätter, 2001; Dominoni et al., 2013). Thus, exposure to ALAN may interfere with circadian rhythms, including sleep and activity patterns (Ruß et al., 2015; Raap et al., 2015; De Jong et al., 2016), and disrupt physiological systems (Dominoni et al., 2013; Jones et al., 2015). As a result, living with abnormal patterns of light and darkness may have wide-reaching, and potentially deleterious, effects on organisms inhabiting urban and suburban environments.

Indeed, research suggests that ALAN can affect an array of behavioral and physiological traits. In birds, behavioral shifts in response to ALAN include initiating singing earlier in the day (Da Silva et al., 2014), prolonged foraging periods (Ruß et al., 2015), and disrupted sleep (Raap et al., 2015). These behavioral changes may reflect shifts in underlying physiological control mechanisms. For example, melatonin is elevated during darkness, promotes restfulness, and is an effective antioxidant (Reiter et al., 2000). Thus, suppression of melatonin by ALAN may lead to restlessness, shifts in behavioral phenotypes, elevated oxidative stress, and pathology (Haus and Smolensky, 2006; Schernhammer et al., 2001). Exposure to artificial light may also interfere with the periodicity of the hypothalamus-pituitary-adrenal (HPA) axis, which helps modulate daily activity schedules and the adrenocortical stress response in vertebrates (Ishida et al., 2005; Mohawk et al., 2007; Navara and Nelson, 2007; Ouyang et al., 2015; Ouyang et al., 2018). Both elevated oxidative stress and increased CORT levels have been shown to accelerate telomere shortening, which could increase rates of biomolecular aging and cellular senescence (Haussmann et al., 2012; Herborn et al., 2014; Angelier et al., 2017; Reichert and Stier, 2017). Telomeres cap the ends of chromosomes, protect coding DNA from damage and malfunction, and regulate senescence by triggering apoptosis (Haussmann et al., 2005, 2012; Monaghan and Haussmann, 2006). Telomeres have been widely employed as markers of physiological stress and biomolecular aging (reviewed in Monaghan, 2014), and accelerated telomere shortening has been linked to disease and reduced survival probability (Haussmann et al., 2005; Heidinger et al., 2012; Boonekamp et al., 2014; Wilbourn et al., 2018). Indeed, a recent meta-analysis demonstrated an association between telomere length and survival across vertebrate taxa (Wilbourn et al., 2018).

Despite increasing and compelling evidence that ALAN can have significant effects on organisms, research on the effects of light pollution has still been limited in scope, primarily focusing on adult organisms in laboratory settings. A particular deficit of knowledge exists on how exposure to ALAN affects developing, wild organisms (but see Raap et al., 2016a, b, 2017a, 2018b; Casasole et al., 2017). This is a critical oversight, because changes in physiology and behavior associated with pollution in general, and ALAN in particular, may have particularly strong effects early in life, when developmental trajectories remain sensitive to stressful conditions (Metcalfe and Monaghan, 2001;

Monaghan, 2008; Spencer et al., 2009; Fonken and Nelson, 2016). Telomere shortening is especially rapid early in life in association with rapid rates of growth and cellular division, and shorter telomeres, or greater telomere shortening, during development has been linked to reduced longevity and later life pathologies (Heidinger et al., 2012; Monaghan, 2014). Natural stressors encountered early in life, including environmental conditions experienced at high altitude (Stier et al., 2016), within-brood competition (Nettle et al., 2015; Stier et al., 2015), and nutritional stress (Nettle et al., 2017), have been shown to accelerate telomere shortening. Other anthropogenic stressors including noise (Meillère et al., 2015; Dorado-Correa et al., 2018) and chemical pollution (Stauffer et al., 2017) have also been linked to early-life telomere loss. In addition, nestlings in urban populations to been shown to have shorter telomeres than nestlings in rural populations, an effect that was independent of natal origin (urban versus rural) (Salmón et al., 2016). However, to our knowledge, no past study has explored the effect of artificial light exposure on telomere shortening in wild nestling birds.

In this experimental study, we used a well-suited model organism, the great tit (*Parus major*) to elucidate the effects of ALAN on developing nestlings. We particularly explored the hypothesis that exposure to ALAN during the nestling stage results in reduced body condition and accelerated telomere shortening. Past work in this study system suggests that even short-term exposure of nestlings to ALAN results in changes in physiological condition that may subsequently affect fitness (Raap et al., 2016a, b). Nestlings exposed to two nights of ALAN displayed decreased nitric oxide (NOx) levels, increased haptoglobin concentrations and lower body mass, although no differences were detected in metrics of oxidative status (Raap et al., 2016a, b). Here, we extended the period of artificial light exposure, and used a repeated measures design to assess change in telomere length and body condition over the course of the nestling period. We also again measured NOx concentrations in the plasma. Nitric oxide plays an adaptive function as a multifaceted signaling molecule involved in inflammatory responses, although very high concentrations can lead to cellular senescence (Sild and Horak, 2009). Stress hormones have been linked to decreased NOx (Vajdovich, 2008), and cell-based studies demonstrate that NOx can delay age-dependent inhibition of telomerase and telomere shortening, counteracting senescence of endothelial cells (Vasa et al., 2000). Thus, reduced NOx could be linked to faster telomere shortening. Given the wide-spread loss of true darkness across the planet (Kyba et al., 2017a), elucidating the effects of ALAN on developing organisms is an urgent research priority.

