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FEEDING ENTRAINMENT OF FOOD-ANTICIPATORY ACTIVITY AND *per1* EXPRESSION IN THE BRAIN AND LIVER OF ZEBRAFISH UNDER DIFFERENT LIGHTING AND FEEDING CONDITIONS

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Food provided on a periodic basis can act as a potent synchronizer, being a stronger zeitgeber than light for peripheral oscillators in mammals. In fish, however, little is known about the influence of feeding time on the circadian pacemaker and the relationship between central and peripheral oscillators. The aim of this research was to investigate the influence of mealtime on the activity rhythms, and on central (brain) and peripheral (liver) oscillators in zebrafish. The authors tested different feeding times under a light-dark (LD) cycle and the endogenous origin of food-anticipatory activity (FAA) by feeding zebrafish at a fixed time under constant bright-light conditions (LL). The authors then measured locomotor activity and the expression of the clock gene *per1* in animals under a LD cycle and fed at random times during the light phase, with restricted feeding at the mid-light phase (ML) or with restricted feeding during the mid-dark phase (MD). Finally, the authors measured locomotor activity and *per1* expression in fish maintained under LL under either random feeding or scheduled feeding. Zebrafish displayed FAA in all the groups fed at a fixed time but not when feeding was randomly scheduled. Under LL, fish entrainment persisted, and when released under fasting conditions FAA free-run with a circa-24-h period. The expression of *per1* in the brain of fish under LD showed a daily rhythm with the acrophase (peak time) at the end of the dark phase regardless of feeding schedule. This brain rhythm disappeared in LL fish under both random feeding and scheduled feeding. Feeding at MD advanced the phase of *per1* in the liver by 7 h compared with the ML-fed group phase (23:54 versus 07:23 h, respectively). In addition, under LL scheduled feeding entrained the rhythms of *per1* expression in the liver. This study reveals for the first time that scheduled feeding entrains peripheral oscillators in a fish species, zebrafish, which is a powerful model widely used for molecular genetics and for the study of basic clock mechanisms of the vertebrate circadian system. (Author correspondence: jflopez@um.es)

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Keywords Central and peripheral oscillators; Clock gene expression; Food-anticipatory activity; Locomotor activity; Zebrafish

INTRODUCTION

When food is periodically restricted, feeding can act as a potent synchronizer of circadian rhythms in vertebrates, eliciting an increase in locomotor activity several hours before mealtime, which is known as food-anticipatory activity (FAA) (Mistlberger, 2009). FAA is driven by a self-sustained oscillator, namely a food-entrainable oscillator (FEO). In mammals, this circadian oscillator is located outside the hypothalamic suprachiasmatic nucleus (SCN), the site of a master circadian light-entrainable oscillator (LEO) (Duguay & Cermakian; 2009; Stephan, 2002).

Circadian rhythms of gene expression are present in several mammalian peripheral tissues, and such rhythms are sustained by autonomous peripheral oscillators (Damiola et al., 2000; Yamazaki et al., 2000; Yoo et al., 2004). Among these, the liver has been the focus of several studies due to its importance in food processing and metabolism and its ability to entrain to restricted food access in a light-independent manner (Damiola et al., 2000; Stokkan et al., 2001). Many studies have suggested that the FEO may not necessarily be located in a single anatomical structure and that multiple FEOs could be present in mammals (Feillet et al., 2006; Stephan, 2002). A recently proposed model suggested a network of interconnected brain structures, entrained by humoral signals derived from the periodic feeding, and which would control the food-entrained overt rhythms (Carneiro & Araujo, 2009).

In fish, the existence of a FEO and FAA has been demonstrated in behavioral studies, with few studies on the feeding anticipation of other variables, such as enzymatic activity and hormones, involved in the control of food intake and stress response (López-Olmeda & Sánchez-Vázquez, 2010). The FEO has been proven to be of endogenous nature in some fish species, such as goldfish and tench, which display FAA in the absence of an external cue other than food, and this rhythm free-runs under constant conditions (Herrero et al., 2005; Sánchez-Vázquez et al., 1997).

The zebrafish has become one of the most important vertebrate models in genetic and developmental studies across various fields, including chronobiology. The zebrafish molecular circadian clockwork has been partially characterized (Cahill, 2002; Pando & Sassone-Corsi, 2002). Although further research in this field is required, as in the better-understood mammalian clock, new components and pathways are being described frequently (Lamia et al., 2009; Robles et al., 2010). Furthermore, seasonal variations in clock gene expression have recently

been reported in a fish species, the Atlantic salmon (Davie et al., 2009). Tissue explants from transgenic zebrafish that express the luciferase protein coupled to a promoter of a clock gene have revealed the existence of molecular oscillators in peripheral tissues (Kaneko et al., 2006); these clock gene oscillations are present in cultured cells and can be entrained directly by the light-dark (LD) cycle (Farhat et al., 2009; Pando et al., 2001; Whitmore et al., 2000). Despite the well-characterized circadian molecular machinery in zebrafish peripheral oscillators, in vivo studies that address the relationship between central and peripheral oscillators are still scarce (Dickmeis et al., 2007). A recent study focused on the effects of scheduled feeding on brain oscillators in zebrafish, showing that light, but not feeding, entrains the daily rhythms of expression of *per1* and *cry1* (Sánchez & Sánchez-Vázquez, 2009). However, feeding entrainment of peripheral oscillators and its endogenous nature remains unexplored.

The aim of the present study was to assess the influence of restricted feeding (as opposed to random feeding throughout the day) on zebrafish locomotor activity and on the expression of the clock gene *per1* in central (brain) and peripheral oscillators (liver) under a LD cycle and continuous bright light (LL).

MATERIALS AND METHODS

Animals and Housing

Adult zebrafish (*Danio rerio*) were reared at the facilities of the University of Murcia. Fish were kept in well-aerated 60-L aquaria equipped with mechanical and biological filters. Light was provided by fluorescent bulbs (F15W/GRO; Sylvania Gro-Lux, Germany) placed 20 cm over the water surface, where light intensity was 400 lux. Water temperature was maintained at 25°C by means of a water heater (100 W; Askoll) located in each aquarium. **Q1**

Experimental Design

The experiments were designed to investigate the influence of restricted food access and mealtime on zebrafish behavior and the expression of *per1* clock gene on both the central (brain) and peripheral (liver) oscillators. Fish were reared and manipulated following the Spanish legislation on Animal Welfare and Laboratory Practices. The experiments were conducted ethically and fulfilled the standards required by the journal (Portaluppi et al., 2008).

