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EVOLUTION OF THE CIRCADIAN CLOCK IN EXTREME ENVIRONMENT: LESSONS FROM CAVEFISH

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CONTENTS

Abstract (English)

EVOLUTION OF THE CIRCADIAN CLOCK IN EXTREME ENVIRONMENT: LESSONS FROM CAVEFISH

Evolution has been strongly influenced by the daily cycles of temperature and light imposed by the rotation of the Earth. Fascinating demonstrations of this are seen in extreme environments such as caves where some animals have remained completely isolated from the day-night cycle for millions of years. Most of these species show convergent evolution, sharing a range of striking physical properties such as eye loss. One fundamental issue is whether "hypogean" species retain a functional circadian clock. This highly conserved, physiological timing mechanism allows organisms to anticipate daily environmental changes and is synchronized primarily by light. The Somalian cavefish, *Phreatichthys andruzzii* does possess a circadian clock that is entrained by a daily regular feeding time but strikingly, not by light. Under constant conditions the *P. andruzzii* clock oscillates with an extremely long period and also lacks normal temperature compensation. We document multiple mutations affecting a light-induced clock gene, *Period2* as well as the genes encoding the extra-retinal photoreceptors *Melanopsin* (*Opn4m2*) and *TMT-opsin*. Remarkably, we show that ectopic expression of zebrafish homologs of these opsins rescues light induced clock gene expression in *P. andruzzii* cells. Thus, by studying this natural mutant we provide direct evidence for a peripheral light-sensing function of extra-retinal opsins in vertebrates. Furthermore, the properties of this cavefish illustrate that evolution in constant darkness leads not only to anatomical changes but also to loss of gene function linked with the detection and anticipation of the day-night cycle**.**

Keywords: circadian clock, cavefish, zebrafish, light pathway, clock mechanism, temperature compensation, opsins.

Abstract (Italiano)

EVOLUZIONE DELL'OROLOGIO CIRCADIANO NEI PESCI IPOGEI

L'evoluzione degli esseri viventi è sempre stata fortemente influenzata dai cicli giornalieri derivanti dalla rotazione della Terra introno al proprio asse, come l'alternanza luce-buio e le variazione di temperatura. Gli oscillatori circadiani sono dei meccanismi molecolari capaci di misurare lo scorrere del tempo e che permettono agli organismi di anticipare i cambiamenti ambientali sincronizzando svariati processi fisiologici al ciclo luce-buio giornaliero. Questo meccanismo si è ampiamente conservato ed è presente in pressoché tutte le specie studiate. Un modello particolarmente interessante per lo studio dell'evoluzione dell'orologio circadiano nei vertebrati è rappresentato da animali che si sono adattati ad ambienti estremi come quelli ipogei dove sono rimasti completamenti isolati dall'alternanza del giorno e della notte per milioni di anni. La maggior parte di queste specie è accomunata da un'evoluzione convergente, dove si evidenziano un'ampia gamma di adattamenti fisici comuni, come la perdita degli occhi e del pigmento. Ad oggi non è ancora completamente chiaro se le specie ipogee conservano un orologio endogeno circadiano funzionante. A questo scopo abbiamo investigato il sistema circadiano del pesce ipogeo della Somalia, *Phreatichthys andruzzi*. I nostri risultati hanno dimostrato che *P. andruzzii* possiede un orologio circadiano che non viene sincronizzato dall'alternanza luce-buio, ma da un regime giornaliero di alimentazione. In condizioni costanti l'orologio di *P. andruzzii* oscilla con un periodo estremamente lungo e manca della normale compensazione del periodo al variare della temperatura. Nel corso della nostra indagine molecolare abbiamo riscontrato molteplici mutazioni a carico di un gene orologio la cui espressione è indotta dalla luce, *Period2*, così come nei geni codificanti per i fotopigmenti extra-oculari *Melanopsin* (*Opn4m2*) e *TMT-opsin*. In particolare, abbiamo dimostrato che l'espressione ectopica delle opsine omologhe di zebrafish ristabilisce l'espressione indotta dalla luce del gene orologio *Period2* nelle cellule di *P. andruzzii*.

Attraverso lo studio di questo mutante naturale abbiamo dimostrato che l'evoluzione in buio costante ha portato non solo a cambiamenti anatomici, ma anche alla perdita di funzione di geni circadiani collegati alla sincronizzazione ai cicli luce-buio giornalieri. Inoltre, abbiamo ottenuto per la prima volta in un vertebrato non-mammifero evidenze dirette della funzione circadiana di due opsine extra-oculari.

I. INTRODUCTION

Life and environment have always been in close relation, one influencing the other, along the evolution: alternating days and nights, the cycles of the tides, lunar phases and the seasons represented a considerable selective pressure on all living organisms. Extensive programming of biological activity, that meet and exploit the challenges and opportunities offered by the periodic nature of the environment, provide a valuable selective advantage for survival (Pittendrigh, 1993).

The ability to vary on a daily basis the behaviour is one of the most important adaptive phenomenons that developed during evolution. Many biochemical, physiological and behavioural functions show daily fluctuations; they have been observed at all levels of organization, from the behaviour of mammals (sleep-wake rhythm and locomotor activity) to the specific activity of enzymes (Brown & Schibler, 1999; Gachon et al., 2004).

The mechanisms developed in order to adapt and even anticipate the environmental stimuli are the circadian oscillators or clocks. The circadian clocks are endogenous, self-sustaining time-keeping systems (Pittendrigh, 1960). The oscillation or rhythm generated by the clock has a period length of approximately 24 hours, hence it is termed circadian (*circa-diem*; about a day) and it persists when the organism is placed under constant environmental conditions (aperiodic conditions). Pervasive features of endogenous circadian oscillators are: i) the capacity to synchronize (entrain) to environmental signals (*zeitgeber*, from German Zeit: "Time"; geben: "to give") including light, temperature and food availability ensuring that it remains synchronized with the natural 24-hour cycle (Pittendrigh, 1993); ii) the temperature compensation (Tsuchiya et al., 2003), the period length remains constant over physiological temperature shifts.

All these characteristics of circadian rhythms are unique and have been highly conserved by living organisms during evolution.

The circadian clock can be considered to be composed of three parts (Fig. 1) (Menaker et al., 1978):

1) The core oscillator that autonomously generates circadian rhythms.
2) The input pathways that detect zeitgebers and can entrain and reset the central oscillator.
3) The output pathways through which the circadian rhythm directs physiological and behavioural activities.

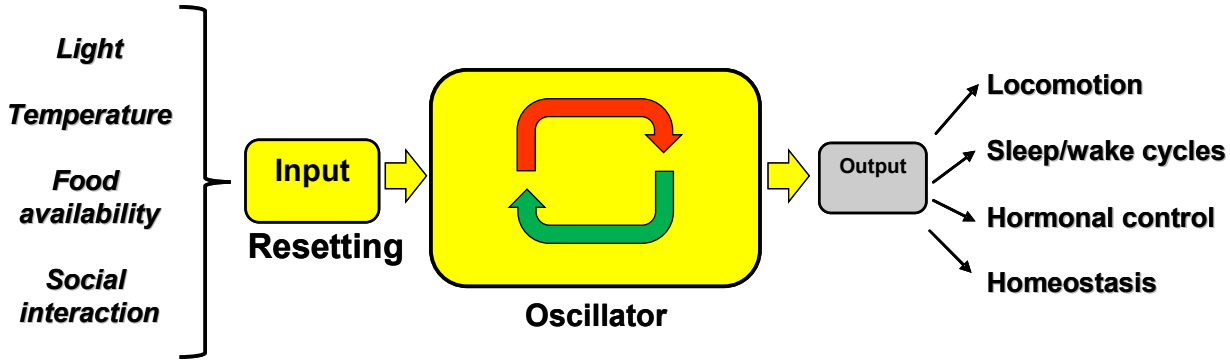

Fig. 1: Schematic presentation of biological timekeeping systems. The core oscillator autonomously generates circadian rhythm. Daily resetting by environmental signals, via the input pathway, ensures that it remains synchronized with the natural 24-hour cycle. The pacemaker then drives the expression of output involved in many aspect of physiology.

In the past 40 years a variety of model systems have been successfully developed to gain essential knowledge of the molecular and cellular basis of the circadian clock and its regulation (Harmer et al., 2001). Most significant progress has been made by forward genetic analysis in *Drosophila*, *Neurospora*, cyanobacteria and more recently in vertebrate models, such as mouse and zebrafish.

I.1 Organization of circadian clocks

Vertebrate circadian timing system comprised of a central pacemaker located in the brain and of peripheral oscillators in most body cells. It is now the challenge to understand how all these multiple clocks communicate with each other and remain synchronized at the whole animal level.

I.1.1 Central and peripheral pacemakers in vertebrates

In mammals, the suprachiasmatic nucleus (SCN) is a region in the anterior hypothalamus of the brain; it has been shown through lesion and transplantation experiments (Moore and Eichler, 1972; Ralph et al., 1990) to be the master circadian pacemaker. It is responsible for the

generation and regulation of rhythms in behaviour, hormonal secretion and various physiological functions (Klein, 1991). The SCN consists of around 20.000 neurons which differ in their pacemaking ability, response to environmental time cues, neuropeptide expression and the rhythms they control (Antle and Silver, 2005).

Two regions within the SCN could be clearly separated from each other: the dorsal shell and the ventral core. The neurons of the dorsal shell reach their daily peak in clock gene expression earlier than those from the ventral core (Yamaguchi et al., 2003). Studies in mice have revealed that the ventral SCN neurons play a major role in synchronizing the two SCN regions with each other (Yamaguchi et al., 2003). *In vivo* experiments have shown that the ventral core neurons were able to synchronize faster to shifted light-dark (LD) cycles than those from the dorsal shell (Albus et al., 2005). Candidate neurotransmitters for the synchronization of the SCN neurons could be VIP (Aton et al., 2005), GABA (Liu and Reppert, 2000) and also gap junctions (Colwell, 2000).

In the nervous system of vertebrates, peripheral pacemakers have been identified in the retina and in the pineal gland. In the retina the circadian clock controls the local synthesis of dopamine and melatonin (Tosini et al., 2008). In non-mammalian vertebrates is present an additional central circadian pacemaker in the pineal gland, that drives rhythms of melatonin release and responds directly to light signal entrainment in culture (Menaker et al., 1997; Takahashi et al., 1980).

Peripheral clocks are also present in various tissues of the mammalian systems, such as liver, kidney, heart, skeletal muscle and lung. Studies in peripheral tissues of transgenic rats explanted into culture showed circadian oscillation over several cycles, while the SCN continues to oscillate for at least 32 days in culture (Yamazaki et al., 2000). Moreover, peripheral organs show tissue specific differences in circadian period and phase, leading to the conclusion that peripheral tissues exhibit self-sustained, rather than driven circadian oscillations (Yoo et al., 2004).

Mammalian peripheral clocks are not directly entrained by light in contrast to the situation in *Drosophila* and zebrafish (Whitmore et al., 1998). Therefore, mammalian peripheral clocks seem to be synchronized with central clocks through a complex combination of different signals including metabolites and body temperature (Brown et al., 2002; Damiola et al., 2000; Schibler, 2007).

I.2 Molecular clockwork

Following the identification of circadian pacemaker structures, the next challenge was to identify the molecular basis of the clocks they contain.
Forward genetic analyses in a variety of model organisms, such as *Drosophila*, *Neurospora*, cyanobacteria, *Arabidopsis* and mouse, have led to the identification of several "clock genes".

I.2.1 Molecular clock mechanism in vertebrates

At the molecular level the vertebrate circadian clock mechanism consists of interacting positive and negative transcriptional/translational feedback loops that operate in a cell-autonomous and self-sustained manner.
The bHLH-PAS transcription factors CLK and BMAL1 are essential positive elements in the vertebrate clock. The *clk* gene has been identified through forward genetic screens. The *Clk* mutant mice exhibit aberrant locomotor activity rhythms under constant darkness (DD) conditions (Vitaterna et al., 1994). Positional cloning then led to the characterization of the m*Clk* locus (King et al., 1997). BMAL1 was identified because of its close interaction with CLK (Gekakis et al., 1998; Hogenesch et al., 1998) and its disruption in mice caused an immediate loss of locomotor activity rhythms under DD (Bunger et al., 2000).
Heterodimers of CLK and BMAL1 activate transcription by binding to conserved elements (E-box, 5'-CACGTG-3') in the promoter regions of clock controlled genes (*ccg*), the three *Period* (m*Per1*, m*Per2*, m*Per3*) and two *Cryptocrome* (m*Cry1*, m*Cry2*) genes (Dunlap, 1999) which also encode for the regulatory elements of the negative limb of the clock mechanism. The binding of negative regulators such as PER and CRY to CLK-BMAL1 inhibit the activation of *ccg* and their own transcription (Fig. 2), determinig the period and the amplitude of the oscillation.
Mutations in only one of the m*per* genes results in no loss of circadian clock function, whereas double mutants of m*Per1* and m*Per2* do result in a complete loss of rhythmicity (Zheng et al., 2001). The same was observed in double mutant m*Cry1/*m*Cry2* mice which lose their circadian rhythmicity in wheel running behaviour under DD conditions (Vitaterna et al., 1999; van der Horst et al., 1999).

An additional feedback loop involves the nuclear receptors *Rev-erbα* and *Rorα*. The expression of REV-ERBα is activated by CLK and BMAL1 and repressed by factors from the negative limb. REV-ERBα in turn represses expression of BMAL1 (Fig. 2). Furthermore, RORα competes with REV-ERBα for the binding of the same elements in the promoter region of BMAL1. Genes containing these REV-ERB/RORE elements in their promoter are repressed by REV-ERBα, whereas RORα activates their transcription. This REV-ERBα/RORα feedback loop interconnects the positive and negative limbs of the clock (Duez and Staels, 2008) and seems to add precision and stability to the core loops in the mammalian clock (Emery and Reppert, 2004). In case of *Rev-erbα*, heme group has recently been implicated as its ligand (Raghuram et al., 2007; Yin et al., 2007). This finding suggests that heme regulation of *Rev-erbα* might be a possible link between the control of metabolism and the mammalian clock (Raghuram et al., 2007).

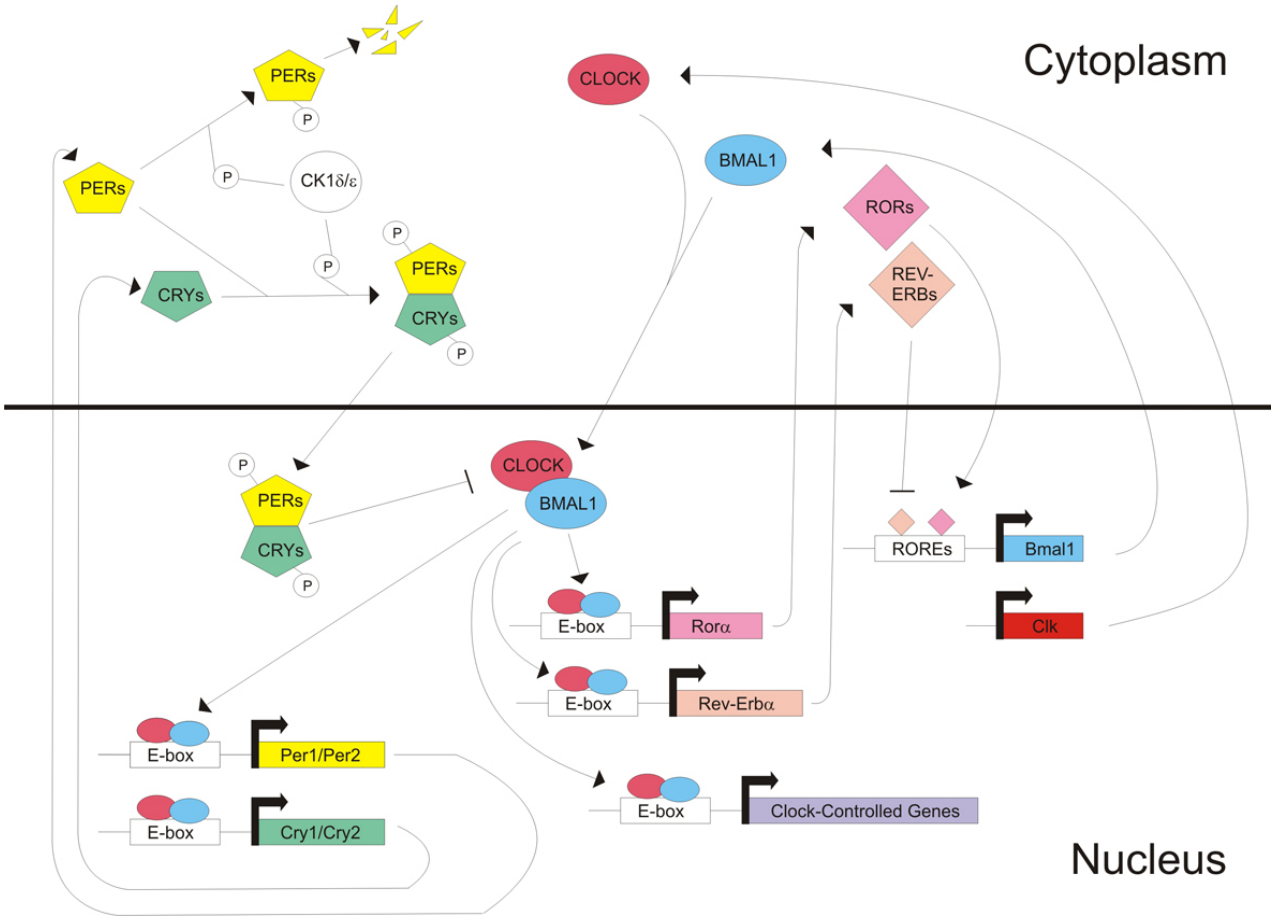

Fig. 2: Molecular clock in mammals. The clock is composed of two interconnecting loop: In the core loop heterodimers of CLK and BMAL1, positive limb, activate transcription of Period, Cryptocrome and clock-controlled genes. Heterodimers of PER and CRY, negative limb, bind to CLK-BMAL1 inhibiting their own transcription. In the stabilizing loop CLK and BMAL1 induce the expression of REV-ERBα and RORα which subsequently regulate transcription of Bmal1.

