Effects of artificial light at night on the behaviour and physiology of free-living songbirds

Proefschrift voorgelegd tot het behalen van de graad van doctor in de wetenschappen aan de Universiteit Antwerpen te verdedigen door

Thomas Raap

Promotoren
prof. dr. Marcel Eens
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Faculteit Wetenschappen
Departement Biologie
Antwerpen 2018

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Cover illustrations: by Hilda Raap

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Effecten van kunstmatig licht tijdens de nacht
op het gedrag en fysiologie van in het wild levende zangvogels

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When the light begins to change

I sometimes feel a little strange

A little anxious when it's dark

Steve Harris

Iron Maiden - Fear of the Dark

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Summary

Urbanization is a serious threat to biodiversity affecting various biological processes. It has a wide range of impacts including habitat destruction and degradation, as well as altered temperatures and exposure to chemical, noise and light pollution. Organisms in urban areas are often exposed simultaneously to a multitude of these anthropogenic pressures which may further exacerbate effects of a single stressor. Compared to other stressors, light pollution has until recently received relatively little attention. Artificial light at night (ALAN) has provided substantial benefits to humankind, such as extending the time that can be used for work. The rapid increase of ALAN, expansion of lit areas and increased light intensity over the last 100 years have also resulted in a worldwide loss of darkness with largely unknown consequences for biodiversity, ecosystems, and ecological and evolutionary processes. Light-dark cycles have driven the development of small and large scale biological phenomena, including metabolic and physiological pathways, the behaviour of individuals, geographical patterns of adaptation and species richness, as well as ecosystem cycles. The alteration of natural light and dark cycles is likely to be problematic for many species. ALAN disrupts circadian rhythms such as sleep and can lead to a multitude of direct and indirect physiological consequences. However, the biological consequences of ALAN are primarily studied under laboratory settings. Comparable research on free-living animals is urgently needed as environmental conditions outside of the laboratory may affect behaviour and physiology.

The general aim of this research was to gain fundamental insights into the behavioural and physiological effects of ALAN in free-living animals. Great tits, and to a lesser extent blue tits, were used as model species. This PhD is primarily based on experimental work in which we illuminated the inside of nest boxes in which these cavity-nesting birds roost and breed using a newly developed LED lighting system. For our experiments we investigated potential changes in behaviour and physiology from a dark night compared to when animals were exposed to ALAN for one or two nights, depending on the experiment.

From these experiments it became clear that ALAN disrupts sleep behaviour of wild animals and that these effects are more pronounced during the breeding season. Sleep loss could impact individual health and performance which during the breeding season could subsequently affect developing nestlings. Chronic sleep disruption by ALAN may thus have severe consequences. Interestingly, while great and blue tits are ecologically closely related species, the sleep behaviour of great tits was more disrupted by ALAN than that of blue tits.

This indicates that results obtained from a single species are not easily extrapolated to other species. The extent of sleep disruption in great tits was not personality-dependent; slow explorers were equally disrupted in their sleep behaviour when exposed to ALAN than fast explorers. However, to what extent other behavioural and physiological effects of ALAN are personality-dependent is unknown. Personality is heritable and associated with variation in fitness thus it is important to study whether light pollution may select for a certain personality type as it may affect population dynamics.

We also investigated the effect of ALAN on nestling physiology as early life experiences may have long lasting effects. We found that after only two nights of ALAN exposure nestlings did no longer gain any body mass and haptoglobin and nitric oxide, two important measures of physiological condition and immunity, were also affected. However, oxidative status, which is related to life-history trade-offs, was unaffected. To what extent this physiological disruption may affect survival needs to be examined but an inappropriate immune response may be costly and detrimental. Furthermore, a lower body mass may reduce survival, and sleep disruption by ALAN may further exacerbate physiological effects.

In our behavioural experiments we found that nestlings exposed to ALAN started begging for food at night implying that they also suffered from disrupted sleep. However, in contrast to adults it is difficult to quantify sleep behaviour in nestlings. Potentially sleep loss can be physiologically quantified using oxalate, which is a recently discovered cross-species marker of sleep debt, and can be determined in small blood samples. We found that ALAN increased oxalate, but only in male nestlings, contrary to a reduction in oxalate in sleep-deprived humans and rats. Future studies should determine whether oxalate is a reliable marker of sleep loss in developing great tits but our work may provide a foundation for future work with free-living animals.

In a different type of experiment we exposed the entrance of nest boxes to ALAN and found that nest boxes shield great tits from the direct effects of artificial light which has implication for field studies using free-living animals. Furthermore, in great tits (and other cavity-nesting songbirds) the usage of nest boxes/ cavities differs between sexes and season. Exposure to light pollution is therefore highly variable in cavity-nesting species. This might also explain why we found that great tit nestlings in nest boxes exposed to high levels of ambient light pollution did not have any different levels of haptoglobin and nitric oxide than those at low levels of light pollution. In that study we examined whether nestling physiology was

associated with light and/or noise pollution as they often co-occur. Noise but not light pollution, was associated with elevated levels of haptoglobin. Our results suggest that the urban environment, through noise exposure, may entail important physiological costs for developing organisms. However, effects of light pollution, such as disrupted sleep behaviour of the adults, may also play an important role but requires further investigation.

While much remains to be examined, we found that short-term exposure to ALAN may have severe behavioural and physiological consequences. Sleep disruption by ALAN may eventually be detrimental for an individual's health. Furthermore, the physiological effects during development may negatively affect short and long-term survival. In this thesis we have also discussed the limitations of our experimental light system such as the short-term exposure that we used. However, our system is highly adaptable and offers many opportunities to examine how ALAN affects free-living animals. We can therefore use it to expand on our current results, for example by using longer periods of exposure to ALAN. Furthermore, we can examine the potential of new lighting strategies to mitigate environmental effects, such as part-night lighting where lights are turned off during part of the night. Taken together, given that ALAN is steadily increasing even with the use of energy efficient LED lights, it is now vitally important to experimentally examine how long-term exposure to ALAN impacts behaviour and physiology and ultimately reproduction and survival and how these effects can be mitigated.

Samenvatting (Dutch Summary)

Verstedelijking vormt een serieuze bedreiging voor de biodiversiteit en beïnvloedt verschillende biologische processen. Het heeft een breed scala aan effecten, zoals habitat vernietiging, gewijzigde temperaturen en blootstelling aan chemische-, geluids- en lichtvervuiling. Organismen in stedelijke gebieden zijn vaak tegelijkertijd blootgesteld aan een groot aantal van deze antropogene stressoren wat de effecten van een enkele stressor verder kan vergroten. In vergelijking met andere stressoren heeft lichtvervuiling maar relatief weinig aandacht gekregen. Kunstmatig licht tijdens de nacht (Engelse afkorting ALAN; artificial light at night) heeft substantiële voordelen voor de mensheid opgeleverd, bijvoorbeeld dat er na zonsondergang gewerkt kan worden. De snelle toename van ALAN, de uitbreiding van verlichte gebieden en de toename van lichtintensiteit over de laatste 100 jaar hebben een wereldwijd verlies van de duisternis veroorzaakt met grotendeels onbekende effecten voor biodiversiteit, ecosystemen en ecologische en evolutionaire processen als gevolg. Licht en donker cycli hebben de ontwikkeling van biologische fenomenen op kleine en grote schaal aangestuurd, inclusief metabolische en fysiologische pathways, geografische patronen van adaptatie en soortenrijkdom, alsook ecosysteem cycli. De verandering van natuurlijke licht-donker cycli is waarschijnlijk problematisch voor veel soorten. ALAN verstoort circadiaanse ritmes zoals slaap en leidt tot een groot aantal directe en indirecte fysiologische gevolgen. De biologische gevolgen van ALAN worden voornamelijk in het laboratorium onderzocht. Vergelijkbaar onderzoek op in het wild levende dieren is dringend nodig omdat omstandigheden buiten het laboratorium gedrag en fysiologie kunnen beïnvloeden.

Het algemene doel van dit onderzoek was om fundamentele inzichten te verkrijgen in de gedragsmatige en fysiologische effecten van ALAN bij in het wild levende dieren. Koolmezen en in mindere mate pimpelmezen werden gebruikt als modelsoorten. Dit doctoraat is voornamelijk gebaseerd op experimenteel werk waarin we de binnenkant van de nestkasten, waarin deze holenbroeders slapen en broeden, hebben verlicht met behulp van ons nieuw ontwikkelde LED-verlichtingssysteem. Voor onze experimenten onderzochten we de veranderingen in gedrag en fysiologie van een donkere nacht in vergelijking met wanneer de dieren werden blootgesteld aan ALAN voor één of twee nachten, afhankelijk van het experiment.

Uit deze experimenten werd duidelijk dat ALAN het slaapgedrag van in het wild levende dieren verstoort en dat deze effecten sterker zijn tijdens het broedseizoen.

Slaapgebrek kan invloed hebben op de individuele gezondheid en prestaties welke tijdens het broedseizoen vervolgens de ontwikkeling van nestvogels kunnen beïnvloeden. Een chronische verstoring van de slaap door ALAN kan dus grote gevolgen hebben maar dit moet nog onderzocht worden. Interessant is, alhoewel kool- en pimpelmezen nauw aan elkaar verwante soorten zijn, dat het slaapgedrag van koolmezen meer verstoord was door ALAN dan dat van pimpelmezen. Dit wijst erop dat de resultaten die bekomen zijn bij een enkele soort niet gemakkelijk te extrapoleren zijn naar andere soorten. De mate van slaapverstoring bij koolmezen was ook persoonlijkheidsafhankelijk en stoutmoedige individuen waren meer verstoord in hun slaapgedrag door ALAN dan schuwe individuen. Persoonlijkheid is erfelijk en geassocieerd met variatie in fitness, daardoor kan lichtvervuiling zorgen voor selectie van bepaalde persoonlijkheid types en de populatiedynamiek beïnvloeden. In welke mate andere gedrags-en fysiologische effecten van ALAN persoonlijkheidsafhankelijk zijn, moet nog onderzocht worden.

We onderzochten ook de effecten van ALAN op de fysiologie van koolmeesjongen aangezien blootstelling tijdens de ontwikkeling schadelijk kan zijn. Na slechts twee nachten met blootstelling aan ALAN kwamen de jongen niet meer aan in gewicht. Haptoglobine en stikstofoxide, twee belangrijke maten van fysiologische conditie en immuniteit, werden ook beïnvloed. Oxidatieve status, wat gerelateerd is aan “life-history tradeoffs” veranderde niet. In welke mate deze fysiologische verstoring overleving kan beïnvloeden moet onderzocht worden maar een incorrecte immuunrespons kan kostbaar en schadelijk zijn. Bovendien kan een lager lichaamsgewicht overleving beïnvloeden en slaapverstoring door ALAN kan deze fysiologische effecten nog verder versterken. In onze gedragsexperimenten vonden we dat de jongen die blootgesteld werden aan ALAN tijdens de nacht begonnen te bedelen om voedsel wat er op wijst dat ook zij last hadden van een verstoorde slaap. In tegenstelling tot bij volwassenen kunnen we bij jongen het slaapgedrag moeilijk kwantificeren. Mogelijk kan dit wel fysiologisch gekwantificeerd worden met oxaalzuur wat een recent ontdekte merker van slaapgebrek is in meerdere soorten en wat bepaald kan worden in kleine volumes van bloed. We vonden dat ALAN oxaalzuur verhoogde maar alleen in mannelijke kuikens, in tegenstelling tot de vermindering van oxaalzuur in mensen en ratten met slaapgebrek. Of oxaalzuur een betrouwbare merker van slaapgebrek is in jonge koolmezen moet verder onderzocht worden maar ons werk kan een basis vormen voor toekomstig werk met in het wild levende dieren.

In een ander soort experiment, waarin we de nestkast opening belichtten, ontdekten we dat nestkasten de koolmezen kunnen beschermen tegen de directe effecten van kunstlicht wat gevolgen heeft voor veldstudies met in het wild levende dieren. Verder verschilt het gebruik van nestkasten/ holtes bij koolmezen (en andere holtebroeders) tussen geslachten en seizoenen. Blootstelling aan lichtvervuiling is daarom zeer variabel bij holtebroeders. Dit zou ook kunnen verklaren waarom de koolmezen in nestkasten die werden blootgesteld aan hoge niveaus van lichtvervuiling, geen verschillende concentraties van haptoglobine en stikstofoxide hadden vergeleken met die in nestkasten met lage niveaus van lichtvervuiling. In deze studie onderzochten we of de fysiologie van de jongen gerelateerd was aan licht en/of geluidsvervuiling omdat deze vaak tegelijk voorkomen. Geluidsvervuiling maar niet lichtvervuiling was wel geassocieerd met hogere haptoglobine concentraties. Onze resultaten suggereren dat de stedelijke omgeving door geluidsvervuiling belangrijke fysiologische gevolgen kan hebben voor de ontwikkelende organismes. Ook effecten van lichtvervuiling, zoals de verstoring van slaapgedrag in volwassen individuen, kunnen een belangrijke rol kunnen spelen maar dit moet verder onderzocht worden.

Hoewel er nog veel onderzoek noodzakelijk is, vonden we dat een korte blootstelling aan ALAN mogelijk grote gedragsmatige en fysiologisch gevolgen kan hebben. Slaapverstoring door ALAN kan mogelijk schadelijk zijn voor de gezondheid. Bovendien kunnen de fysiologische effecten tijdens de ontwikkeling negatief zijn voor korte en lange termijn overleving. In deze thesis bespreken we ook de beperkingen van ons experimenteel systeem zoals de korte blootstellingstijd die we gebruikten. Ons systeem is echter zeer flexibel en biedt veel mogelijkheden om te onderzoeken hoe ALAN in het wild levende dieren beïnvloedt. We kunnen het daarom gebruiken om onze huidige resultaten uit te breiden, zoals het gebruik van langere blootstelling aan ALAN. Bovendien kunnen we de effectiviteit onderzoeken van nieuwe verlichtingsstrategieën om de effecten op de omgeving te verminderen, zoals verlichting welke tijdens een gedeelte van de nacht is uitgeschakeld. Aangezien lichtvervuiling gestaag toeneemt, zelfs met het gebruik van energie efficiënte LED verlichting, is het nu van vitaal belang om te onderzoeken wat de gevolgen zijn van lange termijn blootstelling aan ALAN op het gedrag en fysiologie en uiteindelijk op de voortplanting en overleving, alsook hoe deze effecten gemitigeerd kunnen worden.

Chapter 1

General introduction

Urbanization

We are currently living in the "anthropocene" (Crutzen 2006) as the rapid worldwide urbanization has led to a human-dominated planet (Vitousek et al. 1997). Over half of the global population already lives in cities, with the developed world being about 80% urban. This percentage is expected to further increase as more than 2 billion people will move to cities in the coming decades (UN-Habitat 2010). Urbanization poses a serious threat to biodiversity affecting various biological processes as it has a wide range of impacts including habitat destruction and degradation, as well as altered temperatures and exposure to chemical, light and noise pollution (Grimm et al. 2008; Van Dyck 2012). For instance, anthropogenic noise alters physiology (increased stress, decreased immune response), behaviour (communication, foraging, sleep) and affects animal abundance through its effects on survival and reproductive success (Cox et al. 2018; Francis and Barber 2013). Organisms are often exposed to a multitude of anthropogenic pressures which may further exacerbate the effects of a single factor (Halfwerk and Slabbekoorn 2015). Research that investigates multiple stressors such as light and noise pollution simultaneously is rare (Swaddle et al., 2015; but see McMahon et al., 2017).

Losing the darkness

Anthropogenic environments expose organisms to novel stressors that have not been experienced over the course of their evolutionary history, such as light pollution. The rapid increase of artificial light at night (ALAN), expansion of lit areas and increased light intensity over the last 100 years, results in a worldwide loss of darkness (Cinzano et al. 2001; Falchi et al. 2016) with largely unknown consequences for biodiversity, ecosystems, and ecological and evolutionary processes (Hölker et al. 2010b; Rich and Longcore 2005). The recent change to solid-state lighting such as LED has further increased the area of earth which is artificial lit (Kyba et al. 2017a). The alteration of natural levels of night lighting is caused by anthropogenic sources of light such as street-, advertising-, architectural-, security-, domestic- and vehicle lighting. Street lighting can easily be more than a 1000 times as bright (10-40 lux; Gaston et al. 2017) than light at night produced by natural sources such as moon light (about 0.05-0.1 lux; Kyba et al. 2017b; see also Figure 1 for natural and anthropogenic levels of light at night). Life on earth has evolved under natural light-dark cycles and only recently is the light intensity at night much higher due to this light pollution than it would be under natural "dark conditions".

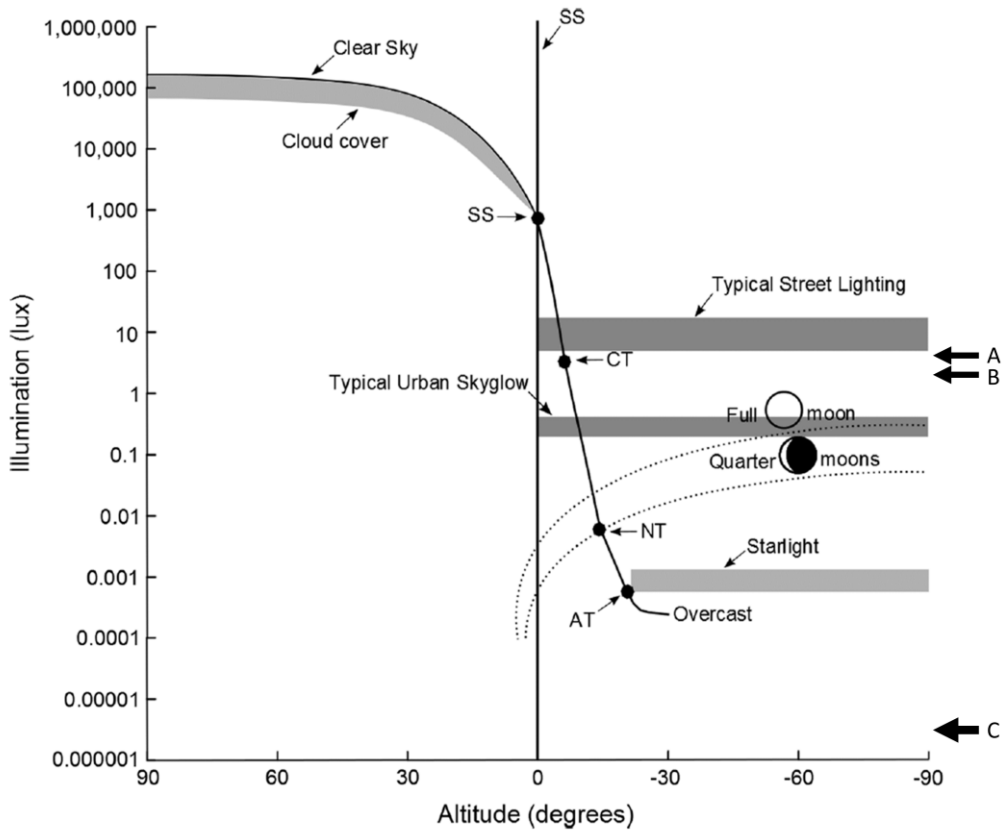

Figure 1: Typical levels of artificial and natural light at night. From left to right the change in illumination with positive solar and negative lunar altitude above the horizon. Typical street lighting is around 10-40 lux, while a full moon is about 0.05-0.1 lux (Kyba et al. 2017b). Skyglow is ALAN that is emitted or reflected upwards and scattered in the atmosphere by water, dust and gas molecules. Often laboratory studies use 5 lux (or higher) as "dim" ALAN (e.g. Cissé et al. 2017; Stenvers et al. 2016); arrow A. Our experimental studies used light intensities of 1.6 and 3.0 lux; arrow B. Levels as low as < 0.00001 lux can already have biological effects (see references in Gaston et al. 2013); arrow C. Figure adapted from Gaston et al. (2014). SS Sunset, CT civil twilight, NT nautical twilight, AT astronomical twilight.

The alteration and disruption of natural light and dark cycles is likely to be problematic for many species. Life on earth has evolved under daily and seasonal cycles of light and dark (Kronfeld-Schor et al. 2013; Tan et al. 2010). These light-dark cycles have a major influence on organismal behaviour and have a strong biological relevance for the daily and annual rhythms of life (Bradshaw and Holzapfel 2007; Dawson et al. 2001; Helm et al. 2013). Light-dark cycles have driven the development of small and large scale biological phenomena, including metabolic and physiological pathways, the behaviour of individuals, geographical patterns of

adaptation and species richness, and ecosystem cycles (see e.g. Arendt 1998; Bennie et al. 2014b; Hays 2003; Urbanski et al. 2012). Animals can already be sensitive to very low levels of light (see references in Gaston et al. 2013). For example, the circadian rhythm of Syrian hamsters (*Mesocricetus auratus*) is already affected when exposed to an intensity of 0.01 lux at night (Evans et al. 2007). Clearly the introduction of ALAN into a natural dark environment will probably impact animal behaviour and physiology.

Altering behaviour

Artificial light has a wide range of behavioural effects in animals ranging from invertebrates to vertebrates including not only insects, reptiles and mammals but also birds (Davies and Smyth 2018; Gaston et al. 2017). For example, many people might be aware that sea turtles are attracted by light pollution which draws them away from the sea when they are hatching (Salmon et al. 1995). Another perhaps well-known example of animal attraction by ALAN is that of moths (see e.g. Frank 1988) which in turn may attract bats (see e.g. Rydell 1992). Short-term positive effects of ALAN would, therefore, be increased foraging opportunities for bats (Blake et al. 1994; Rydell 1991). However, this effect differs among bat species, with slow flying bats avoiding ALAN even though it may offer a higher food abundance, given that fast flying bats showed a higher number of hunting calls with white LED (Spoelstra et al. 2017). Despite that some bat species such as the common pipistrelle (*Pipistrellus pipistrellus*), may take advantage of increased prey abundance near street lights, it is also negative for their distribution and overall there do not seem to be positive effects on the most abundant European bat species (Mathews et al. 2015). ALAN can also disorient and attract migratory birds, drawing them towards brightly lit objects such as offshore platforms (Poot et al. 2008). Urban sources of light pollution also disrupt the migratory behaviour of birds. This probably has serious consequences due to increased costs in time and energy (La Sorte et al. 2017). Furthermore, light pollution attracts fledglings of short-tailed shearwaters (*Ardenna tenuirostris*; a seabird) which causes high mortality as they leave their nest at night but become disoriented by ALAN (Rodriguez et al. 2014; 2015). In the case of the black-tailed godwits (*Limosa limosa*) it was shown that they preferred to breed far away from artificial street light (de Molenaar et al. 2000). A lot of avian light pollution research has, however, focused on how song is affected. In several songbird species, including the great tit (*Parus major)*, ALAN advanced the onset of activity and/ or dawn song (Da Silva et al. 2014; Dominoni et al. 2013b; 2014; Kempenaers et al. 2010; Nordt and

Klenke 2013; Schlicht et al. 2014). Song is an important sexually selected trait which plays a role in male-male interactions and female choice (Searcy and Andersson 1986). The timing of dawn song is in many species an indicator of male quality, which may be disrupted by ALAN (Kempenaers et al. 2010; see also "*Light pollution and life-history*"). Interestingly, songbird species that naturally sing early at dawn, such as robins (*Erithacus rubecula*) and blackbirds (*Turdus merula*), had more advanced singing in the morning with ALAN compared to species that normally start singing later, such as blue tits (*Cyanistes caeruleus*) and chaffinches (*Fringilla coelebs*; Kempenaers et al. 2010). Dawn and dusk singing developed earlier in the year due to ALAN in robins, blackbirds, great and blue tits. Likewise, this effect was most pronounced in robins and blackbirds (Da Silva et al. 2015). It seems that the behavioural response to ALAN differs among bird species. However, few studies have experimentally studied in the wild whether individuals of different species respond differently to ALAN. This is important as species-specific effects can have implications for community dynamics. There are several examples that broad spectrum lights such as LED have the potential to alter species interactions (Davies et al. 2013). An outdoor mesocosm experiment showed that ALAN changes aphid-parasitoid population dynamics (Sanders et al. 2015) and another study by McMahon et al. (2017) showed that the urban habitat can alter host-parasite interactions. Light and noise pollution did not affect Túngara frogs (*Engystomops pustulosus*) while abundance of the parasitic frog-biting midges (*Corethrella* spp.) was reduced. Furthermore, the effect that ALAN has on the activity of animals during the night may have consequences for other organisms that are active during the day. For example, light pollution affects nocturnal pollinators thereby negatively affecting pollination of their host plants leading to less fruits with cascading effects to diurnal pollinators (Knop et al. 2017). Further research is necessary to clarify how and to what extent light pollution alters interactions between species and ecosystem functioning (Sanders and Gaston 2018).

Light pollution may also extend activity into the night. The common redshank (*Tringa totanus*) takes advantage of light pollution because it increases visibility thereby extending foraging into the night (Dwyer et al. 2013). There have also been reports of other species opportunistically taking advantage of increased visibility to continue foraging, or in the case of the northern goshawks (*Accipiter gentilis*), hunting (Rutz 2006). For example, Lebbin et al. (2007) reported that several nocturnally migrating species (>15 wood-warbler species (Parulidae), one tyrant-flycatcher (Tyrannidae), and one mimid (Mimidae)) foraged at night due

to ALAN. ALAN also had a positive effect on nocturnal foraging of waders although there may be a trade-off with increased predation risk (Santos et al. 2010). During winter the effect on foraging for day active songbirds seemed limited (Da Silva et al. 2017a) but during the breeding season Northern mockingbirds (*Mimus polyglottos)* were able to feed their nestlings after dark when exposed to light pollution (Stracey et al. 2014). Light pollution also caused blackbirds to extend their foraging (Russ et al. 2014). However, Dominoni et al. (2014) found that in blackbirds onset of activity was advanced but offset was unaffected. As ALAN affects the activity patterns of birds it is reasonable to assume that it also affects sleep behaviour. Hence, light pollution may cause animals to wake up earlier and potentially sleep less and/or, as cessation of activity can be delayed, also fall asleep later. In laboratory studies ALAN has been shown to disrupt sleep behaviour in pigeons (*Columba livia;* Rattenborg et al. 2005). However, this has not yet been studied in free-living birds and experiments involving physiology and behaviour are particularly susceptible to the environment in which they are performed (Calisi and Bentley 2009).

The behavioural response to anthropogenic disturbance may not only vary among species but also among individuals. Variation in personality may play an important role in explaining among individual variation (Sih et al. 2012). Personality or behavioural syndromes appear to be widespread across the animal kingdom including birds (Gosling 2001). Novel environment exploration (exploration behaviour) is commonly used in great tits to determine personality types (Dingemanse et al. 2002; Verbeek et al. 1994). Exploration behaviour of great tits ranges from slow to fast explorers: those that explore an area slowly but thoroughly or rapidly but superficially (Verbeek et al. 1994). This behaviour is related to other ecologically relevant behaviours (boldness, aggressiveness), differences in stress physiology and fitness (see e.g. Baugh et al. 2017; Carere et al. 2010; Dingemanse et al. 2003; Dingemanse and Reale 2005; Hollander et al. 2008; Thys et al. 2017; van Overveld and Matthysen 2010). Fast explorers are less neophobic and more readily accept and approach novel objects (Baugh et al. 2017; Carere et al. 2005; Cole and Quinn 2014; Stuber et al. 2013; Verbeek et al. 1994), which is particularly relevant considering anthropogenic disturbances (Tryjanowski et al. 2016), such as ALAN. For example, in great tits the effect of anthropogenic noise differed between slow and fast explorers with slow explorers being more affected as they took longer to enter a noise exposed nest box (Naguib et al. 2013). Furthermore, fast explorers were more likely than slow explorers to sleep in the same nest box on subsequent winter nights when a video camera (novel object)

had been installed inside (Stuber et al. 2013). Similarly, the response to ALAN could be stronger in slow exploring great tits but this remains to be examined.

Sleep

Sleep, an important behaviour observed in nearly all animal species (Cirelli and Tononi 2008; Siegel 2008; Tougeron and Abram 2017), is probably affected by light pollution. It is not always obvious when an animal is asleep but the general definition is a rapidly reversible state of immobility and greatly reduced sensory responsiveness. Sleep is thus a state with reduced environmental awareness and has been found in animals ranging from worms to humans. Sleep can be observed through an animal's behaviour (see Figure 2) but can also be defined by its neurophysiological state, changes in brain activity or waves measured using an electroencephalogram (EEG).

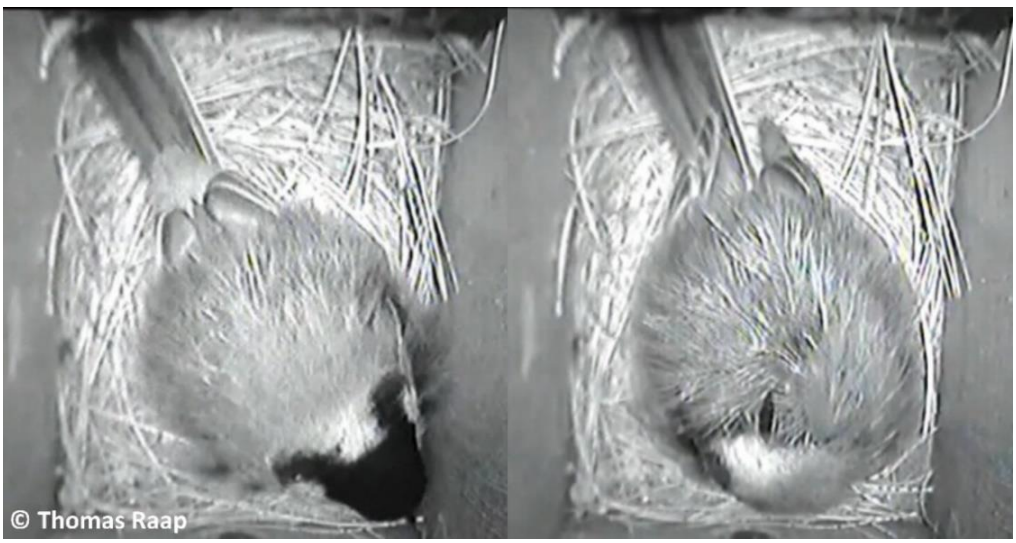

Figure 2: Typical sleeping posture of a songbird. A free-living great tit inside a nest box when it is awake (left) and when it assumes the typical sleep posture (right). In songbirds, such as great tits, typical sleep behaviour with the head backwards indicates increased sleep depth and reduced vigilance (Amlaner and Ball 1983). Pictures are stills from a video where sleep behaviour was recorded in the field with the use of an infrared camera.

Most, if not all, bird species show sleeping behaviour (Lesku and Rattenborg 2014; Libourel and Herrel 2016; Roth II et al. 2006) and avian sleep shares many characteristics of mammalian sleep (Rattenborg and Martinez-Gonzalez 2015). For example they both show two types of

sleep, slow wave sleep (SWS) and rapid-eye movement (REM; Siegel 2008). With the use of EEG sleep phases and sleep intensity can be studied in greater detail, while studying sleep via behavioural observation is less accurate and more limited to measuring quantity, but is also less invasive (Aulsebrook et al. 2016).

While its exact function is still unclear (Rattenborg et al. 2017), sleep serves multiple purposes including energy conservation and memory consolidation (Gobes et al. 2010; Roth II et al. 2010). Sleep deprivation can have negative effects (Cirelli and Tononi 2008; Siegel 2008) such as reduced cognitive functioning (Rattenborg et al. 2004), and can ultimately affect reproduction and survival. In rats it was shown that sleep deprivation eventually leads to death (Rechtschaffen and Bergmann 2002). Other species such as pigeons appeared to be more resistant to sleep deprivation although they also tried to compensate for lost sleep (Newman et al. 2008). Sleep also has its costs because as animals are typically immobile and less aware of the local environment, it makes them vulnerable and unable to perform other essential tasks. However, both in mammals and in birds, sleep deprivation is followed by compensation (increased sleep duration or depth), indicating the necessity of sleep (Lima et al. 2005; Martinez-Gonzalez et al. 2008; Rattenborg et al. 2009; Roth II et al. 2010).

Most sleep research has been performed in laboratories, however, environmental conditions outside of the laboratory may affect behaviour including sleep (Calisi and Bentley 2009; Daan 2011). For example, wild great tits initiate sleep earlier than captive tits (Stuber et al. 2015b). It would, therefore, be informative to study the neurophysiological state of free-living animals in ecologically relevant situations. However, studying sleep in free-living animals is very challenging (Rattenborg et al. 2017). Innovative lightweight EEG data loggers can be used for animals larger than 100 g (Vyssotski et al. 2009). For smaller animals such as great tits (approx. 16-18 g) researchers are still dependent on behavioural observations when aiming to study sleep in the wild. Although measuring brain activity might be preferable, quantifying sleep behaviour is nevertheless ecologically relevant and has been linked to amongst others, basal metabolic rate, extra pair paternity, predation risk and furthermore variation in sleep behaviour has a genetic basis (see e.g. Christe et al. 1996; Steinmeyer et al. 2010; 2013; Stuber et al. 2014; 2015b; 2016; 2017). Moreover, previous work in blackbirds shows a close correspondence between behaviourally observed and electrophysiological measured sleep (Szymczak et al. 1993).

Physiology

The earlier mentioned behavioural effects may have physiological consequences and ALAN may also directly affect an individual's physiological condition (Dominoni 2015). ALAN probably disrupts circadian rhythms such as sleep, which are modulated by the light-sensitive release of melatonin (Tan et al. 2010). Melatonin has multiple biological functions (Tan et al. 2010) and its suppression by ALAN (Dominoni et al. 2013d) may not only lead to disrupted sleep but also have cascading effects on an animal's physiology. For example, melatonin is, amongst others, involved in the regulation of oxidative status and immunological modulation (Tan et al. 2010). Oxidative status is the balance between pro-oxidants and antioxidants. Oxidative stress is a biochemical condition of the cell that occurs when there is an imbalance between pro-oxidants and antioxidants in favour of pro-oxidants leading to oxidative damage (Costantini and Verhulst 2009; Halliwell and Gutteridge 1985). The variable nature of interactions among oxidative status biomarkers makes it very difficult to generalise the oxidative status based on one or two measures (Cohen and McGraw 2009; Costantini et al. 2013; Dotan et al. 2004). Therefore, to adequately characterise oxidative status multiple indicators of antioxidant capacity, redox state and oxidative damage should be measured. Because increased molecular damage and depletion of antioxidants may influence growth, reproductive strategies and survival, oxidative stress may be a mediator of some life-history trade-offs (Costantini 2014). Through this pathway ALAN may affect reproduction and survival. Recent reviews by Russart and Nelson (2018) and Ouyang et al. (2018) further illustrate the multitude of physiological and endocrinal effects caused by ALAN. These effects include disruption of reproductive hormones as well as the suppression of the immune system. For example in crickets (*Teleogryllus commodus*), ALAN had a negative impact on melatonin as well as on several immune parameters such as haemocyte concentration and lytic activity (Durrant et al. 2015). In great tits and other songbirds, haptoglobin (Hp) and nitric oxide (NOx) are important and often used measures of the immune system (Hegemann et al. 2017; Matson et al. 2012; Sild and Horak 2009). Assays to measure Hp and NOx require only a limited blood plasma volume and can therefore be done (repeatedly) on small songbirds including nestlings (<10 g). Haptoglobin is an acute phase protein that plays an important role in inflammation, infection and trauma, and it can also act as an antioxidant (Quaye 2008). 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 Hp and NOx also provide

useful information on changes in physiological condition and health state (Matson et al. 2012; Sild and Horak 2009). ALAN may affect the immune system in free-living birds which might be quantified by changes in Hp and NOx. Outside of the laboratory altered physiology together with demands of limited resources and harsh environmental conditions may seriously impact survival. There is therefore a pressing need for field studies on free-living animals (Gaston et al. 2017).

Early life exposure

The impact of ALAN on physiology may be especially relevant when the organism is exposed in early life (Fonken and Nelson 2016), as the environment in which a young individual develops has profound, long-lasting and often irreversible consequences throughout an individual's lifetime (Harris and Seckl 2011; Henriksen et al. 2011; Lindstrom 1999; Monaghan 2008). In mice, early life exposure to ALAN increased anxiety-like and fearful behaviour throughout life and decreased growth rates although these normalised during adolescence (Borniger et al. 2014). In free-living birds, body mass at fledging is a good predictor of survival as well as recruitment because body energy reserves help individuals to cope with the adverse conditions of winter (Horak et al. 1999; Magrath 1991; Naef-Daenzer et al. 2001; Perrins and McCleery 2001). In songbirds ALAN can influence the foraging behaviour of parents as it may allow them to feed nestlings after sunset (Russ et al. 2014; Stracey et al. 2014), and it is plausible to expect an impact of ALAN on individual health, condition and survival of nestlings through its effects on body mass. On the other hand, sleep disruption by ALAN may reduce the performance and provisioning behaviour of parents and thus nestling body mass. Moreover, direct physiological effects such as those described above are likely but have rarely been studied in developing animals or in free-living individuals.

Light pollution and life-history

Light pollution may affect an individual's behaviour and physiology, including life-history decisions which may eventually impact populations. ALAN advances dawn song in several songbird species which might be beneficial (Kempenaers et al. 2010). The correct timing of a sexually selected trait such as dawn song is important as males that sing earlier may obtain more fertilizations but viability selection favours later dawn song as it increases energetic costs and predation risks (Hau et al. 2017). In blue tits light pollution advanced dawn song in males

and laying date in females (Kempenaers et al. 2010). There is, however, uncertainty about whether this is due to the earlier advancement of female gonads or because females responded to earlier dawn song by the males. te Marvelde et al. (2012) showed that while in winter a single night of ALAN did advance gonadal development in captive female great tits, it had no effect on the laying date in free-living birds. However, even at low intensities (0.3 lux) ALAN advanced gonadal growth and reproductive physiology in captive male blackbirds (Dominoni et al. 2013a). Sleep disruption by ALAN could also potentially affect life-history traits. Sleep probably plays an important role in energy conservation or recovery from activity during daytime (Rattenborg et al. 2017; see also "*Sleep*"). Furthermore, sleep appears to be especially important during early life of some animals (especially for altricial animals) with REM sleep playing a role in brain development (Siegel 2005). Light pollution could increase energy expenditure due to prolonged foraging (Stracey et al. 2014) and, combined with the likelihood of reduced and disrupted sleep, can affect survival. Within the same species and populations, individuals differ in how strongly they respond to environmental stressors, such as light pollution (Sih et al. 2012). For example, in blue tits, yearling males appeared to show a stronger response to ALAN than older individuals, as light pollution increased extra pair paternity rates more in yearling males than in older males (Kempenaers et al. 2010). Light pollution would in this case lead to maladaptive mate choice as it disrupts the link between quality and dawn song as yearling males would seem of higher quality than they would be otherwise. Taken together, individual level effects of light pollution on life-history traits and sleep are thus likely to cascade to population-level effects. Light pollution may, therefore, affect several life-history traits although this remains to be determined. It is therefore important to examine how ALAN affects behaviour and physiology in free-living animals.

Aim, model species and thesis outline

The general aim of this research was to gain fundamental insights into the behavioural and physiological effects of ALAN in free-living animals. Because animals in nature face a multitude of trade-offs lacking under laboratory conditions, such as the trade-off between predation risk, antipredator vigilance and sleep (Roth II et al. 2010), research on free-living animals is crucial (Gaston 2013). As main model species we used the great tit and all research was performed using the semi-urban population of great tits at the University of Antwerp (51°9'44''N, 4°24'15''E; see Figure 3), which has been continuously monitored since 1997 (see e.g. Thys et al. 2017; Van Duyse et al. 2000).

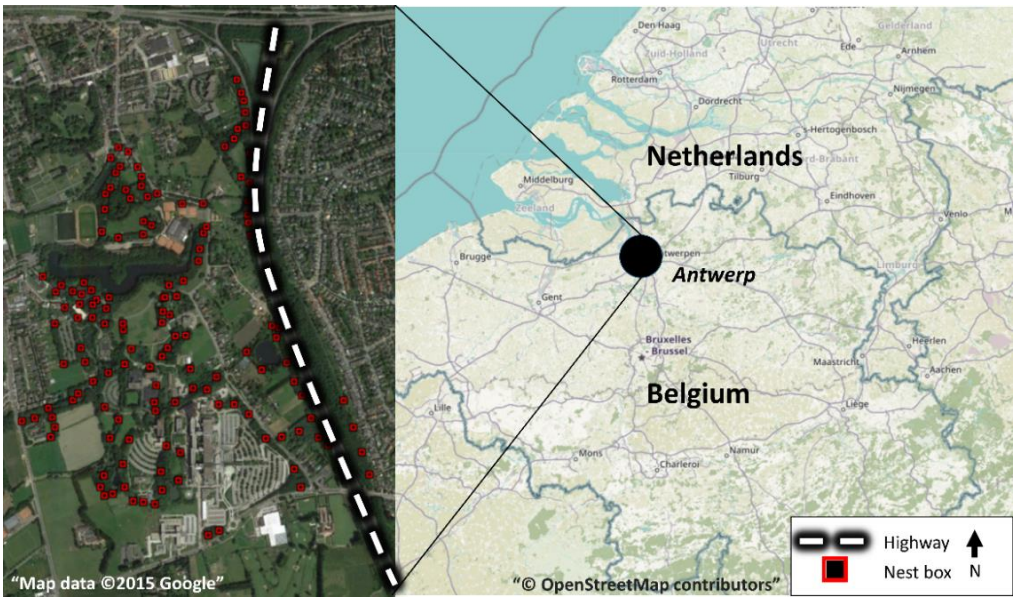

Figure 3: Study area and its location in Belgium. Street lights are the main source of light and the highway is the main source of noise pollution.

Great tits in this population have been caught inside nest boxes during previous winter and breeding seasons, and were sexed and ringed after capture. Since 2011 all adults have been provided with a ring or implant containing a PIT tag (passive integrated transponder), thereby enabling the detection of individual birds without physical disturbance when they were roosting inside nest boxes. Blue and great tits are ecologically closely-related sympatric species (Dhondt 1977) and we used them both to examine differences in the behavioural response to

ALAN (**Chapter 5**). The blue tit population at the University of Antwerp has also continuously been monitored since 1997 similar to the great tit population (see e.g. Eens et al. 1999).

The great tit is an important model species in evolutionary and environmental research, and is increasingly being used to study the effects of ALAN on behaviour and physiology (e.g. Da Silva et al. 2017b; de Jong et al. 2016; Kempenaers et al. 2010; Sprau et al. 2017). Both males and females sleep in nest boxes in winter and nest boxes are readily used for breeding. During the breeding season the female sleeps with the nestlings in the nest box (or cavity) but males usually roost in foliage or on a branch against a tree trunk (Hinde 1952; Kluijver 1950). Great tits are relatively short-lived (1-5 years) and can be observed and captured year round throughout their entire life.

Cavity-nesting species like great tits are ideal to gain fundamental insights into the effects of ALAN on free-living animals as it is possible to manipulate light conditions within a nest box. This enables experimentation and observations in a more natural environment compared to that in the laboratory. Using free-living open-nesting birds would be very challenging due to the difficulty of experimental manipulation of light conditions to which they are exposed as well the difficulty of obtaining a reliable sample size. Studies using wild animals have often compared urban versus non-urban populations. In these types of studies effects of light pollution may be confounded by other urban stressors such as noise (Swaddle et al. 2015) and chemical pollution (Isaksson 2015). Using great tits as a model species enables us to manipulate the light conditions to which they are exposed while keeping all other influencing factors equal, which is necessary to fully comprehend the effects of ALAN on animals. As they are highly site-faithful, we can associate environmental conditions/stressors at their territory, such as ambient light and noise pollution, with their behaviour and physiology. Thus, they can be used in correlational studies as well as in experiments where the light levels to which they are exposed throughout the night, can be manipulated.

The research described in this thesis focuses on two main themes: *I Effects on Sleep and Activity,* and *II Effects on Nestling Physiology*. This thesis reports the results of my PhD research but I will mainly use "we" as many people have helped and contributed to this work (see also "*Acknowledgements - Dankwoord"*).

I Effects on Sleep and Activity

Using new technological developments, such as small infrared sensitive cameras and a new LED light system to light the inside of the nest box (which was developed in our research group, the Behavioural Ecology & Ecophysiology Group; Figure 4), we studied for the first time the effects of ALAN on sleep behaviour in free-living animals during the winter (**Chapter 2**).

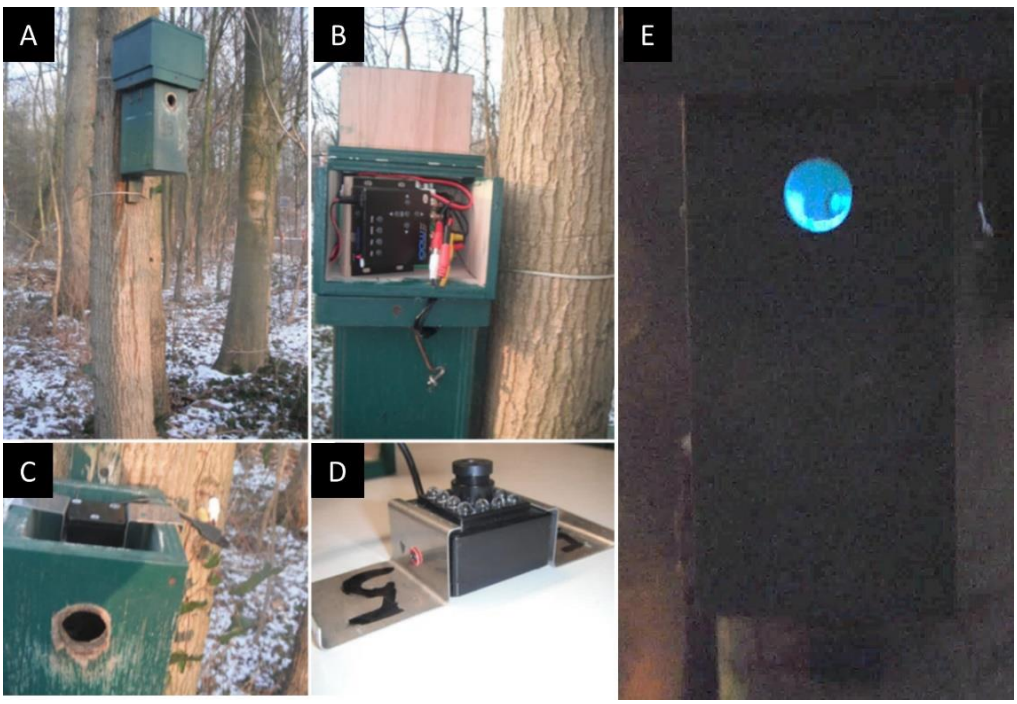

Figure 4: Experimental camera system and LED light for nest boxes. A) nest box with the camera system on top; B) the video recording device; C) camera placed inside the nest box; D) the infrared camera; E) nest box lit from within with LED light (picture brightness enhanced to show contour of the nest box, we used a broad spectrum white LED light).

We used a within-individual design (as for most other experiments) and looked at changes in behaviour from a dark night compared to when animals were exposed to ALAN. Birds served as their own control due to this design. Another experiment was carried out during the breeding season when the great tits had nestlings (**Chapter 3**). We studied the effects of artificial light inside the nest box on the sleep behaviour of adult females and compared these with the results of the first experiment (performed during winter). We also studied the effects of artificial light on the begging behaviour of the nestlings at night. Laboratory research showed

that in great tits behavioural effects towards ALAN may differ between light intensities (de Jong et al. 2016). Similarly, sleep behaviour under natural conditions differed between December and February (Stuber et al. 2015b). We therefore further explored the potential seasonal and light intensity dependent effects by conducting another experiment during December and February and with a higher light intensity than our previous experiment (**Chapter 4**).

The response to pollution (such as light pollution) may also differ between species, even those that are closely related (Sih et al. 2011). We therefore experimentally examined sleep behaviour of congeneric great and blue tits (**Chapter 5**). We first compared their sleep behaviour while they slept in a natural dark environment. Subsequently we observed changes in sleep behaviour caused by ALAN inside the nest boxes, and compared the response to ALAN between species. Great tits use cavities and nest boxes to roost during the night, however, local light conditions outside the nest box seem to affect sleep behaviour (Stuber et al. 2015b), although this has not been experimentally tested. Therefore, in **Chapter 6**, we examined whether sleep behaviour is affected by ALAN from outside the nest box (opposed to the experimental treatment used in **Chapters 2-5**) and to what extent nest boxes (and by extension cavities) may shield animals from the effects of ALAN.

ALAN affects many aspects of animal behaviour and physiology but the response to ALAN varies widely, not only among species (**Chapter 5**) but also among individuals (**Chapter 3**). Individual differences in this response might be related to consistent individual differences in other behaviours (i.e. personality). This has, however, rarely been examined. Personality is heritable and associated with variation in fitness (Dochtermann et al. 2015; Smith and Blumstein 2008) and light pollution may thus select for a certain personality type thereby affecting population dynamics (Sih et al. 2012). We therefore assessed whether personality types differed in their avoidance behaviour towards ALAN (slow versus fast explorers; Reale et al. 2007; Verbeek et al. 1994) and whether experimental exposure to ALAN induced personality-dependent changes in sleep behaviour (**Chapter 7**).

II Effects on Nestling Physiology

Changes in physiology associated with ALAN may be detrimental (Russart and Nelson 2018), especially for developing animals (Fonken and Nelson 2016). Using our experimental setup with light inside the nest box we aimed to gain insight into physiological effects of ALAN in

developing nestlings (**Chapters 8 and 9**). To investigate the physiological effects, we focused on oxidative status and Hp and NOx as measures of physiological condition and immunity.

