Early-life exposure to artificial light at night elevates physiological stress in free-living songbirds

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2

3   **Artificial light at night elevates feather CORT**

4

5   **Melissa L. Grunst[1*], Thomas Raap[1], Andrea S. Grunst[1], Rianne Pinxten[1,2], Charline Parenteau[3],**

6   **Frédéric Angelier[3], Marcel Eens[1]**

7

8   **[1]Department of Biology, Behavioural Ecology and Ecophysiology Group, University of Antwerp,**

9   **2610 Wilrijk, Belgium**

10

11   **[2]Faculty of Social Sciences, Didactica Research group, University of Antwerp, 2000 Antwerp,**

12   **Belgium**

13

14   **[3]Centre d'Etudes Biologiques de Chizé, CNRS-ULR, UMR 7372, Villiers en Bois, France**

15

16   **[*]Corresponding author. Email: melissa.grunst@uantwerpen.be, Telephone: +32 (0)466 16 65 74**

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18   **ABSTRACT**   Artificial light at night (ALAN) can disrupt adaptive patterns of physiology and behavior

19   that promote high fitness, resulting in physiological stress and elevation of steroid glucocorticoids

20   (corticosterone, CORT in birds). Elevated CORT may have particularly profound effects early in life,

21   with the potential for enduring effects that persist into adulthood. Research on the consequences of early-

22   life exposure to ALAN remains limited, especially outside of the laboratory, and the effects of early-life

23   light exposure on CORT concentrations in wild nestling birds remain to be elucidated. We used an

24   experimental setup to test the hypothesis that ALAN elevates CORT concentrations in developing free-

25   living birds, by exposing nestling great tits (*Parus major*) to ALAN inside nest boxes. We measured

26   CORT in feathers grown over the timeframe of the experiment (7 nights), such that CORT concentrations

27   represent an integrative metric of hormone release over the period of nocturnal light exposure, and of

28   development. We also assessed the relationships between feather CORT concentrations, body condition,

29   nestling size rank and fledging success. In addition, we evaluated the relationship between feather CORT

30   concentrations and telomere length. Nestlings exposed to ALAN had higher feather CORT

31   concentrations than control nestlings, and nestlings in poorer body condition and smaller brood members

32   also had higher CORT. On the other hand, telomere length, fledging success, and recruitment rate were

33   not significantly associated with light exposure or feather CORT concentrations. Results indicate that

34   exposure to ALAN elevates CORT concentrations in nestlings, which may reflect physiological stress. In

35   addition, the organizational effects of CORT are known to be substantial. Thus, despite the lack of effect

36   on telomere length and survivorship, elevated CORT concentrations in nestlings exposed to ALAN may

37   have subsequent impacts on later-life fitness and stress sensitivity.

38

39   **Capsule**: In nestlings of a common songbird, exposure to artificial light at night elevated feather

40   corticosterone concentrations, but did not affect telomere length, fledging success, or recruitment rates.

41

42   **Keywords**: Artificial light at night; corticosterone; telomeres; body condition; fledging success

43

44   **1. INTRODUCTION**

45   Novel disturbance factors experienced in urbanized areas create the possibility for maladaptive or

46   adaptive responses, which can be mediated through behavioral plasticity or rapid evolutionary change

47   (Marzluff 2001; Sih 2011, 2013; Atwell et al. 2012; Sol et al. 2013). Thus, urban environments are

48   increasingly becoming model systems for studies of how free-ranging organisms adjust behavior and

49   physiology to changing environmental conditions (Hu and Cardoso 2009; Stillfried et al. 2017; Morelli et

50   al. 2018). Artificial light at night (ALAN) is one anthropogenic disturbance factor that is ubiquitous

51   within the urban matrix, and is affecting a growing proportion of the planet (Hölker et al. 2010; Gaston et

52   al. 2013; Swaddle et al. 2015). Introduction of ALAN into the environment by humans lacks a strong

53   parallel in natural systems, and thus has the potential to disrupt biological systems and result in

54   physiological stress. Indeed, organisms have evolved with the periodicity of natural light-dark cycles,

55   such that light plays an integral role in coordinating adaptive daily and seasonal patterns of physiology

56   and behavior (Gwinner et al. 2001; Dominoni et al. 2013).

57   Exposure to ALAN may disrupt the internal clock controlled by the suprachiasmatic nucleus of the

58   hypothalamus, with consequent effects on production of hormones, activity, sleep and recovery cycles,

59   and health (Navara and Nelson, 2007; Dickmeis, 2008; Figueiro and Rea, 2010). One hormonal system

60   that helps synchronize internal conditions and external stimuli and which may be particularly sensitive to

61   exposure to ALAN, is the hypothalamus-pituitary-adrenal (HPA) axis (Ouyang et al. 2018; Dickmeis

62   2008). The HPA axis helps mediate diurnal and season cycles of behavior in vertebrates, with peaks in

63   hormone production coinciding with the onset of daily activity and the nadir in hormone concentrations

64   occurring at night (Breuner et al. 1999; Ouyang et al. 2018). The effect of the HPA axis on activity levels

65   may interact with patterns of release of melatonin, a pineal hormone which is both light sensitive and

66   which has been shown to be affected glucocorticoid concentrations in complex ways (Persengiev et al.,

67   1991; Navara and Nelson, 2007). The HPA axis also primes organisms to cope with predictable life-

68   history challenges, such as reproduction and molt (Sapolsky et al. 2000; Romero 2002; Landys et al.

