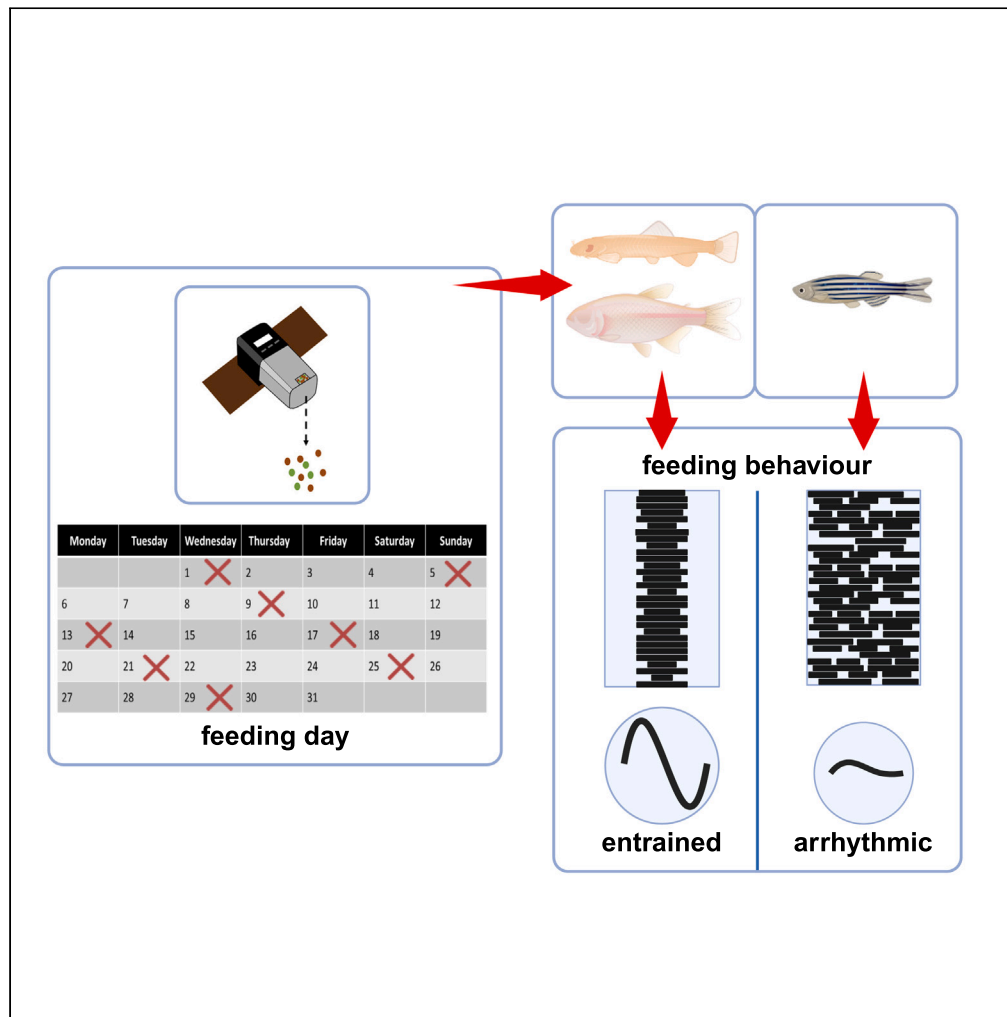**Article**

Sporadic feeding regulates robust food entrainable circadian clocks in blind cavefish

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Highlights

Feeding represents a
strong circadian
synchronizer for blind
cavefish

One meal every 4 days is
sufficient to entrain
circadian rhythmicity in
cavefish

Feeding entrainment in
zebrafish occurs only when
food is available daily

feeding behaviour

entrained   arrhythmic

feeding day

Article

Sporadic feeding regulates robust food entrainable circadian clocks in blind cavefish

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SUMMARY

The circadian clock represents a key timing system entrained by various periodic signals that ensure synchronization with the environment. Many investigations have pointed to the existence of two distinct circadian oscillators: one regulated by the light-dark cycle and the other set by feeding time. Blind cavefish have evolved under extreme conditions where they completely lack light exposure and experience food deprivation. Here, we have investigated feeding regulated clocks in two cavefish species, the Somalian cavefish *Phreatichthys andruzzii* and the Mexican cavefish *Astyanax mexicanus*, in comparison with the surface-dwelling zebrafish *Danio rerio*. Our results reveal that feeding represents an extremely strong synchronizer for circadian locomotor rhythmicity in subterranean cavefish. Indeed, we showed that consuming just one meal every 4 days is sufficient to entrain circadian rhythmicity in both cavefish species, but not in zebrafish. These profound adaptations to an extreme environment provide insight into the connections between feeding and circadian clocks.

INTRODUCTION

The circadian clock represents a key biological timing system that enables organisms to anticipate and thereby adapt to the progression of the day-night cycle.[1] It is composed of a network of central and peripheral oscillators distributed in cells and tissues throughout the body.[2] The interaction between these oscillators and their entrainment by external timing cues coordinates physiological and behavioral rhythms allowing animals to anticipate environmental changes and optimally time physiological processes in order to enhance survival.[3,4] The entraining effects of photic and non-photic environmental signals indicative of the time of day (so-called *zeitgebers* or time givers) have been intensively studied in both laboratory and natural conditions. In natural conditions, multiple *zeitgebers* act in concert and so the circadian timing system must integrate this complex combination of timing information in order to precisely ascertain the time of day. Via this process the phase of the endogenous circadian oscillator is set accordingly to accurately match that of the environmental day-night cycle.[5,6]

One of the characteristic properties of the circadian clock is its tight interconnection with metabolism. Circadian rhythmicity is a characteristic feature of many metabolic pathways, and these rhythms can in turn provide regulatory feedback to stabilize core clock function. During the past 50 years, the results from many studies have pointed to the existence of a food-entrainable oscillator (FEO), an additional circadian oscillator which is set specifically by feeding time, independently of the light dark cycle. Subsequently, mainly using rodent models, a major goal has been to identify the location and molecular components of the FEO.[7–9] Despite the use of various physiological and molecular approaches, the location and mechanism of the FEO has remained elusive. Therefore, a better understanding of the mechanisms whereby circadian clocks respond to feeding cues represents an important goal for future studies and requires the study of appropriate animal models.

