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ORIGINAL ARTICLE



Artificial light at night decreases the pupillary light response of dark-adapted toads to bright light

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Abstract

Artificial light at night (ALAN) is expanding worldwide. Many physiological effects have been reported in animals, but we still know little about the consequences for the visual system. The pupil contributes to control incoming light onto the retina. Sudden increases in light intensity evokes the pupil light reflex (PLR). Intrinsically photosensitive retinal ganglion cells (ipRGC) affect PLR and melatonin expression, which largely regulate circadian rhythms and PLR itself. IpRGC receive inputs from various photoreceptors with different peak sensitivities implying that PLR could be altered by a broad range of light sources. We predicted ALAN to enhance PLR. Contrary to our prediction, dark-adapted cane toads *Rhinella marina*, exposed to ALAN (5 lx) for 12 days, exhibited a lower PLR than controls and individuals exposed to 0.04 lx, even after 1 h in bright light. We cannot conclude whether ALAN induced a larger pupil size in dark-adapted toads or a slower initial contraction. Nevertheless, the response was triggered by a light source with an emission peak (590 nm) well above the sensitivity peak of melanopsin, the main photoreceptor involved in PLR. Therefore, ALAN alters the capacity of toads to regulate the incoming light in the eye at night, which may reduce the performance of visually guided behaviors, and increase mortality by predators or road kills at night. This first study emphasizes the need to focus on the effect of ALAN on the vision of nocturnal organisms to better understand how this sensory system is altered and anticipate the consequences for organisms.

Key words: amphibian, anuran, light pollution, pupillary light reflex, vision

INTRODUCTION

Artificial light at night (ALAN) is increasing worldwide and concerns all ecosystems (Bennie *et al.* 2015b; Kyba *et al.* 2017). This phenomenon generated by urban areas and road networks now raises night brightness across large continental expanses (Falchi *et al.*

2016). In animals, ALAN disturbs many physiological processes such as metabolism, immune system, reproduction, and behaviors such as foraging, orientation, and social interactions, which effects can scale up to the population or the community level (Rich & Longcore 2006; Gaston *et al.* 2015; Bennie *et al.* 2015a; Knop *et al.* 2017; Touzot *et al.* 2020). Studies have highlighted the importance of photoreception on the changes in biological rhythms, behavior, and physiology induced by ALAN (Salmon 2003; Stone *et al.* 2009; Dominoni *et al.* 2013; Brüning *et al.* 2015). Vision is known to be important for orientation toward light sources in insects (Frank 2006), birds (Jones & Francis 2003),

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or amphibians (Hailman & Jaeger 1974), as well as for foraging (Thomas *et al.* 2006) and social interactions in many groups of animals (Kurvers & Hölker 2015). However, we have a limited understanding of how ALAN affects visual performance in exposed organisms, especially at the sensory level. One unaddressed issue is that physiological disruption may cause the inability to accurately control light input onto the retina during the nocturnal period and eventually affect many visually guided behaviors.

Different mechanisms control the light level entering the eye in vertebrates. Eyelids reduce the light input. In fish, amphibians, and birds, retinomotor movements shift photoreceptors away or closer to the retina surface depending on the time of the day (Burnside 2001). In most groups, the eye contains a diaphragm, the iris, which determines the area through which the light enters the eye, the pupil. The iris sphincter contracts or dilates to control the amount of light reaching the retina through the pupil to avoid glare, enhance image perception, and thus optimize the efficiency of visual tasks like foraging (Douglas 2018). The importance of the pupillary light response (PLR) for organisms has been poorly appreciated in ecological studies and the implication of ALAN on this mechanism has never been addressed to our knowledge. Any alteration of nocturnal vision may have negative effects for individuals in terms of orientation, foraging, or the detection of threats.

