SYMPOSIUM

Biological Clocks and Visual Systems in Cave-Adapted Animals at the Dawn of Speleogenomics

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Synopsis Cave-adapted animals are characterized by extreme reduction or complete absence of eyes, reflecting their lack of exposure to daylight. Given the overall constancy of abiotic variables in the cave environment, one would also expect the relaxation and eventual loss of circadian rhythms, and with it, the regressive evolution of the underlying genetic biological clock. Remarkably, however, recent behavioral and molecular studies converge with earlier evidence that the biological clock remains conserved in cave-adapted species. Reviewing the structural and behavioral data on the relationship between the visual system and the biological clock in cave species, I assess the potential of deep sequencing for elucidating their evolutionary conservation and adaptive significance in the subterranean fauna. The combined evidence confirms the widespread conservation both of the visual system and of the behavior regulated by the biological clock in cave species. The data from over 40 vertebrate and arthropod species further reveal that the absence of activity rhythms is correlated with the regression of the visual system. At the same time, the network of biological clock genes is likely to be generally conserved in cave species, regulating rhythmic behaviors in response to non-visual cycling variables as well as organismal homeostasis. Arrhythmic, eyeless species of cave beetles emerge as the most stringent choice for using deep-sequencing approaches to test and explore the conservation of the biological clock, independently of the visual system.

Introduction

Since its discovery by de Mairan in 1729, the biological clock is usually associated with the autonomous cycling of behaviors as its output, adjusted to the circadian change of daylight, setting the clock as zeitgeber. This “surface perspective” has led to the prediction of increasing dispensability of both the biological clock and the visual system when species become established in environments, such as caves, that are devoid of daylight (Poulson and White 1969; Lamprecht and Weber 1992; Trajano et al. 2009). The loss of the biological clock as a behavioral organizer is further predicted in cave species based on the general constancy of both abiotic and biotic variables in subterranean environments, implying a lack of directive information from the zeitgeber. Troglomorphs, animals that are characterized by the signature morphological characteristics of cave adaptation, such as the loss of eyes and body pigmentation (Christiansen 2005), thus have special significance in discussions of the biological clock (Yerushalmi and Green 2009).

Even the first experiments addressing the hypothesized correlation of troglomorphy with regression of the biological clock revealed that the situation might be more complex. The initial test for temporal activity patterns in the cave crayfish _Orconectes pellucidus_ resulted in the conclusion that rhythmic elements were lacking (Park et al. 1941). The later analysis of the same data with improved statistical tools, however, detected significant circadian rhythmicity in the activity of this species (Brown 1961). In support of this result, a new series of experiments on _O. pellucidus_ confirmed rhythmic patterns in activity and also found oxygen consumption to be rhythmic (Jegla and Poulson 1968). These findings were
network of transcription factor genes such as period (per), cycle (cyc), Clock (Clk), and timeless (tim) are conserved not only in the Metazoa but also in unicellular eukaryotes such as yeast (Konopka and Benzer 1971; Bell-Pedersen et al. 2005; Stöckel et al. 2008; Simonetta et al. 2009; Eelderink-Chen et al. 2010; Reitzel et al. 2010; van der Linden et al. 2010). Further, both in vertebrates and invertebrates the biological clock not only regulates major behavioral routines from central pacemaker cells but is an important part of optimal performance by cells and organs that is contingent on the control via endogenous, and thus peripheral, clocks (Yamazaki et al. 2000; Ivanchenko et al. 2001; Nagoshi et al. 2004; Yoo et al. 2004; Dijk and von Schantz 2005; Innominato et al. 2010). It has therefore been argued that conservation of the clock in cave organisms could be explained by a need for coordinating metabolic processes (Oda et al. 2000). This scenario implies that the regression of the visual system in troglomorphs may not be obligatorily linked to a regression of the biological clock. In addition, it is conceivable that the biological clock remains conserved in cave species due to its role in the regulation of sleep and resting patterns (Allada and Siegel 2008; Wisor et al. 2008; Mongrain et al. 2011). In support of this, recent studies in fish confirmed the conservation of sleep in troglomorphs as well as of circadian patterns of sleep allocation (Duboué and Borowsky 2012).

Given the forthcoming integration of high-throughput sequencing technologies in the experimental design of genetic studies of cave adaptation (Juan et al. 2010), it is reasonable to predict that genomic methods are about to rapidly advance our insights into the evolution of vision and of the biological clock of cave species. It therefore seems timely to review the available phenotypic evidence for a relationship between vision and the conservation of the biological clock in cave species. By helping to develop predictions and working hypotheses, the comprehensive compilation and analysis of the phenotypic evidence will help to identify promising species for genomic analyses.

**The spectrum of regression in the visual systems of cave species**

Eutrogophilic species (facultative soil-inhabiting or water-inhabiting cave dwellers, *sensu* Sket 2008) and troglobiotic species (obligate soil-inhabiting or water-inhabiting cave dwellers, *sensu* Sket 2008) usually are characterized by some form of reduction of the major lateral photosensory organs, the eyes. The quantitative nature of this reduction has led to sorting into three categories: (1) In macrophthalmic species, the visual system is not substantially smaller than that in allied surface-dwelling (epigean) species. (2) In microphthalmic species, reduction of the eye ranges from mild, but significant, to close to complete. (3) Anophthalmic species are defined by the complete lack of the visual system. An example of this range can be found in the small carrion beetles of the genus *Ptomaphagus*, which includes macrophthalmic eutroglophiles (Fig. 1a), mildly microphthalmic eutroglophiles (Fig. 1b), severely microphthalmic troglobionts (Fig. 1c–e), and anophthalmic troglobionts (Fig. 1f). Besides this morphological classification, it is necessary to discriminate among species in which the visual system never develops versus species in which development proceeds to the formation of functional visual tissues but is aborted at some point, leading to adult-specific anophthalmia. Further, in many species troglomorphy constitutes an extreme morphotype or ecotype. In this case, fully-eyed surface populations co-exist with eyeless cave populations, and these populations can interbreed despite their extensive phenotypic and genetic differentiation. Importantly, this situation implies the potential for gene flow between eyed surface forms and anophthalmic cave forms (Gross 2012). The above cases of adult-specific and population-specific anophthalmia contrast with what will be referred to as primary anophthalmia, defined as the lack of eyes at any stage of the life cycle and across populations. Finally, it needs to be noted that anophthalmia does not rule out the presence of other light-sensing mechanisms. This includes extraretinal photoreceptors such as in the pineal organ or dermal photoreceptors (Shand and Foster 1999; Xiang et al. 2010; Ullrich-Lüter et al. 2011). Moreover, the assumption of anophthalmia is usually based on the absence of peripherally visible visual organs, which may overlook internalized, extremely reduced vestiges of visual organs.

