THE CIRCADIAN CLOCK OF THE MEXICAN BLIND CAVEFISH, *ASTYANAX MEXICANUS*

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I, Andrew David Beale, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

ABSTRACT

The prevalence of circadian clocks in most, if not all, organisms on the planet implies they are an adaptive trait. However, only a few studies provide evidence to support this. To address this issue directly, we examined circadian clock function in the Mexican cavefish, *Astyanax mexicanus*. This species has spent a significant period of its evolutionary history in constant darkness, away from the daily cycles of the surface environment. Many cavefish populations have adapted for life in the dark by reducing eyes and pigmentation and enhancing sensory processes relevant in darkness, such as mechanosensation. Have cavefish retained the circadian clock architecture in the absence of the primary entraining cue, light?

This thesis presents evidence for:

i. The presence of a light-entrainable molecular circadian clock in *Astyanax* cavefish. Robust circadian rhythms of *per1* expression are exhibited in cavefish, but with reduced amplitude and altered phase relative to their surface fish ancestors.

ii. Alterations in the light input pathway, especially the raised expression of a putative clock repressor, *per2b*. We propose that this dampens the amplitude of core clock gene expression rhythms observed in cavefish.

iii. Absence of circadian rhythmicity in wild cavefish.

iv. A developmental delay in the appearance of light-detection in cavefish embryos

v. Upregulation of two DNA repair genes, *CPD phr* and *ddb2*, in cavefish. This effect is accentuated in the wild, leading us to propose that dampening of the circadian clock in cavefish may be a by-product of selection for raised expression of DNA repair family genes and light-induced genes.

By examining multiple aspects of the circadian biology of *A. mexicanus* surface and cavefish, the data presented in this thesis supports the hypothesis that the clock gives a fitness advantage through the provision of internal temporal order but suggests the clock may be partially reduced in aperiodic environments.

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1 GENERAL INTRODUCTION

There is a grandeur in this view of life, with its several powers, having been originally breathed into a few forms or into one; and that, whilst this planet has gone on cycling according to the fixed law of gravity, from so simple a beginning endless forms most beautiful and most wonderful have been, and are being, evolved.

(Darwin, 1859)

Life on Earth is enormously diverse, with a wonderful array of unique adaptations. From the unicellular to the multicellular, plant to animal, evolution has taken its course amongst the many challenges that the environment places upon it. The geophysical properties of this planet have resulted in cycles in biotic (e.g. food availability, predation, reproduction timing) and abiotic (e.g. light and temperature) factors, which impose considerable periodicity on organisms. In response most, if not all, animals and plants have evolved an endogenous timing system, known as the circadian clock, to temporally co-ordinate their biology (Pittendrigh, 1993; Sharma, 2003). Since circadian clocks are ubiquitous in nature, it is proposed that co-ordinating biological activity has provided a selective advantage for survival (Pittendrigh, 1993; Sharma, 2003; Hut and Beersma, 2011). This selective advantage can be conferred in two ways: (i) synchrony of the internal with the external world through synchronisation to external cycles, and; (ii) provision of temporal order within an organism.

For these clocks to serve a useful biological purpose, they need to be set or entrained. Perhaps the most fundamental and palpable external cyclic event to which clocks entrain is the daily cycle of light and dark. The most common resetting signal, or zeitgeber (time-giver), is the environmental light-dark (LD) cycle. Constantly dark environments, caves or the depths of the ocean being two of the few examples, are not subject to such fundamental periodic events as the LD cycle and animals that exist here represent an interesting opportunity to study the origin, evolution and adaptive value of circadian clocks. It has therefore been the generally held view that animals that live in constantly

dark environments have no need for such a timing mechanism and thus have allowed it to regress. This thesis will address this statement by investigating the circadian clocks of an obligate cave-dwelling fish, *Astyanax mexicanus*.

1.1 General introduction to the circadian clock

Many physiological, cellular and behavioural processes show oscillations in a daily fashion. Daily oscillations are observed at all levels of the organism, from the whole – such as sleep and wake cycles – to the enzymatic, such as alcohol dehydrogenase activity in the liver. These oscillations continue even in the absence of environmental time cues, and were first described nearly 300 years ago (De Mairan, 1729). Behind such oscillations is the endogenous, self-sustaining time-keeper known as the circadian clock (Pittendrigh, 1960). The circadian clock system can be depicted in its most simple form as an input-oscillator-output pathway and is illustrated in Figure 1.1.

Figure 1.1: Simplified model of the circadian clock system.
Inputs (zeitgebers) such as light are received by the circadian clock. This entrains the circadian clock, which then regulates the rhythmic outputs such as the expression of genes and other cellular and biological functions (adapted from Roenneberg and Merrow, 2005). *Reprinted by permission from Macmillan Publishers Ltd: Nat Rev Mol Cell Biol 6: 965-971, copyright (2005).*

The principal component is the core of the system: the endogenous clock generating free-running rhythms with a period, tau (τ), of approximately 24 hours. This clock requires resetting by external signals, the zeitgebers, to maintain a stable relationship to the external 24-hour daily cycle. This process is known as entrainment. The timing information held by the core oscillator is then passed on to outputs, from the transcription

of genes to behaviour. This conceptual pathway applies at all levels of organisation of the organism, and has aided the dissection of the physical and genetic basis of the circadian clock (Roenneberg and Merrow, 2005).

A number of fundamental properties or generalisations have been described for the circadian clock system, which were originally formalised at a Cold Spring Harbour conference on Circadian Biology in 1960. One such property is temperature compensation, which was known from very early experiments by Pittendrigh and others (Pittendrigh, 1993). In this case, the period of the clock, which is both characteristic to the individual and can exhibit deviations dependent on conditions and even stochastic variations between cells, remains stable within a range of temperatures (Pittendrigh, 1960; Carr and Whitmore, 2005). Temperature compensation is important for a biological clock to be a reliable measure of time, as it must be able to keep the same time over a range of temperatures and not follow the Q10 law, where the speed of the reaction doubles with increases of 10°C, unlike most biochemical reactions.

Pittendrigh defined a number of further general properties of the circadian clock and the circadian rhythms it generates. Circadian rhythms are ubiquitous in living systems – across all kinds of organisms and within all processes of the organism; their period is dependent on the entrainment regime and light intensity; and they are susceptible to phase shifting as a function of the intensity and duration of the perturbing stimulus, the free-running period and the phase at which the stimulus is given (Pittendrigh, 1960). This work in formalising fundamental properties of the circadian clock by Pittendrigh, Aschoff and others in the early part of the 20th century has provided a firm foundation for circadian clock research.

1.2 CIRCADIAN CLOCK ORGANISATION

Locating the circadian clock within the organism was a significant direction of research early in the circadian field. Central master clocks, or pacemakers, were discovered in invertebrates, birds and mammals. When these regions were ablated, the organism lost the ability to generate circadian rhythms (Stephan and Zucker, 1972). Furthermore, when these regions were transplanted from animals with mutant circadian periodicities to animals who had had these regions ablated, the recipient animals took on the period of the donor, including 'mutant' donor periods (Ralph et al., 1990), indicating the significance of the transplanted tissue in generating circadian rhythms (Page, 1982; Ralph et al., 1990).

A centralised model of circadian clock organisation thus developed where these structures contained the master clock, the sole region where circadian rhythms are generated to drive the rhythms of the whole animal. Though a hierarchy of organisation does exist, the centralised model was challenged by the subsequent discovery of cell-autonomous molecular oscillators in explanted tissues and cells in culture (Plautz et al., 1997; Balsalobre et al., 1998; Whitmore et al., 1998). Further evidence in mammals supported a less centralised organisation: a lesion of the central pacemaker of mice, the suprachiasmatic nucleus (SCN), did not destroy the circadian rhythms of the animal but rather caused the numerous peripheral oscillators of organs and tissues to become out of synchrony with each other (Yoo et al., 2004). The master clock is not the sole possessor of circadian rhythms, but rather more of a 'conductor', keeping the multitude of peripheral oscillators in synchrony.

1.3 THE MOLECULAR CIRCADIAN CLOCK

Identification of the molecular basis of circadian rhythms came some time after the discovery of the physiological basis. Early progress was made in *Drosophila*, with isolation and sequencing of *period* (*per*) which, when mutated, alters or abolishes the period of the

free-running circadian activity rhythm (Konopka and Benzer, 1971; Reddy et al., 1984). Subsequently, the genetic basis of vertebrate circadian rhythms was explored, with the mapping of *tau* in the hamster and *Circadian locomotor cycles kaput* (*Clock*) in mice (Ralph and Menaker, 1988; Vitaterna et al., 1994; King et al., 1997; Lowrey et al., 2000). With the exception of *tau*, which was a discovery based on a natural mutation of the circadian clock in hamsters, these genes were discovered using forward genetic screens and analysis of the circadian phenotypes of many animals. Further screens have isolated many more genes involved in the circadian clock, recently including a novel circadian role for Fblx3 revealed by the *Afterhours* mutant mouse (Godinho et al., 2007), and a feedback loop mechanism for their interaction has been established.