Sleep disruption by ALAN is likely to occur in developing songbirds (**Chapter 3**) but is difficult to quantify. Oxalic acid or oxalate is a recently discovered cross-species biomarker for sleep debt (Weljie et al. 2015). In humans and in rats, loss of sleep was associated with a decrease in blood plasma oxalate levels. Potentially it can be used in free-living nestlings and we therefore examined the effect of ALAN on oxalate in great tit nestlings (**Chapter 10**).

As mentioned earlier, ambient light pollution may interact with noise pollution (Halfwerk and Slabbekoorn 2015). However, studies examining simultaneously whether the effects of light and noise pollution are additive or synergistic are rare (Swaddle et al. 2015). Thus, we examined whether light pollution interacts with noise pollution and their relation to nestling physiology (**Chapter 11**). In this correlative study we took a small blood sample from all nestlings from our semi-urban population of great tits during one breeding season. In this population, nest boxes (animals) are exposed to different levels of ambient light and noise pollution. Physiological parameters were determined and we subsequently analysed whether they were associated with the level of light and/or noise pollution.

Finally, in the “General discussion” all the results of this thesis are discussed as well as their limitations and potential implications.

Chapter 2

Light pollution disrupts sleep in free-living animals

Thomas Raap, Rianne Pinxten & Marcel Eens

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Abstract

Artificial lighting can alter individual behaviour, with often drastic and potentially negative effects on biological rhythms, daily activity and reproduction. Whether this is caused by a disruption of sleep, an important widespread behaviour enabling animals to recover from daily stress, is unclear. We tested the hypothesis that light pollution disrupts sleep by recording individual sleep behaviour of great tits, *Parus major,* that were roosting in dark nest-boxes and were exposed to light-emitting diode light the following night. Their behaviour was compared to that of control birds sleeping in dark nest-boxes on both nights.

Artificial lighting caused experimental birds to wake up earlier, sleep less (-5%) and spend less time in the nest-box as they left their nest-box earlier in the morning. Experimental birds did not enter the nest-box or fall asleep later than controls. Although individuals in lit nest-boxes did not wake up more often nor decreased the length of their sleep bouts, females spent a greater proportion of the night awake. Our study provides the first direct proof that light pollution has a significant impact on sleep in free-living animals, in particular in the morning, and highlights a mechanism for potential effects of light pollution on fitness.

Introduction

Our natural environment is dramatically altered by increasing urbanization. One of its consequences is light pollution, which is defined as the alteration of natural light levels due to the introduction of artificial light at night. The rapid increase of artificial light at night, expansion of lit areas and increased light intensity, result in a loss of darkness with largely unknown consequences for biodiversity, ecosystems and ecological and evolutionary processes (Hölker et al. 2010b; Rich and Longcore 2005). Light has a strong biological relevance for the daily and annual rhythms of life, given its periodic changes and/or seasonal fluctuations (Bradshaw and Holzapfel 2007; Dawson et al. 2001; Helm et al. 2013), and there is accumulating evidence that light at night is not as harmless as previously thought. Laboratory studies found major disruptive effects from artificial light on a wide range of behavioural aspects such as reproduction, foraging, sleep and migration (Bedrosian et al. 2011; 2013b; Brainard et al. 1984; Cho et al. 2013; Dominoni et al. 2013c; Navara and Nelson 2007; Rodriguez et al. 2015; Shuboni and Yan 2010). In addition, they also reported physiological effects including alterations in immune response (Bedrosian et al. 2011), cortisol levels (Bedrosian et al. 2013b), melatonin levels (Brainard et al. 1984; Dominoni et al. 2013d; Santhi et al. 2012), testosterone levels (Dominoni et al. 2013a) and glucose metabolism (Dolgin 2013; Knutson et al. 2007). There are, however, hardly any field studies on free-living animals and experimental manipulations of light conditions are almost entirely lacking (but see de Jong et al. 2015; Schlicht et al. 2014; Titulaer et al. 2012).

Artificial light has a wide range of behavioural effects in birds. A study on black-tailed godwits (*Limosa limosa*) showed that they preferred to breed far away from artificial street light (de Molenaar et al. 2000). Light at night can also disorient and attract migratory birds, drawing them towards brightly lit objects such as offshore platforms (Poot et al. 2008). Light pollution also attracts seabird fledglings which causes high mortality (Rodriguez et al. 2014; 2015). In several songbird species, including the great tit (*Parus major)*, it was shown that artificial light advanced the onset of activity and/ or dawn song (Da Silva et al. 2014; Dominoni et al. 2013b; 2014; Kempenaers et al. 2010; Nordt and Klenke 2013; Schlicht et al. 2014). As artificial light at night affects activity patterns of birds it is reasonable to assume that it also affects sleep behaviour. Blue tits (*Cyanistes caeruleus*) have been shown to adjust their awakening time according to local light conditions (Steinmeyer et al. 2010). Hence, light pollution may cause animals to wake up earlier and potentially sleep less or, as cessation of

activity can be delayed (Russ et al. 2014), also fall asleep later. In contrast to laboratory studies (e.g. Rattenborg et al. 2004), whether and how artificial light affects sleep behaviour in free-living birds has not yet been studied.

However, sleep is an important animal behaviour widespread across the animal kingdom (Cirelli and Tononi 2008; Siegel 2008). There is clear evidence in many species that sleep allows animals to recover from daily stress (Siegel 2009; Weljie et al. 2015) and that sleep deprivation has major negative effects (Cirelli and Tononi 2008; Siegel 2008). Sleep is common in bird species (Lesku and Rattenborg 2014; Roth II et al. 2006) where it may not only serve to consolidate memory but also to conserve energy (Gobes et al. 2010; Roth II et al. 2010; Vorster and Born 2015). White-crowned sparrows (*Zonotrichia leucophrys gambelii*) can reduce sleep during migration without negative effects, however, outside the migratory season loss of sleep reduced cognitive functioning (Rattenborg et al. 2004). The few studies that have been carried out to study effects of natural or experimentally induced variation in sleep on fitness have produced mixed results until now. In the blue tit males that sleep longer are more likely to sire extra-pair offspring but otherwise there was no strong effect of variation in sleep behaviour on fitness (Steinmeyer et al. 2013). Pectoral sandpipers (*Calidris melanotos)*, which breed in the arctic, can almost completely eliminate sleep without negative effects during the breeding season and males that sleep less sire more offspring (Lesku et al. 2012). There is also indirect and partial evidence from studies on activity patterns which suggest that variation in sleep might affect some aspects of fitness although much remains unknown. In blue tits animals that had an earlier dawn song (because of light pollution), suggesting that they slept less, had an advanced laying date and increased male extra-pair paternity (Kempenaers et al. 2010). Male great tits who had their activity experimentally delayed in the morning (through melatonin implementation, suggesting that they slept longer), had their fitness reduced through an increased risk of cuckoldry (Greives et al. 2015). However, negative effects have also been reported. A case report on zebra finches (*Taeniopygia guttata)* suggests that sleep deprivation from exposure to continuous light led to increased mortality (Snyder et al. 2013). Although effects of sleep loss and disruption on fitness are largely unclear, it is an important first step to evaluate whether and how artificial light at night affects species in the wild.

Here we studied for the first time the impact of artificial light on sleep in free-living animals by quantifying its effects on sleep behaviour of great tits during the pre-breeding season. We experimentally provided male and female great tits, sleeping in nest-boxes, with

artificial light to investigate the change in sleep behaviour compared to the natural dark situation. Individual sleep behaviour was, therefore, observed over two subsequent nights. We used a within-subject design in which the treatment group was provided with artificial light during the second night, the first night being used as a control. As an additional control we observed birds that slept in a natural dark situation during both nights.

Methods

Study area and general procedures

We collected data between February 17 and March 4 2014 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 this free-living population has been continuously monitored since then (Van Duyse et al. 2005). Great tits were caught inside nest-boxes during winter and breeding seasons after which they were sexed and ringed. Since 2012, all adults have been provided with a ring containing a passive integrated transponder (PIT) tag. This enables the individual detection of birds sleeping in nest-boxes without physically disturbing the birds.

Experimental procedure

A paired design was used in which sleep behaviour was observed over two subsequent nights in a control (dark) treatment and a light treatment (Table S1). In the control group birds were observed over two nights sleeping in a naturally dark situation, while birds in the light group slept without a light turned on the first night and with a light turned on (see below) during the second night.

Observations of sleep behaviour in the control and light group were always performed simultaneously during one recording session (of two consecutive nights) with a total of six sessions. Paired data were obtained from nine individuals (three males and six females) in the control group and of 18 individuals (eleven males and seven females) in the light group. We expected minor differences in sleep behaviour between nights in the control group and therefore recorded fewer individuals in this group, compared to the light group.

Recording of sleep behaviour and light treatment

Prior to the night of the first recording, all nest-boxes were checked during the night (at least one hour after sunset) for presence of a sleeping great tit by moving a handheld transponder reader (GR-250 RFID Reader, Trovan, Aalten, Netherlands) around the outside of the nest-box.

Nest-boxes in which great tits had been sleeping were used in the experiment. During the experiment, nest-boxes were also checked every night with the transponder reader to ensure that the same individual slept in the nest-box on both nights. Infrared sensitive cameras (Pakatak PAK-MIR5, Essex, UK) were installed under the nest-box roof lid at least two hours before sunset and removed, at the earliest, two hours after sunrise the next morning (recordings started after installation). Ten infrared LED lights (which are invisible for great tits; Hart 2001) around the objective served as a light source for the camera.

Simultaneously with the video camera, we placed in each nest-box a small LED light (15 mm x 5 mm, taken from a RANEX 6000.217 LED headlight, Gilze, Netherlands) above the nest-box entrance hole on the inside, pointing downwards. These LEDs were standardized to produce 1.6 lux on the bottom of the nest-box as measured with an ISO-Tech ILM 1335 light meter (Corby, UK). In light polluted areas, birds are exposed to similar and higher light intensities, especially outside of nest-boxes or cavities (Dominoni et al. 2013a; Gaston et al. 2013). In our population, those nest-boxes which are located near street lights, experience light intensities of more than 8 lux at the front of the nest-box opening. We chose white LED light because there is now a shift towards energy efficient broad spectrum light sources such as LED (Davies et al. 2013; Schubert and Kim 2005).

We made recordings of the control group on two consecutive nights with a turned off LED light inside the nest-box. The first night that sleep behaviour was recorded in the light group, a LED light was present in the nest-box but turned off, thus birds slept in their normal dark situation similar to the control group. On the subsequent night the LED light was turned on at least two hours before sunset until at least two hours after sunrise the next morning. Thus, the light was turned on several hours before the birds entered the nest-box to go to sleep.

Sleep parameters

Sleep of great tits was quantified in detail using 10 parameters: (1) entry time, (2) sleep onset, (3) evening latency, (4) awakening time, (5) leaving time, (6) morning latency, (7) sleep amount, (8) sleep proportion, (9) frequency of sleep bouts and (10) sleep bout length. We focused on these parameters as most have been used previously to study sleep behaviour in the closely related blue tit and have been associated with fitness related traits (Steinmeyer et al. 2010; 2013).

We followed the definition of sleep parameters as described in Steinmeyer et al. (2010). In short, a bird was considered to be sleeping when it showed the classical sleep position

(beak pointing backwards and tucked under the scapulars). Whether a bird was asleep or awake was usually easily distinguished. Only rarely was this distinction more difficult when individuals would occasionally sit quietly for some time with their head pointing forwards or not completely tucked under the scapular. These periods were defined as awake periods also because often they were followed by tucking the head under the shoulder. We define entry time and leaving time as the time when the bird entered or respectively left the nest-box. Sleep onset was defined as the first time a sleep bout of minimum 30 seconds had started. The time between entry time and sleep onset was defined as evening latency. Awakening time was defined as the last time the bird was asleep for at least 10 seconds. The sum of all sleep bouts was defined as sleep amount. We calculated sleep proportion as sleep amount divided by the total time spent inside the nest-box. The number of sleep bouts was calculated per hour as frequency of sleep bouts. All birds remained in the nest-box for the duration of the night after they had entered it in the evening. Some birds sat on the nest-box entrance hole several times before leaving in the morning, but only the moment when the bird had completely left the nest-box was used as leaving time. The time between awakening and leaving time was defined as morning latency.

In addition to the sleep parameters, we recorded activity during morning latency. During morning latency, the total time a bird spent on the nest-box entrance hole was used as "time on entrance" and the number of times it sat on the nest-box entrance hole was counted and used as "number of times on entrance".

Data analysis

Entry time, sleep onset, awakening time and leaving time were all converted to times relative to sunset or sunrise (reference data from Antwerp were used). For all statistical analyses we used R 3.0.2 (R Core Team 2013).

We performed separate linear mixed effects analyses with the different sleep parameters as response variables (using the lme4 package; Bates et al. 2013). As fixed effects, we entered treatment, date (Julian day), sex and night as well as the interactions between them (with the exception of interactions with date to avoid overfitting the model). Sex, as well as date, may influence sleep behaviour (Steinmeyer et al. 2010) and were, therefore, entered in the model. As random effect, we entered bird identity nested in (recording) session to control for the repeated measures.

Results are presented as marginal means with one standard error from the mean (S.E.; unless stated otherwise).

Ethical statement

This study was approved by the ethical committee of the University of Antwerp (ID number 2011-31) and performed in accordance with Belgian and Flemish laws. The Belgian Royal Institute for Natural Sciences (Koninklijk Belgisch Instituut voor Natuurwetenschappen) provided ringing licences for authors and technical personnel.

Results

In addition to the eighteen birds that we observed over two nights in the light group, there were nine birds who slept in a dark nest-box the first night but did not enter the nest-box during the second evening/ night when the LED light was on (these nine observations were excluded from further analyses). The proportion of birds not entering the nest-box the second evening was significantly higher in the light group compared to the control group (none of the nine birds; Fisher Exact Test, $P = 0.026$).

Male birds ($N = 14$) entered the nest-box later compared to female birds ($N = 13$) and their sleep amount was also reduced because of a later sleep onset. Other sleep parameters did not differ between sexes (see Table S2 for details).

Effects of artificial light on sleep

While several aspects of sleep behaviour differed between sexes, the three way-interaction between sex, treatment and night was not significant for all but one sleep parameter, sleep proportion ($N = 27$, $\chi^2_1 = 13.123$, $P = <0.001$; Table S2). The proportion of time spent sleeping in a lit nest-box was reduced for females (by about 4%; $N = 27$, $\chi^2_1 = 62.536$, $P = <0.001$) but not for males ($N = 27$, $\chi^2_1 = 2.774$, $P = 0.096$; Figure 1 and Table S2).

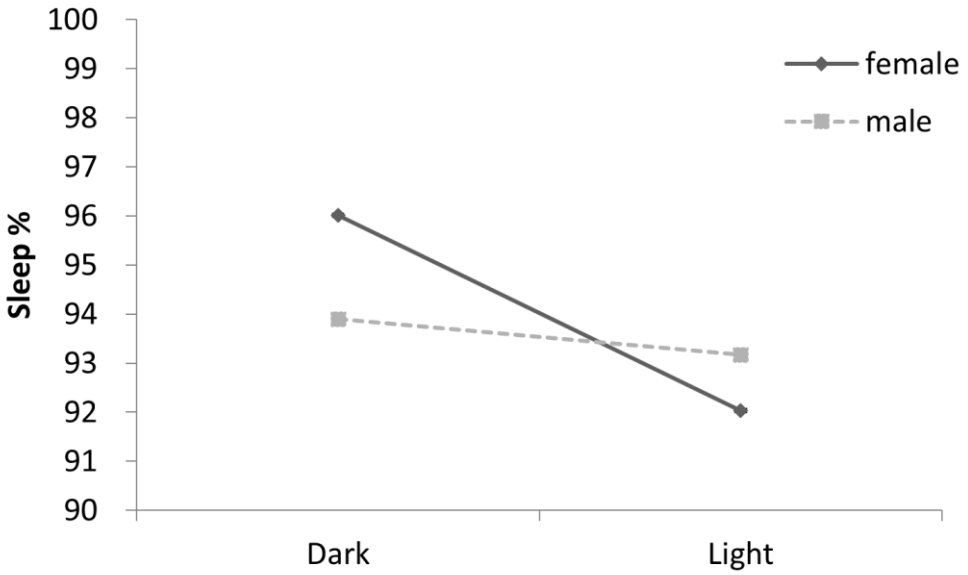

Figure 1: Effect of artificial light on the percentage of time spent asleep in the nest-box. Effects are shown for females (solid line) and males (dotted line) of the treatment group that first slept in a natural dark situation (on night 1) and subsequently with an artificial light turned on (during night 2). The difference was significant for females ($Z = 8.65$, $P < 0.001$), but not for males. P- value is obtained from a GLMM with bird identity nested in recording session (data were collected during six sessions; see Methods) as random factor to correct for repeated measurements. Mean and S.E. are shown (obtained from raw data).

Artificial light significantly affected most sleep parameters as indicated by the significant night*treatment interactions (Figure 2, Tables S2 and S3). Exposed to the influence of artificial light, great tits woke up half an hour earlier (–26.3 min ± 4.5, $N = 27$, $t = 5.79$, $P < 0.001$) and left the nest-box 20 minutes earlier (–18.3 min ± 4.6, $N = 27$, $t = 3.96$, $P < 0.001$). Total sleep amount was reduced by almost three quarters of an hour (–39.4 min ± 8.6, $N = 27$, $t = –4.56$, $P < 0.001$) which amounts to a reduction of more than 5%. There was a small increase of several minutes in evening and morning latency (respectively 1.4 min ± 0.1, $N = 27$, $t = -2.15$, $P = 0.04$ and 2.2 min ± 0.2, $N = 27$, $t = 3.91$, $P < 0.001$). There was however no effect on sleep bout length or frequency (respectively: $\chi^2_1 = 0.512$, $P = 0.474$ and $\chi^2_1 = 0.780$, $P = 0.377$).

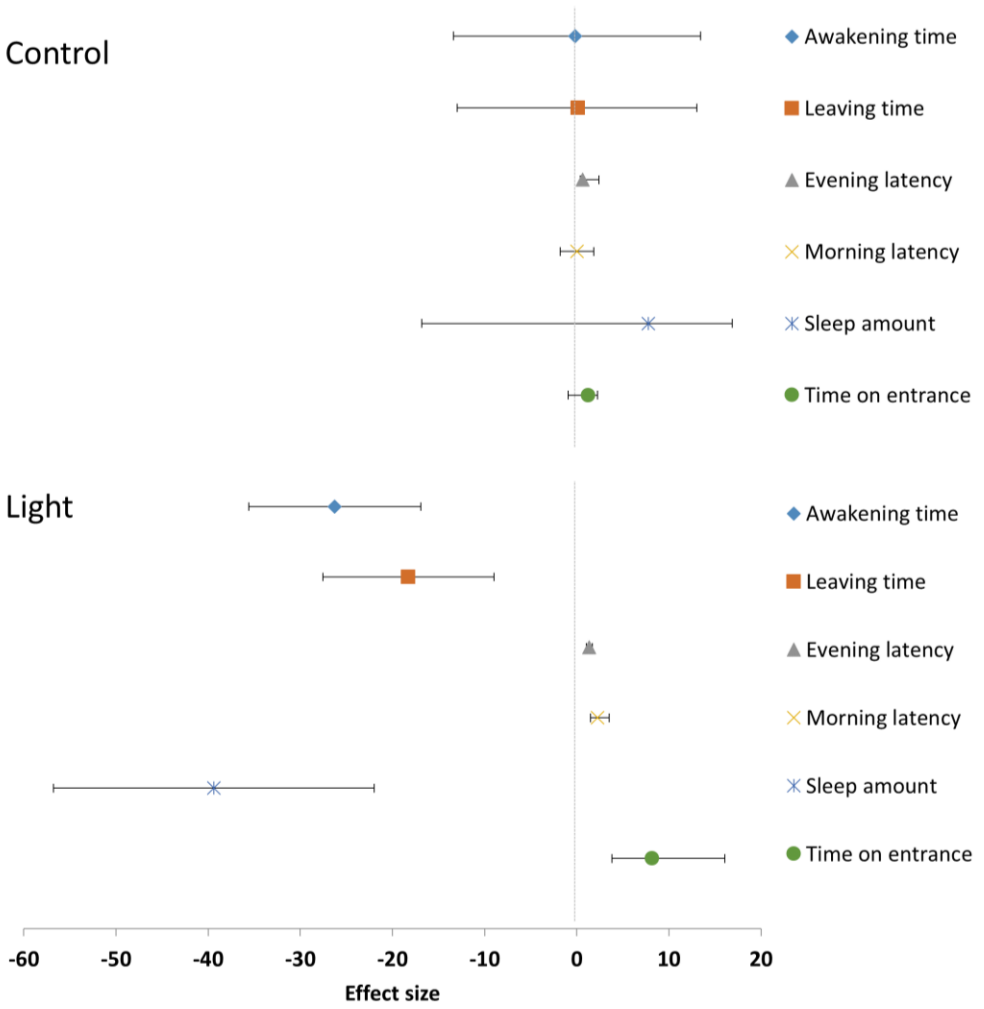

Figure 2: Effect of artificial light at night on sleep parameters. Shown are effect sizes and 95% confidence intervals of the contrast between the first and second night of sleep behaviour. Only sleep parameters that were significantly affected by artificial light are shown, with the top panel showing the effect sizes in the control group (sleeping in a natural dark situation on both nights), and the lower panel showing the effect sizes in the treatment group. Effect sizes are given in minutes, except for 'time on entrance' which is given in seconds, and are from a GLMM with bird identity nested in recording session (data were collected during six sessions; see Methods) as random factor to correct for repeated measurements.

Effects of artificial light on behaviour in the nest-box after awakening

Besides the effect of artificial light on morning latency, it significantly affected activity in the nest-box after awakening (see Figure 2, Tables S2 and S3). When exposed to artificial light, birds went significantly more often to the nest-box entrance (1.5 ± 0.4, $Z = 3.80$, $P < 0.001$) and spent more time on it before leaving the nest-box (8.2 sec ± 0.4, $t = -5.76$, $P < 0.001$) compared to birds in the dark.

Discussion

Previous studies reported that artificial light significantly affected activity patterns and the onset of dawn chorus in songbirds (Da Silva et al. 2014; Dominoni et al. 2013b; 2014; Kempenaers et al. 2010; Schlicht et al. 2014), making it likely that sleep would also be affected. To our knowledge, our study is the first to demonstrate experimentally that artificial light does indeed disrupt sleep behaviour in free-living animals. Light at night caused birds to wake up earlier and leave the nest-box earlier in the morning, and as a result sleep less. Although individuals did not wake up more often at night females, but not males, spent a greater proportion of the night awake. During the night, sleep bout length and frequency were unaffected. In the evening, there was no direct effect of artificial light on sleep as birds did not fall asleep or enter the nest-box later. Nonetheless there was a small but significant increase in the time spent between entering the nest-box and falling asleep. Great tits were also less likely to enter an artificially lit nest-box.

Artificial light affected sleep in particular in the morning, with more subtle effects in the evening and during the night. We found that great tits woke up and left the nest-box earlier as a consequence of artificial light, perhaps because they perceived it as if the sun had already risen (Steinmeyer et al. 2010; Titulaer et al. 2012). Birds in artificially lit nest-boxes also went more often to the nest-box entrance and spent more time on it during morning latency, which could be interpreted as birds being confused by the artificial light as it did not match with the light levels outside. In urban areas, similar behaviour might be found where there are street lights which are turned on in the morning which could “confuse” the birds. Additionally, the birds spent more time between waking up and leaving the nest-box, as demonstrated by the small but consistent increase in morning latency.

The increase in evening latency suggests that animals took longer to fall asleep after they entered the artificially lit nest-box. Although we found no direct evidence that artificial light affects sleep onset, Da Silva et al. (2014) found that street lights can prolong activity, which

could thereby indirectly cause animals to fall asleep later. Although most studies found that light pollution causes songbirds to advance the onset of activity (Da Silva et al. 2014; Dominoni et al. 2013b; 2014; Kempenaers et al. 2010) the effects on cessation of activity are inconsistent between studies and species (see Byrkjedal et al. 2012; Dominoni et al. 2013b; 2014 and Da Silva et al. 2014; Dominoni and Partecke 2015; Russ et al. 2014). Da Silva et al. (2014) showed that great tits continue singing longer at dusk because of light pollution. However an experiment on activity patterns of great tits did not find artificial light to cause a later cessation of activity or an earlier onset (Titulaer et al. 2012). There are two reasons which could explain this discrepancy in the results found on cessation of activity between our study and those of Da Silva et al. (2014) and Titulaer et al. (2012). First, the methodologies that were used differed between the studies. We used an experimental approach using lights installed inside nest-boxes, while the study of Titulaer et al. (2012) used lights which were installed outside of the nest-box (white LED lights, 10 lux). This potentially reduced the light intensity that actually reached the birds while roosting inside the nest-box. Da Silva et al. (2014) used a correlational approach comparing light polluted areas against dark areas. Our lights did not illuminate a substantial part of the environment outside of the nest-box and hence did not allow animals to be active for an extended period, contrary to light pollution caused by street lights (Da Silva et al. 2014). Second, our study was conducted before the peak of the breeding season while the study of Da Silva et al. (2014) was conducted before and during the breeding season and the study of Titulaer et al. (2012) used nests with 1-16 day old chicks, which may have influenced leaving time (Schlicht et al. 2014).

A study on the relation between emergence time and extra-pair paternity showed that female blue tits emerged 20 minutes earlier when an artificial light (white LED), also placed inside the nest-box, was switched on one hour before sunrise (Schlicht et al. 2014). Whether, except for emergence time, also sleep was affected was not studied. Although the light we used was switched on for the entire night, the effect on leaving time appears very similar, suggesting that the effect of artificial light on awakening and leaving time could depend mainly on light intensity directly before sunrise. We used a relatively low light level (compared to street lights) which did not affect sleep bout length or frequency (sleep quality). However, females significantly reduced the proportion of time spent asleep in the nest-box. Turning lights off during part of the night, e.g. from midnight to 05:00 (as an alternative lighting strategy) may, therefore, still produce profound negative effects on sleep (Gaston et al. 2012) also because

we found most effects in the morning. Nonetheless it could mitigate part of the effects on sleep behaviour of birds and mitigate other effects on a large diversity of other organisms (Gaston et al. 2012; 2013).

In general, the effect of artificial light on sleep did not differ between sexes in our study. However, we did find that females, unlike males, reduced the proportion of time spent asleep while in an artificially lit nest-box. It is difficult to explain this small but consistent effect, although it is known that male and female blue tits differ in their sleeping behaviour (Steinmeyer et al. 2010) and we also found that in a natural dark situation male great tits entered the nest-box later, fell asleep later and slept less than females. Such sex differences could perhaps explain why the effect of artificial light differed between sexes for this aspect of sleep.

This study was performed before the breeding season. Other differences in how artificial light affects males and females could perhaps be found during the breeding season. Given that great tits sleep proportionally less during the breeding season than before the breeding season, (between 48 and 74% during the breeding season (Christe et al. 1996) and around 94% in our study) and that the difference in sleep between blue tit males and females is greater near the breeding season (Steinmeyer et al. 2010), it is important to carry out additional experiments at different periods of the year. However, during the breeding season mainly females occupy the nest-boxes making it more difficult to collect data on male sleep behaviour in great tits.

Previous research on effects of artificial light on activity patterns and onset of dawn chorus in songbirds (Da Silva et al. 2014; Dominoni et al. 2013b; 2014; Kempenaers et al. 2010; Schlicht et al. 2014) provided only some clues that artificial light may reduce sleep as the results may have been caused either by birds falling asleep later and/or waking up earlier. Our results show that artificial light did not cause individuals to enter the nest-box or fall asleep later and that the effect on evening latency, although highly significant, only amount to a few minutes. We can therefore conclude that the reduction in sleep amount of almost three quarter of an hour (more than 5% reduction compared to a natural dark situation) mainly results from animals waking up earlier and that most effects occur in the morning. Experimental birds did not only spend less time in the nest-box but they also slept less because of artificial light. Individuals in the light group did leave the nest-box earlier and the advancement of awakening time was even larger. Surprisingly, animals did not wake up more often at night, nor did they

spend longer periods awake between sleep bouts, indicating that artificial light during the night did not disrupt these aspects of sleep once birds were asleep.

With this study we provide the first direct experimental proof that light pollution can have a significant impact on several aspects of sleep behaviour. These results point out to a mechanism through which light pollution may affect fitness (Steinmeyer et al. 2013) which requires further investigation. In blue tits it was shown that light pollution advanced their dawn song and that males had more extra-pair paternity (Kempenaers et al. 2010). Advancement of dawn song may indicate that light pollution also affected sleep, which is then correlated with increased male extra-pair paternity. In a different study it was shown that for female blue tits, artificial light advanced emergence time but did not affect extra-pair paternity (Schlicht et al. 2014). However, experimentally delayed awakening and leaving time (through administration of melatonin and not light pollution) increased the risk of cuckoldry in great tits (Greives et al. 2015). Quite clearly, further research is needed to assess the costs and benefits of disruption of sleep by light pollution.

As this is the first study on the effects of artificial light on sleep behaviour in the wild, we used a cavity-nesting bird as a model species. Because it is possible to manipulate light conditions within a nest-box, they are ideal study species to study effects of light on sleep in the wild. Experimental manipulation of light conditions of open-nesting birds is much more difficult. We believe that our results offer a first indication of how artificial light affects sleep in free-living birds and that these results could also be relevant for other animals exposed to light pollution as they are exposed to similar and even higher light intensities (Dominoni et al. 2013a; Gaston et al. 2013). However, we recognise that besides similarities there are also differences in sleep between mammals, birds and invertebrates (Lesku et al. 2011; Rattenborg et al. 2011; Rattenborg and Martinez-Gonzalez 2015) as well as between different bird species (e.g. Lesku et al. 2012; Steinmeyer et al. 2013). For instance, sleep could differ between species because of differences in exposure to predators (Lima et al. 2005). Although sleep quantity was affected, we did not find any effect on sleep quality (measured behaviourally) during the pre-breeding season using a light intensity of 1.6 lux. However, there is now an urgent need to perform similar studies in different periods of the year, using different light intensities and to look at the effects of this sleep disruption on fitness. A proper assessment of short and long-term effects on fitness would require a larger sample size then we used here and perhaps also a longer period of light exposure.

Our experimental setup has great potential for further research to elucidate short-term physiological effects as well as short- and long-term fitness effects of artificial light in free-living birds. Using our experimental setup, the light regime could also be manipulated in terms of light intensity, duration and spectra (light colours) in future studies. This is necessary for crucial research on the effects of light pollution (Gaston et al. 2013) and can be used to investigate the effectiveness of management options to reduce consequences of artificial light at night (Gaston et al. 2012). Our experimental setup and approach can be used with other cavity-nesting species and at different times of the year (outside versus within the breeding season), which will undoubtedly yield new insights about the effects of light pollution under natural conditions.

Supplementary material

Methodology

Selection of nest-boxes

Nest-boxes with a maximum light intensity of 0.3 lux (range: 0.01 - 0.26 lux, average: 0.12 lux) at the entrance hole of the nest-box were selected for this experiment (light intensity inside nest-boxes: ± 0.01 lux). These nest-boxes were not under the direct influence of street lights but under a natural light regime. Light measurements were carried out from January 27 till January 31 2014 at least one hour after sunset with a light meter (ISO-Tech ILM 1335, Corby, UK; range 0.01 to 30000 lux) at the nest-box entrance hole. The light meter was placed vertically on the entrance and the maximum value of light intensity was obtained.

Statistical analysis

We analysed the relationship between sleep parameters using a Spearman rank correlation test (in the psych package; Revelle 2014) on the sleep behaviour of the first night (dark control) of both treatment groups. To avoid pseudoreplication the second night of the control group was not used. Although we found significant correlations between some sleep parameters (sleep onset and entry time as well as sleep bout and sleep bout per hour; Table S4), we chose to analyse each parameter separately as artificial light could influence sleep parameters independently from each other. We followed Steinmeyer et al. (2014) in testing all sleep parameters even though they can be correlated, as they found that they can be affected independently from each other (natural light affected awakening time but not leaving time).

Since each bird was tested more than once, and the birds are tested within sessions, we cannot assume independence between the observations in this dataset. This violates a key assumption of the classic ANOVA and regression analysis, that the error terms are independent. Also non-parametric tests assume that all observations are independent. Mixed models are a widely used technique to account for the non-independence between observations in a dataset, by including random effect terms into the regression equation. The significance of the independent variables (“fixed effects”) is hereby calculated, accounting for the non-independence of the observations within the same individual (or session; Fitzmaurice et al. 2004).

Evening and morning latency and time on entrance were log transformed to reduce right-sided skew. To test whether artificial light changed sleep behaviour from the first night compared to the second night, we tested the interaction night*treatment. Activity during morning latency (time on entrance and number of times on entrance) was analysed with a linear mixed effects analysis (see main text). A generalized linear mixed model with a Poisson error structure was used to analyse the effect of artificial light on "number of times on entrance". We were mainly interested in the interactions sex*treatment*night and night*treatment to show the effect of artificial light on sleep and in the main effects sex and date as control variables.

Model selection was done by backward elimination of non-significant ($P > 0.05$) factors and interactions, starting with the highest order interaction. P-values were obtained by likelihood ratio tests of the full model against the reduced model.

Table S1: Experimental design. The number of individuals is given per treatment. A total of 54 observations from 27 individuals were obtained and used in the analysis of differences in sleeping behaviour.

Treatment	Night (light off/ on)		Observations	Individuals total (N)	Male (N)	Female (N)
	1	2				
Control	OFF	OFF	18	9	3	6
Light	OFF	ON	36	18	11	7
Total			54	27	14	13

Table S2: Effect of sex, date and artificial light (treatment) on sleep parameters. To correct for changes in day length, response variables were standardised to civil sunset (entry time, sleep onset) or sunrise (awakening time and leaving time). GLMM models were used with bird identity nested in recording session (data were collected during six sessions; see Methods) as random factor to correct for repeated measurements. Significant P values are shown in bold, *N* = 27.

Sleep parameter	Sex[a]			Date[b]			Night*Treatment		Sex*Treatment *Night	
	Estimate ± SE	X^2	p-value	Estimate ± SE	X^2	p-value	X^2	p-value	X^2	p-value
Entry time	15.05 ± 3.96	12.389	**<0.001**	0.04 ± 0.68	0.004	0.950	1.055	0.304	1.368	0.242
Sleep onset	15.00 ± 3.94	12.757	**<0.001**	0.05 ± 0.69	0.006	0.937	2.418	0.120	1.104	0.293
Awakening time	-7.71 ± 4.36	3.1269	0.096	0.06 ± 0.70	0.006	0.941	9.949	**0.002**	0.900	0.353
Leaving time	-4.94 ± 3.88	1.755	0.185	0.18 ± 0.38	0.247	0.619	5.429	**0.020**	1.426	0.232
Evening latency[c]	-0.03 ± 0.13	0.086	0.770	0.01 ± 0.01	0.596	0.440	10.891	**0.001**	0.523	0.470
Morning latency[c]	0.12 ± 0.22	0.402	0.526	0.02 ± 0.06	0.242	0.623	5.153	**0.023**	1.425	0.233
Time on entrance[c]	0.05 ± 0.38	0.072	0.789	0.08 ± 0.11	0.860	0.354	8.633	**0.003**	1.337	0.248
Nr times on entrance	-0.12 ± 0.26	0.251	0.616	0.01 ± 0.04	0.135	0.714	13.814	**<0.001**	0.043	0.836
Sleep amount	-4.01 ± 8.22	4.294	**0.038**	-3.17 ± 1.19	7.047	**0.008**	9.669	**0.002**	1.553	0.213
Sleep bout (sec)	-42.06 ± 61.73	0.512	0.474	11.06 ± 6.05	3.192	0.074	2.479	0.115	0.261	0.609
Sleep bout/h	0.39 ± 0.49	0.780	0.377	-0.09 ± 0.06	2.981	0.084	1.835	0.176	1.4429	0.230
Sleep %	-	-	-	-0.01 ± 0.01	0.827	0.363	-	-	13.123	**<0.001**
Sleep % male	-	-	-	-	-	-	2.774	0.096	-	-
Sleep % female	-	-	-	-	-	-	62.536	<0.001	-	-

[a] Effects of sex (females-males) were estimated as differences in means.

[b] The effects of date are given as slopes.

[c] Values were log transformed to reduce skew.

Table S3: Descriptive statistics of sleep in great tits under natural dark conditions and the effect of artificial light on them. Entry time and sleep onset are standardised to civil sunset and awakening, and leaving time are standardised to sunrise. SD is standard deviation, CI is 95% confidence interval. See also Table S2 and Figure 2.

	Natural conditions				Effect of artificial light		
Sleep parameter (min)[#]	Average	Min	Max	SD	Estimate	CI low	CI high
Entry time	-4.9	-33	22	15.2			
Sleep onset	-0.2	-30	24	15.1			
Awakening time	-26	-43	-10	8.8	-26.3	-16.9	-35.6
Leaving time	-21.8	-34	-9	7.4	-18.3	-9.0	-27.6
Evening latency	4.7	2	13	2.5	1.3	1.7	1.0
Morning latency	4.7	0	11	3.5	2.2	3.5	1.5
Time on entrance*	47.3	2	261	63.9	8.2	16.0	3.8
Nr times on entrance	1.8	1	5	1.0			
Sleep amount	731.5	694	807	29.9	-39.4	-22.0	-56.8
Sleep bout*	678.5	317	1040	183.4			
Sleep bout/h	5.4	3.3	10.1	1.6			
Sleep proportion	94%	89%	98%	2%			

[#] Unless stated otherwise

* Values in seconds.

Table S4: Correlation coefficients describing the relationship between sleep parameters. Spearman rank correlation tests with P-values adjusted for multiple testing (Holm method) were used on the averaged data from control nights. Significant correlations ($P < 0.05$) are shown in bold, trends ($P < 0.10$) are underlined, other correlations are not significant, $N = 27$.

Sleep parameters	Entry time	Sleep onset	Evening latency	Awakening time	Leaving time	Morning latency	Sleep amount	Sleep %	Sleep bout/h
Sleep onset	**0.99**								
Evening latency	-0.01	0.06							
Awakening time	-0.27	-0.26	-0.05						
Leaving time	-0.59	-0.59	-0.03	0.60					
Morning latency	0.34	0.35	0.31	0.00	-0.26				
Sleep amount	-0.58	-0.57	-0.06	0.13	0.53	-0.40			
Sleep %	0.14	0.12	-0.45	-0.02	0.09	-0.44	0.38		
Sleep bout/h	0.23	0.21	-0.09	-0.12	-0.17	0.12	-0.39	-0.19	
Sleep bout (sec)	-0.24	-0.22	0.05	0.13	0.22	-0.15	0.41	0.25	**-0.99**

Chapter 3

Artificial light at night disrupts sleep in female great tits (*Parus major*) during the nestling period, and is followed by a sleep rebound

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Abstract

Artificial light at night has been linked to a wide variety of physiological and behavioural consequences in humans and animals. Given that little is known about the impact of light pollution on sleep in wild animals, we tested how experimentally elevated light levels affected sleep behaviour of female songbirds rearing 10 day old chicks. Using a within-subject design, individual sleep behaviour was observed over three consecutive nights in great tits (*Parus major*), with females sleeping in a natural dark situation on the first and third night, whereas on the second night they were exposed to a light-emitting diode (1.6 lux).

Artificial light in the nest box dramatically and significantly affected sleep behaviour, causing females to fall asleep later (95 min; while entry time was unaffected), wake up earlier (74 min) and sleep less (56%). Females spent a greater proportion of the night awake and the frequency of their sleep bouts decreased, while the length of their sleep bouts remained equal. Artificial light also increased begging of chicks at night, which may have contributed to the sleep disruption in females or vice versa. The night following the light treatment, females slept 25% more compared to the first night, which was mainly achieved by increasing the frequency of sleep bouts. Although there was a consistent pattern in how artificial light affected sleep, there was also large among-individual variation in how strongly females were affected. When comparing current results with a similar experiment during winter, our results highlight differences in effects between seasons and underscore the importance of studying light pollution during different seasons. Our study shows that light pollution may have a significant impact on sleep behaviour in free-living animals during the reproductive season, which may provide a potential mechanism by which artificial light affects fitness.

Introduction

Life on earth has evolved under daily and seasonal cycles of light and dark (Kronfeld-Schor et al. 2013; Tan et al. 2010). Humans profoundly alter these natural light cycles through the introduction of artificial light at night (ALAN), also known as light pollution. For a long time, exposure to ALAN was considered to be an innocuous environmental manipulation for humans without serious repercussions. However, the loss of darkness is a potentially important threat for wildlife, biodiversity and humans (Duffy et al. 2015; Gaston et al. 2013; Hölker et al. 2010b; Navara and Nelson 2007; Rich and Longcore 2005). Recently there is increasing evidence that disruption of our naturally evolved light and dark cycles can result in a wide range of biological responses (Bedrosian et al. 2016; Swaddle et al. 2015).

For example, ALAN can affect individual animal behaviour (Longcore and Rich 2004; Rich and Longcore 2005). In several bird species, light pollution has been shown to extend daily activity (Da Silva et al. 2014; 2015; Dominoni et al. 2014; Dominoni and Partecke 2015; Schlicht et al. 2014), including foraging activity (Russ et al. 2014; Stracey et al. 2014). Furthermore, ALAN appears to affect reproductive behaviour, by advancing the laying date of some songbirds (de Jong et al. 2015; Kempenaers et al. 2010), and/or by affecting the singing behaviour of males in the morning (Kempenaers et al. 2010). Blue tit males (*Cyanistes caeruleus*) that sing earlier because of light pollution are able to increase extra-pair paternity gain (Kempenaers et al. 2010).

Light pollution is also likely to negatively affect nocturnal sleep (Raap et al. 2015), which is an important animal behaviour widespread across the animal kingdom (Cirelli and Tononi 2008; Siegel 2008), and serves multiple purposes including energy conservation and memory consolidation (Gobes et al. 2010; Roth II et al. 2010). Consequently, the deprivation of sleep can have negative effects (Cirelli and Tononi 2008; Siegel 2008) such as reduced cognitive functioning (Rattenborg et al. 2004), and can ultimately affect fitness. For example, a case report on zebra finches (*Taeniopygia guttata)* suggested that continuous light led to sleep deprivation and increased mortality (Snyder et al. 2013). Nevertheless, also positive effects of reduced sleep have been reported under natural conditions in the pectoral sandpiper (*Calidris melanotos),* where males that slept less sired more offspring (Lesku et al. 2012).

Two recent studies used an experimental approach with artificial light in the nest box to study the effects of light pollution. In blue tits, emergence time was advanced by artificial light in order to study the effects on extra-pair paternity (Schlicht et al. 2014). More recently,

using the same approach, it was shown that continuous light at night disrupts sleep during winter in free-living great tits (*Parus major*; Raap et al. 2015). Effects were mainly found during the morning, when artificial light caused animals to wake up and leave the nest box earlier. Sleep behaviour does, however, differ between seasons, with animals sleeping more during winter and less during spring, as was found in blue tits (Steinmeyer et al. 2010) and great tits (Stuber et al. 2015b). ALAN could therefore affect sleep differently during the nestling period compared to the winter period, making it important to consider seasonal effects of light pollution. Specifically, sleep disruption by artificial light may be particularly costly during the breeding season as breeding is a costly individual investment (Nur 1984) and sleep is necessary for its restorative functions (Siegel 2009; Weljie et al. 2015; Xie et al. 2013). Additional "stress" from artificial light during the breeding season could therefore potentially lead to fitness effects.

Here, we used an experimental approach to explore the effects of artificial light on sleep behaviour in a free-living bird. Given that the effects of sleep disruption may be particularly deleterious during the breeding season, we studied the effects of ALAN on sleep behaviour of female great tits rearing 10 day old chicks. Great tits readily sleep in nest boxes enabling us to manipulate light conditions and to study their sleeping behaviour in a way that, at present, is probably not possible in wild open-nesting birds. In addition to observing female sleeping behaviour, our approach enabled us for the first time to assess whether artificial light (indirectly) affected the nestlings, which potentially also could be linked to effects on the females. We used a within-subject design to observe individual sleep behaviour, with females sleeping in a natural dark situation the first and third night (control nights), while artificial light was provided during the second night. Using a within-subject approach is important as this effectively controls for the large variability in sleep behaviour among individual great tit females (Stuber et al. 2015b) and for confounding variables, which may differ between individuals/nests such as, for example, differences in brood size (Ruxton and Colegrave 2010). To investigate potential seasonal differences in the effects of artificial light, we compared current results with our previous study which was done during winter (Raap et al. 2015). Additionally, we investigated whether the light manipulation during the second night was followed by sleep recovery during the third night. Several laboratory studies have shown in (amongst others) humans (Beersma et al. 1990), rats (Marinesco et al. 1999) and pigeons (*Columba livia*; Newman et al. 2008) that sleep deprivation is followed by recovery sleep or

"sleep rebound". Recovery of sleep is important in biochemical and molecular events that restore balance and decrease cell injury (Everson et al. 2014). Therefore, we expected that females that lose sleep due to the light treatment will sleep more the following dark night (i.e. they show a "sleep rebound"), which may indicate that sleep is essential and sleep disruption is costly.

Methodology

Study area and general procedures

We collected data between April 27th and May 10th 2014 in a resident suburban nest box population of great tits in the surroundings of Wilrijk, Belgium (51°9'44''N, 4°24'15''E). This nest box population has been established in 1997 and has been monitored continuously since then (Rivera-Gutierrez et al. 2012; Van Duyse et al. 2005). During the breeding season, nest boxes were checked every other day, to monitor the different breeding stages, and every day just before egg-laying. During previous breeding seasons and winters nest boxes were also regularly checked to capture, ring and monitor individuals. Female age was determined by using hatching records for resident birds and by comparing the coloration of primary and secondary coverts for individuals that did not hatch in our nest box population. Using the latter method, individuals could be classified as either yearling (grey primary coverts) or adult/ older (bluish primary coverts; Gosler 1993; Rivera-Gutierrez et al. 2010).

Experimental procedure

A paired design was used in which sleep behaviour of 19 females was observed over three subsequent nights. During the first and third night the nest box was dark while on the second night birds slept with a light turned on (first night: pre-control, second night: light and third night: post-control).

Recording sleep behaviour and light treatment

Recording of female sleep behaviour started when chicks were 10 days of age (day of hatching is day 1). Sleep behaviour was recorded as described in Raap et al. (2015). In brief, infrared sensitive cameras (Pakatak PAK-MIR5) were installed under the nest box roof lid before 16:00 in the afternoon and removed at the earliest at 10:00 the next morning (recordings started after installation). In each nest box a small white LED light (15 mm x 5 mm, taken from a RANEX 6000.217 LED headlight) was placed (simultaneously with the video camera) above the nest

box entrance hole on the inside, pointing downwards. These LEDs were standardized to produce 1.6 lux on the bottom of the nest box as measured with an ISO-Tech ILM 1335 light meter. Especially outside of nest boxes or cavities, birds living in light polluted areas are exposed to similar and higher light intensities (Dominoni et al. 2013a; Gaston et al. 2013). Because there is now a shift towards energy efficient broad spectrum light sources such as LED (Davies et al. 2013; Schubert and Kim 2005) we chose to work with white LED light. Because LED light is very energy efficient there is no temperature/ warming effect of the lights inside the nest boxes. On the first and third night the LED was present in the nest box but turned off. On the second day/night of recording the LED was turned on simultaneously when recordings started, before 16:00 roughly five hours before sunset, allowing animals to become accustomed to changed light conditions. The following morning the light was turned off when the recordings ended (after 10:00).

Sleep parameters

Sleep of great tits was quantified in detail using 10 parameters: (1) entry time, (2) sleep onset, (3) evening latency, (4) awakening time, (5) leaving time, (6) morning latency, (7) sleep amount, (8) sleep proportion, (9) frequency of sleep bouts and (10) sleep bout length (see also supplementary material Figure S1). We followed the definition of sleep parameters as described in earlier studies on great tit and blue tit sleep behaviour (e.g. Raap et al. 2015; Steinmeyer et al. 2010).

In short, when a bird showed the classical sleep position (beak pointing backwards and tucked under the scapulars), it was considered to be sleeping. It was usually easy to distinguish whether a bird was awake or asleep. Only in rare cases when an individual would occasionally sit quietly for some time with the head pointing forwards or not completely tucked under the scapular, this was more difficult. Because these periods were often followed by the classical sleep position we defined them as awake (conform Steinmeyer et al. 2010).

Entry and leaving time were defined as when the female entered (in the evening) or respectively left the nest box (in the morning). The first time a sleep bout of minimum 30 seconds had started was used as sleep onset. Evening latency is the time between entry time and sleep onset. The last time a bird was asleep, after which it went to the nest box entrance hole, was used as awakening time. The last sleep bout was almost always more than 30 seconds long. Morning latency was calculated as the time between awakening time and leaving time. The sum of all sleep bouts was defined as sleep amount. We calculated sleep proportion as

sleep amount divided by the total time spent inside the nest box. The number of sleep bouts was calculated per hour (spent inside the nest box) as frequency of sleep bouts.

Begging

We scored begging behaviour of chicks during the time when the female was present in the nest box, between entry time and leaving time. Begging was defined as the moment when one or more chicks had their beak wide open and pointed upwards (Kolliker et al. 1998). The time between when one or more chicks started begging until the last one closed its beak was defined as a begging bout. Begging amount (sum of begging bouts) was scored for the entire nest as one unit because individual recognition of chicks was impossible. Begging frequency was calculated as begging/ hour: number of begging bouts divided by the time when the female was present inside the nest box.

Data analysis

For all statistical analyses we used R 3.0.2 (R Core Team 2013). We converted entry time, sleep onset, awakening time and leaving time to times relative to sunset or sunrise (reference data from Antwerp were used).

A key assumption of the classic ANOVA and regression analysis is that the error terms are independent. We cannot assume independence between the observations in this dataset since each bird/ nest was observed more than once. We therefore used mixed models (LMM) which are a widely used technique to account for the non-independence between observations in a dataset, by including random effect terms into the regression equation. The significance of the independent variables ("fixed effects") is hereby calculated, accounting for the non-independence of the observations within the same individual (Fitzmaurice et al. 2004).

