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# LIGHT AND TEMPERATURE CYCLES AS *ZEITGEBERS* OF ZEBRAFISH (*DANIO RERIO*) CIRCADIAN ACTIVITY RHYTHMS

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Light and temperature cycles are the most important synchronizers of biological rhythms in nature. However, the relative importance of each, especially when they are not in phase, has been poorly studied. The aim of this study was to analyze the entrainment of daily locomotor activity to light and/or temperature cycles in zebrafish. Under two constant temperatures (20°C and 26°C) and 12:12 light-dark (LD) cycles, zebrafish were most active during the day (light) time and showed higher total activity at the warmer temperature, while diurnalism was higher at 20°C than at 26°C (87% and 77%, respectively). Under thermocycles (12:12 LD, 26:20°C thermophase:chryophase or TC), zebrafish daily activity synchronized to the light phase, both when the thermophase and light phase were in phase (LD/TC) or in antiphase (LD/CT). Under constant dim light (3 lux), nearly all zebrafish synchronized to thermocycles ($\tau = 24$ h), although activity rhythms (60% to 67% of activity occurred during the thermophase) were not as marked as those observed under the LD cycle. Under constant dim light of 3 lux and constant temperature (22.5°C), 4 of 6 groups of zebrafish previously entrained to thermocycles displayed free-running rhythms ($\tau = 22.9$ to 23.6 h). These results indicate that temperature cycles alone can also entrain zebrafish locomotor activity.

**Keywords** Zebrafish, LD cycle, Thermocycle, Conflicting *zeitgeber*, Locomotor activity, Circadian rhythm, Entrainment

## INTRODUCTION

In the natural environment, daily light and temperature cycles are the most important synchronizers of circadian rhythms. The light phase coincides with the thermophase (the phase of higher temperature) and the dark phase with the chryophase (lower temperature); thus, transitions from cold to warm temperature are roughly associated with dawn, and transitions from warm to cold temperature with dusk (Johnson et al.,

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2004). Although light cycles have been studied in depth and are regarded as the major synchronizer of biological rhythms, temperature cycles are also known to be strong entraining cues of circadian clocks that can synchronize circadian rhythms in most organisms, from cyanobacteria to higher vertebrates (Rensing and Ruoff, 2002). Indeed, temperature may sometimes be a stronger synchronizer than light, as observed, for example, in *Neurospora* sporulation rhythms (Liu et al., 1998) or in lizard activity (Evans, 1966). Furthermore, moderately high temperature pulses can elicit phase-response curves (PRC) that are similar to those generated by light pulses (Barrett and Takahashi, 1995; Ruby et al., 1999). In addition, seasonal variations in temperature also influence a wide variety of biological rhythms, including reproduction and maturation (Davies and Bromage, 2002; Shimizu, 2003). On the other hand, a functional prerequisite of the circadian pacemaker is that the period lengths are temperature-compensated and remain constant over a wide range of constant temperatures (Pittendrigh, 1954), with a $Q_{10}$ value for tau ($\tau$) around 1.

In vertebrates, mammals (endothermic), and reptiles (ectothermic) have been the main subjects of research on the influence of temperature change on circadian rhythmicity. Thermocycle effects were first investigated in regard to lizard locomotor activity (Evans, 1966). Recent research has focused on temperature effects on melatonin circadian rhythms, both *in vitro* (Barrett and Takahashi, 1995; Moyer et al., 1997; Valenciano et al., 1997; Zachmann et al., 1991) and *in vivo* (Firth et al., 1999; García-Allegue et al., 2001; Masuda et al., 2003; Wright and Bruni, 2004). In fish, the temperature influence on melatonin production *in vitro* (Bolliet et al., 1994; Samejima et al., 2000; Zachmann et al., 1991) and the seasonal rhythms of melatonin (García-Allegue et al., 2001; Iigo and Aida, 1995; Masuda et al., 2003) were the main targets of previous studies while only few studies have examined temperature and behavioral rhythms (Aranda et al., 1999; Hurd et al., 1998; Reebs, 2002). Therefore, we aimed in our research to contribute to the understanding of the relative role played by light- and thermocycles in the entrainment of fish locomotor activity rhythms.

