Early life exposure to artificial light at night affects the physiological condition : an experimental study on the ecophysiology of free-living nestling songbirds

Early life exposure to artificial light at night affects the physiological condition: an experimental study on the ecophysiology of free-living nestling songbirds

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Light pollution or artificial light at night (ALAN) is increasingly recognised to be an important anthropogenic environmental pressure on wildlife, affecting animal behaviour and physiology. Early life experiences are extremely important for the development, physiological status and health of organisms, and as such, early exposure to artificial light may have detrimental consequences for organism fitness. We experimentally manipulated the light environment of free-living great tit nestlings (*Parus major*), an important model species in evolutionary and environmental research. Haptoglobin (Hp) and nitric oxide (NOx), as important indicators of immunity, health, and physiological condition, were quantified in nestlings at baseline (13 days after hatching) and after a two night exposure to ALAN. We found that ALAN increased Hp and decreased NOx. ALAN may increase stress and oxidative stress and reduce melatonin which could subsequently lead to increased Hp and decreased NOx. Haptoglobin is part of the immune response and mounting an immune response is costly in energy and resources and, trade-offs are likely to occur with other energetically demanding tasks, such as survival or reproduction. Acute inhibition of NOx may have a cascading effect as it also affects other physiological aspects and may negatively affect immunocompetence. The consequences of the observed effects on Hp and NOx remain to be examined. Our study provides experimental field evidence that ALAN affects nestlings' physiology during development and early life exposure to ALAN could therefore have long lasting effects throughout adulthood.

Keywords: artificial light at night; light pollution; physiology; development; early-life

Capsule: Artificial light at night affects the physiological condition of developing free-living songbirds

Introduction

Over the last 100 years, the night-time environment in much of the world has greatly been disrupted through the introduction of artificial light at night (ALAN), also known as light pollution. It is increasingly being recognised as a widespread and important anthropogenic environmental pressure on wildlife (Hölker et al. 2010). Recent studies (reviewed in Swaddle et al. 2015) have documented effects of ALAN on a wide variety of behavioural traits, such as reproduction, foraging, and migration. Several physiological effects have also been reported, including alterations in immune response, melatonin, and testosterone levels (reviewed in Swaddle et al. 2015) .

The immature circadian system may be particularly sensitive to circadian disruption through artificial light as experiences during early life profoundly affect the developing brain, influence adult behaviour, physiology, health, and disease (Fonken and Nelson 2016). ALAN can influence foraging behaviour of adult songbirds (Stracey et al. 2014) as well as begging behaviour of nestlings (Raap et al. 2016). These behavioural effects may have physiological consequences. Moreover, laboratory studies showed that ALAN can cause direct changes in physiology, including a decreased immune response to challenges, an increase in stress hormones, and a decrease in melatonin levels (reviewed in Fonken and Nelson 2016; Swaddle et al. 2015). Effects of ALAN on individual physiological condition and health state of developing birds in the wild are therefore likely (Fonken and Nelson 2016; Isaksson 2015; Salmon et al. 2016) but are unknown at present.

Studies to examine the effects of ALAN in the wild are important but rare. Nonetheless, experiments using laboratory animals and wild derived animals in captivity have provided useful insights into how artificial light may affect animal physiology (see Table 1 for some particularly relevant studies on wild derived animals). Even though studying ecophysiology in the wild remains challenging, examining the effects of ALAN on developing animals in

ecologically realistic situations is urgently needed because the laboratory is a simplified environment that fails to capture the complexity of natural conditions. To the best of our knowledge, experimental studies on effects of ALAN on the physiology of developing animals in the wild are completely lacking. Altered physiology together with demands of limited resources and harsh environmental conditions may however seriously impact survival outside of the laboratory. Studies in the wild have often compared urban versus non-urban populations and focused on adult individuals (see Table 2). In these types of studies effects of light pollution may be confounded by other major urban stressors such as noise (Swaddle et al. 2015) and chemical pollution (Isaksson 2015). Experimental studies in the wild that manipulate light conditions but keep all other influencing factors as stable as possible (e.g. Ouyang et al. 2015) are therefore necessary to fully comprehend the effects of ALAN on developing animals.

ALAN may directly affect an individual's physiological condition, e.g. increased oxidative stress (Navara and Nelson 2007), and indirectly as it decreases melatonin (Swaddle et al. 2015) which may lead to a cascade of other physiological effects (Tan et al. 2010). Melatonin has multiple functions and is involved in regulation of oxidative stress and immunological modulation (Tan et al. 2010). We therefore hypothesized that artificial light at night would affect the physiological condition of developing animals.

