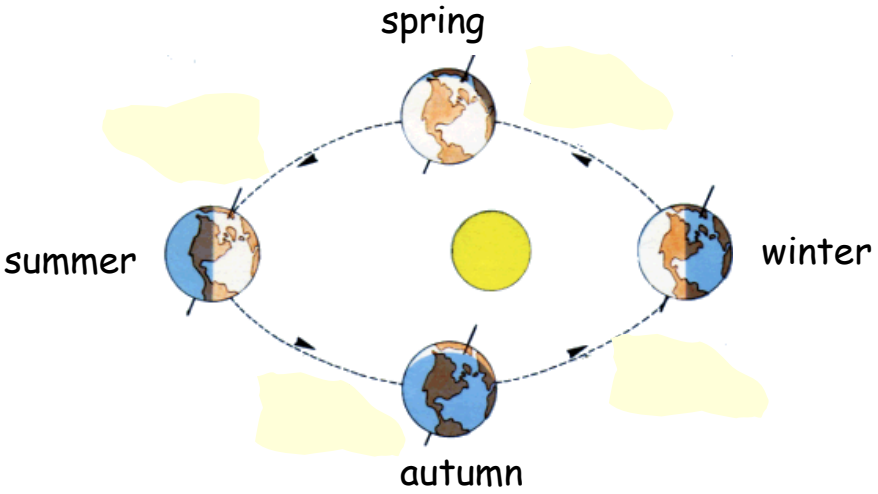


Life on earth is under the influence of basic environmental periodic changes such as day-night cycles or the fluctuation of seasons

Organisms have adapted to these rhythms in order to maximally benefit from the limited natural resources

The mechanism that keeps track of time, and therefore allows the organism to anticipate upcoming daily changes is termed

CIRCADIAN CLOCK

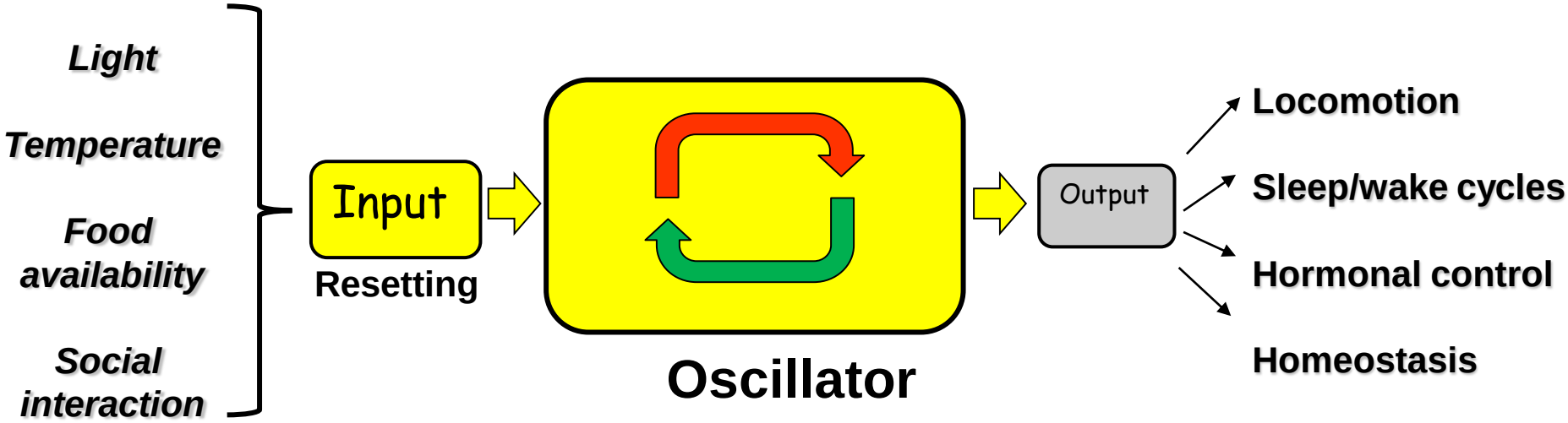
C.Bertolucci
Dip. Biologia ed Evoluzione

Circadian clocks are molecular time-keeping mechanisms that reside in a wide range of cell types in a variety of organisms

The key feature of a circadian clock is its ability to synchronize (***entrain***) to environmental time cues (so-called ***zeitgebers***; "time-givers") and to maintain rhythmic function when placed in constant conditions

C.Bertolucci
Dip. Biologia ed Evoluzione

General mechanism of biological oscillators

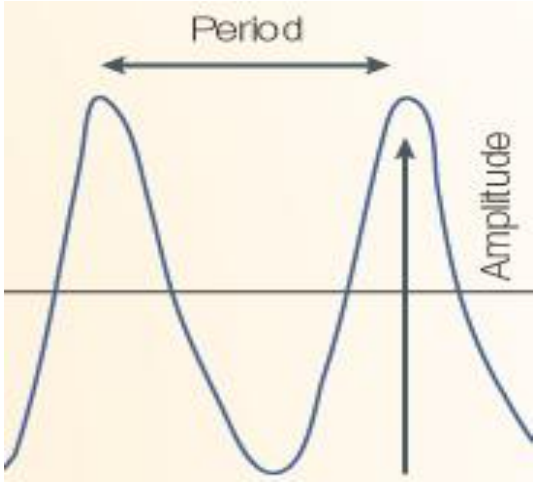


FEATURES OF BIOLOGICAL CLOCKS:

- Reset by environmental cues *(Zeitgebers)*:
Light, temperature…..

- Circadian oscillation:
Period approx. 24h (circa diem = about a day)
Persists in constant conditions

- Temperature compensation:
Period constant over physiological temperature range



C.Bertolucci
Dip. Biologia ed Evoluzione

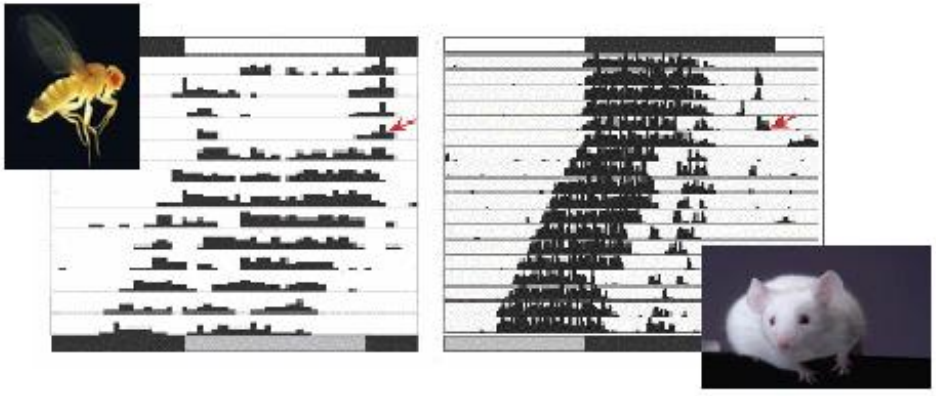
Endogenous clock systems
(circadian, circannual, ultradian)
are present in all organisms known.

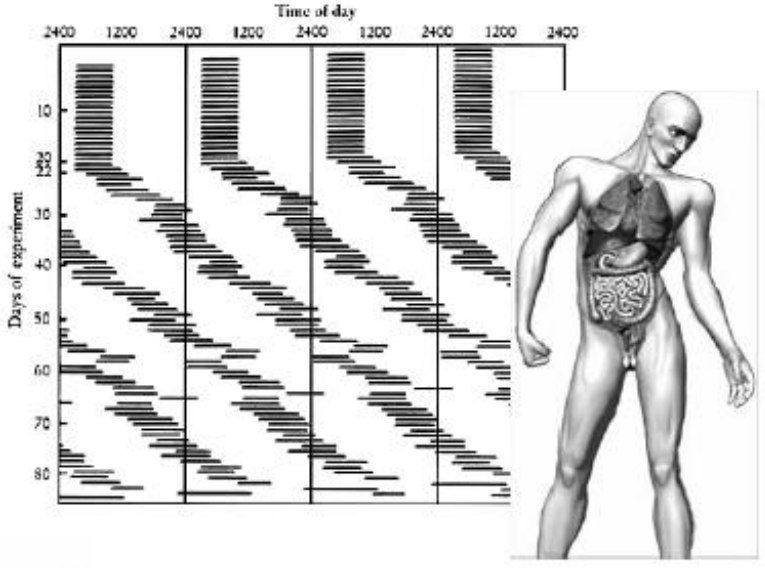
They have evolved to measure time and to keep the organism in *entrainment* with the environment.

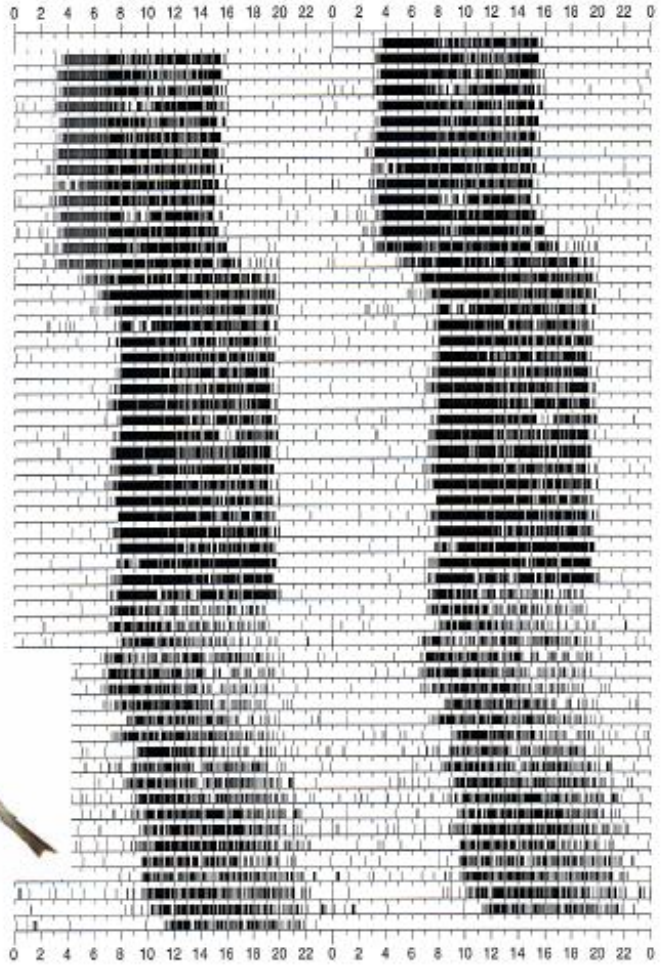
Light, feeding and temperature are the strongest *Zeitgebers* for circadian rhythms.