2. Methods

2.1. Study population and general methods

We studied a population of great tits breeding in the immediate vicinity of the University of Antwerp's Campus Drie Eiken (Wilrijk, Belgium; 51°9′44″N, 4°24′15″E). This population consists of >120 resident breeding pairs, and has been continuously studied since 1997 (e.g. Van Duyse et al., 2000, 2005; Rivera-Gutierrez et al., 2010, 2012; Raap et al., 2016a, b, 2017a, b; Vermeulen et al., 2016). Individuals in the population are intensively monitored both during the breeding season and through nest box checks in the winter. To determine laying date, hatching date, and brood size, we checked nest boxes every other day beginning in late March.

2.2. Experimental design

Nest boxes in the experimental (ALAN) and control (CTR) treatments (N = 26 nest boxes (12 ALAN, 14 CTR); 206 nestlings (93 ALAN, 113 CTR)) were paired according to hatching date and spatial location (unequal sample sizes reflect failure of some nests). Of the 93 nestlings in the ALAN group, 37 were females, 43 were males, and 13 were unsexed. Of the 113 nestlings in the CTR group, 51 were females, 42 were males and 10 nestlings were unsexed. Nest boxes used in the experiment were located in areas with minimal disturbance from anthropogenic noise or light pollution, and our previous work suggests that the external light environment at nest boxes has no detectable effect on nestling physiology (Casasole et al., 2017; Raap et al., 2017a) or adult sleep behavior (Raap et al., 2018a). All clutches were initiated over a narrow time frame (hatching dates between April 13 and April 23, 2017), sampling was completed between April 20 and May 8, 2017, and only first nesting attempts were used in the experiment.

We exposed nestlings to ALAN from day 8 to day 15 of the nestling stage (hatch day = day 1) using a system of 4 small LED lights (Diameter: 5 mm, Cree® Round LED C535A-WJN, Durham, North Carolina, USA) that produce broad-spectrum white light, with a sharp peak in relative luminous intensity around 450 nm and a lower peak around 550 nm (see Supplementary Appendix; Fig. S1 for color spectrum specifications). This period of light exposure is substantial given the short developmental time of great tits within the nest box, constituting ≈ 2/5 of the ≈21 day-long nestling period. The LED system was fitted under the nest box lid, and standardized to produce 1 lx at the average nest height of great tits, 8 cm above the bottom of the box, using a ISO-Tech ILM 1335 light meter (Corby, UK). This light intensity is within the range of light levels experienced by birds in light exposed areas, and is similar to the intensity used in a previous study in this population (Raap et al., 2015). The light intensity associated with a full moon is about 0.05–0.2 lx (Kyba et al., 2017b), and light levels as low as <0.00001 lx can already have biological effects (see Gaston et al., 2013). We chose to use white LEDs as a source of ALAN due to the current shift towards energy efficient, broad spectrum light sources, such as LEDs (Schubert and Kim, 2005; Davies et al., 2013). Note that this experiment was not meant to mimic natural conditions inside artificial or natural cavities, which are largely impermeable to light (Casasole et al., 2017; Raap et al., 2017a, 2018a, b). Rather, great tit nestlings serve as convenient models to study likely effects of light exposure on other open-cup nesting species, for which manipulating light levels at nests would be very challenging. We used a timer inside a homemade enclosure to automatically turn on light systems at 19.00 h in the evening (≈2 h before sunset) and to turn the lights off at 0700 in the morning (≈1 h after sunrise). In addition, the system was also turned off during the night from 2400 to 0200. We maintained this period of darkness during the central portion of the night to reduce the risks of nest abandonment, particularly by the female. Control boxes were equipped with LED systems, but no electronics.

2.3. Field sampling

To assess the effect of ALAN on telomere length and body condition, we used a repeated measures design that controlled for potential differences in the physiological condition of nestlings prior to onset of the experiment. On day 8 of the nestling stage (before the first night of light exposure), we obtained a ≈50 μL blood sample to assess pretreatment, and early stage, telomere length. This blood sample was immediately dispensed into glycerol buffer (50 mM Tris-Cl, 5 mM MgCl, 0.1 mM EDTA, 40% glycerol) in the field, stored on ice, flash frozen in liquid nitrogen within 4 h, and stored at −80 °C. At the time of blood sampling, we also measured mass (±0.1 g) and tarsus length (0.01 mm), providing a pre-treatment, or early-stage, metric of body condition. Body condition was calculated as the residuals of a regression predicting body mass from tarsus length (Schulte-Hostedde et al., 2005). On day 8,

we uniquely marked nestlings with a metal band (the majority of nestlings) or color band (nestlings too small for a metal ring). We repeated the blood sampling and body measurement procedures on day 15. Thus, we were able to assess within-individual changes in telomere length, body mass, tarsus length, and body condition over the course of the light manipulation. All measurements on nestlings were completed between 0800 and 1230.

2.4. Laboratory assays

We determined telomere length, and molecularly sexed nestlings (Griffiths et al., 1998), using DNA extracted from blood samples using the Macherey-Nagel NucleoSpin® blood kit. We measured the concentration and purity of DNA using a Nanodrop. All samples consisted of high quality DNA with acceptable 260/280 (mean ± SE: 1.902 ± 0.011) and 260/230 ratios (mean ± SE: 2.143 ± 0.36), indicative of purity.