I.3 Output pathways

Key components of the circadian timing system are the output pathways whereby the molecular clock machinery is linked with diverse aspects of physiology and behaviour. It is now clear that clock output pathways operate on the whole system level involving central pacemakers and on the cellular level where peripheral clock components are able to differentially regulate the expression of non-clock genes.

Most animals have circadian rhythms in behavioural processes such as locomotor activity, alertness and feeding as well as coupled physiological functions, e.g. hormonal rhythms and metabolic gene regulation.

In mammals the circadian rhythmicity of sleep-wake cycles and hormone production is linked to the functional integrity of the SCN. Thus, via indirect adrenergic innervations the SCN controls the nocturnal synthesis of melatonin in the mammalian pineal gland. In turn, circadian rhythms of circulating melatonin affect many aspects of physiology.

The conserved regulatory system of the cell cycle represents an important circadian clock output. Proper timing of this system is of highest importance for most organisms because it represents one strategy for avoiding the potentially damaging effect to UV exposure of sunlight upon key steps of the cell cycle such as S phase (Mori et al., 1996).

I.4 Zebrafish as a circadian clock animal model

The zebrafish (*Danio rerio*) has been established as one of the most important models for studying vertebrate embryogenesis and early development. Thus, both, the easy access of early developmental stages and its proven utility in large scale forward genetic analysis have made it an attractive vertebrate to study the origin and function of the circadian clock.

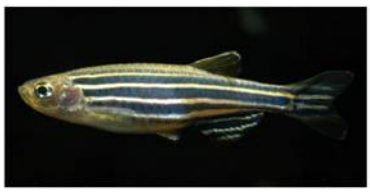

Fig. 3: The zebrafish, Danio rerio, is a tropical freshwater fish belonging to the Cyprinidae family and is an important vertebrate model organism in scientific research.

In contrast to mammalian model organisms such as the mouse, zebrafish has several advantages to offer. For instance, its transparency allows the observation of individual cells during the earliest stages of development and embryogenesis. Peripheral clocks in zebrafish are directly light responsive and make it a powerful tool for studying light input to the vertebrate clock and peripheral clock regulation (Vallone et al., 2004; Whitmore et al., 2000). Furthermore, zebrafish cell lines which posses directly light entrainable circadian clock function have been successfully used to study light input pathways at the cellular and molecular levels (Vatine et al., 2009). Zebrafish represents an ideal genetic model to study the role of temperature as a clock zeitgeber, mainly because of its poikilothermic properties. Zebrafish primary cell lines contain both light and temperature entrainable clocks and therefore provide a unique opportunity to study crosstalk between the two zeitgebers (temperature and light) at the cell culture level (Vallone et al., 2004; Lahiri et al., 2005). Zebrafish cells remain viable for up to 2 months as confluent cultures without sub-culturing and do not require a CO_2 gassed humidified environment because of the buffering properties of their preferred medium. Transfection protocols allow transient and stable introduction of clock and light regulated promoter-luciferase reporter constructs (Vallone et al., 2004). Following addition of luciferin to the culture medium, bioluminescence can then be monitored automatically *in vivo* under different light and temperature conditions over extended time courses.

I.4.1 The circadian clock in zebrafish

Zebrafish basic circadian clock cellular and molecular elements are very similar to those of mammals. However, there are some important differences. As in most of the non-mammalian vertebrates, the zebrafish pineal gland (the major site of synthesis of the hormone melatonin) is a photoreceptive organ which contains an intrinsic circadian oscillator. The pineal clock is directly light regulated and controls the nocturnal expression of melatonin. It is contrasts with the situation in the mammalian pineal where there is no directly light regulated clock and nocturnal melatonin synthesis is indirectly driven by the SCN pacemaker. The expression of the rate-limiting enzyme for melatonin synthesis, arylalkylamine-N-acetyltransferase (*Aanat2*), is both light and clock regulated in the zebrafish pineal (Ziv and Gothilf, 2006). Neuroanatomical studies of the adult zebrafish brain have revealed a structural counterpart of the SCN in the

hypothalamus. However, it remains an unanswered question whether this putative SCN shares a similar central pacemaker role with the mammalian SCN.

As in mammals, rhythmic clock gene expression in zebrafish is not restricted to classical pacemaker structures such as the pineal gland and retina but is also present in most other tissues. These oscillations continue even if the tissues are explanted *in vitro*. This demonstrates the existence of self-sustaining circadian oscillators in different organs and cell types which, unlike mammals, are entrained by direct exposure to light-dark cycles.

Documented clock output pathway targets of the zebrafish circadian clock are rhythmic locomotor activity in larvae and adult fish (Hurd and Cahill, 2002), rhythmic melatonin release (Kazimi and Cahill, 1999) and cell cycle timing (Dekens et al., 2003; Dickmeis et al, 2007).

Clock genes have been isolated from zebrafish either by sequence homology with their mammalian counterparts or by two-hybrid screens for interacting partners of the CLK protein. So far three *Clock* (*Clock1, 2, 3*) (Kobayashi et al., 2000; Whitmore et al., 1998), three *Bmal* (*Bmal1, 2, 3*) (Kobayashi et al., 2000; Cermakian et al., 2000), four *Period* (*Period 1a, 1b, 2, 3*) (Delaunay et al., 2000; Delaunay et al., 2003; Vallone et al., 2004), six *Cryptochrome* genes (*Cry1a, Cry1b, Cry2a, Cry2b, Cry3, Cry4*) (Kobayashi et al., 2000) and one *Rev-Erbα* gene (Kakizawa et al., 2007) have been identified. Interestingly, the zebrafish genome contains far more copies of different clock genes than the mammalian genome. It has been proposed that a genome duplication event early in teleost evolution was a major determinant of this greater number of clock genes (Postlethwait et al., 1998; Meyer and Schartl, 1999; Semon and Wolfe 2007).

At the molecular level expression of zebrafish clock genes show peculiar behaviour. While in mammals the temporal expression of *Clk* is constant, in zebrafish it exhibits daily fluctuation (Whitmore et al., 1998) while *Cry1a*, *Cry5* (*6-4 photolyase*) and *Per2* are light induced (Hirayama et al., 2005; Kobayashi et al., 2000, Vatine et al. 2009). Moreover temperature cycles induce rhythmic expression of *Cry2a*, *Cry3* and *Per1b* (Lahiri et al., 2005).

I.5 Light input pathways

Light is considered to be the most important zeitgeber. It represents in a very reliable way, the daily changes in photoperiod in most environments (Roenneberg and Foster, 1997). Other zeitgebers, such as temperature changes, feeding time (Damiola et al., 2000) and social

interactions (Levine et al., 2002) play also a significant role in many circadian systems but they still indirectly depend on the earth's daily exposure to sunlight. The mechanism and the factors through which light entrains the clock have been extensively studied.

I.5.1 Ocular and extraocular light input pathways in vertebrates

In mammals, the retina has been shown to represent the only photoreceptive structure for the circadian clock (Foster, 1998). In the case of the rod and cone cells, which are localized in the outer layer of the retina, transgenic studies in mice have shown that these retinal cell types have no circadian photoreceptive role. In these studies the genetic ablation of rod and cone cells does not eliminate the photo-entrainment of the clock (Freedman et al., 1999; Lucas et al., 1999). However, the retinal ganglion cells localized in the inner retinal layer were sufficient to maintain this photoentrainement (Ebihara and Tsuji, 1980; Van Gelder et al., 2003). For that reason humans or mice which suffer blindness caused by degradation of the outer retinal layer are still able to synchronize their circadian clock by exposure to daily LD cycles (Ebihara and Tsuji, 1980; Czeisler, 1995).

Subsets of intrinsically photosensitive retinal ganglion cells (ipRGCs) that express the photopigment *Melanopsin* were identified to be the principal circadian photoreceptors (Berson et al., 2002; Hattar et al., 2002). However, gene knockout of *Melanopsin* only showed a slight reduction in the ability of the circadian clock to be entrained by light (Ruby et al., 2002; Hattar et al., 2002). The complete elimination of circadian photoreception was only achieved through the combined ablation of melanopsin, rod and cone cells (Lucas et al., 2003; Panda et al., 2003).

Therefore, the rod and cone cells seem to play some supporting role in circadian photoreception depending upon the light intensity (Hattar et al., 2003).

Subsequently, the ipRGCs convey light information to the SCN. This signalling is thought to be achieved through both glutaminergic and the pituitary adenylate cyclase-activating peptide (PACAP) pathways via the retino hypothalamic tract (RHT) (Hattar et al., 2003; Hannibal, 2006). Perception of light leads to an induction of the immediate early genes *c-Fos* and *c-Jun*, as well as the clock genes *Per1* and *Per2* within the SCN (Kornhauser et al., 1996; Zylka et al., 1998). The MAPK signaling pathway (via CREB mediated transcription) seems to induce the transcription of these genes (Obrietan et al., 1998). Recent studies report that many light induced genes in the SCN posses evolutionary conserved CRE elements in their promoter regions, with

the strongest light inducible responses coming from the combination of CRE elements and associated TATA boxes (Porterfield et al., 2007).
Other candidate photoreceptor molecules include the blue-light photoreceptive cryptochromes, however whether they play an active role as photoreceptors in vertebrates is still open an open question.
In mammals, cryptochromes are expressed in both the outer and inner nuclear layer as well as the ganglion cell layer of the retina (Sancar, 2000). Cryptochrome knockout mice have a 20-fold less *c-Fos* expression induced by light than WT mice (Selby et al., 2000). Moreover, mice lacking both cryptochromes and retinaldehyde (component of the visual photopigments in the eye) no longer show phototransduction to the SCN (Thompson et al., 2004). However, these cryptochrome knockout mice are arrhythmic in constant darkness (DD) and therefore their actual involvement in photoentrainment is hard to test, suggesting their role primarily as a central clock component and not as a component of the light input pathway (van der Horst et al., 1999).

In non-mammalian vertebrates like birds, reptiles and fish, circadian photoreception occurs in several specialized photoreceptive organs which develop from the embryonic forebrain: For example the eyes, the pineal gland and deep brain photoreceptors. In the case of zebrafish, observations in cultured cells and tissues have revealed that clock gene expression rhythms can be entrained by direct exposure to LD cycles (Whitmore et al., 2000). This suggests that in zebrafish beside the “classical” identified photoreceptive organs there also exists a widespread photosensitivity of peripheral tissues. This has confirmed that the zebrafish represents an ideal system to study the circadian clock and particularly the light entrainment pathway. Good candidates for peripheral tissue circadian photoreceptors in zebrafish are cryptochromes (Cermakian et al., 2002) and opsins. Opsins belong to the G protein coupled receptor superfamily and they are characterized by a conserved seven transmembrane domain motif (Fig. 4). The mechanism of light perception is mediated by the binding of the opsin protein with a chromophore, a vitamin A-based retinaldehyde, through a lysine residue in the seventh transmembrane alpha helix (Bownds, 1967). The absorption of a single photon results in the photoisomerisation of the chromophore from the cis to an all-trans conformation. This change involves the opsin structure, causing the activation of the transduction cascade. When the opsin is bound to the trans form is then inactivated.

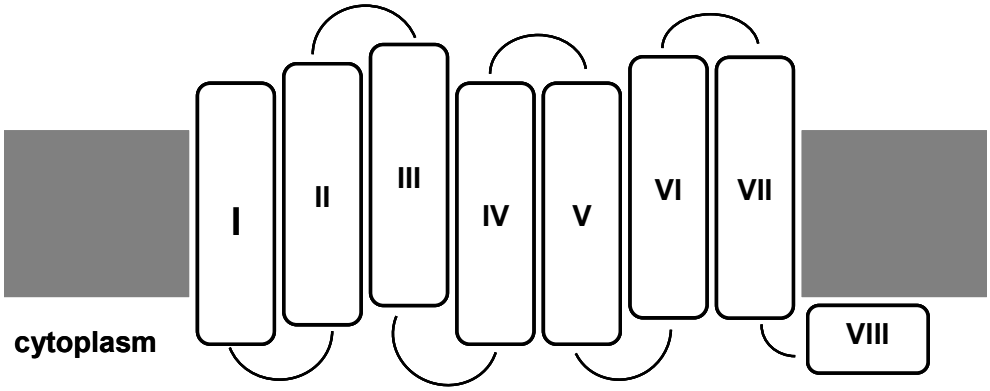

Fig. 4: Schematic representation of the membrane spanning structure of a generic opsin protein. The seven transmembrane domains are labelled I - VII. The light perception is mediated by the binding of the opsin protein with retinaldehyde, a derivative of vitamin A, through a lysine residue in the seventh transmembrane alpha helix while the signal transduction occurs in the first three domain where is located a G protein binding site.

Over the last decade, several novel opsin groups have been identified in different organisms and especially in non-mammalian vertebrates. The function of many of these new opsins is still unknown. The classical opsins are expressed solely in the retina, whereas the new opsins, like *Exorhodopsin*, *Teleost Multiple Tissue* (*TMT*) *opsin* and *Melanopsin* (*Opn4m2*) have a wide range of expression patterns (Bellingham et al., 2002; Mano et al., 1999; Moutsaki et al., 2003).

I.6 Cavefish as animal model system

The study of subterranean species provides valuable and unique insight into how organisms evolve in response to an extreme environment.

An heterogeneous group of animals has adapted to life in constant darkness in the caves. Remarkably, all these different animals evolved convergent "regressive" phenotypes including many aspects of behaviour, such as negative phototaxis (a kind of taxis which occurs when a whole organism moves in response to a light stimulus in the opposite direction) and changes in anatomy, such as loss of the eyes and pigmentation, which appear to represent key adaptations to life in these environments. The mechanism of regressive evolution is still unclear and cave animals offer an opportunity to study convergent and parallel evolution of regressive traits that are driven by the same environmental cue (Jeffery, 2009). Due to differences in the time that

species have been isolated in subterranean habitats and in the precise ecological conditions, comparative studies involving subterranean species are particularly valuable.

Troglobitic (exclusively subterranean) fish evolved under conditions that contrast with those of their epigean (surface dwelling) ancestors mainly by the absence of daily cycles of light (and in many cases, also of temperature cycles). These species develop the same autapomorphic characters as the other cave-dwelling animals, such as loss of eyes and melanic pigmentation.

The teleost *Astyanax mexicanus* is perhaps the most common studied cavefish. This species is characterized by an eyed surface-dwelling form (surface fish) and many blind cave forms (cavefish) (Fig. 5). This make *A. mexicanus* particularly valuable for studying evolution of eye degeneration. In the blind fish small eye primordia are formed during embryogenesis, which later arrest in development, degenerate and sink into the orbits. Eye degeneration is caused by apoptosis of the embryonic lens due to the increasing in hedgehog genes signalling and the consequent iperactivation of downstream genes (Yamamoto et al., 2004).

Fig. 5: The Mexican blind cavefish (Astyanax mexicanus) accompanied by two surface-dwelling morph (Niven, 2007).

I.6.1 Somalian cavefish

Phreatichthys andruzzii (Ercolini et al., 1982) is a phreaticolous fish from Somalia, a tropical cyprinid that inhabits the subterranean waters under the central Somalian desert (Fig. 6). Its habitat consists of large phreatic layers that develop in Eocene horizontal limestone rock formations. The ontogenetic recapitulation of phylogenetic history suggests that troglobitic forms of *P. andruzzii* originated from normal-eyed ancestors, which lived in surface water when Somalia had a wetter climate. The change of climate in Somalia towards a very dry surface

habitat probably forced *P. andruzzii*'s ancestors to adopt a subterranean life in the phreatic waters. The underground water can only be reached through sink holes or shallow wells.