69   2006; Romero et al. 2009), and mediates the vertebrate response to acute, unpredictable stressors

70   (Wingfield et al. 1998; Romero et al. 2009; Angelier and Wingfield, 2013). Furthermore, elevation of

71   glucocorticoid stress hormones (GCs; corticosterone, CORT, in birds) via the HPA axis plays complex

72   and adaptive functions in glucose metabolism and energy balance, flight responses, and the immune

73   system (Sapolsky et al. 2000; Romero 2004; Wingfield and Romero 2001), whereas prolonged elevation

74   of GCs may result in chronic stress, deterioration in health status and fitness declines (McEwen and

75   Wingfield 2003). Thus, disruption of the HPA axis by anthropogenic disturbance factors such as ALAN

76   could have wide-reaching effects.

77   Previous studies have demonstrated that exposure to ALAN can eliminate the natural circadian rhythm

78   of GC release or elevate GC concentrations, both extensively in the laboratory (Scheving and Pauly 1966;

3

79   Mohawk et al. 2007; Fonken et al. 2012; Bedrosian et al. 2013; Martynhak et al. 2017; Alaasam et al.

80   2018; Emmer et al. 2018), and to a limited extent in free-living animals (Ouyang et. al. 2015). For

81   instance, Siberian hamsters (*Phodopus sungorus*) and rats exposed to ALAN in the laboratory displayed

82   increased cortisol concentrations (Scheving and Pauly 1966; Bedrosian et al. 2013), with the rats also

83   displaying an altered diurnal pattern of hormone release. In addition, in the first study to explore the

84   effects of ALAN in a wild animal, Ouyang et al. (2015) reported that adult great tits (*Parus major*)

85   exposed to white light at night had elevated CORT concentrations relative to control birds and birds

86   exposed to red or green light.

87   However, a critical deficit in knowledge exists regarding whether exposure to ALAN results in

88   elevated GC concentrations (or otherwise altered HPA activity) in developing organisms. Elevation of

89   GCs may have particular potent effects during development, when the phenotype remains sensitive to

90   organizational programming effects (Metcalfe and Monaghan 2001; Spencer et al. 2009). For instance,

91   past research has demonstrated that elevated GC concentrations early in life can be correlated with

92   heightened sensitivity to stressors later in life (Chung et al. 2005; Cottrell and Seckl 2009; Spencer et al.

93   2009; Banerjee et al. 2012; although the reverse has also been reported; Love and Williams 2008; Zimmer

94   et al. 2013), declines in the expression of sexually selected traits (Spencer et al. 2003; Husak and Moore

95   2008; Spencer and MacDougall-Shackleton 2011; Schmidt et al. 2014; Dupont et al. 2019), and reduced

96   life expectancy (Monaghan et al. 2012; Grace et al. 2017), with effects potentially being mediated by life-

97   history tradeoffs (Hau et al., 2010; Vitousek et al., 2018).

98   The effects of early-life elevation of GCs on later-life fitness may, in part, be mediated through effects

99   on telomere dynamics (Monaghan 2014; Angelier et al. 2018). Telomeres are conserved repeats of

100   nucleotides in organisms that cap the ends of chromosomes and protect DNA from damage and

101   malfunction (Haussmann et al. 2005, 2012; Monaghan and Haussmann 2006; Monaghan 2014), and

102   accelerated telomere loss has been linked to reduced longevity and disease (Blackburn et al. 2006).

103   Telomere shortening is especially rapid early in life, and has been shown to be accelerated by stressful

104   conditions and elevation of GCs (Haussmann et al. 2012; Herborn et al. 2014; Reichert and Stier 2017;

105   Angelier et al. 2018). In addition, both exposure to ALAN and elevation of GCs may elevate oxidative

106   stress. ALAN may result in higher oxidative stress by suppressing the concentrations of melatonin, a

107   potent antioxidant, and through down-stream effects of sleep deprivation and heightened activity on the

108   production of pro-oxidants and the concentrations of antioxidants (Navara and Nelson, 2007). GCs may

109   increase metabolic rate and alter patterns of activity, thus increasing the generation of reactive oxygen

110   species (Angelier et al. 2018). Telomeres are especially sensitive to damage by reactive oxygen species

111   due to a high guanine content, and although its importance has been debated, elevated oxidative stress has

112   been linked to increased rates of telomere shortening (Reichert and Stier, 2017).

113   We experimentally investigated the effect of ALAN on great tit nestlings, using a system of LEDs to

114   produce light inside of nest boxes. In an earlier publication, we report that this manipulation had no effect

115   on nestling telomere length, but that body condition changed differently in control and experimental

116   nestlings over the course of the experiment (significant interaction term), with control nestlings tending to

117   gain condition and experimental nestlings tending to experience condition declines (Grunst et al., 2019).

118   Here we incorporate new data from hormonal assays to explore the effects of exposure to ALAN on

119   CORT concentrations. Our study differs from the earlier Ouyang et al. (2015) study, also on the effect of

120   ALAN on CORT release in great tits, in that we focused on nestlings instead of adults. We also used a

121   lower light intensity than Ouyang et al. (2015) (1 lux relative to ~8 lux), but since nestlings are not mobile

122   they are also less able to avoid the light manipulation than are free-ranging adults. Unlike Ouyang et al.

123   (2015), we did not consider the effects of different wavelengths of light.

124   We exposed nestlings to light for 7 nights, constituting a substantial proportion of the nestling cycle,

125   and assessed CORT release using hormone concentrations from feathers (feather CORT; Bortolotti et al.