For each sleep parameter, as well as for "begging amount" and "begging frequency" a separate linear mixed effect analysis was performed (using the lme4 package; Bates et al. 2013). The full model was constructed with as fixed effects: night (pre-control/ light/ post-control), date (Julian day), brood size (number of chicks) and female age (yearling/ older). We included nest identity as a random factor (to include repeated measures). In order to meet model assumptions, outliers for awakening time (two observations, on night two with light, which were more than six hours before sunrise) were removed based on Cleveland plots (Zuur et al. 2010). Although females always entered their nest box in the evening and left after midnight, one individual did not sleep in the nest box on the second night and another did not

on the third night. While sleep behaviour could not be scored in these two cases, both individuals only left after being inside for more than three hours, therefore these nests were used in the analyses on begging behaviour and entry and exit time. Morning latency and begging amount were log transformed, begging frequency was square root transformed. Model selection was done by backward elimination of non-significant factors ($P > 0.05$, using the lmerTest package; Kuznetsova et al. 2014).

Where applicable, Least Squares Means were used for post-hoc analyses (using the lmerTest package; Kuznetsova et al. 2014). We also analysed whether begging amount was correlated with the amount of sleep of the female and whether a loss of sleep (amount) due to artificial light was related to a sleep rebound (Hmisc package; Harell and Dupont 2015). Results are presented as estimates which had been corrected for other significant factors with one standard error (unless stated otherwise).

We also compared our results with earlier results obtained during winter in the same population (Raap et al. 2015). This study used a similar design but without a post-control. From the winter study we had data from six females, which we used in a comparison to the results obtained from the current study.

Ethical statement

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. The Belgian Royal Institute for Natural Sciences (Koninklijk Belgisch Instituut voor Natuurwetenschappen) provided ringing licenses for all authors and the assisting technicians involved.

Results

Most sleep parameters were significantly affected by artificial light except entry time, morning latency and sleep bout length (Figure 1, 2, Table 1, 2 and Table S1). Artificial light reduced the amount of sleep by more than half (112.9 ± 23.4 minutes, $t = -4.80$, $P < 0.001$) as females fell asleep later (94.8 ± 21.6 minutes, $t = 4.39$, $P < 0.001$) and woke up earlier (-74.3 ± 13.1 minutes, $t = -5.70$, $P < 0.001$; values given are the differences between the first and second night). There was, however, large variation in the effect on awakening time (Figure 2). In order to meet model assumptions, two females that woke up six hours before sunrise had been excluded from the analysis (see Methods). Including these individuals would give a large increase in the estimate (-110.4 ± 23.87 minutes, $t = -4.62$, $P < 0.001$). Individual variability in effects of light on other

sleep parameters can also be seen in Figures 1 and 2. The proportion of sleep during the night was also reduced by almost half (reduction of 0.20 ± 0.04, $t = 3.60$, $P = 0.001$; Figure 3).

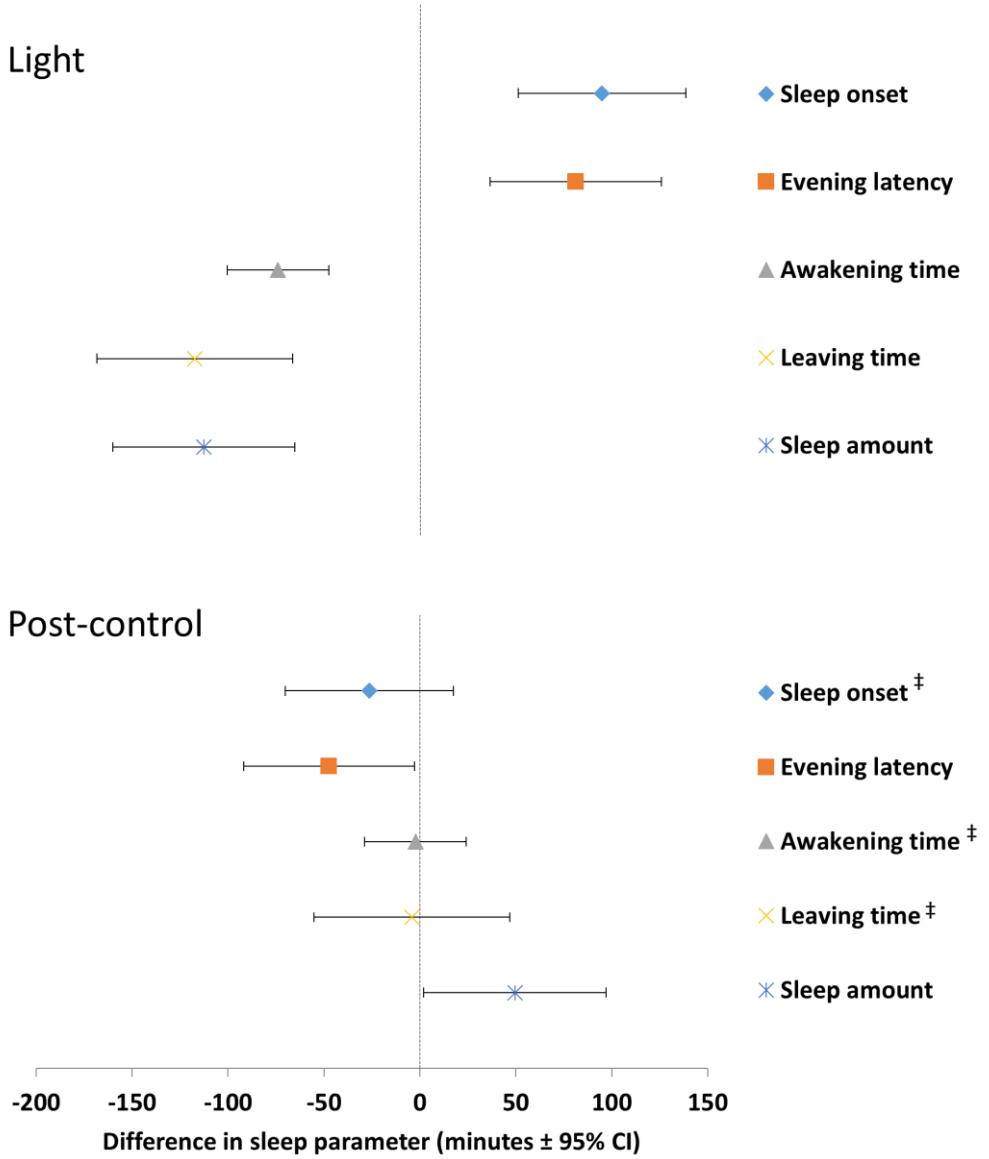

Figure 1: Effect of light on sleep and subsequent rebound. Effect of light (top panel) is the difference between night one and night two. The rebound during the post-control (lower panel) is the difference between night one and night three. Effect sizes and 95% confidence interval are obtained by using LMMs with nest identity as random factor to correct for repeated measurements (N = 19). ‡ indicates that a sleep parameter was not significantly different ($P > 0.05$), all other parameters were.

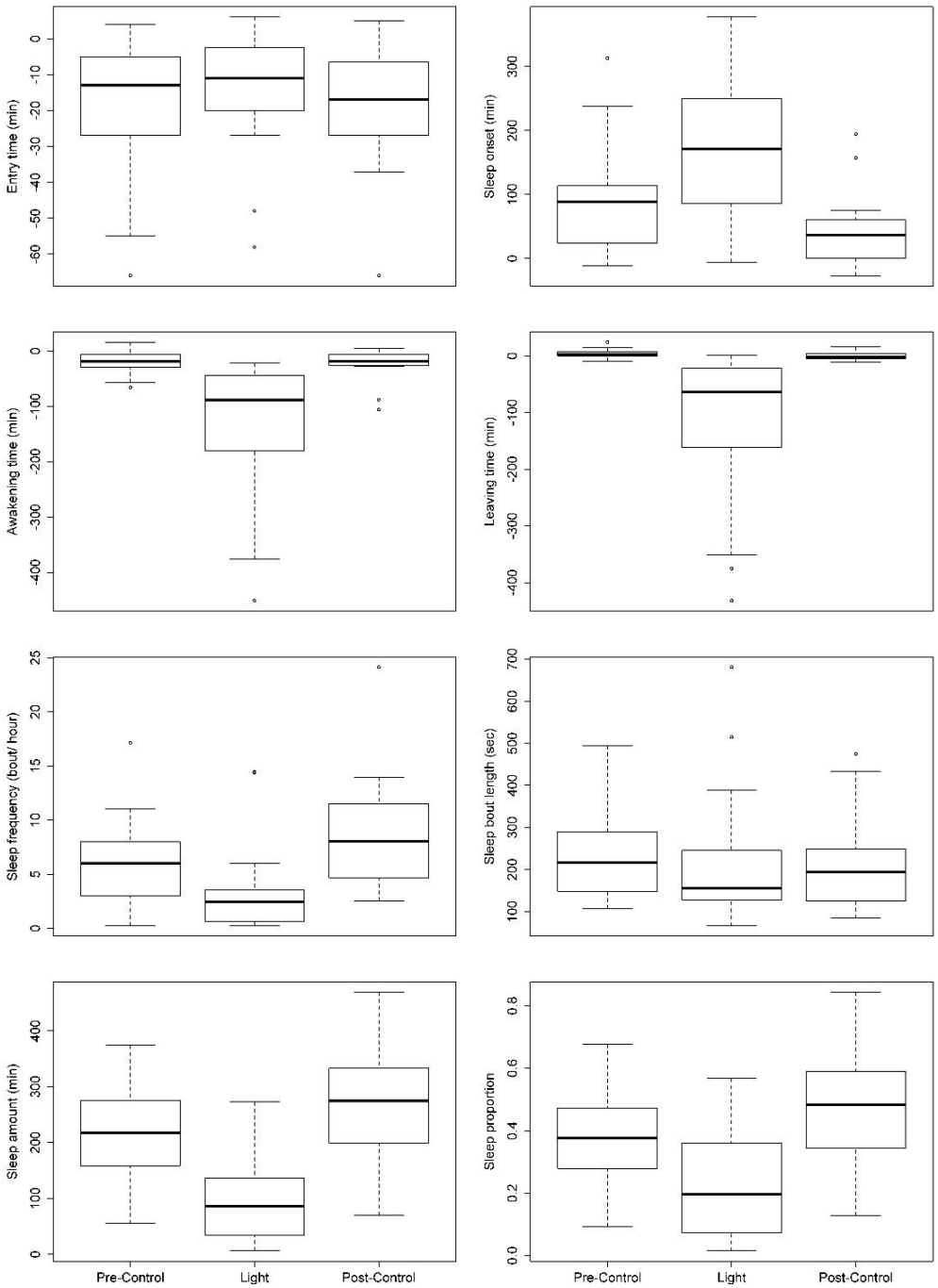

Figure 2: Differences in sleep behaviour between pre-control, light and post-control. Boxplots of raw data are presented with horizontal lines as medians, boxes show the interquartile range (IQR), whiskers show the full range excluding outliers (dots) defined as being more than ± 1.5 IQR outside the box. See Figure 1 and Table 2 for significantly affected sleep parameters.

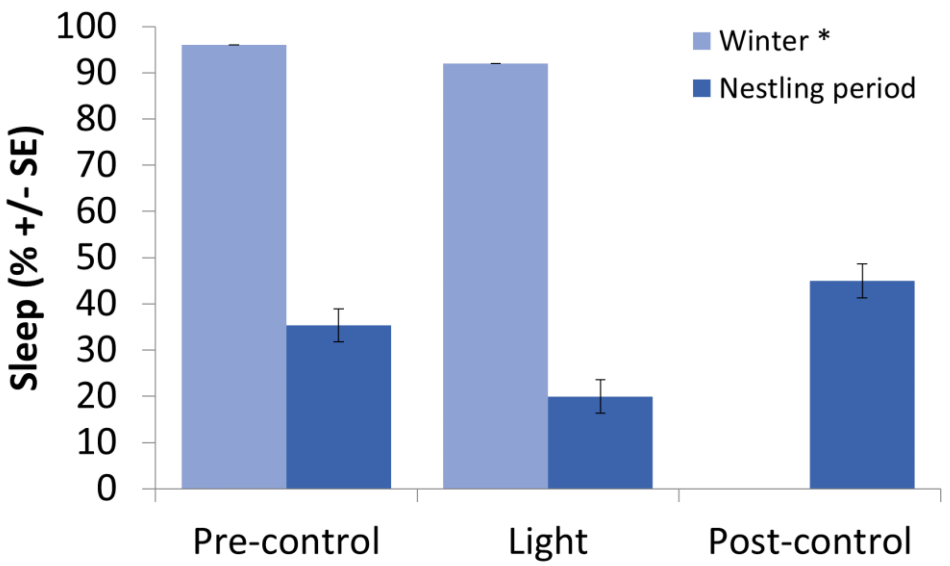

Figure 3: Effect of light on percentage of time spent asleep in the nest box. Estimates were obtained by using LMMs with nest identity (for winter data female identity) as random factor to correct for repeated measurements (N = 19). Differences between pre-control and light and pre-control and post-control are significant during the nestling period ($P < 0.05$; Table 2). During winter the difference between pre-control and light was also significant ($P < 0.001$).

* The data from the winter period (N = 6 females) come from Raap et al. (2015), in which a similar experimental set up was used but without a post-control.

Females took almost twice as long to fall asleep after entering the nest box (evening latency; 81.1 ± 22.0 minutes, t = -3.70, $P < 0.001$) and sleep frequency was reduced (with 2.7 ± 1.0 sleep bouts/hour, t = 2.72, P = 0.010; Figure 4). Artificial light caused females to leave the nest box almost two hours earlier (-117.7 ± 25.5 minutes, t = 4.62, $P < 0.001$).

In the night after the light treatment (post-light), sleep onset, awakening and leaving time returned to the same levels as measured during the first night (pre-control; Figure 1, 2, Table 2). The amount of sleep, however, increased compared to the first night (with 49.5 ± 23.4 minutes, t = -2.10, P = 0.04). Likewise, sleep frequency (with 2.8 ± 1.0 sleep bouts/hour, t = -2.80, P = 0.008) and sleep proportion (0.1 ± 0.0, t = -2.30, P = 0.010) also increased compared to the first night. The increase in sleep amount (rebound) was, however, not correlated with the loss of sleep through artificial light (r_s = -0.14, P = 0.578). Evening latency decreased to almost half the amount of the first night (-47.3 ± 21.9 minutes, t = 2.16, P = 0.038).

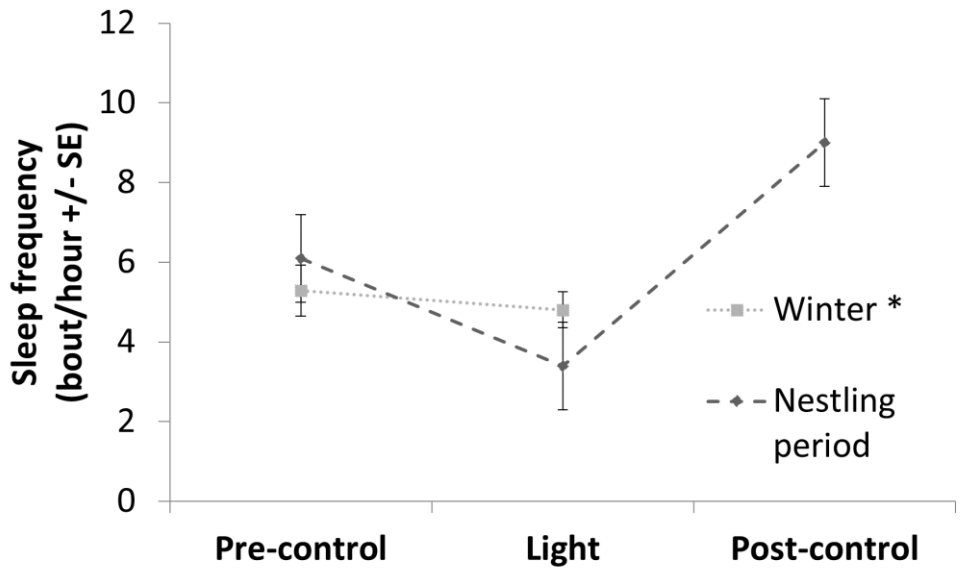

Figure 4: Effect of artificial light on sleep frequency and subsequent rebound. Estimates were obtained by using LMMs with nest identity (for winter data female identity) as random factor to correct for repeated measurements (N = 19). For the nestling period, differences between pre-control and light and pre-control and post-control are significant ($P < 0.05$; Table 2). During winter the difference between pre-control and light was not significant.

* Winter data of females (N = 6) from Raap et al. (2015) in which a similar experimental set up was used but without a post-control, were used for comparison.

Artificial light caused chicks to beg longer (about 5 minutes, t = -7.210, $P < 0.0001$) and with a higher frequency (an increase of about 4 begging bouts/hour, t = -8.21, $P < 0.0001$; Tables 1 and 2) during the night. There was, however, large variation in both the amount and frequency of begging between nests and how ALAN affected this behaviour (Figure 5). During the following dark (third) night, both the amount of begging (t = 1.390, P = 0.20) and the frequency of begging (t = 0.50, P = 0.6) returned to similar levels as during the first night. Female sleep amount was not correlated with the begging amount of their nestlings during the pre-control, light or post-control ($P > 0.18$ in all cases).

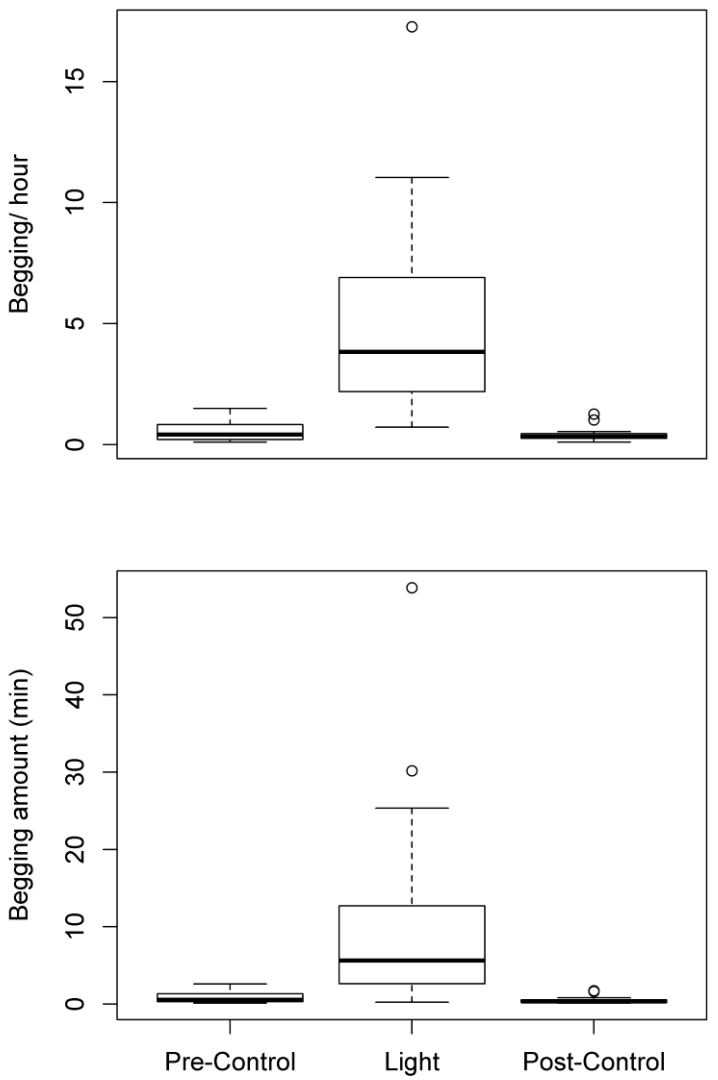

Figure 5: Variation in begging behaviour of chicks. Begging behaviour of the entire nest as a whole was scored during the time that the female was present inside the nest box, during all three nights for 19 nests with a total of 57 observations. Boxplots of raw data are presented with horizontal lines as medians, boxes show the interquartile range (IQR), whiskers show the full range excluding outliers (dots) defined as being more than ± 1.5IQR outside the box. Differences between pre-control and light are significant, differences between pre-control and post-control are not.

Table 1: Statistical output of the final mixed effect models, effect of night (pre-control, light, post-control), age, brood size and date on sleep behaviour and begging. To correct for changes in day length, response variables were standardized to civil sunset (entry time, sleep onset) or sunrise (awakening time and leaving time). LMM models were used with nest identity as random factor to correct for repeated measurements. *N* is the number of observations while Ind is the number of individuals (female great tits), for begging amount and begging/ hour Ind is the number of nests. Only significant effects from the final model are shown, statistical output of the full model can be found in the supplementary material Table S1.

	N	Ind	Night		Age		Brood size		Date	
			F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Entry time	57	19	-	-	-	-	-	-	5.377	0.031
Sleep onset	55	19	17.236	<0.001	-	-	-	-	13.152	0.002
Evening latency	55	19	16.953	<0.001	10.668	0.005	-	-	-	-
Awakening time	53	19	20.257	<0.001	-	-	-	-	-	-
Leaving time	56	19	13.624	<0.001	-	-	-	-	-	-
Morning latency	55	19	-	-	-	-	-	-	9.478	0.006
Sleep bout/ hour	55	19	14.788	<0.001	-	-	-	-	-	-
Sleep bout length	55	19	-	-	-	-	-	-	-	-
Sleep amount	55	19	24.472	<0.001	4.502	0.049	-	-	-	-
Sleep proportion	55	19	16.545	<0.001	14.918	0.001	-	-	-	-
Begging amount	57	19	42.662	<0.001	-	-	5.605	0.030	-	-
Begging/ hour	57	19	47.876	<0.001	-	-	-	-	-	-

Table 2: Post-hoc analysis of treatment on significantly affected sleep parameters and begging. Sleep onset, awakening time and leaving time are adjusted for sunrise/sunset. All parameters are given in minutes ± SE and 95% confidence interval (lower, upper) except for sleep frequency, sleep proportion and begging/ hour. Significant differences between light and pre-control and between post-control and pre-control are indicated with * $P < 0.05$ and ** $P < 0.001$.

	Pre-control	Light	Post-control
Sleep onset	76.1 ± 17.4 (41,0, 111.2)	170.9 ± 17.6 (135.5, 206.2)**	49.8 ± 17.8 (14.1, 85.6)
Evening latency	116.8 ± 18.2 (80.1,/ 154.0)	197.9 ± 18.8 (160.1, 236.0)**	69.5 ± 18.6 (32.0, 107.0)*
Awakening time	-20.3 ± 9.8 (-40.0, -0.6)	-94.6 ± 10.6 (-116.0, -73.3)**	-22.6 ± 10.0 (-42.8, -2.4)
Leaving time	3.8 ± 18.0 (-32.3, 39.9)	-113.9 ± 18.0 (-150.0, -77.8)**	-0.4 ± 18.5 (-37.5, 36.7)
Sleep frequency	6.1 ± 1.1 (3.98, 8.26)	3.4 ± 1.1 (1.2, 5.6)*	9.0 ± 1.1 (6.8, 11.1)*
Sleep amount	201.3 ± 22.0 (156.8, 246.0)	88.4 ± 22.5 (42.8, 134.0)**	250.8 ± 22.3 (205.6, 296.0)**
Sleep proportion	0.35 ± 0.04 (0.28, 0.43)	0.20 ± 0.04 (0.13, 0.27)*	0.45 ± 0.04 (0.38, 0.52)*
Begging amount [a]	3.52 ± 0.23 (3.06, 3.97)	5.74 ± 0.23 (5.28, 6.20)**	3.09 ± 0.23 (2.63, 3.55)
Begging / hour [b]	0.69 ± 0.13 (0.44, 0.95)	2.12 ± 0.13 (1.86, 2.37)**	0.61 ± 0.13 (0.35, 0.86)

[a] Original values in seconds are log transformed

[b] Values are square root transformed

Discussion

The unprecedented increase of light pollution is now being recognised as a potential threat for wildlife and biodiversity (Gaston et al. 2013; Hölker et al. 2010b; Rich and Longcore 2005). Using great tits as a model species, we documented for the first time the effect of artificial light on sleep during the nestling period in a free-living songbird. Large effects on almost all sleep parameters were found during the evening, morning and entire night. Sleep onset was delayed and the time between entering the nest box and falling asleep was increased in the evening. Females woke up and left the nest box earlier in the morning. The total amount of sleep was reduced by more than half, and although the length of sleep bouts was unaffected, their frequency was decreased during the night. All the above observations generally indicate that artificial light at night had a large effect on sleep behaviour in female great tits during the nestling period. Additionally, we found that artificial light caused nestlings to beg more at night. Females also showed a strong sleep rebound, which might be a physiological response to a lack of sleep during the previous night (Marinesco et al. 1999; Siegel 2009) caused by artificial light,

suggesting that sleep disruption is costly. There was, however, large variation in how strongly light affected sleep and to what extent individuals showed a subsequent sleep rebound.

Light dramatically affects sleep

In our study, ALAN did not cause great tits to enter the nest box later, in contrast to findings of other previous studies (Da Silva et al. 2014; Russ et al. 2014), probably because our light treatment inside the nest box did not illuminate the surroundings. However, ALAN caused females to fall asleep much later, either because after entering the box, the birds were subjected to the LED light, or indirectly because artificial light affected nestling behaviour as indicated in Figure 6.

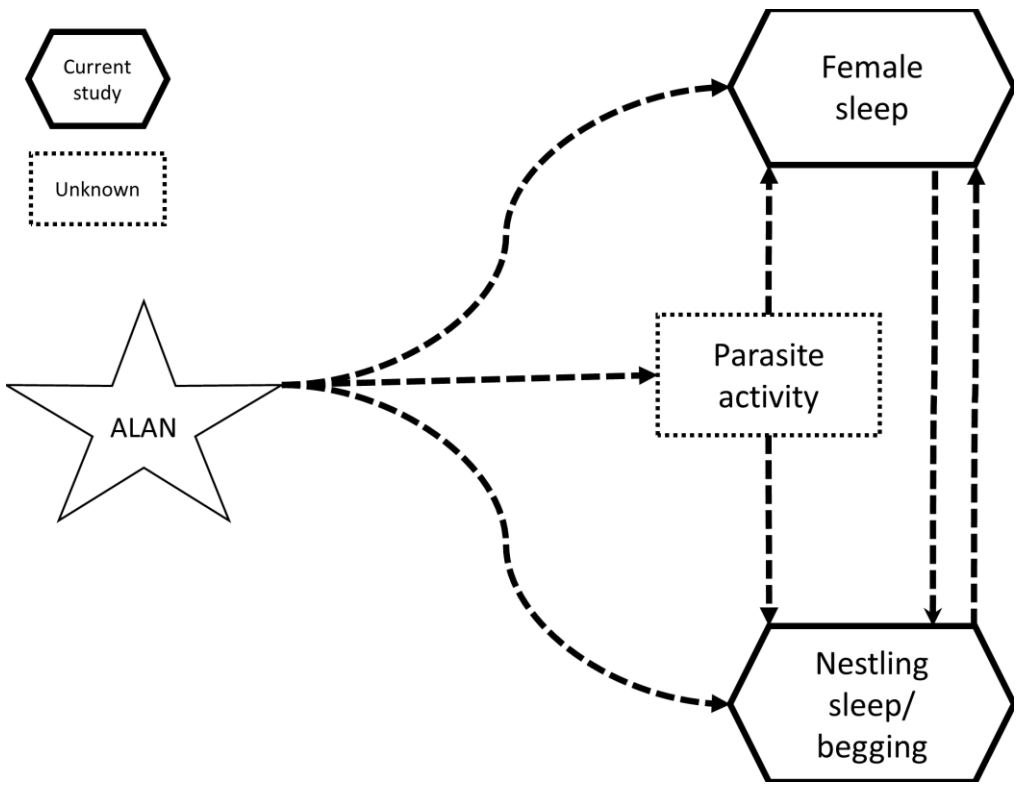

Figure 6: Schematic and simplified representation of direct and indirect effects of artificial light at night (ALAN). Effects of ALAN on female sleep and nestling begging can be caused directly. Disruption of female sleep can effect nestling begging and vice versa. ALAN may affect parasite activity and thus affect female sleep and or nestling begging behaviour, although this remains to be studied.

During the morning ALAN had additional large effects causing females to wake up one and a half hour earlier and leave the nest box almost two hours earlier. However, this is an underestimation of the true effect, as two females who left much earlier had to be excluded from the statistical analysis to meet model assumptions. The substantial effect on leaving time may be explained by females being triggered to start feeding as soon as possible. In a natural situation, great tits and blue tits leave the nest box around sunrise which enables them to search for food. Light and photoperiod will therefore serve as a cue to leave the nest box (Steinmeyer et al. 2010; Stuber et al. 2015b). Our experimental artificial light probably disrupted this cue and caused females to leave much earlier, even while it was still dark outside.

ALAN strongly reduced the total amount of sleep and the proportion of time spent asleep (time spent asleep divided by time spent in the nest box) was also significantly reduced. The reduction of sleep by light was therefore not solely due to females leaving the nest box earlier. Although females did not decrease the length of their sleep bouts, they did decrease the frequency of sleep bouts. Hence, while females during the dark control night slept already very little, less than four hours, artificial light reduced their sleep amount by more than 50%, resulting in less than one and a half hour of total sleep. In a laboratory study, light suppressed sleep in pigeons, while darkness promoted sleep in both a 12:12 LD cycle and 3:3 LD cycle (Rattenborg et al. 2005). During the hours of the subjective night, darkness had the greatest sleep promoting effect. In our study, we used a continuous light during the entire night and in agreement with Rattenborg et al. (2005) found that it suppressed sleep.

ALAN increased nestlings begging frequency and their amount of time spent begging. Under natural dark conditions, chicks hardly beg during the night but begging might perhaps be induced by the movements of the female and the other chicks, which is enhanced by ALAN. The increased movements and vocalizations of the chicks during begging (Kolliker et al. 1998) may have caused sleep disturbance of the female in addition to the direct effects of artificial light discussed above (see also Raap et al. 2015). The effect on begging behaviour was limited compared to the effect of ALAN on female sleep and the lack of a correlation between nestling begging and female sleep would also argue against a strong effect of ALAN through nestling begging on female sleep behaviour. However, restlessness of the chicks induced by ALAN may still contribute to female sleep disruption and vice versa.

Under normal conditions nestling begging serves as a cue for the female to feed them (Hinde et al. 2010). How increased begging at night would affect feeding activity of the females

the following day is unknown, although Titulaer et al. (2012) hypothesized that it would increase the feeding rate of the parents. We hypothesize that when light pollution increases nestling activity and begging at night, this increased energy expenditure may negatively affect growth (Kilner 2001; Rodriguez-Girones et al. 2001; Soler et al. 2014) especially if the parents cannot compensate for this energy loss. However, if there is increased feeding of the parents this may perhaps affect their individual fitness (Davies et al. 2012a), the more because disrupted sleep through artificial light may increase their energy expenditure and reduce their capacity to recover from stress (Siegel 2009; Weljie et al. 2015).

Seasonal differences

The effects of artificial light on sleep behaviour during the nestling period (around May), found in the present study, were much larger compared to during the late winter period (around February; Raap et al. 2015). Around February, the largest effects were found on awakening time, advanced by more than 20 minutes, and sleep amount, reduced by about 40 minutes. Although the sample size in winter was relatively small, the effects found were very consistent. During the nestling period, loss of sleep amount was more than twice as large and the effect on awakening time was more than four times as large. While effects during winter were found particularly in the morning, we found additional effects in the evening and at night during the nestling period as it took females more than an hour longer to fall asleep while sleep bout frequency was decreased.

Under natural conditions, sleep behaviour is strongly related to season in blue tits (Steinmeyer et al. 2010) and great tits (Stuber et al. 2015b), with animals sleeping longer during winter and going to sleep later (closer to sunset) as the breeding season approaches. Such seasonal differences in sleep behaviour along with the differential effect of artificial light can partly be explained by the increase in day length and differences in temperature (Steinmeyer et al. 2010; Stuber et al. 2015b). During the nestling period, females sleep on nests with chicks which are therefore likely to have more parasites compared to the winter when nest boxes are without nest material and chicks. Parasites reduce sleep (Christe et al. 1996) and may thus contribute to seasonal differences in sleep. Therefore, the greater number of affected behavioural aspects and the stronger effect of ALAN during the nestling period might be explained by a combination of multiple drivers (see also Figure 6). These seasonal differences in the response to ALAN emphasize that studies to elucidate (fitness) consequences of light exposure should be done in different seasons. Another important aspect to consider is that

light pollution may have a different effect on males and females of cavity-nesting species such as the great tit. During incubation and the nestling period, females sleep in nest boxes/cavities where they are exposed to multiple stressors such as nestlings and parasites (Christe et al. 1996), while males rarely sleep in nest boxes during this period, but they may be exposed to higher light intensities.

Rebound

To the best of our knowledge, we are the first to show that a sleep rebound also occurs in free-living animals after sleep deprivation (due to artificial light at night). Female great tits showed a rebound effect (Cirelli and Tononi 2008) as they appeared to attempt to recover from their lost sleep by sleeping more the following night. However, compared to the first night, females did not fall asleep earlier or wake up later although they did decrease the time between entering the nest box and falling asleep. While the length of sleep bouts remained equal, their frequency increased during the recovery night. This, together with the increase in sleep proportion, indicates that females reduced the time between sleep bouts and therefore slept more within the same time.

Loss of sleep can be recovered by several mechanisms: sleeping longer, deeper and/or more consolidated (Cirelli and Tononi 2008). Following short-term sleep deprivation, the most consistent effect is an increase in the intensity of short wave sleep (SWS), as reflected in the level of slow-waves in the electroencephalographic power spectrum (Jones et al. 2008; Martinez-Gonzalez et al. 2008). Increases in REM sleep may appear later, after the increase in SWS intensity (Martinez-Gonzalez et al. 2008). The lack of a relationship between sleep loss and recovery sleep (amount) in our study may indicate that some birds recovered faster by sleeping deeper in SWS than others. This intensity dimension to SWS is thought to give birds and mammals flexibility in how they recover lost sleep (Rattenborg et al. 2009). Although we did not measure SWS, we could conclusively show that female great tits tried to recover from sleep loss due to artificial light by sleeping more (sleep amount) and in a more consolidated manner (increased sleep bout frequency) compared to the night before the light manipulation. Compared to the first night, females also fell asleep faster after they had entered the nest box, as illustrated by the reduced evening latency. The need of females to compensate for their lost sleep suggests that sleep has an important function in great tits, for instance in energy conservation (Gobes et al. 2010; Roth II et al. 2010) and/or recovery from damage occurred during waking, such as oxidative stress (Siegel 2009; Weljie et al. 2015). Sleep disruption

through artificial light might therefore have additional effects besides increased energy expenditure, which in its turn could affect individual fitness.

In the present study, we exposed females to artificial light during one night only and we therefore cannot make any conclusions about effects of long-term exposure to ALAN. In rats, long-term sleep deprivation suppressed reparative sleep rebound, which might impair recovery (Marinesco et al. 1999). Similar effects could be expected in great tits and other bird species that are sleep deprived by light pollution for a longer period, but this remains to be investigated.

Individual variation

We found that there was large variation in 'normal' sleep behaviour among individuals and in the way they responded to ALAN. For example under natural dark conditions awakening time varied with more than an hour. Consistent with other studies on great and blue tits (Steinmeyer et al. 2010; Stuber et al. 2015b), we found that age partly explained such variation in sleep behaviour (Table 1). Interestingly, there was a consistent but highly variable effect of artificial light on sleep behaviour. For example, artificial light caused females to wake up one to two hours earlier with some individuals waking up more than six hours earlier. Large differences among individuals were also found for other affected sleep parameters. This variation might be due to differences in natural sleep patterns and/or sensitivity of individuals, with some animals being "better" sleepers, suggesting that light pollution might select for specific chronotypes in birds (Swaddle et al. 2015), but this should be addressed in future studies.

Study limitations

We studied sleep behaviour in free-living birds and can therefore not make any conclusions about the brain state of birds, such as that based on electroencephalography. Nevertheless, sleep behaviour can be considered to be ecologically relevant as it has been linked to behavioural changes, genetic variation and fitness (Christe et al. 1996; Steinmeyer et al. 2010; 2013; Stuber et al. 2014; 2015a; 2015b; 2016).

We recognise that our experimental manipulation may not necessarily allow for a direct comparison with effects of light pollution, as effects from our short-term light treatment may differ from long-term effects. However, an aviary experiment on peahens (*Pavo cristatus*) showed little habituation of animals towards artificial light at night (Yorzinski et al. 2015). Moreover, long-term exposure to ALAN in comparison to our short-term exposure may even

elicit larger effects than the ones we already found. This seems likely as a laboratory experiment using great tits who were subjected to low light levels (0.5 lux), found that effects on activity at night actually increased during several days (de Jong et al. 2016). However, although it remains to be studied, poor sleep caused by artificial light may also cause birds to move away to a darker nest/ roosting site. While occupancy of a nest/roost site may depend on many factors, deterrence by ALAN would seem unlikely to occur when birds already have eggs or nestlings. Nevertheless, our results provide a first indication of how light pollution affects free-living birds during the nestling period and could also be relevant for other animals exposed to ALAN who are exposed to similar or potentially even higher light intensities (Dominoni et al. 2013a; Gaston et al. 2013).

Conclusions

We have provided the first experimental evidence that artificial light has large disruptive effects on sleep in free-living female songbirds during the nestling period. Not only was the effect of artificial light much more pronounced compared to during the pre-breeding season, it also affected a greater variety of sleep parameters, showing that it is crucial to study the consequences of light pollution during different periods of the year. We also showed that artificial light increased the amount of nestling begging behaviour during the night. Additionally, we found that females tried to recover from their lost sleep by sleeping more the next night. Loss of sleep of the female and increased begging of nestlings could both lead to increased energy expenditure. The disruption of sleep by artificial light at night may therefore be a mechanism through which it affects individual fitness. Future research should focus on the effect of sleep disruption through artificial light and on how it affects fitness of both parents and young. This should best be studied using a longer period of light exposure and a larger sample size. To be able to make a comprehensive evaluation of the fitness consequences of light pollution, it is important to assess all potential costs of altered behaviours, and effects on sleep deserve more attention. It is also important to note that we found large variation in how females reacted to artificial light at night. Understanding these differences will be necessary as it may be a potential driver of selection.

Supplementary Material

Table S1: Statistical output of the full mixed effect models, effect of night (pre-control, light, post-control), age, brood size and date on sleep parameters and begging. To correct for changes in day length, response variables were standardized to civil sunset (entry time, sleep onset) or sunrise (awakening time and leaving time). LMM models were used with nest identity as random factor to correct for repeated measurements. *N* is the number of observations while Ind is the number of individuals (female great tits), for begging amount and begging/ hour Ind is the number of nests. Significant P values are shown in bold ($P \leq 0.05$) and trends are underlined ($P < 0.10$).

	N	Ind	Night		Age		Brood size		Date	
			F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Entry time	57	19	1.096	0.344	1.166	0.297	3.961	0.065	8.104	**0.012**
Sleep onset	55	19	18.004	**<0.001**	3.336	0.089	1.295	0.274	3.470	0.083
Evening latency	55	19	15.662	**<0.001**	3.842	0.070	2.218	0.158	1.361	0.263
Awakening time	53	19	19.938	**<0.001**	0.530	0.478	0.472	0.502	0.042	0.841
Leaving time	56	19	13.452	**<0.001**	0.060	0.811	2.211	0.161	0.000	0.988
Morning latency	55	19	1.978	0.152	0.425	0.524	1.436	0.249	4.550	0.050
Sleep bout/ hour	55	19	14.412	**<0.001**	2.357	0.145	0.379	0.547	0.013	0.912
Sleep bout length	55	19	0.091	0.913	1.455	0.247	0.798	0.386	1.101	0.311
Sleep amount	55	19	24.197	**<0.001**	3.094	0.099	0.423	0.526	0.041	0.843
Sleep proportion	55	19	16.505	**<0.001**	9.665	**0.007**	0.008	0.931	0.204	0.659
Begging amount	57	19	42.855	**<0.001**	0.532	0.477	4.898	**0.043**	0.729	0.407
Begging/ hour	57	19	47.909	**<0.001**	0.013	0.912	2.479	0.136	0.392	0.541

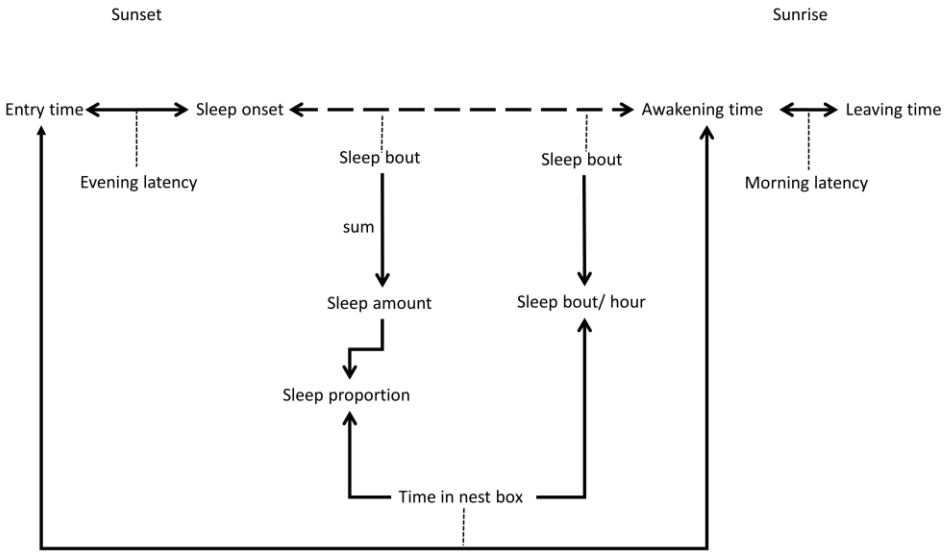

Figure S1: Schematic representation of how sleep behaviour was defined. Entry time is around sunset which is followed by sleep onset. The time between entering the nest box and falling asleep is defined as evening latency. Animals sleep in sleep bouts and the end of the final bout is defined as awakening time. The animals usually leave the nest box around sunrise. The time between awakening and leaving time is defined as morning latency. The sum of all sleep bouts is sleep amount. Sleep proportion is defined as the time that the animal was asleep between time of entry and leaving time. The number of sleep bouts per hour is calculated using the total time in the nest box.

Chapter 4

Disruptive effects of light pollution on sleep in free-living birds: season and/or light intensity dependent?

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Abstract

Light pollution or artificial light at night (ALAN) is an increasing anthropogenic environmental pollutant posing an important potential threat for wildlife. Evidence of its effects on animal physiology and behaviour is accumulating. However, in order to effectively mitigate light pollution it is important to determine which factors contribute to the severity of effects of ALAN. In this experimental study we explored whether there are seasonal-dependent effects of ALAN on sleep in free-living great tits (*Parus major*), an important model species. Additionally, we looked at whether light intensity determined the severity of effects of ALAN on sleep. We therefore exposed animals to artificial light inside the nest box (3 lux) in December (winter) and February (pre-breeding season). Results from February were compared with the results from a previous study in February, using a lower light intensity (1.6 lux).

We found little evidence for a season-dependent response. Effects of ALAN hardly differed between high and low light intensity. ALAN disrupted sleep with as main effect a decrease in sleep duration (≈-40 min) as animals woke up earlier (≈-24 min). However, compared to a natural dark situation sleep onset was delayed by high but not by low light intensity of ALAN. Our study underlines earlier found disruptive effects of ALAN on sleep of free-living animals. While we found no conclusive evidence for seasonal or light intensity-dependent effects of ALAN, additional experimental work using lower light intensities might show such differences. Examining potential management options is crucial in mitigating disruptive effects of light pollution, which will be an important focus for future studies.

Introduction

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

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

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

While ALAN affects a range of animal behaviours (reviewed by Swaddle et al. 2015), the present study is focused on the effects of ALAN on sleep in songbirds in the wild, more specifically in free-living great tits (*Parus major*), a widely used model species. Studying the effects of light pollution on sleep in birds is of major importance for several reasons. First, sleep is an important animal behaviour widespread across the animal kingdom (Cirelli and Tononi 2008; Siegel 2008), and most if not all bird species show sleeping behaviour (Lesku and Rattenborg 2014; Libourel and Herrel 2016; Roth II et al. 2006). Second, it serves multiple purposes including energy conservation and memory consolidation (Gobes et al. 2010; Roth II et al. 2010). Third, avian sleep shares many characteristics of mammalian sleep, for example both consist of two types of sleep, REM and non-REM (Siegel 2008). Sleep is important for many organisms, plays a role in maintaining high levels of physical and cognitive functioning and is ideally suited to examine differences in effects of ALAN in the wild.

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

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

Here, we tested for a seasonal-dependent and light intensity-dependent effect of ALAN on sleep in free-living great tits. First, using a field experiment, we compared the effect of ALAN on sleep between December (winter) and February (pre-breeding season). We expected larger disruptive effects on sleep in February. Second, we tested whether light

intensity and sleep disturbance by ALAN are associated. We compared results obtained from the current study using a light intensity of 3 lux in the nest box with our previous study, which was also done in February but used a lower light intensity of 1.6 lux (Raap et al. 2015). Under laboratory conditions, great tits' responses of daily activity rhythms to ALAN have been shown to be dose-dependent (de Jong et al. 2016) and so we expected that a higher light intensity (similar to those used by de Jong et al. 2016) would increase the disruptive effect of ALAN on sleep behaviour of free-living animals.

Method

Study Area and General Procedures

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

Experimental Procedure

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

Sleep Behaviour Recordings and Light Treatment

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

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

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

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

Defining Sleep Behaviour

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

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

Sleep of great tits was quantified in detail as described in earlier studies on great and blue tit sleep behaviour (see e.g. Raap et al. 2015; Steinmeyer et al. 2010). We used 10 parameters: (1) entry time, (2) sleep onset, (3) evening latency, (4) awakening time, (5) leaving time, (6) morning latency, (7) sleep duration, (8) sleep duration/night duration, (9) frequency of sleep bouts and (10) sleep bout length. For a detailed description of scoring sleep behaviour, see Raap et al. (2015) and/or other articles on sleep behaviour in great and blue tits (Raap et al. 2016b; Steinmeyer et al. 2010; Stuber et al. 2014; 2015a; 2015b). During morning latency, we additionally recorded “time on entrance”: total time a bird spent on the nest box entrance hole and “number of times on entrance”: the total number of times it sat on the nest box entrance (Raap et al. 2015).

Statistical Analysis

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

Testing a seasonal-dependent response of sleep behaviour to ALAN

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

As we wanted to avoid overfitting our models, we did not include interactions with sex except in the model with sleep duration/night duration as dependent parameter because in a previous study an effect was found (Raap et al. 2015). This enabled us to make a more complete comparison between studies with a low and high light intensity (see *Testing a light intensity-dependent response of sleep behaviour to ALAN*).

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

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

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

In the previous study we used sleep amount (sum of all sleep bouts) instead of sleep duration (time between sleep onset and awakening time). However, sleep duration has been used in studies of blue and great tits (Steinmeyer et al. 2010; Stuber et al. 2014; 2015a; 2015b; 2016). To make our results more comparable to these studies we examined the correlation between sleep amount and duration on a subset of individuals for which we had both sleep parameters. Therefore, the number of birds for which we had data on sleep bout length and

frequency was limited to 12. Sleep duration and amount were correlated (Pearson's correlation: $r = 0.82$, $P < 0.0001$, $N = 12$; see also Stuber et al. 2015b) and so we used sleep duration throughout the manuscript and compared sleep duration at 3 lux with sleep amount under 1.6 lux (data from our previous study: Raap et al. 2015).

Ethical Note

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 and adhere to the ASAB/ABS guidelines for the use of animals in behavioural research and teaching. The Belgian Royal Institute for Natural Sciences provided ringing licenses for all authors and technicians. Because of the short duration of the manipulation (one night of artificial light per experiment) and because no individuals were caught during the course of the study the disturbance was assumed to be minimal.

Sleep behaviour data accessible at Zenodo DOI: 10.5281/zenodo.845332.

Results

Individuals Not Entering a Lit Nest Box

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

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

Effect of ALAN on Sleep Behaviour

Independent of month (December/February), ALAN (3 lux) reduced the time that animals were asleep (≈-40 min) due to awakening earlier (≈-24 min), and caused earlier leaving of the nest box (≈-18 min). In addition, the frequency of sleep bouts during the night decreased but their length increased, while the proportion of time that animals were asleep during the night decreased (-5%). Several other small effects on other sleep parameters were also found (see Tables 1 and 2). Animals fell asleep about 15 minutes later and the time between entering the nest box and falling asleep was slightly increased (evening latency). Likewise the time spent between waking up and leaving the nest box in the morning slightly increased (morning latency) with animals going more often to the entrance and spending more time on it. Sex and age explained little of the variation in sleep behaviour (sex was significant for sleep duration: F = 4.482, P = 0.036; sleep duration/night duration: F = 5.452, P = 0.028 and sleep bout/h: F = 5.909, P = 0.046; age was significant for sleep bout/h: F = 12.733, P = 0.008; Table 2).