The Somalian cavefish presents a more extreme specialized phenotype than the other more commonly studied American blind cavefish *Astyanax* spp. In fact, *P. andruzzii* shows more rapid eye degeneration with respect either to *A. mexicanus* (Yamamoto et al., 2004) or to other cave-dwelling vertebrates, such as *Proteus anguinus* (Durand, 1971) and *Typhlotriton spelaeus* (Besharse and Brandon, 1974). Furthermore, *Astyanax'*s populations show different degrees of eye degeneration, and in some cases still possess rudimental eyes.

Despite the degeneration of the eye, *P. andruzzii* still shows clear behavioral responses to light stimulation. Specifically, it shows a notable photic sensitivity in behavioural tests where it tends to avoid illuminated areas in preference for completely dark areas. The photophobic response is enhanced under blue light (wavelength of 480 nm), but it is also present under green (539 nm), orange (615 nm) and red (692 nm) light (Ercolini and Berti, 1975). Furthermore, the fish seem capable of perceiving not only the presence of light but are also able to discriminate between different wavelengths.

Fig. 6: Phreatichthys andruzzii (left) is a species of ray-finned fish in the Cyprinidae family, and the only species of the genus Phreatichthys. Ancestors of P.andruzzii entered the large phreatic layers of the Somalian desert, that developed in Eocene horizontal limestone formations, at the end of the Pliocene (1.4-2.6 million years ago) and became isolated with the extinction of epigean sister species as the result of extreme climatic changes. The Adult cavefish were collected in the wild, in the oasis of Bud-Bud in the centre of the Somalian desert during several expeditions to Africa (1960-1980).

Many studies have shown that circadian locomotor rhythmicity is regressed in troglobites, corroborating the hypothesis that daily cycles are mainly selected for by external factors such as photoperiod cycles (Lamprecht and Weber, 1992; Trajano et al., 2005).

Unlike the more famous Mexican cavefish *Astyanax*, *P. andruzzii* belongs to the cyprinid family and so is a close relative of the zebrafish (*Danio rerio*). This peculiarity allowed many of the tools, experimental approaches and informations that have been developed for zebrafish studies to be also applied to the Somalian cavefish.

Furthermore, the ease with which these fish can be raised in the laboratory as well as their high fecundity make them particularly attractive for studies using embryonic, larval or juvenile stages.

I.7 AIM

The evolution of the circadian clock has been intricately linked with the natural day-night cycle. To provide the first clues about the consequences of the absence of this canonical selective pressure on the conserved circadian oscillator, we applied a comparative analysis to take advantage both of the divergent evolution of the cavefish and of their common origin amongst surface-dwelling teleosts.

We chose to study a troglomorphic cave-dwelling fish that have been isolated for million of years in a totally dark environment, the Somalian cavefish *Phreatichthys andruzzii*.

For this purpose we established *P. andruzzii* as a model which by comparison with a normal sighted species such as the zebrafish (*Danio rerio*) can be used to investigate the molecular, cellular and anatomical adaptations to life in constant darkness.

The first aim was to identify cavefish clock genes homologues, in order to perform functional analysis of the mechanisms regulating the circadian timekeeping system. The emerging differences between *P. andruzzi* and *D. rerio* were invaluable to assign a function to cardinal circadian clock elements participating in light-dependent regulation. Furthermore, for the first time we developed specific primary cell lines from the Somalian cavefish to facilitate the molecular investigation of the directly light entrainable cellular clocks, an intrinsic property of teleost cells.

The present research also aimed to investigate the adaptations of extraocular photoreceptors during evolution in complete darkness. Isolation of non-visual opsin coding sequences and their physiological characterization will help to clarify the roles of these photopigments in the circadian photoentrainment system.

An increased understanding of the molecular processes underpinning circadian rhythmicity furnished by the present vertebrate models, provide valuable new insight into how the molecular mechanism of the clock is regulated by light. Furthermore, with a longer term perspective, the development of *P. andruzzii* as a cavefish model system will enable the detailed investigation of many other fascinating cavefish adaptations to life in constant darkness and so how light has shaped the evolution of living systems.

II. MATERIALS AND METHODS

II.1 Animals

Somalian cavefish (*P. andruzzii*) were collected from several sites around the locality of Bud-Bud (04°11'19"N–46°28'27"E) during the years 1968-1982. Fish were transferred alive to the laboratories of the University of Florence, Italy, where they have been bred repeatedly using standard methods (Chang, 1983b; Sokolowska et al., 1984). The fish were raised in aquaria (100 x 40 x 50 cm) containing 160 l of dechlorinated tap water and equipped with an adsorbing charcoal filter and aerator. They were maintained in darkness except during food administration and aquaria maintenance. Three to 6 times per week the fish were fed with frozen chironomid larvae. Water temperature was maintained constant at 29±1°C. The present study used both adult and larva raised in the Florence laboratory. Fertilized eggs were collected every 30 minutes and aliquots of 10 or 20 were transferred into 75cm^2 tissue culture flasks (Falcon). Flasks were sealed and submerged horizontally in a large volume, thermostatically controlled water bath (Tetraht, Tetra, Milano, Italy) to maintain a constant temperature of 29±1°C. Larvae were illuminated with a fluorescent light source (100 lux, full-spectrum cool fluorescent tubes, Osram, Germany). From the third/fourth day after hatching, larvae were fed once a day. RNA extracts were prepared from pools of 20 embryos or 10 larvae per time point. 14 years old adults, belonging to the offspring of wild stock collected in 1982 were used as a source of adult tissues for LD cycle and restricted feeding regime experiments. To analyse the effects of LD cycles on clock gene expression *in vivo*, adult fish were starved for 10 days prior to (and during) the sampling period. Dissections in dark conditions were performed under dim red light (<1 lux). Tissues and whole larvae were immediately frozen in dry ice to avoid RNA degradation.

II.2 Cell lines

CF-1, CF-2 and CF-3 cell lines were obtained from fin clips of different adult fish adapting the protocol from (Vallone et al., 2007). Fin clips were treated with trypsin and plated in 50mm cell culture dishes (Falkon) with Leibovitz's L-15 medium (Gibco) supplemented with 20% fetal calf

serum (Biochrom KG), 100 units/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamycin (Gibco BRL) and maintained in an atmospheric CO_2 and non-humidified cell culture incubator at 25°C. Once cells were confluent they were seeded in flasks (Falkon). These fibroblast-like cells grow optimally as an adherent monolayer culture on normal tissue culture-treated plastic substrates. Cells were typically passaged once every two weeks by first using trypsin to induce detachment from the substrate followed by dilution in culture medium at a ratio of 1:2 and then seeding in fresh culture flasks. Typically, cells proliferated and returned to confluence within two weeks of passaging and confluent cultures could be maintained for up to one month without significant loss of viability.

A subline derived from the zebrafish adult cell line AB9 (Kwok et al., 1998) was propagated at 25°C in L-15 (Leibovitz) medium (Gibco BRL) supplemented with 20% Fetal Calf Serum (Biochrom KG), 100 units/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamycin (Gibco BRL). Cells were typically passaged once every two weeks and diluted in culture medium at a ratio of 1:4 and then seeding in fresh culture flasks. Cells proliferated and returned to confluence within one week of passaging.

II.3 Genomic DNA extraction

Confluent flasks (Falkon) of CF and AB9 cells were treated with 500 µl of lysis Buffer (100 mM Tris-HCl pH 8; 200 mM NaCl; 5 mM EDTA pH 8.0; 0.2% SDS) with 2.5 µl of Proteinase K (20 mg/ml) after removing the culture medium and incubated 1 hours at 55°C.

Samples were centrifuged 15 min at 12.000xg, room temperature, and the supernatants were transferred in new tubes and extracted once with phenol-chloroform. The aqueous phase was incubated with RNase A 50 µg/ml for 30 min at 37°C and then precipitated with 300 µl isopropanol, by centrifugation for 15 min at 12.000xg at room temperature. Pellet was then washed with 700 µl 70% ethanol, air dried and resuspended in 50 µl DNase free water.

II.4 RNA extraction and reverse transcription

Total RNA was extracted from tissues, total larvae or confluent cell monolayers of *P. andruzzii* and from AB9 cell line by lysing in Trizol Reagent (Gibco, BRL). Addition of chloroform and

subsequent centrifugation lead to phase separation under conditions where RNA remained water-soluble and proteins or DNA were partitioned in the lower, organic phase or at its interface. Total RNA was subsequently isolated from the aqueous phase by isopropanol precipitation followed by centrifugation and then rinsing the pellet using 75% ethanol.

Zebrafish tissues RNA samples were kindly provided by Prof. Dr. Nicholas S. Foulkes (Universität Heidelberg und Institut für Toxikologie und Genetik Forschungszentrum Karlsruhe, Germany) .

A reverse transcription was performed with the total RNA to produce cDNA using Superscript III RT (Invitrogen), according to the manufacturer's conditions.

II.5 Cloning cavefish cDNA sequences

To initially obtain partial cDNA sequences, single-stranded cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen, Milano, Italy). Cavefish genes were amplified by PCR using Taq DNA Polymerase (Invitrogen, Milano, Italy) with primers designed by Primer3 software on the basis of sequence of the *Danio rerio* (zebrafish) homologs. Bands of the predicted sizes were cloned into the pGEM-T Easy Vector (Promega, Madison, WI). The cavefish gene cDNA fragments were sequenced (QIAGEN GmbH) and compared with the GenBank database by using the BLAST algorithm. Additional cDNA sequences were subsequently obtained using a 5'-3'SMART RACE cDNA amplification kit (BD Bioscience-Clontech, Palo Alto, CA), and then coding sequences were deposited in Gen-Bank. Nucleotide coding sequences were converted into amino acid sequences by ExPASy Proteomics Server (http://www.expasy.org/tools/dna.html), and protein domains were predicted using SMART (http://smart.embl-heidelberg.de). By this approach, we cloned 13 clock genes and 3 opsins from *P. andruzzi*: *Per1, Per2, Per3, Cry1a, Cry1b, Cry2a, Cry2b, Cry3, Cry4, Cry5 Clk1, Clk2, Clk3, Opn4m2, TMT-opsin, Exo-rhodopsin* based on their homology with zebrafish homologous genes (*Beta-actin* GQ404475; *Cryptochrome1a* GQ404476; *Cryptochrome1b* GQ404477; *Cryptochrome2a* GQ404478; *Cryptochrome2b* GQ404479; *Cryptochrome3* GQ404480; *Cryptochrome4* GQ404481; *Cryptochrome5* GQ404482; *Clock1* GQ404483; *Clock2* GQ404484; *Clock3* GQ404485; *Period1* GQ404486; *Period2* GQ404487; *Period3* GQ404488; *Melanopsin* GQ404489; *TMT-opsin* GQ404490; *Exo-rhodopsin* GQ404491).

II.6 Phylogenetic analysis

Sequences from the cavefish PER protein family have been aligned with homologs from other teleost species (*Takifugu rubripes, Tetraodon nigroviridis, Danio rerio, Gasterosteus aculeatus* and *Oryzias latipes*) (Wang, 2008) using ClustalW (Thompson et al., 1994). Alignments were manually verified and phylogenetic trees were generated using Neighbour-joining methods (Tamura et al., 2007) with a complete deletion mode. Bootstrap tests were performed with 1000 replications. Poisson correction distance was adopted and rates among sites were set as uniform. The PER sequence from *Drosophila melanogaster* was used as an outgroup to root the tree.

II.7 LD experiment (adults and larvae)

Adults (n=24, 14 years old, belonged to the offspring of a wild stock collected in 1982 from the wells of Bud-Bud) and larvae (1st day and 4th weeks post hatching) were used. Adults were maintained at 29±1°C under a 12:12-h light-dark cycle (lights on from zeitgeber time (zt) 0 to 12 with an intensity of 100 lux). Adult were starved for 10 days and tissues (brain and fin) collected from ZT3 every 6 h for 24 h. Larvae were maintained at 29±1°C under a 14:10-h light-dark cycle. Larvae were collected during the 1st day post hatching at 5 different time points (24, 29, 36, 44 and 48 hours post fertilization, 20 larvae for time point) and 4 weeks post hatching at ZT3, 9, 15 and 21 (10 larvae per time point). Dissections in dark conditions were performer under dim red light (<1 lux). Tissues and whole larvae were immediately frozen in dry ice to avoid RNA degradation.

II.8 Feeding experiment in adult cavefish

Cavefish (n=36, 14 years old, belonged to the offspring of a wild stock collected in 1982 from the wells of Bud-Bud) were kept in DD at 29±1°C and food was supplied daily at 12:00. Food was available for a short period to avoid the possibility to eat out of the established time window resulting in a non-homogeneous synchronization in the group. After 30 days, samples from different tissues (brain and heart) were collected at six different time points (N=3 for time point) in the 24h during the 1st and the 2nd day of fasting (every 4 hours from CT21), for a total of 48

hours, in order to test for the persistence of circadian clock driven gene expression in constant conditions.

II.9 Gene expression analysis

Quantitative RT PCR was performed for the various *P. andruzzii* clock genes using the following pairs of primers: CF *Clock1* Fwd: 5'-GTACTGTGGAGGAGCCCAAT-3' Rev: 5'-GGGTCTCCAGGTCATCCAC-3' (162bp fragment); CF *Period1* Fwd: 5'-GGCAACATCTCAACCAGTGG-3' Rev. 5'-GGGCTTCATAACCCGCGTGC-3' (145bp fragment); CF *Period2* Fwd: 5'-TTCCAGCTGTGTGTTTCAGG-3' Rev: 5'-AGAAGCGGAAAGAGTGGTCA-3' (187bp fragment); CF *Cry1a* Fwd: 5'-TGATGAGAAGTTCGGGGTTC-3', Rev: 5'-GCGAATTGGCATTCATTCTT-3' (173bp fragment); CF *Cry5* Fwd: 5'-CTGCAGAGGTCCTTCCAAAG-3', Rev: 5'-GCTTTCCGTTGTTCTCTTCG-3' (198bp fragment) and CF *β-actin* Fwd: 5'-AGGACCTGTATGCCAACACA-3', Rev: 5'-AATCCACATCTGCTGGAAGG-3' (200bp fragment). Quantitative RT PCR was performed for the various zebrafish clock genes using the following pairs of primers: ZF *Clock1* Fwd: 5'-CTGGAGGATCAGCTGGGTAG-3' Rev: 5'-CACACACAGGCACAGACACA-3'; ZF *Period1* Fwd: 5'-CCGTCAGTTTCGCTTTTCTC-3' Rev. 5'-ATGTGCAGGCTGTAGATCCC-3'; ZF *Period2* Fwd: 5'-ATGTCGATGGCTTTAGGCAG-3' Rev: 5'-CGAGACATCCAGAAGGTGCT-3'; ZF *Cry1a* Fwd: 5'-TCCGCTGTGTGTACATCCTC-3', Rev: 5'-CAAACACTGCAGCAAAAACC-3'; ZF *Cry5* Fwd: 5'-AATGGCAAGACTCCCATGAC-3', Rev: 5'-GTGGCCCTAAGGATGACGTA-3' and ZF *β-actin* Fwd: 5'-GCCTGACGGACAGGTCAT-3', Rev: 5'-ACCGCAAGATTCCATACCC-3'. 4µl of 1:5 diluted cDNA was pipetted in each well of a 96well plate together with according SYBRgreen-Primer-MasterMix. Quantitative Real-Time PCR was performed using an Applied Biosystems StepOnePlus Real-Time PCR System (Applied Biosystems). PCR conditions were: 15 min at 95 °C, then 40 cycles of 15 s at 95 °C, 30 s at 60 °C. The relative levels of each RNA were calculated by the $2^{-\Delta\Delta CT}$ method (CT standing for the cycle number at which the signal reaches the threshold of detection). Relative expression levels were normalized to cavefish or zebrafish *β-actin* respectively.

II.10 Cells in light-dark cycles

For LD experiment CF and AB9 cells were seeded in culture flasks approximately 70% of confluency. Flasks containing cells were submerged in 60 l water baths with circulating heating and cooling units (Lauda, Lauda-Königshofen, Germany) to keep the temperature constant and illuminated with a tungsten light source (11 $\mu W/cm^2$). Cells were raised at 25°C under a 12:12-h light-dark cycle and then collected during the 4^{th} day in LD cycle at 4 different time points (ZT3, 9, 15, 21; N=4 for CF cells and N=4 for AB9 cells).

II.11 Reporter and expression vectors

***zfPer1b-luc*:** The zf*Per1b* promoter was amplified by using Genome Walker PCR (Clontech), according to the manufacturer's instructions, and subcloned into pGL3Basic (Promega) (*zfper4:luc*, Vallone et al. PNAS 2004).

***zfPer2-luc*:** The light responsive promoter of zebrafish Period 2 (zf*Per2*) containing a fragment of 1,571 bp of the 5' flanking region and 129 bp of the 5'UTR of the per2 gene was PCR amplified using the Universal GenomeWalker Kit (Clontech) by using Genome Walker PCR (Clontech) and subcloned into pGL3Basic (Promega) (*-1.7per2:Luc*, Vatine et al., 2009).

***zfPer2* LRM:** The per2 minimal promoter, containing 431 bp of the 5' flanking region and 164 bp of 5' UTR of the per2 gene was subcloned into pGL3basic (Promega) upstream of the luciferase reporter gene (*-0.43per2:Luc*,Vatine et al.,2009).

