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

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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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 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.

Keywords: artificial light at night, developmental stress, telomeres, nitric oxide, body condition, *Parus major*

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 et al. 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 (Haussman 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.

105 In this experimental study, we used a well-suited model organism, the great tit (*Parus major*) to
106 elucidate the effects of ALAN on developing nestlings. We particularly explored the hypothesis that
107 exposure to ALAN during the nestling stage results in reduced body condition and accelerated telomere
108 shortening. Past work in this study system suggests that even short-term exposure of nestlings to ALAN
109 results in changes in physiological condition that may subsequently affect fitness (Raap et al. 2016a, b).
110 Nestlings exposed to two nights of ALAN displayed decreased nitric oxide (NOx) levels, increased
111 haptoglobin concentrations and lower body mass, although no differences were detected in metrics of
112 oxidative status (Raap et al. 2016a, b). Here, we extended the period of artificial light exposure, and used
113 a repeated measures design to assess change in telomere length and body condition over the course of the
114 nestling period. We also again measured NOx concentrations in the plasma. Nitric oxide plays an
115 adaptive function as a multifaceted signaling molecule involved in inflammatory responses, although very
116 high concentrations can lead to cellular senescence (Sild and Norak 2009). Stress hormones have been
117 linked to decreased NOx (Vajdovich 2008), and cell-based studies demonstrate that NOx can delay age-
118 dependent inhibition of telomerase and telomere shortening, counteracting senescence of endothelial cells
119 (Vasa et al. 2000). Thus, reduced NOx could be linked to faster telomere shortening. Given the wide-
120 spread loss of true darkness across the planet (Kyba et al. 2017a), elucidating the effects of ALAN on
121 developing organisms is an urgent research priority.
122
123 **2. METHODS**
124 ***2.1. Study population and general methods***: We studied a population of great tits breeding in the
125 immediate vicinity of the University of Antwerp's Campus Drie Eiken (Wilrijk, Belgium; 51°9'44"N,
126 4°24'15"E). This population consists of >120 resident breeding pairs, and has been continuously studied
127 since 1997 (e.g. Van Duyse et al. 2000, 2005; Rivera-Gutierrez et al. 2010, 2012; Raap et al. 2016a, b,
128 2017; Vermeulen et al. 2016). Individuals in the population are intensively monitored both during the
129 breeding season and through nest box checks in the winter. To determine laying date, hatching date, and
130 brood size, we checked nest boxes every other day beginning in late March.

131

132 ***2.2 Experimental design***: Nest boxes in the experimental (ALAN) and control (CTR) treatments (N = 26
133 nest boxes (12 ALAN, 14 CTR); 206 nestlings (93 ALAN, 113 CTR)) were paired according to hatching
134 date and spatial location (unequal sample sizes reflect failure of some nests). Of the 93 nestlings in the
135 ALAN group, 37 were females, 43 were males, and 13 were unsexed. Of the 113 nestlings in the CTR
136 group, 51 were females, 42 were males and 10 nestlings were unsexed. Nest boxes used in the
137 experiment were located in areas with minimal disturbance from anthropogenic noise or light pollution,
138 and our previous work suggests that the external light environment at nest boxes has no detectable effect
139 on nestling physiology (Casasole et al. 2017; Raap et al. 2017a) or adult sleep behavior (Raap et al.
140 2018a). All clutches were initiated over a narrow time frame (hatching dates between April 13 and April
141 23, 2017), sampling was completed between April 20 and May 8, 2017, and only first nesting attempts
142 were used in the experiment.
143 We exposed nestlings to ALAN from day 8 to day 15 of the nestling stage (hatch day = day 1) using a
144 system of 4 small LED lights (Diameter: 5 mm, Cree® Round LED C535A-WJN, Durham, North
145 Carolina, USA) that produce broad-spectrum white light, with a sharp peak in relative luminous intensity
146 around 450 nm and a lower peak around 550 nm (see Supplementary Appendix; Fig. S1 for color
147 spectrum specifications). This period of light exposure is substantial given the short developmental time
148 of great tits within the nest box, constituting ≈ 2/5 of the ≈ 21 day-long nestling period. The LED system
149 was fitted under the nest box lid, and standardized to produce 1 lux at the average nest height of great tits,
150 8 cm above the bottom of the box, using a ISO-Tech ILM 1335 light meter (Corby, UK). This light
151 intensity is within the range of light levels experienced by birds in light exposed areas, and is similar to
152 the intensity used in a previous study in this population (Raap et al. 2015). The light intensity associated
153 with a full moon is about 0.05-0.2 lux (Kyba et al. 2017b), and light levels as low as < 0.00001 lux can
154 already have biological effects (see Gaston et al. 2013). We chose to use white LEDs as a source of
155 ALAN due to the current shift towards energy efficient, broad spectrum light sources, such as LEDs
156 (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 19.00h in the evening (≈2 hrs before sunset) and to turn the lights off at 0700 in the morning (≈1 hr 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 pre-treatment, 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 hours, 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.