We determined telomere length using a relative real-time qPCR assay modified from Criscuolo et al. (2009), which measures telomere length relative to a single copy reference gene. We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as our reference gene. We amplified GAPDH using primers specific to the great tit: GAPDH-F (5′-TGTGATTTCAATGGTGACAGC-3′) and GAPDH-R (5′-AGCT TGACAAAATGGTCGTTC-3′) (Atema et al., 2013). We amplified telomere sequences using the primers Tel1b (5′CGGTTTGTTTGGGTTTGGGTTTGG GTTTGGGTTTGGGTT-3′) and Tel2b (5′-GGCTTGCCTTACCCTTACCCTTAC CCTTACCCTTACCCT-3′), which amplify telomere sequences across avian species. For both telomeres and GAPDH, we ran 15 μL qPCR reactions containing 7.5 μL of FastStart Essential DNA Green Master (Roche Diagnostic Corporation, Indianapolis, IN). Telomere reactions contained 0.9 μL each of forward and reverse primers at a concentration of 10 μM (final concentration: 600 nM), 2.325 μL of water, 0.375 μL of 100% DMSO (2.5% of total reaction volume), and 3 μL of 1 ng/μL DNA. GAPDH reactions contained 0.3 μL each of forward and reverse primers at a concentration of 10 μM (final concentration: 200 nM), 3.9 μL of water, and 3.0 μL of 1 ng/μL DNA.

We performed qPCR using a LightCycler®480 System (Roche). We ran telomere and GAPDH reactions on separate 480-well plates. Telomere thermocycling conditions were: 10 min preincubation at 95 °C, followed by 40 cycles of 15 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C. GAPDH conditions were: 10 min preincubation at 95 °C, followed by 40 cycles of 15 s at 95 °C, 20 s at 60 °C, and 20 s at 72 °C. We used a ramp speed of 4.4 °C/s, and followed both amplification programs with high resolution melting curve analysis. Melting curve analysis confirmed amplification of a single product of appropriate length.

We included a serial dilution (12 ng, 6 ng, 3 ng, 1.5 ng, 0.75 ng, and 0.375 ng) of DNA from the same reference bird, run in duplicate, on each plate. The standard curve derived from this dilution series was used to determine and control for the qPCR's amplification efficiency. Amplification efficiency was within the acceptable range for both the telomere (mean ± SD: 103.51 ± 5.08) and GAPDH (mean ± SD: 101.06 ± 3.36) reactions, and standard curves displayed coefficients of determination close to 1 (telomere: r^2: 0.993 ± 0.005; GAPDH: r^2: 0.990 ± 0.004). We also included a "golden standard" reference sample on each plate, derived by pooling the DNA samples of multiple individuals. We ran all samples in duplicate and in the same position on the telomere and GAPDH plates. Negative controls were included on each plate.

To calculate calibrator-normalized relative telomere length (RTL; amount of telomere sequence relative to GAPDH; T/S ratio), we used the formula: $RTL = E_T^{CtT(C)-CtT(S)} * E_R^{CtR(S)-CtR(C)}$ (Pfaffl, 2001). In qPCR, the C_T (crossing threshold) is the number of amplification cycles needed for products to exceed a threshold florescent signal. E_T is the efficiency of the telomere qPCR reaction, CtT(S) is the C_T of each sample, and CtT (C) is the C_T of the calibrator (golden standard). E_R is the efficiency of the GAPDH qPCR reaction, CtR(S) is the C_T of each sample, and CtR (C) is the C_T of the calibrator (Pfaffl, 2001). The mean intra-plate

coefficient of variation of C_T values was 1.04% and 0.39%, and inter-plate variation was 2.47% and 0.69%, for the telomere and GAPDH reactions, respectively. For RTL, mean intra- and interplate variation were 11.37% and 4.30%, respectively, and within-plate repeatability was 0.873 (95% CI: [0.847, 0.894]).

Finally, we measured NOx in plasma samples from day 15 nestlings using a spectrophotometric assay based on reduction of nitrate to nitrite by copper-coated cadmium (Sild and Horak, 2009). This assay is routinely run in our laboratory (Vermeulen et al., 2016; Raap et al., 2017a; Sebastiano et al., 2018) and has been shown to be highly repeatable (Sild and Horak, 2009), so we did not run samples in duplicate.

2.5. Statistical analyses

All statistical analyses were conducted in R 3.4.1 (R Core Team, 2017). We used linear mixed effects models (LMMs) in R package lme4 (Bates et al., 2015) to investigate the effect of exposure to ALAN on RTL, body condition (mass-size residuals) and NOx concentrations. To facilitate interpretation of our results regarding body condition, we also constructed two additional models to elucidate whether exposure to ALAN affected body mass and tarsus length. We used Satterthwaite approximations for degrees of freedom, using R package lmerTest (Kuznetsova et al., 2016). All models were reduced via a stepwise reduction process, by first removing nonsignificant interaction terms and then sequentially removing terms with the highest p-value. Post-hoc tests were performed using R package lsmeans, with Tukey tests between factor levels (Lenth, 2016). Terms retained in final models were significant at the $\alpha = 0.05$ level. Sample sizes are reduced in some cases (for RTL and NOx) due to failure to obtain high quality DNA or plasma samples from some nestlings.