***cfPer2-luc*:** The promoter fragment of the cavefish *Period* 2 gene, containing 876 bp of the 5' flanking region and 112 bp of the 5'UTR, was amplified by using Genome Walker PCR (Clontech) from cavefish genomic DNA with the primer CFPer1GW1 (5'-TCTGTCCGGCTAAAAAGTCCGACTAAAT-1) in combination with the AP1 primer (Clontech) and sequenced. CFPer2SacIF (5'-ACTG**GAGCTC**TGCGATTTTCACCTTAGATTCC-3') CFPer2XhoIR (5'-ACTG**CTCGAG**TCTGTCCGGCTAAAAAGTCC-3') introducing the restriction site for the enzyme SacI and XhoI (Fermentas) were used to clone the fragment into PGL3 basic vector (Promega).

Expression vectors were generated using a PCR-based strategy. The zebrafish per2 minigene (zfPER2) was constructed by first amplifying the full length coding sequence of zebrafish *per2*

(genbank NM_182857.1), eliminating the stop codon by introducing an artificial XbaI restriction site and cloning into the expression vector pcDNA3.1 Myc/His A. The CMV promoter was eliminated from this expression vector by digestion with the restriction enzymes MfeI and BamHI (New England Biolabs). CMV promoter fragment was replaced with the light responsive promoter fragment of the zebrafish *Period 2* gene (Vatine et al., 2009); the fragment contains 1,457 bp of the 5' flanking region and 164 bp of the 5' untranslated region (UTR) of the zebrafish *Per2* gene, amplified from zebrafish genomic DNA with the primers ZFPer2MfeIF (5'-ATCG**CAATTG**ACCATGCATAGCAGAAACTT-3') and ZFPer2BamHIR (5'-ATCG**GGATCC**CTGACAACTTCAGCAAATCTT-3').

The full-length zebrafish *TMT-opsin* (ENSDART00000081729) was amplified with the primers 5'TMTfor (5'-ATCG**GAATTC**GCTTTCCCCTCAGAAAACAA-3') and 3'TMTrev (5'-ATCG**GAATTC**TCGCCTGTAAACAAATCCAA-3') introducing restriction sites for EcoRI (Fermentas) and cloned into the pcDNA3.1 (+) to generate the *TMT-opsin* expression vector. In the case of *Opn4m2* (ENSDART00000018501), appropriate restriction sites were introduced (BamHI, XhoI, Fermentas) and stop codon abolished using the PCR primers ZFOpn4m2BamHIF **(**5'-GATC**GGATCC**GCATGAGCCATCACTCTTCA-3') and ZFOpn4m2MycXhoIR **(**5'-GATC**CTCGAG**GTTCCCTCCAAGCAAAGCCT-3'). Amplified *Opn4m2* was then used to create an expression vector introducing the cDNA into the pcDNA3.1 Myc/His A.

These PCR reactions were performed using the Perkin Elmer Gene Amp XL PCR kit according to manufacturer's instructions. All PCR reactions were temperature cycled using a Perkin Elmer 9700 thermal cycler. Obtained cDNA and DNA inserts were digested and purified by agarose gel electrophoresis followed by Qiaquick column purification from agarose gel slices (gel extraction kit, Qiagen). Vectors were transformed into competent *E.coli* bacteria (TOP10F', InVitrogen) using the manufacturer's reagents and instructions.

II.12 Plasmid DNA extraction

Liquid cultures of ampicillin-resistant bacteria were prepared by inoculating 100ml aliquots of Luria Bertani (LB) medium using either single bacterial colonies or frozen bacterial glycerol stocks (Sambrook et al., 1989). Plasmid DNA was extracted from saturated overnight cultures using a column-based extraction system according to the manufacturer's instructions (Midi prep, Qiagen) and the final DNA pellets were dissolved in 100–200µl of Millipore filtered, glass

distilled water. Yields of DNA were calculated based on optical density measurements of dilutions made from the stocks. The optical density was measured at both 260nm and 280nm and so used to assess DNA purity as well as concentration (Sambrook et al., 1989). Glycerol stocks were prepared for each plasmid produced according to standard methods (Sambrook et al., 1989). Minipreps of plasmid DNA from 1.5ml cultures were prepared using a standard "boiling method" (Sambrook et al., 1989).

II.13 Transfection using Fugene HD™ transfection reagent (Roche Diagnostics)

CF cells were transfected using Fugene HD™ transfection reagent according to the manufacturer's conditions with a 3:1 ratio of Fugene6™(in µl):DNA(in µg) (Roche Diagnostics, Fugene6™) and subsequently stored in the incubator over night at 25°C.

II.14 *In vivo* luciferase assay LD

CF and AB9 cells were grown with Leibovitz's L-15 medium (Gibco) supplemented with 20% fetal calf serum (Biochrom KG), 100 units/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamycin (Gibco BRL) and approximately $6x10^4$ CF cells and $3x10^4$ AB9 cells per well were seeded into a 96-well fluoro-assay plate (Nunc Rochester) 1 day before the experiment. The plate was then incubated overnight at 25°C to ensure proper attachment of the cells to the surface of the well. The day after, grown medium was replaced with serum free Leibovitz's L-15 medium, to avoid any induction of oscillation driven by the culture serum. Cells were transfected with the reporter vectors *zfPer1b-luc*, *zfPer2-luc* or *cfPer2-luc* using FuGENE HD (Roche) according to the manufacturer's protocol. In the experiments with the minigene, zfPER2 minigene was co-tranfected at different concentration in combination with the *zfPer1b-luc* reporter vector.
24h after transfection medium was replaced with 250µl fresh culture medium containing 0.5mM beetle luciferin, potassium salt solution (Promega). The plate was sealed using an adhesive "Top Seal" sealing sheet (Packard). Plates were then transferred into a Packard Top-count NXT scintillation counter (2-detector model, Packard) and exposed to LD cycles (12:12).

Bioluminescence was measured and expressed as the frequency of photon emission (cps) counted during 4-6 sec/well at intervals of 30-60 mins. In the experiments the plate was illuminated during the intervals between counting. To ensure uniform illumination of wells across the plate, each sample plate was positioned below a transparent, empty 96 well plate when inserted into the counter's plate-stacking unit. The counter was located in a thermostatically controlled dark room and illuminated using a tungsten light source (20 $\mu W/cm^2$) that was connected to a programmable timer. Luciferase assay data was analysed by first storing the data as ASCII data files using the Packard "Hologram" software system and then importing these files directly into Microsoft Excel using the Import and Analysis macro (I&A, Plautz et al., 1997). Subsequently data was plotted graphically using I&A Excel macro software.

II.14.1 Rescue of light induction on zf*Per2* promoter-reporter in CF cells

CF-1 cells were grown with Leibovitz's L-15 medium (Gibco) supplemented with 20% fetal calf serum (Biochrom KG), 100 units/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamycin (Gibco BRL) and approximately $6x10^4$ CF cells per well were seeded into a 96-well fluoro-assay plate (Nunc Rochester) 1 day before the experiment. The plate was then incubated overnight at 25°C to ensure proper attachment of the cells to the surface of the well. The day after, grown medium was replaced with serum free Leibovitz's L-15 medium. Cells were transfected with the reporter vector *zfPer2-luc* and the opsins expression vectors using FuGENE HD (Roche) according to the manufacturer's protocol. 24h after transfection medium was replaced with 250µl fresh culture medium containing 0.5mM beetle luciferin, potassium salt solution (Promega) and also retinaldehyde isoforms (100 nM; 9-cis-Retinal and all-trans-Retinal from Sigma-Aldrich) under dim red light. The plate was sealed using an adhesive "Top Seal" sealing sheet (Packard).

Plates were then transferred into a Packard Top-count NXT scintillation counter (2-detector model, Packard) and exposed to LD cycles (12:12). Cells were kept in the dark or under dim red light during the experiment. Bioluminescence was measured and expressed as the frequency of photon emission (cps) counted during 4-6 sec/well at intervals of 30-60mins. In the experiments the plate was illuminated during the intervals between counting. To ensure uniform illumination of wells across the plate, each sample plate was positioned below a transparent, empty 96 well plate when inserted into the counter's plate-stacking unit. The counter was located in a

thermostatically controlled dark room and illuminated using a tungsten light source (20 μW/cm^2) that was connected to a programmable timer. Luciferase assay data was analysed by first storing the data as ASCII data files using the Packard "Hologram" software system and then importing these files directly into Microsoft Excel using the Import and Analysis macro (I&A, Plautz et al., 1997). Subsequently data was plotted graphically using I&A Excel macro software.

II.15 Induction of cycling *Per1b* promoter-reporter vector by Dexamethasone

CF-1 cells were grown with Leibovitz's L-15 medium (Gibco) supplemented with 20% fetal calf serum (Biochrom KG), 100 units/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml gentamycin (Gibco BRL) and approximately X cells/96 well plate were plated 1 day before the experiment into a 96-well fluoro-assay plate (Nunc Rochester). The plate was then incubated overnight at 25°C to ensure proper attachment of the cells to the surface of the well. After 24h grown medium was replaced with serum free L-15 medium. Cells were transfected with the reporter vector *zfPer1b-luc* using FuGENE HD (Roche) according to the manufacturer's protocol. In the experiments with the minigene, zfPER2 minigene was co-tranfected at different concentration in combination with the *zfPer1b-luc* reporter vector.

The day after cells were treated with Dexamethasone (Sigma) added in L-15 at a final concentration of 100 nM which replaced the transfection medium. After 2 hours the Dexamethasone was removed by washing the plate twice with PBS and substituted with 250μl fresh culture medium containing 0.5mM beetle luciferin, potassium salt solution (Promega) and the plate sealed using an adhesive "Top Seal" sealing sheet (Packard). Plates were then transferred into the scintillation counter (Envision Top Count; Perkin Elmer) and incubated at constant temperature (22°, 25°, 29°C). Bioluminescence was measured once per hour directly after the treatment. Data were exported with the Wallac EnVision Manager 1.12 software and analysed by first storing the data as CSV data file and then importing directly into Microsoft Excel.

II.15.1 Phase Response Curve

CF-1 cells were grown with Leibovitz's L-15 medium (Gibco) supplemented with 20% fetal calf serum (Biochrom KG), 100 units/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamycin (Gibco BRL) and approximately $6x10^4$ CF cells per well were seeded into a 96-well fluoro-assay plate (Nunc Rochester) 1 day before the experiment. The samples on the plate were distributed in five group on the left part (I-V; Light pulse) and five group on the right part (I-V; Dark control). The plate was then incubated overnight at 25°C to ensure proper attachment of the cells to the surface of the well. After 24h grown medium was replaced with serum free L-15 medium. Cells were transfected with the reporter vector *zfPer1b-luc* using FuGENE HD (Roche) according to the manufacturer's protocol. The day after each group of cells were treated with dexamethasone (Sigma) added in L-15 at a final concentration of 100 nM with an interval of approximately 8 hours per group. After 2 hours the medium was replaced with 250µl fresh culture medium containing 0.5mM beetle luciferin, potassium salt solution (Promega). At the end of the treatments the plate was sealed using an adhesive "Top Seal" sealing sheet (Packard) and half of the plate was exposed to 15 min light pulse. Plate was then transferred into the scintillation counter (Envision Top Count; Perkin Elmer) and incubated at constant temperature (25°C). Cells were kept in the dark or under dim red light during the experiment. Bioluminescence was measured once per hour directly after the treatment. Data were exported with the Wallac EnVision Manager 1.12 software and analysed by first storing the data as CSV data file and then importing directly into Microsoft Excel.

III. RESULTS

III.1 Cavefish clock genes cloning

The first part of the study focused on the molecular characterisation of the *P. andruzzii* circadian oscillator. With this aim, an analysis of the transcriptome was performed where various clock and clock-controlled genes were identified.

cDNA sequences of vertebrate clock genes, cloned from various species, have revealed regions of high conservation. A RT-PCR approach with primers designed against conserved domains of zebrafish clock gene homologs was used to clone many positive and negative elements of the clock machinery in the Somalian cavefish. Clock genes such as *Clock*, *Period* and *Cryptochrome* have all been isolated and characterised (Table 1).

Large sections of the coding sequences of members of the *Cryptochrome* family have been identified: as in other teleosts, this group of genes is composed by six *Cry*s (*Cry1a*, *Cry1b*, *Cry2a*, *Cry2b*, *Cry3*, *Cry4*) together with *6-4 photolyase*, that is also called *Cry5*. DNA photolyase is an enzyme that repairs UV damaged DNA in a light-dependent manner (Todo et al., 1993). Specifically, (6–4) photolyases repair (6–4) pyrimidine photoproducts. *Cryptochromes* are evolutionary descendents of photolyases; they have lost the DNA repair activity and function as photoreceptors or transcriptional repressors (Cashmore et al., 1999). CRYs share the same structure with a conserved N-terminal alpha/beta domain homologous to photolyases and a divergent C-terminal helical domain, connected by an interdomain loop. CRYs have been implicated as photoreceptors in zebrafish, as in the case of Drosophila, where CRY is a directly photosensitive nuclear protein that interacts with the negative feedback loop in the fruitfly clock.

In zebrafish, *Cry1a* is a light inducible gene (Hirayama et al., 2005) and a strong repressor of the positive limb via its binding with the heterodimer CLOCK-BMAL in the nucleus (Ishikawa et al., 2002). The *P. andruzzii Cry1a* cDNA contains an 1872-bp open reading frame (ORF) encoding a 623 amino acid protein. The predicted CRY1a protein shares very high sequence similarity with other vertebrate orthologs (96% with zebrafish, 82% with human, 80% with mouse, 79% with chicken), revealing that functional domains are conserved. They include the two characteristic chromophore-binding domains (FAD-binding domain and pterin-binding domain) also shared with photolyases.

In zebrafish *Cry1b*, *Cry2a* and *Cry2b* are also able to repress transactivation mediated by

CLOCK-BMAL. On the contrary, zebrafish *Cry3* and *Cry4* are more divergent and they do not inhibit this transcriptional activation. The *P. andruzzii* coding regions for *Cry1b* (1824 bp, full length cds), *Cry2a* (1465 bp), *Cry2b* (542 bp) and *Cry3* (1426 bp) share very high sequence similarity with zebrafish (>90%). In the case of cavefish *Cry4*, only a part of the coding sequence was cloned (825 bp) where the 3' end represented an unspliced intron-exon junction. *Cry5* (*6-4 photolyase*) expression in zebrafish is induced upon exposure to light and in constant darkness, low basal levels of expression are observed. In *P. andruzzii* a 1235 bp fragment of the *Cry5* coding sequence was cloned. Analysis of the sequence confirmed high homology with the DNA photolyases (aa 3-178) as well as specifically the FAD binding domain (aa 212-410).

Period gene homologs were cloned using primers directed against the conserved PAS (A and B) and PAC domains involved in the binding of CLOCK-BMAL. The *P. andruzzii Per1* (4203 bp, full-length cds), *Per2* (3252 bp) and *Per3* (1172 bp) genes were cloned. Cavefish *Per1* encodes a 1400 amino acid protein (PAS A, aa 244-311; PAS B, aa 384-450; PAC, aa 458-501).

The *Clock* genes are members of the bHLH-PAS protein family of transcription factors. In *P. andruzzii* homologs of *Clk1* (2655 bp, full-length cds), *Clk2* (2421 bp) and *Clk3* (943 bp) were identified. The deduced sequence of *Clk1* encodes for a 884 amino acid protein and it includes in the N-terminal region a basic helix loop helix motif (bHLH, aa 31-81) involved in DNA binding as well as the PAS domains (PAS A, aa 103-169; PAS B, aa 258-324; PAC, aa 330-373) mediating protein-protein interactions, essential for heterodimeric interaction with its partner factor, BMAL.

A comparison of the *P. andruzzii* clock gene sequences with the orthologues in other vertebrates revealed high homology of the amino acid sequences, especially with the zebrafish, *D. rerio*, together with conservation of the functional domains in the proteins (Table 1; Fig. 7).

Gene name	**Cds (bp)**	**% of homology with *D. rerio***			
Cry1a	1872	96	*Clk1*	2655	90
Cry1b	1824	90	*Clk2*	2421	91
Cry2a	1465	95	*Clk3*	943	92
Cry2b	542	92	*Per1*	4203	86
Cry3	1426	97	*Per2*	3252	82
Cry4	825	86	*Per3*	1172	92
Cry5 (6-4 photolyase)	1235	89			

Table 1: P. andruzzi and D. rerio both belong to the cyprinid family and show very high sequence homology within the clock gene coding sequences.

Specific couples of primers were then designed to allow Real Time RTPCR analysis of the expression of these clock genes, and thereby to study clock and light regulated gene expression in *P. andruzzii*.

