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TITLE:

An Experimental Approach to Investigating Effects of Artificial Light at Night on Free-Ranging Animals: Implementation, Results, and Directions for Future Research

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SUMMARY:

Artificial light at night (ALAN) has wide-reaching biological effects. This article describes a system for manipulating ALAN inside nest boxes while monitoring behavior, consisting of LED lights coupled to a battery, timer, and audio-capable infrared video camera. Researchers could employ this system to explore many outstanding questions regarding the effects of ALAN on organisms.

ABSTRACT:

Animals have evolved with natural patterns of light and darkness. However, artificial light is being increasingly introduced into the environment from human infrastructure and recreational activity. Artificial light at night (ALAN) has the potential to have widespread effects on animal behavior, physiology, and fitness, which can translate into broader-scale effects on populations and communities. Understanding the effects of ALAN on free-ranging animals is non-trivial due to challenges such as measuring levels of light encountered by mobile organisms and separating the effects of ALAN from those of other anthropogenic disturbance factors. Here we describe an approach that allows us to isolate the effects of artificial light exposure on individual animals by experimentally manipulating light levels inside nest boxes. To this end, a system can be used consisting of light-emitting diode (LED) light(s) adhered to a plate and connected to a battery and timer system. The setup allows exposure of individuals inside nest boxes to varying intensities and durations of ALAN while simultaneously obtaining video recordings, which also include audio. The system has been used in studies on free-ranging great tits (*Parus major*) and blue tits (*Cyanistes caeruleus*) to gain insight into how ALAN affects sleep and activity patterns in adults and physiology and telomere dynamics in developing nestlings. The system, or an adaptation

thereof, could be used to answer many other intriguing research questions, such as how ALAN interacts with other disturbance factors and affects bioenergetic balance. Furthermore, similar systems could be installed in or near the nest boxes, nests or burrows of a variety of species to manipulate levels of ALAN, evaluate biological responses, and work towards building an interspecific perspective. Especially when combined with other advanced approaches for monitoring the behavior and movement of free-living animals, this approach promises to yield ongoing contributions to our understanding of the biological implications of ALAN.

INTRODUCTION:

Animals have evolved with the natural patterns of light and darkness that define day and night. Thus, circadian rhythms in hormonal systems orchestrate rest and activity patterns and allow animals to maximize fitness[1–3]. For instance, the circadian rhythm in glucocorticoid hormones, with a peak at the onset of daily activity, primes vertebrates to behave appropriately across the 24-h period via effects on glucose metabolism and responsiveness to environmental stressors[4]. Similarly, the pineal hormone melatonin, which is released in response to darkness, is integrally involved in governing patterns of circadian rhythmicity and also has antioxidant properties[5,6]. Entrainment of many aspects of circadian rhythmicity, such as melatonin release, is affected by the photoreception of levels of light in the environment. Thus, the introduction of artificial light into the environment to support human activity, recreation, and infrastructure has the potential to have wide-reaching effects on the behavior, physiology and fitness of free-ranging animals[7,8]. Indeed, diverse effects of exposure to artificial light at night (ALAN) have been documented[9,10], and ALAN has been highlighted as a priority for global change research in the 21st century[10].

Measuring the effects of ALAN on free-ranging animals poses non-trivial challenges for a number of reasons. First, mobile animals moving through the environment constantly experience different levels of light. Thus, how does one quantify the level of light that individual animals are exposed to? Even if levels of light on the territory of the animal can be quantified, the animal may employ avoidance strategies that affect patterns of exposure, thus demanding simultaneous tracking of animal location and light levels. Indeed, in most field studies, the mean and variation in light exposure levels are unknown[11]. Second, exposure to ALAN is often correlated with exposure to other anthropogenic disturbance factors, such as noise pollution, chemical exposure, and habitat degradation. For instance, animals occupying habitats along the margins of roadways will be exposed to light from street lamps, noise from vehicular traffic, and air pollution from vehicular emissions. How then does one effectively isolate the effects of ALAN from the effects of confounding variables? Rigorous field experiments that enable good measurements of both light exposure levels and response variables are essential to evaluating the severity of the biological effects of ALAN, and to developing effective mitigation strategies[11].

This article describes an experimental approach that, although not without its limitations (see discussion section), helps assuage, if not eliminate the difficulties identified above. The approach entails experimentally manipulating ALAN levels inside the nest boxes of a free-living, diurnal bird species, the great tit (*Parus major*), using a system of light-emitting diode (LED) lights and an infrared (IR) camera installed within nest boxes. The setup enables simultaneous acquisition of video recordings, including audio, which allows researchers to assess effects on behaviors and vocalizations. Great tits utilize nest boxes for breeding, and sleep in the nest boxes between November and March. Females also sleep inside the nest boxes during the breeding season[12]. The system has also been used to a lesser extent to study effects of ALAN on blue tits (*Cyanistes caeruleus*). The first difficulty, involving knowing levels of light level encountered by the animal, is mitigated in that, given that an individual is willing to enter the nest box (or is already in the nest box in the case of immobile nestlings), light levels can be precisely determined by the researcher. The second difficulty, involving correlations to confounding variables, can be controlled by using nest boxes in similar environments, and/or measuring the levels of confounding variables near nest boxes. In addition, in cavity-nesting birds adopting an experimental approach is powerful because nest boxes or natural cavities can shield nestlings and adults from ALAN[13], which may explain why some correlative studies find little effect of ALAN (or anthropogenic noise)[14], whereas experimental studies more often find clear effects (see below). Moreover, a repeated measures experimental design can be adopted in which individuals serve as their own control, which further increases statistical power, and the probability of detecting meaningful biological effects. The sections below: (1) explain the details of the design and implementation of the system, (2) summarize the important results that have been thus far derived using the system, and (3) propose future research directions that could be pursued, both in tits and other animals.

PROTOCOL:

All applications of this system to animal experiments were approved by the University of Antwerp's ethical committee and conducted in accordance with Belgian and Flemish laws. Methodology adhered to the ASAB/ABS guidelines for the use of animals in behavioral research. The Belgian Royal Institute for Natural Sciences (Koninklijk Belgisch Instituut voor Natuurwetenschappen; KBIN) provided licenses for all researchers and personnel.

1. Creating the experimental system

1.1 Obtain broad-spectrum LED(s) to use in creating ALAN. Take LED light(s) from a LED headlight. Use either a single LED light or multiple (e.g., 4) broad-spectrum LED lights for more diffuse lighting (**Figure 1**).

NOTE: As a modification, LEDs with different spectral properties (e.g., red versus blue) could be used but would have to be obtained from a different source (see the Supplementary material of Grunst et al. 2019[15] for the spectral properties of the LEDs used in past studies using this system).

