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► **To cite this version:**

Morgane Touzot, Tristan Lefebure, Thierry Lengagne, Jean Secondi, Adeline Dumet, et al.. Transcriptome-wide deregulation of gene expression by artificial light at night in tadpoles of common toads. *Science of the Total Environment*, 2022, 818, pp.151734. 10.1016/j.scitotenv.2021.151734 . hal-03440101

HAL Id: hal-03440101

<https://hal.science/hal-03440101>

Submitted on 30 Aug 2022

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Transcriptome-wide deregulation of gene expression by artificial light at night in tadpoles of common toads

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Abstract

Artificial light at night (ALAN) affects numerous physiological and behavioural mechanisms in various species by potentially disturbing circadian timekeeping systems and modifying melatonin levels. However, given the multiple direct and indirect effects of ALAN on organisms, large-scale transcriptomic approaches are essential to assess the global effect of ALAN on biological processes. Moreover, although studies have focused mainly on variations in gene expression during the night in the presence of ALAN, it is necessary to investigate the effect of ALAN on gene expression during the day. In this study, we combined *de novo* transcriptome sequencing and assembly, and a controlled laboratory experiment to evaluate the transcriptome-wide gene expression response using high-throughput (RNA-seq) in *Bufo bufo* tadpoles exposed to ecologically relevant light levels. Here, we demonstrated for the first time that ALAN affected gene expression at night (3.5% and 11% of differentially expressed genes when exposed to 0.1 and 5 lux compared to controls, respectively), but also during the day with a dose-dependent effect (11.2% of differentially expressed genes when exposed to 5 lux compared to controls). ALAN globally induced a downregulation of genes (during the night, 58% and 62% of the genes were downregulated when exposed to 0.1 and 5 lux compared to controls, respectively, and during the day, 61.2% of the genes were downregulated when exposed to 5 lux compared to controls). ALAN effects were detected at very low levels of illuminance (0.1 lux) and affected mainly genes related to the innate immune system and, to a lesser extent to lipid metabolism. These results provide new insights into understanding the effects of ALAN on organism. ALAN impacted the expression of genes linked to a broad range of physiological pathways at very low levels of ALAN during night-time and during daytime, potentially resulting in reduced survival under environmental immune challenges.

Keywords: Light pollution, amphibian, transcriptome, RNA-seq, immune system, lipid metabolism.

1. Introduction

Photoperiod is one of the major environmental cues that regulates biological processes in living organisms (Longcore and Rich 2004, Ouyang et al. 2017) and allows them to anticipate daily and seasonal changes and adapt their biochemical, physiological and behavioural activities. In the last century, artificial light at night (ALAN), generated by the surge in urbanized areas and transport infrastructures (Gaston et al. 2013), has dramatically expanded worldwide (Falchi et al. 2016) and endangered biodiversity on the global scale (Hölker et al. 2010, Gaston et al. 2015, Guetté et al. 2018, Secondi et al. 2019).

In the last decade, numerous studies have reported the effects of ALAN on many physiological processes in a large panel of animals. This anthropogenic light modifies illuminance levels so that the perception of fine changes in natural light level can be impaired, thus desynchronizing daily or seasonal activities (Robert et al. 2015, de Jong et al. 2016). Hence, the internal circadian timekeeping system is impacted by ALAN mainly through alterations in the expression of clock genes (Bedrosian et al. 2013, Fonken et al. 2013, Honnen et al. 2016, 2019, Khan et al. 2018). However, many other genes that are regulated by photoperiod, such as genes related to metabolic detoxification, immunity, nutrient sensing and reproductive processes, could also be impacted by ALAN (Brüning et al. 2016, Honnen et al. 2016). In many taxa, ALAN reduces the nocturnal expression of melatonin (Brüning et al. 2015, Grubisic et al. 2019), a hormone involved in the regulation of the immune system, energy metabolism, antioxidant defences and sex hormone synthesis (Reiter et al. 2010, Zhao et al. 2019). Short-term exposure to ALAN was, however, not associated with alterations in immunity or oxidative stress in adult fishes (Kupprat et al. 2021) and birds (Raap et al. 2016b), suggesting that longer exposure and/or a more vulnerable developmental stage might be required to have deleterious effects if any. Accordingly, in humans and rodents, prolonged

ALAN exposure is associated with an increased prevalence of obesity, type 2 diabetes, hypertension, affective disorders or specific cancers (Wyse et al. 2011, Haim and Portnov 2013, Fonken and Nelson 2014).

At the molecular level, the impact of ALAN is beginning to be studied, but the studies target mainly specific candidate genes (Bedrosian et al. 2013, Fonken et al. 2013, Brüning et al. 2018, Honnen et al. 2019). However, given the multiplicity of ALAN effects on organisms, integrative approaches are required to simultaneously investigate all these effects at the gene level and better understand the causes of physiological and behavioural changes triggered by this stressor. A pioneering study using a large-scale transcriptomic approach revealed a sex-specific response of urban adult mosquitoes to ALAN, with reduced expression in males of genes mostly related to gametogenesis, lipid metabolism and immunity (Honnen et al. 2016). A second study characterizing the transcriptomes of coral reefs showed alterations of the pathways associated with the cell cycle, growth, proliferation and protein synthesis in ALAN-exposed coral reefs (Rosenberg et al. 2019). However, both studies used rather high experimental light levels to mimic direct illumination at night. To our knowledge, no transcriptomic study has yet been conducted using low light levels representative of levels recorded in peri-urban or rural areas, although the low light levels would be more relevant from an ecological point of view (Luginbuhl et al. 2014). In addition, while transcriptomic analysis of ALAN impacts was restricted to the night period, several studies showed that ALAN had long-lasting impacts on animal physiology and behaviour during the daytime (Dominoni et al. 2020, Latchem et al. 2021).

Freshwater ecosystems are particularly concerned with ALAN exposure (Secondi et al. 2017, Grubisic 2018) since lit road networks, urban development and industrial infrastructure are frequently located along rivers and lakes (Reid et al. 2019). However, these habitats are home to rich fauna (Dudgeon et al. 2006), which will thus suffer the effects of ALAN. This is particularly the case for amphibians, such as the common toad, *Bufo bufo*. This species is one of the most common amphibians in western Europe, and frequently colonizes urban areas (Beebee 1979). Unlike

adults, who may try to escape from light sources at night, earlier stages, such as tadpoles, cannot escape ALAN given their dependence on the aquatic environment and will therefore be exposed to ALAN throughout their embryonic and larval development. Moreover, it is suggested that the impacts of ALAN may be especially relevant when the organism is exposed in early life, since the immature circadian system may be particularly sensitive to rhythm disruptions through ALAN (Fonken and Nelson 2016). Therefore, in the present study, we combined *de novo* transcriptome sequencing and assembly, and a controlled laboratory experiment to investigate the transcriptome-wide gene expression response using Illumina RNA-seq in common toad tadpoles following prolonged exposure to ALAN. This is the first study using ecologically relevant ALAN levels at night and evaluating ALAN effects for each light treatment, both at night and during daytime. We first expected ALAN to affect tadpole gene expression and more specifically with a dose dependent effect. Moreover, we predicted effects of ALAN on night-time and daytime gene expression.

2. Material and Methods

2.1 Animal collection and housing conditions

Nine fragments of approximately 20 eggs each were randomly sampled from a clutch of common toads in March at the beginning of the breeding season in La Burbanche, France (45°N, 5°E). This site was chosen for its low levels of ALAN regardless of weather conditions and lunar phase (≤ 0.01 lux). Upon arrival at the animal care facility (EcoAquatron, University of Lyon), the 9 fragments were each individually placed in an aquarium (32 cm x 17.5 cm x 18.5 cm) containing 7.4 L of dechlorinated and bubbled water and immediately randomly assigned to one of the three experimental treatments (control and two light treatments, 3 aquariums per treatment). From stage 25 of Gosner, the stage at which

independent feeding is possible (Gosner 1960), to the end of the experiment, tadpoles were fed every two days with boiled organic green salad. Each week, one-third of the water of the aquarium was renewed with dechlorinated and oxygenated water. Ambient temperature and relative humidity were kept constant during the whole experiment at $16.1 \pm 0.9^{\circ}\text{C}$ and $60.0 \pm 11.2\%$, respectively. As soon as the individuals had hatched, tadpole mortality was measured and was equal to zero.

