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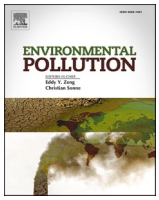

Transcriptome-wide deregulation of gene expression in zebrafish exposed to artificial light at night☆

Chiara Vergata [a], Giuditta Codogno [a,c], Gaia De Russi [b], Elena Frigato [b], Tyrone Lucon-Xiccato [b], Stefano Cannicci [a], Cristiano Bertolucci [b,**], Sara Fratini [a,*]

[a] Department of Biology, University of Florence, Via Madonna del Piano 6, Sesto Fiorentino, 50019, Italy
[b] Department of Life Sciences and Biotechnology, University of Ferrara, Via Luigi Borsari 46, 44121, Ferrara, Italy
[c] Department of Earth and Marine Sciences, University of Palermo, Via Archirafi 22, 90123, Palermo, Italy

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ABSTRACT

Urbanization and human activities are increasing global levels of artificial light at night (ALAN). Several studies have shown that ALAN negatively impacts animals, altering their perception, physiology, and behaviour, eventually leading to reduced fitness. ALAN also disrupts biological rhythms, affecting animals' ability to synchronize with natural cycles. There still is a critical lack of knowledge on ALAN's effects on freshwater ecosystems, which are highly threatened biodiversity hotspots. Our study aimed to understand the molecular effect of prolonged ALAN exposure in the zebrafish, a model species in this field. Using high-throughput RNA sequencing, we investigated the transcriptome-wide gene expression responses in whole brains of ALAN-exposed zebrafish. Samples were collected at four different timepoints along the day, two at daytime and two at nighttime, to identify daily changes in gene expression and metabolic pathways. A one month exposure to ALAN varied the expression of several genes and metabolic pathways, both at night, when zebrafish were directly exposed to artificial light, and during the day, at natural light conditions. The highest number of differentially expressed genes were found at the early hours of the day and at night. Overall, circadian clock genes and those related to immunity, stress response, responses to sensory stimuli, energy production, motor activity, and reproductive processes changed in expression levels. These results provide new insights into the mechanisms through which ALAN affects animal biology, suggesting broader impacts than previously recognised. To safeguard aquatic ecosystems and their biodiversity, it is paramount to mitigate light pollution.

1. Introduction

Urbanization and human activities are increasing globally, impacting all environments. As a result, wildlife species are subject to numerous anthropogenic stressors, including physical pollutants such as artificial light at night (ALAN; Falchi et al., 2016; Davies and Smyth, 2018; Gaston et al., 2021). There is growing evidence that the disruption of natural light-dark (LD) regimes by ALAN impacts all levels of biological organization, from individuals to species to ecosystem. Many studies have shown that ALAN can alter phenotypic traits related to perception, physiology and behaviour in a wide range of taxa, including terrestrial and aquatic animals (Halfwerk and Slabbekoorn, 2015; Jagerbrand and Spoelstra, 2023), eventually affecting their fitness (for a review see Bumgarner and Nelson, 2021; Dickerson et al., 2023; McLay et al., 2019; Stanton and Cowart, 2024).

One of the most significant effects of the exposure to ALAN is its impact on biological rhythms, which in turn affect the animals' ability to synchronize with daily, lunar, and seasonal cycles (Gaston et al., 2017). The impacts of ALAN on biological clocks have been documented in marine and terrestrial organisms at molecular and physiological levels. Animals' circadian rhythms are regulated by the cyclical transcription and translation system of a set of core clock genes, which includes members of the *Clock/Bmal* (which encode activators) and *Per/Cry* (which encode repressors) families. Among vertebrates, alteration in the expression of *Per2* and *Bmal1* was showed in zebra finches *Taeniopygia guttata* and great tits *Parus major* when exposed to ALAN (Batra et al.,

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* Corresponding author.
** Corresponding author.
E-mail addresses: bru@unife.it (C. Bertolucci), sara.fratini@unifi.it (S. Fratini).

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2019; Dominoni et al., 2022). *Per1*, *Per2*, and *Bmal1* expression was changed in the hippocampus and central nervous system of ALAN-exposed Siberian hamsters *Phodopus sungorus* (Bedrosian et al., 2013). The expression of circadian clock genes was also affected in invertebrates exposed to ALAN, such as the cricket *Gryllus bimaculatus* (Levy et al., 2022) and the Pacific oyster *Crassostrea gigas* (Botté et al., 2023). ALAN can alter the expression pattern of other photoperiod-regulated genes, such as those related to immunity, energy production, motor activity, and reproductive processes (Bumgarner and Nelson, 2021; Dominoni et al., 2022).

The mechanisms by which these effects occur are not fully understood, and more focus should be placed on freshwater ecosystems, which are among the most threatened on the planet (Dudgeon, 2019; Williams-Subiza and Epele, 2012). In fact, ALAN is particularly prevalent near (<3 km) freshwater ecosystems, as this is where about half of the world's population is concentrated (Kummu et al., 2011). In addition, inland waters, although covering less than 1 % of the Earth's surface, are rich in biodiversity, with 10 % of all known animal species, which also provide crucial ecosystem services (Tickner et al., 2020).

To bridge this gap, we investigated the effect of ALAN on the transcriptome of the zebrafish *Danio rerio* across the 24h cycle. Zebrafish are small tropical freshwater fish belonging to the family Ciprinidae. They are a model species in many research areas, such as ecotoxicology, genetics, behaviour, physiology, nutrition and human diseases, primarily thanks to their high fecundity, short generation time, rapid embryonic development and a fully sequenced and annotated genome (Choi et al., 2021; Horzmann and Freeman, 2018; Krylov et al., 2021; Patton et al., 2021; Sacksteder and Kimmey, 2022). Within the last decade, zebrafish have been used as a research model to investigate the effects of exposure to ALAN on physiology and behaviour (Cao et al., 2024; Khan et al., 2018; Labala et al., 2024; Li et al., 2024; Lucon-Xiccato et al., 2023; De Russi et al., 2024, 2025). ALAN impairs simple learning abilities and cognitive lateralisation in zebrafish larvae while it determines hyperactivity (Lucon-Xiccato et al., 2023; De Russi et al., 2025). ALAN also affects complex cognitive functions in adults, such as cognitive flexibility (De Russi et al., 2024). Furthermore, zebrafish exposed to ALAN exhibited anxiety-like behaviour, and F1 offspring born to ALAN-exposed mothers displayed reduced movement (Li et al., 2024). At the molecular level, the effect of ALAN has been studied in zebrafish ovarian tissues, where the treatment induced desynchronization of clock-associated genes, melatonin arrhythmicity, and reproductive hormonal imbalance (Khan et al., 2018).

In this study, we used zebrafish to investigate the effects of ALAN exposure from a molecular circadian perspective. To achieve this goal, we examined the transcriptome-wide gene expression response using Illumina RNA-sequence (RNA-seq) methodology in whole brain of zebrafish exposed to nocturnal light for four weeks (treatment group), compared to individuals experiencing a natural light-dark cycle (control group). After one month, all treated and control samples were sacrificed at four different timepoints along the day, two at daytime and two at nighttime. Our time-series transcriptome analysis allowed us to identify modification of gene expression and metabolic pathways after prolonged ALAN exposure, both at night and at daytime.

