CAVEFISH CLOCKS

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TELLING TIME IN THE DARK

NICHOLAS S. FOULKES

The circadian clock is the chronometer of life. Among other things, this 'biological clock' determines our waking, sleeping and mealtimes, and it influences our heart rate, blood pressure and body temperature. The clock is set by external stimuli, usually light, to follow a 24-hour rhythm. But what about organisms whose habitats are so isolated from the earth's surface that they are never reached by a single ray of sunlight? In their research, Heidelberg zoologists address the question whether these animals have no sense of time at all, or whether they use other natural 'time givers' to regulate their activity and biological processes.

Amongst extreme habitats on earth, some of the most fascinating and enigmatic are deep, subterranean caves where perpetual darkness reigns. Despite the reliance of life on sunlight, these environments are home to a strange collection of organisms ranging from bacteria to vertebrates. Many of these cave inhabitants share a set of striking physiological and anatomical adaptations for life under complete darkness, so-called troglomorphisms. These are subdivided into constructive and regressive traits. Constructive traits are frequently linked with enhancing non-visual senses which allow animals to navigate without light, as well as metabolic adaptations for surviving with limited sources of food. By contrast, regressive traits are associated with loss of light-dependent functions that are predicted to serve no purpose in complete darkness such as eye loss and reduced body pigmentation.

Why do animals inhabit such hostile environments? In the case of aquatic animals, it is thought that major colonisation of cave environments came under the selective pressure of climate change that left surface habitats uninhabitable. For example, major colonisation events have been linked with the Messinian salinity crisis during the Miocene (7 million years ago) and Pliocene (5 million years ago) eras, and inter-glacial aridity in the Pleistocene era (2.5 until 0.5 million years ago).

A group of several hundred unrelated fish species distributed throughout the world are amongst the most impressive examples of cave dwelling animals. Their aquatic habitats not only lack light, but also frequently feature high salinity, high temperatures, lack of oxygen, and high concentrations of natural toxins. As a result, these cavefish also tend to show a significantly enhanced tolerance of harsh environments. In this regard, it is misleading to consider all cave habitats equivalent. The geology of each cave is unique. It is defined by the degree and duration of isolation from surface water, the properties of the water, as well as food availability. Thus each cavefish population faces a very different set of challenges to its survival.

Isolation from the day-night cycle

One fundamental consequence of life in constant darkness is that it involves isolation from the day-night cycle. This

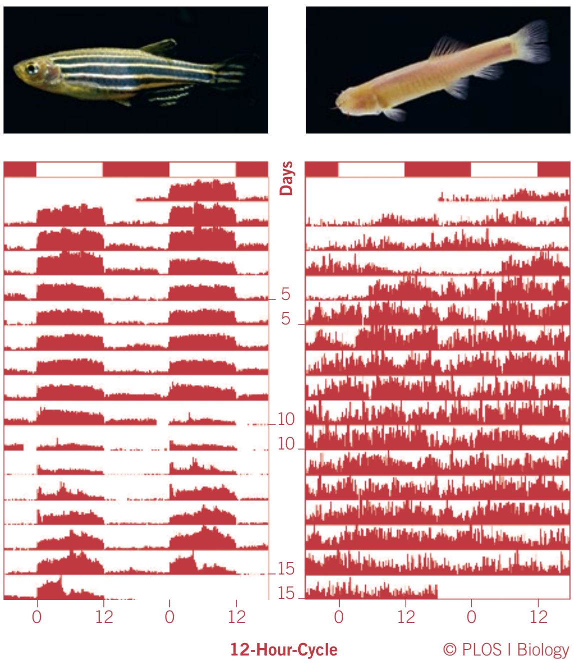

Figure 1: Actograms of cavefish (Phreatichthys andruzzii) and zebrafish (Danio rerio) showing locomotor activity under light-dark cycles aproportional to the level of activity. Each horizontal bar represents activity during a 48-hour period with each day double-plotted to facilitate analysis.

