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

3

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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

40 **Abstract:**

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

60

61

62 **Keywords:**

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

64 circadian rhythm

## 65        **1. Introduction**

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

78            Most of these alterations could be linked to a disruption of circadian timekeeping,  
79 mainly regulated by melatonin. In most vertebrates, this hormone, which is only synthesized  
80 during nocturnal periods in the absence of light, is defined as the chemical expression of  
81 darkness (Reiter, 1991; Zhao et al., 2019). In addition to its function of relaying information  
82 about changes in the photoperiod, melatonin plays important physiological roles in the  
83 regulation of oxidative stress (Reiter et al., 2016; Tan et al., 2010), immune system (Calvo et  
84 al., 2013; Carrillo-Vico et al., 2013; Pohanka, 2013) or sexual hormones (Mayer et al., 1997;  
85 Tan et al., 2010). Melatonin is synthesized from tryptophan through four successive  
86 enzymatic steps, two of them involving rate-limiting enzymes (Hardeland, 2008). Indeed,  
87 melatonin production is limited by the availability of 1) aralkylamine N-acetyltransferase  
88 (AANAT), that catalyses the transfer of the acetyl group of Acetyl-CoA to the primary amine  
89 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

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

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

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



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

## 145 **2. Methods**

### 146 *2.1. Animal collection, housing conditions*

147 Six fragments of approximately 20 eggs each were randomly sampled from a clutch  
148 of both species, *R. dalmatina* and *B. bufo*, in March during the breeding season *in natura* in  
149 Saint André de Corcy (46°N, 5°E) and Sainte-Croix (45°N, 5°E), France, respectively. Those  
150 sites were chosen as they are located approximately 20 km from Lyon, reducing ALAN  
151 exposure *in natura*. Upon arrival at the animal care facility (EcoAquatron, University of  
152 Lyon), the 6 fragments per species were individually placed in an aquarium (32 cm X 17.5 cm  
153 X 18.5 cm) containing 7.4 L of dechlorinated and oxygenated water. Each fragment was  
154 immediately randomly assigned to one of the two experimental treatments (control, *i.e.*, <0.01  
155 lx, and ALAN night light treatment, *i.e.*, 3 lx), resulting in 3 aquariums per experimental  
156 treatment and per species. We used the classification of Gosner (Gosner, 1960) to distinguish  
157 the different stages of tadpoles. From stage 25 of Gosner, the stage at which independent  
158 feeding is possible, to the end of the experiment, tadpoles were fed every two days with  
159 boiled organic green salad. Each week, one-third of the water of the aquarium was renewed  
160 with dechlorinated and oxygenated water. Ambient temperature and relative humidity were  
161 kept constant during the whole experiment at  $16.0 \pm 0.3$  °C and  $55.3 \pm 0.3\%$ , respectively  
162 (mean  $\pm$  SEM). After egg hatching, tadpole mortality was monitored and was equal to zero  
163 during our experiments.

