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1 **Disruptive effects of light pollution on sleep in free-living birds:**

2 **season and/or light intensity-dependent?**

3

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20

21 Light pollution or artificial light at night (ALAN) is an increasing anthropogenic
22 environmental pollutant posing an important potential threat for wildlife. Evidence of its effects
23 on animal physiology and behaviour is accumulating. However, in order to effectively mitigate
24 light pollution it is important to determine which factors contribute to the severity of effects of
25 ALAN.

26 In this experimental study we explored whether there are seasonal-dependent effects of
27 ALAN on sleep in free-living great tits (*Parus major*), an important model species.
28 Additionally, we looked at whether light intensity determined the severity of effects of ALAN
29 on sleep. We therefore exposed animals to artificial light inside the nest box (3 lux) in December
30 (winter) and February (pre-breeding season). Results from February were compared with the
31 results from a previous study in February, using a lower light intensity (1.6 lux).

32 We found little evidence for a season-dependent response. Effects of ALAN hardly
33 differed between high and low light intensity. ALAN disrupted sleep with as main effect a
34 decrease in sleep duration (\approx -40 min) as animals woke up earlier (\approx -24 min). However,
35 compared to a natural dark situation sleep onset was delayed by high but not by low light
36 intensity of ALAN.

37 Our study underlines earlier found disruptive effects of ALAN on sleep of free-living
38 animals. While we found no conclusive evidence for seasonal or light intensity-dependent
39 effects of ALAN, additional experimental work using lower light intensities might show such
40 differences. Examining potential management options is crucial in mitigating disruptive effects
41 of light pollution, which will be an important focus for future studies.

42

43

44

45	KEYWORDS
46	Dose-dependent
47	Mitigation
48	Season
49	Urbanization
50	Wildlife

51 1. INTRODUCTION

52 Light pollution or artificial light at night (ALAN) is an increasing worldwide
53 anthropogenic environmental pollutant (Falchi et al. 2016). The loss of darkness poses a
54 potentially important threat for wildlife, biodiversity and humans (Rich et al. 2005; Navara et
55 al. 2007; Hölker et al. 2010; Gaston et al. 2013; Kyba et al. 2013; Duffy et al. 2015). This
56 disruption of our natural light and dark cycles, to which animals and plants have evolved, results
57 in a wide range of physiological and behavioural responses. For example in songbirds, ALAN
58 has been shown to reduce melatonin levels, advance dawn song (reviewed in Swaddle et al.
59 2015; Bedrosian et al. 2016) and to disrupt sleep (Raap et al. 2015, 2016c).

60 It is crucial to understand which factors contribute to the severity of negative
61 environmental impacts of light pollution, in order to effectively mitigate them (Gaston et al.
62 2012; 2013; 2015). However, what determines the extent of these impacts on free-living
63 animals is still unknown. Seasonal variability and intensity of light are both likely to be
64 important and must be better understood to develop short and long-term solutions.

65 Variability in responses to artificial light across the year (see e.g. Meyer et al. 2013)
66 may influence management options during a particular period. Reducing the intensity of
67 lighting is another possible strategy to reduce effects of light pollution. Studies of effects of
68 ALAN at different light intensities are of vital importance (Gaston et al. 2013) but are
69 uncommon (but see e.g. Newman et al. 2015; de Jong et al. 2016), especially those using free-
70 living animals.

71 While ALAN affects a range of animal behaviours (reviewed by Swaddle et al. 2015),
72 the present study is focused on the effects of ALAN on sleep in songbirds in the wild, more
73 specifically in free-living great tits (*Parus major*), a widely used model species. Studying the
74 effects of light pollution on sleep in birds is of major importance for several reasons. First, sleep

75 is an important animal behaviour widespread across the animal kingdom (Cirelli et al. 2008;
76 Siegel 2008), and most if not all bird species show sleeping behaviour (Roth II et al. 2006;
77 Lesku et al. 2014; Libourel et al. 2016). Second, it serves multiple purposes including energy
78 conservation and memory consolidation (Gobes et al. 2010; Roth II et al. 2010). Third, avian
79 sleep shares many characteristics of mammalian sleep, for example both consist of two types
80 of sleep, REM and non-REM (Siegel 2008). Sleep is important for many organisms, plays a
81 role in maintaining high levels of physical and cognitive functioning and is ideally suited to
82 examine differences in effects of ALAN in the wild.

83 The severity of effects of ALAN may vary over time during the year (e.g. Meyer et al.
84 2013) and strategies for mitigating light pollution may need to be adjusted accordingly. Day
85 length is an important cue for seasonal time-keeping in animals (Bradshaw et al. 2010). For
86 example, as the season progresses from December to February onwards, sleep behaviour of
87 great and blue tits (*Cyanistes caeruleus*) changes, with birds waking up earlier (relative to
88 sunrise) in both species (Steinmeyer et al. 2010; Stuber et al. 2015a). Under natural conditions
89 light initiates a cascade of physiological effects associated with day length (Bradshaw et al.
90 2010) and at the end of winter as day length starts to increase, this cascade prepares the animal
91 for reproduction (Helm et al. 2013). In contrast with December, in February great tits are near
92 the breeding season and therefore physiological events already prepare them for reproduction.
93 Previously, we found that effects of ALAN on sleep were more severe during the nestling
94 period, such as a 50% reduction in sleep of female great tits, instead of a reduction of about 5%
95 in February. This may have been due to multiple factors (Raap et al. 2016c). For example,
96 differences might have been due to direct effects of ALAN on female sleep or indirectly through
97 increased nestling begging and parasite activity during the nestling period. The severity of
98 ALAN due to season or other drivers (e.g., nestling or parasite activity) remains unclear and
99 requires study.