The clock consists of transcription-translation feedback loops involving positive and negative elements that interact to produce a cycle time of approximately 24 hours. In *Drosophila*, the positive element consists of the basic helix-loop-helix (bHLH) per-arnt-sim (PAS) domain transcription factors Clock (Clk) and Cycle (Cyc) (Allada et al., 1998; Rutila et al., 1998). These bind as heterodimers to activate transcription from E-box elements (CACGTG), which are found in the promoters of *period* and *timeless* (*tim*). The protein products of these genes are degraded in the light: the degradation of Per and Tim is mediated in the light by Double-time (Dbt) kinase and a Cryptochrome-Jetlag (Cry and Jet) mechanism respectively (Kloss et al., 1998; Price et al., 1998; Koh et al., 2006; Peschel et al., 2009). After dark, the proteins accumulate, the Per protein is stabilised by Tim, and the two proteins form a heterodimer to bind to Clk/Cyc in the nucleus. This prevents Clk/Cyc from binding to DNA and thus inhibits Clk/Cyc mediated transcription, including that of *per* and *tim*, and the negative loop is completed (reviewed in Peschel and Helfrich-Förster, 2011). A secondary feedback loop exists in *Drosophila* and regulates the rhythmic expression of *clk*. Clk/Cyc directly activate the expression of *vrille* (*vri*) and *par domain protein 1* (*pdp1*), which in turn directly regulate *clk* expression. Vri promotes and Pdp1 represses *clk* transcription (Cyran et al., 2003).

The circadian clocks of *Drosophila* and vertebrates are in essence very similar. Both clocks consist of transcription-translation feedback loops, which involve very similar positive and negative elements to produce a 24-hour period. In vertebrates, the positive element consists of homologues of the *Drosophila clock* and *cycle*, known as *Clock* and *Bmal*. Like *Drosophila*, these bind as heterodimers to E-box elements in the promoters of *period* (*Per*) and *cryptochrome* (*Cry*) genes (Gekakis et al., 1998; Hogenesch et al., 1998). In turn, the translated PERIOD and CRYPTOCHROME heterodimerise and inhibit the transcriptional activation of CLOCK-BMAL within the nucleus, and thus inhibit their own transcription, completing the feedback loop (Griffin et al., 1999; Kume et al., 1999). A secondary, stabilising loop similar to that of *Drosophila* also exists in mammals and regulates the transcription of *Bmal* genes. The CLOCK-BMAL heterodimer promotes the transcription of nuclear receptors *Rev-Erbα* and *Rorα*, which in turn bind retinoic acid-related orphan receptor response elements (RORE) in the promoter of *Bmal*. REV-ERBα represses and RORα promotes *Bmal* transcription.

Thus, the fundamental elements of the circadian clocks of vertebrates and *Drosophila* are very similar, in many cases using homologous genes in homologous roles despite the large evolutionary distance between them. However, in other cases the orthologues have diverged in function and occupy different roles in the circadian clock. The cryptochromes are one example of this. Cryptochromes are often divided into two groups or types based on their function and role in the clock mechanism. Though there is evidence for a core clock role in the periphery, *cry* predominately acts as the circadian photoreceptor in *Drosophila* and is designated as a light-responsive, Type I cryptochrome (Stanewsky et al., 1998; Emery et al., 2000; Krishnan et al., 2001). In mammals, the photoreceptive function of Cry proteins is lost, and *Cry* genes act as core clock components in mammals (van der Horst et al., 1999). *Cry1* and *Cry2* inhibit the transcriptional activation of Clock-Bmal and are thus designated light-irresponsive Type II cryptochromes (Kume et al., 1999; and

reviewed in Chaves et al., 2011b). Interestingly, some animals have both Type I and Type II cryptochromes; this will be discussed with reference to zebrafish below.

In contrast to *Drosophila*, the vertebrate clock has multiple copies of the *clock* genes: *Drosophila clock* is the invertebrate homologue of at least two vertebrate *Clock* genes, for example *Clock* and *Npas2* in mice (Hogenesch et al., 1997; King et al., 1997); *Drosophila cycle* is the homologue of the vertebrate *Bmal* genes (Hogenesch et al., 1998); and *Drosophila period* has homologues in the vertebrate *Period* genes (Sun et al., 1997; Zylka et al., 1998). In a similar way to the duplication of the *Hox* genes, the duplication of clock genes within vertebrates is proposed to allow the development of a diverse and complex system of circadian regulation (Tauber et al., 2004). Firstly, paralogues that have persisted may give functional redundancy in the vertebrate system (as highlighted by the circadian locomotor rhythms of mice mutant for *Clock* and *Npas2* (Debruyne et al., 2006; 2007a)). Secondly, the presence of paralogous genes allows the generation of high levels of specificity: between tissues and between developmental stages. Though CLOCK and NPAS2 have overlapping functions in the nervous system of mice, NPAS2 is unable to maintain rhythmicity in the periphery in the absence of CLOCK (Debruyne et al., 2007b; 2007a). Finally, the expression kinetics and binding partners can vary amongst paralogues, for example the *Period* genes in mice (Zylka et al., 1998), further supporting the idea that multiplicity of genes allows both functional redundancy and spatial or temporal specificity.

1.4 ZEBRAFISH AND THE CIRCADIAN CLOCK

The zebrafish, *Danio rerio*, started its life as a scientific model in the field of developmental biology. Its quick generation time, transparent body and amenability to forward genetic screens make it ideal for studying early events during development, such as gastrulation.

The first instance of zebrafish as a circadian clock model was in 1996, with a description of rhythmic melatonin release from pineal glands in response to light and dark cycles (Cahill, 1996). The use of zebrafish as a model for circadian clock research accelerated when, in 1998, Whitmore and colleagues cloned a zebrafish homologue of *clock* (Whitmore et al., 1998). *Clock* is expressed rhythmically, not only in the brain and pineal gland, but also in peripheral tissues even when cultured outside of the body (Whitmore et al., 1998). Concurrently peripheral oscillators were shown to exist in *Drosophila* and mouse (Plautz et al., 1997; Balsalobre et al., 1998), and very quickly the circadian world moved from a centralised to a decentralised model. This work demonstrated the existence of self-sustaining oscillators in non-neural organs in non-vertebrates and vertebrates alike and, in 2000, the situation in zebrafish was developed to show that the peripheral oscillators in zebrafish are directly entrainable by a light-dark cycle in a cell-autonomous manner, resembling the clocks of *Drosophila* (Whitmore et al., 2000). Thus, zebrafish offered several unique advantages for the study of vertebrate circadian clocks, and has proved a very useful model organism.

More recently, the ability to synchronise a peripheral circadian clock with light alone has provided an extremely useful tool for dissecting the vertebrate circadian clock mechanism and downstream regulation (Tamai et al., 2005; Vatine et al., 2009; Tamai et al., 2012). In addition, zebrafish have been useful for the study of the very early stages of circadian clocks during development. The earliest signs of functional circadian clocks have been the appearance of circadian rhythms of melatonin synthesis and *aanat2* gene expression in the pineal gland on the second day of development and of clock gene rhythms in the whole embryo (Kazimi and Cahill, 1999; Ziv et al., 2005; Dekens and Whitmore, 2008). The insights on how light is perceived by the zebrafish circadian clock, gained from investigation of the development of the light response, will be discussed further in Section 1.5.3.

1.4.1 THE MOLECULAR CLOCK OF ZEBRAFISH

After a genome duplication event during teleost evolutionary history (around 320 million years ago (Meyer and Van de Peer, 2005)), zebrafish (and other teleosts) possess multiple copies of genes compared to mammals, including genes involved in the circadian clock (Postlethwait et al., 1998; Kobayashi et al., 2000; Wang, 2008a). Three *clock*, three *bmal*, six *cryptochrome*, and four *period* genes have been identified in zebrafish (Kobayashi et al., 2000; Wang, 2008b; 2008a; 2009). These genes are suggested to have similar functions in zebrafish to those in the mammalian model. However, the duplication events during teleost evolution have allowed even greater subdivision of function and tissue expression between paralogues beyond that demonstrated in mice.