The zebrafish is currently one of the most attractive animal models in genetic and developmental studies. In addition, this fish constitutes a useful tool for chronobiological studies on the molecular bases of vertebrate circadian clocks, in which behavioral and melatonin rhythms are being used to identify and characterize clock mutants (Cahill, 2002). Moreover, the molecular clock of the zebrafish has characteristics that show similarities to both the *Drosophila* and mammalian systems (Pando and Sassone-Corsi, 2002). Circadian rhythms of melatonin and locomotor activity in zebrafish have been analyzed both in larvae and in adult fish (Cahill, 1996; Cahill et al., 1998; Hurd and Cahill, 2002; Hurd et al., 1998; Kazimi and Cahill, 1999). Hurd and colleagues (1998) showed that temperature

changes affect the rhythmicity under constant dark (DD) or constant light (LL) conditions. Furthermore, water temperature also influences the development of *per3* rhythmic expression in zebrafish larvae in DD conditions (Kaneko and Cahill, 2005). Recently, the effect of temperature cycles on the entrainment of the zebrafish circadian clock has been investigated, showing that temperature changes influence transcriptional rhythms, as well as phosphorylation and function of clock protein (Lahiri et al., 2005). However, the entraining effects of thermocycles on zebrafish overt rhythms (such as locomotor activity) have yet to be explored.

Therefore, the aim of this study was to evaluate the influence of light and temperature cycles, as well as the strength of each when provided in antiphase, on the daily pattern of locomotor activity of zebrafish. In addition, we studied the ability of thermocycles alone to entrain locomotor activity under constant light conditions.

## MATERIALS AND METHODS

### Animals and Housing

Zebrafish (*Danio rerio* L.) were obtained from a local provider (Jumipez S.A., Murcia, Spain) and reared in the facilities of the University of Murcia. Fish were an average length of $37 \pm 2$ mm (mean $\pm$ SD) and were kept in a well aerated 100 L tank equipped with biological and mechanical filters. The aquarium was divided into 6 sections (each 17 cm wide) using white plastic plates with 2 mm holes. Ten zebrafish were placed in each section. Light was provided by a fluorescent bulb (F15W/GRO, Sylvania Gro-Lux, Germany), placed 15 cm from the water surface, where light intensity was 700 lux. Thermocycles were set by means of two heaters (100 W, Askoll) and a cooler (Cubigel, E-500). Fish were fed with a standard diet (Wardley Premium) at random times, both during day and at night.

### Experimental Procedure

The experiments were conducted according to the ethical standards of the Journal (Touitou et al., 2004) and designed to investigate the influence and strength of temperature cycles as a synchronizer of the daily activity rhythms of zebrafish. The thermophase consisted of a 12 h period of 26°C, while the chryophase was maintained for 12 h at 20°C.

#### *Experiment 1. Effect of Different Constant Temperatures (26°C vs. 20°C) and LD Cycles*

The aim of this experiment was to evaluate the effect of constant high/low water temperature on locomotor activity rhythms of zebrafish.

The temperature was maintained at 26 ± 0.5°C and the photoperiod was 12:12 LD, with lights on at 8:00 h. The locomotor activity was registered for 4 wks. Then the temperature was changed to 20 ± 0.5°C, and locomotor activity was registered for another 4 wks.

### *Experiment 2. Entrainment of Activity Rhythms to LD and Thermocycles*

This experiment was designed to test the influence of light and temperature cycles on the activity rhythm of zebrafish, and the strength of each zeitgeber when given in reversed order. At the end of Experiment 1, the temperature changes occurred in phase with the LD cycle, the thermophase (26°C) coinciding with the light phase, and the chryophase (20°C) with the dark phase. This thermocycle was maintained for 2 wks. At the end of that period, the thermocycle was reversed by extending the thermophase, so that this phase coincided with the dark phase and the chryophase coincided with the light phase. This reversed light and temperature schedule was maintained for another 2 wks.

### *Experiment 3. Entrainment to Thermocycles Under Constant Light (LL)*

This experiment was designed to evaluate the capacity of thermocycles to entrain the activity rhythms of zebrafish when LD was lacking. The thermocycle was maintained as in Experiment 2, but the light was constant, with a low intensity of 3 lux, adjusted by covering the fluorescent tubes with aluminium foil and punching holes in it until the desired light intensity was attained. Zebrafish were kept under these conditions for 2 wks, during which locomotor activity was registered. Then, the thermocycle was reversed by extending the thermophase, and activity was registered for another 2 wks. After this period, temperature was kept constant at 22.5 ± 0.5°C, and locomotor activity was analyzed to evaluate the existence of free-running rhythms previously entrained by the thermocycle.