126   2008; Lattin et al. 2011; Jenni-Eiermann et al. 2015; Romero and Fairhurst 2016). CORT is deposited in

127   feathers over the period of feather growth, and the period over which these feathers grew corresponded to

128   the period of light exposure. Thus, feather CORT concentrations provide an integrative metric of CORT

129   release during the timeframe of the experiment. We predicted that CORT concentrations would be

130   elevated in experimental nestlings, due to disruptive effects of ALAN on physiology and behavior

131 (Swaddle et al. 2015; Ouyang et al. 2018). To our knowledge, this is the first study to investigate the

132 effect of exposure to ALAN on CORT concentrations in a developing, free-living bird. In addition, there

133 may be a component of variation in GCs concentrations that is independent of exposure to ALAN, based

134 energetic state, competitive environment, or genetics (Wada et al., 2008; Jenkins et al., 2014). Thus, we

135 also assessed whether CORT concentrations could independently predict telomere length, body condition,

136 or fledging success, or whether CORT concentrations interacted with exposure to ALAN to predict these

137 variables. We predicted that nestlings with higher feather CORT concentrations would be in poorer body

138 condition, have shorter telomeres, and have reduced fledging success. With respect to the predicted

139 negative relationship between body condition and CORT concentrations, the causality is not necessarily

140 unidirectional. Elevated CORT might act to reduce body condition (although high CORT concentrations

141 might also facilitate maintenance of body condition in some cases), but low body condition could also

142 lead to higher CORT concentrations. With respect to the interaction between CORT and ALAN, we

143 hypothesized that relationships might differ in directionality or magnitude in the two treatment groups,

144 since CORT might play different functions given exposure to ALAN. Finally, to gain additional insight

145 into potential fitness ramifications, we evaluated whether exposure to ALAN or feather CORT

146 concentrations were negatively related to rates of recruitment into the population.

147

148 **2. METHODS**

149 **2.1. Study population and general methods**: Our study population of great tits breeds in the immediate

150 vicinity of the University of Antwerp's Campus Drie Eiken (Wilrijk, Belgium; 51°9'44"N, 4°24'15"E),

151 and consists of >120 resident breeding pairs (e.g. Van Duyse et al. 2000, 2005; Rivera-Gutierrez et al.

152 2010, 2012; Raap et al. 2015, 2016a, b, 2017, 2018a,b; Vermeulen et al. 2016). We checked nest boxes

153 every other day beginning in late March, and continuing through the end of the breeding season in mid-

154 May, to determine laying date, hatching date, brood size, and fledging success. Nestlings were

155 considered to have recruited into the population, which is extensively monitored, if they were re-sighted

156 or recaptured on the study sight in subsequent years. This study was approved by the ethical committee

157   of the University of Antwerp (ID number: 2017-90) and conducted in accordance with Belgian and

158   Flemish laws.

159

160   **2.2. Experimental design**: Details of the experimental design are given in Grunst et al. (2019). In brief,

161   we exposed nestlings to ALAN from day 8 to day 15 of the nestling stage (hatch day = day 1) using a

162   system of 4 small LED lights (Diameter: 5 mm, Cree® Round LED C535A-WJN, Durham, North

163   Carolina, USA) that produce broad-spectrum white light (see Grunst et al. 2019 for color spectrum

164   specifications). The LED system was fitted under the nest box lid, and standardized to produce 1 lux at

165   the average nest height of great tits (8 cm above the nest box bottom; ILM 1335 light meter, ISO-TECH,

166   Northamptonshire, UK). We used a timer inside a homemade enclosure to turn light systems on at 1900

167   in the evening and off at 0700 in the morning. In addition, to reduce chances of nest abandonment, the

168   system was turned off during the night from 2400 to 0200. Control nest boxes were fitted with LED

169   systems, but no electronics. The experiment was completed between April 20 and May 8, 2017 on first

170   nesting attempts (N = 26 nest boxes; 12 ALAN, 14 CTR; 206 nestlings; 93 ALAN, 113 CTR). Control

171   and experimental nests did not differ significantly in hatching date (t_{21} = -0.231, P = 0.820) or brood size

172   (t_{19} = -0.384, P = 0.705). Broods included in the experiment had an average (mean ± SE) of 7.96 ± 0.296

173   (range: 5 to 12) nestlings, and had hatching dates between April 13 and April 23, 2017. For one control

174   nest box, feather samples were of insufficient mass for use in the CORT assay.

175

176   **2.3. Field sampling**: To assess the effect of ALAN on CORT concentrations, we gently removed ≈ 15-20

177   contour (breast) feathers from each nestling on day 15 of the nestling stage, following the last night of

178   light exposure. Feathers were stored in small envelops in a dark and dry location.

179   To assess the effect of ALAN on telomere length and body condition, we used a repeated measures

180   design, detailed in Grunst et al. (2019). In brief, blood samples and body measurements were taken on

181   day 8 and 15 of the nestling stage, and were completed between 0800 and 1230. Body condition was

182 calculated as the residuals of a regression predicting body mass from tarsus length (Schulte-Hostedde et

183 al., 2005). On day 8, we uniquely marked nestlings with a metal ring or color band.

184

185 **2.4. Laboratory assays**:

186 *2.4.1. Feather CORT radioimmunoassay***:** Following feather collection, we used a high precision

187 balance (Mettler Toledo XS205 Dual Range) to weigh feather samples, with a target mass of $\approx$ 20 mg.

188 Masses of feather samples ranged from 19.1 to 20.7 mg, and sample mass was not related to CORT

189 concentrations (mean $\pm$ SE: -0.197 $\pm$ 0.153, t_{157} = -1.29, P = 0.200). A non-linear relationship between

190 feather mass and the amount of CORT/mg detected may occur, especially at low feather masses ($<$ 20

191 mg), and using similar feather masses avoids this problem (Kennedy et al. 2013; Grunst et al. 2015).

192 Feather samples were transported to the Centre d'Etudes Biologiques de Chizé, where radioimmunoassay

193 was performed.

