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Effects of light and circadian clock on the antiviral immune response in zebrafish

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ABSTRACT

The circadian clock mechanism, which is evolutionarily conserved across various organisms, plays a crucial role in synchronizing physiological responses to external conditions, primarily in response to light availability. By maintaining homeostasis of biological processes and behavior, the circadian clock serves as a key regulator. This biological mechanism also coordinates diurnal oscillations of the immune response during infections. However there is limited information available regarding the influence of circadian oscillation on immune regulation, especially in lower vertebrates like teleost fish. Therefore, the present study aimed to investigate the effects of light and the timing of infection induction on the antiviral immune response in zebrafish. To explore the relationship between the timing of infection and the response activated by viral pathogens, we used a zebrafish model infected with tilapia lake virus (TiLV). Our findings demonstrated that light availability significantly affects the antiviral immune response and the functioning of the molecular clock mechanism during TiLV infection. This is evident through alterations in the expression of major core clock genes and the regulation of TiLV replication and type I IFN pathway genes in the kidney of fish maintained under LD (light-dark) conditions compared to constant darkness (DD) conditions. Moreover, infection induced during the light phase of the LD cycle, in contrast to nocturnal infection, also exhibited similar effects on the expression of genes associated with the antiviral response. This study indicates a more effective mechanism of the zebrafish antiviral response during light exposure, which inherently involves modification of the expression of key components of the molecular circadian clock. It suggests that the zebrafish antiviral response to infection is regulated by both light and the circadian clock.

1. Introduction

The circadian clock is highly conserved timing mechanism that generates circadian rhythms in animal physiology, behavior, and biochemistry and regulates their timing. Thus, this internal mechanism enables organisms to keep track of time, coordinate the internal systems, and anticipate predictable daily events, such as periods of activity and rest [1–3]. In mammals, circadian rhythms are controlled by the suprachiasmatic nucleus (SCN) in the hypothalamus, which coordinates the activities of various peripheral oscillators [4]. In fish, however, circadian rhythms are driven by multiple coupled central circadian clocks, located in the retina, pineal gland, and hypothalamus, and are synchronized with light cycles [5,6]. The rhythmic activity of the central oscillators is manifested by circadian changes in the expression of several key clock genes such as: *clock*, *arntl*, *period* (*per*) and *cryptochrome* (*cry*). Positive clock elements such as CLOCK-ARNTL1 heterodimers induce expression of the genes encoding PER and CRY through E-box elements (6 bp DNA fragments of CANNTG consensus sequence) in their promoters, while PER-CRY heterodimers interact with CLOCK-ARNTL1 to repress their transcriptional activation function [7,8]. In a similar way, the molecular clockwork in peripheral oscillators is formed by autoregulatory transcription-translation feedback loops orchestrated by

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circadian clock genes [9]. They facilitate the transcription of various genes involved in cell growth, differentiation, survival as well as immune functions [10–12].

The clock mechanism have long been known to play crucial role in physiology. However, increasing evidence suggests that it serves an important regulator of the immune system [2,13]. Many specific immune functions or properties of the immune system are rhythmic, for example leukocyte recruitment, phagocytosis, and cytokine synthesis [2, 13,14]. Daily oscillations in the number of circulating leukocytes, levels of cytokines, and chemokines regulate innate and adaptive immune response. This enables organisms to anticipate daily behavioral changes, thereby reducing the risk of infection and tissue damage [15]. For example, in mice higher efficiency in clearing bacterial infection, accompanied by increased production of pro-inflammatory cytokines, was found during the active phase (at night) [16]. Circadian activity of the host immune response, as well as the different virulence of the pathogen during the day and night, result in a different risk of infection at certain times of the day [13]. For example, the daily survival rate of mice varied depending on the time of induction of bacterial infection or the time of administration of bacterial endotoxins [17,18]. However, little is known about the impact of the timing of infection on its development in fish, which could be crucial to the welfare and treatment of these animals, as well as for understanding the evolution of these processes.

The various manifestations of immune activity in fish have been shown to be under the control of the circadian clock, which ensures that fish can effectively neutralize the pathogen and recover optimally from infection or injury [13]. Therefore, circadian rhythms greatly determine the effective immune response and survival during infection in fish. This is inherently related to the phase of the circadian cycle in which infection/immune stimulation occurs, as well as light conditions. For example, when rainbow trout (*Oncorhynchus mykiss*) reared under LL were challenged with *Argulus foliaceus* (fish lice), their ability to clear the infection was significantly reduced compared to trout raised under LD conditions. It was accompanied by arrhythmic expression of genes involved in mucosa anti-microbial and Th1-driven responses, as well as phase-shifted expression rhythms of genes involved in the differentiation and regulation of T-cells [19]. In turn, LL exposure of Atlantic cod (*Gadus morhua*) resulted in increased expression of antioxidant enzymes: superoxide dismutase, catalase and glutathione reductase in the liver [20]. Extended day length also increased susceptibility to ectoparasites and altered expression in specific immune genes in sticklebacks [21], as well as resulted in higher antibody titer in sea ruff (*Sebastiscus marmoratus*), what explains the higher antibody levels in fish immunized in the summer, when photoperiods are longer, compared to the fish treated in the winter months [22]. These results show that the effectiveness of the immune response depends on the light conditions and varies according to the host species and pathogen.

Most studies mainly describe the effect of the photoperiod and the time of infection on the immune response of fish during parasitic and bacterial challenge or stimulation with bacterial factors/compounds, but there are virtually no attempts to explain the interaction between the circadian clock and the response activated by viral pathogens. Therefore, we decided to study the relationship between the biological clock and immune mechanisms activated during the antiviral response using zebrafish, which is a well known animal model to study host-pathogen interactions during infections with viruses associated with mass mortality of farmed fish but also human viruses [23–25]. Recently, we developed the zebrafish-tilapia lake virus (TiLV) infection model to better understand the immunology behind the TiLV infection, which threatens the global Nile tilapia aquaculture and negatively impacts global food security [26,27]. The model shown that TiLV replicates in multiple organs of zebrafish including kidney. It clearly activates type I interferon (IFN) and the inflammatory response which is associated with the induction of expression of genes encoding the main molecules of the type I IFN pathway, including receptors recognizing viral dsRNA (RIG-I), transcription factors IRF-3 and -7, type I IFN, the antiviral protein MXA, and the pro-inflammatory cytokine IL-1β [26,28,29].

Therefore, in the present study, we used zebrafish-TiLV infection model to investigate the effects of light and/or circadian clock activity on the antiviral immune response. We demonstrated that light availability clearly affects the antiviral immune response and the functioning of the molecular clock mechanism during TiLV infection.

2. Materials and methods

2.1. Virus and cells

Tilapia lake virus (TiLV) (VETKU-TV01 isolate) was previously isolated from red hybrid tilapia in Thailand [30] and was multiplied in E−11 cells isolated from the striped snakehead (*Channa striata*) as described previously [26]. The $TCID_{50}$/ml was calculated following the method of Reed and Muench [31].