Feeding represents one of the best-investigated temporally organized behaviors in many animal models including fish species. When food is provided in a cyclic manner most fish species can synchronize their behavioral rhythms to feeding time.[10] These rhythms are controlled by an endogenous pacemaker since they persist when the food signal is removed and fish are fasted.[11] The endogenous origin of the feeding rhythms has been demonstrated in different fish species such as the European sea bass (*Dicentrarchus labrax*), goldfish (*Carassius auratus*), and zebrafish (*Danio rerio*).[4,12–16]

Animals that have evolved under certain extreme environmental conditions, such as at polar latitudes, in deep-sea, or subterranean environments, have allowed us to investigate the consequence of the absence of one or more *zeitgebers* upon the entrainment mechanisms of the circadian clock mechanism.[17–22] Thereby, these species provide a unique opportunity to explore zeitgeber-specific clock input pathways

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as well as how evolution shapes the mechanisms which integrate timing information from multiple zeitgebers. Among species that have evolved in extreme environments, cavefish represent particularly interesting models.[23] The long-term exposure to high environmental stability characterized by perpetual darkness, constant temperature, food scarcity, and low biodiversity have resulted in the evolution of troglomorphic characteristics (i.e., loss of photoreceptive organs, reduction of pigmentation, and resistance to hypoxia) and the alteration of biological mechanisms such as the circadian clock, DNA repair, sleep, and metabolism.[18,20,23–32] Previous studies revealed that the Somalian cavefish *Phreatichthys andruzzii* no longer exhibits photic entrainment, but has retained feeding entrainment of the clock.[18] While cavefish show a functional clock entrainable by feeding but not by light, they display other abnormal clock properties such as a characteristic infradian rhythm in peripheral cells entrained by alternative *zeitgebers* like serum or dexamethasone and the absence of temperature compensation.[18] This points to the existence of multiple genetically distinct clock mechanisms in this species, targeted by different zeitgebers. Furthermore, given the absence of a light-entrainable oscillator (LEO), this cavefish species represents a particularly attractive model for studying the mechanisms and regulation of the FEO. In a recent study of food-seeking and nose-poking behavior, Ehichioya et al.[33] showed that food anticipatory behavior is regulated by a LEO located outside the primary circadian pacemaker within the suprachiasmatic nucleus (SCN). Therefore, the cavefish *P. andruzzii* potentially represents an exciting model to investigate the FEO because they have completely lost LEO function.

Food supply in a subterranean environment is scarce and a number of adaptations have evolved in cavefish to survive with this highly restricted food supply including profound changes in the rate and properties of metabolism. One notable behavioral adaptation is the use of the circadian clock to anticipate regular, daily food availability with increased food anticipatory activity (FAA) (i.e., an increase of locomotor activity levels before the anticipated mealtime) and thereby to more effectively exploit the limited food supply. *P. andruzzii* show a strong entrainment of rhythmic locomotor activity to regular daily feeding and exhibits highly robust FAA.[18] Laboratory and field studies on the Mexican cavefish *Astyanax mexicanus* have also shown entrainment of activity to daily food administration.[34,35]

FAA is considered a *bona fide* circadian rhythm because it persists under constant conditions (e.g., fasting), it is entrainable and also does not instantaneously adjust its phase following an abrupt shift of the mealtime, instead being adjusted over the course of several transient cycles.[36,37] The classical experimental protocol to study feeding entrainment is designed with one feeding time per day. However, the adaptive plasticity of the feeding clock has only been tested using a zeitgeber period close to the endogenous free-running period (e.g., in rodents close to 24 h). In the wild, food availability may be scarce in particular environments or during certain periods of the year and it may be uncommon for an animal to regularly obtain one or more meals per day especially in the case of carnivores. To our knowledge, experimental protocols involving the administration of food at lower frequencies of one meal each 48 or more hours have not yet been developed. Cavefish are ideal models to test the adaptative plasticity of the feeding clock because it is possible to reduce the frequency of food administration and to fast fish for longer periods.[38] Furthermore, given the restricted food availability within subterranean cave environments, these lower frequency feeding regimes are likely to be physiologically relevant for cavefish species. They also offer the chance to explore at a holistic level the metabolic adaptations associated with specific troglomorphic phenotypes and more generally, to improve our understanding of adaptations to extreme environments.[39] In addition, since cavefish share similar traits and conserved mutations with several human metabolic diseases without exhibiting pathological effects,[38] a more in-depth study of the temporal control mechanisms associated with feeding behavior could expand our understanding of disease pathology in humans.

Here, we have used an interspecific comparative approach to investigate feeding entrainment of the FEO in three well studied fish model species, namely the cavefish *P. andruzzii* and *A. mexicanus* and the surface-dwelling zebrafish *Danio rerio*. With this aim, we exposed fish to various feeding cycles with different periods ranging from 24 to 96 h, under constant darkness (DD) and temperature, and revealed that in both cavefish species, feeding serves as a remarkably strong zeitgeber, able to entrain circadian rhythms of locomotor activity even when its frequency is reduced to a single meal per week delivered at the same time of day.

RESULTS

Previous investigations revealed that *P. andruzzii* retains a feeding-entrained clock which is synchronized by regular food administration every 24 h.[18] To test how sensitive the *P. andruzzii* circadian clock is to entrainment by periodic food availability, we exposed Somalian cavefish to feeding schedules in which food was regularly available with a period of 24, 48, 72, and 96 h in order to test the behavioral entrainment in response to feeding cycles inside and outside of the circadian range (i.e., between 20 and 30 h). These regular feeding schedules, all with periods of multiples of 24 h, induced a clear entrainment of behavioral circadian rhythms (Figures 1A–1D). Some days after the start of periodic feeding, fish concentrated their daily activity around feeding time, with an increase in activity 3–5 h before the anticipated food delivery that corresponds to FAA (Figures 1A–1D). Periodogram analysis indicated a strong entrainment to all feeding cycles (Figures 1A–1D). To confirm the endogenous origin of this rhythmicity, it is important to test for persistence of rhythmicity during extended periods of fasting, when the food zeitgeber is completely absent. This is a challenging experiment for many model organisms such as rodents which cannot survive long periods of fasting. However, blind cavefish are eminently well suited to these experiments due to their well-documented tolerance of fasting in their food-poor environments. Therefore, after regular feeding entrainment, we subjected *P. andruzzii* to fasting for about 20 days. During fasting, locomotor rhythms persisted with free-running circadian periods (F_{24}: τ = 24,1 $\pm$ 0,2; F_{48}: τ = 24,7 $\pm$ 0,8; F_{72}: τ = 23,9 $\pm$ 0,4; F_{96}: τ = 24,1 $\pm$ 0,23), independently from the period of the previous feeding schedule (Figures 1A–1D).

