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Artificial light at night triggers slight transcriptomic effects on melatonin signalling but not synthesis in tadpoles of two anuran species

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34 **Highlights:**

- 35 • Melatonin-related gene expression tested at night under 2 light levels in 2 anurans
- 36 • Gene analysis based on respective species transcriptome and valid reference genes
- 37 • Preliminary description of a **nocturnal** rhythm in gene expression in tadpoles
- 38 • In both species, light pollution did not affect melatonin synthesis at gene level
- 39 • Melatonin signaling was slightly altered by light pollution during the night

Abstract:

The worldwide expansion of artificial light at night (ALAN) is acknowledged as a threat to biodiversity through alterations of the natural photoperiod triggering the disruption of physiological functions. In vertebrates, melatonin production during the dark phase can be decreased or suppressed by nocturnal light as shown in many taxa. But the impact of ALAN at low intensity mimicking light pollution in periurban area has never been investigated in amphibians. We filled this gap by studying the impact of low ALAN levels on the expression of genes related to melatonin synthesis and signaling in two anurans (agile frog, *Rana dalmatina*, and common toad, *Bufo bufo*). Circadian expression of genes encoding enzymes catalyzing melatonin synthesis (aralkylamine N-acetyltransferase, AANAT and acetylserotonin O-methyltransferase, ASMT) or melatonin receptors (Mel1a, Mel1b and Mel1c) was investigated using RT-qPCR after 23 days of exposure to control (< 0.01 lx) or low ALAN level (3 lx) at night. We showed that the relative abundance of most transcripts was low in late afternoon and early evening (06 pm and 08 pm) and increased throughout the night in *R. dalmatina*. However, a clear and ample nocturnal pattern of target gene expression was not detected in control tadpoles of both species. Surprisingly, a low ALAN level had little influence on the relative expression of most melatonin-related genes. Only Mel1c expression in *R. dalmatina* and Mel1b expression in *B. bufo* were affected by ALAN. This target gene approach therefore provides experimental evidence that the melatonin signaling pathway was slightly affected by low ALAN level in anuran tadpoles.

62 **Keywords:**

63 Amphibian; anthropogenic light pollution; gene expression; melatonin receptors; night-time

64 circadian rhythm

1. Introduction

The natural light/dark cycle leads to the rhythmic development of biological processes, from molecules to organisms (Gaston et al., 2013). However, for the last decades, due to the worldwide use and spread of artificial light at night (ALAN), a dramatic alteration of the photoperiod regime beyond its natural range has been observed especially in urbanised areas (Bennie et al., 2016; Cinzano et al., 2001; Gaston et al., 2014, 2012). In both terrestrial and aquatic ecosystems (Davies and Smyth, 2018; Desouhant et al., 2019; Gaston et al., 2013; Jechow and Hölker, 2019; Longcore and Rich, 2004; Secondi et al., 2017), ALAN was shown to impact numerous behavioural and physiological mechanisms altering reproduction and survival (Gaston et al., 2017, 2014; Longcore and Rich, 2004; Schroer and Hölker, 2017; Touzot et al., 2020, 2019). ALAN is therefore likely to threaten population dynamic of wild species (Sanders et al., 2015) and community composition (Davies et al., 2015, 2012; Sanders et al., 2021).

Most of these alterations could be linked to a disruption of circadian timekeeping, mainly regulated by melatonin. In most vertebrates, this hormone, which is only synthesized during nocturnal periods in the absence of light, is defined as the chemical expression of darkness (Reiter, 1991; Zhao et al., 2019). In addition to its function of relaying information about changes in the photoperiod, melatonin plays important physiological roles in the regulation of oxidative stress (Reiter et al., 2016; Tan et al., 2010), immune system (Calvo et al., 2013; Carrillo-Vico et al., 2013; Pohanka, 2013) or sexual hormones (Mayer et al., 1997; Tan et al., 2010). Melatonin is synthesized from tryptophan through four successive enzymatic steps, two of them involving rate-limiting enzymes (Hardeland, 2008). Indeed, melatonin production is limited by the availability of 1) aralkylamine N-acetyltransferase (AANAT), that catalyses the transfer of the acetyl group of Acetyl-CoA to the primary amine of serotonin, thereby producing CoA and N-acetylserotonin, and 2) N acetylserotonin O-

90 methyltransferase (ASMT) that catalyses the methylation of N-acetylserotonin to generate
91 melatonin. The rate of both enzymatic steps depends on the light/dark cycle and therefore
92 impacts melatonin synthesis, but differences in the circadian expression of these enzymes
93 exist between species (Borjigin et al., 1995; Coon et al., 2002; Ribelayga et al., 1999).
94 Melatonin actions are mediated through transmembrane G-protein-linked receptors, with two
95 subtypes identified in mammals (Mel1a and Mel1b) and three subtypes characterized in
96 amphibians, fishes, and birds (Mel1a, Mel1b and Mel1c). In vertebrates, these receptors exist
97 in many structures within the brains and in peripheral tissues, including kidney, liver,
98 intestine, blood, gills and skin (Confente et al., 2010; Hardeland, 2008; Jones et al., 2012).
99 The expression of these receptors also varies along the light/dark cycle (Li et al., 2013).
100 However, slight differences in circadian gene expression pattern exist depending on subtypes,
101 tissues and species (Confente et al., 2010; Jones et al., 2012; Maugars et al., 2020; Rada and
102 Wiechmann, 2006), which could partly be explained by the fact that to exert full physiological
103 action, melatonin receptors may form functional homo or heterodimers (Ayoub et al., 2002).