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

Sleep parameter	Treatment:Month		Treatment		Month	
	F	*P*	*F*	*P*	*F*	*P*
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	3.325	0.076	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	3.325	0.076	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

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

Table 2: Post-hoc analyses of the effect of artificial light at night on sleep behavior during December and February. Control is sleep behavior during the first night when animals slept under natural dark conditions. Sleep onset, awakening time and leaving time are adjusted for sunrise/sunset, with negative values indicating minutes before sunrise/sunset. Light effect is the estimated difference between the first dark night and the subsequent night with artificial light. For every sleep parameter the estimate with their 95% confidence interval (lower, upper) is given. Sleep onset, awakening time, leaving time, sleep duration and sleep bout length are given in minutes. Evening and morning latency, as well as time on entrance and number of times on entrance were log transformed. Only significantly affected/different sleep 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)

Seasonal Difference in Sleep Behaviour

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

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

Seasonal-Dependent Response of Sleep Behaviour to ALAN

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

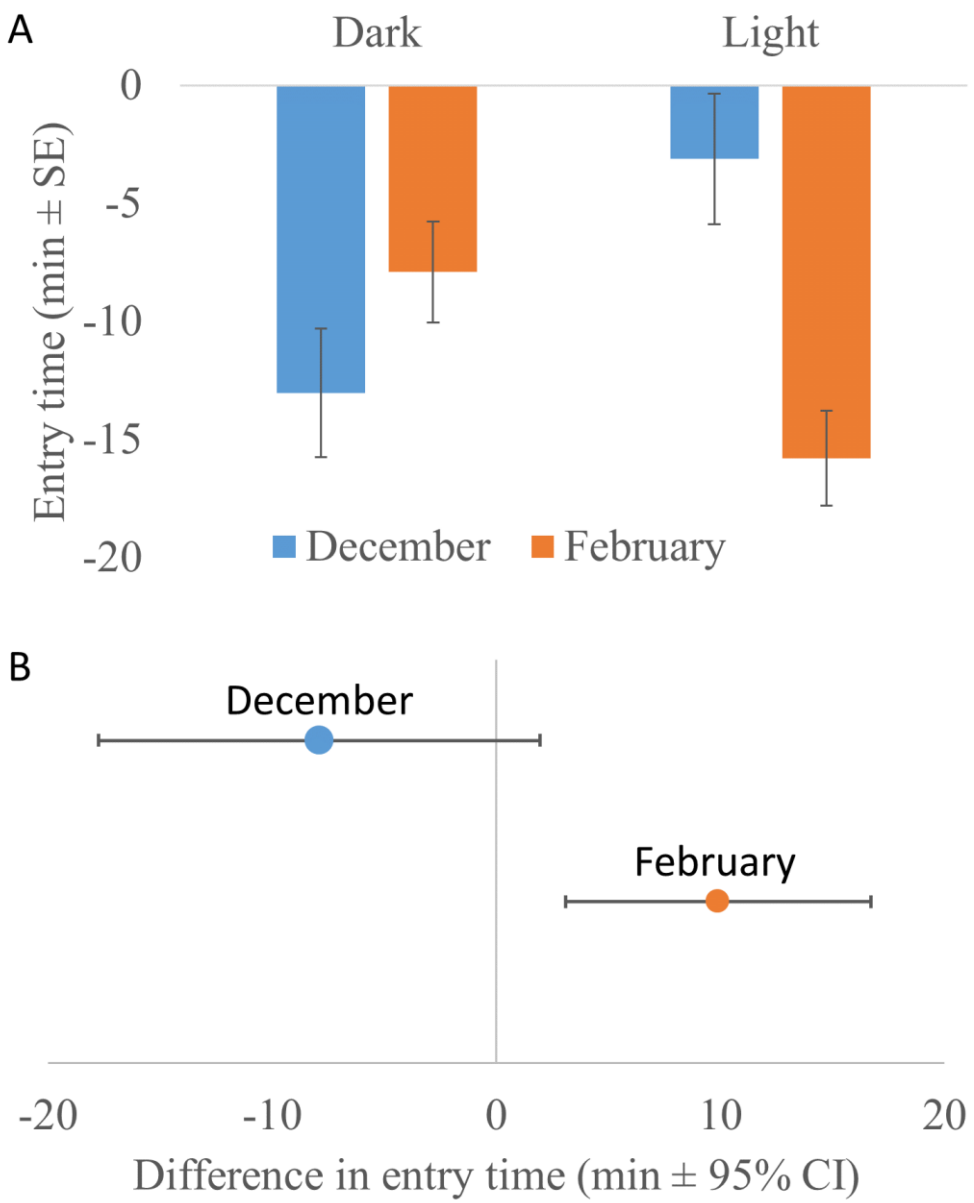

Figure 1: Seasonal d**ifference in effect of artificial light at night on sleep behaviour.** Artificial light at night delayed the time at which great tits entered the nest box in February but not in December. In panel A the raw average of when the birds entered the nest box is shown during December and February when birds slept in a "Dark" nest box or with a "Light" turned on. Negative numbers indicate before sunset. In panel B estimates of effect sizes, the difference caused by ALAN, and 95% confidence interval were obtained by using linear mixed models with bird identity as random factor to correct for repeated measurements. When the 95% CI does not cross zero (vertical line) it indicates that there was an effect/difference.

Light Intensity-Dependent Response of Sleep Behaviour to ALAN

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

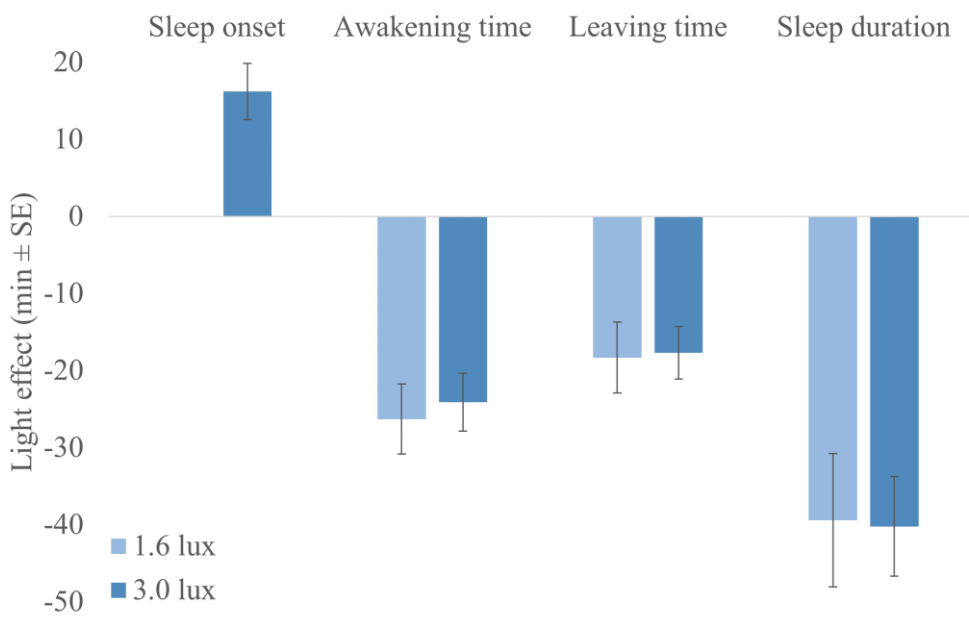

Figure 2: Difference in effect of low and high light intensity of artificial light at night on sleep behaviour. Effect of artificial light at night on sleep behaviour did not differ between low (1.6 lux) and high light intensity (3 lux) except for sleep onset. Significantly affected sleep parameters ($p < 0.05$) are shown. Sleep onset, awakening time and leaving time were adjusted for sunrise/sunset (line at 0 minutes). Estimates at light intensity of 1.6 lux were obtained from Raap et al. (2015), N = 18). Estimates at light intensity of 3.0 lux were obtained from 26 individuals. Sleep onset was not significantly affected by a light intensity of 1.6 lux and therefore no estimate is shown.

Discussion

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

No Seasonal-Dependent Response of Sleep Behaviour to ALAN

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

We thus found little evidence for changes in effects of ALAN (using 3 lux) between December and February, although consistent with earlier studies on blue and great tits (Steinmeyer et al. 2010; Stuber et al. 2015b) sleep behaviour under natural conditions did differ. Differences in response to ALAN between December and February may only become apparent when using a lower light intensity than what we used in the current study. When animals are more sensitive towards light in February, a lower light intensity may cause a disruption of sleep behaviour, while in December it would not disrupt sleep. This remains to be tested.

Light Intensity-Independence of Sleep Behaviour to ALAN

We found little evidence for light intensity-dependent differences in sleep behaviour using 1.6 and 3 lux. Given that de Jong et al. (2016) found that in captive great tits night-time activity increased with higher light intensities, we had expected to find similar results with respect to

sleep behaviour in free-living great tits. Interestingly, higher light intensity caused great tits to fall asleep later in the evening (sleep onset, in relation to sunset), while this effect was not found at a lower light intensity.

However, we believe that the difference in effect on sleep onset between low and high doses of ALAN is not very convincing for several reasons (as explained in *No seasonal-dependent response of sleep behaviour to ALAN*). First, we would also expect differences in other sleep parameters besides sleep onset, which we did not find. Second, de Jong et al. (2016) showed that in the evening, activity offset did not differ between 1.5 and 5.0 lux, while in the morning there was a difference of more than three hours in start of activity. This seems to be in line with our current and earlier results (Raap et al. 2015) where in February most effects are found in the morning. However, in the laboratory study of de Jong et al. (2016) individuals had access to *ad libitum* food and effects of ALAN on activity may be related to the fact that the birds could feed. In our study, birds only experienced increased light inside the nest box and were unable to forage in the darkness outside of the nest box. This difference may also affect the impact of light intensity on sleep behaviour/activity. It is also important to mention that the light spectrum that we used and that used by de Jong et al. (2016) may differ. We used regular white LED light and de Jong et al. (2016) used warm white LED light. There is evidence that differences in spectral composition may also elicit a different response to ALAN in great tits (see e.g. Ouyang et al. 2015). We have no explanation for the observed difference in effect on sleep onset between low and high doses of ALAN.

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

While we found no light intensity-dependent response, we found additional support that ALAN disrupts sleep of wild birds (Raap et al. 2015, 2016b). Animals slept less as they woke up almost three quarters of an hour earlier and left the nest box earlier. The proportion of time that animals spent asleep during the night was reduced (about 5%), while the length of their

sleep bouts increased and sleep bout frequency decreased. Interestingly effects of ALAN were often similar or larger than the difference in sleep behaviour under natural condition between December and February.

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

Study limitations

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

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

otherwise provided more information on why some animals did not enter an artificially lit nest box.

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

Conclusion

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

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

Chapter 5

Artificial light at night affects sleep behaviour differently in two closely related songbird species

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Abstract

Artificial light at night (ALAN) or light pollution is an increasing and worldwide problem. There is growing concern that because of the disruption of natural light cycles, ALAN may pose serious risks for wildlife. While ALAN has been shown to affect many aspects of animal behaviour and physiology, few studies have experimentally studied whether individuals of different species in the wild respond differently to ALAN. Here, we investigated the effect of ALAN on sleep behaviour in two closely related songbird species inhabiting the same study area and roosting/breeding in similar nest boxes. We experimentally exposed free-living great tits (*Parus major*) and blue tits (*Cyanistes caeruleus*) to artificial light inside their nest boxes and observed changes in their sleep behaviour compared to the previous night when the nest boxes were dark.

In line with previous studies, sleep behaviour of both species did not differ under dark conditions. ALAN disrupted sleep in both great and blue tits. However, compared to blue tits, great tits showed more pronounced effects and more aspects of sleep were affected. Light exposed great tits entered the nest boxes and fell asleep later, woke up and exited the nest boxes earlier, and the total sleep amount and sleep percentage were reduced. By contrast, these changes in sleep behaviour were not found in light exposed blue tits. Our field experiment, using exactly the same light manipulation in both species, provides direct evidence that two closely related species respond differently to ALAN, while their sleep behaviour under dark conditions was similar. Our research suggests that findings for one species cannot necessarily be generalised to other species, even closely-related species. Furthermore, species-specific effects could have implications for community dynamics.

Introduction

The increase in the human population and the associated urbanization has caused a dramatic increase in artificial light at night (ALAN), or light pollution (Bennie et al. 2014a; Falchi et al. 2016; Hölker et al. 2010b). Light pollution is potentially an ecological threat due to the impact it can have on natural light cycles (Gaston et al. 2012; 2015b; Longcore and Rich 2004; Rich and Longcore 2005). ALAN has been shown to disrupt animal physiology, e.g. levels of melatonin, testosterone, haptoglobin and nitric oxide (see e.g. Bedrosian et al. 2011; Dominoni et al. 2013a; Jones et al. 2015; Raap et al. 2016a; 2016c; Russ et al. 2015; Schoech et al. 2013), as well as animal behaviour (reviewed in Swaddle et al. 2015). For example, ALAN causes changes in the timing of singing behaviour in songbirds (Da Silva et al. 2014; 2015; Kempenaers et al. 2010; Miller 2006; Nordt and Klenke 2013; but see Da Silva and Kempenaers 2017), changes daily activity patterns (e.g. Dominoni et al. 2013b; 2014; Dominoni and Partecke 2015; Russ et al. 2015; but see Welbers et al. 2017) and alters breeding behaviour (de Jong et al. 2015; Kempenaers et al. 2010; Russ et al. 2015).

Light at night also disrupts sleep, an important and widespread animal behaviour (Cirelli and Tononi 2008; Siegel 2008), which plays an important role in many biological functions (Schmidt 2014; Vorster and Born 2015). For example, ALAN caused great tits (*Parus major*) to wake up earlier and sleep less during winter (Raap et al. 2015). The effect of ALAN was even more disruptive during the breeding season with female great tits showing a reduction in sleep of more than 50% (Raap et al. 2016b). However, to what extent the sleep of other free-living animal or bird species is affected is largely unknown.

Several studies suggest that the effects of light pollution differ among species. For example, songbird species that naturally sing early at dawn, e.g. blackbirds (*Turdus merula*) and robins (*Erithacus rubecula*), had more advanced singing in the morning with ALAN, compared to species that normally start singing later, e.g. blue tits (*Cyanistes caeruleus*) and chaffinches (*Fringilla coelebs*; Kempenaers et al. 2010). Dawn and dusk singing developed earlier in the year due to ALAN in robins, blackbirds, great and blue tits. Likewise, this effect was most pronounced in robins and blackbirds (Da Silva et al. 2015). As a consequence of ALAN, dawn song may potentially no longer be a reliable indicator of male quality (Kempenaers et al. 2010). Hence, species that naturally sing earlier at dawn appear to be the most affected by artificial light in terms of singing behaviour.

Despite the evidence that the effects of ALAN may differ among species, few studies have experimentally examined the difference in response among species in a standardised way. Here, for the first time, we studied the difference in response of sleep behaviour to ALAN in two closely related songbirds inhabiting the same study area. We experimentally provided free-living great and blue tits, sleeping in nest boxes, with artificial light to investigate whether there is a difference in their response to ALAN. In both species, we used a repeated measures design in which we looked at differences within an individual. Such a within-individual design effectively controls for the large variability in sleep behaviour among individuals and also for potential confounding factors.

In this experimental field study, we used free-living great and blue tits as model species to get more insight in possible differences in response towards ALAN. Due to the influence of the environment, there are likely differences in behaviour between free-living and captive animals (Calisi and Bentley 2009). The laboratory is a simplified environment that fails to capture the complexity of natural conditions, which is an important aspect in behavioural and sleep studies (Aulsebrook et al. 2016). Our field experiment, that uses free-living animals, may represent a more ecologically realistic situation compared to the laboratory. We used cavity-nesting birds because we can manipulate light conditions within nest boxes thereby enabling experimentation and observations in a more natural environment compared to that in the laboratory. Our experimental light treatment is not intended to mimic a situation that could be encountered by birds roosting inside cavities or nest boxes, but with it we try to gain a more fundamental insight into possible differences in response to ALAN between closely related species.

We expected great tits to respond stronger than blue tits towards ALAN. While in a naturally dark environment great and blue tits appear to have a very similar sleep behaviour (Stuber et al. 2015b), studies on singing behaviour indicate that great tits are more sensitive than blue tits to ALAN (Da Silva et al. 2015; Kempenaers et al. 2010). In both species, ALAN caused advancement of dawn song but the effect was greater in great tits than in blue tits (about a half-hour difference in response to ALAN). Thus, the disruptive effect of ALAN on sleep could be expected to be greater in great tits than in blue tits.

Methods

Study species and populations

The experiment was carried out during December 2015 in a study area containing a population of great and blue tits. Nest boxes of both species are situated in a semi-rural area with deciduous trees at the University of Antwerp, Wilrijk, Belgium (51°9'44"N, 4°24'15"E). They have been monitored since their installation in 1997 (e.g. Casasole et al. 2017; Eens et al. 1999; Janssens et al. 2001; Raap et al. 2017a; Rivera-Gutierrez et al. 2010, 2012; Van Duyse et al. 2000; 2005; Vermeulen et al. 2016b). The same nest boxes were used for both great and blue tits. Nest boxes were made out of plywood with a metal ceiling (120 mm × 155 mm × 250 mm) and an opening of 30 mm ø for great tits. For blue tits, nest box openings were reduced to 26 mm ø with the use of a plastic plate to prevent great tits from entering. Both species roost in nest boxes at night. Prior to the experiment, nest boxes were regularly checked throughout the year to enable us to capture and ring the birds using them. Since 2012, great and blue tits have been provided with a ring containing a passive integrated transponder (PIT) tag. This enabled us to identify the birds sleeping inside the nest boxes without physically disturbing them.

Experimental design

Approximately one week before we started with the video recordings, we checked, after sunset, for the presence of great and blue tits in nest boxes using a transponder reader (GR-250 RFID Reader, Trovan, Aalten, Netherlands). We measured light levels at the nest box by covering the entrance of the nest box with the sensor (ISO-Tech ILM 1335, Corby UK), and noise levels were measured by holding the sound meter (DVM401, Velleman Inc. Texas USA) in four places outside of the nest box: front, back, left and right (highest value of background noise amplitude; see for details: Casasole et al. 2017; Raap et al. 2017a). These light and noise levels were used to pair great and blue tits with similar light and noise exposure.

We filmed great and blue tits simultaneously to control for environmental factors that may affect sleep behaviour, such as temperature (Steinmeyer et al. 2010; Stuber et al. 2015b; 2017). Video recordings were conducted for each individual bird during two consecutive nights with the first night being used as control night, while birds were exposed to artificial light the following night. Recordings were spread out over eight nights in total. When we recorded sleep behaviour, bird identity was confirmed using the transponder reader. Animals sleeping in a dark nest box do not differ in their sleep behaviour from one night to the next (Raap et al. 2015). Sleep behaviour is highly repeatable from one night to the next and little variation in sleep

behaviours is to be expected in unmanipulated individuals (Stuber et al. 2017). Moreover, we used a within-individual design therefore individuals in our experiment served as their own control (as in: Raap et al. 2016b) and an additional separate control group is unnecessary in this case. Using a within-individual (observations of the same individual over subsequent nights) with a paired design (observing simultaneously great and blue tits) controls for variability in sleep behaviour and for other confounding variables (e.g. temperature; Ruxton and Colegrave 2010). Such a design where an individual acts as its own control increases the statistical power (Seltman 2013).

Recording sleep behaviour and light treatment

To record sleep behaviour we used an infrared camera (Pakatak PAK-MIR5). A small LED light was attached to the camera, similar to in our earlier studies on great tit sleep behaviour (15 mm × 5 mm, taken from a RANEX 6000.217 LED headlight, Gilze, Netherlands; Raap et al. 2015; 2016b). The camera and the LED were installed underneath the nest box lid. All LED lights were standardized to ensure a light intensity of 3 lux of white light at the bottom of the nest box. We used a higher light intensity than our previous studies on great tits (1.6 lux; Raap et al. 2015; 2016a) thereby increasing the likelihood of a response in both great and blue tits towards our treatment. Our experimental approach ensures that the light treatment is effective in terms of exposure, which is crucial when experimentally exposing animals in the wild to ALAN (Raap et al. 2017c).

Before the first recording (dark control night), the recording systems were installed at least two hours before sunset. Only the cameras were connected to the batteries while the LED lights and timers were not (no artificial light was provided). Recorders were turned on directly after installation and removed, at the earliest, two hours after sunrise the following morning. The following night animals were exposed to ALAN. The procedure was the same as that used for the first recording, but the LED lights were connected to the batteries and the timers. Timers were programmed to turn the lights on at 16:00 (30 - 40 min before sunset) and off at 09:00 (30 - 40 min after sunrise).

We obtained a total of 40 observations from 11 great tits and nine blue tits. Among these 20 individuals, there were three females of each species. This skewed sex ratio is probably because more males than females roost in the nest boxes during winter in our study populations. Our study was conducted at the beginning of winter (1 December - 30 December) and as most sleep parameters of great and blue tits showed intersexual difference only at the

beginning of their breeding season (Steinmeyer et al. 2010; Stuber et al. 2015b), we expected no sex-dependent response to ALAN in our experiment.

Sleep behaviour

While ecologically relevant research has been performed using sleep behaviour in both blue and great tits (e.g.; Christe et al. 1996; Steinmeyer et al. 2010; Stuber et al. 2014; 2015b; 2016), we recognise that sleep behaviour remains a behavioural proxy for sleeping brain activity (Aulsebrook et al. 2016). Unfortunately, it remains technologically impossible to conduct neurophysiological studies on small passerines such as great and blue tits in the wild. Therefore, we focused on sleep behaviour. Studying sleep behaviour and disruption of this behaviour due to ALAN is relevant as changes in sleep behaviour have been linked to other behavioural changes (during daytime and night-time), genetic variation and individual fitness (e.g. Christe et al. 1996; Steinmeyer et al. 2010; 2013; Stuber et al. 2014; 2015b; 2016; 2017).

We considered the birds to be asleep when they were in the classic sleep position: their beak pointed backwards and tucked under their scapular (Amlaner and Ball 1983). In the present study, we followed the definition and quantification of sleep parameters according to previous sleep behaviour research in great and blue tits (e.g. Raap et al. 2015; 2016b; Steinmeyer et al. 2010; Stuber et al. 2015b). In short, first we looked at the timing of sleep: entry time, sleep onset (the start time of the first sleep bout longer than 30s), awakening time (the end time of last sleep bout longer than 30s), exit time, and midpoint of sleep (from onset time to awakening time). Second, we analysed sleep latencies: evening latency (time between entry and sleep onset) and morning latency (time between awakening and exit). Third, we looked at sleep quantity: sleep amount (sum of all sleep bouts), sleep percentage (sleep amount divided by the total time spent inside the nest box) and awake duration (total time spent awake). Finally, we analysed sleep continuity: sleep bout (average length of all sleep bouts) and sleep frequency (number of sleep bouts per hour).

Statistical analyses

All statistical analyses were conducted using R 3.2.2 (R Core Team 2015). Entry time, sleep onset, awakening time and exit time were converted to time relative to sunset or sunrise (reference data from Antwerp were used). Separate linear mixed effect analyses (LMM) were used to analyse sleep parameters (lme4 package; Bates et al. 2015). We set “Treatment” (control/light), “Species” (great tit/blue tit) and “Species : Treatment” interaction as fixed

factors. We used a within-individual design (repeated measures) and therefore included "bird identity" as a random factor.

To meet model assumptions, evening latency and morning latency were log-transformed. One blue tit was treated as a statistical outlier based on Cleveland plots (Zuur et al. 2010) and was removed from the analyses on awakening time, exit time, midpoint and sleep amount. It left the nest box almost three hours earlier than other blue tits and may exert undue influence on the analyses (Zuur et al. 2010).

In our models we kept the interaction "Species : Treatment", irrespective of their significance, and performed post-hoc analyses (of the interaction) for all sleep parameters, as this analysis enables us to detect possible difference in effects of ALAN between species. First, the post-hoc analysis enables us to compare each sleep behaviour between the two species during dark control nights. Second, we can analyse whether ALAN affected sleep behaviour in great tits and in blue tits (within the same dataset). If an aspect of sleep is affected in one species but not in the other, this indicates that the species differed in their response. If both species respond to ALAN in the same way (e.g. both show a reduction in sleep amount), we can compare between species the estimated effect of ALAN. Post-hoc analyses were performed using Tukey HSD tests (lmerTest; Kuznetsova et al. 2016). Results are presented as mean model estimates ± standard error (SE) unless otherwise stated.

We preferred to use one dataset with both species in it and use the post-hoc analysis of our interaction (Species : Treatment) over an alternative approach of analysing effects of ALAN on each species separately. Analysing effects of ALAN on both species separately (splitting our dataset) necessitates multiple testing and increases the chance of a type I error which we wished to avoid. Using the post-hoc analysis is a more straightforward approach.

Ethical Note

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. The Belgian Royal Institute for Natural Sciences provided ringing licences for authors and technical personnel.

Results

During naturally dark nights (control situation), none of the 12 examined sleep parameters differed significantly between great and blue tits (Table 1). Post-hoc analyses showed that in great tits ALAN significantly affected all sleep parameters except for midpoint of sleep. Great tits fell asleep later (≈20 min), woke up earlier (≈-30min) and left the nest box earlier (≈-24 min) and lost more than 50 min of sleep (Table 2 and Figure 1). By contrast, in blue tits only evening latency, sleep bout length and frequency were significantly affected (see for full statistical details Table 2 and Figure 1). While the time spent in the nest box in the evening prior to falling asleep (evening latency) was significantly longer for both light exposed great tits and blue tits, this effect was larger in great tits (about 10 minutes). The increase in sleep bout length and the decrease in sleep bout frequency was similar for both great and blue tits. The effect of ALAN on sleep continuity did not differ between great and blue tits.

Table 1: Sleep behaviour of great and blue tits under naturally dark conditions. Results are from linear mixed effect models with treatment (control/light), species, and their interaction as fixed factors and bird identity as a random factor. From post-hoc analyses *t* and *P* values were obtained. All parameters are shown in minutes ± SE except for sleep percentage and sleep frequency (which are shown in percentage and number per hour). Entry time, sleep onset, awakening time and exit time were standardized according to sunset/sunrise. Midpoint of sleep is relative sleep onset plus relative awakening time (see Stuber et al. 2015b). Sample sizes for great tits *N* = 11 and for blue tits *N* = 9, except for awakening time, exit time, midpoint and sleep amount where *N* = 8 for blue tits.

Sleep parameters	Great tit	Blue tit	*t*	*P*
Entry time	7.9 ± 2.2	5.3 ± 2.4	0.78	0.442
Sleep onset	15.3 ± 4.4	9.7 ± 4.8	0.87	0.388
Evening latency [a]	7.4 ± 3.7	4.3 ± 4.1	1.84	0.076
Awakening time	-38.0 ± 6.3	-32.5 ± 7.4	-0.57	0.573
Exit time	-28.0 ± 4.9	-26.9 ± 5.8	-0.14	0.892
Morning latency [a]	10.0 ± 3.7	5.1 ± 4.1	0.51	0.610
Sleep amount	845.5 ± 11.2	863.0 ± 13.1	-1.01	0.318
Sleep bout	9.9 ± 0.9	10.7 ± 1.0	-0.54	0.596
Sleep percentage	0.92 ± 0.01	0.93 ± 0.01	-1.23	0.228
Sleep frequency	6.0 ± 0.5	5.6 ± 0.5	0.62	0.540
Awake duration	75.6 ± 8.9	59.3 ± 9.8	1.23	0.230
Midpoint	-22.6 ± 4.7	-23.4 ± 5.5	0.10	0.920

[a] Original values in minutes are log transformed.

Table 2: Difference in response to ALAN in sleep behaviour between great and blue tits. Post-hoc analyses of treatment (control/light) effect were performed from models (LMMs) with treatment (control/light), species, and their interaction as fixed effects, bird identity as a random factor. All parameters are given as their differences between the first (control) and second (ALAN treatment) night (in minutes ± SE except for sleep percentage and awake frequency, which are shown in percentage and numbers per hour). Entry time, sleep onset, awakening time and exit time are adjusted to sunset/sunrise. Midpoint of sleep is relative sleep onset plus relative awakening time (see Stuber et al. 2015b). The differences between control and light nights for great tits and blue tits are presented respectively. Significant difference are shown in bold ($P < 0.05$). Sample sizes (N) are indicated for both species.

Sleep parameter	Great tit				Blue tit			
	Differences	*t*	*P*	*N*	Differences	*t*	*P*	*N*
Entry time	7.9 ± 3.1	2.53	**0.016**	11	5.6 ± 3.4	1.62	0.113	9
Sleep onset	23.8 ± 6.0	3.94	**0.001**	11	9.1 ± 6.7	1.36	0.190	9
Evening latency [a]	15.9 ± 4.6	3.57	**0.002**	11	3.5 ± 5.1	2.61	**0.018**	9
Awakening time	-29.4 ± 7.4	-3.98	**0.001**	11	-3.0 ± 8.7	-0.35	0.731	8
Exit time	-24.2 ± 6.9	-3.52	**0.003**	11	-3.0 ± 8.1	-0.37	0.714	8
Morning latency [a]	5.2 ± 2.6	2.20	**0.040**	11	0.0 ± 2.8	0.63	0.540	9
Sleep amount	-52.1 ± 13.6	-3.84	**0.001**	11	-11.4 ± 15.9	-0.71	0.485	8
Sleep bout	2.7 ± 1.0	2.80	**0.012**	11	3.2 ± 1.1	2.98	**0.008**	9
Sleep percentage	-2.6 ± 1.0	-2.71	**0.014**	11	-0.5 ± 1.0	-0.52	0.611	9
Sleep frequency	-1.3 ± 0.5	-2.78	**0.010**	11	-1.5 ± 0.5	-2.72	**0.010**	9
Awake duration	19.5 ± 8.1	2.41	**0.030**	11	0.8 ± 8.9	0.09	0.930	9
Midpoint	-5.6 ± 5.1	-1.10	0.280	11	8.3 ± 6.0	1.39	0.180	8

[a] Original values in minutes are log transformed.

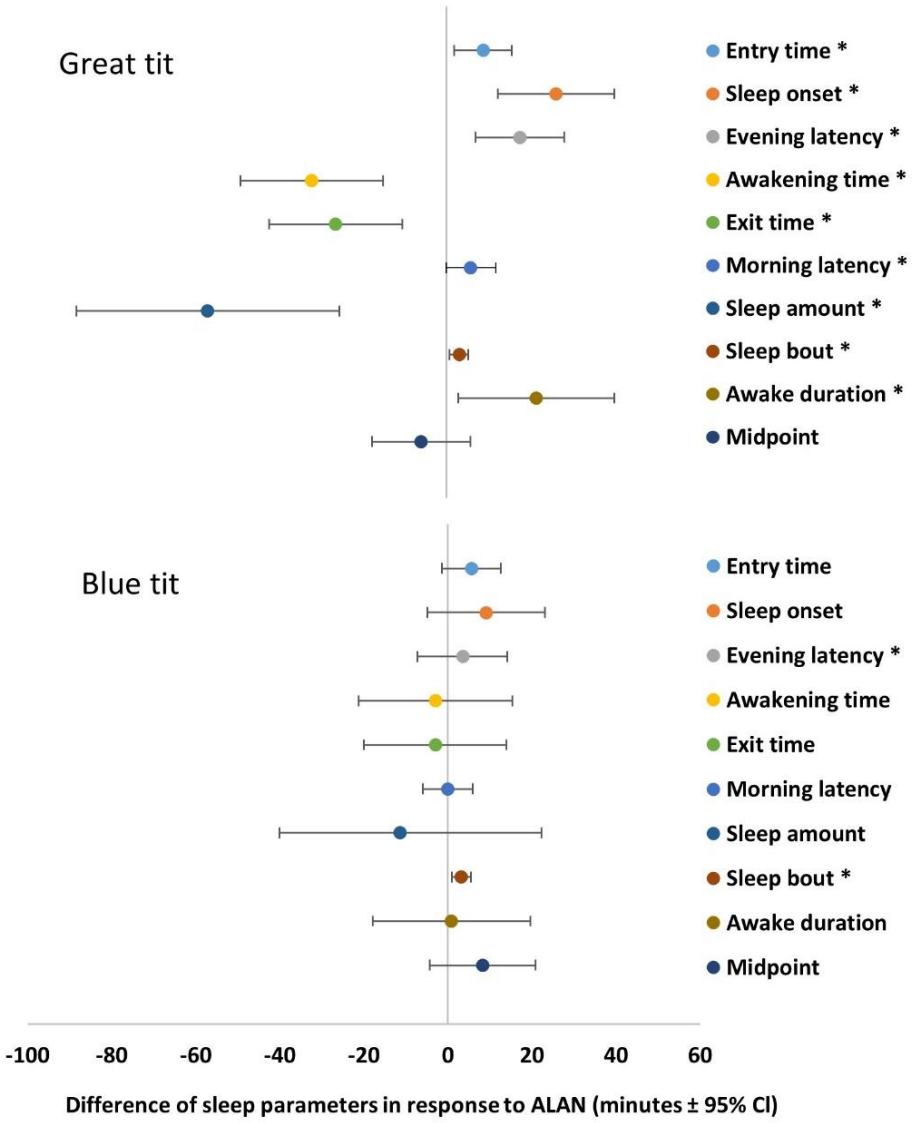

Figure 1: Sleep behaviour of light exposed great and blue tits. Effect sizes and 95% confidence intervals are obtained from post-hoc analyses of LMMs with treatment (control/light), species, and their interaction as fixed factors, and bird identity as a random factor. Evening and morning latency were log-transformed to meet model assumptions, here they are presented with original values in minutes ± 95% CI. Sleep percentage (GT: $P = 0.014$, BT: $P = 0.611$) and sleep frequency (GT: $P = 0.010$, BT: $P = 0.010$) are not presented as they were not quantified in minutes. Asterisks after the sleep parameters indicate significant differences between control (dark) nights and light nights. Sample sizes for great tits $N = 11$ and for blue tits $N = 9$, except for awakening time, exit time, midpoint and sleep amount where $N = 8$ for blue tits.

Discussion

To our knowledge, this study is the first to experimentally demonstrate a difference in response to ALAN in sleep behaviour between closely related species. We found that ALAN had a more disruptive effect and affected more aspects of sleep in great tits compared to blue tits. We first discuss the effects of ALAN, followed by a discussion of possible explanations for the difference in response between great and blue tits and its implications.

Timing of sleep disrupted by ALAN in great tits but not in blue tits

In accordance with Stuber et al. (2015b), we found that under naturally dark conditions great and blue tits were similar in their sleep behaviour. However, the response to ALAN differed between great and blue tits. In great tits, ALAN delayed the time when they entered the nest box and fell asleep as well as advancing the time when they woke up and left the nest box. However, ALAN had no such effects on blue tits, although we used exactly the same light manipulation for both species.

Our results are in accordance with several non-experimental studies, showing a greater response towards ALAN by great tits than by blue tits. For example, ALAN affected the start of dawn song to a greater degree in great tits than blue tits (Da Silva et al. 2014; Kempenaers et al. 2010). Great tits were also more likely to start singing their dawn song earlier in the year than blue tits confronted with ALAN (Da Silva et al. 2015). We found that ALAN affected great tits (in accordance with Raap et al. 2015, 2016b) causing birds to wake up and leave the nest box about half an hour earlier while blue tits were unaffected.

An effect of ALAN in the evening has been observed in blackbirds, with light pollution causing animals to be active longer after sunset (Dominoni and Partecke 2015; Russ et al. 2015). Contrary to our previous studies in great tits (Raap et al. 2015, 2016b), ALAN delayed the time when they entered the nest box or fell asleep. Potentially, the stronger light intensity (3 lux) which we used in the present study compared to the previous ones (1.6 lux; Raap et al. 2015, 2016b) explains the discrepancy in results. Nonetheless, it is of interest that great tits but not blue tits were affected by ALAN in the evening.

Sleep quantity affected by ALAN in great tits but not in blue tits

We found that the effect of ALAN on sleep quantity differed between great and blue tits. In accordance with Stuber et al. (2015b), we found that under naturally dark conditions the amount of sleep was similar in great and blue tits. However, ALAN caused a reduction of sleep

amount in great tits of almost one hour and a nearly 3% reduction in sleep percentage, while in blue tits, ALAN did not affect the amount of sleep or sleep percentage. The reduction of sleep amount in great tits was mainly caused by the delay in when animals fell asleep and because they woke up earlier which blue tits did not.

Sleep latency and continuity disrupted by ALAN in both great and blue tits

Under naturally dark conditions great and blue tits did not differ with regard to the amount of time taken from entering the nest box to falling asleep (evening latency) and from waking up to exiting the nest box (morning latency). In both great and blue tits, ALAN increased their evening latency, although this effect was also larger in great tits. Morning latency was only affected in great tits.

Contrary to the findings of Stuber et al. (2015b), in which great tits woke up less frequently than blue tits, we found that under naturally dark conditions the sleep bouts of great and blue tits were of similar length and frequency. In both great and blue tits, ALAN induced a similar increase in sleep bout length, and decrease in frequency. Animals appeared to wake up less often. Great tits were previously found to wake up less frequently in brighter locations (natural light) than in darker locations (Stuber et al. 2015b). Although ALAN did not affect blue tit timing of sleep nor sleep quantity, the altered evening latency and sleep continuity suggests that the disruptive effect of ALAN during winter might be subtle but still present.

Possible explanations for the different response to ALAN

There are several possible explanations for the difference between great and blue tits in their response to ALAN, such as a difference in sensitivity to ALAN, in physiology and/or ecological strategies.

The sensitivity of great and blue tits when reacting to ALAN may differ (see e.g. effects on dawn song: Da Silva et al. 2014; 2015; Kempenaers et al. 2010). For example, the advancing effect of ALAN on dawn song is about half an hour greater in great tits than in blue tits (Kempenaers et al. 2010). When comparing effects on activity obtained in laboratory studies, there is evidence that the response to ALAN is greater in great tits than in blue tits. For example, the response in activity onset is more than twice as large in great tits than in blue tits when exposed to 5 lux white LED light (respectivitely about -325 and -125 min advancement in onset; de Jong et al. 2016). Our findings on sleep behaviour of great and blue tits seem to be in line with these previous studies, with great tits appearing to be more responsive in the morning.

Whether this is related to a difference in spectral sensitivity between blue and great tits is unknown because to the best of our knowledge only the spectral sensitivity in blue tits has been measured (Hart 2001).

The observed difference could also be driven by physiological differences between the two species, especially under winter conditions. For example, smaller birds have higher thermoregulatory energy demands (Kortner and Geiser 2000) and the relatively smaller blue tits (Dhondt 1977) may, therefore, lose more heat by staying awake compared to larger great tits. Because blue tits could have been downregulating their body temperature to conserve energy during cold nights (Nord et al. 2009) and because the awakening threshold is associated with nocturnal hypothermia (McKechnie and Lovegrove 2002), blue tits might, as a result, have been less responsive than great tits to the same light exposure in their sleep behaviour during winter.

The difference between great and blue tits in their response to ALAN could also be driven by differences in ecological strategies. In great tits, both females and males emerging earlier in the morning had higher extra-pair success rates (Greives et al. 2015; Helm and Visser 2010). By contrast, in blue tits, female extra-pair success was not related to the timing of sleep (Schlicht et al. 2014; Steinmeyer et al. 2013), but males that slept longer appeared to be more successful in extra-pair paternity (Steinmeyer et al. 2013). However, male blue tits with earlier dawn song had more mating partners (Poesel et al. 2006), and ALAN advanced blue tit dawn song and increased extra pair paternity (Kempenaers et al. 2010). While it is unclear to what extent there may be a difference in motivation for great and blue tits for an early start of activity, we have to take into account that our experiment took place in winter in contrast to the above-mentioned studies.

The effect of ALAN on sleep behaviour in great tits was reported to be more profound during the breeding season compared to effects during February (Raap et al. 2016b). Potentially, during the breeding season also more aspects of blue tit sleep behaviour are affected by ALAN. How this affects differences in response between great and blue tits remains to be examined.

Species-specific effects could have implications for community dynamics. Earlier studies showed species-specific effects in invertebrates in which ALAN changed community compositions (Davies et al. 2012b; Sanders et al. 2015). Other experimental work showed that slow-flying bat species avoided white and green light while more agile bat species were

attracted to it (Spoelstra et al. 2017). Here, we experimentally show that two closely related songbird species may respond very differently to ALAN. This would imply that ALAN might have important potential effects on avian community compositions, which will need to be examined. Moreover, as the response differed between two-closely related species, generalisation based upon one species might be inappropriate.

Study limitations

In this study we used a short-term light exposure (1 night) at an intensity of 3 lux to gain fundamental insights into the response of ALAN and how it may differ between species. While birds roosting in cavities would rarely experience this light intensity, 3 lux and more are commonly experienced (at night) by animals outside of cavities. Blackbirds living in the city of Munich (Germany) were exposed to light levels as high as 2.2 lux (Dominoni et al. 2013a) and street lighting often reaches 15 lux (Gaston et al. 2013) or even much more (Riley et al. 2013). In laboratory studies light levels of 5 lux (i.e. more than the 3 lux we used in the current study) are often used and referred to as "dim light" (e.g. Aubrecht et al. 2014; Bedrosian et al. 2013a; Borniger et al. 2013; Cissé et al. 2017; Ikeno et al. 2014; Stenvers et al. 2016). Also the laboratory studies on great and blue tits by de Jong et al. (2016; 2017) used light intensities up to 5 lux. Thus, the light intensity that we used is well within the range of other research on the effects of ALAN and therefore useful in gaining fundamental knowledge in the effects of ALAN.

Our study focussed on the effects of short-term light exposure and more research is therefore necessary to test whether long-term exposure to ALAN may increase the effects or if birds can habituate. Yorzinski et al. (2015) found little habituation of peahens (*Pavo cristatus*) towards ALAN, but here again species may react differently. Indeed, de Jong et al. (2016) found in a laboratory experiment that activity at night actually increased during longer-term exposure to ALAN in great tits. For our future understanding of the impacts of light pollution, it will therefore be important to understand these chronic (rather than acute) effects, to study effects of lower light intensities and whether birds/animals habituate to ALAN. Continuing from our current research, our experimental system could be used to examine long-term effects of ALAN in the field using free-living animals.

Conclusions

Our experiment shows a clear species-specific response to ALAN in the sleep behaviour of two closely related species. In winter, great and blue tit sleep behaviour in a natural dark nest box is similar, however, great tits' sleep behaviour was more disrupted by ALAN compared to that of blue tits. This is potentially driven by differences in sensitivity, physiology and/or ecological strategies. Our findings call attention towards the difference in how ALAN or light pollution may affect different species, even those that are closely related, which may be important when mitigating effects of light pollution. Our research suggests that findings on one species cannot necessarily be generalised to other species, even closely-related species. Furthermore, species-specific effects could have implications for community dynamics.

Chapter 6

Cavities shield birds from effects of artificial light at night on sleep

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Abstract

Light pollution is an ever increasing worldwide problem disrupting animal behaviour. Artificial light at night (ALAN) has been shown to affect sleep in wild birds. Even cavity-nesting bird species may be affected when sleeping inside their cavity. Correlational studies suggest that light from outside the cavity/nest box, for example from street lights, may affect sleep. We used an experimental design to study to what extent nest boxes shield animals from effects of ALAN on sleep. We recorded individual sleep behaviour of free-living great tits (*Parus major*) that were roosting in dark nest boxes and exposed their nest box entrance to ALAN the following night (1.6 lux white LED light; a similar light intensity as was found at nest boxes near street lights). Their behaviour was compared to that of control birds sleeping in dark nest boxes on both nights.

Our experimental treatment did not affect sleep behaviour. Sleep behaviour of birds in the control group did not differ from that of individuals in the light treated group. Our results suggest that during winter cavities shield birds from some effects of ALAN. Furthermore, given that effects of ALAN and exposure to artificial light are species-, sex- and season-dependent, it is important that studies using wild animals quantify individual exposure to light pollution, and be cautious in the interpretation and generalisation of the effects, or lack thereof, from light pollution. Rigorous studies are necessary to examine individual light exposure and its consequences in cavity- and open-nesting birds.

Introduction

Light pollution or artificial light at night (ALAN) is an increasing worldwide environmental alteration (Falchi et al. 2016) and we are just beginning to explore the multitude of its effects. Light pollution disrupts natural light cycles and potentially poses an important threat for wildlife, biodiversity and humans (Duffy et al. 2015; Gaston et al. 2013; Hölker et al. 2010b; Kyba and Hölker 2013; Navara and Nelson 2007; Rich and Longcore 2005) since it results in a wide range of physiological and behavioural responses (see e.g. Da Silva and Kempenaers 2017; Dominoni et al. 2013a). For example, in two cavity-nesting songbird species, blue tits (*Cyanistes caeruleus*) and great tits (*Parus major*), sleep behaviour was disrupted by experimental light inside the nest box (Raap et al. 2015, 2016b; 2017b; Sun et al. 2017). Sleep is an important animal behaviour with multiple possible functions, enabling animals to recover from daily stress (Siegel 2009; Weljie et al. 2015), to consolidate memory and to conserve energy (Gobes et al. 2010; Roth II et al. 2010; Vorster and Born 2015).

There are several indications why ambient light pollution could be expected to affect sleep behaviour of birds inside cavities/ nest boxes. First, blue and great tits sleeping in nest boxes which were exposed to more (natural) light outside the nest box had an earlier awakening time and leaving time (Steinmeyer et al. 2010; Stuber et al. 2015b). However, due to the correlative nature of these studies confounding effects (e.g. noise) cannot be excluded. Furthermore effects of ALAN were not examined. Second, light pollution may affect sleep as it allows some bird species to forage longer for food (Stracey et al. 2014) which can come at the cost of reduced sleep. There are, however, also indications that suggest that nest boxes may shield animals from direct effects of ALAN. Experimental ALAN inside a nest box affected nestling physiology (Raap et al. 2016c), but ambient light pollution at the nest box was unrelated to nestling physiology (Raap et al. 2017a). Whether ambient light pollution leads to altered sleep behaviour of birds inside nest boxes (cavities), similar to what has been found in experiments with ALAN inside a nest box using free-living blue and great tits (Raap et al. 2015, 2016b; 2017b; Sun et al. 2017), needs to be examined.

Because correlational relationships between ambient light and the expression of behaviour may reflect indirect effects, we performed an experiment in which we exposed the entrance of great tit nest boxes from the outside to ALAN during the winter period. Dawn singing of one species may affect that of another (Xia et al. 2018) thereby confounding possible effects of light pollution on sleep. However, during winter most species, including great tits, do

not yet have a dawn chorus (see e.g. Da Silva et al. 2015), excluding the possibility that sleep is affected by dawn song of other species and other great tits. Our experiment more closely resembles light conditions inside cavities experienced in the wild in urban areas, without manipulating daytime behaviours such as extended foraging. We used an outside light source to produce 1.6 lux at the nest box entrance (white LED light). There are several reasons why this treatment could affect sleep behaviour. First, experimental ALAN inside the nest box disrupts sleep behaviour of great tits (Raap et al. 2016b; 2017b). Second, during the night great tits wake up several times per hour (Stuber et al. 2015b) and when a bird sits at the bottom of a nest box it can observe light shining in through the entrance, which could subsequently affect its behaviour. Finally, very low levels of ALAN (0.05 lux) have been found to affect activity onset and offset in great tits in the laboratory (de Jong et al. 2016). However, nestling physiology was unaffected by ambient light pollution (Casasole et al. 2017; Raap et al. 2017a). Therefore, our aim was to test the hypothesis that nest boxes shield birds from the detrimental effects of ambient ALAN on sleep. We recorded individual sleep behaviour of great tits that were roosting in dark nest boxes and exposed their nest box from the outside to ALAN the following night. Their behaviour was compared to that of control birds sleeping in dark nest boxes on both nights. We expected effects in the morning, especially on awakening time (last time the bird was asleep) and leaving time (when the bird leaves the nest box), as natural light in the morning has been shown to relate to these parameters in both blue and great tits (Steinmeyer et al. 2010; Stuber et al. 2015b). Furthermore, during winter our previous experiments with ALAN inside nest boxes also showed most effects to occur during the morning (Raap et al. 2015; 2017b). We used a light intensity for our experimental treatment that was similar to intensities measured at nest boxes located near street lights (≤16m; average 1.6 ± SE 0.6 lux, N = 16; Casasole et al. 2017; Raap et al. 2017a), with street lights themselves often having intensities of around 10-40 lux (Gaston et al. 2017). The intensity that we used may therefore be experienced by animals near street lights. Getting a better understanding of the effects of light from outside the nest box is highly relevant as it provides insights into the effects of light pollution caused by street lights.

Methods

Study area and general procedures

Data was collected between February 12th and March 4th 2014 in a resident suburban nest box population of great tits in the surroundings of Wilrijk, Belgium (51°9'44''N, 4°24'15''E). This nest box population has been established in 1997 and has been continuously monitored (see e.g. Rivera-Gutierrez et al. 2010, 2012; Thys et al. 2017; Van Duyse et al. 2000; 2005; Vermeulen et al. 2016b). Nest boxes were made out of plywood with a metal ceiling, had outer dimensions of 120 × 155 × 250 mm (width × depth × height) and an opening of 30 mm ø. During previous winter- and breeding seasons great tits were caught inside nest boxes after which they were sexed and ringed. Since 2011 all birds have been provided with a ring/implant containing a passive integrated transponder (PIT) tag, enabling the individual detection of birds sleeping in nest boxes without physically disturbing them.

Experimental procedure

Nest boxes with a maximum nighttime light intensity of 0.3 lux at the entrance hole were selected for this experiment (range: 0.01 - 0.26 lux, average: 0.12 lux; ISO-Tech ILM 1335 light meter). After sunset the light intensity inside these nest boxes was ± 0.01 lux, which is the minimum that the light meter can measure. These experimental nest boxes were located far from street lights (>30 m), and experienced a natural light regime. Light intensity from street lights quickly declines within several meters to almost dark levels (Gaston et al. 2017; Raap et al. 2017c).

A within-individual design was used in which sleep behaviour was observed over two subsequent nights in a control (dark) treatment and a light treatment. Because of the high variability between individuals in sleep behaviour (Raap et al. 2016b), we used a within-individual design which "controls" for this variation (Ruxton and Colegrave 2010). This design, where an individual acts as its own control, also increases the statistical power (Seltman 2013).