Surprised by the cavefish's ability to entrain their circadian clock in response to meals delivered once every 3–4 days, we decided to repeat all tests in zebrafish to understand if this ability is widespread among teleosts (Figures 2A–2D). As expected, we observed canonical strong entrainment of rhythmic locomotor in zebrafish fed every 24 h (Figure 2A). FAA is clearly visible where the onset of activity was around 5–6 h before feeding (Figure 2A). During a relatively short period of fasting (15 days), locomotor rhythmicity persisted with a free-running circadian
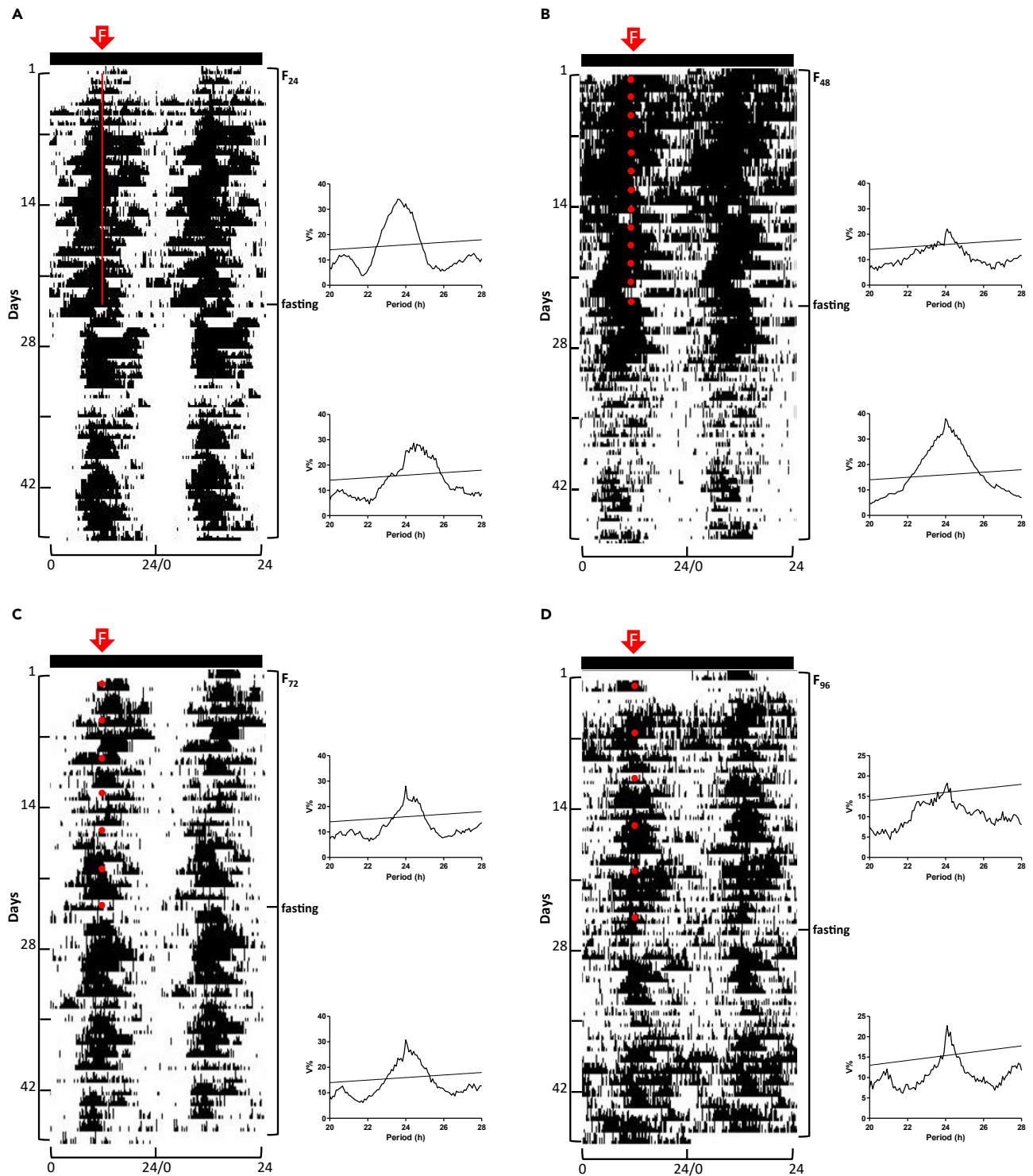
Figure 1. Analysis of locomotor activity of Somalian cavefish subjected to periodic feeding

Representative actograms and χ^2 periodogram analysis of locomotor activity of groups of Somalian cavefish *Phreatichthys andruzzii* subjected to periodic feeding of T = 24 h (A), T = 48 h (B), T = 72 h (C) and T = 96 h (D). Actograms are double plotted on a 48 h timescale to help the interpretation. Each horizontal line is a record of 48 h of activity; each subsequent 48 h cycle is plotted beneath the previous one. 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 height of each point represents the number of infrared light-beam interruptions in 10 min. The number of days is indicated on the left and the time of day is plotted on the bottom of each actogram. The black bar on the top

Figure 1. Continued

of each actogram represents the constant darkness (DD) conditions. Red arrows on the actograms indicate the start of scheduled feeding, the continuous lines indicate feeding time in the T = 24 h group. Red dots indicate feeding time in the T = 48 h, 72 h, and 96 h groups. Activity records in the last 10 days of feeding and fasting were subjected to χ^2 periodogram analysis and each graph is presented on the right of corresponding actogram. The χ^2 periodogram indicates the percentage of variance (%V) of the rhythm explained by each analyzed period within a range of 20–28 h. The sloped dotted lines represent the threshold of significance, set at $p = 0.05$. The start and the end of the feeding schedules are reported on the right hand of the actograms. F_{24}, F_{48}, F_{72}, and F_{96} indicate the day of start of periodic feeding and its frequencies.

period (F_{24}: $\tau = 23,8 \pm 0,6$), confirming its endogenous origin. However, when food was provided every 48 or 72 h, zebrafish concentrated most of their activity after mealtime and FAA was absent or shorter than 40 min (Figures 2B and 2C). Furthermore, during fasting we observed significant free-running rhythms in only 15% of the aquaria for both feeding conditions (Figures 2B and 2C, representative examples of arrhythmicity after F_{48} and rhythmicity after F_{72}, respectively). These results reveal weak feeding entrainment in zebrafish when food was provided once every 48 or 72 h.

Thus, feeding entrainment occurred in Somalian cavefish when food was available with a period of 24, 48, 72, or 96 h, while in zebrafish, feeding entrainment mainly occurred only when food was available with a period of 24 h. To further explore the relationship between feeding frequency and clock entrainment, we chose to test the feeding entrainment in both Cyprinid species using feeding schedules where food was available regularly with a period of 36 and 44 h, two periods in the range of entrainment previously tested, but not multiples of 24 h and outside of the circadian range. Both Somalian cavefish and zebrafish that received meals with a period of 36 and 44 h did not display any FAA, but only a post-feeding increase of activity (Figure S1).

