



# Impact of artificial light at night on zebrafish circadian rhythms: Insights from behavioural and molecular data

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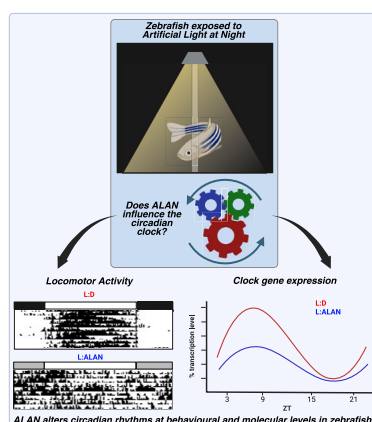
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## HIGHLIGHTS

- Artificial light at night (ALAN) affects circadian rhythms.
- Daily locomotor activity of zebrafish exposed to ALAN is altered.
- ALAN dysregulates the daily expression of clock genes and opsins.
- Integrative research is crucial for understanding ALAN's impact.

## GRAPHICAL ABSTRACT



## ARTICLE INFO

### Keywords:

Circadian clock  
*Danio rerio*  
 Light pollution  
 Locomotor activity  
 ALAN

## ABSTRACT

Artificial light at night (ALAN) is an emerging environmental stressor that alters natural light cycles and disrupts circadian regulation across a wide range of species and ecosystems. By interfering with the environmental cues that have shaped circadian timekeeping mechanisms, ALAN poses a growing concern for circadian physiology and behaviour. In this study, we investigated the effects of ALAN on behavioural and molecular rhythmicity in the diurnal teleost *Danio rerio*, the zebrafish. Fish were exposed to either a natural light-dark (L:D) cycle or a light-ALAN (L:ALAN) regimen. Locomotor activity recordings revealed alterations in behavioural pattern mostly associated to arrhythmicity under ALAN, whereas L:D exposed fish exhibited robust circadian rhythms with clear diurnal patterns. To explore the molecular basis of this disruption, we performed RNA sequencing on brain tissue collected at four time points across the day. Transcriptomic analysis revealed significant alterations in the expression of core circadian clock and opsin genes in ALAN exposed zebrafish. Both positive and negative regulators of the molecular clock exhibited reduced rhythmic amplitude, indicating a dampening of endogenous circadian oscillations. These results demonstrate that ALAN profoundly affects both behavioural and molecular

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<https://doi.org/10.1016/j.scitotenv.2025.181077>

Received 4 July 2025; Received in revised form 15 November 2025; Accepted 25 November 2025

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circadian rhythms in zebrafish. Given the widespread use of artificial lighting and its known ecological impacts, our findings highlight the need for integrative approaches, linking molecular, physiological, and behavioural data, to better understand the biological impacts of light pollution. Such insights are critical for informing conservation strategies and mitigating the effects of ALAN on wildlife and ecosystems.

## 1. Introduction

Life on Earth is synchronized with natural geophysical cycles, such as the day-night cycle and seasons (Pittendrigh, 1993; Roenneberg and Merrow, 2002). Almost all organisms have evolved internal timekeeping systems, like the circadian clock, to coordinate biochemical, physiological, and behavioural processes. Such internal clocks allow them to anticipate and synchronize (*entrain*) their rhythms with environmental signals (*zeitgeber*), such as the light-dark cycle, indicative of the actual time of day (Roenneberg et al., 2003; Hut and Beersma, 2011). In vertebrates, circadian clocks are organized in a multi-oscillatory network including a central oscillator located in the brain, and many peripheral oscillators found in other brain nuclei and tissues throughout the body. Despite the differences occurred during evolution, the basic properties of the circadian clock have been conserved in eukaryotes and, at molecular level, the circadian clock mainly consists of overlapping transcription-translation feedback loops composed by positive and negative regulators (Mihut et al., 2025). In vertebrates, the positive regulators involve the bHLH PAS transcription factors CLOCK and BMAL (also known as ARNTL), which form heterodimers and act as transcriptional activators of the negative regulators, the *per* and *cry* genes, via binding to E-box enhancer elements in their promoter. Subsequently, the PER:CRY heterocomplexes translocate into the nucleus and inhibit the CLOCK:BMAL complex transcriptional activity and their own transcription. The resulting reduction in PER and CRY protein levels ultimately releases CLOCK and BMAL to drive a new cycle of activation. A second feedback loop involves two orphan nuclear receptors, REV-ERB $\alpha/\beta$  (also known as NR1D1/2) and ROR $\alpha$ , which compete to either inhibit or promote the rhythmic expression of *bmal*. Oscillations in gene expression resulting from these feedback loops have period lengths of approximately 24 h, ultimately giving rise to overt circadian rhythms (Takahashi, 2017).

The photic entrainment of locomotor activity is one of the most studied temporally organized behaviours at molecular and physiological levels in vertebrates (Roenneberg and Foster, 1997; Ashton et al., 2022). In non-mammalian vertebrates, including most teleosts, light directly entrains central pacemaker structures and peripheral organs and tissues (Bertolucci and Foà, 2004). This process is mediated by different signalling pathways that involves visual and non-visual photoreceptive organs (Steindal and Whitmore, 2019). In these organs the light-sensitive complexes that absorb photons and trigger a signalling cascade are photopigments composed of an opsin, a 7-domain transmembrane G-protein-coupled receptor, bound to a light-sensitive chromophore. Opsins have also been extensively characterized in zebrafish, where 42 different opsin genes have been identified, with 10 corresponding to visual opsins and 32 to non-visual opsins, which are expressed in ocular and extraocular tissues (Davies et al., 2015). Furthermore, light-sensitive circadian pacemakers have been found in all tissues and in many cells of most fish species examined to date (Steindal and Whitmore, 2019, 2020). For instance, zebrafish *per2* and *cry1* mRNA expression is induced by direct light exposure through the action of D-box enhancer promoter elements (Vatine et al., 2009; Mracek et al., 2012; Steindal and Whitmore, 2020). This induction, which has been also found in other teleosts (Cuesta et al., 2014; Vergès-Castillo et al., 2024), directly mediates photoentrainment and resets the zebrafish clock (Idda et al., 2012; Vatine et al., 2011).

Given the importance of natural light-dark cycles as *zeitgeber*, artificial light at night (ALAN) pollution, defined as the “increased volume concentration of photons in the nocturnal environment above naturally

expected values” (Linares Arroyo et al., 2024), has become an emerging environmental stressor affecting virtually all habitats (Cox and Gaston, 2023; Davies and Smyth, 2018; Marangoni et al., 2022; Moore et al., 2006). In a circadian perspective, the presence of ALAN changes the night-time environment, impacting circadian physiology and behaviour of freshwater, marine and terrestrial species, including humans (Christoforou et al., 2023; Stanton and Cowart, 2024; Duarte et al., 2019; Pulgar et al., 2019; Wolkoff et al., 2023; Hölker et al., 2023; Marangoni et al., 2022). ALAN has shown to impact the biology of many species, especially the nocturnal ones, which represent a large portion of the metazoans (Hölker et al., 2010). Like all other anthropogenic disturbances (Palumbi, 2001), light pollution represents a new evolutionary force, but its impact on organisms that evolved in consistent light and dark cycles is still largely unknown (Hölker et al., 2021).

The impact of ALAN on fish colonising both freshwater and marine habitats has been well documented at different levels, from physiology to behaviour (Bassi et al., 2022). For instance, ALAN changes foraging activity in Japanese eels (*Anguilla japonica*) in urbanized estuarine and riverine habitats (Matsushige and Hibino, 2023) and alters the migration of smolts and the dispersal of fry of Atlantic salmon (*Salmo salar*) (Riley et al., 2012, 2013). ALAN also reduced blood concentration of sex steroids (estradiol and testosterone) and mRNA expression of gonadotropins in the roach (*Rutilus rutilus*) and the European perch (*Perca fluviatilis*) (Brüning et al., 2018).