In all experiments, fish were fed with a standard commercial diet (Nutron Hi-Fi; Prodac, Italy), which was provided by means of automatic

feeders (Eheim GmbH & Co. KG, Germany). The feeders for randomly fed fish were coupled to programmable timers (Data Micro; Orbis, Spain), allowing adjustment of random feeding times that were programmed weekly for each day of the week.

Experiment 1: Endogenous Origin of Food-Anticipatory Activity in Zebrafish

In the first experiment, 10 aquaria of zebrafish (20 fish/aquarium) were maintained under a 12:12-h LD cycle, with lights on at 08:00 h, and fed once a day at a fixed hour, in the middle of the light phase (ML), at 14:00 h. When fish were synchronized to feeding time and displayed FAA under these conditions, they were transferred to LL (400 lux), and the feeding schedule was maintained at the previous mealtime (14:00 h) for 12 days. LL conditions were selected over constant darkness (DD) because fish were previously feeding at the light phase of the LD cycle. Finally, to test the free-running nature of the locomotor activity rhythm and to confirm entrainment to the scheduled feeding, animals were food-deprived for 15 days. Throughout this experiment, locomotor activity was registered continuously by means of an infrared photocell (model E3S-AD62; Omron, Japan) placed at the aquarium wall 22 cm from the bottom and 10 cm from the water surface, in the corner where food was provided. The number of light-beam interruptions was counted and stored every 10 min by a computer connected to the photocell.

Experiment 2: Random Versus Scheduled Feeding Under LD

Zebrafish were maintained under a 12:12-h LD cycle, with lights on at 08:00 h. Three experimental groups were designated: (i) RF group, fish fed once a day at a random time within the light phase (between 08:00 h and 19:00 h); (ii) SL group, fish fed at a fixed time in the middle of the light phase (14:00 h); and (iii) SD group, fish fed at a fixed time in the middle of the dark phase (02:00 h). Zebrafish were divided into two aquaria/group (a total of six aquaria being used in this experiment), and 40 fish were placed in each aquarium. Locomotor activity was registered continuously during the experimental period, as described for Experiment 1.

After 28 days under the feeding regimes, fish from the three groups were sacrificed by decapitation every 3 h during a complete 24-h cycle, collecting four replicates/sampling point and group. Fish were food-deprived during the sampling day to avoid possible food stimulation of clock gene expression. At every sampling point, eight fish were removed from an aquarium of each group, pooling the head and liver of two replicates ($n = 4$). As two aquaria were used per group, sampling was

performed alternately after 6 h. After decapitation, tissues were collected in a Petri dish on ice under sterile conditions. Heads were enucleated (the eyes and part of the optic nerves were removed) to avoid contamination of clock gene expression in the retina, and the jaw was removed so the tissue collected was constituted mainly by the brain, surrounded by part of the skull and skin. This extract was dominated by brain mRNA; therefore, the results obtained are likely to represent the *per1* expression in the whole brain. The liver and the head were placed each in 0.5 ml of Trizol solution (Invitrogen, Carlsbad, CA, USA) and stored at −80°C for posterior analyses of *per1* expression. Fish manipulation and tissue collection during the dark phase were performed under a dim red light.

Experiment 3: Random Versus Scheduled Feeding Under LL

In the third experiment, zebrafish were maintained under LL conditions. Two experimental groups were designed: (i) RF group, fish fed once a day at a random clock time (between 00:00 and 23:00 h); and (ii) SF group, fish fed once a day at a fixed time (14:00 h). Zebrafish were divided into one aquarium/group, with 40 fish being placed in each aquarium. Locomotor activity was registered continuously during the experimental period, as described for Experiment 1. Fish were maintained under these conditions for 28 days. At the end of this period, samples from head and liver were collected every 6 h during a complete 24-h cycle (n = 4; 2 pooled fish/n), as described for Experiment 2.

Real-Time Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) Analysis

Samples of head and liver were mechanically homogenized. RNA extraction with Trizol was performed according to manufacturer's instructions (Invitrogen). The RNA pellet was dissolved in sterile TE buffer prepared with diethylpyrocarbonate (DEPC)-treated water. In the next step, total RNA (1 μg) was pretreated with DNase I (1 unit/μg RNA) (Applied Biosystems, Foster City, CA, USA) and reverse-transcribed into cDNA using the High Capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. The cDNA was subjected to quantitative PCR analyses using a light thermocycler (MJ Research Chromo4; Bio-Rad, Hercules, CA, USA), following the next protocol: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. Quantitative PCR reactions were performed using the TaqMan Gene Expression Assay (Applied Biosystems), with custom TaqMan probes labeled with 6-carboxyfluorescein (FAM). The final volume of the PCR reaction was 20 μl: 9 μl of cDNA, 10 μl of the TaqMan Master Mix, and 1 μl of the Assay Mix (primers and probe), with each primer at a final

concentration of 900 nM and the TaqMan probe at 250 nM. Values of *per1* expression were calculated as relative expression by the $\Delta\Delta C_T$ method, using *β-actin* as the endogenous reference. Relative expression calculations were performed using the Opticon Monitor 3 software (Bio-Rad). All samples were run in triplicate. The primers used were as follows: 5′-TGAACCCCAAGGCCAACAG-3′ and 5′-GCCTGGATGGCAACGTACAT-3′ for *per1*, and 5′-GAAAAGGCTCAGCCACAGAGA-3′ and 5′-CGCTCAAAA GACTGAATGACACTGA-3′ for *β-actin*. The probes were designed to cross an exon-exon boundary. Probes were labeled with FAM, and their sequences were as follows: 5′-ACATGATCTGTGTCATCTTT-3′ for *per1*, and 5′-CATTTGAGCTCTTGCTTTC-3′ for *β-actin*. Primers and probes were designed by means of the software Custom TaqMan Assay Design Tool, available on the Web page of Applied Biosystems. The relative amplification efficiencies of *per1* and *β-actin* in both tissues were verified to be approximately equal; therefore, the data were analyzed by the $\Delta\Delta C_T$ method.