2. Material and methods

2.1. Study animals and housing conditions

Zebrafish were derived from a wild-type strain "Ariosto" established from 100 individual purchased from a local shop and maintained at the University of Ferrara's facility since 2011. The population currently consists of ~1500 individuals and has a 50:50 sex ratio. It is maintained by both routinely performing reproduction with standard procedures, using breeders randomly selected from the different tanks and introducing newly purchased subjects. All aquaria had a recirculating water system equipped with mechanical, chemical and biological filters. They were maintained at a constant water temperature (28 ± 1 °C) and exposed to a 12 h light/12 h dark photoperiod using light-emitting diodes (LED) lamps placed on the ceiling of the facility and controlled by an electronic timer. Food was provided twice a day. A total of 80 adult zebrafish (8–12 months old; sex ratio 50:50) were randomly collected from their aquarium and used in this study. All used subjects were naive to experimentation and never encountered light conditions different to their rearing one (12:12 L:D).

2.2. Experimental conditions

Ten aquaria (41 × 25 × 24 cm; water level: 20 cm; N = 8 individuals for each aquarium) were kept in a chronobiology room, 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 ± 1 °C by means of an automatic air conditioning system. Food (Tropical fish flakes, Prodac, Italy) was provided daily at random times to prevent any synchronization. Zebrafish were exposed to two photic regimes, 12:12 light-dark (Control group) and 12:12 light-ALAN (ALAN group) cycles (Fig. 1a), for one month. This period is sufficient to warrant entrainment of the circadian clock to a new photic condition (Idda et al., 2012; Morbiato et al., 2019), to prevent internal desynchronization between central and peripheral oscillators that could alter gene expression (Ashton et al., 2022). This experimental setup was previously used to test the effect of ALAN in other freshwater anamniotes (Touzot et al., 2022). In all aquaria light-emitting diode sources (white light, 6000 K; TMR, distributed by ELCART, Italy), providing a natural daylight, were used.

Irradiance was measured with a radiometer (DO9721, Probe LP9021 RAD, Spectral range 400–950 nm, DeltaOHM, Padova, Italy). The irradiance was set at 45.3 lux (0.358 W/m^2) during the light phase. In the 12:12 light-dark cycle the LEDs were turned off during the dark phase (i.e., night). In the ALAN aquaria, a small LED strip was turned on during the night phase, providing 3.3 lux (0.026 W/m^2) in line with field measures reported for urban freshwater habitats impacted by ALAN (Brüning et al., 2015; Perkin et al., 2014) and corresponding to the light level of a residential street (Gaston et al., 2013).

2.3. Brain sampling

Zebrafish from both the control and the ALAN groups were anesthetized with ice, and their brains were dissected using sterile scissors and forceps, snap-frozen in liquid nitrogen and immediately stored at −80 °C to prevent any possible degradation of nucleic acid. The circadian clock is composed of a network of central and peripheral oscillators distributed in cells and tissues throughout the body of zebrafish (Idda et al., 2012; Foulkes et al., 2016). The interaction between these oscillators coordinates physiological and behavioural rhythms allowing animals to anticipate environmental changes and optimally time physiological processes in order to enhance survival (Pittendrigh, 1993). The brain was selected as the target tissue for gene expression analysis since it is one of the primary light-signal targets, via retinal and encephalic photoreceptors, and contains circadian pacemakers that regulate physiological and behavioural rhythmicity (Idda et al., 2012).

All treated and control zebrafish were sacrificed at four different timepoints across the day, every 6 h, from 09.00 a.m. It is conventional to divide the 24-h LD cycle into 24-h zeitgeber time (ZT) units and indicate the time of lights on as ZT0 and the time of lights off as ZT12. Zebrafish brains were sampled at ZT3, 9.00 a.m.; ZT9, 3.00 p.m.; ZT15, 9.00 p.m.; ZT21, 03.00 a.m. (Fig. 1a). Following Ching et al. (2014), five biological replicates per group (ALAN and Control) were collected at the four timepoints selected (ZT03, ZT09, ZT15 and ZT21), for a total of 40 samples, selected from the initial 80 zebrafish exposed to the lighting regime. The surplus zebrafish in the experimental aquaria (four per aquarium) minimized potential stress effects due to reduced social

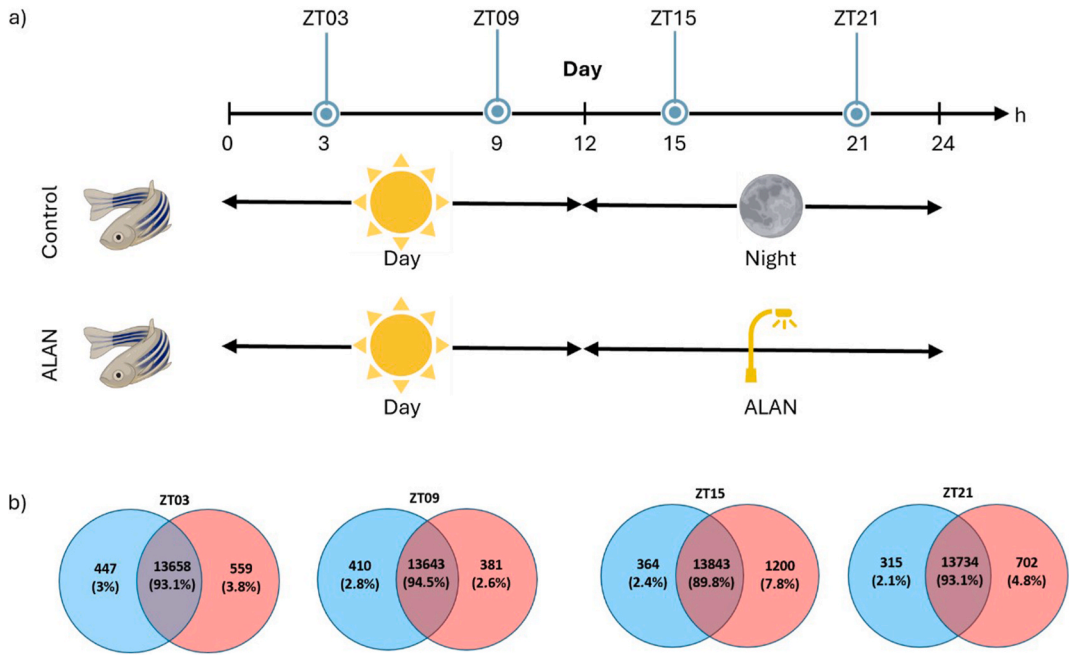

Fig. 1. a) Graphical representation of the experimental design set up for this study. Sampling timepoints for RNA-seq analysis were marked (ZT03, ZT09, ZT15 and ZT21). b) Venn diagrams comparing differentially expressed genes of ALAN (red circle) and control (blue circle) samples at the four timepoints. The irradiance was set at 45.3 lux (0.358 W/m^2) during the light phase and at 3.3 lux (0.026 W/m^2) during ALAN conditions. ZT: Zeitgeber time. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

companionship during sampling.