2.2. Experimental animals

Zebrafish (*Danio rerio*) (Tübingen line) were grown in 8 L tanks on a ZebTEC Stand Alone system (Tecniplast, Buguggiate, Italy) at water temperature of 28 °C, day/night cycle of 12/12 h and fed twice a day with commercially available dry zebrafish food (Gemma Micro 300 ZF, Skretting) and/or brine shrimp (*Artemia salina*) hatched using commercially available artemia cysts (Artemia cysts, Artemia Koral). Before the infection, adult fish were adapted for 3 weeks to different light regimes: 12L:12D (LD) photoperiod (12 h of light and 12 h of darkness) and 0L:24D (DD) (0 h of light and 24 h of darkness). Under the LD photoperiod, the light was turned on at ZT 0 and turned off at ZT 12 (ZT, Zeitgeber Time). The light intensity at the water surface level during the day was 250 lux, while at night, as well as under the DD regime, the light intensity was 0 lux. In both light regimes fish were divided into three groups: intact (non-manipulated fish), control (intraperitoneally (i.p.) injected with culture medium) and infected (fish i.p. injected with medium containing TiLV) and placed into separate aquaria with aeration and water temperature of 28 °C (Fig. 1). Fish were fed twice a day using dry zebrafish food (Gemma Micro 300 ZF, Skretting). At a given time point, all fish in the mock- and TiLV-infected groups were anesthetized with 0.168 g/L tricaine methanesulfonate (TMS; Sigma-Aldrich, St. Louis, MO, USA) buffered with 0.336 g/L $NaHCO_3$ (POCH, Gliwice, Poland) and intraperitoneally injected either with 10 μL medium collected from non-infected cells or 10 μL of medium containing TiLV at a concentration of 1×10^7 $TCID_{50}$/mL. All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies, and all experiments were conducted according to license no. 200/2021 from the Local Ethical Committee (Krakow, Poland).

2.3. Experiment design

Based on the previous results [32], infection experiments were conducted at two different time points of the day: at ZT/CT 2 and ZT/CT 14 (ZT/CT, Zeitgeber/Circadian Time). Experiments were performed as two independent replicates as shown in Fig. 1 to ensure reproducible results. Fish kept under DD light regime, and sampled at nighttime point were anesthetized and injected in complete darkness under the dim red light. Both under LD and DD light regime, an additional groups of fish, were used to monitor the survival during infection.

2.4. Organ sampling

At selected time points: 3-, 6- and 14-days post-infection (dpi) fish (4 fish per each time point) were euthanized using tricaine methanesulfonate (TMS, 0.2 g/L; Sigma-Aldrich, St. Louis, MO, USA) buffered with 0.4 g/L $NaHCO_3$ (POCH, Gliwice, Poland) and the brain and kidney were

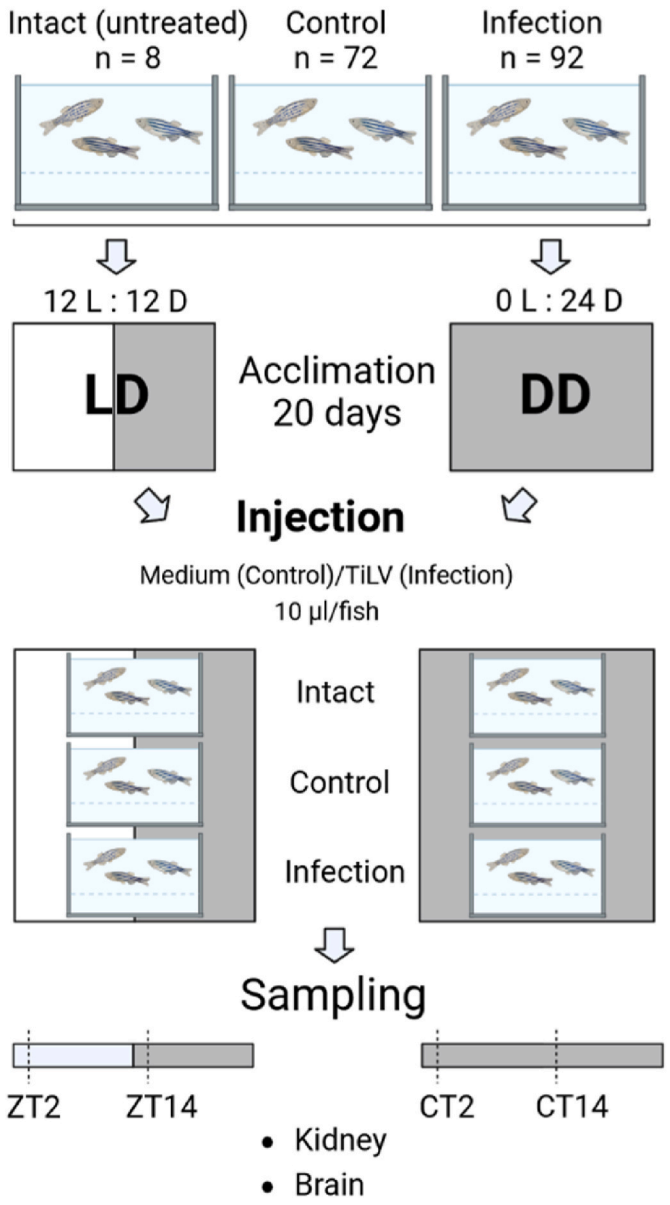

Fig. 1. The layout of the infectious experiments. Fish were maintained under LD (12 h of light and 12 h of darkness) and DD (0 h of light and 24 h of darkness) lighting conditions for 20 days. Under the LD photoperiod, the light was turned on at ZT 0 and turned off at ZT 12. After acclimation, fish were intraperitoneally injected with TiLV-containing medium (the "Infection" group), TiLV-free medium (the "Control" group) or left untreated (the "Intact" group). After an appropriate time from the induction of infection (3-, 6- and 14-days post-infection), fish were euthanized at points ZT/CT 2 and 14, and then the brain and kidney were isolated for further analysis. LD, light/dark, ZT/CT, zeitgeber/circadian time where ZT/CT 0 represents start of the light phase/subjective light phase.

isolated. During the nighttime point (ZT 14) in LD and both time points in DD, organs were isolated in dimmed light. The organs were placed in FIX RNA solution (EURx, Gdansk, Poland) and kept in the dark, then placed at −80 °C until RNA extraction was performed.

2.5. RNA extraction

The total RNA was isolated from tissues using ReliaPrep™ RNA Tissue Miniprep System (Promega, Madison, WI, USA) according to the manufacturer's protocol. RNA concentration and purity were checked spectrophotometrically using a Tecan Spark NanoQuant Plate™ spectrophotometer (Tecan, Männedorf, Switzerland). RNA samples were stored at −80 °C.

2.6. cDNA synthesis

The cDNA synthesis was performed using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Waltham, MA, USA) according to the manufacturer's protocol using 500 ng of total RNA from each tissue. For each reaction, a negative control sample (non-reverse transcriptase) was included. The reaction was conducted in a thermal cycler (Ditabis AG, Pforzheim, Germany) for 10 min at 25 °C, followed by 120 min at 37 °C. The reverse transcriptase was inactivated for 5 min at 85 °C and then cDNA samples were 5 × diluted using RNase-free water (Ambion, Waltham, MA, USA) and stored at −20 °C until further analysis.