164        2.2. *Light treatments*

165            To mimic natural photoperiod, all aquariums were kept under a photoperiod  
166 corresponding to the latitude of Lyon (45°N) at the date of the experiment (at the beginning of  
167 the experiment photoperiod of 12.5L/11.5D and at the end of the experiment photoperiod of  
168 14L/10D). During the daytime, illuminance provided by light tubes (Philips Master TL-D 58  
169 W/865 and Exo Terra Repti Glo 2.0, 40 W T8) for all aquariums was  $1906 \pm 159$  lx (mean  $\pm$   
170 SEM). The spectrum of our daylight is characteristic of fluorescent lamps (Touzot et al.,  
171 2021) and is recommended for the housing of amphibians in animal care facility. Until  
172 hatching, during night-time, individuals remained under dark conditions ( $<0.01$  lx). From the  
173 day of hatching, *i.e.*, 5 days since the start of our study under our conditions, the aquariums  
174 were exposed to their respective light treatment at night. During night-time, to simulate  
175 ALAN, we used white light-emitting diode (LED) ribbons (white cold Light Plus, 6000-6500  
176 K, 14 W, 60 LED/m) whose spectrum is different from our daylight (Touzot et al., 2021).  
177 White LEDs were chosen because they are increasingly used for street lighting worldwide  
178 (Falchi et al., 2016). A LED ribbon of 95 cm (57 LEDs) combined with a light diffuser was  
179 suspended horizontally 41.5 cm above the bottom of the aquariums. Each LED ribbon was  
180 connected to a dimmer (manual dimmer, 12 V max, 8 A) and a laboratory power supply (15  
181 V/DC max, 3 A), which allowed illuminance to be set for each light treatment. The aquariums  
182 belonging to one light treatment were isolated from the others with tarpaulins to avoid light  
183 spilling over from another treatment. At night, the control group was exposed to dark  
184 conditions, *i.e.*,  $<0.01$  lx, hereafter called “control-group”, corresponding to the illuminance  
185 of a sky under clear conditions with a quarter moon (Gaston et al., 2013). The experimental  
186 group was exposed to 3 lx, hereafter called “ALAN-group”, which corresponds to levels  
187 encountered in areas hosting amphibians (Secondi et al., 2017) and in urban parks (Segrestin  
188 et al., 2021). The daylight and 3 lx illuminances were measured with a luxmeter (Illuminance

189 meter T-10A, Konica Minolta). Illuminances for the control-group were set using a highly  
190 sensitive light meter (Sky Quality Meter SQM-L, Unihedron). Consequently, to compare  
191 illuminances, SQM-measured values were converted into lx according to the relationship  
192 curve between SQM and lx measurements (Touzot *et al.*, 2020), as lx is the main unit used in  
193 ALAN studies (Longcore and Rich, 2004). Illuminances were measured at the water surface  
194 of each aquarium (24 cm from the LED) and checked every week ( $0.005 \pm 0.001$  lx for the  
195 control-group and  $3.26 \pm 0.75$  lx for the ALAN-group, mean  $\pm$  SEM).

### 196 2.3. *Experiment*

197 When tadpoles reached development stage 31 of Gosner, corresponding to 23 days of  
198 experiment, for each species, 5 tadpoles of each treatment were randomly sampled every two  
199 hours between 06 pm and 08 am, resulting in 8 timepoints (random sampling on the 3  
200 aquariums of each light treatment). For each species, this resulted in 80 tadpoles sampled.  
201 Development stage 31 of Gosner was chosen, because it is an easily identifiable stage as the  
202 foot is paddle-shaped at this stage only Gosner. Development stage 31 also allowed long-term  
203 exposure to ALAN, while being distant enough from metamorphosis, *i.e.*, stage 46 of Gosner,  
204 the ultimate stages of development during which thyroid hormones are strongly produced to  
205 trigger many functional and morphological changes in the organisms (Galton, 1992;  
206 Kikuyama *et al.*, 1993). Tadpoles were individually sampled and immediately placed in liquid  
207 nitrogen and then stored at  $-80^{\circ}\text{C}$  until molecular analyses. As the main source of melatonin  
208 synthesis is located in the pineal gland and eyes in amphibians (Delgado and Vivien-Roels,  
209 1989), RNA extractions were realised on tadpole head, after tissues collection using a section  
210 behind the eyes.