**Common approaches in the behavioral study of the biological clock in cave species**

Previous work on the consequences of cave adaptation on the biological clock has focused on three
aspects: (1) the identity of zeitgeber variables, which, alternatively or in addition to light, may include temperature, food, and social experience (Collins and Blau 2007), (2) clock output in the form of rhythmic patterns in behaviors like locomotion, and also in a few cases of metabolic and cellular processes to explore the possible conservation of peripheral clocks, (3) the possible evolution of non-circadian clocks due to the lack of diurnal zeitgeber cycles. The latter includes the phasing either at a lower frequency (infradian), which results in longer cycles than the circadian rhythm of the surface world, or phasing at a higher frequency (ultradian), resulting in shorter cycles.

Fig. 1 Different degrees of eye regression in leiodid cave beetles (Ptomaphagus). (a) Ptomaphagus delsur: macrophthalmic surface species. (b) Ptomaphagus geomys: a mildly microphthalmic species. (c) Ptomaphagus shapardi: a moderately microphthalmic species. (d) Ptomaphagus alleghenyensis: a moderately microphthalmic species. (e) Ptomaphagus hirus: a strongly microphthalmic species. White arrow points toward the lateral eye. (f) Ptomaphagus troglodytes: a nearctic anophthalmic species. White arrow indicates area of ancestral eye.
For all of these investigations, the test of the control of cycling by a biological clock is the continuation of cycling in the absence of input from a zeitgeber. With respect to light, this means that cycling entrained by diurnal changes of light and dark phases (LD) continues to “run free” in continuous darkness (DD) or light (LL). In addition, the biological clock can be indicated by anticipatory activity at LD. This describes the reproducible initiation or termination of activity preceding the switch of zeitgeber qualities (Lamprecht and Weber 1992). The majority of studies of cave species have explored activity patterns in DD and LD. Some have also investigated how these patterns develop in response to the zeitgeber’s phase changes. In the most extreme cases, this can lead to an effect called masking, which describes the immediate adjustment of the activity rhythm to the new phasing of the zeitgeber (Aschoff 1988). The observation of masking indicates the effect of mechanisms overruling the clock in the adjustment of behavioral rhythms to environmental rhythms. If behavioral rhythms are strongly responsive to light in this way, the behavior can become diagnostic of visual sensitivity in presumed anophthalmic species.

Structural and behavioral data on the visual system and the biological clock in cave species

Following up on the work on O. pellucidus, a large number of vertebrate and arthropod troglobionts have been investigated with respect to both output of the biological clock and the organization of the visual system. This section explores the data from 27 arthropod species and 14 vertebrate species, sorted into four groups by the degree of retention of the visual system (macrophthalmic, microphthalmic, adult-specific or population genetic anophthalmy, and primary anophthalmy species). Although not exhaustive, this compilation is informative regarding correlated trends in the evolution of the biological clock and the visual system.

Macrophthalmic troglobionts

Although microphthalmal and anophthalmy arguably dominate the cave fauna, important macrophthalmic forms exist. Examples are found among ground beetles (Carabidae) and crickets (Orthoptera) (Table 1). In the former, a selection of eutroglophile species has been investigated in detail, revealing, without exception, free-running circadian locomotor activity consistent with a mild degree of cave adaptation and continued dispersal outside caves (Weber 1980; Rusdea 1992).

Microphthalmic cave species

The cave salamander Proteus anguinus

There are two species among the 11 microphthalmic cave inhabitants covered here (Table 2) which have exceptional features. This includes the European cave salamander, Proteus anguinus, which represents a paradigm example of troglomorphy. The evolution of its extremely extended life span, coupled with low reproductive and metabolic rates, has given this species a special place in the subterranean world (Durand 1973; Kos et al. 2001; Schlegel et al. 2009; Voituron et al. 2011). Importantly, the troglo-morphic P. anguinus forms represent morphotypes of a subspecies complex, which includes epigean forms. The cave populations have been proposed to constitute the subspecies P. anguinus anguinus versus the pigmented and eyed form P. anguinus parkelf that
inhabits surface streams (Grillitsch and Tiedemann 1994; Sket and Arntzen 1994; Sket 1997; Goricki and Trontelj 2006). The latter possesses a regular vertebrate retina with rods, red-sensitive cones, and an as yet uncharacterized UV-sensitive photoreceptor (Kos et al. 2001; Schlegel et al. 2009). In contrast, the visual system of the troglobiotic _P. anguinus anguinus_ is described as “degenerated.” Eyes do develop in the embryo and degenerate photoreceptors have been found to still express rhodopsin and red-sensitive cone opsin (Kos et al. 2001). Further, both physiological and neuroanatomical data indicate continued functionality of the postembryonically regressed eyes, thus constituting extreme microphthalmwy (Hawes 1946; Gogala et al. 1965; Durand 1971; Zener 1973). In support of this notion, _P. anguinus anguinus_ exhibits negative phototactic reactions to stimulation by light in the laboratory (Schlegel et al. 2009). Also noteworthy is the immunohistochemical evidence for dermal photosensitivity in the cave form (Schlegel et al. 2009).