2.7. Real-time quantitative polymerase chain reaction (RT-qPCR)

2.7.1. Viral load analysis

The approximation of virus load was performed by quantification of normalized gene copies as described earlier [26,33]. For quantification, a recombinant DNA plasmid-based standard curve from 10^1 to 10^7 gene copies were prepared and used for quantifying the copy number from each sample. The approximation of virus load is shown as the copy number of the TiLV segment 3 (*qtilv s3*) normalized against 1 × 10^5 copies of the host reference gene, *elongation factor 1 alpha* (*ef1α*) (Table 1). The RT-qPCR reactions were performed in duplicate for each sample using a reaction mix prepared as follows: 1 × Maxima Probe (quantification of viral segment 3) or 1 × Maxima SYBR Green master mix (quantification of zebrafish gene) with 100 nM of ROX (Thermo Fisher Scientific), 0.2 µM of each *ef1α* primer or 0.2 µM of each TiLV primer and 0.5 µM of probe, 5.0 µL of 50 × diluted cDNA and nuclease-free water to a final volume of 20 µL. The amplification protocol included an initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and elongation at 72 °C for 30 s. A melting curve analysis was performed at the end of each Maxima SYBR Green run. No-RT control run was performed for each sample using *ef1α* primers.

2.7.2. Gene expression

Zebrafish-specific primers (5′ to 3') for gene expression of immune activity genes were used (Table 1). The *rps11* gene served as an internal standard. This housekeeping gene showed a constant expression level in both organs tested.

All reactions were conducted on the thermal cycler Rotor-Gene Q (Qiagen, Venlo, Netherlands). The reaction mixture included 7 µL of SYBR® Select Master Mix (Applied Biosystems, Waltham, MA, USA), 2 µL of both forward and reverse primers (2 µM for *cry1a* and 1 µM for others) and 4 µL of 25 × diluted the analysed cDNA sample. For each gene and each sample RT-qPCR was done in duplicate. Amplification was specific and no amplification was observed in negative control samples (non-template control and non-reverse transcriptase control). The real-time qPCR conditions were as follows: preheating at 50 °C for 2 min, denaturation at 95 °C for 2 min, and 40 cycles of amplification and quantification (15 s at 95 °C and 60 s at 60 °C), followed by melt curve analysis (at ramp +0.5 °C). The constitutive expression of target genes was rendered as a ratio of the reference gene (*rps11*) relative to the target gene and was calculated according to the equation:

$$Ratio = \frac{(E_{reference})^{Ct_{reference}}}{(E_{target})^{Ct_{target}}}$$

where E is the amplification efficiency and Ct is the threshold cycle (the number of PCR cycles needed for the signal to exceed a predetermined threshold value).

Table 1
Primer sequences with corresponding accession numbers used for gene expression and virus load studies.

Gene	Primer forward (5′-3′)	Primer reverse (5′-3′)	Acc. no.
rig-I	TTGAGGAGCTGCATGAACAC	CCGCTTGAATCTCCTCAGAC	JX462558.1
irf3	CAAAACCGCTGTTCGTGCC	CATCGTCGCTGTTGGAGTCCT	NM_001143904
irf7	AGGCAGTTCAACGTCAGCTACCAT	TTCCACCAAGTTGAGCAATTCCAG	NM_001313779
ifn1	GAGCACATGAACTCGGTGAA	TGCGTATCTTGCCACACATT	NM_207640.1
mxa	GACCGTCTCTGATGTGGTTA	GCATGCTTTAGACTCTGGCT	AJ544823.1
il-1β	GAACAGAATGAAGCACATCAAACC	ACGGCACTGAATCCACCAC	NM_212844.2
il-10	ATTTGTGGAGGGCTTTCCTT	AGAGCTGTTGGCAGAATGGT	NM_001020785.2
clock1	GGTTCAAGGACAGGGTTTACAGATG	GGTCGACCTCTGAGACTGCTGG	XM_009294633.1
clock3	GAGAGTACAGGGACCTCAGATGATC	ATACACAGGACCGCACTGAGTTAC	XM_005168339.2
arntl1a	GTCACAGACAAGTGCTACAGATGCG	TCCCTCCGCCATCTCCTGA	XM_009297921.1
arntl1b	TGACGGCTCAGGGAAAACC	GAGAATTGTCACTTAAAATGGAGCTG	XM_009303573.1
arntl2	GTGTCAACCAACACGGTTGTATCC	TGGAACTTGTTGGGATTTCTTGGC	XM_005169955.2
per1a	ATGCGTGCAAGAAGTGGTG	ACGTCCTCATTTAGCGGACTC	XM_005172626.2
per1b	CCTCCTGAGTCAGATATCGTAATGG	GCAGCGCACACCTCTTGATAA	NM_212439
per2	GTGGAGAAAGCGGGCAGC	GCTCTTGTTGCTGCTTTCAGTTCT	XM_009298837
per3	CCACAGCCTGAGTCCGAAGTC	CCCCTCTGTGATGTGAATGTGC	NM_131584.1
cry1a	CTACAGGAAGGTCAAAAAGAACAGC	CTCCTCGAACACCTTCATGCC	NM_001077297.2
cry1b	CTACAGGAAGGTAAAGAAGAACAGCA	CAACAACTCCTCAAACACCTTCAT	NM_131790.4
cry2a	GGACCAATACACCAGCACCAG	CAGCAAGTGTCCTGCCATGTC	XM_005166893.2
cry2b	ATCGTCTTATACAGGGGTCAGGAG	CTTCCCGCCTCTCGTTGTC	XM_005168280.2
rps11	TAAGAAATGCCCCTTCACTG	GTCTCTTCTCAAAACGGTTG	NM_213377.1
ef1a	TGCCAGTGTTGCCTTCGT	GCTCAATCTTCCATCCCTTG	NM_131263
qtilv s3	AGCCTGCCACACAGAAG	CTGCTTGAGTTGTGCTTCT	Multiple sequences[a]
qtilv s3 probe	FAM-CTCTACCAGCTAGTGCCCCA-BHQ		Multiple sequences[a]

[a] KU751816, MF574205, MF582636, MF502419, KX631923, MH213039 – MH213047, and MH213048.

2.8. Statistical analyses

Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Shapiro-Wilk normality test was performed to ensure the suitability of the data for parametric significance tests. When the data were normally distributed, differences in the gene expression between fish in the intact group and fish in the mock-infected group were assessed by one-way ANOVA followed by Tukey's multiple comparison test. When the data were not normally distributed, the differences in expression were analysed by the non-parametric Kruskal-Wallis's test followed by Dunn's test. Significant differences in the gene expression between the mock-infected and TiLV-infected fish were assessed by two-way ANOVA followed by Bonferroni test. The level of significance was set at $p < 0.05$.

Multiple group results were compared using one-way ANOVA followed by Tukey's post hoc test when the data sets met Gaussian distribution or the equal variance (Brown-Forsythe test). If the data did follow a Gaussian distribution, but did not have equal variance, the Brown-Forsythe and Welch ANOVA were used, followed by Dunnett's T3 multiple comparisons test. If the results did not follow the Gaussian distribution, the Kruskal-Wallis's test followed by Dunn's multiple comparison test were used. For all tests significance level of $p < 0.05$ was used.