1.2 Design a system to mount the LEDs along with an IR camera to allow for behavioral monitoring. Researchers can accomplish this end in a number of ways.

1.2.1 Option 1. Insert a single broad-spectrum LED into the nest box separately in a plastic tube adjacent to an IR camera mounted with adhesive on a plastic or metal plate that fits within the nest box (**Figure 1A**,**B**).

1.2.2 Option 2. Mount an IR camera in a central position on a plastic or metal plate and then mount LED lights in fixed positions on the plate surrounding the IR camera (Fig. 1C).

1.3 Design a means to connect the system to a power source (battery) and timer.

1.3.1 Use a knife or drill to make groves in the side of the top of the nest through which wire connectors can extend to connect the system to a Fe-battery (12 V; 120 Wh) and homemade timer (12 V).

1.3.2 Design a dark green wooden enclosure that matches the nest box in coloration, length, and width (e.g., nest boxes used in past studies had the dimensions: 120 mm x 155 mm x 250 mm), and with one side opening via a hinge to house the battery, recorder for the video, and timer system for the LEDs (**Figure 2**; **Supplementary Figure 1** and **Supplementary Figure 2**).

1.4 Design a means through which to adjust ALAN intensity.

1.4.1 Obtain a resistor (value contingent on battery voltage and illumination) and connect it in series with the LED(s).

1.5 Design “dummy” boxes with the same dimensions as the enclosures that house the timer and battery for use in habituating birds to the system (i.e., as in **Figure 2A**, but without the internal electronics).

[Insert **Figure 1** and **Figure 2** here]

NOTE: Section 2 and section 3 discuss the step-by-step methods used to study the effects of ALAN on the focal organism

2. Planning the experiment and adjusting ALAN intensity and timing

2.1 Determine the desired light intensity to which to expose animals.

2.1.1 Carefully consider which experimental light intensity to use so as to produce meaningful results that answer the research question. In general, this will mean selecting an ecologically relevant light intensity, which free-ranging animals are likely to encounter (see **Table 1** for guidance).

2.2 Adjust the LED lights to the desired light intensity (e.g., 1-3 lux, as used in past studies; **Table 1** and **Table 2**).

2.2.1 Prior to placement in the field, place the system on a nest box taken into the laboratory to calibrate the light intensity. Connect the LEDs to the power source, as described further below (Protocol section 3).

2.2.2 Adjust the light emitted by the LEDs to the desired intensity (lux) by placing a light meter at the level of the bird within the nest box (~8 cm from the bottom) and simultaneously adjusting the resistor in series with the LEDs.

NOTE: it is possible to achieve very low light intensities (e.g., rural sky glow levels; 0.01 lux).

2.3 Determine the timeframe over which to expose animals to ALAN.

2.3.1 Determine the length and timing of exposure across the night. For instance, one can expose animals to ALAN across the entire night, for only part of the night, or leave a period of darkness in the middle of the night to reduce the degree of perturbation.

2.3.2 In cases that an animal must enter the nest box (or a specific area) to be exposed to the ALAN, also consider whether the light should be turned on before or after the entry event is likely to occur.

2.4 Set the timer to control the period of light exposure during the night.

2.4.1 Set the timer connected to the broad spectrum LEDs so that the light turns on and off at specified periods (e.g., on at least 2 h before sunset; off 2 h after sunrise).

NOTE: The IR camera allows the behavior of the animal to be recorded simultaneously for the duration of the light exposure and will be on as long as it is connected to a charged battery.

2.5 Determine the appropriate experimental design to use for the target research question(s).

NOTE: For some questions, a repeated measures experimental design will be the most powerful option (e.g., How does exposure to ALAN affect sleep behavior?). For others, paired control and experimental groups will be needed (e.g., How does exposure to ALAN affect telomere loss in developing nestlings?).

[Insert **Table 1** here]

3. Implementing the exposure to ALAN

3.1 Habituate the animals to the experimental setup.

3.1.1 If possible within the context of the experiment, habituate animals to the setup by placing dummy boxes on the top of the nest boxes at least 1 day prior to the experiment to minimize the effects of novelty aversion.

3.2 Survey the focal individuals.

3.2.1 Fit animals in the study population with a passive integrative transponder (PIT) tags to allow for identification within nest boxes without disturbing the birds.

3.2.2 In experiments involving the effect of ALAN on sleep behavior, visit the nest boxes on the night before the experiment and scan the boxes with a radio-frequency identification (RFID) reader to determine which birds are roosting inside.

3.2.3 In experiments during the breeding season involving exposure of developing nestlings to ALAN, consistently monitor (e.g., every other day) nest boxes, and check for nest contents and adult identity. Carefully select nest boxes containing broods with certain characteristics (i.e., modal brood size, both parents present, and feeding) for use in the experiment.

3.3 Select and implement the experiment.

3.3.1 For experiments involving sleep behavior, implement a repeated measures design by first recording individuals sleeping under conditions of darkness for at least one night to record undisturbed sleep in the absence of ALAN (control treatment) following steps 3.3.2–3.3.21.

3.3.2 To this end, make sure to synchronize the time on the IR cameras with the local time prior to taking them into the field.

3.3.3 Insert an SD card into the SD slot into the mini DVR recorder adjacent to the battery (**Figure 2B**; **Supplementary Figure 2**). Check to make sure that the SD card is empty, and if not, erase the data it contains.

3.3.4. At least 2 h prior to the onset of darkness, remove the dummy box from on top of the nest box.

3.3.5 Open the nest box lid.

3.3.6 Place the plate containing the IR camera inside the nest box with the camera objective oriented downward.

3.3.7 Extend the electronic connectors out of the grove in the nest box.

3.3.8 Close the nest box lid.

3.3.9 Place the enclosure containing the battery, recorder, and timer on top of the nest box.

3.3.10 Connect the battery power connectors. Connect the red connector from the recorder to the white connector from the camera (audio), the yellow connector from the recorder to the yellow connector from the camera (video), and the black connector from the battery to the red connector from the camera (power) (**Supplementary Figure 1** and **Supplementary Figure 2**).

3.3.11 Push the record button to initiate the camera recording.

NOTE: The timer will not be set and/or the power will not be connected to the timer controlling the LEDs so that no ALAN will be produced on control nights.

3.3.12 Check with a small tft screen to ensure the recording has started and that the image is correct. A port to connect the tft screen is located below the recorder (**Supplementary Figure 2**).

3.3.13 Approximately 1 h after dark, return to the nest box and check the identity of the bird sleeping inside by moving a RFID transponder reader around the bottom and sides of the nest box and recording the unique identification number communicated from the PIT tag.