104 Amphibian species, which are among the most endangered vertebrates today
105 (International Union for Conservation of Nature, IUCN), are particularly exposed to ALAN
106 due to the fact that wetlands are subjected to light pollution (Secondi et al., 2017). Among
107 amphibians, anurans are the most likely to be affected by ALAN (Buchanan, 2006). In
108 anurans, ALAN was shown to increase stress level (Forsburg et al., 2021), alter larval
109 development (Dananay and Benard, 2018), reduce nocturnal activity affecting daily energy
110 allocation (Touzot et al., 2019), impact breeding behaviour and depress male reproductive
111 success (Touzot et al., 2020). Moreover, in adult cane toads, *Rhinella marina*, ALAN reduced
112 and delayed nocturnal activity peak and decreased body mass gain (Secondi et al., 2021).
113 When ALAN intensity is sufficient to alter photoperiod perception, in early stages of
114 development, tadpoles with a relatively immature circadian system and incomplete

differentiation of organs and tissues (Gosner, 1960) might be particularly sensitive to rhythm disruption through ALAN (Fonken and Nelson, 2016).

Although amphibians are an ecological relevant model group to focus on the effects of light pollution studies, consequences of such pollution on melatonin synthesis and signalling have not been investigated yet (Grubisic et al., 2019). The lack of data in amphibians may be explained by the difficulty in measuring melatonin production in non-model animal species. Among the classical techniques for measurements, radio-immuno assay (RIA) and more recently enzyme immunoassay (EIA) can be used (e.g., Delgado and Vivien-Roels, 1989; Edwards and Pivorun, 1991; Jessop et al., 2014; Wright et al., 2006; Wright and Alves, 2001; Bayarri et al., 2004; Rebollar et al., 1999), but they consume a large amount of plasma samples. Conversely, nowadays such invasive blood sampling on adults is not feasible because it involves injury risk on protected species, and the small size of tadpoles precludes RIA analysis. Since proteins, such as enzymes and receptors, are encoded by genes, assessment of gene expression by quantitative reverse transcription polymerase chain reaction (RT-qPCR) appears as a relevant method, with the advantages of being high throughput, repeatable and allowing the assay of multiple genes per sample, thus limiting animal sample size (Ginzinger, 2002).

Thus, this study focused on the influence of ALAN on the expression of genes encoding enzymes catalyzing melatonin synthesis or its receptors in two species of amphibians. Because *Ranidae* and *Bufo* are two of the most widespread and species families of amphibians worldwide (<https://amphibiaweb.org/lists/>), we selected *Rana dalmatina* and *Bufo* to investigate whether a pattern of common consequences exists in two species of anurans. During the breeding season, adults of these species are frequently found in urban and peri-urban areas with wetlands (Beebee, 1979), subjected to light pollution, and laying eggs, themselves exposed to ALAN. In this study, we aimed at (a) validating a RT-qPCR method

valuable on both anuran species, and (b) assessing by RT-qPCR the nocturnal expression of five target melatonin-related genes (AANAT, ASMT, Mel1a, Mel1b and Mel1c) on tadpoles of both species exposed during one third of their aquatic life to low light illuminances: <0.01 lx, which correspond to a control light level, and 3 lx, representing levels encountered in areas hosting amphibians (Secondi et al., 2017).

2. Methods

2.1. Animal collection, housing conditions

Six fragments of approximatively 20 eggs each were randomly sampled from a clutch of both species, *R. dalmatina* and *B. bufo*, in March during the breeding season *in natura* in Saint André de Corcy (46°N, 5°E) and Sainte-Croix (45°N, 5°E), France, respectively. Those sites were chosen as they are located approximatively 20 km from Lyon, reducing ALAN exposure *in natura*. Upon arrival at the animal care facility (EcoAquatron, University of Lyon), the 6 fragments per species were individually placed in an aquarium (32 cm X 17.5 cm X 18.5 cm) containing 7.4 L of dechlorinated and oxygenated water. Each fragment was immediately randomly assigned to one of the two experimental treatments (control, *i.e.*, <0.01 lx, and ALAN night light treatment, *i.e.*, 3 lx), resulting in 3 aquariums per experimental treatment and per species. We used the classification of Gosner (Gosner, 1960) to distinguish the different stages of tadpoles. From stage 25 of Gosner, the stage at which independent feeding is possible, 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 16.0 ± 0.3 °C and $55.3 \pm 0.3\%$, respectively (mean \pm SEM). After egg hatching, tadpole mortality was monitored and was equal to zero during our experiments.

2.2. Light treatments

To mimic natural photoperiod, all aquariums were kept under a photoperiod corresponding to the latitude of Lyon (45°N) at the date of the experiment (at the beginning of the experiment photoperiod of 12.5L/11.5D and at the end of the experiment photoperiod of 14L/10D). 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 1906 ± 159 lx (mean \pm SEM). The spectrum of our daylight is characteristic of fluorescent lamps (Touzot et al., 2021) and is recommended for the housing of amphibians in animal care facility. Until hatching, during night-time, individuals remained under dark conditions (<0.01 lx). From the day of hatching, *i.e.*, 5 days since the start of our study under our conditions, the aquariums were exposed to their respective light treatment at night. During night-time, to simulate ALAN, 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 (Touzot et al., 2021). White LEDs were chosen because they are increasingly used for street lighting worldwide (Falchi et al., 2016). A LED ribbon of 95 cm (57 LEDs) 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 belonging to one light treatment were isolated from the others with tarpaulins to avoid light spilling over from another treatment. At night, the control group was exposed to dark conditions, *i.e.*, <0.01 lx, hereafter called “control-group”, corresponding to the illuminance of a sky under clear conditions with a quarter moon (Gaston et al., 2013). The experimental group was exposed to 3 lx, hereafter called “ALAN-group”, which corresponds to levels encountered in areas hosting amphibians (Secondi et al., 2017) and in urban parks (Segrestin et al., 2021). The daylight and 3 lx illuminances were measured with a luxmeter (Illuminance

meter T-10A, Konica Minolta). Illuminances for the control-group were set using a highly sensitive light meter (Sky Quality Meter SQM-L, Unihedron). Consequently, to compare illuminances, SQM-measured values were converted into lx according to the relationship curve between SQM and lx measurements (Touzot *et al.*, 2020), as lx 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 (0.005 ± 0.001 lx for the control-group and 3.26 ± 0.75 lx for the ALAN-group, mean \pm SEM).