182 ***2.4. Laboratory assays***: We determined telomere length, and molecularly sexed nestlings (Griffiths et al.
183 1998), using DNA extracted from blood samples using the Macherey-Nagel NucleoSpin® blood kit. We
184 measured the concentration and purity of DNA using a Nanodrop. All samples consisted of high quality
185 DNA with acceptable 260/280 (mean ± SE: 1.902 ± 0.011) and 260/230 ratios (mean ± SE: 2.143 ±
186 0.36), indicative of purity.
187 We determined telomere length using a relative real-time qPCR assay modified from Criscuolo et al.
188 (2009), which measures telomere length relative to a single copy reference gene. We used
189 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as our reference gene. We amplified GAPDH
190 using primers specific to the great tit: GAPDH-F (5'-TGTGATTTCAATGGTGACAGC-3') and
191 GAPDH-R (5'-AGCTTGACAAAATGGTCGTTC-3') (Atema et al. 2013). We amplified telomere
192 sequences using the primers Tel1b (5'CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-
193 3') and Tel2b (5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'), which amplify
194 telomere sequences across avian species. For both telomeres and GAPDH, we ran 15 µL qPCR reactions
195 containing 7.5 µL of FastStart Essential DNA Green Master (Roche Diagnostic Corporation, Indianapolis,
196 IN). Telomere reactions contained 0.9 µL each of forward and reverse primers at a concentration of 10
197 µM (final concentration: 600 nM), 2.325 µL of water, 0.375 µL of 100% DMSO (2.5% of total reaction
198 volume), and 3 µL of 1 ng/µL DNA. GAPDH reactions contained 0.3 µL each of forward and reverse
199 primers at a concentration of 10 µM (final concentration: 200 nM), 3.9 µL of water, and 3.0 µL of 1
200 ng/µL DNA.
201 We performed qPCR using a LightCycler®480 System (Roche). We ran telomere and GAPDH
202 reactions on separate 480-well plates. Telomere thermocycling conditions were: 10 min preincubation at
203 95°C, followed by 40 cycles of 15 sec at 95°C, 30 sec at 58°C and 30 sec at 72°C. GAPDH conditions
204 were: 10 min preincubation at 95°C, followed by 40 cycles of 15 sec at 95°C, 20 sec at 60°C, and 20 sec
205 at 72°C. We used a ramp speed of 4.4°C/sec, 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.

231

232 ***2.5. Statistical analyses***: All statistical analyses were conducted in R 3.4.1 (R Core Team 2017). We
233 used linear mixed effects models (LMMs) in R package lme4 (Bates et al. 2015) to investigate the effect
234 of exposure to ALAN on RTL, body condition (mass-size residuals) and NOx concentrations. To
235 facilitate interpretation of our results regarding body condition, we also constructed two additional models
236 to elucidate whether exposure to ALAN affected body mass and tarsus length. We used Satterthwaite
237 approximations for degrees of freedom, using R package lmerTest (Kuznetsova et al., 2016). All models
238 were reduced via a stepwise reduction process, by first removing nonsignificant interaction terms and
239 then sequentially removing terms with the highest p-value. Post-hoc tests were performed using R
240 package lsmeans, with Tukey tests between factor levels (Lenth 2016). Terms retained in final models
241 were significant at the $\alpha = 0.05$ level. Sample sizes are reduced in some cases (for RTL and NOx) due to
242 failure to obtain high quality DNA or plasma samples from some nestlings.

