

Diversity of zebrafish peripheral oscillators revealed by luciferase reporting

Maki Kaneko[†], Nancy Hernandez-Borsetti, and Gregory M. Cahill

Department of Biology and Biochemistry, University of Houston, 4800 Calhoun, Houston, TX 77204-5001

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In various multicellular organisms, circadian clocks are present not only in the central nervous system, but also in peripheral organs and tissues. In mammals peripheral oscillators are not directly responsive to light, but are entrained by the central oscillator in the suprachiasmatic nucleus. These individual oscillators are diverse in their free-running periods and phases. In contrast, cultured peripheral tissues and cell lines from zebrafish are not only rhythmic, but can also be directly entrained by light. Because of the convenience of studying rhythms in cultured cells, however, little has been known about properties of individual oscillators in intact zebrafish. Here, we show the remarkable diversity and consistency of oscillator properties in various peripheral organs and tissues from the *period3-luciferase* (*per3-luc*) transgenic zebrafish. Tissue-dependent differences were found in free-running period, phase, response to light, and temperature compensation. Furthermore, cycling amplitudes were reduced at lower temperatures in some, but not all, of the organs tested. Finally, we found that *per3-luc* rhythms can free run in both constant dark and constant light with remarkably similar amplitudes, phases, and periods, despite the fact that the mRNA of *per2* and *per1* has been shown not to oscillate in constant light.

circadian | period3 | bioluminescence | temperature compensation

Circadian oscillators are essentially intracellular phenomena where transcriptional feedback loops regulate cycling expression of various genes (1–3). They are consistently present in unicellular organisms such as cyanobacteria. In higher metazoan species, it has been believed for a long time that circadian rhythms are present in specialized tissues, such as the suprachiasmatic nucleus (SCN) in mammals, the pineal gland in some lower vertebrates, and the optic lobe of some insects. This view changed radically after cloning of so-called clock genes and studies of their expression patterns. Various tissues all over the body of *Drosophila* have been shown to express the *period* (*per*) gene rhythmically (4, 5). Similarly, many tissues in mammals express *per1* and *per2* rhythmically (6, 7). However, among all of the tissues that possess oscillator properties in these organisms, there is a central oscillator that affects other oscillators, such as the SCN in mammals and some clock neurons in *Drosophila* (8, 9). The common properties of these central oscillators are: (i) they are neuronal cells in the central nervous system that are responsible for locomotor activity rhythms; and (ii) they can affect phases and/or amplitudes of other oscillators, possibly by chemical, physiological, or behavioral factors (10).

However, the extent to which the central oscillators affect peripheral oscillators varies among organisms. In *Drosophila*, most peripheral oscillators are directly responsive to light (5) and can be entrained independently of the central pacemakers in the brain (11, 12). The blue-light photoreceptor Cryptochrome (CRY) is supposed to be the molecule responsible for entrainment of peripheral oscillators (13, 14). Only some clock neurons in the brain and the prothoracic gland have been shown to depend on the small ventral lateral neurons for persistent rhythms in constant dark (DD) (15–17). Many of the peripheral oscillators seem to dampen quickly in DD (18), whereas some

brain neurons other than the small ventral lateral neurons show persistent and independent molecular rhythms in DD (19).

In mammals, central and peripheral oscillators cannot be reset by light directly. Light-induced signals from the retina reset the clock in the SCN (20, 21), which in turn resets and/or sustains rhythmicity in many peripheral tissues (6, 7, 22, 23). Such tight regulation of peripheral oscillators by the central one in mammals may be the evolutionary consequence or, more likely, the prerequisite for the loss of direct photic response in peripheral oscillators. Yet, other cues such as food availability can entrain certain peripheral oscillators independently from the SCN (24), making the hierarchy of oscillators questionable. The persistence of rhythms in peripheral tissues can vary depending on the output studied; rat *Per1-luciferase* (*luc*)-mediated bioluminescence rhythms in peripheral tissues damp in several days, whereas those mediated by mouse *Per2-luc* persist for at least 20 cycles (6, 7). Recently, single-cell imaging techniques revealed that damping of bioluminescence rhythms in cultured cells is caused by desynchronization of individual cellular oscillators (25–27). However, the above-mentioned diversity in peripheral tissues suggests that damping in some peripheral oscillators could be caused by intrinsic properties of clocks in those tissues.

In zebrafish it has been shown that peripheral tissues and cell lines rhythmically express various clock-gene homologues such as *clocks*, *bmals*, *crys*, and *per* (reviewed in ref. 28). It is also known that zebrafish possess more genes (including clock genes) than do mammals, because of a genomewide duplication during early teleost evolution (29). For example, there are six *cry* genes in zebrafish, whereas only two such genes are present in mammals (30). On the other hand, only three transcripts have been identified for zebrafish *per* genes, *per1*, *per2*, and *per3* (*per1* is also called *per4*) (31–34). Like in *Drosophila*, molecular rhythms in peripheral tissues from zebrafish are directly resettable by light even in culture (32, 35), although the photoreceptor molecule responsible for this resetting is not yet known. The location of the central pacemaker or even the presence of it in zebrafish is not yet known, but there may be such a pacemaker in the brain (28). Simultaneous ocular enucleation and pinealectomy in larval fish still leaves their locomotor activity rhythms intact, suggesting that neither the retina nor the pineal gland is necessary for this rhythm (28).