The PLR is triggered when individuals experience a sudden change in the incoming light level. In the context of ALAN, this situation can be encountered in multiple situations, for instance when individuals move from darker to brighter areas, when crossing an open habitat or when moving through brighter patches caused by trespassing light even under vegetation cover. Intermittent light sources such as cars passing on a nearby road may also generate strong variation of light levels. Visual impairment generated by ALAN may particularly affect nocturnal species that perform visually guided tasks, like orientation or foraging, in very dim light (Dacke *et al.* 2003; Somanathan *et al.* 2008). For instance, the common toad *Bufo bufo* (Larsen & Pedersen 1982; Aho *et al.* 1993) attempts to capture prey at light levels as low as 10^{-4} lx, which is an order of magnitude lower than stellar light (Gaston *et al.* 2013), and much lower than the levels generated by ALAN. However, individuals adapted to dark conditions, experience lower foraging efficiency when exposed to light (Buchanan 1993). Like in other vertebrates, a sudden increase of light intensity induces the PLR in dark-adapted amphibians and the response is dependent on light intensity (Cornell &

Hailman 1984; Henning *et al.* 1991). Time to complete pupil constriction varies from a few seconds to about an hour in amphibians (Cornell & Hailman 1984; Henning *et al.* 1991), but generally the PLR seems relatively slow compared with other vertebrates (Douglas 2018), which suggests, given the small number of studies, that changes in ambient light may affect individuals over a longer time period.

Current knowledge suggests that physiological disruption caused by ALAN may affect the PLR. Intrinsically photosensitive retinal ganglion cells (ipRGC) are luminance detectors transducing information about ambient light from the retina to various parts of the brain involved in the control of circadian rhythms and the pupillary reflex (Tring Hoang Do & Yau 2010). ipRGC receive inputs from melanopsin and cone and rod photoreceptors, melanopsin having the major contribution (Gooley *et al.* 2012; Spitschan 2019), and its depolarization by light at night suppresses melatonin expression (Fonken & Nelson 2014). Melatonin is a neurohormone that synchronizes circadian clocks in the organism (Fonken & Nelson 2014). Its expression is suppressed in fishes, birds, and mammals exposed to ALAN. Data are scarce for amphibians, but available evidence suggests a similar effect in this group (Grubisic *et al.* 2019). Because PLR in humans is influenced by the endogenous circadian clock (Münch *et al.* 2012; Bonmati-Carrion *et al.* 2016), ALAN is expected to affect PLR. Melatonin suppression and PLR are maximal at wavelengths corresponding to the melanopsin absorption peak but longer wavelengths are effective (Brainard *et al.* 2001; Gooley *et al.* 2012) supporting the role of different photoreceptor types and, thus, the effectiveness of light sources with various characteristics individuals encounter in natural conditions.

We investigated the effect of ALAN on PLR in a bufonid anuran, the cane toad *Rhinella marina*. This intertropical species experiences a year-round stable photoperiod and may therefore be particularly sensitive to ALAN. As a general prediction, light cues are expected to be more reliable and thus more important than other types of cues for entraining circadian rhythms at lower than higher latitudes (Secondi *et al.* 2020). In humans, PLR correlates positively with physiological proxies of circadian disruption, stronger PLR being associated with higher motor activity and lighter sleep during the rest period (Bonmati-Carrion *et al.* 2016). We do not have as detailed knowledge on processes in amphibians, but we can suspect similar physiological responses. Thus, we predict stronger iris contraction in individuals exposed to ALAN.

MATERIALS AND METHODS

Housing and experimental exposure to ALAN

We caught 60 adult cane toads at Puerto Viejo de Sarapiquí (Costa Rica) in La Selva Biological station (10°25'57.7"N, 84°00'24.9"W) on 24–26 October 2017. The research station is surrounded by forest, and open habitat is present on the station ground between the buildings. Both habitats experience low levels of ALAN. We used a Sky Quality Meter (precision ± 0.10 mag/arcsec²; Unihedron, Grimsby, Canada) to measure light level at night at both sites. The average light level measured across the research station on 10 nights at 6 locations was 19.83 ± 2.49 SD mag/arcsec² ($n = 53$). It ranged from (mean \pm SD) 17.81 ± 1.82 to 22.28 ± 1.08 depending on the location. On the Bortle scale (Bortle 2006), that is, an ordinal scale based on the assessment of sky quality by a naked eye observer, the location in the open and close to a lit building (17.81) corresponded to Bortle class 9 (equivalent to city sky, i.e. a bright environment) and the location under the canopy 22.28 to class 1 (excellent sky, i.e. a very dark environment) (Secondi *et al.* 2020).