### 211 2.4. *Molecular analyses*

#### 212 2.4.1. *Target and housekeeping gene selection*

213 We examined the relative abundance of the transcripts of five target genes related to  
214 melatonin; two enzymes: AANAT and ASMT, and three receptors: Mel1a, Mel1b and Mel1c.  
215 Finding appropriate housekeeping genes was a prerequisite to limit the biases related to the  
216 daily variation in their expression. The majority of studies in amphibians have focused on the  
217 stability of housekeeping genes during development (Dhorne-Pollet et al., 2013; Lou et al.,  
218 2014), but not at the circadian scale. Since we were interested in temporal monitoring of gene  
219 expression, housekeeping genes selected for normalization should have a constant level of  
220 expression over 24h (Hellemans et al., 2007). In a first step, using genes selected from  
221 developmental studies in amphibians (Dhorne-Pollet et al., 2013; Lou et al., 2014), *i.e.*, rpl8  
222 (60S ribosomal protein L8) and tbp (TATA-binding protein) or circadian rhythm studies in  
223 rodents (Kamphuis et al., 2005), *i.e.*,  $\beta$ -actin and GluR2 (AMPA-type glutamate receptor), we  
224 performed an analysis of gene expression stability overnight. For each target and  
225 housekeeping gene, existing amphibian nucleotide sequences have been searched in the NCBI  
226 Nucleotide database (Appendix Table A) and blasted on the respective *de novo* transcriptome  
227 of each species (see Touzot et al., 2021 for *B. bufo* transcriptome and see Appendix Text A  
228 for *R. dalmatina* transcriptome). After validation of the gene sequences (identity percentages  
229 greater than 70% and minimum length of 200 bp), specific primer pairs of 20 bp were created  
230 for both species based on their respective *de novo* transcriptome, except for *R. dalmatina* for  
231 which GluR2 has not been tested, as it had not been chosen as housekeeping gene in *B. bufo*  
232 (Table 1).

233

## 234 **Table 1**

235

### 236 2.4.2. RNA extraction and cDNA synthesis

237

238 Total RNA was extracted by adding 500  $\mu$ L of TRI Reagent (Molecular Research  
239 Center MRC, TR118) to the 160 samples and by homogenising tissues with a tissue lyser  
240 during 2 minutes at 30Hz (Retsch, MM200) and then placing them immediately on ice. All  
241 remaining steps were carried out according to the manufacturer's protocol (Molecular  
242 Research Center MRC, TR118). Then, the RNA was treated with Turbo DNase enzyme  
243 (Turbo DNA free kit, Invitrogen, AM1907) and assayed by fluorescence using a Qubit  
244 fluorometer (Qubit® RNA HS Assay Kits Molecular Probes, Invitrogen, Q32855). RNA  
245 integrity was assessed using a Bioanalyzer 2100 with Agilent RNA 6000 Nano kit (Agilent,  
246 5067-1511). Total RNA was reverse transcribed into cDNA using the transcriptase  
247 SuperScript® III (Invitrogen, 18080-044) according to the manufacturer' protocol and using  
248 oligo(dt) to isolate mRNAs. cDNA obtained were then treated with Ribonuclease H (RNase  
249 H, Invitrogen, AM1907).

#### 250 2.4.3. RT-qPCR realisation

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

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

#### 266 2.4.4. Selection of housekeeping genes, and relative mRNA quantification and analysis

267 The stability of the four housekeeping genes was analysed using software programs  
268 GeNorm v3.5 (Vandesompele et al., 2002) and Normfinder v5 (Andersen et al., 2004), in  
269 order to select genes showing the most stable expression necessary for normalization  
270 (Hellemans et al., 2007). The two software programs evaluate stability slightly differently.  
271 GeNorm estimates the expression stability of a gene, considered as a housekeeping, by  
272 calculating the average variation of the expression levels ratios between this gene and another  
273 one, also considered as housekeeping gene, in all samples (Vandesompele et al., 2002). Genes  
274 with the lowest average variation (*i.e.*, lowest calculated stability value) have the most stable  
275 expression. Stability in GeNorm is estimated for the entire dataset without distinction between  
276 factors (*i.e.*, light treatment or timepoint). On the other hand, Normfinder estimated the  
277 expression stability of a target gene using the Cq values and allows to consider factors effect  
278 (*i.e.*, light treatment or timepoint). Target genes were normalised with the selected  
279 housekeeping genes using *easyqPCR* v1.21.0 package (Hellemans et al., 2007; Le Pape,  
280 2013), which allows the target genes to be normalized by several housekeeping genes. The  
281 normalisation of each target gene was done with the three selected housekeeping genes  
282 measured at the same timepoint. Specifically, it converts the Cq values of each sample into a  
283 normalized relative quantity, considering the variation in Cq between genes, the efficiency of  
284 PCR and the number of housekeeping genes. Relative target gene expression was analysed  
285 with a generalised additive model (GAM) as it is the standard and most relevant model for