3.3.14 On the morning following the control recording, at least 2 h after sunrise, return to the nest box and collect the battery system and IR camera.

3.3.15 Again, place a dummy box on top of the nest box.

3.3.16 In the laboratory or office, charge the battery and remove and download the SD card from the recorder to collect the behavioral data.

NOTE: Batteries have a life span of ~30 h in cold conditions to enable recording for the entire night but need to be fully recharged between consecutive nights of recording.

3.3.17 After successfully downloading the data, erase the data from the SD card and then reinsert it into the mini DVR recorder.

3.3.18 On the subsequent night, implement the light exposure treatment (e.g., 1–3 lux, as used in past experiments using the system; **Table 1** and **Table 2**).

3.3.19 Set the timer system for the desired time period of light exposure.

3.3.20 Follow the same steps (3.1.2–3.3.17) described above for the control recording, but also connect the timer to the power and the LEDs to the timer (**Supplementary Figure 1** and **Supplementary Figure 2**).

3.3.21 If desired, repeat the control recording (of sleep behavior under conditions of darkness, i.e., absence of ALAN) on night three.

3.3.22 For experiments involving exposure of nestlings to ALAN, use control and experimental broods as described in steps 3.3.23–3.3.25.

3.3.23 Place dummy boxes (lacking electronics) on top of the nest boxes of control broods and handle both control and experimental nestlings in equivalent ways.

3.3.24 Implement the experimental ALAN exposure for experimental boxes. During the experimental period, mount the LED system and IR camera within the nest box, as described above, and set the timer to control the desired period of light exposure.

3.3.25 Recharge the batteries. For experiments involving multiple nights of light exposure and video recording, collect the systems each morning to recharge the batteries during the day and then replace the system in the evening.

3.4 Collect data on the response variable(s) of interest.

3.4.1 If behavior within the nest box is the variable of interest, the IR camera will allow simultaneously documenting behavior (e.g., sleep behavior; **Figure 3**).

3.4.2 Collect any other data of interest via additional monitoring methods, with sampling occurring at variable points in time (e.g., blood samples taken before and after light exposure[15]).

[Insert **Figure 3** here]

REPRESENTATIVE RESULTS:

The peer-reviewed research articles published using this system are summarized in **Table 2**. Several other manuscripts are in progress. These studies address three major suites of research questions. First, the system has been used to study the effects of light exposure on sleep behavior and activity levels in adults. To this end, a repeated measures experimental design was employed, in which the same individual was first recorded sleeping under natural conditions and subsequently recorded sleeping in a lighted nest box. All individuals used in these studies were fitted with PIT tags, allowing researchers to verify that the same individual is sleeping in the nest box between subsequent nights using a handheld transponder-reader without disturbing the birds.

Dramatic effects of ALAN exposure on sleep behavior have been documented. For example, great tits exposed to ALAN at an intensity of 1.6 lux, a relatively low intensity that is likely to be experienced by free-ranging animals, woke up half an hour earlier, left the nest box 20 min earlier, and slept 40 min less than the control birds (**Figure 4**)[16]. Interestingly, the effects of ALAN on sleep may be contingent on other variables, such as light intensity and season. In line with this hypothesis, the effects of ALAN on the sleep behavior of female great tits was much greater during the nestling period than during winter, with the effect on sleep loss being more than two times as large and the effect on awaking time being more than four times as large[17]. On the other hand, there was little difference in the effect of light exposure at an intensity of 1.6 versus 3 lux, suggesting that even low-intensity ALAN may have deleterious effects[12]. The system has also been used to document a sleep rebound following sleep disruption by ALAN, wherein individuals responded to ALAN-induced sleep deprivation by sleeping more the following night[17]. Furthermore, significant individual variation in the extent to which sleep is disrupted by ALAN has been observed, which may be important to predicting population responses and the scope for selection[17], although the effect of ALAN on sleep was not modified by exploratory personality type[18]. Substantial effects of ALAN on sleep are likely to have cascading effects on waking behavior, physiology, and fitness. However, studies to date have been relatively short in duration. Examining broader ramifications and longer-term effects is critical to elucidating the repercussions of ALAN for free-ranging animals and is an important area for further research (see below).

[Insert **Figure 4** here]

Second, the system has been used to examine how exposure to ALAN affects developing nestlings, using a range of physiological response variables (**Table 2**). These experiments have exposed nestlings to ALAN during a portion of the nestling stage, ranging from 2–7 days, contingent on the aims of the study. Documented effects of light exposure on nestlings include effects on body mass or condition[19], feather corticosterone levels[20], haptoglobin concentrations[21], and oxalate levels[22]. However, this research also suggests that some parameters, such as telomere degradation rate and oxidative stress[15,19], may be unaffected by exposure to ALAN (**Table 2**). In sum, these studies suggest that exposure to ALAN early in life may alter the course of development and potentially have enduring effects in adulthood, but more research is needed to determine the extent to which the characteristics of developing organisms are sensitive or resilient to light exposure.

Third, the system has been used to assess effects on fitness, including reproductive success and survivorship rates. As of yet, no strong evidence for such effects has emerged. However, and importantly, this work is still very much in progress since effectively assessing fitness effects demands longer-term monitoring of light-exposed individuals.

Lastly, work has been done comparing the effects of exposure to ALAN on the sleep behavior of great tits and blue tits. ALAN had much lesser effects on the sleep behavior of blue tits when compared to great tits, which draws attention to the potential for interspecific differences in light sensitivity, even between closely related species (**Table 2**)[23]. Notably, other research groups have also recently begun to adopt this approach of manipulating light levels within nest boxes, illustrating the strength of the methodology, and the potential for its wider application[24,25].

[Insert **Table 2** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Two systems consisting of IR cameras and LED light(s) used to manipulate ALAN inside nest boxes. (**A**) Top view of the nest box with plate holding the older system in place. (**B**) Older system with 1 broad-spectrum LED to manipulate ALAN and central camera with 10 IR LEDs (c) Newer system with 4 broad-spectrum LEDs and central IR camera with 4 IR LEDs.

Figure 2: The homemade battery and timer unit used to manipulate ALAN and video-record behavior. (**A**) The unit is enclosed within a wooden box that is mounted on top of the nest box. (**B**) View of the electronics inside the unit. Connectors extend from inside the nest box up into the wooden enclosure to connect the electronics to the IR camera and broad-spectrum LEDs.