100 Light intensity may influence the extent of sleep disturbance mediated by ALAN and is
101 especially relevant due to the variation of exposure in free-living animals (Gaston et al. 2014).
102 While laboratory studies showed dose-dependent effects of light on daily activity rhythms of
103 great tits (de Jong et al. 2016), whether this is also true for free-living great tits and for other
104 behaviours is not yet known. Environmental conditions outside of the laboratory may affect
105 physiology and behaviour (Daan 2011) and experiments involving behaviour (such as sleep)
106 are particularly susceptible to environmental influences (Calisi et al. 2009; Stuber et al. 2015a;
107 Aulsebrook et al. 2016). Sleep behaviour of captive animals can thus vary tremendously from
108 the behaviour of wild individuals (Rattenborg et al. 2008). Consequently, responses to ALAN
109 may differ between wild and captive animals and comparing behavioural responses to ALAN
110 recorded in laboratory conditions to natural environments is necessary.

111 Here, we tested for a seasonal-dependent and light intensity-dependent effect of ALAN
112 on sleep in free-living great tits. First, using a field experiment, we compared the effect of
113 ALAN on sleep between December (winter) and February (pre-breeding season). We expected
114 larger disruptive effects on sleep in February. Second, we tested whether light intensity and
115 sleep disturbance by ALAN are associated. We compared results obtained from the current
116 study using a light intensity of 3 lux in the nest box with our previous study, which was also
117 done in February but used a lower light intensity of 1.6 lux (Raap et al. 2015). Under laboratory
118 conditions, great tits' responses of daily activity rhythms to ALAN have been shown to be dose-
119 dependent (de Jong et al. 2016) and so we expected that a higher light intensity (similar to those
120 used by de Jong et al. 2016) would increase the disruptive effect of ALAN on sleep behaviour
121 of free-living animals.

122 **2. METHOD**

123 *2.1 Study Area and General Procedures*

124 Data was collected during December 2015 (November 30th – December 28th) and
125 February 2016 (February 22nd – March 3rd) in a resident nest box population of great tits in the
126 surroundings of Wilrijk, Belgium (51°9'44''N, 4°24'15''E). This nest box population was
127 established in 1997 and has been continuously monitored since then (see e.g. Van Duyse et al.
128 2000; 2005; Rivera-Gutierrez et al. 2010, 2012; Vermeulen et al. 2016; Thys et al. 2017).
129 During previous winter- and breeding seasons great tits were caught inside nest boxes after
130 which they were sexed and ringed (see e.g. Rivera-Gutierrez et al. 2010; 2012; Vermeulen et
131 al. 2016; Casasole et al. 2017; Raap et al. 2017a). Since 2012, all adults have been provided
132 with a ring containing a passive integrated transponder, also known as a PIT tag. This enabled
133 the individual detection of birds sleeping in nest boxes without physically disturbing them.

134

135 *2.2 Experimental Procedure*

136 Similar to a previous study on effects of ALAN on sleep behaviour (Raap et al. 2015),
137 we used a within-individual design (or repeated measures) with two sequential nights of
138 observed sleep behaviour. Using a within-individual design “controls” (Ruxton et al. 2010) for
139 the large variation between individuals in sleep behaviour (Stuber et al. 2015a; Raap et al.
140 2016c). Birds slept with the light in the nest box turned off on the first night and turned on
141 during the second night, which allowed us to observe the change in sleep behaviour caused by
142 ALAN (see paragraph 2.3). In total we obtained paired data from 11 individuals (three females
143 and eight males) in December and from 23 individuals (12 females and 11 males) in February.
144 No individuals from our previous study (Raap et al. 2015) were re-used.

145

146 *2.3 Sleep Behaviour Recordings and Light Treatment*

147 We measured sleep behaviour and exposed great tits to artificial light following Raap et
148 al. (2015). In short, nest boxes were checked for presence and identity of sleeping great tits
149 prior to the first recording and during the experiment with a handheld transponder reader (FR-
150 250 RFID Reader, Trovan, Aalten, Netherlands). To record sleeping behaviour we installed
151 infrared sensitive cameras (Pakatak PAK-MIR5, Essex, UK) under the nest box roof lid. These
152 were installed at least two hours before sunset and removed at the earliest about an hour after
153 sunrise the next morning. In a previous study (Raap et al. 2015) we did not find a difference in
154 sleep behaviour for great tits sleeping in a dark nest box on two subsequent nights. A masking
155 effect would therefore seem unlikely.

156 Under each nest box roof lid we also placed a small white LED light (15 x 5 mm, taken
157 from a RANEX 6000.217 LED headlight, Gilze, Netherlands). We successfully used this
158 system to study the effects of ALAN on sleep and physiology (Raap et al. 2015; 2016a; 2016b;
159 2016c).