In our study, we investigated the effect of ALAN on behavioural and molecular rhythmicity of a small teleost, the zebrafish *Danio rerio*. Although the effects of ALAN on biological rhythms are increasingly documented in various model and non-model species, significant gaps remain in the literature. Among these, for example, the impact of ecologically relevant levels of ALAN on the fundamental properties of the circadian system in a well-established vertebrate model such as zebrafish has never been investigated. Zebrafish provides a fascinating opportunity to assess such concern because its molecular circadian clock and behavioural circadian rhythms have been well-characterized (Steindal and Whitmore, 2019; Idda et al., 2012; Vatine et al., 2011). To reach our research goal, we first investigated the daily locomotor activity of zebrafish exposed to ALAN, to understand the effect of this stressor on the fundamental properties of an entrained, circadian clock-controlled behavioural rhythm. We then evaluated the transcriptional expression pattern of specific genes, including circadian clock genes and opsins, which are notoriously involved in the temporal regulation of various behavioural and physiological processes. We hypothesized that long-term exposure to ecologically relevant ALAN levels in microcosms could impact the photic entrainment of locomotor activity and alter clock gene and opsin expression rhythms in adult zebrafish.

## 2. Materials and methods

### 2.1. Ethics statement

All husbandry and experimental procedures complied with European Legislation for the Protection of Animals used for Scientific Purposes (Directive 2010/63/EU) and the Italian (D.lgs. 26/2014) animal protection standards. General license for fish maintenance and breeding at the University of Ferrara: 29/2023-UT. The experimental protocol was authorized by the University of Ferrara Institutional Animal Care and Use Committee (auth. n. 128/2022-PR and TLX-2022-1).

## 2.2. In-house animals' condition

The zebrafish were derived from a wild-type strain ('Ariosto') established and maintained at the University of Ferrara's facility since 2011. The population consists of approximately 1500 individuals (sex ratio 50:50) and is maintained by routinely performing reproduction with standard procedures, using breeders randomly selected from the different tanks. All aquaria had a recirculating water system equipped with mechanical and biological filters. A constant water temperature in aquaria at  $28 \pm 1$  °C was maintained by means of an automatic air conditioning system. Food was provided twice per day, consisting in live nauplii of *Artemia salina* and commercial flakes (Staple food Vipan, Sera, Heinsberg, Germany). Zebrafish were exposed to a 14 h light/10 h dark photoperiod using light-emitting diodes (LED) lamps placed on the ceiling of the facility and controlled by an electronic timer. A total of 136 zebrafish (8–12 months old; sex ratio 50:50) were used in the experiments (February–March 2024).

## 2.3. Experimental set up

In all experiments, 22 aquaria ( $41 \times 25 \times 24$  cm; water level: 20 cm) were kept in a chronobiology chamber, completely isolated, where light and temperature were controlled. The aquaria had walls covered in black plastic and a black plastic lid to prevent external disturbances and were equipped with water filters and aeration. The temperature was maintained at  $28 \pm 1$  °C in both the chamber and the aquaria by means of an automatic heat pump air conditioning (heating/cooling) system (SUZ-SA71VA3 and PCA-M KA2, Mitsubishi Electric, Japan). The water temperature was recorded every 10 min using underwater data loggers (Hobo Pendant®, Onset Computer Corporation, Massachusetts, USA). Food (Staple food Vipan, Sera, Heinsberg, Germany) was provided daily at random times to prevent any behavioural synchronisation.

Zebrafish were maintained under two lighting regimes: a standard 12:12 light-dark (L:D) cycle and a 12:12 light-artificial light at night (L:ALAN) cycle. In both treatments, illumination was provided by light-emitting diodes (natural daylight, 6000 K; TMR, distributed by ELCART, Italy). Irradiance was measured using a radiometer (DO9721, Probe LP9021 RAD, spectral range 400–950 nm; DeltaOHM, Padova, Italy). During the light phase, irradiance was set at 45.3 lx ( $0.358 \text{ W m}^{-2}$ ) in all aquaria, representing a daytime condition previously used in behavioural experiments in zebrafish (De Russi et al., 2024, 2025; Lucon-Xiccato et al., 2023). In the L:D treatment, LEDs were completely turned off during the dark (D) phase (0 lx). In contrast, in the L:ALAN treatment, a short LED strip provided continuous low-level illumination ( $3.3 \text{ lx}$ ;  $0.026 \text{ W m}^{-2}$ ) throughout the night phase. This nocturnal light intensity falls within the range reported for urban and peri-urban freshwater environments affected by artificial light at night (Brüning et al., 2015; Gaston et al., 2013; Perkin et al., 2014) and may therefore be considered ecologically realistic for impacted aquatic habitats. Locomotor activity was measured throughout the entire experiment, using infrared photocells (Omron, mod E3S-AD62, Japan) placed against the aquarium wall and connected to a computer (Di Rosa et al., 2024; Morbiato et al., 2019). Every time a fish interrupted the infrared light beam, the photocells sent an output signal that was recorded and stored at 10-min intervals using specialized software (DIO98USB; University of Murcia, Spain).

## 2.4. Experimental treatments

A first batch of zebrafish was exposed to 12:12 L:D or 12:12 L:ALAN cycles (12 aquaria in total, 6 aquaria and 48 zebrafish per condition,  $N = 96$  zebrafish in total) for 50 days, and their activity was continuously recorded (Fig. S1). During the first 25 days lights was turned on at 06:00 (L:D<sub>1</sub> and L:ALAN<sub>1</sub>). During the next 25 days of recording, zebrafish from both groups were subjected to a 6-h delay in the switching on of the lights from 06:00 to 12:00 (L:D<sub>2</sub> and L:ALAN<sub>2</sub>) to assess the fish's ability

to readjust their rhythms to the new light cycle.

In parallel, a second batch of adult zebrafish ( $N = 80$ ) was exposed to the 12:12 L:D or 12:12 L:ALAN lighting conditions (8 aquaria, 4 aquaria per treatment,  $N = 10$  per aquarium,  $N = 40$  per condition) for 30 days (Fig. S1). Forty zebrafish ( $N = 5$  per aquarium;  $N = 20$  per conditions) were randomly selected from these 80 zebrafish for gene expression analyses (RNA-seq analysis and qRT-PCR), as explained hereafter. The excess zebrafish in the experimental aquaria ( $N = 5$  per aquarium,  $N = 40$ ) minimized potential stress effects caused by reduced social companionship during sampling. At the end of this experiment, they were returned to the fish housing tanks.