Figure 3: Infrared image of a great tit inside a nest box exposed to ALAN. (**A**) Sleeping and (**B**) Alert great tit

Figure 4: Effect sizes and 95% confidence intervals comparing sleep behaviors of great tits on a first undisturbed night and on a second night. On the second night, birds were either again left undisturbed (control; top panel) or were exposed to 1.6 lux ALAN (light; bottom panel). Effect sizes are given in minutes, with the exception of 'time on the entrance', which is given in seconds. See details in Raap et al. (2015)[16]. This figure has been adapted with permission from Raap et al.[16].

Table 1: Characteristic light intensities in the environment[3,9], exposure levels of free-ranging birds[41], and intensities used in past studies using this system (references in Table 2).

Table 2: Summary of studies published based on exposure to ALAN using the experimental system. Note: 0 = no effect of ALAN on the response variable.

Supplementary Figure 1: The plate containing the infrared (IR) camera and light-emitting diode (LED) lights, additionally showing the cables that connect the system to the power source.

Supplementary Figure 2: An internal view of the chamber containing the battery, recorder, and homemade time system, additionally showing cables connecting various parts of the system.

DISCUSSION:

This nest box-based system of LED lights and a paired IR camera has allowed researchers to assess a range of intriguing questions regarding the biological effects of ALAN. Moreover, there are many more research directions that can be pursued with the system. In addition, expanding the use of the system to other species could help foster an understanding of interspecific differences in sensitivity to ALAN. Below some non-exhaustive possibilities for future research are presented in the hope that this paper will help motivate research in this important field. The conclusion briefly reiterates the strengths of this experimental approach and addresses the limitations of the system.

This system could be employed to answer many outstanding questions regarding how ALAN affects free-ranging animals or animals in semi-captivity. First, studies to date have involved relatively short periods of ALAN exposure and short-term monitoring of biological effects. Consequently, little is known regarding longer-term effects of short-term ALAN exposure, or what would happen if birds were exposed to ALAN for many days, many weeks, or for their entire lifespan (see[26] for a recent paper demonstrating the importance of long-term exposure to ALAN in crickets, *Gryllus bimaculatus*). For instance, does short-term ALAN exposure have long-term effects on health status and biological aging rates? Does long-term ALAN exposure result in physiological stress and accelerated senescence, and are effects similar or distinct from those of short-term ALAN exposure? This system could be used to tackle these questions. Indeed, many great tits and blue tits (and also other species) use the same nest box across their entire lifespans. Second, there is a need to examine interactive effects of ALAN with other anthropogenic disturbance factors (e.g., adopting a multi-stressor perspective as in[27]), and differential effects of ALAN with different properties. This system could be used in combination with other experimental manipulations or in combination with natural variation in anthropogenic disturbance levels to investigate how different anthropogenic disturbance factors (e.g., light, noise, chemical pollution) might interact to affect a range of response variables. For example, nestlings could be simultaneously exposed to ALAN and anthropogenic noise to test whether these two disturbance factors have additive or synergistic effects on corticosterone levels or telomere shortening. The system could also be modified to examine the effects of ALAN with different properties by adjusting the characteristics of the LEDs used. For instance, it would be interesting to employ the system to investigate how ALAN of different wavelengths (e.g., red versus blue wavelengths) affect sleep behavior or nestling development. It has been hypothesized and experimentally supported that different wavelengths of light may induce biological responses that differ in intensity[28,29]. For instance, in a recent study, white versus green light differentially affected the incubation behavior of great tits[29].

Third, this system could be used to explore the effects of ALAN on response variables that have been underexplored to date, including bioenergetics, cognitive processes, social dynamics, and parental care (but see[30] for effects on bioenergetics). To study effects on bioenergetics, ALAN exposure could be combined with respirometry to measure resting or basal metabolic rate (RMR, BMR)[31], the doubly labeled water approach to measuring field metabolic rate (FMR; also known as daily energy expenditure)[30,32], or accelerometry to measure activity patterns and energy expenditure[33]. Effects of ALAN on bioenergetics may have non-trivial effects on fitness, given that metabolic rate and energy expenditure have been proposed to underlie life histories variation and pace of life[34]. To explore the effects of ALAN on cognitive traits, researchers could either utilize field-based cognitive tests following ALAN exposure or capture adults after exposure and conduct cognitive tests in the laboratory. The system is designed to allow research on free-ranging birds, and removing birds to captivity introduces its own complications. Thus, cognitive testing on wild birds is particularly appealing, although also challenging. For instance, recent work examined problem-solving ability at nest boxes using a modified nest box trap[35]. Brooding females exposed to ALAN could be presented with this cognitive test. Another possibility would be using “smart feeders” designed to assess spatial memory or associative learning to explore whether exposure of sleeping adults to ALAN affects these cognitive traits[36]. Finally, to examine the effects of ALAN on social interactions and parental care, researchers could pair the LED system with other technology, some of which have already been commonly employed in studies using the setup. For instance, PIT tag systems at nest boxes allow entries and exits of adult birds fitted with PIT tags to be recorded[37]. Therefore, during the breeding period, researchers could explore whether exposure of brooding females and nestlings to ALAN modifies nestling provisioning rates or affects the balance in parental effort between the sexes. In addition, various radio-telemetry platforms have been miniaturized, facilitating use in small animals, and could be used to assess whether exposure of sleeping adults to ALAN modifies interactions with conspecifics[38].

A system similar to the one described here could be used to study the effects of ALAN on any avian species that use nest boxes for breeding. This includes several well-studied passerines, such as tree swallows (*Tachycineta bicolor*), western and eastern bluebirds (*Sialia mexicana* and *Sialia sialis*), chickadees (Poecile sp.), house wrens (*Troglodytes aedon*), European pied (*Ficedula hypoleuca*), collared (*Ficedula albicollis*) flycatchers, and house sparrows (*Passer domesticus*). European starlings (*Sturnus vulgaris*) are also an especially suitable species since they can be studied in captivity and the wild and are large enough to study sleep behavior using electroencephalographic techniques[39]. Raptors, such as barn owls (*Tyto alba*) and American kestrels (*Falco sparverius*), also utilize nest boxes and could serve as study subjects. Effects of ALAN on nestling development could readily be assessed in these species. The extent to which effects of ALAN on sleep behavior could be investigated depends on whether adults sleep inside nest boxes during the breeding or non-breeding season, but there is likely substantial scope for investing effects of ALAN on sleep in females during the incubation stage.