## Data Analysis

Locomotor activity was measured by means of an infrared photocell (Omron, mod E3S-AD62, Japan) placed at the aquarium wall. Data from two photocells, one placed 5 cm from the bottom and the other placed 25 cm from the bottom and 5 cm from the surface, were pooled and used for each group of animals. The number of light-beam interruptions was counted and stored every 10 min by a computer. Total locomotor activity during the day was calculated and analyzed for differences between each experimental period.

Water temperature was registered continuously by means of a temperature sensor (iButton DS1921H-F50, IDC S.A., Barcelona, Spain). When thermocycles were performed, temperature shifts from high to low temperature and *vice versa* lasted between 1 and 3 h.

Statistical analysis was performed using Excel® and SPSS® software. Percentage values of activity were transformed to arcsine for statistical analysis. The analysis and representation of locomotor activity records were performed using the chronobiology software *El Temps* (Version 1, 192;© Prof. Díez-Noguera, University of Barcelona).

## RESULTS

### Experiment 1. Effect of Different Constant Temperatures and LD Cycles

At constant temperature, zebrafish showed a predominantly diurnal behavior under the 12:12 LD cycle, with most of their activity being displayed during the L phase (Figure 1). When the constant temperature was changed from 26°C to 20°C, total activity counts during the day decreased to about 30% of the activity at 26°C (14200 ± 2300 *vs.*

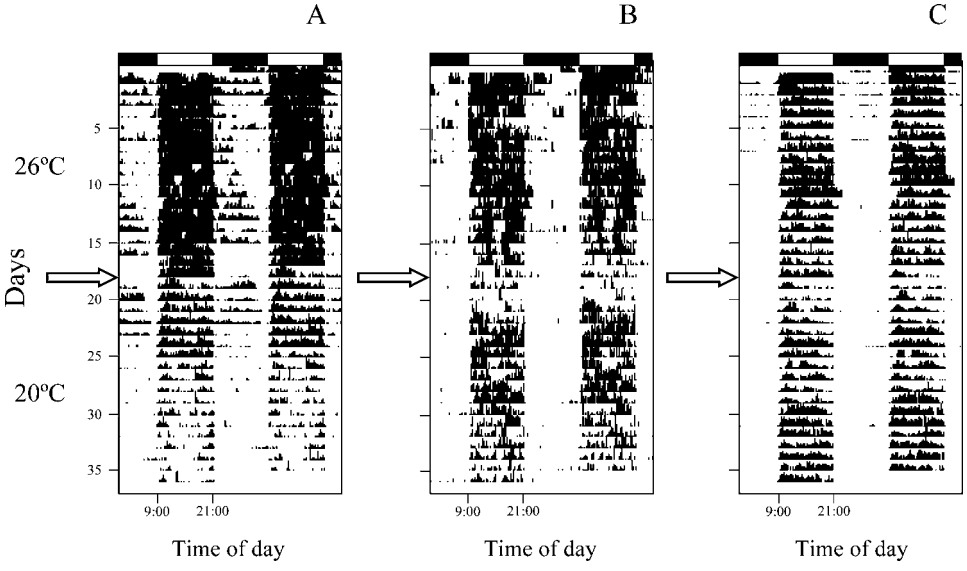


**FIGURE 1** Actograms of locomotor activity of 3 representative groups (*A*, *B*, and *C*) of zebrafish maintained under constant temperature (26 and 20°C) and a 12:12 LD cycle. The bars above each actogram represent the light regime; open and black bars represent the light and dark phase of the cycle, respectively. For convenient visualization, the data were double plotted (48 h) at a resolution of 10 min, the height of each point representing the number of interruptions of the infrared lightbeam. Fish were first kept at constant 26°C, and thereafter the temperature was changed to constant 20°C. Temperature change is indicated by the arrow at the left of the actogram.