First, to investigate the effect of exposure to ALAN on telomere dynamics, we entered RTL as the dependent variable, with RTL at day 8 and 15 entered for each individual. We then predicted RTL from the interaction between treatment (ALAN, control) and: nestling age (day 8 or 15), body condition, nestling sex, NOx concentrations, and nestling size rank (largest nestling within a nest coded as rank 1). Nestling ID, nest ID, and assay number were included as random effects. RTL was log-transformed to normalize model residuals.

Second, we tested whether body condition, body mass and tarsus length were affected by exposure to ALAN. We entered body condition, mass or tarsus length as the dependent variable, with values at day 8 and 15 entered for each individual. We then predicted body condition, body mass, or tarsus length from the interactions between treatment, nestling age, nestling sex, and nestling size rank. Nest ID and nestling ID were included as random effects.

Third, we examined whether exposure to ALAN affected NOx concentrations (measured on day 15). We predicted NOx levels from the interaction between treatment and sex. Nest ID was included as a random effect.

Fourth, we assessed whether fledging success was affected by exposure to ALAN. To this end, we used a general linear model with a binomial error structure to predict whether or not a nestling fledged (1, 0) from treatment, RTL, body condition at day 8, or nestling sex. We used only body condition at day 8, and did not test the effect of NOx, because very few nestlings (8) that survived to day 15 died before fledging. We also did not test interactions in this model since the overall number of nestlings that died was limited (28), and we wanted to avoid over-fitting. We included nest ID as a random effect.

Finally, for telomere length and body condition, for which we had repeated measures, we also assessed within-individual repeatability using the measurements taken on day 8 and 15 using R package rptR (Stoffel et al., 2017). When calculating repeatability, we retained nestling age in the model.

2.6. Ethical statement

This study was approved by the ethical committee of the University of Antwerp (ID number: 2017-90) and conducted in accordance with Belgian and Flemish laws. We made all possible efforts to minimize the stress experienced by nestlings during removal from the nest box. The Belgian Royal Institute for Natural Sciences (Koninklijk Belgisch Instituut voor Natuurwetenschappen) provided banding licenses for all authors and technical personnel.

3. Results

3.1. Relative telomere length (RTL)

RTL ranged from 0.329 to 2.944 (mean ± SE: 1.492 ± 0.031), and did not differ between the control and experimental treatments (Table 1; Fig. 1). There was no interaction between treatment and age (day 8 versus 15; Table 1), suggesting that the rate of telomere shortening was similar in the two treatment groups. However, nestling RTL decreased between day 8 and 15 (Table 1). Other two-way interactions with treatment were non-significant (Table 1). Independent of treatment, nestlings in better body condition had longer telomeres (Table 1; Fig. 2a), and telomere length decreased with nestling age (Table 1). Nestling sex and nitric oxide levels had no effect on telomere dynamics (Table 1). Nestling size rank also had no effect on telomere length when entered in our initial model (Table 1). However, we found that size rank was highly correlated with body condition (see below), and was thus collinear with body condition in the model predicting telomere length. When predicting telomere length from size rank alone (with the interaction with treatment also initially included), we found that nestlings that were smaller than brood mates had shorter telomeres, as reflected by a negative correlation between size rank and RTL ($\beta = -0.010 \pm 0.004$, $t_{257} = -2.345$, $p = 0.019$). The relationship between size rank and telomere length was consistent across the treatment groups (non-significant treatment × size rank interaction: $\beta = -0.002 \pm 0.008$, $t_{255} = -0.252$, $p = 0.801$; Fig. 2b).

3.2. Body condition, body mass and tarsus length

Nestling body condition ranged from −3.899 to 5.076 (mean ± SE: 0 ± 0.044), and depended on treatment in an age-specific fashion. Specifically, there was an interaction between treatment and nestling age (Table 2), reflecting the fact that nestlings exposed to ALAN tended to decline in condition between day 8 and day 15 (Fig. 3), whereas nestlings in the control group tended to gain condition between day 8 and day 15 (Fig. 3). Post-hoc comparisons indicated that nestlings did not significantly differ in body condition between day 8 and day 15 in either the ALAN ($\beta = 0.300 \pm 0.141$, $t_{367} = 2.128$, $p = 0.146$) or CTR ($\beta = -0.223 \pm 0.125$, $t_{364} = -1.785$, $p = 0.282$) treatment groups after adjusting for multiple comparisons. However, note that the difference in body condition between day 8 and 15 was larger in nestlings exposed to ALAN, and that the slopes in the two treatment groups were in the opposite direction. Nestlings in the two treatment groups did not differ in body condition at day 8 (before the beginning of the experiment, $\beta = 0.250 \pm 0.329$, $t_{28} = 0.759$, $p = 0.872$). The difference in body condition between treatment groups at day 15 increased, as expected given the significant treatment × age interaction, but was also statistically non-significant at the 0.05 level after adjusting for multiple comparisons ($\beta = -0.773 \pm 0.329$, $t_{28} = 2.346$, $p = 0.111$). Nestlings that were smaller than their nest mates were in poorer body condition, and this relationship was similar between treatment groups (Table 2).