Early studies generated mixed results regarding circadian rhythms in _P. anguinus anguinus_. One set of experiments demonstrated circadian and ultradian activity rhythms (Briegleb and Schatz 1974), while an independent study following activity over nearly 350 days produced only tentative evidence of a circadian rhythm as well as ultradian and infradian rhythms (Schatz et al. 1977). Testing a larger number of individuals under DD did not indicate significant behavioral rhythms (Hervant et al. 2001). However, response to zeitgebers was not investigated and the analysis in _P. anguinus anguinus_ was hampered by the low levels of activity in a species with extremely low metabolic rates. Moreover, it is possible that animals from different populations differ in their expression of activity rhythms, considering the broad distribution and phenotypic range of _P. anguinus_. The classification as arrhythmic microphthalmic troglobiont suggested by the combined studies is therefore somewhat preliminary.

### The catfish _Rhamdia enfurnada_

The vertebrate examples of microphthalmic troglobionts further include the catfish _R. enfurnada_. This species is characterized by extensive population-genetic variation of troglomorphic traits. Based on external morphology of the eyes, the visual system can be highly reduced but is never fully missing. Consistent with the variable expression of troglomorphic traits, individuals that were tested for behavioral output of the biological clock varied by expressing either circadian or ultradian activity patterns in DD (Trajano et al. 2009).
Table 2 Circadian rhythms in microphthalmic cave species with severe (~50%) to near complete reduction of the eye

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Class</th>
<th>Order</th>
<th>Family</th>
<th>Species</th>
<th>Ecology</th>
<th>Morphotype</th>
<th>Phototaxis</th>
<th>Readout</th>
<th>DD</th>
<th>LL</th>
<th>LD</th>
<th>Reference</th>
</tr>
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<tr>
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<td>Hexapoda</td>
<td>Coleoptera</td>
<td>Carabidae</td>
<td><em>Loemasterus navarius</em></td>
<td>Troglobion</td>
<td>NR</td>
<td>Not tested but dark active</td>
<td>LA</td>
<td>Circadian</td>
<td>Circadian</td>
<td>Circadian</td>
<td>Weber (1980); Lamprecht and Weber (1977); Lamprecht and Weber (1975)</td>
</tr>
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<td>Coleoptera</td>
<td>Carabidae</td>
<td><em>Loemasterus schreibersi</em></td>
<td>Eutrogophile</td>
<td>NR</td>
<td>Not tested but dark active</td>
<td>LA</td>
<td>Circadian</td>
<td>Circadian</td>
<td>Weber (1980); Rusdea (1992)</td>
<td></td>
</tr>
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<td>Hexapoda</td>
<td>Coleoptera</td>
<td>Carabidae</td>
<td><em>Rhodine rubra</em></td>
<td>Troglobion</td>
<td>NR</td>
<td>NR</td>
<td>LA</td>
<td>Circadian</td>
<td>NR</td>
<td>NR</td>
<td>Wiley (1973)</td>
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<td>Hexapoda</td>
<td>Coleoptera</td>
<td>Carabidae</td>
<td><em>Sphodropsis ghikani</em></td>
<td>Troglobion</td>
<td>NR</td>
<td>NR</td>
<td>LA</td>
<td>Circadian</td>
<td>NR</td>
<td>NR</td>
<td>Weber et al. (1994)</td>
</tr>
<tr>
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<td>Hexapoda</td>
<td>Coleoptera</td>
<td>Carabidae</td>
<td><em>Typhlochoromus stolzi</em></td>
<td>Troglobion</td>
<td>NR</td>
<td>Weak</td>
<td>LA</td>
<td>Largely periodic</td>
<td>Largely aperiodic</td>
<td>Mostly circadian</td>
<td>Barkowiak et al. (1991); Lamprecht and Weber (1983)</td>
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<td>Diptera</td>
<td>Keroplatidae</td>
<td><em>Arachnocampa tasmaniensis</em></td>
<td>Troglobion</td>
<td>NR</td>
<td>NR</td>
<td>Bioluminescence</td>
<td>Circadian</td>
<td>NR</td>
<td>Circadian</td>
<td>Merritt and Clark (2011)</td>
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<td>Decapoda</td>
<td>Cambaridae</td>
<td><em>Orconectes pellicus</em></td>
<td>Troglobion</td>
<td>NR</td>
<td>Negative</td>
<td>LA</td>
<td>Circadian and infradian</td>
<td>Circadian</td>
<td>Circadian</td>
<td>Park et al. (1941); Jegla and Poulson (1968)</td>
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<td>Decapoda</td>
<td>Cambaridae</td>
<td><em>Procambarus caemicola</em></td>
<td>Troglobion</td>
<td>NR</td>
<td>NR</td>
<td>Close to circadian</td>
<td>Close to circadian</td>
<td>Circadian</td>
<td>De La O-Martinez et al. (2004); Mejía-Ortíz et al. (2003); Mejía-Ortíz and Hartnoll (2005)</td>
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<td>Siluriformes</td>
<td>Hepapteridae</td>
<td><em>Rhamda enfumada</em></td>
<td>Troglobion</td>
<td>Yes</td>
<td>NR</td>
<td>LA</td>
<td>Circadian, ultradian, individual variation</td>
<td>NR</td>
<td>NR</td>
<td>Trajano et al. (2009)</td>
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<td>Amphibia</td>
<td>Caudata</td>
<td>Proteidae</td>
<td><em>Proteus anguinus anguinus</em></td>
<td>Troglobion</td>
<td>Yes</td>
<td>Negative</td>
<td>LA, oxygen consumption</td>
<td>Inconsistent evidence</td>
<td>NR</td>
<td>NR</td>
<td>Schlegel et al. (2009); Hervant et al. (2001); Briegleb and Schatz (1974)</td>
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</table>