Data Analysis

Analysis of locomotor activity records, representation of actograms and waveforms, and Cosinor and periodogram analyses were performed using the chronobiology software El Temps (version 1, 228; Prof. Díez-Noguera, University of Barcelona; www.el-temps.com). **Q3**

Data of *per1* expression were subjected to Cosinor analysis for each group and treatment. Cosinor analysis is based in the least squares approximation of time series data with a cosine function of known period of the type $Y = \text{Mesor} + \text{amplitude} \times \cos\ [2\pi(t - \text{acrophase})/\text{period}]$, where Mesor is the time series mean; amplitude is a measure of the amount of temporal variability explained by the rhythm; period (τ) is the cycle length of the rhythm, i.e., 24 h for circadian rhythms; t is the time of day; and acrophase is the time of the peak value relative to the designated time scale. Cosinor analysis also provides the statistical significance of the rhythm through an *F*-test of the variance accounted for by the waveform versus a straight line of zero-amplitude (null hypothesis). Therefore, if under a statistical significance of $p < .05$ the null hypothesis was rejected, the amplitude could be considered as differing from 0, thereby constituting evidence for the existence of a statistically significant rhythm of the given period under consideration.

Data of locomotor activity from Experiment 1 were subjected to Sokolove-Bushell periodogram analysis to determine the period of the locomotor activity rhythm in each of the three experimental phases. The periodogram analysis relies on the chi-square distribution to distinguish stochastic oscillations from true rhythms, providing Q_P value for a period (P) that has a probability distribution of chi-square with $P - 1$ degrees of

freedom (Refinetti, 2004). Q_P indicates the percentage of variance of the rhythm explained by the period. The level of significance was set at $p < .05$.

Data of *per1* relative expression were transformed to logarithm for graphic representations and were subjected to a two-way analysis of variance (ANOVA), followed by a Tukey's post hoc test, to check for significant differences between groups and the time-of-day. Statistical analyses were performed using SPSS software. The significant threshold (α) was set at .05 in all statistical test performed.

RESULTS

Experiment 1: Endogenous Origin of Food-Anticipatory Activity in Zebrafish

Zebrafish displayed diurnal activity and FAA under an LD cycle when feeding was restricted to a fixed time of the day (ML) (Figure 1), with 36.1% ± 6.4% (mean ± SD) of the total daily activity taking place within the 4 h before feeding. After release into LL, FAA persisted, but the daily activity patterns became more diffuse than under LD conditions, with FAA accounting for 28.2% ± 2% of the total daily activity within the 4 h before feeding. The period (τ) of activity rhythms under a fixed feeding time was 24 h in all groups, both under LD and LL. Finally, when food was suppressed, FAA displayed significant free-running rhythms in 6 of the 10 groups (chi-square periodogram, confidence level of 95%) (Table 1, Figure 1). The endogenous τ of the groups that showed free-running rhythms was heterogeneous, with three groups displaying values <24 h and three groups displaying values >24 h (Table 1). The average (±SD) of the significant τ values was 23.9 ± 1.2 h.

Experiment 2: Random Versus Scheduled Feeding Under LD

Zebrafish displayed different patterns of locomotor activity depending on the feeding regime. Under LD, RF fish showed diurnal behavior, with most of their activity (81.7% ± 15.3% of the daily total) being displayed within the light phase (Figure 2A). Whereas SL fish displayed 77.9% ± 10.6% of their total daily activity within the light phase (Figure 2B), SD fish displayed 62.3% ± 10.1% of their daily activity within the dark phase (Figure 2C). Thus, fish displayed most of their daily activity within the phase in which food was provided, leading to a nocturnal activity pattern in fish with restricted feeding during the dark phase.

FAA was evident when fish were periodically fed during the light phase (Figure 2B) or during the dark phase (Figure 2C). However, the FAA profile differed depending on the mealtime. Under LD, the FAA of

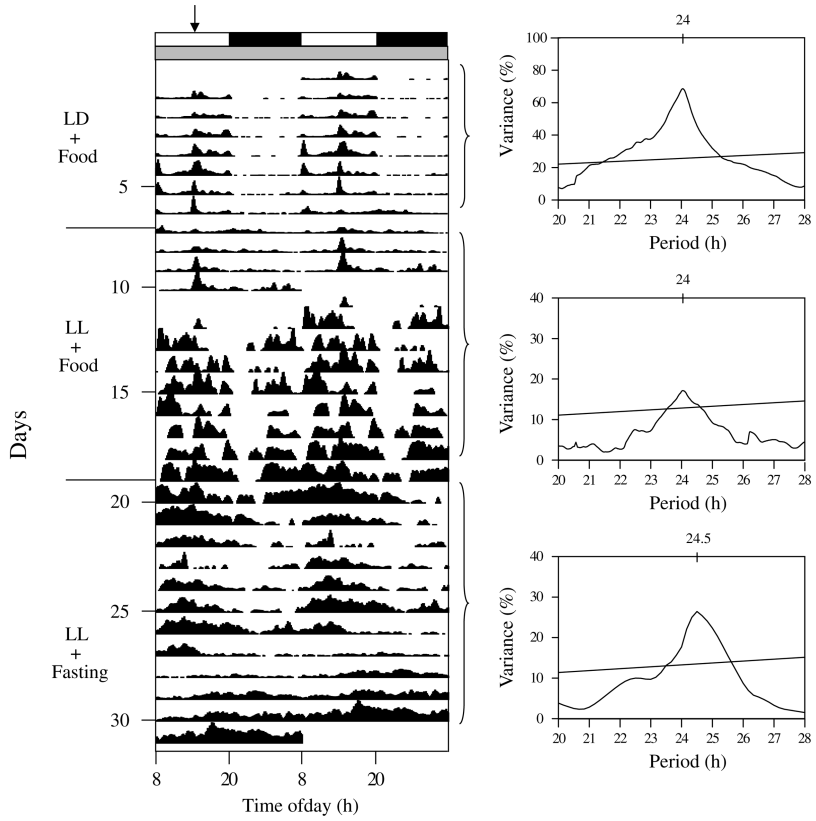

FIGURE 1 A circadian rhythm of food-anticipatory activity in zebrafish. A representative actogram of a group of zebrafish is represented (A). Fish were maintained under an LD cycle and scheduled feeding (LD + Food), then fish were transferred to LL and scheduled feeding was maintained (LL + Food), and they were finally kept under LL and deprived of food (LL + Fasting). Mealtime in the first two stages of the experiment is indicated by the arrow at the top of the actogram. For convenient visualization, the data have been double plotted (48 h); the y-axis progresses in single days, with each day being plotted twice (day 1 on the right side is repeated on day 2 on the left side). The activity was binned every 10 min, the height of each point representing the number of interruptions of the infrared light beam. The bars above each actogram represent the light regime; open and black bars represent light and dark, respectively, during the LD stage of the experiment; the grey bar indicates the continuous lighting conditions during the LL stages. Chi-square periodogram analysis (confidence level, 95%) for each stage of the experiment shown in the actogram are also shown (B). The periodogram indicates the percentage of variance of the rhythm explained by each analyzed period within a range of 20 to 28 h. The highest percentage is associated with the real value of the period (τ). Brackets indicate the days included in each periodogram, and the significant τ (in h) is indicated at the top of each plot. The horizontal line represents the threshold of significance, set at $p = .05$.