2.3. Experiment

When tadpoles reached development stage 31 of Gosner, corresponding to 23 days of experiment, for each species, 5 tadpoles of each treatment were randomly sampled every two hours between 06 pm and 08 am, resulting in 8 timepoints (random sampling on the 3 aquariums of each light treatment). For each species, this resulted in 80 tadpoles sampled. Development stage 31 of Gosner was chosen, because it is an easily identifiable stage as the foot is paddle-shaped at this stage only Gosner. 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 produced to trigger many functional and morphological changes in the organisms (Galton, 1992; Kikuyama *et al.*, 1993). Tadpoles were individually sampled and immediately placed in liquid nitrogen and then stored at -80°C until molecular analyses. As the main source of melatonin synthesis is located in the pineal gland and eyes in amphibians (Delgado and Vivien-Roels, 1989), RNA extractions were realised on tadpole head, after tissues collection using a section behind the eyes.

2.4. Molecular analyses

2.4.1. Target and housekeeping gene selection

We examined the relative abundance of the transcripts of five target genes related to melatonin; two enzymes: AANAT and ASMT, and three receptors: Mel1a, Mel1b and Mel1c. Finding appropriate housekeeping genes was a prerequisite to limit the biases related to the daily variation in their expression. The majority of studies in amphibians have focused on the stability of housekeeping genes during development (Dhorne-Pollet et al., 2013; Lou et al., 2014), but not at the circadian scale. Since we were interested in temporal monitoring of gene expression, housekeeping genes selected for normalization should have a constant level of expression over 24h (Hellemans et al., 2007). In a first step, using genes selected from developmental studies in amphibians (Dhorne-Pollet et al., 2013; Lou et al., 2014), *i.e.*, rpl8 (60S ribosomal protein L8) and tbp (TATA-binding protein) or circadian rhythm studies in rodents (Kamphuis et al., 2005), *i.e.*, β -actin and GluR2 (AMPA-type glutamate receptor), we performed an analysis of gene expression stability overnight. For each target and housekeeping gene, existing amphibian nucleotide sequences have been searched in the NCBI Nucleotide database (Appendix Table A) and blasted on the respective *de novo* transcriptome of each species (see Touzot et al., 2021 for *B. bufo* transcriptome and see Appendix Text A for *R. dalmatina* transcriptome). After validation of the gene sequences (identity percentages greater than 70% and minimum length of 200 bp), specific primer pairs of 20 bp were created for both species based on their respective *de novo* transcriptome, except for *R. dalmatina* for which GluR2 has not been tested, as it had not been chosen as housekeeping gene in *B. bufo* (Table 1).

Table 1

2.4.2. RNA extraction and cDNA synthesis

Total RNA was extracted by adding 500 μ L of TRI Reagent (Molecular Research Center MRC, TR118) to the 160 samples and by homogenising tissues with a tissue lyser during 2 minutes at 30Hz (Retsch, MM200) and then placing them immediately on ice. All remaining steps were carried out according to the manufacturer's protocol (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). RNA integrity was assessed using a Bioanalyzer 2100 with Agilent RNA 6000 Nano kit (Agilent, 5067-1511). Total RNA was reverse transcribed into cDNA using the transcriptase SuperScript® III (Invitrogen, 18080-044) according to the manufacturer' protocol and using oligo(dt) to isolate mRNAs. cDNA obtained were then treated with Ribonuclease H (RNase H, Invitrogen, AM1907).

2.4.3. RT-qPCR realisation

RT-qPCR was performed on the 160 samples with Universal SYBR Green Supermix (BIORAD, 1725274) according to the manufacturer's protocol. Samples were run in duplicates on a thermocycler CFX96 (BIORAD) with the following program: initial denaturation and enzyme activation for 30 s at 95 °C, followed by 40 cycles of 10 s at 95 °C and 30 s at 60 °C. A melting curve was then created for validation of the PCR products. Quantification cycle (Cq) values were obtained and analyzed with Biorad CFX Manager 3.0. software, using the regression method. Cq values were considered valid if duplicate measurements (Δ Cq) differed by less than 1 cycle, otherwise, both Cq values were removed from the analysis. For each pair of primers, the amplification efficiency was calculated from a standard curve, generated from 6 pre-dosed amplicon range points with a dilution factor of 10, and was comprised between 90 and 110%. For technical reasons, mainly related to an assay

plate effect, some samples had a Δ Cq higher than 1 and had to be removed from the analysis, reducing the sampling size from 80 to 77 samples for AANAT, ASMT and Mel1b and to 75 samples for Mel1c in *R. dalmatina*, and from 80 samples to 65 samples for AANAT, ASMT, Mel1a and Mel1c and to 63 samples for Mel1b in *B. bufo*.