LA, locomotory activity; NR, not reported.
Invertebrates, behavioral data on activity patterns are available for eight eutroglophilic or troglobiotic species of arthropods, which are microphthalmic (Table 2). This includes the above-mentioned cave crayfish *O. pellucidus*, the first troglobiont tested for the loss of circadian activity (Park et al. 1941; Brown 1961; Jegla and Poulson 1968). The microphthalmic state of *O. pellucidus* is supported by the description of vestiges of the eyes (Hobbs and Barr 1972) and by experimental documentation of photonegative responses (Park et al. 1941). Similar to *O. pellucidus*, almost all of the investigated microphthalmic arthropods expressed circadian activity periodicities at both DD and LD, consistent with a light-responsive biological clock. Two exceptions are the microphthalmic ground beetle species *Laemostenus navaricus* (Lamprecht and Weber 1977) and *Typhlochoromus stolzi* (Lamprecht and Weber 1983; Bartkowiak et al. 1991). Both of these species are characterized by a very strong degree of microphthalmyn. In *L. navaricus*, a substantial proportion of tested individuals expressed aperiodic DD activity patterns. In *T. stolzi*, the ancestral compound eyes are compacted to subcuticular clusters of photoreceptors that lack ommatidial organization (Lamprecht and Weber 1983; Bartkowiak et al. 1991), very similar to the situation in highly troglophilic leiodid beetles (Fig. 1d and e). *Typhlochoromus stolzi* lacks free-running activity cycles at DD but exhibits weak circadian activity at LD.

Evidence for conservation of a biological activity clock, independent of the visual system, comes from the microphthalmic carabids *L. navaricus*, *Laemostenus schreiberi*, and *Sphodropsis ghilianii*. In these species, temperature changes also functioned as Zeitgeber although with a lower penetrance than LD cycles (Weber et al. 1994, 1995). Taken together, the evidence from microphthalmic beetles speaks in favor of a correlation between conservation of the visual system and the control of activity by the biological clock.

**Glowsworms: Arachnocampa luminosa**

The second exceptional example of microphthalmic troglobionts is represented by cave-adapted species in the keratoplatid fly genus *Arachnocampa* of Australia and New Zealand. *Arachnocampa* larvae are predatory. They produce sticky threads that capture prey attracted by bioluminescence of the larvae. Epigean *Arachnocampa* luminesce nocturnally under the control of their circadian clock. Troglobiotic *Arachnocampa* also luminesce in a circadian manner but exhibit diurnal or nocturnal cycles of producing light (Merritt and Clarke 2011). They have the capacity to maintain free-running rhythms and change their circadian or nocturnal shine in the laboratory and in the field. It has been proposed that the biological clock helps to coordinate the cycling within a population of closely neighboring animals forming carpets of synchronized luminescent glow. While this model remains to be further explored, it is clear that the biological clock in glowworms represents a special case in which its evolutionary conservation is tied to the inheritance of the clock-dependent predatory behavior of the ancestral surface form.

As to the organization of the visual system, the larvae of the New Zealand glowworm (*A. luminosa*) are equipped with small eyes (stemmata) (Gatenby 1959; Morley 2010), typical of the larval stage of holometabolous insects. Glowworms can therefore be considered microphthalmic members of the subterranean world although not exhibiting specifically troglophilic modifications compared with surface *Arachnocampa*.

**Adult-specific and population-specific anophthalmic troglobionts**

The Mexican cavefish: *Astyanax mexicanus*

Obligatory cave inhabitants that are characterized by adult-specific anophthalm, population-genetic variation of the anophthalmic state, or both, include many of the most deeply studied models of adaptation to caves to date (Table 3). Forefront among these is the Mexican cave tetra, *A. mexicanus*, which encompasses a complex of highly troglophilic cave populations as well as surface populations. The combined evidence from molecular data suggests that the 29 currently characterized cave populations originated from three major waves of colonization during the past 2–8 million years (for review, see Gross 2012). Consistent with the multiple independent colonizations of caves, the size and structural organization of rudiments of the eyes vary extensively among *A. mexicanus* cave populations. However, they are uniformly characterized by an early phase of embryonic eye development that is indistinguishable from that of surface forms, which leads to the formation of a transient stratified retina with photoreceptors (Wilkens 1988). In the cave forms, this is followed by the complete loss of the lens as well as partial loss and reduction of the retina due to programmed cell death (for review, see Protas and Jeffery 2012). As a result, only submerged...
Table 3  Circadian rhythms in adult-stage or population-genetic variable anopthalmic species