## 2.5. RNA extraction and gene expression analysis

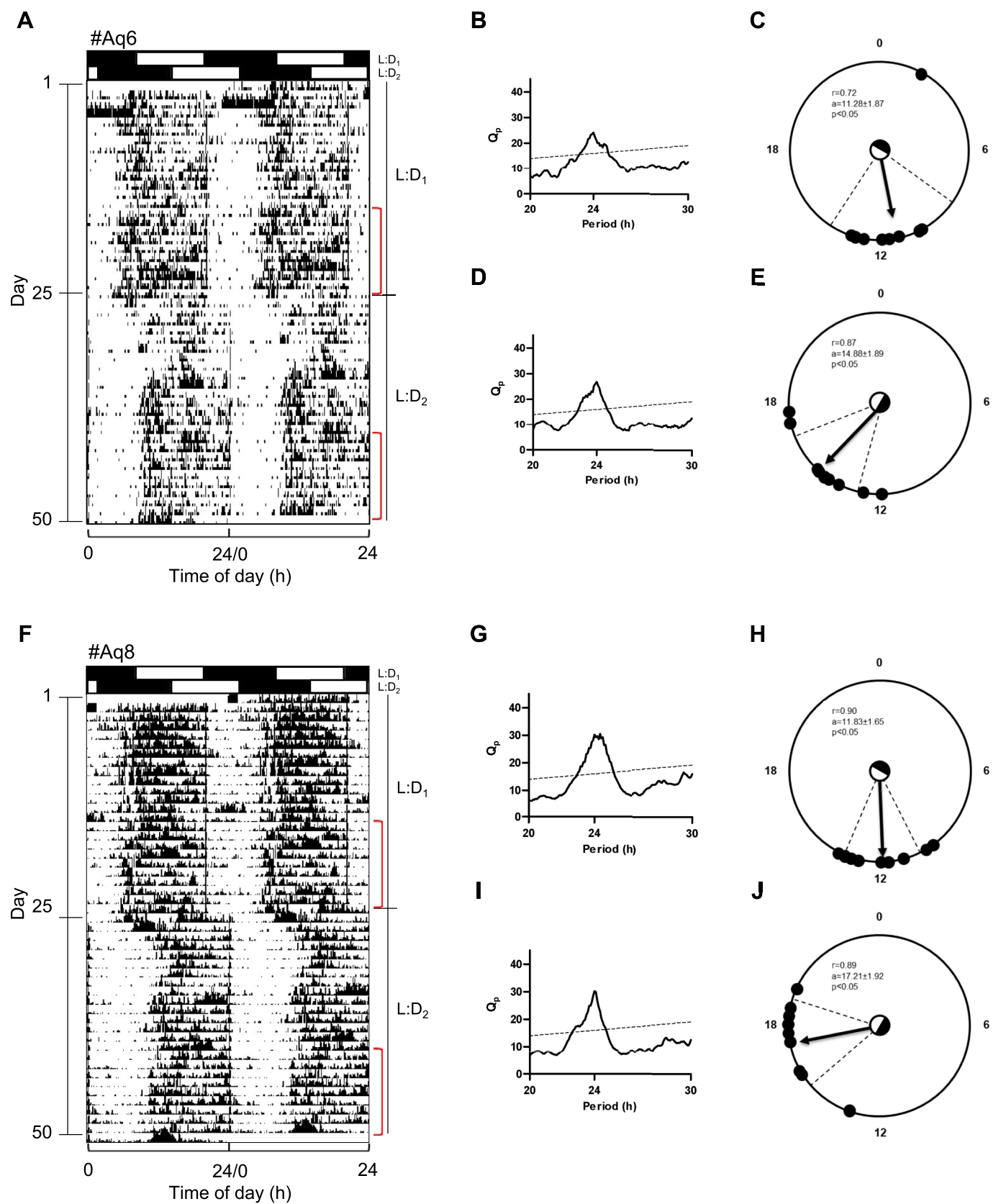
RNA-seq analysis was conducted using the whole brains of zebrafish, sampled at four timepoints, spanned along the 24 h every 6 h, from 09:00. It is conventional to divide the 24-h L:D cycle into 24-h Zeitgeber Time (ZT) units and indicate the time of lights on as ZT0 (corresponding to 06:00) and the time of lights off as ZT12 (corresponding to 18:00). Zebrafish brains were sampled at ZT3 (09:00), ZT9 (15:00), ZT15 (21:00) and ZT21 (03:00), two timepoints during the light phase and two during the dark phase. We applied the same convention also to the 24-h L:ALAN cycle. Total RNA extraction was performed as explained in Vergata et al. (2025), included DNaseI treatment and quantitative and qualitative assessment. RNA samples were then shipped to Novogene UK Company Ltd. (Cambridge, UK) for RNA-seq library preparation and sequencing with the Illumina Novaseq6000 platform (150 bp paired-end). RNA-seq raw data were handled using Novogene in-house pipelines to remove adapters and low-quality data. The cleaned RNA reads were aligned to the *Danio rerio* GRCz11 reference genome assembly using Hisat2 v2.0.5 (Kim et al., 2019). To count the number of reads mapped to each gene, the mapping files were processed using FeatureCounts v1.5.0-p3 (Liao et al., 2014). In order to explore a possible lack of rhythmicity at molecular level in zebrafish exposed to ALAN, we selected 22 genes from the total transcriptomic analysis (Vergata et al., 2025), including circadian clock genes, light-inducible genes and opsins (Table 1), to investigate daily variation in their expression levels at the four timepoints across the day. We analysed normalized read count values from each sample for this purpose.

## 2.6. Validation of rhythmic expression by qRT-PCR

RT-qPCR was performed on a subset of six clock genes (*clock1a*, *arr11a*, *cry5*, *per1b*, *per2* and *cry1a*) that are cardinal components for both generation of circadian rhythmicity and photic entrainment (Idda et al., 2012; Vatine et al., 2011; Mracek et al., 2012; Steindal and Whitmore, 2019, 2020), to validate the transcriptomic results. DNase I-treated RNAs were reverse transcribed to cDNA with iScript cDNA Synthesis Kit (Biorad, Italy) according to manufacturer instructions. cDNAs were then used as the template for qRT-PCR amplification of the six selected genes. The 10 µL PCR reaction mix contained 2 µL of cDNA, 5 µL SsoAdvanced Universal SYBR Green Supermix (Biorad, Italy), 0.4 µL of each primer and 2.2 µL of ddH<sub>2</sub>O. The Real-time qPCR cyclers (CFX Real-Time PCR Systems) program was 10 min at 95 °C, 40 cycles of annealing and extension at 60 °C for 30 s and denaturation at 95 °C for 5 s. Dissociation curve was used to confirm the specificity of the amplicon. The values obtained were used to calculate gene expression by the  $2^{-\Delta\Delta C_t}$  method and normalized to the housekeeping gene 18S. All primer sequences are provided in Table S1.

## 2.7. Data analysis

Representation of actograms, calculation of periodogram and daily acrophases were performed using the chronobiology software El Temps (version 1.275; Prof. Díez-Noguera, University of Barcelona). Data were reported as mean and standard errors (mean  $\pm$  SE) and analysed by



**Fig. 1.** Daily activity rhythms of adult zebrafish. Representative actograms (A, F),  $\chi^2$  periodogram analysis (B, D, G, I) and circular diagrams (C, E, H, J) of locomotor activity of groups of zebrafish subjected to 12:12 L:D cycles. Actograms are double plotted on a 48 h timescale to help the interpretation. Each horizontal line is a record of 48 h of activity; each subsequent 48 h cycle is plotted beneath the previous one. The y axis progresses in single days with each day being plotted twice (day 1 on the right side is repeated on day 2 on the left side). The height of each point represents the number of infrared light-beam interruptions in 10 min. The number of days is indicated on the left and the time of day is plotted on the bottom of each actogram. Starting and ending day of each L:D cycle (L:D1 and L:D2) is shown on the right of the actogram. Bars at the top of each actogram indicate light (white) and dark (black) phases of the L:D cycles. Activity records in the last 10 days of each L:D cycle (delimited by red square brackets) were subjected to  $\chi^2$  periodogram analysis and each graph is presented on the right of corresponding actogram (B, D, G, I). The  $\chi^2$  periodogram indicates the ratio of variance ( $Q_p$ ) of the rhythm explained by each analysed period within a range of 20–30 h. The sloped dotted lines represent the threshold of significance, set at  $p = 0.05$ . Circular diagrams showing acrophases for the last 10 days of each L:D period are plotted (C, E, H, J). Each black dot shows the daily acrophase, while arrows indicate the average acrophase represented as a vector. In each circle the mean vector length ( $r$ ) and mean acrophase ( $A$ ) are shown. The circle inside each circular diagram represents critical values of the Rayleigh test ( $p < 0.05$ ) and the black part of the circle shows the duration of the dark phase. The dotted lines represent the confidence intervals.

parametric (Rayleigh test, Hotelling's paired tests) and non-parametric (Mann-Whitney  $U$  test, Wilcoxon signed rank test, Kruskal-Wallis rank sum test) tests to determine significant differences using the software Prism 5 (GraphPad Inc., La Jolla, CA, USA) and R 3.6.1 (<https://www.R-project.org/>).  $P$ -values  $\leq 0.05$  were considered statistically significant. Periodicity analysis in the activity rhythms was determined by means of the  $\chi^2$  periodogram at a confidence level of 95 % (Sokolove and Bushnell, 1978) using the chronobiology software El Temps. Periodogram analyses were performed with intervals of 10 days. The  $Q_p$  (ratio of variances) values obtained from periodogram analysis were used as measures of the strength and regularity (i.e., robustness) of entrained activity rhythms (Refinetti, 2004). The daily acrophase (i.e., the time at which the peak of a rhythm occurs) of the locomotor activity rhythm was calculated using the chronobiology software El Temps and the average acrophase was determined by vector addition. The Rayleigh test was used to assess whether the acrophases deviated from uniform and whether they were concentrated at a given time of the day (Di Rosa et al., 2024; Morbiato et al., 2019). Hotelling's paired tests were performed to test for differences among average acrophases of different photoperiods in the same group of fish (Di Rosa et al., 2024; Morbiato et al., 2019). The duration of LAA was determined as the time elapsed between lights on time and the rise of anticipatory activity, which was defined as a 150 % increase over baseline activity sustained for at least 30 min and not followed by any inflection for more than 1 h, as described elsewhere for the food-anticipatory activity (Stephan, 1997; Di Rosa et al., 2024). The baseline activity was defined as the median of the daily locomotor activity.