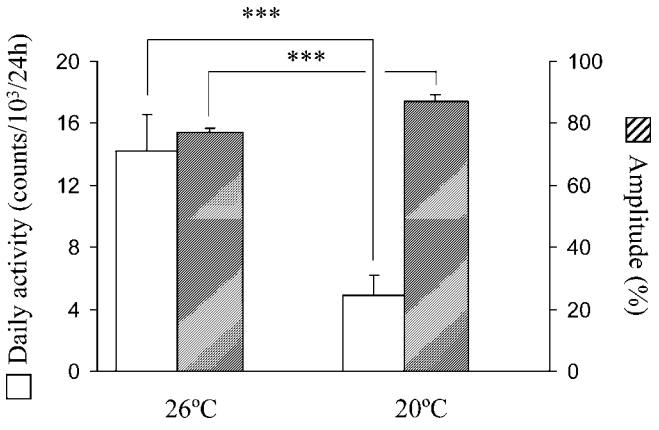


**FIGURE 2** Total daily activity (*open bars*) and the amplitude of the rhythm (*striped bars*) of zebrafish under constant 26°C and 20°C. Data are represented as mean ± SEM. Total daily activity was about three-fold higher at 26°C than at 20°C. Relative locomotor activity during the light phase was higher at 20°C. Asterisks indicate statistically significant differences between groups (paired *t*-test, $p < 0.005$).

4900 ± 1300 counts/day, respectively; paired *t*-test, $p < 0.005$) (Figure 2). The decrease of total daily activity differed widely between groups, from groups that reduced their daily activity by a substantial degree (Figure 1A) to groups where the decrease could hardly be detected in the actogram (Figure 1C).

Diurnalism was defined by more than 65% locomotor activity occurring during the daytime (Sánchez-Vázquez et al., 1996). Following this criterion, all groups of fish showed diurnal activity both at constant high (26°C) and constant low (20°C) temperature (Figure 1). Nocturnal activity varied between groups, with some groups showing basal activity during the night time (Figure 1A), while other groups concentrated their daily activity strictly to the L phase (Figure 1, B–C). When the water temperature was changed from 26°C to 20°C, nocturnal activity decreased (Figure 1A). The mean percentage of activity during the daytime (Figure 2) was significantly different between the two constant temperatures studied, with a more pronounced rhythmicity at 20°C than at 26°C (86.9 ± 2.1% *vs.* 77.2 ± 1.4% of daytime activity, respectively; paired *t*-test, $p < 0.005$).

### Experiment 2. Effect of LD and Thermocycles

Zebrafish held under a thermocycle in phase with the LD cycle (thermophase coinciding with light phase: LD/TC) showed most of their activity during the L phase (Figure 3). When the thermocycle was inverted (thermophase coinciding with the D phase: LD/CT), the activity rhythm remained entrained to L in 5 of the 6 groups, only 1 group lost its daily rhythmicity. In some groups, the locomotor activity profile after