In this study, we experimentally investigated whether artificial light at night affected haptoglobin (Hp) and nitric oxide (NOx) in free-living developing great tits (*Parus major*). Haptoglobin is an acute phase protein that plays an important role in inflammation, infection and trauma. It is part of the non-specific immune response but also acts as an antioxidant (reviewed in Matson et al. 2012). Plasma NOx is an easily measurable multifunctional signalling molecule involved in inflammatory processes but uncontrolled production may lead to cell damage and death (reviewed in Sild and Horak 2009). Changes in haptoglobin and nitric

93 oxide are especially interesting as they provide useful information on changes in physiological
94 condition, health state and innate immunity (Matson et al. 2012; Sild and Horak 2009).

95 In the present study, we experimentally exposed wild great tit nestlings to two nights of
96 artificial light (3.0 lux) and compared these to nestlings which were not exposed to ALAN. We
97 then assessed individual changes in Hp and NOx to determine the effects of ALAN on the
98 physiological condition of developing animals.

Method

Study area and general procedures

Our study was performed during the 2015 breeding season (between 8 and 25 May) in a resident suburban nest box population of great tits in the surroundings of Wilrijk, Belgium (51°9'44''N, 4°24'15''E). Nest boxes were put up in 1997 and ever since this free-living population is continuously monitored (see e.g. Rivera-Gutierrez et al. 2010; Rivera-Gutierrez et al. 2012; Van Duyse et al. 2005; Vermeulen et al. 2016b). During winter and breeding seasons, great tits are caught inside the nest boxes after which they are ringed. This study was approved by the ethical committee of the University of Antwerp (ID number 2014-45) and performed in accordance with Belgian and Flemish laws.

Experimental design

While field studies on physiology often rely on single point measurements and experiments on free-living animals are often unfeasible (van de Crommenacker et al. 2010), we used an experimental field study with repeated measurements. We looked at individual changes in physiology of wild animals caused by ALAN as this takes into account that physiological condition likely differs between individual nestlings (e.g. Vermeulen et al. 2015). We randomly assigned 32 nests to one of the two treatment groups: a control (dark) and a light treated group, which was exposed to two consecutive nights of light (day 13 and 14). We obtained a blood sample ($\leq$ 150 μL) from all nestlings of a nest when they were 13 days old, to determine their baseline Hp and NOx levels and subsequently weighed them (digital balance; Kern TCB 200-1). To quantify changes in physiological condition, this procedure was repeated after two nights when the nestlings were 15 days old. Taking repeated measurements is crucial for understanding physiological responses (van de Crommenacker et al. 2010) and eliminates many

potential confounding variables (Ruxton and Colegrave 2010). However, repeated blood sampling of small songbird nestlings can only be done using small blood samples. We determined Hp and NOx as these assays are especially suitable for small birds (lowest body mass of a nestling in our study: 10.4 g). These assays require a limited plasma volume and can therefore be done on a within individual basis using songbird nestlings (Matson et al. 2012; Sild and Horak 2009).

In the light group, nestlings were exposed to two consecutive nights of light. Under the nest box roof lid of each nest box we placed a small LED light (15 mm x 5 mm, taken from a RANEX 6000.217 LED headlight, Gilze, Netherlands). Lights were standardized to produce 3 lux of broad spectrum white light on the nest box bottom (ISO-Tech ILM 1335 light meter; Corby, UK). Light systems were installed during the morning between 08:00 and 12:00. With the use of a timer, lights were automatically turned on at 19:00 in the evening (about two hours before sunset) and turned off at 07:00 the following morning (about one hour after sunrise). This light system has been successfully used by us in previous studies on the effects of ALAN on sleep (Raap et al. 2015; Raap et al. 2016). There is no warming effect of the lights inside the nest boxes because of the high energy efficiency of the small LED light. The control group had lights installed inside the nest box but these were always turned off, leaving these nests in a natural dark situation. Both groups were otherwise treated the same. Lights located in the light treated nest boxes could not be observed from or influence the control nests.

We used a paired design in which nests from the control and light group with a similar nestling hatching date and brood size (about seven nestlings) were paired. These pairs were always sampled on the same morning immediately after each other (between 8:00 and 12:00). Order of sampling was kept the same within a pair but alternated between pairs. This ensured that there would not be a bias in sampling time between nestlings from the light and control

147 group. In total, we obtained blood samples of 115 nestlings in the control group (16 nests) and
148 112 in the light group (16 nests).