2.2 Light treatments

Upon arrival at the animal care facility, the 9 aquariums were exposed to their respective light treatment at night. During the daytime, illuminance provided by light tubes (Philips Master TL-D 58 W/865 and Exo Terra Repti Glo 2.0, 40 W T8) for all aquariums was 1995 ± 257 lux (mean \pm SEM). The spectrum of our daylight is characteristic of fluorescent lamps (Appendix A) and is recommended for the housing of amphibians in animal care facility. During night-time, to reproduce artificial light by night, we used white light-emitting diode (LED) ribbons (white cold Light Plus, 6000-6500 K, 14 W, 60 LED/m), whose spectrum is different from our daylight (Appendix A). White LEDs were chosen because they are increasingly used for street lighting worldwide (Falchi et al. 2016). An LED ribbon of 95 cm (57 LED) combined with a light diffuser was suspended horizontally 41.5 cm above the bottom of the aquariums. Each LED ribbon was connected to a dimmer (manual dimmer, 12 V max, 8 A) and a laboratory power supply (15 V/DC max, 3 A), which allowed illuminance to be set for each light treatment. The aquariums of one light treatment were isolated from the others with tarpaulins to avoid light spilling over from another treatment. Tadpoles were exposed to the same photoperiod as the natural photoperiod at the date of the experiment. At night, the control group was exposed to 0.01 lux, corresponding to the illuminance of a sky under clear conditions with a quarter moon (Gaston et al. 2013). The first experimental group was exposed to 0.1 lux, corresponding to the illuminance of urban skyglow (Gaston et al. 2013). The second experimental group was exposed to 5 lux, which corresponds to the light level of a

residential street (Gaston et al. 2013). The daylight and 5 lux illuminances were measured with a luxmeter (Illuminance meter T-10A, Konica Minolta). Illuminances for the 0.1 lux and control illuminances were set using a highly sensitive light meter (Sky Quality Meter SQM-L, Unihedron). Consequently, to compare illuminances, SQM-measured values were converted into lux according to the relationship curve between SQM and lux measurements (Touzot et al. 2020), as lux is the main unit used in ALAN studies (Longcore and Rich 2004). Illuminances were measured at the water surface of each aquarium (24 cm from the LED) and checked every week (0.007 ± 0.001 lux for the control group, 0.02 ± 0.004 lux for the 0.1-lux group and 4.09 ± 1.30 lux for the 5-lux-group, mean \pm SEM).

2.3 Experiment

When tadpoles reached stage 31 of Gosner (Gosner 1960), corresponding to 27 days of exposure to ALAN, 5 tadpoles of each treatment were randomly sampled during daytime (13:00) or night-time (01:00) (random sampling on the 3 aquariums of each treatment). This results in 10 tadpoles sampled per light treatment and thus in 30 tadpoles sampled for the whole experiment. Six hours prior to sampling, tadpoles were fasted so that the nutritional status was the same for all animals and did not bias gene expression (Steiner 2007). Development stage 31 was chosen, as it is an easily identifiable stage, and the foot is paddle-shaped at this stage only (Gosner 1960). Development stage 31 also allowed long-term exposure to ALAN, while being distant enough from metamorphosis, *i.e.*, stage 46 of Gosner, the ultimate stages of development during which thyroid hormones are strongly expressed and involve many functional and morphological changes in the organisms. Tadpoles were individually sampled and immediately placed in liquid nitrogen and then stored at -80°C until molecular analyses. Although all individuals were at the same Gosner stage, the tail size differed between individuals. Thus, to limit very large variations in the amount of RNA extracted between samples, we removed

before molecular analyses the tadpole tail. Although gene expression can differ among tissues, we chose to work on the body without distinguishing tissues to investigate the global effects of ALAN across as broad a range of molecular processes as possible.

2.4 Molecular analyses

Total RNA was extracted by adding TRI Reagent (Molecular Research Center MRC, TR118) to the 30 samples and by homogenizing tissues on ice with a tissue lyser (Retsch, MM200). All remaining steps were carried out according to the manufacturers' protocols (Molecular Research Center MRC, TR118). Then, the RNA was treated with Turbo DNase enzyme (Turbo DNA free kit, Invitrogen, AM1907) and assayed by fluorescence using a Qubit fluorometer (Qubit® RNA HS Assay Kits Molecular Probes, Invitrogen, Q32855). Library construction was carried out using the NEBNext Ultra II RNA Library Prep Kit for Illumina (Biolabs New England, NEB E7770, E7490, E7600). Libraries were randomly sequenced on three lanes with an Illumina® HiSeq4000™ machine on the GenomEast platform hosted at the IGBMC (GenomEast Platform – Institut de Génétique et de la Biologie Moléculaire et Cellulaire IGBMC, Illkirch, France), resulting in single-end samples with reads 50 bp long.

2.5 B. bufo de novo transcriptome assembly

We built a transcriptome assembly from the sequenced RNA samples (Appendix A) using Trinity software (version 2.5.1) (Haas et al. 2013) with default parameters. All samples were processed in a single run with the Trinity default read normalization procedure to improve the sensitivity of transcript detection. The resulting assembly was assessed using Busco software (version 3.0.2) (Seppey et al. 2019). We ran Busco using the Tetrapoda dataset (available at https://busco-archive.ezlab.org/v2/datasets/tetrapoda_odb9.tar.gz) and with an E-value threshold of 0.01 (--evalue option). Protein sequences and valid coding sequences (CDSs) were derived from assembled transcripts using the Transdecoder program (version

3.0.1) (Haas et al. 2013). We thus obtained a filtered set of transcripts that was subsequently fed to the Diamond program (Buchfink et al. 2015) with default arguments to discover homologies with genes from the reference genome of *Xenopus tropicalis*, and proteins from the UniProt/Swiss-Prot database. In addition, the CDSs were annotated using PFAM (El-Gebali et al. 2019) domains using the hmmsearch program (Wheeler and Eddy 2013) with default arguments.

2.6 Differential gene expression analysis

Expression levels were quantified using Kallisto (Bray et al. 2016) with options -l 200 -s 20 for single-end samples. We subsequently used rounded effective counts as input for differential gene expression, which was performed using the *DESeq2* package (Love et al. 2014). We specified light treatment, timepoint and their interaction in the model (design formula: “design = ~ timepoint + light treatment + timepoint * light treatment). The *DESeq2* package provides differential gene expression by use of negative binomial distribution and shrinkage estimation for dispersions and fold changes to improve the stability and interpretability of estimates (Love et al. 2014). P-Values were used to test the significance of the false **discovery rate** (FDR) using the Benjamini and Hochberg procedure. Genes with an FDR < 0.05 were considered significantly differentially expressed. We also reported numbers of genes with an absolute Log₂ Fold Change (|LFC|) greater than 1 and 2. Moreover, to represent globally the gene expression variations between light treatments and periods, we ran a principal component analysis (PCA) for the 30 samples using the *ggplot2* package. Proportional Venn diagrams were constructed using the *eulerr* package and bar plots were constructed using the *ggplot2* package.

2.7 Gene enrichment analysis

Functional annotations and overrepresentation of Gene Ontology (GO) terms were analysed using the *topGO* package (Alexa and Rahnenfuhrer 2020). Gene enrichment analyses were performed using a hypergeometric test for overrepresentation of GO terms (Fisher test) from the differentially expressed gene set ($FDR < 0.05$ and $|LFC| > 1$). Again, p-values were used to test FDR significance using the Benjamini and Hochberg procedure and GO terms with $FDR < 0.01$ and odds-ratio > 1 were regarded as significantly different. Then, GO term redundancy was removed first using REVIGO (Supek et al. 2011) and second by manual simplification as GO terms were still redundant. The second simplification occurred when two GO terms were parents/descendants and were significantly enriched with the same number of annotated and significantly differentially expressed genes. We evaluated GO terms from the biological process, molecular function, and cellular component domains.

The analyses of differential gene expression and gene enrichment, and graphics were performed with R software (v3.6.0) (Anon 2018).

2.8 Ethical note

The sampling of common toad clutch fragments was authorized by the Préfecture de l'Ain (DDPP01-18-271) and by the French government (APAFIS#27760-2020102115402767) in accordance with the ethical committee of Lyon 1 University. The animal care structure “EcoAquatron” (University of Lyon) received an agreement of veterinary services (approval DSV 692661201). At the end of the experiment, all the remaining tadpoles were released on the capture site.