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

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

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

283 our initial model (Table 1). However, we found that size rank was highly correlated with body condition
284 (see below), and was thus collinear with body condition in the model predicting telomere length. When
285 predicting telomere length from size rank alone (with the interaction with treatment also initially
286 included), we found that nestlings that were smaller than brood mates had shorter telomeres, as reflected
287 by a negative correlation between size rank and RTL (β = -0.010 ± 0.004, t_{257} = -2.345, p = 0.019). The
288 relationship between size rank and telomere length was consistent across the treatment groups (non-
289 significant treatment × size rank interaction: (β = -0.002 ± 0.008, t_{255} = -0.252, p = 0.801; Figure 2b).
290
291 ***3.2. Body condition, body mass and tarsus length***: Nestling body condition ranged from -3.899 to 5.076
292 (mean ± SE: 0 ± 0.044), and depended on treatment in an age-specific fashion. Specifically, there was an
293 interaction between treatment and nestling age (Table 2), reflecting the fact that nestlings exposed to
294 ALAN tended to decline in condition between day 8 and day 15 (Figure 3), whereas nestlings in the
295 control group tended to gain condition between day 8 and day 15 (Figure 3). Post-hoc comparisons
296 indicated that nestlings did not significantly differ in body condition between day 8 and day 15 in either
297 the ALAN (β = 0.300 ± 0.141, t_{367} = 2.128, p = 0.146) or CTR (β = -0.223 ± 0.125, t_{364} = -1.785, p =
298 0.282) treatment groups after adjusting for multiple comparisons. However, note that the difference in
299 body condition between day 8 and 15 was larger in nestlings exposed to ALAN, and that the slopes in the
300 two treatment groups were in the opposite direction. Nestlings in the two treatment groups did not differ
301 in body condition at day 8 (before the beginning of the experiment, β = 0.250 ± 0.329, t_{28} = 0.759, p =
302 0.872). The difference in body condition between treatment groups at day 15 increased, as expected
303 given the significant treatment × age interaction, but was also statistically non-significant at the 0.05 level
304 after adjusting for multiple comparisons (β = -0.773 ± 0.329, t_{28} = 2.346, p = 0.111). Nestlings that were
305 smaller than their nest mates were in poorer body condition, and this relationship was similar between
306 treatment groups (Table 2).

307 Among day 8 nestlings, body mass ranged from 4.90 to 12.80 g (mean ± SE: 9.266 ± 0.118), and did
308 not differ between the two treatment groups before initiation of experiment (β = -0.866 ± 0.489, t_{23} = -
309 1.773, p =0.090). Among day 15 nestlings, body mass ranged from 7.70 to 18.10 g (mean ± SE: 14.62 ±
310 0.156). Body mass was not related to exposure to ALAN (β = -0.747 ± 0.581, t_{24} = -1.285, p = 0.211),
311 and none of the interactions between treatment, nestling age, size rank, or sex were significant (p > 0.40
312 in all cases). Male nestlings were heavier than females (β = 0.668 ± 0.168, t_{152} = 3.966, p < 0.001), and
313 nestlings of larger size rank were lighter than nestlings of lower size rank (β = -0.378 ± 0.022, t_{317} = -
314 17.174, p < 0.001).
315 Among day 8 nestlings, tarsus length ranged from 8.88 to 17.58 mm (mean ± SE: 14.13 ± 0.101), and
316 did not differ significantly between the two treatment groups before initiation of the experiment (β =
317 0.439 ± 0.349, t_{27} = -1.257, p = 0.597). Among day 15 nestlings, tarsus length ranged from 14.69 to
318 20.47 mm (mean ± SE: 18.68 ± 0.079). As for body condition, there was a significant interaction
319 between treatment and nestling age in predicting tarsus length (β = 0.458 ± 0.184, t_{314} = 2.494, p =
320 0.0131). This interaction reflected the fact that nestlings in the ALAN treatment group increased more in
321 tarsus length than nestlings in the control group. However, nestlings in the two treatment groups did not
322 differ in tarsus length at day 15 (β = 0.019 ± 0.347, t_{27} = 0.055, p = 0.999). The interactions between
323 treatment, size rank, and sex were non-significant (p > 0.40 in both cases). Male nestlings had longer
324 tarsi than females (β = 0.356 ± 0.121, t_{154} = 2.933, p = 0.004), and nestlings of larger size rank had
325 smaller tarsi than nestlings of lower the size rank (β = -0.205 ± 0.020, t_{320} = -10.459, p < 0.001).
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Table 1. Linear mixed effect model predicting relative telomere length from treatment (ALAN versus control) and covariates. Significant p-values (α=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.