2.4. RNA extraction

Total RNA extraction was performed using the entire brain of each sample, following the RNeasy Mini Kit (Qiagen, Hilden, Germany) manufacturer's standard protocol (including the DNAse I treatment carried out with the RNase free DNase Set - Qiagen, Hilden, Germany) with a final elution volume ranging between 30 and 40 μL RNAse-free water. The integrity of extracted RNAs was assessed using the Agilent 2100 BioAnalyzer (RNA 6000 Nano Kit - Agilent Technologies, Inc.). Qubit™ 4 Fluorometer (kit RNA BR - Thermo Fisher Scientific) was used for RNA quantification. All RNAs were of good quality (average RIN = 8.3 ± 0.5) with a concentration varying between 6 and 93 ng μl^{-1}. 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).

2.5. Raw data processing, quality evaluation and differential expression analyses

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). Fragments Per Kilobase per Million mapped fragments (FPKM) were calculated for each gene by considering the length of a specific gene and the reads count mapped to that gene.

Differential expression analysis between ALAN and control groups at the four timepoints was performed using *DESeq2* R package v. 1.20.0 (Love et al., 2014), through a model based on the negative binomial distribution. Genes were considered as differentially expressed (DEGs) when FPKM values had a $|\log_2(\text{Fold Change})| \geq 1$ and a false discovery rate (FDR) Benjamini and Hochberg's adjusted P-value (P-adj) ≤ 0.05. DEGs were classified into different clusters by applying soft clustering using the fuzzy c-means algorithm of *Mfuzz* package in R (Kumar and Futschik, 2007) DAVID 2021 webtool (Sherman et al., 2022) was then used (with default parameters) to identify significant KEGG pathways including genes belonging to the same cluster.

For co-expression analyses, R package *ggvenn* (Gao et al., 2024) was used to construct Venn diagrams and the VolcaNoseR webtool (Goedhart and Luijsterburg, 2020) to produce volcano plots. ClustVis (Metsalu and Vilo, 2015) was used to reconstruct heatmaps for target genes.

2.6. Functional enrichment analysis

To explore the functions of the DEGs, annotations of gene ontology (GO) terms and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways were performed using *ClusterProfiler* R package (Young et al., 2010). GO annotations are divided into 3 categories: Molecular Function (MF), Biological Process (BP) and Cellular Components (CC). The GO terms and KEGG pathways with a P-adj ≤0.05 were considered significantly enriched. Graphical representations of significantly expressed GO terms and KEGG pathways were created using the *ggplot2* R package (Wickham, 2016).

2.7. Protein-protein interaction analysis

Search Tool for the Retrieval of Interacting Genes (STRING, v. 12.0, https://string-db.org/) database was used to investigate direct and indirect protein-protein interaction (PPI) starting from DEGs retrieved for each timepoint comparison. Interactions with a high confidence score (at least 0.70) were retained. The generated PPI networks (PPIN) were manipulated using Cytoscape software v. 3.10.2 (Shannon et al., 2003) with STRING app (v. 2.1.1) and MCODE app (v. 2.0.3) to obtain the densely connected clusters of each PPIN.

3. Results

3.1. Raw data and gene expression analysis

The RNA-seq sequencing process generated a number of raw reads for library ranging from 39,688,752 to 60,943,682, with a Q30 ≥ 92 %.

After low-quality reads filtering, a percentage of remaining reads higher than 98 % was retained for all the 40 samples sequenced (Table A1). Mapping the samples' transcripts to the GRCz11 reference genome yielded alignment percentages between 86.55 % and 89.07 % (Table A1).

Co-expression levels of transcripts in ALAN-treated samples compared to control ones at each timepoint are displayed in Fig. 1b. Overall, a similar number of co-expressed genes between ALAN and control groups were recorded at all timepoints (Fig. 1b). The highest number of DEGs was registered at ZT15 and the lowest at ZT09 (Fig. 1b). ALAN-treated samples shared 13,365 co-expressed genes across the four timepoints. The most affected timepoint was ZT15, with 795 (5 %) exclusively up- and down regulated genes in comparison to its respective temporal control samples (Appendix A - Figure A1).

The number of DEGs for each pairwise timepoint comparison (i.e., ALAN *versus* Control) is showed in Table 1, and the complete list of significant DEGs at each timepoint is reported in Table A2. A total of 75 DEGs, of which 15 up- and 60 down-regulated, were identified at ZT03 (Fig. 2a, Table A2). Up-regulated genes included homeobox group genes (*hoxc3a*, *hoxc4a*, *hoxc5a, hoxc8a*) and nuclear receptor subfamily genes involved in circadian rhythm regulation (*nr1d4a, nr1d4b*). Down-regulated genes were mostly involved in transmembrane trafficking (*aqp3a*, *slc29a1b*), hemopoiesis regulation (*hbba1*, *gata1a*), keratin and intermediate filament activity (*krt1-19d*, *krt97*, *krt91*, *smyhc2*), apoptotic processes (*casp3b*, *hdr*), and circadian clock rhythm (*cry1ab*).

At ZT09, six up-regulated and 12 down-regulated DEGs were identified (Fig. 2b and Table A2). Most of the up-regulated genes were involved in circadian regulation (*nr1d1*, *nr1d2b*, *ciarta, per1a)*, while most of the down-regulated genes were implicated in cellular signalling (*rorcb, fbxw7*), ion binding (*tyrp1a, pkd2l1*) and enzyme regulation (*scg5*).

At the nocturnal timepoint ZT15, among the 37 over-expressed genes, we found genes involved in the modulation of circadian rhythm (*cry1aa, cry1ab, cry2, cry5, per3, ciarta, bhlhe40, nr1d2b*), regulation of transcription (*cipcb, mycla, dbpb*), stress response (*hsf2, hsp70l, hsp70.3, hsp70.2, ddb2*, *cry-dash*, *bco2a*), transmembrane transport (*slc7a10b, slc14a2*, *apoa4b.1*), and cellular metabolism (*znf395b*, arg*2*, *lonrf1l*, *s100s*, *cyp2p6*, *cyp2ad6*) (Fig. 2c and Table A.2). Down-regulated genes were primarily involved in defence responses (*fbxl3l*, *slc7a5*).

A total of 72 DEGs, of which 31 up- and 41 down-regulated, were found at ZT21 (Fig. 2d and Table A2). Up-regulated genes were involved in motor and carrier activities (*myh7bb*, *slc46a3*), energy homeostasis (*mrap2a, lonrf1l*, *btr23*, *mctp2b*) and differentiation of immune cells (*il4r.1*). Down-regulated genes had a function in transcriptional regulation (*foxg1b*, *dbpa*, *dbpb*, *smu1b*), protein biosynthesis and catabolism (*itm2cb*, *usp2a*, *usp2b*, *fkbp5*, *gpt2l*, *stk35*), cell response to light stimulus and stress (*tefb*, *hsd20b2*). At this nocturnal timepoint, genes implicated in circadian regulation were both significantly up-regulated (*per2*, *cry1bb*, *cry1ba*, *cry5*) and down-regulated (*per1a*, *ciarta*, *cry1ab*, *per1b*, *nocta*).