2.4.4. Selection of housekeeping genes, and relative mRNA quantification and analysis

The stability of the four housekeeping genes was analysed using software programs GeNorm v3.5 (Vandesompele et al., 2002) and Normfinder v5 (Andersen et al., 2004), in order to select genes showing the most stable expression necessary for normalization (Hellemans et al., 2007). The two software programs evaluate stability slightly differently. GeNorm estimates the expression stability of a gene, considered as a housekeeping, by calculating the average variation of the expression levels ratios between this gene and another one, also considered as housekeeping gene, in all samples (Vandesompele et al., 2002). Genes with the lowest average variation (*i.e.*, lowest calculated stability value) have the most stable expression. Stability in GeNorm is estimated for the entire dataset without distinction between factors (*i.e.*, light treatment or timepoint). On the other hand, Normfinder estimated the expression stability of a target gene using the Cq values and allows to consider factors effect (*i.e.*, light treatment or timepoint). Target genes were normalised with the selected housekeeping genes using *easyqPCR* v1.21.0 package (Hellemans et al., 2007; Le Pape, 2013), which allows the target genes to be normalized by several housekeeping genes. The normalisation of each target gene was done with the three selected housekeeping genes measured at the same timepoint. Specifically, it converts the Cq values of each sample into a normalized relative quantity, considering the variation in Cq between genes, the efficiency of PCR and the number of housekeeping genes. Relative target gene expression was analysed with a generalised additive model (GAM) as it is the standard and most relevant model for

analysing data with cyclicity (Faraway, 2006; Zuur et al., 2009). The GAM analysis was conducted using the *mgcv* v1.8-28 package (Wood, 2006) with a gamma distribution and inverse function, with timepoint, light treatment and their interaction as explanatory factors. We selected the best models based on the lowest Akaike's Information Criterion (AIC) when the difference in AIC score between two models was higher than 2. A significance threshold of 0.05 was adopted for all statistical analyses. All analyses and graphics were performed with R software v3.5.3 ("R Core Team," 2018).

2.5. Ethical note

The sampling of *R. dalmatina* and *B. bufo* clutches was authorized by the Préfecture de l'Ain (DDPP01-20-369 and 16-271) and by the French government in accordance with the ethical committee of the University of Lyon (APAFIS#27760-2020102115402767 v2). 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 sites.

3. Results

3.1. Housekeeping gene selection

The analysis of housekeeping gene expression stability with GeNorm and Normfinder recommended to select three housekeeping genes in *B. bufo* (Table 2). β -actin, rpl8 and tbp presented the lowest variation in relative expression (*i.e.*, lowest stability value) for both software while GluR2 always showed the highest variability (see Table 2 and Appendices Table B and Table C for details). Therefore, GluR2 has been excluded from our housekeeping gene selection. The three housekeeping genes selected for *B. bufo* also showed a low variation in expression in *R. dalmatina* (Table 2). Therefore, for both species we selected β -actin, rpl8 and tbp as housekeeping genes.

Table 2

3.2. Influence of timepoint and ALAN on melatonin-related gene expression

3.2.1. In *R. dalmatina*

In *R. dalmatina*, the GAM analyses revealed that the relative abundance of transcripts encoding AANAT ($F_{1,76} = 3.64$, $p = 0.028$) and ASMT ($F_1 = 13.32$, $p < 0.001$) was affected by timepoint (see Table 3 and Appendix Table D for details). A low relative abundance of transcripts encoding AANAT and ASMT was observed in the late afternoon and early evening (06 pm and 08 pm, respectively), while it increased throughout the night, nearly doubling in the early morning and 2 hours after the beginning of the day period (06 am and 08 am, respectively) (Fig. 1a and Fig. 1b). Although all melatonin receptor subtypes have been assayed in *R. dalmatina*, only the relative abundance of transcripts encoding Mel1b and Mel1c have been analysed, as the relative abundance of transcripts encoding Mel1a was too low to be exploited (Cq values between 30 and 35, *i.e.*, within the detection limit of RT-qPCR). Timepoint ($F_1 = 21.24$, $p < 0.001$) had a significant effect on the relative abundance of transcripts encoding Mel1b (see Table 3 and Appendix Table D for details). We observed a progressive increase throughout the night to reach its highest value 2 hours after the beginning of the day period (08 am) (Fig. 1c). Light treatment significantly affected the relative abundance of transcripts encoding for melatonin-related gene through an interaction with timepoint in Mel1c (see Table 3 and Appendix Table D for details). In control conditions, the relative abundance of transcripts encoding Mel1c varied with timepoint ($F_{1,32} = 7.72$, $p = 0.002$). Indeed, the relative abundance of transcripts encoding Mel1c was weak in the late afternoon and early evening (06 pm and 08 pm) and increased throughout the night, more than doubling in the early morning and 2 hours after the beginning of the day period (06 am and 08

am) (Fig. 1d). Exposure to ALAN inhibited night-time variation of the relative abundance of transcripts encoding for Mel1c ($F_1 = 0.36, p = 0.549$), as it remained statistically stable throughout the night (Fig. 1d).

Table 3

Figure 1

3.2.2. In *B. bufo*

The GAM analyses showed that the relative abundance of transcripts encoding for Mel1a was significantly affected by timepoint ($F_1 = 6.53, p = 0.013$) (see Table 4 and Appendix Table E for details). The relative abundance of transcripts encoding for Mel1a was low in the late afternoon and early evening (06 pm and 08 pm) and progressively increased throughout the night, to its highest value 2 hours after the beginning of the day period (08 am) (Fig. 2c). Light treatment significantly affected the relative abundance of transcripts encoding for melatonin-related gene through an interaction with timepoint in Mel1b in *B. bufo* (see Table 4 and Appendix Table E for details). Exposure to ALAN induced night-time increase ($F_1 = 7.15, p = 0.009$) in the relative abundance of transcripts encoding for Mel1b (Fig. 2d). While in control conditions, this parameter remained statistically stable throughout the night ($F_{3,51} = 1.06, p = 0.387$). However, the relative abundance of transcripts encoding for AANAT, ASMT and Mel1c (Fig. 2a, 2b and 2e, respectively) was not affected by timepoint (see Table 4 and Appendix Table E for details).