In particular, the division of function of the cryptochromes is highlighted in zebrafish. As discussed above, cryptochromes are often designated Type I or Type II depending on their role within the circadian clock. *Drosophila* and mice possess Type I and Type II cryptochromes respectively, based on the predominate roles of those genes in the molecular clock: type I are light-responsive photoreceptors and type II are light-irresponsive clock repressors. Whilst this classification system is appropriate for mice and *Drosophila* cryptochromes, the cryptochromes of other animals, such as zebrafish, do not always neatly partition into the two classes. Five of the six zebrafish cryptochromes show repressing activity: c*ry1a*, *cry1b*, *cry2a, cry2b*, and *cry3* (Kobayashi et al., 2000). In contrast, the other zebrafish cryptochrome, *cry4*, does not repress Clock-Bmal activation, and so may be classed as a *Drosophila*-type cryptochrome or Type I cryptochrome. However, the distinction of roles in zebrafish is not quite that clear, especially in the case of *cry1a*. Cry1a in particular has been shown to be a potent repressor of the circadian clock by binding to Clock and Bmal to prevent heterodimerisation and transactivation of downstream targets (Tamai et al., 2007; Chaves et al., 2011b). However, *cry1a* is also strongly induced by light, and seems to be able to mimic the repressive action of constant light on the clock suggesting it has a key role in zebrafish clock entrainment (Pittendrigh,

1993; Tamai et al., 2007). This suggests *cry1a* has a light-sensing role, though there is no evidence that the repressive function of the protein is light dependent (Tamai et al., 2007). Zebrafish *cry1a* fits both Type I and Type II designations and, with the discovery in other organisms (such as butterflies (Zhu et al., 2005)) of cryptochromes with both functions, this classification system may be inappropriate.

Functional diversity is also present amongst the *period* genes. *Per1* and *per3* expression is driven by the circadian clock as indicated by persistence of oscillations after transfer into constant darkness (DD) (Pando et al., 2001; Vallone et al., 2004). In contrast, *per2* expression is acutely induced by light, and oscillations do not persist in constant darkness (Pando et al., 2001; Vatine et al., 2009). Although the mechanism of Per2 is largely unknown, there is some evidence that it, together with Cry1a, regulates the transcription activity of the Clock-Bmal complex by regulating its sub-cellular distribution (Hirayama et al., 2003). Per2 is also important for the light-dependent development of the circadian clock in embryos (Ziv et al., 2005). These results suggest that *per2* is involved in the light input pathway to the clock together with *cry1a*, and recent studies have concentrated on its regulation by light to gain insight into light entrainment of the clock in zebrafish (Vatine et al., 2009).

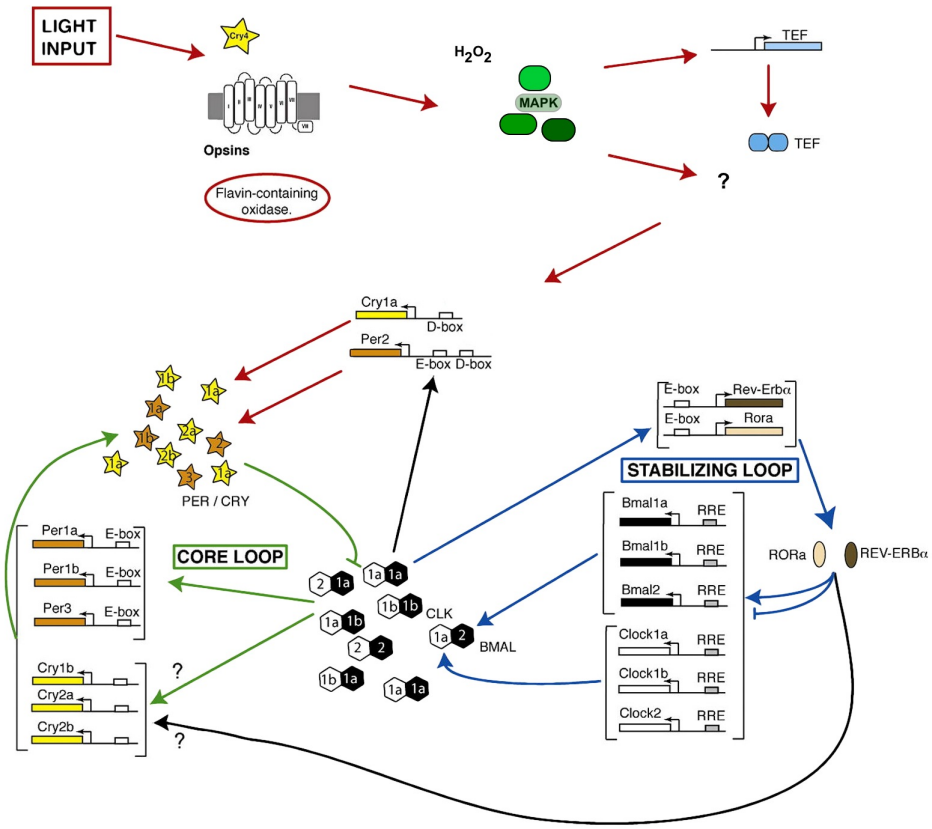

Figure 1.2: Current model of the zebrafish circadian clock.
The multiplicity of clock genes is seen in the "core" and "stabilising" loops. It has been proposed that candidate photoreceptors, opsin, Cry4, and a flavin containing oxidase, signal to the D-boxes of *cry1a* and *per2* via H_2O_2 and MAPK signalling and tef D-box binding (Hirayama et al., 2007; 2009; Vatine et al., 2009; Weger et al., 2011; Mracek et al., 2012). Unknown factors downstream of the photoreceptor are shown by a '?'. The transduction of light to the zebrafish circadian clock is discussed in Section 1.5 (adapted from Vatine et al., 2011). *Reprinted from FEBS Letters 585, Vatine et al., "It's time to swim! Zebrafish and the circadian clock", 1485-1494, copyright (2011), with permission from Elsevier.*

The organisation of the circadian clock in other teleost species is generally thought to reflect that of zebrafish. Clock genes and their expression patterns have been documented for the goldfish (*Carassius auratus*), reef fish (*Siganus guttatus*), sole (*Solea senegalensis*), and others, and are very similar to the expression seen in zebrafish (Park et al., 2007; Velarde et al., 2009; Martín-Robles et al., 2011; 2012).

1.5 LIGHT INPUT TO THE ZEBRAFISH CIRCADIAN CLOCK

1.5.1 DETECTION OF LIGHT BY THE ZEBRAFISH CLOCK

Light is the main environmental cue for the circadian clock. In mammals, light is transduced via the eyes to the SCN. A mouse with no eyes or no intact retina cannot entrain its circadian clock (Foster et al., 1991; Freedman et al., 1999). Instead of the visual photoreceptors, photoresponsive retinal ganglion cells (pRGC) expressing *Melanopsin* are required to entrain the circadian clock of mice (Provencio et al., 2000; Lucas et al., 2001; Hattar et al., 2003).

In contrast, zebrafish possess a highly decentralised circadian clock in which all organs and cells are directly light responsive and can be synchronised by a single light pulse (Whitmore et al., 1998; 2000; Carr and Whitmore, 2005). Therefore, all cells and organs must possess photoreceptive elements that are able to entrain the circadian clock.

In order to demonstrate that a candidate molecule acts as a photopigment rather than a component of the phototransduction cascade, a number of complimentary approaches and their results have been applied for characterisation of the molecule:

- Expression – the candidate must be expressed in the photoresponsive tissue under test.
- Spectral analysis – the absorbance spectrum of the candidate must match the action spectrum of the light-dependent response.
- Functional – the candidate should be able to confer light-sensitivity on an unresponsive tissue or cell in the presence of the necessary signalling pathway components.

These approaches have been applied to zebrafish tissues and cell culture in order to identify the photoreceptive molecule. A number of molecules have been suggested, and candidates include opsins, cryptochromes and flavin-containing oxidases.

Extra-retinal opsins are good candidates for the photoreceptive elements. There are a multitude of opsin genes in the zebrafish, with diverse expression patterns. Though they have only been characterised in the retina, the presence of five melanopsin genes in a non-overlapping expression pattern is a clear example of the diversity of the opsin gene family (Davies et al., 2011). The best candidate for an opsin involved in circadian photoreception is *teleost multiple tissue (tmt) opsin*, which shows a broad expression across the whole zebrafish body and in cell lines (Moutsaki et al., 2003). This opsin possesses the key residues required for photoreceptive function, though to date no functional data has been produced (Moutsaki et al., 2003).