An unpaired *t*-test was used to compare the data collected from fish infected at the different time points (ZT/CT 2 vs ZT/CT 14) when datasets were Gaussian and met the requirement of equal variance. If the data were Gaussian but not equal to standard deviation, the unpaired *t*-test with Welch's correction was used. If the results did not follow the Gaussian distribution, the Mann-Whitney test was used. The level of significance was set at $p < 0.05$. Grubb's test was employed to remove outliers. The n values indicate the number of analysed individuals. Data were presented as means ± SEM.

3. Results

3.1. Light induces rhythmic expression of clock genes in the brain of zebrafish

As the brain is the main center of the biological clock in various animal species [5,34,35], we first investigated whether our light treatments affected the constitutive expression of key clock genes at various points throughout the day in the brain of zebrafish. We observed that under DD conditions, the expression level of all tested genes was constant at all sampled time points (Supplementary Fig. 1). Light, in turn, contributed to gene-specific circadian rhythmicity of the gene expression, supporting the role of light in regulating the rhythmicity of clock gene expression in the brain of zebrafish. The *clock1* and *arntl* genes forming the positive loop of the clock mechanism showed similar circadian rhythms of expression (Supplementary Fig. 1A, C – E) and reached the highest expression level at the end of the light phase (ZT 10) and at the beginning of the dark phase (ZT 14) and it was clearly higher than at the other points of the day (ZT 2 and 22) and higher than in DD (Supplementary Fig. 1A, C – E). The expression of *clock3* was at the same level at all analysed time points of the day (Supplementary Fig. 1B). On the other hand, the negative loop components of the biological clock (*per1a, per1b*, *per3,* and *cry1b* genes) showed a diurnal rhythm of expression opposite to the *clock* and *arntl* genes, reaching the highest mRNA levels at ZT 2 and 22, and the lowest at ZT 10 and 14. The expression level of *per2*, like *cry1a*, was highest at ZT 2 and lowest at ZT 14 (Supplementary Figs. 1F–K). Both *cry2* genes showed a different diurnal rhythm than *per* and *cry1* (Supplementary Fig. 1L – M).

3.2. Light conditions dictate the gene-specific circadian expression pattern of clock genes in the kidney of zebrafish

We further investigated the effect of different light conditions on the regulation of the biological clock mechanism in the immune system of zebrafish. In view of the fact that the kidney is the main immunocompetent organ in fish [36,37], we decided to study the constitutive expression of clock genes at different times of the day in the kidney of zebrafish. We observed that under LD conditions, genes representing the positive arm of the molecular clock (*clock1*, *arntl1a, arntl2*) showed similar circadian variability in expression levels, reaching a maximum during the day (at ZT 2 or 10) and a marked decrease at night, especially at ZT 14. A similar trend was observed for the *clock3* and *arntl1b* genes (Supplementary Figs. 2A–E). In constant darkness, this circadian expression pattern was attenuated, but still noticeable for some genes (*arntl1a*, *arntl2*) (Supplementary Figs. 2C and E). This suggests that the expression of key core clock genes in the zebrafish kidney is endogenously regulated, and light further enhances this effect. Moreover, the

negative loop genes (*per, cry1a* and *cry1b*) exhibited reverse diurnal rhythms of expression under LD conditions relative to *clock* and *arntl*, with a nighttime peak (at ZT 14 or 22) and a daytime trough (ZT 2 or 10). Under DD conditions, the level of expression of these genes remained constant throughout the day (Supplementary Figs. 2F–K). Both subtypes of the *cry2* gene (*cry2a* and *b*) showed a different expression pattern compared to *per* and *cry1* genes. While *cry2a* expression was highest during the day (ZT 2 and 10) and lowest at night (ZT 14 and 22), *cry2b* expression remained at a constant low level throughout the day (Supplementary Fig. 2L – M).

3.3. Light and time of infection affect the rhythmic expression of clock genes in the kidney of zebrafish during TiLV infection

To investigate the mechanism behind the effect of light on the immune response to viral infection, the expression of clock genes in the kidney of TiLV-infected zebrafish was assessed under the different light conditions. Expression analysis of the zebrafish core clock genes in the kidney showed that in fish maintained under DD conditions and infected at CT 2 the expression of *arntl1b* was higher, on 14 dpi than on 3 and 6 dpi (Fig. 2D). In fish maintained under LD and infected at ZT 2, *arntl2* expression was highest on 6 dpi, both compared to the expression of this gene in control fish, DD-maintained fish and fish infected at ZT 14. Moreover, such upregulation was not observed on 6 dpi in fish kept under the LD regime but infected at ZT 14 (Fig. 2E). In the case of genes forming a negative clock loop, it was found that in fish kept under LD conditions and infected at ZT 2, *per1a* expression on 14 dpi was significantly higher than in fish kept under the same light conditions but infected at ZT 14 (Fig. 2F). In fish kept under the DD regime and infected at CT 2, *cry1a* expression was reduced on 14 dpi compared to uninfected control fish (Fig. 2J). In turn, in fish kept under the same light conditions (DD) but infected at CT 14, *cry2b* mRNA level on 6 dpi was significantly lower than on 3 dpi in fish kept under LD. In fish maintained under LD conditions and infected at ZT 14, the expression of *cry2b* on 3 dpi was higher than in fish infected at ZT 2 (Fig. 2M).

3.4. Light contributes to the slower replication of TiLV, regardless of the time of infection

We examined whether the time of infection and light conditions affect the intensity of virus replication in infected fish. RT-qPCR analyses revealed that on 6 dpi, the virus load in fish kept under the LD regime and infected at ZT 2 was significantly higher than in fish from the same time point and light conditions but infected at ZT 14. Moreover, on 3 dpi, the virus load in fish kept under LD and infected at ZT 2 was lower than in fish from the same time point and infected at the same time (CT 2), but kept under the DD regime (Fig. 3).

3.5. Light and time of infection modify the rhythmic expression of genes involved in antiviral immune response in the kidney of zebrafish during TiLV infection

We examined whether light and timing of infection had any impact on the antiviral immune response during TiLV infection. This was achieved by analyzing the expression of the genes involved in type I IFN-dependent response as well as genes encoding pro- and anti-inflammatory cytokines. This analysis revealed that on 3 dpi both fish kept under LD and DD light conditions and infected at ZT/CT 2 and at ZT/CT 14 showed an increase in *rig-1* expression compared to control fish. Such upregulation was also observed on 6 dpi in fish kept under LD light conditions and infected at ZT 2 and in fish kept under DD conditions and infected both at CT 2 and CT 14 (Fig. 4A). Similar changes were observed in the expression of *irf3* and *irf7*. In fish kept under LD conditions and infected at ZT 14, an increased level of *irf7* mRNA was found on both 3 and 6 dpi, while expression of *irf3* was higher compared to control fish only on 3 dpi (Fig. 4B and C). The highest expression of *ifn1* was observed on 6 dpi in fish kept under LD and injected at ZT 2. Interestingly, both under LD and DD light conditions TiLV infection upregulated *ifn1* expression on 3, 6 and 14 dpi in fish infected at ZT/CT 2 only (Fig. 4D). In contrast significant upregulation of *mxa* was mainly observed in fish infected at ZT/CT 14. The highest increase in *mxa* expression was observed on day 3 post infection in fish kept under LD and infected at ZT 14. Infection at ZT 14 also resulted in an increase in *mxa* expression on 6 dpi under LD and on all post-infection points under DD. In fish infected at ZT 2 an increase in mRNA of *mxa* was found only on 3 dpi, in fish kept under DD (Fig. 4E). TiLV decreased *il-1β* expression in fish kept under LD conditions and infected at ZT 14 on 14 dpi. In turn, upregulation of *il-10* expression was observed in fish kept under LD light conditions and infected at ZT 2 on day 6 post infection and in fish infected at ZT 14 on 14 dpi. In fish kept under DD light conditions such upregulation was found only on day 6 post infection in animals injected at CT 14 (Fig. 4G). These results suggest that the time of day of viral infection and light conditions in which fish are kept play an important role in the speed and intensity of the antiviral response, and thus the likelihood of overcoming the infection.