Analyses of daily variation of gene expression data (obtained from both RNA-seq analysis and RT-qPCR) of target genes were performed in R using MetaCycle with the function *meta2d* (Wu et al., 2016). MetaCycle applied ARSER, JTK\_CYCLE, and Lomb-Scargle algorithms to detect periodic signals and the Fisher method for  $p$ -value integration (Wu et al., 2016). *meta2d* computed integrated phase values via a circular average and relative amplitude value (rAMP), which could be taken as the ratio between amplitude and baseline and is used to compare the amplitude values among genes with different expression levels (values indicated in Table 1 and 2) (<https://cran.r-project.org/web/packages/MetaCycle/vignettes/implementation.html>).

For both RNA-seq analysis and RT-qPCR, group differences were assessed using the non-parametric Kruskal-Wallis test (R version 4.5.1). When significant differences were detected, post-hoc pairwise comparisons were performed using Wilcoxon rank sum exact test. A  $p$ -value  $\leq 0.05$  was considered statistically significant.

### 3. Results

#### 3.1. Daily rhythms of locomotor activity

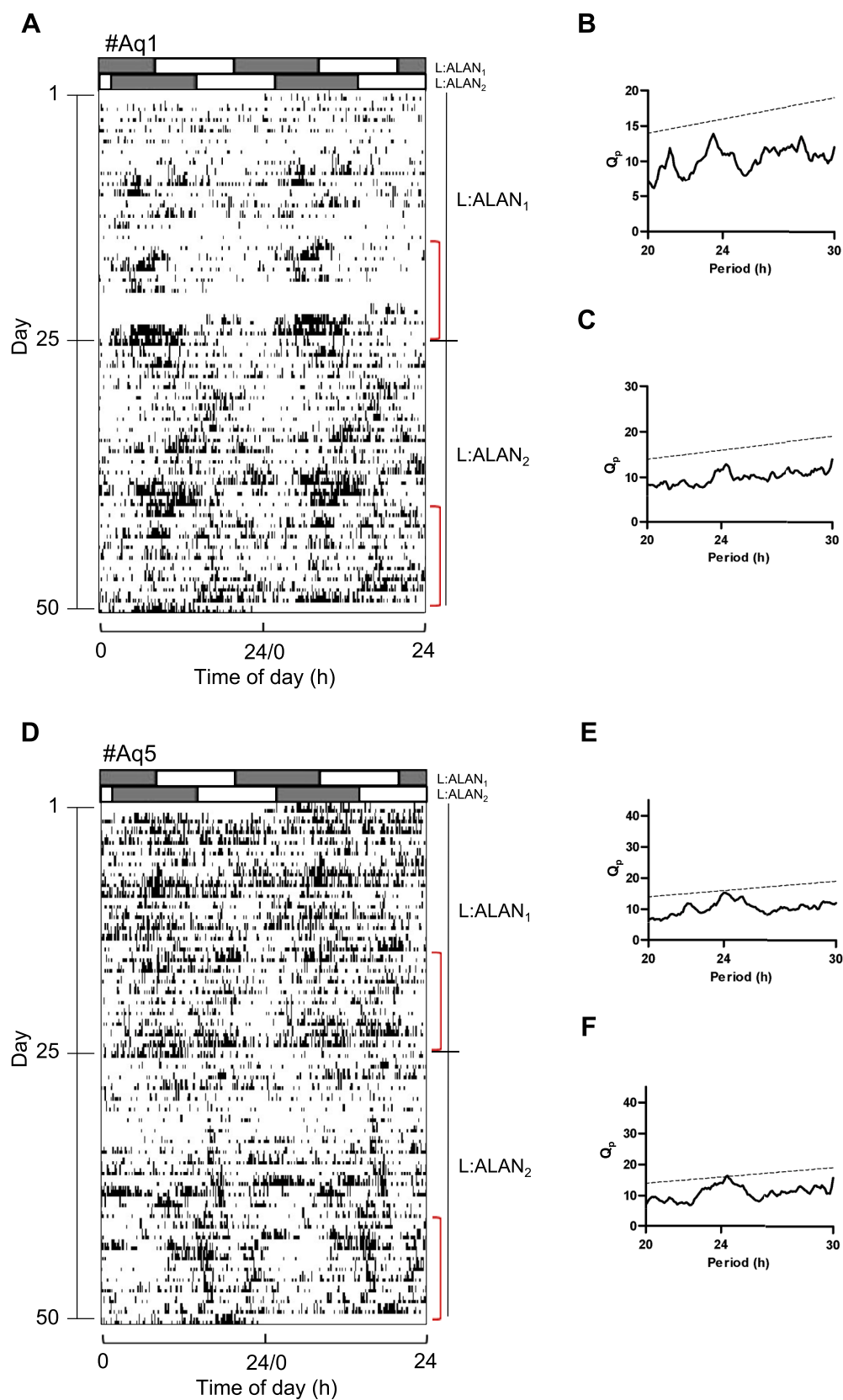
The first experiment was designed to investigate the effect of ALAN on the daily zebrafish's locomotor activity and on the photic entrainment. We evaluated the key characteristics of an entrained, circadian clock-controlled rhythm which included 1) the presence of the anticipation of the light-dark transition (light-anticipatory activity, LAA), 2)

the entrainment in response to lighting cycles, and 3) the evidence that the activity rhythm shifts gradually over several transient cycles following a light-on transition (Aschoff and Pohl, 1978). We continuously measured locomotor activity in zebrafish exposed to a 12:12 L:D or a 12:12 L:ALAN cycles (L:D<sub>1</sub> and L:ALAN<sub>1</sub>, respectively) for 25 days. As expected, the L:D<sub>1</sub> cycles induced a strong entrainment of behavioural circadian rhythms with a diurnal pattern as demonstrated by actograms (Fig. 1A, F; Fig. S2A-D), periodogram analysis (Fig. 1B, G), and the LAA, the characteristic increase in locomotor activity encountered a few hours prior to the lights on ( $1.86 \pm 0.33$ ; Fig. 1A, F; Fig. S2A-D). Next, to test the fish ability to readjust their rhythm to a new L:D cycle (L:D<sub>2</sub>), we subjected zebrafish to a 6-h shift of the lights on, from 06:00 to 12:00, and we recorded the locomotor activity for other 25 days. In the L:D<sub>2</sub> group, the 6-h shift of the lights on induced shifts of the activity onsets, which resulted, after 4–5 days of transient, in entrainment to the new L:D cycle (Fig. 1A, F; Fig. S2A-D). Furthermore, the LAAs were always present ( $2.53 \pm 0.57$ ; Fig. 1A, F) and did not significantly differ from the L:D<sub>1</sub> ( $W_6 = -11$ ,  $p > 0.05$ , Wilcoxon signed rank test). The robustness of entrained locomotor rhythms ( $Q_{p(L:D1)}: 30.32 \pm 3.48$ ;  $Q_{p(L:D2)}: 32.17 \pm 3.54$ ) also did not significantly differ ( $W_6 = -13$ ,  $p > 0.05$ , Wilcoxon signed rank test). To verify the accuracy of the entrained rhythm we estimated the acrophase for the last 10 days of recording in both L:D<sub>1</sub> and L:D<sub>2</sub> cycle (representative example in Fig. 1C, E, H, J). We found that the distribution of acrophases deviated from uniform ( $p < 0.05$ , Rayleigh test), and the mean acrophase fell between ZT2 and ZT5. The mean acrophase significantly changed after the 6-h shift of the lights on ( $Aq\#2$ ,  $F_{2,8} = 263.76$ ,  $p < 0.0001$ ;  $Aq\#3$ ,  $F_{2,8} = 11.06$ ,  $p < 0.01$ ;  $Aq\#6$ ,  $F_{2,8} = 9.13$ ,  $p < 0.01$ ;  $Aq\#8$ ,  $F_{2,8} = 8.23$ ,  $p < 0.01$ ;  $Aq\#10$ ,  $F_{2,8} = 244.52$ ,  $p < 0.0001$ ;  $Aq\#12$ ,  $F_{2,8} = 172.67$ ,  $p < 0.0001$ ; Hotelling's paired test).