Table 4

Figure 2

4. Discussion

In this study, we developed a RT-qPCR method to investigate the nocturnal changes in the relative expression of two melatonin-synthesis related enzymes (AANAT and ASMT) and three melatonin-related receptors (Mell1a, Mell1b and Mell1c) in tadpoles of two non-model amphibian species under low levels of artificial light at night. Despite variations in relative abundance of most transcripts during the night in *R. dalmatina*, a marked night-time circadian pattern could not be demonstrated in control tadpoles of both species. Contrary to our working hypotheses and surprisingly, a low level of ALAN had little influence on most melatonin-related genes. Only Mell1c expression in *R. dalmatina* and Mell1b expression in *B. bufo* were affected by low levels of ALAN at night.

4.1. Robust molecular methodology based on use of respective transcriptomes and validated housekeeping gene stability

For our RT-qPCR analysis, we gave special attention to the methodological issue to ascertain the correct assessment of relative gene expression in tadpoles. Firstly, considering that our study was conducted on two non-model species, but which are representative of the most widespread and species families of amphibians worldwide, primers were designed with the respective transcriptome of each species, thus favoring sequence identity and detection of the target mRNAs. Each target gene sequences were validated by identity percentages greater than 70% when compared to sequences obtain in different taxa (amphibian, fish, birds, and

mammals) and when the minimum length of the sequence was of 200 bp. In addition, to confirm the correct amplification of the sequences corresponding to our target genes, gene amplification was checked by Sanger sequencing and melting curves confirmed the specificity of amplification. Secondly, finding appropriate housekeeping genes for normalization was a prerequisite to limit the biases related to the daily variation in gene expression. Indeed, the normalization of relative quantities of target genes with housekeeping genes relies on the assumption that these housekeeping genes are stably expressed across all tested samples, stability which is most of the time lacking in the studies (Hellemans et al., 2007). In our study, we tested and verified the stable expression across circadian scale and light treatments of genes commonly used for amphibians, such as rpl8 and tbp (Dhorne-Pollet et al., 2013; Lou et al., 2014) or used in circadian rhythm studies in rodents, such as β -actin and GluR2 (Kamphuis et al., 2005). Among the genes tested, we selected those with the most stable expression across circadian scale and light treatments and opted for a normalization based on multiple housekeeping genes, known to produce more reliable data (Hellemans et al., 2007). Our approach highlights the need to validate housekeeping genes for each biological model and specific experimental conditions. This step is crucial especially when the expression of the targeted genes varies with time. Considering all these methodological parameters, our robust molecular biology methodology gave confidence about the results obtained in this study.

4.2. Lack of marked effects of low ALAN levels on melatonin synthesis

In a recent study, we showed that tadpole global gene expression, through transcriptomic approach, was largely impacted by low levels of ALAN illuminance (Touzot et al., 2021). In the present study, we used target-specific approaches to decipher potential effects of ALAN on melatonin-related gene expression, but these effects were weak and complex. Indeed, we did not detect a marked impact of ALAN on the relative expression of

406 genes encoding melatonin synthesis enzymes. This unexpected result weakens the hypothesis
407 that in tadpoles the negative impact of ALAN could result from an alteration in the level of
408 melatonin synthesis and/or induce a shift in the rhythm of melatonin synthesis as shown in
409 fishes (Brüning et al., 2016, 2015; Kupprat et al., 2020; Vera et al., 2005), in birds (de Jong et
410 al., 2016; Dominoni et al., 2013; Jiang et al., 2020) and in mammals (Le Tallec et al., 2016;
411 Rahman et al., 2019). Several hypotheses could be put forward to explain our results. First,
412 tadpoles could have been exposed to a lower light illuminance than expected (as 3 lx was the
413 illuminance measured at the upper water level). However, melatonin production can be
414 suppressed by very low light illuminances (reviewed in Grubisic et al., 2019). Melatonin
415 production, measured in water tank or measured on individual plasma, is inhibited in fishes
416 and rodents, respectively, as early as 0.01 – 0.03 lx, and in human saliva from 6 lx. Because
417 in this study we only tested one level of ALAN (3 lx), we could also have missed a non-
418 monotone dose-response relationship (inverted U-shaped curve), where the effect of ALAN
419 on relative expression of genes increases up to a certain light level, and then decreases past
420 this threshold. Such pattern have already been observed for physiological parameters (Yang et
421 al., 2018, 2016) and behavioural variables (Gomes et al., 2021; Touzot et al., 2020), whose
422 responses to light exposure is non-linear. Still, our results support the possibility that
423 ecologically relevant and low levels of ALAN may have a masking effect on molecular
424 pacemakers, *i.e.*, circadian clock genes, in vertebrates, as it has been suggested in the diurnal
425 songbird, *Zebra finch* (Alaasam et al., 2021). The masking effect, which appears to occur at
426 low ALAN levels, corresponds to a situation in which the behavior and the physiology are
427 altered by the external environment (*i.e.*, low light intensity) without any change in the central
428 circadian clock genes (Mrosovsky, 1999; Rietveld et al., 1993). Second, the faint nocturnal
429 pattern in the expression of genes encoding melatonin synthesis enzymes in control tadpoles
430 could suggest that at this early stage of development, mechanisms regulating circadian rhythm