The fact that zebrafish rhythms can be studied in cultured cells facilitated studies of photic responses, promoter dissections of clock genes, single-cell monitoring of bioluminescence rhythms, and entrainment by temperature shift (25, 33, 36, 37).

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Abbreviations: SCN, suprachiasmatic nucleus; *per*, *period*; *luc*, *luciferase*; DD, constant dark; LL, constant light; CRY, Cryptochrome; LD, light-dark; FFT-NLLS, fast Fourier transform-nonlinear least squares; MESA, maximum entropy spectral analysis; PRC, phase response curve; ZT, Zeitgeber time; CT, circadian time.

[†]To whom correspondence should be sent at the present address: Neurosciences Institute, 10640 John Jay Hopkins Drive, San Diego, CA 92121. E-mail: kaneko@nsi.edu.

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However, the use of cell lines also harbors the risk that these cells may not represent various cell types present in intact organisms, and the findings in cell lines may not be applicable to some tissues. Therefore, we used the *per3-luc* transgenic zebrafish to study the properties of various peripheral oscillators in adult zebrafish (38). Tissues and organs from this transgenic fish were dissected, cultured, and monitored for bioluminescence rhythms in various lighting and temperature conditions. Many tissues and organs showed bioluminescence rhythms in culture that were reset by light. These rhythms varied in free-running period, phase, response to light, and temperature compensation. Cycling amplitude was also affected by lower temperatures only in some organs tested. Furthermore, bioluminescence rhythms in DD and constant light (LL) were remarkably similar in period, amplitude, and phase. This is different from *per1-luc*-mediated bioluminescence rhythms in cultured cells, where the rhythm and overall level of luminescence were acutely suppressed by light (33).

Results and Discussion

Bioluminescence Rhythms in Isolated Tissues and Organs from *per3-luc* Zebrafish. To test whether various peripheral organs from adult zebrafish produce *per3-luc*-mediated bioluminescence rhythms, they were dissected and monitored for bioluminescence as organ cultures in DD. Robust free-running rhythms that persisted for at least 6.5 days were found in the heart, spleen, and gall bladder (Fig. 1 C, E, and G, respectively). The retina also showed fairly robust rhythms, although amplitudes and periods varied from experiment to experiment possibly because of the susceptibility of this organ to different batches of serum (Fig. 1A). The rhythms damped in DD and recovered during subsequent exposure to light-dark (LD) conditions (Fig. 1A, C, E, and G). The phases and periods of the bioluminescence rhythms in DD varied depending on the organ. The gall bladder consistently had longer periods than did the heart and spleen (Table 1; $P < 0.0001$, $\alpha = 0.0051$, Wilcoxon-Kruskal/Wallis test). The periods of retinal rhythms seemed more variable; they were >25 h in some experiments (see Fig. 5). The periods of the heart and spleen were not significantly different [$P = 0.0094$ by fast Fourier transform–nonlinear least squares (FFT-NLLS), and $P = 0.2892$ by maximum entropy spectral analysis (MESA), $\alpha = 0.0051$, Wilcoxon-Kruskal/Wallis test]. Peaks in different organs occurred at various circadian times (CTs) in DD (Table 1). The retina peaked at later phases, and the gall bladder peaked at slightly earlier phases ($P < 0.0001$, $\alpha = 0.0051$, Watson-Williams test). Peaks of these organs in LD conditions did not vary as much as in DD: retina, Zeitgeber time (ZT; ZT 12 corresponds to lights off, and ZT 22 to lights on) 4.4 ± 0.1 , $n = 23$; heart, ZT 2.8 ± 0.2 , $n = 12$; spleen, ZT 3.2 ± 0.1 , $n = 12$; gall bladder, ZT 3.1 ± 0.2 , $n = 11$.