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Class</th>
<th>Order</th>
<th>Family</th>
<th>Species</th>
<th>Morphotype</th>
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<td>Maxillopoda</td>
<td>Mysida</td>
<td>Lepidomyidae</td>
<td>Speleoemyris bontazzii</td>
<td>NR</td>
<td>Not tested</td>
<td>LA</td>
<td>Circadian and ultradian</td>
<td>NR</td>
<td>Pasquali et al. (2007); Ariani and Wittmann (2010)</td>
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<td>Cypriniformes</td>
<td>Balitoridae</td>
<td>Nemocheilus evezardi</td>
<td>Yes</td>
<td>Nutrition-dependent photophobia</td>
<td>LA, phototaxis, air gulping, burying</td>
<td>Reduced penetration circadian</td>
<td>Circadian/ultradian/infradian</td>
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<td>Yes</td>
<td>Mildly photophobic</td>
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<td>Infraadian</td>
<td>NR</td>
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<td>Yes</td>
<td>Yes</td>
<td>LA, retinomotor</td>
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<td>No</td>
<td>Yes but through peripheral visual system</td>
<td>LA, peripheral clock, hatching rate</td>
<td>Arrhythmic but circadian in response to periodic feeding, infradian peripheral rhythm</td>
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<td>Hepapteridae</td>
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<td>Yes</td>
<td>Not tested</td>
<td>LA</td>
<td>Individually variable circadian and ultradian rhythms</td>
<td>NR</td>
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<td>Not tested</td>
<td>Individually variable circadian and ultradian rhythms</td>
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<td>Trichomycteridae</td>
<td>Trichomycterus sp.</td>
<td>Yes</td>
<td>Not tested</td>
<td>LA</td>
<td>Individually variable circadian and ultradian rhythms</td>
<td>NR</td>
<td>Trajano and Menna-Barreto (1996)</td>
</tr>
</tbody>
</table>

This includes species in which the adults lack peripheral visual organs, which, however, are transiently formed during embryogenesis; or species which are represented both by surface forms and by cave forms. All cave forms are troglobiotic. LA, locomotory activity; NR, not reported.
rudiments of the eye persist into the adult stage, along with relics of disorganized retinal tissues of varying size (Wilkens 1988). In *A. mexicanus* from the Molino Cave population, the transient formation of fully developed photoreceptor cells has been documented (Wilkens 2007). Of further note is the fact that the potentially light-sensitive pineal organ differs only moderately between surface and cave populations (Wilkens 1988).

Observations on two output processes, activity of the animal and movement of the granules of eye pigment, indicate the conservation of the biological clock in *A. mexicanus* cave populations. In LD, animals from surface populations have opposite circadian rhythms with respect to depth in the water (Erckens and Martin 1982a). Activity close to the surface of the water peaks in the D-phases while activity at the bottom peaks in the L-phases. Compared with surface animals, Pachon cave animals had similar, but much less pronounced, circadian rhythms in surface-directed and bottom-directed activities in LD, suggesting that some form of visual input facilitated entrainment (Erckens and Martin 1982b). For Pachon cave animals, these circadian oscillations of activity in LD have been confirmed by the recent analysis of overall resting and activity patterns. Considerably weaker to arrhythmic activity patterns were found in animals from the Tinaja and Molino caves (Duboué et al. 2011). In DD, surface animals expressed a short-term (two cycles) free-running circadian activity rhythm of both bottom-directed and surface-directed activity after entrainment in LD (Erckens and Martin 1982a, 1982b; Duboué et al. 2011). In six animals tested from the Pachon cave population, a weak free-running rhythm of surface-directed activity was detected but not any rhythm of bottom-directed activities. Taken together, these findings were interpreted to indicate that control of activity by the biological clock has been retained in troglobomorphic populations of *A. mexicanus* (Erckens and Martin 1982b).

The second line of evidence supporting retention of the biological clock comes from the observation of free-running circadian cycles in movement of granules in the retinal pigment epithelium of the rudimentary retina of animals from Chica cave. In surface fish, the movement of retinal pigment epithelium granules is part of the retinomotor mechanism that adjusts the sensitivity of the retina to the circadian change of daylight intensity (Espinasa and Jeffery 2006).

The Somalian cavefish: *Phreatichthys andruzzi*  
Both behavioral and genetic data document the conservation of the biological clock in the cyprinid Somalian cave fish, *P. andruzzi*. In contrast to *Astyanax*, this species is endemic to a completely enclosed cave habitat (Cavallari et al. 2011). Based on molecular estimates of the time since divergence from its most closely related surface ally, *P. andruzzi* is assumed to represent the result of potentially 1.4–2.6 million years of adaptation to caves (Colli et al. 2008). The morphological consequences of the presumed long-term adaptation to the cave environment include the loss of scales, eyes, and pigmentation. Nonetheless, *P. andruzzi* develops eyes during embryogenesis, which subsequently degenerate, much as in *A. mexicanus*, to the extent that histological analysis failed to detect any remnants of the lens or optic nerve (Ercolini and Berti 1975; Berti et al. 2001). *Phreatichthys andruzzi* thus constitutes an adult-specific anophthalmic species. However, *P. andruzzi* exhibits photonegative behavior in tests involving choices between light and dark (Ercolini and Berti 1975; Tarttellin et al. 2012). This response was significant for four different wavelengths (480, 539, 615, and 692 nm) and most pronounced to blue light. Given the strong degree of eye reduction in the adult, it has been proposed that this behavior is mediated by extraretinal photoreceptors (Ercolini and Berti 1975). In support of this viewpoint, the expression of two putatively functional full-length opsin transcripts (orthologs of zebrafish rod opsin and exo-rod opsin) has been recovered specifically from brain tissue (Tarttellin et al. 2012). Two further opsin genes have been found in *P. andruzzi*: TMT-opsin and melanopsin, which mediate light perception by the peripheral clock in zebrafish. In *P. andruzzi*, however, the TMT-opsin and melanopsin orthologs are non-functional based on the presence of nonsense mutations (Cavallari et al. 2011). Correlated with this condition, laboratory testing revealed that neither the central nor the peripheral clocks of *P. andruzzi* can be entrained by light. However, *P. andruzzi* possesses a functional biological clock based on the conservation of major core clock genes (*Clk1a, Clk2, Per1*, and *Per1b*) (Cavallari et al. 2011). Further study has shown that the *P. andruzzi* clock can be entrained by non-visual zeitgebers. Periodic feeding entrains free-running circadian activity as well as cycling of the peripheral clock in various tissues. Further, exposure to glucocorticoid triggers infradian periods of the peripheral clock, detectable by expression of reporter genes in cultured cells. Considering finally the loss of capacity for temperature compensation in the biological clock (Cavallari et al. 2011), *P. andruzzi* can be classified as an example of an adult-specific anophthalmic species with a partially degenerated biological clock.
The cave fish Nemacheilus evezardi