194   We extracted CORT from feathers by adding 10 ml of methanol (HPLC grade) to each sample, placing

195 samples in a sonicating water bath at room temperature for 30 minutes, and incubating at 50°C in a

196 shaking water bath overnight. Feathers were very small, so it was unnecessary to pulverize samples

197 before the extraction process. We separated methanol from feather residue using filtered syringes (see

198 details in Meillére et al. 2016), dried extracts under air in a 50°C water bath placed in a fume hood, and

199 reconstituted extract residues in a small volume of the phosphate buffer system (PBS; 0.05 m, pH 7.6).

200 We used previously described radioimmunoassay techniques to determine CORT concentrations in

201 reconstituted extracts (Lormée et al. 2003). We confirmed the linearity of the assay with respect to

202 nestling great tit feathers before running the assay. Samples were run in 5 assays, with all samples

203 assayed in duplicate. The intra- and inter-assay coefficients of variation were 7.96% and 15.6%,

204 respectively. Although the inter-assay coefficient of variation is somewhat high, samples were randomly

205 distributed between assays, so we do not expect this to affect results.

206

207 *2.4.2. Telomere qPCR and nestling sex determination*

208 We determined telomere length and molecularly sexed nestlings (Griffiths et al. 1998) using DNA

209 extracted from blood samples using the Macherey-Nagel NucleoSpin® blood kit. We measured the

210 concentration and purity of DNA using a Nanodrop (2000c; Thermo Scientific; Merelbeke, Belgium).

211 Samples were of high purity, with 260/280 and 260/230 ratios close to recommended values (mean ± SE:

212 1.90 ± 0.01 and 2.14 ± 0.36, respectively) (Desjardins and Conklin, 2010). We determined telomere

213 length using a relative real-time qPCR assay modified from Criscuolo et al. (2009), and developed for the

214 great tit by Atema et al. (2013), which measures telomere length relative to a single copy reference gene

215 (in our case glyceraldehyde-3-phosphate dehydrogenase (GAPDH)). See Grunst et al. (2019) for details

216 on the telomere assay.

217

218 **2.5. Statistical analyses**: We performed all statistical analyses in R 3.4.1 (R Core Team 2017). We used

219 linear mixed effects models (LMMs) in R package lme4 (Bates et al. 2015) to investigate the effect of

220 exposure to ALAN on feather CORT concentrations, with Satterthwaite approximations for degrees of

221 freedom (R package lmerTest; Kuznetsova et al. 2016). We used the scale function in R to center and

222 standardize all continuous predictor variables to a mean of zero and a standard deviation of one.

223 Performing this operation aids in interpretation of beta estimates, especially when interaction terms are

224 included in the model (Schielzeth, 2010). We report statistics from global models in all cases. We

225 predicted feather CORT concentrations (log-transformed) from treatment (ALAN, control), with nest ID

226 as a random effect, and brood size, size rank within a brood (largest nestling = size rank 1 on the basis of

227 day 15 mass), nestling sex and hatching date as fixed-effect covariates. To assess the possibility of size-

228 or sex-dependent effects of ALAN on feather CORT concentrations, we also included the two-way

229 interactions between treatment and sex and treatment and size rank. We used a separate LMM to test

230 whether body condition was associated with feather CORT. We did not include body condition in the

231 initial model because body condition is reduced at day 15 in nestlings exposed to ALAN (see Grunst et al.

9

232   2019). Thus, in the analysis presented here, treatment and body condition were collinear in the model

233   predicting feather CORT.

234   Second, we tested whether RTL (log-transformed) or body condition were associated with feather

235   CORT concentrations. We predicted RTL from the interaction between feather CORT and treatment,

236   with nestling age (day 8 or day 15) and size rank as covariates, and Nest ID, nestling ID, and qPCR assay

237   number as random effects. We used the same fixed and random effects in the model predicting body

238   condition, with the exception of additionally including the interaction between nestling age and treatment

239   (we previously found that this interaction was significant for body condition, but not for telomere length;

240   Grunst et al. 2019), and excluding the assay number random effect. We also previously found that sex

241   had no effect on telomere dynamics or body condition in nestling great tits (Grunst et al. 2019).

242   Finally, we assessed whether fledging success or recruitment rates were associated with exposure to

243   ALAN, feather CORT concentrations or body condition at day 15. To this end, we used general linear

244   models with a binomial error structure to predict whether or not a nestling fledged or recruited (both 1, 0).

245   We did not test the interaction between feather CORT and treatment in these models since the number of

246   nestlings that died before fledging (28/206) and recruited (20/206) were limited, and we wanted to avoid

247   over-fitting. We included nest ID as a random effect.

248

249   **3. RESULTS**

250   **3.1. Feather CORT concentrations**: Feather CORT concentrations ranged from 2.91 to 24.0 pg/mg

251   (mean $\pm$ SE = 8.16 $\pm$ 0.25) and were significantly higher in nestlings exposed to ALAN than in nestlings

252   in the control group (Table 1; Figure 1). Nestlings that were smaller than their brood mates (higher size

253   rank) had higher feather CORT (Table 1; Figure 2a), whereas nestlings in better body condition had lower

254   ($\beta \pm$ SE = -0.081 $\pm$ 0.020, t_{134} = -3.97, p < 0.001, N = 159 nestlings, 25 nest boxes; Figure 2b), feather

255   CORT. The interactions between treatment and size rank and treatment and sex were non-significant, and

256   brood size and hatching date were also unrelated to feather CORT concentrations (Table 1).