149

150 *Quantification of haptoglobin and nitric oxide*

151 Following earlier research on great tits (Vermeulen et al. 2015; Vermeulen et al. 2016a;
152 Vermeulen et al. 2016b) we determined nestling sex and quantified haptoglobin and nitric
153 oxide. In short, nestling sex was determined with the use of PCR (Griffiths et al. 1998). We
154 quantified plasma haptoglobin concentrations (µg/mL) using the manufacturer's instructions
155 provided with the commercially available colorimetric assay (PHASE Haptoglobin assay,
156 Tridelta Development Ltd). We used the spectrophotometric assay based on the reduction of
157 nitrate to nitrite by copper-coated cadmium granules to quantify nitric oxide concentrations
158 (µmol/L) (Sild and Horak 2009). Due to plasma limitations, sample sizes vary between
159 physiological parameters and sampling day (Hp control d13 $N = 94$, d15 $N = 109$, light d13 N
160 $= 93$, d15 $N = 100$; NOx control d13 $N = 106$, d15 $N = 115$, light d13 $N = 102$, d15 $N = 109$).

161

162 *Statistical analyses*

163 We used R 3.1.2 (R Core Team 2014) for all statistical analyses. For both Hp and NOx
164 we performed a linear mixed effect analysis (LMM) using the lme4 package (Bates et al. 2014).
165 As fixed effects, "treatment" (control, light), "day" (13, 15), "sex", "brood size", "body mass"
166 as well as the two-way interactions and the three-way interaction between "treatment", "sex"
167 and "day" were used. As random effect we used "bird identity" which was nested in "nest
168 identity" which was nested in "pair" (bird identity:nest identity:pair) to take the experimental
169 design into account. Both Hp and NOx were square root transformed to meet model
170 assumptions.

171 P-values obtained by a stepwise backward regression are given in results and Tukey
172 HSD tests were used for post-hoc analyses, using the lmerTest package (Kuznetsova et al.
173 2014). We analysed the relationship between Hp and NOx on day 13 and changes in both
174 measures (difference between day 13 and day 15) using a Spearman rank correlation test.

Results and Discussion

Artificial light at night increased Hp and decreased NOx

Using a sophisticated field experiment, in which our within-individual and paired design is likely to eliminate many confounding variables, we find that early life exposure to two nights of artificial light at night (ALAN) was sufficient to alter nestlings' physiological condition (see below). No differences were found between male and female nestlings (haptoglobin (Hp): F = 0.825, P = 0.364; nitric oxide (NOx): F = 0.077, P = 0.7816). Sex, brood size and body mass did not affect Hp or NOx.

Nestling Hp was increased and NOx was decreased by a two night exposure to artificial light. ALAN had a significant effect on Hp ("treatment x day interaction": F = 6.138, P = 0.014). Nestlings in the control group showed no difference in Hp between day 13 and day 15 (t = -1.04, P = 0.30), while light exposed nestlings had an increased Hp concentration (t = -2.44, P = 0.01; Figure 1). Light at night also had a significant effect on NOx ("treatment x day interaction": F = 3.901, P = 0.049). Nestlings in the control group showed no difference in NOx between day 13 and day 15 (t = 0.07, P = 0.948), while NOx concentrations decreased in nestlings of the light group (t = 2.70, P = 0.007; Figure 1). Interestingly, while ALAN affected both Hp and NOx, it did so in opposite directions and effects were uncorrelated (r = 0.038, P = 0.734). On day 13 (natural dark situation), Hp and NOx were also uncorrelated (r = -0.116, P = 0.119).

Increased stress may lead to increased Hp and decreased NOx

Wild songbirds that are exposed to ALAN may suffer from acute or chronic stress as shown by elevated levels of corticosterone (Ouyang et al. 2015; Russ et al. 2015). Stress is known to stimulate haptoglobin production (reviewed in Downs and Stewart 2014).

Furthermore, there is evidence that NOx production is decreased by stress hormones (Vajdovich 2008). Acute stress from artificial light at night may thus increase haptoglobin while decreasing nitric oxide.

Increased oxidative stress may lead to increased Hp and decreased NOx

Light pollution can have both direct and indirect adverse effects on oxidative status and antioxidant defence (Navara and Nelson 2007). Haptoglobin has, besides anti-inflammatory, also antioxidative properties. Plasma Hp might therefore have been elevated to counteract increases in oxidative damage compounds (Jelena et al. 2013) as part of a compensatory mechanism to maintain the oxidative balance (Costantini and Verhulst 2009).