Besides the organs shown in Fig. 1, the brain, pineal gland, gill, kidney, liver, oocyte–follicle complex, caudal fin, and testis have been tested for bioluminescence rhythms. Only the oocyte–follicle complex and the pineal gland showed robust rhythmicity comparable to the heart, spleen, and gall bladder (see Fig. 5 and Fig. 6, which is published as supporting information on the PNAS web site). The gill, kidney, and caudal fin produced relatively low-amplitude rhythms in at least some specimens (see Figs. 5 and 6). The brain did not show rhythmicity when cultured as a whole brain, probably because of its large size. Different parts of the brain, however, showed bioluminescence rhythms in cultures (H. Borsetti and G.M.C., unpublished data). Bioluminescence from the testis did not last >3 days in culture, suggesting that this organ dies in the culture conditions used. The liver had very low bioluminescence that oscillated very little (Fig. 6). Importantly, similar bioluminescence rhythms in the retina, heart, spleen, gall bladder, and gill were observed in another line of *per3-luc* (line

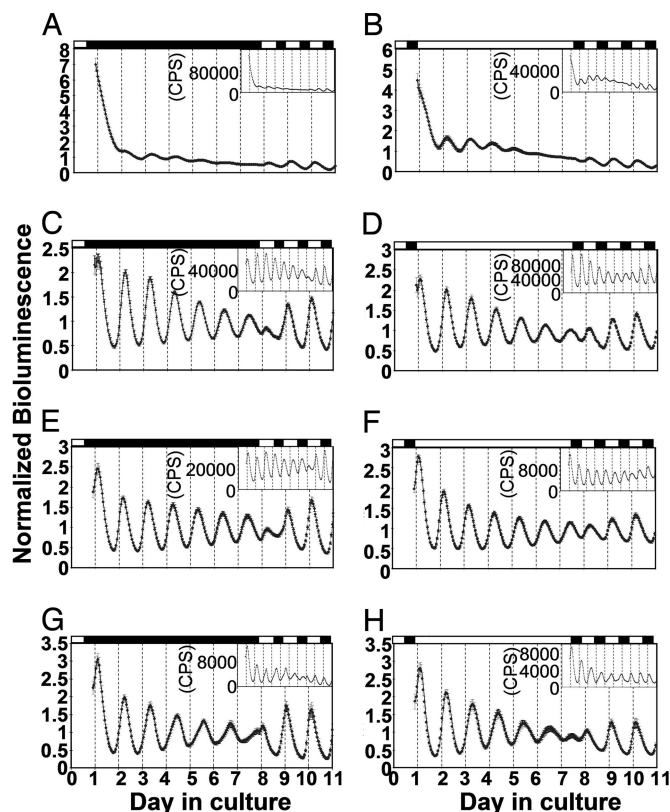


Fig. 1. Bioluminescence rhythms in cultured organs in DD (A, C, E, and G) and LL (B, D, F, and H). The retina (A and B), heart (C and D), spleen (E and F), and gall bladder (G and H) were tested for bioluminescence rhythms in DD and LL. After ~ 6.5 days in constant conditions the cultured organs were transferred to 14:10 LD cycles and tested for 3 more days. For each type of organ, an averaged plot from an experiment is presented. Error bars represent SEM. The number of samples used for each averaged plot was: 19 (A), 15 (B), 11 (C–F and H), and 12 (G). (Insets) Representative single plots are shown for each type of organ. The first 12 h of each record was deleted from the plot. White and black bars represent the time when the lights were on and off, respectively. The 0 point on the x axis corresponds to ZT 0 (2 h after lights on) on the day the fish were dissected.

86) with much lower overall luminescence counts (data not shown).

The low amplitude of the rhythms in some of these tissues and organs may be caused by their susceptibility to the culture condition used or to endogenous properties of their oscillators, such as weak coupling among cells in a given tissue and cells with variable periods, phases, and amplitudes within a tissue. Variability in cycling phase among different tissues has been documented in zebrafish for *bmal1*, *bmal2*, and *clock* (39). Tissue-dependent diversity in period and phase was also observed in the *mPer2-luc* knockin mouse (7). As has been suggested in their case, quantitative or qualitative difference in clock gene expression and/or cellular environment among different tissues may be responsible for the diversity. The tissue-dependent variability of clock-gene expression in zebrafish is not surprising considering that there are more clock genes present in zebrafish than in mammals (28).

Bioluminescence Rhythms in LL. In LL, these organs showed bioluminescence rhythms remarkably similar to those in DD (Fig. 1). However, there were differences between DD and LL; the periods of the retinal rhythms were shorter in LL than in DD (Table 1), and the spleen showed slightly, but significantly, longer periods in LL than in DD (Table 1; $P < 0.0006$, $\alpha =$

Table 1. Rhythmicity, periods, and phases of bioluminescence rhythms in cultured peripheral organs in DD and LL

Organ	Light condition	<i>n</i> , tested	<i>n</i> , rhythmic	Relative-amplitude error	Period by FFT-NLLS	Period by MESA	Phase (CT)
Retina	DD	41	15	0.56 ± 0.05	24.0 ± 0.3	24.6 ± 0.1 (21)	8.3 ± 0.7
Heart	DD	25	25	0.12 ± 0.00	25.0 ± 0.1	25.0 ± 0.1	4.1 ± 0.2
Spleen	DD	22	22	0.10 ± 0.00	24.7 ± 0.1	25.0 ± 0.1	3.8 ± 0.1
Gall bladder	DD	22	22	0.18 ± 0.01	26.4 ± 0.2	26.6 ± 0.2	2.9 ± 0.2
Retina	LL	32	22	0.45 ± 0.03	22.2 ± 0.2	23.5 ± 0.2 (14)	8.8 ± 0.5
Heart	LL	20	20	0.18 ± 0.01	24.7 ± 0.1	24.9 ± 0.2	4.0 ± 0.1
Spleen	LL	20	20	0.12 ± 0.01	25.3 ± 0.1	25.5 ± 0.1	3.0 ± 0.1
Gall bladder	LL	19	19	0.18 ± 0.01	26.5 ± 0.2	26.7 ± 0.2	3.0 ± 0.2