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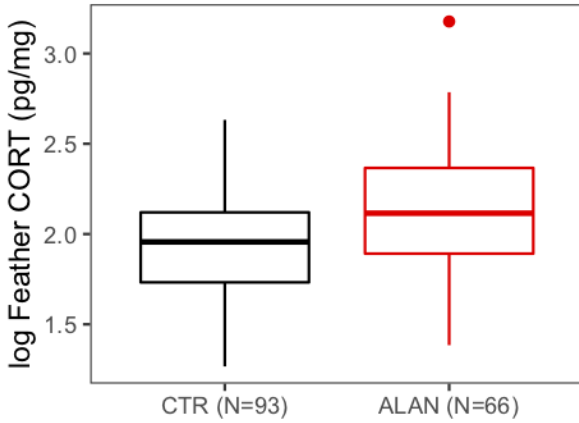
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258 **Figure 1**. Box plot of feather CORT concentrations in the control (CTR) and light (ALAN) treatment

259 groups. CORT concentrations were significantly higher in nestlings exposed to ALAN ($\beta \pm SE = 0.209 \pm$

260 0.100; $t_{39} = 2.09$; p = 0.043). N = the number of nestlings in each treatment group. Whiskers extend from

261 the first and third quartiles to the highest value within 1.5 times the interquartile range.

262

263 **Table 1**. Linear mixed effect model predicting feather CORT concentrations from treatment and

264 covariates. Significant p-values appear in bold.

	Estimate ($\beta \pm SE$)	Df	T	P > |t|
Intercept	2.00 ± 0.09	29.6	21.5	**< 0.001**
Treatment[a]	0.209 ± 0.10	38.8	2.09	**0.043**
Size rank	0.096 ± 0.03	129	2.99	**0.003**
Sex[b]	0.063 ± 0.062	133	1.02	0.310
Brood size	0.035 ± 0.042	23.0	0.842	0.408
Hatching date	-0.063 ± 0.076	25.5	-0.83	0.414
Treatment × size rank	0.039 ± 0.048	136	0.82	0.413
Treatment × sex	-0.054 ± 0.098	141	-0.55	0.582
Random effects	**Variance**	**SD**	**N**	

| Nest | 0.035 | 0.186 | 25 |
| Residual | 0.060 | 0.246 | 151 |

265 [a]**ALAN relative to control nestlings**

266 [b]**Males relative to females**

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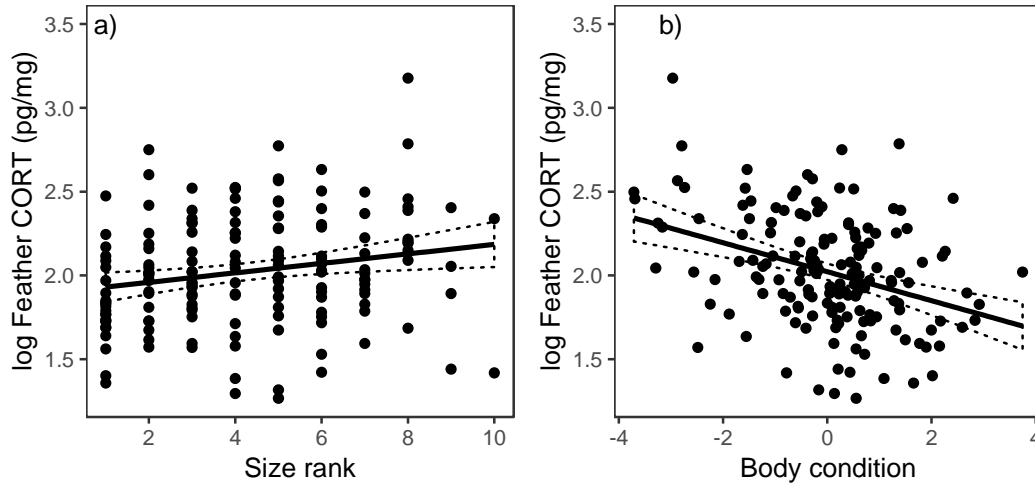
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269

270 **Figure 2.** Relationships between feather CORT concentrations and (a) size rank and (b) body condition.

271 Dotted lines show 95% confidence intervals.

272

273 **3.2. Telomere length and body condition**: Relative telomere length was not associated with treatment or

274 nestling feather CORT concentrations, and the interaction between light exposure and feather CORT was

275 also non-significant (Table 2). Nestlings that were smaller than their brood mates (which are also in

276 poorer body condition) had shorter telomeres, and nestling telomere length also declined with age,

277 although this effect was not significant in this model (Table 2).

278 Feather CORT concentrations positively interacted with treatment to predict body condition, reflecting

279 that feather CORT concentrations were negatively related to body condition only within the control group

280 ($\beta \pm SE = -0.227 \pm 0.108$, $t_{170} = -2.11$, $p = 0.037$, N = 187 observations, 93 nestlings, 13 nest boxes) and

281 not among nestlings exposed to ALAN ($\beta \pm SE = 0.101 \pm 0.088$, $t_{122} = 1.148$, $p = 0.253$, N = 129

282 observations, 66 nestlings, 12 nest boxes) (Figure 3). There was also a negative interaction between

283 feather CORT concentrations and nestling age, reflecting that feather CORT concentrations were only

284 significantly negatively related to body condition in day 15 ($\beta \pm SE = -0.104 \pm 0.048$, $t_{148} = -2.16$, p =

285 0.032, N = 159 nestlings, 25 nest boxes), and not in day 8 ($\beta \pm SE = -0.045 \pm 0.037$, $t_{152} = -1.21$, p =

286 0.230, N = 157, 24 nest boxes), nestlings. Nestlings that were smaller than their brood mates (higher size

287 rank) were also in poorer body condition (Table 3). The interaction between treatment and nestling age

288 was not significant in this model (Table 3).