NOx concentrations may have been reduced by the increased oxidative stress (Price et al. 2000) caused by ALAN. The increase of Hp which is also considered an antioxidant may have contributed to the decrease of NOx which is also a potent oxidant (Schaer et al. 2013). However, effects on Hp and NOx were uncorrelated and we therefore have little evidence of a direct effect of Hp on NOx.

Decreased melatonin may lead to decreased NOx and perhaps increased Hp

Artificial light at night may decrease melatonin (see Tables 1 and 2) and subsequently decrease NOx and increase Hp. Plasma NOx may have been reduced through the reducing effect of ALAN on melatonin as melatonin normally stimulates NOx production (Tan et al. 2010). The reduction of melatonin may also have a cascading effect on haptoglobin. Melatonin has immunomodulation and anti-inflammatory activities (Tan et al. 2010) and artificial

reduction in melatonin levels may thus affect haptoglobin concentration. However, this pathway remains to be examined.

Potential negative effect of increased haptoglobin and decreased nitric oxide

The observed changes in haptoglobin and nitric oxide caused by ALAN may have a negative effect on nestling survival and fitness. Oxidative stress is a potentially very important mediator of life-history trade-offs. Raising antioxidant defences such as haptoglobin may be costly and could affect an individual's fitness and long term survival (reviewed in Monaghan et al. 2009). Moreover, haptoglobin is part of the immune response and mounting an immune response is costly in energy and resources and trade-offs are likely to occur with other energetically demanding tasks, such as survival or reproduction (Downs and Stewart 2014).

Acute inhibition of NOx may have a cascading effect as it also affects other physiological aspects, e.g. testosterone (reviewed in Vargas et al. 2007). Although Hp was increased, the decrease in NOx may indicate a negative effect of ALAN on immunocompetence of nestlings (Bichet et al. 2012; Vajdovich 2008). This would be in line with earlier laboratory studies on adult animals that showed a negative effect of ALAN on immune responses (e.g. Bedrosian et al. 2011).

While here we present a study on the effects of a short term light treatment on the physiology of animals, behavioural studies showed either no habituation of animals to ALAN or even larger effects as a consequence of long term exposure to light at night (de Jong et al. 2016; Yorzinski et al. 2015). Additional studies are needed to evaluate whether the effects that we found are enhanced or ameliorated over longer treatments or if additional effects would appear.

Conclusions and perspectives

We found that a short term exposure to ALAN had a significant effect on the physiological condition of wild developing nestlings. This could also be relevant for other animals that are exposed to similar or higher intensities of light at night. Experiences during early life may profoundly affect an individual's fitness (Fonken and Nelson 2016; Salmon et al. 2016). However, it remains to be examined what the long term consequences are of the ALAN-induced increase in Hp and decrease in NOx. Yet, the multitude of behavioural and physiological aspects affected by ALAN (reviewed in Swaddle et al. 2015), including an altered physiological condition, suggests early exposure to ALAN in nestlings developing in urban or otherwise light-exposed areas may be detrimental (see also Salmon et al. 2016) and carry over later in life (i.e. reduced fitness).

While in this study we measured only a limited number of physiological parameters, our experimental design shows great potential for further research. For example it could be used to elucidate physiological and behavioural effects (see also Raap et al. 2015; Raap et al. 2016) as well as short- and long term fitness effects of artificial light at night in free-living birds. It would be useful to study the (possible) interrelationships between stress hormones, melatonin and Hp and NOx in future light manipulation studies. Our experimental design may also prove useful in future research on light pollution necessary to determine effects at different light intensities, wavelengths and duration of artificial light at night (Gaston et al. 2013).

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405 **Table 1** Examples of particularly relevant experiments on the physiological effects of artificial light at
406 night on wild derived animals in captivity. All studies used adult animals.

Species	Physiological measurement	Light intensity used (lux)	Main results	References
Siberian hamsters *Phodopus sungorus*	Delayed-type hypersensitivity Blood plasma bactericidal capacity	5	*ALAN supressed immune responses* • Delayed-type hypersensitivity response was reduced • Blood plasma bactericidal capacity was reduced	Bedrosian et al. (2011)
Indian weaver birds *Ploceus philippinus*	Melatonin	Different intensities between 0.1-100	*ALAN supressed melatonin levels*	Singh et al. (2012)
Western scrub-jay *Aphelocoma californica*	Luteinizing hormone Testosterone Estradiol Melatonin	3.2	*ALAN did not stimulate the reproductive axis* • Luteinizing hormone was reduced in males, but not in females • Testosterone was reduced in females but not in males • ALAN increased melatonin • ALAN disrupted the correlation between testosterone and estradiol	Schoech et al. (2013)
Blackbird *Turdus merula*	Testosterone	0.3	*ALAN advanced reproductive physiology* • Earlier increase in testosterone secretion	Dominoni et al. (2013a)
Blackbird *Turdus merula*	Melatonin	0.3	*ALAN decreased melatonin secretion*	Dominoni et al. (2013c)
Blackbird *Turdus merula*	Testosterone	0.3	*Long term exposure to ALAN affects the reproductive system* • Long term exposure to ALAN caused testosterone to remain at baseline (non-reproductive state)	Dominoni et al. (2013b)