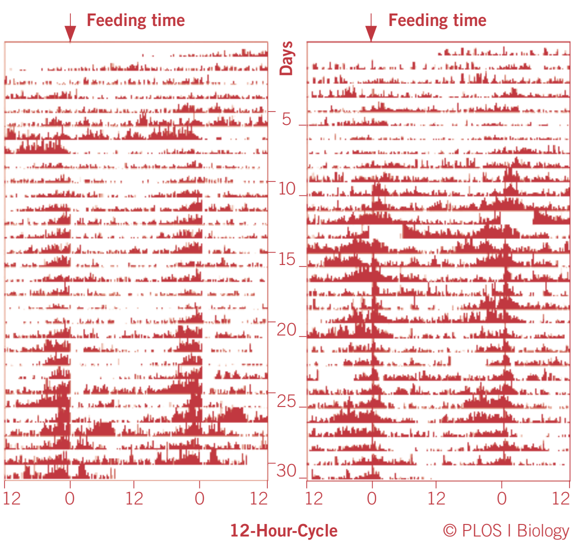

Figure 2: Actograms of cavefish (Phreatichthys andruzzii) and zebrafish (Danio rerio) showing locomotor activity under constant darkness but with daily feeding (indicated as feeding time). The height of each vertical bar is proportional to the level of activity. Each horizontal bar represents activity during a 48-hour period with each day double-plotted to facilitate analysis.

24-hour cycle has dominated life since its very origin and has shaped many aspects of the physiology and behaviour of organisms. One of the most ancient adaptations was the evolution of the circadian clock, an endogenous timing system that allows animals – and humans – to anticipate the progression of the day-night cycle. These clocks rely on daily resetting by environmental signals such as light and food availability to ensure that they remain perfectly synchronised with the environmental cycle. Do cavefish that live in completely dark, relatively unchanging environments still have clocks? If so, do they still fulfil some important function? Do cavefish clocks still respond to light or other environmental signals? Fundamental to addressing these questions is an understanding of the extent to which individual cave habitats are isolated from the day-night cycle. For example, although no regular sunlight may reach the cave water systems, it is still possible that food might be carried into the cave periodically by roosting bats, thereby imposing a 24-hour cycle on the cave environment.

In order to answer some of these basic questions, my group at the Heidelberg Centre for Organismal Studies (COS) and the Karlsruhe Institute of Technology – in collaboration with Dr Cristiano Bertolucci from the University of Ferrara, Italy – is studying an extreme cavefish species from Somalia, *Phreatichthys andruzzii.* This fish inhabits layers of water contained within limestone rock formations deep beneath the Somalian desert. The only contact between the water layers where *P. andruzzii* lives and the surface are small, deep natural wells in the desert. Based on the geology of the region, it is predicted that these animals have been isolated from the surface for around two million years as a result of desertification in that region of Africa. Consistent with the relatively long period of isolation in their cave environment, these fish show extreme troglomorphisms such as a complete loss of eyes and body pigmentation as well as an ability to survive long periods of starvation.

Of course, collecting and studying these animals in their natural habitat is currently impossible. However, during the 1970s fish were collected from Somalia by Italian scientists and have since been maintained in aquaria at the universities of Florence and Ferrara in Italy. As a result, we have access to a large colony of adult fish. One characteristic of cave animals is longevity. Indeed, some of the adult *P. andruzzii* originally collected in Somalia around 40 years ago are still alive and reproductively active. However, we have also optimised methods for inducing reproduction in this species and so we also have access to eggs, embryos, larvae and juvenile forms.

Food as an alternative 'timer giver'

Our first experiments revealed that upon artificial exposure to light-dark cycles, *P. andruzzii* failed to display activity rhythms that are typical of other surface fish such as the

PROF. DR NICHOLAS S. FOULKES has been a member of the Centre for Organismal Studies (COS), Heidelberg as well as a group leader at the Institute of Toxicology and Genetics at the Karlsruhe Institute of Technology since 2007. He originally trained as a zoologist at the University of Oxford, England, where he obtained his master and doctoral degrees. Following that, Nicholas Foulkes carried out his post-doctoral training at the 'Institut de génétique et de biologie moléculaire et cellulaire' in Strasbourg, France and held a Directeur de Recherche position at the 'Centre national de la recherche scientifique'. In 2000 he came to Germany and launched his own research group at the Max Planck Institute for Developmental Biology in Tübingen.