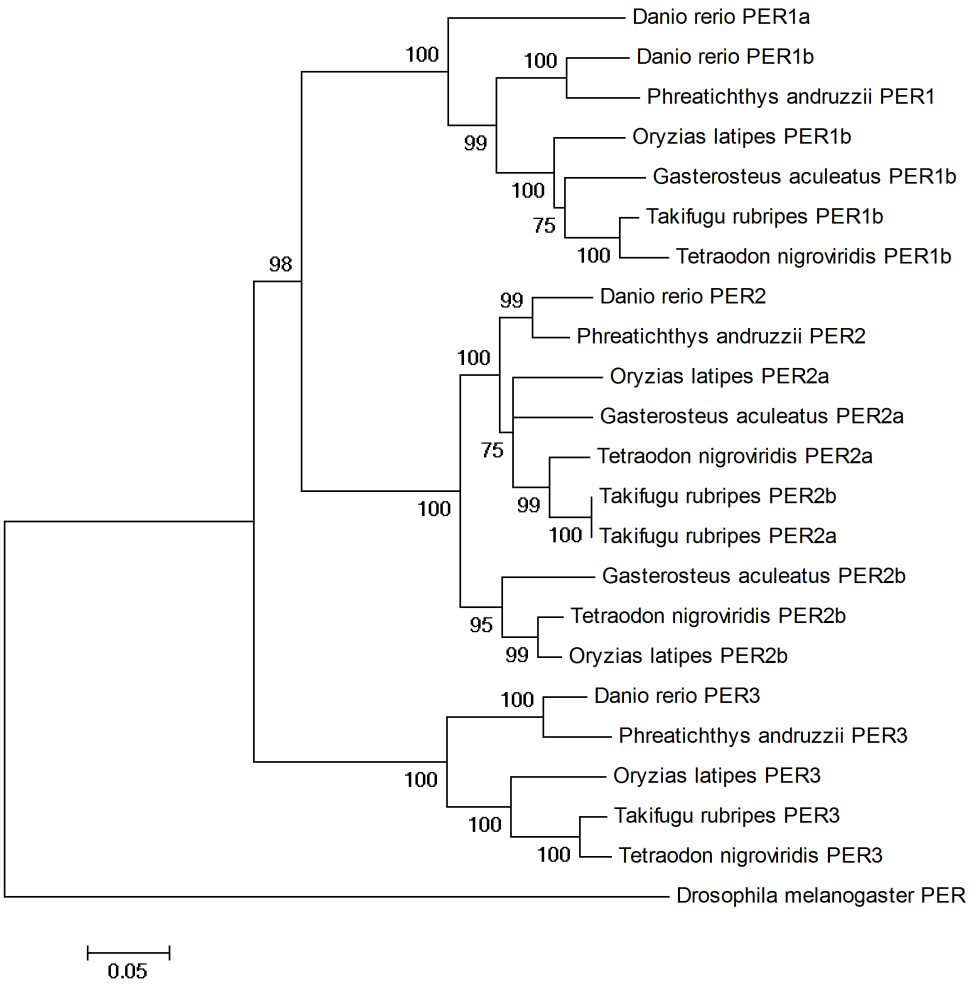

Fig. 7: By alignment with different animal models and using phylogenetic tree analysis it was possible to allocate the genes cloned from the Somalian cavefish within the Period genes families. High sequence conservation was found between P. andruzzi and D. rerio. Notably Per1 gene from cavefish is a closer relative of Per1b of zebrafish.

III.2 Quantitative analysis of daily expression of clock genes

In contrast to mammals, teleosts such as the zebrafish are remarkable since in most tissues and even cell lines the circadian clocks are synchronized by direct exposure to light (Whitmore et al., 2000).

During million of years of evolution, *P. andruzzii* did not experience regular day-night changes so the first question of the study was whether these fish still retain a functional circadian clock and if so, what is the effect of light exposure on this internal oscillator.

A pool of adult fish (N=12) and larvae (N=300 1st day; N=72 4th week) from cavefish were exposed for 1 month to a 24h light-dark (LD) cycle (12h:12h). Samples from different tissues (brain and fin) and total larvae RNA were collected at four different time points, every 6 hours, during one 24h cycle. Clock gene expression was subsequently measured by Real Time RTPCR and then the expression pattern was compared with that of zebrafish tissues (brain and fin, collected from fish exposed to the same experimental conditions).

The preliminary experiment focused on the expression pattern of the following genes: *Per1*, *Clk1*, *Per2*, *Cry1a* and *Cry5*. These genes were selected to form a representative panel of the molecular clock. *Clk1* and *Per1* are core clock components of the positive and the negative limb respectively. *Per2*, *Cry1a* and *Cry5* are light-induced genes in zebrafish (Hirayama et al., 2005; Ziv et al., 2005; Tamai et al., 2007; Kobayashi et al., 2000; Vatin et al. 2009).

Rhythmic expression of these genes was encountered in zebrafish tissues (Fig. 8a,b,e,f). *Clk1* and *Per1b* showed daily variations of mRNA: *Clk1* peaked around ZT15 and ZT9 in fin and brain respectively and *Per1b* peaked at ZT21, (Fin: one-way ANOVA, *Per1b*: $F_{3,15}$=248.4, p<0.0001; *Clk1*: $F_{3,15}$=35.4, p<0.001. – Brain: one-way ANOVA, *Per1b*: $F_{3,15}$=247.2, p<0.0001; *Clk1*: $F_{3,15}$=6.8, p<0.007.). In *Per2*, *Cry1a* and *Cry5* upregulation in the expression was observed only following a few hours of light exposure (Fin: one-way ANOVA, *Per2*: $F_{3,15}$=79.68, p<0.0001; *Cry1a*: $F_{3,15}$=66.56, p<0.0001; *Cry5*: $F_{3,15}$=248.4, p<0.0001. – Brain: one-way ANOVA, *Per2*: $F_{3,15}$=33.54, p<0.0001; *Cry1a*: $F_{3,15}$=173.2, p<0.0001; *Cry5*: $F_{3,15}$=62.28, p<0.0001.).

Remarkably arrhythmic expression of *Per1*, *Cry1a* and *Per2* was encountered in the fin and brain of *P. andruzzii* (Fig. 8c,d,g,h) (Fin: one-way ANOVA, *Per1*: $F_{3,15}$=1,08, p>0.3, *Per2*: $F_{3,21}$=2.5, p>0.07; *Cry1a*: $F_{3,25}$=2.7, p>0.06. – Brain: one-way ANOVA, *Per1*: $F_{3,19}$=0,78, p>0,4; *Per2*: $F_{3,23}$=2.9, p>0,07; *Cry1a*: $F_{3,16}$=2,9, p>0,06.), and no upregulation in the expression of *Per2* or *Cry1a* was observed during the light phase (Fig. 8d,h). Interestingly, in the larvae collected during the 1st day after hatching, when they still possess eye rudiments (Berti et al., 2001), and in the 4 weeks old group where eye loss is complete, this expression pattern was the same (Fig. 8i,j).

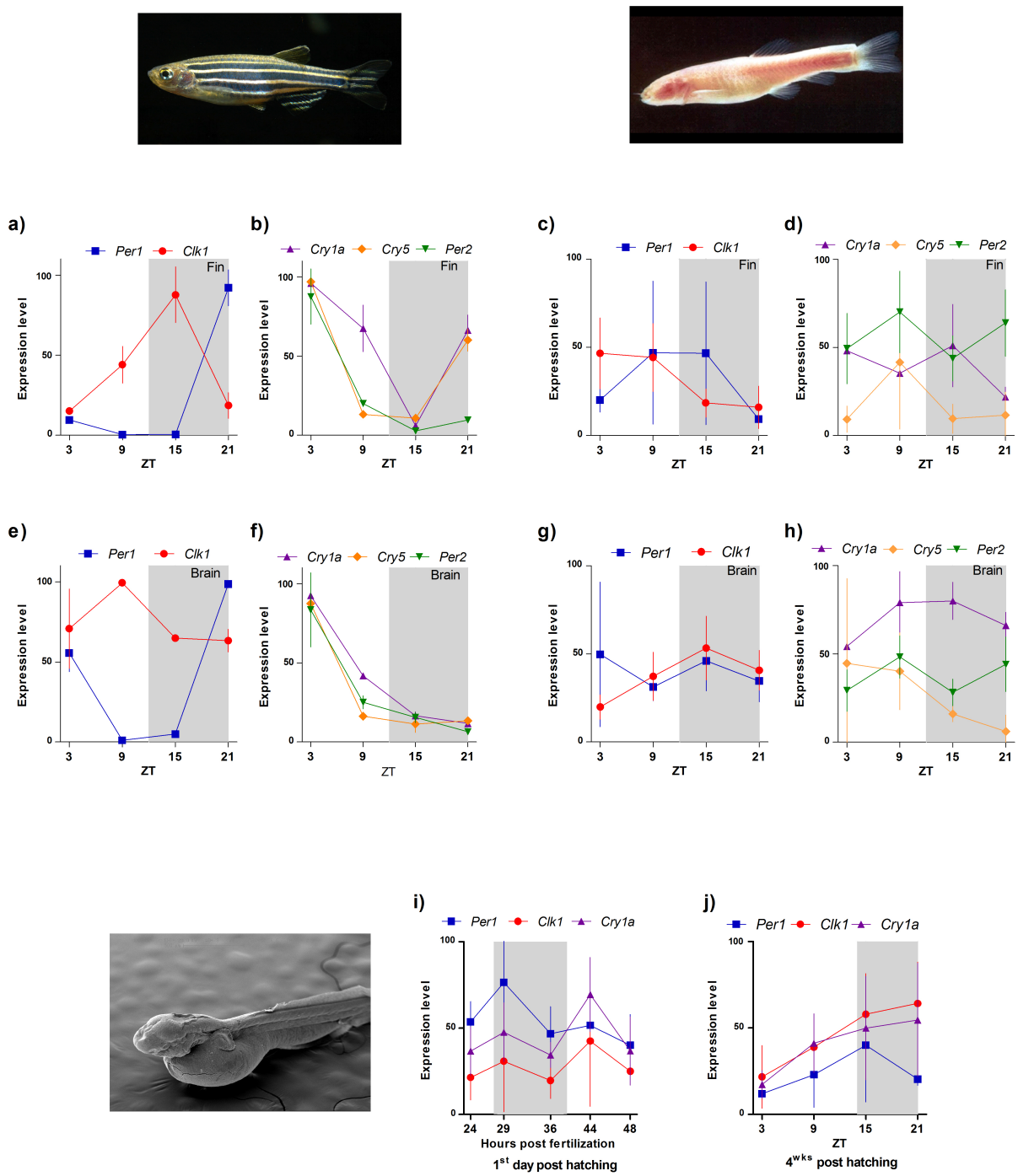

Fig. 8: ***a,b,e,f*** *Quantitative RT-PCR of clock-regulated and light-inducible gene expression in zebrafish tissues (fin and brain) during one 24h period exposed to a 12:12 LD cycle. Mean and standard error are plotted in the graphs. Analysis of mRNA levels confirmed the antiphase relationship between cycling of the core clock componenst Clk1 and Per1b (**a,e**) and the early upregulation during the light phase of light-inducible genes Per2, Cry1a and Cry5 (**b,f**).*
c,d,g,h *Quantitative RT-PCR of clock-regulated and light inducible gene expression in cavefish adult tissues (n=16) exposed to a 12:12 LD cycle. There was no evidence for antiphase daily rhythms of Clk1 and Per1 either in the fin (**c**) or in the brain (**g**). Furthermore light inducible genes are not significantly upregulated upon light exposure (**d,h**).*
i,j *Cavefish larvae gene expression levels. The genes investigated did not exhibit circadian rhythms of expression either during the first day after hatching (**i**) or after 4 weeks (**j**). (The lighting conditions were identical to those used for adults).*

III.3 Cell line experiments

Based on these preliminary results the investigation continued to explore the regulation of the various clock genes in comparison with zebrafish. Cell lines derived from the cave fish were chosen as a model to perform a more detailed analysis of the clock and light regulation of gene expression. Several primary cell lines (CF-1, CF-2, CF-3) were obtained from fin clips of adult *P. andruzzii*. These cells are derived from fibroblasts and epidermal cells and represent a useful tool to broaden the molecular study. Combined with the use of *in vitro* assays and transfections, these cells allow a level of functional analysis that is not feasible with this cavefish species *in vivo*.

When the cell lines were established, Real Time RTPCR was performed to confirm that clock gene mRNA expression was also not rhythmic after 4 days under LD cycle conditions (one-way ANOVA, *Per1*: $F_{3,18}$=0.55, p>0,6; *Clk1*: $F_{3,23}$=2.17, p>0,1; *Per2*: $F_{3,14}$=0.68, p>0,5; *Cry1a*: $F_{3,20}$=1.7, p>0,1; *Cry5*: $F_{3,15}$=0.665, p>0,5) (Fig. 9c,d). To validate the results, in parallel we analysed a similar cell line derived from adult zebrafish fin clips (AB9) (Kwok et al., 1998), that showed robust rhythms of clock gene oscillation (one-way ANOVA, *Per1*: $F_{3,15}$=32.9, p<0.0001; *Clk1*: $F_{3,15}$=44.8, p<0.0001; *Per2*: $F_{3,15}$=81.25, p<0.0001; *Cry1a*: $F_{3,15}$=131.3, p<0.0001; *Cry5*: $F_{3,15}$==85.8, p<0.0001) (Fig. 9a,b).

In addition both cell lines were transfected with clock and light regulated luciferase reporter constructs, based on the zebrafish *Per1b* and *Per2* promoters respectively, which were positioned upstream of the luciferase reporter gene. .Subsequently, reporter gene expression was measured using an *in vivo* luciferase assay. *Period1b* transcription is directly clock controlled; the promoter region of *Per1b* contains four E-boxes, two canonical (5'-CACGTG-3') and two non canonical (5'-AACGTG-3'), and so provides a direct measure of clock activity (Vallone et al., 2004). *Period2* is a light regulated gene; its promoter contains a light responsive module (LRM), constituted by an E-box coupled with a D-box (Vatine et al., 2009). The D-box (5'-RT(G/T)AYGTAAY-3') is the binding site for transcription factors implicated in conferring light driven gene expression, such as TEF and E4BP4 (Doi et al., 2001; Cowell, 2002).

The *in vivo* reporter assay allowed monitoring of the response of the cells to the lighting regime at the level of transcriptional control of these two key clock genes. The AB9 cells showed rhythmic expression of luciferase in both constructs with a period close to 24 hours (Fig. 9e). In contrast, in CF cells neither reporter showed any significant daily rhythmic expression in luciferase activity during the course of the experiment (Fig. 9f).