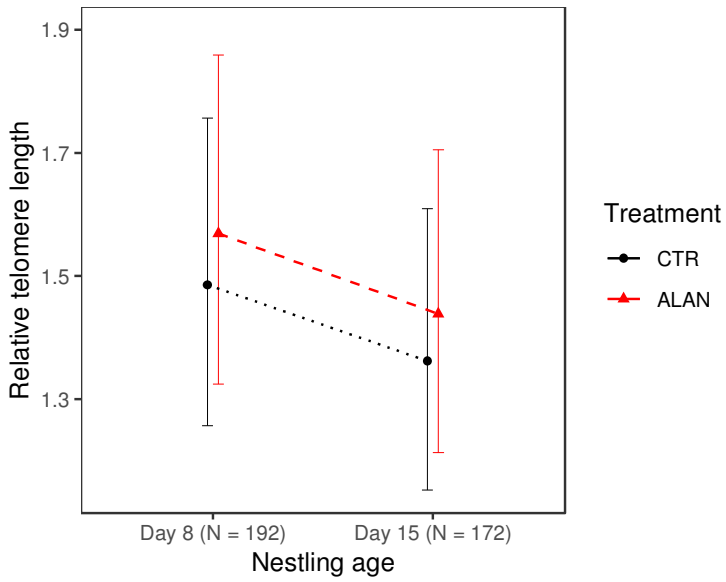
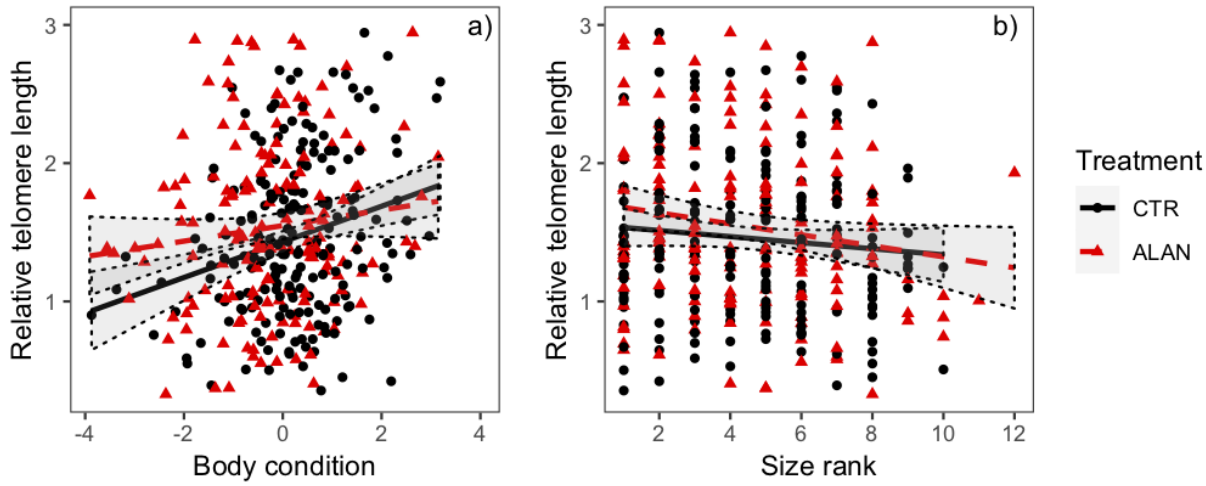


Figure 1. Change in relative telomere length between day 8 and day 15 in nestlings exposed, versus not exposed, to ALAN. Bars show 95% confidence intervals. ALAN = artificial light at night; CTR = control.