160 All LED lights were standardized to produce 3 lux at the bottom of the nest box (ISO-
161 Tech ILM 1335 light meter; Corby, UK). Birds living in light polluted areas are exposed to
162 similar and higher light intensities outside of nest boxes or cavities (Dominoni et al. 2013;
163 Gaston et al. 2013). In the laboratory, large differences in daily activity rhythms were found
164 when comparing 1.5 and 5 lux (de Jong et al. 2016). Using a 5 lux light intensity might cause
165 not enough birds to enter the nest box as free-living great tits tend to not enter a nest box when
166 it was lit with an interior light of 1.6 lux (Raap et al. 2015). Therefore, instead of the 5 lux light
167 intensity (de Jong et al. 2016) we used 3 lux. With this light intensity we still expected to find
168 differences in sleep behaviour but also that sufficient animals would enter the nest box when
169 the light was turned on.

170 On the first night of recording the LED was present but off. During the second day/night
171 the LED and the recording system were turned on, before 15:00 (at least two hours before
172 sunset). This allowed animals to become accustomed to changed light conditions. The following
173 morning the light was turned off when the recordings ended (about an hour after sunrise). In
174 December sunrise is around 8:40 and sunset around 16:40, in late February sunrise and sunset
175 are at respectively 7:30 and 18:20.

176

177 *2.4 Defining Sleep Behaviour*

178 Great tits are an ideal model species as they readily sleep in nest boxes, making it
179 possible to study their sleep behaviour and manipulate light conditions to which they are
180 exposed during the night (Raap et al. 2015). Great tits are too small to be fitted with modern
181 data loggers for recording brain activity (necessary for defining sleep). While using behaviour
182 as a proxy for sleep (as we did in our current study) has its limitations (Aulsebrook et al. 2016),
183 it can still be considered to be ecologically relevant as it has been linked to behavioural changes,
184 genetic variation and fitness (Christe et al. 1996; Tripet et al. 2002; Steinmeyer et al. 2010;
185 Amo et al. 2011; Steinmeyer et al. 2013; Stuber et al. 2014; 2015a; 2015b; 2016).

186 A bird was considered to be sleeping when in the classical sleep position, with the beak
187 pointing backwards and tucked under the scapulars (Amlaner et al. 1983). Otherwise it was
188 considered to be awake. In rare cases, the condition was ambiguous, and these periods were
189 defined as awake. While some sleep might have occurred with the head facing forwards, it is
190 impossible with a top view (camera is located above the bird) to distinguish this from a resting
191 posture.

192 Sleep of great tits was quantified in detail as described in earlier studies on great and
193 blue tit sleep behaviour (e.g. Steinmeyer et al. 2010; Raap et al. 2015). We used 10 parameters:

194 (1) entry time, (2) sleep onset, (3) evening latency, (4) awakening time, (5) leaving time, (6)
195 morning latency, (7) sleep duration, (8) sleep duration/night duration, (9) frequency of sleep
196 bouts and (10) sleep bout length. For a detailed description of scoring sleep behaviour, see Raap
197 et al. (2015) and/or other articles on sleep behaviour in great and blue tits (Steinmeyer et al.
198 2010; Stuber et al. 2014; 2015a; 2015b; Raap et al. 2016c). During morning latency, we
199 additionally recorded “time on entrance”: total time a bird spent on the nest box entrance hole
200 and “number of times on entrance”: the total number of times it sat on the nest box entrance
201 (Raap et al. 2015).

202

203 *2.5 Statistical Analysis*

204 For all statistical analyses we used R 3.2.3 (R Core Team 2015). We converted entry
205 time, sleep onset, awakening time and leaving time to times relative to sunset or sunrise
206 (reference data from Antwerp were used).

207

208 *2.5.1 Testing a seasonal-dependent response of sleep behaviour to ALAN*

209 For each sleep parameter a separate linear mixed effect analysis was performed (using
210 the lme4 package, Bates et al. 2013). The full models included as fixed effects “Treatment”
211 (control night/light night), “Month” (December/February), “Sex”, “Age” (yearling/adult) and
212 the interaction “Treatment:Month” which would indicate whether there was a seasonal effect
213 of light on sleep behaviour. Because we used a within-individual design (repeated measures),
214 we included bird identity as a random factor. This also takes into account that six birds were
215 observed in both December and February.

216 As we wanted to avoid overfitting our models, we did not include interactions with sex
217 except in the model with sleep duration/night duration as dependent parameter because in a

218 previous study an effect was found (Raap et al. 2015). This enabled us to make a more complete
219 comparison between studies with a low and high light intensity (see paragraph 2.5.2).

220 Where applicable, Tukey HSD tests were used for post-hoc analyses (lmerTest,
221 Kuznetsova et al. 2016). P-values were obtained by a stepwise backward elimination of non-
222 significant factors (Zuur et al. 2009) and are given in results. Results are presented as marginal
223 means with one standard error, unless stated otherwise.

224

225 2.5.2 Testing a light intensity-dependent response of sleep behaviour to ALAN

226 To test our second hypothesis, whether there is a light intensity-dependent response of
227 avian sleep to artificial light at night, we compared a light intensity of 3 lux (February 2016)
228 with earlier results (February 2015) with a light intensity of 1.6 lux (Raap et al. 2015). Data on
229 sleep behaviour of 18 individuals, 11 males and seven females, were available from this study.
230 Estimates about the effect of light were compared between studies. From this comparison it was
231 obvious that formal statistical testing was unnecessary (see paragraph 3.5 and Fig 2).