For each group, the results of two experiments were pooled. The numbers of rhythmic samples judged by FFT-NLLS are given. Mean ± SEM of relative-amplitude error (53), periods, and peak phases were calculated for rhythmic samples only. Only the samples judged as rhythmic by autocorrelation (such numbers are given in parentheses in case they are different from those done by FFT-NLLS) were averaged for periods by MESA.

0.0051). Therefore, the effect of LL on period depends on the organ studied. The peaks occurred at similar CT times in LL and DD except that the spleen showed slightly, but significantly, earlier phases in LL than in DD ($P = 0.0003$; Table 1). Like in DD, rhythms damped gradually in LL, then recovered in subsequent LD cycles. The fact that *per3-luc*-mediated bioluminescence rhythms in some organs were not affected by LL was remarkable, because the other two zebrafish *per* genes are not rhythmic in LL at least in cell lines and some tissues; *per2* mRNA is induced by light (32, 36), whereas *per1* transcription is suppressed by light [*per1* is also called *per4* (33, 34)]. Although the exact elements responsible for *per3* mRNA cycling are not known, three E-boxes have been found within 1-kb upstream sequences of this gene (38). If these E-boxes are responsible for *per3* mRNA rhythms, constitutive levels of PER2 and PER1 could possibly dampen *per3* mRNA rhythms. There are several possibilities to explain the results: (i) unlike in cell lines, *per2* and *per1* mRNA may cycle in LL in the organs studied here, although the light-dependent suppression of *per1* was observed in the brain, and larval fish and the PAC-2 cell line (33), and induction of *per2* has been observed in embryos (40); (ii) *per2* and *per1* mRNA may not cycle, but their protein products may do so by posttranscriptional mechanisms, although such mechanisms are unlikely to compensate for the very low level of *per1* mRNA in LL; (iii) *per2* and *per1* may not be expressed in these organs, although *per1* mRNA has been detected in the retina, heart and spleen (ref. 34 and M.K., unpublished data); and (iv) PER2 and PER1 are expressed, but do not affect the *per3* promoter.

In any case, this study clearly shows that circadian clocks persist in LL in many zebrafish tissues. Adult zebrafish have persistent locomotor activity rhythms in LL (41). Because it is the only known zebrafish *per* gene that shows mRNA rhythms in LL, *per3*, which is a homologue of mammalian *per3*, may be the most important zebrafish *per* gene for the central pacemaker responsible for locomotor activity rhythms. If this is true, it is different from mammals where knocking out *mPer3* has little effect on behavioral rhythms (42). Alternatively, there may be additional zebrafish *per* genes that are part of the central oscillator. In fact, there is a second *per1*-like sequence in the zebrafish genome although no cDNA corresponding to that sequence has been identified (G.M.C., unpublished data).

Resetting *per3-luc* Rhythms by Light. It has been shown that clocks in cultured tissues and cells from zebrafish are resettable by light (Fig. 1) (32, 35). We have also tested whether *per3-luc*-mediated bioluminescence rhythms can be re-entrained to an opposite LD cycle. The retina, heart, spleen, and gall bladder were entrained in two almost-opposite 14:10 LD cycles for 3 days in culture (lights on at 8 a.m. or 9 p.m.), and then tested for bioluminescence rhythms in DD (Fig. 2A). As a result, these two groups of

organs had almost opposite phases, suggesting that these organs can be entrained by light in culture (Fig. 2B–E). The phases of the spleen in the 9 p.m. to 11 a.m. LD group were significantly earlier than those of the other organs from the same group ($P = 0.0008$, $\alpha = 0.025$), suggesting that spleen may not have been wholly shifted to a new phase by the 25-lux light used in this experiment (Fig. 2D). In another experiment that used 200-lux light all four organ types were shifted completely (Fig. 7, which is published as supporting information on the PNAS web site).