289

290 **Table 2**. Linear mixed effect model predicting relative telomere length from treatment, feather CORT

291 concentrations and covariates. Significant p-values appear in bold.

	Estimate ($\beta \pm SE$)	Df	T	P > |t|
Intercept	0.458 ± 0.095	16.8	4.82	<0.001
Treatment	0.034 ± 0.051	25.9	0.67	0.510
Feather CORT	0.055 ± 0.037	112	1.48	0.141
Nestling age	-0.038 ± 0.020	153	-1.87	0.064
Size rank	-0.020 ± 0.01	202	-2.18	**0.026**
Treatment × CORT	-0.077 ± 0.047	120	-1.65	0.100
CORT × age	-0.019 ± 0.021	157	-0.90	0.371

Random effects	Variance	SD	N	
Individual ID	0.0003	0.018	159	
Nest box	0.003	0.059	25	
qPCR Plate	0.061	0.248	11	
Residual	0.123	0.351	295	

292 **[a]ALAN relative to control nestlings**

293

294   **Table 3**. Linear mixed effect model predicting body condition from treatment, feather CORT

295   concentrations and covariates. Significant p-values appear in bold.

	Estimate (β ± SE)	Df	T	P > |t|
Intercept	0.609 ± 0.236	29.7	2.58	0.015
Treatment[a]	-0.448 ± 0.315	21.5	-1.43	0.168
Feather CORT	-0.221 ± 0.103	308	-2.13	**0.034**
Nestling age	0.169 ± 0.063	283	2.65	**0.008**
Size rank	-0.104 ± 0.022	299	-4.54	**<0.001**
Treatment × CORT	0.338 ± 0.127	307	2.66	**0.008**
Treatment × age	-0.143 ± 0.103	286	-1.39	0.167
CORT × age	-0.167 ± 0.051	284	-3.31	**0.001**

Random effects	Variance	SD	N
Individual ID	0	0	159
Nest box	0.535	0.731	25
Residual	0.735	0.857	316

296   **[a]ALAN relative to control nestlings**

297

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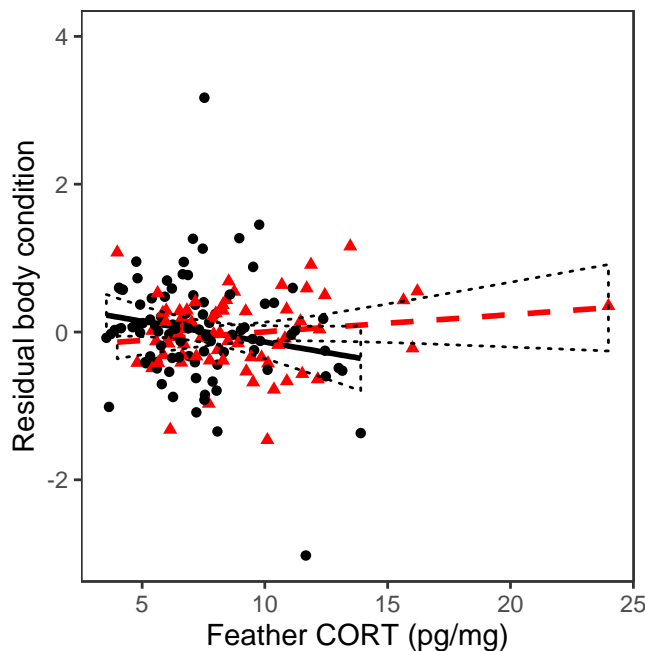
299   **Figure 3**. Relationship between
300   body condition (mass-size
301   residuals) and feather CORT
302   concentrations in the two
303   treatment groups. The values
304   used for body condition are

305   residuals that control for the other variables included in the model. Dotted lines show 95% confidence

306   intervals.

307

308   **3.3. Fledging success and recruitment rate**: Exposure to ALAN did not affect fledging success ($\beta \pm$

309   SE: -1.15 $\pm$ 1.18, z = -0.969, p = 0.333, N = 159 nestlings, 25 nest boxes). Of nestlings that reached day

310   15 and had feather CORT measured, 4/93 and 5/66 nestlings did not fledge, in the control versus ALAN

311   group, respectively. Feather CORT concentrations ($\beta \pm$ SE = -0.754 $\pm$ 0.450, z = -1.68, p = 0.094), and

312   body condition at day 15 ($\beta \pm$ SE = 0.336 $\pm$ 0.344, z = 0.975, p = 0.330) were also unrelated to fledging

313   success, although the coefficient estimate for feather CORT was negative. Exposure to ALAN was

314   unrelated to recruitment rate ($\beta \pm$ SE = -0.627 $\pm$ 0.862, z = -0.728, p = 0.467). Of nestlings that reached

315   day 15 and had feather CORT measured, 11/93 and 9/66 nestlings recruited, in the control versus ALAN

316   group, respectively. Feather CORT ($\beta \pm$ SE = 0.746 $\pm$ 0.442, z = 1.69, p = 0.091) was also unrelated to

317   recruitment into the population, with the coefficient estimate in this case being positive. However,

318   nestlings that were in better body condition at day 15 were more likely to recruit ($\beta \pm$ SE = 0.686 $\pm$ 0.319,

319   z = 2.15, p = 0.032).

320

321

322   **4. DISCUSSION**

323   We used an experimental setup to expose free-living songbird nestlings to ALAN. We demonstrate that

324   exposure to ALAN elevates feather CORT concentrations in these developing organisms. In the context

325   of our study, feather CORT concentrations reflect an integrative metric of hormone release over both the

326   period of light exposure and of development. Nestlings with higher feather CORT concentrations were

327   also in poorer body condition, and smaller brood members had higher feather CORT, both of which are

328   consistent with higher feather CORT being indicative of physiological stress (Bortolotti et al. 2008).