Thus, our results indicate that *P. andruzzii* possesses a feeding-regulated circadian clock entrainable by periodical food administration at significantly lower frequencies (one meal every 4 days) than in zebrafish (one meal each day). Is this a characteristic property of cavefish species? To tackle this question we decided to investigate feeding entrainment in the most investigated cavefish species, the Mexican cavefish *A. mexicanus* originating from the Pachón cave. We exposed Mexican cavefish to 3 feeding schedules in which food were available with a period of 24, 72, and 96 h. Results show a clear feeding entrainment of the behavioral circadian rhythms with a marked FAA response (3–5 h) to all feeding cycles (Figures 3A–3C). During fasting, locomotor rhythmicity persisted with a free-running circadian period (F_{24}: $\tau = 24,1 \pm 0,1$; F_{72}: $\tau = 24,4 \pm 0,6$; F_{96}: $\tau = 24,3 \pm 0,3$), confirming its endogenous origin (Figures 3A–3C).

Finally, in all 3 fish species we tested for the existence of a "*transient cycle*" following a shift of mealtime. This property represents important evidence that the daily activity rhythmicity is generated by circadian oscillators entrained by daily meals. All species showed a clear FAA during the first daily feeding cycle (Figures 4A–4C). In order to verify the accuracy of the entrained rhythm we estimated the time of acrophase (the time at which the rhythm peak occurred) for the last 10 days of recording in the feeding cycle. Using a circular statistic approach, we showed that the distribution of acrophases deviated from uniform (Rayleigh test, *D. rerio*: Z = 8,42, $p < 0,0001$; *A. mexicanus*: Z = 9,1, $p < 0,0001$; *P. andruzzii*: Z = 8,3, $p < 0,0001$), and the mean acrophases fell 1–3 h before ZT0, the feeding time (*D. rerio*: $F_{24} = 126,02° \pm 23,3°$; *A. mexicanus*: $F_{24} = 165,7° \pm 16,9°$; Figures 4A–4C). When mealtime was shifted abruptly by 8 h, zebrafish and Mexican cavefish showed a transient of 4–6 days (Figures 4A and 4B) to entrain to the new feeding cycle. The distribution of acrophases deviated from uniform (Rayleigh test, *D. rerio*: Z = 9,54, $p < 0,0001$; *A. mexicanus*: Z = 8,79, $p < 0,0001$), and the mean acrophases fell 1–4 h before the new feeding time (*D. rerio*: $F_{24S} = 290,3° \pm 12,3°$; *A. mexicanus*: $F_{24S} = 232,7° \pm 15,1°$; Figures 4A–4C). As expected, the mean acrophases significantly changed after the 8-h shift of the feeding time (Hotelling's paired test; *D. rerio*: F = 1155,5, $p < 0,00001$; *A. mexicanus*: F = 25,8, $p < 0,00001$). In contrast, Somalian cavefish did not respond to the shift as expected. Specifically, they lacked a transient, days after the shift of mealtime and the daily activity maintained the phase associated with the initial daily feeding cycle (Mean acrophase, $F_{24} = 159,7° \pm 9,1°$; $F_{24S} = 189,6° \pm 10,2°$; Hotelling's paired test, F = 1,83, $p > 0,2$). Furthermore, the length of the activity period significantly increased from 3–5 to 6–8 h (Figure 4C).

DISCUSSION

In the wild, daily rhythms of light, temperature, food availability, and other ecological factors (i.e., predators, parasites) serve as zeitgebers. In extreme environments such as cave, deep-sea, polar latitudes, or high altitudes some entrainment cues may be absent or do not exhibit daily rhythmicity. Cavefish are one of the more intensively investigated animal models which have evolved under extreme environmental conditions. Indeed, during subterranean isolation the evolution of cavefish has led to the development of extreme troglomorphic phenotypes such as complete anophthalmy, albinism, altered metabolism (high levels of body fat and blood sugar), and an aberrant circadian clock.[17,23,39–41] These profound adaptations to extreme subterranean environments provide unique insight into the function of many aspects of physiology and metabolism as well as the circadian clocks which time them.