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343

344 **Figure 2.** Relative telomere length increased with body condition (mass-size residuals) (a) and decreased

345 with nestling size rank (largest nestling = rank 1) (b), with these effects being similar between the control

346 (CTR) and light (ALAN) treatment groups. Shaded regions show 95% confidence intervals.

347

348 **Table 2**. Linear mixed effect model predicting body condition from treatment (ALAN versus control)

349 and covariates. Significant p-values ($\alpha = 0.05$) appear in bold.

a. Initial model	**Estimate ($\beta \pm$ 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.

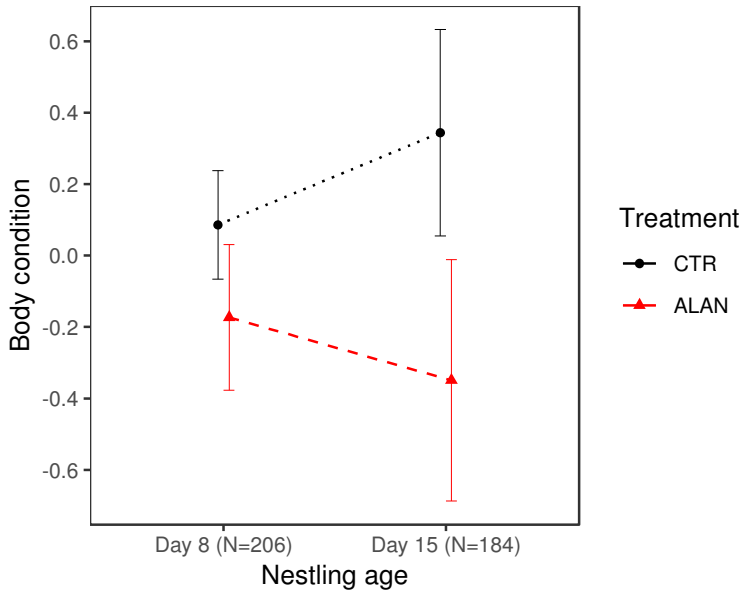
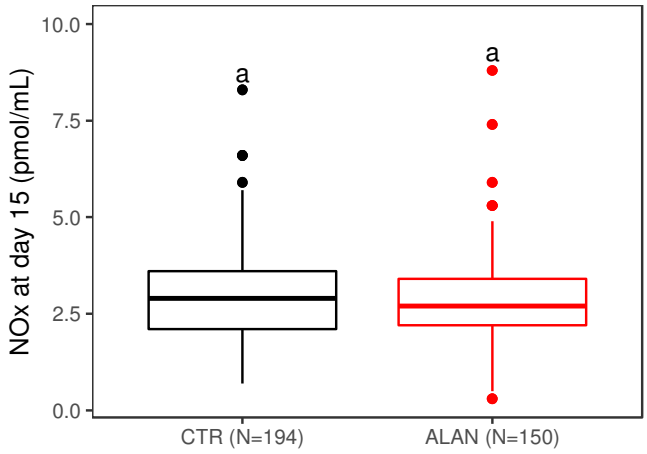


Figure 3. Change in body condition (mass, size residuals) between day 8 and 15 in nestlings exposed, versus not exposed to ALAN. Bars show 95% confidence intervals. ALAN = artificial light at night; CTR = control.