3. Results

3.1 ALAN affected gene expression in *B. bufo* tadpoles

The sequencing of 30 individual tadpoles bred experimentally with nocturnal light mimicking three levels of ALAN (0.01, 0.1 or 5 lux) and randomly sampled during night-time or daytime yielded a total of 863.8 million 50 bp single-end reads, averaging approximately 28.8 million reads per sample (range 19,700,000 – 38,700,000 reads). Since no genetic data were available for this non-model species, we first assembled a *de novo* transcriptome from all sequenced RNA from *B. bufo* samples, which yielded 38,692 contigs (coding sequences *i.e.*, genes) and 95,483 total transcripts (isoforms).

A PCA representation of the transcriptome-wide gene expression variations clearly discriminated the samples according to nocturnal illuminance treatment and sampling period (Fig 1). This global representation showed that control individuals are separated from the individuals exposed to 5 lux, indicating a clear effect of ALAN on gene expression. Moreover, within these two light illuminances, a day/night effect was observable, distinctly separating individuals collected during night-time from those collected during daytime (Fig 1). The samples from the 0.1-lux group overlapped both with the 5-lux group at night and with the control group in the daytime (Fig 1). This complex distribution indicated a different effect of 0.1 lux on day/night gene expression compared to the 5-lux group. This suggests that at a light intensity of 0.1 lux, major effects occurred at night, whereas with a higher light intensity of 5 lux, gene expression was affected regardless of the time period.

(Fig. 1)

3.2 ALAN altered nocturnal gene expression regardless of the illuminance level and also altered diurnal gene expression

When focusing on the variation in gene expression at night, we showed that exposure to ALAN at both 0.1 and 5 lux induced differential expression of genes compared to controls (Fig 2A). The higher the ALAN illuminance, the greater the number of differentially expressed genes compared to controls. Indeed, 3.5% (1,194 genes) and 11% (3,676 genes) of the tested genes were differentially expressed (false discovery rate, $FDR < 0.05$) in the 0.1- and 5-lux groups compared to the controls, respectively (Fig 2Aa). Among those genes, 284 and 786 had an absolute \log_2 fold change ($|LFC|$) greater than 1, and 79 and 82 had an $|LFC|$ greater than 2 in the 0.1- and 5-lux groups, respectively. However, and in accordance with the PCA (Fig 1), far fewer genes were differentially expressed at night between the two ALAN treatments (331 genes with an $FDR < 0.05$, 125 of which had an $|LFC|$ greater than 1 and 51 of which had an $|LFC|$ greater than 2) (Fig 2Aa). Furthermore, among the 1,194 genes affected by the 0.1-lux group, 76% were also affected by the 5-lux exposure. Among the genes differentially expressed between light treatments at night compared to controls, the majority were downregulated (58% and 62% in the 0.1- and 5-lux groups when compared to controls, respectively) (Fig 2Ab). Once again, the higher the illuminance at night, the greater the under- and overexpression of differentially expressed genes at night compared to controls.

During the daytime, our results showed that exposure to 5 lux at night resulted in differential gene expression relative to the controls and 0.1-lux group (Fig 2B). We observed differential expression in 11.2% (3,794 genes) and 11.8% (3,983 genes) of the tested genes ($FDR < 0.05$) in individuals exposed to 5 lux relative to controls or 0.1 lux, respectively (Fig 2Ba). Among those genes, 808 and 952 had an $|LFC|$ greater than 1, and 117 and 153 had an $|LFC|$ greater than 2 in tadpoles exposed to 5 lux compared to controls or 0.1 lux, respectively. Moreover, among the 3,794 affected genes when comparing the 5-lux group to controls, 55.5% were also differentially expressed between 5 lux and 0.1 lux. However, and in accordance with the PCA (Fig 1), far fewer genes were differentially expressed during daytime when comparing the 0.1-lux group to the controls

(237 genes with an FDR < 0.05, 90 of which had an |LFC| greater than 1 and 42 of which had an |LFC| greater than 2) (Fig 2Ba). Furthermore, among the differentially expressed genes between light treatments during the daytime, we again observed an excess of downregulated genes in the 5-lux group (61.2% and 64.2% were downregulated when compared to the controls or the 0.1-lux group, respectively) (Fig 2Bb).

(Fig. 2)

3.3 ALAN exposure affected different physiological pathways, mainly the immune system

Functional annotations and overrepresentation of GO terms were analysed on the most strongly differentially expressed genes (FDR < 0.05 and |LFC| > 1) of the 30 samples. The analysis of the GO term enrichment showed that several physiological pathways were affected by ALAN and revealed that genes linked to the immune system corresponded to the physiological pathway mainly affected by ALAN (Fig 3 and appendix Table A). Indeed, among the 54 most enriched GO terms (FDR < 0.01 and odds-ratio > 10 for at least one light treatment comparison), 17 were linked to the immune system (Fig 3 and appendix Table A). The overrepresentation of GO terms linked to the immune system was observable at night for two light treatment comparisons, *i.e.*, when comparing the 5-lux to the controls and the 5-lux to the 0.1-lux group, and in a lesser extend when comparing the 0.1-lux to the controls (Fig 3 and appendix Table A). Indeed, at night, enriched GO terms included antibacterial humoral response when comparing the 0.1-lux group to the controls. When we compared the 5-lux group to the controls or the 0.1-lux group, the 17 GO terms linked to the immune system were enriched, such as inflammatory response, C5a anaphylatoxin chemotactic receptor binding, regulation of

membrane attack complex, complement-dependent cytotoxicity and complement activation, except for the GO term antibacterial humoral response when comparing the 5-lux group to the 0.1-lux group. The same result was observable during the daytime, with the 17 GO terms related to the immune system that were enriched when comparing the 5-lux group to the controls or the 0.1 lux group (Fig 3 and appendix Table A). Globally, at night or daytime, the genes involved in immunity were always downregulated under the higher light level, regardless of the light treatment comparison (0.1 lux vs controls, 5 lux vs controls or 5 lux vs 0.1 lux) (appendix Table B). More specifically, the genes that were downregulated at night and daytime under ALAN were, for instance, coding for complement C3, complement C3 alpha chain and complement factor H, therefore indicating potential impairment of the innate immune system under ALAN.

(Fig. 3)

In addition to the immune system, ALAN also affected lipid metabolism and transport pathways (11 of the 54 most enriched GO terms). This effect was observable whatever the period, and mainly during daytime, and involved GO terms such as steroid and triglyceride biosynthetic processes and lipid storage and transport (Fig 3 and appendix Table A). Genes involved in these pathways were globally always downregulated under the higher light level, regardless of the light treatment comparison (0.1 lux vs controls, 5 lux vs controls or 5 lux vs 0.1 lux) at night or daytime (appendix Table B). In addition to these two main physiological pathways affected by ALAN, several other GO terms were also enriched in presence of ALAN. These overrepresented GO terms were linked to various physiological processes, including synapse pruning, oxidative

balance, zymogen granule binding and cellular glucuronidation (Fig 3 and appendix Table A). Most of them were affected regardless of the light treatment comparison (0.1 lux *vs* controls, 5 lux *vs* controls or 5 lux *vs* 0.1 lux) at night or daytime. GO terms implicated in oxidative balance and cellular glucuronidation were further enriched when comparing 0.1 lux *vs* controls at daytime (Fig 3 and appendix Table A). In contrast, among the differentially expressed genes mentioned previously (FDR < 0.05 and |LFC| > 2), we did not find any gene related to circadian rhythm, *i.e.*, clock genes or genes coding for enzymes involved in melatonin synthesis, which we presumed to be affected by ALAN. Therefore, no GO terms involved in circadian rhythms were found to be enriched (appendix Table C).

4. Discussion

Our study of 30 transcriptomes from *B. bufo* tadpoles exposed to light treatments during two thirds of larval development demonstrated that ALAN induced a strong disruption of gene expression both at night and daytime. In addition, we clearly showed that most of the differentially expressed genes in ALAN exposed tadpoles were downregulated, leading to changes in many physiological pathways, including their innate immune system and their lipid metabolism.