3.6. Both light and time of infection induction do not affect the survival of zebrafish to infection with TiLV

To investigate whether light and time of infection induction influence fish survival, we monitored fish for 14 days after infection with TiLV. No differences in the survival of fish kept under the different light settings, regardless of the time of infection induction were observed (Fig. 5).

4. Discussion

A growing body of data points to a direct effect of light on the immune system and the regulation of the immune response during infection in mammals. However, our knowledge about the influence of light on the course of infectious diseases is still limited in fish. Understanding the circadian rhythms of the immune response during infection in fish, as well as the regulation of this response by the biological clock, would be extremely helpful in developing optimal conditions for fish farming to protect against infections, which would improve the welfare of these animals and reduce significant losses for fish farmers. It would also help in the development of future effective therapeutic strategies. To understand the mechanisms linking the effect of light and molecular clock on the antiviral response in fish, we used a model of infection of adult zebrafish with TiLV. Our study demonstrates that light clearly affects the expression of key core clock genes and regulates the antiviral immune response during TiLV infection in zebrafish.

We show that not only the brain, which is the main site of the central circadian clock in various groups of animals [5,34,35], but also the crucial lymphoid organ in fish, the kidney [38], express key circadian clock genes such as: *clock, arntl, per* and *cry*, forming autoregulatory feedback loops of the clock mechanism. Kidney of zebrafish has been shown to harbour peripheral clocks as well as the photoreceptive mechanisms required for entrainment by light/dark cycles [39,40]. Thus, this organ is a clear link between the biological clock and immune functions, which makes it extremely interesting from the point of view of circadian regulation of the immune response.

In the present study, we show that in both studied organs (brain and kidney), light triggers the rhythmic expression of clock genes, and its absence leads to disturbances in the regulation of the circadian pathway in zebrafish. However, it should be noted that the light-induced rhythmicity of expression is tissue-dependent and its pattern for specific genes is different in the central nervous system and the immune system of fish. This is probably related to the different light-activated signaling pathways and the different course of the light pulse to individual structures of the clock system in the case of the central (brain) and peripheral (kidney) clocks. Interestingly, the circadian changes in the expression of

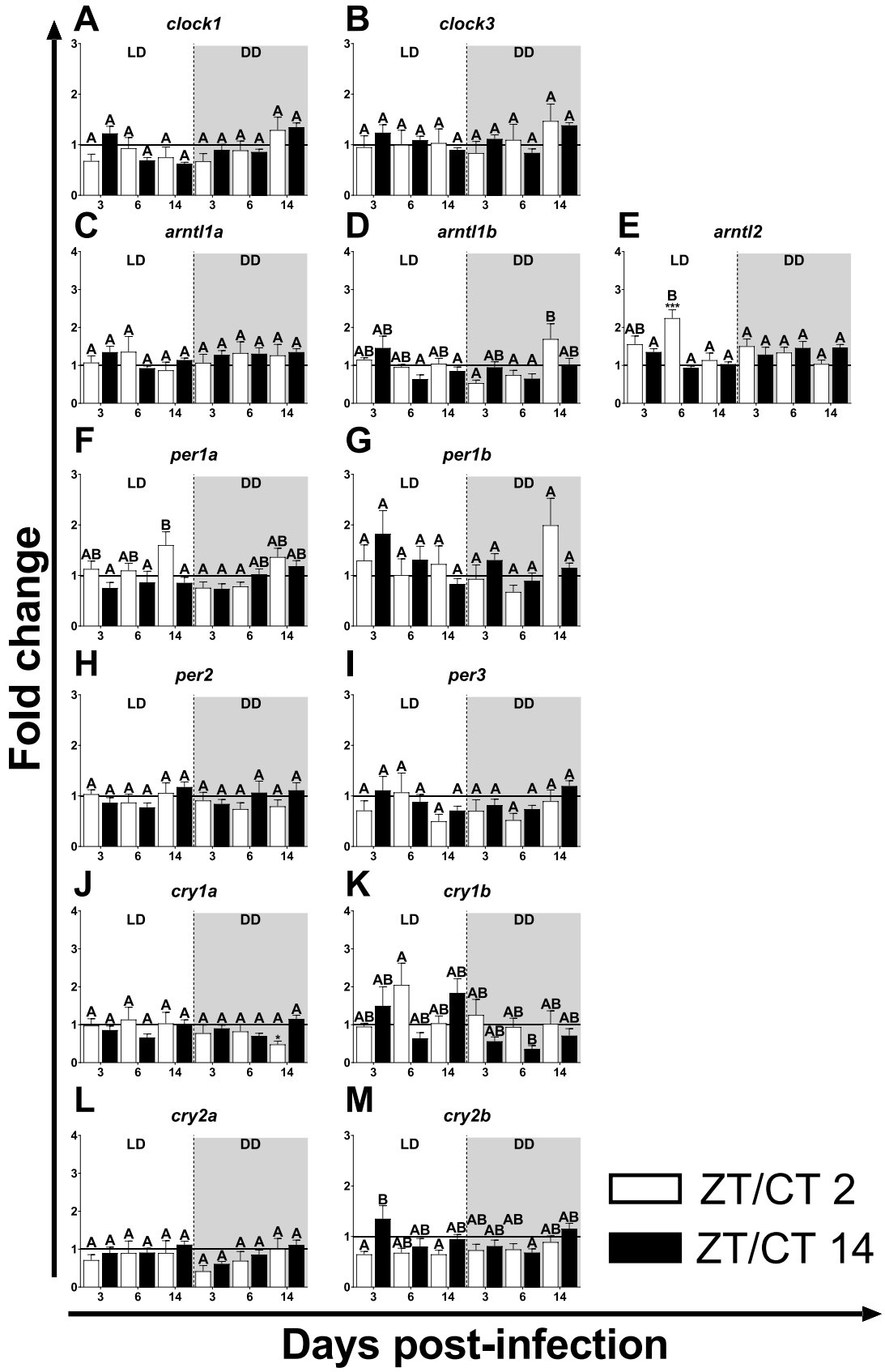

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Fig. 2. Changes in the expression of core clock genes in the kidney of zebrafish during TiLV infection. Relative mRNA levels of the clock genes: *clock1*, *clock3*, *arntl1a*, *arntl1b*, *arntl2*, *per1a*, *per1b*, *per2*, *per3*, *cry1a*, *cry1b*, *cry2a* and *cry2b* at different time points of infection induction/sampling (ZT/CT 2 and 14) on 3-, 6- and 14-days post-infection in the kidney of fish kept under the LD (12L:12D) and DD (0L:24D) light regimes. Data normalized to the control group (medium-injected, uninfected fish). Data obtained from RT-qPCR analysis are shown as mean ± SEM (n = 7–10). The *s11 ribosomal protein* gene (*rps11*) served as the reference housekeeping gene. Differences between different post-infection times and light regimes are indicated by letters (A, B) using two-way ANOVA, $p < 0.05$. When significant (Kruskal-Wallis's test or one-way ANOVA, $p < 0.05$), differences between control and infected groups are marked with asterisks: *$p \leq 0.05$, ***$p \leq 0.001$. ZT/CT, zeitgeber/circadian time where ZT/CT 0 represents start of the light phase/subjective light phase, dpi, days post-infection.