Cane toads were kept outdoors under the canopy of a wooded area in the Biological Station of La Selva. Individuals were housed singly in opaque boxes (length \times width \times height: $64 \times 46 \times 40$ cm) partly topped with a lid. Boxes were enriched with a 4-cm layer of natural soil that was kept permanently wet and a semi-circular section of a PVC tube (5 cm radius, 15 cm long) for shelter, and were covered with a net. Food (crickets and cockroaches) was provided every other day. No group of toads lost weight during the course of the experiment. Individuals were exposed to the natural light conditions during the day. The natural light level at night measured above unlit boxes was 21.43 mag/arcsec² ± 1.30 SD (about 0.001 lx), which corresponds to a dark sky.

For 12 days, we exposed individuals in their box to 1 of the 3 experimental light intensities at night: <0.001 lx (control), 0.04 lx (corresponding to skyglow level), and 5 lx (corresponding to side street lighting, (Gaston *et al.* 2013). Mass is often age dependent in amphibians (Duellman & Trueb 1994). To avoid any bias between groups, toads were randomly assigned to a treatment so that the average mass of individuals did not differ between groups (ANOVA, $P > 0.05$). The light was emitted by yellow LEDs ribbons (2800–3200 K; Sysled, Marseille, France) with a peak wavelength at 590 nm close to the yellow-orange peak of sodium lamps that still generates the dominant color of skyglow across the world (Fig. S1, Supporting Information, for spectrum characteristics).

The LED ribbon was placed horizontally across 3 sides of each box, about 15 cm above the soil, so that toads were not able to hide away from light at night. LEDs were lit during nighttime according to the natural photoperiod at the time of the experiment (12: 12 LD). Daylength ranged between 11:46 and 11:41 during the course of the experiment (October–mid-November). More details on the protocol can be found elsewhere (Secondi *et al.* 2020). We measured activity, mass and stress response in the same individuals before carrying out this study.

Experimental setup and pupillary constriction test

Cane toads were acclimatized in their individual box for 2 days following their capture before the experiment started. After 13 days of exposure to ALAN, we measured pupillary constriction in dark-adapted toads exposed to light (Fig. 1). Dark adaptation of retinal photoreceptors takes about 90 min (Donner & Reuter 1965); therefore, toads were kept in a transparent box in a dark room at sunset for at least 90 min. We carried each toad from the dark room to a lit room as fast as possible. Light intensity at the toad level was 450 ± 20 lx (mean \pm SD) and light was provided using the same LED ribbons as during the experiment that were coiled around the boxes. Toads were held in contention in a circular container and maintained in a still position by hand. We used a digital camera (Lumix DMC-FZ200 12 Mpx) to take one picture of the eye for each individual within 2 min, but in less than a minute for most individuals. Another picture of the same eye was taken after 60 min. We tested one group of 30 individuals (10 from each experimental group) on one day and a second group of 30 individuals the day after. We could not take pictures of all individuals within an hour, so we halved each group in 2 batches of 15 individuals. The toads of the second batch experienced a longer time (from 2.0 h for the first individual of the second batch to 2.75 h for the last individual) in the dark before testing. Images were analyzed using Image J (version 1.51j8, <http://imagej.nih.gov/ij>). The picture scale was determined by a ruler placed on the same height as the toad eye and parallel to the camera lens. In order to standardize the pictures, the contention device, the ruler, and the camera were fixed in the same position throughout the experiment. At 2 and 60 min, we measured eye size and pupil size in order to compute the relative pupil size, which is the ratio of pupil diameter to eye diameter (Fig. 1). We used relative pupil size (pupil size/eye size) in the analyses, as this measure is independent of toad and eye size.