To our knowledge, cavefish are the first animals in which consuming one pulse of food every 4 days, is sufficient to entrain circadian rhythmicity. This result suggests that feeding represents an extremely strong zeitgeber for circadian rhythms of locomotor activity in subterranean cavefish. In a classical view, a zeitgeber periodic signal has been considered "strong" when it can entrain circadian rhythms to a larger range of periods.[42] In our case the period of the zeitgeber is very large (up to 96 h) indicating how strong the feeding signal is for the cavefish. It is tempting to speculate that this result is linked with the specialized metabolic status shown by cavefish that includes low metabolic rate, increased appetite, fat storage, and fasting resistance.[43–45] Given the lack of primary production due to the absence of sunlight,[46] food availability is paramount to survival in subterranean environments, especially for species that feed sporadically or only at limited times of the day. The capacity to maintain entrainment of a 24 h rhythm in response to a pulse of food delivered every 96 h and the absence of transient cycles in
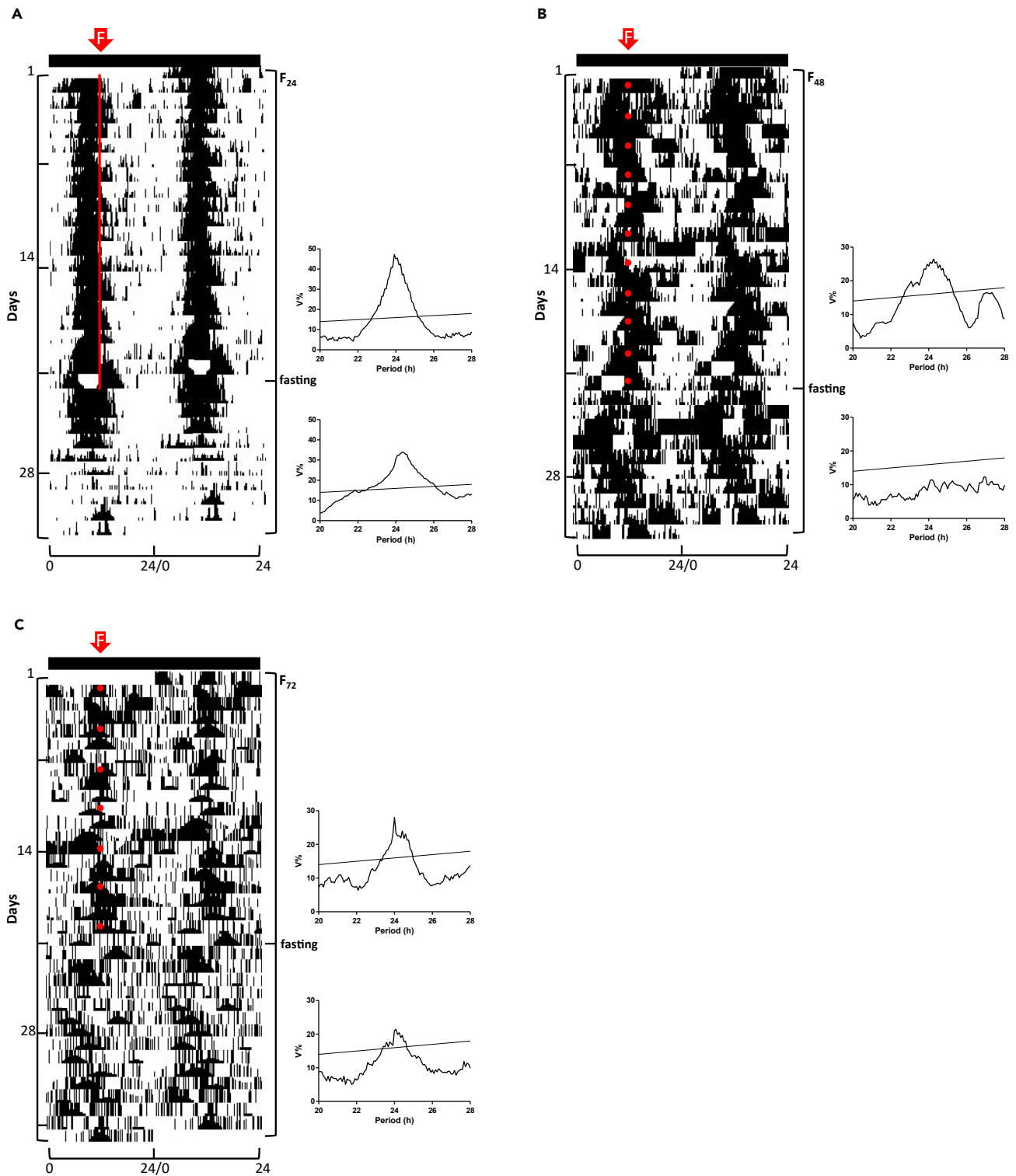
Figure 2. Analysis of locomotor activity of zebrafish subjected to periodic feeding

Representative actograms of locomotor activity of groups of zebrafish *Danio rerio* subjected to periodic feeding of T = 24 h (A), T = 48 h (B), T = 72 h (C). Activity records in the last 10 days of feeding and fasting were subjected to χ^2 periodogram analysis. For more details, see Figure 1.
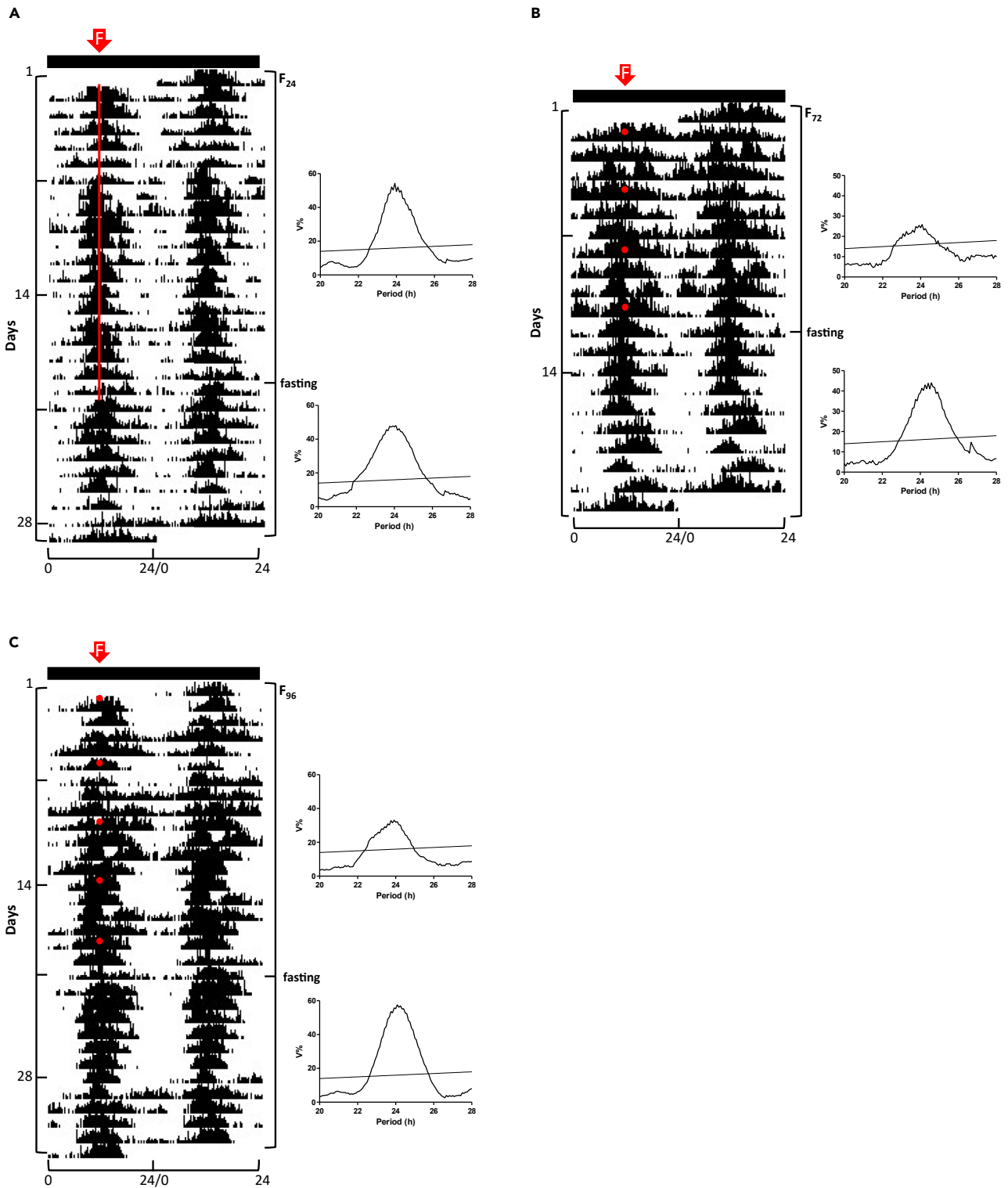
Figure 3. Analysis of locomotor activity of Mexican cavefish subjected to periodic feeding