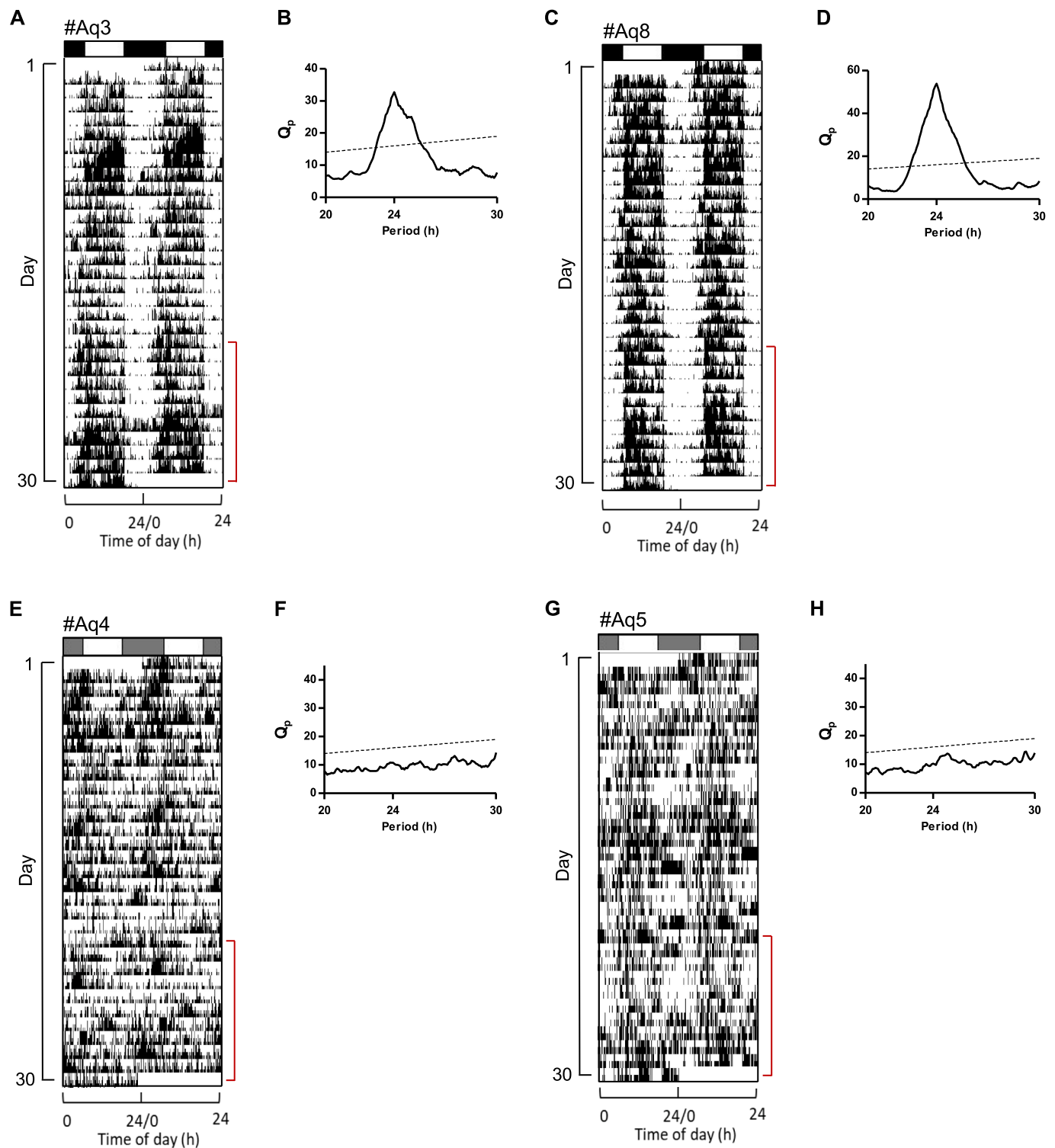
Conversely, we documented an alteration in the daily behavioural pattern in zebrafish exposed to ALAN treatments (Fig. 2; Fig. S2E-H). In three out of six aquaria (Fig. 2; Fig. S2E), zebrafish exposed to both L:ALAN cycles exhibited a complete loss of behavioural rhythmicity ( $\chi^2$  periodogram,  $p > 0.05$ ). In the other three aquaria (Fig. S2F-H), we observed a tendency toward the loss of rhythmicity, confirmed by the absence of a clear LAA and by the lack of a transient after the 6-h shift of the lights on. Furthermore, consistent nocturnal activity distributed across all ALAN hours was observed (Fig. 2A, D; Fig. S2E-H).

#### 3.2. Daily expression profiles of circadian clock genes and opsins

Before sampling, adult zebrafish were subjected to each lighting regime for 30 days, and their locomotor activity was monitored, confirming a robust circadian rhythmicity in the L:D group (Fig. 3A-D) and alterations in behavioural rhythmicity in the L:ALAN group (Fig. 3E-H). Indeed, in the L:D group, analyses of the actograms (Fig. 3A, C) and periodograms (Fig. 3B, D) confirmed the strong entrainment of behavioural circadian rhythms with a diurnal pattern. The LAA ( $1.23 \pm 0.24$ ) and the robustness of the rhythms ( $Q_{p(L:D)} = 39.15 \pm 5.10$ ) were not statistically different from those obtained in the other behavioural experiment (LAA: LD vs. LD<sub>1</sub>,  $U_{4,6} = 6$ ,  $p > 0.05$ ; LD vs. LD<sub>2</sub>,  $U_{4,6} = 2.5$ ,  $p > 0.05$ ; Robustness: LD vs. LD<sub>1</sub>,  $U_{4,6} = 6$ ,  $p > 0.05$ ; LD vs. LD<sub>2</sub>,  $U_{4,6} = 6$ ,  $p > 0.05$ , Mann-Whitney  $U$  test).



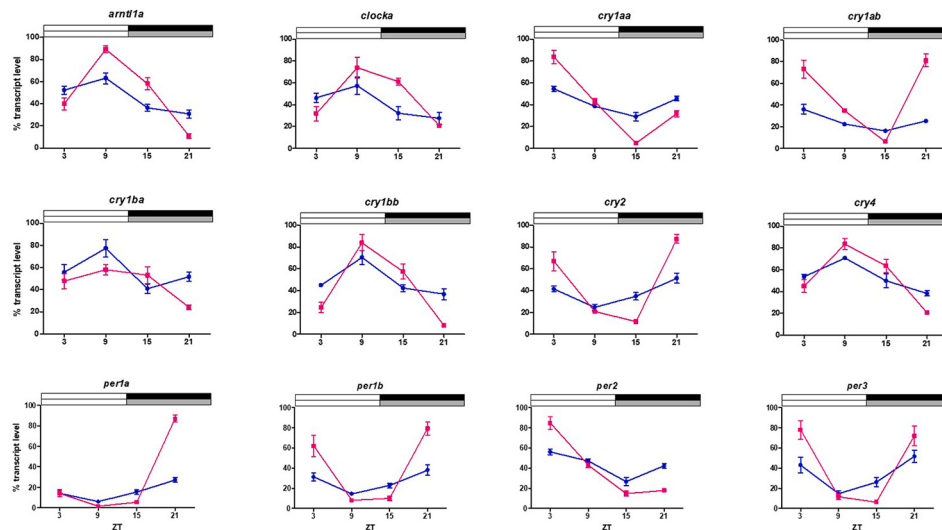
**Fig. 2.** Representative actograms of locomotor activity of groups of zebrafish subjected to 12:12 L:ALAN cycles. Starting and ending day of each L:ALAN cycle (L:ALAN1 and L:ALAN2) is shown on the right of the actogram. Activity records in the last 10 days of 12:12 L:ALAN cycles were subjected to  $\chi^2$  periodogram analysis. For more details, see Fig. 1.