Contact: nicholas.foulkes@kit.edu

zebrafish *(Danio rerio)* (see Figure 1, page 102). Importantly, this lack of an endogenous rhythm was also observed at the level of clock regulated gene expression in many tissues. Does this mean that *P. andruzzii* lack a functional clock or alternatively, do they have a blind clock? We addressed this question by establishing cell cultures from *P. andruzzii* fin clips and then artificially inducing clock function in these cells by transient treatment with serum.

These in vitro experiments revealed that *P. andruzzii* cells do indeed contain circadian clocks and confirmed that these clocks are effectively blind. By cloning and sequencing candidate photoreceptors, we were able to show that *P. andruzzii* carries mutations in two opsins: melanopsin (Opn4m2) and TMT opsin. Opsins are the key light-sensitive proteins of the rod and cone photoreceptor cells in the retina but are also widely expressed in fish tissues. Our data indicates that melanopsin and TMT opsin normally enable fish cell clocks to respond to light. However, our cell culture experiments also revealed another strange property of these cavefish clocks: they tick with an abnormally long, so-called infradian rhythm of around 40 hours instead of the normal rhythms of approximately 24 hours observed in most other organisms.

It is tempting to interpret this striking result as evidence that this species is in the process of losing its normal circadian clock. Alternatively, might there be some advantage to having a clock with these abnormal properties? The story takes a complex twist with our discovery that regular feeding (one meal provided at the same time each day) is sufficient to establish robust 24-hour activity rhythms (Figure 2, page 102). More specifically, each day the fish become more active just before food is available. Furthermore, in these regularly fed fish, robust 24-hour rhythms of clock gene expression are observed in most tissues. This striking result conflicts with the cell culture data that show abnormally long rhythms, and indicate that *P. andruzzii* does possess a strongly food-regulated circadian clock.

We speculate that it is of utmost importance for the survival of *P. andruzzii* to maintain a food-responsive 24-hour clock in their subterranean environment. Specifically, food might be available at a regular time each day and so it is vital that the fish are prepared to consume it. One key clue that would tend to support this idea is that the original collectors in Somalia were only able to catch the fish in the desert wells during the night. Could it be that the fish were driven to these wells each night where they may be able to catch food, but were prevented from going there during the day, when their bright pink colour would make them easy targets for predators? In such a scenario, having a robust 24-hour food-regulated clock would make the difference between life and death.

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Blind, yet sensitive to light

So far, it would be tempting to conclude that *P. andruzzii* is unable to detect or respond to light. However, this is far from the truth. The behaviour of these fish, like that of many other cave animals, is strongly influenced by light, although with a significant wavelength dependence. We have shown that *P. andruzzii* exhibits a strong avoidance of green light, a so-called photophobic response. This is based on the normal expression of the photoreceptor *exorhodopsin* in certain brain structures of many fish species, including *P. andruzzii*. The significance of this behavioural response is unclear, however it is tempting to speculate that green light avoidance may be linked with controlling the movement of the fish in their habitat, possibly preventing their access to the wells during the day. In this regard, photophobic behaviour might be essential to modulate their feeding or possibly even their reproductive behaviour. The quality of the water, perhaps enriched with algae, may lead to a filtering of light and a consequent enrichment of green wavelengths in the well water.

Different environments, different adaptations

An obvious question is whether the adaptations we have described in *P. andruzzii* are encountered in other cavefish species which inhabit different habitats. So far, the circadian clock has only been studied in detail in one other cavefish species: the Mexican tetra *(Astyanax mexicanus)*. The cave environments of this species differ fundamentally from those of *P. andruzzii* in that they are not located beneath a desert. Instead they are in a mountainous region where surface water and rivers are connected with the cave water systems. Thus in this environment, the cave water systems are significantly less isolated than in the case of the Somalian fish.