232 In the previous study we used sleep amount (sum of all sleep bouts) instead of sleep
233 duration (time between sleep onset and awakening time). However, sleep duration has been
234 used in studies of blue and great tits (Steinmeyer et al. 2010; Stuber et al. 2014; 2015a; 2015b;
235 2016). To make our results more comparable to these studies we examined the correlation
236 between sleep amount and duration on a subset of individuals for which we had both sleep
237 parameters. Therefore, the number of birds for which we had data on sleep bout length and
238 frequency was limited to 12. Sleep duration and amount were correlated (*Pearson's correlation*:
239 $r = 0.82$, $p < 0.0001$, $N = 12$; see also Stuber et al. 2015b) and so we used sleep duration
240 throughout the manuscript and compared sleep duration at 3 lux with sleep amount under 1.6
241 lux (data from our previous study: Raap et al. 2015).

242

243 *2.6 Ethical Note*

244 This study was approved by the ethical committee of the University of Antwerp (ID
245 number 2014-45) and performed in accordance with Belgian and Flemish laws and adhere to
246 the ASAB/ABS guidelines for the use of animals in behavioural research and teaching. The
247 Belgian Royal Institute for Natural Sciences provided ringing licenses for all authors and
248 technicians. Because of the short duration of the manipulation (one night of artificial light per
249 experiment) and because no individuals were caught during the course of the study the
250 disturbance was assumed to be minimal.

251

252 3. RESULTS

253 3.1 Individuals Not Entering a Lit Nest Box

254 Not all birds slept in the nest box during the second night when the light was turned on.
255 In addition to the eleven birds that we recorded both nights in December, five birds slept in a
256 dark nest box the first night but not the second evening/night when the LED light was on. In
257 February, of the 39 birds entering the first night, 23 entered and 16 did not on the second,
258 artificially lit, night. The proportion of birds not entering the nest box the second evening did
259 not differ between December and February (respectively 0.31 and 0.41; *Fisher's Exact Test*, p
260 = 0.556).

261 In the previous study nine out of 27 birds did not enter the nest box (Raap et al. 2015).
262 There was no difference in the proportion of birds not entering with high light intensity in
263 February compared to the previous study with a lower light intensity in the same month
264 (respectively 0.41 and 0.33; *Fisher's Exact Test*, $p = 0.453$). Birds that did not enter the nest
265 box on the second evening were excluded from further analyses.

266

267 3.2 Effect of ALAN on Sleep Behaviour

268 Independent of month (December/February), ALAN (3 lux) reduced the time that animals
269 were asleep (\approx -40 min) due to awakening earlier (\approx -24 min), and caused earlier leaving of the
270 nest box (\approx -18 min). In addition, the frequency of sleep bouts during the night decreased but
271 their length increased, while the proportion of time that animals were asleep during the night
272 decreased (-5%). Several other small effects on other sleep parameters were also found (see
273 Tables 1 and 2). Animals fell asleep about 15 minutes later and the time between entering the
274 nest box and falling asleep was slightly increased (evening latency). Likewise the time spent

275 between waking up and leaving the nest box in the morning slightly increased (morning latency)
276 with animals going more often to the entrance and spending more time on it. Sex and age
277 explained little of the variation in sleep behaviour (sex was significant for sleep duration: $F =$
278 4.482 , $p = 0.036$; sleep duration/night duration: $F = 5.452$, $p = 0.028$ and sleep bout/h: $F =$
279 5.909 , $p = 0.046$; age was significant for sleep bout/h: $F = 12.733$, $p = 0.008$; Table 2).

280

281 *3.3 Seasonal Difference in Sleep Behaviour*

282 There were several differences in sleep behaviour between December and February (see
283 Table 1 and 3). In February, animals fell asleep earlier in relation to sunset (half an hour) and
284 woke up later in relation to sunrise (quarter of an hour). Overall, animals slept about two hours
285 less in February and a smaller part of the night was spent asleep (-7%).

286

287 *3.4 Seasonal-Dependent Response of Sleep Behaviour to ALAN*

288 The effect of light at night (3 lux) on the time that great tits entered the nest box (entry time)
289 differed between December and February ($F = 8.822$, $p = 0.004$; Table 1, Fig 1). In December
290 time of entering the nest box was unaffected by light at night ($t = 1.60$, $p = 0.114$), while in
291 February entry time was delayed (-9.9 ± 3.41 minutes, $t = 2.91$, $p = 0.005$; Fig 1). For other
292 sleep parameters, there was no season-dependent effect of ALAN (Table 1).

293

294 *3.5 Light Intensity-Dependent Response of Sleep Behaviour to ALAN*

295 Previously, we found a sex-dependent effect on sleep proportion with a light intensity
296 of 1.6 lux in February, with the proportion of time spent asleep being reduced for females but

297 not for males (Raap et al. 2015). However, we found no sex-dependent effect on sleep
298 duration/night duration ($F = 0.058$, $p = 0.811$) with a higher light intensity of 3 lux. A light
299 intensity of 3 lux delayed the time when animals fell asleep (≈ 16 minutes), while with a light
300 intensity of 1.6 lux no effects on sleep onset were found (Fig 2). Effects of ALAN on awakening
301 time, leaving time or sleep duration did not differ between birds exposed to two different light
302 intensities as can be observed from Fig 2. There was also no difference in effects on evening or
303 morning latency (all about 1-2 minutes longer).