Among day 8 nestlings, body mass ranged from 4.90 to 12.80 g (mean ± SE: 9.266 ± 0.118), and did not differ between the two treatment groups before initiation of experiment ($\beta = -0.866 \pm 0.489$, $t_{23} = -1.773$, $p = 0.090$). Among day 15 nestlings, body mass ranged from 7.70 to 18.10 g (mean ± SE: 14.62 ± 0.156). Body mass was not

Table 1
Linear mixed effect model predicting relative telomere length from treatment (ALAN versus control) and covariates. Significant p-values ($\alpha = 0.05$) appear in bold.

a. Initial model	Estimate ($\beta \pm$ SE)	Df	T	P > |t|
Intercept	0.319 ± 0.132	87.593	2.412	0.018
Treatment[a]	0.254 ± 0.157	152.890	1.623	0.107
Nestling age	-0.151 ± 0.054	152.423	-2.819	**0.005**
Sex[b]	0.073 ± 0.062	149.838	1.175	0.242
Body condition	0.078 ± 0.028	216.412	2.767	**0.006**
NOx	0.142 ± 0.212	124.023	0.671	0.504
Size rank	0.006 ± 0.014	221.166	0.412	0.681
Treatment × age	0.073 ± 0.080	151.025	0.909	0.365
Treatment × sex	-0.126 ± 0.092	151.162	-1.363	0.175
Treatment × condition	-0.042 ± 0.038	150.486	-1.105	0.271
Treatment × NOx	-0.245 ± 0.340	133.644	-0.719	0.474
Treatment × size rank	-0.019 ± 0.019	216.782	-0.974	0.331

Random effects	Variance	SD	N
Individual	0.006	0.079	159
Nest box	0.002	0.045	26
Assay number	0.044	0.211	11
Residual	0.114	0.338	300

b. Reduced model	Estimate ($\beta \pm$ SE)	Df	T	P > |t|
Intercept	1.253 ± 0.044	11.724	28.420	<0.001
Nestling age	-0.044 ± 0.020	195.910	-2.196	**0.029**
Body condition	0.028 ± 0.009	145.305	3.043	**0.002**

Random effect	Variance	SD	N
Individual	0.002	0.048	206
Nest box	0.008	0.028	26
Assay number	0.017	0.131	11
Residual	0.035	0.189	364

[a] ALAN relative to CTR treatment.
[b] Males relative to females.

related to exposure to ALAN ($\beta = -0.747 \pm 0.581$, $t_{24} = -1.285$, p = 0.211), and none of the interactions between treatment, nestling age, size rank, or sex were significant (p > 0.40 in all cases). Male nestlings were heavier than females ($\beta = 0.668 \pm 0.168$, $t_{152} = 3.966$, p < 0.001), and nestlings of larger size rank were lighter than nestlings of lower size rank ($\beta = -0.378 \pm 0.022$, $t_{317} = -17.174$, p < 0.001).

Among day 8 nestlings, tarsus length ranged from 8.88 to 17.58 mm (mean $\pm$ SE: 14.13 ± 0.101), and did not differ significantly between the two treatment groups before initiation of the experiment ($\beta = 0.439 \pm 0.349$, $t_{27} = -1.257$, p = 0.597). Among day 15 nestlings, tarsus length ranged from 14.69 to 20.47 mm (mean $\pm$ SE: 18.68 ± 0.079).

Fig. 1. Change in relative telomere length between day 8 and day 15 in nestlings exposed, versus not exposed, to ALAN. Bars show 95% confidence intervals. ALAN = artificial light at night; CTR = control.

As for body condition, there was a significant interaction between treatment and nestling age in predicting tarsus length ($\beta = 0.458 \pm 0.184$, $t_{314} = 2.494$, p = 0.0131). This interaction reflected the fact that nestlings in the ALAN treatment group increased more in tarsus length than nestlings in the control group. However, nestlings in the two treatment groups did not differ in tarsus length at day 15 ($\beta = 0.019 \pm 0.347$, $t_{27} = 0.055$, p = 0.999). The interactions between treatment, size rank, and sex were non-significant (p > 0.40 in both cases). Male nestlings had longer tarsi than females ($\beta = 0.356 \pm 0.121$, $t_{154} = 2.933$, p = 0.004), and nestlings of larger size rank had smaller tarsi than nestlings of lower the size rank ($\beta = -0.205 \pm 0.020$, $t_{320} = -10.459$, p < 0.001).

3.3. Nitric oxide

Nitric oxide levels ranged from 0.300 to 8.800 pmol/mL (mean $\pm$ SE: 2.993 ± 0.075 pmol/mL), and did not vary with treatment group ($\beta = -0.015 \pm 0.383$, $t_{31} = -0.040$, p = 0.969, N = 327 nestlings, 26 nest boxes; Fig. 4). Male nestlings had lower nitric oxide levels relative to females ($\beta = 0.399 \pm 0.187$, $t_{309} = -2.134$, p = 0.034), with this effect being similar in both the ALAN and CTR groups (Treatment × sex interaction: $\beta = -0.045 \pm 0.286$, $t_{314} = -0.155$, p = 0.877).

3.4. Fledging success

Body condition at day 8 was a strong positive predictor of fledging success ($\beta = 1.565 \pm 0.496$, z = 3.152, p = 0.001, N = 206 nestlings, 26 nests). However, fledging success was not affected by exposure to ALAN ($\beta = -0.162 \pm 2.826$, z = -0.057, p = 0.954), RTL ($\beta = -1.176 \pm 0.905$, z = -1.301, p = 0.193), or nestling sex ($\beta = 1.101 \pm 1.042$, z = 1.056, p = 0.291).