TABLE 1 Free-running circadian period of FAA under LL and fasting conditions in 10 groups of zebrafish

	1	2	3	4	5	6	7	8	9	10
Tau (h)	24.5*	26.3	28.5	23*	24.6*	22.3*	23.5*	28.3	25.5*	27.2

*Statistically significant periods (95% confidence level, chi-square periodogram).

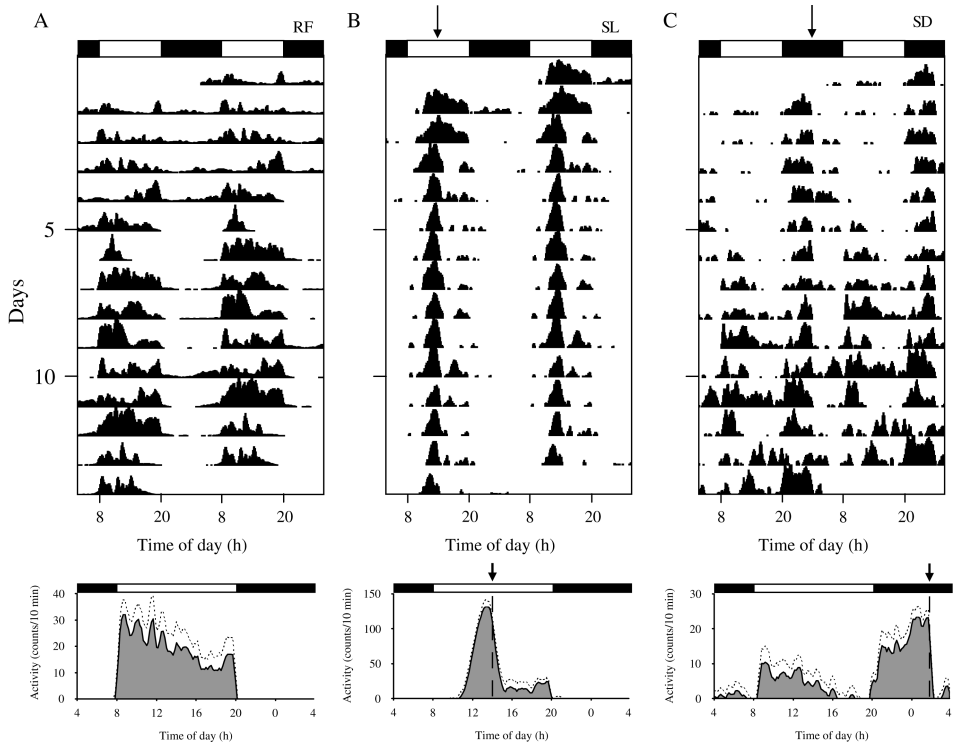

FIGURE 2 Food-anticipatory activity in zebrafish is present when feeding is scheduled during the day or during the night. Top: Representative actograms for groups of fish, maintained under a LD cycle, that were random fed (RF) (A), scheduled fed at 14:00 h (mid-light) (SL) (B), or scheduled fed at 02:00 h (mid-dark) (SD) (C). Mealtime of scheduled-fed groups is represented by the arrow at the top of the actogram. For convenient visualization, the data have been double plotted (48 h); the y-axis progresses in single days, with each day being plotted twice (day 1 on the right side is repeated on day 2 on the left side). The activity was binned every 10 min, the height of each point representing the number of interruptions of the infrared light beam. The bars above each actogram represent the light regime; open and black bars represent light and dark phases, respectively. Bottom: Mean waveforms of locomotor activity for each actogram. Each point has been calculated as the mean ± SD from 10-min binned data across all the experimental days shown on each actogram. The continuous line and grey area indicate the mean light-beam interruptions, and the dotted line indicates the SD. Bars above mean waveforms represent light (open bars) and dark (black bars) phases of the LD cycle. Mealtime in scheduled-fed groups is represented by an arrow and a dashed line.

SL fish was evident as a sharp increase in the locomotor activity concentrated within the few hours immediately preceding mealtime, with fish displaying 39.8% ± 3.6% of their total daily activity occurring within the 4 h before feeding. In contrast, the FAA in SD fish was present several hours before mealtime, with 29.8% ± 3.3% of the total daily activity within the 4 h before feeding. In addition, SD fish displayed a splitting of their locomotor activity into two components: a night component synchronized to the feeding time and a day component synchronized to the light phase of the LD cycle (Figure 2C). Activity in SD fish decreased immediately after mealtime, whereas it decreased more gradually in SL

fish, with 2.8% ± 0.4% and 10.6% ± 1.1% of the total daily activity displayed during the hour after feeding, respectively, for each group.

Daily expression of *per1* in the brain displayed similar variations in the three experimental groups (Figure 3). Statistically significant differences were found between the times-of-day (two-way ANOVA, $p < .05$), but neither the group nor the interaction between time and groups yielded significant differences (two-way ANOVA, $p > .05$). When *per1* values were analyzed within each group, low expression levels were observed during the light phase, increasing during the transition from light to dark, and showing the highest levels towards the end of the dark phase (Figure 3). A significant daily rhythm was observed in all groups, with the acrophases located at the end of the dark phase, between 04:51 and 05:18 h (Cosinor, $p < .05$) (Table 2).

Analysis of *per1* expression in the liver revealed differences that depended on the feeding regime to which the fish were subjected (Figure 4). Statistically significant differences were found across time-of-day and in the interaction between the group and the time-of-day (two-way ANOVA, $p < .05$), but not between groups (two-way ANOVA, $p > .05$). In every group, a significant daily rhythm was observed, but the acrophases differed among treatments (Table 2). Under LD, the rhythm in both RF and SL showed the acrophase at the end of the dark phase (06:31 and 07:23 h, respectively). In contrast, the phase of *per1* expression in the liver of animals fed at MD (SD group) was displaced to 23:54 h, approximately 7 h prior to the acrophase of each of the daytime-fed groups.

Experiment 3: Random Versus Scheduled Feeding Under LL

Locomotor activity of zebrafish fed once a day at a random time under LL (RF) became arrhythmic, with no clear pattern being observed during the 24 h (Figure 5A). Because this rhythm is drawn from several individuals, it cannot be determined whether arrhythmicity emerges from a sample of animals, each one of which is arrhythmic, or from a sample of rhythmic animals, each with a different phase. In contrast, fish fed at a fixed time (SF) displayed FAA (Figure 5B). In this case, 27.9% ± 7.8% of total daily activity was displayed in the 4 h prior to feeding time (Figure 5B).