may not be fully matured. Indeed, it is known that in addition to a circadian rhythm, the activity of AANAT varies during the development of tadpoles, with a low AANAT activity in the first development stages (Alonzo-Gómez et al., 1994). For instance, in the Perez's frog, *Pelophylax perezi*, an increase of 26% of AANAT activity was observed between the Gosner development stages 26/27 and 40 (Alonzo-Gómez et al., 1994). However, many studies showed that the circadian rhythm of melatonin synthesis is strongly dependent on the light/dark cycle in tadpole (Green et al., 1999) and appears very early in the development of amphibians (Alonzo-Gómez et al., 1994). Indeed, in *P. perezi*, a significant day/night rhythm of AANAT was detected in the eyes at development stage 25 of Gosner and at development stage 43/44 of Nieuwkoop and Faber in the *Xenopus laevis* (Alonzo-Gómez et al., 1994). The small differences in overnight gene expression in tadpoles in the present study could also be partly related to the use of the whole head for the RNA extraction which could contain other melatonin-synthesizing tissues. Indeed, melatonin production was detected in the gut of adult frogs, *Rana pipiens* and *Lithobates catesbeianus* (Bubenik and Pang, 1997) and in various tissues and glands in *L. catesbeianus* tadpoles (Wright et al., 2001). These extra pineal and extra retinal sites of melatonin production could be independent of the light/dark cycle and be rather regulated by tissue demands, such as the local defense against oxidative stress (Acuña-Castroviejo et al., 2014). Alternatively, although a positive correlation between retinal AANAT activity and retina melatonin level has been demonstrated during the light/dark cycle in adult green frogs, *P. esculenta* (d'Istria et al., 1994; Serino et al., 1993), this was not the case in adult European green toads, *Bufo viridis* (Serino et al., 1993). Similarly, in adult Perez's frogs, *P. perezi*, high retina AANAT activity during winter at any time of the day was associated with low retina melatonin synthesis (Delgado et al., 1993). This could also be the case in our species, *i.e.*, enzyme activity did not reflect melatonin synthesis. Indeed, even if

proteins come from the translation of mRNAs, it would be approximate to consider the proteome as the exact reflect of the transcriptome.

4.3. Slight effects of low ALAN levels on melatonin signalling pathway

Present data also showed that the expression of genes encoding melatonin receptor subtypes could be detected in tadpoles of both *R. dalmatina* and *B. bufo*, in accordance with the ontogenic profile of melatonin binding sites in tadpoles of *P. perezi* (Isorna et al., 2005). For both species, we observed weak changes in the relative expression of melatonin receptor subtypes over time, with a global trend to overexpress at the end of the night/early morning. A recent comparison of transcriptomic levels during the day and the night in a freshwater teleost, *Oryzias latipes*, mainly revealed an up-regulation of melatonin receptors during the night, however, photoperiod has also been shown to act in a gene-specific and tissue-specific manner (Maugars et al., 2020). While it is at night that Mel1a increased in the pituitary and the heart, and Mel1c increased in the eye and the skin, Mel1b was significantly higher during the day in the adipose tissue of *O. latipes* (Maugars et al., 2020). Our results also showed for the first time to our knowledge that the expression of genes encoding for membrane melatonin receptors was impacted by ALAN, but without a clear pattern. In *R. dalmatina*, ALAN inhibited the nocturnal increase of Mel1c, a receptor of the quinone reductase 2 family that has only been identified in fish, amphibians, and birds (Li et al., 2013). Sparse studies in amphibians revealed that the Mel1c receptor is located in the retina, brain and peripheral tissues in amphibians (Ebisawa et al., 1994; Rada and Wiechmann, 2006; Wiechmann et al., 2009, 1999) and a reduction of its expression could contribute to an alteration of the overall regulation of melatonin signal transduction (Maugars et al., 2020). Further studies are required to confirm this statement. In *B. bufo*, Mel1b, a receptor which is present in all vertebrates (Dubocovich and Markowska, 2005; Reppert et al., 1994; Slominski et al., 2005) and whose expression is stable over time in the control individuals of our study, was increased

when tadpoles were exposed to ALAN. It therefore appears that low levels of ALAN act on the expression of genes encoding melatonin receptor in a gene-specific and a species-specific manner in amphibians. Such an observation is consistent with previous studies showing that the expression of melatonin receptors varies according to species, geographical area, lighting regime, time of day, stage of neuronal development and endocrine status (Jones et al., 2012; Vanecek, 1998; Witt-Enderby et al., 2003). Most of these studies concern adult organisms and present results suggest that studies on juvenile are necessary to better understand physiological impact of ALAN especially at early stage of organism development.

5. Conclusion

Through the development of a rigorous RT-qPCR method, we have described the nocturnal changes in the relative expression of two melatonin-synthesis related enzymes and three melatonin-related receptors in tadpoles of two non-model amphibian species, *R. dalmatina* and *B. bufo*. For the first time, the results provided experimental evidence that a 3 lx ALAN illuminance, which is encountered in urban and peri urban areas, had weak effects on melatonin-related genes and melatonin signaling at this stage of development in contrast to the deleterious effects of such illuminance on adult physiology and behavior.

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8. Data availability

R. dalmatina de novo transcriptome reads are in the process of being deposited on the of the Sequence Read Archive (SRA) and *de novo* transcriptome assembly is in the process of being deposited on the Transcriptome Shotgun Assembly (TSA) database of the SRA. All data will be available on SRA BioProject.