Birds in the light group slept with the flashlight (see "*Light treatment*") turned off on the first night and turned on during the second night, while in the control group birds were observed over two nights sleeping in a naturally dark situation. Flashlights were turned on when infrared sensitive cameras were installed, at least two hours before sunset (lights were on for about 18 hours in total; see "*Sleep behaviour recordings*"). We performed observations of sleep behaviour in the control and light group simultaneously over a period of 20 days/nights, with not all individuals being observed during the same night. In total we obtained paired data from

seven individuals (three females and four males) in the control group and from ten individuals (four females and six males) in the light group. Because we expected smaller differences in sleep behaviour between nights in the control group (Raap et al. 2015) we recorded fewer individuals in this group compared to the light group.

Light treatment

Birds were first allowed to sleep in their normal dark situation and a pole with a dummy flashlight was put up at 5 meters from the nest box (at the same time the camera was installed; at the latest two hours before sunset). The following night we replaced the dummy with a similar sized flashlight (white LED, Xtar R30 XML U2) calibrated to produce about 1.6 lux at the nest box entrance. We used white LED as these light types are increasingly used as street lights (Kyba et al. 2017a; Schubert and Kim 2005). Animals in the control group slept in the dark on both nights with a dummy, similar to the flashlight, installed outside. We used a light intensity of 1.6 lux which is lower than the maximum values in our population of nest boxes near street lights (≈ 8 lux at the outside of the nest box opening) but represents a light intensity which can be found for nest boxes (and cavities) exposed to ALAN from street lights (see also Dominoni et al. 2013a; Gaston et al. 2013; 2017). While nest boxes close to street lights (8m; not used in this experiment) can experience light intensities as high as 8 lux on the nest box opening, the light intensity inside at the bottom of the nest box is negligible (0.01 lux, N = 20).

Sleep behaviour recordings

We used the procedure for recording sleep behaviour as previously described by Raap et al. (2015). Nest boxes were checked for presence and identity of sleeping great tits prior to the first recording and during the experiment with a handheld transponder reader (FR-250 RFID Reader, Trovan, Aalten, Netherlands). To record sleeping behaviour we installed infrared sensitive cameras (Pakatak PAK-MIR5, Essex, UK) under the nest box roof-lid, at least two hours before sunset and removed them, at the earliest, two hours after sunrise the next morning. Recordings started after the cameras were installed. Birds were never present inside the nest box during the time of installation.

Defining sleep behaviour

As great tits readily sleep in nest boxes, they are an ideal model species to study sleep behaviour (and physiology) in free-living animals and to manipulate the light conditions to which they are exposed to during the night (e.g. Raap et al. 2017b). Unfortunately they are too small to be fitted with modern data loggers, which would otherwise enable recording of their brain activity (necessary for defining sleep). We acknowledge that sleep behaviour remains a proxy for sleep and has its limitations (Aulsebrook et al. 2016) but it is difficult to study sleep in the wild (Rattenborg et al. 2017). Nonetheless, sleep behaviour is ecologically relevant as it has been linked to behavioural changes, genetic variation and fitness-related traits (Amo et al. 2011; Christe et al. 1996; Steinmeyer et al. 2010; 2013; Stuber et al. 2014; 2015a; 2015b; 2016; Tripet et al. 2002). Previous work in blackbirds (*Turdus merula*) also showed close correspondence between behaviourally observed and electrophysiological measured sleep (Szymczak et al. 1993).

Similar to other relevant work on great and blue tits, we thus defined sleep entirely by using sleep behaviour (Raap et al. 2015, 2016b; Steinmeyer et al. 2010; 2013; Stuber et al. 2014; 2015b; Sun et al. 2017). When a bird showed the classical sleep position (beak pointing backwards and tucked under the scapulars), it was considered to be sleeping (Amlaner and Ball 1983). However, in rare cases, individuals sat quietly for some time with the head pointing forwards or not completely tucked under the scapular. These periods were defined as awake as they were often followed by the classical sleep position. Sleep of great tits was quantified in detail, as described in earlier studies on great and blue tit sleep behaviour (e.g. Raap et al. 2015; Steinmeyer et al. 2010), using 12 parameters: entry time (min), sleep onset (min), evening latency (min), awakening time (min), leaving time (min), morning latency (min), time on entrance (min), number of times on entrance, sleep proportion, sleep bout length (min), sleep bout/ hour, sleep amount (min). For a detailed description of these 12 parameters and how they were scored, please see Raap et al. (2015) and the supplementary material in Raap et al. (2016b).

Data analysis

For all statistical analyses we used R 3.2.2 (R Core Team 2016). We converted entry time, sleep onset, awakening time and leaving time to times relative to sunset or sunrise (reference data from Antwerp were used; Royal Observatory Belgium).

For each sleep parameter a separate linear mixed effect model was constructed (using the lme4 package; Bates et al. 2013). As dependent variable we used the different sleep parameters. The full model was constructed with "Sex", "Date" (Julian day), "Treatment" (control, light), "Night" (1 or 2) and the interaction "Night:Treatment" as fixed effects, to look at whether the light treatment affected sleep behaviour. We did not take into account a possible sex-dependent effect of our treatment because this is unlikely to be the case (Raap et al. 2017b). Because we used a within-individual design (repeated measures) we included individual identity as a random factor. We tested whether our light treatment affected sleep behaviour by using likelihood ratio tests to compare the full models against the models without the interaction "Night:Treatment". Generalized linear mixed models were used for "numbers of time on the entrance" (visits on entrance; Poisson distribution) and proportion of time asleep (binomial distribution). We checked normality of dependent variables using histograms (Zuur et al. 2010) and validated models by inspecting residual plots (Zuur et al. 2009). Based on the variation inflation factor there was no multicollinearity.

Ethical statements

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. The Belgian Royal Institute for Natural Sciences provided ringing licenses for all authors and field technicians.

Results

There was no effect of our experimental light, which only exposed the entrance of the nest box to ALAN and not the environment, on any of the sleep parameters, as indicated by non-significant "Night:Treatment" interactions (all $P > 0.255$; Table 1; Figure 1).We obtained estimates and confidence intervals for visualisation purposes which clearly showed that the sleep behaviour of birds in the control group did not change from night one to night two (Figure 1). Likewise, the sleep behaviour of animals sleeping in a nest box exposed to our ALAN treatment did not differ between the dark versus illuminated night or from the control group (Figure 1). Birds spent about one minute on the nest box opening (1.1 ± 0.3 minutes; Table 1) and this was not affected by our treatment.

Birds slept less as the season progressed (sleep amount, -3.7 ± 0.8 minutes/ day, $F = 23.709$, $P < 0.001$). Males slept less than females (-26.9 ± 8.3 minutes, $F = 10.570$, $P = 0.009$), woke up earlier (5.7 ± 5.8 minutes, $F = 7.266$, $P = 0.012$), left the nest box earlier (-18.9 ± 6.2

minutes, F = 9.216, P = 0.005) and took slightly longer to leave the nest box after waking up (morning latency, 3.7 ± 1.3 minutes, F = 7.635, P = 0.010).

Table 1: Results of the mixed effect models on sleep parameters. To correct for changes in day length, response variables were standardized to civil sunset (entry time, sleep onset) or sunrise (awakening time and leaving time). LMM models were used with nest identity as random factor to correct for repeated measurements (N = 17). EST=estimate. Sleep parameters have been abbreviated. Entry time: ET; Sleep onset: SO; Evening latency: EL; Awakening time: AT; Leaving time: LT; Morning latency: ML; Time on entrance: TE; Number of times on entrance: NE; Sleep proportion: SP; Sleep bout length: SL; Sleep bout/ hour: SH; Sleep amount: SA.

	Intercept		Date					Sex					Night:Treatment				
	Est	SE	Est	SE	DF	*F*	*P*	Est	SE	DF	*F*	*P*	Est	SE	DF	*F*	*P*
ET	**44.1**	**46.2**	**-0.8**	**0.8**	**7.6**	**0.861**	**0.382**	**8.2**	**8.3**	**26.3**	**0.969**	**0.334**	**10.7**	**14.9**	**21.2**	**0.521**	**0.479**
SO	**36.2**	**45.6**	**-0.7**	**0.8**	**7.8**	**0.718**	**0.422**	**7.8**	**8.1**	**26.2**	**0.940**	**0.341**	**10.0**	**14.4**	**21.4**	**0.478**	**0.497**
EL	**8.2**	**4.1**	**-0.1**	**0.1**	**7.8**	**1.191**	**0.308**	**0.3**	**0.8**	**26.6**	**0.204**	**0.655**	**0.8**	**1.4**	**21.3**	**0.325**	**0.575**
AT	**29.5**	**27.5**	**-0.8**	**0.5**	**28.0**	**2.384**	**0.134**	**-15.7**	**5.8**	**28.0**	**7.266**	**0.012**	**0.2**	**10.8**	**28.0**	**0.000**	**0.984**
LT	**30.5**	**29.4**	**-0.8**	**0.5**	**28.0**	**2.494**	**0.126**	**-18.9**	**6.2**	**28.0**	**9.216**	**0.005**	**1.0**	**11.6**	**28.0**	**0.007**	**0.934**
ML	**-0.5**	**8.4**	**0.1**	**0.2**	**8.9**	**0.131**	**0.726**	**3.7**	**1.3**	**25.9**	**7.635**	**0.010**	**-0.8**	**2.4**	**22.4**	**0.102**	**0.753**
TE	**-3.2**	**2.0**	**0.1**	**0.0**	**7.8**	**4.069**	**0.079**	**1.3**	**0.4**	**8.9**	**9.466**	**0.013**	**-0.6**	**0.6**	**15.0**	**0.975**	**0.339**
NE	**0.8**	**1.3**	**0.0**	**0.0**	**26.0**	**0.215**	**0.643**	**0.5**	**0.3**	**26.0**	**2.864**	**0.091**	**-0.1**	**0.5**	**26.0**	**0.023**	**0.880**
SP	**-2.8**	**0.7**	**0.0**	**0.0**	**26.0**	**0.002**	**0.962**	**0.1**	**0.2**	**26.0**	**0.855**	**0.355**	**-0.1**	**0.3**	**26.0**	**0.052**	**0.820**
SL	**20.6**	**8.3**	**-0.1**	**0.2**	**13.0**	**0.770**	**0.396**	**-2.5**	**1.8**	**13.0**	**1.980**	**0.183**	**1.0**	**1.2**	**15.0**	**0.749**	**0.400**
SH	**-0.1**	**7.5**	**0.1**	**0.1**	**3.4**	**0.531**	**0.513**	**0.8**	**1.0**	**2.2**	**0.558**	**0.506**	**-0.6**	**0.5**	**15.0**	**1.402**	**0.255**
SA	**953.6**	**41.4**	**-3.7**	**0.8**	**8.9**	**23.709**	**<0.001**	**-26.9**	**8.3**	**9.8**	**10.570**	**0.009**	**-6.1**	**11.1**	**15.0**	**0.306**	**0.588**

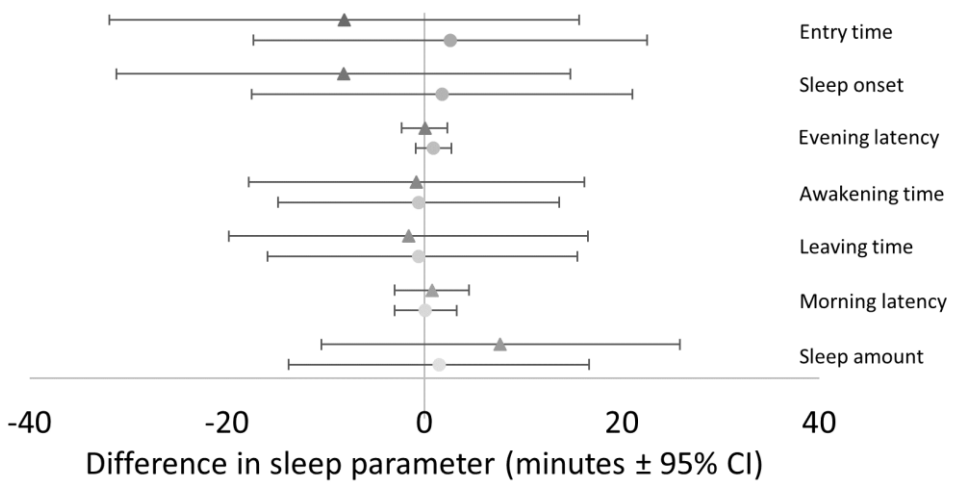

Figure 1: The experimental light treatment, of 1.6 lux at the nest box entrance, did not affect sleep behaviour. Differences in sleep behaviour between nights for animals in the control group (triangles) and in the light treated group (circles) are given. We used mixed models with nest identity as random factor to correct for repeated measurements (*N* = 17). For visual purposes we extracted effect sizes with 95% confidence intervals, therefore we used Least Squares Means for post-hoc analyses on all normally distributed sleep parameters (using the lmerTest package; Kuznetsova et al. 2014).

Discussion

We found no evidence that sleep behaviour of free-living great tits was affected by our experimental light, which only exposed the entrance of the nest box to ALAN and not the environment. With our experimental treatment, we wanted to isolate effects of light on sleep behaviour from any other possible confounding effects which may indirectly affect sleep behaviour, such as those through extended foraging behaviour (Stracey et al. 2014). Based on correlational studies showing that great tits that slept in brighter nest boxes woke up earlier (Stuber et al. 2015b) and because experimental ALAN inside the nest box advanced awakening time (Raap et al. 2015; 2017b), we could expect effects. Furthermore very low light intensities (0.05 lux) also caused great tits to advance their activity (de Jong et al. 2016). However, both the timing and duration of sleep behaviour were unaffected. In the following we discuss our results and their possible implications.

Although we used a within-individual design, which is powerful to detect changes in behaviour (Seltman 2013), it might be that our relatively small sample size made it difficult to detect changes in sleep behaviour. From a power analysis it seems that, for example for

awakening time, we would need about double the sample size to obtain a 80% power to detect a 20 minute difference in the light treated group. This is an effect size similar to our experimental studies with ALAN inside the nest box (Raap et al. 2017b). We therefore recommend experiments with larger sample sizes to validate our results.

The light source (flashlight) in our experimental design was set perpendicular to the nest box opening, which differs from street lights. However, how we exposed the nest box to ALAN is unlikely to explain the lack of effect, as even in nest boxes which are exposed to higher light intensities from street lights (≈ 8 lux on the opening) we measured no light on the bottom of the nest box (pers. obs.). The size of the nest box opening and its relatively high position in the nest box make it very difficult for any light to directly reach the bottom of the nest box, where great tits roost during the winter. However, birds sitting at the bottom of the nest box can observe whether light shines in through the entrance, which could subsequently affect their behaviour. Our experimental treatment lasted only for a single night and therefore we cannot exclude the possibility that a longer light treatment might have elicited effects on sleep behaviour. However, our findings showing no effect of ALAN seem to be in line with a previous study by Titulaer et al. (2012). They used an experimental setup with a light on top of great tit nest boxes during the nestling period for 9 consecutive days (due to nest material great tits will be closer to the nest box opening). They used a white LED light with an intensity of 10 lux at the entrance. In their study they also did not find an effect on activity offset or onset, two behavioural parameters similar to the parameters 'entry and leaving time' that were used in the present study.

Effects of light pollution on sleep are likely because the external environment is manipulated, causing changes in dawn song (Kempenaers et al. 2010) or extended foraging (Stracey et al. 2014), rather than direct exposure to ALAN while inside the cavity/ nest box. Sleep behaviour of, for example, great tits might also be affected by the presence of other species that are active earlier in the morning, as dawn song of one species may affect that of another (Xia et al. 2018). Several songbird species, such as robins (*Erithacus rubecula*) and blackbirds, have a naturally earlier (about 20-30 minutes) dawn song than great tits. Exposure to light pollution further advances their dawn song (30-60 minutes or more; Da Silva et al. 2014; Kempenaers et al. 2010). The singing behaviour of other species could affect the sleep behaviour of great tits under natural conditions explaining why natural variation in morning light intensity influences leaving time (Stuber et al. 2015b). The study by Stuber et al. (2015b)

included data from March when more bird species sing around dawn (Da Silva et al. 2015), which can help in explaining why birds in brighter box locations exited their boxes earlier in the morning in their study. Light pollution is, however, often associated with noise pollution (Halfwerk and Slabbekoorn 2015) and can also advance dawn song (Fuller et al. 2007; Gil et al. 2015) and effects of light and noise pollution are therefore difficult to disentangle. Da Silva et al. (2014) found that light but not noise advanced dawn song in the European robin, the common blackbird, the song thrush (*Turdus philomelos*), the great tit and the blue tit. Arroyo-Solis et al. (2013) on the other hand, found the opposite for the spotless starling (*Sturnus unicolor*) and the house sparrow (*Passer domesticus*). Whether light or noise pollution affects the timing of dawn song may in part be species-dependent. Our treatment did not expose the larger area around the nest box to ALAN thereby isolating effects of light on sleep behaviour from any other possible confounding effects. Our experimental treatment therefore unlikely affected the singing behaviour of other birds. Furthermore, as our experiment was performed during winter, dawn song of most species should still have been very limited at that moment (see e.g. Da Silva et al. 2015).

Effects of light pollution on cavity-nesting species are potentially not only species-dependent (Sun et al. 2017) but also sex and season may play an important role and interact with each other. Although our study was done during winter when both male and female great tits roost inside cavities and nest boxes, during the breeding season mainly females sleep inside nest boxes (Hinde 1952; Kluijver 1950). Males are therefore possibly exposed to higher levels of light pollution, which could explain results observed on dawn song, a typical male behaviour in great tits (Da Silva et al. 2014; 2015; 2016; Da Silva and Kempenaers 2017). In our current experimental study performed during the winter period we did not find any effects of our light treatment on sleep behaviour of male and female great tits. Previously we also found no effects of ambient light pollution (caused by street lights) on great tit nestlings' physiology (Casasole et al. 2017; Raap et al. 2017a) while several important indicators of immunity, health, and physiological condition were affected in nestlings experimentally exposed to two nights of ALAN inside the nest box. ALAN caused elevated haptoglobin levels, decreased nitric oxide levels, and nestlings did no longer gain any body mass (Raap et al. 2016a; 2016c) and in male nestlings oxalate, a cross-species biomarker for sleep debt (Weljie et al. 2015), seemed to be affected (Raap et al. 2018). Thus during winter, nest boxes/cavities may provide shielding for both sexes while during the breeding season exposure to light pollution is likely sex-dependent

for adults. Exposure to light pollution is not only highly variable for cavity-nesting species light but also for open-nesting species. For example, Dominoni et al. (2013a) showed that urban blackbirds were exposed to a large range of light intensities. While city street lights had a light intensity of around 6 lux, males were exposed to a mean intensity of 0.3 and maximum of about 2.5 lux. Furthermore, not only may exposure to light pollution vary greatly among individuals but also from one night to another (Dominoni et al. 2014). It is therefore important that studies using wild animals quantify individual exposure to light pollution (Raap et al. 2017c), and be cautious in the interpretation and generalisation of the effects, or lack thereof, from light pollution.

We conclude that our light treatment, in which we experimentally exposed the entrance of the nest box to ALAN (1.6 lux white LED), independent of the rest of the environment, had no effect on great tit sleep, while direct exposure to ALAN does disrupt sleep (Raap et al. 2015; 2017b). We therefore hypothesize that artificial light at night resulting from street lights may have a limited direct effect on sleep of birds inside cavities during winter. Light pollution is a growing problem which disrupts the timing of a wide variety of animals (Gaston et al. 2017) but under certain circumstances animals might, due to limited exposure, not suffer from direct effects. Future studies should examine individual light exposure and its consequences for cavity and open-nesting birds throughout different seasons.

Chapter 7

Disruptive effects of artificial light at night on sleep behaviour are not personality-dependent in a free-living songbird

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Abstract

Light pollution or artificial light at night (ALAN) is an increasing, worldwide problem that affects many aspects of animal behaviour, but the response to ALAN varies widely among individuals of the same species. Variation in personality (consistent individual differences in behaviour) may be one important factor explaining individual-level differences in responses to ALAN, but this has rarely been examined. Here, we assayed exploration behaviour in a novel environment as a proxy for personality variation in great tits (*Parus major*), with individual personality types ranging from slow to fast explorers. Using a within-subject design, we observed individual sleep behaviour over two consecutive nights, with animals sleeping under natural dark conditions the first night and exposed to ALAN inside the nest box on the second night. First, we tested whether the likelihood to enter a nest box when confronted with a camera (novel object) was personality-dependent, as this could potentially create a sampling bias. Next, we assessed whether personality types differed in their avoidance behaviour towards ALAN. Finally, we assessed whether experimental exposure to ALAN induced personality-dependent changes in sleep behaviour.

Slow and fast explorers were equally likely to enter a nest box with either a camera or artificial light inside, indicating the absence of personality-dependent sampling bias or avoidance of exposure to ALAN. Slow explorers were also equally disrupted in their sleep behaviour when exposed to ALAN as fast explorers. Whether other effects of ALAN are personality-dependent remains to be determined. Nonetheless, in our increasingly urbanized world, determining whether the effects of anthropogenic stressors depend on personality type will be of paramount importance as it may affect population dynamics.

Introduction

Urbanization presents organisms with a variety of novel and challenging situations, as human disturbance often drastically changes environments. These human-induced environmental changes include climate change, habitat loss and fragmentation, and the spread of exotic species (Wong and Candolin 2015). Moreover, increasing urbanization has led to a dramatic worldwide increase in artificial light at night (ALAN), or light pollution (Falchi et al. 2016). Light pollution impacts natural light dark cycles, posing a potential global threat for wildlife, biodiversity and humans (Davies and Smyth 2018). Increasing evidence shows disruptive effects of ALAN on animal physiology, such as changes in levels of melatonin, testosterone, and immune parameters, as well as animal behaviour (reviewed in Gaston et al. 2017). For instance, in songbirds ALAN affects the timing of singing behaviour, daily activity patterns, sleep behaviour, and breeding behaviour.

Individuals of the same species and population may differ in how strongly they respond to environmental disturbances such as ALAN (Sih et al. 2012). For example, in blue tits (*Cyanistes caeruleus*), yearling males appeared to show a stronger response to ALAN than older individuals, as light pollution increased extra pair paternity rates more in yearling males than in older males (Kempenaers et al. 2010). Similarly, the disruptive effect of ALAN on sleep behaviour in great tits (*Parus major*) varied greatly among females during the nestling period (Raap et al. 2016b). When exposed to ALAN, some individuals seemed unaffected while others did not sleep at all. Moreover, about one in three great tits will not enter a nest box with artificial light inside (Raap et al. 2017b).

Variation in personality may be an important factor explaining individual-level differences in behavioural responses to anthropogenic disturbance in general (Sih et al. 2012), and ALAN in particular. Personality refers to individual differences in behaviour that are consistent across time and/or context (Reale et al. 2007). Growing evidence indicates that personality differences can influence a wide range of ecological processes (Wolf and Weissing 2012), have a heritable component and are associated with variation in fitness (Dochtermann et al. 2015; Smith and Blumstein 2008). Hence, variation in personality might be adaptive and subject to selection (Dingemanse and Wolf 2010), and could influence patterns of behaviour in the urban matrix (Lowry et al. 2013; Sih et al. 2012).

In the great tit, novel environment exploration (henceforth exploration behaviour) is commonly used as an operational measure of variation in personality (Dingemanse et al. 2002;

Verbeek et al. 1994). Exploration behaviour has been shown to be a heritable (Dingemanse et al. 2002; Nicolaus et al. 2012; Quinn et al. 2009) and repeatable trait in different European great tit populations (Dingemanse et al. 2012; Stuber et al. 2013), including in our population (Thys et al. 2017). Individuals differ in how they explore a novel environment, ranging from those that explore an area slowly but thoroughly (so-called slow explorers), to those that explore rapidly but superficially (so-called fast explorers; Verbeek et al. 1994). Exploration behaviour covaries with other personality traits, such as boldness (e.g. Hollander et al. 2008; van Oers et al. 2004) and aggressiveness (Thys et al. 2017), other ecologically relevant behaviours (e.g. Dingemanse et al. 2003; van Overveld and Matthysen 2010), differences in stress physiology (Baugh et al. 2017; Carere et al. 2010), and has been shown to influence fitness (Dingemanse and Reale 2005). Fast explorers are also more likely than slow explorers to accept and approach novel objects (i.e. less neophobic), both in the lab (Baugh et al. 2017; Carere et al. 2005; Verbeek et al. 1994) and field (Cole and Quinn 2014; Stuber et al. 2013), and responses to novelty may be particularly relevant when considering responses to anthropogenic modifications of the environment (Tryjanowski et al. 2016), such as ALAN. For instance, fast explorers were more likely than slow explorers to sleep in the same nest box on subsequent winter nights when a video camera (novel object) was installed inside (Stuber et al. 2013), and breeding great tits with different personality types differed in their response to noise when feeding nestlings, with slow explorers taking longer to enter the nest box during noise playback than fast explorers (Naguib et al. 2013). Along this line, we previously demonstrated that about one in three birds do not enter a nest box with light inside, whereas birds always enter when the light is off (Raap et al. 2017b). Hence, there is the possibility that slow explorers, given they are generally more neophobic and sensitive to disturbances, will be more deterred or actually avoid exposure to light at night, but this remains to be tested.

For those individuals that do enter their nest box with light inside, ALAN caused disruptive effects on sleep behaviour, but interestingly, these effects varied greatly among individuals (Raap et al. 2015, 2016b). Sleep is an important and widespread animal behaviour and, although its functions remain poorly understood, it seems to serve multiple crucial purposes including energy conservation and memory consolidation (reviewed in Rattenborg et al. 2017; Siegel 2008; Tougeron and Abram 2017). Given the importance of sleep and the fact that the disruptive effects of ALAN on sleep behaviour can be highly variable among individuals (Raap et al. 2015, 2016b), it is important to understand what causes this variation. As outlined

above, it seems plausible that a component of this variation may reflect differences in personality, with the most intuitive, but untested, hypothesis being that slow explorers avoid and/or are more disrupted by light.

In this study, we tested the hypothesis that consistent individual differences in exploration behaviour predict how individuals respond to novelty and ALAN, in the context of roosting decisions and sleep behaviour. First, both as a metric of responses to novelty, and as a means of assessing the potential for sampling bias in our subsequent analysis, we examined whether slow exploring great tits in our population were less likely to enter a nest box with a camera (novel object) installed, as previously reported in another population (cf. Stuber et al. 2013). Second, we examined whether slow explorers were less likely to enter a nest box with artificial light inside. Third, of those individuals that did enter the nest box with artificial light, we examined whether slow explorers exposed to ALAN showed more disrupted sleep behaviour compared to fast explorers.

Methodology

Study population and standard procedures

We collected data in a resident semi-urban nest box population of great tits in the surroundings of Wilrijk, Belgium (51°9'44''N, 4°24'15''E), which has been continuously monitored since 1997 (see e.g. Casasole et al. 2017; Raap et al. 2017a; Thys et al. 2017). The great tits used in this study were caught inside nest boxes during previous winter and breeding seasons, and were sexed and ringed after capture. Since 2011 all adults have been provided with a ring or implant containing a PIT tag (passive integrated transponder), thereby enabling the detection of individual birds without physical disturbance when they were roosting inside nest boxes. Age was determined using either hatching records or colour differences of primary coverts to distinguish yearlings (grey) from older birds (bluish; Gosler 1993). Great tits are an important model system for evolutionary and environmental research. Because they readily sleep in nest boxes, we can study their sleep behaviour in the wild and experimentally manipulate the light conditions to which they are exposed to during the night (Raap et al. 2015).

Exploration behaviour in a novel environment

We tested birds for their exploration behaviour as described in Dingemanse et al. (2002). In brief, birds roosting in nest boxes in the winters (November - February) of 2010 until and including 2015 were caught and transported to the laboratory. The morning after capture,

individuals were released separately into a novel environment room (4.0 x 2.4 x 2.3 m) containing five artificial trees. We used the total number of flights and hops within two minutes upon arrival in the room to calculate exploration scores (Dingemanse et al. 2002). Low and high scores represent slow and fast explorers, respectively. After the test, we released all birds near the nest box where they had been captured. Since exploration scores have been shown to increase with repeated testing (Dingemanse et al. 2012; Stuber et al. 2013; Thys et al. 2017), we used only the first measured exploration score (i.e. exploration behaviour in a completely novel environment) for those individuals that were tested multiple times.

Novel object test

Novel object (miniature video camera inside nest box) tests were performed during the winters (November-February) of 2011 until and including 2015. On the first night, prior to the installation of the video camera, the presence and identity of sleeping great tits was checked with a handheld transponder reader (FR-250 RFID Reader, Trovan, Aalten, Netherlands). The next day, miniature infrared sensitive video cameras (Pakatak PAK-MIR5, Essex, UK) were installed under the nest box roof lid (similar to Stuber et al. 2013). Cameras were installed at least two hours before sunset and removed at the earliest about an hour after sunrise the next morning. We had data on both exploration behaviour and the response to the novel object for 86 individuals. This specific dataset was used to examine whether slow explorers were less likely to enter a nest box with a camera inside, similar to Stuber et al. (2013). We used only the first response to the novel object for those individuals that were tested multiple times. The median number of days between novel environment and novel object test was 97, the shortest period between tests was six days. On three occasions the novel environment test was performed after the novel object test (12 days later at the earliest).

Exposure to ALAN and recording sleep behaviour

We constructed a second dataset, in which we combined data on exploration behaviour with data from our previous experimental studies on sleep behaviour and the response to ALAN (Raap et al. 2015, 2016b; Sun et al. 2017). The experimental procedure was generally as follows. Individuals served as their own control in all experiments. Video recording and experimental light systems were installed several hours before sunset when great tits normally go to roost. During the first night when sleep behaviour was recorded, birds slept in a dark nest box. During the second night of recording, a white LED light (15 x 5 mm, taken from a RANEX 6000.217 LED

headlight, Gilze, Netherlands) underneath the nest box roof lid was turned on (see for details Raap et al. 2015; 2017b). This approach ensures effective exposure to the light treatment, which is crucial when exposing animals in the wild to ALAN (Raap et al. 2017c). Our earlier studies during the winter (Raap et al. 2015) and nestling season (Raap et al. 2016b) both used a light intensity of 1.6 lux, while data from Raap et al. (2017b) includes observations obtained with a 3 lux light intensity. However, no differences in the response in sleep behaviour were found between 1.6 and 3 lux (Raap et al. 2017b). Our experimental approach with ALAN inside the nest box does not intend to mimic ambient light pollution to which animals inside cavities/ nest boxes could be exposed to. However, street lighting can easily be more than 10 times as bright (10-40 lux Gaston et al. 2017) as the intensity we used in our experiments on sleep behaviour (1.6 and 3.0 lux; Raap et al. 2015; 2016b), indicating that animals outside cavities/nest boxes can be exposed to similar intensities (see e.g. Dominoni et al. 2013a). Moreover, this field-based experimental approach with free-living animals (contrary to laboratory studies), can offer useful insights into behavioural and physiological effects of ALAN on wild animals (Raap et al. 2016a; 2016c; 2018). Especially since sleep behaviour differs between captive and free-living animals, with great tits sleeping less in captivity (Stuber et al. 2015b).

To examine whether exploration behaviour was associated with the likelihood to enter a nest box with ALAN inside, we used winter data from our 2015 and 2017 publications (Raap et al. 2015; 2017b; Sun et al. 2017), including all individuals that slept in the nest box under natural dark conditions the first night and were confronted with ALAN inside their nest box the following night (N = 68). Data from the experiment performed during the breeding season (Raap et al. 2016b) was omitted from this analysis, as the motivation to enter the nest box greatly differs when individuals need to take care of their nestlings compared to the winter period.

To examine whether exploration behaviour was associated with the degree to which ALAN disrupted sleep behaviour, we used all observations of sleep behaviour from individuals that slept inside a nest box with a light inside (N = 47 of which there was exploration data available for N = 41). This includes data on sleep behaviour from both the winter and nestling season (Raap et al. 2015; 2016b; 2017b; Sun et al. 2017). We recorded sleep behaviour because great tits are too small to be fitted with modern data loggers for recording brain activity (necessary for conclusively defining sleep). Although using this proxy for sleep has its limitations

(Aulsebrook et al. 2016), it can be considered to be ecologically relevant as it has been linked to amongst others, predation risk and extra pair paternity, and has a genetic basis (Christe et al. 1996; Steinmeyer et al. 2010; 2013; Stuber et al. 2014; 2015a; 2015b; 2016; 2017; Tripet et al. 2002). Moreover, in blackbirds behaviourally observed and electrophysiological measured sleep shows close correspondence (Szymczak et al. 1993). In great tits, sleep disturbance resulted in recovery sleep the following night (sleep rebound; Raap et al. 2016b) indicating that sleep behaviour is associated with important functions which cannot be performed otherwise (Lesku and Rattenborg 2014). Sleep phases and sleep intensity can be studied in greater detail by recording brain activity and while behavioural observation is less accurate and more limited to measuring quantity, it is also less invasive and allows the study of sleep in small free-living animals (Aulsebrook et al. 2016). Based on the findings in our previous studies we focussed here on the sleep parameters awakening time and sleep duration, since disruptive effects of ALAN were strongest for these parameters and both were affected during the winter and nestling season (Raap et al. 2015; 2016b; 2017b; Sun et al. 2017). Sleep duration is the time between when an animal falls asleep for the first time in the evening and wakes up in the morning before leaving the nest box, the latter referring to awakening time. The response to ALAN is the difference in sleep behaviour during the first control night and the second night when birds were exposed to ALAN. As in other sleep behaviour studies on birds (Christe et al. 1996; Steinmeyer et al. 2010; 2013; Stuber et al. 2014; 2015a; 2015b; 2016; 2017; Tripet et al. 2002), we defined an individual as asleep when the bird was in the typical sleep position, with the beak pointing backwards and tucked under the scapulars (Amlaner and Ball 1983).

Statistical analysis

All statistical analyses were performed in R 3.3.2 (R Core Team 2016) and were performed in three steps to answer the following questions:

1) Is exploration behaviour associated with the likelihood to enter a nest box with a novel object (camera)? (cf. Stuber et al. 2013)
2) Is exploration behaviour associated with the likelihood to enter a nest box with ALAN inside?
3) Is exploration behaviour associated with the degree to which ALAN disrupts sleep behaviour?

The sim function (package arm; Gelman et al. 2015) was used throughout to simulate values (2000 simulations) of the posterior distribution of all model parameters. Results are

presented as estimated means with 95% credible intervals (CrI), unless stated otherwise, and effects were considered significant in the frequentist sense when CrIs did not overlap zero.

1) Is exploration behaviour associated with the likelihood to enter a nest box with a novel object (camera)?

First, we established that our dataset (N = 86) formed a representative sample of our study population regarding exploration behaviour. That is, the relative frequency distribution of exploration scores in the dataset used in the subsequent analysis was not different from that of the whole population (N = 621, including only the first measured exploration score per individual; X^2 = 7.76; df= 7; P = 0.35; Supplementary Fig. S1 & S2).

To determine whether the likelihood to enter the nest box with a camera was related to exploration score we constructed a generalized linear model (GLM) with binomial error distribution (no overdispersion) and logit-link function (lme4 package; Bates et al. 2015). Whether or not birds entered the nest box was the response variable and the model included sex as a factor and exploration scores as a standardized covariate.

2) Is exploration behaviour associated with the likelihood to enter a nest box with ALAN inside?

Our second dataset (N = 68) also formed a representative sample of our study population regarding exploration behaviour (no significant differences in relative frequency distributions: X^2 = 5.88; df= 7; P = 0.55; Supplementary Fig. S1 & S2). Of the 68 individuals that slept in the nest box under natural dark conditions the first night and were confronted with ALAN the second night, there were only nine for which we had more than one observation of whether they entered the nest box with ALAN inside. Hence, due to the small proportion of repeated measures compared to the overall sample size, we could not use repeated measures in our statistical analysis as model assumptions could not be met (Bolker et al. 2009). We therefore chose to use only the first observation of whether a bird entered the nest box, reflecting the initial response.

To determine whether the likelihood to enter the nest box with ALAN inside was related to exploration score we constructed a generalized linear model (GLM) with binomial error distribution (no overdispersion) and logit-link function. Whether or not birds entered the

nest box was the response variable and the model included sex as a factor and exploration scores as a standardized covariate.

3) Is exploration behaviour associated with the degree to which ALAN disrupts sleep behaviour?

Our past experiments showed that ALAN in winter affected awakening time and sleep duration, which were also affected during the nestling period (Raap et al. 2016b). Therefore, we focused on those effects in this analysis, which also helps avoid multiple testing. Since the degree of response towards ALAN has been shown to differ between periods (winter versus nestling; Raap et al. 2016b), we standardized sleep parameters per period.

The relationship between disruption in sleep parameters by ALAN and exploration score was then modelled using linear models with either awakening time or sleep duration as the response variable. Both models included sex and age (first-year versus older) as a factor and exploration score as a standardized covariate.

Ethics

We compiled a dataset from previous experiments and behavioural data and therefore did not need to expose new individuals to experimental treatments. All experiments were approved by the ethical committee of the University of Antwerp (ID number 2014-45), performed in accordance with Belgian and Flemish laws, and adhered to the ASAB/ABS guidelines for the use of animals in behavioural research and teaching. The Belgian Royal Institute for Natural Sciences provided ringing licenses for all authors and technicians. Because of the short duration of the manipulation (one night of artificial light per experiment) the disturbance was assumed to be minimal.

Data accessible at Zenodo DOI: 10.5281/zenodo.1138586

Results

1) *Is exploration behaviour associated with the likelihood to enter a nest box with a novel object (camera)?*

Exploration score was not associated with the likelihood to enter a nest box with a camera (Table 1; Supplementary Fig. S3). The relative frequency distribution of exploration scores in the population (individuals roosting in nest boxes) was similar before and after camera installation (Supplementary Fig. S2). Nonetheless, the percentage of individuals that entered the nest box when a camera was present dropped to 81%. Overall, there were no sex differences in the likelihood to enter the nest box with a camera (Table 1), with a mean likelihood of 0.86 for females and 0.76 for males.

Table 1: Predictors of the likelihood to enter the nest box with a camera (*N* = 86). Point estimates (β) are given with 95% credible intervals (CrI).

	β[a]	q2.5%[b]	q97.5%[b]
Intercept	1.87	0.99	2.72
Sex[c]	-0.70	-1.87	0.50
Exploration score	-0.10	-0.67	0.47

[a] estimated mean of the posterior distribution

[b] 2.5% and 97.5% quantiles of the posterior distribution (95% CrI)

[c] female as reference category

2) *Is exploration behaviour associated with the likelihood to enter a nest box with ALAN inside?*

Exploration score was not associated with the likelihood to enter a nest box with ALAN inside (Table 2; Supplementary Fig. S4). The relative frequency distribution of exploration scores in the population (individuals roosting in nest boxes) was similar before and after turning on the light inside the nest box (Supplementary Fig. S2). Nonetheless, the percentage of individuals that entered the nest box with ALAN inside dropped to 71% (67% entered in winter 2014, *N* = 27; 73% entered in winter 2015, *N* = 41). Overall, there were no sex differences in the likelihood to enter the nest box with ALAN inside (Table 2), with a mean likelihood of 0.67 for females and 0.74 for males.

Table 2: Predictors of the likelihood to enter the nest box with ALAN inside (*N* = 68). Point estimates (*β*) are given with 95% credible intervals (CrI).

	β[a]	q2.5%[b]	q97.5%[b]
Intercept	0.73	-0.04	1.49
Sex[c]	0.30	-0.82	1.35
Exploration score	-0.18	-0.71	0.34

[a] estimated mean of the posterior distribution

[b] 2.5% and 97.5% quantiles of the posterior distribution (95% CrI)

[c] female as reference category

3) Is exploration behaviour associated with the degree of disruption in sleep behaviour when exposed to ALAN?

Exploration score was not associated with the degree of disruption in sleep behaviour when exposed to ALAN. That is, neither the response in awakening time, nor the response in sleep duration was predicted by exploration score (Table 3; Fig. 1). Overall, there were no differences between the sexes or age classes (first-year versus older) in the degree of sleep disruption by ALAN (Table 3).

Table 3: Predictors of the degree of disruption by ALAN on Awakening time and Sleep duration (*N* = 41).

Point estimates (β) are given with 95% credible intervals (CrI).

	Awakening time			Sleep duration		
	β[a]	q2.5%[b]	q97.5%[b]	*β*[a]	q2.5%[b]	q97.5%[b]
Intercept	0.07	-0.31	0.53	0.09	-0.33	0.55
Sex[c]	0.09	-0.61	0.80	0.07	-0.64	0.74
Age[d]	-0.22	-0.88	0.47	-0.25	-0.91	0.44
Exploration score	-0.21	-0.53	0.13	-0.10	-0.45	0.23

[a] estimated mean of the posterior distribution

[b] 2.5% and 97.5% quantiles of the posterior distribution (95% CrI)

[c] female as reference category

[d] first-year as reference category

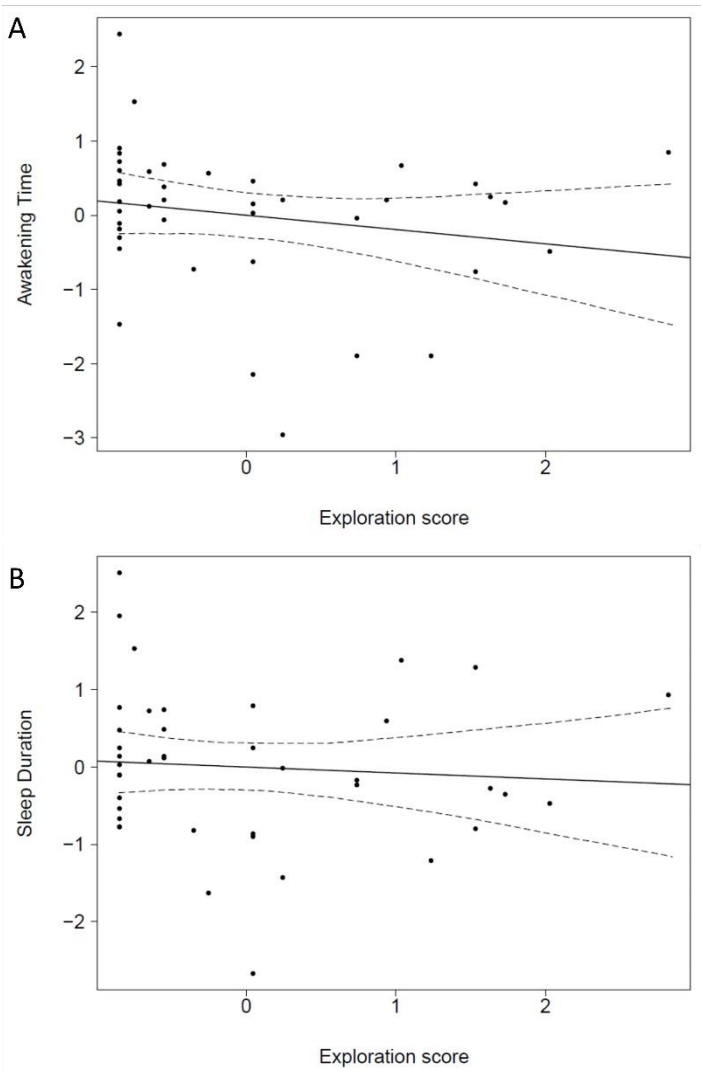

Figure 1: Disruptive effects of artificial light at night are not personality-dependent. Slow and fast explorers were equally disrupted by ALAN in awakening time (A) and sleep duration (B). Exploration score (standardized) in relation to the disruption by ALAN, average regression line (bold) including the 95% credible interval (dashed lines). Observations are shown in circles (*N* = 41).

Discussion

We show that the disruptive effects of artificial light at night on sleep behaviour (awakening time and sleep duration) were not associated with consistent individual differences in exploration behaviour, as a proxy of personality variation, in free-living great tits. Contrary to expectations and findings in Stuber et al. (2013), we found that slow and fast explorers were equally likely to enter a nest box with a camera inside, as well as equally likely to enter a nest box with ALAN inside. Hence, although the overall percentage of birds entering their nest box decreases after installation of a camera or light, we found no evidence for personality-dependent sensitivity to the novel object or avoidance of artificial light. Consequently, no sampling bias arose due to this experimental treatment. In the following paragraphs, we discuss our results and their possible implications.

No evidence for personality-dependent sampling bias or avoidance of ALAN

We find no evidence that nest boxes with a camera or LED light inside induced a personality-dependent sampling bias or avoidance of artificial light, since slow and fast explorers were equally likely to enter their nest box in both cases. This contradicts predictions, since compared to fast explorers, slow exploring great tits are generally found to be less willing to approach and accept novel objects (e.g. Baugh et al. 2017; Carere et al. 2005; Cole and Quinn 2014; Stuber et al. 2013; Verbeek et al. 1994), and are expected to be more sensitive to anthropogenic disturbances (Lowry et al. 2013; Naguib et al. 2013; Sih et al. 2012). Indeed, Stuber et al. (2013) previously found that slow exploring great tits were less likely than fast explorers to enter a nest box when confronted with a camera inside.

There are several possible explanations for this discrepancy in results, such as a difference in the environment. Of particular note, Stuber et al. (2013) studied a rural population of great tits, whereas our study was done in a semi-urban population. Increasing evidence suggests that the level of urbanization can influence behaviour, both within and among species (Wong and Candolin 2015), and that certain behavioural phenotypes might be more successful in settling in more urbanized environments (review in Lowry et al. 2013). For example, when compared to rural counterparts, male song sparrows (*Melospiza melodia*) in urban populations were found to be bolder towards humans, and more aggressive during territorial intrusion (Evans et al. 2010; Scales et al. 2011). Similarly, urban great tits were on average more exploratory than their rural conspecifics (Charmantier et al. 2017) and different great tit personality types have been found to be non-randomly distributed along urbanization

gradients (Charmantier et al. 2017; Sprau and Dingemanse 2017). Hence, the difference in findings in our semi-urban population compared to the rural population of Stuber et al. (2013) could be caused by the overrepresentation of fast exploring, bold individuals in our population. However, this was not the case, as the relative frequency distributions of exploration behaviour in both populations seem very similar, and if anything, slow explorers might be more represented in our population (relative frequency exploration score 0-5, our population ≈ 0.42, Supplementary Fig. S2; Stuber et al. 2013 ≈ 0.23).

Another possibility is that differences in selection pressures between (semi-) urban and rural environments influence the relationship between exploration behaviour and the response to novelty (Sih et al. 2012). Higher human disturbance levels in our population (relative to rural populations) might result in habituation to disturbance and/or select for on average less neophobic birds (Tryjanowski et al. 2016), regardless of differences in exploration behaviour. Additionally, the availability of natural cavities might be lower in our population, leaving less alternatives for roosting sites and increasing motivation to use nest boxes. Together this might explain why the percentage of birds entering the nest box with a camera inside is higher in our population (81%) compared to the population of Stuber et al. (2013; 60%). However, whether particular behavioural trait associations are influenced by urbanization (e.g. Bokony et al. 2012; Scales et al. 2011), and whether this is due to phenotypic plasticity (e.g. habituation) and/or adaptive evolution, requires further research (see e.g. Charmantier et al. 2017; Sih et al. 2012).

It should also be noted that Stuber et al. (2013) showed short-term nest box fidelity of great tits, with individuals roosting in the same nest box on subsequent nights. We have no data to formally test nest box fidelity in our population. However, our population has been continuously monitored since 1997 and individuals appeared to be site faithful and can often be found in the same nest box throughout the winter and even in subsequent years (personal observation).

While about one in three birds did not enter a nest box with a LED light inside (Raap et al. 2017b), our study suggests that this variation in behaviour is not personality-dependent. That is, slow explorers are not more likely than fast explorers to avoid exposure to artificial light when roosting. As a result, our experimental treatment (ALAN inside a nest box) did not limit itself to a specific sample of the population based on exploration behaviour. Potentially this generally means that in great tits light pollution does not deter slow explorers to a greater

extent than fast explorers. A recent study in great tits demonstrated that individual differences in exploration behaviour were not associated with variation in urbanization features of the breeding territory, including artificial light (Charmantier et al. 2017). This indicates that artificial light does not necessarily influence personality-dependent decisions of breeding locations. However, whether ambient light conditions influence personality-dependent roosting decisions in free-living birds remains to be determined. Moreover, it should be noted that occupancy of a nest/roost site depends on many factors such as availability of natural cavities and nest boxes and that great tits (and other cavity-nesting species) are not entirely "free" to choose where to roost. They may therefore roost in suboptimal conditions due to a lack of alternatives.

No evidence for personality-dependent effects of ALAN on sleep behaviour

We find no evidence that ALAN induced personality-dependent effects on sleep behaviour, since sleep behaviour of slow and fast explorers was equally disrupted. More exploratory and bolder individuals are generally expected to be better able to cope with challenges and to have a higher tolerance to disturbance (e.g. Lowry et al. 2013) implying that we could expect a difference in their response to ALAN. That is, fast exploring great tits are predicted to be more successful in inhabiting disturbed environments, including those with high levels of (light) pollution. In line with this prediction, when compared to fast explorers, slow exploring great tits were more disturbed by noise, taking longer to restart feeding their nestlings after the initiation of experimental playback (Naguib et al. 2013). Moreover, rural great tits have been found to be less exploratory and more neophobic than their urban counterparts (Charmantier et al. 2017; Tryjanowski et al. 2016) suggesting that slow exploring and neophobic individuals are less successful in inhabiting environments with higher anthropogenic disturbance. Similarly, in another study on great tits, bold individuals (with boldness measured as flight initiation distance) were overrepresented in areas with more cars and fewer pedestrians, while shy individuals were more likely to be found in areas with less cars and more pedestrians (Sprau and Dingemanse 2017). However, the sensitivity to disturbance of different behavioural types might depend on the behavioural context and the specific type of disturbance or component of urbanization in question, making broad generalizations difficult.