Indeed, *A. mexicanus* has successfully colonised both surface and cave environments. The surface forms of this species have normal body pigmentation and eyes and they superficially look like different species when compared with their eyeless, pink-white cave relatives. The possibility of crossing these surface forms with the cave forms and generating fertile hybrid offspring has led to this species being used as a powerful model to study the genetic basis of troglomorphic phenotypes. However, the incomplete isolation of the cave and surface forms has also led to a genetic flow between the two. Combined with a shorter period of isolation in the cave habitat (predicted to be in the order of 100,000 years), this has resulted in less extreme troglomorphic phenotypes than those observed in *P. andruzzii*.

To add even more complexity to this situation, these Mexican habitats represent a complex network of partially interconnected caves, and individual Astyanax colonies exist within each cave that experience different degrees of isolation and show different degrees of troglomorphic phenotypes. Another important difference between the cave habitats in Somalia and Mexico is that the Mexican caves are also a roosting site for bats. These fly in and out of the cave systems at dawn and dusk in order to feed, and their return to the roosting site and the associated production of guano represents a regular supply of food for the fish. Thus in many ways, *A. mexicanus* has a closer link with the day-night cycle than *P. andruzzii*.

Possibly reflecting these environmental differences, the cave forms of *A. mexicanus* still possess light-entrainable circadian clocks that show relatively subtle differences compared with normal surface fish. More specifically, under artificial laboratory light-dark cycle conditions, both surface and cave *Astyanax* exhibit cycles of clock gene expression which are more dampened in the cave forms. Curiously, the particular expression profiles of a range of clock genes are reminiscent of those observed under exposure to constant light. In the actual cave environments, certain *Astyanax* populations show no rhythmic clock gene expression but again display sustained upregulation of clock genes normally driven by light. The significance of these molecular changes remains unclear, but these findings reinforce the notion that cavefish adaptations related to circadian rhythms and photoreception are very much environment-specific.

The loss of eyes

Of course, the most striking phenotype related to light sensing in cavefish is the loss of eyes and visual photoreception. Characteristically, in most cavefish studied to date a complete eye is formed during early embryonic development, but subsequently lost. The mechanisms underlying this eye loss have been the subject of many studies in *Astyanax*. In this species, eye loss has been attributed

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From molecules to living systems

The Centre for Organismal Studies (COS) is the largest life science research centre at Heidelberg University. Researchers at the COS want to investigate the complex biological mechanisms of living systems across all scales and organisational levels: from the molecular and cellular level to the analysis of entire organisms within the context of their environment. The centre has 15 departments and is home to nine independent junior research groups; all in all, it consists of 40 research groups with nearly 400 members. The Centre for Organismal Studies was founded in 2010 through the merging of the Heidelberg institutes of zoology and plant sciences; it is one of the University's central research institutions.

www.cos.uni-heidelberg.de

DIE INNERE UHR DER HÖHLENFISCHE

ZEITMESSUNG IM DUNKELN

NICHOLAS S. FOULKES

Zu den extremsten Lebensräumen auf der Erde gehören unterirdische, ewig dunkle Höhlen, in die kein Lichtstrahl vorzudringen vermag. Und doch sind diese unwirtlichen Höhlen, in denen es weder Tag noch Nacht gibt, Heimat zahlreicher Organismen – unter anderem einer Reihe außergewöhnlicher Fischspezies. Sie haben sich an ihre Umgebung angepasst und zeichnen sich durch auffallende sogenannte troglomorphe Merkmale aus, etwa fehlende Augen und Körperpigmentation sowie die Fähigkeit, lange Zeit ohne Nahrung auszukommen. Für Zoologen ergibt sich hieraus eine spannende Frage: Besitzen diese Höhlenfische ebenfalls einen endogenen Tag-Nacht-Rhythmus – die sogenannte „innere Uhr" –, und wenn ja, wie wird dieser ohne den Einfluss des Sonnenlichts reguliert?