To test how bioluminescence rhythms respond to light stimuli, cultured organs were monitored in DD for 2–3 days and then transferred to LL. The results depended on the organ studied. The damped rhythms of the retina were somewhat recovered when they were shifted to LL, and the initial response was always a slight increase in bioluminescence (Fig. 3A and B). Bioluminescence in many of the hearts and gall bladders typically increased 1–2 h after the LL shift, and then decreased after a few hours (Fig. 3C, D, G, and H). This response was observed

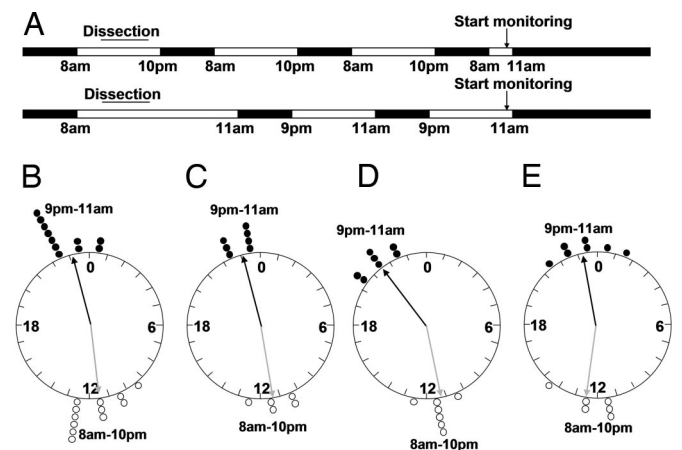


Fig. 2. Entrainment of bioluminescence rhythms of cultured organs. The retina, heart, spleen, and gall bladder were cultured under 14:10 LD cycle of two almost opposite phases for ≈ 2.5 days. The intensity of lights used in this experiment was 25 lux. (A) The exact entrainment schedule is shown. The 8 a.m. to 10 p.m. LD cycle shown on top is the lighting condition in which the fish were kept. (B–E) The phase values of the peaks in real time (CST) were plotted on circular graphs for the retina (B), heart (C), spleen (D), and gall bladder (E) in empty circles (8 a.m. to 10 p.m.) and filled circles (9 p.m. and 11 a.m.). Gray and black arrows represent mean vectors for the 8 a.m. to 10 p.m. group and the 9 p.m. to 11 a.m. group, respectively. The lengths of mean vectors are: 8 a.m. to 10 p.m. group, 0.96; 9 p.m. to 11 a.m. group, 0.99 (B); 8 a.m. to 10 p.m. group, 0.99; 9 p.m. to 11 a.m. group, 0.98 (C); 8 a.m. to 10 p.m. group, 0.99; 9 p.m. to 11 a.m. group, 0.98 (D); and 8 a.m. to 10 p.m. group, 0.91; 9 p.m. to 11 a.m. group, 0.96 (E).

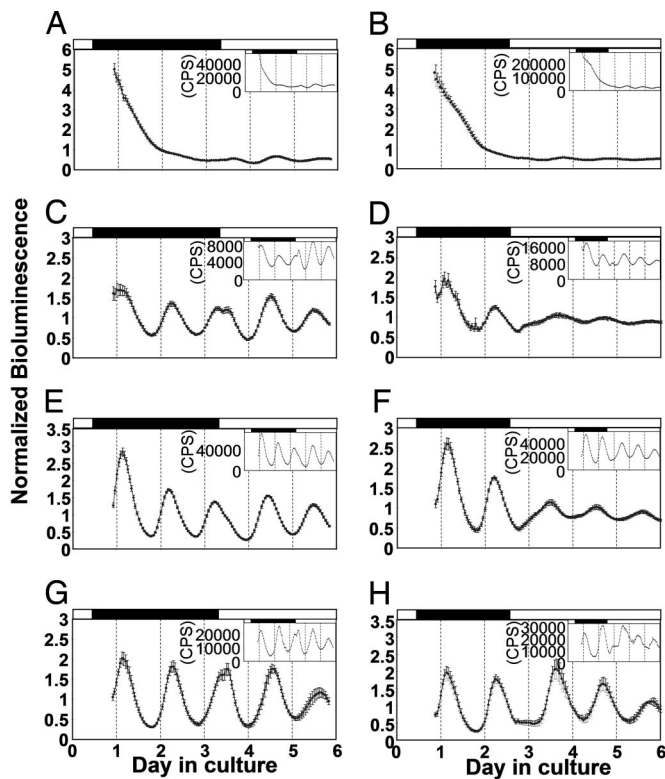


Fig. 3. Responses of bioluminescence rhythms to LL shifts. The retina (*A* and *B*), heart (*C* and *D*), spleen (*E* and *F*), and gall bladder (*G* and *H*) were tested for bioluminescence rhythms in DD for 2–3 days, shifted to LL at approximate peak time points (*A*, *C*, *E*, and *G*) and trough time points (*B*, *D*, *F*, and *H*) and tested for 2–3 more days. The number of samples used for each averaged plot was: 20 (*A*), 15 (*B*), 10 (*C*, *E*, and *G*), 9 (*D* and *F*), and 7 (*H*).

whether LL shift occurred near the peak or trough time point in 10 of 19 hearts and 12 of 17 gall bladders. The rest of the hearts and gall bladders showed milder effects, slowing down of the decrease in bioluminescence in response to a shift that occurred near the peak time point, or delay of the increase in response to a shift near the trough time point. These milder effects were observed for all of the spleen samples ($n = 10$ for peak time point and $n = 9$ for trough; Fig. 3 *E* and *F*). Taken together, the initial responses of many of the organs to light seemed to be an increase in bioluminescence. However, this is not a long-lasting effect, but rather a transient effect that leads to a rhythm with a new phase. Initial induction of *luc* RNA by light was also suggested in *per3-luc* larval fish (38).