Expression of *per1* in the brain remained constant throughout the 24-h cycle (Figure 6A) (two-way ANOVA, $p > .05$ for group, time, and the interaction between factors), with no daily rhythm being observed in any of the groups (Cosinor, $p > .05$) (Table 2). Constant levels of *per1* expression were also observed in the liver of randomly fed fish (Figure 6B, Table 2) (Cosinor, $p > .05$). In contrast, a circadian rhythm of *per1* expression was observed in the liver of fish fed once a day at a fixed

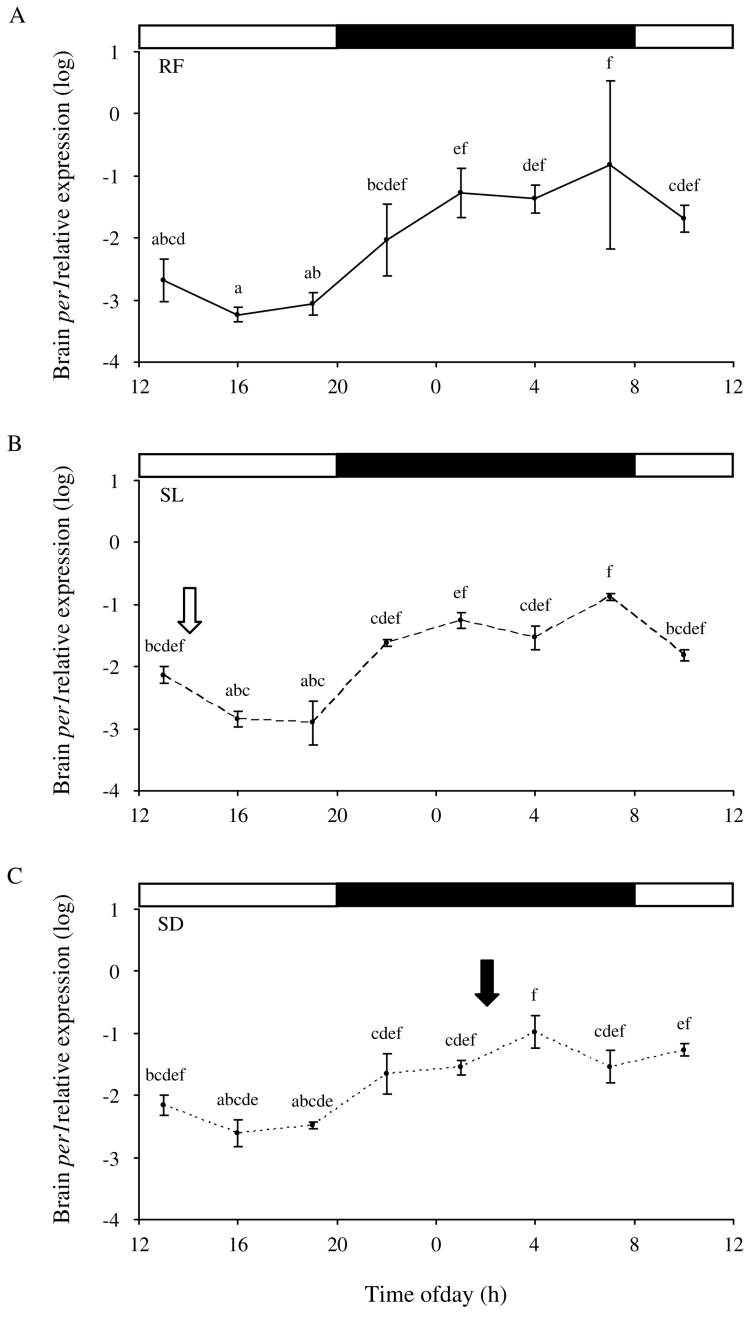

FIGURE 3 *per1* expression oscillates in the brain of zebrafish irrespective of feeding schedule. Relative mRNA levels in the brain of fish maintained at LD and random feeding (RF), continuous line (A); scheduled fed at 14:00 h (mid-light) (SL), dashed line (B); and scheduled fed at 02:00 h (mid-dark) (SD), dotted line (C). Data (mean ± SEM) were calculated as the logarithm of the relative *per1* expression using *β-actin* as the housekeeping gene (n = 4 for each point). Data were analyzed by two-way ANOVA ($p < .05$), followed by a Tukey's post hoc test. Different letters indicate significant differences between groups. The white and black arrows indicate the time of feeding for SL and SD groups, respectively.

TABLE 2 Parameters of the cosine function calculated by means of Cosinor for data of *per1* expression in brain and liver of fish maintained at LD or LL

	LD						LL			
	Brain			Liver			Brain		Liver	
	RF	SL	SD	RF	SL	SD	RF	SF	RF	SF
Mesor	−2.060	−1.967	−1.758	−1.920	−1.674	−1.844	−1.713	−1.678	−1.687	−1.112
Amplitude	1.108	0.850	0.703	1.156	0.867	0.679	0.125	0.217	0.168	1.017
Acrophase (hh:mm)	4:51	5:18	4:54	6:31	7:23	23:54	11:53	11:04	1:14	6:55
Significance	*	*	*	*	*	*	n. s.	n. s.	n. s.	*
Variance (%)	50.9	78.5	58.3	73.0	48.8	45.8	9.7	23.4	21.9	90.8

Note. LD fish were fed randomly during the light phase (RF), fed once a day at mid-light (SL), or fed once a day at mid-dark (SD). Fish maintained in LL were either fed randomly (RF) or fed once a day at a fixed clock time (SF). Mesor and amplitude are given as relative expression values; the acrophase is given as time-of-day relative to local midnight; significance of the rhythm was calculated through *F*-test of the variance accounted for by the waveform versus a straight line of zero-amplitude. The percentage of variance indicates the percentage of experimental data explained by the cosine equation calculated by the Cosinor method.

*$p < .05$; n.s. = nonsignificant.

time, with the acrophase located at 06:55 h (Figure 6B, Table 2) (Cosinor, $p < .05$). A two-way ANOVA of liver *per1* expression yielded statistically significant differences between groups (RF versus SF) and across time-of-day, as well as a significant interaction (two-way ANOVA, $p < .05$).