**Fig. 3.** Representative actograms of locomotor activity of groups of zebrafish subjected to 12:12 L:D (A, C) and 12:12 L:ALAN (E, G) cycles, and used for the brain transcriptomic analysis. Activity records in the last 10 days of each recording were subjected to  $\chi^2$  periodogram analysis (B, D for 12:12 L:D and F, H for 12:12 L:ALAN). For more details, see Fig. 1.

RNA-seq analysis of the ALAN-exposed group revealed a reduction in the amplitude of core clock gene oscillations, while gene expression profiles under L:D conditions exhibited oscillatory patterns, with positive (*clock*, *arntl*) and negative (*per*, *cry*) regulatory elements peaking in the expected phases (Fig. 4). Table 1 listed the statistical significance of rhythmicity and amplitude variation for circadian clock genes under both lighting conditions. Under ALAN exposure, the amplitudes of core

clock components, including both positive regulators (*arntl1a*, *clocka*) and negative regulators (*per* and *crys*), were markedly reduced, exhibiting a 41–66 % decrease relative to L:D conditions, except for *cry1ba* (Fig. 4). Notably, light-inducible clock gene *per2* showed only a modest reduction (18 %) in amplitude. These attenuated oscillations were statistically significant for all investigated clock genes (Table 1;  $p < 0.001$ , Kruskal-Wallis rank sum test). We also found that the two melanopsin



**Fig. 4.** Oscillation patterns of clock genes in the brain of zebrafish maintained in L:D or L:ALAN cycle for 30 days. Brains were harvested every 6 h for a day starting at ZT3 ( $n = 5$  per ZT per condition). RNA-Seq data was presented as mean  $\pm$  SEM of normalized reads count expressed in percentage (100 % is the maximum level detected for each gene in L:D and L:ALAN condition). On the x-axes time is expressed as zeitgeber time (ZT, where ZT0 represents lights on). White, grey and black bars represent light, ALAN and dark periods, respectively. L:D cycle = squares and pink line; L:ALAN cycle = circles and blue line.

genes *opn4b* and *opn4.1* lost daily oscillating expression profile in L:ALAN cycle (Fig. 5; Table 1; meta2d,  $p > 0.1$  for both *opn4b* and *opn4.1*). We also investigated other nonvisual opsins members of *tmt opsins* (*tmtopsb*, *tmtops3b*, *tmtops2b*, *tmtops3a* and *tmtops2a*) and *va opsins* (*valopa*) families that showed arrhythmic patterns (Fig. 5; Table 1; meta2d,  $p > 0.05$ ) and no changes in expression levels (Fig. 5; Table 1; Kruskal-Wallis rank sum test,  $p > 0.05$  for all opsins) in both lighting conditions. Among the light-inducible genes, both *cry5* (also called 6,4photolyase) and *dash*, two genes belonging to the cryptochrome family with photolyase function, were still oscillating, but decreased by 50 % in L:ALAN (Fig. 5; Kruskal-Wallis rank sum test,  $p < 0.001$  for both *cry5* and *dash*).

To validate RNA-seq results, RT-qPCR was performed on a subset of six clock and clock-controlled genes (Fig. 6; Table S2). Core clock genes (*clock1a*, *arntl1a*, *per1b*) confirmed rhythmic expression pattern in L:D (Table S2; meta2d,  $p < 0.0001$ ), which was maintained in L:ALAN albeit with a drastic amplitude reduction (Table S2; meta2d,  $p < 0.05$ ). Similar trends were observed for the light inducible genes *per2* and *cry1a*, and for *cry5* photolyase mRNA levels (Table S2; meta2d,  $p < 0.0001$  in L:D and  $p < 0.001$  in L:ALAN).

#### 4. Discussion

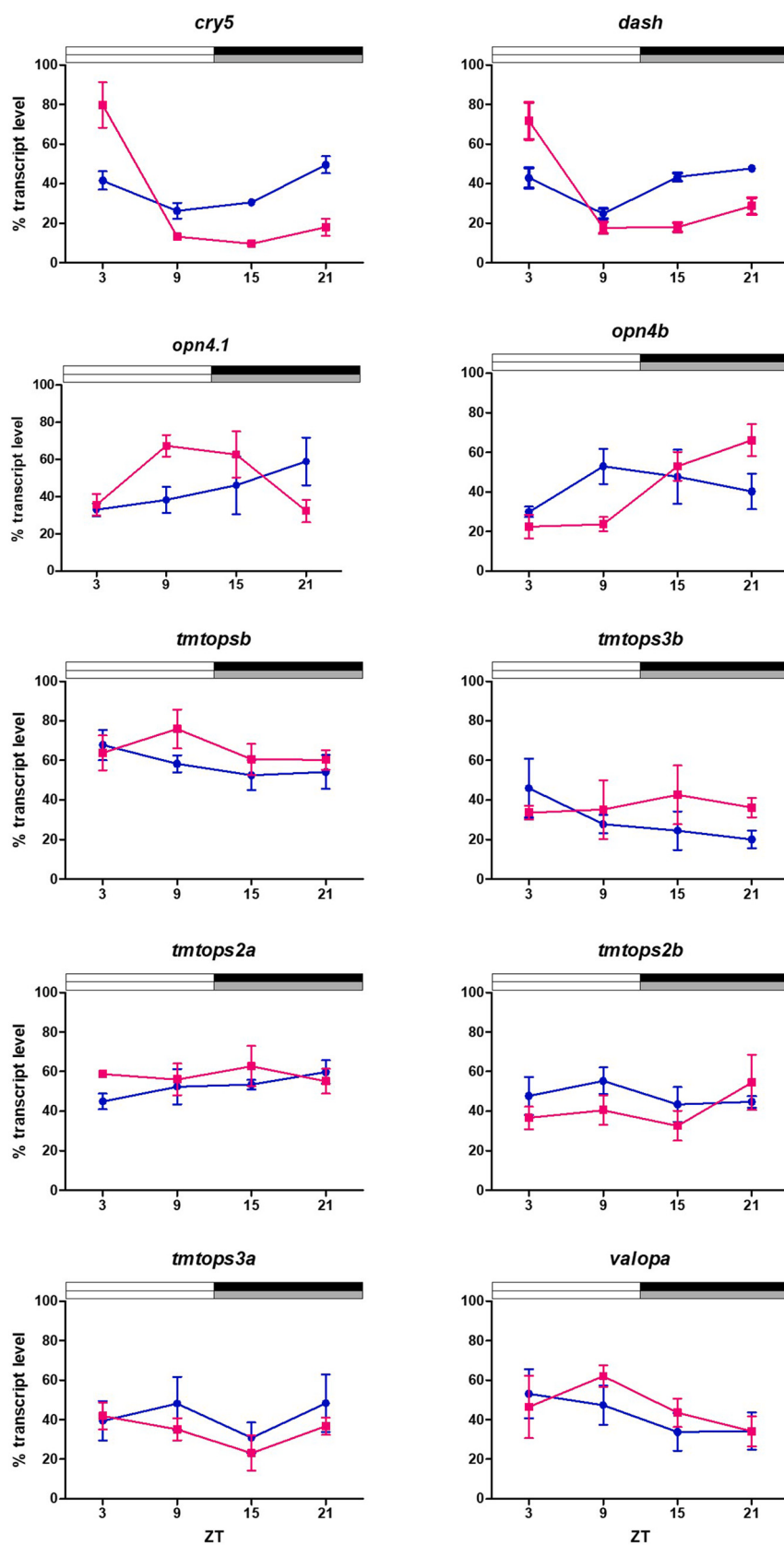
In this study we investigated how ALAN exposure affects the circadian clock of adult zebrafish. We found a marked tendency to arrhythmic locomotor activity in the ALAN group that contrasts with the rhythmic, diurnal pattern observed for zebrafish under L:D cycle. We also explored the drivers of such alteration of behavioural rhythmicity at molecular level, performing a brain transcriptomic analysis across the 24 h. The analyses revealed significant variations in the expression of clock and opsins genes after 30 days of ALAN exposure. Both positive and negative regulators of the molecular circadian clock mechanisms were influenced by the ALAN exposure, showing a decrease in their amplitude. Zebrafish has been previously used as a model-species to study the effects of ALAN on physiology, behaviour and gene expression (Khan et al., 2018; Labala et al., 2024; Li et al., 2024; Lucon-Xiccato et al., 2023; De Russi et al., 2024, 2025). A recent study showed that genes related to immunity, stress response, responses to sensory stimuli, energy production, motor activity, and reproductive processes were impacted by ALAN exposure (Vergata et al., 2025).