A third cave fish, for which activity patterns have been investigated in depth, is the Indian loach, *N. evezardi* (Kumar Pati 2001). As in *A. mexicanus*, troglomorphism in *N. evezardi* has an extreme variation across populations: there are surface forms, microphthalmic forms, and presumed anophthalmic forms (Pradhan et al. 1989). The microphthalmic forms exhibit a complex variability of phototactic behavior, which is influenced by daytime, feeding status, illumination of food source (light versus dark area), and light intensities (Agrawal 2008). The data indicate the preservation of functional vision as well as the retention of a biological clock. Consistent with this conclusion, additional behavioral routines exhibit free-running circadian rhythmicity, including burrowing and air gulping (specific to cave forms) (Biswas et al. 1990a, 1990b; Pradhan and Biswas 1994; Pati 2001). Finally, as in the Somalian cave fish, scheduled feeding can assume zeitgeber function in *N. evezardi* (Biswas and Ramteke 2008). Other cave-adapted species of fish

Besides *A. mexicanus, P. andruzzi*, and *N. evezardi*, there is a considerable number of cave-adapted species of loach and catfish that likewise meet the criteria of adult-specific or population-specific anophthalmy and that have been investigated for circadian behavior (Table 3). In all of these cases, evidence of free-running circadian or modified rhythms was observed under DD. One of the most noteworthy activity rhythms has been reported for the hillcrest loach, *Schistura oedipus*. The anophthalmic morphotype of this species is non-responsive to light–dark changes but exhibits a 38.5-h infradian activity rhythm (Duboué et al. 2011).

Arthropods

The only invertebrate currently known to fall into the category of adult-specific anophthalmy is the opossum shrimp, *Spelaeomysis bottazii*, which undergoes embryonic development of the visual system (Ariani and Wittmann 2010). The locomotory activity of this inhabitant of groundwater exhibits circadian and ultradian periodicities in DD (Pasquali et al. 2007).

Anophthalmic troglobions

Fish

There are currently two species of catfish (*Imparfinis* sp. and *Taunayia* sp.) and one characiform species (*Stygichthys typhlops*) that can be considered candidates for primary anophthalmy (Table 4). However, the absence of information on development and population-genetic variation recommends verification. Most intriguing is the virtual absence of rhythmic activity in the characiform *S. typhlops* both under DD and LD conditions (Trajano et al. 2012), i.e., in line with a correlation between the regression of the visual system and the control of behavior by the biological clock. The catfish species are characterized by non-circadian phasing at DD, consistent with having behavior affected by a regressed biological clock resulting from advanced adaptation to caves. At the same time, however, both species exhibit circadian activity in LD, implying responsiveness to light and recommending further caution in accepting their status as anophthalmic.

Arthropods

The most compelling evidence of anophthalmy coupled with a loss of ancestral rhythmic behavior and of response to light comes from six eyeless species of ground beetles and one eyeless small carrion beetle (Table 4) (Wiley 1973; Weber 1980). The lack of detectable rhythmic activity patterns in all of these species under DD as well as LD and LL, when tested, indicates a correlation between circadian behavior and presence of a functional visual system. Notably, these data represent consistent results from independent studies of Nearctic and Palearctic species. The apparent convergent evolution of arrhythmic activity correlated with anophthalmy suggests that this is a general evolutionary trajectory for anophthalmic troglobions, at least in the Coleoptera.

Another putative anophthalmic representative in the cave-adapted arthropod fauna is the amphipod *Niphargus puteanus*, based on the reduction of its peripheral and central visual system (Blume et al. 1962; Günzler 1964). However, *N. puteanus* are specifically night-active under LD conditions and exhibit photonegative behavior, cautioning against uncritically accepting this classification. A weak free-running follow-up response in DD following LD entrainment suggests that *N. puteanus* maintains a biological clock that regulates activity patterns.