Finally, to test the effects of short light pulses on bioluminescence rhythms, phase response curves (PRCs) were constructed for the heart and spleen. The PRC for the heart showed large phase shifts and was close to type 0 (Fig. 4*A*). The melatonin production rhythm from cultured pineal glands, however, showed a typical type 1 PRC (34). The spleen showed much smaller phase shifts in most time points than did the hearts (Fig. 4). At one time point in the middle of the subjective night, the spleen showed all kinds of responses (Fig. 4*B*). The light pulse at this time point also made the rhythms in the spleen weaker (data not shown). These results are in contrast to the flat PRC of the *cry^b* mutant flies (43), which are also rhythmic in LL. In the case of *Drosophila*, light degrades TIMELESS (TIM), possibly through the action of CRY, which leads to constantly low TIM, which in turn causes PER to be cytoplasmic and low (44). As a vertebrate, zebrafish may have molecular light responses very different from those in *Drosophila*. For instance, zebrafish

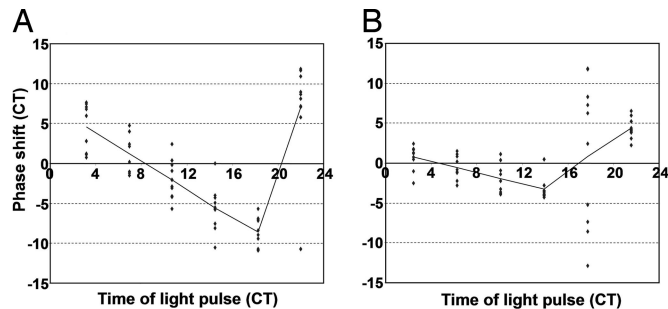


Fig. 4. PRCs for the heart and spleen. Phase responses to 1-h light pulses were determined in CT for the heart (*A*) and spleen (*B*) and plotted against the time of light pulse in CT. Each dot represents a phase shift for each organ tested. The lines in each plot connect the average response for each time point. The results of two experiments are pooled.

crys may not function as circadian photoreceptors, and zebrafish *tim* may not be involved in the clock mechanism.

As has been documented (32, 35), our data strongly support that cultured peripheral tissues from zebrafish are directly responsive to light. The response of *per3-luc*, however, is more transient in nature compared with those responses of the other zebrafish *per* genes. Our results on entrainment, LL shift, and PRC all point to lower light sensitivity of *per3-luc* rhythms in the spleen compared with photic responses of clocks in other organs such as the heart. This difference between the spleen and other organs may be caused by difference in photosensitivity, photo-transduction pathways, or the pacemaking properties.

Effects of Temperature on Bioluminescence Rhythms. Period lengths of circadian rhythms in various organisms are relatively invariant at varying temperatures. This is one of the basic characteristics of circadian clocks and is called temperature compensation. The *per3-luc*-mediated bioluminescence rhythm was also temperature-compensated in the six tissues tested (Fig. 5). As has been shown for the rhythms of melatonin production in cultured zebrafish pineal glands (34) and *per1* promoter- and four E-box-mediated bioluminescence rhythms in cultured cells (37), bioluminescence rhythms in the retina, gill, heart, gall bladder, and pineal gland showed slight, but significant, overcompensation with Q_{10} values ranging from 0.89 in the retina to 0.96 in the gill (Fig. 5). These Q_{10} values are within the range of circadian rhythms in various organisms (45). The spleen showed almost perfect temperature compensation with a Q_{10} value of 0.99. The two-way ANOVA, performed on all of the six tissues and temperatures as variables, showed significant effects of tissue, temperature, and tissue \times temperature interaction on period lengths ($P < 0.0001$ for each of the three effects).

Bioluminescence rhythms mediated by luciferase in *Drosophila* showed earlier phases at higher temperatures, and unstable luciferase activity in higher temperatures has been suggested as the mechanism responsible for this phenomenon (43). In zebrafish tissues, it is possible that lower enzyme stability at higher temperatures might have caused different period lengths. However, it is unlikely that this also caused tissue-dependent Q_{10} differences, because luciferase stability should be the same in all of the tissues at a given temperature.