C.Bertolucci
Dip. Biologia ed Evoluzione

Biological rhythms are ubiquitous

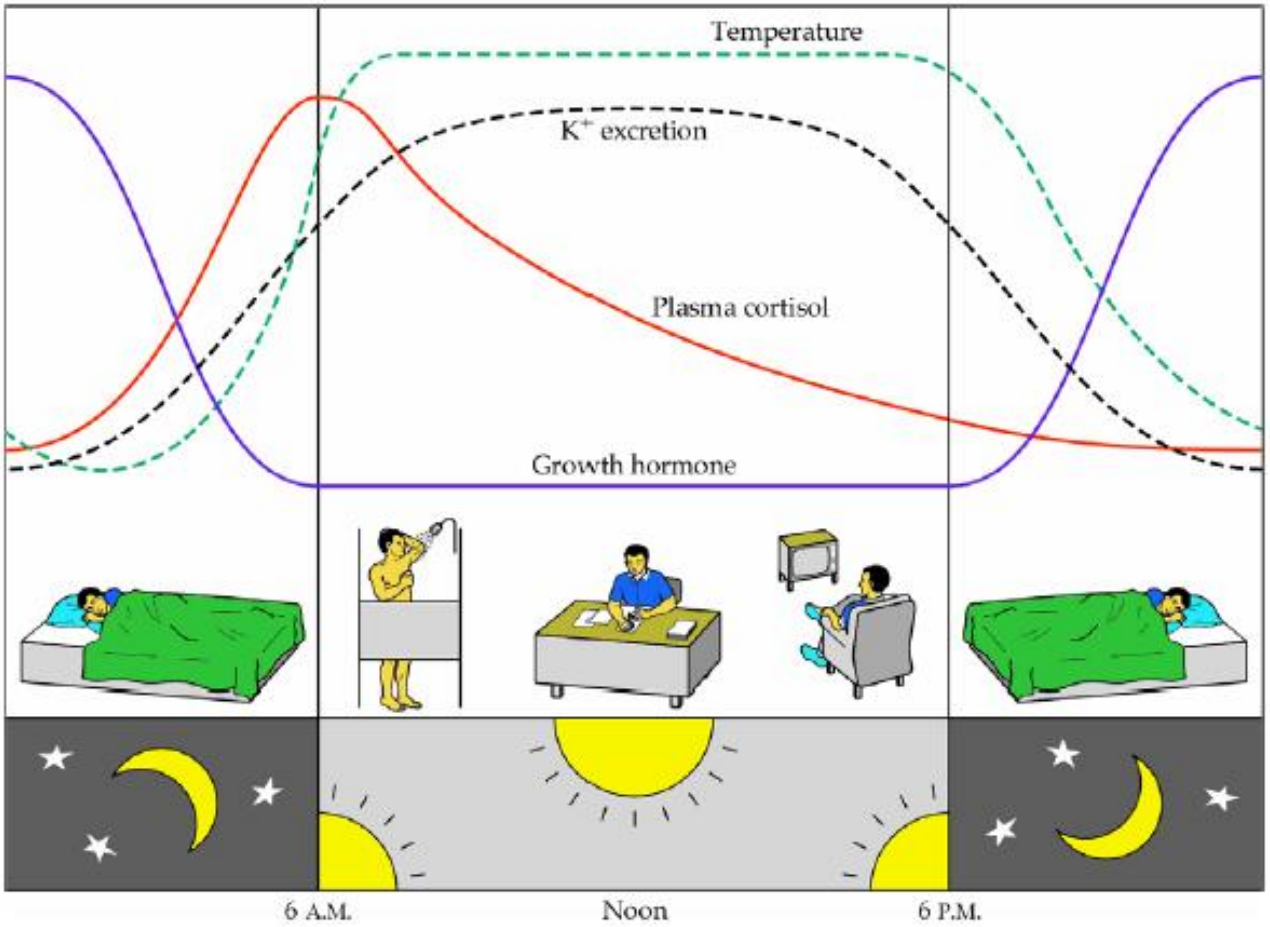






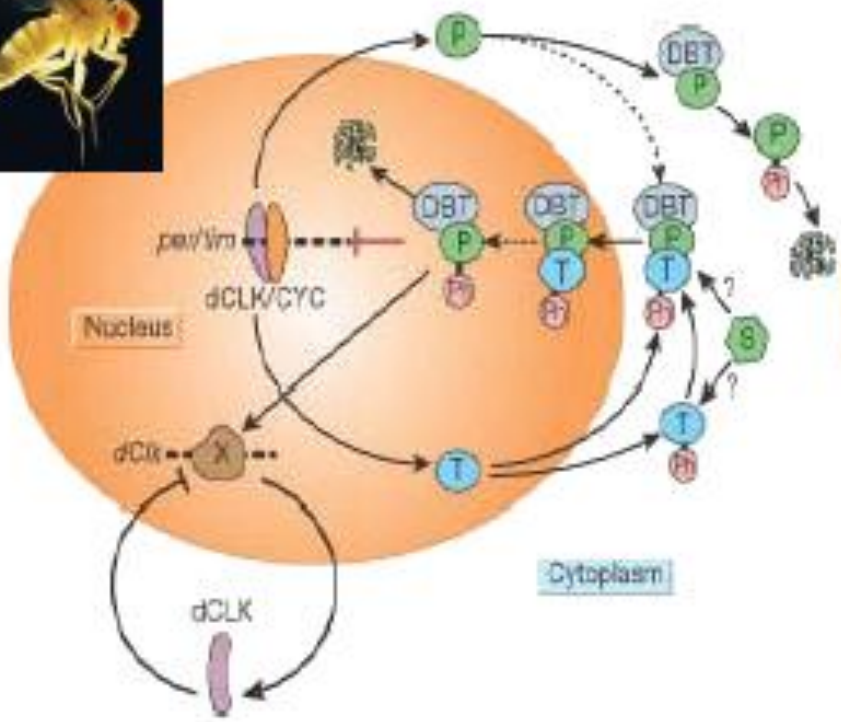
C.Bertolucci
Dip. Biologia ed Evoluzione

Rhythms of body function in humans

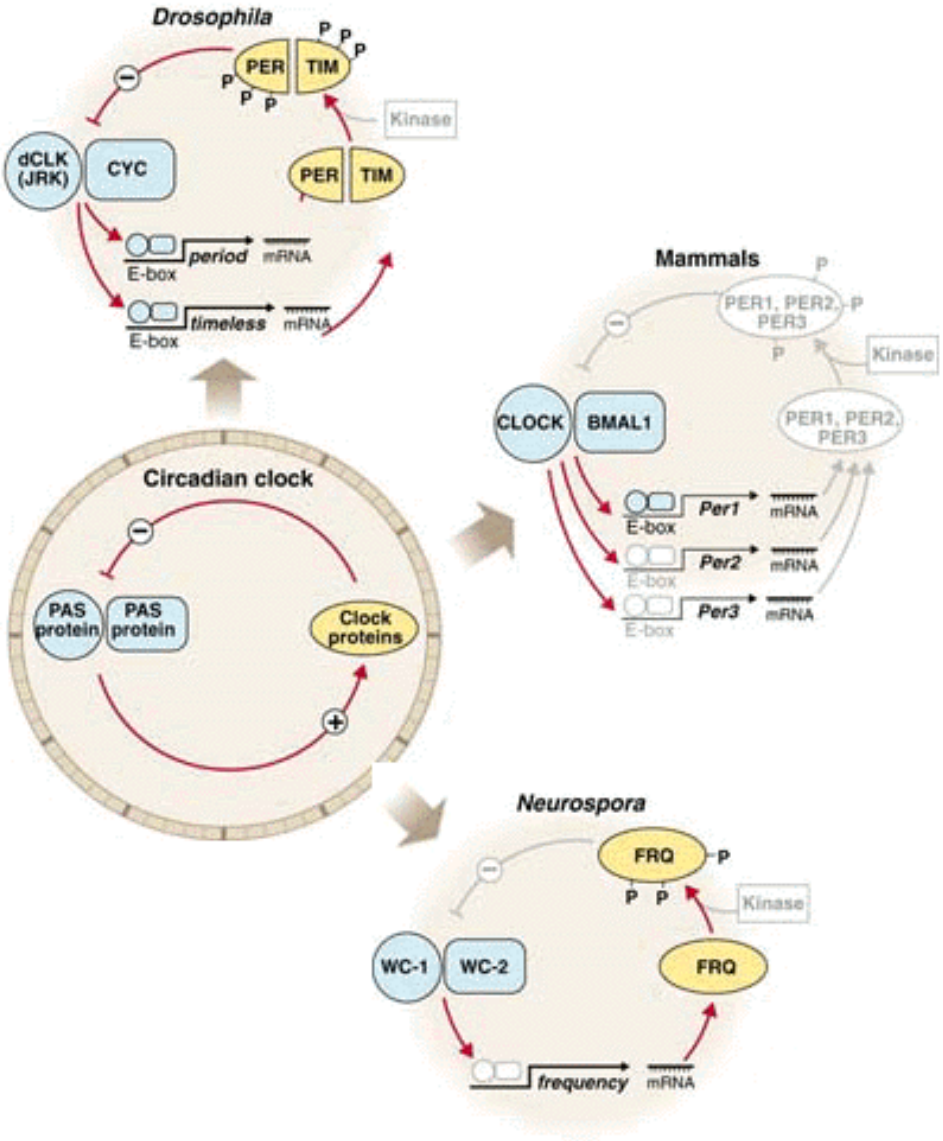


C.Bertolucci
Dip. Biologia ed Evoluzione

Circadian rhythms generation consists of interacting positive and negative transcriptional/translational feedback loops



C.Bertolucci
Dip. Biologia ed Evoluzione



The molecular circadian mechanism (molecular transcriptional and translational feedback loop) has been conserved during the evolution

Molecular oscillations result in modifications of cellular activity

Cells within a given tissue synchronize with each other via particular output signals

Neurotransmitters, neuropeptides, diffusable factors, hormones, etc.

Organs communicate with each other (Coordination of organ function)

Whole-organism feedback system (From genes to cells to tissues to organs to behavior and back)

C.Bertolucci
Dip. Biologia ed Evoluzione

Body physiological functions have to be coordinated in a way that:

Physiology and behaviour correspond to the environment

Interdependent metabolic functions are synchronised with each other

Incompatible metabolic functions are separated from each other

C.Bertolucci
Dip. Biologia ed Evoluzione

Key questions:

Where are the regulatory centres localised?

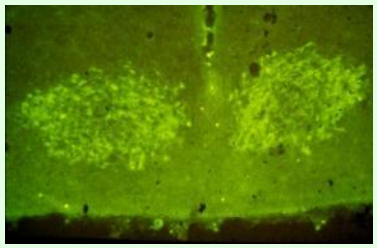
How and where is the light signal , the most powerful zeitgeber, detected?

VERTEBRATE CIRCADIAN SYSTEM

MULTIOSCILLATORY SISTEM

Central Oscillator

Peripheral Oscillators



Suprachiasmatic Nuclei of the Hypothalamus (SCN)

Retine

Pineal

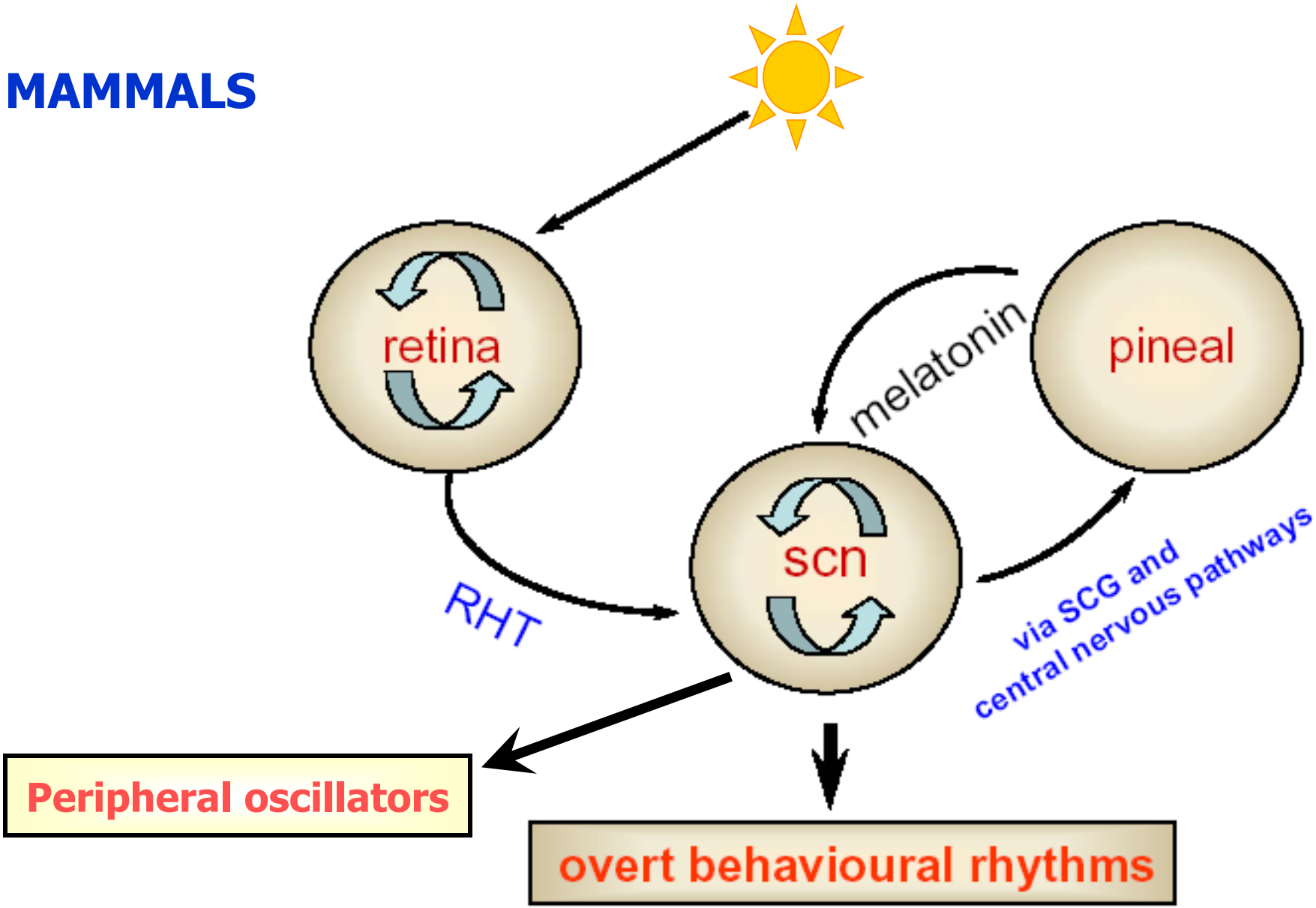
Liver

Kidney

Heart .....