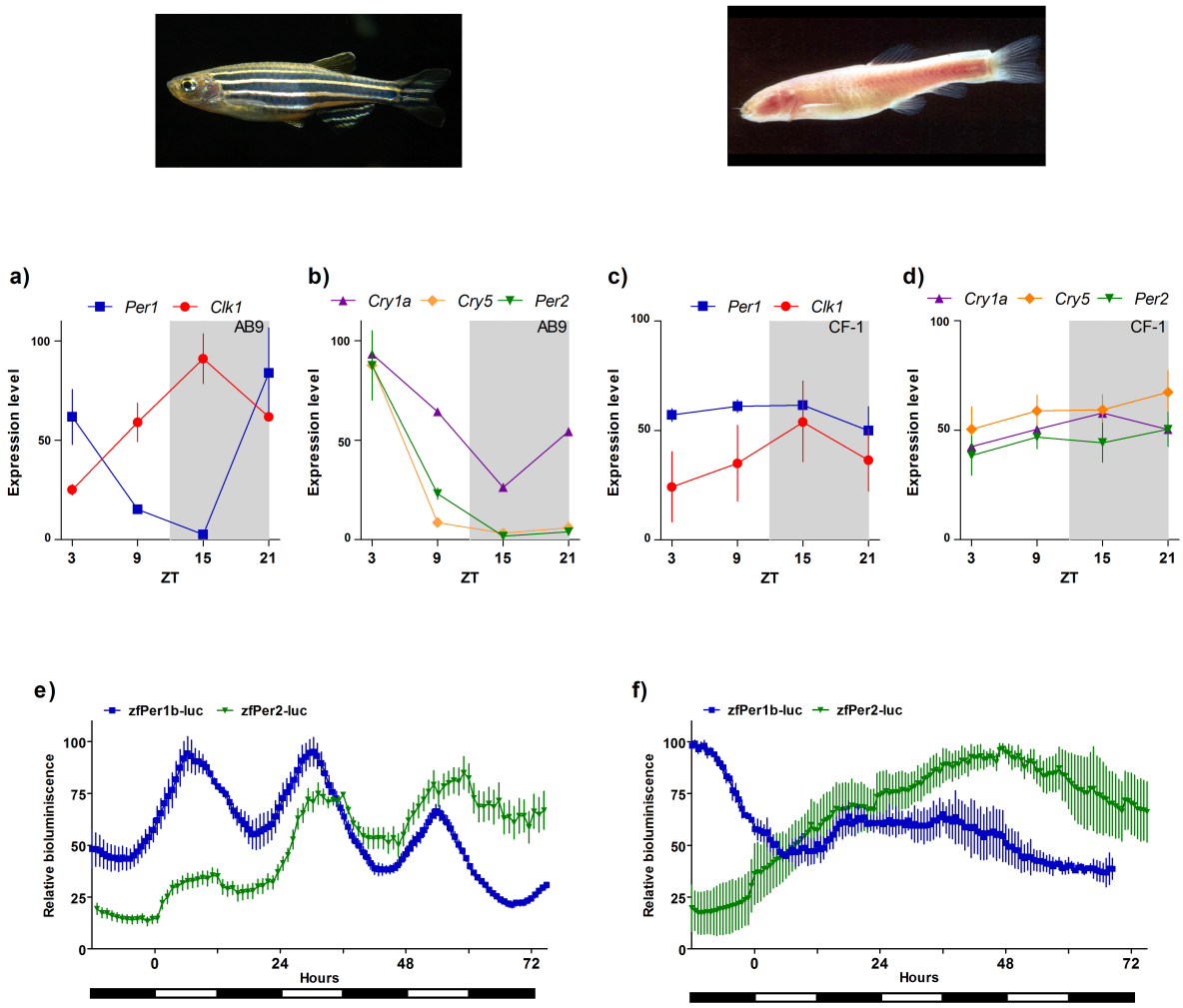

Fig. 9: ***a,b*** *Quantitative RT-PCR of clock-regulated and light-inducible gene expression in a zebrafish cell line derived from an adult fin clip (AB9) during a 24h period exposed to a 12:12 LD cycle. Mean and standard errors are plotted on the graphs. Analysis of mRNA levels confirmed the antiphase cycling of the core clock components Clk1 and Per1b (**a**) and the early upregulation during the light phase of the light-inducible genes Per2, Cry1a and Cry5 (**b**).*
c,d *Quantitative RT-PCR of clock-regulated and light-inducible gene expression in CF-1 cavefish cells exposed to a 12:12 LD cycle. There was no evidence for antiphase daily rhythms of Clk1 and Per1 expression (**c**). Furthermore, light inducible genes are not significantly upregulated after light exposure (**d**). In the cavefish CF-1 cells the levels of the genes investigated did not change during the LD cycle (p>0.1, one-way ANOVA). (The lighting conditions were identical to those used for adults).*
e,f *zfPer1b and zfPer2 reporter vectors were transfected into AB9 and CF-1 cells and then a Packard Topcount scintillation counter was used to assay for bioluminescence in the transfected cells under 12:12 LD cycles. In the AB9 cells there was a pronounced zfPer1b reporter expression rhythm that showed an increase in expression with anticipation of the beginning of the light phase while the light-inducible zfPer2 promoter, directed a strong induction of reporter gene expression immediately following the beginning of the light phase (**e**). In CF-1 cells both reporter vectors failed to show any oscillation or light induction even after exposure to several LD cycles (**f**). Values of reporter are mean ± standard error.*

In contrast to the robust rhythms of clock gene expression documented in different cell lines from zebrafish (Vallone et al., 2004; Farhat et al., 2009), all *in vivo* and *in vitro* experiments showed a complete absence of rhythmic clock gene expression in *P. andruzzii*, implicating either absence of a functional circadian clock or a defect in the light input pathway for what is otherwise a normal clock.

III.4 Feeding entrainment in adult *P. andruzzii*

To distinguish between these two possibilities, the use of an alternative zeitgeber was tested for inducing rhythmic gene expression in adult fish. If the clock mechanism can be synchronized by an alternative input, this would point to a defect in the light input pathway explaining the observed cavefish phenotype.

Considering the available data on the ecology of this fish, regular feeding time was implicated as a reasonable candidate to act as a zeitgeber. Scheduled feeding time is well documented to serve as a strong zeitgeber that can entrain the clocks in the brain as well as peripheral organs and tissues in mammals and teleosts (Stephan, 2002; Stokkan et al. 2001; Sanchez et al., 2009). Regular feeding time has even been reported to uncouple peripheral clocks such as that in the liver from the central pacemaker (Hara et al., 2001).

To perform this experiment, a pool of adult cavefish (N=36) was entrained for 1 month to a scheduled feeding regime (Fig. 10a). Animals were kept in constant darkness, to avoid any interference from light. Food was administrated once per day at the same time (12:00 a.m.) and for a short period to avoid the possibility that fish might continue to eat outside of the designated feeding time window, thus resulting in a non-homogenous synchronization within the group. Samples from different tissues (brain and heart) were collected at six different time points (N=3 for time point) during the 1st and the 2nd day of fasting, for a total of 48 hours , in order to test for the persistence of circadian clock driven gene expression in constant conditions (free-running period). In this case the zeitgeber time (ZT) is then substituted by the circadian time (CT). Following mRNA extraction, levels of clock genes transcript were then quantified in the different organs by Real time RTPCR.

The resulting data showed a strong response to the feeding entrainment as revealed by a robust

oscillation of clock gene expression in both tissues (Brain: Kruskal-Wallis ANOVA, *Per1*: W_{12}=41.6, p<0.0001; *Clk1*: W_{12}=39.75, p<0.0001. – Heart: Kruskal-Wallis ANOVA, *Per1*: W_{12}=42.15, p<0.0001; *Clk1*: W_{12}=24.71, p=0.01). The *Per1* transcript peaked at CT1 during the first day both in brain and heart (Fig. 10b,c). During the second day, the peak of expression shifted to CT33 for the brain and to CT29 for the heart. The deduced period length, measured on the oscillation of *Per1*, was between 32 and 28 hours and so outside of the circadian range. The *Clk1* mRNA expression rhythm was antiphase to that of *Per1*; however, on the second day it dampened very rapidly and so the precise location of the second peak was difficult to determine (Fig. 10b,c).

These results indicate for the first time the presence of a functional circadian clock in *P. andruzzii*.

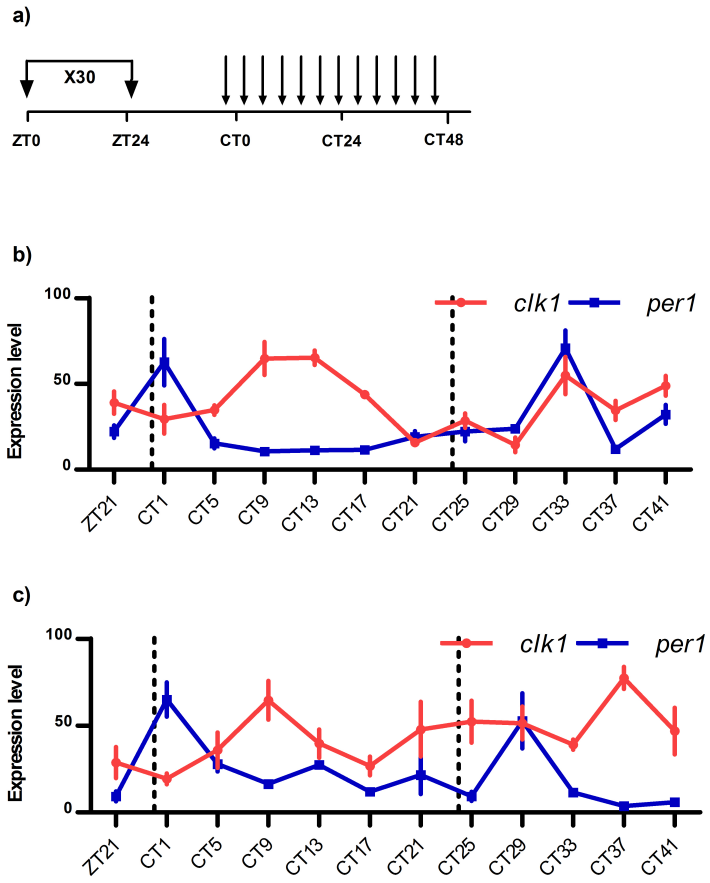

Fig. 10: ***a,*** *scheme of the feeding cycle used to entrain adult cavefish. Food was available once per day at the same time (ZT0) and for 30 days (X30). Samples were collected during the 1st and the 2nd day of fasting at six different circadian time (CT, black arrows) during 24 hours.*
b,c *Quantitative analysis of Clk1 and Per1 expression in the brain (**b**) and in the heart (**c**) of cavefish exposed to feeding entrainment. Dashed lines indicate time of food availability. The profile of the mRNAs were rhythmic (p<0.01 one-way ANOVA) and the Per1 transcript rhythm was antiphase to that of Clk1, suggesting entrainment in the clock machinery.*

III.5 Induction of rhythmic gene expression in cavefish cell lines

The next issue to be addressed was whether other signalling pathways could also activate the rhythmic expression of clock genes in the cavefish cell lines.

Transient treatments with glucocorticoids or high serum concentrations are widely used to induce rhythmic gene expression in mammalian cultured cells (Balsalobre et al., 2000). On the contrary a similar approach has not been reported for teleost cell lines. CF cells were transiently transfected with the zf*Per1b* luciferase reporter construct. The expression of the reporter was tested following a transient, 2 hours treatment with Dexamethasone (an agonist of the glucocorticoid receptor, widely used to synchronize circadian clocks in cell culture). After the dexamethasone pulse, CF cells were then incubated at constant temperature inside the luminescence counter and luciferase activity was assayed once each hour.

The Dexamethasone shock induced a bioluminescence rhythm that persisted for 3 cycles with an extremely long period of about 43 hours at 25°C (one-way ANOVA, $F_{119,991}$=21.99, $p<0.0001$) (Fig. 11a). These data confirmed the *in vivo* feeding entrainment results that this clock displays an abnormal, non-circadian period under constant conditions.

III.5.1 Temperature compensation of the clock

Many artificially generated clock mutations that confer an altered period length also affect temperature compensation (Price, 1997), an essential property of the circadian clock whereby the period length remains relatively constant over a physiological range of temperatures (Izumo et al., 2003). The temperature compensation of the cavefish clock was tested by measuring the period lengths of rhythms induced by Dexamethasone pulses in cells held at a constant 22°C, 25°C or 29°C. The period length of oscillations that persisted for almost 3 cycles is about 43 hours at 25°C, while it is reduced to 38 hours at 29°C (one-way ANOVA, $F_{119,1309}$=30.24, $p<0.0001$) and reaches 47h at 22°C (one-way ANOVA, $F_{119,2499}$=55.73, $p<0.0001$) (Fig. 11b,c). The resulting Q_{10}, a value which describes the period change when the temperature is increased by 10°C, is 0.7(Fig. 11d). For temperature compensated circadian rhythms typical Q_{10} values lie between 0,8 and 1,2.

Taken together these results indicate that the *P. andruzzii* circadian timing system not only lacks a functional light input pathway but also has an “exotic” core clock mechanism displaying an abnormally long free running period that is not efficiently temperature compensated.

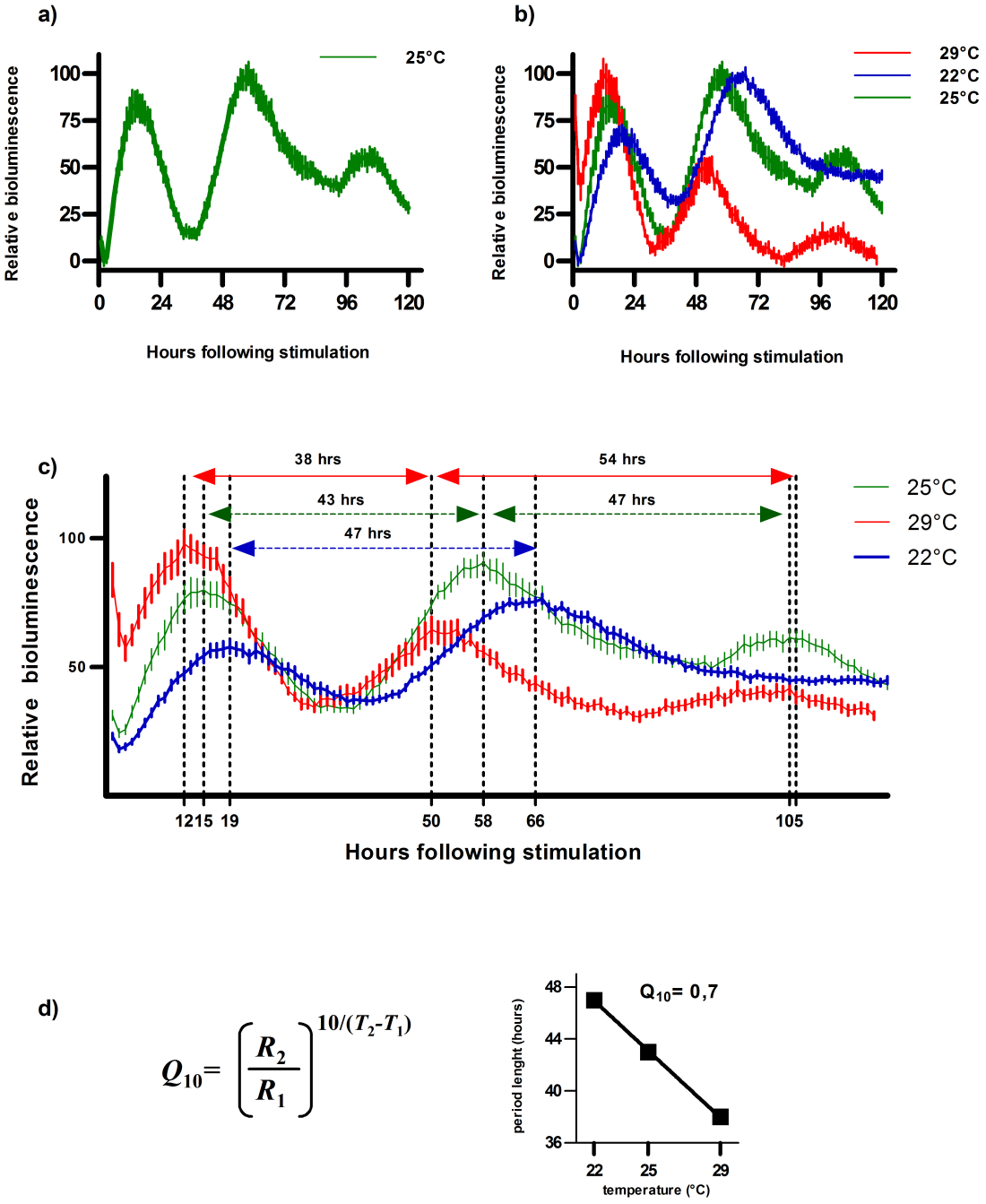

Fig. 11: ***a-c*** *CF-1 cells transfected with the zfPer1b luciferase reporter vector and then bioluminescence was measured on an Envision multilabel reader, after transient treatment with dexamethasone. Cells were kept at constant temperatures and bioluminescence was measured once per hour. Values of reporter activity are expressed as mean ± standard error.*

a, *Reporter gene expression rhythms were measured at constant temperature (25°C) following transient dexamethasone treatment. The oscillation persisted for almost 3 cycles, covering a time period of more than 120 hours.*

b, *Reporter gene expression rhythms measured over a temperature range of 7 degrees (29°-22° C).*

c, *Analysis of the period lengths. The deduced period lengths lie outside of the circadian range (τ ≥ 38h).*

d, *Variation of the temperature (29°, 25°, 22° C) in different experiments revealed the inability of the cavefish circadian clock to maintain a constant period over a physiological temperature range (τ = 38h, 43h, 47h; Q_{10}<0.8).*

III.6 Phase response curve (PRC)

Given the unusually long free running period of the cavefish clock, failure to detect rhythmic gene expression under a 24 hours light-dark cycle could result from the large discrepancy between the period length of the internal oscillator and the period length of the light cycle. To exclude this possibility and so to prove that light cannot entrain the *P. andruzzii* clock, single, short light pulses were delivered to CF cells where rhythmic clock gene expression had already been established by Dexamethasone treatment. Light pulses were delivered at different time points distributed through one oscillation cycle in order to plot a phase response curve (PRC). A PRC is obtained by plotting the magnitudes of shifts in the phase of the clock (either advances or delays) induced by single zeitgeber stimuli against the time of these stimuli measured relative to the circadian cycle.

Based on previous measurements, the period of the first peak was divided into 5 different time windows (I-V; induction, rising, peak, decreasing and trough), each window spanning 8 hours. Five groups of cells were transiently treated with dexamethasone at 5 different time points, separated by 8 hours. Then all the cells were exposed to a single 15 minute light pulse, The phase of the rhythm was then subsequently analysed for each group of cells compared with non-light pulsed control cells. In this way the effect of one light pulse delivered at different phases of the oscillation was tested (Fig. 12a). In zebrafish cell lines, an exposure of 15 minutes to light is sufficient to induce a robust phase shift for an established oscillation (Carr and Whitmore, 2005; Vallone et al., 2004).

To perform the experiments CF-1 cells were previously transfected with the *zfPer1b-luc* reporter construct and then subsequently maintained at constant temperature.

Comparison of the phase between the light pulsed samples and the “dark control” did not reveal any light induced phase difference, thus confirming that light is not able to regulate the clock (I: two-way ANOVA $F_{1,1096}=0.79$, $p>0.3$ – II: two-way ANOVA $F_{1,685}=0.7$, $p>0.7$ – III: two-way ANOVA $F_{1,548}=0.46$, $p>0.4$ – IV: two-way ANOVA $F_{1,822}=0.36$, $p>0.5$ – V: two-way ANOVA $F_{1,1096}=0.07$, $p>0.7$) (Fig. 12b,c,d,e,f).