The system could also be adapted for use in species other than nest box nesting birds. Besides birds, a number of mammal species also nest or sleep in nest boxes. Thus, the system could be adopted to study the effects of ALAN on these species. For example, several lemur species will occupy boxes, and artificial nest boxes are already being employed to study their breeding behavior[40]. In addition, although challenging, the system has the potential to be adopted by innovative scientists to study the effects of ALAN on open-cup nesting birds and avian or mammal species that nest or sleep in crevasses or burrows. For open-cup nesting birds, this would involve creating a means via which LED lights, and IR cameras could be mounted above the nest. Given the need to secure the LED system and camera above nests, such a system would probably most easily be implemented for species that nest on or near to the ground. For burrow or crevasse nesting species, the researcher would need to fit the LED system and camera inside the cavities. For instance, for some species that nest in rocky crevasses, it could be possible to remove rock to create space in which to secure the light system and camera.

As discussed above, the primary strength of this methodology for manipulating ALAN levels inside nest boxes is the ability to expose study subjects to predetermined light levels over specific time frames during the night. The ability to accurately control light exposure levels and durations allows the researcher to overcome many of the limitations inherent to non-experimental studies regarding the biological impacts of ALAN. However, the methodology also has limitations, especially in that animals can be exposed to light only when resting, sleeping, or caring for young inside the nest box. Direct effects of ALAN on behaviors that occur outside the nest box, such as singing and foraging, cannot be explored (although indirect effects of exposure of ALAN inside the nest box on these behaviors could be investigated). To explore such direct effects of ALAN outside of the nest box, researchers will need to employ larger-scale experimental networks of artificial lighting or non-experimental approaches.

In addition, a major criticism of the approach of manipulating light levels inside nest boxes is that nest boxes, or natural cavities, would normally shield individuals from external sources of anthropogenic ALAN. However, it is important to note that not all great tits will have nest boxes, or cavities, available to sleep in, as they are a limited resource. Thus, it is possible, if not likely, that adult birds in urban areas are exposed to the low levels (1–3 lux) of ALAN that have been used in past studies using this system (**Table 2**). Nest boxes in our population are exposed to between 0.01–6.4 lux at the nest box opening[13], suggesting that birds sleeping outside of the nest boxes could be exposed to comparable levels of light than have been used in the manipulations. Indeed, although in a different species, Dominoni et al. 2013[41] used light loggers to measure the levels of ALAN experienced by free-ranging European blackbirds (*Turdus merula*), and found that urban birds experienced significantly higher levels of ALAN than rural birds, although exposure levels were highly variable (0.7–2.2 lux)[41]. Moreover, in an experiment using these low levels of ALAN (0.3 lux), they demonstrated a significant effect of these very low levels of ALAN on the timing of reproduction and molt[41]. On the other hand, de Jong et al. 2016[42] found that male great tits breeding on artificial lit transects within a forest area did not experience higher levels of ALAN than control birds, suggesting avoidance behavior. Nevertheless, they note that such evasion might be more difficult to accomplish in urban areas with pervasive light exposure[42]. Thus, if experiments are properly designed using ecologically relevant levels of ALAN, the approach of manipulating light levels inside nest boxes has the potential to yield ecologically relevant results. Preferably this will involve first measuring ALAN exposure of free-ranging birds in the target study population(s) or an urban population of the same species.

With respect to the relevancy of exposing nestlings to ALAN inside nest boxes, it is true that the levels of ALAN used are much higher than those that would normally be experienced inside cavities (in the studied population, light levels are ~0.08 lux at the bottom of the nest box during the day, and between 0 and 0.01 lux at night). Rather, cavity-nesting species such as great tits and blue tits serve as convenient model species for effects that may occur for open nesting species, whose nestlings will be more exposed[14,18,20,24]. More research is now urgently needed to document the levels of ALAN experienced by the nestlings of open-cup nesting species. Based on such research, this system has the potential to be adapted to manipulate ALAN levels at open cup nests, as suggested above.

To conclude, the approach of manipulating light levels inside nest boxes has both its strengths and weakness. However, when properly applied, the approach makes a solid contribution to the diverse body of experimental and correlational approaches that are needed to build a coherent understanding of the biological effects of ALAN.

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DISCLOSURES:

The authors declare that they have no conflicts of interest.