Mfuzz grouped DEGs into six different clusters based on their temporal expression patterns in ALAN-treated samples compared to control samples (Fig. A2, Table A3). Cluster 1 counts for 606 DEGs with pronounced downregulation at ZT15, while Cluster 5 shows a peak of up-regulation at this timepoint (Fig. A2). The DEGs in Cluster 1 were significantly enriched in "phototransduction" (dre04744), and those in Cluster 5 in pathways involved in energy production, such as "oxidative phosphorylation" - dre00190 and "Ribosome" - dre03010. An opposite temporal pattern was also observed for Cluster 3 and 6, respectively up- and down-regulated at ZT03 compared to the other timepoints (Fig. A2). Cluster 3 comprised DEGs involved in nucleic acid regulation, such as "DNA binding" and "Transcription regulation", while Cluster 6 was mainly enriched with pathways associated with movement ("muscle protein", "skeletal muscle contraction", and "troponin complex"). Cluster 6 was also up-regulated at nighttime ZT09. Cluster 2 included 810 DEGs with a notable down-regulation peak at ZT21, whereas in Cluster 4 the expression profile showed a down-regulation apex at ZT09 (Fig. A2). These two clusters were not associated with any significantly enriched pathways.

Table 1
Number of Differentially Expressed Genes (DEGs) in ALAN-treated samples compared to control samples at each timepoint (ZT03, ZT09, ZT15, ZT21). Up-regulated, down-regulated and total DEGs are reported. Only DEGs with P-adj value ≤ 0.05 and $|\log_2 (\text{Fold Change})| \geq 1$ were counted.

Pairwise Alan vs Control Comparisons	Upregulated in ALAN	Downregulated in ALAN	Total
ZT03	15 (20 %)	60 (80 %)	75
ZT09	6 (33 %)	12 (67 %)	18
ZT15	37 (84 %)	7 (16 %)	44
ZT21	31 (43 %)	41 (57 %)	72

3.2. Functional enrichment analyses

GO enrichment analyses were carried out to underline the regulatory pathways in which DEGs were involved at each timepoint. Overall, 282 significant GO categories were found, mainly related to BP term (Table 2). All the details on the annotated GO terms significantly regulated at the four timepoints are available in Table A4. ZT03 and ZT15 were the most enriched timepoints, with 72 (eight over- and 64 under-represented) and 193 (184 over- and nine under-represented) GOs, respectively (Table 2).

As shown in Fig. 3a–c, at ZT03 several under-represented GO terms in the three categories BP, CC and MF were related to movement and circadian rhythm. A minor number of GOs were involved in gas transport both in BP and MF categories (Fig. 3a, b, 3c). ALAN-ZT09 samples, compared to controls, showed only few non-specific enriched GO terms, except for four over-represented DEGs included in the BP category "regulation of circadian rhythm" (Fig. 3f). At ZT15 over-represented GO terms were linked to stress response, especially in BP category (immune response; response to toxic substance; defence response; response to oxidative stress; response to external biotic stimulus: Fig. 4a). At this timepoint, circadian rhythm-related and energy-metabolism related GOs were also highly enriched. Under-represented GO terms, instead, were chiefly involved, among the others, in detection and response to light, response to abiotic stimuli, and visual perception (Fig. 4d). At ZT21 gene regulation was mainly associated with BP and CC. Enriched GOs were exclusively associated with muscle components and contraction, while under-represented GO terms were mainly related to circadian rhythm (Fig. 4e and f).

Differentially expressed pathways under ALAN condition compared to natural light-dark cycle condition were then searched based on the KEGG database. Overall, across the four timepoints, DEGs were enriched in 11 pathways, mainly involved in muscle contraction, phototransduction and oxidative stress (Fig. 5 and Table A5). At ZT03 an up-regulation of 24 genes involved in "regulation of actin cytoskeleton" was observed, whereas "adrenergic signalling in cardiomyocytes", "motor proteins" and "cardiac muscle contraction" were found to be repressed (Fig. 5). At ZT09 no pathways were up- nor down-regulated (Fig. 5). Consistent with DEGs evidences, changes in pathways expression were mainly at ZT15 (four enriched pathways and one repressed) (Fig. 5). Up-regulation was observed in genes involved in "oxidative phosphorylation", "ribosome", "glutathione metabolism" and "nucleotide metabolism", while the "phototransduction" pathway was significantly down-regulated. "Arachidonic acid metabolism" and "foxO signalling pathway" were respectively up- and down-regulated in treated samples at ZT21 (Fig. 5).

3.3. Protein-protein interaction (PPI)

PPI analysis was performed to deeply investigate the interaction of the DEGs in response to ALAN exposure at each sampled timepoint