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

### 293 2.5. Ethical note

294 The sampling of *R. dalmatina* and *B. bufo* clutches was authorized by the Préfecture  
295 de l'Ain (DDPP01-20-369 and 16-271) and by the French government in accordance with the  
296 ethical committee of the University of Lyon (APAFIS#27760-2020102115402767 v2). The  
297 animal care structure "EcoAquatron" (University of Lyon) received an agreement of  
298 veterinary services (approval DSV 692661201). At the end of the experiment, all the  
299 remaining tadpoles were released on the capture sites.

## 300 3. Results

### 301 3.1. Housekeeping gene selection

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

310

311 **Table 2**

312

313 *3.2. Influence of timepoint and ALAN on melatonin-related gene expression*

314 *3.2.1. In R. dalmatina*

315 In *R. dalmatina*, the GAM analyses revealed that the relative abundance of transcripts  
316 encoding AANAT ( $F_{1,76} = 3.64, p = 0.028$ ) and ASMT ( $F_1 = 13.32, p < 0.001$ ) was affected  
317 by timepoint (see Table 3 and Appendix Table D for details). A low relative abundance of  
318 transcripts encoding AANAT and ASMT was observed in the late afternoon and early  
319 evening (06 pm and 08 pm, respectively), while it increased throughout the night, nearly  
320 doubling in the early morning and 2 hours after the beginning of the day period (06 am and 08  
321 am, respectively) (Fig. 1a and Fig. 1b). Although all melatonin receptor subtypes have been  
322 assayed in *R. dalmatina*, only the relative abundance of transcripts encoding Mel1b and  
323 Mel1c have been analysed, as the relative abundance of transcripts encoding Mel1a was too  
324 low to be exploited (Cq values between 30 and 35, *i.e.*, within the detection limit of RT-  
325 qPCR). Timepoint ( $F_1 = 21.24, p < 0.001$ ) had a significant effect on the relative abundance  
326 of transcripts encoding Mel1b (see Table 3 and Appendix Table D for details). We observed a  
327 progressive increase throughout the night to reach its highest value 2 hours after the beginning  
328 of the day period (08 am) (Fig. 1c). Light treatment significantly affected the relative  
329 abundance of transcripts encoding for melatonin-related gene through an interaction with  
330 timepoint in Mel1c (see Table 3 and Appendix Table D for details). In control conditions, the  
331 relative abundance of transcripts encoding Mel1c varied with timepoint ( $F_{1,32} = 7.72, p =$   
332  $0.002$ ). Indeed, the relative abundance of transcripts encoding Mel1c was weak in the late  
333 afternoon and early evening (06 pm and 08 pm) and increased throughout the night, more than  
334 doubling in the early morning and 2 hours after the beginning of the day period (06 am and 08



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

338

### 339 **Table 3**

340

### 341 **Figure 1**

342

#### 343 *3.2.2. In B. bufo*

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

357

358 **Table 4**

359

360 **Figure 2**

361

#### 362 **4. Discussion**

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

##### 372 *4.1. Robust molecular methodology based on use of respective transcriptomes and* 373 *validated housekeeping gene stability*

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

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

#### 400 *4.2. Lack of marked effects of low ALAN levels on melatonin synthesis*

401 In a recent study, we showed that tadpole global gene expression, through  
402 transcriptomic approach, was largely impacted by low levels of ALAN illuminance (Touzot et  
403 al., 2021). In the present study, we used target-specific approaches to decipher potential  
404 effects of ALAN on melatonin-related gene expression, but these effects were weak and  
405 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