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Figure 1

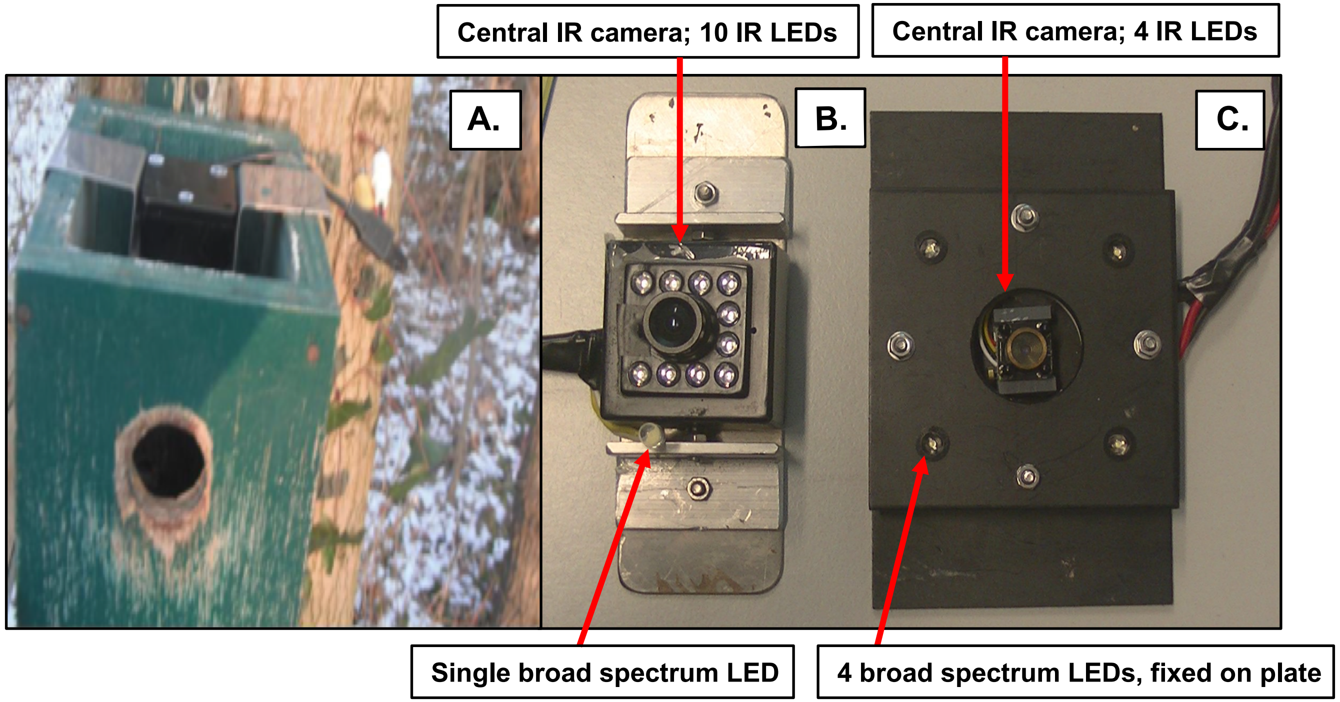
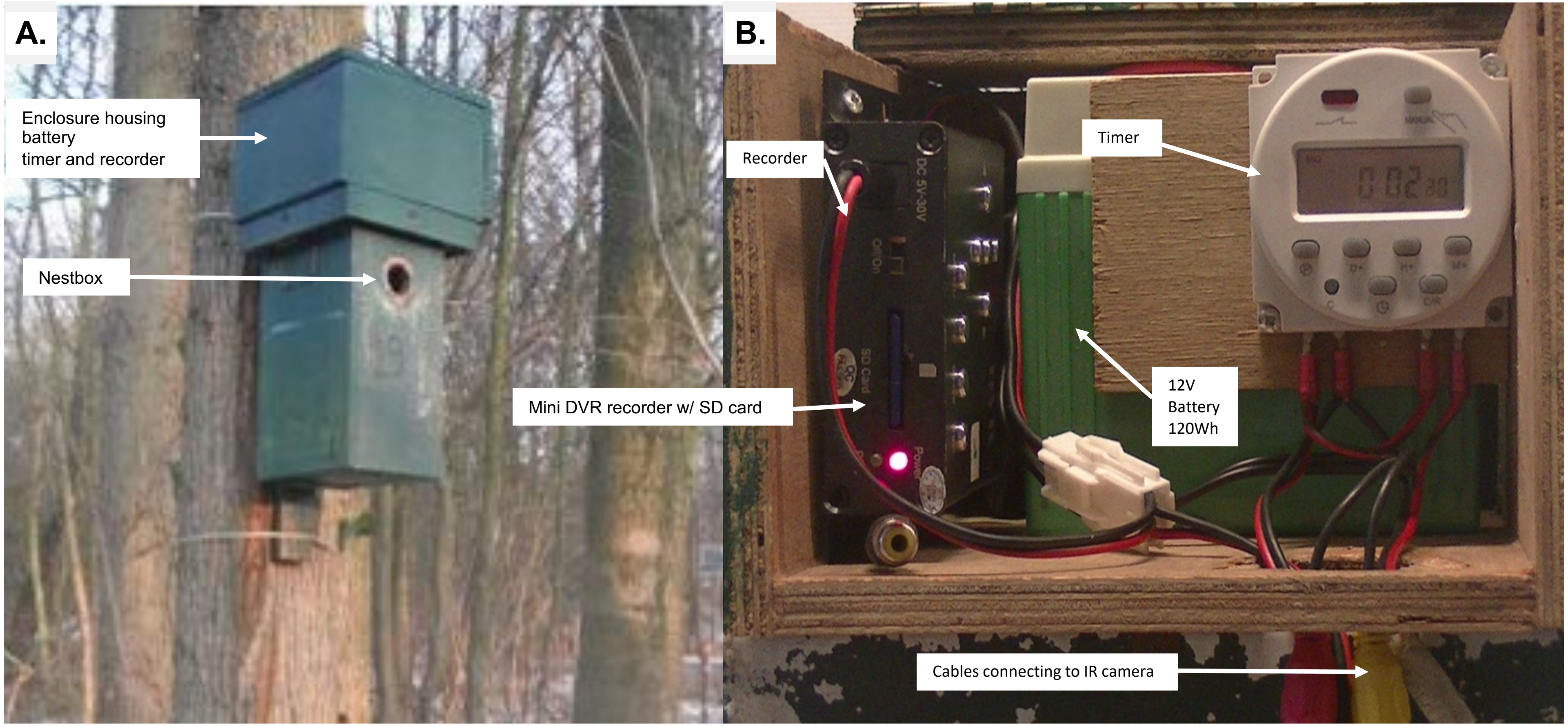
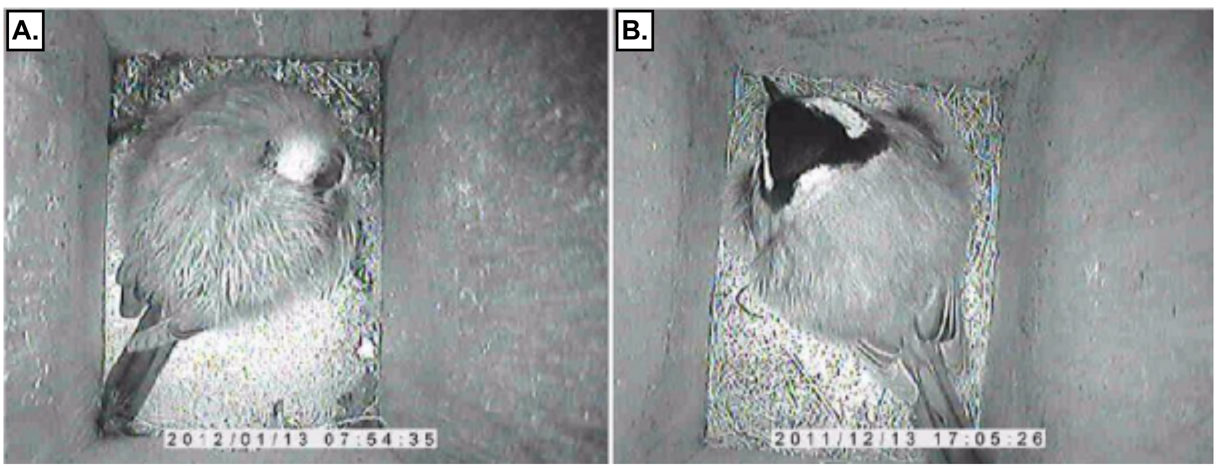

Central IR camera; 10 IR LEDs
Central IR camera; 4 IR LEDs
A.
B.
C.
Single broad spectrum LED
4 broad spectrum LEDs, fixed on plate

Figure 2


A.
Enclosure housing
battery
timer and recorder
Nestbox
B.
Recorder
Timer
Mini DVR recorder w/ SD card
12V
Battery
120Wh
Cables connecting to IR camera


