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MINIREVIEW

# Immunity, Infection, and the Zebrafish Clock

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**ABSTRACT** Circadian clocks are universally used to coordinate biological processes with the Earth's 24-h solar day and are critical for the health and environmental success of an organism. Circadian rhythms in eukaryotes are driven by a cell-intrinsic transcription-translation feedback loop that controls daily oscillations in gene expression which regulate diverse physiological functions. Substantial evidence now exists demonstrating that immune activation and inflammatory responses during infection are under circadian control, however, the cellular mechanisms responsible for this are not well understood. The zebrafish (*Danio rerio*) is a powerful model organism to study vertebrate circadian biology and immune function. Zebrafish contain homologs of mammalian circadian clock genes which, to our current knowledge, function similarly to impart timekeeping ability. Consistent with studies in mammalian models, several studies in fish have now demonstrated a bidirectional relationship between the circadian clock and inflammation: the circadian clock regulates immune activity, and inflammation can alter circadian rhythms. This review summarizes our current understanding of the molecular mechanisms of the zebrafish clock and the bi-directional relationship between the circadian clock and inflammation in fish.

**KEYWORDS** bacteria, circadian, clock, cry, immunity, infectious disease, light, melatonin, *per2*, zebrafish

From cyanobacteria to humans, circadian rhythms allow organisms to anticipate and prepare for daily changes in their environment. Circadian rhythms (*circa* + *dia "about a day"*) are driven by a self-sustaining transcription-translation feedback loop which oscillates once every 24-h and persists in the absence of outside stimuli. Importantly, circadian rhythms can align with certain external cues such as light or temperature through a process called entrainment, which allows organisms to anticipate regular changes in their environment and coordinate biological function with the time of day. Advances in murine systems have defined the genetic and biochemical mechanisms by which the molecular clock controls gene expression in mice (1–3). Zebrafish (*Danio rerio*) are another advantageous vertebrate model system due to their genetic tractability, short generation time, and optical transparency. The core components of the mammalian clock are conserved in zebrafish, and both light and temperature serve as primary entraining cues to synchronize the clock with external time. However, in mammals, light information can only be received via photoreceptors in the eye, which synchronize a master clock in the brain called the superchiasmatic nucleus (SCN) (4). Peripheral cells in mammals are not responsive to light, but can be synchronized by oscillations in body temperature which are controlled by the SCN (5). In contrast, all zebrafish cells are directly responsive to both light and temperature (6, 7).

Studies in both mice and zebrafish are now beginning to connect the circadian clock to outcomes of disease and infection. As vertebrates, zebrafish are also a valuable model to study the immune system, as many key aspects of infection and inflammation are conserved (8). These characteristics have made zebrafish an intriguing model to study the relationship between circadian rhythms and immunity. This review will discuss our current understanding of the molecular zebrafish clock, and the ways in which the clock is linked to the immune system in zebrafish and other teleost fish species.

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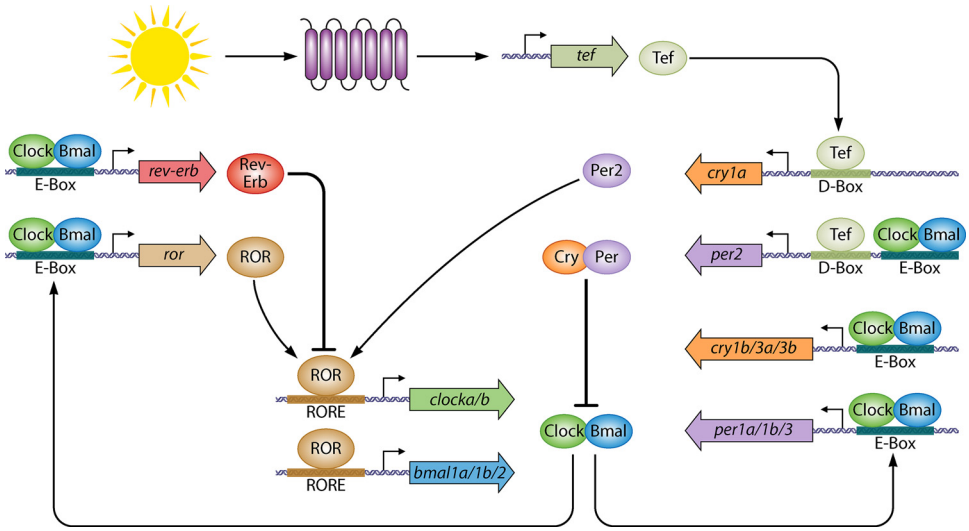