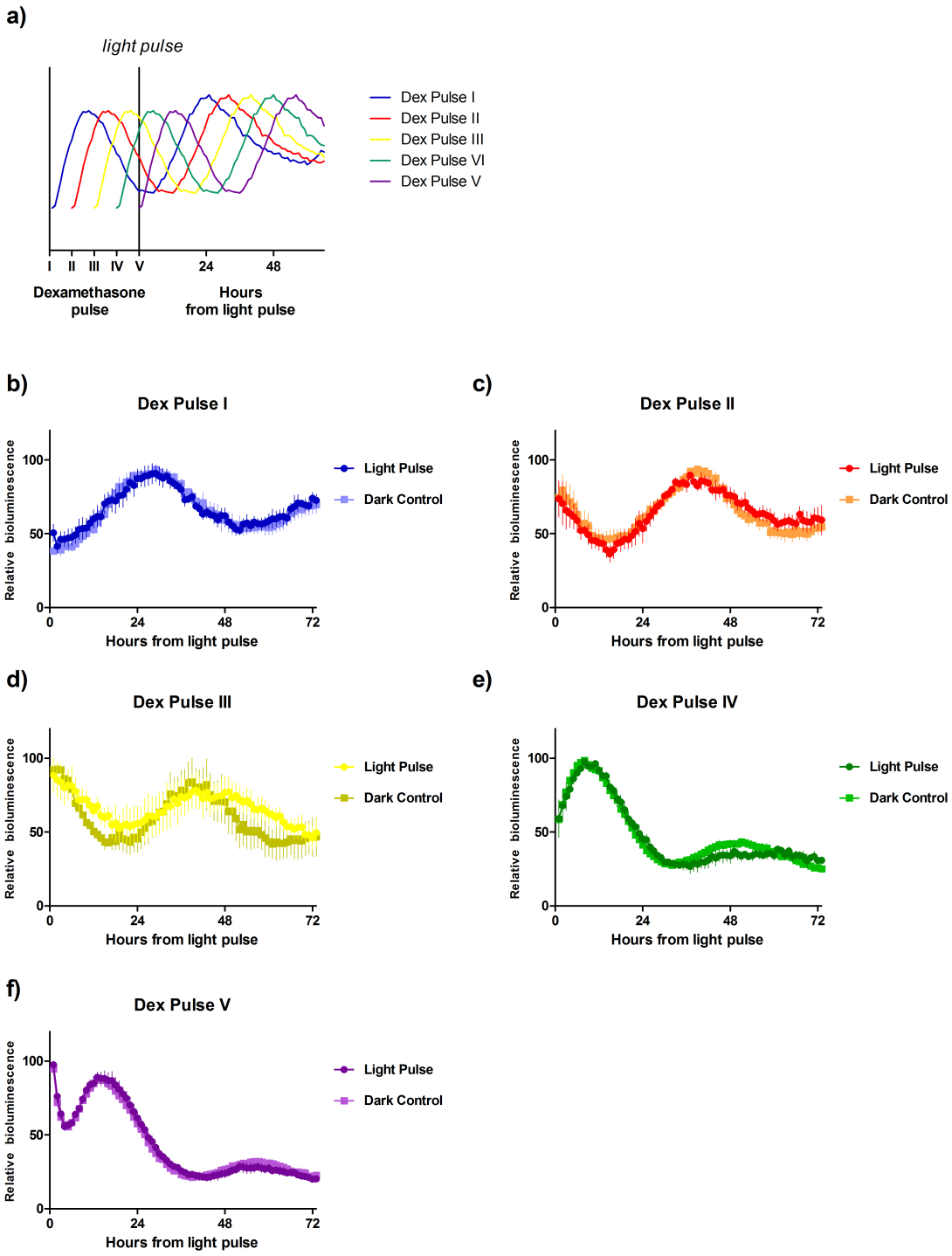

Fig. 12: ***a,*** *scheme of the phase response curve (PRC) experiment. Oscillation is induced in different groups of CF-1 cells (I-V) by Dexamethasone treatment with an interval of one group every 8 hours before delivering a 15 minutes light pulse. In the graph the oscillation of CF-1 cells at 25°C is represented. The time the "light pulse" is delivered corresponds to ZT0 and the beginning of the measurements.*
b-f, *Comparison of the phase between the light pulsed samples and the "dark control". CF-1 cells were transfected with the of zfPer1b reporter vector and subsequently assayed for luciferase activity on an Envision multilabel counter at a constant temperature of 25°C. Values of reporter are mean ± standard error.*

III.7 Mutations in genes involved in the light transduction pathway

Mutations affecting the coding or regulatory sequences of clock gene elements could explain the defect in the light signal transduction, the loss of temperature compensation or the non-circadian period. For this reason 5´-3´ RACE PCR was used to extend the sequences from the cDNA fragments to both extremities of the transcripts for most of the genes cloned (Table 1).

Initially two core clock genes *Cry1a* and *Per2* were selected for a more complete sequence analysis. These genes are light inducible and so in the case of zebrafish may participate in resetting of the clock by light, and also encode negative transcriptional regulators at the core of the clock mechanism.

In the case of *Cry1a*, alignment of the full coding sequences for zebrafish and cavefish revealed high conservation along its entire length. A partial cDNA of cavefish *Per2*, containing a 3252 bp ORF, encodes for a 1084 amino acid protein. As in all other PER2 homologs, the N-terminal region, contains PAS (A and B) and the PAC motifs (PAS A, aa 247-312; PAS B, aa 387-451; PAC, aa 460-501) followed by a nuclear export signal (NES, aa 526-535) and the phosphorylation target site for casein kinase 1 epsilon (CK1ε, aa 747-760), involved in the degradation pathway. Surprisingly downstream of these functional domains, at position +2836 from the transcription start site, is inserted a sequence of 225bp, similar to a fish reverse transcriptase-like gene (transposon) that preserves the transcripts open reading frame and thereby inserts 75 aminoacids (Fig. 13).

The full length cDNAs of cavefish *Clk1* and *Per1* were also sequenced and failed to show any significant differences with their zebrafish counterparts. Furthermore the sequence of many other clock components has been extended.

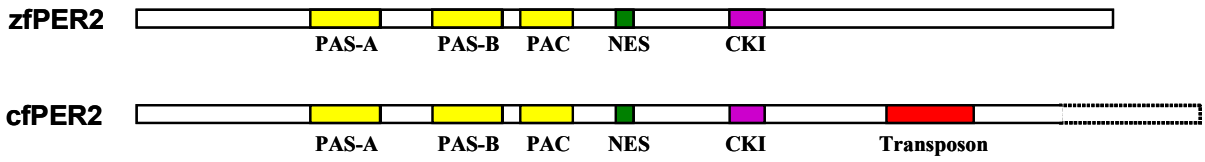

Fig. 13: Schematic representation of cavefish and zebrafish PER2 protein. Cavefish homolog present the same conserved domain in the peptide as in zebrafish plus a characteristic insertion of a transposon in the C-terminal region.

To determine if the presence of a mutated PER2 could explain the lack of photic entrainment as well as the abnormal clock in *P. andruzzii*, ectopic expression of zebrafish PER2 in CF cells was tested in an attempt to "rescue" a normal zebrafish-like phenotype. For this purpose a *Per2* minigene construct (zfPER2) was generated, where the zebrafish *Per2* promoter transcribes the zebrafish *Per2* cDNA (see materials and methods). The zfPER2 minigene was then co-transfected with the *zfPer1b-luc* reporter construct into CF cells. When the cells were exposed to LD cycles no light driven rhythmic reporter gene expression was observed (Fig. 14a). However, following a Dexamethasone pulse, the transfection of increasing concentrations of the minigene was able to shorten the period by 7,5% (3 hours at 25°C) (Fig. 14b).

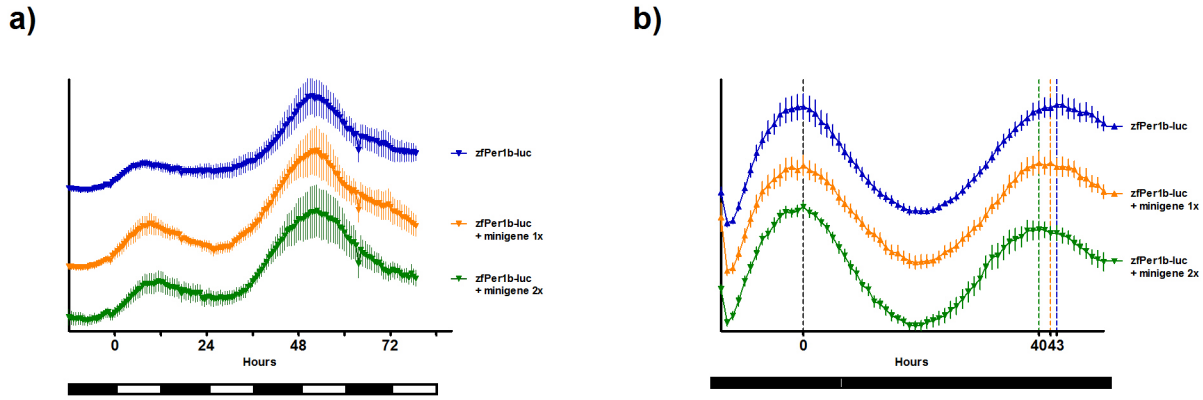

Fig. 14: ***a,*** *CF-1 cells transfected with the zfPER2 minigene and zfPer1b-luc reporter vector and exposed to 12:12 LD cycles. Co-transfection of the zfPer1b reporter construct with the zfPER2 minigene construct resulted in an arrhythmic reporter gene expression.*
b, *CF-1 cells transfected with the zfPER2 minigene and zfPer1b-luc reporter vector and treated with Dexamethasone. Increasing concentrations of the minigene shortened the period length up to 3 hours at 25°C.*