The invertebrate section of anophthalmic cave species with behavioral documentation also includes two millipedes (Table 4), one of which, *Glyphiulus caver-nicolus sulu*, has been investigated in detail (Koilraj et al. 2000). Circadian activity patterns were recorded under LD, DD, and LL conditions. In each case, only a fraction of the tested individuals expressed rhythmic components.
<table>
<thead>
<tr>
<th>Phylum</th>
<th>Class</th>
<th>Order</th>
<th>Family</th>
<th>Species</th>
<th>Morphotype</th>
<th>Phototaxis</th>
<th>Readout</th>
<th>DD</th>
<th>LL</th>
<th>LD</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
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<td>Diplopoda</td>
<td>Julida</td>
<td>Blaniulidae</td>
<td>Blaniulus lichtensteini</td>
<td>No</td>
<td>NR</td>
<td>LA</td>
<td>Low penetrance circadian</td>
<td>NR</td>
<td>NR</td>
<td>Mead and Gilhodes (1974)</td>
</tr>
<tr>
<td>Arthropoda</td>
<td>Diplopoda</td>
<td>Spirostreptida</td>
<td>Glyphiulidae</td>
<td>Glyphius cavernicolus</td>
<td>No</td>
<td>NR</td>
<td>LA</td>
<td>Low penetrance circadian</td>
<td>Very low penetrance circadian</td>
<td>High penetrance circadian</td>
<td>Koilraj et al. (2000)</td>
</tr>
<tr>
<td>Arthropoda</td>
<td>Hexapoda</td>
<td>Coleoptera</td>
<td>Carabidae</td>
<td>Rhodina subterrana</td>
<td>NR</td>
<td>NR</td>
<td>LA</td>
<td>Aperiodic</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Wiley (1973)</td>
</tr>
<tr>
<td>Arthropoda</td>
<td>Hexapoda</td>
<td>Coleoptera</td>
<td>Carabidae</td>
<td>Rhodina tenebrosa</td>
<td>NR</td>
<td>NR</td>
<td>LA</td>
<td>Aperiodic</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Wiley (1973)</td>
</tr>
<tr>
<td>Arthropoda</td>
<td>Hexapoda</td>
<td>Coleoptera</td>
<td>Leiodidae</td>
<td>Speonomus diecki</td>
<td>NR</td>
<td>NR</td>
<td>LA</td>
<td>Aperiodic</td>
<td>Aperiodic</td>
<td>Aperiodic</td>
<td>Weber (1980); Lamprecht and Weber (1977)</td>
</tr>
<tr>
<td>Arthropoda</td>
<td>Malacostraca</td>
<td>Amphipoda</td>
<td>Niphargidae</td>
<td>Niphargus puteanus</td>
<td>NR</td>
<td>Negative phototaxis</td>
<td>LA</td>
<td>Ultradian</td>
<td>NR</td>
<td>Circadian</td>
<td>Günzler (1964); Blume et al. (1962)</td>
</tr>
<tr>
<td>Chordata</td>
<td>Actinopterygi</td>
<td>Characiformes</td>
<td>Characidae</td>
<td>Stygichthys typhlops</td>
<td>No</td>
<td>Not tested</td>
<td>LA</td>
<td>Arrhythmic</td>
<td>NR</td>
<td>Low penetrance circadian</td>
<td>Trajano et al. (2012); Trajano et al. (2009)</td>
</tr>
<tr>
<td>Chordata</td>
<td>Actinopterygi</td>
<td>Siluiformes</td>
<td>Hepapteridae</td>
<td>Imporfins</td>
<td>No</td>
<td>Positive phototaxis based on field observation</td>
<td>LA</td>
<td>Some circadian, ultradian</td>
<td>NR</td>
<td>Circadian</td>
<td>Trajano and Menna-Barreto (1995); Trajano et al. (2005); Mendes (1995)</td>
</tr>
<tr>
<td>Chordata</td>
<td>Actinopterygi</td>
<td>Siluiformes</td>
<td>Hepapteridae</td>
<td>Taunayia spec</td>
<td>No</td>
<td>No</td>
<td>LA</td>
<td>Infradian and arrhythmic</td>
<td>NR</td>
<td>Circadian</td>
<td>Trajano and Menna-Barreto (2000)</td>
</tr>
</tbody>
</table>

All of these species are troglobsyns. LA, locomotory activity; NR, not reported.
Gene-specific studies

At this point, genetic studies of the consequences of cave adaptation have been more numerous for the visual system than for the biological clock. Most of the work on visual systems attempted to understand the corollaries and mechanisms of eye regression at the level of developmental gene regulation. This aspect has been explored in depth in *A. mexicanus* (Protas and Jeffery 2012) but satellite models are being established for crustaceans (Protas et al. 2011; Aspiras et al. 2012). The first genetic evidence of conserved vision in troglobionts came from the comparative analysis of conservation of a long wavelength opsin gene in three cave crayfish species in comparison to closely related epigean allies (Crandall and Hillis 1997). Various molecular phylogenetic tests failed to detect differences between surface species versus cave species with regard to the selection pressure acting on the opsin genes. It was therefore speculated that opsin might serve additional functions besides detection of light. Indeed, lately opsin was found to be involved in thermosensation by *Drosophila* larvae (Shen et al. 2011). However, considering the mounting evidence of functional vision in many microphthalmic and anophthalmic troglobionts (Tables 2–4), including the cave crayfish *O. pellucidus*, the possibility of photosensory function of the opsins of cave crayfish needs to be given new consideration.

Other studies uncovered evidence for the evolutionary loss of visual-system-specific genes. A molecular signature of this is the accumulation of nucleotide changes that compromise the translation of a gene into a functional protein, and turning the genetic locus into a pseudogene as in the case of the above-mentioned TMT-opsin and melanopsin genes of the Somalian cavefish (Cavallari et al. 2011). Establishing an analogous case, multiple independent cases of pseudogenization of the eye-specific pigmentation gene *cinnabar* have been found in troglobiotic diving beetles of Australia (Leys et al. 2005). These data show that changes in molecular sequences can be effective indicators of regression of the visual system at the genetic level.

The conservation of biological clock genes in troglobiotic species has thus far only been investigated in the Somalian cavefish *P. andruzzi* (Cavallari et al. 2011). This scarcity of data points at a critical need for studying the genetic components of the biological clock in troglobionts.

First deep-sequencing studies

The summary view of the behavioral literature suggests that the central regulatory function of the biological clock often has remained preserved in cave species. Interestingly, this state is not strictly associated with the preservation either of eyes (*A. mexicanus*) or of light as a zeitgeber (*P. andruzzi*). The results of the first large-scale sequencing studies of the genetic organization of troglobionts appear to support this notion.

Fish: Astyanax mexicanus

Two major transcriptome studies have been launched in the Mexican cavefish. Hinaux et al. (2013) compiled a transcriptome database of 44,145 contigs assembled from expressed sequence tags generated by Sanger sequencing. This database contains sequences of eight cDNA libraries; four from a surface stream population in Texas and four from the Pachon cave population in Mexico. For both populations, libraries were generated from tissue harvested from four different embryonic and postembryonic developmental stages. Over 6400 of the contigs showed orthologous relationships to zebrafish genes. These were analyzed in detail for informative variations in sequence, which revealed 31 non-conservative amino-acid changes specific to the lineage of the cave population. The significant majority of the affected genes function in carbohydrate metabolism but no enrichment of eye developmental genes was found. The authors did note, however, that a significant proportion of 79 non-conservative amino-acid differences between the genes of surface and cave populations were observed in the transcripts of conserved genes that have been documented to be expressed in the developing eye of zebrafish (Hinaux et al. 2013). This finding suggested accelerated evolution of genes related to vision in either of the two populations.