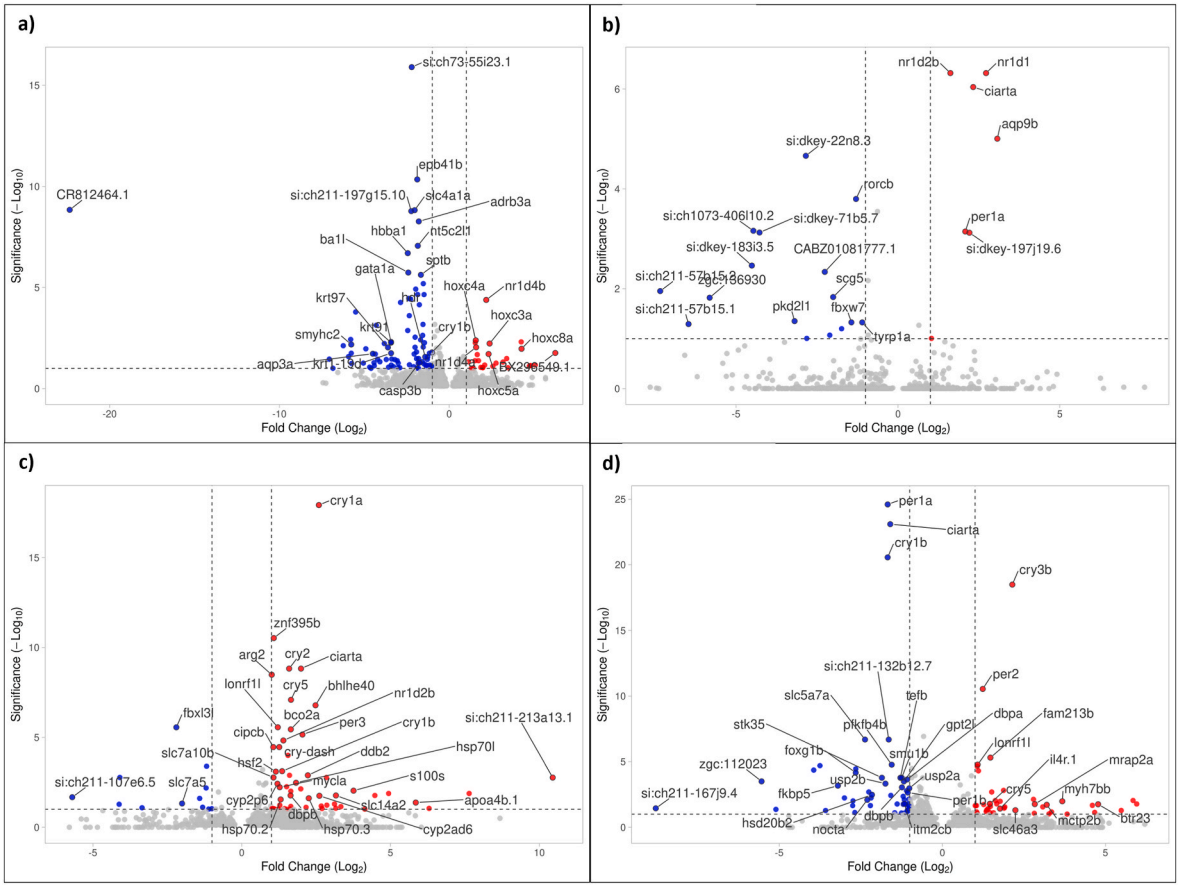

Fig. 2. Volcano plots of the differentially expressed genes (DEGs) between ALAN and control groups at the four timepoints: a) ZT03, b) ZT09, c) ZT15, d) ZT21. X axis and Y axis represent $\log_2$ transformed fold change and -$\log_{10}$ transformed significance, respectively. Non-significant DEGs are in grey. Significantly upregulated and downregulated DEGs are in red and blue, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Summary of statistically significant over- and under-represented Gene Ontology (GO) terms in the four sampled timepoints, divided by GO category (Biological Process, BP, Cellular Component, CC, and Molecular Function, MF).

Pairwise Alan vs Control Comparisons	BP	CC	MF	Total GOs
ZT03_up-regulated	8	0	0	8
ZT03_down-regulated	27	24	13	64
ZT09_up-regulated	1	0	3	4
ZT09_down-regulated	0	2	2	4
ZT15_up-regulated	106	37	41	184
ZT15_down-regulated	9	0	0	9
ZT21_up-regulated	3	16	1	20
ZT21_down-regulated	7	0	0	7

(Fig. 6). At ZT03, a major core (22 nodes, 70 edges) was identified, comprising two communities, one connected to stress-related proteins and the other related to proteins involved in transcriptional activities (Fig. 6a). PPI analysis also identified other small clusters referred to proteins involved in muscle activities, hormones homeostasis and circadian regulation. A minor core including only two proteins (Txn2 and Txnipa) was also observed, among the others, at this time point (Fig. 6a).

In line with the functional enrichment analysis, the PPIN at ZT09 timepoint showed only one hub of proteins involved in the regulation of the circadian rhythm (Fig. 6b). A high number of protein interactions were recorded at ZT15, with a main community of 51 nodes and 381 edges, along with two hubs (Fig. 6c). In this cluster proteins involved in electron transfer and transcriptional activities were identified. Three minor hubs implicated in stress responses, circadian regulation and fatty acid metabolism were also identified (Fig. 6c). At ZT21, the cluster with the highest MCODE score included stress-induced proteins. Hubs referred to circadian rhythm and transcriptional activities were also predominant at this late-night timepoint (Fig. 6d).

4. Discussion

In this work, we studied the transcriptome-wide gene expression response in the zebrafish brain to a one-month exposure to ALAN. We performed a time-series transcriptome analysis, sampling RNA at four timepoints evenly distributed across the 24-h cycle, covering both diurnal and nocturnal periods. Such experimental design allowed us to highlight the time course of changes in gene expression patterns induced by the exposure to ALAN and understand the transcriptional modification over a 24-h period. Exposure to an altered light-dark cycle induced alteration in the transcription of several genes and metabolic pathways both at night, when zebrafish were directly exposed to light, and during the day, when they experienced the natural light conditions. Overall, the impacted genes included both circadian-clock genes and those related to immunity, stress response to sensory stimuli, energy production, motor activity and reproductive processes, consistent with previous findings (Bumgarner and Nelson, 2021; Dominoni et al., 2022; Alaasam et al., 2024). Our results ultimately confirm that ALAN induces significant changes of gene expression in zebrafish during both night and daytime.

Previous studies on terrestrial, marine and freshwater species have documented alterations - varying in intensity between day and night - in behavioural, physiological and genetic traits due to ALAN exposure,