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



364

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

368

369 ***3.4. Fledging success***: Body condition at day 8 was a strong positive predictor of fledging success (β =
370 1.565 ± 0.496, z = 3.152, p = 0.001, N = 206 nestlings, 26 nests). However, fledging success was not
371 affected by exposure to ALAN (β =-0.162 ± 2.826, z = -0.057, p = 0.954), RTL (β = -1.176 ± 0.905, z = -
372 1.301, p = 0.193), or nestling sex (β = 1.101 ± 1.042, z = 1.056, p = 0.291).

373

374 ***3.5. Repeatability in telomere length and body condition***:
375 Nestling telomere length was not significantly repeatable between day 8 and day 15 (R = 0.065, 95% CI:
376 [0, 0.192], p = 0.124, N = 364 observations, 206 nestlings). Repeatability of RTL was higher within the
377 ALAN than within the CTR group (ALAN: R = 0.097, 95% CI: [0, 0.302], p = 0.154, N = 163
378 observations, 93 nestlings; CTR: R = 0, 95% CI: [0, 0.139], p = 1, N =201 observations, 113 nestlings),
379 but 95% confidence intervals extensively overlapped suggesting that repeatability did not significantly
380 differ depending on light exposure. In contrast to RTL, body condition was individually repeatable (R =
381 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 artificial light at night (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.

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

408 condition associated with the ALAN treatment might not have been large enough to translate into
409 physiological stress and induce telomere shortening.
410 Indeed, we found that nestlings exposed to ALAN tended to deteriorate in body condition between
411 days 8 and 15, whereas nestlings in the control treatment tended to gain body condition, leading to a
412 statistically significant interaction between the ALAN treatment and nestling age. However, this effect
413 was not large enough to translate into a statistically significant difference in body condition at day 15,
414 suggesting a relatively modest effect on nestling condition. Moreover, this pattern was driven by longer
415 tarsus lengths relative to body mass in nestlings exposed to ALAN, rather than overall reductions in body
416 mass, suggesting that nutritional stress was not severe. Why exposure to ALAN would induce nestlings
417 to gain more in tarsus length than in mass is unclear, but could reflect disruption of physiological control
418 systems, such as the pineal hormone melatonin and hormones related to food-intake and growth rate
419 (Fonken and Nelson 2014; Durrant et al. 2017; Ouyang et al. 2018).
420 We also found no evidence that exposure to ALAN affected NOx or fledging success, again
421 suggesting relatively low stress levels. Rather, the only significant predictor of NOx levels was nestling
422 sex, with males having higher NOx than females, a finding consistent with one of our previous studies
423 (Raap et al. 2017a). Body condition is often a strong predictor of fledging success (Both et al. 1999;
424 Tilgar et al. 2010; Rodríguez et al. 2016), and has also been shown to subsequently affect juvenile
425 survival rates and later-life fitness metrics (Perrins 1979; Tinbergen and Boerlijst 1990; Naef-Daenzer et
426 al. 2001; Perrins and McCleery 2001; Rodríguez et al. 2016), including in our population (Vermeulen et
427 al. 2016). Body condition was also a good predictor of fledging success in our study. However, there
428 was no detectable effect of light exposure on fledging success, perhaps due to relatively low death rates
429 before fledging and the relatively small difference between body condition at day 15 in the two treatment
430 groups.
431 Earlier work in our population of great tits suggested a stronger effect of ALAN on nestling body
432 condition and NOx levels, with nestlings exposed to ALAN failing to gain mass and showing suppressed
433 NOx levels after only two nights of light exposure (Raap et al. 2016a). One possible explanation for this