C.Bertolucci
Dip. Biologia ed Evoluzione

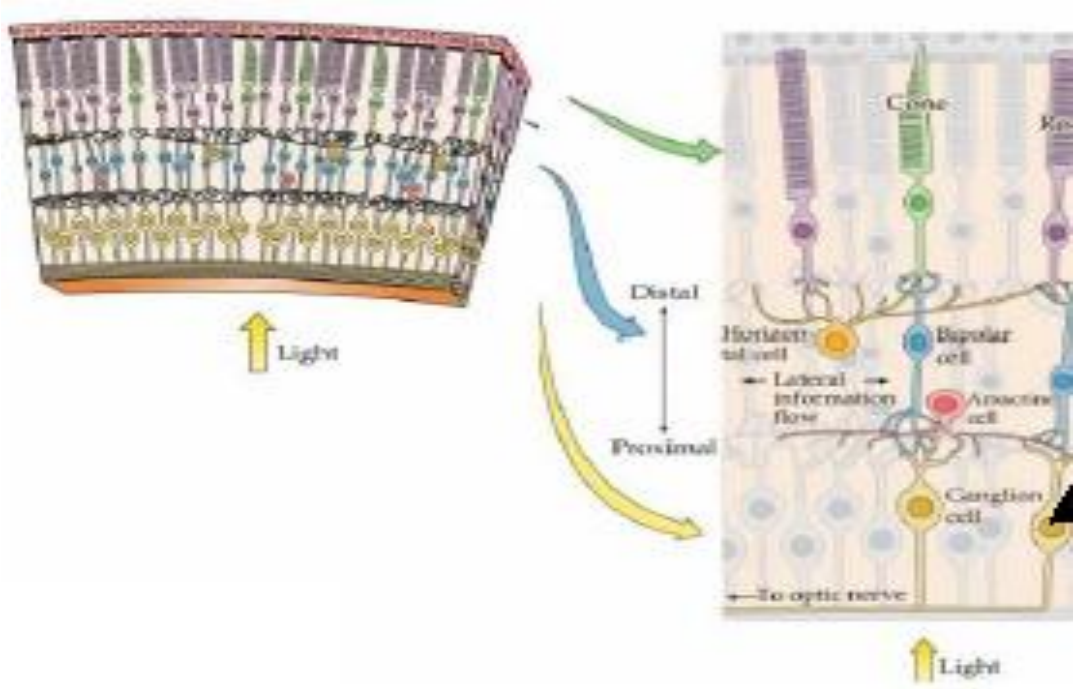
MAMMALS



C.Bertolucci
Dip. Biologia ed Evoluzione

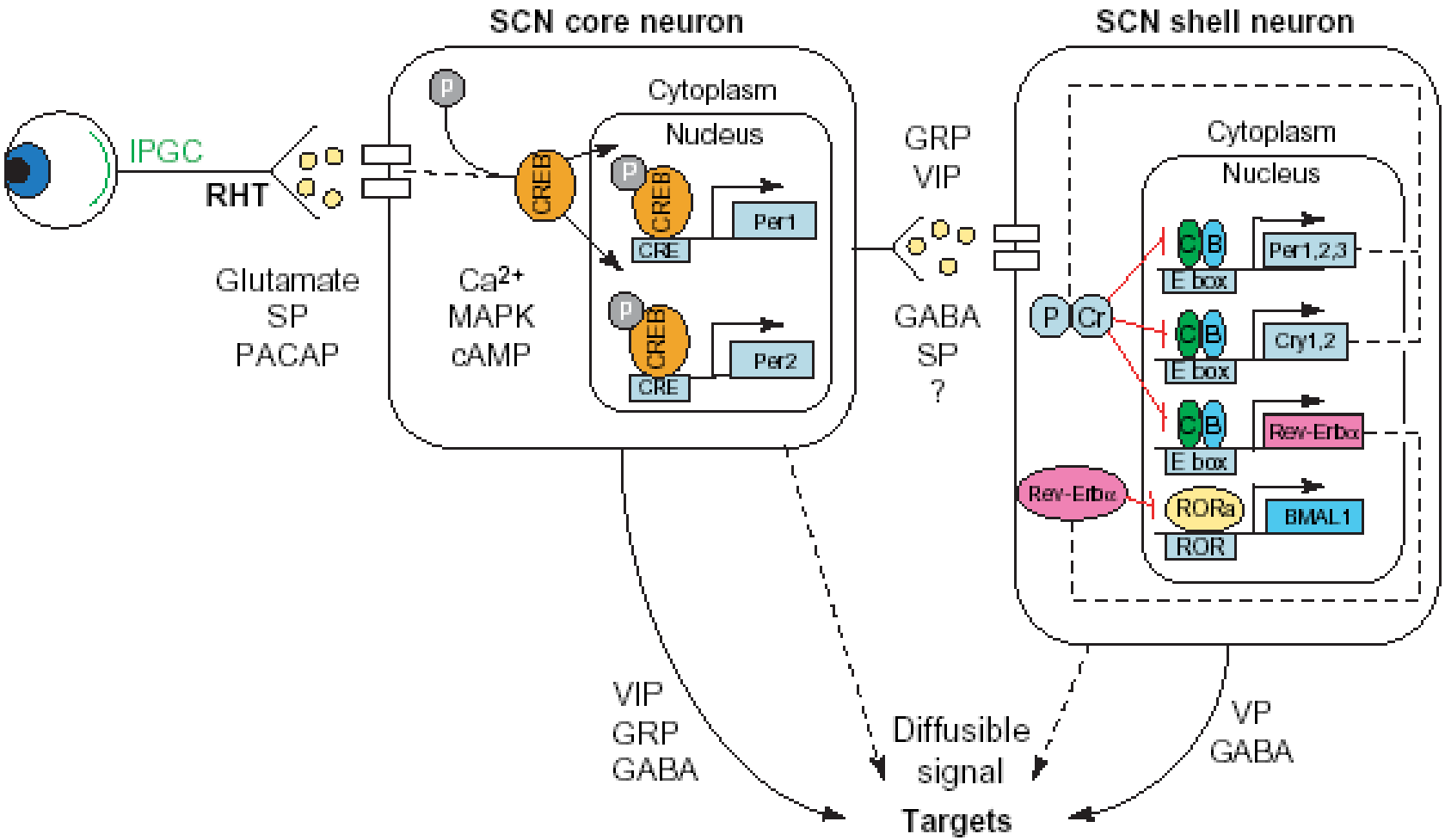
In mammals

Light input to the circadian system comes exclusively from the retina, mediated via a subset of ganglion cells that project to the suprachiasmatic nucleus.



Melanopsin

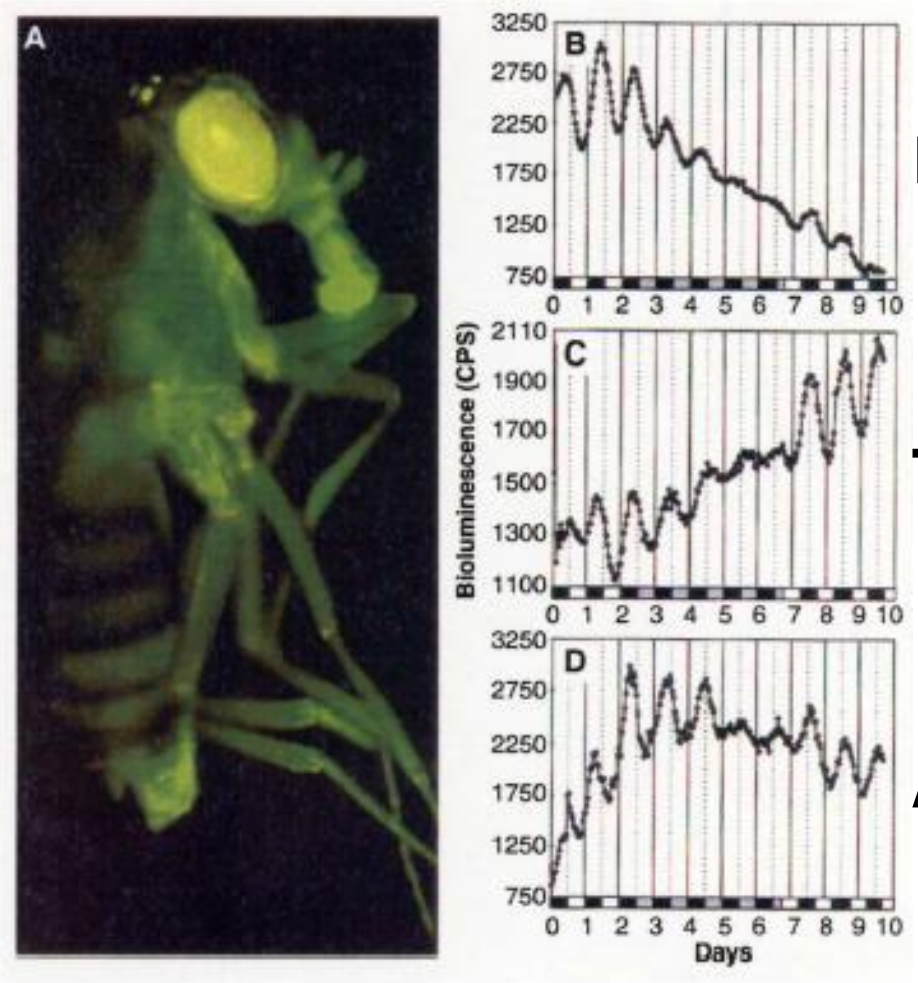
C.Bertolucci
Dip. Biologia ed Evoluzione



C.Bertolucci
Dip. Biologia ed Evoluzione

Light responsive circadian oscillators

Drosophila melanogaster



Head

Thorax

Abdomen

***Period* gene driven bioluminescence**

- present throughout the whole fly
- rhythms maintained in various body parts in culture

C.Bertolucci
Dip. Biologia ed Evoluzione

Drosophila

Rhythmic clock gene expression can be found in any tissue

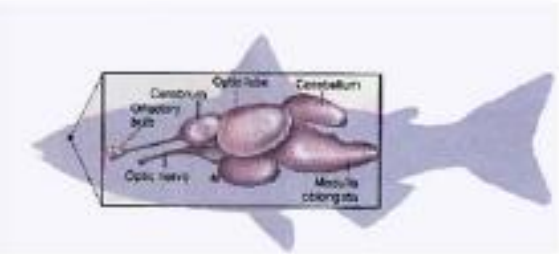
Rhythmic clock gene expression is maintained in culture

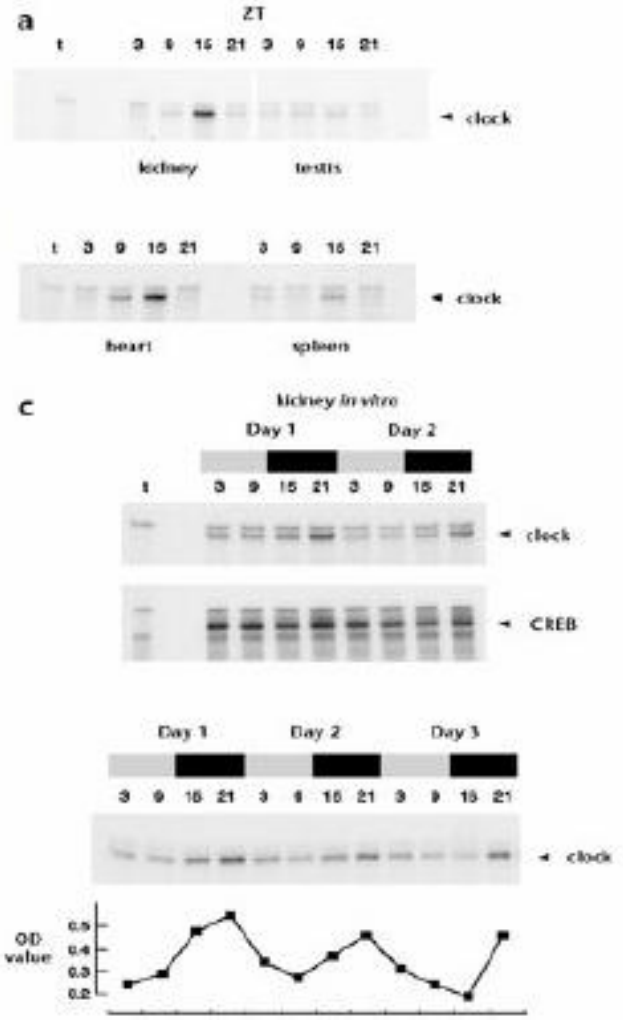
All cultured tissues are light responsive

C.Bertolucci
Dip. Biologia ed Evoluzione

Light responsive circadian oscillators

ZEBRAFISH





C.Bertolucci
Dip. Biologia ed Evoluzione

ZEBRAFISH

Rhythmic clock gene expression can be found in almost any tissue

Rhythmic clock gene expression is maintained in culture

All rhythmic tissues are directly light responsive

Use evolution to explore the input pathways to the circadian clock

C.Bertolucci
Dip. Biologia ed Evoluzione

CAVEFISH AS MODEL SYSTEMS

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).

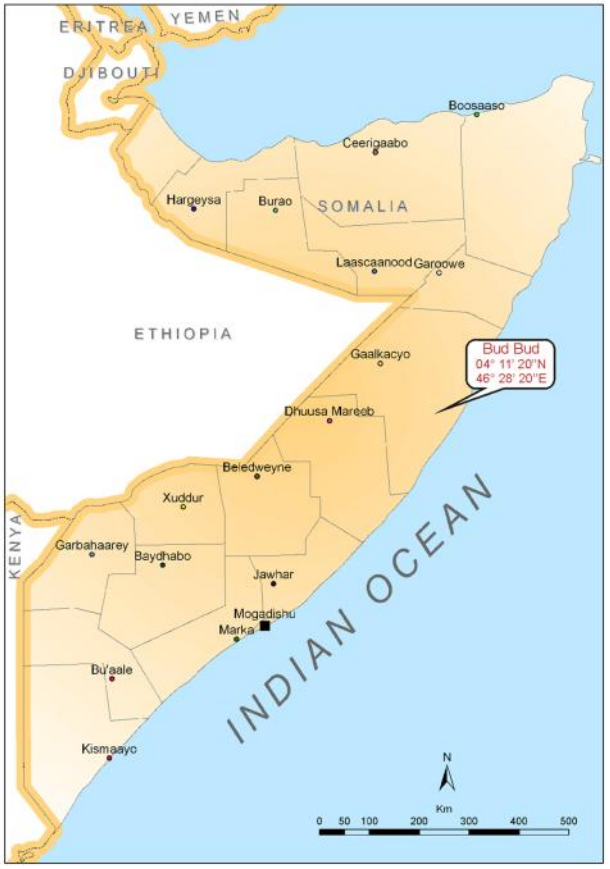
Astyanax mexicanus

Phreatichthys andruzzii

CONVERGENT EVOLUTION

C.Bertolucci
Dip. Biologia ed Evoluzione

Phreatichthys andruzzii



- **Anophtalmic (eye loss 36 hours post-fertilisation)**
- **Scales not present**
- **Complete depigmentation**
- **Reduced metabolism and oxygen consumption**
- **Negative phototaxis**

C.Bertolucci
Dip. Biologia ed Evoluzione

What are the advantages to use *Phreatichthys andruzzii*?

- Cyprinids (close relatives)

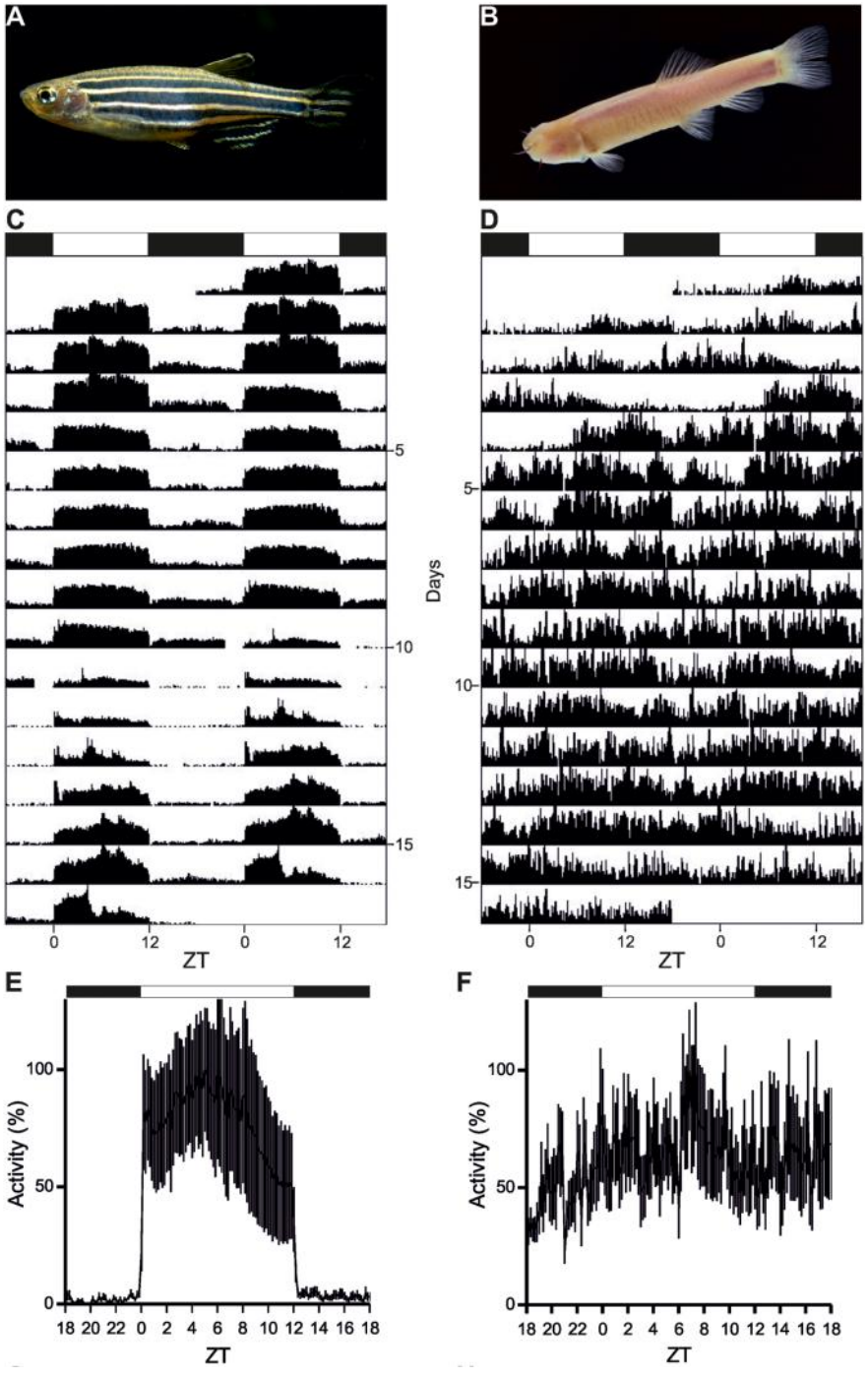
- Isolated for 2 million of years

Comparative analysis between zebrafish and the Somalian cavefish

C.Bertolucci
Dip. Biologia ed Evoluzione

What is the effect of light exposure on the behavioural activity in the cavefish?