**FIG 1** The zebrafish molecular clock. Light drives the expression of the transcription factor Tef, which activates the expression of the *cry1a* and *per2* genes via D-box elements. The Clock and Bmal proteins dimerize and bind to E-box elements in the *per* and *cry* promoters (with the exception of *cry1a*) to stimulate their expression. Per and Cry proteins dimerize and interact with Clock/Bmal to repress their transcriptional activation function, turning off their own expression. A second loop consisting of Rev-Erb and Ror regulates the expression of the *clock* and *bmal* genes. Clock/Bmal activates the transcription of *rev-erb* and *ror* through E-boxes. Rev-Erb and Ror then bind to ROREs in the promoters of *clock* and *bmal*, repressing and stimulating their expression, respectively. Per2 also interacts with Ror$\alpha$ to increase the Ror$\alpha$-dependent expression of *clock* and *bmal*.

## THE ZEBRAFISH CLOCK

**The core clock loop.** Circadian rhythms in zebrafish are generated by a cell autonomous transcription-translation feedback loop consisting of the positive regulators Clock (Circadian Locomotor Output Cycles Kaput) and Bmal (Brain and Muscle ARNT-Like 1), and the negative regulators Per (Period) and Cry (Cryptochrome) (Fig. 1). Clock and Bmal proteins heterodimerize to bind to E-box elements in the promoters of the *per* and *cry* genes, driving their expression. Per and Cry are then transcribed, translated, and translocated back into the nucleus as a Per/Cry complex. The Per/Cry complex then interacts with the Clock/Bmal complex to block its transcriptional activation function, repressing their own expression and closing the feedback loop (9, 10). An additional loop involving Rev-Erb and Ror family proteins is also present, where Clock/Bmal drives the transcription of the *rev-erb* and *ror* genes. The better studied murine model contains 2 *Rev-erb* genes (*Rev-erba* and *Rev-erb*$\beta$, also known as *Nr1d1* and *Nr1d2*) and 3 *Ror* genes (*Ror*$\alpha$, *Ror*$\beta$, and *Ror*$\gamma$). Zebrafish contain homologs of each of these genes, as well as several additional homologs of both *rev-erb* and *ror* (11). Rev-Erb and Ror bind to Ror response elements (ROREs) in the promoters of several circadian controlled genes including *clock* and *bmal* genes. Upon binding to ROREs, Rev-Erb family proteins act as a transcriptional repressors while Ror family proteins act as transcriptional activators (12). This loop is present in all vertebrates studied, and creates distinct rhythms in the expression of each clock gene over an approximate 24-h period (1).

Genome duplications in zebrafish have led to additional copies of core clock factors (*per*, *cry*, *clock*, *bmal*) compared to mammalian systems (13). For example, mice contain 3 *per* genes (*Per1*, *Per2*, and *Per3*), while zebrafish have four (*per1a*, *per1b*, *per2*, and *per3*). As the evolution and function of these genes has been studied, they have been renamed several times in an attempt to follow the nomenclature guidelines in zebrafish. Unfortunately, this has resulted in significant inconsistency in the literature based on the date of publication – for example, the gene currently named *cry3a* was previously known as *cry1ba* and *cry2a*, while the gene currently named *cry2* was formerly known as *cry3*. For clarity, we have included a table describing the current gene names as listed by the Zebrafish International Network (ZFIN) and the naming history of the core circadian genes in zebrafish (Table 1). Within this review, we use the current (June

**TABLE 1** Summary of zebrafish circadian gene nomenclature and function[a]

| Protein name | Current ZFIN gene name | Previous gene names | | Canonical function | Notes |
|---|---|---|---|---|---|
| | | Wang et al., 2008 (14) | Ishikawa et al., 2002 (72) | Clock dimerizes with Bmal to drive E-box mediated transcription | |
| Clocka | *clocka* | *clock1a* | *clock1* | | |
| Clockb | *clockb* | *clock1b* | *clock3* | | |
| Npas2 | *npas2* | *clock2* | *clock2* | | |
| | | Wang et al., 2009 (15) | Ishikawa et al., 2002 (72) | Bmal dimerizes with Clock to drive E-box mediated transcription | |
| Bmal1a | *arntl1a* | *bmal1a* | *bmal1* | | |
| Bmal1b | *arntl1b* | *bmal1b* | *bmal3* | | |
| Bmal2 | *arntl2* | *bmal2a* | *bmal2* | | |
| | | Wang et al., 2008 (26) | Vallone et al., 2004 (25) | Per dimerizes with Cry to repress Clock/Bmal activity | |
| Per1a | *per1a* | *per1a* | *per1* | | |
| Per1b | *per1b* | *per1b* | *per4* | | *per1b* is repressed by light |
| Per2 | *per2* | *per2* | *per2* | | *per2* is stimulated by light, Per2 enhances Ror$\alpha$ activity |
| Per3 | *per3* | *per3* | *per3* | | |
| | | Liu et al., 2015 (19) | Kobayashi et al., 2000 (18) | Cry dimerizes with Per to repress Clock/Bmal activity | |
| Cry1a | *cry1a* | *cry1aa* | *cry1a* | | *cry1a* is stimulated by light |
| Cry1b | *cry1b* | *cry1ab* | *cry1b* | | |
| Cry3a | *cry3a* | *cry1ba* | *cry2a* | | |
| Cry3b | *cry3b* | *cry1bb* | *cry2b* | | |
| Cry2 | *cry2* | *cry2* | *cry3* | | Cry2 cannot bind Clock/Bmal |
| Cry4 | *cry4* | *cry3* | *cry4* | | Cry4 cannot bind Clock/Bmal |
| Cry5 | *cry5* | –[b] | 6-4 photolyase | | Cry5 is a photolyase |