At night, ALAN induced strong differential gene expression compared to controls. More unexpectedly, we showed noticeable differential gene expression during the daytime between tadpoles exposed to ALAN and controls. While our low intensity, 0.1 lux, affects gene expression only during night-time, at our high intensity, 5 lux, gene expression is altered both at night-time and daytime. To our knowledge, molecular scale studies to date have always focused on variations in gene expression occurring at night during ALAN exposure (Honnen et al. 2016, Rosenberg et al. 2019), but never during the day, when light levels are similar between exposed individuals and controls. Our results suggest that alterations in

gene expression are a direct long-lasting result of ALAN exposure at night, and an indirect effect of physiological disorders impacting gene expression during the day in absence of light pollution. Moreover, our results of gene expression analysis and GO term enrichment showed that ALAN has a rather monotonic dose-dependent effect on molecular parameters. The higher the ALAN illuminance, the greater the deregulation of transcriptome expression. This observation had previously been noted in several species exposed to a range of ALAN levels on biological processes, such as hormonal synthesis, (Brüning et al. 2015, Grubisic et al. 2019, Kupprat et al. 2020), activity rhythms (de Jong et al. 2016, Barré et al. 2020, Secondi et al. 2021), reproductive physiology (Dominoni et al. 2018) and individual's density (Sanders et al. 2018). In addition, in our study, strong molecular impacts were obtained even at very low illuminance, 0.1 lux, corresponding to the skyglow level recorded in rural areas (Gaston et al. 2013).

Analysis of differentially expressed genes and GO term enrichment showed a reduced expression of genes involved in innate immune system both at night and daytime (e.g.. Complement C3, complement C3 alpha chain and complement factor H). In vertebrates, activation of the complement system acts by promoting the inflammatory response to eliminate microorganisms (Demas et al. 2011). The complement is also involved in the lysis of foreign cells through the formation of membrane attack complexes and the mediation of phagocytosis (Demas et al. 2011, Juul-Madsen et al. 2014). A nocturnal downregulation of genes encoding peptidoglycan-recognition proteins was also observed under ALAN. These proteins play a key role in innate immunity, as they belong to a group of receptors involved in the recognition of the bacterial peptidoglycan wall (Dziarski and Gupta 2006). Consistent lower expression of genes linked to immunity in ALAN exposed tadpoles could be explained by a modulation of melatonin synthesis with ALAN. Indeed, for several years it has been documented that low ALAN intensities are responsible for a reduction of melatonin synthesis, a hormone regulating immune and inflammatory processes (reviewed in Grubisic et al. 2019). Because the

maintenance and use of the immune system are costly (McKean and Lazzaro 2011), ALAN-induced downregulation of genes linked to immunity could also result from an altered energy allocation in tadpoles. It would, therefore, be relevant to investigate whether the downregulation of genes involved in immune function results in depressed immunocompetence in tadpoles exposed to ALAN. Several studies have shown an alteration in the immune capacity of organisms exposed to ALAN, although these effects seem not to be uniform across species, with heterogeneity possibly related to light intensity, duration of treatment, and potential developmental window of sensitivity (Bedrosian et al. 2011, Saini et al. 2019). In the wild, amphibians encounter a variety of immune challenges due to the presence parasites, pathogens and infectious fungal species (Grogan et al. 2018), representing a major cause of the decline in amphibians worldwide (Carey et al. 1999). Although infection with fungal species is generally not lethal before metamorphosis, infected tadpoles may exhibit sublethal effects on growth and development (Blaustein et al. 2005). Thus, by reducing the innate immune response, exposure to ALAN *in natura* would probably impair the capacity of tadpoles to overcome immune challenges.

GO terms linked to lipid metabolism and transport were another important physiological pathway affected by ALAN. Others experimental studies demonstrated an impaired balance of lipid biosynthetic pathways, but with high intensity of ALAN (Honnen et al. 2016; Okuliarova et al. 2020). A modification of the expression of genes related to lipid metabolism and transport is likely to affect body lipid homeostasis, to limit the availability and use of lipid resources, and thus be of particular concern in developing individuals. Lipids have the potential of serving as a source of metabolic energy and seems to form an important constituent of the tadpole bodies, as they are known to increase during larval growth and prometamorphosis (Sawant and Varute 1973). During metamorphosis, when histolytic events appear, *e.g.*, internal gills degeneration, tail regression, and when tadpoles do not feed, anuran tadpoles seem to make use of lipids that have accumulated during the growth period (Sawant

and Varute 1973). Thus, studies on the impacts of ALAN on physical activity and growth in juveniles in relation to metabolism should further investigate this issue.

Most ALAN studies have suggested that light at night affects circadian rhythms through modifications of clock gene regulation and melatonin synthesis. However, when comparing our transcriptomes at night or daytime neither clock genes (*e.g.*, Clock, Period, Bmal 1, Cryptochrome) nor melatonin-related genes (*i.e.*, genes encoding enzymes catalysing melatonin synthesis) were differentially expressed between light treatments. Present results contrast with those obtained by others at 5 lux, where melatonin plasma levels were reduced by more than a quarter in European sea bass, *Dicentrarchus labrax* (Bayarri et al. 2002), in great tit (de Jong et al. 2016) or in zebra finch, *Taeniopygia guttata* (Batra et al. 2019). An absence of changes in melatonin-related genes could possibly be related to the fact that enzyme expression did not reflect melatonin synthesis in *B. Bufo* tadpoles. Indeed, any correlation between retinal aralkylamine-N-acetyltransferase (AANAT) activity, an enzyme catalysing melatonin synthesis, and retinal melatonin levels during the light/dark cycle was observed in adult European green toads, *Bufo viridis* (Serino et al. 1993). A positive correlation was however reported in adult green frogs, *Pelophylax esculenta* (Serino et al. 1993, d'Istria et al. 1994). Alternatively, because of the low levels of ALAN intensities used in our study, this could have resulted in discrete changes in the transcription of these genes that were not detected by our large-scale analysis. An absence of changes in clock genes or melatonin-related genes could also partly be related to the use of the whole body for RNA extraction. Indeed, extra pineal and extra retinal tissues are known to synthesize melatonin, such as the gut in adult amphibians and other glands in tadpoles (Bubenik and Pang 1997, Wright et al. 2001). Clarifying this point warrants further investigations with

specific tissue approaches. The whole-body approach carried out in this study, allows us to avoid any potential organ effect and reflects at the individual scale the global molecular effect of ALAN.

5. Conclusion

In conclusion, the present study revealed that ecologically relevant illuminances at night mimicking anthropic emerging pollution markedly affected the global gene expression pattern in tadpoles of common toads. Transcriptomic changes suggested that ALAN would result in depressed capacity to overcome immune environmental challenges. The impacts of ALAN are multifarious, and we only slowly disentangle its multiple negative effects on organisms, populations, and ecosystems. Present data advocate the need for integrative studies with molecular approaches to identify and characterize pathways that may link physiological and behavioural disruption caused by light at night and potential health and *fitness* consequences.

6. Acknowledgements

We thank A. Clair, J. Ulmann and L. Averty for their technical assistance in the EcoAquatron and M. Sémon for her advice on the experimental design and data analysis.

7. Funding

This work was supported by the French Government [PhD grants 2017–2020], by the ‘LABEX IMU Laboratoire d’Excellence Intelligence des Mondes Urbains’ and by IDEX Initiative d’Excellence Université Lyon (IDEX, ANR-16-IDEX-0005, ALAN Project), and was conducted under the aegis of the "Ecole Universitaire de Recherche" H2O'Lyon (ANR-17-EURE-0018).

8. Data availability

Transcriptomes reads have been deposited on the NCBI Gene Expression Omnibus (GEO). *B. bufo de novo* transcriptome reads have been deposited on the of the Sequence Read Archive (SRA) and *de novo* transcriptome assembly has been deposited on the Transcriptome Shotgun Assembly (TSA) database of the SRA. All data are available on the SRA BioProject accession number PRJNA769252.

9. Author contributions

M. Touzot: Conceptualization, Formal Analysis, Investigation, Writing – Original Draft Preparation. T. Lefebure: Data Curation, Formal Analysis, Writing – Review and Editing.

T. Lengagne and J. Secondi: Funding Acquisition, Writing – Review and Editing.

A. Dumet and L. Konecny-Dupre: Investigation.

P. Veber and V. Navratil: Data Curation, Writing – Review and Editing.

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10. Competing interests

The authors declare no competing or financial interests.