C.Bertolucci
Dip. Biologia ed Evoluzione

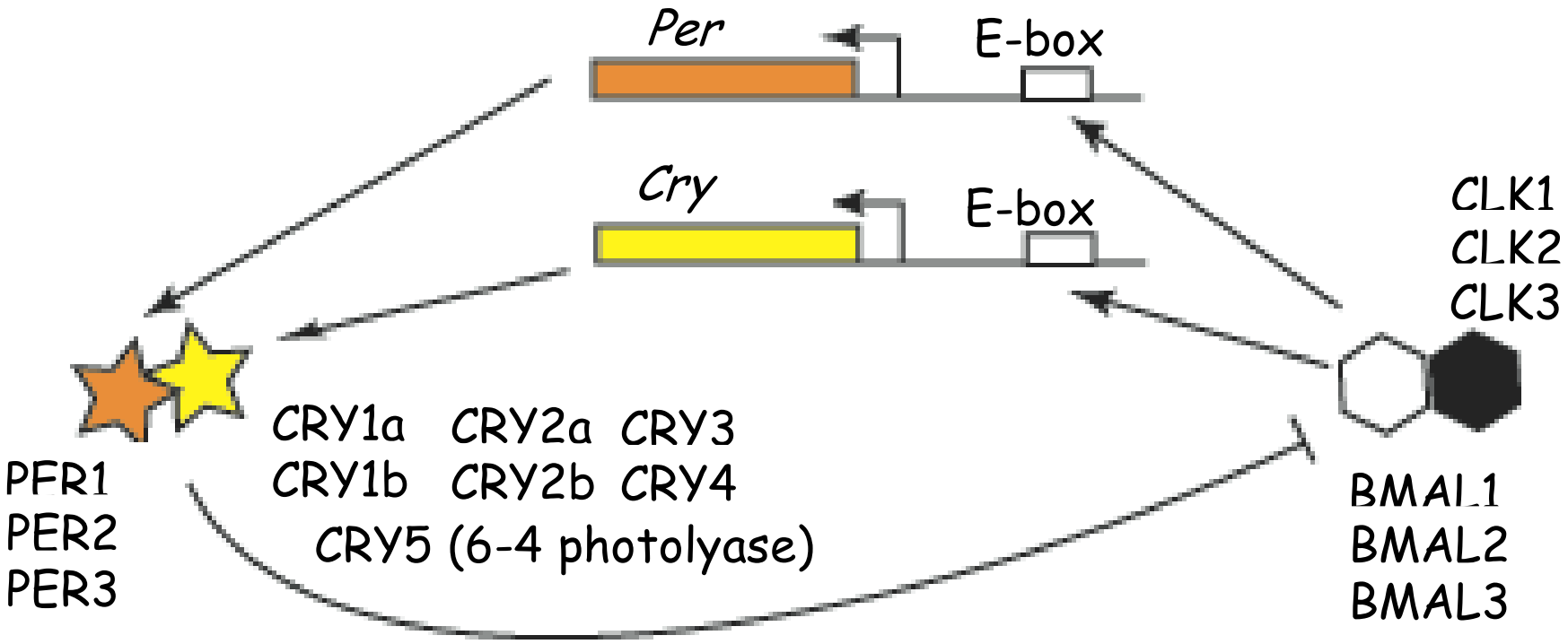


C.Bertolucci
Dip. Biologia ed Evoluzione

What is the effect of light exposure on clock gene expression in the cavefish?

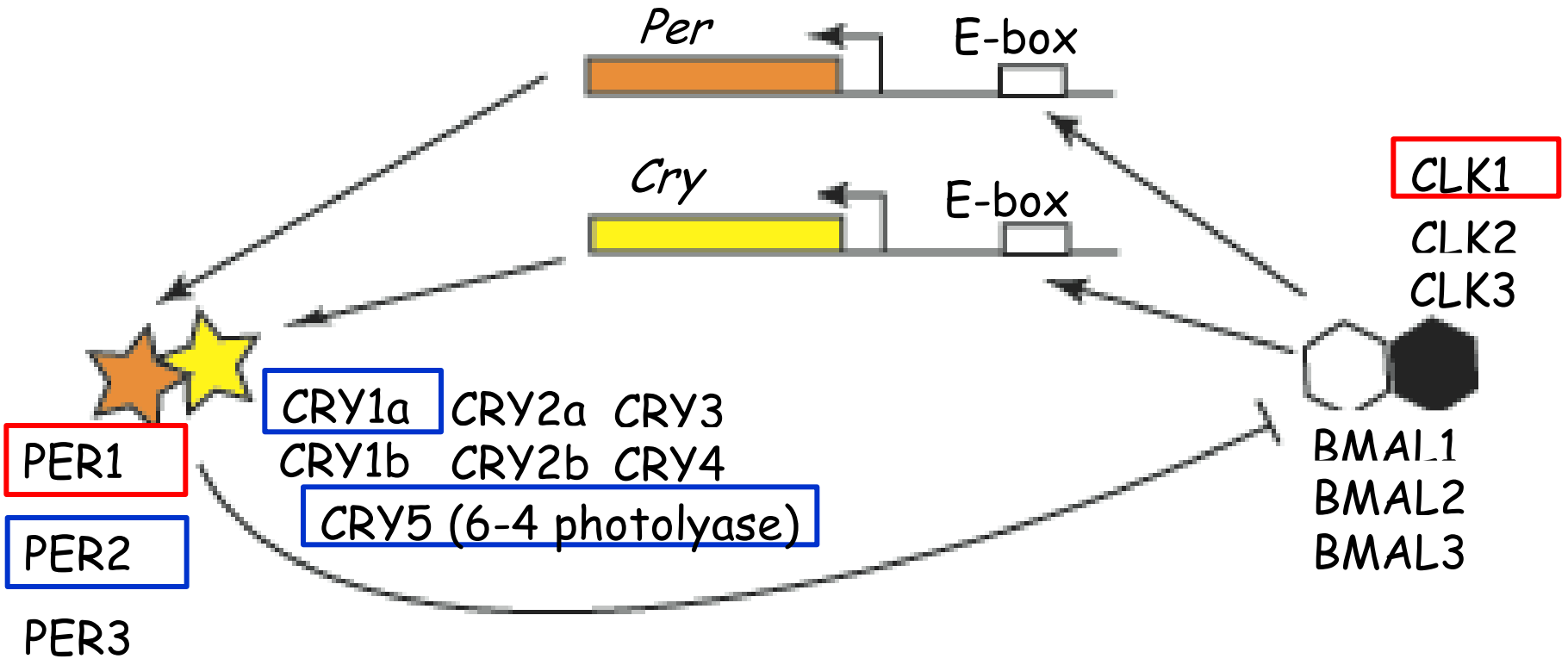
C.Bertolucci
Dip. Biologia ed Evoluzione

FIRST STEP: cloning of cavefish clock gene homologs



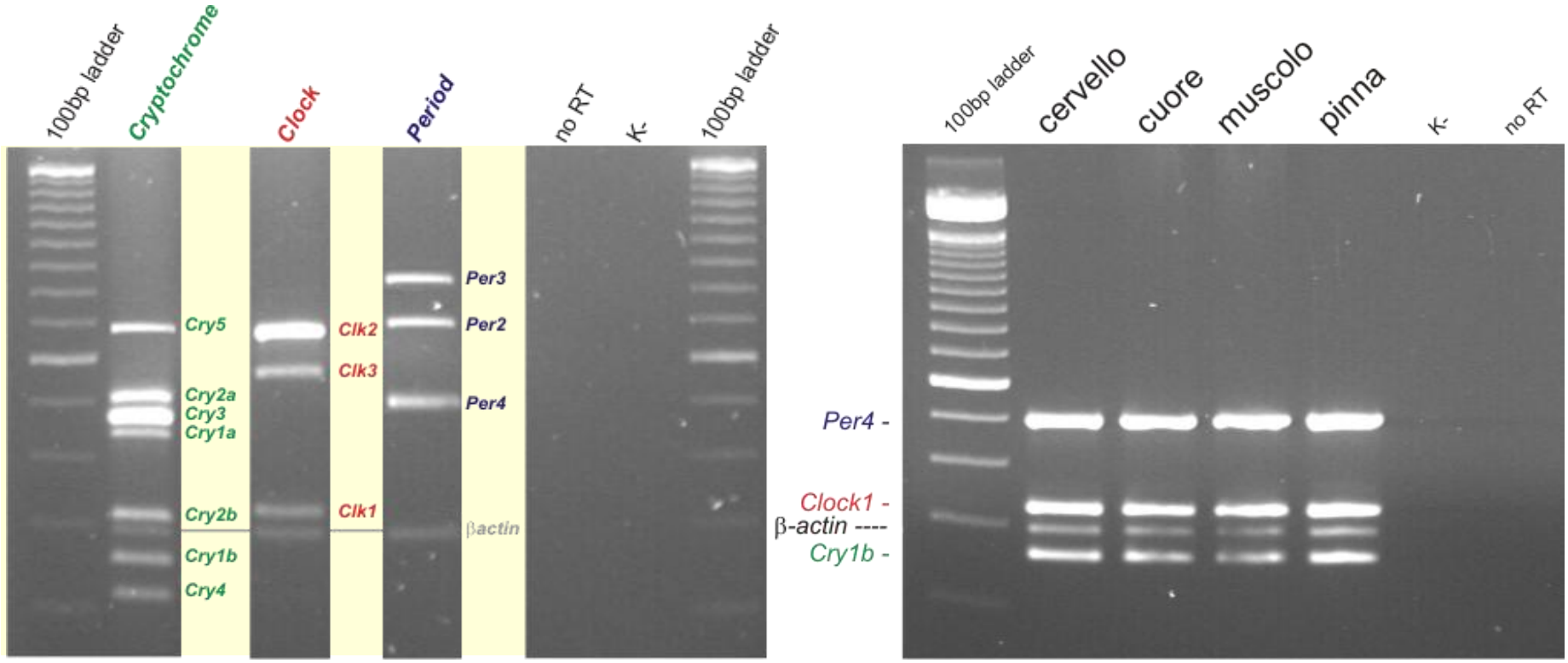
C.Bertolucci
Dip. Biologia ed Evoluzione

FIRST STEP: cloning of cavefish clock gene homologs



C.Bertolucci
Dip. Biologia ed Evoluzione

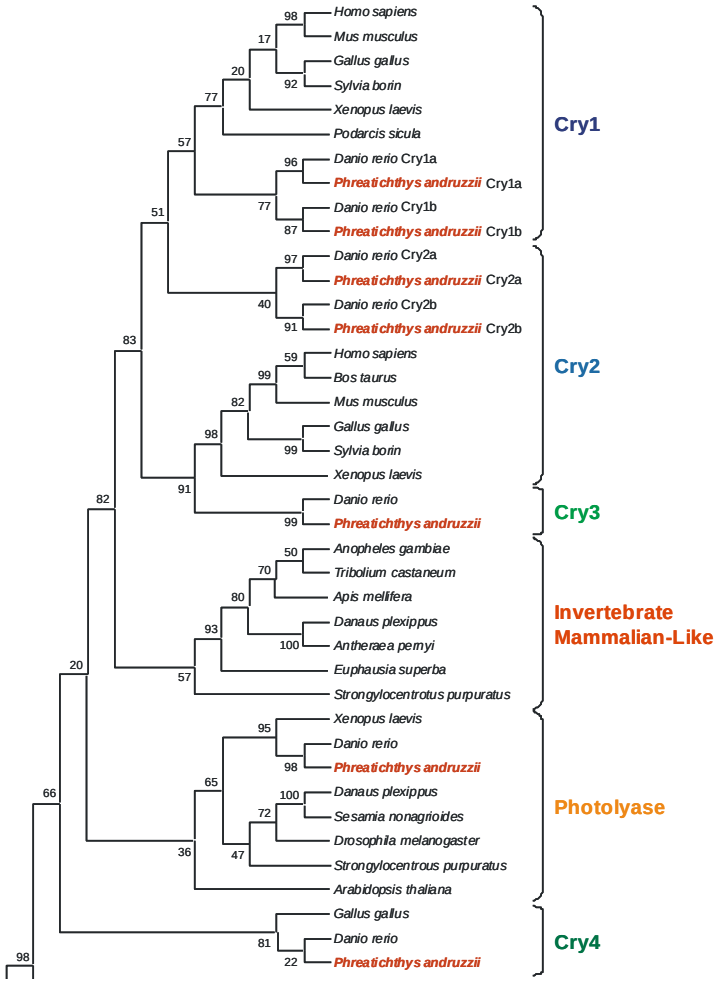
FIRST STEP: cloning of cavefish clock gene homologs