[a]Circadian genes in zebrafish have been renamed several times in order to remain consistent with nomenclature guidelines. However, this can result in confusion when referencing the literature and we have therefore summarized these changes here. The protein name is noted in the first column and the current gene name is listed in the second column, as per the Zebrafish Information Network (ZFIN). For previous names, the citation in which the name change occurred is included. While ZFIN convention dictates that the genes previously known has *bmal* are called *arntl*, the common name for the Arntl proteins is Bmal. To avoid confusion, we refer to these genes as *bmal* throughout this review.

[b]–, Cry5 is not discussed in Liu et al., 2015 (19).

2022) ZFIN name for all genes and proteins with the exception of the *bmal* homologs (*bmal1a*, *bmal1b*, *bmal2*) which are officially named *arntl1a*, *arntl1b*, and *artnl2* on ZFIN. The vast majority of circadian literature in fish, murine, and human systems refer to this factor as Bmal despite official gene names of *Arntl* (mouse) and *ARNTL* (human). We have therefore used the most common and recognizable name of Bmal within the text and figures to maintain consistency within the literature but have noted all names in Table 1. The approximate rhythms of the expression of each core circadian gene in light exposure and constant darkness is depicted in Fig. 2.

A total of 3 *clock* and 3 *bmal* genes have been identified in zebrafish: *clocka*, *clockb*, and *npas2* (*npas2* was previously named *clock2*), and *bmal1a*, *1b*, and *2* (officially named *arntl1a*, *arntl1b*, and *arntl2*) (14, 15). The daily rhythmic expression of all the *clock* and *bmal* genes are similar, with a peak right before lights off at the end of the day (16, 17) (Fig. 2A). These duplicate Clock and Bmal proteins interact in various heterodimer combinations to bind to E-box elements in the promoters of clock-controlled genes (including *per* and *cry*) and drive their expression, and represents the positive arm of the circadian clock. The negative arm of the clock is mediated by the repressive activities of Cry and Per, which directly bind Clock/Bmal to inhibit their transcriptional activation function.

Zebrafish contain 6 *cry* genes, *cry1a*, *cry1b*, *cry2*, cry*3a*, *cry3b*, *cry4*, and *cry5*. Only 4 of the Cry proteins act as Clock/Bmal repressors in the core circadian loop: Cry1a, Cry1b, Cry3a, and Cry3b. Cry2 and Cry4 lack repressive function due to a non-functional Clock/Bmal interaction domain, and Cry5 is a photolyase (an enzyme that repairs DNA damaged by UV light) (18, 19). The rhythmic expression of each repressive *cry* gene varies; *cry1a* and *cry1b* peak early in the morning following lights on, while *cry3a* and *cry3b* peak later near the light to dark transition