11. Appendices

Appendix A: Sequencing and assembly of *B. bufo de novo* transcriptome

Table S1: Enrichment level of the 54 most enriched GO terms

Table S2: The top 10 most overrepresented GO terms

Table S3: Enrichment level of GO terms related to circadian rhythm

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Figure legend

Figure 1 . 1. PCA plot of all the genes of the 30 *B. bufo* transcriptomes. Individuals were exposed to one of three different light treatments: control (dark blue), 0.1 lx (royal blue) or 5 lx (light blue) and sampled at different timepoints: daytime (open symbols) or night-time (closed symbols). Each point represents one individual sample and ellipses represent a 95% confidence level for the multivariate normal distribution of samples in a light treatment. Distance of points to its centroid according to light treatment and day/night effect is represented by a line. PCA plot was illustrated on the two principal axes, explaining 32% of the variability. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Gene expression variation among light treatments during night-time (A) or daytime (B). a.: Venn diagram showing the number of differentially expressed genes (FDR < 0.05) for light treatment comparisons (0.1 lx vs control, 5 lx vs control, and 5 lx vs 0.1 lx), as well as the possible intersections between these light treatment comparisons. The three colours (red, yellow, green) correspond to one of the three light treatment comparisons, and the different shades of the same colour indicate an intersection between two or three light treatment comparisons. The surfaces are proportional to the number of differentially expressed genes. b.: Bar plot representing, among the differentially expressed genes, the

number of up- (closed bar plot) or downregulated (open bar plot) genes (FDR < 0.05) for all light treatment comparisons (0.1 lx vs control, 5 lx vs control, and 5 lx vs 0.1 lx). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. Heatmaps illustrate the enrichment level of the 54 most enriched GO terms (rows, FDR < 0.01 and odds-ratio > 10 for at least one light treatment comparison) for each light treatment comparisons (columns) at either night-time (A) or daytime (B). The colour scale represents the odds-ratio levels; a darker colour indicated a higher enrichment, and a lighter colour indicates a lower enrichment. Please note the difference in scale for the level of enrichment between the two panels. The red insert groups the GO terms associated to the immune system and the blue insert groups the GO terms associated to the lipid metabolism. The data used to generate this heatmap are available in S1 Table. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