A.
2012/01/13 07:54:35
B.
2011/12/13 17:05:26

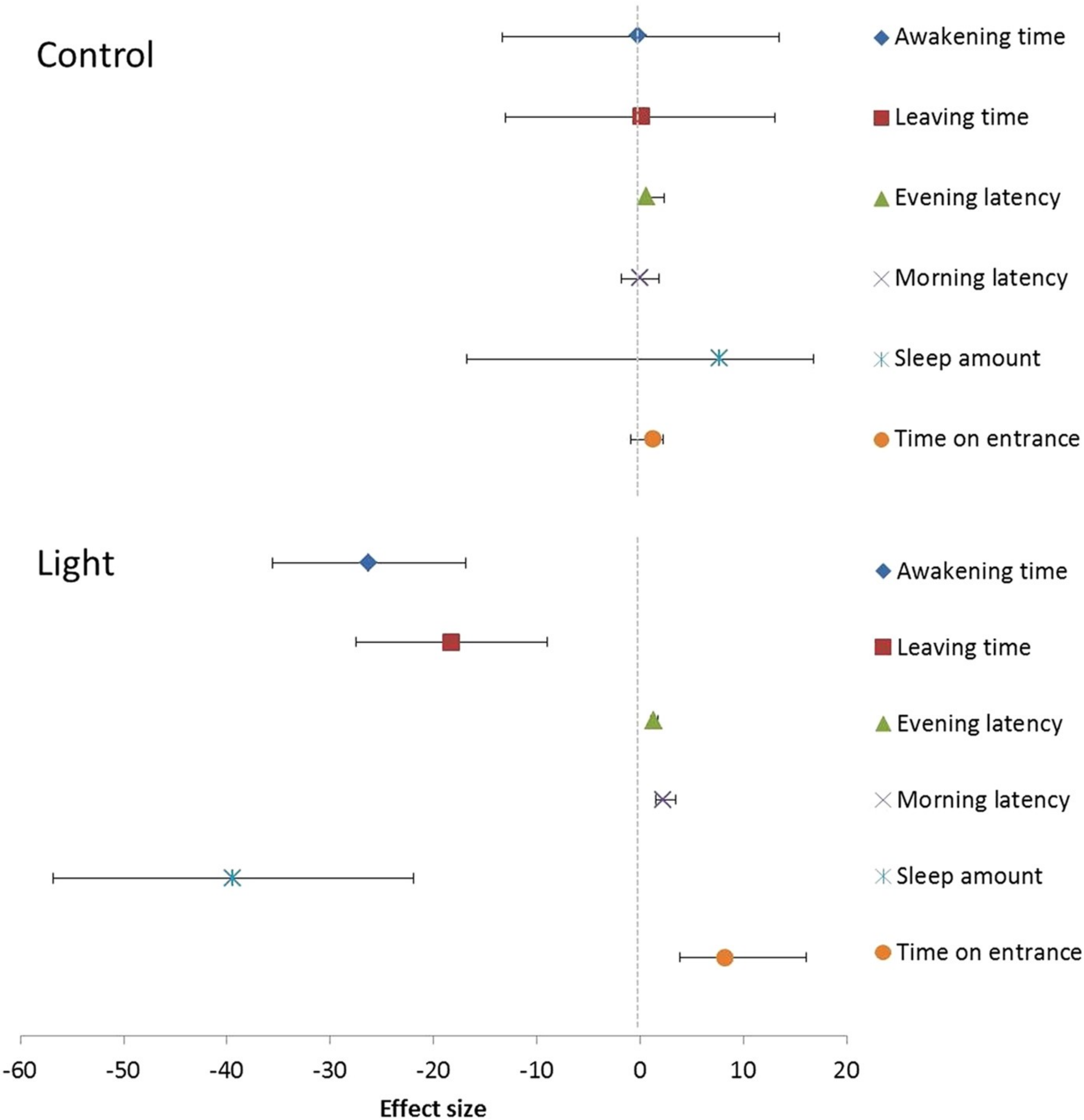

Control
Awakening time
Leaving time
Evening latency
Morning latency
Sleep amount
Time on entrance
Light
Awakening time
Leaving time
Evening latency
Morning latency
Sleep amount
Time on entrance
-60
-50
-40
-30
-20
-10
0
10
20
Effect size

Table 1

Source/exposure level	Intensity (lux)
Full sunlight	103000
Full moonlight	0.05–1
Urban Sky glow	0.2–0.5
Exposure of free-living European blackbirds	0.2 (0.07–2.2)
Past experimental studies using the system	1–3
LED street lights	~10
Low pressure sodium street lights	~10
High pressure sodium	~10
Florescent lighting	300
Metal halide	400–2000

Table 2

Species	Life stage	ALAN intensity used (lux)
Great tit (*Parus major*)	Nestling	1
Great tit	Nestling	1
Great tit	Adult	1.6, 3
Great tit	Nestling	3
Great tit	Nestling	1.6, 3
Great tit/blue tit (*Cyanistes caeruleus)*	Adult	3
Great tit	Adult	1.6
Great tit	Nestling	3
Great tit	Nestling	3
Great tit	Adult	1.6

Note: 0 = no effect of ALAN on the response variable. Numbers prc

Response variables	Effect of ALAN
Feather corticosterone (fCORT), body condition, telomere length, fledging success, recruitment	(+) fCORT (-) Body condition (0) Other response variables
Telomere length, body condition, fledging success, nitric oxide	(-) Body condition (0) Telomere length, other response variables
Sleep personality (exploratory behavior)-dependent response?	(-) Sleep behavior Not modified by personality
Oxalate & whether response modified by sex	(+) Oxalate, males (0) Oxalate, females
Sleep behavior & whether response modified by season or light intensity	(-)Sleep behavior Little effect of season Sleep onset delayed only by high intensity ALAN
Sleep behavior	Less (-) effect on sleep in blue tits
Sleep behavior of females	(-) Sleep behavior Sleep rebound after ALAN exposure More (-) effect in nestling period
Change in body mass, blood oxidative status, fledging success	(-) Body mass (0) Oxidative status, fledging success
Haptoglobin (Hp), nitric oxide (NO)	(+)Hp (-) NO
Sleep behavior	(-) Sleep behavior

›ceeding the reference entries refer to the order in the reference list.