No information on genes of the circadian clock network could be extracted from the dataset by Hinaux et al. (2013), despite the behavioral and physiological evidence of retention of the biological clock by *A. mexicanus* (Erckens and Martin 1982a, 1982b; Espinasa and Jeffery 2006). However, *A. mexicanus* orthologs of the core clock genes *Clk*, *tim*, and *per* have been recovered in the transcriptome of 22,596 contigs assembled in a parallel study using 454 sequencing data from Pachon and surface-stream animals (Gross et al. 2013). More than 600 transcripts were unique to the cave or surface populations, over 10% of which were related to genes known from zebrafish. Two classes of gene functions were overrepresented in the conserved population-specific subsamples. Metabolic genes were specific for, or transcriptionally elevated in, the cave population, consistent with the findings by Hinaux et al.
The complementary picture emerged for 16 homologs associated with visual functions, which were specifically missing or reduced in the cave population, consistent with the adult-specific anophthalmy of the cave population’s morphotype. Biological clock genes, however, did not appear to be expressed in a morphotype-specific manner. In combination, these data indicate that the visual system has experienced regressive evolution to a greater extent than have biological clock network genes in the cave population A. mexicanus.

Finally, deep-genome sequencing has been deployed to define 698 new genetic markers, improving the map of the A. mexicanus genome (O’Quin et al. 2013). These data were used to search for loci that are associated with the compromised retinal development of the Pachon cave morphotype, leading to the detection of four QTLs that are linked to candidate genes for eye development.

**Arthropods: Ptomaphagus hirtus**

The studies in A. mexicanus exemplify how transcriptomic and genomic methods advance the research progress in a well-established experimental model. Similarly, dramatic progress can be expected in lesser-studied or new models of cave adaptation. A case in point comes from a transcriptome study of the small carrion beetle P. hirtus (Fig. 1e). This troglobiont is endemic to the Mammoth cave system (Peck 1975, 1986). Early histological and ecological work concluded that P. hirtus was characterized by a relict visual system that is non-functional (Packard 1888). Deep sequencing of the transcriptome of the adult head assembled over 5500 contigs with orthology to characterized insect genes (Friedrich et al. 2011). This database included orthologs of all genes known to be essential for phototransduction in insects. Moreover, behavioral tests for light–dark choice revealed a strong photonegative behavior in P. hirtus (Friedrich et al. 2011). Thus, the genetic and behavioral data converged on revising the characterization of P. hirtus from anophthalmic to microphthalmic troglobiont. Remarkably, the assembled transcripts also included all members of the biological core clock gene network of insects. The possible regulation of activity patterns by a central biological clock in P. hirtus must still be defined.

**Summary and perspectives**

Giving credit to the notion that caves can be considered natural laboratories for studying the adaptive significance of the biological clock (Poulson and White 1969), the completed survey reveals that information on clock output as well as the regressive state of the visual system has become available for over 40 cave-dwelling species. Two of these will not be included in the analysis of correlative trends because of ambiguous (P. anguinis) or insufficient documentation (S. ghilianii) (Table 2). The comparison across groups defined by the state of regression of the visual system is fraught with further caveats. For one, there is the bias towards arthropods and vertebrates, which likely represents a research bias as well as the relative abundance of arthropods in the cave fauna (Culver et al. 2000). Secondly, in some anophthalmic species, more information is desirable for increasing the confidence in the absence of any relict visual system, e.g., Imparfinis spp., Taunayia spp., and N. puteanus (Table 4). Finally, the activity rhythms of some species are expressed only in some of the tested animals. With the data available, it is not possible to decide whether this reflects low expressivity of the biological clock gene network as a result of function-comprising mutations or an inadequate design of zeitgeber stimuli in the laboratory.

Notwithstanding these limitations, the ratio of rhythmic versus arrhythmic species in the four classes of reduction of the visual system reveals a compelling trend towards correlation of regression of the visual system with clock-regulated rhythmic behaviors (Fig. 2). Behaviorally arrhythmic species appear to be absent in all categories in which the visual system is reduced but not completely absent, including even adult-specific or population-specific anophthalmic species. In the group of primary anophthalmic species, in contrast, there is a
conspicuous representation of both rhythmic and arrhythmic species. It will be interesting to investigate in future studies how ecological variables or the evolutionary time-span of cave adaptation impact the correlation of the visual system with the evolution of the biological clock.

A second important aspect emerging from the data is the large number of microphthalmic troglobionts (11), suggesting that highly regressed visual systems persist in a considerable fraction of the subterranean fauna, possibly utilizing the diffuse light in the twilight zones of caves. Finally, the data document that the biological clock persists even in physiologically blind species. This is most clearly demonstrated in the Somalian cavefish *P. andruzzi*, in which processes regulated both by central and peripheral clocks have been retained but the responsiveness to input by the *zeitgeber* light has been lost (Cavallari et al. 2011). This finding is consistent with the hypothesis that the biological clock is retained independent of the visual system in the regulation of homeostasis (Oda et al. 2000). Arrhythmic anophthalmic troglobionts are the logical choice to test this further, taking advantage of the efficacy and sensitivity of high-throughput sequencing for exploring the genetic organization of troglomorphs. The survey specifically identifies the comparatively well-studied anophthalmic cave beetles as a promising test case. Understanding the function of the hypothesized biological clock in these, and other, troglobionts will eventually be further propelled through the identification of output processes by virtue of correlated transcript fluctuations in circadian time-series of transcriptome samples (Doherty and Kay 2010).

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**References**