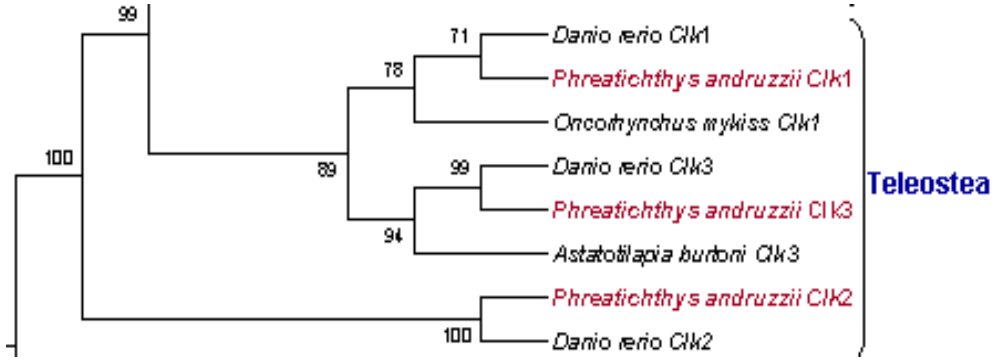
C.Bertolucci
Dip. Biologia ed Evoluzione

PHYLOGENETIC ANALYSIS

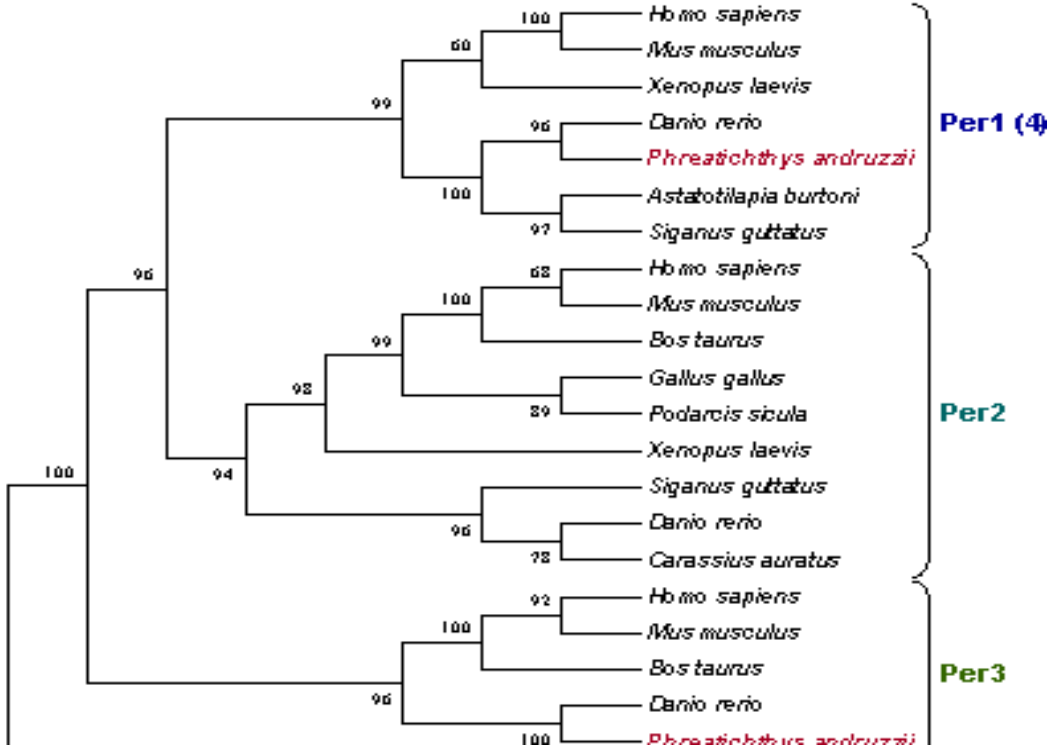
CRYPTOCROME



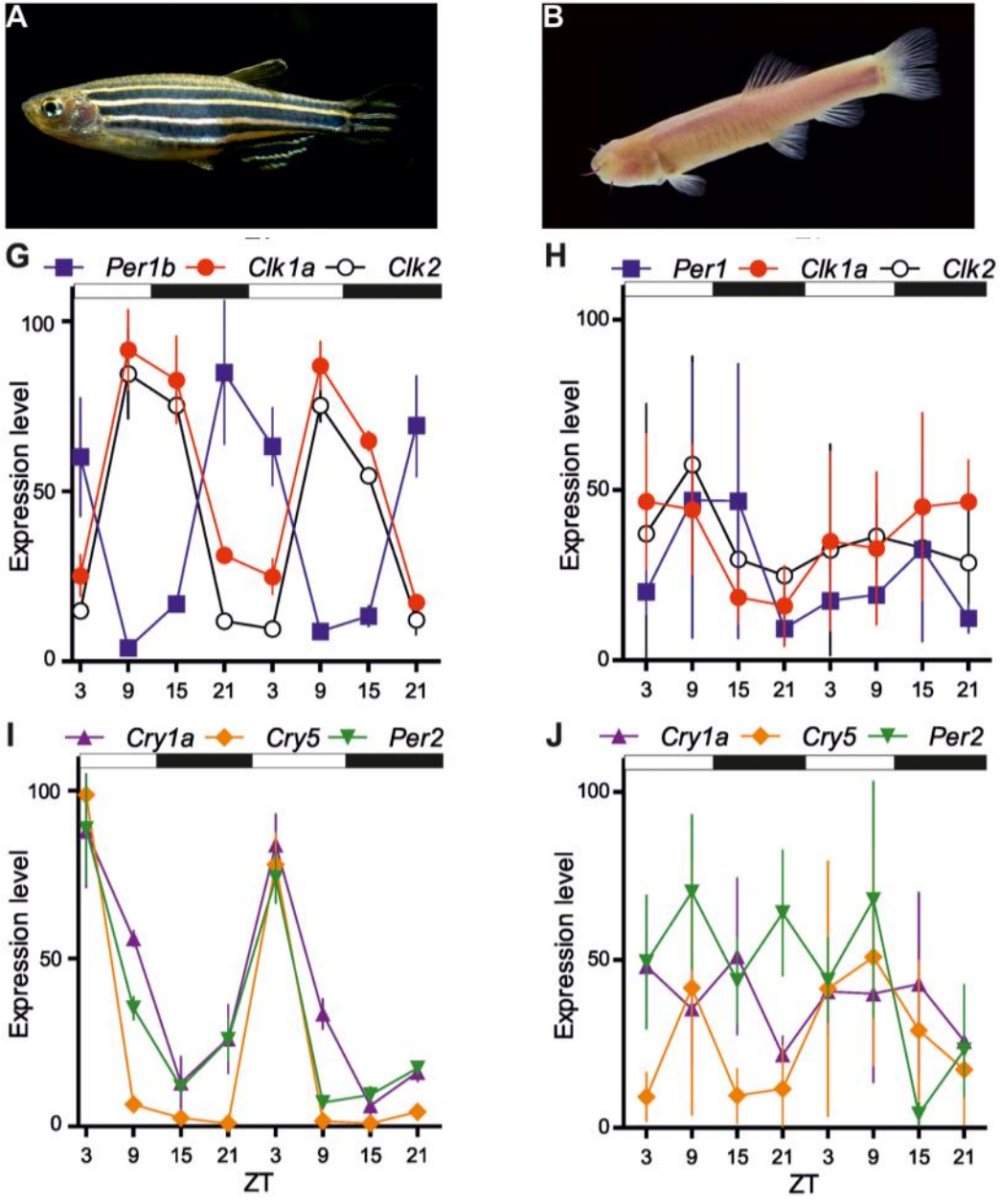
CLOCK



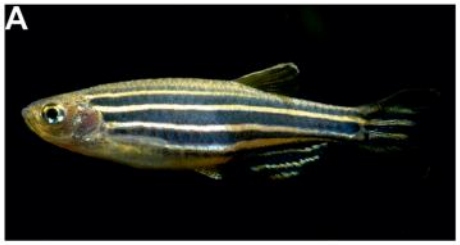
PERIOD

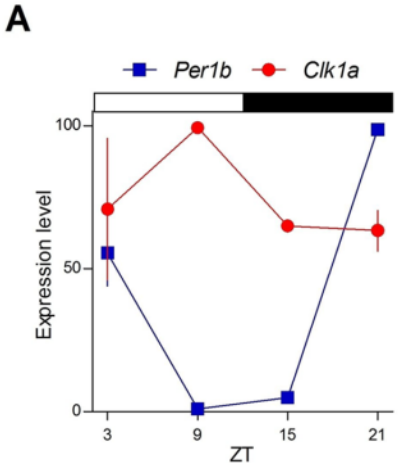


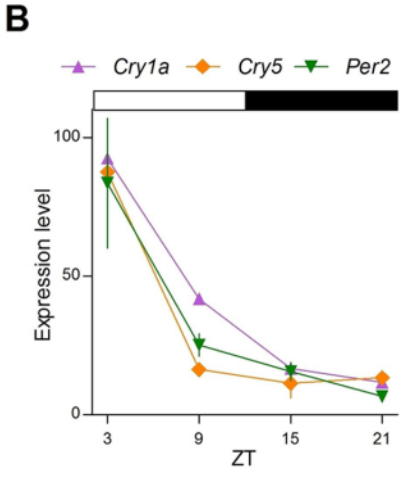
C.Bertolucci
Dip. Biologia ed Evoluzione

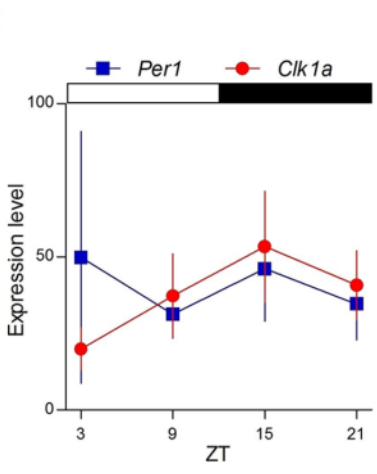


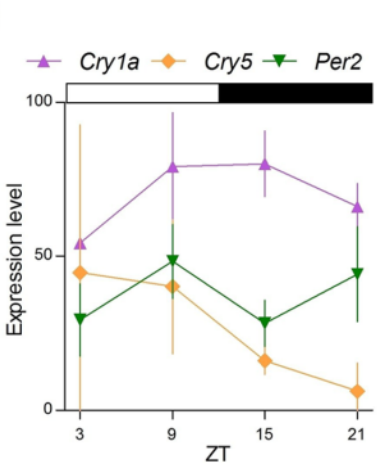
C.Bertolucci
Dip. Biologia ed Evoluzione