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Tables and Figures

Table 1: List of primers used for RT-qPCR for each target and housekeeping gene according to species. Target genes are AANAT: aralkylamine N-acetyltransferase, ASMT: N acetylserotonin O-methyltransferase, Mel1a, Mel1b and Mel1c: melatonin receptors. Housekeeping genes are β -actin, rpl8: 60S ribosomal protein L8, tbp: TATA-binding protein, and GluR2: AMPA-type glutamate receptor.

Table 2: Housekeeping gene stability analysis with GeNorm and Normfinder for *B. bufo* and *R. dalmatina*. GeNorm stability values are given for the entire dataset. Normfinder stability was analysed according to light treatment or timepoint. A low stability value indicates a low variation in gene expression either globally or in each of the tested conditions of light treatment or timepoint.

Table 3: GAM results for each target gene in *R. dalmatina* with the AIC scores for the complete model (Timepoint * Light treatment) and the best model. The « * » character indicates the interaction and the additive effects between the explanatory terms. Bold P-values are significant.

Figure 1: Relative abundance of transcripts encoding for target gene in *R. dalmatina* exposed to two light treatments of different night-time illuminance: control-group (black) or ALAN-group (orange) and sampled over 8 timepoints. Each panel corresponds to one melatonin-related target gene with a) AANAT, b) ASMT, c) Mel1b, and d) Mel1c. When the light treatment is significant, both treatments are distinguished (solid dots and lines), while when the light treatment is not significant, the treatments are not distinct (one dashed line). Darkblue shaded areas comprised between 20 and 06 hours correspond to night-time period. Dots represent relative abundance of transcripts of each sample, lines represent the regression from the GAM, and shaded areas correspond to the 95% confidence interval. Relative abundance of transcripts was normalised based on the transcription of β -actin, rpl8 and tbp housekeeping genes.

Table 4: GAM results for each target gene in *B. bufo* with the AIC scores for the complete model (Timepoint * Light treatment) and the best model. The « * » character indicates the interaction and the additive effects between the explanatory terms. Bold P-values were significant.

Figure 2: Relative abundance of transcripts encoding for target gene in *B. bufo* exposed to two light treatments of different night-time illuminance: control-group (black) or ALAN-group (orange) and sampled over 8 timepoints. Each panel corresponds to one melatonin-related target gene with a) AANAT, b) ASMT, c) Mel1a, d) Mel1b and e) Mel1c. When the light treatment is significant, both treatments are distinguished (solid dots and lines), while when the light treatment is not significant, the treatments are not distinct (one dashed line). Darkblue shaded areas comprised between 20 and 06 hours correspond to night-time period. Dots represent relative abundance of transcripts of each sample, lines represent the regression

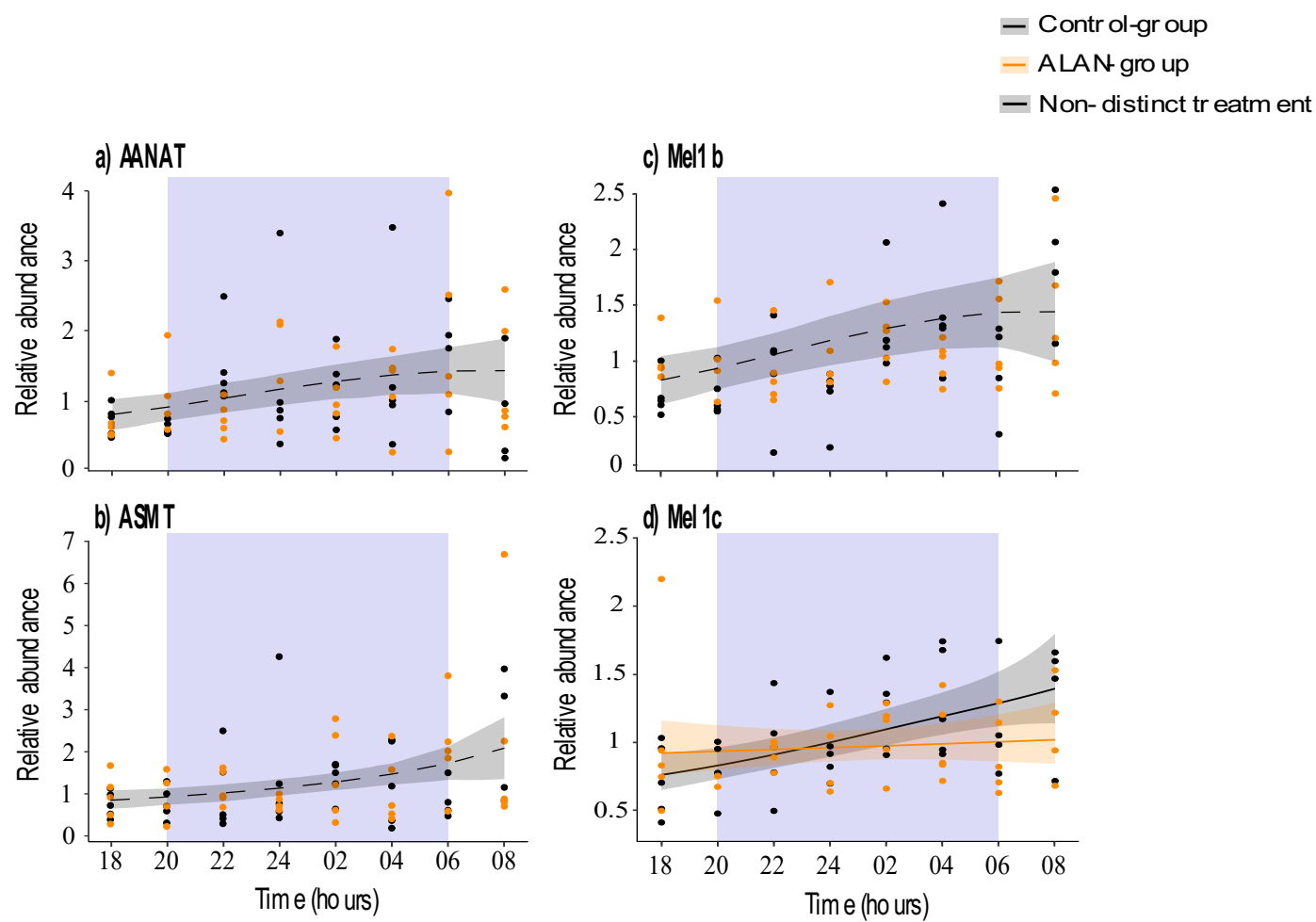
857 from the GAM, and shaded areas correspond to the 95% confidence interval. Relative
858 abundance of transcripts was normalised based on the transcription of β -actin, rpl8 and tbp
859 housekeeping genes.