As previously mentioned, the cryptochromes in zebrafish have diverse expression patterns and functions in the circadian clock (Kobayashi et al., 2000). Due in part to the photoreceptive role of the *cry* in the circadian system of *Drosophila* (Stanewsky et al., 1998), and the similarity of zebrafish *cry4* to *Drosophila cry* in sequence, *cry4* has been suggested as a further candidate photoreceptor. Functional evidence for this candidate is also sparse, although an action spectrum on a zebrafish cell line suggested peak sensitivity to blue light wavelengths, which is consistent with a cryptochrome photoreceptor and seems to exclude an opsin photoreceptor (Cermakian et al., 2002). The validity of this data has, however, been frequently questioned. Similar unpublished data in the Whitmore lab shows a broad spectral response, with the induction of light responsive genes being remarkably similar for wavelengths between 400-700nm.

Finally, studies in zebrafish cell lines comparing the induction of *per2* and *cry1a* in light with the induction in response to hydrogen peroxidase and the subsequent block of the induction by light in the presence of over-expressed catalase, lead to the suggestion that a photoresponsive oxidase mediates the light signal (Hirayama et al., 2007; Osaki et al., 2011). Furthermore, the blue light sensitivity shown by Hirayama (2007) is consistent with the involvement of a flavin-containing oxidase, and treatment with DPI, an inhibitor

of the flavin containing enzyme NADPH oxidase, suppresses light induction of *cry1a* and *per2* in zebrafish cells (Cermakian et al., 2002; Hirayama et al., 2007; Osaki et al., 2011).

Unfortunately conclusive evidence for each candidate is thus far absent, and the evidence that has been published is often called into question. None of the suggested candidate molecules has been subject to the full range of characterisation approaches for photoreceptive molecules as presented above. In part, this is due to the difficulty in finding a reliable response to measure the action spectrum, especially in cell lines with their very broad spectral response. It is possible that a combination of these candidates may be involved, at least in cell lines, which has complicated the identification of single photoreceptive molecules.

1.5.2 TRANSDUCTION OF LIGHT

How is the light signal transduced within the zebrafish cell? Similar to the diversity of candidate photoreceptors, it is possible that there are a number of diverse light-signalling pathways. Again, evidence is far from conclusive, but there is some a suggestion that H_2O_2 and the MAPK pathway are involved in the immediate response to light, at least in cell lines (Cermakian et al., 2002; Hirayama et al., 2007; 2009).

H_2O_2 has been suggested as the second messenger for light signalling to the circadian clock based on the similarities between H_2O_2 and light on the induction of *cry1a* and *per2*. This response is abolished when catalase is overexpressed, adding further evidence to H_2O_2's role (Hirayama et al., 2007). Inhibitors of the MAPK pathway, such as U0126 (an inhibitor of MEK), abolish *per2*, *cry1a* and *6-4 photolyase* induction in response to light (Cermakian et al., 2002; Hirayama et al., 2007; 2009), leading to the suggestion that the MAPK pathway acts downstream of H_2O_2 in the photoreception of the circadian clock. However, though the suggestion is made that H_2O_2 can serve to synchronise the circadian clock of Z3 cells upstream of MAPK, no control treatment is presented and so its role in the circadian clock remains unresolved (Hirayama et al., 2007). The light induction of *catalase* (*cat*) is

proposed to regulate the response to H_2O_2 and MAPK, by removing the second messenger (Hirayama et al., 2007). However, light induction of *cat* is not affected by the MEK inhibitor, U0126, unlike both *cry1a* and *per2*, and so must be receiving the light input independently from the mechanism proposed to signal to the circadian clock, raising further questions about the identity of the light input pathway in zebrafish (Cermakian et al., 2002; Hirayama et al., 2007).

Thus, though there is some evidence for the identity of the photoreceptor and the pathways involved in the signalling of light to the circadian clock in zebrafish, definitive evidence has not been produced. In contrast, much more progress has been made in downstream pathways, including the transcriptional control of light-responsive genes (Weger et al., 2011).

Studies have focussed on the expression of the downstream clock genes *cry1a* and *per2*, since they form the early response of the circadian clock in cells and embryos to light and interact with the core clock mechanism through their repressive function (Pando et al., 2001; Hirayama et al., 2003; Ziv et al., 2005; Tamai et al., 2007). How are they transcriptionally regulated by light? Analysis of the *per2* promoter revealed a Light Responsive Module (LRM) containing D and E-box elements that are able to recapitulate the light response of *per2* (Vatine et al., 2009). The key role for D-boxes in light driven expression was revealed through the expression profile of luciferase reporter constructs of artificial multimerised D-box promoters or with deletions of the *per2* promoter (Vatine et al., 2009). This led to the suggestion of D-box binding factors as the transcriptional regulators of the light response. A candidate D-box binding factor is Thyrotroph embryonic factor (Tef), a member of the PAR (proline and acidic amino acid-rich) subfamily of basic region/leucine zipper (bZIP) transcription factors, which mediates the light induced transcriptional activation of *per2* and is transcriptionally upregulated by light (Vatine et al., 2009). Later work revealed a broader role for D-box and *tef1* in the

mediation of light-induced transcription (Gavriouchkina et al., 2010; Weger et al., 2011). These transcriptome screens searched for genes that respond to light and identified many other genes in addition to *per2* and *cry1a* that are transcriptionally upregulated. Many of these genes have E- and D-box elements in their promoters (Weger et al., 2011). Antisense morpholino knock down of *tef1* attenuates the induction of these genes in response to light, and overexpression of *tef1* increases expression even in samples kept in darkness (Vatine et al., 2009; Gavriouchkina et al., 2010; Weger et al., 2011).

However, the regulatory mechanisms revealed for *per2* as investigated by these papers might not be universal. Firstly, the residual light response even after *tef* knock down suggests that *tef* may not be the sole mediator of light signalling (Gavriouchkina et al., 2010; Weger et al., 2011). Secondly, light activation of the AP-1 complex and AP-1 enhancer elements in the *cry1a* promoter mediate at least some light induction of this gene, though AP1 elements were not enriched in the motif search of the broader light-responsive transcriptome, and deletion of the AP1 element in the cry1a promoter in a luciferase reporter has no effect on its response to light (Hirayama et al., 2005; Weger et al., 2011; Mracek et al., 2012). Thirdly, the light induction of *cry1a*, *tef1*, *6-4 phr*, *e4bp4-6* and *lonrf1* show a sensitivity to cyclohexamide treatment, implicating the requirement of protein synthesis, whereas *per2* induction is not sensitive to the same treatment (Hirayama et al., 2005; Mracek et al., 2012). It is likely that a combination of these mechanisms produces the wide-ranging transcriptional induction to light in the zebrafish across all the various light-induced genes or perhaps even within the regulation of a single gene.

The transcriptome screens revealed that light-driven gene expression is present across multiple pathways, gene families and mechanisms, indicating that an extensive aspect of zebrafish biology is influenced by light (Gavriouchkina et al., 2010; Weger et al., 2011). A significant functional cluster of genes that are induced by light are those involved in DNA

repair (Tamai et al., 2004; Weger et al., 2011). This includes the photolyases *6-4 photolyase* (*6-4 phr*) and *CPD photolyase* (*CPD phr*), *damage-specific DNA binding protein 2* (*ddb2*), *xeroderma pigmentosum C* (*xpc*), *x-ray repair cross-complementing protein 1* (*xrcc1*) and *nei endonuclease VIII-like 1* (*neil1*) (Gavriouchkina et al., 2010; Weger et al., 2011). Whereas the photolyases require light for their catalytic actions (Tamai et al., 2004; Brettel and Byrdin, 2010), the latter DNA repair genes belong to different DNA repair pathways, including nucleotide excision repair (NER) and base excision repair (BER), which do not require light for their function.

Interestingly, the link between the regulation of light induction in different gene families has been investigated previously, especially regarding *cry1a* and *6-4 phr*, two closely related genes. Light-induction of *cry1a* and *6-4 phr* is blocked by the same MAPK pathway inhibitor, U1026, and potentiated by the same p38 inhibitor, SB203580, indicating that similar mechanisms direct the expression of these two genes (Hirayama et al., 2009). The evidence for a common regulatory pathway for light-detection by the clock (*cry1a*) and DNA repair (*6-4 phr*) gives the possibility of cross-talk and close links between these two influences on physiology. Finally, light detection early in zebrafish development improves survival when embryos are treated with UV light (Tamai et al., 2004), further signifying the importance of light signalling on broad aspects of the biology of the zebrafish. DNA repair will be discussed in more detail in Section 6. In summary, light can affect physiology indirectly through the circadian clock, but also directly, with one example being the cellular response to DNA damage.

1.5.3 Development of the light input pathway and circadian clock

For the same reasons zebrafish were established as a model for developmental biology, zebrafish have proved amenable to study the development of the circadian clock. It had been suggested that, as in the case for mammals, circadian clock phase and timing is inherited in zebrafish embryos (Reppert and Schwartz, 1983; Delaunay et al., 2000).