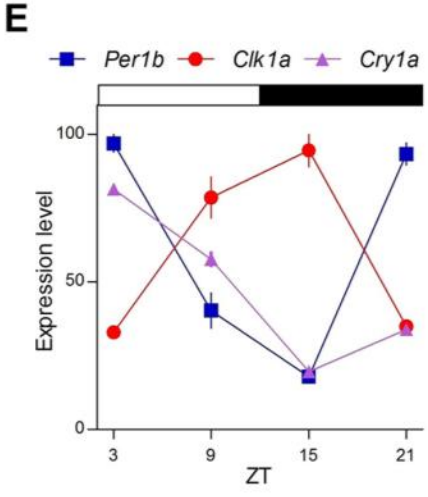


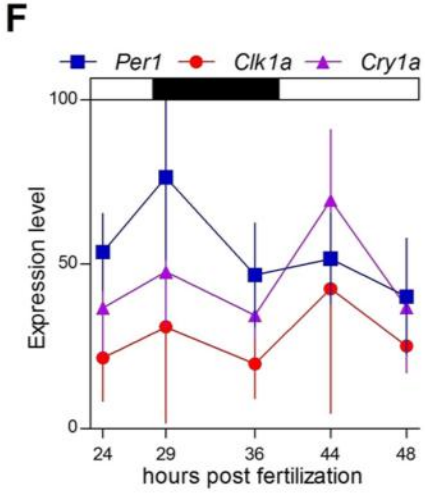


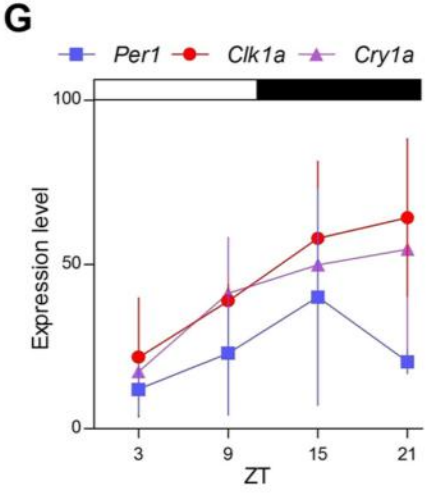


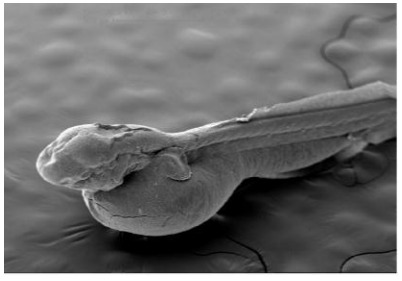










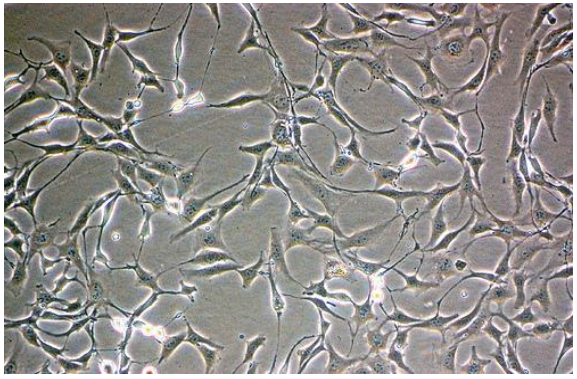


C.Bertolucci
Dip. Biologia ed Evoluzione

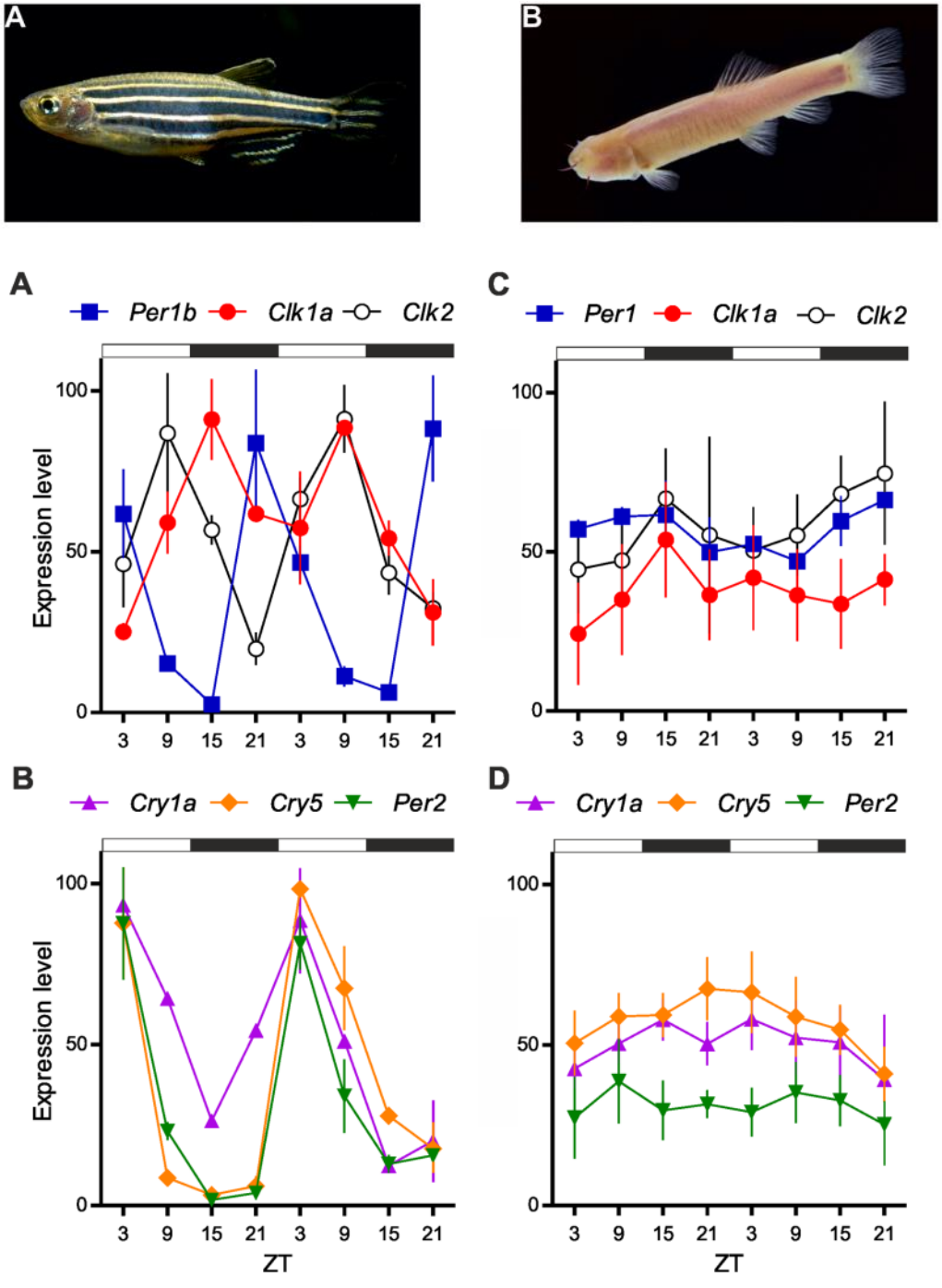
CELL LINE CREATION

Comparative study to dissect the mechanisms of light input pathway

CF cell



C.Bertolucci
Dip. Biologia ed Evoluzione

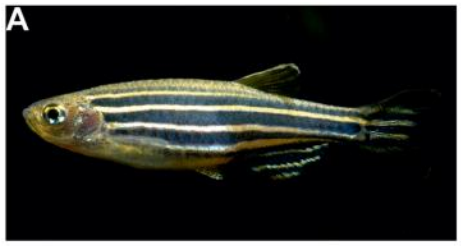


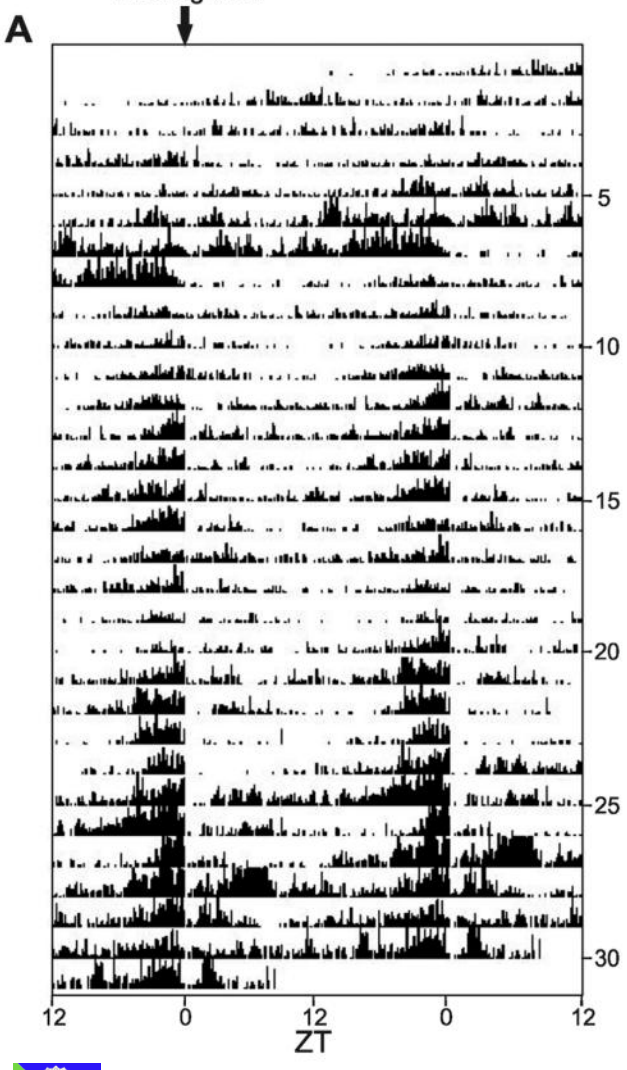
C.Bertolucci
Dip. Biologia ed Evoluzione

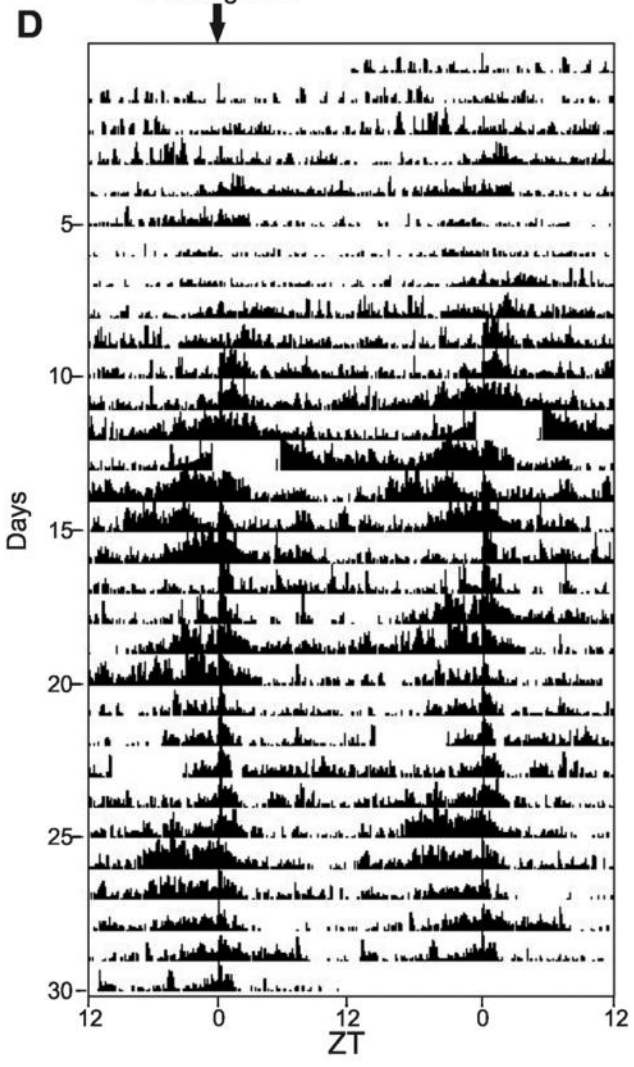
Our data reveal that either *P.andruzzii* lacks the circadian clock itself or it has a clock lacking a functional light input pathway

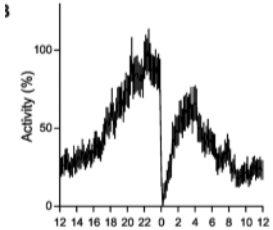
Could the cavefish circadian clock be entrained by an alternative environmental time signal (*zeitgeber*) like the periodic food availability.

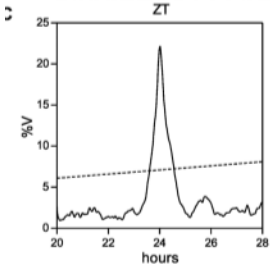
C.Bertolucci
Dip. Biologia ed Evoluzione