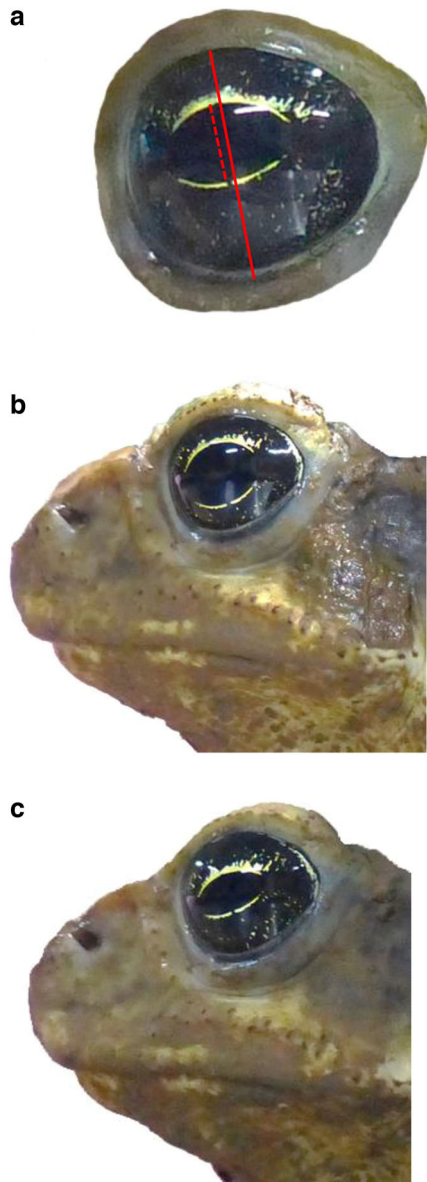


Figure 1 (a) Measurement of relative pupil size in the cane toad *Rhinella marina*. Illustration of the pupil light response within 2 min (b) and after 60 min (c) of exposure to light in dark-adapted adults.

Statistical analyses

We computed mixed linear models to test the effect of ALAN treatment (<0.001, 0.04, 5 lx), time (2' vs 60') and their interaction on eye size and relative pupil size. We expect variation in relative pupil size but not in eye size. Batch number was also included as a predictor in the model and toad identity was considered as a random

factor. All analyses were carried out using R studio (R Studio Team 2021) and R (R Core Team 2021) and statistical analyses with lme4 and emmeans packages. Graph was drawn using ggplot2.

Ethical approval

Experiments in Costa Rica were carried out with the authorization of the Ministerio de Ambiente y Energía, Sistema Nacional de Áreas de Conservación (N° SINAC-ACC-054-2017, SINAC-ACC-053-2017, SINAC-ACC-052-2017). This research adhered to the National Institutes of Health standards regarding the care and use of animals in research. All toads were released on their site of capture after the completion of the experiments.

RESULTS

We first checked that there was no effect of treatment and measurement time (2' vs 60') on eye size, which was confirmed by the fact that batch number was the only predictor retained in the minimal model retained (lmm: LRT = 4.453, $P < 0.034$). In contrast, we found an effect of treatment (lmm: LRT = 29.326, $P < 0.001$) and time (lmm: LRT = 66.206, $P < 0.001$) on relative pupil size, but not of their interaction (lmm: LRT = 3.965, $P = 0.140$) or batch number (lmm: LRT = 0.198, $P = 0.656$).

For all treatments, relative pupil size was lower at 2' than at 60' (0.084 ± 0.008 SE, $t_{59} = 10.902$, $P < 0.001$). At both times, we found the same pattern when comparing pupil contraction across light treatments (Fig. 2). We observed no difference in relative pupil size between 0.001 and 0.4 lx (-0.019 ± 0.02 SE, $t_{57} = -0.865$, $P = 0.390$), while this variable was lower in individuals exposed to 5 lx than to 0.001 lx (11.225 ± 0.022 SE, $t_{57} = -5.569$, $P < 0.001$). Relative pupil size was also lower in individuals exposed to 5 lx than to 0.04 lx (-1.034 ± 0.022 SE, $t_{57} = -4.704$, $P < 0.001$) (Fig. 2).

DISCUSSION

Rate of contraction independent of treatment

Our results clearly demonstrate an effect of ALAN intensity on the capacity of toads to regulate pupil constriction. However, contrary to our prediction, we observed no enhancement of the PLR in adults exposed to ALAN, but the opposite response. Iris constriction was less complete in individuals exposed to the brighter light treatment after both, 2 and 60 min. At both measurement points, relative pupil size was larger in individuals treated with 5 lx than