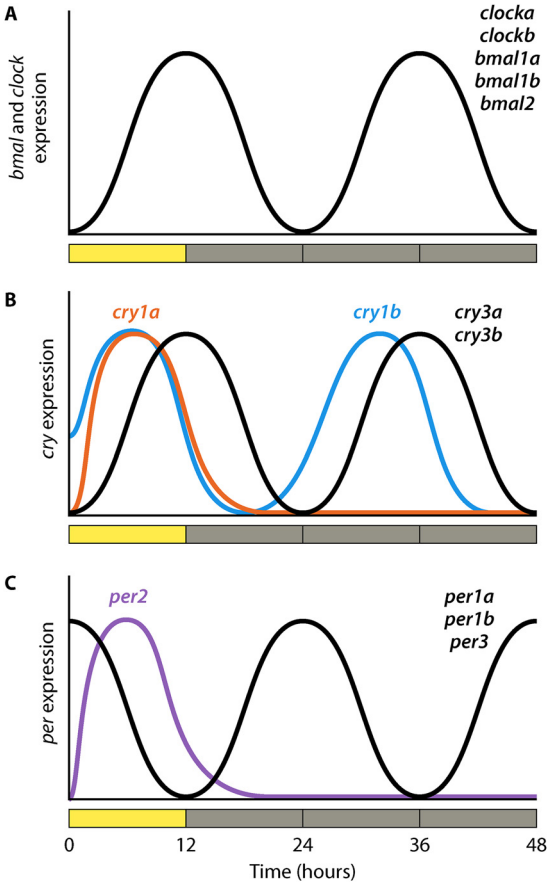


**FIG 2** Daily rhythms of the zebrafish core clock genes. (A) The *clock* and *bmal* genes all display similar expression rhythms, with a peak during the light to dark transition and a trough right at lights on. (B) In light:dark conditions, *cry1a* (orange) and *cry1b* (blue) display similar oscillation patterns, with a peak following lights on and a trough at night. *cry1b* continues to oscillate in constant darkness, while *cry1a* is mainly expressed in the presence of light. *cry3a* and *cry3b* (black) show similar rhythmic expression to that of *clock* and *bmal*, with a peak at the end of the light phase and trough at the end of the dark. (C) *per1a*, *per1b*, and *per3* (black) peak during the transition from dark to light and oscillate in both light:dark conditions and constant darkness, while *per2* (purple) peaks following lights on and is minimally expressed in the dark. Yellow bars indicate light exposure; gray bars indicate darkness.

(18–20) (Fig. 2B). Notably, unlike the other *cry* genes, *cry1a* is directly induced by light and has not been reported to oscillate in constant darkness.

There are 4 *per* genes in zebrafish: *per1a*, *per1b*, *per2*, and *per3*. Based on homology to mammalian counterparts, it is predicted that the Clock/Bmal repression function of all 4 Per proteins is conserved, but an additional, positive function for zebrafish Per2 has been identified in the second clock loop, where Per2 directly interacts with Ror$\alpha$ (gene *roraa*) to enhance *bmal1b* expression (21). Similar to the *cry* genes, different patterns of rhythmic expression can be seen for each *per* gene. *per2* is directly induced by light and displays minimal expression in constant darkness, a pattern similar to *cry1a* (22–24) (Fig. 2C). The expression of *per1a*, *per1b*, and *per3* also peak during the light period, but unlike *per2*, their expression diminishes following sustained light exposure and they continue to oscillate clearly in constant darkness (22, 25, 26).

## THE LIGHT-RESPONSIVE CIRCADIAN GENES

By definition, for a gene to be circadian, its expression must rhythmically oscillate without the presence of outside cues. However, in zebrafish, the expression of *per2* and *cry1a* are largely dependent on light, a distinct property of the zebrafish molecular clock. The ability of light to directly induce *per2* and *cry1a* is an important mechanism by which light entrains the zebrafish clock. The current model for the light-induced expression of *per2* and *cry1a* begins

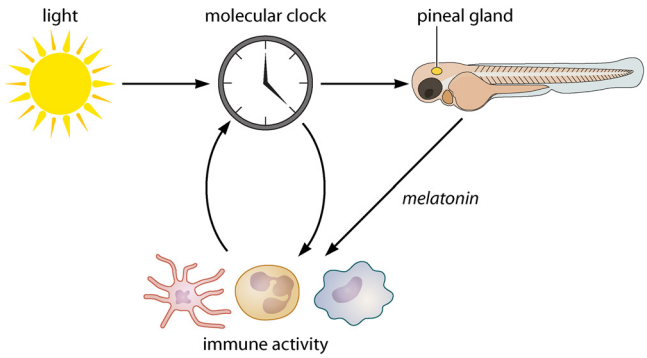


**FIG 3** A bi-directional relationship between immunity and circadian rhythms in zebrafish. Light sets the molecular clock, which regulates many aspects of the zebrafish immune system. The molecular clock also regulates the production of melatonin in the pineal gland, which directly alters immune function. Immune activity can also regulate the molecular circadian clock in zebrafish.

with light stimulation of an opsin. Multiple opsins have been implicated in the expression of *per2* and *cry1a*, including the flavin-containing oxidase NADPH oxidase, TMT-opsin, and Melanopsin, but the relative contributions of each opsin in this light input pathway are currently unknown (27–29). Activation of the opsin (or opsins) then triggers transcription of the thyrotroph embryonic factor gene, *tef*. Tef is a bZIP PAR domain transcriptional activator that binds to D-box sequences in the promoters of *per2* and *cry1a* to activate the expression of these genes (23). There are 2 *tef* isoforms in zebrafish, *tefa* and *tefb*, both of which have been implicated in the circadian light response pathway in zebrafish (24). Other bZIP PAR transcription factors can also activate D-box driven expression in zebrafish (such as HLF and DBP), though Tef is the most well studied thus far (24).