Given the wide-ranging effects of ALAN on animals, integrative

approaches that span multiple scales, from omics and physiology to behaviour, ecology, and environmental science, are essential for a deeper understanding of the physiological and behavioural changes induced by this anthropogenic stressor. Although the extensive use of ALAN has improved the quality of human activities, several scientific studies have shown that it is a significant anthropogenic pressure on natural biological systems (Sanders et al., 2021; Gaston et al., 2014). In nature, the brightest light in the nights of full moon is up to 0.3 lx (Kyba et al., 2017), while light pollution can cause nocturnal illumination on water surfaces to reach levels as high as 30 lx (Bennie et al., 2016). ALAN can thus represent a significant departure from natural conditions, potentially impacting terrestrial as well as aquatic organisms.

Suppression or increase of locomotor activity due to ALAN has been reported in different fish species, including zebrafish. ALAN-impacted locomotor activities were observed in the Bluegill (*Lepomis macrochirus*), which reduced its swimming activity (Latchem et al., 2021), in guppies (*Poecilia reticulata*), which left their shelters more quickly and spent more time in open areas (Kurvers et al., 2018), and in humbug damselfish (*Dascyllus aruanus*) that became more active at night (Georgiou et al., 2024). Our previous work on zebrafish larvae also showed that ALAN determines hyperactivity (Lucon-Xiccato et al., 2023). Only an investigation on a fish species (the rockfish *Girella laevis*, an intertidal fish living along the Southeastern Pacific coasts) showed the abolition of daily activity rhythms during a short period of 10 days of ALAN exposition (Pulgar et al., 2019).

For what concerns the molecular bases of the observed alterations in rhythmicity, our molecular analyses suggested that a potential driver was a decrease in the amplitude of expression of clock and opsin genes. Recent investigations on bird species, the zebra finch *Taeniopygia guttata* and the tree sparrow *Passer montanus*, reported similar results, indicating that a downregulation of clock gene expression is sufficient to induce arrhythmic behavioural patterns (Alaasam et al., 2021; Renthlei et al., 2020). In zebrafish, the observed downregulation of the light-responsive genes *Cry1s* and *Per2*, which are key mediators in transferring photic information to the fish circadian timekeeping mechanism (Tamai et al., 2007; Ziv et al., 2005; Okano et al., 2020; Steindal and Whitmore, 2020), could contribute to the behavioural pattern altered by ALAN. In addition, the loss of rhythmic expression of *opn4.1* and *opn4b*, two melanopsin homologs that are orthologs of mammalian OPN4 (Matos-Cruz et al., 2011), may be critical for the alteration of photic entrainment. Indeed, both opsins are expressed in zebrafish brain, and



**Fig. 5.** Oscillation patterns of photolyase and nonvisual opsin genes in zebrafish maintained in L:D or L:ALAN cycle for 30 days. Zebrafish brains were harvested every 6 h for a day starting at ZT3 (n = 5 per ZT per condition). For more details, see Fig. 4.

**Table 1**

Comparisons of statistical significance, phase and relative amplitude under L:D and L:ALAN conditions, for circadian clock genes, photolyase and opsins expression level obtained from RNA-Seq data, using MetaCycle R package and Kruskal-Wallis test. Bold indicates statistically significant values. Index of amplitude variation were calculated as percentage change in rAMP in L:ALAN compared to rAMP in L:D.

| Gene            | Lighting conditions |                 |                |                  |                 |                | K-W test                                       | rAMP variation<br>L:ALAN vs L:D |
|-----------------|---------------------|-----------------|----------------|------------------|-----------------|----------------|------------------------------------------------|---------------------------------|
|                 | L:D                 |                 |                | L:ALAN           |                 |                |                                                |                                 |
|                 | meta2d<br>pvalue    | meta2d<br>phase | meta2d<br>rAMP | meta2d<br>pvalue | meta2d<br>phase | meta2d<br>rAMP |                                                |                                 |
| <i>arntl1a</i>  | < 0.0001            | 10.5            | 0.76           | <0.0001          | 9.0             | 0.25           | H <sub>7</sub> = 32.70<br><b>p &lt; 0.001</b>  | −67 %                           |
| <i>clocka</i>   | < 0.0001            | 10.5            | 0.66           | < 0.01           | 9.0             | 0.28           | H <sub>7</sub> = 25.15<br><b>p &lt; 0.001</b>  | −58 %                           |
| <i>cry1aa</i>   | < 0.0001            | 4.5             | 1.03           | < 0.0001         | 3.0             | 0.36           | H <sub>7</sub> = 35.53<br><b>p &lt; 0.0001</b> | −65 %                           |
| <i>cry1ab</i>   | < 0.0001            | 3.0             | 1.12           | < 0.0001         | 3.0             | 0.41           | H <sub>7</sub> = 37.06<br><b>p &lt; 0.001</b>  | −63 %                           |
| <i>cry1ba</i>   | < 0.01              | 10.5            | 0.31           | < 0.01           | 7.5             | 0.51           | H <sub>7</sub> = 22.80<br><b>p &lt; 0.01</b>   | +64 %                           |
| <i>cry1bb</i>   | < 0.0001            | 10.5            | 0.97           | < 0.01           | 9.0             | 0.40           | H <sub>7</sub> = 31.43<br><b>p &lt; 0.001</b>  | −59 %                           |
| <i>cry2</i>     | < 0.0001            | 3.0             | 1.18           | < 0.0001         | 22.5            | 0.34           | H <sub>7</sub> = 35.31<br><b>p &lt; 0.001</b>  | −71 %                           |
| <i>cry4</i>     | < 0.0001            | 10.5            | 0.59           | < 0.0001         | 9.0             | 0.30           | H <sub>7</sub> = 31.22<br><b>p &lt; 0.001</b>  | −49 %                           |
| <i>cry5</i>     | < 0.0001            | 3.0             | 0.89           | < 0.0001         | 22.5            | 0.35           | H <sub>7</sub> = 32.83<br><b>p &lt; 0.001</b>  | −61 %                           |
| <i>per1a</i>    | < 0.0001            | 22.5            | 1.22           | < 0.0001         | 21.0            | 0.60           | H <sub>7</sub> = 33.08<br><b>p &lt; 0.001</b>  | −51 %                           |
| <i>per1b</i>    | < 0.0001            | 22.5            | 1.14           | < 0.0001         | 22.5            | 0.47           | H <sub>7</sub> = 34.93<br><b>p &lt; 0.001</b>  | −59 %                           |
| <i>per2</i>     | < 0.0001            | 4.5             | 0.57           | < 0.0001         | 4.5             | 0.47           | H <sub>7</sub> = 34.47<br><b>p &lt; 0.001</b>  | −18 %                           |
| <i>per3</i>     | < 0.0001            | 3.0             | 1.33           | < 0.0001         | 22.5            | 0.61           | H <sub>7</sub> = 33.56<br><b>p &lt; 0.001</b>  | −54 %                           |
| <i>dash</i>     | < 0.001             | 24.0            | 0.67           | < 0.01           | 21.0            | 0.34           | H <sub>7</sub> = 31.79<br><b>p &lt; 0.001</b>  | −49 %                           |
| <i>opn4b</i>    | < 0.01              | 21.0            | 0.21           | > 0.1            | –               | –              | H <sub>7</sub> = 19.45<br><b>p &lt; 0.01</b>   |                                 |
| <i>opn4.1</i>   | < 0.01              | 10.5            | 0.47           | > 0.1            | –               | –              | H <sub>7</sub> = 9.90<br><b>p &gt; 0.05</b>    |                                 |
| <i>valopa</i>   | > 0.1               | –               | –              | > 0.1            | –               | –              | H <sub>7</sub> = 8.38<br><b>p &gt; 0.05</b>    |                                 |
| <i>tmtopsb</i>  | > 0.1               | –               | –              | > 0.1            | –               | –              | H <sub>7</sub> = 6.06<br><b>p &gt; 0.05</b>    |                                 |
| <i>tmtops3b</i> | > 0.1               | –               | –              | > 0.1            | –               | –              | H <sub>7</sub> = 6.06<br><b>p &gt; 0.05</b>    |                                 |
| <i>tmtops3a</i> | > 0.1               | –               | –              | > 0.1            | –               | –              | H <sub>7</sub> = 4.08<br><b>p &gt; 0.05</b>    |                                 |
| <i>tmtops2b</i> | > 0.1               | –               | –              | > 0.1            | –               | –              | H <sub>7</sub> = 5.66<br><b>p &gt; 0.05</b>    |                                 |
| <i>tmtops2a</i> | > 0.1               | –               | –              | > 0.1            | –               | –              | H <sub>7</sub> = 7.84<br><b>p &gt; 0.05</b>    |                                 |