Fig. 2. Relative telomere length increased with body condition (mass-size residuals) (a) and decreased with nestling size rank (largest nestling = rank 1) (b), with these effects being similar between the control (CTR) and light (ALAN) treatment groups. Shaded regions show 95% confidence intervals.

3.5. Repeatability in telomere length and body condition

Nestling telomere length was not significantly repeatable between day 8 and day 15 (R = 0.065, 95% CI: [0, 0.192], p = 0.124, N = 364 observations, 206 nestlings). Repeatability of RTL was higher within the ALAN than within the CTR group (ALAN: R = 0.097, 95% CI: [0, 0.302], p = 0.154, N = 163 observations, 93 nestlings; CTR: R = 0, 95% CI: [0, 0.139], p = 1, N = 201 observations, 113 nestlings), but 95% confidence intervals extensively overlapped suggesting that repeatability did not significantly differ depending on light exposure. In contrast to RTL, body condition was individually repeatable (R = 0.369, 95% CI: [0.24, 0.487], p < 0.001, N = 390 observations, 206 nestlings). As for RTL, repeatability of body condition was higher within the ALAN than CTR group, (ALAN: R = 0.447, 95% CI: [0.26, 0.616], p < 0.001, N = 175 observations, 93 nestlings; CTR: R = 0.277, 95% CI: [0.103, 0.447], p = 0.002, N = 215 observations, 113 nestlings), but 95% confidence intervals overlapped, again suggesting similar repeatability regardless of light exposure.

4. Discussion

We paired an innovative experimental set-up with a repeated-measures design to investigate the effects of exposure to ALAN on the physiology, telomere attrition, and fledging success of free-living nestlings. Our results suggest that exposure to artificial light inside the nest box may affect developmental trajectories of nestlings, as reflected by differential changes in body condition and tarsus length in nestlings exposed to ALAN relative to in the control group. However, effects on body condition were not strong, and telomere shortening appeared unaffected by light exposure. On the other hand, across both the control and ALAN groups, there was a robust correlation between body condition and telomere length, and nestlings that were smaller than their brood mates also had shorter telomeres. In addition, as predicted, we also found that telomere length declined between day 8 and 15, suggesting that the duration of our study (7 nights) was long enough to detect a decline in telomere length over the course of development. We proceed to discuss possible reasons for our results, as well as potential implications.

Table 2
Linear mixed effect model predicting body condition from treatment (ALAN versus control) and covariates. Significant p-values (α = 0.05) appear in bold.

a. Initial model	Estimate (β ± SE)	Df	T	P > |t|
Intercept	0.830 ± 0.291	60.652	2.851	0.006
Treatment[a]	−0.539 ± 0.436	64.027	−1.238	0.220
Nestling age	0.304 ± 0.134	311.949	2.253	0.025
Sex[b]	−0.002 ± 0.153	319.543	−0.015	0.988
Size rank	−0.170 ± 0.031	318.260	−5.481	**<0.001**
Treatment × age	−0.519 ± 0.200	313.515	−2.592	**0.009**
Treatment × sex	−0.016 ± 0.230	322.949	−0.070	0.944
Treatment × size rank	0.069 ± 0.045	318.883	1.539	0.124

Random effects	Variance		SD	N
Individual	0		0	174
Nest box	0.659		0.812	26
Residual	0.846		0.920	344
b. Reduced model	Estimate (β ± SE)	Df	T	P > |t|
Intercept	0.684 ± 0.239	37.145	2.859	0.007
Treatment[a]	−0.250 ± 0.329	28.325	−0.760	0.454
Nestling age	0.220 ± 0.127	357.947	1.731	0.084
Size rank	−0.121 ± 0.020	362.062	−6.099	**<0.001**
Treatment × age	−0.515 ± 0.191	359.707	−2.693	**0.007**

Random effects	Variance		SD	N
Individual	0		0	206
Nest box	0.581		0.762	26
Residual	0.856		0.925	386

[a] ALAN relative to CTR treatment.
[b] Males relative to females.
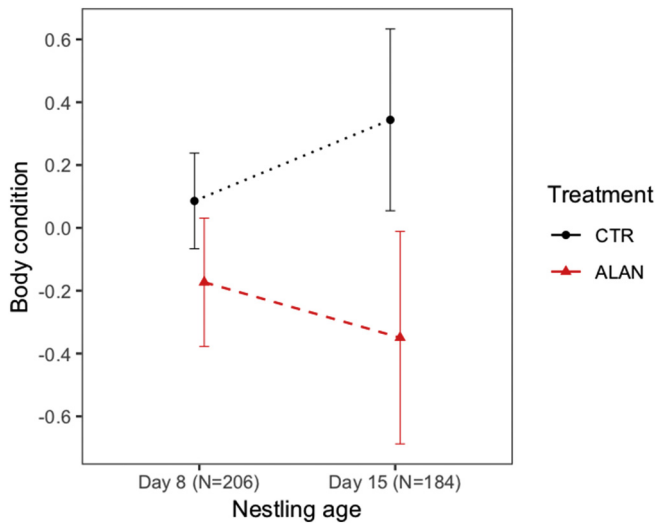
Fig. 3. Change in body condition (mass, size residuals) between day 8 and 15 in nestlings exposed, versus not exposed to ALAN. Bars show 95% confidence intervals. ALAN = artificial light at night; CTR = control.