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<u>Response to the Editor's Comments and Suggestions (R1)</u>

Ms. Ref. No.:	JoVE63381_R1
Title:	An experimental approach to investigating effects of artificial light at night on free-ranging animals: implementation, results, and future directions
Journal:	JoVE
Authors:	Melissa L. Grunst, Andrea S. Grunst, Rianne Pinxten, Geert Eens, Marcel Eens

Dear Editors,

I am writing to submit a revision of the JoVE article titled, "An experimental approach to investigating effects of artificial light at night on free-ranging animals: implementation, results, and future directions" by Melissa Grunst, Andrea Grunst, Rianne Pinxten, Geert Eens and Marcel Eens. The article has been thoroughly revised in-line with the Editor's suggestions on the first revision. We provide a response to each of the Editor's comments below. However, please note that we are still waiting to receive copyright permission to republish a figure for the representative results. We are resubmitting without this permission since the dead line for resubmission has passed. Again, we would like to thank you for the valuable feedback provided through the peer review process, and we look forward to working with you further to improve the quality of this work

Best regards,
Melissa Grunst (on behalf of all coauthors)

<u>Response to Editorial comments</u>

Editorial comments:

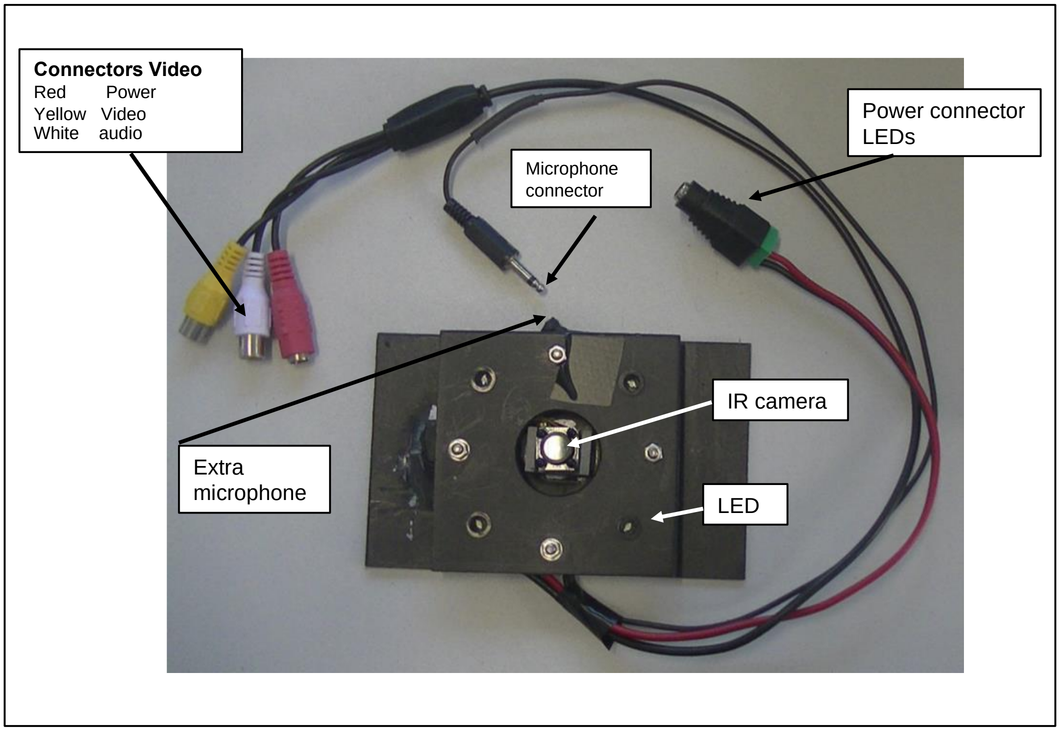
Comment 1. Please note that the manuscript has been formatted to fit the journal standard. Consider retaining the changes. Comments to be addressed are included within the manuscript. Please review and revise accordingly.

Response 1. We have retained the formatting changes that were made. With respect to the comments embedded in the manuscript, we have checked the numbers referenced on L309. We now use the term "electroencephalographic" (L509)

Comment 2. Please include at least one representative result obtained using this protocol in addition to the details of the studies summarized in the tables (Table 1 and Table 2). You can include data from any of your previously published manuscripts and discuss it in the Representative Results section. However, please obtain explicit copyright permission to reuse any figures. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Response 2. We now include a figure (Figure 4) from Raap et al. *Sci Rep* 2015 showing the effects of ALAN on various aspects of sleep behavior. We are currently waiting to receive the appropriate copyright permission.

Supplementary Figure 1.



Supplementary Figure 2

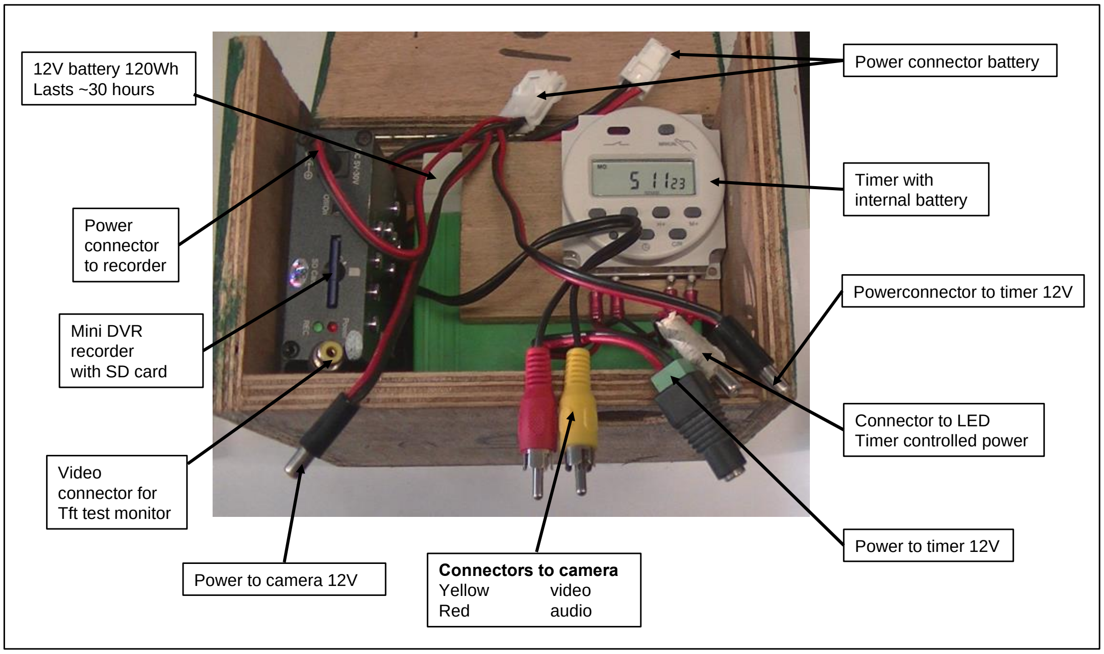


Figure 4

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Light pollution disrupts sleep in free-living animals

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Publication: Scientific Reports

Publisher: Springer Nature

Date: Sep 4, 2015

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