We have documented tissue-dependent differences of Q_{10} values for circadian rhythms within species. The only comparable example may be a Q_{10} value of 0.99 for neuronal rhythms of the rat SCN (46) compared with a Q_{10} of 0.85–0.88 for *mPer1::luc*-mediated bioluminescence rhythms in Rat-1 fibroblasts (47). However, those studies dealt with rhythms of different biological processes in a homeothermic organism. Our studies showed that rhythms of the same output can vary in terms

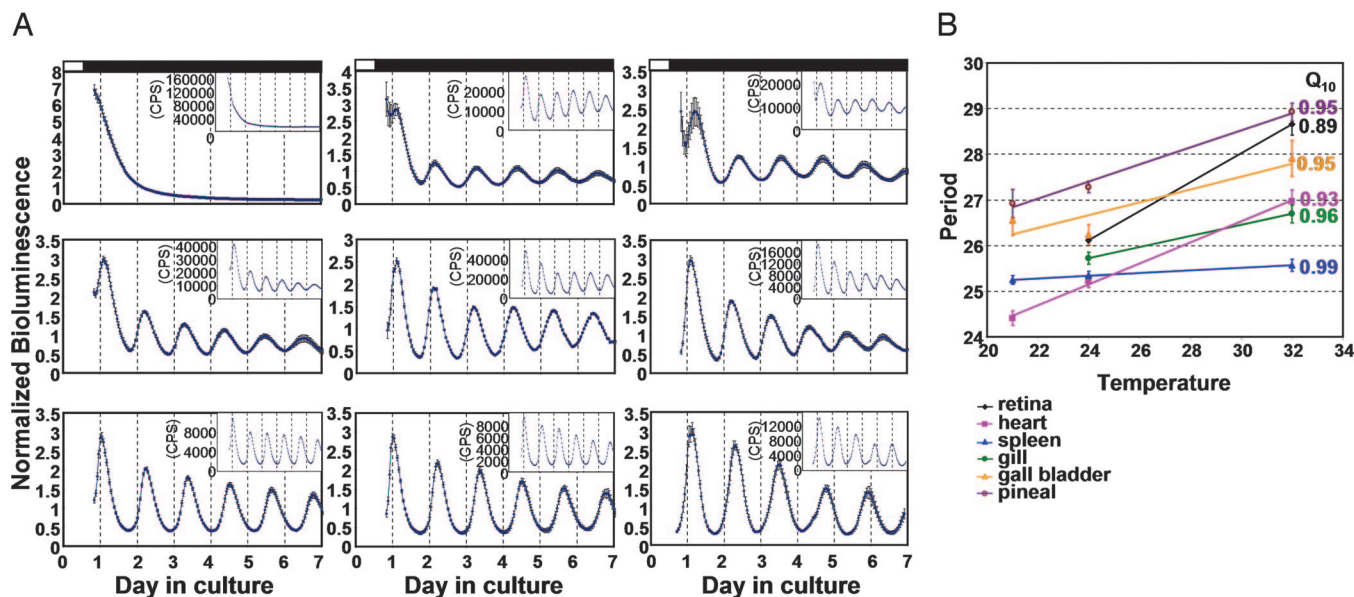


Fig. 5. Effects of temperatures on periods of bioluminescence rhythms in various cultured tissues. (A) Averaged plots from an experiment for the retina (Top), spleen (Middle), and pineal gland (Bottom) at 21°C (Left), 24°C (Center), and 32°C (Right) are shown. The number of samples averaged for each plot was: retina, 9 at 21°C, 16 at 24°C, and 12 at 32°C; spleen, 6 at 21°C, 8 at 24°C, and 8 at 32°C; pineal gland, 6 at 21°C, 6 at 24°C, and 5 at 32°C. (B) Average period lengths for the six tissues tested are plotted. Period lengths were estimated by FFT-NLLS. Similar results were obtained by MESA (data not shown). For each temperature, results from two to three experiments are combined. The number of samples for each group was: retina, 24 at 24°C and 33 at 32°C; heart, 9 at 21°C, 25 at 24°C, and 23 at 32°C; spleen, 10 at 21°C, 24 at 24°C, and 22 at 32°C; gill, 21 at 24°C and 21 at 32°C; gall bladder, 6 at 21°C, 12 at 24°C, and 15 at 32°C; pineal gland, 7 at 21°C, 12 at 24°C and 9 at 32°C. The Q_{10} value for each tissue is given to the right. The retina and gill were mostly arrhythmic at 21°C. Error bars represent SEM. Q_{10} values that were obtained by MESA were: retina, 0.89; heart, 0.93; spleen, 0.99; gill, 0.94; gall bladder, 0.95; pineal, 0.95.

of temperature compensation in a poikilothermic organism. Because the mechanisms responsible for temperature compensation of circadian rhythms are not known, it is not easy to speculate what caused tissue-dependent variability of Q_{10} values. Because zebrafish has multiple homologues of many mammalian clock genes (28), it is possible that these different isoforms vary in terms of their sensitivity to temperature changes. In fact, changes in clock genes can affect temperature compensation, because some period-altering mutations in clock genes of *Neurospora*, *Drosophila*, and hamster also cause defects in temperature compensation (44, 48, 49). In *Drosophila*, even natural polymorphisms in a clock gene that are distributed as a highly significant latitudinal cline may account for differences in temperature compensation (50). A circadian period-altering mutation in zebrafish has been demonstrated to affect Q_{10} values of melatonin production rhythms from cultured pineal glands (34). Although the mutated gene has not been identified in this case, it has been speculated that the gene is involved in pace-making mechanisms of the zebrafish circadian rhythm, because the mutation affects both larval swimming and melatonin rhythms (34). Differences in relative expression levels of different clock genes in various tissues may account for the tissue-dependent variability in Q_{10} values.