Representative actograms of locomotor activity of groups of Mexican cavefish *Astyanax mexicanus* subjected to periodic feeding of T = 24 h (A), T = 72 h (B), T = 96 h (C). Activity records in the last 10 days of feeding and fasting were subjected to χ^2 periodogram analysis. For more details, see Figure 1.
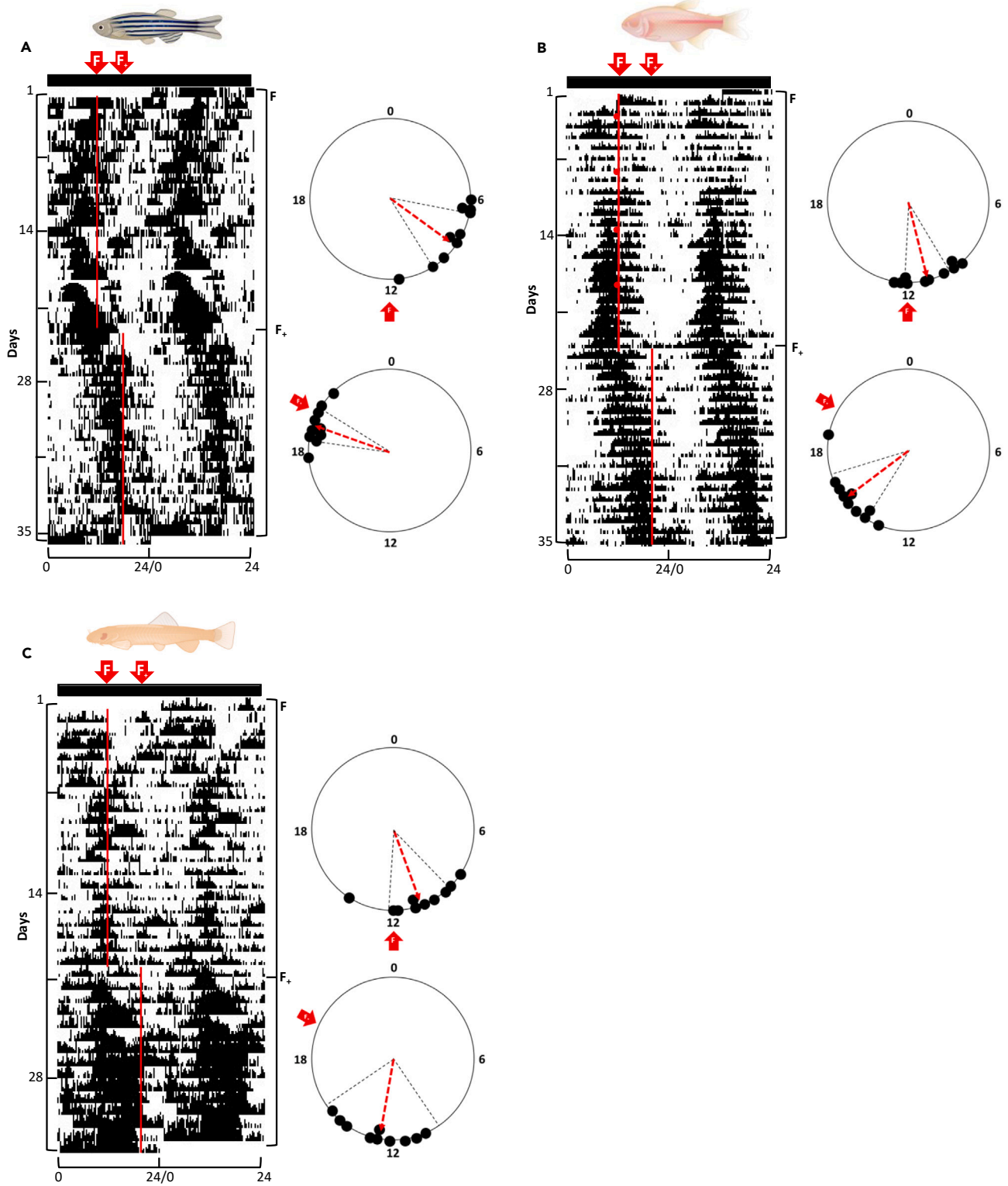
Figure 4. Locomotor activity of zebrafish, Somalian cavefish, and Mexican cavefish subjected to a shift of feeding time
Representative actograms of locomotor activity of groups of *D. rerio* (A), *A. mexicanus* (B), and *P. andruzzii* (C) subjected to a periodic mealtime of T = 24 h, which later was shifted (delayed) by 8 h. Circular diagrams showing acrophases for the last 10 days of each LD period are plotted. Each black dot shows the daily acrophase, while dashed line indicates the average acrophase represented as a vector. Red arrows indicate food administration time. The dotted lines represent the confidence intervals. Confidence limits were chosen at 99% level. For more details, see Figure 1.

P. andruzzii could be an indispensable strategy to forage for food in an environment where there is almost nothing to eat. This capacity can now be added to the list of metabolic adaptations observed in troglomorphic species, such as loss of pigmentation, enhanced lipogenesis, and hyperglycemia that do not exhibit pathological effects, but instead may provide a survival advantage in a nutrient-poor environment. A recent study[38] has highlighted the metabolic adaptations observed in cavefish species because they share conserved traits with several human diseases such as obesity and diabetes. Therefore, cavefish are potentially valuable models to study the mechanisms underlying these important metabolic diseases. As well as these metabolic adaptations, cavefish also exhibit radical alterations in the function of the circadian timing system which include significant changes in the sleep/wake cycle and a switch from photic to exclusively feeding entrainment. It has been well documented that artificial disruption of the circadian clock increases the susceptibility to a range of important human diseases such as cancer and cardio-vascular diseases.[47] For these reasons cavefish could also improve our understanding of functional links between the circadian clock mechanisms and physio-pathological alterations. In this way, detailed knowledge of the mechanisms underlying the cavefish phenotype could represent a translational tool to guide therapeutic strategies for various human pathologies.