However, these reports have not been reproducible in zebrafish. The presence of light or temperature cycles, or even a single transition in light or temperature, is necessary to generate synchronised whole embryo circadian rhythms (Ziv et al., 2005; Vuilleumier et al., 2006; Dekens and Whitmore, 2008).

Without a stimulus the circadian clock oscillates asynchronously within the embryo (Dekens and Whitmore, 2008). Hence, the effect of light on development, how that light is perceived and how the light response develops are fascinating problems in the development of the circadian clock system as a whole. The circadian system of zebrafish is able to respond to light extremely early in its development; a light pulse from 0 to 6 hours post fertilisation (hpf) is able to synchronise *aanat2* rhythms in the pineal gland two days later, and induction of *per2* and *6-4 phr* is seen after a light pulse between 5 and 10 hpf (Tamai et al., 2004; Ziv and Gothilf, 2006). These responses, after the mid-blastula transition but before any anatomical structures have developed, are able to synchronise the clock and further demonstrate the peripheral and decentralised nature of the teleost clock. Clearly transcription is important for the light response and the phototransduction cascade is in place very early. However, the appearance of clock outputs, and thus how the circadian clock becomes coupled to those outputs appears later in development. Circadian rhythms in the cell cycle begin independent of the developmental stage, but require four light-dark cycles, which is also the minimum number of cycles required for the presence of circadian rhythms of locomotor activity (Hurd and Cahill, 2002; Dekens et al., 2003). Interestingly this timing appears to correlate with the onset of rhythmic expression of the transcriptional activators, *clock* and *bmal* (Dekens and Whitmore, 2008). Thus, the establishment of a "complete" clock mechanism may be required for the regulation of the clock outputs, but this is yet to be tested.

Light is clearly a very important external influence on the biology of the zebrafish. Whether this is a characteristic of teleosts in general is, therefore, a very interesting question.

1.6 CAVE ANIMALS

Of particular interest to circadian biologists are organisms that live under unusual lighting conditions: where the cycles of light and dark are highly irregular throughout the year or completely absent. Caves represent one such habitat where light and dark cycles are absent, and are in general more constant and stable environments than the surface (Poulson and White, 1969). Temperature is stable, being approximately equal to the mean annual temperature of the surrounding region, humidity is high as evaporation rates are low, water quality is unchanging with a steady concentration of dissolved inorganic compounds and constant low organic matter content (Poulson and White, 1969). Though the principle aim of this thesis is to investigate circadian clocks in cave animals, the perpetual darkness in caves presents a suite of challenges rising from the absence of light: these include limitations in food (absence of photosynthesis), absence of visual information and challenges for spatial information, and challenges for reproduction (Poulson and White, 1969; White and Culver, 2012). Therefore, organisms that live in extreme environments and constant darkness, like caves, have proved to be very interesting subjects for many other aspects of evolutionary study, as discussed below.

A diverse group of animals from many taxa and geographical locations have adapted and evolved to exploit caves (Jeffery, 2009; White and Culver, 2012). Some species use caves for shelter, but are not restricted to the caves, for example many bats. These species are known as troglophiles. Many species are obligate cave-dwellers, or troglobites, fully removed from the surface environment spending their entire life cycle underground. Remarkably, many of these animals have converged on highly similar phenotypes during adaptation to the cave environment. These phenotypes include degenerate features such

as the reduction or loss of eyes and pigmentation, and constructive features such as increased sensitivity in olfaction and mechanosensation, and longer life spans (Gross, 2012). An advantage to the study of cave animals is that the evolutionary and developmental mechanisms behind these changes can be studied in the context of a known environmental change: the cave environment. Nevertheless, the evolutionary mechanisms behind the regressive phenotypes have puzzled biologists for many years. Charles Darwin, in 'On the Origin of Species', was tentative in his application of natural selection to this particular example of evolution:

> *As it is difficult to imagine that eyes, though useless, are in any way injurious to animals living in darkness, I attribute their loss solely to disuse.*
>
> *(Darwin, 1859)*

In recent years, evidence has accumulated supporting two evolutionary processes for the presence of regressive cave phenotypes (Jeffery, 2009; Wilkens, 2010). The first, natural selection, accounts for the regressive evolution by suggesting the losses are beneficial in some way to the animals. Selection could be acting directly, as for constructive changes, or indirectly due to antagonistic pleiotropy, i.e. the reduction of one trait due to the selection for another, linked trait. The second process is genetic drift, where relaxed selection has allowed the accumulation of mutations that become fixed in a population by genetic drift and lead to the regression and loss of the trait over time. Evidence supporting either process has come from recent advances in genetic and developmental approaches. These approaches, in particular quantitative trait loci (QTL) analysis, have been especially successful in the study of the regression of eyes and pigmentation. In one species of cavefish, *Astyanax mexicanus*, QTL studies suggest that selection is responsible for the regression of eyes, as cave alleles at all 'eye' trait loci are negative and result in smaller eyes (Protas et al., 2007). This result is in contrast to the prediction made by neutral mutation theory which would predict both positive and negative trait values with cave alleles giving eye sizes both larger and smaller than the parent fish. QTL analysis predicts

a neutral mutation mechanism for the regression of pigmentation, as cave alleles are associated with both an increase and decrease in pigmentation (Protas et al., 2007; Jeffery, 2008). Despite this evidence, there is still debate about whether regressive evolution in these cases is produced by selection or drift (Jeffery, 2010; for example, Wilkens, 2010). The evolutionary processes leading to the changes in other traits such as body size, metabolism, feeding behaviour and the circadian clock are less well studied.

1.6.1 CLOCKS IN CAVE ANIMALS AND THE ADAPTIVE VALUE OF CLOCKS

The endogenous circadian clock can provide temporal organisation in two ways:

1. Synchrony of the internal with the external world through synchronisation to external cycles such as light and dark, and;
2. Provision of temporal order within an organism.

These two levels of organisation both provide pressures for the evolution of endogenous clocks (Pittendrigh, 1993). Despite the ubiquitous nature of circadian clocks implying that they provide a fitness advantage to the organism, only a few studies have been performed that provide evidence for the adaptive value of the clock. Important examples are life-span studies in SCN-lesioned chipmunks (DeCoursey et al., 2000), and in *Arabidopsis* plants fitness studies (Green et al., 2002) and competition studies between different clock mutants on matched external light and dark cycles (Dodd et al., 2005), which show that possession of a clock is advantageous compared to the clock-less organisms. Investigating the circadian clocks of animals that live in aperiodic environments may obtain further evidence for the advantage of circadian clocks, especially with regard to the provision of internal temporal order. While the polar regions are one such environment that lacks regular 24-hour rhythmicity during parts of the year, perhaps more extreme environments are the perpetual darkness of the deep sea and caves.

It is suggested that animals that exist in an aperiodic environment gain no selective advantage from a functional circadian clock and so it is a likely target for regression as in

the case of eyes and pigmentation (Sharma, 2003). Circadian clock studies in these animals are therefore of great importance to address this hypothesis. Unfortunately, the empirical evidence for this standpoint is relatively sparse: only a few studies have examined circadian clocks in cave animals, and most have examined activity or locomotion Table 1.1. Examples include studies on cave crayfish (Brown, 1961; Jegla and Poulson, 1968); cave amphipods (Blume et al., 1962); cave crickets (Reichle et al., 1965); cave millipedes (Mead and Gilhodes, 1974; Koilraj et al., 2000); cave beetles (Lamprecht and Weber, 1978); cave fish (Erckens and Martin, 1982b; Pati, 2001); and cave salamanders (Hervant et al., 2001). Only a few have investigated the molecular nature of the clock (Cavallari et al., 2011), and none have investigated the molecular clock in the wild cave environment.