434 discrepancy is that nestlings are able to habituate to ALAN over a longer time frame, perhaps via
435 adjustments in other physiological systems, such that body condition and NOx levels recover. In
436 addition, although shorter in duration, Raap et al. 2016a used a light intensity 3 times higher than the
437 current study (3 as compared to 1 lux) and did not maintain a two-hour period of darkness during the
438 central period of the night. Thus, it is possible that a higher light intensity might lead to larger reductions
439 in nestling condition and a significant effect on telomere length, especially given the significant
440 relationship between body condition and telomere length that we observed in this study. In addition, the
441 year in which we conducted our study was abnormally cold, with low nestling mass at day 15 and high
442 nestling mortality rates. Hence, it might be easier to detect an effect of ALAN on nestling stress levels in
443 a more moderate year.
444 Another potential explanation for our results is that exposure to ALAN does not affect telomere
445 dynamics, despite having effects on patterns of growth and other physiological systems. Indeed, a recent
446 study on adult great tits also found no effect of ALAN on telomere length (Ouyang et al. 2017; but see
447 Raap et al. 2017b). Animals exposed to ALAN might be able to maintain telomere length despite
448 increases in stress levels by investing in defense mechanisms, such as antioxidant enzymes or telomerase
449 activity. However, in a previous study, we found no differences in oxidative stress levels or antioxidant
450 activity in nestlings exposed to two nights of ALAN (Raap et al. 2016a). It is also possible that ALAN
451 induces a unique cascade of physiological and behavioral responses that combine to cause no overall
452 effect on telomere length. For example, increased activity levels and reduced sleep may elevate
453 metabolism and oxidative stress, but a slower gain in body mass may reduce energy expenditure and
454 production of free radicals, thus neutralizing the effect on oxidative stress and telomere shortening.
455 Surprisingly, other research on the relationship between exposure to ALAN and telomere length is largely
456 absent, even in humans and laboratory animals, although sleep deprivation and shift work has been linked
457 to shorter telomere length in humans (Liang et al. 2011) and circadian disruption in mice leads to shorter
458 telomeres (Chen et al. 2014). Thus, more research is needed to assess the generality of our results, and
459 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.

485 The timing of the transition between shortening of class III and class II shortening is unclear (Atema et al.
486 *In Press*), and this transition could perhaps be accelerated by stress exposure during development. Thus,
487 further research examining the effect of ALAN on telomere shortening in nestlings, while employing a
488 technique that allows discrimination between telomere classes (Terminal Restriction Fragment (TRF);
489 Haussmann and Vleck 2002; Atema et al. *In Press*), is warranted and could yield intriguing results.
490 In contrast to some past work in great tits (Stier et al. 2015, 2016), telomere length was not
491 significantly repeatable between the day 8 and 15 sampling point in the nestlings included in our study.
492 Although this could be taken as reflecting methodological issues, we do not feel that this is likely since
493 we did find several expected, biologically meaningful results, namely the decline in telomere length
494 between day 8 and day 15, and the strong correlation between telomere length and body condition.
495 Rather, we suggest that the low repeatability estimate for telomere length in our study could reflect
496 differential rates of telomere shortening in different individuals. In contrast to telomere length, body
497 condition was repeatable, suggesting that nestlings that were in poorer condition at day 8 were also in
498 poorer condition at day 15.
499 Finally, also in contrast to some past studies in great tits (Stier et al. 2015), but in agreement with
500 others (Salmón et al. 2016; Stier et al. 2016), we found that nestling sex did not affect telomere length.
501 This suggests that, at least in our population, mechanisms controlling telomere attrition or maintenance
502 have been similarly selected in males and females, and that neither sex is more sensitive to condition
503 declines associated with exposure to ALAN.
504 In conclusion, we found that telomere dynamics of free-living nestlings were not affected by exposure
505 to ALAN inside the nest box, although body condition tended to decline over the timeframe of the
506 experiment. This suggests that the physiological stress induced by exposure to 1 lux of ALAN over a 7-
507 night timeframe was not severe enough to accelerate telomere shortening. Nestlings may have been able
508 to prevent deleterious effects of ALAN on telomeres via plasticity in other physiological systems, or
509 telomeres may be less sensitive to light exposure than other phenotypic traits. However, exposure to a
510 higher light intensity over a longer time period could lead to higher stress levels and telomere shortening.

511 Given ever-increasing levels of light pollution world-wide (Davies et al. 2014; Falchi et al. 2016),
512 resolving which phenotypic traits are sensitive to ALAN, and the intensity and duration of light exposure
513 that constitutes a threat, remains an important area for further research.
514
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524
525 **Data availability**: Data will be available in the Dryad Digital Repository.
526
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