304

305 **Table 1 Statistical output of the mixed effect models.** Linear mixed models were used with
 306 bird identity as random factor to correct for repeated measurements. Main factors of treatment
 307 (control/light 3 lux) and month (December/February) and their interaction are shown. The
 308 interaction between treatment, month and sex was not significant and is not shown. To correct
 309 for changes in day length, response variables were standardized to civil sunset (entry time,
 310 sleep onset) or sunrise (awakening time and leaving time). Evening and morning latency were
 311 log transformed as well as time and number of times on entrance. Significant effects are
 312 depicted in bold ($p \leq 0.05$) and trends are underscored ($p < 0.1$).

Sleep parameter	Treatment:Month		Treatment		Month	
	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>
Entry time	8.822	0.004	0.114	0.737	1.589	0.212
Sleep onset	2.084	0.154	19.703	<0.001	58.707	<0.001
Awakening time	0.965	0.332	41.708	<0.001	7.372	0.009
Leaving time	1.807	0.187	26.878	<0.001	8.459	0.005
Evening latency	<u>3.325</u>	<u>0.076</u>	29.329	<0.001	19.618	<0.001
Morning latency	0.081	0.777	8.402	0.006	0.263	0.610
Time on entrance	1.741	0.202	7.493	0.012	2.109	0.159
Nr on entrance	<u>3.325</u>	<u>0.076</u>	29.329	<0.001	19.618	<0.001
Sleep duration	0.313	0.579	38.617	<0.001	232.662	<0.001
Sleep duration/ night duration	0.283	0.598	38.915	<0.001	57.500	<0.001
Sleep bout length*	0.016	0.900	14.350	0.001	0.897	0.354
Sleep bout/h*	0.147	0.705	11.355	0.003	2.190	0.152

313

314 * For all sleep parameters we had 28 individuals (68 observations in total) except for sleep
 315 bout length and sleep bout/h for which we had 12 individuals (34 observations in total).

316

317

318 **Table 2 Post-hoc analyses of the effect of artificial light at night on sleep behavior during**
319 **December and February.** Control is sleep behavior during the first night when animals slept
320 under natural dark conditions. Sleep onset, awakening time and leaving time are adjusted for
321 sunrise/sunset, with negative values indicating minutes before sunrise/sunset. Light effect is
322 the estimated difference between the first dark night and the subsequent night with artificial
323 light. For every sleep parameter the estimate with their 95% confidence interval (lower,
324 upper) is given. Sleep onset, awakening time, leaving time, sleep duration and sleep bout
325 length are given in minutes. Evening and morning latency, as well as time on entrance and
326 number of times on entrance were log transformed. Only significantly affected/different sleep
327 parameters are shown ($p \leq 0.05$; see Table 1).

Sleep parameter	Control	Light effect
Sleep onset	4.15 (-1.19, 9.49)	16.20 (8.92, 23.50)
Awakening time	-26.48 (-35.50, -17.50)	-24.10 (-31.70, -16.60)
Leaving time	-19.54 (-27.70, -11.30)	-17.70 (-24.62, -10.80)
Evening latency	1.57 (1.32, 1.81)	0.60 (0.36, 0.78)
Morning latency	1.32 (0.86, 1.77)	0.70 (0.20, 1.13)
Time on entrance	-0.28 (-1.22, 0.66)	1.10 (0.26, 1.92)
Nr on entrance	0.57 (0.04, 1.11)	1.30 (0.88, 1.72)
Sleep duration	844 (833, 855)	-40.20 (27.12, 53.20)
Sleep duration/ night duration	1.01 (1.00, 1.03)	0.05 (0.03, 0.06)
Sleep bout length	10.14 (8.12, 12.20)	2.70 (1.23, 4.19)
Sleep bout/h	5.33 (4.37, 6.28)	-1.30 (-2.05, -0.49)