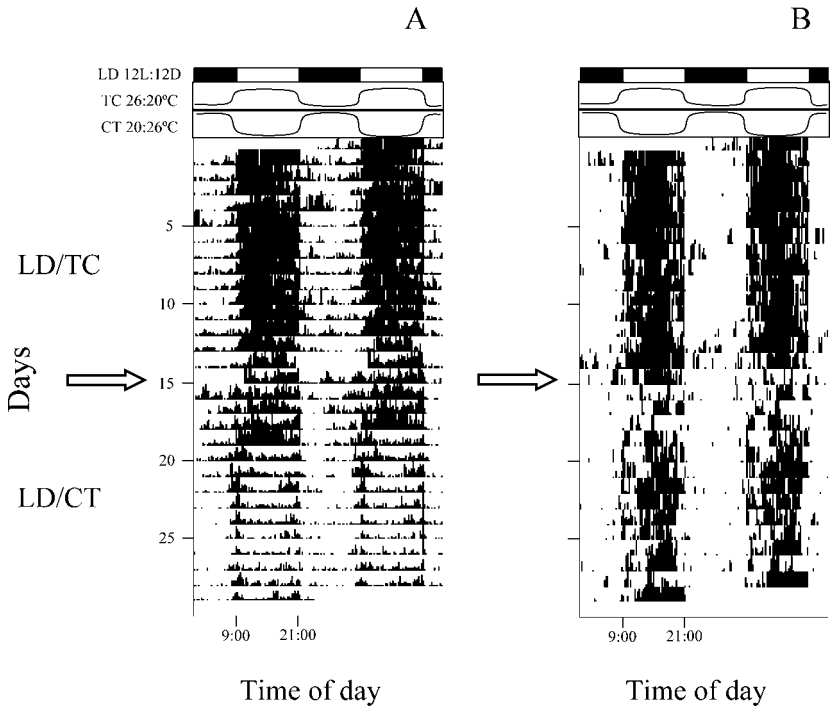


**FIGURE 3** Actograms of locomotor activity of two representative groups (*A* and *B*) of zebrafish maintained under a 12:12 LD cycle and a thermocycle of 26°C during the thermophase (*T*) and 20°C during the chryophase (*C*). The bars above each actogram represent the light regime; *open bars* and *black bars* represent the light and dark phases of the cycle, respectively. The curves above the actograms represent the temperature cycle. For other details, see Figure 1. At the beginning, the thermophase coincided with the light phase. After 15 d, the thermocycle was inversed and the thermophase coincided with the dark phase. The change in the position of the thermocycle is indicated by the arrows to the left of the actograms.

thermophase inversion changed and became more irregular and crepuscular than in the LD/TC phase (Figure 3A), although activity still coincided predominantly with the L phase (Figure 3B).

Total daily activity, measured as total number of interruptions of the infrared lightbeam, did not differ significantly ($6400 \pm 1900$ *vs.* $5400 \pm 1600$ counts/d, respectively; paired *t*-test, $p = 0.53$). When the photocycle and thermocycle were in phase, activity levels were closer to those observed under constant low temperature (20°C), as shown in Figure 2, although the average water temperature during the day was higher (23°C).

The amplitude of the rhythm was almost equal when comparing light and thermocycles in phase and antiphase ($82.8 \pm 5.5\%$ *vs.* $78.1 \pm 5.1\%$, respectively; paired *t*-test, $p = 0.12$), although activity profiles seemed to be less robust when both environmental cycles were in antiphase (Figure 3).

### Experiment 3. Entrainment to Thermocycles Under LL

When animals were held under constant dim light (3 lux), five of the six groups of zebrafish entrained to the daily thermocycle (T = 24 h), with 2 groups showing higher activity during the thermophase (Figure 4A), while 3 groups synchronized, surprisingly, to the chryophase (Figure 4B). The mean percentage of activity of these groups during the active phase (thermo- or chryophase) was 66.6 ± 2.5%. When the