To the best of our knowledge, this is only the first study to assess whether the degree of disruption by ALAN is dependent on the personality type of free-living animals and therefore there are many opportunities for further research. First, our light treatment consisted of a

single night, while light pollution is a long-term disturbance. Hence, we cannot exclude the possibility that long-term exposure to ALAN might result in personality-dependent avoidance or other behavioural and physiological responses. Indeed, laboratory and aviary experiments suggest that habituation towards ALAN is unlikely, as has for example been demonstrated in peahens (*Pavo cristatus*; Yorzinski et al. 2015). Moreover, light exposed great tits in a laboratory experiment actually showed increased effects on nightly activity during longer-term exposure to ALAN (de Jong et al. 2016). Due to differences in behaviour and especially sleep behaviour between captive and wild animals (Aulsebrook et al. 2016; Stuber et al. 2015b) it will be of interest to examine effects of long-term exposure to ALAN in free-living great tits. Second, our light treatment does not attempt to mimic natural variation in the light conditions experienced by cavity-nesting species at night (see also Methodology). Hence, while we find that personality does not affect whether an individual enters a nest box with ALAN, the extent to which ambient light conditions affects personality-dependent roosting decisions in the wild remains to be determined. Preferably our results would be validated using other populations since between-population differences might occur (Tryjanowski et al. 2016), as for example found here for the response to novelty between our semi-urban population and the rural population used by Stuber et al. (2013). Third, although we find no evidence that the degree of disruption by ALAN is dependent on personality type, future studies should aim at larger samples sizes to validate our results. Finally, while our study is based on a widely used model species, differences in response to ALAN may occur even between closely related species (Sih et al. 2012). For example, earlier we found that ALAN differentially affects the sleep behaviour of congeneric blue and great tits, with blue tits hardly being affected by ALAN, in contrast to great tits (Sun et al. 2017). Hence, whether differences in personality-dependent responses to ALAN occur between populations and even closely related species requires further research. If ALAN would differentially affect certain behavioural or physiological phenotypes across ecologically relevant contexts, this might have cascading effects on energy balance and fitness, and might affect population dynamics in urbanized and light polluted areas (Sih et al. 2012; Wong and Candolin 2015).

Conclusions

Slow and fast personality types were equally affected by ALAN. Furthermore, experimentally placing a light inside the nest box to study the effects of ALAN did not cause personality-dependent sampling bias or avoidance behaviour. Therefore, this setup ideally bridges the gap between laboratory and field studies exploring fundamental questions about the effects of ALAN in free-living birds. However, our results need to be validated as personality-dependent effects of ALAN and other anthropogenic stressors could alter population dynamics in urbanized areas. Therefore, in our increasingly urbanized world, determining whether the effects of these stressors depend on personality

Supplementary Material

Table S1: Predictors of the likelihood of a strong response of ALAN on Awakening time and Sleep duration for females (*N* = 26). Point estimates (β) are given with 95% credible intervals (CrI). Effects where CrIs do not overlap with zero are considered significant.

	Awakening time			**Sleep duration**		
	β[a]	q2.5%[b]	q97.5%[b]	*β*[a]	q2.5%[b]	q97.5%[b]
Intercept	-0.69	-1.90	0.59	1.01	-0.35	2.37
Period[c]	0.73	-0.94	2.44	-0.57	-2.39	1.27
Exploration score	0.58	-0.34	1.48	1.44	0.05	2.81

[a] estimated mean of the posterior distribution

[b] 2.5% and 97.5% quantiles of the posterior distribution (95% CrI)

[c] nestling period as reference category

Table S2: Predictors of the likelihood of a strong response of ALAN on Awakening time and Sleep duration in the winter period (*N* = 28). Point estimates (β) are given with 95% credible intervals (CrI). Effects where CrIs do not overlap with zero are considered significant.

	Awakening time			**Sleep duration**		
	β[a]	q2.5%[b]	q97.5%[b]	*β*[a]	q2.5%[b]	q97.5%[b]
Intercept	0.06	-1.03	1.22	0.41	-0.77	1.60
Sex[c]	-1.14	-2.73	0.50	-1.57	-3.29	0.15
Exploration score	0.53	-0.27	1.30	0.76	-0.03	1.58

[a] estimated mean of the posterior distribution

[b] 2.5% and 97.5% quantiles of the posterior distribution (95% CrI)

[c] female as reference category

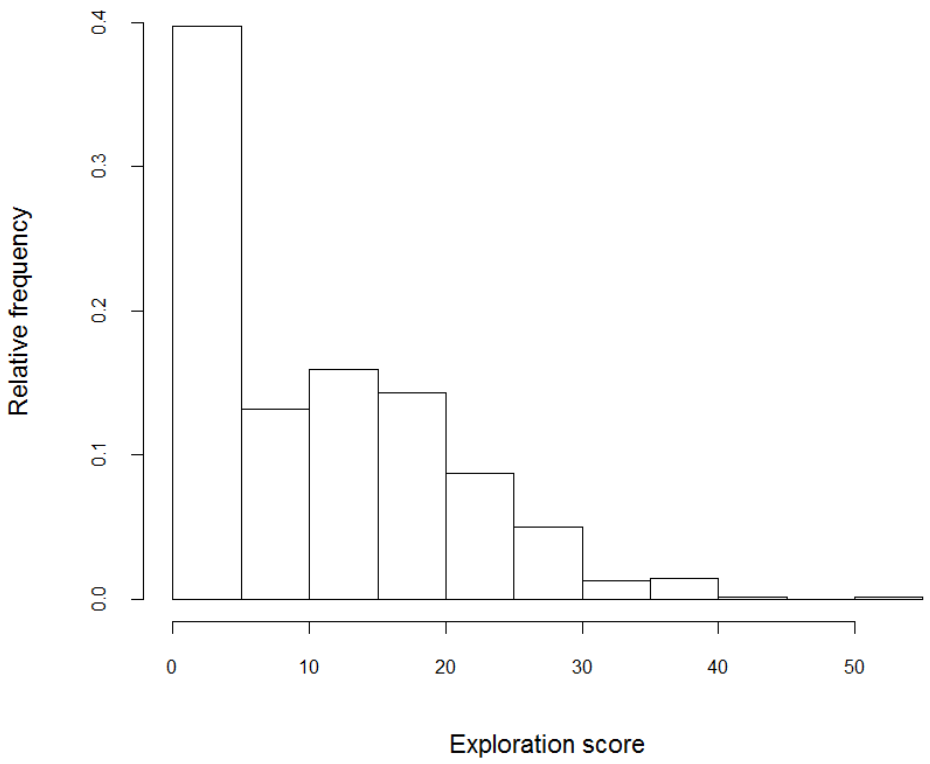

Figure S1: The relative frequency distribution of exploration scores from the whole population (*N* = 621), including only first measured exploration score per individual.

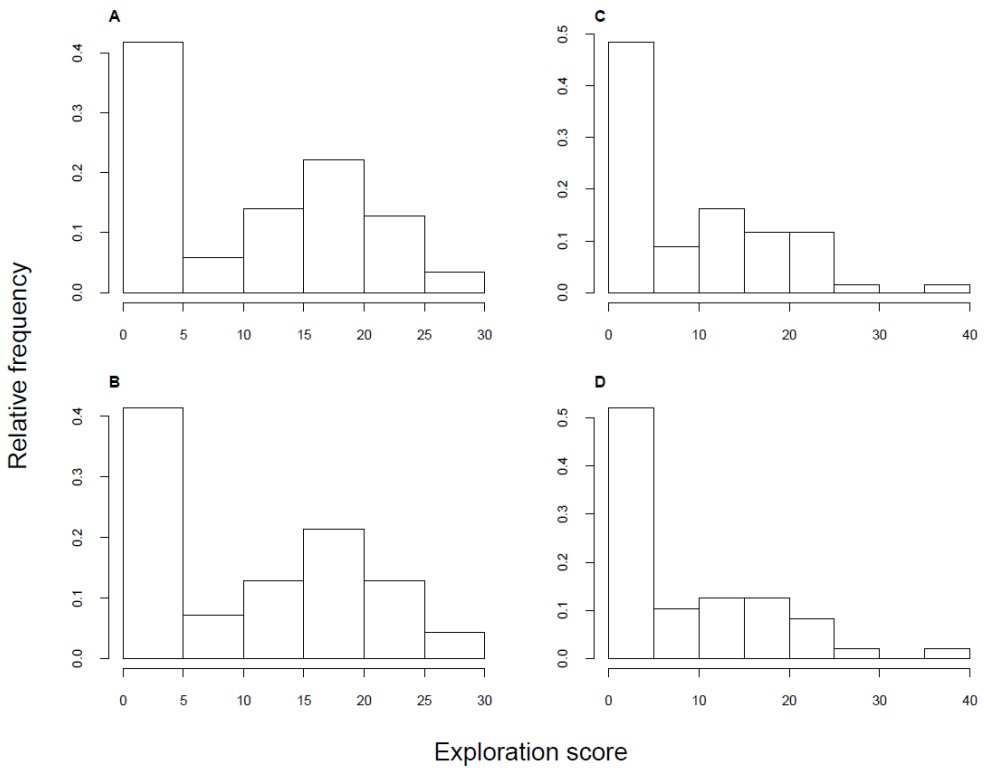

Figure S2: Relative frequency distribution of individuals' exploration scores. A) Individuals that utilise nest boxes to roost without handling (*N* = 86). B) Individuals that utilise nest boxes to roost with a camera inside (*N* = 70). C) Individuals that utilise nest boxes to roost under natural dark conditions (*N* = 68). D) Individuals that utilise nest boxes to roost with the LED light turned on (*N* = 48).

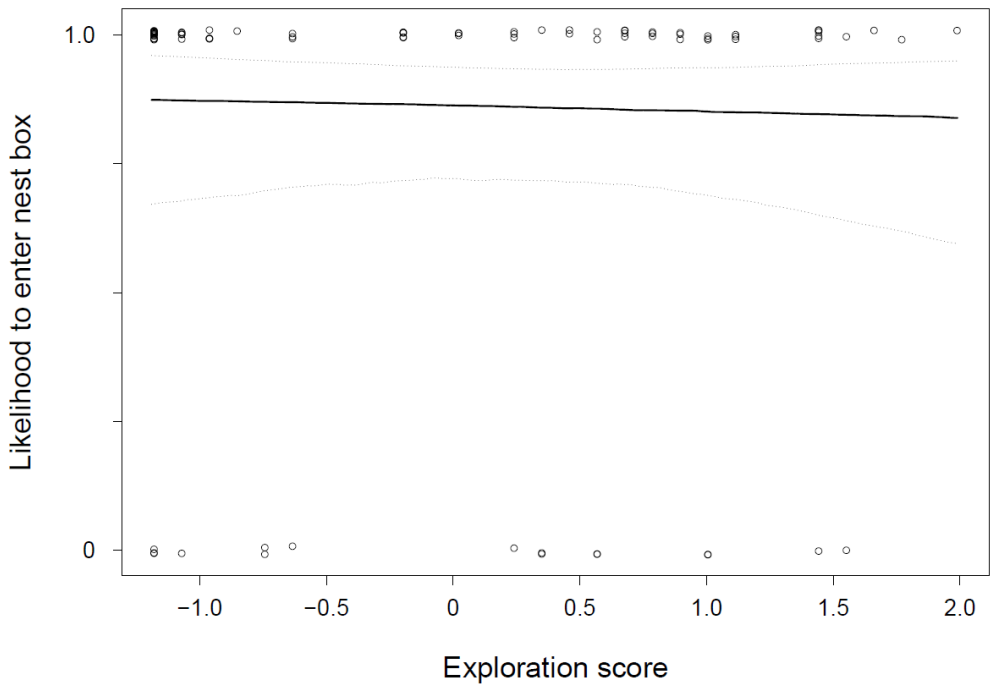

Figure S3: Slow explorers are equally likely as fast explorers to enter a nest box with a camera inside. Exploration score (standardized) in relation to the likelihood to enter a nest box with a camera inside is indicated with an average regression line (bold) including the 95% credible interval (dotted lines). Observations are shown in circles (*N* =86).

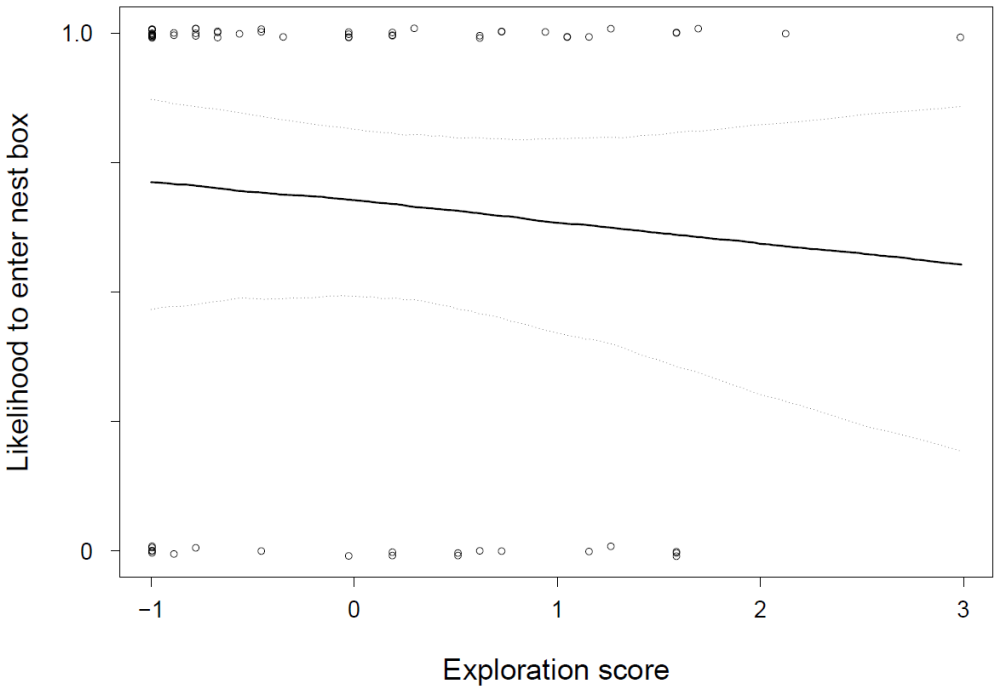

Figure S4: Slow explorers are equally likely as fast explorers to enter a nest box with ALAN inside. Exploration score (standardized) in relation to the likelihood to enter a nest box with ALAN inside is indicated with an average regression line (bold) including the 95% credible interval (dotted lines). Observations are shown in circles (*N* = 68).

Chapter 8

Artificial light at night affects body mass but not oxidative status in free-living nestling songbirds: an experimental study

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Abstract

Artificial light at night (ALAN), termed light pollution, is an increasingly important anthropogenic environmental pressure on wildlife. Exposure to unnatural lighting environments may have profound effects on animal physiology, particularly during early life. Here, we experimentally investigated for the first time the impact of ALAN on body mass and oxidative status during development, using nestlings of a free-living songbird, the great tit (*Parus major*), an important model species. Body mass and blood oxidative status were determined at baseline (=13 days after hatching) and again after a two night exposure to ALAN. Because it is very difficult to generalise the oxidative status from one or two measures we relied on a multi-biomarker approach. We determined multiple metrics of both antioxidant defences and oxidative damage: molecular antioxidants GSH, GSSG; antioxidant enzymes GPX, SOD, CAT; total non-enzymatic antioxidant capacity and damage markers protein carbonyls and TBARS.

Light exposed nestlings showed no increase in body mass, in contrast to unexposed individuals. None of the metrics of oxidative status were affected. Nonetheless, our study provides experimental field evidence that ALAN may negatively affect free-living nestlings' development and hence may have adverse consequences lasting throughout adulthood.

Introduction

The rapid increase of artificial light at night (ALAN), termed 'light pollution', is leading to a loss of darkness with largely unknown consequences for biodiversity, ecosystems and ecological and evolutionary processes (Hölker et al. 2010b; Rich and Longcore 2005). This is likely to be problematic for many species as light has a strong biological relevance for the daily and annual rhythms of life (Bradshaw and Holzapfel 2007). Recently it is becoming clear that ALAN affects a wide variety of behavioural traits, such as reproduction, foraging, sleep and migration (Swaddle et al. 2015), and has also physiological effects, including alterations in immune response, cortisol levels, testosterone levels and glucose metabolism (Bedrosian et al. 2016; Swaddle et al. 2015).

The impact of ALAN on individual status may be especially relevant when the organism is exposed in early life (Fonken and Nelson 2016), as the environment in which a young individual develops has profound, long-lasting and often irreversible consequences throughout the individual lifetime (Harris and Seckl 2011; Henriksen et al. 2011; Lindstrom 1999; Monaghan 2008). For example, in the wild, body mass of young birds at fledging is a good predictor of survival and recruitment because body energy reserves help individuals to cope with the adverse conditions of winter (Horak et al. 1999; Magrath 1991; Naef-Daenzer et al. 2001; Perrins and McCleery 2001). Given that ALAN can influence foraging behaviour of parents (Stracey et al. 2014) and sleep behaviour of nestlings (Raap et al. 2016b), it is plausible to expect an impact of ALAN on individual health and condition through its effects on body mass. However, it may impact individual status (other than body mass) also through other mechanisms, such as changes in oxidative status, especially since ALAN affects the immune response and cortisol levels (Swaddle et al. 2015).

In recent years, ecologists have been studying antioxidants and oxidative damage in free-living organisms and have integrated principles of oxidative stress into several core evolutionary concepts, such as life-history trade-offs (e.g. survival versus reproduction), senescence and sexual selection (Costantini 2014). Oxidative stress is a biochemical condition of the cell that occurs when there is an imbalance between pro-oxidants and antioxidants in favour of pro-oxidants leading to oxidative damage to biomolecules (Costantini and Verhulst 2009; Halliwell and Gutteridge 1985). It is thought that oxidative stress is an important candidate mechanism underlying the effects of environmental changes on organism fitness because of its effects on growth (Stier et al. 2015), fertility (Costantini et al. 2010), immune

protection (Costantini 2008) and cellular senescence (Finkel and Holbrook 2000). It was shown in European shags (*Phalacrocorax aristotelis*) that fledglings with higher oxidative stress had a lower recruitment probability (Noguera et al. 2012) and in great tits (*Parus major*) that red blood cell resistance to oxidative stress predicted fledging success (Losdat et al. 2013). Laboratory work also showed that ALAN could influence the individual oxidative status (Navara and Nelson 2007), as ALAN reduces melatonin (Dominoni et al. 2013d; Reiter et al. 2011) which is an enhancer of antioxidant enzymes gene expression and known as a reactive oxygen species scavenger (Cruz et al. 2003; Hardeland et al. 2003; Reiter et al. 2000; Tan et al. 2010). However, whether and how light pollution affects oxidative stress in the wild is still a major research gap (Isaksson 2015).

The variable nature of interactions among oxidative status biomarkers makes it very difficult to generalise the oxidative status from one or two measures (Cohen and McGraw 2009; Costantini et al. 2013; Dotan et al. 2004). The low correlations among biomarkers that are commonly found also imply that each biomarker reveals independent information on the oxidative status (Cohen and McGraw 2009; Costantini et al. 2013; Dotan et al. 2004). Moreover, one must take into consideration the fact that there is a vast array of antioxidant molecules that might respond to greater production of reactive species, as well as a large number of damage compounds can be produced (Halliwell and Gutteridge 2007). In addition, either low or high antioxidant levels do not necessarily indicate whether damage is, or, is not occurring (Costantini and Verhulst 2009), thus it is important to measure more than one type of marker of antioxidant protection along with markers of oxidative damage. To this end, in this study, we have relied on a multi-biomarker approach in order to obtain a better understanding of oxidative status.

In this study, we assessed for the first time the impact of disturbance induced by ALAN on body mass and multiple metrics of oxidative status (including antioxidant defences and oxidative damage) in nestlings of a free-living songbird, the great tit. The great tit is an important model species in evolutionary and environmental research. Although laboratory studies have often focused on one sex (see e.g. Ashkenazi and Haim 2013), we took into account that in birds, and especially in great tits, there may be sex-specific differences in oxidative status, growth rate (Giordano et al. 2015; Speakman et al. 2015) and environmental sensitivity (reviewed in Jones et al. 2009), and we therefore used both male and female nestlings.

Methods

Study area and general procedures

Data were collected between 8 and 25 May 2015 in a resident suburban nest box population of great tits in the surroundings of Wilrijk, Belgium (51°9'44''N, 4°24'15''E). In 1997, nest boxes were installed and since then this free-living population has been continuously monitored (Rivera-Gutierrez et al. 2012; Van Duyse et al. 2005). Nest boxes made out of plywood with a metal ceiling were of standard size with outer dimensions of 120 × 155 × 250 mm (width × depth × height) and a nest box opening of 30 mm ø. During the breeding season, we checked nest boxes every other day, and every day when close to hatching, to determine laying date, clutch size, hatching day and fledging day. Nestlings were provided with a unique metal ring when they were between 11 and 13 days old (hatch day = day 1).

Experimental procedure

While field studies on oxidative status (OS) often rely on single point measurements and experiments on free-living animals are often unfeasible (van de Crommenacker et al. 2010), we experimentally investigated effects of ALAN on OS using wild great tits and took repeated measurements as the latter is important for understanding physiological responses (van de Crommenacker et al. 2010) as well as to control for confounding variables (e.g. brood size) and variation generated by individuals. We randomly assigned 32 nests to one of the two treatment groups: a control (dark) and a light treated group. When nestlings were 13 days old, we collected a blood sample (≤ 150 µL) to determine their baseline levels of oxidative status and subsequently weighed them (0.1 g; digital balance; Kern TCB 200-1). We repeated this procedure after two nights when the nestlings were 15 days old, to assess changes in oxidative status and body mass. In the light group, nestlings were exposed to two consecutive nights of light (see *Light treatment*), from day 13 to day 15.

Nests from the control and light group were always paired, primarily based on hatching date and similar brood size (7.0 ± 1.2 SD nestlings) and sampled on the same morning immediately after each other (between 8:00 and 12:00). The order of sampling the control and experimental nest(s) was kept the same within a pair but alternated between pairs so that there was no bias in the timing of sampling between the light and control group. Using a within individual and paired design is important as it eliminates many potential confounding variables (Ruxton and Colegrave 2010). In total, we obtained paired data on body mass and oxidative status from 16 nests in the control and 16 nests in the light group. From 115 nestlings in the

control group and 109 in the light group we obtained body mass measurements. To get a representative sample on the oxidative status of each nest, we used blood samples of three nestlings for each nest, the heaviest, lightest and the median (N = 96), for further laboratory analyses. However, due to limitations in blood availability, sample size varies per oxidative status measurement (between 89-96; see Supplementary Table S1).

Light treatment

In each nest box we placed a small LED light (15 mm x 5 mm, taken from a RANEX 6000.217 LED headlight, Gilze, Netherlands), which was standardized to produce 3 lux on the bottom of the nest box (ISO-Tech ILM 1335 light meter; Corby, UK), under the nest box roof lid. We have used this light system successfully in earlier studies on effects of ALAN on sleep behaviour (Raap et al. 2015, 2016b).

In the light-treated group, lights were turned on at 19:00 in the evening (about two hours before sunset) and turned off at 07:00 (about one hour after sunrise) the following morning. 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.

We based the length (two nights) and light intensity (3 lux) of our experimental treatment on previous laboratory studies because experiments as ours have not been done in the wild until now. Previous experimental studies on the physiological effect of ALAN (Bedrosian et al. 2016; Davies et al. 2013; de Jong et al. 2016; Dominoni et al. 2013a; 2013d; Gaston et al. 2013; Raap et al. 2016c) used light intensities ranging from 0.05 to 5 lux and higher (Raap et al. 2016b; see for an overview Raap et al. 2016c). In the laboratory, effects of light manipulations on melatonin levels were difficult to detect at lower light levels ≤ 0.5 lux and were more obvious using 1.5 and 5.0 lux in great tits (de Jong et al. 2016). We chose a light intensity of 3 lux with which we still expected to find differences in oxidative status but which was not too high so that parents would abandon their nests when the light was turned on (Raap et al. 2015). Animals living in light polluted areas are exposed to similar and/or higher light intensities, especially outside nest boxes or cavities (Dominoni et al. 2013a; Gaston et al. 2013; Raap et al. 2015). Because there is now a shift towards energy efficient broad spectrum light sources, such as LED for street lighting, we chose white LED light which has a broad spectrum (Davies et al. 2013; Schubert and Kim 2005). Because of the energy efficiency of LED light there is no warming effect of the lights inside the nest boxes. Laboratory studies often use

experimental periods of several weeks or months (see e.g. de Jong et al. 2016; Dominoni et al. 2013d), which is unfeasible with free-living developing nestlings. Short-term light treatments (e.g. two nights of half an hour) have also been used in combination with high light intensity (450 lux; Ashkenazi and Haim 2013). Two nights of ALAN may thus induce effects on oxidative status but limit the risk of any nest abandonment (Raap et al. 2015).

Laboratory analyses

With the use of PCR we determined the sex of nestlings (Griffiths et al. 1998). We measured seven parameters of oxidative status (Beaulieu and Costantini 2014) in red blood cells and one in plasma. Using HPLC, we measured two molecular antioxidants in red blood cells: reduced glutathione (GSH) and oxidised glutathione (GSSG) after which we calculated the ratio GSH/GSSG, which is used as an index of redox state, with lower values indicating higher oxidative stress (Jones 2006). We also estimated the total non-enzymatic antioxidant capacity (TAC) and measured activity of three major antioxidant enzymes in red blood cells that differ in the way they protect cells against oxidative stress (Beaulieu and Costantini 2014; Moreno et al. 2005): glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase (CAT). Finally, we measured protein carbonyls (marker of protein oxidation) in red blood cells, as well as thiobarbituric acid reactive substances (TBARS; marker of lipid peroxidation) in plasma, as markers of oxidative stress. Further details are given in the supplementary material.

Data analysis

For all statistical analyses we used R 3.1.2 (R Core Team 2016). We performed a linear mixed effect analysis (LMM) on the effect of ALAN on nestling body mass (using the lme4 package (Bates et al. 2015); see the supplementary material about the justification of the use of LMM). As fixed effects, we entered “treatment” (control, light), “day” (13, 15), “sex”, “brood size” as well as the interaction between “treatment“ and “day” and the three-way interaction “treatment”, “sex” and “day”. We used as random effect “bird identity” which was nested in “nest identity” which was nested in “pair” (bird identity:nest identity:pair) to control for the repeated measures and to take the experimental design into account (see experimental procedure).

We performed separate LMMs with the different metrics of oxidative status as dependent parameters. As fixed effects, we entered “treatment” (control, light), “day” (13, 15), “sex”, “brood size”, “body mass” and the three-way interaction “treatment”, “sex” and “day”.

The same random structure was used as for the analysis on body mass (bird identity:nest identity:pair). In order to meet model assumptions the parameters GSH/ GSSG, GSSG, TAC, GPX, CAT, SOD and protein carbonyls were square root transformed and TBARS was log transformed. P-values obtained by a stepwise backward regression are given in results (full model output is given in Supplementary Tables S2 and S3) and where applicable, Tukey HSD tests were used for post-hoc analyses (lmerTest; Kuznetsova et al. 2016).

Ethical statement

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. The Belgian Royal Institute for Natural Sciences (Koninklijk Belgisch Instituut voor Natuurwetenschappen) provided ringing licences for authors and technical personnel.

Results

Artificial light at night had a significant effect on nestling body mass ($F = 7.209$, $P = 0.009$, Fig 1; full model output is given in Supplementary Table S2). Nestlings from the control group gained body mass between day 13 and day 15 (0.5 ± 0.06 gram, $t = 7.14$, $P < 0.001$), while body mass of individuals from the light group did not change ($t = 0.47$, $P = 0.6$). Males ($N = 107$) had on average larger body masses than females ($N = 117$; average of day 13-15 respectively 15.9 ± 0.230 and 15.2 ± 0.231 gram; $t = 5.38$, $P < 0.001$).

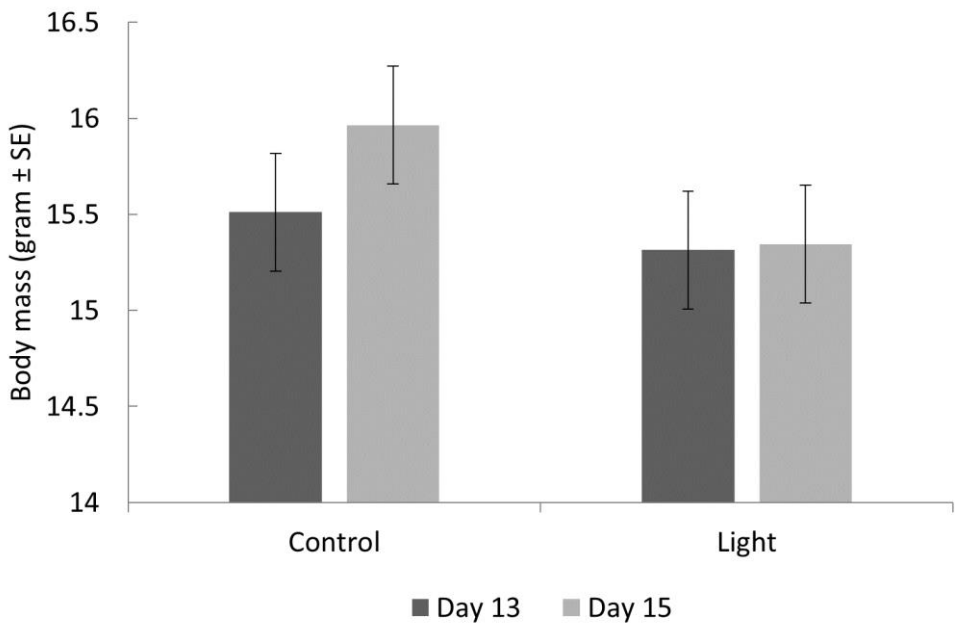

Figure 1: Effect of artificial light at night on nestling body mass. Estimates were obtained from linear mixed models with individual (*N* = 224) nested in nest (32) nested in pair as random factor (bird identity:nest identity:pair). Nestlings in the control group gained body mass between day 13 and day 15 (t = 7.41, P < 0.001) contrary to individuals in the light group whose body mass did not change (t = 0.047, P = 0.6).

There was no sex dependent effect of artificial light on any of the oxidative status metrics and ALAN did not affect any of the oxidative status biomarkers measured (see Table 1 for full model results and Supplementary Table S3 for final models). Individuals in the control group had higher levels of GSH than those in the light group but because this was independent of time (no time:treatment interaction) it is not an effect of ALAN (0.6 ± 0.22 mmol/ gram RBCs square root transformed; t = 2.54, P = 0.02). Males in the control group had lower levels of GSSG than those in the light group but this is not an effect of ALAN because it was independent of time (no time:treatment:sex interaction; see Supplementary Table S4 for estimates). There was no difference in GSSG between females in the control or light group (t = -1.01, P = 0.32). There was a small decrease in TAC, GPX and CAT over time (Supplementary Table S5). While there was no observable effect of ALAN on oxidative damage, there was a difference between sexes in TBARS (F = 7.914, P = 0.005; Supplementary Table S6) with an increase over time for males but not for females.

Four individuals did not fledge after the experiment, one individual from the control group and three from the light group. This difference was not significant ($X^2 = 1.131$, $P = 0.288$).

Table 1: Statistical output of the full mixed effect models, effect of artificial light on oxidative stress parameters. Linear mixed models with "bird identity" nested in "nest" nested in "pair" as random factor were used (bird identity:nest identity:pair). Significant values ($P < 0.05$) are depicted in bold), see Supplementary Table S1 for sample sizes per parameter (between 89-96 individuals). P-values obtained after a stepwise backward regression are mentioned in the main text (see also Supplementary Table S3).

		GSH	GSSG	GSH/GSSG	TAC	GPX	SOD	CAT	Protein carbonyls	TBARS
Sex: Treatment: Time	*F*	0.024	0.310	0.005	0.098	0.213	2.155	1.768	0.585	0.116
	P	0.877	0.579	0.946	0.754	0.645	0.144	0.185	0.445	0.733
Treatment: Sex	*F*	0.039	6.400	0.426	0.439	0.252	0.354	0.188	1.311	0.002
	P	0.843	**0.013**	0.516	0.508	0.616	0.553	0.665	0.254	0.961
Sex: Time	*F*	3.185	0.003	0.097	0.881	0.701	0.056	2.220	0.125	7.657
	P	0.078	0.958	0.756	0.349	0.404	0.814	0.138	0.724	**0.006**
Treatment: Time	*F*	1.479	0.376	1.735	2.811	0.237	0.288	1.973	0.189	0.761
	P	0.227	0.541	0.191	0.095	0.627	0.592	0.162	0.665	0.384
Treatment	*F*	5.444	0.885	0.000	1.785	0.535	0.337	0.378	0.018	0.754
	P	**0.033**	0.362	0.986	0.183	0.465	0.569	0.540	0.895	0.386
Sex	*F*	1.509	0.614	0.039	0.615	0.010	0.304	0.289	0.002	0.708
	P	0.223	0.436	0.843	0.434	0.922	0.582	0.591	0.964	0.401
Time	*F*	0.263	0.012	0.062	9.564	11.416	0.002	22.191	2.666	3.686
	P	0.609	0.915	0.803	**0.002**	**0.001**	0.964	**0.000**	0.104	0.056
Brood size	*F*	3.397	0.450	2.171	0.888	0.036	0.707	0.378	1.246	0.321
	P	0.076	0.508	0.147	0.351	0.851	0.407	0.541	0.266	0.571
Weight	*F*	1.920	3.430	0.038	0.007	0.346	3.542	0.234	0.143	0.074
	P	0.170	0.069	0.846	0.933	0.557	0.063	0.629	0.705	0.786

Discussion

Using a sophisticated experiment, in which the within-individual and paired design is likely to eliminate many confounding variables, we show that artificial light at night (ALAN) affects the development of free-living nestlings. ALAN had a significant negative effect on body mass gain of nestlings. Markers of oxidative status (OS) appeared to be unaffected by our short-term light treatment.

We found that nestlings exposed to artificial light, contrary to those in the control group (who gained body mass in accordance with results from earlier studies; e.g. Gebhardt-Henrich and Noordwijk 1994), did not gain any body mass during a period of two days. An earlier study in our population showed that artificial light inside the nest box significantly increased nestlings' activity as they started begging during the night while in the dark they hardly begged at all (Raap et al. 2016b). This implies that ALAN causes not only adults (Raap et al. 2015, 2016b) but also nestlings to be more awake and thus more active. There are two possible and not mutually exclusive explanations of how increased begging and or activity could lead to the observed lack of gain in body mass.

Firstly, this increased activity may lead to increased energy expenditure and a deterioration of body condition (Kilner 2001; Neuenschwander et al. 2003; Soler et al. 2014). Rodriguez-Girones et al. (2001) experimentally showed that increased begging (during the day) of ring dove (*Streptopelia risoria*) and magpie (*Pica pica*) nestlings can indeed lead to a decreased growth rate. If in our case the parents were unable to compensate for the increased energy expenditure, through an increased feeding rate or time the following day, it could explain why the chicks did not gain any body mass. It is not clear whether ALAN effectively enhances foraging and or food provisioning (see e.g. Stracey et al. 2014; Titulaer et al. 2012) and even if it does, it may not be sufficient to compensate energy loss of the nestlings. For example in adult blackbirds (*Turdus merula*) extension of foraging time did not affect body mass (Russ et al. 2014). Moreover, in our study, LED lights were installed inside the nest box and this light does not create an environment outside the nest box with sufficient light to be used by the parents to extend their feeding time. It is therefore unlikely that our treatment could have extended feeding time of the parents. Using the same light treatment inside a nest box during a different experiment, we did not find any effect of ALAN on the time of entry of the female in the evening and although females did leave the nest box earlier in the morning (Raap et al.

2016b), it remains to be examined whether this time, when it is still dark outside, can effectively be used to feed nestlings.

Secondly, a long-term experimental study on house sparrow (*Passer domesticus*) nestlings showed that costs of begging also include physiological costs besides affecting growth (Soler et al. 2014). Therefore, an alternative explanation of how increased begging (and activity) could lead to reduced growth is provided by the energy allocation hypothesis. This hypothesis predicts that an increased maintenance cost reduces the proportion of energy spent on growth (Dawson and Evans 1957, 1960), which was found to be true for nestlings of red-winged blackbirds (*Agelaius phoeniceus*; Olson 1992). Increased begging (activity) could also increase metabolic demand thereby leading to oxidative stress (Metcalfe and Alonso-Alvarez 2010). In magpie nestlings, increased begging reduced growth but nestlings maintained their oxidative status (Moreno-Rueda et al. 2012). Maintenance of oxidative status could therefore potentially reduce the proportion of energy spent on increases in body mass (see discussion on effects on OS below and Casagrande et al. 2016).

Given that our short-term light treatment already affected body mass gain in a period of two days, significant differences in body mass may possibly arise at fledging if nestlings are exposed during their entire development to ALAN. Although we did not find an effect on fledging with the current short light treatment, the effect of ALAN on early development of nestlings may not be limited to body mass, but could also affect metabolism, immune competence and sexual attractiveness in adulthood (Lindstrom 1999). Moreover, body mass seems to be a good proxy for condition as heavier nestlings have higher nutritional reserves (Peig and Green 2009) resulting in higher survivorship and recruiting probabilities (Both et al. 1999; Bowers et al. 2014; Maness and Anderson 2013). Verhulst et al. (1997) showed that body condition during early development (weight on day 16) correlates with the quality of the breeding habitat that the birds later occupy, which is another indication that a reduced body condition through artificial light could have effects into adulthood.

We did not find any effect of ALAN on oxidative status. There was no effect on molecular antioxidants or oxidative status as measured by the ratio between glutathione and reduced glutathione. Activity of antioxidant enzymes (GPX, CAT and SOD) were not affected nor was the total antioxidant capacity (TAC). Neither did we find any evidence that ALAN increased oxidative damage as measured by the amount of protein oxidation or lipid peroxidation.

Nonetheless, we could have expected our treatment to affect oxidative status. Firstly, our experimental treatment which consisted of two nights of light inside the nest box was sufficient to reduce body mass gain of nestlings. Secondly, our earlier studies showed that a single night and a lower light intensity (1.6 lux instead of 3 lux) had profound effects on sleep of adult great tits as well as on begging behaviour/ sleep of nestlings (Raap et al. 2015, 2016b). ALAN therefore likely causes nestlings to be more active during the night and this may increase metabolic demand leading to oxidative stress (Metcalfe and Alonso-Alvarez 2010). Thirdly, the same treatment as we used here (3 lux during two nights) significantly increased haptoglobin while decreasing nitric oxide (Raap et al. 2016c), which are two important indicators of immunity, physiological condition and health state (Matson et al. 2012; Sild and Horak 2009).

There are several possible explanations as to why ALAN did not affect blood oxidative status in our experiment. 1) The great tits in our population are from a semi-urbanized area which could have already affected their oxidative status physiology (through adaptation to their environment), making them less susceptible to the stress induced by artificial light. 2) The length of our treatment might not be sufficient to affect blood oxidative status. 3) ALAN may have affected OS in other tissues than blood. 4) The increase in haptoglobin (Raap et al. 2016c) and reduced growth rate (lack of gain in body mass) may mask other effects of ALAN on OS.

Rural blackbirds have been shown to experience higher blood oxidative damage and higher baseline blood antioxidant defences compared to city blackbirds (Costantini et al. 2014). To show differences in OS between adult city and rural blackbirds, Costantini et al. (2014) used, during an 11 month period, a repeated immune challenge and chronic disturbance which is a much longer and stronger disturbance than our two days of artificial light. It could be that a longer period of light exposure is necessary to induce oxidative stress (but see Methodology and below of why this is difficult to do with free-living nestlings). Differences between blood and liver oxidative stress measurements have been observed earlier in Brandt's voles (*Lasiopodomys brandtii*) where experimental effects on SOD differed between serum and liver measurements (Xu et al. 2014). A study using Mongolian gerbils (*Meriones unguiculatus*) also showed that measures of oxidative stress, antioxidant and damage are tissue dependent (Yang et al. 2013). However, a recent study found that, except for oxidised glutathione (GSSG) and the ratio between GSH and GSSG, there was generally good qualitative and quantitative agreement between blood and tissue oxidative stress measurements (malondialdehyde, GSH, SOD, CAT, GPX, vitamin C and E; Margaritelis et al. 2015). It is therefore unlikely that oxidative

status in other tissues was affected by ALAN. In order to counteract increases in oxidative damage compounds, haptoglobin might have been elevated (Jelena et al. 2013). This might have eliminated the need to upregulate other antioxidants like GPX and CAT. Light exposed nestlings did not gain any body mass indicating a reduced growth rate, thus lowering metabolic activity, which might have masked to some extent any impact of ALAN on (some) metrics of oxidative status.

While we are the first to experimentally study the effect of ALAN on the oxidative status of free-living developing animals, our study has some limitations. Firstly, we used a cavity-nesting bird as a model species because we can experimentally manipulate its light environment while the experimental manipulation of light conditions of open-nesting birds is much more difficult. Although the light intensity which we used (3.0 lux) may not always be experienced by nestlings of cavity-nesting birds, behavioural changes have already been observed in adult male great tits using very low light intensities of 0.05 lux (de Jong et al. 2016). Future studies may build upon our results and examine effects on oxidative status and body mass using lower light intensities and longer experimental treatments. Nonetheless, we believe that our results offer insight in how ALAN affects free-living birds during development and that these results can also be relevant for other animals exposed to light pollution as they are exposed to similar and even higher light intensities (Dominoni et al. 2013a; Gaston et al. 2013). Secondly, we used a short-term light treatment as a long-term treatment in a free-living population presents several practical and ethical issues. For example, while a short-term light treatment of one night during winter may already deter adult birds from entering the nest box (Raap et al. 2015), a long-term artificial light treatment would then increase the risk of deterring adult birds which would have fatal consequences for the nestlings. Our design allowed us to sample nestlings twice. If we had taken samples from nestlings before the age of 13 days to obtain a longer treatment, the nestlings might have been too small to draw a sufficient amount of blood taking blood (and opening the nest box) from nestlings older than 15 days might induce early fledging in nestlings which would likely decrease their chances of survival. While we used a short-term light treatment 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). We showed that a short-term light treatment affects nestlings' body mass but subsequent studies may show if these effects are enhanced or ameliorated over longer treatments or if additional effects, also with regard to oxidative status, would appear.

Supplementary material

Laboratory analysis

The choice of biomarkers was based on previous work on the effect of environmental light on the oxidative status (Ashkenazi and Haim 2013; Baydas et al. 2001; Cruz et al. 2003; Hardeland et al. 2003), as well as on literature showing the sensitivity of biomarkers to environmental stressors (Costantini 2014). Markers of damage included protein carbonyls (well-established indicator of oxidative damage to proteins (Dalle-Donne et al. 2003) and thiobarbituric acid reactive substances (TBARS, formed as a byproduct of lipid peroxidation (Halliwell and Gutteridge 2007). In terms of antioxidants, we measured the enzyme glutathione peroxidase (GPX) alongside the reduced (GSH) and oxidized (GSSG) forms of glutathione because GPX uses GSH to catalyse the breakdown of hydrogen peroxide and organic hydroperoxides into water and alcohol, respectively (Yu 1994). We have also measured the two antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD), which detoxify cells from hydrogen peroxide (when it occurs at high concentrations) and superoxide anion, respectively (Halliwell and Gutteridge 2007). Given that the antioxidant barriers also relies on non-enzymatic antioxidants, we have used the FRAP assay to estimate the non-enzymatic antioxidant (Halliwell and Gutteridge 2007).

For detection of molecular antioxidants in red blood cells: reduced glutathione (GSH) and oxidised glutathione (GSSG), we used high-performance liquid chromatography with electro-chemical detection by a reversed-phase HPLC of Shimadzu (Shimadzu, 's Hertogenbosch, The Netherlands), following the protocol as described by Sinha et al. (2014). Concentrations of GSH and GSSG were expressed as micromole per gram fresh weight of red blood cells. The ratio between GSH/ GSSG was used as an index of redox state with lower values indicating higher oxidative stress (Jones 2006).

Activity of antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) were determined from haemolysates of red blood cells. Red blood cells were homogenized by MagNALyser (Roche, Vilvoorde, Belgium) in 250 µl of extracting buffer (pH 7.4; 1.15% KCl and 0.02 M EDTA in 0.01 M PBS). All measurements were scaled down for semi-high throughput using a micro-plate reader (Multiskan RC plate reader type 351; Synergy Mx, Biotek Instruments Inc., Vermont, USA). SOD activity was determined by measuring the inhibition of nitroblue tetrazolium (NBT) reduction at 560 nm (ε_{530} = 12.8 mM^{-1} cm^{-1}), following Dhindsa et al. (1981). CAT activity was measured following Aebi (1984), by

monitoring the rate of decomposition of H_2O_2 (ε_{240} = 39.4 $M^{-1}cm^{-1}$). Activity of GPX was determined following Drotar et al. (1985) by measuring the decrease in NADPH absorbance measured at 340 nm and calculated from the 6.22 mM^{-1} cm^{-1} extinction coefficient. A modified ferric ion reducing antioxidant power (FRAP) assay was used to estimate the total antioxidant capacity (TAC; Benzie and Strain 1996). Homogenised red blood cells were mixed with the FRAP reagent, and the absorption was measured at 600 nm after 30 min. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was used as the standard.

Finally, we measured protein carbonyls (marker of protein damage) in red blood cells, as well as thiobarbituric acid reactive substances (TBARS, marker of lipid peroxidation) in plasma, as oxidative stress markers. We followed the procedure explained in the "Protein Carbonyl Colorimetric Assay Kit" by Cayman Chemical's (Ann Arbor, MI, USA;, see also Levine et al. 1990) to measure protein carbonyl content after samples had been diluted with buffer extract to 2 mg protein ml−1. We estimated the concentration of TBARS as a measure of lipid peroxidation following El-Shafey and AbdElgawad (2012). Plasma was mixed with 0.5% (w/v) thiobarbituric acid (TBA) in 20% TCA. After incubation at 90°C for 45 minutes, samples were left to cool and then centrifuged at 10.000g. Absorbance was measured at 532, 600 and 450 nm and amount of MDA equivalents was calculated using the following formula: $6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}$ and expressed as nmol MDA equivalents g^{-1} plasma.

Data analysis

Error terms are assumed independent in order to use the classic ANOVA and regression analysis. Since each bird/ nest was measured more than once we cannot assume independence between measurements. Mixed models were therefore used as they are a widely used technique to account for the non-independence between measurements in a dataset, by including random term effects into the regression equation. This ensures that significance of independent variables (fixed effects) is calculated while accounting for the non-independence of the measurements within the same individual/ nest (Fitzmaurice et al. 2004). Please see for averages of raw data Supplementary Table S7.

Table S1: Number of individuals analysed for the different oxidative stress parameters. Individuals are from 16 nests in the control and 16 nests in the light group.

	Control	Light	Total
GSH	48	48	96
GSSG	48	48	96
GSH/GSSG	48	48	96
GPX	45	44	89
CAT	46	43	89
SOD	47	45	92
TAC	47	45	92
Protein carbonyls	47	45	92
TBARS	48	48	96

Table S2: Statistical output of the full mixed effect model, effect of artificial light at night on nestling weight. Linear mixed models with "bird identity" nested in "nest" nested in "pair" as random factor were used (bird identity:nest identity:pair). Significant values ($P < 0.05$) are depicted in bold, $N = 224$ individuals. P-values obtained after a stepwise backward regression are mentioned in the main text.

	F	*P*
Treatment: Day: Sex	0.177	0.675
Treatment: Day	21.472	**<0.001**
Brood size	2.085	0.160
Sex	35.193	**<0.001**
Day	27.853	**<0.001**
Treatment	1.131	0.675

Table S3: Statistical output of the final mixed effect models, effect of artificial light on oxidative stress parameters. Linear mixed models with "nest" nested in "pair" as random factor were used (bird identity:nest identity:pair). See Supplementary Table S1 for sample sizes per parameter (between 89-96 individuals). P-values are obtained after a stepwise backward.

		GSH	GSSG	GSH/GSSG	TAC	GPX	SOD	CAT	Protein carbonyls	TBARS
Sex: Treatment: Time	*F*	-	-	-	-	-	-	-	-	-
	P	-	-	-	-	-	-	-	-	-
Treatment: Sex	*F*	-	6.287	-	-	-	-	-	-	-
	P	-	0.014	-	-	-	-	-	-	-
Sex: Time	*F*	-	-	-	-	-	-	-	-	7.914
	P	-	-	-	-	-	-	-	-	0.005
Treatment: Time	*F*	-	-	-	-	-	-	-	-	-
	P	-	-	-	-	-	-	-	-	-
Treatment	*F*	6.446	1.139	-	-	-	-	-	-	-
	P	0.022	0.302	-	-	-	-	-	-	-
Sex	*F*	-	0.004	-	-	-	-	-	-	0.522
	P	-	0.947	-	-	-	-	-	-	0.471
Time	*F*	-	-	-	10.598	11.042	-	22.726	-	3.772
	P	-	-	-	0.001	0.001	-	<0.0001	-	0.053
Brood size	*F*	-	-	-	-	-	-	-	-	-
	P	-	-	-	-	-	-	-	-	-
Weight	*F*	-	-	-	-	-	4.038	-	-	-
	P	-	-	-	-	-	0.047	-	-	-

Table S4: Results of post-hoc analyses for the interaction between treatment and sex for GSSG. Significant values ($P < 0.05$) are depicted in bold.

					estimate	se	*t*	*P*
F	control	-	M	control	-0.2	0.098	-1.83	**0.07**
F	control	-	F	light	-0.1	0.096	-1.01	0.32
F	control	-	M	light	0.1	0.100	0.73	0.47
M	control	-	F	light	0.1	0.100	0.82	0.42
M	control	-	M	light	0.3	0.104	2.41	**0.02**
F	light	-	M	light	0.2	0.098	1.74	0.09

Table S5: Results of post-hoc analyses for the difference in TAC, GPX and CAT between day 13 and 15 independent of light treatment. Estimates are given and *t* and *P* values for the difference between days.