Gene	Species	Forward 5'-3' primer sequence	Reverse 5'-3' primer sequence	Amplicon size (bp)
<i>Target genes</i>				
AANAT	<i>R. dalmatina</i>	CCTGCCAGTGAATTCCGTTG	CCTGACTTCATCCAGATGGA	116
	<i>B. bufo</i>	CCCGCCAGTGAATTCCGCTG	TCTAACTTCATCCAGGTGGA	116
ASMT	<i>R. dalmatina</i>	TTTCTTCAGTATATCTGATC	TAGCTTGAGGTAGGAATTG	104
	<i>B. bufo</i>	TTTCCACCACGTACTIONTGGTC	CAGCCTGCGGTAAGAAGTG	104
Mel1a	<i>R. dalmatina</i>	TTAATGGGAATAAGTGTCAT	ATCCAACAAATAAATTTGGC	184
	<i>B. bufo</i>	TTAATGGGAATAAGTGTCAT	ATCCAACAAATAAATTTGGC	184
Mel1b	<i>R. dalmatina</i>	TGCTGTTATATACGGATTGC	GCTCTTTGGGCATTCTGTTC	126
	<i>B. bufo</i>	TGCTGTGATTTATGGACTTC	GCTTTTAGGGCACTCTGTTCG	126
Mel1c	<i>R. dalmatina</i>	CAAGCACAGAGTAAGACAAG	GCCACCGCTAGGCCAATAAA	139
	<i>B. bufo</i>	CAAGTGAAGCACAGAGTAAG	GCTACAGCAAGGCCAATGAA	139
<i>Housekeeping genes</i>				
β-actin	<i>R. dalmatina</i>	GAAAATTCTGACTGAGAGGG	TGACCTGCCCATCAGGCAAC	140
	<i>B. bufo</i>	GAAGATTCTTACTGAGAGAG	TGACCTGCCCATCAGGCAAC	140
rpl8	<i>R. dalmatina</i>	GCTAAGCTCAGGGCTGTCTGA	AAGGCAACTTTAGCAAGTGG	109
	<i>B. bufo</i>	GCTAAGCTCAGGGCTGTCTGA	AAGGCAACCTTGGCAAGAGG	109
thp	<i>R. dalmatina</i>	TGAAGAGCAGTCGCGCTTGG	TTACATCACAGCTCCCTACC	115
	<i>B. bufo</i>	AACAATCTCGGTTAGCAGCC	GATGCGTGAGCACGAGACCC	149
GluR2	<i>B. bufo</i>	ACCTTGATTATCATTTTCATC	GAATTCCTTTGTTGACCCTG	149

Species	Gene	GeNorm stability value	Normfinder stability value	
			Light treatment effect	Timepoint effect
<i>B. bufo</i>	β -actin	0.806	0.04	0.23
	rpl8	0.938	0.07	0.26
	tbp	0.993	0.07	0.28
	GluR2	1.006	0.09	0.36
<i>R. dalmatina</i>	β -actin	0.424	0.03	0.10
	rpl8	0.446	0.03	0.10
	tbp	0.439	0.04	0.11

862 **Table 3**

Gene	Complete model AIC	Best model AIC	Explanatory term in the fixed part of the best model	Explained deviance (%)	Factor in the fixed part of the model	F-value	edf	P-value
AANAT	142.3	139.6	Timepoint	12.0	Timepoint	3.64	1.76	0.028
ASMT	168.6	165.0	Timepoint	16.2	Timepoint	13.32	1	<0.001
Mel1b	76.0	77.8	Timepoint	20.3	Timepoint	21.24	1	<0.001
Mel1c	37.5	37.5	Timepoint * Light treatment	19.6	Control-group * Timepoint ALAN-group * Timepoint	7.72 0.36	1.32 1	0.002 0.549



Gene	Complete model AIC	Best model AIC	Explanatory term in the fixed part of the best model	Explained deviance (%)	Factor in the fixed part of the model	F-value	edf	P-value
AANAT	54.2	55.0	Timepoint	5.16	Timepoint	1.48	1.77	0.241
ASMT	115.4	111.5	Timepoint	2.05	Timepoint	0.47	1.42	0.684
Mel1a	136.9	133.3	Timepoint	10.10	Timepoint	6.53	1.00	0.013
Mel1b	132.7	132.7	Timepoint * Light	22.80	Control-group * Timepoint ALAN-group * Timepoint	1.06 7.15	3.51 1	0.387 0.009
Mel1c	101.7	102.7	Timepoint	0.03	Timepoint	0.016	1	0.900