While the *cry1a* promoter contains both E-boxes and a D-box, the E-boxes are not required for the response to light (24). For *per2*, the story is a bit more complicated, as the promoter region termed the Light-Responsive Module (LRM), which is both necessary and sufficient to drive *per2* expression in the presence of light, contains an E-box and a D-box (23). This LRM is conserved in other vertebrate *per2* genes, even in species without light-sensitive peripheral clocks (23). Vatine et al. showed that both the E-box and D-box with their respective binding partners Clock/Bmal and Tef are required for normal LRM activation *in vitro* (23). Thus, while light-driven *cry1a* expression is exclusively Tef dependent (24), some *per2* expression can be driven by either Tef or Clock/Bmal alone (23). Regulation of *per2* expression may even contain an additional level of control via another *per* gene, *per1b*. Multiple studies have found that deletion of *per1b* increases the expression of *per1a*, *per2*, and *per3* in zebrafish raised in either light:dark (light exposure during the day and darkness at night) or dark:dark (constant darkness during the day and night) conditions, pointing to a repressive role for Per1b in *per2* expression (30, 31). The increased expression of *per2* in constant darkness in *per1b*$^{-/-}$ zebrafish suggests that Per1b may be able to block transcription of *per2* via Clock/Bmal binding in darkness, but not in the light, as *per1b* expression is repressed following light exposure (25).

## IMMUNITY AND THE FISH CLOCK

The circadian clock has been shown to regulate various aspects of immunity in zebrafish. Circadian regulation of immunity is thought to prepare fish for times when they are most likely to encounter pathogens during their active phase, and allows them to recover from infection or injury during their resting phase (32). Thus, the ability for zebrafish to produce the optimal immune response is directly dependent on their circadian rhythms. Furthermore, immune activation and inflammation can regulate circadian gene expression, indicating a bidirectional relationship between the circadian clock and immunity (Fig. 3).

## EFFECT OF LIGHT ON FISH IMMUNITY

As discussed, light is potent and commonly used signal to entrain zebrafish circadian clocks. However, given that light can also directly activate gene expression, one must note that patterns observed during a regular light cycle may result from circadian rhythms or may be a direct response to light. Many studies have demonstrated light-associated effects on immune function. For example, phagocytosis of *Escherichia coli* by myeloid cells in zebrafish has been reported to oscillate over the course of a light:dark cycle, peaking during the light phase and declining to a trough at night (33). Interestingly, the same study found that phagocytosis of *Staphylococcus aureus* did not show significant oscillations in zebrafish. The authors suggest that this may be due to rhythmic expression of innate immune receptors involved in recognition of Gram-negative, but not Gram-positive bacteria, though no specific receptors were identified (33). This is consistent with a result seen in human peripheral blood mononuclear cells, where cytokine production stimulated by lipopolysaccharide (LPS, present in Gram-negative bacteria) oscillated in a circadian manner, while cytokine release in response to lipoteichoic acid (LTA, present in Gram-positive bacteria) did not (34).

The light-regulated response to infection has also been shown to dictate survival in zebrafish. Zebrafish larvae infected with *Salmonella enterica* during the light phase showed enhanced survival compared to those infected during the dark phase (35). This effect occurred regardless of whether these fish were raised in light:dark conditions or constant light, suggesting that the enhanced survival is driven by a direct effect of light on immunity, and not the effect of light on the circadian clock. The increased survival of zebrafish infected during light exposure was associated with increased bacterial clearance, inflammatory cytokine expression, and neutrophil and macrophage recruitment to the site of infection (35).

A light-associated response to infection in other teleost fish species has also been shown. The Japanese medaka (*Oryzias latipes*) is a popular aquarium fish that is also used as model species for research. In Japanese medaka injected with LPS, *tnfα* induction was higher in fish stimulated during the dark period compared to those injected during the light period (36). This induction is consistent with a peak in *tnfα* rhythms during the dark phase in both Japanese medaka and zebrafish (31, 36).

The pattern recognition receptor gene *tlr9*, which recognizes hypomethylated CpG DNA, also exhibits daily rhythms in Japanese medaka, with a peak during the light period and a trough at night (37), allowing enhanced detection of DNA from bacteria and viruses while the fish is active. In response to *Aeromonas hydrophila* infection, Japanese medaka head kidney cells significantly increase the expression of *tlr9* and the TLR-adaptor *myd88*, however this response was only observed during the light period, consistent with the normal daytime expression of *tlr9* (37).