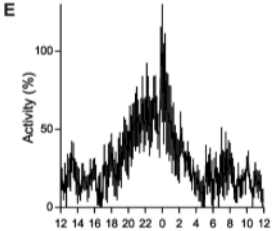


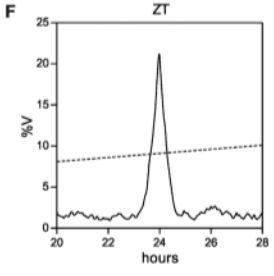




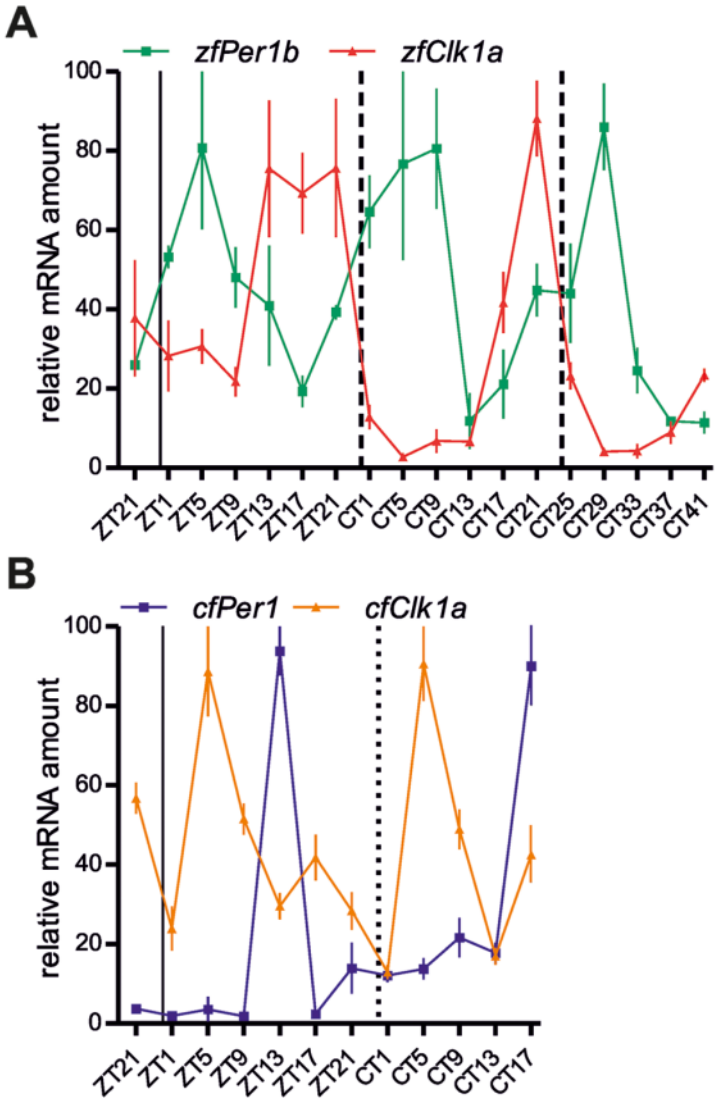


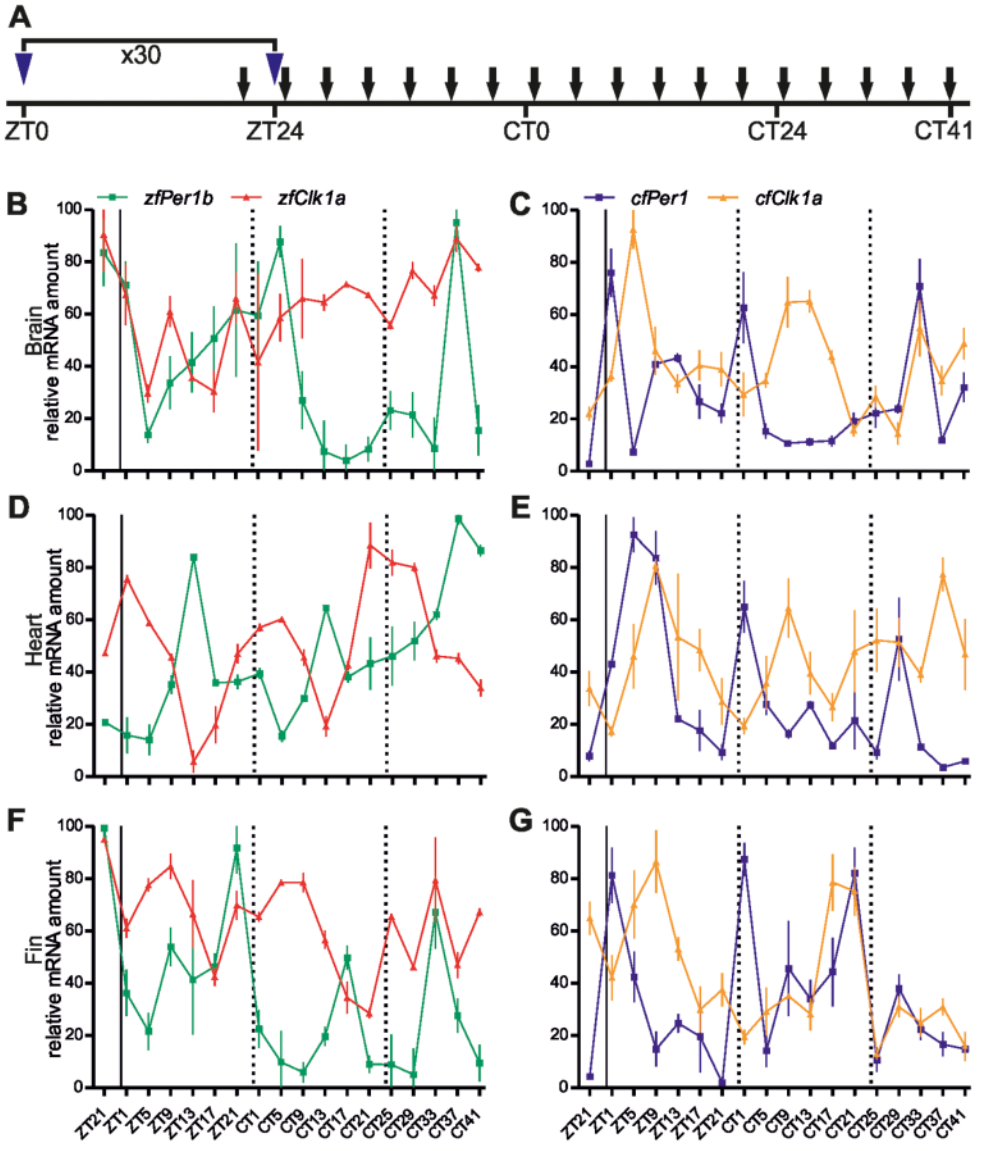






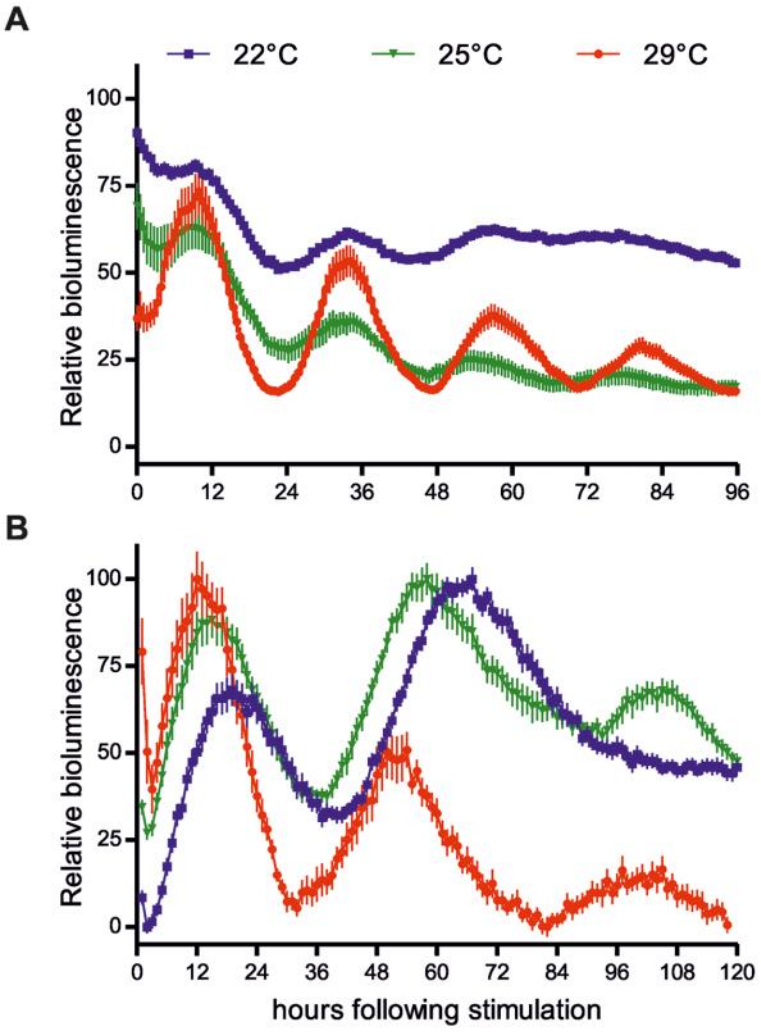
C.Bertolucci
Dip. Biologia ed Evoluzione





C.Bertolucci
Dip. Biologia ed Evoluzione

Transient treatments with glucocorticoids are widely used to induce rhythmic gene expression in cultured cells



τ=47 (22°C), 43 (25°C), and 38 h (29°C) revealing reduced temperature compensation with Q_{10}≈1.35

In zebrafish τ=23.6 (22°C), 24.2 (25°C), and 24.6 (29°C) h, respectively Q_{10}≈ 1

C.Bertolucci
Dip. Biologia ed Evoluzione

These results point to *P. andruzzii* having a functional clock that is entrainable by feeding but not by LD cycles.

This contrasts with the situation in zebrafish where both light- and food-entrainable oscillators are present.

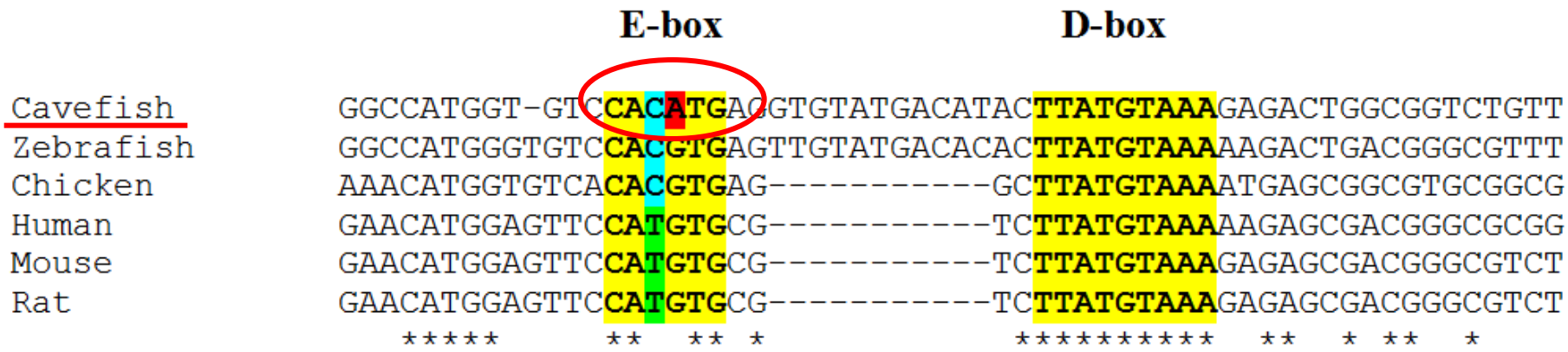
P.andruzzii represents a powerful complementary model for exploring the function of the light input pathway in vertebrates

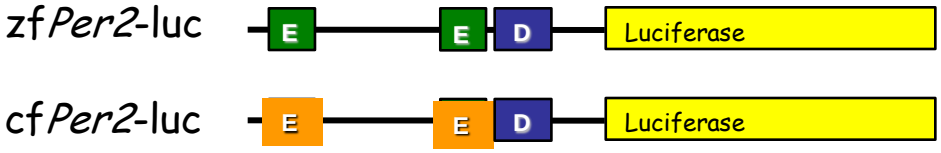
Light-induced transcription of clock genes represents a key step in photic entrainment of the zebrafish clock.

Could mutations in promoter sequences of light-inducible clock genes account for the cavefish blind clock phenotype?

C.Bertolucci
Dip. Biologia ed Evoluzione

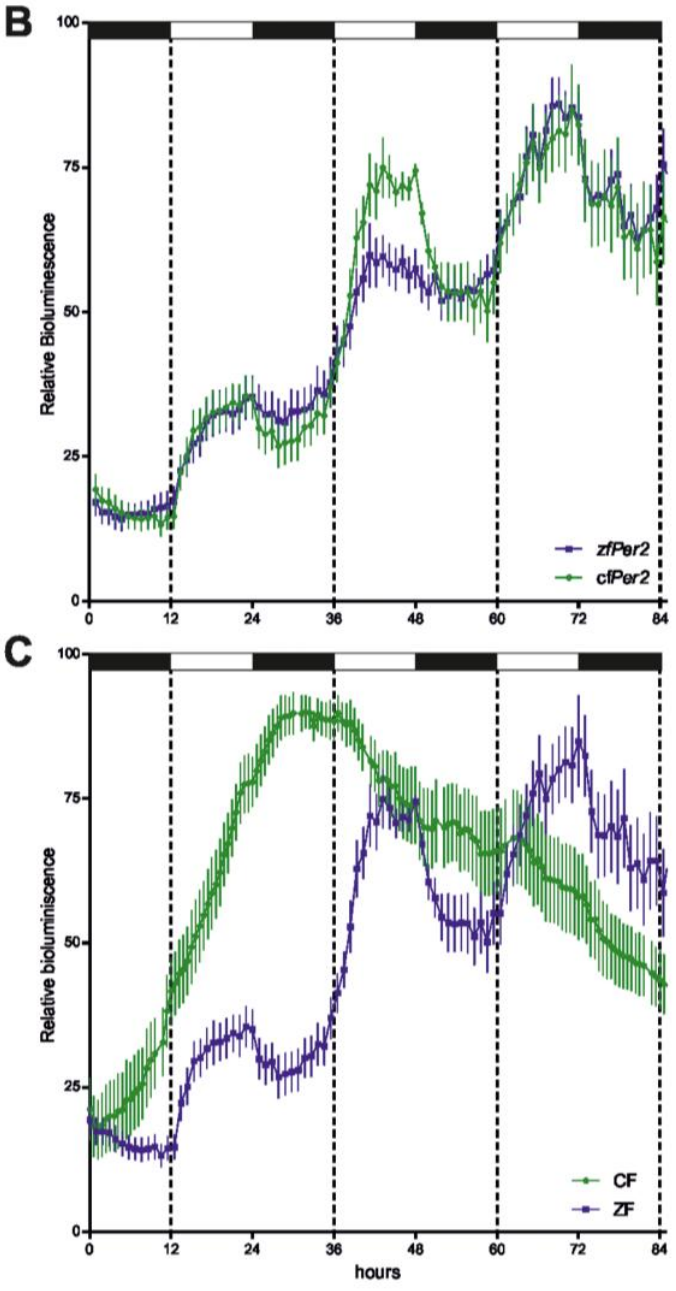
Cavefish *Period2* promoter analysis





C.Bertolucci
Dip. Biologia ed Evoluzione

Luciferase reporter assay of cf*Per2*-luc in zebrafish cells (AB9)



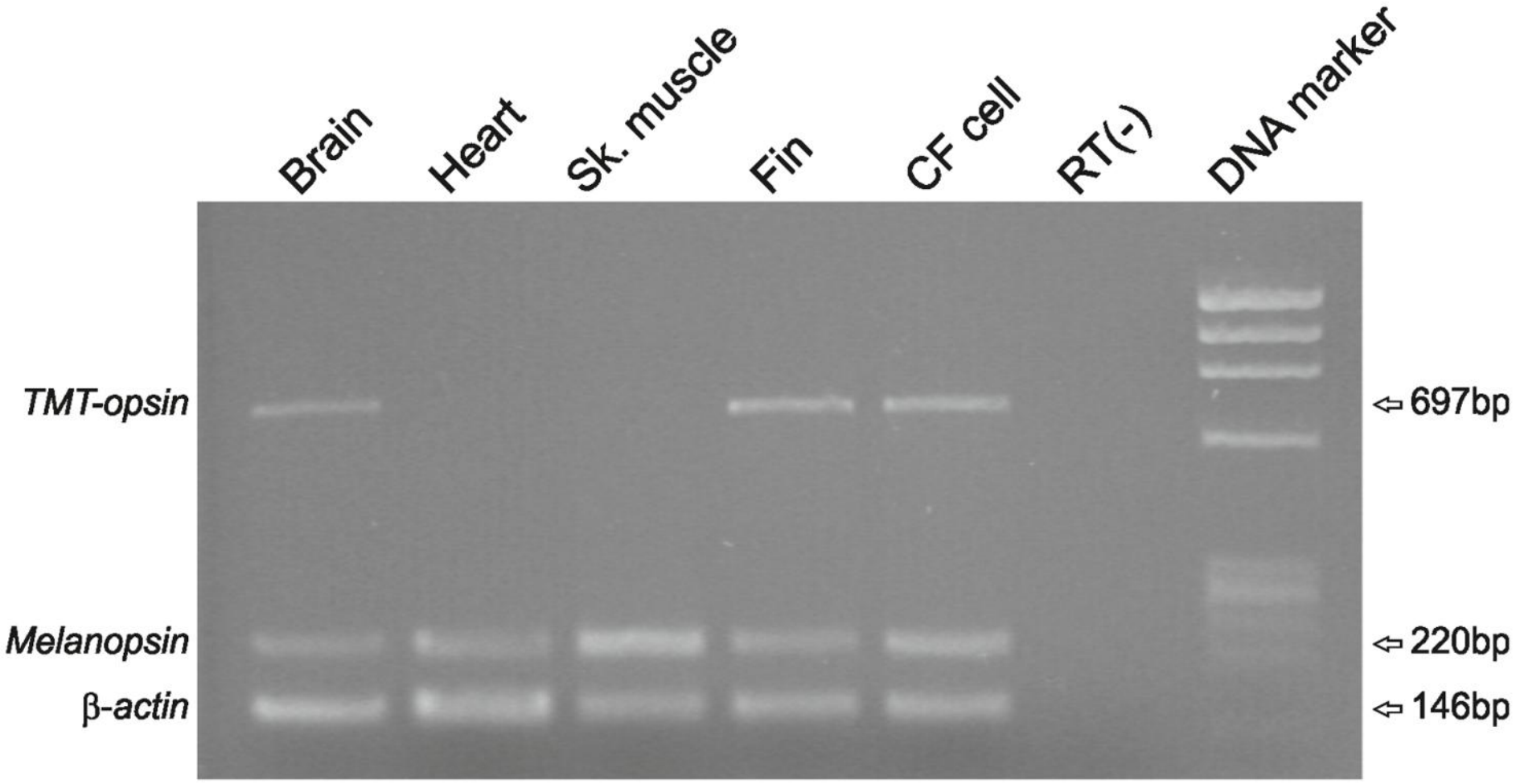
C.Bertolucci
Dip. Biologia ed Evoluzione

These results indicate that mutations disrupting the cavefish light input pathway should lie upstream of directly light-regulated clock gene promoters.

Mutations affecting peripheral photoreceptors could also account for the blind cavefish clock.