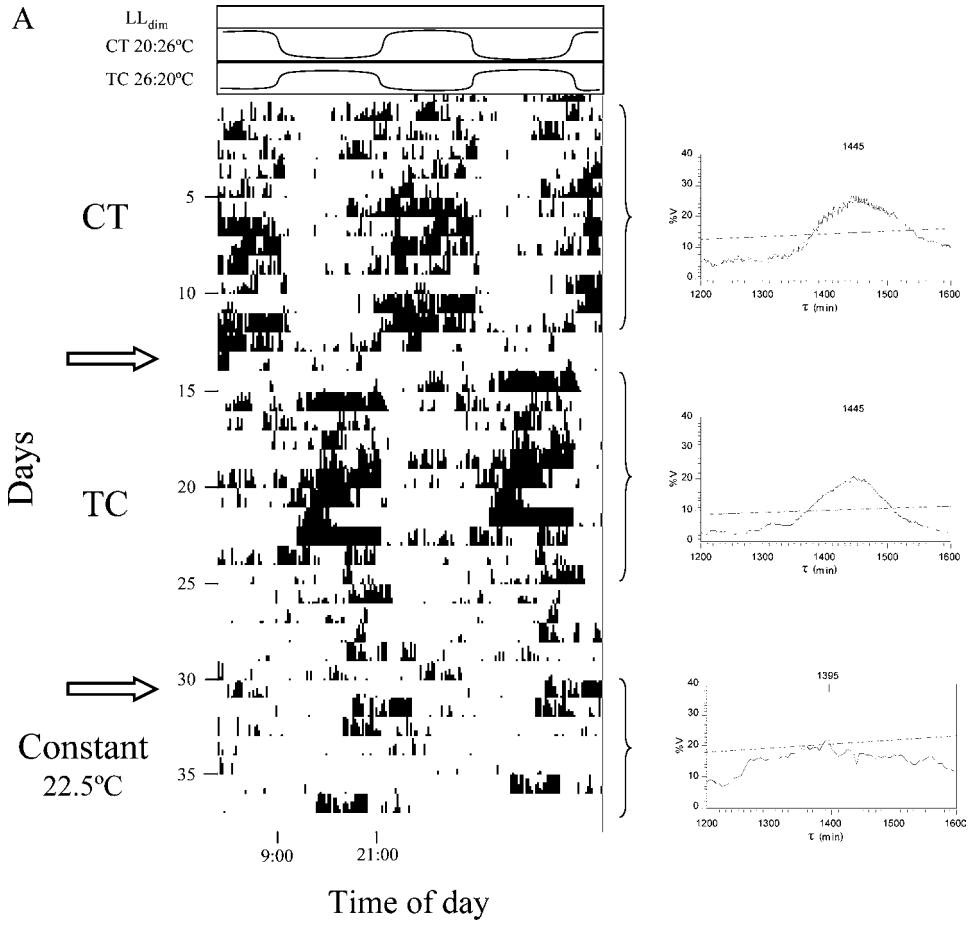


**FIGURE 4** Actograms of locomotor activity of two zebrafish groups maintained under constant dim light (3 lux) and a 26°C and 20°C thermocycle. Under these conditions, zebrafish entrained either to the thermophase (*A*) or chryophase (*B*). The curves above the actograms represent the temperature cycle. For other details, see Figure 1. After 15 d, the thermocycle was reversed (*arrow* to the left of the actogram). After another 15 d the temperature was kept constant at 22.5 ± 0.5°C. Chi-square periodogram analysis (confidence level, 95%) of the 3 parts of this experiment have been represented to the right of each actogram. The brackets indicate the interval of days taken for analysis. The period of each phase (mins) is indicated at the top of each periodogram.

(*continued*)