Because light is a key regulator of circadian rhythms, perturbations in photoperiod, or the amount of daily light received by an organism, can have serious consequences. The Turquoise killifish (*Nothobranchius furzeri*) is a short-lived fish that is often used as a model to study aging. Almaida-Pagán et al. subjected Turquoise killifish to a human-like shift-work schedule by rotating their light-dark cycle every week for 8 weeks. These fish showed disrupted rest-activity rhythms and circadian gene expression of the core clock genes *clock*, *bmal1*, *rora*, and *rev-erba* in the brain and liver. The disruption of circadian genes was coupled with a significant increase in antiprotease activity and a slight decrease in lysozyme and protease activity during the night (38). Photoperiod can also affect the adaptive immune system, as a longer light period results in higher antibody titer in sea ruffe (*Sebastiscus marmoratus*), a small commercially farmed tropical fish (39). This may explain why antibody levels are higher in sea ruffe immunized in the summer, when photoperiods are longer, compared to the winter months (39).

Further experimental photoperiod manipulations include constant light and constant darkness, both of which effect immunity in fish. The immune factors alkaline phosphatase, lysozyme, peroxidase, and protease exhibit significant rhythmicity under a normal light:dark photoperiod in another farmed fish, Nile tilapia (*Oreochromis niloticus*) (40). Under constant darkness, however, the rhythms of all 4 of these immune factors are abolished, indicating the importance of light in regulation of the innate immune system. Additionally, Nile tilapia

raised under constant darkness exhibited decreased survival compared to light:dark raised fish, further highlighting the importance of normal light exposure for fish health (40).

Atlantic cod (*Gadus morhua*) is another commercially farmed fish that is often placed under abnormal photoperiod conditions to delay sexual maturation and prevent weight loss (41). Constant light exposure in Atlantic cod leads to increased expression of the antioxidant enzymes superoxide dismutase, catalase, and glutathione reductase in the liver (42). The authors of this study speculate that the oxidative stress elicited by constant light may be related to decreased melatonin production (which has antioxidant activity) due to the lack of darkness (43). Literature also shows that Rainbow trout (*Oncorhynchus mykiss*) raised in constant light infected with *Argulus foliaceus* (fish lice) are unable to clear the infection as well as trout raised in light:dark conditions (44). This difference may be due to a disruption of the immune response, as *A. foliaceus* infected fish raised in constant light showed decreased expression and rhythms of several immune-related genes (44). These studies show that the immune response is dependent not only on the presence of light, but also the photoperiod the fish has previously been exposed to and varies depending on the species of host and pathogen.

## THE MOLECULAR CLOCK AND FISH IMMUNITY

There are now many immune-related genes known to be regulated by the molecular clock in zebrafish. A mutation in the zebrafish circadian gene *per1b* leads to a decrease in extracellular signal-regulated kinase (ERK) phosphorylation and a subsequent decrease in I$\kappa$B degradation and p65 activation, ultimately resulting in decreased NF-$\kappa$B signaling and decreased production of TNF-$\alpha$, IL-1$\beta$, and IL-8 following tail injury (45). Restoring ERK phosphorylation in the *per1b*$^{-/-}$ fish increased TNF-$\alpha$ production, confirming the positive regulation of inflammatory cytokines by Per1b. It is unknown whether the downregulation of ERK activation occurred as a direct result of *per1b* mutation, changes in the expression of other circadian genes, or alterations in other targets of *per1b*. Another study found opposite roles of *per1b* and *per2* in regulating cytokine gene expression, as *per2*$^{-/-}$ zebrafish showed increased expression of *tnf*$\alpha$, *il1*$\beta$, *il6*, and *il8*, while *per1b*$^{-/-}$ zebrafish displayed significantly reduced expression of *tnf*$\alpha$, *il1*$\beta$, *il6*, and *il8* (31). *per1b*$^{-/-}$ zebrafish also showed a decrease in the number of neutrophils recruited to tail injury, as is expected following reduced expression of the neutrophil chemoattractant *il8* (31).

The Clock/Bmal protein complex can regulate immunity through its effect on *per*, and by directly regulating transcription of immune-related genes. Expression of *tnf*$\alpha$ in Japanese medaka is driven by Clock/Bmal transcriptional activation through an E-box in its promoter (36). The rhythms of *tnf*$\alpha$ are antiphase to that of *clock* and *bmal* expression, and are consistent with that of other E-box driven genes such as *per1b* (36). *tlr9* was also found to be transcriptionally regulated by Clock and Bmal via an E-box in Japanese medaka (37). However, unlike *tnf*$\alpha$ and other E-box regulated genes, *tlr9* expression in Japanese medaka oscillates with the same phase as *clock* and *bmal* expression (37), indicative of another regulatory mechanism besides the E-box. Knockdown of *bmal1* in Japanese flounder (*Paralichthys olivaceus*) reduces the expression of *il1*$\beta$, *il6*, and *il8* (46), likely due to the presence of E-box elements and thus suggesting a role for Clock/Bmal in the regulation of these inflammatory genes as well.