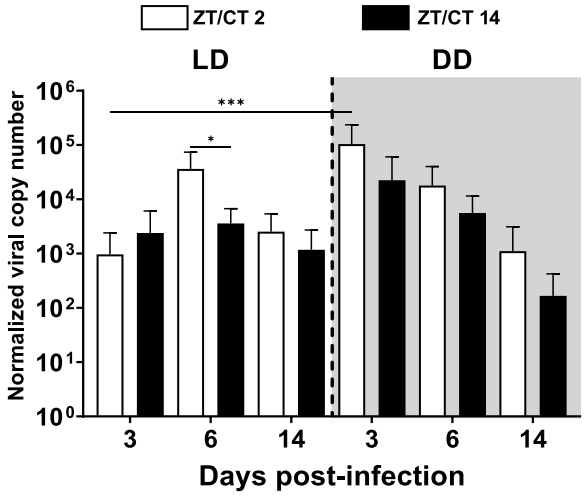

Fig. 3. Normalized copy numbers of TiLV RNA in kidney of intraperitoneally injected zebrafish at different time points post-infection. Results are normalized against mRNA transcript of *ef1α* gene of the host. The data are the mean ± SD of n = 8–10 fish. When significant (Welch's *t*-test or Mann-Whitney *U* test, $p < 0.05$), differences between the data points are indicated by asterisks: *$p \leq 0.05$; ***$p \leq 0.001$. ZT/CT, zeitgeber/circadian time where ZT/CT 0 represents start of the light phase/subjective light phase, dpi, days post-infection.

clock and *arntl* observed under LD conditions in the kidney were also found, albeit to a lesser extent, in constant darkness. However, this was not the case for negative loop genes such as *per* and *cry*, whose diurnal oscillations disappeared in constant darkness. This endogenously regulated expression of positive core clock elements indicates their significant role in the regulation of many genes, not only related to the clock system, but also many other physiological processes, through interactions with E-box sequences in the promoter regions of the relevant genes, which ensures maintaining their circadian rhythm regardless of the influence of external factors [41,42].

Circadian oscillations in the mRNA levels of *clock1, arntl1* and *per1* were also observed in the kidney of medaka raised under LD conditions [43]. Like in our hands, the expression of *clock1* and *arntl1* genes in medaka increased during the light phase, while it decreased during the dark phase of the LD cycle. In both medaka (*clock1* and *arntl1* genes) and zebrafish (*clock1*, *clock3*, *arntl2* genes), acrophases of expressions of respective genes were confirmed at ZT 10 [43]. In both fish species most of the *per* genes showed an opposite expression pattern and reached acrophases at the end of the night (ZT 22) [43]. Our results also corroborated the patterns of the expression of key clock genes, previously reported in medaka tissues (heart, fin, and liver) [44,45], rainbow trout neural retina [46], Atlantic cod fast skeletal muscle (*clock*) [47], and Japanese flounder (*Paralichthys olivaceus*) caudal fin (*per1*) [48]. Our results confirm the presence of a biological clock mechanism in fish like that in mammals, where the expression profile of many relevant genes was maintained by autoregulatory transcriptional/post-translational feedback loops with molecular components such as *arntl*, *clock*, *per* and *cry* genes [49]. Moreover, we found strong down-regulation of *clock1, clock3, arntl1a, arntl1b* and *arntl2* in the brain of animals exposed to constant darkness at ZT/CT 10, which is consistent with the results of Purushothaman and co-workers [50] under similar conditions. We also observed a similar rhythmic pattern at this time of day in the kidney of zebrafish (an increase in the expression levels of *per1*, *per3* and *cry1* genes and a decrease in the expression of *clock1* and *arntl2*), indicating a strong effect of light on the CLOCK/ARNTL-PER/CRY regulatory loops and their association with the light/dark disturbances. This also suggests the synchronization of the central clock with the peripheral clocks in the immune system and thus the influence of the clock system on the immune response at sensitive times of the day.

Various aspects of immune response in zebrafish are known to be regulated by a circadian clock that is thought to prepare fish for increased exposure to pathogens during the active phase and allows them to recover from infection or injury during the resting phase [13]. On the other hand, the immune system during its activation can modulate the circadian clock activity by regulating the expression of key circadian genes, reflecting the bidirectional interaction between the circadian clock and the immune system [51]. Interestingly, most of the data indicating these bidirectional interactions in fish are related to bacterial or parasitic infections and according to our best knowledge there is no information of such regulation of the immune response during viral infections. In the present studies, we demonstrated that the aquaculture-important TiLV virus tends to modify the expression levels of some clock genes in the kidney of zebrafish (upregulation of *arntl2* on 6 dpi under LD conditions) and that this effect depends on the time of infection induction (higher expression levels of *arntl2* and *per1a* for daytime-induced infection than for nighttime infection). This is the first report on the changes of clock gene expression during viral infection in fish.

Recently, the interaction between viral infections and the circadian clock has received increasing attention, as evidenced by the development of work in this field [52–55]. In mammals, important clockwork components such as CLOCK, ARNTL1, and REV-ERB are known to affect nucleic acid detection during viral infections by regulating the expression of pattern recognition receptors (PRRs) [56,57]. Disruption of the circadian clock in mice enables the reactivation of latent murine γ-herpesvirus, resulting in increased viral load and changes in lung cytokine and chemokine concentrations [58]. The circadian control of influenza infection has also been shown to be related to the time-dependent proportion of natural killer T cells, natural killer cells, and inflammatory monocytes ($Ly6c^{hi}$ monocytes), while ARNTL1 has been identified as a key regulator of the diurnal oscillations of $Ly6C^{hi}$ monocytes [53,59]. The observed rhythmicity of the immune response is related not only to innate, but also to adaptive immune mechanisms, for example previous studies described rhythmicity in the development and trafficking of lymphocytes [60]. Lymphocyte numbers fluctuate throughout the day, and disruption of their circadian rhythmicity dysregulated the rhythmic adaptive immune responses important for influenza A virus neutralization [61]. In contrast, little is known about these interactions in fish. In fish, parasite infection has been shown to affect host circadian gene expression, which is ambiguous, gene-specific and photoperiod-dependent. For example, in the skin of rainbow trout infected with *Argulus foliaceus* under LD conditions, increased expression of *arntl2*, *clock1a*, *cry1* and *per1* genes was observed, while under constant light, expression of *arntl1*, *clock1a*, *clock1b* and *clock3* genes was reduced [19]. On the other hand, in zebrafish infected with *Pseudoloma neurophilia* under the LD light regime, a decrease in the expression of important clock genes, e.g., *per1b* or *nr1d1* was found [62]. Importantly,