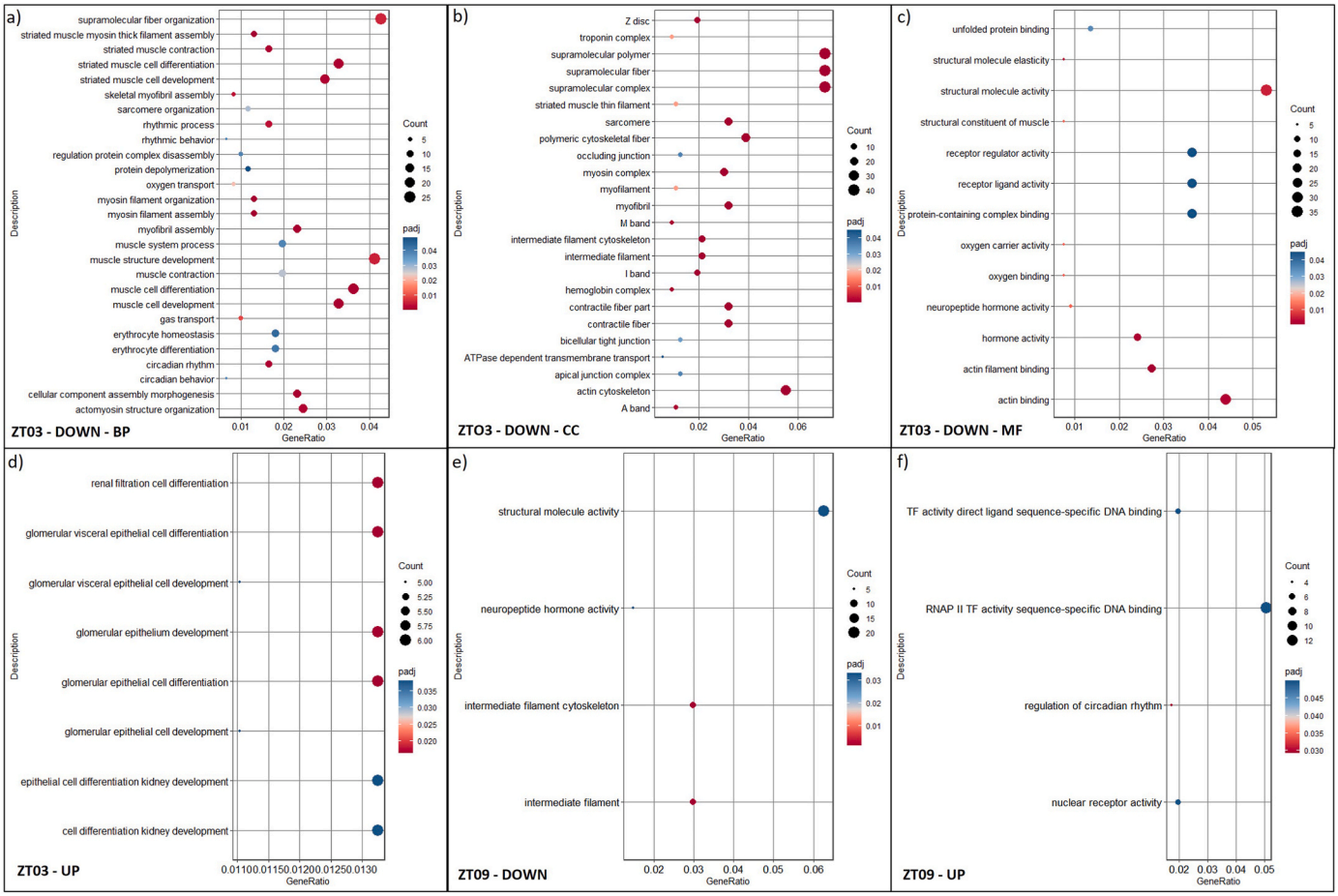

Fig. 3. Bubble plots of the differentially expressed genes enriched in Gene Ontology (GO) terms at the two daytime timepoints: a) ZT03, downregulated Biological Process genes; b) ZT03, downregulated Cellular Component genes; c) ZT03, downregulated Molecular Function genes; d) ZT03, upregulated genes; e) ZT09, downregulated genes; f) ZT09, upregulated genes.

which ultimately resulted in lowered fitness (Bumgarner and Nelson, 2021; Ferretti et al., 2025; Georgiou et al., 2024; Marangoni et al., 2022; Stanton and Cowart, 2024; Touzot et al., 2022). Our results are in line with those evidence, as adult zebrafish exposed to ALAN showed significant alteration in gene expression due to increased stress responses. We recorded upregulation and downregulation of several genes related to circadian clock, homeostasis, motility, and visual perception in both the early hours of the day (ZT03, corresponding to 9 a.m.) and at night (ZT15 and ZT21, corresponding to 9 p.m. and 3 a.m., respectively). Conversely, at ZT09 (3 p.m.), very few numbers of genes and pathways were differentially expressed in ALAN-treated samples. In detail, at ZT03 we observed an up-regulation of genes related to transcriptional regulation, which were then down-regulated at ZT15 and again up-regulated at ZT21. At ZT03 we also observed a down regulation of genes related to mobility, conversely up-regulated at night, i.e. during ALAN exposure. Furthermore, gene expression showed an almost complete convergence at ZT09 with the few down- and up-regulated genes mainly related to phototransduction and energy production, respectively. At this time, the effects of ALAN may be less pronounced compared to other timepoints, such as ZT15 which occurs 3 h after the beginning of ALAN exposure. Since the circadian system has mechanisms that compensate for disruptions and maintain homeostasis, it is possible that at ZT09 the system has partially buffered, or corrected the transcriptional changes induced by ALAN. Finally, at ZT21 genes related to movement remained up-regulated, and those involved in the circadian system continued to be significantly down-regulated.

Given that the expression levels of many genes change across the light-dark cycle, we emphasize the importance to include multiple sampling points along the day to better understand how ALAN exposure may impact organisms at molecular level. In addition, given the recent findings by Alaasam and coworkers (2024), new studies should focus on how gene expression patterns may change under acute or chronic exposure to artificial light, and whether ALAN-exposed animals may show habituation to repeated light pollution exposure. Analysis of DEGs and GOs for all timepoints showed a widespread and significative modification in the transcription profile of genes involved in circadian rhythm. In zebrafish, the circadian system is a 24-h loop self-regulated feedback entrained by both light and heat zeitgebers (Sacksteder and Kimmey, 2022). Along with many factors involved in the regulation of transcription, we recorded a differential expression of Cry and Per family genes (*cry1, cry2, per1, per2* and *per3*), which act as repressors by directly binding CLOCK:BMAL proteins (Sacksteder and Kimmey, 2022). This result has not been reported in previous studies on fish and amphibians (Labala et al., 2024; Touzot et al., 2022). Interestingly, at ZT15 we found the overexpression of *cry1aa* and *cry1ab*, which, in normal conditions, peak early in the morning. In contrast, the expression profile of *cry3a* and *cry3b*, which usually peaks near the transition from light to dark, was not modified with respect to controls (Liu et al., 2015; Sacksteder and Kimmey, 2022). Moreover, *cry1* is a strong modulator of the circadian clock by inhibiting CLOCK:BMAL and can stop the clock on its own during prolonged photoperiods (Tamai et al., 2007). We also found at ZT21 the upregulation of *per2*, a central component of the zebrafish molecular circadian clock that has been proposed to play an important role in the photic entrainment mechanism (Ruggiero et al., 2021). Characterization of *per2* mutant zebrafish showed that this gene is essential for maintaining behavioural rhythmicity, photic entrainment