Parameter	Day	estimate	se	*t*	*P*
TAC	13	0.31	0.01		
	15	0.29	0.01	3.26	0.001
GPX	13	0.07	0.00		
	15	0.06	0.00	3.32	0.001
CAT	13	0.61	0.02		
	15	0.53	0.02	4.77	<0.0001

Table S6: Results of post-hoc analyses for the interaction between sex and treatment for TBARS. Significant values ($P < 0.05$) are depicted in bold.

					estimate	se	*t*	*P*
F	13	-	M	13	0.00	0.03	1.40	0.163
F	13	-	F	15	0.00	0.03	0.63	0.527
F	13	-	M	15	-0.10	0.03	-1.86	0.064
M	13	-	F	15	0.00	0.03	-0.81	0.418
M	13	-	M	15	-0.10	0.03	-3.28	**0.001**
F	15	-	M	15	-0.10	0.03	-2.48	**0.014**

Table S7: Raw average for metrics of oxidative status. Average obtained from raw data are given with their standard deviation per treatment group, control or light exposed nestlings.

	Control		Light	
	Day 13	Day 15	Day 13	Day 15
GSH µmol/g fresh weight	2.808 ± 1.388	3.033 ± 1.297	2.398 ± 1.042	2.314 ± 1.263
GSSG µmol/g fresh weight	0.884 ± 1.272	0.912 ± 1.274	0.653 ± 0.731	0.708 ± 0.707
GSH/ GSSH	16.877 ± 44.972	30.304 ± 107.009	11.276 ± 13.574	10.078 ± 13.134
TAC µmol trolox/g fresh weight	0.126 ± 0.106	0.094 ± 0.065	0.103 ± 0.087	0.088 ± 0.061
GPX µmol NADPH/mg prot/min	0.005 ± 0.005	0.004 ± 0.004	0.005 ± 0.005	0.004 ± 0.004
CAT µmol H2O2/mg prot/min	0.462 ± 0.371	0.335 ± 0.252	0.42 ± 0.363	0.32 ± 0.217
SOD U/mg prot/min	0.414 ± 0.368	0.396 ± 0.152	0.43 ± 0.224	0.409 ± 0.15
Protein carbonyls nmol/mg prot	1.663 ± 1.993	1.856 ± 2.04	1.571 ± 1.765	1.832 ± 1.716
TBARS nmol MDA/ g plasma	66.398 ± 15.575	68.025 ± 19.445	85.37 ± 159.466	69.32 ± 21.785

Chapter 9

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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Abstract

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.

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. 2010b). 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. 2016b). 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 (see e.g. Ouyang et al. 2015), are therefore necessary to fully comprehend the effects of ALAN on developing animals.

Table 1: Examples of particularly relevant experiments on the physiological effects of artificial light at 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. (2013d)
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. (2013c)

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)

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

In the present study, we experimentally exposed wild great tit nestlings to two nights of artificial light (3.0 lux) and compared these to nestlings which were not exposed to ALAN. We then assessed individual changes in Hp and NOx to determine the effects of ALAN on the 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; 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 (see 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 (≤ 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, 2016b). 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

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

Quantification of haptoglobin and nitric oxide

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

Statistical analyses

We used R 3.1.2 (R Core Team 2016) for all statistical analyses. For both Hp and NOx we performed a linear mixed effect analysis (LMM) using the lme4 package (Bates et al. 2015). As fixed effects, "treatment" (control, light), "day" (13, 15), "sex", "brood size", "body mass*" as well as the two-way interactions and the three-way interaction between "treatment", "sex" and "day" were used. As random effect we used "bird identity" which was nested in "nest identity" which was nested in "pair" (bird identity:nest identity:pair) to take the experimental design into account. Both Hp and NOx were square root transformed to meet model assumptions.

P-values obtained by a stepwise backward regression are given in results and Tukey HSD tests were used for post-hoc analyses, using the lmerTest package (Kuznetsova et al. 2016). We analysed the relationship between Hp and NOx on day 13 and changes in both measures (difference between day 13 and day 15) using a Spearman rank correlation test.

*Body mass of day 13 and day 15 instead of only day 13 were used. This does not influence the results and body mass was removed from the model during stepwise backward regression.

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).

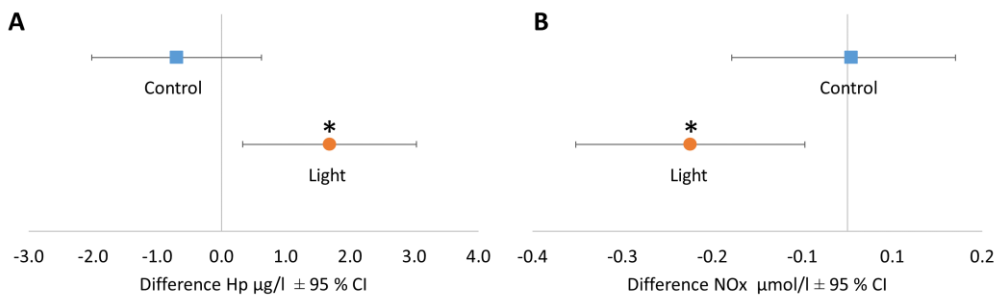

Figure 1: Artificial light at night increased nestling plasma haptoglobin (A) and decreased nitric oxide (B). Estimates (square root transformed ± 95% CI) were obtained from LMMs with individual nested in nest nested in pair as random factor (bird identity:nest identity:pair). Differences in haptoglobin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

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 (see 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; 2016b), 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).

Chapter 10

Artificial light at night causes an unexpected increase in oxalate in developing male songbirds

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Abstract

Artificial light at night (ALAN) is a widespread and increasing environmental pollutant with known negative impacts on animal physiology and development. Physiological effects could occur through sleep disruption and deprivation, but this is difficult to quantify, especially in small developing birds. Sleep loss can potentially be quantified by using oxalate, a biomarker for sleep debt in adult humans and rats. We examined the effect of ALAN on oxalate in free-living developing great tits (*Parus major*) as effects during early life could have long-lasting and irreversible consequences. Nestlings' physiology was quantified at baseline (=13 days after hatching) and again after two nights of continued darkness (control) or exposure to ALAN (treatment).

We found that ALAN increased oxalate levels but only in male nestlings, rather than decreasing it as was found in sleep-deprived humans and rats. Our results using developing birds differ strongly from those obtained with adult mammals. However, we used ALAN to reduce sleep while in rats forced movement was used. Finally, we used free-living opposed to laboratory animals. Whether oxalate is a reliable marker of sleep loss in developing great tits remains to be examined. Potentially the increase of oxalate in male nestlings was unrelated to sleep debt. Nonetheless, our results substantiate physiological effects of ALAN in developing animals and may provide a foundation for future work with free-living animals.

Introduction

Artificial light at night (ALAN), also known as light pollution, has greatly altered the night-time environment. It is a widespread, ever increasing and important anthropogenic environmental pressure on wildlife (Falchi et al. 2016; Hölker et al. 2010b). ALAN affects a wide variety of behavioural traits, such as reproduction, foraging, and migration and has clear deteriorating physiological effects such as a supressed immune response to challenges and increased corticosterone levels (see e.g. Bedrosian et al. 2011; Russ et al. 2015; 2017; reviewed in Russart and Nelson 2018). Disruption of the immature circadian system during development by ALAN may, due to its effects on the developing brain, influence adult behaviour, physiology, health, and disease and could have long-lasting and irreversible consequences (Fonken and Nelson 2016). In wild developing great tits (*Parus major*) ALAN has been shown to affect physiology (Raap et al. 2016a; 2016c) and to cause begging at night (Raap et al. 2016b). Sleep disruption due to ALAN, similar as has been demonstrated in adult great tits (Raap et al. 2015, 2016b), seems likely but is difficult to quantify in nestlings. During the nestling period ALAN reduces female great tit sleep behaviour by about 50% with some females not sleeping at all during the entire night. Furthermore, when nestlings were in natural darkness they never begged for food, while they showed substantial begging behaviour throughout the night when being exposed to ALAN (Raap et al. 2016b). This together with the severe sleep disruption in females gives a strong indication of sleep disruption and loss in great tit nestlings, but this is difficult to quantify as they do not exhibit sleep behaviour like adults. In adults, but not in nestlings, sleep behaviour can be defined as when an individual is in the classical sleep position, with the beak pointing backwards and tucked under the scapulars (Amlaner and Ball 1983). Sleep is an important behaviour widespread across the animal kingdom (Cirelli and Tononi 2008; Siegel 2008) but difficult to study in the field (Rattenborg et al. 2017), especially in small animals.

Recently, a laboratory study showed that sleep loss reduced plasma levels of oxalic acid, also known as oxalate, in humans and in rats (Weljie et al. 2015). Ouyang et al. (2017) claimed that light pollution decreased oxalate levels in adult great tits. However, because of several severe methodological and statistical issues with this study, such as a high uncertainty that their light treatment was effective in terms of exposure and because they did not study sleep or sleep behaviour (Raap et al. 2017c), it is still unknown how ALAN affects oxalate in birds. If oxalate is a cross-species biomarker of sleep debt then this could be used to quantify whether ALAN causes nestlings to suffer from sleep disruption. Moreover, oxalate would be a

particularly useful tool when working with free-living small animals as they are often too small to be fitted with modern data loggers and behaviour is necessarily used as a proxy for sleep and sleep debt (Aulsebrook et al. 2016). Data loggers are used to record brain activity which allows for a more precise and detailed measurement of sleep that cannot be obtained from behaviour. However, great tit nestlings are too small to be fitted with data loggers and sleep cannot be quantified behaviourally as they do not always exhibit the classical sleep position like adults do (beak pointing backwards and tucked under the scapulars; Amlaner and Ball 1983). Nonetheless, they are an important model system in evolutionary and environmental research, and are increasingly being used to study the effects of ALAN on behaviour and physiology (see e.g. Da Silva et al. 2014; Kempenaers et al. 2010; Ouyang et al. 2017; Raap et al. 2017b; Sprau et al. 2017; Sun et al. 2017) and the impact of ALAN on nestling sleep would thus be of particular interest.

Oxalate is decreased in both sleep restricted rats and humans (Weljie et al. 2015) and as ALAN affects nestling behaviour and physiology it is important to test whether it also affects oxalate in developing animals. Sleep likely plays a crucial role in metabolic processes. Weljie et al. (2015) investigated cross-species consequences via comprehensive metabolite profiling and showed that oxalate may provide a potential link between sleep loss and metabolic dysfunction and serve as a biomarker for sleep deprivation. Blood oxalate can decrease through several mechanisms as outlined in Weljie et al. (2015): reduced synthesis, increased gut microbiota processing and/or increased urinary clearance. Increases could occur through degradation of ascorbate, production by the liver and red blood cells or by increased dietary oxalate (Marengo and Romani 2008). To examine whether ALAN affects oxalate in developing birds, we experimentally exposed wild great tit nestlings to two nights of artificial light (3 lux) and compared these to nestlings which were not exposed to ALAN.

Methods

Plasma samples from a previous experiment were used in which free-living great tit nestlings were exposed to ALAN inside their nest box (see details in Raap et al. 2016a; 2016c). In brief, our experiment was performed during the 2015 breeding season (between 8 and 25 May) in a resident nest box population of great tits in the surroundings of Wilrijk (Antwerp), Belgium (51°9’44’’N, 4°24’15’’E). This population was established in 1997 and has been monitored ever

since (see e.g. Eens et al. 1999; Janssens et al. 2001; Rivera-Gutierrez et al. 2010, 2012; Thys et al. 2017; Van Duyse et al. 2000; 2005; Vermeulen et al. 2016b).

The previous experiment included two groups of each 16 nests; a control and a light treated group (see details in Raap et al. 2016a; 2016c). Nests with a similar hatching date and brood size (about seven) were used and otherwise randomly allocated to a treatment. In the light treated group, we obtained a blood sample (≤ 150 µl) from the brachial vein of all nestlings and subsequently weighed them (digital balance; Kern TCB 200-1) at base line (day 13 after hatching) and again after a two night exposure to ALAN (Figure 1). Nestlings from the control "dark" group were not exposed to ALAN, but were otherwise treated the same. Sampling was standardized for time and always occurred during the morning. The light treated group was exposed to 3 lux broad-spectrum white light as measured on the nest box bottom (ISO-Tech ILM-1335 light meter; Corby, UK). This intensity has been shown to disrupt sleep behaviour (Raap et al. 2017b) and affect nestling physiology (Raap et al. 2016a; 2016c).

Figure 1: Fieldwork procedure. General fieldwork included taking great tit nestlings (A) out of their nest box (B) after which their body mass is taken (C) as well as a small blood sample (D).

We examined changes caused by ALAN on a within-individual level by taking two samples from each individual which we used to generate a single 'change in oxalate' measure (one metric per individual). This approach is especially important because there is high heterogeneity in physiological markers on the between-individual level within nests (see e.g. Casasole et al. 2017; Vermeulen et al. 2015). Moreover, effects of ALAN may differ greatly among individuals (see e.g. Raap et al. 2016b). Our field-based experimental approach with free-living animals (contrary to laboratory studies), may offer useful insights about possible physiological effects of ALAN on developing animals.

Oxalate was quantified in 4 µl of plasma using the manufacturer's instructions provided with the commercially available colorimetric assay (BioVision inc, USA), similar to Ouyang et al. (2017) who also used great tits. Plasma samples had been stored at -80°C but had also been defrosted twice for previous analyses (see details in Raap et al. 2016a; 2016c). In brief, we diluted samples with assay buffer to a total of 50 µl. In the same assay samples to generate a standard curve were included. Samples were subsequently incubated at 37°C for one hour and absorbance was measured at 450 nm. We selected those samples of which sufficient plasma remained after previous analyses. Due to sample limitations we could not run duplicates although there was always some plasma left-over. All samples were within the assays limit. Samples of day 13 and 15 from the same individual were kept on one plate, and two plates/ assays were used in total. Overall we obtained repeated measurements (day 13 and 15) of oxalate for 23 females and 21 males in the control group (14 nests) and 22 females and 22 males in the light group (13 nests). So, in total 88 great tit nestlings of known sex were sampled twice. Nestling sex was determined by molecular sexing (Griffiths et al. 1998).

All statistical analyses were conducted in R 3.3.2 (R Core Team 2016). We compared changes in oxalate from day 13 to day 15, between the control and light group. Changes in oxalate (expressed as a percentage from baseline, similar to Weljie et al. 2015) were analysed by constructing a linear mixed model as data were normally distributed (lme4 package; Bates et al. 2015). The change in oxalate is unrelated to the level of oxalate on day 13. Nest identity (NestID) was included as random factor to avoid pseudoreplication. The full model contained weight on day 13 (covariate), sex (factor), treatment (factor) and the interaction between sex and treatment as explanatory variables. We included the interaction with sex as there may be sex-specific differences in physiology (Giordano et al. 2015; Speakman et al. 2015) and environmental sensitivity (reviewed in Jones et al. 2009). P-values obtained by a stepwise backward model reduction (Zuur et al. 2009) are given in the results and Tukey HSD tests were used for post-hoc analyses (lmerTest package; Kuznetsova et al. 2016).

Ethics

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. The Belgian Royal Institute for Natural Sciences provided ringing licences for authors and technical personnel.

The dataset supporting this article was uploaded to the Zenodo digital repository DOI: 10.5281/zenodo.831995.

Results

There was a sex-dependent effect of ALAN on oxalate (sex:treatment interaction: $F = 4.994$, $P = 0.028$; Table 1 and 2). Light exposed males, but not females, showed increased levels of oxalate (≈15%; see Figure 2 and Table 3). Male and female nestlings in a natural dark situation showed no change in oxalate from day 13 to day 15. Weight at day 13 did not affect changes in oxalate ($F = 0.050$, $P = 0.823$).

Table 1: Statistical output of the full mixed effect model, effect of artificial light at night on oxalate. A linear mixed model with "nest" as random factor was used (lme4 package Bates et al. 2015). In bold the significant p-value is indicated ($P < 0.05$), $N = 88$ individuals. P-values obtained after a stepwise backward regression can be found in Table 2. Estimates with their standard error (SE) are given. NumDF is nominator degrees of freedom, DenDF is denominator degrees of freedom.

	Estimate	SE	NumDF	DenDF	F-value	P-value
Intercept	-0.077	0.401				
Weight	0.006	0.026	1	83	0.050	0.823
Sex	-0.122	0.110	1	83	0.405	0.526
Treatment	-0.082	0.108	1	83	1.355	0.248
Sex:Treatment	0.343	0.154	1	83	4.978	**0.028**

Table 2: Statistical output of the model reduction, effect of artificial light at night on oxalate. A linear mixed model with "nest" as random factor was used. Stepwise model reduction was performed (lmerTest package Kuznetsova et al. 2016). In bold the significant p-value ($P < 0.05$) is indicated, $N = 88$ individuals. NumDF is nominator degrees of freedom, DenDF is denominator degrees of freedom, elim.num is the order in which a variable is removed from the model.

	NumDF	DenDF	F-value	elim.num	P-value
Weight	1	83	0.0503	1	0.8231
Sex	1	84	0.4542	kept	0.5022
Treatment	1	84	1.4112	kept	0.2382
Sex:Treatment	1	84	4.9941	kept	**0.0281**

Table 3: Results of post-hoc analyses for the interaction between sex and treatment for the difference in oxalate between day 13 and day 15. Tukey HSD tests were used for post-hoc analyses (lmerTest package Kuznetsova et al. 2016). Estimates give the difference between oxalate from day 13 to day 15 as a % from baseline (day 13) and p values indicate whether this differs from 0. Sample sizes: ♀dark = 23; ♂dark = 21; ♀light = 22; ♂light = 22 nestlings. In bold the significant p-value ($P < 0.05$) is indicated. Standard error (SE) and confidence interval (CI) of the estimates are given. See Figure 1 for the raw data of the change in oxalate.

	Estimate	SE	DF	t-value	Lower CI	Upper CI	P-value
♀ dark	1.12	7.46	84	0.15	-13.72	15.97	0.8808
♂ dark	-10.80	7.81	84	-1.38	-26.33	4.74	0.1706
♀ light	-6.87	7.63	84	-0.9	-22.05	8.31	0.3705
♂ light	15.34	7.63	84	2.01	0.16	30.52	**0.0476**

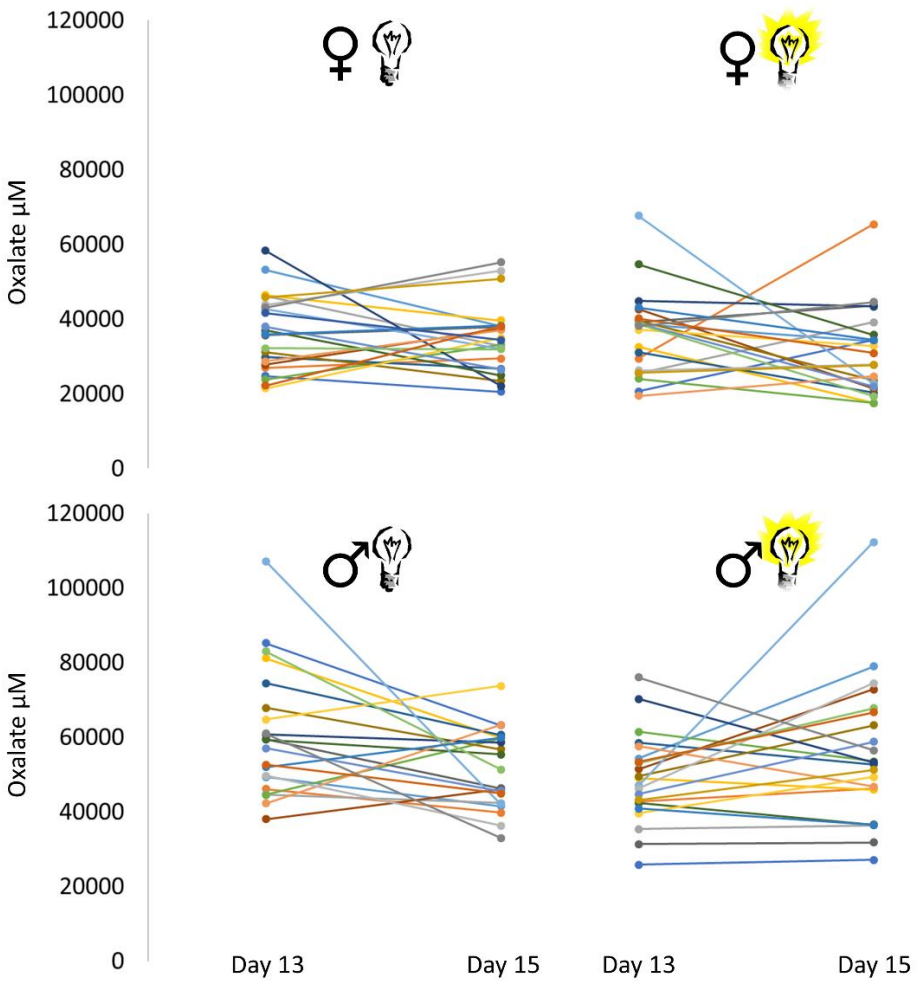

Figure 2: ALAN increased oxalate in male nestlings. Raw data of the change in oxalate after two nights is shown for females (top panels) and males (lower panels), in a natural dark situation (left panels) and for light exposed animals (right panels). Lines indicate unique individuals; sample sizes: ♀dark = 23; ♂dark = 21; ♀light = 22; ♂light = 22 nestlings. In males there was a significant effect of artificial light at night on oxalate levels (t=2.01, P=0.048; Table 3).

Discussion

We found a sex-dependent effect of ALAN on oxalate, a potential biomarker for sleep debt (Weljie et al. 2015). The effect of ALAN on oxalate only manifested itself in male nestlings and in the opposite direction as we had expected, an increase instead of a decrease. In the following we discuss these sex-dependent effects of ALAN on oxalate and the potential of oxalate as a proxy for sleep debt in developing birds.

Sex-dependent effect of ALAN on oxalate

Males and females differ in their behaviour, physiology and their response to the environment which may explain that effects on oxalate were only found in males. Oxalate could increase through degradation of ascorbate, production by the liver and red blood cells or by increased dietary oxalate (Marengo and Romani 2008) and there are several indications why oxalate could be affected in a sex-dependent manner. First, sleep behaviour (including its duration) is strongly sex-dependent in great (Stuber et al. 2015b; 2017) and blue tits (Steinmeyer et al. 2010), and adult male and female great tits use different sleep strategies depending on their metabolic requirements (Stuber et al. 2015a). Contrary to females, males decrease their sleep duration with an increased basal metabolic rate. Second, male and female great tit nestlings may also differ in their physiology besides possible behavioural differences. For example, male nestlings have higher NOx levels (Raap et al. 2017a), and nutritional conditions during development had a sex-dependent effect on the oxidative status of great tit nestlings, with females being more sensitive to nutritional stress indicating sex-specific allocation priorities (Giordano et al. 2015). Third, the response towards ALAN is potentially sex-dependent as adult females showed a slightly stronger reduction than males in sleep amount (% of sleep; amount divided by the total time spent inside the nest box) when subjected to ALAN in February (Raap et al. 2015). However, whether this is true during the breeding season and for developing nestlings remains to be tested. Generally speaking males are often more susceptible to environmental conditions than females although effects are small (reviewed by Jones et al. 2009). Overall, this implies that male and female nestlings may differ in their sleep behaviour, physiology and response to the environment, which could contribute to the observed differences in oxalate. Finally we need to consider that as this is the first study in developing animals in the wild, our results may also be false positive. The effect is not particularly strong and in the direct opposite of predictions. However, we used a repeated measures design to look at changes within the same individuals, which increases statistical power (Seltman 2013) and gives confidence in the obtained results. Nonetheless, further experimental and comparative work is needed to determine and validate our results.

Increased begging might have led to increased food provisioning by the parents and thus higher oxalate levels in males. When nestlings are exposed to ALAN they start begging for food which they never do during a dark night. Although this needs to be examined it could trigger females (and to an extent indirectly also males) to feed their nestlings more, especially

during the morning. Our samples were obtained during the morning between 08:00 and 12:00. During this period nestlings could have received a substantial amount of food and because male nestlings are larger than females (average of day 13–15 respectively 15.9 ± 0.230 and 15.2 ± 0.231 g; Raap et al. 2016a) they might have received more food (e.g. Anderson et al. 1993) and thus more dietary oxalate. However, whether male nestlings did effectively obtain more food, that also contains oxalate, needs to be determined. Doing so will be challenging because, besides other practical issues, nestling sex can only be determined genetically. Another potentially contributing mechanism could be through the disruption of the gut microbiota by ALAN. This could have led to reduced processing and thus accumulation of oxalate in light exposed nestlings but this requires further investigation and does not explain the sex-dependent effect. To conclude, the increase of oxalate by ALAN may therefore be unrelated to any changes in sleep.

Oxalate as a proxy for sleep debt in developing birds

Because of the inability to directly measure sleep amount or disruption in nestlings we cannot be entirely certain about the relationship between sleep loss and oxalate levels in developing nestlings. While nestlings showed more begging at night when exposed to ALAN (Raap et al. 2016b), we cannot be sure that they spent more time awake. Awakenings at night are a normal part of the adult tits' sleep patterns, however, ALAN clearly disrupts adult sleep behaviour (Raap et al. 2015; 2016b). It therefore seems very likely that great tit nestlings disturbed by ALAN (Raap et al. 2016b) also effectively slept less, but whether an increase in oxalate is related to sleep loss remains to be tested. Furthermore, whether oxalate can be used as a reliable biomarker of sleep loss in (developing) birds and whether it is direct or indirectly affected by ALAN will require further research.

Effects of sleep loss on oxalate in developing animals may differ from those in adults, where in rats and humans a decrease was found. Sleep differs between developing and adult animals in both mammals and birds (reviewed in Rattenborg and Martinez-Gonzalez 2015), with for example the amount of REM (rapid eye movement) sleep being higher in developing animals than in adults. Following sleep deprivation a rebound mainly occurs in REM sleep. Sleep loss may thus be different for developing animals compared to adults. This difference may subsequently be reflected in differences in how oxalate is affected. Ouyang et al. (2017) examined effects of light pollution on oxalate levels in adult great tits. They claimed that great

tits exposed to white light had higher nightly activity and linked this to a decrease in oxalate from March to May. However, there are several serious issues with these results (Raap et al. 2017c), warranting further study. For example, there is high uncertainty that their light treatment was effective in terms of exposure and the claims about a relationship between sleep loss (which was not measured) and physiological effects seems to be premature. Furthermore, Weljie et al. (2015) showed that in rats (but not in humans) the reduction of oxalate was related to increased oxidative stress due to sleep deprivation. We would thus expect that sleep deprivation in developing great tits might be associated with oxidative status as well as reduction in oxalate. However, we previously showed that our treatment of two nights ALAN did not affect the oxidative status of developing great tits (Raap et al. 2016a).

Our experimental treatment may not have restricted sleep sufficiently to be detected by decrease in oxalate. The laboratory study by Weljie et al. (2015) revealed that oxalic acid is depleted after sleep restriction in male rats over a period of 5 days, while allowing them to sleep for only 4 hours per day. In our study nestlings were exposed to ALAN continuously during two nights and the amount of sleep loss each night might be more severe than the sleep reduction in the study by Weljie et al. (2015) although their treatment was three days longer. Therefore, the reduction in sleep by ALAN over two nights could be insufficient to be detected using oxalate, although it affected body mass gain, haptoglobin and nitric oxide (Raap et al. 2016a; 2016c). Future studies may, however, build upon our results and use longer periods of sleep restriction.

Conclusions

We have shown here for the first time that a brief exposure of only two nights to ALAN affects oxalate levels in developing wild animals. This provides further evidence of the physiological effects of ALAN during early-life, such as increased Hp and decreased NOx (Raap et al. 2016c), in addition to the behavioural effects, such as increased begging behaviour and likely disrupted sleep (Raap et al. 2016b). The sex-dependent effect on oxalate might indicate different physiological coping mechanisms by developing great tits, which requires further investigation. Experiments using humans and rats showed that oxalate is a potential biomarker of sleep loss (Weljie et al. 2015), and while its use in free-living birds and developing animals is promising it requires further investigation. The increasing illumination of the night is a serious threat as it disrupts circadian rhythms, physiology and behaviour. Early-life exposure could have long

lasting effects throughout adulthood, reducing survival and reproduction. With the progressive, worldwide increase of light pollution, the physiological effects of this stressor, especially during development, should receive greater attention. Urbanization is not only associated with light pollution but with many other anthropogenic stressors such as noise pollution, and new methods and approaches are necessary to understand the consequences of increasing human pressure on free-living animals.

Chapter 11

Ambient anthropogenic noise but not light is associated with the ecophysiology of free-living songbird nestlings

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Abstract

Urbanization is associated with dramatic increases in noise and light pollution, which affect animal behaviour, physiology and fitness. However, few studies have examined these stressors simultaneously. Moreover, effects of urbanization during early-life may be detrimental but are largely unknown. In developing great tits (*Parus major)*, a frequently-used model species, we determined important indicators of immunity and physiological condition: plasma haptoglobin (Hp) and nitric oxide (NOx) concentration. We also determined fledging mass, an indicator for current health and survival. Associations of ambient noise and light exposure with these indicators were studied.

Anthropogenic noise, light and their interaction were unrelated to fledging mass. Nestlings exposed to more noise showed higher plasma levels of Hp but not of NOx. Light was unrelated to Hp and NOx and did not interact with the effect of noise on nestlings' physiology. Increasing levels of Hp are potentially energy demanding and trade-offs could occur with life-history traits, such as survival. Effects of light pollution on nestlings of a cavity-nesting species appear to be limited. Nonetheless, our results suggest that the urban environment, through noise exposure, may entail important physiological costs for developing organisms.

Introduction

As a consequence of urbanization, anthropogenic noise and light have dramatically increased over the recent decades and they pose a worldwide environmental challenge (Barber et al. 2010; Davies et al. 2014; Falchi et al. 2016; Hölker et al. 2010a; 2010b; Swaddle et al. 2015). Mounting evidence raises concerns about their environmental and health impacts, and a wide variety of behavioural, physiological and fitness effects have been reported (reviewed in Swaddle et al. 2015). While most studies have investigated these pressures in isolation, urbanization is often associated with an increase in both noise and light. It is therefore crucial to study these anthropogenic pressures simultaneously to determine whether they have an additive effect or whether the combined effects are stronger than the sum of their parts (synergistic effect; Halfwerk and Slabbekoorn 2015; Isaksson 2015; McMahon et al. 2017; Swaddle et al. 2015). Such studies are urgently needed for effective mitigation and management of protected areas especially because anthropogenic noise and light may amongst other effects lead to a loss of species and have negative consequences for populations, communities and ecosystems (Barber et al. 2010; Gaston et al. 2015b; Spoelstra et al. 2015; Swaddle et al. 2015).

Most studies on anthropogenic noise and light have focused on effects in adults in the laboratory (but see e.g. Meillère et al. 2015; Raap et al. 2016a; 2016c; Salmon et al. 2016), but experiences during early-life in the wild may profoundly alter individual physiology and health in later life. Environmental conditions experienced during development can shape individual life histories and therefore potentially lifetime reproductive success (Monaghan 2008). Noise exposure can have a major impact on behaviour and physiology (Francis and Barber 2013). Noise can increase stress (Blickley et al. 2012), reduce the immune response (Kight and Swaddle 2011) and may entail important costs for developing organisms. For example, experimental noise exposure reduced telomere length of free-living house sparrows (*Passer domesticus)* which likely affects their longevity (Meillère et al. 2015). The immature circadian system may be particularly sensitive to circadian disruption through artificial light and experiences during early-life may have profound negative effects on the developing brain, influence adult behaviour, physiology, health and disease (Fonken and Nelson 2016). While studies have shown that noise and light can have negative behavioural and physiological effects on adult birds, results on nestlings are almost completely missing (but see e.g. Meillère et al. 2015; Raap et al.

2016a; 2016c; Salmon et al. 2016). These are equally important, especially as early-life experiences will have long-term effects on these individuals.

Therefore, we studied simultaneously the variation in noise and light exposure of free-living great tit (*Parus major*) nestlings in an urban population and related exposure levels to important indicators of short-term survival, physiological condition and health: fledging mass, haptoglobin (Hp) and nitric oxide (NOx). Fledging mass is a good proxy for condition as heavier nestlings have higher nutritional reserves (Peig and Green 2009), resulting in higher survivorship and recruiting probabilities (Both et al. 1999; Bowers et al. 2014; Maness and Anderson 2013). Haptoglobin plays an important role in inflammation, infection and trauma. It acts as an antioxidant and is part of the non-specific immune response (reviewed in Matson et al. 2012). Plasma nitric oxide is a multifunctional signalling molecule and involved in inflammatory processes, although uncontrolled production may lead to cell damage and death (reviewed in Sild and Horak 2009). Haptoglobin and NOx have also previously been shown to be affected by light at night in an experimental field study (Raap et al. 2016c). Haptoglobin and NOx may therefore provide useful information on physiological condition, health state and innate immunity (Matson et al. 2012; Sild and Horak 2009). This may generate a better understanding of underlying physiological mechanisms that may link anthropogenic noise and light exposure to potential health and fitness consequences. While in a previous experimental study we have shown that artificial light at night inside the nest box affects body mass gain, Hp and NOx, little is known about how ambient levels of light pollution affect developing great tits. Moreover it is unknown whether noise pollution also has an effect and whether the combined effect of noise and light is additive or synergistic.

We expected negative effects of the combined effect of noise and light pollution. Noise exposure alone appears not to affect fledging mass (Meillère et al. 2015) but effects on the immune response have been reported (Kight and Swaddle 2011). Developing great tits exposed to artificial light at night had increased Hp and decreased NOx levels (Raap et al. 2016c) and a reduced growth rate (Raap et al. 2016a). Given that noise and light in songbirds can influence foraging behaviour of parents (Quinn et al. 2006; Stracey et al. 2014) and sleep behaviour of nestlings (Raap et al. 2016b), and noise may impair parent offspring communication (Lucass et al. 2016), we anticipated a negative impact of noise and light on individual health and condition through direct and/or indirect effects. Streets are often associated with noise and light pollution. For example, in our study population the highway

represents the main source of noise pollution. However, roads may have negative effects on animals other than those through noise and/or light pollution (Crino et al. 2011; Fahrig and Rytwinski 2009). For example, road-related chemical pollution may affect oxidative stress and inflammatory responses (reviewed in Isaksson 2015). Therefore, distance to the nearest road or to the highway was considered as an alternative explanation.

Methods

Study site and data sampling

Data were collected 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 (Antwerp), Belgium (51°9'44''N, 4°24'15''E). This nest box population was established in 1997 and has been continuously monitored since then (e.g. Rivera-Gutierrez et al. 2010, 2012; Van Duyse et al. 2000; 2005; Vermeulen et al. 2016b). In order to determine laying date, hatching date and brood size, we checked nest boxes every other day. Nestlings that were 15 days old (hatch day = day 1) were weighed to obtain fledging mass (conform Halfwerk et al. 2011; 0.1g; digital balance; Kern TCB 200-1) and blood sampled (≤ 150 µl) from the brachial vein. Blood samples were kept cool and were centrifuged within a few hours after sampling to separate red blood cells from plasma. We did not obtain sufficient amounts of blood from all nestlings in order to perform all analyses, resulting in different sample sizes (fledging mass: 562 nestlings from 85 nests; Hp: 475 nestlings from 78 nests; NOx: 344 nestlings from 58 nests). Sixteen nests from the current study had also been used in a previous experiment as a control group but these were not manipulated (see Raap et al. 2016a; 2016c). Nests that were exposed to experimental artificial light inside the nest box during that experiment were excluded from the current study. This study was approved by the ethical committee of the University of Antwerp (ID number 2014-88) and performed in accordance with Belgian and Flemish laws.

Nestlings' ambient noise and light exposure were measured at each nest box after sunset. In order to minimize disturbance of nestlings and parents, both noise (DVM 401 environmental meter, Velleman Inc., Fort Worth, TX, USA) and light intensity (ILM 1335 light meter, ISO-TECH, Northamptonshire, UK) were measured at the nest box opening. These measurements were taken as a proxy for nestling exposure to anthropogenic noise and light. The main source of light pollution comes from street lights while the main source of noise pollution is from the highway adjacent to the study area. Nightly noise measurements (> 1 hour

after sunset to 1 hour after midnight) were taken during spring of 2012-2015 at mostly the same nest boxes (Table 1).

Table 1: Average noise levels in our study population. Average noise levels are given per year. Sample sizes (*N*) varied between years but consisted mostly of the same nests, with 67 nests being measured in all four years.

Year	Average noise (dB) ± SE	*N*
2012	51.5 ± 0.6	69
2013	54.3 ± 0.8	74
2014	53.4 ± 0.8	84
2015	53.1 ± 0.8	79

In 2015 also daytime noise measurements were taken (between 8:30-12:30). We registered the highest value of background noise amplitude, measured during 10 s. Measurements were made only when there was no car passing (except for those on the highway) or other extreme source of noise and therefore measurements represent background noise. As is the case in many other studies (Swaddle et al. 2015), we relied on a relatively simple and inexpensive metric of noise. However, noise measurements were highly correlated among years (Table 2) and between day (N = 79) and night time (N = 79) measurements in 2015 (Pearson r = 0.6, P < 0.001) which confirms the reliability of our measurements. Moreover, according to a report by the Flemish government, noise levels in our study area are similar throughout the day and between working days and weekends (Departement Leefmilieu Natuur en Energie 2016).

Table 2: Noise measurements in our study population are highly correlated over the years. Spearman rank correlation coefficient (adjusted for multiple tests; Holm correction) are given for nightly noise measurements between 2012-2015. All correlations were significant (P < 0.001).

	2012	2013	2014
2013	0.69		
2014	0.61	0.66	
2015	0.54	0.52	0.57

Our measurements are also consistent with those taken by the Flemish government, implying that we can be confident that areas with high levels of noise in their report correspond

with nest boxes exposed to high noise levels in our study. The main source of noise pollution in our study area, the highway, is one of the busiest highways of Belgium and noise levels are therefore similar throughout the day and among years. Average noise measurements from 2015 were subsequently used as an approximation of the level of noise pollution to which the nestlings were exposed. Light levels ranged between 0.01 and 6.4 lux (0.01 lux is the lower limit of the light meter).

Sexing, haptoglobin and nitric oxide determination

Following earlier research on great tits, we determined nestling sex (from red blood cells) and quantified Hp and NOx concentrations (from blood plasma; Vermeulen et al. 2015; 2016a; 2016b). Nestling sex was determined genetically (Griffiths et al. 1998). Plasma Hp concentrations (µg/ml) were quantified using the manufacturer's instructions provided with the commercially available colorimetric assay (PHASE Haptoglobin assay, Tridelta Development Ltd; Matson et al. 2012). To quantify NOx concentrations (µmol/l) we used the spectrophotometric assay based on the reduction of nitrate to nitrite by copper-coated cadmium granules (Sild and Horak 2009). The inter assay coefficient of variability was 4.5% for Hp and 3.9% for NOx.

Statistical analyses

All statistical analyses were conducted in R 3.1.2 (R Core Team 2016). We first tested whether our data exhibited spatial autocorrelation to avoid possible pseudoreplication (Zuur et al. 2009) which was not the case; models with and without an auto-correlation structure (ratio, spherical, exponential, Gaussian and linear correlation structure) were compared using AIC and inclusion of an auto-correlation structure did not improve the model (AIC increased). We then examined whether light and/or noise (average of day and night time noise measurements in 2015) explained variation in fledging mass, Hp or NOx, by constructing a set of linear mixed models (LMM) for each of these three dependent offspring parameters (lme4 package; Bates et al. 2015). Noise and light levels were not correlated with each other (Spearman rank $r = 0.2$, $P = 0.2$, $N = 85$). Nest identity (NestID) was included in all models as random factor to avoid pseudoreplication. The model with fledging mass as dependent variable contained brood size (covariate), laying date (covariate) and noise (covariate), light (log +1 transformed; covariate), sex (factor) and all possible three-way and two-way interactions between noise, light and sex as explanatory variables. We used interactions with "sex" as there may be sex-specific

differences in physiology (e.g. oxidative status), growth rate (Giordano et al. 2015; Speakman et al. 2015) and environmental sensitivity (reviewed in Jones et al. 2009). For the models on Hp and NOx, we additionally included fledging mass, bleeding time and weather condition as covariates. Time of day and temperature might influence Hp and NOx levels (Matson et al. 2012; Sild and Horak 2009) and body mass is a measure of condition and may therefore be related to physiological measurements. Data on weather conditions (daily average rain in mm, wind speed in km/h and temperature in °C) were obtained for the day of sampling from a local meteorological station in Antwerp. These weather data were used in a Principal Component Analysis (PCA) to obtain an overall variable for weather condition which explained 50.4% of the variance for temperature, rain and wind.

We furthermore also constructed alternative models where noise and light (and their interactions) were replaced by either distance to the nearest road or distance to the highway (covariates). Distance to road/highway was not used in combination with noise and light in one model in order to prevent collinearity and overfitting the model. Distance to the nearest road and distance to the highway were correlated with each other (Spearman rank $r = 0.3$, $P = 0.02$, $N = 85$). Distance to the nearest road/ highway were also correlated with noise (Spearman rank $r = -0.44$, $p < 0.01$, $N = 85$; $r = -0.57$, $P < 0.01$, $N = 85$) but not to light levels (Spearman rank $r \leq -0.14$, $P \geq 0.23$, $N = 85$). To meet model assumptions, both Hp and NOx were square root transformed.

Finally, all models were compared (per dependent parameter) using a model selection approach based on Akaike's information criterion for small sample sizes (AICc; Anderson and Burnham 2002; MuMIn package; Barton 2016). We used all models within Δ AICc < 2 of the top model to obtain model-averaged estimates and standard errors for each explanatory variable and relative variable importance is calculated (Anderson and Burnham 2002; MuMIn package; Barton 2016). Models within Δ AICc < 2 have substantial support or evidence (Burnham and Anderson 2004).

Results

Fledging mass was unrelated to anthropogenic noise and/or light

Noise, the interaction between noise and light, and distance to the road/highway received no support in the fledging mass models (Table 3). The top ranked model included only the variable sex, with male nestlings being heavier than female nestlings (15.8 ± 0.2 g and 15.2 ± 0.2 g, respectively). The model ranked second additionally included light (Δ AICc = 1; there were no other models with Δ AICc < 2 of the top model). However, light did not contribute substantially to variation in fledging mass (β = 0.08 ± 0.67 g).

Haptoglobin, nitric oxide and relationships with anthropogenic noise and/or light

The interaction of noise and light received support in the Hp models but did not contribute substantially in explaining variation in Hp (Tables 3 and 4). Distance to the road/highway received no support in the Hp models (Table 3). All supported models to explain variation in Hp (within Δ AICc < 2 of the top model) contained noise as an explanatory covariate, and nestlings exposed to higher noise levels had higher Hp levels (β = 0.20 ± 0.06 µg/ml Hp square root transformed; Figure 1 and Tables 3 and 4). Light was also included in some of the supported models, but based on its estimated effect it did not contribute substantially to Hp variation (β = -8.54 ± 12.47 µg/ml Hp square root transformed; Tables 3 and 4).

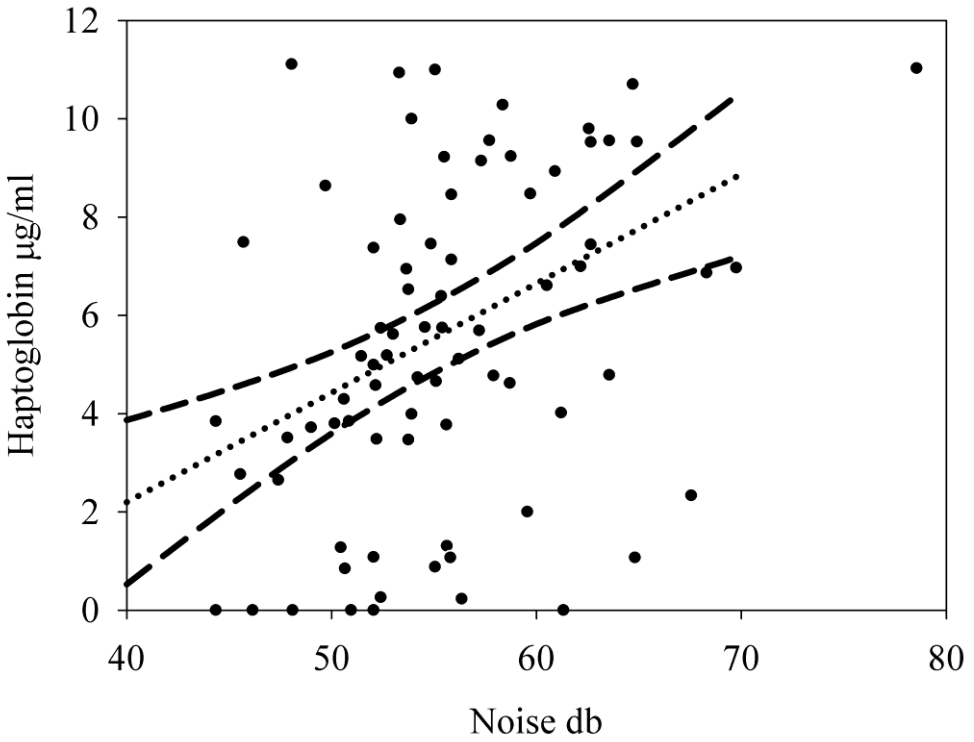

Figure 1: Nestlings exposed to higher levels of noise had higher levels of haptoglobin. Raw data of average Hp concentration per nest (square root transformed) in response to measured noise levels at the nest entrance. Lines represent model-averaged estimate and 95% confidence intervals (see *Statistical analysis* and Tables 2 and 3; partial R^2 = 0.116), based on data from 475 nestlings of 78 nests.

Nitric oxide models including anthropogenic effects as explanatory variables (light, noise, and distance to the road/highway) received no support (Table 3). The top ranked models for nitric oxide contained fledging mass and sex as explanatory variables (there was only one model within Δ AICc < 2 of the top model). Heavier nestlings had lower levels of NOx (β = -0.096 ± 0.022 µmol/l square root transformed) and males tended to have higher levels of NOx (β = 0.12 ± 0.06 µmol/l square root transformed).

Table 3: Results of the fledging mass, Hp and NOx model selection procedure based on AICc. Linear mixed models with "NestID" as random factor were used to avoid pseudoreplication. Top ranked models included noise in combination with sex (s), light, brood size (b), laying date (ld), fledging mass, the interaction light:sex (light:s) and/or the interaction noise:light. Fledging mass models were run on data from 562 nestlings from 85 nests; Hp models on data from 475 nestlings from 78 nests and NOx models on data from 344 nestlings from 58 nests. Only models within Δ AICc < 2 of the top model are shown.

Fledging mass model	AICc	Δ AICc	Akaike weight
s	1834.9	0	0.622
light+s	1835.9	1	0.378
Haptoglobin model	AICc	Δ AICc	Akaike weight
b+ld+s+noise+light+light:s	2651.3	0.00	0.093
b+s+light+noise light:s+noise: light	2651.3	0.07	0.090
b+ld+s+light+noise+light:s+noise: light	2651.6	0.38	0.077
ld+s+light+noise+light:s	2651.7	0.44	0.074
b+s+light+noise+light:s	2651.9	0.60	0.069
b+ld+s+light+noise	2651.9	0.60	0.069
b+ld+s+noise	2652.1	0.81	0.062
b+s+light+noise+noise: light	2652.3	1.06	0.055
ld+s+light+noise	2652.4	1.11	0.053
b+s+light+noise	2652.4	1.18	0.052
b+ld+light+noise	2652.6	1.35	0.047
b+ld+s+light+noise+noise: light	2652.6	1.38	0.047
ld+s+noise	2652.7	1.44	0.045
b+s+noise	2652.7	1.47	0.045
ld+s+light+noise+light:s+noise: light	2652.8	1.50	0.044
b+ld+noise	2652.8	1.52	0.044
ld+light+noise	2653.2	1.93	0.035
Nitric oxide model	AICc	Δ AICc	Akaike weight
fledging mass	612.8	0	0.73
fledging mass+s	614.8	1.99	0.27

Table 4: Results from the Hp model selection procedure showing parameter estimates and selection probabilities (see *Statistical analysis* and Table 2). Only factors that were used for model averaging are shown. Models were run on data from 475 nestlings of 78 nests. Haptoglobin levels had been square root transformed. Relative variable importance (RVI) are shown as well as effect sizes (partial R^2's) which were calculated following Edwards et al. (2008).

Parameter	Estimate ± SE	RVI	Effect size R^2
Brood size	0.46 ± 0.20	0.75	0.059
Laying date	-0.12 ± 0.04	0.69	0.072
Sex	-0.69 ± 0.38	0.87	0.008
Light	-8.54 ± 12.47	0.80	0.044
Noise	0.20 ± 0.06	1.00	0.116
Light:Sex	0.83 ± 1.41	0.45	0.001
Noise:Light	0.35 ± 0.19	0.31	0.043

Discussion

In this study we show that ambient anthropogenic noise was associated with the physiology of free-living great tit nestlings. However, noise, light, and their interaction were unrelated to fledging mass. Noise exposure did explain a significant part of the variation in nestling haptoglobin (Hp) concentration but not in nitric oxide (NOx) concentration. Light was unrelated to Hp and NOx. Against our expectations, we found no additive or synergistic effect of noise and light on nestling physiology. Distance of the nest to the road or highway was also unrelated to nestling fledging mass and physiology (Hp and NOx).