Previous studies have shown that the timing of food intake entrains the cavefish circadian timekeeping system.[18,34,35] Anticipation of daily meals (FAA) and the persistence of post-entrainment free-running periods are two of the canonical properties of behavioral rhythms generated by circadian oscillators entrainable by periodic food availability.[7,37] Our study revealed the presence of both properties in the two cavefish species analyzed: a strong FAA and the persistence of rhythmicity for several cycles during total food deprivation. Our results demonstrate that this food-regulated mechanism is self-sustaining and generated by circadian oscillators. Interestingly, a comparison between epigean (zebrafish) and hypogean (cavefish) fish reveals some fundamental differences in feeding entrainment. Specifically, while the cavefish circadian clock is entrainable when food is available regularly with a period of 72 or 96 h, zebrafish feeding entrainment only occurs when the feeding cycle has a period of 24 h. It has already been documented that circadian rhythms are entrained to zeitgeber with periods which are close to their free-running periods.[48] If the zeitgeber period differs greatly from the free-running period, then entrainment does not emerge, an effect termed "*limits to entrainment*".[49] For example, the mammalian circadian clock has a free-running period (τ) of about 24 h and it can become entrained to zeitgeber periods within an entrainment range of about $\pm$ 1–2 h.[50,51] Indeed, the fact that the Somalian cavefish and zebrafish behavior was not entrained by a feeding cycle with a period of 36 or 44 h indicates that this process is driven by a circadian clock. Furthermore, the fact that feeding cycles with periods of 72 and 96 h which are multiple of 24 h, successfully entrained circadian rhythmicity is another indication that feeding entrainment in cavefish is mediated by circadian oscillators. With regards to the molecular mechanisms that underlie the food entrainable clock, our previous studies revealed the presence of an infradian oscillator in a *P. andruzzii* primary cell line derived from the caudal fin that ticks with a period of 43 h.[18] The striking difference in the free-running period between this *P. andruzzii* cell culture clock and the feeding entrained clocks which regulate FAA described here, indicates that *P. andruzzii* might possess two distinct molecular clock mechanisms. These observations raise the intriguing possibility that functionally distinct clock mechanisms may underlie the response to different zeitgebers.

FAA is a clear output of the interaction between the circadian clock, feeding-related signals and learning.[52] Subterranean environments are frequently characterized by scarce food availability and these environmental conditions could potentially explain both a strong motivation to food-seeking behaviors and a robust associative learning (e.g., a classical conditioning in which connections are made between unconditioned and conditioned stimuli[53]). In cavefish, a rapid and long-lasting learning of food availability could be an important adaptation for survival in extreme hypogean conditions where food is scarce. This inference is also supported by the lack of "*transient cycles*" following a shift of mealtime in the daily rhythm of locomotor activity in *P. andruzzii*. Normal epigean fish species typically show transients after a shift of feeding time, which start the first days at the previous feeding time and progressively shift to the new feeding time.[54] This was the case here observed in zebrafish and Mexican cavefish, which took 3-5 days to progressively resynchronize to the new feeding time. In contrast Somalian cavefish did not display clear "*transient cycles*", but they showed a "*memory*" of the feeding time prior to the shift confirming the retention of the learning.

It is tempting to speculate about the possible selective advantage of a feeding regulated circadian clock that coordinates a high level of FAA every single day, in response to the timing of rare meals which may only be consumed once every few days. The presence of feeding rhythmicity gives a clear indication that in the wild, the trophic resources for cavefish might be restricted to particular times of day. Unfortunately, ecological data for *P. andruzzii* are very scarce because of the logistic challenges of performing field studies in the Somalian desert region. We do know that the water table where this cavefish lives is relatively superficial, lying at a depth varying from some meters to a few tens of centimetres beneath the ground surface. The original specimens that were identified were collected exclusively during the night either in hand dug wells and springs or sites where the water table was exposed after the caliche (i.e., a sedimentary rock, a hardened natural cement of calcium carbonate that binds other materials—such as gravel, sand, clay, and silt) had collapsed.[55] We predict that *P. andruzzii* is an opportunistic feeder that feeds on animals, plants or decaying organic matter when swimming close to the surface. However, the pale pink color together with the lack of visual sensing in this species makes it particularly vulnerable to predation during the daytime. It would therefore seem logical that these cavefish would restrict their foraging and feeding activities to the night period. Furthermore, given the impressive tolerance of this species to long periods of fasting, the fish would not necessarily need to visit the surface on a regular daily basis. A robust

FEO would then play a critical role in timing their foraging trips and thereby avoiding the risk of randomly timed visits to the surface. *Astyanax mexicanus* cavefish on the other hand are obligate cave dwellers, they live in isolated, deep caves, and they feed in the dark in the ponds where they swim. In the Pachón cave where the Mexican cavefish tested in the present study originate from, the adult feeding regime is diversified and mainly opportunist and detritivorous.[56] Interestingly, the Pachón cave hosts a colony of bats and their feces represent a major energetic input into the cave and source of food for the fish. Daily rhythms of defecation associated with bats moving to and from their roosting sites could entrain the feeding activity of cavefish.[35,56,57]

Our results reveal that feeding represents a potent zeitgeber for subterranean cavefish and also provide compelling evidence for the existence of genetically distinct light and feeding entrainable clocks. In this regard, cavefish represent excellent models both to study the evolution of the circadian timekeeping system and to deepen our knowledge on the differential involvement of molecular and physiological pathways in clock entrainment in response to diverse environmental signals.

Limitations of the study

Probably the most important limitation of our study is the current impossibility to acquire ecological data from the Somalian desert sites where *P. andruzzii* lives. It is clear that a detailed knowledge of the ecology of organisms and the ecological niches that they occupy is essential for a more comprehensive understanding of how particular combinations of zeitgebers entrain their circadian clocks.

The focus of this work was *P. andruzzii* due to the extreme adaptations of its circadian timing system. The Pachón form of *A. mexicanus* served as an additional blind cavefish model in this initial comparative study. For future studies aimed at exploring the genetics and mechanisms underlying the FEO and FAA, *A. mexicanus* would be a more suitable model. Its advantages include the existence of "normal sighted" epigean forms as well as forms with intermediate troglomorphic phenotypes which can all be inter-crossed and yield fertile offspring. However, the inevitably large scale of these functional experiments would place them outside the scope of the current manuscript.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.110171.

AUTHOR CONTRIBUTIONS

Conceptualization, J.F.L.O., F.J.S.V., N.S.F., and C.B.; formal analysis, V.D.R., E.F., W.C., J.F.L.O., and C.B.; investigation, V.D.R., E.F., P.N., W.C., and C.B.; resources, S.R., N.S.F., and C.B.; data curation, V.D.R., E.F., P.N., W.C., and J.F.L.O.; writing – original draft, E.F., N.S.F., and C.B.; writing – review & editing, V.D.R., E.F., W.C., J.F.L.O., S.R., F.J.S.V., N.S.F., and C.B.; visualization, E.F.; funding acquisition, J.F.L.O., S.R., F.J.S.V., N.S.F., and C.B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Raw and analyzed data	This paper, Mendeley Data	
Experimental models: Organisms/strains		
Danio rerio	University of Murcia	N/A
Astyanax mexicanus	University of Ferrara	N/A
Phreatichthys andruzzii	University of Ferrara	N/A
Software and algorithms		
El Temps - version 313	Díez-Noguera[58]	http://www.el-temps.com/principal.html
DIO98USB	Cavallari et al.[18]	N/A

RESOURCE AVAILABILITY

Lead contact

Information and requests about this study should be directed to and will be fulfilled by the lead contact, Cristiano Bertolucci (bru@unife.it).