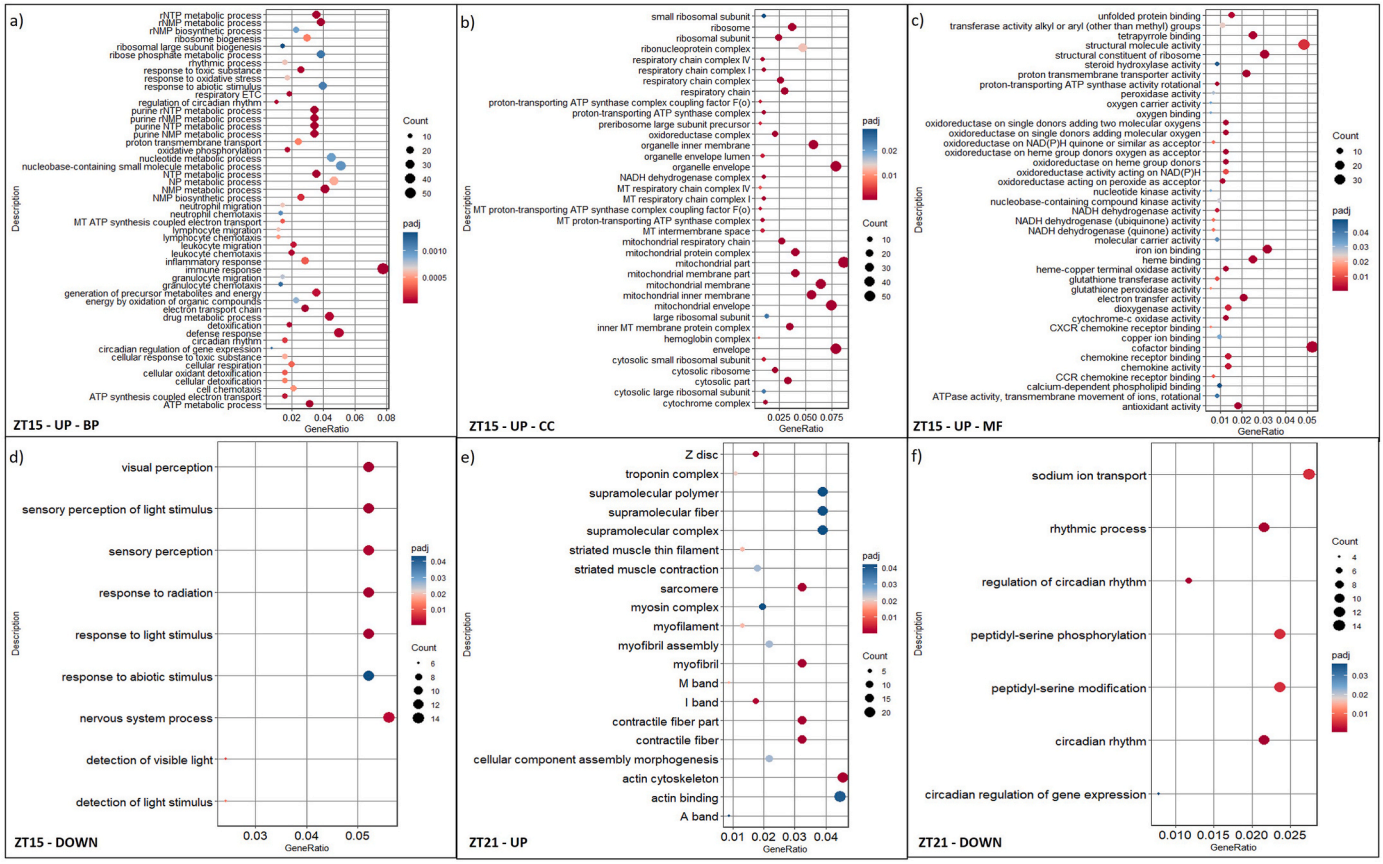

Fig. 4. Bubble plots of the Differentially Expressed Genes enriched in Gene Ontology (GO) terms at the two nighttime timepoints: a) ZT15, upregulated Biological Process genes; b) ZT15, upregulated Cellular Component genes; c) ZT15, upregulated Molecular Function genes; d) ZT15, downregulated genes; e) ZT21, upregulated genes; f) ZT21, downregulated genes.

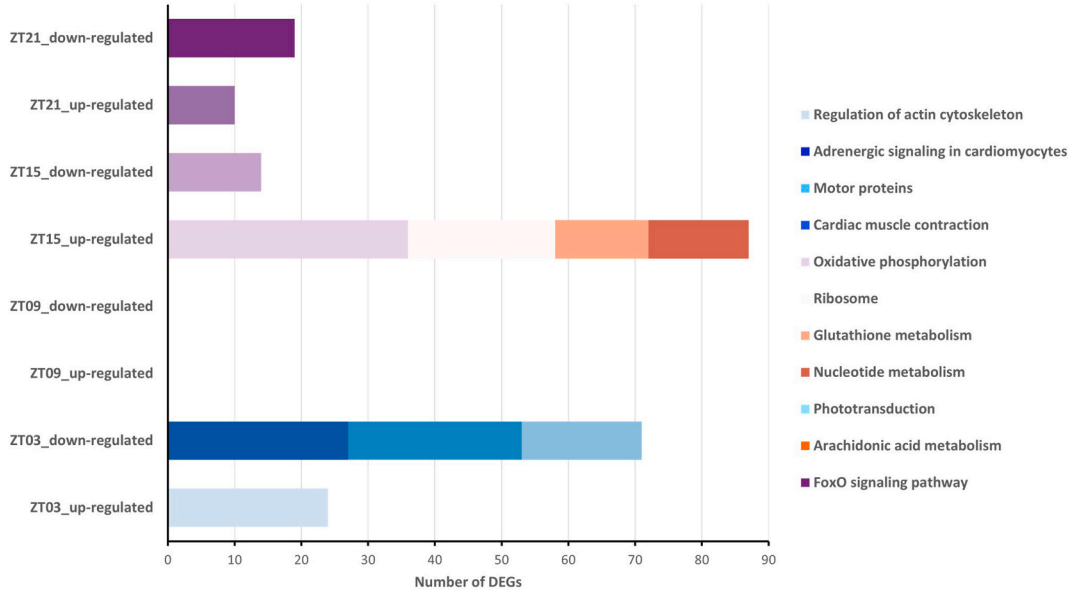

Fig. 5. KEGG pathways of up- and down-enriched significant differentially expressed genes at the four timepoints.

and locomotor activity levels (Ruggiero et al., 2021; Wang et al., 2015). The cardinal role of both *cry1* and *per2* in the generation of daily rhythmicity has been further demonstrated using a zebrafish null mutant for both genes (Hirayama et al., 2019). Our findings confirm how ALAN affects the regulation of the circadian system, which is crucial for maintaining the overall biological homeostasis. In zebrafish, circadian rhythms are known to regulate critical functions such as sleep, feeding, hormonal balance, immunity and infections (Elbaz et al., 2013; Sack-steder and Kimmey, 2022).