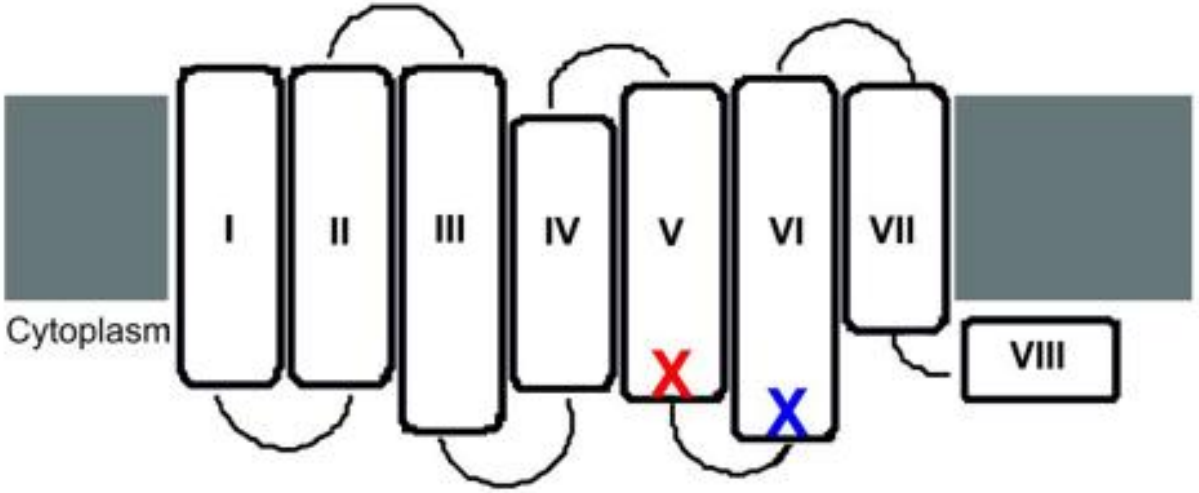
We chose to clone and characterize two opsins (Melanopsin and TMT-opsin) in the cavefish.

C.Bertolucci
Dip. Biologia ed Evoluzione



C.Bertolucci
Dip. Biologia ed Evoluzione

Premature stop-codons were encountered in the coding sequences of both TMT-opsin and Melanopsin at the C-terminus of the 5th transmembrane domain and the N-terminus of the 6th transmembrane domain, respectively.



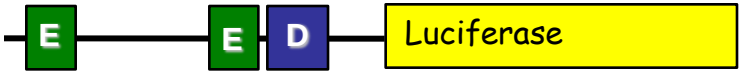

```
TMT-opsin
Cavefish   SSISYIICLFIFCLIVPFFGHYLLLWX----------
Zebrafish  NNISYIICLFIFCLIVPFLVIIFCYGKLLHAIKQVSS
             ..*****************:
```

```
Opn4m2
Cavefish    RAAGKEIRELDCGETLRCMNSPSWSLCFLX----------
Zebrafish   RAAGKEIRELDCGETHKVYERMQNEWKMAKVALVVILLFI
            ***************  :  :
```

C.Bertolucci
Dip. Biologia ed Evoluzione

Rescue of the light input pathway to *Period2* promoter

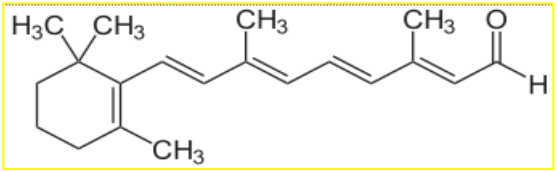
zf*Per2*-luc



zf*TMT* pcDNA 3.1

zf*Opn4m2* pcDNA 3.1

Retinaldehyde
(9-cis and All-trans)



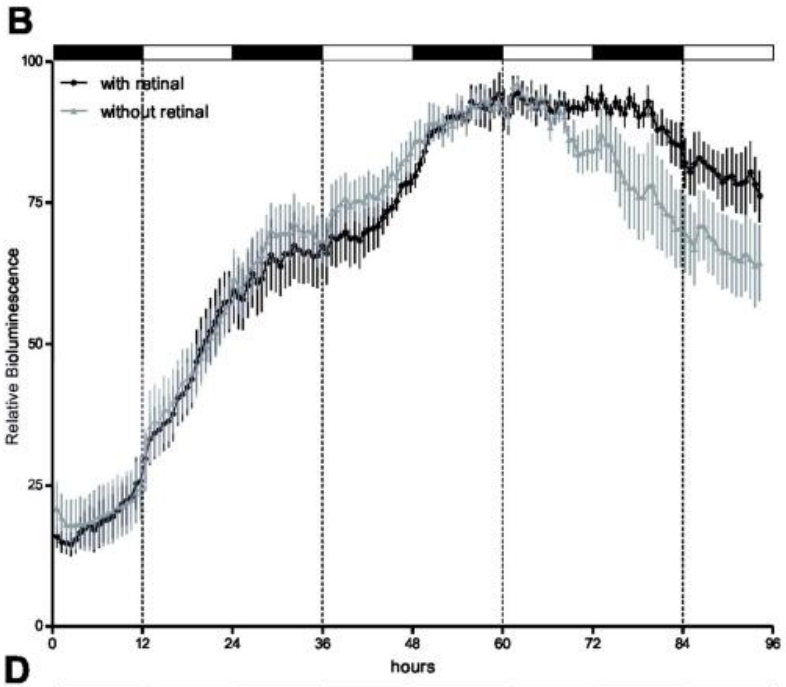
Cavefish cells
CF

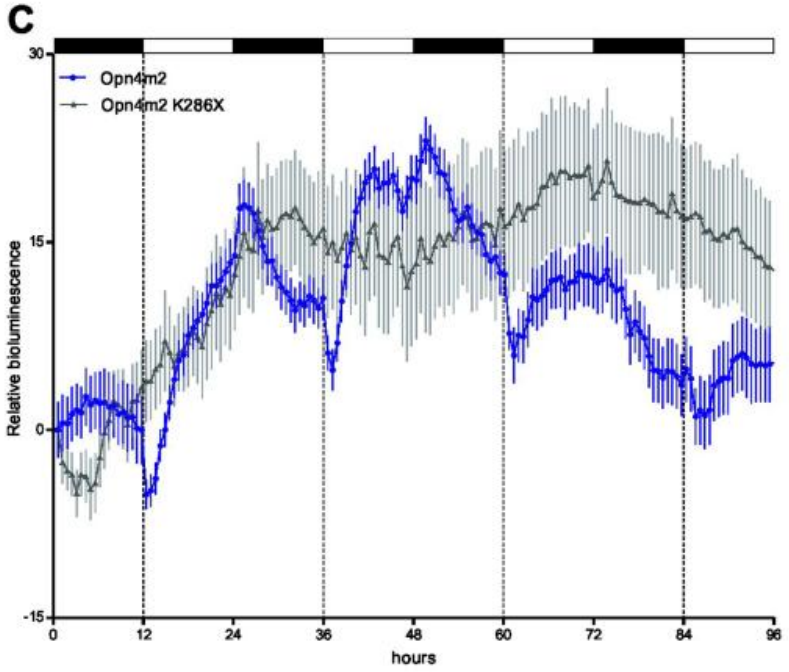
C.Bertolucci
Dip. Biologia ed Evoluzione

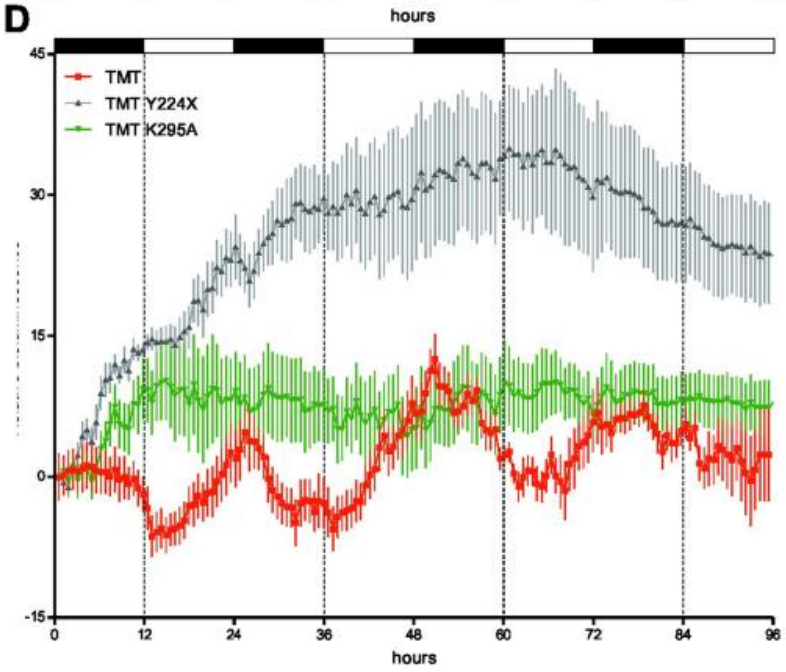
- Supplementing the culture medium with retinaldehyde failed to induce rhythmic expression of zf*Per2*-Luc

- Upon cotransfection with single opsin expression vectors, zf*Per2*-Luc was robustly induced during the light phase and subsequently decreased during the dark phase (C, blue D, red).

- Expression in cavefish cells of zebrafish Melanopsin and TMT-opsin carrying mutations introducing premature stop codons equivalent to the two cavefish opsins (zfOpn4m2K286X and zfTMTY224X) failed to rescue light inducible zfper2-Luc expression (C-D, grey, green).



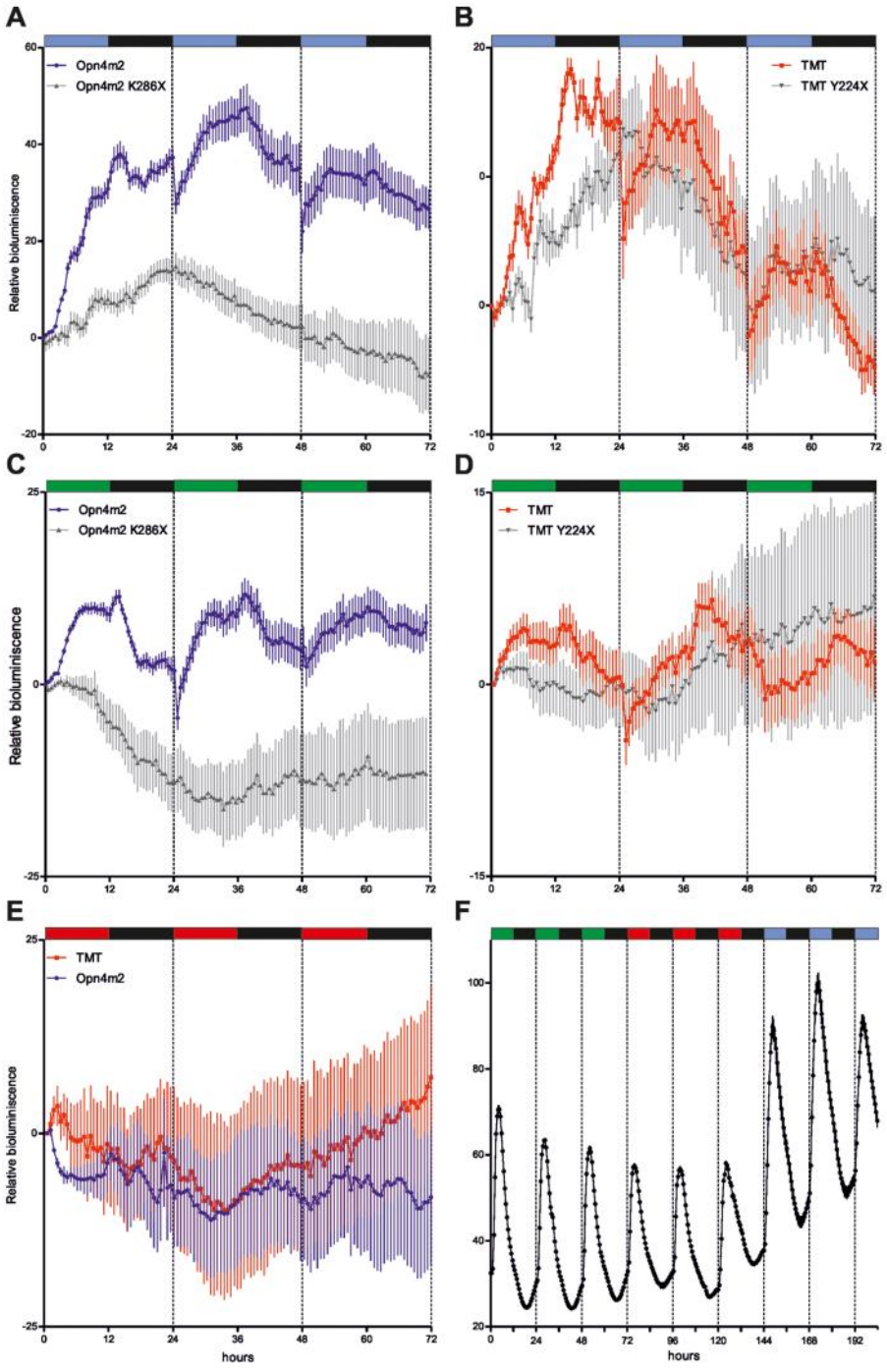




C.Bertolu
Dip. Biologia ed Evoluzione

Exposure of Melanopsin or TMT-opsin transfected cavefish cells to blue (468 nm) or green (530 nm) light is able to activate the *zfper2* promoter (A–D). In contrast, no rescue was observed under red (657 nm) light (E).

Exposure of zebrafish cells to these same monochromatic light sources revealed activation by all three light sources, with the strongest induction by blue (F).



C.Bertolucci
Dip. Biologia ed Evoluzione

SUMMARY

During 1.4–2.6 million years of isolation from the day-night cycle, the evolution of the cavefish P. andruzzii has lead to an aberrant circadian clock.

Contrary to the situation in most organisms, this clock is no longer entrained by light but by food.

It is tempting to speculate that food availability in the subterranean environment of this cavefish might indeed be periodic, and therefore a clock responding to and anticipating feeding time may confer a survival advantage.

C.Bertolucci
Dip. Biologia ed Evoluzione

SUMMARY

TMT-opsin and Melanopsin serve as peripheral tissue photoreceptors in teleosts

The presence of multiple photoreceptors, each one differentially extracting timing information from sunlight, could enable the circadian system to more reliably indicate the timing of dawn and dusk

P. andruzzii serves as a powerful complementary model to dissect the molecular pathways that respond to light

C.Bertolucci
Dip. Biologia ed Evoluzione