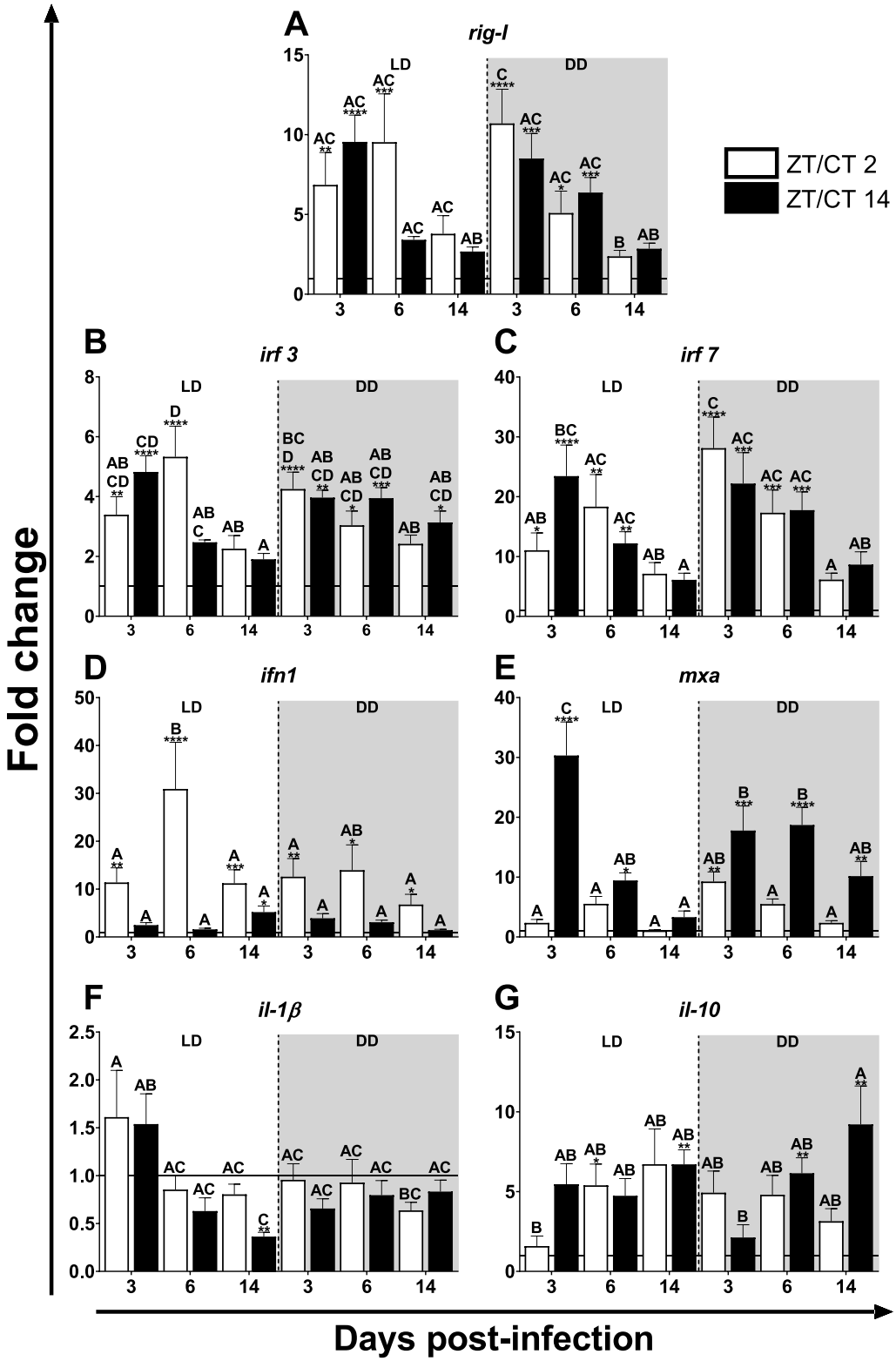

Fig. 4. Changes in the expression of type I IFN pathway and immune response genes in the kidney of zebrafish infected with TiLV at various times of the day. Relative mRNA levels of the genes: *rig-I*, *irf3*, *irf7*, *ifn1*, *mxa*, *il-1β* and *il-10* at different time points of infection induction/sampling (ZT/CT 2 and 14) on 3-, 6- and 14-days-post infection in the kidney of fish kept under the LD (12L:12D) and DD (0L:24D) light regimes. Data normalized to the control group (medium-injected, uninfected fish). Data obtained from RT-qPCR analysis are shown as mean ± SEM (n = 7). The *ribosomal protein s11* gene (*rps11*) served as the reference housekeeping gene. Differences between different post-infection times and light regimes are indicated by letters (A, B) using two-way ANOVA, $p < 0.05$. When significant (Kruskal-Wallis's test or one-way ANOVA, $p < 0.05$), differences between control and infected groups are marked with asterisks: *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$. ZT/CT, zeitgeber/circadian time where ZT/CT 0 represents start of the light phase/subjective light phase, dpi, days post-infection.

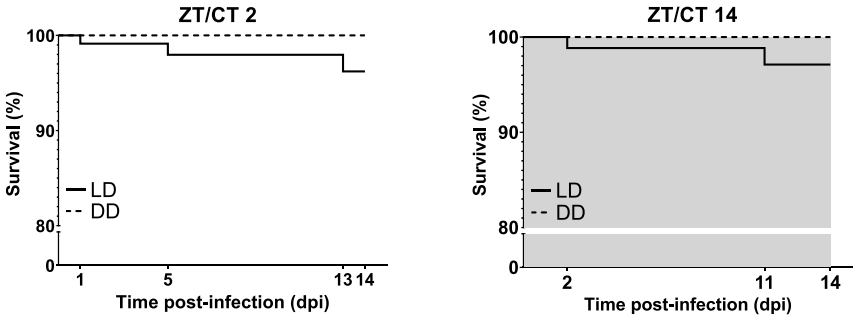

Fig. 5. The survival of zebrafish during 14 days of TiLV infection. Zebrafish kept under LD (12L:12D, solid line) and DD (0L:24D, dashed line) light regimes were infected with TiLV at two different time points: ZT/CT 2 (white background) and 14 (gray background). There were no significant differences in fish survival, regardless of the time of infection induction and the applied light regime (Mantel-Cox test). ZT/CT, zeitgeber/circadian time where ZT/CT 0 represents start of the light phase/subjective light phase, dpi, days post-infection.

the expression of clock genes is closely related to the activation of immune mechanisms, as it was found in zebrafish where knockout of *per1b* reduced the expression of pro-inflammatory cytokines, and impaired neutrophil recruitment towards injury [63]. Moreover, PER and CRY have been identified as clock elements regulating inflammation in mice, supporting the link between the molecular clock and the immune response [2]. In mice, knockout of *per2* has been shown to provide protection against LPS-induced lethality by reducing TNF-α and IL-12 production and suppressing the immune response to pathogens by downregulation of TLR9 expression [56,64]. Therefore, modification of host core clock gene expression is likely to be a mechanism used by pathogens to weaken the host immune system and thus enable successful invasion. It was proofed that some pathogens, by affecting the biological clock of the host, increase their own chances of survival, as in the case of *Trypanosoma brucei*, which can shorten the period of the mice circadian clock by regulating the expression of clock genes to increase the likelihood of their own invasion [65,66].