We found a small effect of the ALAN treatment on the change in body condition from day 8 to 15 of the nestling stage, but no difference in telomere shortening between nestlings in lighted and unlighted boxes. Given the effect on body condition, we might also have expected an effect on telomere length, because past research across a range of taxa has related growth dynamics and body condition to telomere dynamics. For example, king penguin chicks (*Aptenodytes patagonicus*) that engaged in catchup growth at the detriment of somatic maintenance showed accelerated telomere loss (Geiger et al., 2012) and red garter snakes (*Thamnophis sirtalis parietalis*) in poorer body condition had shorter telomeres (Rollings et al., 2017). In fact, we did find a positive correlation between telomere length and body condition, and nestlings that were smaller than brood mates had shorter telomeres. However, the change in body condition associated with the ALAN treatment might not have been large enough to translate into physiological stress and induce telomere shortening.

Indeed, we found that nestlings exposed to ALAN tended to deteriorate in body condition between days 8 and 15, whereas nestlings in the control treatment tended to gain body condition, leading to a statistically significant interaction between the ALAN treatment and nestling age. However, this effect was not large enough to translate into a statistically significant difference in body condition at day 15, suggesting a

Fig. 4. Box plot of nitric oxide (NOx) levels in the control (CTR) and light (ALAN) treatment groups. Whiskers extend from the first and third quartiles to the highest value within 1.5 times the interquartile range.

relatively modest effect on nestling condition. Moreover, this pattern was driven by longer tarsus lengths relative to body mass in nestlings exposed to ALAN, rather than overall reductions in body mass, suggesting that nutritional stress was not severe. Why exposure to ALAN would induce nestlings to gain more in tarsus length than in mass is unclear, but could reflect disruption of physiological control systems, such as the pineal hormone melatonin and hormones related to food-intake and growth rate (Fonken and Nelson, 2014; Durrant et al., 2018; Ouyang et al., 2018).

We also found no evidence that exposure to ALAN affected NOx or fledging success, again suggesting relatively low stress levels. Rather, the only significant predictor of NOx levels was nestling sex, with males having higher NOx than females, a finding consistent with one of our previous studies (Raap et al., 2017a). Body condition is often a strong predictor of fledging success (Both et al., 1999; Tilgar et al., 2010; Rodríguez et al., 2016), and has also been shown to subsequently affect juvenile survival rates and later-life fitness metrics (Perrins, 1979; Tinbergen and Boerlijst, 1990; Naef-Daenzer et al., 2001; Perrins and McCleery, 2001; Rodríguez et al., 2016), including in our population (Vermeulen et al., 2016). Body condition was also a good predictor of fledging success in our study. However, there was no detectable effect of light exposure on fledging success, perhaps due to relatively low death rates before fledging and the relatively small difference between body condition at day 15 in the two treatment groups.

Earlier work in our population of great tits suggested a stronger effect of ALAN on nestling body condition and NOx levels, with nestlings exposed to ALAN failing to gain mass and showing suppressed NOx levels after only two nights of light exposure (Raap et al., 2016a). One possible explanation for this discrepancy is that nestlings are able to habituate to ALAN over a longer time frame, perhaps via adjustments in other physiological systems, such that body condition and NOx levels recover. In addition, although shorter in duration, Raap et al., 2016a used a light intensity 3 times higher than the current study (3 as compared to 1 lx) and did not maintain a two-hour period of darkness during the central period of the night. Thus, it is possible that a higher light intensity might lead to larger reductions in nestling condition and a significant effect on telomere length, especially given the significant relationship between body condition and telomere length that we observed in this study. In addition, the year in which we conducted our study was abnormally cold, with low nestling mass at day 15 and high nestling mortality rates. Hence, it might be easier to detect an effect of ALAN on nestling stress levels in a more moderate year.