329   Feather CORT concentrations were not significantly associated with fledging success (in contrast to

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330 Lodjak et al. 2015) or recruitment rate, such that we have no evidence of longer-term effects of high

331 CORT on fitness. However, our power to detect these effects was limited, and past work suggests that

332 nestlings in poorer condition are less likely to survive the juvenile period, and to recruit into the

333 population (Perrins 1979; Tinbergen and Boerlijst 1990; Naef-Daenzer et al. 2001; Perrins and McCleery

334 2001; Rodríguez et al. 2016; Vermeulen et al. 2016). Indeed, nestlings in better body condition were also

335 more likely to recruit in this study. Moreover, even if unrelated to fledging success and recruitment rates,

336 elevated CORT concentrations during development may have deleterious effects on other phenotypic

337 traits important to fitness, such as song learning and acquisition of sexual coloration (Spencer et al. 2003;

338 Husak and Moore 2008; Spencer and MacDougall-Shackleton 2011; Schmidt et al. 2014), and may affect

339 the function of the HPA axis during adulthood, with implications for stress-sensitivity (Chung et al. 2005;

340 Cottrell and Seckl 2009; Spencer et al. 2009; Banerjee et al. 2012).

341 Exposure to ALAN could result in higher CORT concentrations via a number of mechanisms. Light

342 exposure during the night can disrupt the natural circadian rhythm of the HPA axis (Scheving and Pauly

343 1966; Ouyang et al. 2018). Indeed, light exposure can directly activate the adrenal glands, resulting in

344 higher CORT secretion (Ishida et al. 2005) and photoreceptors are also present in the hypothalamus

345 (Ouyang et al. 2018). Behavioral alterations arising from exposure to ALAN may be mediated by

346 elevated CORT, and may also potentially feedback to promote higher HPA activity. For instance, in an

347 earlier study we found that ALAN increases the nighttime activity levels (begging) of nestlings, which

348 could reflect sustained CORT secretion into the nighttime period (Raap et al. 2016a), and other studies

349 have also found increases in nighttime activity levels, and parallel increases in CORT concentrations, in

350 adult birds (Alaasam et al. 2018). Since CORT supports glucose metabolism, increased activity could in

351 turn enforce elevated release of CORT. ALAN could also induce changes in parental behavior that could,

352 in turn, affect patterns of CORT release in nestlings (e.g. Stracey et al. 2014). For instance, parents may

353 reduce nestling provisioning rates prior to fledging to encourage nestlings to leave the nest, potentially

354 reducing body mass and elevating CORT concentrations (Kern et al. 2001; Lodjak et al. 2015). ALAN

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355   may have expedited or enhanced this late-nestling stage reduction of feeding rates, or could also have

356   reduced provisioning rates for the duration of the experiment.

357   A number of past studies have linked conditions of elevated stress, or disturbance, during development

358   to increases in feather CORT (Lodjak et al. 2015; Johns et al. 2018; Beaugeard et al. 2019). For instance,

359   great tit nestlings in enlarged broods had higher feather CORT concentrations in a poorer-quality

360   coniferous forest habitat (Lodjak et al. 2015), and nestling house sparrows (*Passer domesticus*) in an

361   urban environment had higher feather CORT than rural counterparts (Beaugeard et al. 2019). Since a

362   number of different hypotheses can explain elevation of CORT, different or similar pathways could be

363   leading to elevated feather CORT in these studies. However, this body of work suggests that elevated

364   feather CORT concentrations may reflect a mechanism via which organisms cope with a number of

365   different natural and anthropogenic developmental stressors, with potential implications for life-history

366   trajectories.

367   Somewhat unexpectedly, although body condition was negatively related to feather CORT

368   concentrations, this relationship was stronger, and only statistically significant among control nestlings.

369   This result arose despite the fact that feather CORT concentrations were higher in nestlings exposed to

370   ALAN. Why feather CORT concentrations would more strongly predict body condition in control

371   nestlings is unclear. One possible explanation is that elevated feather CORT in nestlings exposed to

372   ALAN is not merely a reflection of pathology, but may actually aid nestlings in coping with light-

373   associated stress, thus dampening the relationship between low body condition and elevated CORT in

374   comparison to in the control group.   In addition, CORT exerts biological effects via two classes of

375   receptors, mineralocorticoid receptors, which bind the hormone at lower concentrations, and

376   glucocorticoid receptors, which bind the hormone at higher concentrations (Sapolsky et al. 2000; Romero

377   2004). Thus, differences in receptor activation patterns could explain the stronger relationship between

378   feather CORT concentrations and body condition in control versus experimental nestlings.

379   We also found that body condition was only related to feather CORT concentrations in 15-day old, and

380   not in 8-day old nestlings. In great tit nestlings, feathers are just beginning to grow around day 8, and in

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381   the case of our study, the experimental ALAN exposure occurred beginning on day 8 and continuing

382   through day 15. Thus, it is logical that differences in feather CORT between nestlings would be more

383   closely related to body condition at day 15.

384   Finally, with respect to our conclusions regarding body condition, in Grunst et al. (2019) we found a

385   significant interaction term between nestling age and treatment. This interaction reflects a tendency for

386   control nestlings to gain condition and experimental nestlings to lose condition between day 8 and day 15.