## THE PINEAL GLAND, MELATONIN AND CONNECTIONS TO IMMUNITY

The most widely recognized output of the circadian clock is sleep-wake rhythms. One important regulator of sleep in zebrafish is melatonin, a hormone synthesized in a small gland in the brain called the pineal gland (47). The synthesis of melatonin in the pineal gland is tightly regulated, primarily by the enzyme arylalkylamine N-acetyltransferase 2 (Aanat2), a positive regulator of melatonin production (48). Aanat2 synthesis itself is regulated by the circadian clock, as the *aanat2* gene exhibits rhythmic circadian expression and contains E-box elements in its promoter (49). At night, Clock/Bmal binds to E-boxes to drive the expression of *aanat2* and thus melatonin synthesis. During the day, light triggers the expression of *per2* and *cry1a* to interact with Clock/Bmal, turning off *aanat2* expression and the production of melatonin (50, 51).

Historically, the pineal gland was considered the "master circadian regulator" in fish. Like most zebrafish tissues, the pineal gland exhibits self-sustaining circadian rhythms *in vivo* (52), and pineal gland cells can even rhythmically produce melatonin for several days *in vitro* (53, 54). Blocking the circadian rhythms of melatonin producing cells in the pineal gland disrupts clock-controlled rhythms of locomotor activity and sleep behaviors in zebrafish. However, abolishing circadian rhythms in the pineal gland does not appear to affect the molecular clock in other tissues (47, 55), indicating that the pineal gland may be more important in controlling circadian outputs rather than the circadian clock itself.

Across vertebrates, melatonin bridges endocrine-immune system interactions (56). Melatonin is thought to be an "immune buffer," as it can stimulate the immune system under basal conditions while also acting as an anti-inflammatory compound during infection or other acute inflammatory events (43). Melatonin alters the expression of inflammatory cytokines in humans (57), and boosts the innate and adaptive immune response in birds (58). In fish, melatonin produced in the pineal gland binds to leukocytes via the MT1 melatonin receptor to regulate immunity (59). In zebrafish, melatonin induces the rhythmic migration of neutrophils toward an injury, resulting in a peak in their migration during the dark phase (60). Constant treatment with melatonin abolishes this rhythmic migration, as does the removal of the pineal gland (60). A further study found that deletion of *annat2* also reduced the rhythms of neutrophil migration, and that endogenous melatonin promoted the migration of neutrophils by inducing the expression of *il8* and *il1*$\beta$ (61). Intraperitoneal injection of melatonin in Gilthead seabream (*Sparus aurata*), an important aquaculture species in the Mediterranean, upregulates several innate immune processes including peroxidase activity, phagocytosis, ROS production, and cytotoxic activity (62). *il1*$\beta$, *irf1*, *mx*, *tcr*$\alpha$, and *igm* are also upregulated in the head–kidney of these fish 1–3 days post-melatonin injection, highlighting the role of melatonin in both the innate and adaptive immune systems of this fish species (62).

## INFLAMMATION REGULATES FISH CIRCADIAN RHYTHMS

While there is a wide body of literature regarding the influence of circadian rhythms on the immune system, less is known about how the immune system regulates circadian rhythms. However, increasing evidence suggests that the immune system and inflammation can directly alter circadian rhythms in fish. A study in zebrafish identified NF-$\kappa$B as a regulator of the circadian clock, where activation and inhibition of NF-$\kappa$B increased and decreased the circadian amplitude of *per3*, respectively (63). This same study found that the amplitude of *per3* in zebrafish larvae is decreased by pro-inflammatory compounds and increased by anti-inflammatory compounds. Zebrafish lacking microglia, a macrophage-like cell in the brain, also showed an increase in *per3* amplitude, further suggesting a role for inflammation in regulating the zebrafish clock (63). Infection can alter the expression of circadian clock genes in zebrafish, as zebrafish infected with the brain-dwelling parasite *Pseudoloma neurophilia* displayed downregulated expression of *per1b* and *rev-erb*$\alpha$ expression in the brain (64). The authors speculate that the suppression of these genes may be an immune evasion strategy by the parasite, as *per1b* has been implicated in the expression of pro-inflammatory cytokines as discussed earlier (31, 45). These studies indicate that the circadian response to infection and inflammation is not straightforward, and further research is needed to determine whether these responses are advantageous for the host or pathogen.