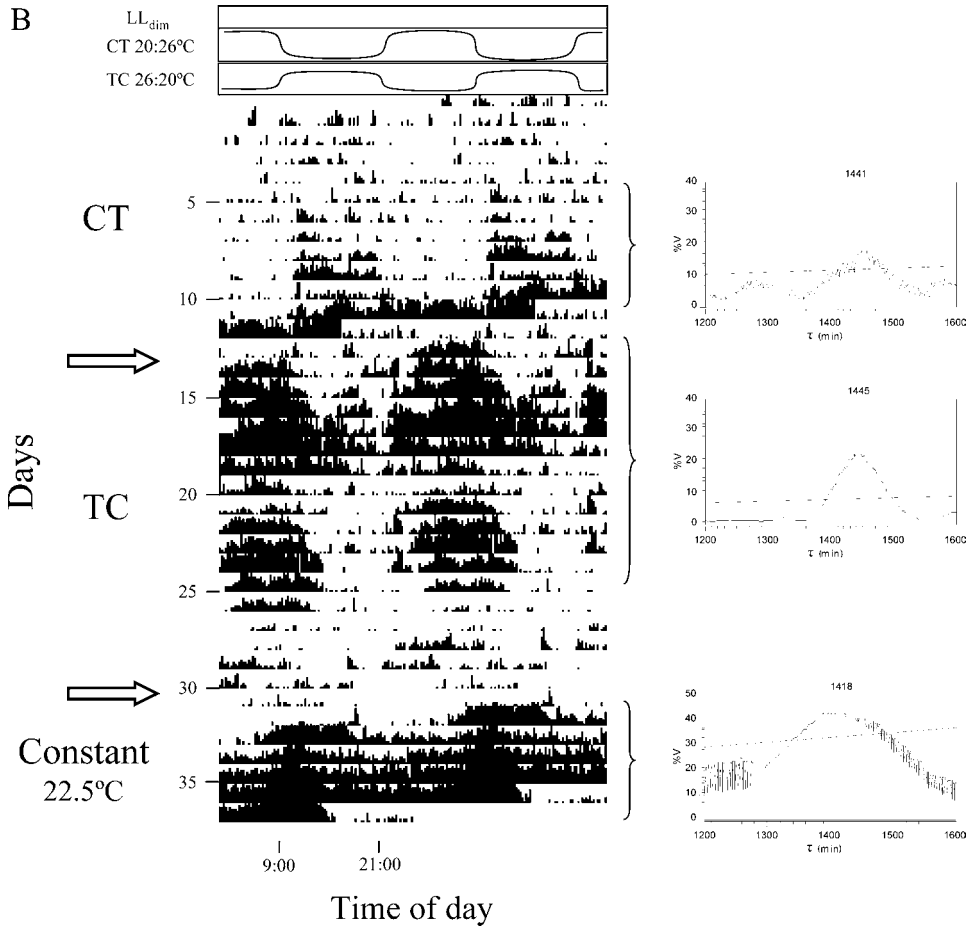


**FIGURE 4** Continued.

thermocycle was reversed, all groups (6 of 6) entrained their activity rhythms to the thermocycle, and the number of groups synchronizing their activity to the thermophase increased (4 of 6) (Table 1). Moreover, group 3, which was previously entrained to the chryophase, entrained to the thermophase. In addition, group 1 lost its rhythmicity during the first period of Experiment 3, but finally entrained to the thermophase.

The activity rhythms obtained from these data were not as clear as those obtained with the light cycle, and locomotor activity, although synchronized by the temperature rhythm (periodogram analysis, $\tau = 24\,\text{h}$), was more disperse, as can be observed in the actograms of Figure 4.

When the thermocycle was eliminated, and the zebrafish were kept at a constant temperature of 22.5°C, 4 of 6 groups showed significant free-running rhythms (Table 1), with an average period ($\tau$) of $23.3 \pm 0.2\,\text{h}$.

**TABLE 1** Periods of Locomotor Activity Rhythms of the 6 Zebrafish Groups in Experiment 3

| | Group | | | | | |
|---|---|---|---|---|---|---|
| Temperature cycle | 1 | 2 | 3 | 4 | 5 | 6 |
| TC (26:20°C) | n.s. | 24T | 24C | 24C | 24.1T | 24C |
| CT (20:26°C) | 24T | 23.9T | 24T | 24C | 24.1T | 24C |
| Constant (22.5°C) | n.s. | 22.9 | 23.6 | 23.6 | 23.25 | n.s. |

Each column corresponds to a single group. TC: thermocycle of 26:20°C; CT: inversion of the previous thermocycle (20 and 26°C); Constant: constant temperature of 22.5°C. Periods were calculated using the chi-square periodogram (software, *El Temps*) with a confidence level of 95% indicated by hours. The letter next to the period indicates the phase to which zebrafish synchronized (showing a percentage of activity of 65% or higher); thermophase (*T*) or chryophase (*C*). n.s. = not significant.

## DISCUSSION

In the present study, zebrafish entrained their circadian rhythms to both light and thermocycles, displaying most of their activity during the daytime regardless of the phase of the temperature cycles. In constant dim light, most zebrafish entrained to the 24 h thermocycles, showing increased activity during the thermophase, although some entrained to the chryophase instead. Under dim LL and constant temperature, zebrafish displayed free-running rhythms with an average $\tau$ of 23.3 h. These observations provide first insights into the influence of daily light- and temperature-related changes on locomotor activity rhythms, and revealing the ability of thermocycles to entrain circadian activity rhythms in fish.

The ability of temperature cycles to act as a zeitgeber for the vertebrate circadian clock has been studied mainly in mammals and reptiles (reviewed in Rensing and Ruoff, 2002). Temperature changes can directly affect the clock mechanism by accelerating or slowing component processes in cells (Dunlap, 1999; Rensing and Ruoff, 2002). Among ectotherms, reptiles have been the main subjects of research that examined the influence of temperature on circadian rhythmicity. In recent years, these studies on the effects of thermocycles have focused on rhythmic melatonin production, both *in vitro* (Barrett and Takahashi, 1995; Moyer et al., 1997; Valenciano et al., 1997; Zachmann et al., 1991) and *in vivo* (Firth et al., 1999; García-Allegue et al., 2001; Masuda et al., 2003; Wright and Bruni, 2004), and more recently on the molecular clock, itself (Lahiri et al., 2005; Liu et al., 1998; Sidote et al., 1998).