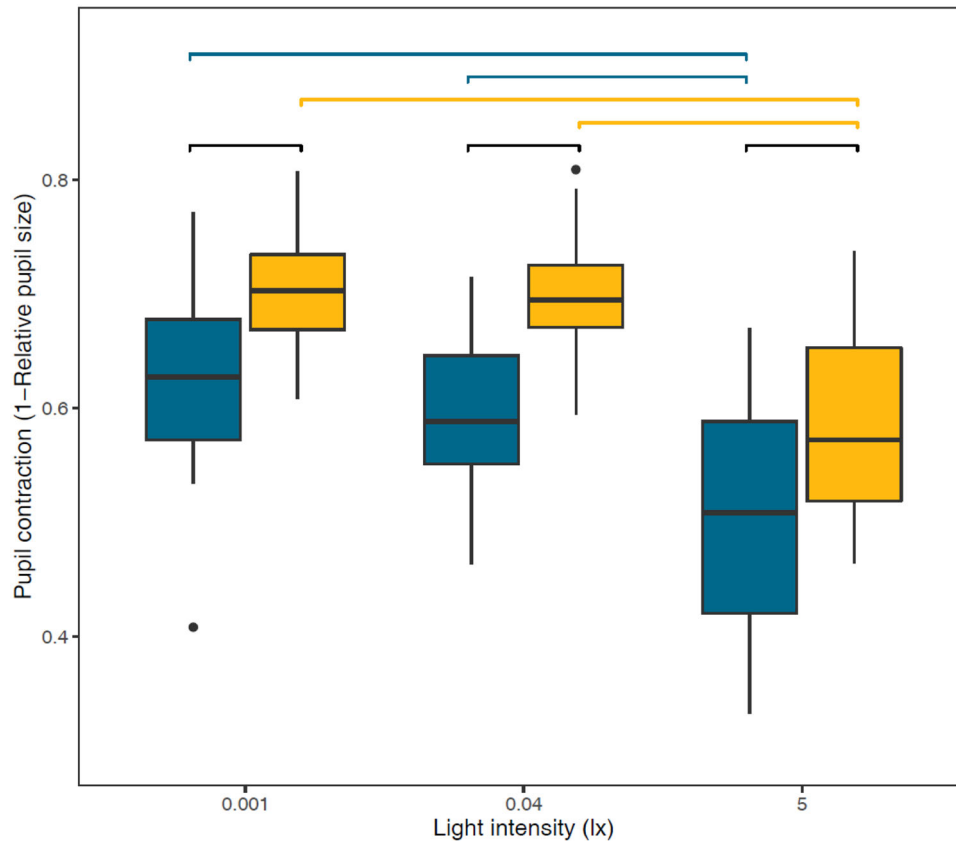


Figure 2 Pupil contraction of cane toads experiencing 3 levels of light at night after 2 min (blue boxes) and 60 min of exposure (orange boxes) to bright ambient light. Contraction is computed as 1-Relative pupil size so that the maximal value of 1 means a complete contraction (the pupil disappears). Lower values represent smaller pupil size. Bars indicate significant differences at $\alpha = 0.05$ between exposure time within a treatment (black lines) and between treatments for each exposure time (blue lines for 2', orange lines for 60').

in individuals from the other treatment groups. The lack of significant interaction between the light treatment and measurement time indicates that the rate of contraction between the 2 measurement points did not depend on the intensity of ALAN individuals were exposed to during the previous 12 days. Toads from the 5 lx group did not manage to compensate the initial constriction lag and did not reach the same level of contraction as the controls and the 0.04 lx group. Thus, the main difference between the groups could be explained by an initial slower contraction rate or an initial larger pupil size in dark-adapted toads exposed to more intense ALAN. We cannot tell which explanation is correct because we could not measure pupil size in the dark. Nevertheless, this study allows to reach solid conclusions. Toads from the 0.04 and 5 lx groups did keep the ability to contract their iris but there is an initial time lag in contraction caused by ALAN that was not compensated after 1 h. A longer time seems necessary to

determine whether maximal contraction has been reached in all groups or whether ALAN-exposed toads would eventually reach the same level of contraction. The pattern cannot be explained by a difference of exposure to light during the 12-day period as no difference in the time spent under the shelter between groups had been detected (Secondi *et al.* 2020). Our results rather suggest that ALAN induces a slower PLR in amphibians. This response is not explained by higher stress levels which would dilate the pupil (Carr 2003). Corticosterone levels decreased with ALAN intensity in this species (Secondi *et al.* 2020).

From an ecological perspective, the alteration of the PLR may have several consequences. First, individuals incur a prolonged susceptibility to glare, which is known to reduce the performance of visually guided behaviors like predator detection in animals (Fernández-Juricic *et al.* 2012). Sudden exposure to similar levels of ALAN as