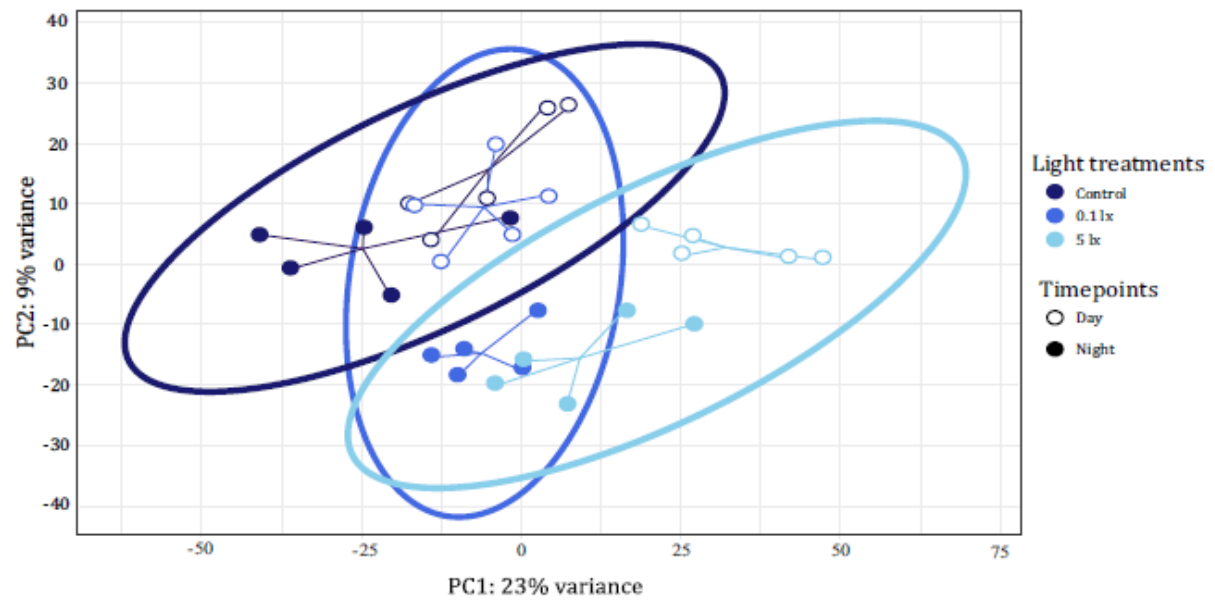


Fig 1

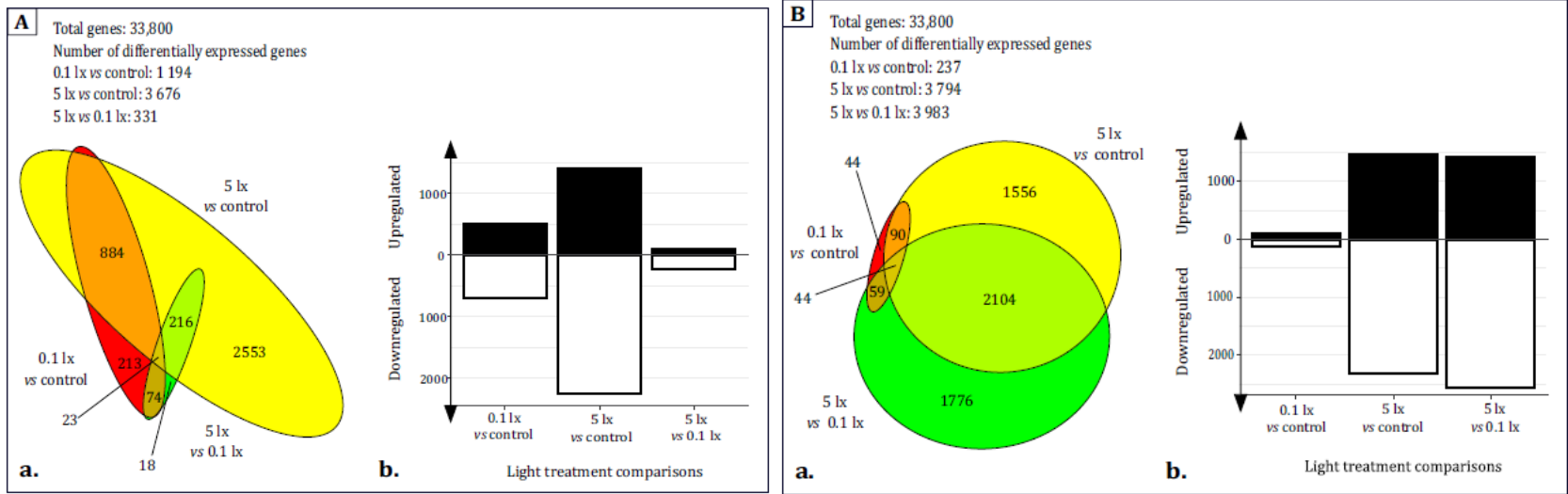


Fig 2

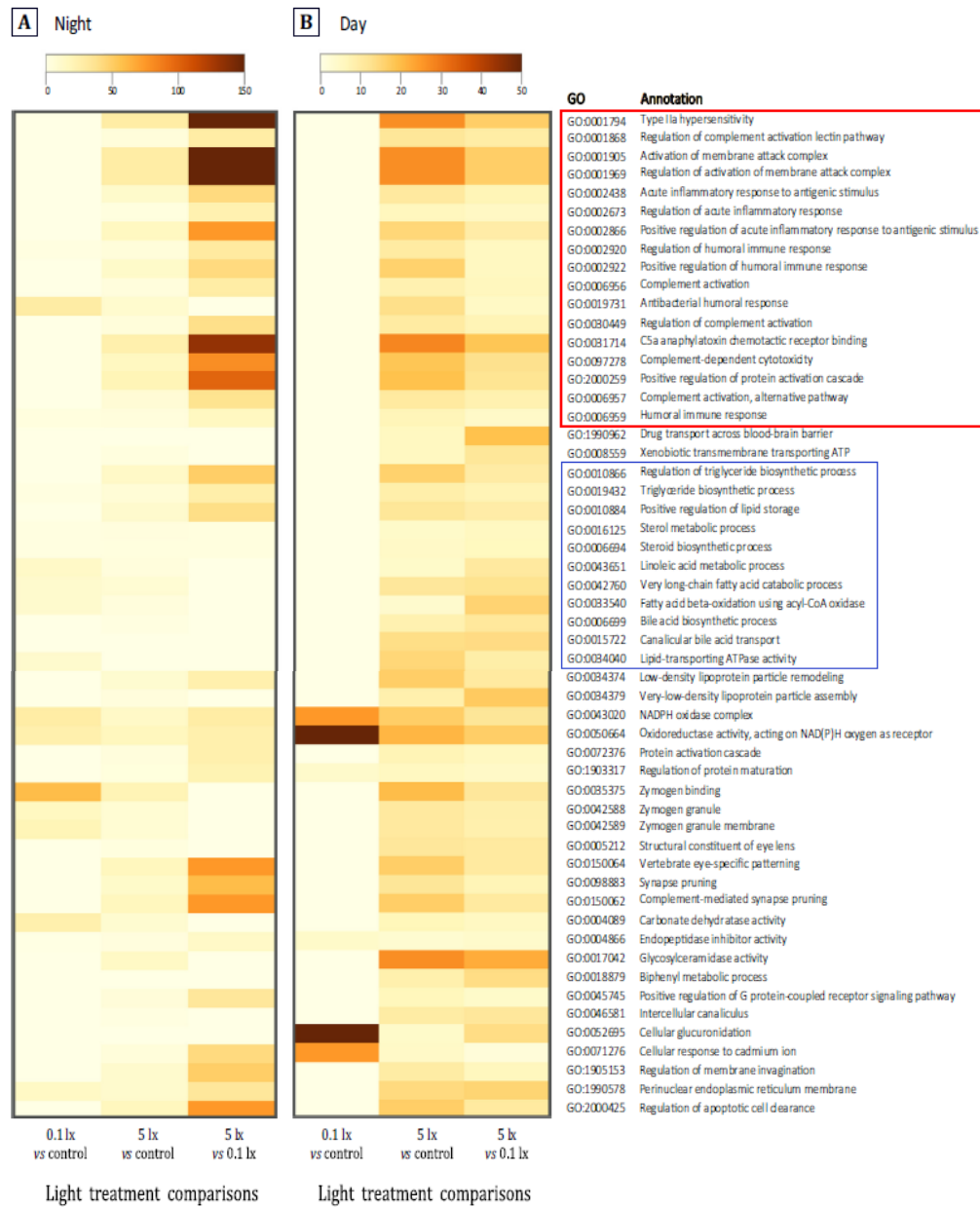


Fig 3

Supplementary Material

Table S1: Enrichment level of the 54 most enriched GO terms (FDR < 0.01 and odds-ratio > 10 at least for one light treatment comparison) for each light treatment comparisons at either night-time or daytime.

GO	Annotation	At night-time			At daytime		
		0.1 lux vs control Odds-ratio (FDR)	5 lux vs control Odds-ratio (FDR)	5 lux vs 0.1 lux Odds-ratio (FDR)	0.1 lux vs control Odds-ratio (FDR)	5 lux vs control Odds-ratio (FDR)	5 lux vs 0.1 lux Odds-ratio (FDR)
GO:0001794	Type IIa hypersensitivity	0.00 (1.10 ¹)	28.57 (2.10 ⁻³)	150.00 (3.10 ⁻⁴)	0.00 (1.10 ¹)	26.67 (4.10 ⁻⁴)	16.67 (2.10 ⁻²)
GO:0001868	Regulation of complement activation lectin pathway	0.00 (1.10 ¹)	4.88 (8.10 ⁻¹)	28.57 (1.10 ⁻¹)	0.00 (1.10 ¹)	11.36 (4.10 ⁻³)	9.43 (7.10 ⁻³)
GO:0001905	Activation of membrane attack complex	0.00 (1.10 ¹)	28.57 (2.10 ⁻³)	150.00 (3.10 ⁻⁴)	0.00 (1.10 ¹)	26.67 (4.10 ⁻⁴)	16.67 (2.10 ⁻²)
GO:0001969	Regulation of activation of membrane attack complex	0.00 (1.10 ¹)	28.57 (2.10 ⁻³)	150.00 (3.10 ⁻⁴)	0.00 (1.10 ¹)	26.67 (4.10 ⁻⁴)	16.67 (2.10 ⁻²)
GO:0002438	Acute inflammatory response to antigenic stimulus	0.00 (1.10 ¹)	11.63 (9.10 ⁻³)	42.86 (5.10 ⁻³)	0.00 (1.10 ¹)	10.64 (5.10 ⁻³)	7.02 (7.10 ⁻²)
GO:0002673	Regulation of acute inflammatory response	0.00 (1.10 ¹)	3.13 (3.10 ⁻¹)	23.68 (1.10 ⁻⁷)	0.00 (1.10 ¹)	6.12 (4.10 ⁻⁶)	5.07 (3.10 ⁻⁵)
GO:0002866	Positive regulation of acute inflammatory response to antigenic stimulus	0.00 (1.10 ¹)	16.67 (1.10 ⁻²)	75.00 (1.10 ⁻³)	0.00 (1.10 ¹)	14.81 (5.10 ⁻³)	9.38 (9.10 ⁻²)
GO:0002920	Regulation of humoral immune response	1.61 (1.10 ¹)	5.20 (10.10 ⁻³)	31.03 (2.10 ⁻⁸)	0.00 (1.10 ¹)	10.05 (4.10 ⁻¹¹)	5.70 (4.10 ⁻⁵)
GO:0002922	Positive regulation of humoral immune response	0.00 (1.10 ¹)	12.20 (7.10 ⁻³)	42.86 (4.10 ⁻³)	0.00 (1.10 ¹)	15.91 (2.10 ⁻⁵)	5.66 (3.10 ⁻¹)
GO:0006956	Complement activation	0.00 (1.10 ¹)	4.40 (5.10 ⁻³)	28.26 (7.10 ⁻¹²)	0.00 (1.10 ¹)	8.05 (4.10 ⁻¹²)	6.11 (3.10 ⁻⁹)
GO:0019731	Antibacterial humoral response	29.41 (3.10 ⁻³)	10.20 (1.10 ⁻²)	0.00 (1.10 ¹)	0.00 (1.10 ¹)	13.21 (6.10 ⁻⁵)	4.69 (4.10 ⁻¹)
GO:0030449	Regulation of complement activation	0.00 (1.10 ¹)	5.19 (4.10 ⁻²)	39.13 (2.10 ⁻⁹)	0.00 (1.10 ¹)	10.14 (6.10 ⁻⁹)	7.30 (3.10 ⁻⁶)
GO:0031714	C5a anaphylatoxin chemotactic receptor binding	0.00 (1.10 ¹)	25.00 (3.10 ⁻³)	133.33 (3.10 ⁻⁶)	0.00 (1.10 ¹)	27.78 (6.10 ⁻⁵)	18.18 (1.10 ⁻³)
GO:0097278	Complement-dependent cytotoxicity	0.00 (1.10 ¹)	16.67 (3.10 ⁻³)	80.00 (7.10 ⁻⁵)	0.00 (1.10 ¹)	18.18 (4.10 ⁻⁵)	12.82 (2.10 ⁻³)
GO:2000259	Positive regulation of protein activation cascade	0.00 (1.10 ¹)	21.05 (5.10 ⁻³)	100.00 (6.10 ⁻⁴)	0.00 (1.10 ¹)	19.05 (2.10 ⁻³)	12.00 (5.10 ⁻²)
GO:0006957	Complement activation, alternative pathway	0.00 (1.10 ¹)	7.22 (8.10 ⁻³)	37.50 (8.10 ⁻⁶)	0.00 (1.10 ¹)	10.38 (1.10 ⁻⁶)	7.81 (4.10 ⁻⁵)
GO:0006959	Humoral immune response	3.09 (1.10 ¹)	3.96 (2.10 ⁻³)	16.88 (2.10 ⁻⁹)	0.00 (1.10 ¹)	6.85 (3.10 ⁻¹⁵)	4.34 (8.10 ⁻⁸)
GO:1990962	Drug transport across blood-brain barrier	0.00 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)	5.56 (1.10 ¹)	19.05 (2.10 ⁻³)
GO:0008559	Xenobiotic transmembrane transporting ATP	0.00 (1.10 ¹)	3.03 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)	5.56 (6.10 ⁻¹)	11.36 (2.10 ⁻³)