DISCUSSION

In the present paper, we showed that restricted food access can entrain a circadian rhythm of FAA in zebrafish. This rhythm is present regardless of whether the food is restricted to daytime, the usual feeding time for zebrafish, or nighttime. Upon release of fish into constant conditions (LL), FAA persisted with a phase predicted by the time of food availability. Importantly, whereas the time of feeding synchronized the circadian profile of *per1* expression in the liver, it was not associated with phase changes in the rhythm of *per1* expression in the brain, even when food was periodically restricted in the absence of any periodic light input.

Circadian rhythms of FAA has been observed in a wide variety of vertebrate and invertebrate species (Stephan, 2002), conferring adaptive value by allowing animals to anticipate the forthcoming meal. FAA has previously been described in several fish species, including goldfish, European sea bass, golden shiner, tench, and zebrafish (Azzaydi et al., 2007; Herrero et al., 2005; Reebs & Lague, 2000; Sánchez & Sánchez-Vázquez, 2009; Sánchez-Vázquez & Madrid, 2001). In fish, FAA persisted under restricted feeding and constant conditions of illumination in tench

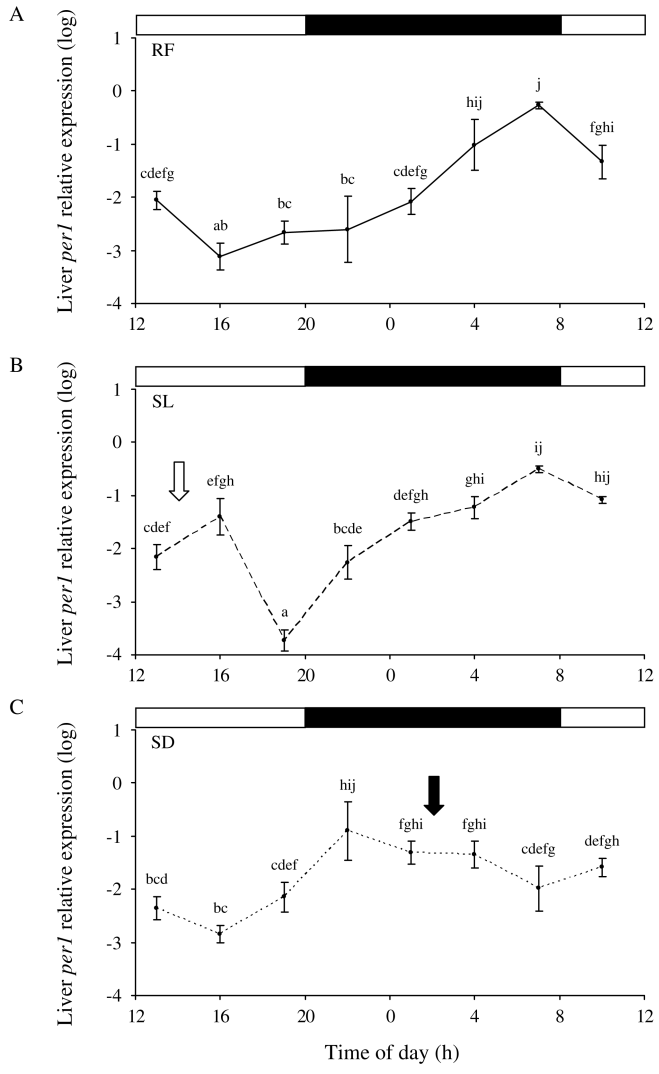

FIGURE 4 *per1* expression oscillation in the liver of zebrafish is shifted by scheduled feeding. Relative mRNA levels in the liver of fish maintained at LD and random feeding (RF), continuous line (A); scheduled fed at 14:00 h (mid-light) (SL), dashed line (B); and scheduled fed at 02:00 h (mid-dark) (SD), dotted line (C). Data (mean ± SEM) were calculated as the logarithm of the relative *per1* expression using *β-actin* as the housekeeping gene (n = 4 for each point). Data were analyzed by two-way ANOVA ($p < .05$), followed by a Tukey's post hoc test. Different letters indicate significant differences within groups and between groups. The white and black arrows indicate the time of feeding for SL and SD groups, respectively.

(Herrero et al., 2005), goldfish (Sánchez-Vázquez et al., 1997), and zebrafish (present study), indicating that FAA can be synchronized to periodic feeding and that this synchronization is not the result of an internal timing mechanism that measures the appearance of food relative to the LD cycle (Sánchez-Vázquez & Madrid, 2001). As previously observed in