In Zusammenarbeit mit Wissenschaftlern der italienischen Universität Ferrara und der Gruppe von Jochen Wittbrodt am Heidelberger Centre for Organismal Studies (COS) untersuchen wir die innere Uhr, die Lichtwahrnehmung und die Mechanismen des Augenverlustes bei „Phreatichthys andruzzii". Dies ist ein Höhlenfisch, der in unterirdischen Gewässern in der somalischen Wüste lebt und eindrucksvoll belegt, wie anpassungsfähig Organismen sind. Unsere Arbeiten zeigen, wie wichtig die genaue Kenntnis von Lebensräumen ist, um die Adaptions-Strategien einer Spezies zu verstehen. Darüber hinaus gilt: Kennen wir die spezifischen Adaptionen eines Lebewesens, lässt dies Rückschlüsse auf die Umwelteinflüsse zu, denen es ausgesetzt ist. Zudem können wir von „Phreatichthys andruzzii" zentrale Erkenntnisse über grundlegende molekulare Mechanismen gewinnen, etwa indem wir verstehen, wie genau es zu dem Verlust der Augen kommt. ●

PROF. DR. NICHOLAS S. FOULKES forscht seit 2007 am Heidelberger Centre for Organismal Studies (COS) sowie als Gruppenleiter am Institut für Toxikologie und Genetik des Karlsruher Instituts für Technologie (KIT). Nach dem Studium der Zoologie und der Promotion an der Universität Oxford in Großbritannien verbrachte er seine Postdoc-Phase am „Institut de génétique et de biologie moléculaire et cellulaire" in Straßburg und arbeitete anschließend als „Directeur de Recherche" am „Centre national de la recherche scientifique". Im Jahr 2000 kam Nicholas Foulkes nach Deutschland und gründete eine eigene Forschungsgruppe am Max-Planck-Institut für Entwicklungsbiologie in Tübingen.

Kontakt: nicholas.foulkes@kit.edu

„Ein Leben in ewiger Dunkelheit bedeutet ein Leben ohne natürlichen Tag-Nacht-Rhythmus. Höhlenfische belegen eindrucksvoll, wie anpassungsfähig Organismen selbst unter widrigsten Umweltbedingungen sind."

to interference during eye field patterning. Furthermore, apoptosis – cell death – in the lens appears to play a coordinating role.

In the case of *P. andruzzii*, Jochen Wittbrodt's lab at COS, University of Heidelberg, have studied the expression of marker genes involved in eye patterning, morphogenesis, differentiation and maintenance. In contrast to *Astyanax*, eye field patterning and evagination of the optic vesicles appears to proceed normally. However, the subsequent differentiation of retinal cell types is arrested during generation of retinal ganglion cells, which also fail to project correctly to the optic tectum – an area of the brain that receives input from the eyes and other sensory systems. The death of retinal cells progresses in a wave-like manner and eliminates progenitor cells that fail to differentiate. Thus, evolution has targeted late retinal differentiation events, indicating that there are several ways to discontinue the development and maintenance of an eye.

Many lessons to be learned

In conclusion, there are many lessons to learn from studying cavefish. First and foremost, these extraordinary eyeless, pink species are fascinating animals which catch the public's attention: Watching them swimming and navigating effortlessly through a complex environment, it is difficult not to be impressed. The fact that fish can successfully colonise such extreme environments is a testament to the adaptability of living systems. Secondly, cavefish are a powerful illustration of how knowledge of the natural habitat of animals is of fundamental importance for understanding the functional significance of specific adaptations. Furthermore, documenting combinations of specific adaptations can also help us to build a better picture of an animal's natural habitat – very much like reconstructing a crime scene in a detective story. For example, detailed knowledge of how *P. andruzzii* times its feeding behaviour and responds to certain wavelengths of light allows us to make predictions about their inaccessible and hostile subterranean environment. Finally, cavefish can teach us a lot about basic molecular mechanisms. Thus, identifying mutations in opsin photoreceptors in *P. andruzzii* helps us identify the photoreceptors that entrain clocks in normal fish species. In addition, through the knowledge of the mechanisms leading to eye loss we are able to understand the fundamental mechanisms that shape the normal development of the vertebrate eye. ●

"Knowledge of the natural habitat of animals is of fundamental importance for understanding the functional significance of specific adaptations."