Table 1.1: Examples of studies on the locomotor activity of cave animals

Cave animal	*Species*	*Study*
Crayfish	*Orconectes pellucidus*	(Brown, 1961; Jegla and Poulson, 1968)
Amphipod	*Niphargus puteanus*	(Blume et al., 1962)
Cricket	*Hadenoecus subterraneus*	(Reichle et al., 1965)
Millipede	*Glyphiulus cavernicolus*	(Mead and Gilhodes, 1974; Koilraj et al., 2000)
Beetle	*Aphaenops cerberus; Geotrechus orpheus; Speonomus diecki*	(Lamprecht and Weber, 1978)
Salamander	*Proteus anguinus*	(Hervant et al., 2001)
Fish	*Nemacheilus evezardi*	(Pati, 2001)
	Astyanax mexicanus	(Erckens and Martin, 1982b; Zafar and Morgan, 1992)

These few studies reveal an array of circadian phenotypes in cave-adapted animals. Some animals can retain circadian rhythms, some show highly variable rhythms within populations, with some members having circadian periodicity only occasionally and others having non-circadian periods, and some animals show a complete absence of circadian rhythms. A full comparison of the published work is difficult due to differences in experimental conditions and animal maintenance (for example, whether the animals were entrained to LD cycles before the experiment, the use of LD cycles of various

photoperiods, and experiments under different length days (T-cycles)). However, circadian rhythms of activity and metabolism can be seen in DD for cave crayfish, *Orconectes pellucidus*, in some individuals (Brown, 1961; Jegla and Poulson, 1968), and entrainment of circadian activity rhythms to 12hr:12hr LD cycles with persistence, albeit very weakly, in DD, is seen in the cave loach *Nemacheilus evezardi* (Pati, 2001) and *Astyanax mexicanus* (Erckens and Martin, 1982b). Circadian rhythms of activity are absent or irregular in cave amphipods (Blume et al., 1962), cave millipedes (Mead and Gilhodes, 1974; Koilraj et al., 2000), cave beetles (Lamprecht and Weber, 1978), and the cave-dwelling salamander *Proteus anguinus* (Hervant et al., 2001).

Though evidence suggests that the cave crayfish is able to show circadian rhythms of activity, interestingly it was first thought not to, and only upon re-analysis of the data were rhythms apparent (Park et al., 1941; Brown, 1961). In another example of analysis method being crucial to these data sets, periodogram analysis of raw activity data in cave millipedes suggested a rudiment of a circadian rhythm, even though these rhythms were not apparent in the raw data (Mead and Gilhodes, 1974; Koilraj et al., 2000). Therefore, the persistence or absence of circadian rhythms in troglobites is not clear and is highly subjective to experimental technique and subsequent analysis. Activity data is sensitive to the type of analysis performed and perhaps a clearer idea of the presence of circadian rhythms and a functional circadian clock in troglobites can be gained from an analysis of their molecular circadian clocks. Recent work using quantitative PCR in the Somalian cavefish, *Phreatichtys andruzzii*, show the absence of molecular clock rhythms after LD cycles, even though these fish still possess photophobic responses (Ercolini and Berti, 1975; Cavallari et al., 2011; Tarttelin et al., 2012). Molecular studies on cave animals should produce very interesting data.

1.6.2 *ASTYANAX* CAVEFISH AS A MODEL SYSTEM

The Mexican tetra, *Astyanax mexicanus,* is a unique and developing model organism that is well suited for studying adaptation to cave life, including the study of troglobite circadian clocks (Jeffery, 2001; 2008). *Astyanax mexicanus* offers many features that set it apart as a cave animal suitable for laboratory study. Firstly, unlike many cave animals, they can be maintained and bred within the laboratory, with a generation time of four to six months (Yamamoto, 2004). Secondly, surface and several cave dwelling forms exist within the same species, a feature that sets *Astyanax* apart from many studied cave animals and gives it great power for evolutionary study (Jeffery, 2009). Despite a divergence time between the forms of the order of millions of years, these forms are still inter-fertile, which allows crossing and genetic complementation analysis (Sadoglu, 1957; Strecker et al., 2004; Protas et al., 2006). Thirdly, like all cave animals, the direction of evolutionary change is known with certainty; cavefish evolved from surface fish-like ancestors and thus development of a phenotype can subsequently be analysed in the context of a specific environmental condition (Jeffery, 2001). Finally, there are currently 30 known caves in north-eastern Mexico holding several forms of *Astyanax* cavefish, which allows comparison between caves and discovery of possible convergent evolution (Mitchell et al., 1977; Jeffery, 2009). The region where *Astyanax* are found and examples of the different cavefish are shown in Figure 1.3.

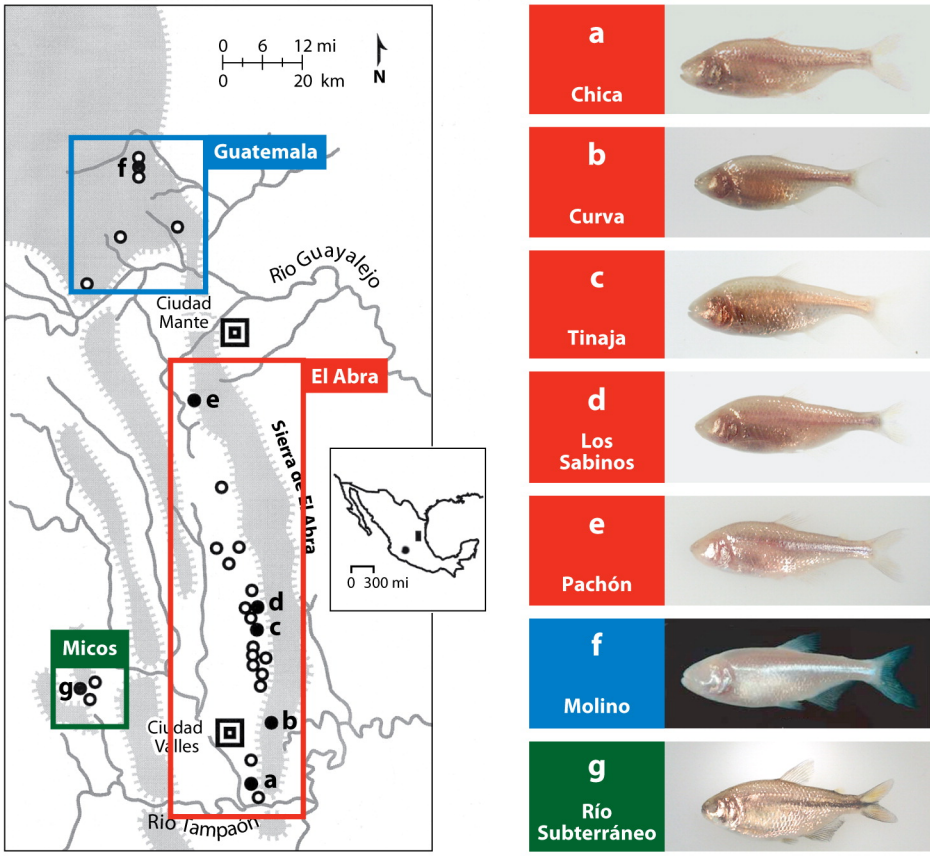

Figure 1.3: Map of the El Abra region of Mexico showing the locations of 29 of the known caves with *Astyanax* populations.
(a-g) examples of different cavefish, named according to the cave in which they are found. Taken from Jeffery (2009). *Republished with permission of Annual Reviews, from "Regressive Evolution in* Astyanax *cavefish", Jeffery, W., 43: 25-47, copyright 2009; permission conveyed through Copyright Clearance Center, Inc.*

Whilst the relationships between populations of *Astyanax* cavefish are still the subject of much debate, there is evidence for independent colonisation of multiple caves and therefore parallel evolution, or convergence, of cave related phenotypes. Genetic and biogeographic evidence suggests that the cavefish populations have evolved independently from at least two to five or more separate invasions of caves by surface fish (Dowling et al., 2002; Strecker et al., 2003; 2004; Borowsky, 2008; Bradic et al., 2012). Other cavefish populations may have become established later during the colonisation of the cave environment as founder cavefish dispersed through closely connected cave systems. Original estimates for the colonisation time of caves by surface fish are as recent

as 10,000 years ago, though modern estimates using the phylogenetic molecular clock are of the order of 700,000 to 3 million years ago (Mitchell et al., 1977; Strecker et al., 2004; Ornelas-García et al., 2008). Exact relationships within *Astyanax* cavefish are complicated by contrasting data from genomic and mitochondrial phylogenetic studies. Recent studies based on microsatellite loci and the subsequent clustering of genotypes have suggested three independent origins for cavefish in the El Abra region: 1) Chica cavefish, 2) Pachón cavefish, and 3) cavefish from the central El Abra region (including Tinaja and Curva cavefish) (Bradic et al., 2012). Results based on mitochondrial DNA suggest a different relationship, with some caves showing closer relationship to the surrounding river fish than neighbouring caves, suggesting a more recent origin (Strecker et al., 2003; Hausdorf et al., 2011). This discrepancy is especially noted with regard to Chica cavefish, as there is the possibility of introgression with surface fish, as the cave is connected to the nearby River Tampaón (Breder, 1942; Mitchell et al., 1977; Strecker et al., 2003).