Lahiri *et al.* (37) demonstrated that amplitudes of *per1* promoter- and four E-box-mediated bioluminescence rhythms in cultured cells vary greatly depending on ambient temperature. In our study, we did not find such a dramatic amplitude differences between 24°C and 32°C except for the gill where significant amplitude difference between these two temperatures was observed (Table 2, which is published as supporting information on the PNAS web site). However, at 21°C, the gill and retina were mostly arrhythmic, and the heart, pineal gland, and spleen showed significant reduction in amplitudes (Fig. 5A and Table 2). The gall bladder did not show significant amplitude differences among the three temperatures. Therefore, cycling amplitudes seem to be stable over at least 8°C in most tissues and quickly

deteriorate below certain temperatures in some tissues. Lahiri *et al.*'s studies on cycling amplitudes involved only two temperatures of rather large differences, 20°C and 30°C. Therefore, it is not clear whether the amplitude differences they found in their cell-based studies were caused by gradual changes over a range of temperature or sudden deterioration of rhythms below certain temperature. Sensitivity of cycling amplitude to temperature is a widespread phenomenon observed in diverse species such as *Gonyaulax* and chicken (51). Furthermore, our study shows that susceptibility of cycling amplitudes to ambient temperature varies depending on the tissue studied.

In summary, we have found that *per3-luc*-mediated bioluminescence rhythms differ among cultured peripheral tissues and organs in various ways such as period, phase, response to light, and temperature compensation. Furthermore, cycling amplitudes were reduced by lower temperatures in some, but not all, of the organs tested. This diversity suggests that those cell line-based studies successfully carried out in zebrafish should be treated as equivalent to studies based on one particular tissue (25, 33, 35, 37). This also raises the question of how these oscillators are orchestrated in intact animals. In zebrafish, individual oscillators can be entrained by light in LD conditions. Therefore, they may not need to be entrained by the central pacemaker in natural conditions. Do these oscillators desynchronize in constant conditions, or are they synchronized by the central oscillator in the brain as has been shown for mammals (6, 7)? This question is of particular interest in light of evolution of mammalian-like hierarchical circadian systems. The zebrafish, a vertebrate with *Drosophila*-like light-entrainable peripheral oscillators, could nonetheless have a more hierarchical system than do insects.

Materials and Methods

Animals. Animals used in this study carried the *per3-luc* transgene in hemizygous condition and were originally derived from the University of Oregon *AB strain (38). Among five independent

germ-line transformants, the strongest glowing line (line 23) was used unless otherwise stated (38). Animals were kept under 14-h light/10-h dark (14:10 LD; lights on 8 a.m.; lights off 10 p.m. CST) cycle, and group-housed in plastic tanks in a Z-MOD holding system (Marine Biotech, Beverly, MA) with recirculating filtered water at $\approx 28.5^\circ\text{C}$. They were fed commercial flake food in the morning, baby brine shrimp at midday, and adult brine shrimp in the evening. Experimental protocols were approved by the Institutional Animal Care and Use Committee.

Monitoring Bioluminescence Rhythms in Cultured Tissues and Organs. Adult zebrafish between 9 and 14 months old were dissected for organs/tissues for cultures. Bioluminescence emitted from cultured organs and tissues were monitored as described (52). Details on culturing conditions and bioluminescence monitoring are in *Supporting Text*, which is published as supporting information on the PNAS web site.

Data Analysis. Bioluminescence data from the Topcount were imported into Microsoft (Redmond, WA) Excel 2000 by the Import and Analysis macro (Steve Kay, The Scripps Institute, La Jolla, CA). The FFT-NLLS multicomponent cosine analysis was

used to estimate rhythmicity, periods, and peak phases (53). Free-running periods were also estimated by MESA, and rhythmicity was estimated by autocorrelation (54). Details on these methods are in *Supporting Text*.

PRCs. PRCs were constructed by a method similar to those described (33, 34). For details, see *Supporting Text*.

Statistics. For period lengths and cycling amplitudes, either the Wilcoxon-Kruskal/Wallis test, one-way ANOVA, or two-way ANOVA was performed on JMP 3.1.5 or JMP 5.1 (SAS Institute, Cary, NC) (55). Phase values were treated by methods of circular statistics using Microsoft Excel 2000 (56–58). Details on statistical methods used are in *Supporting Text*.

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