Materials availability

No new materials were generated in this study.

Data and code availability

- The MS raw data has been deposited on Mendeley Data (https://doi.org/10.17632/hstb94ddn4.1) and is publicly available as of the date of publication. The DOI is listed in the key resources table.
- This paper does not report the original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animals

Adult male and female zebrafish were derived from a wild-type strain established from fish bought at a local shop and maintained at the Chronobiology laboratory of the University of Murcia, where all experiments using zebrafish were performed. Adult male and female cavefish *Phreatichthys andruzzii* and *Astyanax mexicanus* (Pachón cave) were obtained from a colony maintained at the University of Ferrara, where all experiments using cavefish were performed. During acclimation to laboratory conditions, all fish were kept in aquaria with a recirculating water system equipped with mechanical and biological filters. Water pH in the facility was set to 8.0 and regularly checked in the aquaria (8.04 $\pm$ 0.13, mean $\pm$ standard deviation). The temperature of the facility was maintained at 28 $\pm$ 1°C by means of an automatic air conditioning system, thereby ensuring constant water temperature in the aquaria. Zebrafish were fed twice per day with nauplii of *Artemia salina* and commercial flakes (Staple food Vipan, Sera, Heinsberg, Germany), cavefish were fed three times a week with frozen chironomid larvae and commercial flakes. Zebrafish were exposed to a 14 h light/10 h dark photoperiod using light-emitting diodes lamps placed on the ceiling of the facility and controlled by an electronic timer. Cavefish were kept in darkness except during food administration and aquaria maintenance. A total of 180 zebrafish, 88 Somalian cavefish and 64 Mexican cavefish were used in the experiments.

Ethical approval

The present research was carried out in the Chronobiology laboratories of the University of Murcia (Spain) and of the University of Ferrara (Italy). All husbandry and experimental procedures complied with European Legislation for the Protection of Animals used for Scientific Purposes (Directive 2010/63/EU). The experimental protocol was previously authorized by the Committee of Ethics in Animal Research of the University of Murcia and by the University of Ferrara Institutional Animal Care and Use Committee and the Italian Ministry of Health (auth. n. 890/2016-PR).

METHOD DETAILS

Apparatus

In all experiments, aquaria were maintained in completely isolated chronobiology chambers, where light and temperature were tightly controlled. The temperature was maintained at 28 ± 1°C by means of water heaters (50 W, Askoll, Italy) and was recorded every 10 minutes using an underwater data logger (Hobo Pendant®, Onset Computer Corporation, Massachusetts, USA). Food was provided by means of automatic feeders (3581, Eheim, Germany) located in the upper part of the aquaria and controlled by an electronic timer (Data log 2, Orbis, Spain). The food provided in all experiments (Tropical fish flakes, Prodac, Italy) was calculated as 1 % of fish body weight.

Locomotor activity was measured throughout the experimental period by means of infrared photocells (Omron, mod E3S-AD62, Japan) placed against the aquarium wall. The photocells were connected to a computer, and every time a fish interrupted the infrared light beam it induced an output signal that was recorded and stored in 10-min bins using specialized software (DIO98USB; University of Murcia, Spain).

Experimental design

We defined experimental protocols to reveal canonical properties of a feeding-entrained, circadian clock-controlled rhythm including 1) the presence of FAA, 2) the entrainment only in response to feeding cycles in the circadian range, 3) the persistence of the rhythmicity with a free-running period during a fasting period following the feeding cycle, and 4) evidence that the activity rhythm does not instantaneously follow an abrupt shift of the regular mealtime, but instead occurs progressively over the course of several transient cycles.

With this aim, fish were submitted to feeding cycles of different periods. Initially they were maintained in constant darkness (DD) and fed randomly for 1 week to avoid any synchronization. Subsequently, fish were subjected to feeding schedules in which food was available with a period of 24, 36, 44, 48, 72 and 96 hours to test the entrainment in response to feeding cycles within and outside of the circadian range. After 20-30 days of treatment, fish were starved for 15 (zebrafish) or 20 (cavefish) days, in order to check the persistence of free-running rhythms that would confirm the existence of FEO entrainment.

Finally, to test for evidence of a transient cycle following a shift of the mealtime, fish were maintained in DD and fed randomly for 1 week to avoid any synchronization and then submitted to a feeding schedule in which food was available daily at 12:00. Once fish were entrained to the 24-h feeding period, the mealtime was then delayed by 8 hours, from 12:00 to 20:00, in order to test for the presence of a transient and the capacity of the fish to readjust its rhythm to the new feeding time.

We performed 4 biological replicates (4 independent aquaria) for each feeding condition. In each aquarium we kept 7-8 zebrafish or 4-5 cavefish.

Data analysis

Analysis of locomotor activity records, representation of actograms, periodograms and daily acrophases were performed using the chronobiology software *El Temps* (version 313; Prof. Díez-Noguera, University of Barcelona). Under constant darkness and periodic feeding it is conventional to divide the 24-h cycle into 24-h Zeitgeber Time (ZT) units and indicate the time of feeding as ZT0. In the absence of all external signals (light and feeding) the 24-h circadian cycle is divided into 24-h circadian time (CT) units. As a conventional reference point, in DD and fasting the timepoint that would normally coincide with the feeding time, which is called CT0, is used.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data are reported as mean and standard deviation (mean ± S.D.). The length of the endogenous period (τ) was determined by means of the chi-square periodogram analysis at a confidence level of 95%[59] and by eye-fitting methods. Periodogram analyses were performed with intervals of 10 days. The daily acrophase (i.e., the time at which the peak of a rhythm occurs) of the locomotor activity rhythm was calculated and the average acrophase was determined by vector addition. The Rayleigh test was used to test whether the acrophases deviated from uniform and whether they were concentrated at a given time of the day ($p < 0.05$). Hotelling's paired test was performed to test for differences ($p < 0.05$) among average acrophases.[60,61] The duration of FAA was determined as the time elapsed between feeding time and the rise of anticipatory activity, which was defined as a 2.5-fold increase over baseline activity sustained for at least 30 min and not followed by any inflection for more than 1 h, as described elsewhere.[62] The baseline activity was defined as the median of the daily locomotor activity.