GO:0010866	Regulation of triglyceride biosynthetic process	0.00 (1.10 ¹)	13.04 (2.10 ⁻³)	50.00 (3.10 ⁻⁴)	0.00 (1.10 ¹)	16.00 (3.10 ⁻⁶)	9.84 (1.10 ⁻³)
GO:0019432	Triglyceride biosynthetic process	2.44 (1.10 ¹)	7.08 (5.10 ⁻³)	26.32 (4.10 ⁻⁴)	0.00 (1.10 ¹)	8.87 (5.10 ⁻⁶)	7.33 (3.10 ⁻⁵)
GO:0010884	Positive regulation of lipid storage	0.00 (1.10 ¹)	10.53 (5.10 ⁻³)	40.00 (5.10 ⁻⁴)	0.00 (1.10 ¹)	11.29 (2.10 ⁻⁴)	9.33 (5.10 ⁻⁴)
GO:0016125	Sterol metabolic process	1.96 (1.10 ¹)	3.72 (3.10 ⁻³)	2.74 (1.10 ¹)	0.00 (1.10 ¹)	4.26 (9.10 ⁻⁶)	5.64 (8.10 ⁻¹³)
GO:0006694	Steroid biosynthetic process	1.91 (1.10 ¹)	3.18 (2.10 ⁻²)	2.70 (1.10 ¹)	0.00 (1.10 ¹)	4.56 (9.10 ⁻⁷)	5.85 (4.10 ⁻¹⁴)
GO:0043651	Linoleic acid metabolic process	13.33 (1.10 ¹)	4.65 (9.10 ⁻¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)	4.26 (8.10 ⁻¹)	10.53 (10.10 ⁻⁴)
GO:0042760	Very long-chain fatty acid catabolic process	11.11 (1.10 ¹)	8.33 (5.10 ⁻¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)	11.11 (7.10 ⁻²)	12.50 (9.10 ⁻³)
GO:0033540	Fatty acid beta-oxidation using acyl-CoA oxidase	11.11 (1.10 ¹)	4.17 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)	3.70 (1.10 ¹)	15.63 (6.10 ⁻⁴)
GO:0006699	Bile acid biosynthetic process	0.00 (1.10 ¹)	2.25 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)	8.16 (4.10 ⁻⁴)	11.02 (1.10 ⁻⁸)
GO:0015722	Canalicular bile acid transport	0.00 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)	13.33 (8.10 ⁻³)	13.89 (10.10 ⁻⁴)
GO:0034040	Lipid-transporting ATPase activity	11.11 (10.10 ⁻¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)	14.81 (6.10 ⁻³)	9.09 (6.10 ⁻²)
GO:0034374	Low-density lipoprotein particle remodeling	0.00 (1.10 ¹)	9.09 (5.10 ⁻¹)	25.00 (10.10 ⁻¹)	0.00 (1.10 ¹)	16.67 (3.10 ⁻³)	10.34 (7.10 ⁻²)
GO:0034379	Very-low-density lipoprotein particle assembly	0.00 (1.10 ¹)	4.55 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)	8.33 (4.10 ⁻¹)	17.24 (3.10 ⁻⁴)
GO:0043020	NADPH oxidase complex	28.57 (10.10 ⁻³)	13.16 (3.10 ⁻³)	28.57 (5.10 ⁻¹)	25.00 (1.10 ¹)	16.67 (9.10 ⁻⁶)	11.54 (3.10 ⁻⁴)
GO:0050664	Oxidoreductase activity, acting on NAD(P)H oxygen as receptor	25.00 (2.10 ⁻¹)	18.18 (7.10 ⁻³)	25.00 (9.10 ⁻¹)	50.00 (9.10 ⁻¹)	20.83 (2.10 ⁻⁴)	16.67 (2.10 ⁻⁴)
GO:0072376	Protein activation cascade	0.00 (1.10 ¹)	3.93 (10.10 ⁻³)	25.00 (2.10 ⁻¹¹)	0.00 (1.10 ¹)	7.19 (4.10 ⁻¹¹)	5.71 (4.10 ⁻⁹)
GO:1903317	Regulation of protein maturation	0.00 (1.10 ¹)	3.44 (9.10 ⁻²)	22.73 (2.10 ⁻⁸)	3.85 (1.10 ¹)	5.57 (5.10 ⁻⁶)	4.62 (4.10 ⁻⁵)
GO:0035375	Zymogen binding	60.00 (7.10 ⁻³)	21.43 (2.10 ⁻²)	0.00 (1.10 ¹)	0.00 (1.10 ¹)	20.00 (2.10 ⁻²)	11.11 (2.10 ⁻¹)
GO:0042588	Zymogen granule	16.67 (2.10 ⁻²)	8.82 (5.10 ⁻³)	0.00 (1.10 ¹)	0.00 (1.10 ¹)	10.53 (5.10 ⁻⁵)	8.60 (1.10 ⁻⁴)
GO:0042589	Zymogen granule membrane	21.05 (2.10 ⁻²)	7.69 (9.10 ⁻²)	0.00 (1.10 ¹)	0.00 (1.10 ¹)	10.53 (1.10 ⁻³)	8.45 (2.10 ⁻³)
GO:0005212	Structural constituent of eye lens	0.00 (1.10 ¹)	2.80 (10.10 ⁻¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)	11.11 (10.10 ⁻⁸)	10.42 (2.10 ⁻⁹)
GO:0150064	Vertebrate eye-specific patterning	0.00 (1.10 ¹)	18.18 (7.10 ⁻³)	75.00 (9.10 ⁻⁴)	0.00 (1.10 ¹)	16.67 (3.10 ⁻³)	10.34 (7.10 ⁻²)
GO:0098883	Synapse pruning	0.00 (1.10 ¹)	12.50 (3.10 ⁻²)	60.00 (3.10 ⁻³)	0.00 (1.10 ¹)	11.43 (2.10 ⁻²)	6.98 (2.10 ⁻¹)
GO:0150062	Complement-mediated synapse pruning	0.00 (1.10 ¹)	18.18 (7.10 ⁻³)	75.00 (9.10 ⁻⁴)	0.00 (1.10 ¹)	16.67 (3.10 ⁻³)	10.34 (7.10 ⁻²)
GO:0004089	Carbonate dehydratase activity	25.00 (2.10 ⁻³)	9.09 (2.10 ⁻²)	0.00 (1.10 ¹)	0.00 (1.10 ¹)	6.67 (9.10 ⁻²)	5.41 (8.10 ⁻²)
GO:0004866	Endopeptidase inhibitor activity	1.14 (1.10 ¹)	3.73 (2.10 ⁻³)	13.64 (1.10 ⁻⁷)	4.08 (1.10 ¹)	3.23 (2.10 ⁻³)	3.85 (10.10 ⁻⁷)
GO:0017042	Glycosylceramidase activity	0.00 (1.10 ¹)	14.29 (2.10 ⁻¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)	26.67 (7.10 ⁻⁴)	22.22 (5.10 ⁻⁴)
GO:0018879	Biphenyl metabolic process	0.00 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)	8.33 (4.10 ⁻¹)	13.79 (6.10 ⁻³)
GO:0045745	Positive regulation of G protein-coupled receptor signaling pathway	0.00 (1.10 ¹)	5.48 (3.10 ⁻¹)	33.33 (1.10 ⁻³)	0.00 (1.10 ¹)	6.25 (4.10 ⁻²)	4.17 (3.10 ⁻¹)
GO:0046581	Intercellular canaliculus	0.00 (1.10 ¹)	2.17 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)	9.80 (5.10 ⁻³)	11.11 (7.10 ⁻⁵)
GO:0052695	Cellular glucuronidation	0.00 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)	50.00 (1.10 ¹)	4.17 (1.10 ¹)	13.79 (6.10 ⁻³)
GO:0071276	Cellular response to cadmium ion	0.00 (1.10 ¹)	4.88 (8.10 ⁻¹)	42.86 (4.10 ⁻³)	25.00 (1.10 ¹)	4.55 (7.10 ⁻¹)	1.89 (1.10 ¹)

GO:1905153	Regulation of membrane invagination	0.00 (1.10 ¹)	10.53 (4.10 ⁻²)	50.00 (4.10 ⁻³)	0.00 (1.10 ¹)	9.76 (3.10 ⁻²)	6.00 (2.10 ⁻¹)
GO:1990578	Perinuclear endoplasmic reticulum membrane	14.29 (1.10 ¹)	10.53 (4.10 ⁻¹)	33.33 (1.10 ¹)	0.00 (1.10 ¹)	14.29 (3.10 ⁻²)	15.38 (2.10 ⁻³)
GO:2000425	Regulation of apoptotic cell clearance	0.00 (1.10 ¹)	18.18 (7.10 ⁻³)	75.00 (9.10 ⁻⁴)	0.00 (1.10 ¹)	16.67 (3.10 ⁻³)	10.34 (7.10 ⁻²)

Table S2: The top 10 most overrepresented GO terms from the differentially expressed gene sets (FDR <0.05 and |LFC| >1), according to light treatment comparisons and timepoints.