A number of studies have investigated the phenotypic differences between the two forms; some of the characteristics are listed in Table 1.2. Genetic complementation tests between surface and cavefish forms and candidate gene analyses linking to significant QTL have been valuable in identifying the genetic basis of some of these characteristics. These studies have revealed that, in addition to phenotypic convergence in eye and pigment reduction (see Figure 1.3a-g), *Astyanax* cavefish show convergence at the molecular level, as different cavefish populations often carry mutations in the same genes (Protas et al., 2006; Borowsky, 2008; Gross et al., 2009).

Table 1.2: Phenotypic changes in *Astyanax* cavefish
(adapted from Jeffery, 2008).

Regressive changes	*Constructive changes*	*Other changes*
Eyes	Jaw size	Craniofacial skeleton
Visual centres of the brain (e.g. optic tectum)	Tooth number (on maxillary bone)	Brain shape and length
Visual behaviours (e.g. schooling, aggression, fright)	Taste bud number and location	Body shape and length
Pigment cells	Solitary chemosensory cell number and location	Bottom feeding posture
Melanin biosynthesis	Nostril size	Scale size and shape
Number of thoracic vertebrae and ribs	Olfactory bulb size	
Number of anal fin rays	Hypothalamus size	
Sclera ossification	Cranial neuromast size	
	Fat content	
	Food sampling behaviour	

Albinism and pigment reduction are two characteristics that evolved independently through different changes in the same genes: Pachón and Molino cavefish both populations carry mutations in *ocular and cutaneous albinism 2* (*oca2*) (Protas et al., 2006), and Pachón, Yerbaniz and Japonés cavefish carry mutations in *melanocortin type 1 receptor* (*mc1r*) (though albinism is epistatic to the brown phenotype) (Gross et al., 2009). The evolution of the same phenotype in multiple populations by alterations in the same gene is a relatively common phenomenon: a famous example is the regulatory mutation of *pitx1* resulting in the reduction of pelvic spines in sticklebacks (Shapiro et al., 2004). Whether these common genetic alterations are due to selection, or a consequence of genomic size or position (e.g. *oca2*) is an increasing area of research (Chan et al., 2010).

By contrast, eye regression has only been studied extensively in Pachón cavefish, and has not fully exploited the independent origins of cavefish populations. However, the evidence generated from such studies is beginning to support one of the mechanisms by which regressive evolution may occur: pleiotropy. Pachón cavefish show an expansion in midline *shh* expression during development and proapoptotic signals from the cavefish lens (Yamamoto and Jeffery, 2000; Hooven et al., 2004; Yamamoto et al., 2004; Strickler et al., 2007). Interestingly, the expansion of *shh* expression during development not only leads

to eye loss but also an increase in taste bud number (Yamamoto et al., 2009). Yamamoto and colleagues suggest that the pleiotropic function of Shh causes eye loss at the same time as increasing constructive jaw traits. Vision is rescued in some of the F1 hybrid progeny of different cave populations (Tinaja, Molino and Pachón), indicating complementation can occur between the different genes and mechanisms responsible for eye loss in the different cavefish (Borowsky, 2008).

These studies show the power of *Astyanax* cavefish as a model for the study of regressive and convergent phenotypes. Despite complexities in the exact evolutionary history, *Astyanax mexicanus* offers several advantages over alternative models for the study of evolution in the cave environment. *Astyanax mexicanus* is therefore an excellent model for the study of circadian rhythms in the cave.

1.7 THESIS AIMS

This study aims to investigate the circadian clock in the cave using *Astyanax mexicanus* as a model by addressing the following questions:

1. Do cave populations of *Astyanax* possess a molecular circadian clock?
2. To what extent is this circadian clock expressed in the wild?
3. Is the circadian clock expressed early in development in cavefish?
4. What other aspects of light-responsive biology have altered during evolution in the dark?

2 MATERIALS AND METHODS

2.1 BIOLOGICAL MATERIALS

2.1.1 ANIMAL HUSBANDRY

Cave populations of *Astyanax mexicanus* were originally captured in the 1990s from several caves in North East Mexico (Figure 1.3) by a team of cavers and scientists led by Dr William Jeffery (University of Maryland, USA). Pachón fish originated from the Cueva El Pachón, near Ciudad Mante, Mexico and Chica and Tinaja cavefish from Cueva El Chica and Cueva de la Tinaja respectively, near Ciudad Valles, Mexico (Jeffery and Martasian, 1998). River populations (surface fish) were collected from springs in Balmorhea State Park, Texas, USA and streams near Tamaulipas and San Luis Potosi, Mexico (Jeffery and Martasian, 1998). Fish were initially bred in the laboratory of Dr Jeffery at the University of Maryland and then some of these fish were transferred in 2004 to the laboratory of Dr Yoshiyuki Yamamoto at University College London. Fish were raised in 45 litre tanks at 22±1°C (pH 7, conductivity 500 μS), in which the water was passed through carbon and physical filters. Between 8 and 12 fish were kept in each tank. In the laboratory, all fish were kept under light-dark (LD) cycles of 14 hours of light and 10 hours of darkness, and fed flake food once per day during the light phase.

2.1.2 EMBRYO COLLECTION

Spawning behaviour was induced by raising the temperature of the water to 24.5°C in the preceding dark phase. Fish were fed as normal in the light, and embryo trays were placed in the bottom of the tanks 1-2 hours after feeding. Spawning behaviour began in the next dark phase (approximately 1 hour after lights off for surface fish, and 3-8 hours after lights off for cavefish). In order to obtain embryos on consecutive days, the water temperature was increased a further 0.5°C in the next light phase. Fertilised embryos were collected from the trays and sorted under a dissecting microscope. To raise embryos to adulthood, they were cleaned and sorted into 10 cm petri dishes containing E2 medium (15 mM NaCl, 0.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.15 mM KH_2PO_4, 0.05 mM Na_2HPO_4, 0.7 mM

$NaHCO_3$) and placed in an incubator at 24.5±1°C. Embryos hatched at 24 hpf (hours post fertilisation), and were fed brine shrimp twice a day from 5 dpf (days post fertilisation). Embryo development in *Astyanax* appears very similar to zebrafish at the early stages. It is therefore expected that the midblastula transition (MBT) in *Astyanax* occurs at 3 hpf as in zebrafish (Kane and Kimmel, 1993). Since zygotic transcription starts at MBT, only embryos collected at less than 2 hpf were used in experiments. 12-20 embryos were sorted into pre-washed 25 cm^3 tissue culture flasks (Greiner) containing 20 ml E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 0.00001% Methylene Blue) and placed in thermostatically controlled water baths at 25°C.

Hybrid fish of surface and Pachón (surface x Pachón) and Pachón and Chica (Pachón x Chica) were generated by *in vitro* fertilisation. Under normal spawning conditions, eggs were taken from Pachón cavefish and fertilised using sperm from surface fish or Chica cavefish. These embryos were then raised to adulthood or used for experiments as described in Section 2.3.2.

2.1.3 ASTYANAX CELL LINES

Cell lines from surface, Pachón and Chica fish were generated by Dr David Whitmore from 24 hpf embryos as described (Whitmore et al., 2000). Cells were cultured in 25 cm^3 flasks (Greiner) in L15 medium (Gibco) supplemented with 15% Fetal Calf Serum (FCS, Biochrom AG), penicillin (100 U/ml), streptomycin (100 µg/ml) and 0.1% gentamycin (50 µg/ml, Gibco). Cells were maintained in a dry incubator (atmospheric CO_2, non-humidified) at 28±0.5°C, whilst cell experiments were performed at either 28°C or 25°C in thermostatically controlled water baths.

2.1.4 TRANSFECTION OF CELLS

Surface and Pachón cells were grown to 80% confluence. These cells were trypsinised (0.05% solution with 0.5 mM EDTA), resuspended in media, washed with PBS and then resuspended to a final concentration of 10,000,000 cells/ml in Resuspension (R) buffer for

electroporation using the Neon Transfection System (Invitrogen). 10 µl of the cell suspension was transfected with 0.5 µg each of the linearised plasmids, pcDNA3.1, which contains the neomycin resistance gene, and a zebrafish *per1b*-luciferase reporter construct (Vallone et al., 2004). Transfected cells were grown to confluence before selection with neomycin (0.25 µg/ml).

After neomycin selection, transfected cells were sorted by FACS (fluorescence-activated cell sorting) into single wells of a 96-well plate. Clones were tested for expression of the luminescent reporter construct using a Packard TopCount (Perkin Elmer Inc.) by removing the culture medium, replacing it with fresh media supplemented with 0.5 mM luciferin and sealing the plate with a plastic TopSeal (PerkinElmer Inc.). Bioluminescence was measured on a Packard TopCount luminometer (PerkinElmer Inc.). The experimental plate was placed into a temperature-controlled TopCount stacker (at 28°C) along with translucent stacker plates, which allow maximum light from LEDs to reach the experimental plate. Each well was counted approximately every hour. The bioluminescence was recorded as counts per second (CPS) by the TopCount luminometer, and the data was processed using the Import and Analysis macro in Microsoft Excel. The brightest clones showing rhythmic luminescence similar to the endogenous *per1b* gene were kept for experimentation. Unfortunately, no Pachón clones showed high levels of bioluminescence, so only surface cells were analysed.