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

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

#### 457 4.3. Slight effects of low ALAN levels on melatonin signalling pathway

458 Present data also showed that the expression of genes encoding melatonin receptor  
459 subtypes could be detected in tadpoles of both *R. dalmatina* and *B. bufo*, in accordance with  
460 the ontogenic profile of melatonin binding sites in tadpoles of *P. perezi* (Isorna et al., 2005).  
461 For both species, we observed weak changes in the relative expression of melatonin receptor  
462 subtypes over time, with a global trend to overexpress at the end of the night/early morning. A  
463 recent comparison of transcriptomic levels during the day and the night in a freshwater  
464 teleost, *Oryzias latipes*, mainly revealed an up-regulation of melatonin receptors during the  
465 night, however, photoperiod has also been shown to act in a gene-specific and tissue-specific  
466 manner (Maugars et al., 2020). While it is at night that Mel1a increased in the pituitary and  
467 the heart, and Mel1c increased in the eye and the skin, Mel1b was significantly higher during  
468 the day in the adipose tissue of *O. latipes* (Maugars et al., 2020). Our results also showed for  
469 the first time to our knowledge that the expression of genes encoding for membrane melatonin  
470 receptors was impacted by ALAN, but without a clear pattern. In *R. dalmatina*, ALAN  
471 inhibited the nocturnal increase of Mel1c, a receptor of the quinone reductase 2 family that  
472 has only been identified in fish, amphibians, and birds (Li et al., 2013). Sparse studies in  
473 amphibians revealed that the Mel1c receptor is located in the retina, brain and peripheral  
474 tissues in amphibians (Ebisawa et al., 1994; Rada and Wiechmann, 2006; Wiechmann et al.,  
475 2009, 1999) and a reduction of its expression could contribute to an alteration of the overall  
476 regulation of melatonin signal transduction (Maugars et al., 2020). Further studies are  
477 required to confirm this statement. In *B. bufo*, Mel1b, a receptor which is present in all  
478 vertebrates (Dubocovich and Markowska, 2005; Reppert et al., 1994; Slominski et al., 2005)  
479 and whose expression is stable over time in the control individuals of our study, was increased

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

## 488 **5. Conclusion**

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

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## 505 **8. Data availability**

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

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815 **Tables and Figures**

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

821

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

827

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

832

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

843

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

848

849 **Figure 2:** Relative abundance of transcripts encoding for target gene in *B. bufo* exposed to  
850 two light treatments of different night-time illuminance: control-group (black) or ALAN-  
851 group (orange) and sampled over 8 timepoints. Each panel corresponds to one melatonin-  
852 related target gene with a) AANAT, b) ASMT, c) Mel1a, d) Mel1b and e) Mel1c. When the  
853 light treatment is significant, both treatments are distinguished (solid dots and lines), while  
854 when the light treatment is not significant, the treatments are not distinct (one dashed line).  
855 Darkblue shaded areas comprised between 20 and 06 hours correspond to night-time period.  
856 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  $\beta$ -actin, rpl8 and tbp  
859 housekeeping genes.