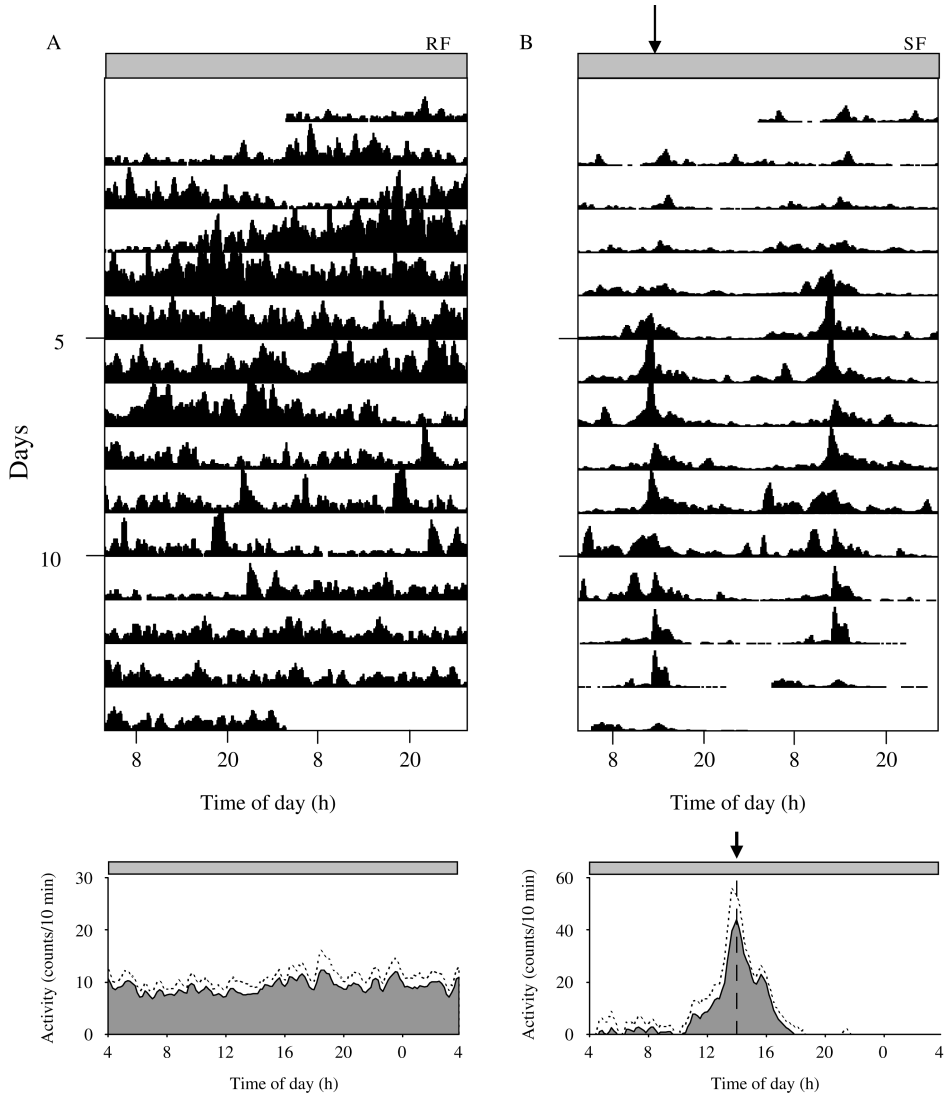

FIGURE 5 Food-anticipatory activity in zebrafish persists under constant light (LL) conditions and scheduled feeding. Top: Representative actograms for groups of fish, maintained at LL, that were random fed (RF) (A) or scheduled fed at 14:00 h (SF) (B). For convenient visualization, the data have been double plotted (48 h), the y-axis progresses in single days, with each day being plotted twice (day 1 on the right side is repeated on day 2 on the left side). The activity was binned every 10 min, the height of each point representing the number of interruptions of the infrared light beam. The grey bar above each actogram represents the constant lighting conditions. Bottom: Mean waveforms of locomotor activity for each actogram. Each point has been calculated as the mean ± SD from 10-min binned data across all the experimental days shown on each actogram. The continuous line and grey area indicate the mean light-beam interruptions and the dotted line indicates the SD. Mealtime of scheduled fed group is represented by an arrow at the top of the actogram, and an arrow and a dashed line in the mean waveform. The grey bar represents LL conditions.

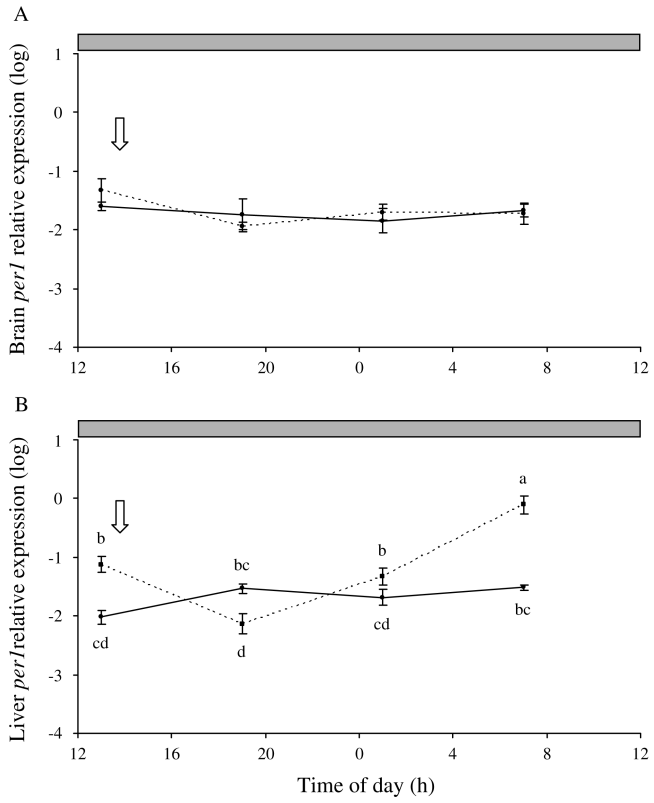

FIGURE 6 *per1* expression oscillation is synchronized to scheduled feeding in the liver but not in the brain of zebrafish. Relative mRNA levels in the brain (A) and liver (B) of fish maintained at LL: fish fed randomly (RF), continuous line; and fish fed once a day at a fixed time (14:00 h) (SF), dotted line. Data (mean ± SEM) were calculated as the logarithm of the relative *per1* expression using *β-actin* as the housekeeping gene (n = 4 for each point). Data from each tissue were analyzed by two-way ANOVA ($p < .05$), followed by a Tukey's post hoc test. Different letters indicate significant differences within groups and between groups. The white arrows indicate the time of feeding for SF group.

other fish, such as goldfish and tench (Herrero et al., 2005; Sánchez-Vázquez et al., 1997), FAA free-ran in zebrafish after periodic feeding was removed by transferring the animals to fasting conditions. This free-running rhythm started from the FAA rhythm synchronized to the previous phase of restricted feeding, indicating that FAA represents a bona fide circadian rhythm that is entrained by periodic feeding (Sánchez-Vázquez & Madrid, 2001). The present research is the first that describes the endogenous nature of a food-entrained rhythm in the zebrafish. Interestingly, the free-running nature of FAA has also been shown in mammals (Stephan, 2002), but in the case of fish longer fasting times are possible, allowing a more rigorous assessment of the free-running robustness of FAA and making zebrafish a very interesting model for studies on food entrainment.

In contrast to what happens with some strictly nocturnal fish (Herrero et al., 2005), which only feed during the dark phase, most diurnal fish seem to be able to feed either during the day or night if feeding time is restricted (López-Olmeda et al., 2009a, 2009b; Reebs & Lague, 2000). Our study shows that the zebrafish, which has been described previously as a diurnal fish (Hurd et al., 1998), can exhibit predominantly nocturnal activity under similar conditions, supporting the notion that this species also exhibits more plasticity regarding its daily activity patterns (López-Olmeda & Sánchez-Vázquez, 2009). In addition, SD zebrafish showed a splitting in the rhythm, displaying a night and a day component. Whereas the night component was clearly synchronized to the feeding schedule, the day component seemed to be synchronized to the light phase of the LD cycle. These components could be the behavioral outputs of the FEO (night component) and LEO (day component), which can be dissociated as has been observed previously in fish under conflicting zeitgebers (Sánchez-Vázquez et al., 1995).