	GO	Annotation	Odds-ratio (FDR)	Differentially expressed genes (down-regulated under higher light level)
0.1 lux vs control	GO:0035375	Zymogen binding	60.00 (7.10 ⁻³)	3 (3)
	GO:0019731	Antibacterial humoral response	29.41 (3.10 ⁻³)	5 (5)
	GO:0043020	NADPH oxidase complex	28.57 (10.10 ⁻³)	4 (2)
	GO:0004089	Carbonate dehydratase activity	25.00 (2.10 ⁻³)	5 (5)
	GO:0017171	Serine hydrolase activity	4.78 (9.10 ⁻³)	11 (9)
5 lux vs control	GO:0006952	Defence response	2.74 (1.10 ⁻³)	32 (23)
	GO:0001969	Regulation of activation of membrane attack complex	28.57 (2.10 ⁻³)	4 (4)
	GO:0031714	C5a anaphylatoxin chemotactic receptor binding	25.00 (3.10 ⁻³)	4 (4)
	GO:2000425	Regulation of apoptotic cell clearance	18.18 (7.10 ⁻³)	4 (4)
	GO:0050664	Oxidoreductase activity, acting on NAD(P)H, oxygen as receptor	18.18 (7.10 ⁻³)	4 (3)
	GO:0097278	Complement-dependent cytotoxicity	16.67 (3.10 ⁻³)	5 (5)
	GO:0043020	NADPH oxidase complex	13.16 (2.10 ⁻³)	5 (4)
	GO:0010866	Regulation of triglyceride biosynthetic process	13.04 (2.10 ⁻³)	6 (6)
	GO:0010884	Positive regulation of lipid storage	10.53 (5.10 ⁻³)	6 (6)
	GO:0042588	Zymogen granule	8.82 (5.10 ⁻³)	6 (6)
5 lux vs 0.1 lux	GO:0030049	Muscle filament sliding	6.30 (7.10 ⁻³)	8 (1)
	GO:0031714	C5a anaphylatoxin chemotactic receptor binding	133.33 (3.10 ⁻⁶)	4 (4)
	GO:0097278	Complement-dependent cytotoxicity	80.00 (7.10 ⁻⁵)	4 (4)
	GO:2000425	Regulation of apoptotic cell clearance	75.00 (9.10 ⁻⁴)	3 (3)
	GO:0098883	Synapse pruning	60.00 (3.10 ⁻³)	3 (3)
	GO:1905153	Regulation of membrane invagination	50.00 (4.10 ⁻³)	3 (3)
	GO:0071276	Cellular response to cadmium ion	42.86 (4.10 ⁻³)	3 (3)
	GO:0010884	Positive regulation of lipid storage	40.00 (5.10 ⁻⁴)	4 (4)
	GO:0045745	Positive regulation of G protein-coupled receptor signalling pathway	33.33 (1.10 ⁻³)	4 (4)
	GO:0006956	Complement activation	28.26 (7.10 ⁻¹²)	13 (12)
5 lux vs control	GO:0019432	Triglyceride biosynthetic process	26.32 (4.10 ⁻⁴)	5 (5)
	GO:0031714	C5a anaphylatoxin chemotactic receptor binding	27.78 (6.10 ⁻⁵)	5
	GO:0001798	Positive regulation of type IIa hypersensitivity	26.67 (4.10 ⁻⁴)	4
	GO:0001905	Activation of membrane attack complex	26.67 (4.10 ⁻⁴)	4
	GO:0017042	Glycosylceramidase activity	26.67 (7.10 ⁻⁴)	4
	GO:0050664	Oxidoreductase activity, acting on NAD(P)H, oxygen as acceptor	20.83 (2.10 ⁻⁴)	5
	GO:2000259	Positive regulation of protein activation cascade	19.05 (2.10 ⁻³)	4
	GO:0097278	Complement-dependent cytotoxicity	18.18 (4.10 ⁻⁵)	6
	GO:0002524	Hypersensitivity	16.67 (3.10 ⁻³)	4
	GO:0034374	Low-density lipoprotein particle remodelling	16.67 (3.10 ⁻³)	4
5 lux vs 0.1 lux	GO:0150062	Complement-mediated synapse pruning	16.67 (3.10 ⁻³)	4
	GO:0017042	Glycosylceramidase activity	22.22 (5.10 ⁻⁴)	4
	GO:1990962	Xenobiotic transport across blood-brain barrier	19.05 (2.10 ⁻³)	4
	GO:0031714	C5a anaphylatoxin chemotactic receptor binding	18.18 (1.10 ⁻³)	4
	GO:0034379	Very-low-density lipoprotein particle assembly	17.24 (3.10 ⁻⁴)	5
	GO:0050664	Oxidoreductase activity, acting on NAD(P)H, oxygen as acceptor	16.67 (2.10 ⁻⁴)	5
	GO:0033540	Fatty acid beta-oxidation using acyl-CoA oxidase	15.63 (6.10 ⁻⁴)	5
	GO:1990578	Perinuclear endoplasmic reticulum membrane	15.38 (2.10 ⁻³)	4
	GO:0015722	Canalicular bile acid transport	13.89 (10.10 ⁻³)	5
	GO:0018879	Biphenyl metabolic process	13.79 (6.10 ⁻³)	4
At night-time	GO:0052695	Cellular glucuronidation	13.79 (6.10 ⁻³)	4
At daytime				

1 **Table S3:** Enrichment level of GO terms related to circadian rhythm according to light treatment
 2 comparisons and timepoints.

3

GO	Annotation	At night-time		
		0.1 lux vs control	5 lux vs control	5 lux vs 0.1 lux
		Odds-ratio (FDR)	Odds-ratio (FDR)	Odds-ratio (FDR)
GO:0007622	Rhythmic behavior	2.78 (1.10 ¹)	1.00 (1.10 ¹)	0.00 (1.10 ¹)
GO:0007623	Circadian rhythm	0.57 (1.10 ¹)	0.40 (1.10 ¹)	1.19 (1.10 ¹)
GO:0009416	Response to light stimulus	0.00 (1.10 ¹)	0.30 (1.10 ¹)	0.88 (1.10 ¹)
GO:0009649	Entrainment of circadian clock	0.00 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)
GO:0010840	Regulation of circadian sleep/wake cycle, wakefulness	0.00 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)
GO:0022410	Circadian sleep/wake cycle process	0.00 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)
GO:0032922	Circadian regulation of gene expression	0.00 (1.10 ¹)	0.00 (1.10 ¹)	3.57 (1.10 ¹)
GO:0042320	Regulation of circadian sleep/wake cycle, REM sleep	0.00 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)
GO:0042745	Circadian sleep/wake cycle	0.00 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)
GO:0042746	Circadian sleep/wake cycle, wakefulness	0.00 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)
GO:0042747	Circadian sleep/wake cycle, REM sleep	0.00 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)
GO:0042749	Regulation of circadian sleep/wake cycle	0.00 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)
GO:0042752	Regulation of circadian rhythm	1.16 (1.10 ¹)	0.83 (1.10 ¹)	0.00 (1.10 ¹)
GO:0042753	Positive regulation of circadian rhythm	0.00 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)
GO:0042754	Negative regulation of circadian rhythm	0.00 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)
GO:0043153	Entrainment of circadian clock by photoperiod	0.00 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)
GO:0045187	Regulation of circadian sleep/wake cycle, sleep	0.00 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)
GO:0045475	Locomotor rhythm	0.00 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)
GO:0045938	Positive regulation of circadian sleep/wake cycle	0.00 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)
GO:0048511	Rhythmic process	0.40 (1.10 ¹)	0.43 (1.10 ¹)	0.85 (1.10 ¹)
GO:0048512	Circadian behavior	2.78 (1.10 ¹)	1.00 (1.10 ¹)	0.00 (1.10 ¹)
GO:0050802	Circadian sleep/wake cycle, sleep	0.00 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)
GO:0060024	Rhythmic synaptic transmission	0.00 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)
GO:1904059	Regulation of locomotor rhythm	0.00 (1.10 ¹)	0.00 (1.10 ¹)	0.00 (1.10 ¹)

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