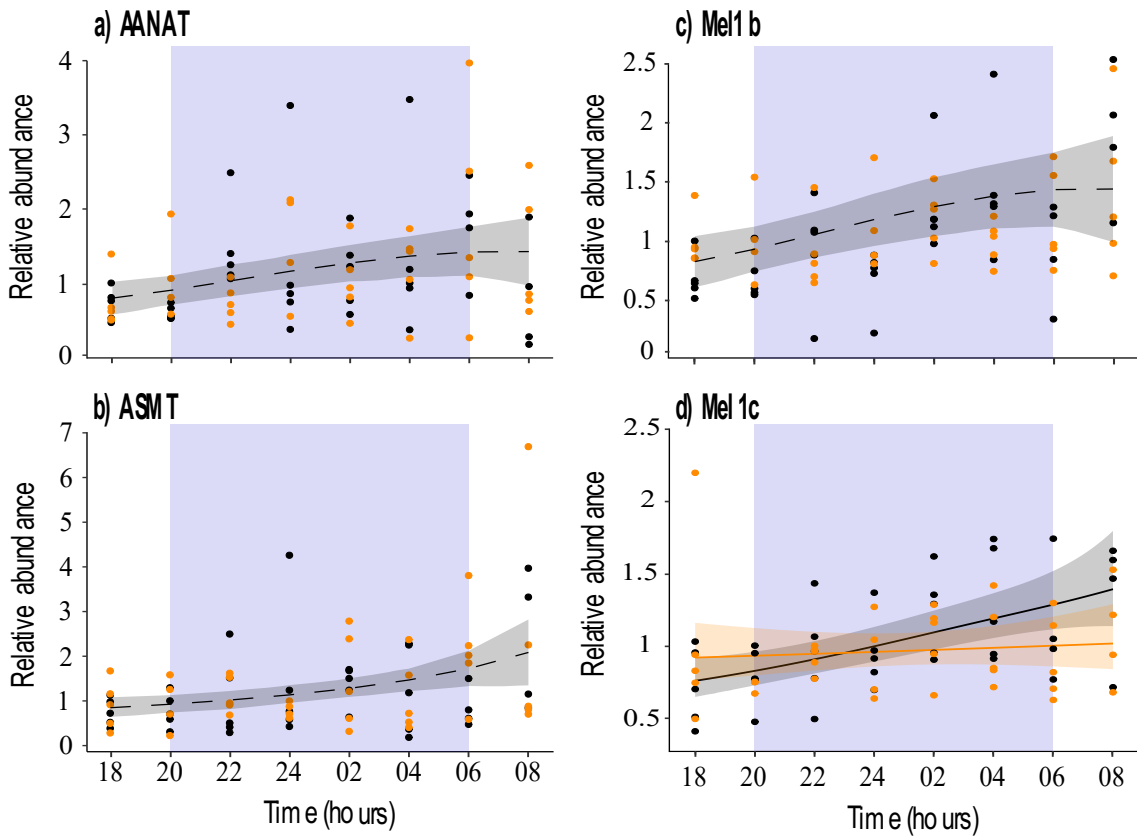
<b>Gene</b>	<b>Species</b>	<b>Forward 5'-3' primer sequence</b>	<b>Reverse 5'-3' primer sequence</b>	<b>Amplicon size (bp)</b>
<i>Target genes</i>				
<b>AANAT</b>	<i>R. dalmatina</i>	CCTGCCAGTGAATTCCGTTG	CCTGACTTCATCCAGATGGA	116
	<i>B. bufo</i>	CCCGCCAGTGAATTCCGCTG	TCTAACTTCATCCAGGTGGA	116
<b>ASMT</b>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

Species	Gene	GeNorm stability value	Normfinder stability value	
			Light treatment effect	Timepoint effect
<i>B. bufo</i>	$\beta$ -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>	$\beta$ -actin	0.424	0.03	0.10
	rpl8	0.446	0.03	0.10
	tbp	0.439	0.04	0.11

862 **Table 3**

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

- Control-group
- ALAN-group
- Non-distinct treatment



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
<b>Mel1a</b>	136.9	133.3	Timepoint	10.10	Timepoint	6.53	1.00	<b>0.013</b>
<b>Mel1b</b>	132.7	132.7	Timepoint * Light	22.80	Control-group * Timepoint ALAN-group * Timepoint	1.06 7.15	3.51 1	0.387 <b>0.009</b>
<b>Mel1c</b>	101.7	102.7	Timepoint	0.03	Timepoint	0.016	1	0.900

— Control-group  
— ALAN-group  
— Non-distinct treatment