328

329

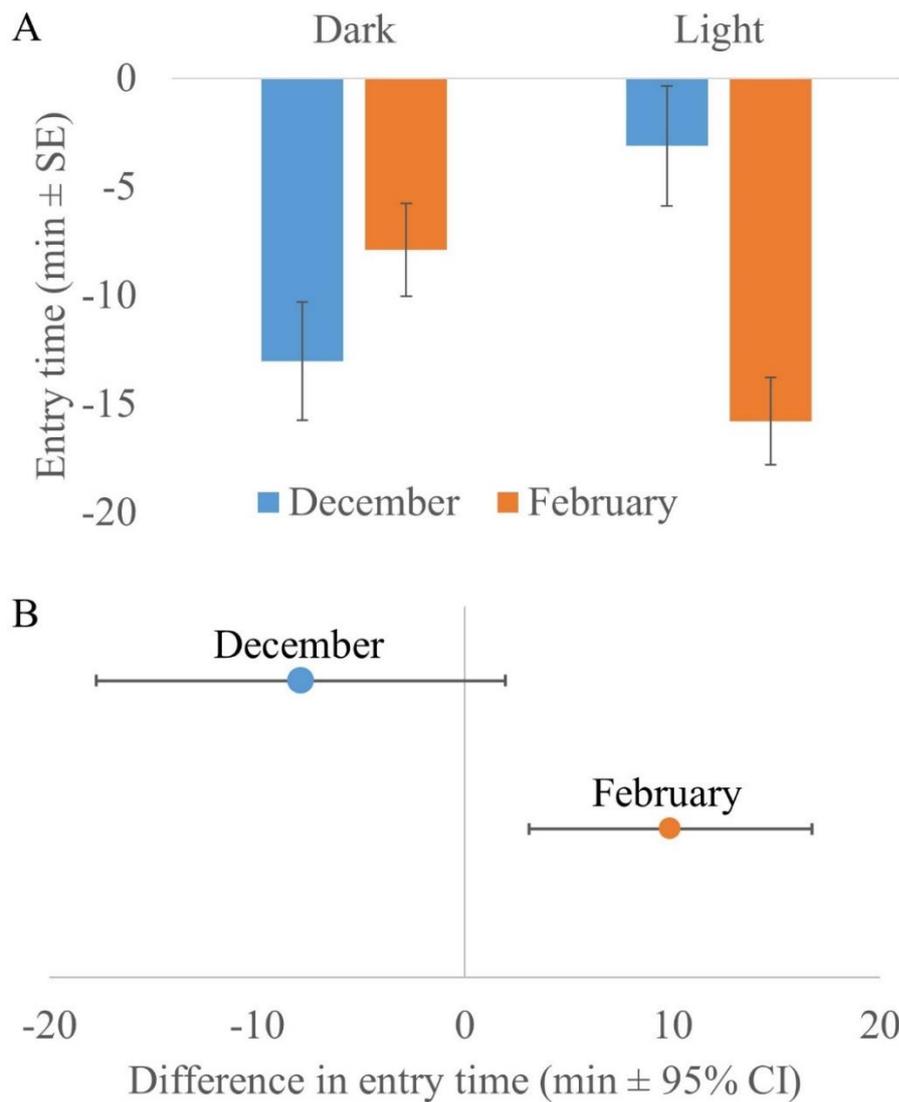
330

331 **Table 3 Post-hoc analyses of the seasonal difference in sleep behavior under a natural**
332 **dark situation.** The column December shows sleep behavior in December. Sleep onset,
333 awakening time and leaving time are adjusted for sunrise/sunset, with negative values
334 indicating minutes before sunrise/sunset. The column Month difference shows the difference
335 between December and February and negative values indicated a decrease compared to
336 December. Sleep onset, awakening time and leaving time are adjusted for sunrise/sunset. For
337 every sleep parameter the estimate with their 95% confidence interval (lower, upper) is given.
338 Sleep onset, awakening time, leaving time and sleep duration are given in minutes. Evening
339 latency was log transformed. Only significantly affected/different sleep parameters are shown
340 ($p \leq 0.05$; Table 1).

Sleep parameter	December	Month difference
Sleep onset	27.22 (20.80, 33.63)	-29.90 (-37.72, -22.10)
Awakening time	-45.80 (-56.70, -34.90)	14.50 (3.82, 25.20)
Leaving time	-35.49 (-45.40, -25.60)	14.20 (4.43, 24.00)
Evening latency	2.18 (1.88, 2.48)	-0.70 (-0.96, -0.36)
Sleep duration	884 (870, 898)	-120 (-104, -136)
Sleep duration/ night duration	1.03 (1.01, 1.04)	-0.07 (-0.09, -0.05)

341

342



343

344 **Figure 1 Seasonal difference in effect of artificial light at night on sleep behaviour.**

345 Artificial light at night delayed the time at which great tits entered the nest box in February but

346 not in December. In panel A the raw average of when the birds entered the nest box is shown

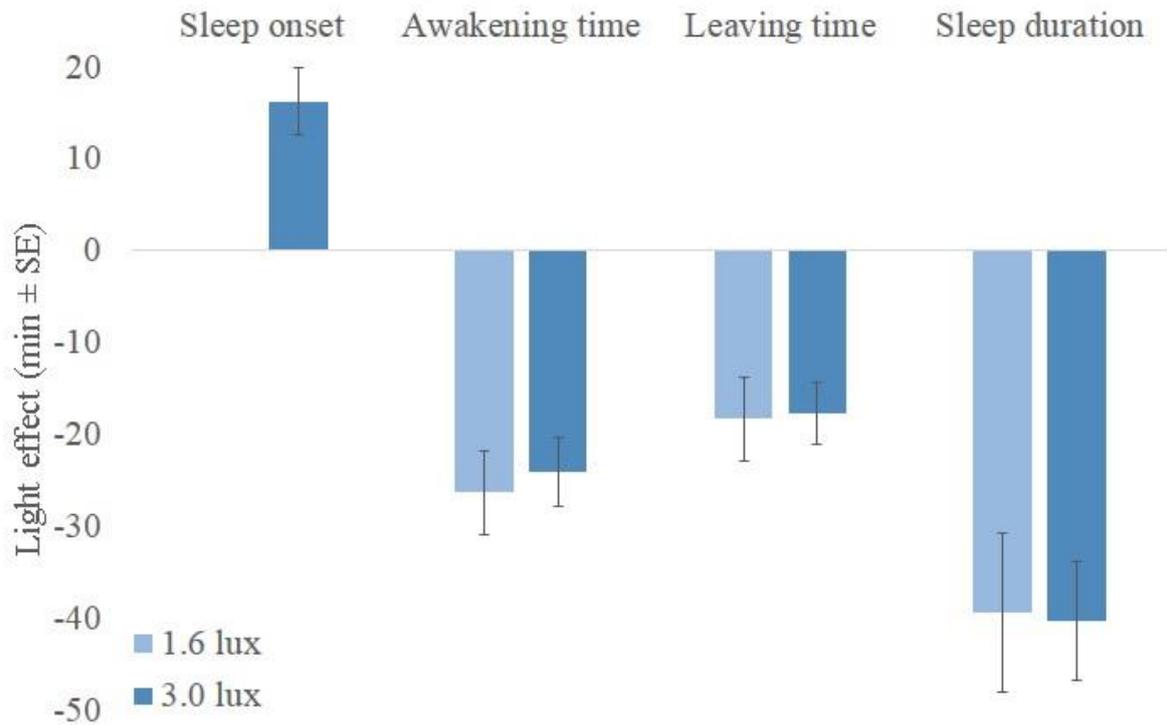
347 during December and February when birds slept in a “Dark” nest box or with a “Light” turned