Another potential explanation for our results is that exposure to ALAN does not affect telomere dynamics, despite having effects on patterns of growth and other physiological systems. Indeed, a recent study on adult great tits also found no effect of ALAN on telomere length (Ouyang et al., 2017; but see Raap et al., 2017b). Animals exposed to ALAN might be able to maintain telomere length despite increases in stress levels by investing in defense mechanisms, such as antioxidant enzymes or telomerase activity. However, in a previous study, we found no differences in oxidative stress levels or antioxidant activity in nestlings exposed to two nights of ALAN (Raap et al., 2016a). It is also possible that ALAN induces a unique cascade of physiological and behavioral responses that combine to cause no overall effect on telomere length. For example, increased activity levels and reduced sleep may elevate metabolism and oxidative stress, but a slower gain in body mass may reduce energy expenditure and production of free radicals, thus neutralizing the effect on oxidative stress and telomere shortening. Surprisingly, other research on the relationship between exposure to ALAN and telomere length is largely absent, even in humans and laboratory animals, although sleep deprivation and shift work has been linked to shorter telomere length in humans (Liang et al., 2011) and circadian disruption in mice leads to shorter telomeres (Chen et al., 2014). Thus, more research is needed to assess the generality of our results, and the potential effect of variation in the intensity and duration of artificial light exposure.
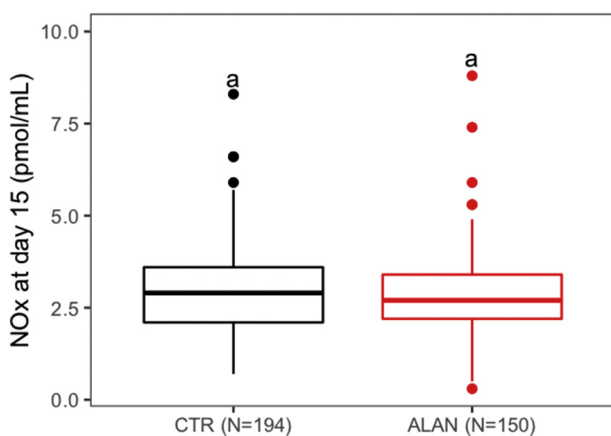
One could also argue that we lacked the statistical power to detect a treatment effect on telomere length, especially given the non-significant overall difference in the body condition, body mass, and tarsus length of day-15 nestlings in the two treatment groups. However, rather than being in the predicted negative direction, the coefficient estimate for the effect of ALAN on telomere length was positive (although non-significant; see Table 1), making it less plausible that a negative effect would have emerged with an increased sample size. Furthermore, using R package simr (Green and MacLeod, 2016), we conducted a power analysis, which suggested that we would have good power (95.00%, 95% CI: [88.72, 98.38]) to detect a slope (β) of −0.10 for the treatment × age interaction, which is similar in magnitude to the slope for the treatment × age interaction reported for great tits by Stier et al. (2015) (β = 0.09), who examined the effect of elevation on telomere shortening, and the treatment effect reported in Meillère et al. (2015) (β = −0.15), who examined the effect of noise exposure on the telomere length of nestling house sparrows (*Passer domesticus*). In past studies, we have also found significant effects of exposure to ALAN on body mass and other physiological variables with comparable sample sizes (Raap et al., 2016a, b). Nevertheless, it could be informative to repeat this study with an expanded sample size and/or with a longer period of light exposure or higher light intensity, as discussed above.

Another consideration is that great tits have two distinct classes of terminal telomeres, type II telomeres and type III (ultra-long) telomeres (Atema et al., In press), that may be affected differently by developmental stress. Shortening of type II telomeres may be undetectable via techniques, such as ours (qPCR), that cannot distinguish between different classes of telomeres, and thus yield a single estimate to calculate telomere length. This is the case because ultra-long telomeres dominate the overall distribution of the telomere sequence. Atema et al. found that class III telomeres shorten with age in nestlings, and thus predict that telomere shortening should be detectable in nestlings via qPCR, as indeed was the case in ours, as well as previous (Stier et al., 2015), studies. However, it is possible that stressors, such as ALAN, could induce premature shortening of class II telomeres, which would then not be detectable via our methodology. Indeed, Atema et al. (In Press) found that only class II telomeres shorten in adult great tits. The timing of the transition between shortening of class III and class II shortening is unclear (Atema et al., In press), and this transition could perhaps be accelerated by stress exposure during development. Thus, further research examining the effect of ALAN on telomere shortening in nestlings, while employing a technique that allows discrimination between telomere classes (Terminal Restriction Fragment (TRF); Haussmann and Vleck, 2002; Atema et al., In press), is warranted and could yield intriguing results.

In contrast to some past work in great tits (Stier et al., 2015, 2016), telomere length was not significantly repeatable between the day 8 and 15 sampling point in the nestlings included in our study. Although this could be taken as reflecting methodological issues, we do not feel that this is likely since we did find several expected, biologically meaningful results, namely the decline in telomere length between day 8 and day 15, and the strong correlation between telomere length and body condition. Rather, we suggest that the low repeatability estimate for telomere length in our study could reflect differential rates of telomere shortening in different individuals. In contrast to telomere length, body condition was repeatable, suggesting that nestlings that were in poorer condition at day 8 were also in poorer condition at day 15.

Finally, also in contrast to some past studies in great tits (Stier et al., 2015), but in agreement with others (Salmón et al., 2016; Stier et al., 2016), we found that nestling sex did not affect telomere length. This suggests that, at least in our population, mechanisms controlling telomere attrition or maintenance have been similarly selected in males and females, and that neither sex is more sensitive to condition declines associated with exposure to ALAN.

In conclusion, we found that telomere dynamics of free-living nestlings were not affected by exposure to ALAN inside the nest box,

although body condition tended to decline over the timeframe of the experiment. This suggests that the physiological stress induced by exposure to 1 lx of ALAN over a 7- night timeframe was not severe enough to accelerate telomere shortening. Nestlings may have been able to prevent deleterious effects of ALAN on telomeres via plasticity in other physiological systems, or telomeres may be less sensitive to light exposure than other phenotypic traits. However, exposure to a higher light intensity over a longer time period could lead to higher stress levels and telomere shortening. Given ever-increasing levels of light pollution world-wide (Davies et al., 2014; Falchi et al., 2016), resolving which phenotypic traits are sensitive to ALAN, and the intensity and duration of light exposure that constitutes a threat, remains an important area for further research.

Acknowledgements

Data availability

Data will be available in the Dryad Digital Repository.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.12.469.

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