One possible explanation for the failure of light induced gene expression in cavefish cells could be the presence of mutations in the promoter regions of these clock genes. Thus, the promoter region of the cavefish *Per2* was subsequently cloned to study the regulation of the endogenous gene. A fragment of 916 bp upstream of the transcription start was amplified from cavefish genomic DNA. Analysis of the promoter revealed strong conservation for certain regulatory elements like the D-box but not the E-box in comparison with the same region in zebrafish (Vatin et al. 2009) (Fig. 15).

```
                                  E-box                    D-box
Cavefish          GGCCATGGT-GTCCACATGAGGTGTATGACATACTTATGTAAAGAGACTGGCGGTCTGTT
Zebrafish         GGCCATGGGTGTCCACGTGAGTTGTATGACACACTTATGTAAAAAGACTGACGGGCGTTT
Chicken           AAACATGGTGTCACACGTGAG----------GCTTATGTAAAATGAGCGGCGTGCGGCG
Human             GAACATGGAGTTCCATGTGCG----------TCTTATGTAAAAAGAGCGACGGGCGCGG
Mouse             GAACATGGAGTTCCATGTGCG----------TCTTATGTAAAGAGAGCGACGGGCGTCT
Rat               GAACATGGAGTTCCATGTGCG----------TCTTATGTAAAGAGAGCGACGGGCGTCT
                     *****     **  ** *           **********  **  * **  *
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Fig. 15: alignment of LRM sequences of cavefish, zebrafish, chicken, human, mouse and rat per2 promoters (modified from Vatine et al., 2009).

The promoter fragment plus part of the 5' UTR (from position -876 to + 112 from the transcription start) was cloned into the PGL3 vector (*cfPer2-luc*). The vector was then transfected into AB9 cells and exposed to LD cycles to test the behaviour of the cavefish promoter. The reporter was light induced in a comparable fashion to the "wt" zf*Per2* promoter (Fig. 16).

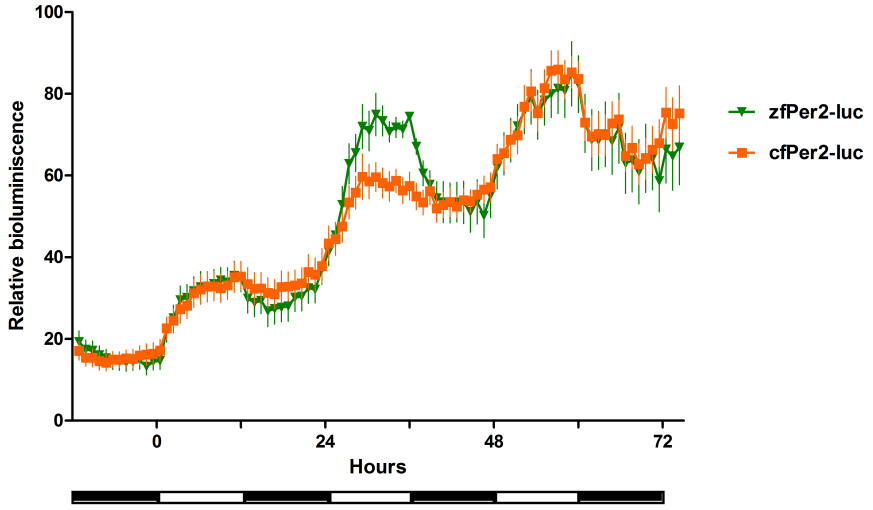

Fig. 16: zfPer2 and cfPer2 reporter vectors were transfected into AB9 and exposed to a 12:12 LD cycles. cfPer2-luc showed a pronounced expression rhythm, increasing in expression following the beginning of the light phase as the zfPer2-luc.

On the contrary, the light-inducible *Per2* promoter of zebrafish was not induced in the cavefish cells under LD cycles (Fig. 9f), suggesting that the defect in light perception resides outside of the core clock mechanism and implies the presence of multiple mutations, or the absence of important elements, in the light activated signal transduction pathway.

III.8 Opsins

Important candidates for fulfilling the function of photoreceptor are encountered in the opsin family, especially in the extra-retinal opsins group, which potentially also provides an explanation for the widespread photosensitivity of peripheral tissues in non-mammalian vertebrates (Whitmore et al., 2000; Vallone et al., 2007).

Candidate extra-retinal photoreceptors include opsins such as *Exorhodopsin*, *Teleost Multiple Tissue* (*TMT*) *opsin* and *Melanopsin* (*Opn4m2*) that are widely expressed in most tissues in zebrafish (Bellingham et al., 2002; Mano et al., 1999; Moutsaki et al., 2003). A similar approach to the one used to clone clock genes was used to isolate cDNA sequences of opsins from cavefish.

Alignment with the homologous sequences of other teleosts revealed strong conservation of the *Exorhodopsin* sequence along its entire length (90% homology with *D. rerio*, 88% homology with *T. rubripes*).

Opn4m2 and *TMT-opsin* instead presented a mutated ORF characterized by a frameshift of the sequence that predicted the translation of a non functional protein. Specifically, the insertion of a thymine in the *TMT-opsin* (position +656 of the cds) results in an early stop codon in the end of the 5th transmembrane domain while a deletion of a guanine (position +816 of the cds) truncates *Opn4m2* at the beginning of the 6th (Fig. 17). These mutations are predicted to affect the binding of the chromophore retinaldehyde, which is located in the 7th transmembrane domain (Bownds, 1967).

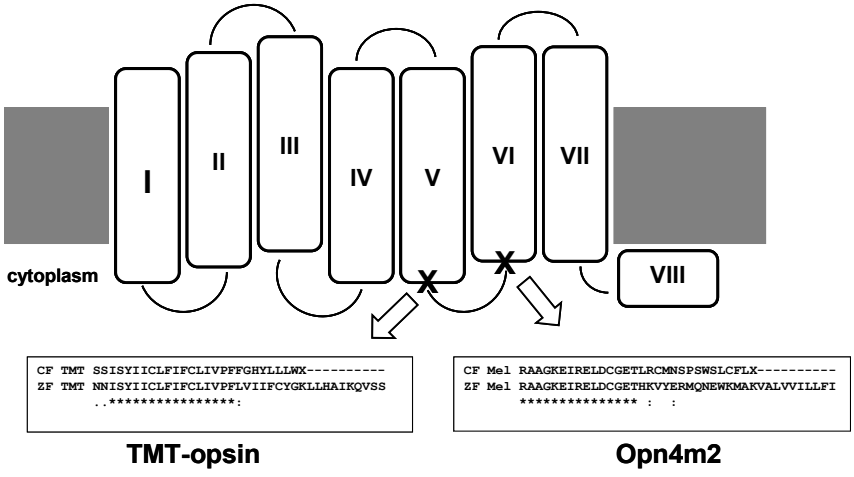

Fig. 17: Schematic representation of the membrane spanning structure of a generic opsin protein. The seven transmembrane domains are labelled I - VII. Crosses denote the position of stop codons in the cavefish TMT and Opn4m2 opsins that are also indicated in an alignment of the zebrafish and cavefish sequences.

III.9 Rescue of light induction of the zf*Per2* promoter

The mutations found in *TMT-opsin* and *Melanopsin* could be responsible for the lack of light induced gene expression in *P. andruzzii*.

To directly test if the re-insertion of these missing opsins by the wildtype zebrafish counterparts is sufficient to repair the signal transduction pathway in cavefish cells, the full cds of the zebrafish homologs of these two opsins were cloned into expression vectors. Analysis of zebrafish clock gene promoters identified the *Per2* promoter as putative target for the light induced signal transduction cascade, and in particular the recently identified light responsive module (Vatine et al. 2009).

CF cells were transiently transfected with the *zfPer2-luc* reporter alone or in combination with opsin expression vectors and exposed to LD cycles (LD 12:12). A solution of retinaldehyde containing both active and in-active (9-cis and All-trans) isoforms was added to the medium to ensure the pool of chromophore was sufficient for opsin function and also to test if the loss of light induction might be due to lack of vitamin A.

Supplementing the culture medium with the chromophore, resulted in no rhythmic expression of *zfPer2-luc* (Fig. 18). However, upon cotransfection with *TMT-opsin*, *Opn4m2* or both opsin expression vectors, *zfPer2-luc* was robustly induced during the light phase (Fig. 18b,c).

An induction of luciferase activity was observed already after only a few minutes of light exposure, reaching its peak around the transition between phases (one-way ANOVA, *Per2* + *TMT-opsin* $F_{151,604}=9.12$, $p<0.0001$; *Per2* + *Opn4m2* $F_{151,2869}=15.55$, $p<0.0001$; *Per2* + *TMT-opsin* & *Opn4m2* $F_{151,3599}=9.15$, $p<0.0001$) and subsequently decreasing during the dark phase. Oscillation in expression persisted for almost 3 light-dark cycles.

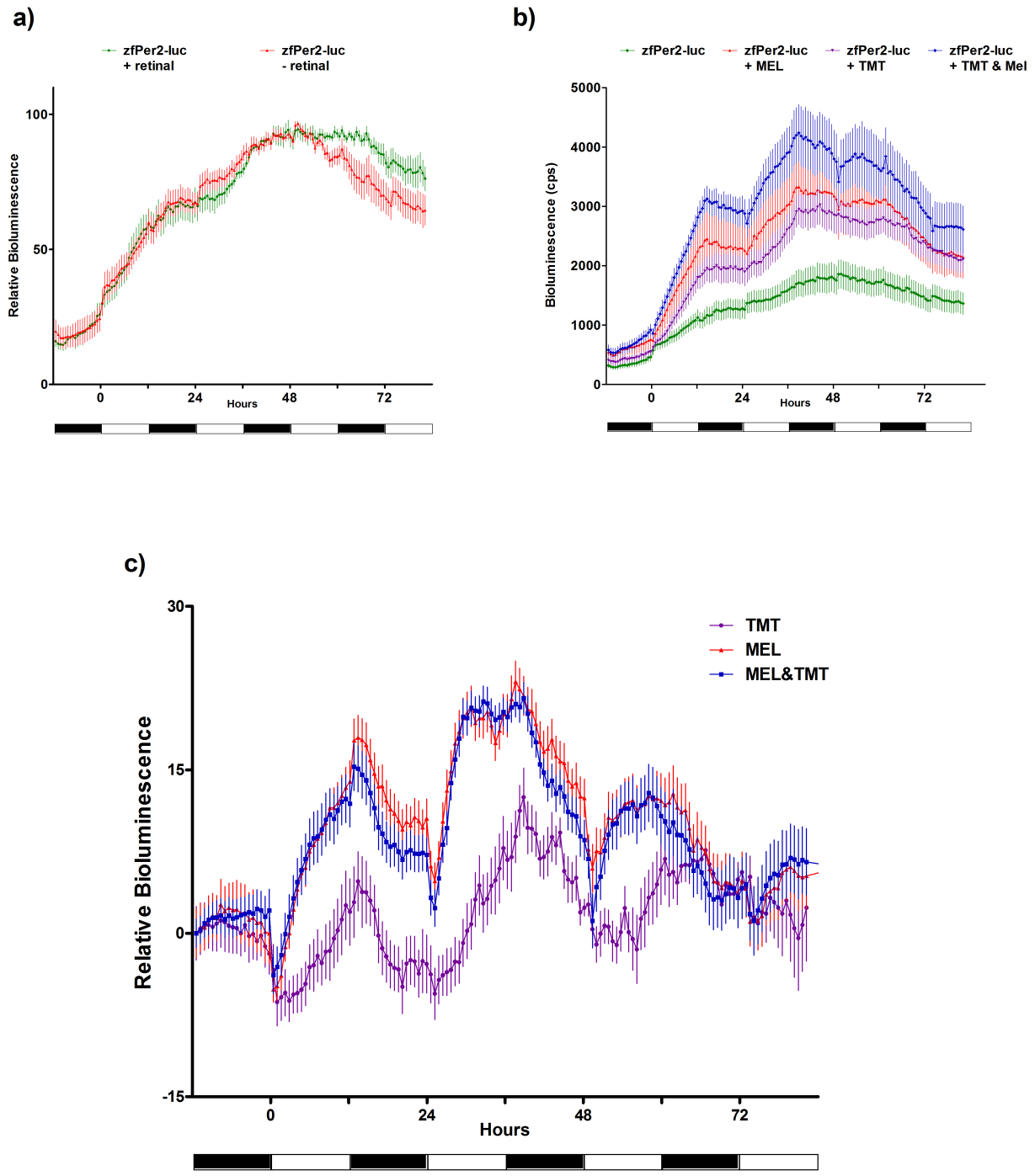

Fig. 18: ***a,*** *Arrhythmic bioluminescence profiles of CF cells transfected with zfPer2-luc in the presence or absence of 9-cis/all-trans retinal and under LD cycle conditions. Bioluminescence was measured and for each time point, mean values ± standard errors were plotted as relative luciferase activity (%).*
b, *Rescued light-inducible expression of zfPer2-luc in CF cells cotransfected with zebrafish TMT and Opn4m2 opsin expression vectors, raw data. Addition of the opsins expression vectors result in a high amplitude of the reporter induction during the light phase.*
c, *To more optimally visualize the effects of light on reporter gene expression, the basal expression levels measured with the zfPer2-luc reporter alone (**b**) were subtracted from the expression levels observed with the co-transfected opsins.*

To determine if the light responsive module (LRM) on the zebrafish *Per2* promoter was effectively the target of the light transduction cascade activated by *TMT-opsin* and *Melanopsin*, cavefish cells where transfected with the minimal promoter of zf*Per2* containing the E-D box region (Vatine et al., 2009) and exposed to LD cycles (LD 12:12).

The *zfPer2* LRM reporter vector alone did not show any significant variation during the measurements while in combination with both opsins expression vectors, luciferase activity continued to be induced in a light dependent manner (Fig. 19).

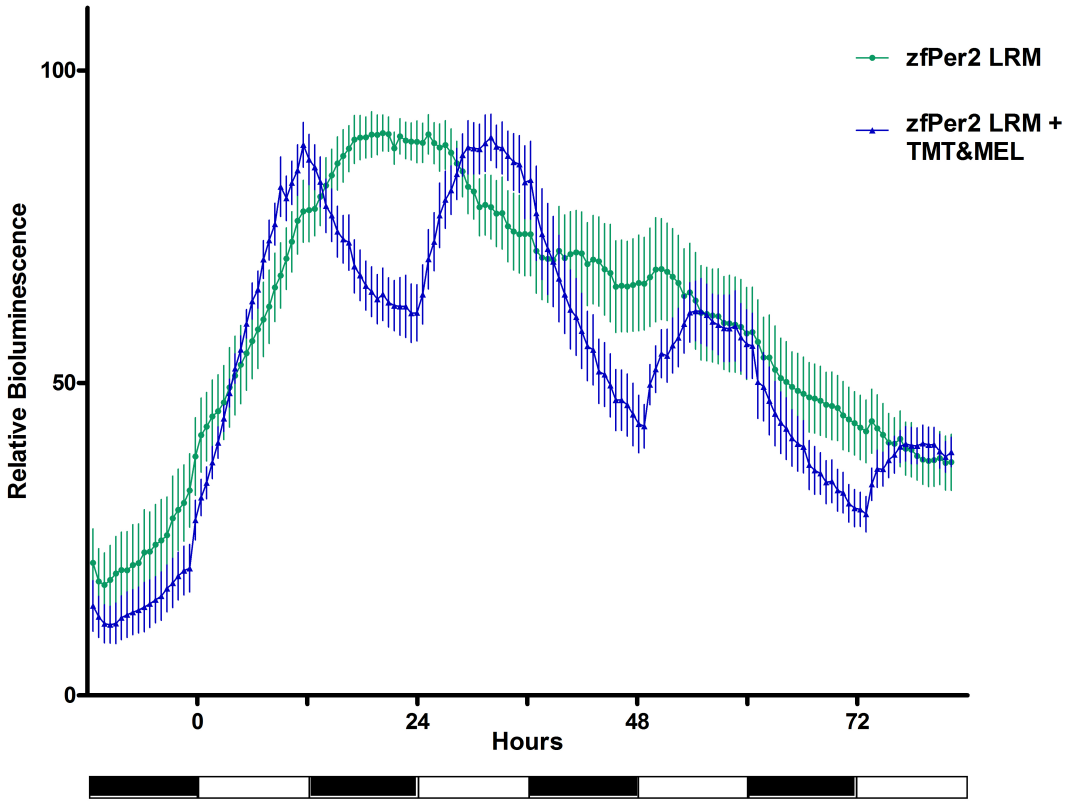

Fig. 19: Bioluminescence profiles of CF cells transfected with zfPer2 LRM alone or in combination with zebrafish TMT and Opn4m2 opsin expression vectors. Luciferase activity continued to be induced in a light dependent manner as it was for the zfPer-luc reporter, identifying the light responsive module as the target of the phototransduction cascade activated by opsins. Bioluminescence was measured and for each time point, mean values ± standard errors were plotted as relative luciferase activity (%).

IV. DISCUSSION

The evolution of *Phreatichthys andruzzii* has been strongly influenced by 1-2 million years of isolation under the desert of Somalia: anophtalmy, depigmentation and loss of the scales are amongst the most striking morphological adaptations to life in this cave environment. The absence of canonical selective pressures, such as day-night cycles, clearly affects many aspects of morphology and physiology of the cavefish via modifications to the underlying molecular control mechanisms.

This comparative study involving the Somalian cave fish and the zebrafish exploited the divergent evolution of these two species as a tool to study the mechanisms constituting the internal timekeeping system and its regulation by light.

Analysis of the *P. andruzzii* transcriptome was facilitated by its close similarity with zebrafish and lead to the identification of many clock and clock-controlled genes. Cloned sequences showed strong conservation of the functional domains in each of the clock gene families (*Clk*s, *Per*s and *Cry*s), consistent with their role as essential circadian clock elements.

Only in the case of cavefish *Cry4* was it not possible to clone a full length coding sequence. By sequence homology *Cry4* is the closest vertebrate *Cryptochrome* to *Drosophila*, although its function is still unknown, it has been implicated in light perception. Life in a constant dark environment would explain a negative or relaxed selection that could lead to disruption of genes encoding photoreceptors. Conversely, the ORF of *Cry5* (*6-4 photolyase*), a strictly light-dependent enzyme involved in the repair of UV damaged DNA, was apparently not disrupted, implying a fundamental role for this enzyme in cell homeostasis, even in the absence of light. Alternatively, the rate of mutation within the cavefish genome might not have been sufficient to result in all genes under negative or relaxed selection to have accumulated disruptive mutations.

Contrary to the situation in almost all organisms, the circadian clock of *P. andruzzii* is not entrained by exposure to light. Strikingly, arrhythmic expression of clock genes was encountered in adult cavefish and larvae exposed to light-dark cycles, in stark contrast to the rhythmic oscillations observed in zebrafish tissues. The variation in the levels of endogenous cavefish gene expression showed that neither the fin (a peripheral tissue) nor even the brain (presumably containing central pacemakers) seemed able to synchronize the endogenous oscillators in response to light. This is unlikely to be a direct consequence of anophthalmy, given that cavefish larvae did not show rhythmic expression of clock gene even before the loss of the eyes.

Furthermore, the clocks of eyeless zebrafish mutants are still entrained normally by LD cycles (Dickmeis et al., 2007). Instead this cavefish phenotype seems to reflect the absence of the general photosensitive property of teleost cells.
Only cavefish *Cry5*, that is not part of the clock machinery, was following a light-dependent-like behaviour: the upregulation after few hours of light exposure may reflect an indirect activation driven by the accumulation of DNA damage by light.
The establishment of primary *P. andruzzii* cell lines from adult fin clips and comparison with the directly light sensitive zebrafish cell lines provided a powerful tool for the exploration of the properties of the cavefish clock. The use of the cavefish cell lines allowed a detailed phase response curve (PRC) analysis of the effects of light on the phase of the endogenous clock demonstrating conclusively the absence of photic entrainment. The cell culture models allowed an exploration of the molecular basis for the loss of light entrainment in cavefish. The combined *in vitro* and *in vivo* results demonstrate that evolution in a costant dark environment can lead to loss of photoentrainment of the circadian clock.

Regular daily feeding proved to be a strong zeitgeber for the cavefish circadian oscillator, entraining both brain and peripheral organ clocks. It is tempting to speculate that food availability in its cave environment might indeed be periodic and therefore a clock responding to and anticipating feeding time may confer a selective advantage. Interestingly, several recent reports have implicated the existence in vertebrates of a food anticipatory oscillator (FAO) that does not rely on the classical circadian clock components (Storch and Weitz, 2009). However, the remarkably long free running period suggests significant similarity exists between the clock in cavefish cells (that can be entrained by transient treatment with dexamethasone) and the FAO.

Now the challenge remains to understand if the core oscillator may be following the same destiny as the light input pathway of the clock and so be slowly loosing its function. It is possible that the extremely long free running period and loss of temperature compensation for this cavefish clock reflects progressive loss of a mechanism that provide no selective advantage for these animals that live under constant darkness and temperature.
One preliminary observation that may potentially explain the aberrant phenotype of this cavefish clock is the presence of a mutated version of PER2 in cavefish. A transposable element is inserted into the C-terminal region of the predicted protein, between the CK1ε phosphorylation site, responsible for PER2 protein degradation, and the putative binding site for CRY, which is

implicated in the translocation of the PER-CRY complex to the nucleus (Tomita et al., 2010). Considerable changes in the three-dimensional structure of the PER2 protein might alter the stability and turnover of this protein as well as its ability to interact with other components of the oscillator. In turn this may have a significant effect on the entire clock machinery, significantly extending the time required to complete one regulatory cycle. Thus, this transposable element insertion might represent a gain of function mutation. Indeed, ectopic expression of zebrafish PER2 in CF cells by transfecting the zfPER2 minigene construct failed to re-establish a normal zebrafish-like phenotype, suggesting that the endogenous cavefish PER2 protein had a dominant effect on the internal timekeeping system. If this is the case, removing the endogenous mutant protein by knocking-down the transcript might be more likely to restore zebrafish clock-like properties on the cavefish circadian system, since the absence of only one of the *Per* genes normally does not affect clock function (Zheng et al., 2001).

Another possible explanation for the failure of light induced gene expression in cavefish cells could be the presence of mutations in the promoter regions of light regulated genes. Promoter analysis of the cavefish *Per2* gene revealed the conservation of regulatory elements, the D-box, directly implicate in light driven gene expression, but less conservation of the adjacent E-box element (CACGTG), the binding sites for the circadian clock activators CLOCK-BMAL (Vatine et al. 2009). The mutated E-box (CACATG) potentially would represent a preferential binding site for another protein involved in the clock mechanism, DEC2 (Noshiro et al., 2004). This protein, differently from CLOCK-BMAL, acts as a repressor of transcription. Inhibition in the expression of *Per2* probably resulted in a selective advantage because, as a light inducible gene, high levels of expression were not required in the dark environment.

Inactivation of the *Per2* gene was probably a consequence of a general relaxed selection also acting on extra-retinal photoreceptors given their loss of function during evolution in the constant dark environment. Remarkably, by employing a comparative approach involving zebrafish and *P.andruzzii*, it was possible to provide direct evidence that *TMT-opsin* and *Melanopsin* serve as peripheral tissue photoreceptors in teleosts. The mutations in the cavefish coding sequences together with the associated loss of photic entrainment of the cavefish clock underline the key role of these two opsins in the teleost circadian clock light input pathway. The restoration of light inducibility of the zf*Per2* promoter by coexpression of the zebrafish homologs of *TMT-opsin* and *Melanopsin* also demonstrate the conservation of light-dependent signal transduction pathways even during evolution of the cavefish in the absence of light.

P. andruzzii represents a fascinating model to explore this novel clock, based on its lack of a light entrainable circadian clock, its tolerance of fasting and the likelihood that the mutations conferring the aberrant circadian clock phenotype are unlikely to affect the FAO in precisely the same way. Thus, in addition to displaying a unique and interesting collection of adaptations to their extreme environment, these animals serve as a powerful model to dissect the many pathways and processes that respond to light.

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