348 on. Negative numbers indicate before sunset. In panel B estimates of effect sizes, the difference

349 caused by ALAN, and 95% confidence interval were obtained by using linear mixed models

350 with bird identity as random factor to correct for repeated measurements. When the 95% CI

351 does not cross zero (vertical line) it indicates that there was an effect/difference.



352

353 **Figure 2 Difference in effect of low and high light intensity of artificial light at night on**

354 **sleep behaviour.** Effect of artificial light at night on sleep behaviour did not differ between low

355 (1.6 lux) and high light intensity (3 lux) except for sleep onset. Significantly affected sleep

356 parameters ($p < 0.05$) are shown. Sleep onset, awakening time and leaving time were adjusted

357 for sunrise/sunset (line at 0 minutes). Estimates at light intensity of 1.6 lux were obtained from

358 Raap et al. (2015), $n = 18$). Estimates at light intensity of 3.0 lux were obtained from 26

359 individuals. Sleep onset was not significantly affected by a light intensity of 1.6 lux and

360 therefore no estimate is shown.

361

362

363

364 4. DISCUSSION

365 Previous studies have reported seasonal differences in sleep behaviour in free-living
366 great tits (Stuber et al. 2015a) and a dose-dependent response of daily activity rhythms to ALAN
367 in captive great tits (de Jong et al. 2016). While ALAN affected several aspects of sleep, we
368 found limited evidence for differences in response of birds between December and February or
369 between low (1.6 lux) and high (3 lux) light intensity.

370

371 *4.1 No Seasonal-Dependent Response of Sleep Behaviour to ALAN*

372 There were no clear differences in response of sleep behaviour to ALAN (using 3 lux)
373 between seasons, except for the time when the birds entered the nest box in the evening (entry
374 time). In December, ALAN did not affect entry time, while in February entry time was delayed
375 by about 10 minutes. There are several possible explanations. First, the effect on entry time
376 might be weaker in December when animals are already roosting relatively close to sunset,
377 while in February animals normally roost earlier and before sunset (Stuber et al. 2015a).
378 Second, it could be that, by chance, more light sensitive individuals were recorded in February
379 compared to December or that in February animals are more sensitive towards light and
380 therefore enter the nest box later. This seems, however, unlikely as we would then also expect
381 differences in other sleep parameters as well. Moreover, the same proportion of individuals did
382 not enter the nest box in February and December. Third, that a bird does not enter the nest box
383 might be more related to neophobia than a direct effect of ALAN.

384 We thus found little evidence for changes in effects of ALAN (using 3 lux) between
385 December and February, although consistent with earlier studies on blue and great tits
386 (Steinmeyer et al. 2010; Stuber et al. 2015a) sleep behaviour under natural conditions did differ.
387 Differences in response to ALAN between December and February may only become apparent

388 when using a lower light intensity than what we used in the current study. When animals are
389 more sensitive towards light in February, a lower light intensity may cause a disruption of sleep
390 behaviour, while in December it would not disrupt sleep. This remains to be tested.

391

392 *4.2 Light Intensity-Independence of Sleep Behaviour to ALAN*

393 We found little evidence for light intensity-dependent differences in sleep behaviour
394 using 1.6 and 3 lux. Given that de Jong et al. (2016) found that in captive great tits night-time
395 activity increased with higher light intensities, we had expected to find similar results with
396 respect to sleep behaviour in free-living great tits. Interestingly, higher light intensity caused
397 great tits to fall asleep later in the evening (sleep onset, in relation to sunset), while this effect
398 was not found at a lower light intensity.

399 However, we believe that the difference in effect on sleep onset between low and high
400 doses of ALAN is not very convincing for several reasons (as explained in paragraph 4.1). First,
401 we would also expect differences in other sleep parameters besides sleep onset, which we did
402 not find. Second, de Jong et al. (2016) showed that in the evening, activity offset did not differ
403 between 1.5 and 5.0 lux, while in the morning there was a difference of more than three hours
404 in start of activity. This seems to be in line with our current and earlier results (Raap et al. 2015)
405 where in February most effects are found in the morning. However, in the laboratory study of
406 de Jong et al. (2016) individuals had access to ad libitum food and effects of ALAN on activity
407 may be related to the fact that the birds could feed. In our study, birds only experienced
408 increased light inside the nest box and were unable to forage in the darkness outside of the nest
409 box. This difference may also affect the impact of light intensity on sleep behaviour/activity. It
410 is also important to mention that the light spectrum that we used and that used by de Jong et al.
411 (2016) may differ. We used regular white LED light and de Jong et al. (2016) used warm white

412 LED light. There is evidence that differences in spectral composition may also elicit a different
413 response to ALAN in great tits (e.g. Ouyang et al. 2015). We have no explanation for the
414 observed difference in effect on sleep onset between low and high doses of ALAN.