407

408

Table 2 Review of existing literature on the physiological effects of artificial light at night in free-living animals. Almost all previous studies (except for Salmon et al. 2016) used adult animals while the current one used developing animals (nestling birds).

Species	Physiological measurement	Study type	Main results	References
Tree sparrows *Passer montanus*	Luteinizing hormone Testosterone Estradiol	Observational Urban versus rural + indoor light experiment	*Reproductive hormone rhythm differed between urban and rural birds* • Urban birds had lower peak luteinizing hormone, testosterone and estradiol • Urban birds secreted luteinizing hormone earlier in the season	Zhang et al. (2014)
Abert's Towhees *Melozone aberti*	Luteinizing hormone Testosterone Lytic and agglutination capacity	Observational Urban versus desert	*Urban birds had advanced seasonal reproductive development* • Urban birds secreted luteinizing hormone earlier in the season • No earlier increase in testosterone secretion • Urban and desert birds had similar lytic and agglutination capacity	Davies et al. (2015)
Great tit *Parus major*	Corticosterone	Experimental field study	*ALAN increased stress* • White light increased corticosterone • Individuals near red light had increased corticosterone • No effect of green light on corticosterone	Ouyang et al. (2015)
Tammar wallaby *Macropus eugenii*	Melatonin	Observational Urbanized versus natural	*ALAN delayed reproductive activity* • Melatonin decreased	Robert et al. (2015)
Blackbird *Turdus merula*	Testosterone Estrone Corticosterone	Observational Rural-urban gradient	*ALAN increased stress* • No effect on testosterone. • Decrease of estrone • Increase of corticosterone	Russ et al. (2015)
Great tit *Parus major*	Telomere length	Experimental field study Rural and urban nests	*Urban environment shortens telomere length* • ALAN, noise and or air pollution may have caused shortening of telomeres	Salmon et al. (2016)

Great tit *Parus major*	Haptoglobin Nitric Oxide	Experimental field study	*ALAN affects physiological condition* • ALAN increased haptoglobin • Alan decreased nitric oxide	Current study

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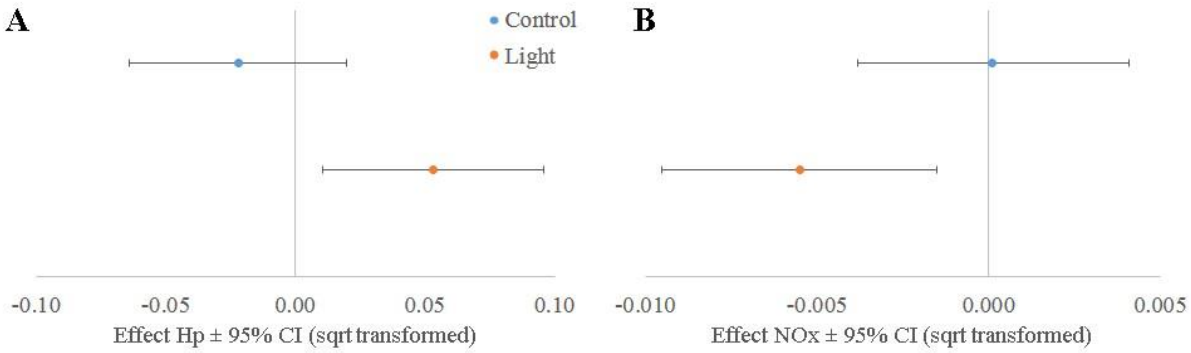
414 **Figure Caption**



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416 **Figure 1** Artificial light at night increased nestling plasma haptoglobin (A) and decreased nitric
417 oxide (B). Estimates (square root transformed ± 95% CI) were obtained from LMMs with
418 individual nested in nest nested in pair as random factor (bird identity:nest identity:pair).
419 Differences in haptoglobin and nitric oxide between day 13 and day 15 were significant in the
420 light group: $P \leq 0.01$(*).

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