In zebrafish, *catalase*, an important antioxidant enzyme that maintains redox balance during inflammation by degrading hydrogen peroxide, displays rhythmic expression that is antiphase to *per2* and *cry1a* expression (27). Overexpression of *catalase* reduced the light-dependent induction of *per2* and *cry1a*, while inhibiting *catalase* increased their induction, indicating negative regulation of these light-activated circadian genes by the catalase enzyme in zebrafish (27). Another study found that the addition of ROS to zebrafish cells was sufficient to induce D-box driven gene expression, and was associated with an induction of JNK and p38 MAP kinase pathways (28). Consistent with the *catalase* study discussed above (27), inhibition of ROS abolished light-driven D-box expression in these cells, as did inhibition of NADPH oxidase-mediated ROS production (28). These findings have

interesting implications for the mechanism of light-regulated circadian gene expression, and show that one photopigment involved in light-driven gene expression in zebrafish is the flavin- containing oxidase NADPH oxidase.

The circadian clock is also altered by the immune response in other species of fish. In a study discussed in the previous section, Ellison et al. found that lice infection of rainbow trout altered the rhythms and expression of immune-related genes, as well as the oscillation of circadian genes (44). The skin of infected fish displayed dampened rhythms of *clock3*, *per1*, and *cry2*, and phase-shifted expression of *clock3*, *per1*, *bmal1*, and *cry1*. Additionally, *bmal1*, *clock1b*, *clock3*, *cry1*, *per1*, and *per2* exhibited significantly different phases of expression between infected fish kept in constant light and light:dark conditions, likely leading to the differences in immune-related gene expression rhythms and the subsequent differences in survival outcome between these treatment groups. Infection also alters the molecular clock in Japanese flounder: in fish infected with the bacterial pathogen *Vibrio harveyi*, *bmal1* expression was significantly decreased (46). Knockdown of *bmal1* decreased the bacterial load and increased cytokine expression (46), suggesting that the downregulation of *bmal1* expression during infection may be an evolutionary mechanism to enhance the defense against pathogens in Japanese flounder.

Melatonin synthesis is also affected by the immune system, as LPS stimulation results in a decrease in melatonin secretion 4-h post-stimulation, followed by an increase 4-h later (40). Whether this alteration occurs via differential expression of circadian genes or proteins (i.e., *per2*, which inhibits melatonin synthesis in zebrafish) or directly modifies melatonin synthesis itself, is unknown. Additionally, the difference in melatonin secretion at 4- and 8-h post-LPS stimulation may be related to the immune buffering effects of melatonin, but whether melatonin is acting as an anti-inflammatory or immune activator during this response remains to be investigated.

## CONCLUSIONS

Most of our current understanding of vertebrate clocks is based on mammalian systems such as mice. However, zebrafish are a genetically tractable vertebrate model which have already proven valuable to study molecular circadian rhythms. By comparing fundamental differences between fish and mammalian clocks, further study of zebrafish clocks will elucidate how evolution has shaped circadian clocks among vertebrates. For example, certain key components, such as Clock/Bmal driven expression of E-box containing genes, are conserved between fish and mammals, while other features, such as the direct induction of *per2* and *cry1a* in response to light, occurs in zebrafish but not in mammalian cells.

Although we have made great strides in our knowledge of circadian rhythms in zebrafish, the molecular mechanisms of the zebrafish clock are still not fully understood. For example, we do not yet know the significance of the genome duplications leading to expanded *per*, *cry*, *clock*, and *bmal* genes, or how they interact to maintain 24-h oscillations in gene expression. Furthermore, several studies in mammalian models have demonstrated that the core components of the molecular clock are regulated post-transcriptionally (3) and the cellular concentration of circadian proteins are ultimately what dictates circadian output. No studies have characterized daily oscillations in clock protein concentration in zebrafish, and future studies are needed to fully understand the mechanisms of the core circadian clock in zebrafish.

The use of zebrafish has also provided advances in the study of immunity, and it is now clear that there is a direct relationship between the circadian clock and the immune system in this model organism. The connection between circadian rhythms, immunity, and infection is not limited to zebrafish – evidence in mammals (65–67), birds (68, 69), and flies (70, 71) also shows a link between these processes across vertebrate animals. This relationship has also been characterized in other species of fish, some of which were covered in this review.

Once we have established a better understanding of the molecular mechanisms of the circadian clock in fish, future research can further dissect the mechanisms by which the clock regulates the immune response and vice versa in zebrafish and other species. Studying these mechanisms will allow us to determine how this link may be beneficial or detrimental for host outcome following infection. Furthermore, given that the use of

zebrafish to study human pathogens is increasing (8), zebrafish may emerge as a valuable model to study the relationship between circadian rhythms and human pathogens. In conclusion, we are only just beginning to understand the exciting connections between the zebrafish clock and the immune system. Much remains to be discovered about our fishy friends!

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