415 We found no difference in response of sleep behaviour between males and females using
416 a higher light intensity. However, under low light intensities there was a sex-dependent effect
417 on sleep proportion (the amount of time spent asleep divided by the night duration), in females
418 but not in males (Raap et al. 2015). The earlier reported effect was small but robust and sleep
419 behaviour in natural dark conditions differs between male and female great and blue tits
420 (Steinmeyer et al. 2010; Stuber et al. 2015a). In order to comprehensively study whether there
421 are sex-dependent effects, it is advisable to have a larger sample size in both males and females.

422 While we found no light intensity-dependent response, we found additional support that
423 ALAN disrupts sleep of wild birds (Raap et al. 2015, 2016c). Animals slept less as they woke
424 up almost three quarters of an hour earlier and left the nest box earlier. The proportion of time
425 that animals spent asleep during the night was reduced (about 5%), while the length of their
426 sleep bouts increased and sleep bout frequency decreased. Interestingly effects of ALAN were
427 often similar or larger than the difference in sleep behaviour under natural condition between
428 December and February.

429 Given that sleep also has an intensity dimension (Rattenborg et al. 2009), birds that spent
430 less time asleep may compensate for this loss by sleeping deeper. However, ALAN might also
431 reduce sleep intensity and the potential impact of ALAN on sleep might be even greater than
432 that reflected in loss of sleep. An earlier study of ours showed that under dark conditions loss
433 of sleep might also be compensated by sleeping more (Raap et al. 2016c). While it is outside
434 the scope of the current study, it will be of interest to examine the effect of ALAN not only on
435 sleep quantity but also on sleep intensity.

436

437 *4.3 Study limitations*

438 Although great tits are an ideal study species to study effects of ALAN in the wild, our
439 study does have its limitations. We are not trying to mimic natural conditions inside cavities (or
440 those exposed to light pollution), but aim for a more fundamental insight into the effects of
441 ALAN. Because it is possible to manipulate light conditions within a nest box, we used a cavity-
442 nesting bird as a model species. This enables experimentation and observations in a more
443 natural environment compared to that in laboratory. Our approach ensures effective exposure
444 to the light treatment which is crucial when exposing animals in the wild to ALAN (Raap et al.
445 2017b). Experimental manipulation of light conditions of free-living open-nesting birds as well
446 as obtaining a sufficient sample size of them is much more difficult. Nonetheless, the intensity
447 which we used (3 lux) could be experienced by animals outside of cavities as street lighting
448 often reaches levels which are much higher (15 lux; Gaston et al. 2013). During the great tit
449 breeding season mainly females sleep inside nest boxes. Males are therefore possibly exposed
450 to levels more similar to that experienced by open-nesting birds. Furthermore, the light
451 intensities which we used are in line with laboratory studies also those using blue and great tits
452 (de Jong et al. 2016; 2017). In such studies often intensities of 5 lux are used (and referred to
453 as dim light; e.g. Stenvers et al. 2016; Cissé et al. 2017).

454 Because our experimental design uses wild animals, we cannot observe individuals that
455 did not enter the nest box when it was lit from inside and these individuals might be more
456 sensitive to artificial light. However, that some individuals did not enter an artificially lit nest
457 box may also be due to neophobia. Unfortunately, we do not have sufficient data on the
458 “personality” (exploration test) of our individuals (David et al. 2015), which might have
459 otherwise provided more information on why some animals did not enter an artificially lit nest
460 box.

461 In our field experiment, we used a model system to create more insight into the effects
462 of ALAN, which is necessary because of the likely differences in behaviour due to the influence
463 of the environment (lab versus field; Calisi et al. 2009). Our experimental system, that uses
464 free-living animals, may represent a more ecologically realistic situation compared to the
465 laboratory, which is a simplified environment that fails to capture the complexity of natural
466 conditions, which is an important aspect in behavioural and sleep studies (Aulsebrook et al.
467 2016). Our model system, using a field-based experimental approach with free-living animals,
468 may therefore offer useful insights about possible seasonal-dependent and/or light intensity-
469 dependent effects of ALAN.

470

471 *4.4 Conclusion*

472 While we found little evidence for seasonal-dependent effects of ALAN on sleep
473 behaviour in the present study, such differences may become apparent at different light
474 intensities. Nonetheless, our study underlines earlier found effects of ALAN on sleep behaviour
475 of free-living animals (animals waking up earlier and sleeping less; Raap et al. 2015).
476 Differences in sleep behaviour caused by ALAN were often similar or sometimes larger than
477 seasonal differences in sleep suggesting that ALAN had a biologically relevant effect on sleep.

478 While we found little evidence for a light intensity-dependent effect on sleep behaviour,
479 such differences may become apparent using other, and a larger range of, light intensities. Given
480 that in a previous study ALAN appeared to have a very disruptive effect on sleep behaviour
481 during the nestling period (Raap et al. 2016c), a potential light intensity-dependent effect of
482 ALAN may become apparent in this period when using a low light intensity.

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492 TR JS RP ME conceived and designed the study. TR JS carried out the fieldwork. TR
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495

496 **DATA ACCESSIBILITY**

497 Sleep behaviour data Zenodo doi: 10.5281/zenodo.845332

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