in our study reduced prey consumption in a hylid frog species (Buchanan 1993). In contrast, more prey were found in the guts of cane toads exposed to ALAN and placed in small enclosures, but only in nights around full moon (Komine *et al.* 2020). However, these studies do not control for the effect on the prey. Results may not be entirely replicable in free-ranging conditions in areas where skyglow is the main light source, which does not lead to a concentration of prey around a punctual light source. The extension of the glare period may also affect the time budget if individuals are less likely to move when their vision is impaired. We actually observed such a pattern in these toads (Secondi *et al.* 2020). It is noteworthy that the alteration of the PLR may combine with the circadian retinomotor movements. If rods are no longer shielded during the natural nighttime, increased glare or photoreceptor saturation may be aggravated by the larger pupil size. Second, a delayed PLR increases the exposure of the retina to light at a time when melatonin expression is inhibited by ALAN. Melatonin inhibition is amplified in humans with pharmacologically dilated pupils (Gaddy *et al.* 1993). A similar phenomenon in toads could generate a positive feedback. The greater light flux on the retina is likely to increase melatonin suppression, aggravate the physiological effects of ALAN, and further alter the PLR, which in turn would elevate the level of incoming light onto the retina. Third, beyond the enhancement of image quality, the PLR provides information during social interactions in some species and the iris contraction also contributes to camouflage by hiding the pupil (Douglas 2018). An iris that is not contracted enough may reduce the efficiency of camouflage and increase predation risk. This mechanism may be relevant for toads. Finally, another conservation issue is related to road mortality. A field experiment showed that the probability of moving in 4 amphibian species was drastically reduced by a light stimulus mimicking an approaching car (Mazzerolle *et al.* 2005). Individuals that stay longer on a road are more likely to be killed. A lower capacity to adapt to light may increase this behavioral response and increase road kills.

We show here for the first time that ALAN alters the aperture control of the visual system in animals. Our data show that the ability of the iris to reduce the incoming light flux is impaired at 5 lx but not significantly at 0.04 lx. However, the process is likely gradual (Fig. 2). Additional intermediate and higher levels of ALAN need to be tested to determine the actual shape of the response. Nevertheless, our results may be conservative for amphibians. Although toads are equipped with a very sensitive night vision (Yovanovich *et al.* 2017), many species in-

cluding the cane toad are known for living close to human settlements and to feed in lit areas. In spite of negative physiological effects caused by ALAN (Touzot *et al.* 2019, 2020), toads may incur a lesser disruption of their visual system than species unable to colonize environments near urbanized areas. Our results should also be viewed as conservative regarding the exposure to various sources of ALAN. Our experimental light source produced an “orange” artificial light (590 nm) similar to those observed in many cities but far from the peak sensitivity of melanopsin, the main photoreceptor involved in PLR and melatonin suppression (Cahill *et al.* 1998; Brainard *et al.* 2001; Prayag *et al.* 2019). Thus, we suggest that shorter wavelengths such as those emitted by LED lights, the occurrence of which spreads rapidly around the world, induce an even stronger response as the one we observed.

Here, it is very likely that other photoreceptors than melanopsin were involved in PLR in the 5 lx treatment, but we cannot exclude that melanopsin was not stimulated at all as the spectral range of this opsin slightly overlaps the emission spectrum of our experimental light source (Fig. S1). It is noteworthy that in amphibians melanopsin is expressed in other tissues than retina (Provencio *et al.* 1998; Bluhm *et al.* 2012), including the iris to which it confers light sensitivity (Barr 1963; Kargacin & Dettwiler 1985) (valid for *Rhinella marina*). Melanopsin expression is down-regulated by melatonin in the dermal melanophores of a frog (Bluhm *et al.* 2012) and ALAN suppresses melatonin expression (Fonken & Nelson 2014). If the same regulation loop is valid for the ocular receptors, melatonin inhibition by ALAN may maintain high levels of melanopsin in the ipRGC or the iris. Thus, we expect melanopsin to largely influence PLR in anurans. Testing this hypothesis would require a more refined protocol able to selectively stimulate target photoreceptors (Spitschan & Woelders 2018) but this is an interesting perspective.

Our results raise questions about the physiological processes involved in the regulation of the PLR in amphibians and how ALAN affects these processes. More investigation is definitively required to understand the implication of the alteration of the PLR on the performance of visually guided behaviors and its consequences on the fitness of individuals in exposed populations. Considering the potential interplay between melatonin and melanopsin but also the possible positive feedback between PLR and melatonin suppression, the consequences of ALAN on the non-image forming component of the visual system certainly deserves more attention given the global rise of nocturnal brightness.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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SUPPLEMENTARY MATERIALS

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Spectral sensitivity of melanopsin and irradiance spectrum of the experimental light source.

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