387   In the current analysis, this interaction term was not significant, although the relationship is in the same

388   direction. We noted in our previous publication that the effect of exposure to ALAN on body condition

389   was modest and did not translate into a significant difference in body condition at day 15. Thus, it is not

390   surprising that this interaction term is not significant when other variables are included in the model. In

391   short, we can conclude that exposure to ALAN had only a minimal impact on nestling body condition in

392   our study. Thus, it seems probable that different pathways underlie the positive relationship between

393   exposure to ALAN and CORT concentrations and the negative relationship between body condition and

394   CORT concentrations.

395   We reported earlier that exposure to ALAN had no effect on telomere dynamics in great tit nestlings

396   (Grunst et al. 2019). Here we additionally show that CORT concentrations are not related to telomere

397   length. Elevated CORT, and associated increases in oxidative stress, have been proposed as mechanisms

398   that mediate accelerated telomere shortening (Angelier et al. 2018; Casagrande and Hau 2019). However,

399   in a previous study we found that there was no effect of exposure to ALAN on oxidative stress in great tit

400   nestlings (Raap et al. 2016c), and in the current study, telomere length appeared resistant to early-life

401   exposure to ALAN and elevation of CORT. As we suggested in our previous publication, it is possible

402   that ALAN induces a unique cascade of physiological and behavioral responses that combine to cause no

403   overall effect on telomere length (Grunst et al. 2019). A past study on great tits also reported no effect of

404   ALAN on telomere shortening (Ouyang et al. 2017; but see Raap et al. 2017b), despite higher CORT

405   concentrations in adults exposed to white light (Ouyang et al. 2015). Although not in the context of light

406   exposure, a number of other studies have also found no relationship between CORT concentrations and

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407   telomere dynamics, or have reported positive relationships (see Angelier et al. 2018 for a review). On the

408   other hand, several past studies have associated conditions of elevated stress and/or elevated CORT

409   concentrations with reduced telomere length (Herborn et al. 2014; Quirici et al. 2016; Pegan et al. 2019;

410   Angelier et al. 2019). As for the relationship between CORT concentrations and fitness, the relationship

411   between CORT concentrations and telomere dynamics is likely to be complex and species- and context-

412   dependent (Angelier et al. 2018). Methodological differences between studies, such as when and how GC

413   concentrations and telomere length were measured and the life-history stage of focus, could also in part

414   explain the mixed results of past studies. Further research and meta-analyses will be necessary to resolve

415   this complexity into overarching patterns.

416   Regarding our specific methodology, and as discussed in greater length in our earlier publication

417   (Grunst et al. 2019), great tits have two classes of terminal telomeres, and attrition of the shorter class

418   cannot be detected via the qPCR technique (Atema et al. 2019). We were able to detect biologically

419   meaningful patterns with respect to telomere dynamics, including shortening between day 8 and 15 and

420   reduced telomere length in nestlings in poor condition (Grunst et al. 2019). Although the relationship

421   between nestling age and telomere length was not significant in the analysis presented in this paper, it was

422   only marginally non-significant ($p = 0.064$). However, it remains possible that presence of these two

423   classes of telomeres could have affected our ability to detect an effect of ALAN and elevated CORT

424   concentrations on telomere dynamics.

425   In addition, details of our experimental design could be implicated in our failure to detect an effect on

426   telomere dynamics. In particular, we included a dark period in the middle of the night, with the aim of

427   preventing complete mortality. Including this dark period could have resulted in recovery of melatonin

428   levels, thus dampening effects on the oxidative status of nestlings. We also used a relatively low light

429   intensity, and we would predict that a higher light intensity would have more pronounced phenotypic

430   effects. Thus, in the future, examining the effects of lighting through the entire night, or exposing

431   nestlings to a higher light intensity, could prove informative, although employing these regimes could

432   also result in total nest abandonment.

433   Great tits serve as a convenient organism to test the effects of ALAN on nestlings since they readily

434   occupy nest boxes, allowing for experimental manipulation and facilitating sampling. Thus, our

435   experimental design holds promise for more detailed studies of the ramifications of early life exposure to

436   ALAN. Future experiments could further investigate the effect of different light intensities, wavelengths

437   and durations of exposure on physiological and behavioral endpoints and fitness outcomes.   However,

438   since great tits are cavity nesters, nestlings may be relatively buffered against ALAN in natural situations,

439   although some cavities may be less impervious to light than man-made nest boxes. Indeed, our past

440   research suggests that nest boxes buffer sleeping adults from ambient ALAN (Raap et al. 2018a), and

441   effects of ambient ALAN on nestlings in nest boxes are similarly low (Casasole et al. 2017; Raap et al.

442   2017a). However, our results are transferable to real-life situations since the nestlings of open-cup bird

443   species are likely to experience light levels comparable to those used in our experiment. In addition,

444   nestlings and adult great tits may experience comparable light levels when sleeping outside of nest boxes,

445   and consequently experience elevated CORT, as indeed suggested by the work on adult great tits cited

446   earlier (Ouyang et al. 2015).

447

448   **5. CONCLUSIONS**

449   In conclusion, our results suggest that exposure to ALAN induces physiological stress in developing

450   songbirds, as indicated by elevated feather CORT concentrations. Elevation of CORT could arise via a

451   number of mechanisms, including direct effects on the circadian rhythmicity of the HPA axis, disruption

452   of sleep and activity patterns of nestlings, and altered parental behavior. These mechanisms are not

453   mutually exclusive. Regardless of mechanistic underpinnings, elevated CORT concentrations could have

454   enduring effects on physiology and fitness that persist into adulthood. In a world where true darkness is

455   increasingly difficult to find, more research is urgently needed to elucidate the extent to which ALAN

456   affects physiology, behavior, and fitness, and whether animals can develop adaptive avoidance or

457   mitigation strategies.

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459 **Data availability**: Data will be available in the Dryad Digital Repository.

460

461 **Declaration of competing interests**: The authors declare that there are no conflicts of interest.

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