Zebrafish showed a marked diurnal activity. Fish entrained to the LD cycle displayed more than 75% of their daily activity during the light time (Figures 1 and 2). This finding is in agreement with those of previous studies by Hurd and colleagues, (1998), in which a strong confinement of zebrafish activity to the light phase was observed. This diurnal pattern appears already in the early stages of larval zebrafish (Cahill et al.,

1998). Moreover, locomotor activity rhythms in larvae are even more robust than those observed in adults. In the present experiments, constant water temperature influenced the total daily activity of zebrafish; activity at 26°C was about three fold higher than activity at 20°C (Figure 2). Thus, the amplitude of the locomotor activity rhythm showed a direct response to water temperature, as has been observed in other rhythms in fish, such as melatonin production (García-Allegue et al., 2001; Iigo and Aida, 1995; Masuda et al., 2003; Samejima et al., 2000), under a range of temperatures similar to those used in this experiment. In contrast light, the percentage of diurnal activity was higher at 20°C. This has been observed also in previous reports (Hurd et al., 1998), which showed that circadian rhythms of zebrafish under constant lighting and temperature conditions are strongest and best defined around 21°C, although most experiments with zebrafish have been performed at a higher water temperature of 26°C to 28°C.

An early study by Evans (1966) showed that lizard activity patterns, when light and thermo-cycles were out of phase, entrained to the thermocycle in the species *Uta stansburiana*, while *Coleonyx variegates* did not show a significant rhythm. Sea bass is a marine fish that shows nocturnal feeding rhythms during winter and diurnal feeding during summer. In a previous study, sea bass feeding activity entrained strictly to the lighting conditions when the photoperiod simulated was similar to that of summer (16L:8D), and water temperature was similar to that of winter (Aranda et al., 1999). In the present study, zebrafish showed a marked diurnal activity when the photophase and the thermophase were in phase (Figure 3). When the two environmental cycles were in antiphase (conflicting zeitgeber), the fish predominantly entrained to the light phase regardless of the phase of the thermocycle (Figure 3). The percentage of diurnal activity was similar in both cases, and the number of groups entraining to light was only slightly higher when light and thermocycles were in phase (6 *vs.* 5 of 6). Thus, light of the applied intensity (lux) is a stronger synchronizer than is a temperature change of 6°C. The relative importance of temperature as a zeitgeber in ectotherms, as was first stated by Hoffmann (1969), seems to depend on species-specific and intraspecific (season, age, and development) variables, since in the studies carried out in the meantime, a variety of responses to light and temperature (when given as conflicting zeitgeber) have been observed. Interestingly, in the two fish species investigated, sea bass (Aranda et al., 1999) and zebrafish (Figure 3), light (700 lux) was a stronger synchronizer than was the temperature change of 6°C.

In the absence of an LD cycle, under constant dim light of 3 lux, zebrafish entrained their circadian activity patterns to thermocycles (Figure 4). However, the rhythms were not as prominent as those obtained with LD cycles and constant temperature (Figure 1), as the percentage of activity in the light phase in Experiment 1 (77% to 87%) was significantly higher

than that observed during the active phase of the thermocycle (65% to 70%) ($p < 0.001$, *t*-test) when thermocycles were the only time cue. These results show that temperature can act as a synchronizer in zebrafish to entrain its circadian activity rhythms, although it is a weaker synchronizer than light at the given amplitudes of the zeitgeber cycles. Similar conclusions have been obtained by other authors: although thermocycles, in the absence of light cycles, can entrain circadian rhythms in several species, these rhythms are usually less marked than those obtained with light cycles (Barrett and Takahashi, 1995; Firth et al., 1999; Moyer et al., 1997; Wright and Bruni, 2004; Yoshi et al., 2002; Zachmann et al., 1991).

It should be noted that 3 groups maintained the previous phase of the rhythm when constant dim light was given (Experiment 3), while 2 groups resynchronized their circadian rhythm of activity to the thermophase (groups 2 and 5). This finding may indicate that the previous light entrainment has initially "phase locked" the activity rhythms and then the thermocycle resynchronized the rhythms afterward. In the second part of the experiment, 2 additional groups locked to the thermophase, one of them (group 3) shifting from the chryophase to the thermophase (Table 1). In a recent study by Lahiri and coworkers (2005), thermocycles were observed to effectively entrain clock gene expression in the absence of light information. Furthermore, the daily profiles of gene expression were similar to those observed under an LD cycle, with the thermophase expression levels being similar to that of the light phase, and expression levels during the chryophase similar to that in the dark phase.

In constant conditions, zebrafish showed significant free-running rhythms, which indicates that circadian locomotor activity was entrained to the thermocycles and was not a result of masking (Table 1). The free-running of temperature-entrained rhythms showed an average $\tau$ of 23.3 h, similar to the free-running period (shorter than 24 h) observed in zebrafish under LL conditions (Hurd et al., 1998).

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