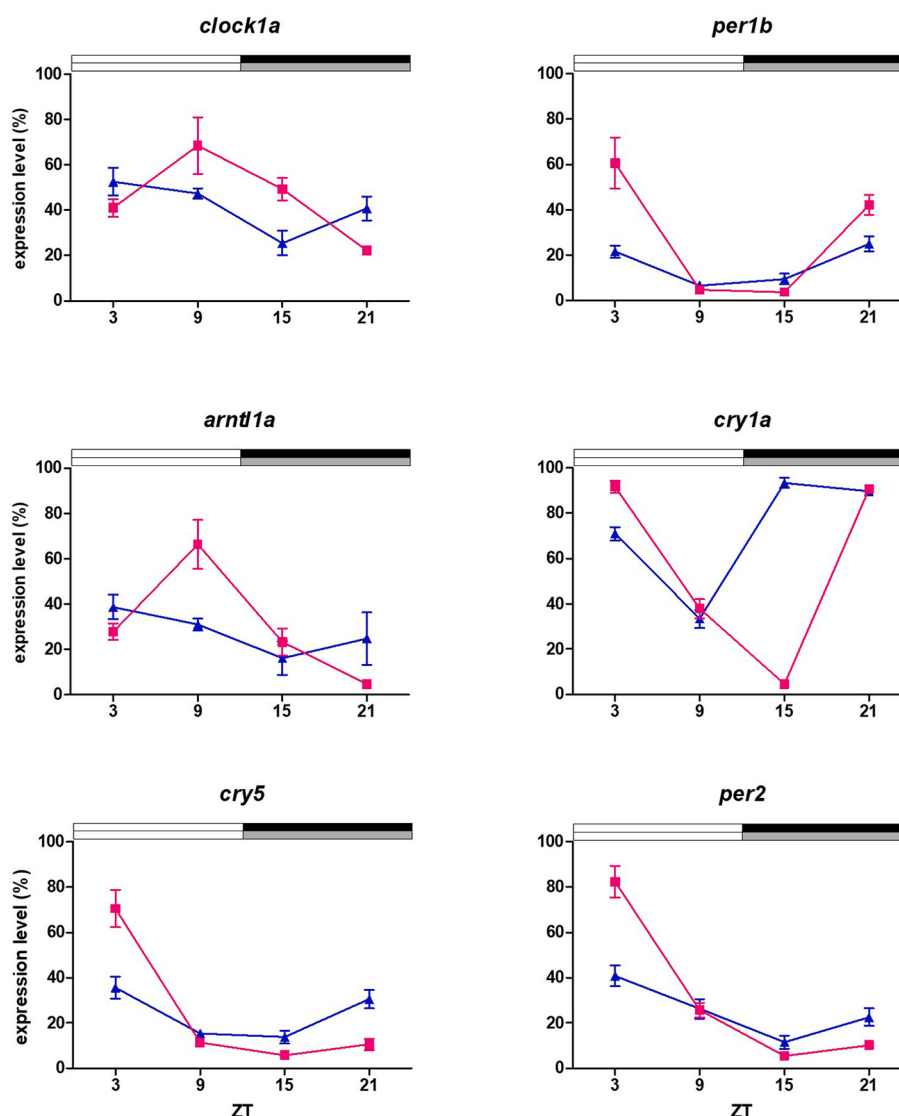
their double knockout attenuates locomotor activity (Dekens et al., 2022; Pan et al., 2023).

In the last decade, numerous studies have reported the effects of ALAN on many biological processes. A meta-analysis of the biological impacts of ALAN on different animal taxa showed that it induces strong responses in physiology, daily activity patterns, and life history traits, including reproduction, predation risk, and cognition (Sanders et al., 2021). Our findings demonstrate that ALAN significantly influences the behaviour and physiology of a diurnal teleost. These alterations have the potential to disrupt both intra- and interspecific interactions, thereby affecting individual fitness and, ultimately, evolutionary processes (Hopkins et al., 2018; Hölker et al., 2023).

The adaptation of a specific temporal niche (diurnal, nocturnal, crepuscular) is an evolutionary consequence of the plasticity of the circadian clock, shaped by natural selection. Diurnality and nocturnality have led to changes in the physiology and behaviour of animals (Gerkema et al., 2013; Doyle and Menaker, 2007; Bertolucci and Foà,

2004). For instance, nocturnality has driven changes in the photolyase DNA protection system, with the loss of photolyase genes, and in the photoreception, with the loss of extraretinal photoreception (Gerkema et al., 2013). Conversely, diurnality helps ectotherms to optimize their thermoregulation and energy balance by aligning activities with the sunniest and warmest part of the day (Gerkema et al., 2013). Zebrafish, being a diurnal freshwater species, strongly rely on light cues to synchronize activity, feeding, and social behaviours with the daylight. Our data confirm that when the photic niche to which zebrafish have adapted over the course of evolution is altered by ALAN, there are significant effects on their physiology and behaviour.

ALAN can stem from many sources (e.g., street and traffic lighting, vehicles and transports, decorative and architectural lighting, residential, commercial, industrial, and recreational lighting) of different types, from LED to halogen to fluorescent, that produce light with varying irradiance and spectral composition. Further research on ALAN should investigate how different wavelengths and intensities can influence



**Fig. 6.** RT-qPCR analysis of selected genes in zebrafish maintained in L:D or L:ALAN cycle for 30 days. Zebrafish brains were harvested every 6 h for a day starting at ZT3 (n = 5 per ZT per condition). Relative expression levels (100 % is the maximum level detected for each gene in L:D and L:ALAN condition) were presented as mean  $\pm$  SEM. For more details, see Fig. 4.

biological processes. Importantly, such research must also align with the study of light perception systems (e.g., retinal and extraretinal photoreceptors) and ecological niches (e.g., nocturnal or diurnal, terrestrial or aquatic) of the target species. By integrating species-specific sensory biology and ecological traits, future studies can generate more ecologically relevant insights into the consequences of ALAN on wildlife.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2025.181077>.

#### CRediT authorship contribution statement

**Elena Frigato:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Gaia De Russi:** Writing – review & editing, Investigation, Formal analysis, Data curation, Conceptualization. **Chiara Vergata:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Giuditta Codogno:** Validation, Methodology, Investigation, Formal analysis, Data curation. **Tyrone Lucon-Xiccato:** Writing – review & editing, Methodology, Conceptualization. **Stefano Cannicci:** Writing – review & editing, Funding acquisition, Conceptualization. **Sara Fratini:** Writing –

review & editing, Validation, Supervision, Software, Methodology, Data curation, Conceptualization. **Cristiano Bertolucci:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Funding acquisition, Conceptualization.

#### Funding sources

Project funded under the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.4 – Call for tender No. 3138 of 16 December 2021, rectified by Decree n.3175 of 18 December 2021 of Italian Ministry of university and Research funded by the European Union – Next Generation EU. Project code CN00000033. Concession Decree No. 1034 of 17 June 2022 adopted by the Italian Ministry of University and Research, CUP: B83C22002910001 (Spoke 3). Project title “National Biodiversity Future Center – NBFC”. C.B., T.L-X. and E.F. were supported by FAR2024 grant from University of Ferrara, Italy.

#### Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgments

The authors gratefully acknowledge Alessio Iannucci for his assistance with the transcriptomic analysis, Chiara Uccello for her help in maintaining the zebrafish, and Andrea Margutti for constructing the experimental apparatus. Graphical abstract and Fig. S1 were created with [BioRender.com](https://www.biorender.com).

## Data availability

Data will be made available on request.

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