In this study, we also found that ALAN-exposed zebrafish showed

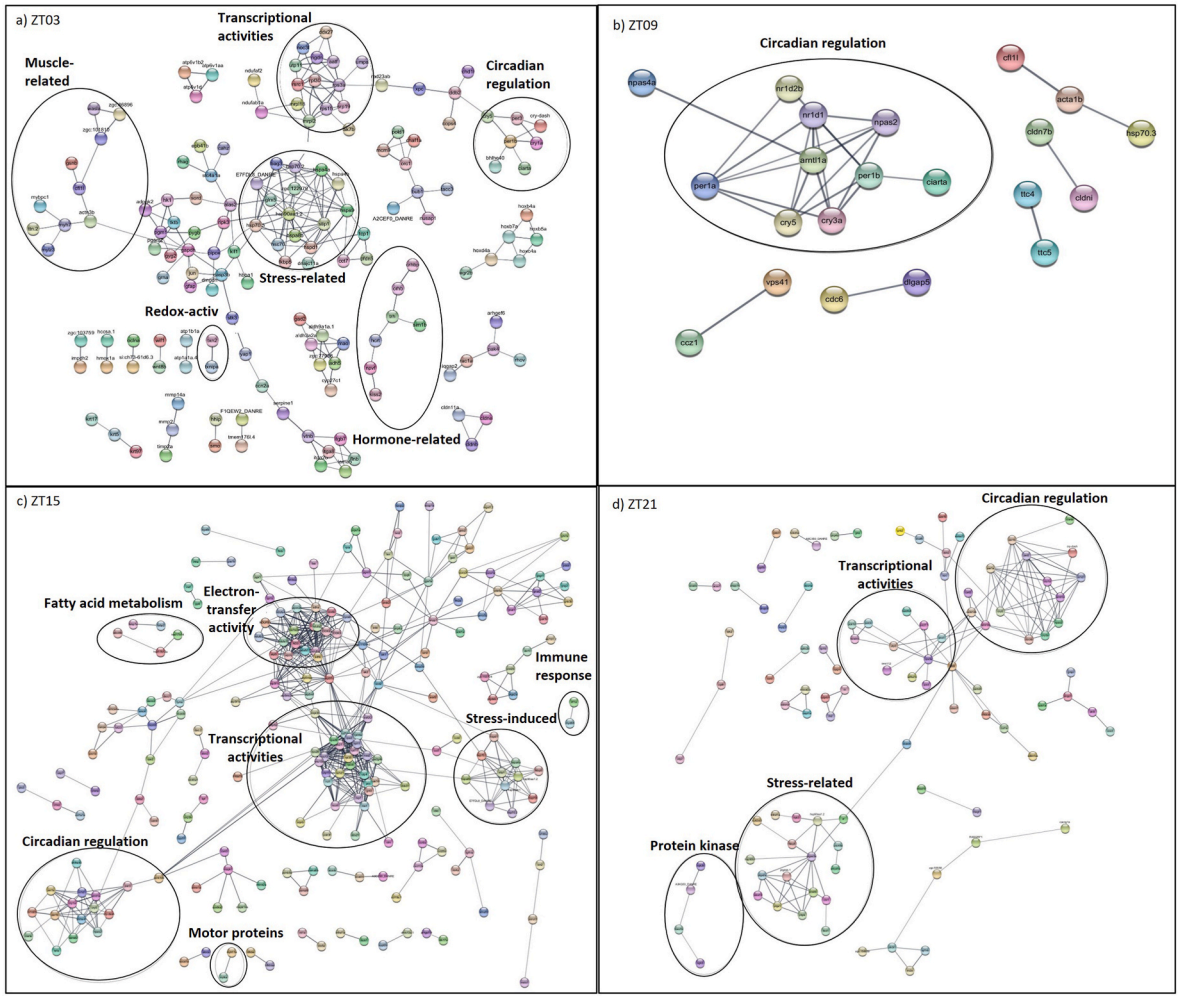

Fig. 6. Protein-protein interaction (PPI) network analysis. Comparisons between ALAN and controls at a) ZT03, b) ZT09, c) ZT15, d) ZT21.

altered expression of genes and pathways related to stress and defence responses as well as apoptosis along the 24-h sampling period. For example, genes such as *caspase 3* and *apoptosis-related cysteine peptidase b* were found to be down-regulated during the day. In contrast, during the night, we found *cryptochrome DASH*, *interleukin 4 receptor*, and various members of the heat-shock proteins (HSP) family among the up-regulated genes. In addition, GO terms linked to stress, immune and defence responses, arachidonic acid metabolism and FoxO signalling pathway were differentially expressed under ALAN exposure. The role of caspases - particularly those related to inflammation and apoptosis - in stress response is well established (Van Opdenbosch and Lamkanfi, 2019). Similarly, various members of the HSP family have been associated with responses to infections by interacting directly with immune cells (Wallin et al., 2002). A link between differential expression profiles of the circadian clock under light pollution and of genes involved in inflammation and infection processes has been reported by various authors. For instance, Touzot et al. (2022) recorded a deregulation of the complement system in *Bufo bufo* tadpoles exposed to ALAN, while NF-kB and cytokines were found altered in ALAN-exposed zebrafish and rats (Khan et al., 2018; Labala et al., 2024; Spengler et al., 2012). Our results corroborate the idea that a compromised circadian rhythm may negatively impact the optimal activity of the immune system (Sacksteder and Kimmey, 2022).

ALAN is known to reduce the nocturnal expression of melatonin (Brüning et al., 2015; Grubisic et al., 2019; but see: Touzot et al., 2022), whose synthesis is regulated by the circadian clock (Grubisic et al., 2019 and references therein). Altered levels of this hormone have been associated with the cognitive impairments observed in birds under ALAN conditions (Buniyaadi et al., 2022; Taufique, 2022). Cognitive impairments have been recently described also in ALAN-exposed zebrafish (Lucon-Xiccato et al., 2023; De Russi et al., 2024; De Russi et al., 2025). We found no evidence of variation in gene expression connected to the metabolism of melatonin, consistent with Touzot et al. (2022). Based on these findings, we speculate that alterations in melatonin levels alone are not sufficient to explain the cognitive impairment observed in zebrafish under ALAN conditions.

GOs related to transmembrane trafficking were also significantly impacted in zebrafish exposed to ALAN, particularly near transitions from dark to light and *vice versa* (i.e, at ZT03 and ZT15). At ZT03, under-representation of biological processes involved in oxygen and gas transport were observed. Consistent with previous studies, this may indicate a disruption of molecular transportation systems due to increased energetic costs under ALAN conditions (Labala et al., 2024; Touzot et al., 2022). Conversely, pathways involved in gas transport were significantly enriched at ZT15. During the night, light exposure compels zebrafish to higher activity levels, as seen with immune and inflammatory responses. Consequently, up-regulation of energy-related pathways is essential (Labala et al., 2024; Lynn et al., 2022). The induction of stress-related proteins and changes in metabolic pathways suggest that ALAN may impose a metabolic burden on ALAN-exposed zebrafish, potentially compromising their energy homeostasis and increasing susceptibility to diseases. Moreover, various authors related disruption of trafficking activities and circadian rhythms to a reduced fitness of the population, defined as lower eggs laying, reduced

oocyte/spermatid development and sperm motility, or decreased concentration in sex hormones (Chen et al., 2021 and references therein).

Several biological processes involved in muscle organization and motility were also found to be differentially enriched during daytime and nighttime. An increase in locomotor activity under ALAN conditions has been widely observed in many animal taxa (Alaasam et al., 2018; Alaasam et al., 2021; Batra et al., 2019; Spoelstra et al., 2018) including zebrafish (Lucon-Xiccato et al., 2023) and other fish species (Georgiou et al., 2024). While part of these alterations is likely related to the circadian modulation of activity, we cannot exclude that they are also the by-product of ALAN impacts on muscular processes.

5. Conclusion

ALAN has recently come under the spotlight for its impact on a wide variety of terrestrial and aquatic species (Chen et al., 2021; Dalle Carbonare et al., 2023; Khan et al., 2018; Labala et al., 2024; Stanton and Cowart, 2024; Touzot et al., 2022). Its effects at the molecular level in animals, however, remain largely unexplored. Our study provides a better understanding of the molecular responses induced by one-month long ALAN exposure in aquatic species and shows how these regulatory responses are activated both during the night, when the animals are directly exposed to ALAN, and during the day, under natural light conditions. Overall, this study demonstrates that ALAN profoundly affects adult zebrafish brains at multiple levels, by disrupting circadian rhythms, inducing stress responses, altering energy metabolism, and potentially impairing reproductive and developmental processes. As zebrafish are an integral part of many freshwater ecosystems, disruptions in their behaviour, physiology, and reproductive success can influence community structure and ecosystem functioning. Changes in feeding behaviour and predator-prey interactions due to altered activity patterns can cascade through the food web, affecting species interactions and biodiversity. Moreover, considering that the evidence in zebrafish has been often predictive of findings in other species, we believe that what we observed in this study may be common in most aquatic ecosystems. These findings highlight the urgent need to address light pollution to safeguard aquatic ecosystems and preserve their biodiversity as well as their functionality. Future research should focus on long-term impacts, potential acclimation mechanisms, and the interactions between ALAN and other anthropogenic stressors to develop comprehensive and effective conservation policies. We also emphasize the need of multidisciplinary research aimed to identify the effects of different LED wavelengths on the physiology of organism to inform biologically based regulations to limit the impact of light exposure on natural populations.

CRediT authorship contribution statement

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

Ethical note

All husbandry and experimental procedures were performed in accordance 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.

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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.

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Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2025.126683.

Data availability

BAM files generated from each sample were deposited in SRA under accession number PRJNA1244314.

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