In the case of TiLV infection, we observed that changes in the expression of clock genes were accompanied by a specific rate of viral replication, which was also reflected in the profile of the expression of type I IFN pathway genes. This indicates a mutual interaction between the response to the pathogen and circadian clock activity. We can speculate that the more pronounced modification of the expression of clock genes (*arntl2*, *per1a*) under LD than DD conditions was associated with different TiLV replication, as well as a change in the expression of relevant genes related to the response to this pathogen in fish kept under LD photoperiod relative to animals from DD regime. It is also interesting that the highest level of expression of most genes of the type I IFN pathway under LD conditions was observed on 6 dpi in the case of daytime-induced infection (ZT 2), while nighttime-induced infection (ZT 14) resulted in a maximum increase in the expression of the relevant genes (comparable to infection in constant darkness) already on the first analysed time point after infection (3 dpi). Therefore, light provides a more effective response of the zebrafish immune system during TiLV infection, although we did not observe differences in fish survival regardless of infection time and light conditions. However, this may be due to the fact that zebrafish is not a natural host of this virus, therefore it would be reasonable to investigate the effect of light on tilapia survival during TiLV infection.

Similarly, infection of zebrafish larvae with *Salmonella enterica* during the light phase of the LD cycle conferred greater resistance to the pathogen than the infection at night. Larvae infected under light exposure showed increased survival, higher bacterial clearance, increased expression of inflammatory cytokines, and greater recruitment of neutrophils and macrophages to the site of infection than their counterparts infected during the dark phase of the LD cycle and under DD conditions [67]. Infection induced during the light phase of the LD cycle also resulted in a higher number of leukocytes recruited into the peritoneal cavity during *A. salmonicida* infection in rainbow trout (*Oncorhynchus mykiss*) [68] and higher expression of pro-inflammatory *interleukin-1β* (*il-1β*) in medaka (*Oryzias latipes*), infected with *Edwardsiella piscicida* or stimulated with lipopolysaccharide (LPS) [69]. Consistent with this line, phagocytosis of *Escherichia coli* by myeloid cells in zebrafish has been reported to oscillate throughout the LD cycle, peaking during the light phase and decreasing at night. However, no diurnal rhythmicity was observed for *Staphylococcus aureus* phagocytosis, which may be related to the rhythmic expression of innate immune receptors involved in the recognition of Gram-negative but not Gram-positive bacteria [70]. As with the observed lower response associated with TiLV infection during the day than at night, the antibacterial response of Japanese medaka associated with *tnf-a* induction was lower when fish were stimulated during the day and higher when stimulated at night. This is also consistent with the diurnal rhythmicity of *tnf-a* levels found in medaka and zebrafish [43,63]. This diurnal rhythmicity of the antimicrobial response may be partially due to the rhythmic expression of *tlr9* receptor that recognizes hypomethylated CpG DNA as Taira and co-workers [71] found that in medaka *tlr9* expression reaches a peak during the day and is at lowest level at night. This ensures more efficient detection of pathogen DNA when fish is active. For example, it was shown that *tlr9* expression increased significantly during *Aeromonas hydrophila* infection, but only when infection was induced during the light phase [71]. However, since TiLV is an RNA virus, this does not explain the differences we observed in the level of virus replication and expression of genes associated with the antiviral response.

Moreover, the response to TiLV was accompanied by the typical inflammatory response associated with the increase of *il-1β* expression under LD conditions. This pro-inflammatory cytokine is crucial in preventing the spread of infection and fighting with pathogens [72,73]. We did not observe differences in *il-1β* response depending on the time of infection throughout the LD cycle, but importantly, in constant darkness, this cytokine was not induced at all. The lack of elevated expression of *il-1β*, and thus the lack of a clear pro-inflammatory response under DD, may explain the higher level of expression of the relevant genes of the type I IFN pathway than under the LD photoperiod. Under the LD cycle, as mentioned, no differences in the level of *il-1β* were observed depending on the time of infection, however, nighttime infection resulted in a much faster anti-inflammatory response, manifested by the level of *il-10* expression, than daytime-induced infection. This one of the most important anti-inflammatory cytokines inhibits the pro-inflammatory activity of the immune system, leading to the suppression of the inflammatory response [74,75]. This may to some extent explain the stronger response to TiLV infection during nighttime-induced infection compared to daytime infection.

Studies in mice have shown that the time of infection consistently determines the outcome of a flu infection. The clock has been shown to exert its effect not by acting directly on the pathogen burden, but by altering the inflammation generated as the host response to the infection. Animals underperformed when infected just prior to the active phase, and this was associated with higher bronchoalveolar lavage cell counts, more severe lung injury, and a distinct transcriptomic signature consistent with increased inflammation [53]. On the other hand, human monocytes infected with SARS-CoV-2 phagocytized the virus more

effectively when the cells were infected at the beginning of the active phase than at the end of this phase. Infection at this time of day also resulted in a higher viral titer but lower level of viral mRNA [55]. Overall, many respiratory viruses have been shown to be circadian-dependent, as evidenced by the markedly higher susceptibility to RSV (respiratory syncytial virus) and PIV3 (human parainfluenza virus type 3) infection associated with ARNTL1 deficiency. It was observed that ARNTL1 participates in the regulation of innate immunity against specific RNA viruses [76] and is also likely to play a key role during TiLV infection in zebrafish.

In conclusion, these data demonstrate that light positively regulates circadian clock activity in zebrafish, acting by inducing rhythmic expression of clock genes in both the nervous and immune systems. This zeitgeber also plays a special role during viral infection in zebrafish. It is important to be aware that the circadian variability of optimal immune system activity is the result of a combination of endogenous circadian rhythm and the diurnal rhythm of environmental cues [77–79]. The data show that light is an important cue that stimulates circadian clock activity and the immune response to viral infection in zebrafish. While the endogenous rhythmicity of the response to viral infection is independent of the time of infection, light clearly modulates this response, providing a more efficient mechanism accompanied by increased clockwork activity. Although we did not observe an effect of light on zebrafish survival during infection, it is highly likely that such an effect will occur in tilapia, the natural host of TiLV. This is all the more justified as the previous studies indicate the presence of a circadian rhythmic humoral response in this fish species, as well as a higher survival of fish reared under LD conditions than in constant darkness [80]. Therefore, our findings may have important practical implications, as they will allow for the optimal timing of treatment methods to take advantage of the maximum natural capacity to effectively neutralize the virus. In light of our results, light modulation should also be further explored in tilapia aquaculture to optimize the timing of other interventions that require an optimal immune response, such as vaccination.

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CRediT authorship contribution statement

Mikolaj Mazur: Methodology, Formal analysis, Investigation, Visualization, Writing – review & editing. **Krzysztof Rakus:** Conceptualization, Methodology, Writing – review & editing. **Mikolaj Adamek:** Investigation, Resources, Writing – review & editing. **Win Surachetpong:** Resources, Writing – review & editing. **Magdalena Chadzinska:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **Lukasz Pijanowski:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2023.108979.

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