Nestlings exposed to higher levels of noise pollution had higher concentrations of Hp. Distance to the road/highway was not associated with concentration of Hp, while it was correlated with noise levels. The association of noise with Hp seems therefore to be independent from other potential confounding variables related to proximity to a road. Haptoglobin has besides anti-inflammatory also antioxidative properties (Jelena et al. 2013). Noise pollution may increase oxidative stress (Cheng et al. 2011) which may have led to the increase in Hp as part of a compensatory mechanism (Costantini and Verhulst 2009). Alternatively, noise exposure may lead to stress (increased corticosterone levels; Bonier 2012). Stress is known to affect baseline innate immunity (Martin 2009; Matson et al. 2006) and may therefore lead to elevated Hp concentrations.

Whether elevated Hp levels at higher noise exposure have long-term fitness consequences is still unclear. Nonetheless, increasing levels of Hp are potentially energy demanding and trade-offs could occur with life-history traits, such as survival or life-time reproduction. In frigatebird nestlings (*Fregata magnificens*) facing a herpesvirus outbreak, plasma concentrations of Hp were predictive for survival (Sebastiano et al. 2017). While innate immunity has been linked to long-term survival (e.g. Bowers et al. 2014; Cichon and Dubiec 2005), Hp concentrations in great tit nestlings were not found to be predictive of local recruitment (Vermeulen et al. 2016a). However, noise has been shown to have a negative effect on great tit reproduction (Halfwerk et al. 2011) and to reduce telomere length in nestling house sparrows (Meillère et al. 2015). Whether elevated levels of Hp which we found here contribute to these negative effects on reproduction and/or survival remains to be examined.

Despite that noise exposure was related to Hp concentrations, neither the combination of noise and light exposure nor light exposure itself were related to Hp. Moreover, noise and light exposure were unrelated to fledging mass and NOx and there was no combined effect. Nestlings inside nest boxes may be exposed to only limited amounts of artificial light which might to an extent explain why we found no effect of light pollution on their physiology. Nonetheless, indirect effects might occur as adult great tits may be affected and, for example, show disrupted activity patterns (de Jong et al. 2016; Raap et al. 2016b), altered foraging behaviour (Titulaer et al. 2012), increased stress levels (Ouyang et al. 2015) or advanced laying dates (de Jong et al. 2015). Interestingly, although noise did not affect daily timing of dawn song (Da Silva et al. 2014), light pollution did appear to affect song behaviour in great tits (Da Silva et al. 2015; 2016; Kempenaers et al. 2010; but see also Da Silva et al. 2017b). These studies indicate that in adult free-living great tits, activity patterns and perhaps foraging behaviour and subsequently nestlings could be (indirectly) affected by light pollution while noise pollution might cause more direct physiological effects in nestlings.

Low light intensities could have been expected to lead to direct physiological changes. Light intensity measured at the nest boxes in our population ranged between 0.01 and 6.4 lux (0.01 lux is the lower limit of the light meter). In experimental studies, sleep behaviour of adult great tits and nightly activity and physiology of nestlings (fledging mass, Hp and NOx) were affected by light intensities of 1.6 (adult sleep behaviour and nestling nightly activity; Raap et al. 2015, 2016b) and 3.0 lux (nestling physiology Raap et al. 2016a; 2016c). However, a low light intensity of 0.3 lux was already sufficient to advance reproductive physiology and decrease

melatonin levels of adult male blackbirds (*Turdus merula*; Dominoni et al. 2013a; 2013d) and even lower light intensities of 0.05 lux affect nightly activity in adult male great tits (de Jong et al. 2016). Very low levels of light exposure could thus potentially still have caused physiological effects, especially in combination with exposure to noise pollution. Nonetheless, despite the potentially low light intensities to which our nestlings were exposed, our results are still relevant for cavity-nesting species such as great tits where it would appear that light pollution would have a limited direct effect on nestling development and physiology.

Although we are one of the first to examine possible additive or synergetic effects of ambient noise and light pollution on free-living developing animals, our study comes with some limitations. First, we studied how ambient levels of light pollution may affect nestling physiology, however, we cannot know the exact light levels to which nestlings were exposed. Females usually sleep inside the nest box (even when nestlings are 15 days old) and to an extent also on top of the nestlings (Raap et al. 2016b) which may limit the amount of light exposure of the nestlings and also severely complicates taking light measurements at the level of the nestlings. Experiments are therefore necessary to examine direct and indirect effects of artificial light at night. However, our study does represent a natural situation for cavity-nesting species exposed to light pollution. Second, another limitation of our study is its inherently correlational nature which may make it difficult (or impossible) to prove causation compared to experimental studies. For example, in the current study we took measurements of Hp and NOx at day 15, while in our experimental studies we took measurements at 13 days after hatching and again after a two night exposure to experimental artificial light inside the nest box (Raap et al. 2016a; 2016c). Such an experimental design (within-individual design with an additional control group) is very powerful to detect possible differences caused by our treatment. Moreover, under natural conditions such as in our current study, the variation in Hp and NOx (amongst other physiological markers) among nestlings of the same nest is higher within the same nest than among nests (Vermeulen et al. 2015). This might increase the difficulty of detecting an effect of pollutants which occurs at the nest level (Vermeulen et al. 2015). Here we have found that noise was associated with higher levels of Hp. Direct effects of noise on nestling physiology have been reported by Meillère et al. (2015) who showed that experimental exposure to traffic noise reduced telomere length in nestling house sparrows, although growth and fledging success was unaffected. In tree frogs (*Hyla arborea*) noise also had a direct effect, increasing stress hormones and inducing an immunosuppressive effect

(Troianowski et al. 2017). There is also evidence that air pollution from roads could affect nestlings (Peach et al. 2008). However, our results appear to suggest that it is the noise from the roads and not air pollution that affected nestling physiology as models including proximity to the road/highway as an explanatory variable had no support. However, whether there is a causal relationship between noise and higher levels of Hp needs to be examined further with experimental studies. Third, here we used nestlings from a cavity-nesting bird as a model species, the great tit, because they readily accept nest boxes to breed. Although these results are perhaps difficult to extend to open-nesting species, a similar study on this scale (more than 500 nestlings were included in the current study) using nestlings of open-nesting birds is much more difficult. However, such nestlings might be exposed to similar or higher levels of noise and light pollution and may experience an additive or synergistic effect of these pollutants, which remains to be studied.

In conclusion, this study demonstrates that, contrary to our expectations, there was no additive or synergistic effect of ambient noise and light on nestling physiology or fledging mass. Anthropogenic noise but not light was associated with the physiology of 15 day old nestlings from a cavity-nesting species. This could have long lasting adverse consequences. Our study on free-living nestlings complements experimental studies (Meillère et al. 2015; Salmon et al. 2016) and suggests that the urban environment may entail important costs for developing animals.

For my part I know nothing with any certainty,

but the sight of the stars make me dream

Vincent van Gogh

Chapter 12

General discussion

Main findings

We found that ALAN had a variety of behavioural and physiological effects in free-living animals. We also found large among individual variation in sleep disruption which could not be explained by a personality-dependent response to ALAN. Laboratory studies showed that the response to ALAN may vary with light intensity but we could not replicate this in free-living animals using a low (1.6 lux) and high (3 lux) light intensity. Using an experimental setup we did find large differences between two ecologically closely related species in their response to ALAN. Furthermore, physiology and condition of developing great tits was affected by experimental ALAN inside their nest box but ambient light pollution was unrelated to our physiological measures. Nest boxes seemed to shield animals from direct effects of light pollution. Below, first the effects of ALAN on sleep and physiology are discussed and how these results are related to each other, followed by study limitations and the opportunities that our experimental system offers.

Sleep disruption

We found that ALAN disrupted sleep in free-living animals but also that there was large variation in these effects depending amongst others on season and species. Laboratory studies already showed that light at night suppresses sleep (Rattenborg et al. 2005). In great tits (*Parus major*) wild individuals initiated sleep earlier (relative to sunrise) than those held in outdoor aviaries (Stuber et al. 2015b). It is therefore necessary to validate results from laboratory and aviary studies as behaviour (and physiology) may differ between captive and wild animals (Calisi and Bentley 2009; see also "*Lab versus field*"). Using an experiment with a within-individual design we studied how exposure to ALAN inside the nest box may change the sleep behaviour of free-living animals (**Chapter 2**). With our experiment we found that in free-living great tits sleep is disrupted by ALAN in winter. Effects were relatively small, with a 5% reduction in sleep as animals woke up earlier. Our treatment lasted only for a single night and cumulative sleep loss from chronic long-term light pollution may therefore be much greater. We repeated our previous experiment, which was done during winter, with females that were raising nestlings of 10 days old (**Chapter 3**). During the nestling period the disruptive effect of ALAN on sleep in females was much larger. ALAN reduced female sleep by about 50% by as individuals woke up more than an hour earlier and also fell asleep more than an hour later. A combination of multiple drivers that are absent during winter may explain the larger disruption during the nestling period. In winter there are less parasites due to a lack of nest material and nestlings

and these parasites can reduce sleep (Christe et al. 1996). During natural dark nights nestlings hardly or never beg for food, however, there is a considerable amount of begging when they are exposed to ALAN. This begging behaviour may in turn affect female sleep behaviour and vice versa. Potentially this behavioural effect is also related to the effects of ALAN that we found on nestling physiology (see *"Nestling physiology"*).

In urban areas light pollution rarely occurs in isolation but is often associated with noise pollution (Swaddle et al. 2015). Noise pollution has many behavioural and physiological effects on animals (Francis and Barber 2013; see also "*Nestling physiology*" and **Chapter 11**). In humans noise disrupts sleep even when there is no conscious perception of being in a noisy environment (see e.g. Muzet 2007). Animals in urban areas may therefore thus suffer from sleep disruption by both light and noise pollution simultaneously. The effect of noise on sleep in wild animals and how this may interact with the effect of light pollution need to be examined as effects may exacerbate each other (Swaddle et al. 2015). For animals roosting in cavities/nest boxes the effects of noise on sleep may be more severe as light exposure might be limited (**Chapter 6**) but this requires further study.

We found that ALAN disrupts sleep especially during the breeding season (**Chapters 2-5**) but how this affects an individual's reproduction and survival needs to be examined. During the nestling season sleep was already less than in winter (respectively about 35% versus 95% of the time spent asleep in the nest box) and a single night of ALAN exposure reduced sleep amount from about 200 to 90 minutes (variation is discussed in the following paragraphs). Potentially, extensive sleep disruption by ALAN over an extended period of time would probably affect an animal's health. In rats extended sleep deprivation led to serious health consequences and eventually death (Rechtschaffen and Bergmann 2002). Interestingly stress and loss of circadian rhythm, which are both potential consequences of ALAN (Russart and Nelson 2018), were suggested as alternatives to the potential cause of death in this experiment. Laboratory research on the other hand showed that birds might be more resilient to sleep loss as pigeons (*Columba livia*) did not suffer from a compromised health condition (Newman et al. 2008; 2009). Whether this is also the case for free-living birds during the breeding season, which are therefore under increased pressures is unknown. Sleep deprivation potentially decreases foraging efficiency in great tits, which might result in decreased nestling body condition and survival. ALAN may through this pathway decrease reproductive output. Research on humans indicates that one of the functions of sleep is to maintain high waking

neurobehavioral performance (Basner et al. 2013). However, research on how sleep loss in other animals and birds affects performance is limited (Aulsebrook et al. 2016). In birds, sleeping less does not necessarily have to be detrimental for performance (Rattenborg et al. 2017). Increased infestations of hen fleas (*Ceratophyllus gallinae*) reduced sleep (73.5% to 48.1%) but did not affect provisioning behaviour of female great tits (Christe et al. 1996). The few studies that have been carried out to study effects of natural or experimentally induced variation in sleep on fitness have produced mixed results until now. In specific cases such as pectoral sandpipers (*Calidris melanotos*), an Arctic breeding and polygynous shorebird, less sleep can be beneficial as it allows males to sire more offspring (Lesku et al. 2012). Males reduced sleep in order to be more active, therefore they had more time to pursue and display to fertile females and gained a competitive advantage on less active males. Costs of ALAN induced sleep disruption are likely for both humans and wild animals as unnatural sleep deprivation is associated with cardiovascular disease, endocrine disruption and has a profound effect on the circadian expression of the transcriptome, especially on genes associated with the immune and stress response (reviewed in Dominoni et al. 2016b). Recent studies show that in humans low levels of light disrupt and reduce sleep (Cho et al. 2016) and that sleep deprivation reduces cognitive function (reviewed by Orzel-Gryglewska 2010) and this seems likely in wild animals as well. In humans deviation from the norm of sleep duration (shorter or longer) has been associated with increased mortality risks (Gallicchio and Kalesan 2009), which may indicate that chronic sleep disruption by ALAN may have severe consequences. Reduction in performance due to sleep loss has mainly been demonstrated in the laboratory and has been difficult to prove in free-living animals (Rattenborg et al. 2017). In several songbird species, light pollution probably reduced their sleep as their dawn song was advanced but it also led to an increase in the extra-pair success of blue tits (*Cyanistes caeruleus;* Kempenaers et al. 2010). Some species such as the common redshank (*Tringa totanus*) also take advantage of light pollution to extend their foraging into the night (Dwyer et al. 2013). To what extent this comes at the expense of sleep needs to be determined. A study on herring gulls (*Larus argentatus*) suggested that foraging may increase the need to sleep (Shaffery et al. 1985). It would thus be of interest to examine to what extent nocturnal foraging comes at the dispense of nocturnal sleep and whether this is compensated by daytime sleep. Some songbird species such as the Northern mockingbird (*Mimus polyglottos*) may take advantage of light pollution to feed their nestlings after dark (Stracey et al. 2014). Whether this results in higher quality nestlings and

breeding success is unclear (Shaffery et al. 1985). In blue tits it was shown that breeding success was lower in an urban area because food quality was lower compared to a natural area (Pollock et al. 2017). Therefore, while light pollution may extend foraging time, it also disrupts sleep (**Chapter 3**) and ultimate costs and benefits in term of reproduction and survival are unclear and need to be examined.

Interestingly, in **Chapter 3** we find that there was large variation among individuals in their response to ALAN. In general ALAN disrupted sleep behaviour, but some individuals appeared to be hardly affected and did not alter their sleeping behaviour. However, physiological sleep as measured by EEG could still be affected and potentially these animals also slept less deep (see also "*Limitations and opportunities"*). Nonetheless, there were also some individuals that did not sleep at all or less than half an hour. Variation in natural sleep behaviour in great tits appears not to be related to their personality (slow versus fast explorers; Stuber et al. 2015b). However, the large variation in sleep behaviour in response to ALAN (see **Chapter 3**) may potentially be personality-dependent and we found that in winter about one in three birds did not enter a nest box with ALAN inside (see **Chapter 4**). Personality is a heritable trait and associated with variation in fitness (Dochtermann et al. 2015; Smith and Blumstein 2008). If a specific personality type is favoured under pressure of light pollution it may thus act as an agent of selection (Swaddle et al. 2015). Personality types of great tits do indeed seem to differ between urban and rural populations with urban great tits being more aggressive and faster explorers (Charmantier et al. 2017). In **Chapter 7** we showed that our experimental light inside the nest box did not deter slow explorers more than fast explorers. Furthermore, slow explorers, compared to fast explorers, did not appear to be more disrupted in their sleep behaviour when exposed to ALAN. Further research is necessary to validate these findings and to examine to what extent behavioural and physiological changes by ALAN are personality-dependent. This type of research is crucial as personality-dependent disruptive effects of ALAN on sleep could have cascading effects on energy balance, reproduction, survival, and eventually population dynamics.

The variation in the response to ALAN which we found especially during the nestling period (**Chapter 3**) could therefore be due to other factors instead of being personality-dependent. Many factors can influence sleep behaviour and the need to sleep, such as the presence of predators or parasites (Rattenborg et al. 2017). Furthermore, the duration that an animal, such as a great tit, sleeps not only differs between sexes but sleep behaviour also

reflects an individual's experience, condition and/or genes and is an individual-specific trait (Stuber et al. 2015b; 2016). As mentioned earlier, foraging may increase the need for sleep (Shaffery et al. 1985). Differences in how much energy is spent during the day may therefore influence differences in the need for sleep among individuals and their response to ALAN may depend on their daily activity (past experiences). Further studies are necessary to determine to what extent the response to ALAN (with respect to sleep behaviour) is repeatable and reflects how an individual responds to ALAN or whether variation in response is due to other biotic or abiotic factors (Stuber et al. 2015b; 2016; 2017).

Besides the individual variation in the response to ALAN we also examined whether there is a season, light intensity and/or species dependent response to ALAN. As the winter season progresses a differential response to ALAN could be expected because natural sleep behaviour of songbirds such as great tits changes, with birds waking up earlier (relative to sunrise; Stuber et al. 2015b). We therefore examined the response to ALAN in December and February with a higher light intensity and compared effects with those that we previously found with a lower light intensity (3.0 and 1.6 lux; **Chapter 4**). There were no clear differences in response, and sleep disruption in December and February was similar. At the end of winter as day length starts to increase, light initiates a cascade of physiological effects associated with day length (Bradshaw and Holzapfel 2010) which prepares individuals for reproduction (Helm et al. 2013). Therefore, in February individuals may become more light sensitive. A lower light intensity than that we used in our experiment may therefore disrupt sleep behaviour in February and not have an effect in December.

Nonetheless, the response to ALAN with regard to sleep behaviour strongly differed between the winter and breeding season (**Chapter 3**). Hence, results based on a single period describe an incomplete picture of the effects of ALAN on an animal. This is important because mitigation strategies to reduce the environmental impact of ALAN may need to be adapted according to the season. Furthermore, a lack of effects during one season cannot be interpreted as a lack of effects year-round. In addition to temporal or seasonal variation, one may need to take geographical variation into account (see *"Latitudinal variation"*).

In **Chapter 4** we also examined whether different light intensities may elicit a different response to ALAN. A laboratory study using great tits found that the response to ALAN, in terms of nightly activity, increased with increasing light intensity (0.05, 0.15, 0.5, 1.5 and 5 lux; de Jong et al. 2016). We could not replicate this result in the field using a low (1.6 lux) and high

(3.0 lux) light intensity. We did not use a higher light intensity than 3 lux as with 1.6 lux already about 1 in 3 birds did not enter the nest box (**Chapter 2**) and using 5 lux may make it more difficult to obtain a reliable sample size. Nonetheless, differences between 1.6 and 3.0 lux could be expected as the laboratory study from de Jong et al. (2016) showed that both the response to ALAN in regard to activity onset and nocturnal activity was about twice as strong with 5 lux compared to 1.5 lux. While sleep patterns (shifts between short wave sleep; SWS and rapid eye moment; REM phase) and how much time was spent in SWS and REM sleep might have been affected (see "*Limitations and opportunities"*), we found no difference in the extent that ALAN disrupted sleep behaviour between the two used intensities. Sleep disruption is larger when great tits have nestlings (**Chapter 3**) and differences in response to different intensities might become more obvious during this period. With our experimental set-up we have so far used relatively low light intensities of 1.6 and 3 lux, which are only a fraction of the intensities found near street lights (10-40 lux; Gaston et al. 2017) although birds in urban areas are exposed to levels similar to what we used (up to 2.2 lux; Dominoni et al. 2013a). Very low light intensities of 0.05 lux appeared to increase nightly activity in great tits (de Jong et al. 2016). However, this study was done in the laboratory and validating these results in the field is necessary as it is unclear to what extent results from laboratory experiments can be generalized to the field (see e.g. Calisi and Bentley 2009). Using different and lower light intensities may also show a dose-dependent response to ALAN in sleep behaviour of wild great tits during the nestling period. Little is known about how relatively low light intensities may influence behaviour and physiology in free-living animals. Using lower light intensities (dimming of light) may potentially mitigate effects of ALAN and should be examined.

We found a clear species-specific response to ALAN in the sleep behaviour of two ecologically closely related species (**Chapter 5**). With our experiment where we looked at changes caused by ALAN within the same individual, in both great and blue tits, we were able to exclude potential confounding variables and account for the large variability in sleep behaviour among individuals (**Chapter 3**). In line with previous studies, during winter great and blue tit sleep behaviour in a dark nest box was similar (Steinmeyer et al. 2010; Stuber et al. 2015b). However, great tits' sleep behaviour was more disrupted by ALAN compared to that of blue tits. Earlier studies already suggested that the behavioural response to light pollution may differ among songbirds (e.g. Kempenaers et al. 2010) and that this might be due to differences in light sensitivity (Da Silva and Kempenaers 2017). Further differences in the extent that ALAN

disrupts sleep behaviour between blue and great tits may become apparent during the nestling period as the disruption of sleep was much greater during this period for great tits (**Chapter 3**).

Species-dependent effects of ALAN may have consequences for the generalization of results from one species to another. The absence of effects in a particular species may not necessarily mean that it does not affect other even closely-related species (**Chapter 5**). Regarding birds it seems that there is a gradient in the extent of disturbance by ALAN, with species such as robins (*Erithacus rubecula*) and blackbirds (*Turdus merula*) that have an earlier dawn chorus are being more affected than great tits (although this depends on the geographical region, see "*Latitudinal variation*"; Da Silva and Kempenaers 2017). Species-dependent effects of light pollution are also clear in other taxa. Slow flying bat species (*Myotis*) and (*Plecotus*) were deterred by white light while fast flying bat species (*Pipistrellus*) were attracted by it (Spoelstra et al. 2017). Similar to light pollution, the effects of noise pollution seem to be species-dependent as well, as some bird species do not show altered dawn songs while others do (Dominoni et al. 2016a). Furthermore urbanization and in particular NOx pollution had a species-dependent effect on oxidative status: tree sparrows (*Passer montanus*) showed higher levels of protein damage compared to blue tits, great tits and house sparrows (*Passer domesticus;* Salmon et al. 2018). Species-dependent effects of urbanization and ALAN may thus not be limited to behaviour but also relate to physiological effects.

Effects of light pollution on cavity-nesting species are not always straightforward and are potentially not only species-dependent, but also sex and season may play an important role and interact with each other. In **Chapter 6** we showed that nest boxes shield animals from the direct effects of ambient light pollution. We exposed nest boxes of great tits to experimental ALAN from outside and observed no changes in sleep behaviour. Furthermore, in **Chapter 11** we found no effects of ambient light pollution (caused by street lights) on great tit nestlings' physiology although individuals experimentally exposed to ALAN had elevated Hp levels, decreased NOx and did no longer gain any body mass (**Chapters 8 and 9**; see also "*Nestling physiology"*). During the great tit breeding season mainly females sleep inside nest boxes (Hinde 1952; Kluijver 1950). Males are therefore possibly exposed to higher levels of light pollution, which could explain results observed on dawn song, a typical male behaviour in great tits (Da Silva et al. 2014; 2015; 2016; 2017). However, during winter, nest boxes/cavities may provide shielding for both sexes (**Chapter 6**) while during the breeding season the amount of exposure to light pollution is likely sex-dependent for adults. It is therefore important that

studies using wild animals quantify individual exposure to light pollution (Raap et al. 2017c), and be cautious in the interpretation and generalisation of the effects, or lack thereof, from light pollution (see also "*Variation in light exposure*").

Exposure to ambient light pollution probably affects sleep (see *"Variation in light exposure"*) and could have serious consequences. Sleep constitutes a behavioural trade-off as it precludes other activities such as foraging and vigilance (Roth II et al. 2010). In a laboratory study, ALAN advanced activity in the morning and led to less activity during the day (de Jong et al. 2016) possibly because animals rested more (Mace 1989) as a response to reduced sleep. For diurnal animals such as great tits it is beneficial to sleep during the night when they cannot forage and by sleeping they can conserve energy. In harsh climates this is crucial for survival. If ambient light pollution disturbs nocturnal sleep and increases daytime rest/sleep it likely comes not only at the cost of increased predation risk but it also reduces the time that individuals can use for foraging. This is important during winter when birds need sufficient energy stores to survive the night but also during the breeding season when they need to feed their nestlings. Although birds such as great tits show certain flexibility in how much sleep they need (see also "*Latitudinal variation"*), the consequences of inappropriate timing of behaviours such as sleep because of its disruption need to be determined.

Nestling physiology

We examined the effects of ALAN on oxidative status and immunity in great tit nestlings using our experimental treatment with light inside the nest box (**Chapters 8 and 9**). While we determined multiple metrics of both antioxidant defences and oxidative damage (using nine different parameters) we found no change in any aspect after two nights of ALAN exposure. However, Hp and NOx, both important indicators of immunity, health, and physiological condition were affected by ALAN, with an increase in Hp and a decrease in NOx. Furthermore, we found that light exposed nestlings showed no natural increase in body mass over the course of the experiment. Haptoglobin is part of the non-specific immune response and it may also act as an antioxidant (reviewed in Matson et al. 2012) and hence play a role in maintaining oxidative status. Plasma Hp might therefore have been elevated to counteract increases in oxidative damage compounds (Jelena et al. 2013), however, we found no other metrics of oxidative status to be affected (**Chapter 8**). Potentially, effects on metrics of oxidative status may become apparent using a longer treatment. ALAN may also affect stress (Russ et al. 2015) which affects the immunological response (Matson et al. 2006) and therefore potentially

haptoglobin as well. The physiological effects that we found here might also be related to increased begging behaviour due to ALAN exposure (see **Chapter 3**). Sleep disruption of the females (**Chapter 3**) may also affect how much and how frequently they provide food for their nestlings. This, in addition to direct effects of ALAN, might also have affected nestling physiology.

ALAN increased oxalate levels, a cross-species marker for sleep debt, but only in male nestlings (**Chapter 10**), rather than decreasing it as was found in sleep-deprived humans and rats (Weljie et al. 2015). This discrepancy in results can potentially be explained by the differences in methodology used between the two studies. We used birds opposed to mammals and developing animals instead of adults. Furthermore, we used ALAN to reduce sleep while in rats forced movement was used. Finally, we used free-living animals opposed to laboratory animals and results obtained from field and laboratory studies may differ widely (Calisi and Bentley 2009). The sex-dependent effect on oxalate might indicate different physiological coping mechanisms by developing great tits, which requires further investigation. A potential explanation of higher levels of oxalate is that increased begging (**Chapter 3**) might have led to increased food provisioning by the parents especially for the (larger) male nestlings (see e.g. Anderson et al. 1993), and thus to more dietary oxalate. The use of oxalate as a biomarker for sleep loss in free-living birds and developing animals requires further investigation. However, our results may potentially provide a foundation for future work with free-living animals.

Relatively low light intensities (1.6 and 3.0 lux) and short exposure had behavioural and physiological effects in developing great tits. A single night of light exposure at night was sufficient to cause nestlings to beg at night (**Chapter 3**) and two nights of ALAN affected their body mass and physiology (**Chapters 8 and 9**). Light at night may thus have potential detrimental effects during development. For instance, when growth is reduced in great tit nestlings (but also other bird species) this will affect their short-term survival (less animals will fledge) but also when they reach adulthood they are less likely to survive (Naef-Daenzer et al. 2001; Perrins and McCleery 2001). We thus showed an important potential pathway through which light pollution may affect reproductive output as well as short and long-term survival. Effects of long term exposure need to be examined (see also "*Exposure time"*)

While we found effects of ALAN exposure on nestling physiology (**Chapters 8-10**), how this translates to effects on lifetime reproductive success and survival should be examined. Laboratory studies showed that ALAN exposure reduces the immune response (Russart and

Nelson 2018). It would thus be of interest to examine whether ALAN exposure also reduces the immune response in adult and developing free-living animals. This is highly relevant as in humans ALAN exposure was shown to have many (detrimental) physiological consequences. Exposure to ALAN has been linked to an increased risk of obesity, diabetes and cancer (Russart and Nelson 2018). Detrimental effects on wildlife are therefore likely. Furthermore, nestling great tits in an urban environment were shown to have shorter telomeres (Salmon et al. 2016), in which ALAN might play a crucial role. Sleep disruption by ALAN (**Chapters 2-5**) may also reduce telomere length (Tempaku et al. 2015). ALAN might hence affect the survival of developing birds as the premature shortening of telomeres reduces longevity (Monaghan 2014).

In a correlative field study (**Chapter 11**) we examined whether ambient levels of light and noise pollution affected nestling physiology. Nestlings exposed to higher noise levels showed higher plasma levels of Hp but not of NOx. However, ambient levels of light pollution were unrelated to Hp and NOx and did not interact with the effect of noise on nestlings' physiology. Increasing levels of Hp are potentially energy demanding and trade-offs could occur with life-history traits, such as survival. Effects of light pollution on nestlings of a cavity-nesting species appear to be limited. Potentially nest boxes may provide some shelter from the effects of ALAN (**Chapter 6**). Indeed, when we experimentally exposed nest boxes to ALAN from the outside we found no effect on the sleep behaviour of great tits. While light exposure at the bottom of the nest box is very low under natural circumstances, great and blue tits adjust their sleeping behaviour according to local light exposure (Steinmeyer et al. 2010; Stuber et al. 2015b). When a bird stays at the bottom of the nest box it can observe light shining in through the entrance, which could subsequently affect its behaviour. However, sleep behaviour was unaffected by our treatment. Potentially when individuals are inside a nest box or a cavity this may shield them from the direct effects of artificial light (**Chapter 6**). Nestlings that were inside a nest box exposed to high levels of ambient light pollution did not have any different levels of Hp and NOx than those at low levels of light pollution (**Chapter 11**) and their oxidative status also showed no differences (Casasole et al. 2017). Light pollution may extend foraging behaviour (Stracey et al. 2014), however, it may therefore reduce sleep which in turn can negatively impact foraging behaviour. Thus while the time that can be spend to forage for food could be increased by light pollution sleep disruption may negatively affect performance and the amount of food given to the nestlings could be reduced which would affect nestling

condition. Indirect effects of light pollution on the parents may thus affect developing animals. For example, in bats (*Rhinolophus ferrumequinum*, *Myotis emarginatus* and *M. oxygnathus*) it was shown that illumination of their roosts disrupts the timing of emergence which likely led to impaired foraging of the adults and juveniles were smaller and lighter (Boldogh et al. 2007). Comprehensive studies are necessary to examine the effect of light pollution on the trade-off between foraging and sleep and its effect on nestling condition and survival as well as how it affects parental health.

Lab versus field

Our study focused on the effects of ALAN on free-living animals due to the differences in behaviour and physiology compared to captive held or laboratory animals. Animals in the wild are exposed to a variety of biotic and abiotic factors which may impact their physiology as well as affect their need and the time that they can spend on sleep. These factors are either lacking or controlled in the laboratory while others factors, such as a confined space and solitary confinement, are often introduced. Animals held in captivity are also released from predation risks which can affect physiology (such as stress) and behaviour (such as sleep). For example, brown-throated three-toed sloths (*Bradypus variegatus*) sleep much less in the wild than in captivity, likely due to higher predation risk (Rattenborg et al. 2008). In great tits captive held animals slept less due to a later sleep onset as well as an earlier awakening time (Stuber et al. 2015b).

Examining effects of ALAN on sleep behaviour in captive birds would thus be confounded by their altered behaviour due to captivity. Using free-living birds, we overcome these issues. Furthermore, it gave us the opportunity to observe changes in sleep behaviour of females with nestlings (**Chapter 3**). This would be challenging to achieve in captivity for non-domesticated animals. It also offers us another opportunity: to examine whether sleep disruption (caused by ALAN) does indeed affect performance in free-living animals, which has rarely been studied in the wild (Rattenborg et al. 2017). Because we exposed birds inside their nest box they have no increased opportunities to extend their foraging time by our manipulation and we could therefore examine whether sleep disruption affects their provisioning behaviour (performance).

Laboratory studies are also limited in the extent that they can mimic natural light-dark cycles and thus the disruption of these cycles. For example, the shift in light spectrum which occurs during twilight affects circadian clocks (in mammals) but is difficult to replicate under

laboratory circumstances (Walmsley et al. 2015). Therefore laboratory animals held under "control" conditions already differ from those in the wild. We do find that the effects of ALAN differed between studies using wild or laboratory animals, although we cannot pinpoint to the exact reason. Captive great tits held under short day conditions showed effects mainly during the morning (on activity onset; de Jong et al. 2016), while we found that during the nestling period (**Chapter 3**) the effect (on sleep onset and awakening time) was similar during the evening and morning. Our study had a more natural setting where nestlings were also present, although this also confounds our results (as explained in **Chapter 3**) making them less comparable to laboratory studies where animals are held in solitary confinement.

Of course the physiological response to ALAN may also depend on the environment of an animal (lab versus field) as baseline physiology (e.g. immunocompetence) already differs between captive and free-living animals (Calisi and Bentley 2009). For example, in captive house sparrows the number of lymphocytes had decreased (Kuhlman and Martin 2010) and stress levels (corticosterone) were higher even after a longer period of captivity compared to wild-caught birds. Using free-living great tits as we did in our physiological experiments (**Chapters 8-10**) can overcome these issues. This does, however, also introduce more variability due to differences among individuals/nests. Therefore, we used a within-individual design and examined changes caused by ALAN within the same individual. With this design we examined effects of ALAN on oxidative status in free-living great tits (**Chapter 8**). Laboratory studies indicate that ALAN decreases melatonin and affects oxidative status but our experimental ALAN treatment did not affect oxidative stress markers in free-living great tit nestlings. Melatonin is expected to play a role in maintaining oxidative status (see e.g. Reiter et al. 2016) and a laboratory study showed that in Golden spiny mice (*Acomys russatus*) exposure to 30 min of ALAN for 2, 7 or 21 nights altered oxidative status markers (Ashkenazi and Haim 2013). Also in rats there is evidence that links the reduction of melatonin by ALAN to effects on oxidative status (Cruz et al. 2003). In birds this also seems the case as low levels of ALAN (0.3 lux) were shown to decrease plasma melatonin levels in blackbirds (Dominoni et al. 2013d) and another laboratory study found evidence for melatonin to affect oxidative status markers in the brains of chickens (Pablos et al. 1998). In our experimental field study great tit nestlings were exposed to 3.0 lux which would likely decrease melatonin levels. Moreover, we found that ALAN caused birds to wake up earlier (**Chapters 2-5**) and in blackbirds earlier onset of activity was related to lower melatonin levels (Dominoni et al. 2013d). Furthermore we did find that our treatment

affected levels of haptoglobin and nitric oxide (**Chapter 9**). Taken together we had expected our ALAN treatment to affect oxidative status but this was not the case. We do not know whether this is due to differences in environment (lab versus field), to the duration of our treatment or to some other factor(s).

Variation in light exposure

We used cavity-nesting species as model species and inside cavities/nest boxes effects of light pollution might be limited due to a lack of direct exposure (**Chapters 6 and 11**). However, during the breeding season male great tits rarely sleep inside nest boxes and their dawn song is strongly affected indicating that light pollution is also relevant in cavity-nesting species such as great and blue tits (see e.g. Da Silva et al. 2014). Unravelling fundamental questions about how ALAN exposure affects free-living great (and blue) tits is therefore also applicable for light pollution research in general. For example, based on our results we can expect that light pollution not only advances male dawn song but that their sleep is likely disrupted as well. It will be of interest to exactly quantify levels of light exposure in free-living great tits especially during the breeding season similar to what was done using tammar wallabies (*Macropus eugenii*; Robert et al. 2015) and blackbirds (Dominoni et al. 2013a). Such research will be very challenging as great tits are much smaller implying that when using a small light logger the sensor is easily covered by feathers which may give unreliable data. Furthermore, the orientation of the bird may also influence the light levels measured.

Light exposure can be highly variable not only for cavity-nesting species but also for open-nesting species. For example, one of the few studies to examine light exposure in free-living animals found that blackbirds were exposed to a large range of intensities (Dominoni et al. 2013a). Exposure to ALAN was determined for eight adult males using miniature light loggers attached to their back. While city street lights had intensities of around 6 lux, males were exposed to a mean intensity of 0.3 and maximum of about 2.5 lux. Another study that compared city and rural blackbirds illustrates that city birds (business district) were exposed to higher light levels at night but also that these levels varied greatly among individuals and that this may also differ from one night to another (Dominoni et al. 2014). The amount of light exposure for nests or individual birds can vary due to several factors (Raap et al. 2017c). First, especially in areas with trees/shrubs light intensity from street light quickly diminishes within several meters (Kempenaers et al. 2010). Height is another key consideration because when street lights only shine light downwards an individual that is higher than the street light would not be directly

exposed to ALAN. However, an animal could still be affected by upwards reflected light and/or skyglow (Kyba and Hölker 2013). The light intensities to which urban blackbirds were exposed (Dominoni et al. 2013a) partly overlap with those that we used to show that sleep behaviour of great tits was disrupted (1.6-3.0 lux; **Chapters 2-5**). Therefore, we could expect sleep behaviour of open-nesting birds to be affected by ambient light pollution. This also seems likely as blackbirds appeared to be more light sensitive or more responsive to ALAN than great tits (Da Silva et al. 2014; 2015), thus they may show altered behaviour at already lower light intensities. Future studies should examine to what extent sleep behaviour of open-nesting birds is affected by light pollution.

Latitudinal variation

Day length changes throughout the year but also differs at different latitudes and effects of ALAN may therefore differ depending on the geographical location and season. During the breeding season there is species specific latitudinal variation in the advancement of dawn song (Da Silva and Kempenaers 2017). For great tits the effect on dawn song was similar at high and low latitudes (among Finland, Germany and Spain). Effects on great tit sleep (**Chapters 2-5**) could therefore be similar as well. For other species that have a naturally earlier dawn song, such as blackbirds, effects of light pollution differed among latitudes. In Germany and Spain dawn song was advanced but not in Finland where dawn song is already relatively earlier due to the longer day length. Differences in light sensitivity may explain these species specific effects across latitudes. During the winter, higher latitudes experience shorter days as well (more similar to lower latitudes) and light pollution does affect activity. For example, during the breeding season dawn song of the robin was not advanced by light pollution at higher latitudes (Da Silva and Kempenaers 2017) but it did advance activity during the winter and birds seemed to actively move towards artificially lighted areas (Byrkjedal et al. 2012). Urban blackbirds in Germany (Leipzig) also showed prolonged foraging under the influence of ALAN during winter although this effect subsided as day length increased (Russ et al. 2014). Nonetheless, effects of light pollution were still present during the breeding season as blackbirds in this area advanced their laying day when exposed to higher light intensities (Russ et al. 2017). Depending on the species, advanced and/or prolonged activity by ALAN may thus be less pronounced in higher latitudes. Even so sleep quality could still be affected and during longer winter nights at higher latitudes exposure to light pollution is also longer and we could expect larger physiological effects (Dominoni et al. 2013a).

Limitations and opportunities

Light intensity

Our research, as most other research, comes with certain limitations or aspects which need to be taken into consideration. Most of our work has focused on great tits, with one study also on blue tits (**Chapter 5**), and both are cavity-nesting species. The light intensity that we used may not necessarily be experienced by birds (animals) in nest boxes or cavities (**Chapter 6**). Nonetheless, free-living animals exposed to ambient light pollution are often exposed to similar and even higher light intensities (street lighting is about 10-40 lux; Gaston et al. 2017), than what we used in our experiments (1.6-3.0 lux; see also Figure 1 in **Chapter 1** and "*Variation in light exposure*"). Using cavity-nesting birds and the experimental light inside a nest box, is a highly valuable study system in which free-living individuals can be exposed to ALAN. It provides a multitude of opportunities to examine fundamental questions on how ALAN affects free-living animals. Unfortunately, as the artificial light treatment is limited to the inside of the nest box potential benefits such as extended foraging cannot be examined. Experimental light pollution studies need to ensure effective light exposure of free-living animals (Raap et al. 2017c), which can be accomplished with our experimental system. Free-living animals may be exposed to a range of light intensities ("*Variation in light exposure*"). Hence, future studies on the exposure of free-living mobile animals to street lighting might have difficulties in obtaining reliable sample sizes due to the variation in light exposure. This is perhaps even further complicated by the large individual variation in the response to ALAN (**Chapter 3**). Quantifying individual light exposure and experimentally induced light at night at territories may offer a more direct comparison to direct and indirect effects from light pollution.

Exposure time

Most of our experiments exposed animals for a short period to ALAN (two nights maximum). Using this short treatment we already found severe disruption in adult sleep behaviour as well as in nestling physiology. Longer exposure to ALAN may lead to habituation and thereby reduced disruption. However, other experimental research on ALAN seems to contradict this and in humans habituation to sleep loss seems subjective as performance remains impaired (Balkin et al. 2008). An aviary experiment on peahens (*Pavo cristatus*) showed little habituation of animals towards ALAN (Yorzinski et al. 2015). Moreover, long-term exposure to ALAN may even elicit larger responses than the ones we already found. A laboratory experiment using

great tits which were subjected to low light levels (0.5 lux) found that effects on activity at night actually increased over the course of several days which could be due to the suppression of ALAN on melatonin (de Jong et al. 2016). Long-term ALAN exposure may also lead to chronic stress and disrupted physiology (Dominoni et al. 2013c; Ouyang et al. 2018). Over a longer period of ALAN exposure sleep debt may therefore accumulate and physiological disruption may be more severe. In **Chapter 3** we show that after sleep disruption by ALAN females tried to recover from their lost sleep the following night. Sleep need would thus accumulate over several nights and increase the effects although arousal thresholds would also increase. Sleep disruption could be due to two aspects which are affected by ALAN, a reduction in melatonin and an increase in stress. Melatonin levels are still reduced after an extended period of ALAN exposure (Dominoni et al. 2013d). However, animals can habituate to stress which could lead to less severe sleep disruption. For example with regard to sleep humans can habituate, to an extent, to noise pollution (Basner et al. 2011). A logical next step would therefore be to use our experimental setup and expose animals to a longer period of ALAN. However, we found that during the winter about one in three birds do not enter a nest box anymore when it is lit from the inside (**Chapter 4**). It may therefore be challenging to obtain a sufficient sample size given that free-living animals might decide to roost elsewhere when exposed to a longer period of ALAN. During the nestling period it may be possible to examine effects of long-term exposure to ALAN. However, then there is the risk of nest abandonment and nest failure, although lower light intensities may potentially reduce this risk.

Sleep disruption due to ALAN or the change at the nest box

With regard to the experiments on sleep behaviour (**Chapters 2-5**) one question that rises is whether disturbance caused by the "change" at the nest box by ALAN, rather than light exposure *per se*, resulted in the observed sleep disruption. If it was only due to the "change" of the nest box we could expect more pronounced effects in the evening when the bird first experiences the ALAN treatment. However, during the winter this was not the case and the effects were most pronounced during the morning (**Chapter 4**). This is in line with other studies on songbirds (Dominoni et al. 2014; Miller 2006) where morning activity was more advanced by ALAN than evening activity was extended. Similarly, in several species including the great tit, the effect of ALAN on dawn song was larger than that on dusk song (Da Silva et al. 2014).

Sleep behaviour versus physiological sleep

Another limitation in our sleep experiments is that we measured sleep behaviour and can therefore not make any conclusions about the brain state of birds, such as that based on electroencephalography (EEG). The amount of time spend sleeping can be greatly underestimated if based on behaviour (Aulsebrook et al. 2016), but this is also true for sleep disruption by ALAN. Electrophysiological studies of sleep in free-living animals especially of small animals such as great tits (about 16-18 grams for adults) are challenging and perhaps impossible with the current technology (Aulsebrook et al. 2016; Rattenborg et al. 2017). However, the continued development of light weight EEG loggers may make this possible in the near future but is currently limited to animals of about 100 g (Vyssotski et al. 2009). While EEG loggers will give us more detailed insight into the effects of ALAN on sleep and different sleep states, sleep behaviour seems ecologically relevant as it is related to amongst others predation risk and has a genetic basis where particularly clock genes are involved (Stuber et al. 2014; 2015b; 2016). Furthermore, sleep is greatly reduced (several hours) after a single night of ALAN exposure (**Chapter 3**) and birds tried to compensate for this loss of sleep by sleeping more the following dark night. Thus it would seem to be an important behaviour and relevant in ALAN research.

Nestling versus adult physiology

Our physiological studies focused on specific markers of immunity and condition in developing animals only (**Chapters 8-10**). Using a within-individual design is preferred in ALAN experiments due to the variation among individuals in their response (**Chapter 3**). Taking multiple blood samples of adults instead of nestlings would be more complicated due to a high risk of nest box abandonment during winter. During the breeding season repeated blood sampling may be possible. However, as was shown in blue tits (Schlicht and Kempenaers 2015), adults likely will not return to their nest for several hours after capture. During this time nestlings are deprived of food which will also affect experimental results. Potentially adding an experimental treatment like ALAN inside a nest box may further deter birds from returning to their nest. There is thus a potentially high risk of nest failure and experimental failure when using adults instead of nestlings in these types of experiments with free-living birds. ALAN may disrupt biological rhythms which are also important for host-parasite interactions (Martinez-Bakker and Helm 2015). Further research is necessary to examine what the consequences are of the

physiological alterations that we observed in nestlings and whether these are larger with a longer period of ALAN exposure.

Mitigating measures

We found that in winter, effects of ALAN on sleep are most prominent during the morning (**Chapters 2, 4 and 5**). Furthermore, during the nestling period effects also became apparent in the evening (**Chapter 3**). It will thus be of interest to examine to what extent these effects of ALAN may be mitigated when exposure to light is limited in time with part-night lighting. With part-night lighting lights are for example turned off between 00:00 and 06:00 (Davies and Smyth 2018) and the lights are still on during the evening and morning. While behavioural responses are potentially similar as with continuous light, the physiological response during development (**Chapters 8-10**) may be weakened. Thus, the effectiveness of mitigating strategies will need to be examined for behavioural and physiological effects of ALAN (Gaston et al. 2012) and preferably during different seasons. Effectiveness of lighting strategies in reducing biological effects may also depend on the species/taxon. For example, foraging of bats often peaks just after sunset and current part-night lighting schemes are not very effective in mitigating effects (Azam et al. 2015; Day et al. 2015). However, while ALAN, such as the beams of the National September 11 Memorial & Museum's "Tribute in Light" in New York, may attract migrating birds, turning these lights off for a short period allowed birds to continue onwards (Van Doren et al. 2017).

Street lighting is a major source of ALAN and the recent shift towards more energy efficient broad spectrum light sources, such as LED, offers lighting managers greater flexibility when it comes to tailoring the spectral power distribution (light colour), timing and intensity of municipal lighting systems (Gaston 2013). The experimental light system that we used for most experiments with ALAN inside the nest box can be adapted to examine these different adaptations of light systems and their effects on free-living animals. In specific cases using a different light colour to reduce ecological impacts, such as red lights for bats (Spoelstra et al. 2017) may be worthwhile and are being used (DutchNews.nl 2018). However, because of perceived and realized benefits of ALAN for society (Gaston et al. 2015a) and because the responses of different species are evolutionarily adapted to utilize different wavelengths, a unified approach is unlikely. A different strategy that reduces energy costs and carbon emissions, and which may reduce effects on the environment is part-night lighting (see above), switching off lighting sources from late at night until the early hours of the morning (Gaston et

al. 2012). Part-night lighting will potentially be more broadly adopted by human communities (Davies and Smyth 2018) as different light spectra might meet with public resistance (DutchNews.nl 2018). The need to cut public expenditure and reduce carbon emissions is associated with part-night lighting being adopted across Europe (Bennie et al. 2014a). However, the effectiveness of part-night lighting in mitigating the effects of ALAN is largely unknown and urgently needs to be examined (Azam et al. 2015; Davies and Smyth 2018; Gaston 2013; 2017). Another mitigating strategy is the use of low light intensities as this may reduce biological effects of ALAN on free-living animals. However, laboratory studies have shown that low light intensities can still affect and disrupt avian physiology and behaviour (see Table 1 in **Chapter 9)**. An intensity of 0.3 lux advanced reproductive physiology in blackbirds (Dominoni et al. 2013a), while 0.05 lux increased nightly activity in great tits (de Jong et al. 2016). As explained above ("*Lab versus field"*), due to the potential discrepancy between field and laboratory studies, there is an urgent need to examine the effect of low light intensities on free-living animals in the field (Gaston et al. 2017).

Policy implications

Our short-term treatment using white LED lights already produced significant behavioural and physiological changes. We used a relatively low light intensity to which animals in urban areas can be exposed (see "*Variation in light exposure*"). Ambient light pollution may lead to long-term exposure to ALAN which makes it probable that animals in urban areas suffer from sleep disruption as well as the physiological consequences that we found. Therefore, policy makers should consider the implications that the use of broad spectrum light (e.g. white LED) can have on the environment especially during the breeding season (**Chapter 3**). In this case, the precautionary principle should be applied in lighting policy and landscape planning. However, LED lights do offer great flexibility in terms of duration and intensity (see "*Mitigating measures*"). Therefore, the use of artificial light during the night can be restricted to when it is needed and to the lowest intensity which is required. Combined with shielding of light fixtures this would reduce unnecessary spilling of ALAN and therefore reduce unwanted alteration of the environment.

Concluding remark

While much remains to be examined, we found that short-term exposure to ALAN had severe behavioural and physiological consequences in great tits. Long-term sleep disruption by ALAN is likely to be detrimental for an individual's health. Furthermore, the physiological effects during development may negatively affect short- and long-term survival. Artificial light during the night has many benefits for humans by extending the period of day that is usable for activity (Gaston et al. 2015a), although ALAN is also linked to increased risk of developing cancer (see e.g. the review by Reiter et al. 2007) and unnecessary spillage is wasted energy with many unintended biological consequences (Gaston et al. 2017). Taken together, given that light pollution is steadily increasing even with the use of energy efficient LED lights (Kyba et al. 2017a), it is now vitally important to experimentally examine in free-living animals how long-term exposure to ALAN impacts behaviour and physiology and ultimately reproduction and survival. Furthermore, whether part-night lighting and low light intensities effectively mitigate these effects, urgently requires investigation (Davies and Smyth 2018).

Exit light

Enter night

James Hetfield

Metallica - Enter Sandman

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Alt har svartnet

Alt lys er svunnet hen

Translation

All has blackened

All light has faded away

Sven Atle Kopperud

Dimmu Borgir – Alt Lys Er Svunnet Hen


HR
2017