Circadian oscillators outside the central nervous system appear to be a common theme to invertebrates and vertebrates (Plautz et al., 1997; Whitmore et al., 1998; Yamazaki et al., 2000). In contrast to mammals, circadian rhythms of gene expression in zebrafish peripheral cells and tissues are directly entrainable by light (Kaneko et al., 2006; Pando et al., 2001), suggesting that the circadian system in this species consists of highly independent oscillators rather than the more hierarchical system found in mammals (Whitmore et al., 2000). Clock gene expression rhythms have been described in zebrafish brain, liver, heart, kidney, spleen, and gall bladder (Cermakian et al., 2000; Kaneko et al., 2006; Whitmore et al., 1998, 2000). However, our study is the first to report oscillations of a clock gene, *per1*, in the liver of food-entrained fish under both LD and LL conditions.

Whereas exposure to daytime random feeding under an LD cycle led to a circadian rhythmic pattern of *per1* expression in both the liver and brain, similar random feeding under LL led to an absence of such rhythmicity in both tissues. This result indicates that when food is not periodically restricted, light has the ability to entrain both the brain and the liver oscillators. In contrast, if food is not periodically restricted and temporal light cues are not available, the oscillators are not entrained. Daily profiles of *per1* in the brain under LD and LL are similar to those previously reported (Sánchez & Sánchez-Vázquez, 2009). As for the absence of rhythmicity under LL, another possible explanation would be that as each timepoint is derived from a sample of fish whose individual phases are unknown, we cannot determine whether the lack of rhythmicity in livers and brains from randomly fed LL animals reflects arrhythmic expression of *per1* in each animal or scattered phases between animals. Interindividual variability in τ seems to be a common feature among fish,

as reported previously for individual locomotor activity and feeding rhythms (Sánchez-Vázquez & Tabata, 1998).

Our study is the first reporting daily variations of *per1* in the liver of adult zebrafish. Daily variations of other clock genes (*clock* and *bmal*) in the zebrafish liver had been previously reported (Cermakian et al., 2000). In addition, *per1* expression has been recently described in the liver of the goldfish (Velarde et al., 2009). In the present research, the rhythm in the liver was consistent with the *per1* rhythm reported for goldfish liver, with a peak towards the end of the dark phase, although in goldfish the rhythm did not reach statistical significance (Velarde et al., 2009). It should be noted that the circadian acrophase of *per1* in zebrafish differs from the circadian acrophases of *period* genes in mammals, which are located in the second half of the light phase (Zylka et al., 1998). Such differences may suggest differences between fish and mammals in the regulation by light of *period* genes expression.

Liver *per1* expression was shifted by 7 h in MD-fed animals compared with the acrophase of the group fed at ML. This contrasts with previous experiments in rodents where the phase shift in *per1* rhythms in the liver under similar experimental conditions was approximately 12 h (Damiola et al., 2000; Stokkan et al., 2001). Two non–mutually exclusive hypotheses could explain this partial shift in *per1* expression in zebrafish. On the one hand, zebrafish cells and tissues can be entrained directly by light (Kaneko et al., 2006; Pando et al., 2001; Whitmore et al., 2000), and it is conceivable that the phase of the *per1* expression rhythm under an LD cycle and night-time restricted feeding is a compromise between each zeitgeber's entraining input to the oscillator. On the other hand, the LEO and FEO in fish present a strong degree of coupling, as demonstrated in previous research in other fish species (López-Olmeda et al., 2009b; Sánchez-Vázquez et al., 1995, 1997), which could explain why the liver *per1* expression phase was not totally reversed in MD-fed fish compared to ML-fed fish.

Interestingly, *per1* expression in scheduled-fed animals under LL was rhythmic in the liver but not in the brain. Taken together, our results are similar to those found in a number of experiments on rodents, in which feeding time resets the phase of clock gene expression rhythms in the liver but not in SCN, both under a LD cycle and under LL (Damiola et al., 2000; Stokkan et al., 2001). Indeed, the SCN is not necessary for the expression of circadian rhythms of either FAA (Stephan, 2002; Stephan et al., 1979) or of clock gene expression in peripheral tissues (Hara et al., 2001; Yoo et al., 2004). The simultaneous presence of FAA and rhythmic *per1* expression in the liver but not in the brain of our scheduled fed animals under LL suggests a similar independence of FAA and food-entrained liver clock gene expression rhythms from the central oscillators. In mammals, however, FAA is controlled by an oscillator located in the brain (Davidson et al., 2003) and, in addition, restricted

feeding can reset the phase of oscillators located in several regions in the brain of the rat (Ángeles-Castellanos et al., 2007; Miñana-Solis et al., 2009). Moreover, the lack of rhythmicity under LL observed in the brain of SF fish could be due to pooling the whole brain for the analyses, so food-entrained regions of the brain could not be identified. Therefore, the possible role of certain brain regions in feeding entrainment cannot be discarded, and further research should be considered in the future.

Since the discovery of food-entrained rhythms in mammals, a number of studies have focused on finding its anatomical substrate; indeed, its putative location in the brain is still a matter of controversy (Carneiro & Araujo, 2009; Escobar et al., 2009; Gooley et al., 2006; Landry et al., 2007; Moriya et al., 2009). Our study and previous studies in rodents suggest a high degree of autonomy of the liver oscillator from oscillators in the central nervous system, at least under temporally restricted food access (Damiola et al., 2000; Hara et al., 2001; Stokkan et al., 2001; Yoo et al., 2004). In line with this view, a recent study has found a direct pathway by which metabolic changes induced by restricted food access could directly reset the molecular clockwork of the liver peripheral oscillator through the protein AMPK, whose activity is regulated by the nutritional state (Lamia et al., 2009). Nevertheless, it is likely that multiple and parallel pathways remain to be discovered (Lamia et al., 2009). It should be noted that zebrafish could emerge as a useful model for the research on food-entrainable rhythms in vertebrates, because this species shows (i) molecular genetic tools comparable to those available for mammalian models; (ii) plasticity of circadian behavior that allows zebrafish to synchronize to food restriction either during the light or the dark phase; and (iii) longer fasting times that confers an advantage for the design of longer experiments under free-running conditions.

In summary, we showed that zebrafish displayed FAA when food was periodically restricted, regardless of whether this restriction was during the day, during the night, or under constant light conditions, and that FAA was of endogenous origin because it persisted after the deprivation of the scheduled feeding. The molecular clockwork within the liver oscillator was entrained by periodic feeding, but not the oscillator(s) in the whole brain, whose clock gene expression rhythms seemed to be synchronized mostly by the LD cycle. Our results point to zebrafish as a reliable model for the study of food entrainment of peripheral oscillators, representing a unique model for unmasking the mechanisms by which food can time physiological and behavioral rhythms.

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