2.2 FIELD STUDIES

Field studies were performed primarily by Drs David Whitmore, Christophe Guibal, Yoshiyuki Yamamoto and Victor Reynoso between 2007 and 2010. Field trips were conducted annually in February/March. In the Micos River (+22° 6' 59.01" N, -99° 10' 16.8" W), adult fish were captured using baited fish traps and nets 2-3 days prior to the sampling period. The fish were then placed in large, perforated tubs, open to natural water flow and allowed to float within the main stream of the river. An individual tub was set up

for each time point to reduce any possible disturbance to other animals during the experiment. Night-time samples were collected using head torches with dim red light. Caudal fin clips of four individual fish were taken at six time points per day over two days, with each fin placed in a separate tube of TRIzol Reagent (Invitrogen). Samples were homogenised, immediately placed on ice and later stored at -20˚C.

Cave samples were collected using a similar approach, with individual open tubs being floated within the cave ponds. Animals were collected (typically 40 fish or more) using dim red light, and sampling was performed in a location away from the main body of fish to reduce any possible disturbance. Caudal fin clips of four individual fish were taken at four time points per day over two days, with each fin placed in a separate tube of TRIzol Reagent (Invitrogen). All clipped animals were kept in a separate container away from the experimental individuals and were not released back into the main body of water until the end of the study. Water quality was monitored using a Multi-Parameter TROLL 9500 probe (In-Situ Inc.) suspended in the water column next to the collected fish. This device provided continuous measurements of water temperature, barometric pressure, pH, dissolved oxygen and nitrates. Bat activity was monitored by video cameras placed within the cave tunnel. Bat activity was filmed over a 24 hour period, starting at 4 pm (16:00) on the first day, and the number of bats crossing the field of view was counted and binned into 1 hour windows.

2.3 LABORATORY EXPERIMENTS

2.3.1 ADULT FISH

Fish were maintained in a 12hr:12hr LD cycle for 7 days before transfer into constant darkness prior to sampling. Fish were not fed during this time. Samples of the caudal fin of fish were taken at 6-hourly intervals, with each fin placed in a separate 1.5 ml tube of TRIzol Reagent (Invitrogen). Fins were homogenised in TRIzol and stored at -20°C until processing.

To measure the effect of acute light pulses on clock gene expression, adult fish were maintained on a LD cycle as above, but given a 3 hour light pulse at ZT16 on day 7, or kept in darkness as a control. A 1 hour light pulse at CT16 has been shown to elicit large inductions of *per2* and *cry1a* in zebrafish cell lines (Tamai et al., 2007). Caudal fin samples were collected at ZT19 (at the end of the light pulse), homogenised in TRIzol (Invitrogen) and stored until processing as above.

2.3.2 EMBRYO EXPERIMENTS

Flasks of 12-20 embryos were kept in thermostatically controlled water baths at 25°C and subjected to 12hr:12hr LD cycles from the point of fertilization. Single flasks of embryos were collected at 6-hourly intervals, with malformed or dead embryos removed. Embryos were then placed directly into a separate 1.5 ml tube, homogenised in TRIzol Reagent (Invitrogen) and stored at -20°C until processing.

To measure the effect of light pulses at different stages of development, and for the expression of the clock genes when embryos are raised in constant darkness, fertilised embryos of less than 2 hpf were sorted into flasks of 12-20 embryos under a dissecting microscope as above. Embryos older than 2 hpf were discarded to eliminate any transcriptional induction by light in these experiments. Flasks were wrapped in foil as an added precaution against light, and placed into thermostatically controlled water baths at 25°C in constant darkness. To examine rhythmic expression of clock genes, single flasks of embryos were collected at 6-hourly intervals, removing dead embryos, and homogenised in TRIzol (Invitrogen) as above. To examine acute light induction at different developmental stages, one flask of a pair was removed from the water bath, unwrapped and replaced in a water bath exposed to light. After three hours, the light-exposed and dark control flasks were collected, and embryos were sorted and homogenised in TRIzol (Invitrogen).

2.3.3 CELL LINES

Cells were seeded onto a 6-well plate (Greiner) at a concentration appropriate for them to reach >90% confluence after 7 days of culture (75,000 cells/ml for surface fish cells, and 50,000 cells/ml for Pachón and Chica fish cells). Plates were put into clear plastic boxes and submerged in thermostatically controlled water baths at either 25°C or 28°C. Cells were subjected to 12hr:12hr LD cycles for 7 days before transfer into constant darkness. Samples were taken at 6-hourly intervals on day 6 in LD and day 7 and 8 in DD. Alternatively, cells were subject to 12hr:12hr LD cycles for 6 days and given a 3 hour light pulse at ZT16 on the sixth day or kept in darkness as a control. Samples were taken at the end of the light pulse, or after 3 hours in the dark.

At each timepoint, a single 6-well plate was removed from the water bath. The media was removed from the wells, and cells were washed once with cold PBS, which was then aspirated. 0.5ml of TRIzol Reagent (Invitrogen) was added to each well, and a sterile cell scraper was used to assist in the removal and breakup of adherent cells. A single 1.5ml tube was used to collect the cells from 2 wells, and stored at -20°C until processing.

2.4 RNA EXTRACTION AND CDNA SYNTHESIS

Frozen samples in TRIzol Reagent (Invitrogen) were thawed and vortexed before 200 µl chloroform was added. The samples were vortexed, incubated at room temperature for 2 minutes and centrifuged at 13,200 rpm at 4°C for 15 minutes. The aqueous phase was extracted into new 1.5 ml tubes and 500 µl of isopropanol was added. The samples were vortexed and incubated at -20°C for 1 hour (for cell lines) or overnight (for fin tissue or embryos). The samples were then centrifuged at 13,200 rpm at 4°C for 20 minutes to collect the precipitated RNA. The RNA was washed with 75% EtOH and spun for another 15 minutes at 13,200 rpm at 4°C. The EtOH was removed, and the samples were spun a final time to remove the final ethanol residue. The samples were air dried and

resuspended in DEPC H_2O. RNA concentration was determined using a NanoDrop 1000 or 2000 Spectrophotometer (Themo Scientific), and samples were stored at -20°C.

2 µg of RNA was used for cDNA synthesis using SuperScript II Reverse Transcriptase with random hexamer and oligo dT primers (Invitrogen). PCR tubes containing RNA, H_2O, primers and dNTPs were first heated to 65°C before a mastermix containing DTT, H_2O, First Strand cDNA synthesis buffer, SuperScript II Reverse Transcriptase and RNAseOUT (Invitrogen) was added. Reverse transcription (RT) conditions were as follows: 65°C, 5 min; held at 4°C before adding the mastermix; 25°C, 10 min; 42°C, 15 min; 70°C, 60 min.

2.5 CLONING *ASTYANAX* GENES

A degenerate RT-PCR approach was taken to initially isolate fragments of clock genes from *Astyanax mexicanus*. PCR primers were designed by aligning gene sequence from several different teleost species (Table 1.1). cDNA was synthesised from RNA obtained from *Astyanax* fin or cell line using SuperScript II Reverse Transcriptase (Invitrogen) with random hexamer and oligo dT primers. PCR products of the appropriate size were obtained using Advantage II Polymerase (Clontech), and subcloned into pGEM-T Easy (Promega).

Plasmids containing the partial gene sequences were transformed into chemically competent *E.coli* bacteria by heat shock. The transformed bacteria were plated on plates containing LB agar medium and 100 µg/ml ampicillin and incubated at 37°C overnight. 3 ml aliquots of LB medium with 100 µg/ml were inoculated with single colonies and incubated at 37°C overnight whilst shaking. Plasmid DNA was extracted using the GeneJET Plasmid Miniprep kit (Fermentas). Plasmid DNA quantity and quality was measured using a NanoDrop 1000 or 2000 Spectrophotometer (Themo Scientific).

The DNA fragments contained in the plasmids were sequenced using BigDye Terminator v1.1 (Applied Biosystems) with T7 and SP6 primers and their identity determined by the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/) and phylogenetic analysis.

The partial gene sequences were then used to design primers for rapid amplification of cDNA ends (RACE) to obtain full-length cDNAs. 5' and 3' RACE libraries were constructed by Dr David Whitmore using poly A^+-purified RNA from Astyanax embryonic cell lines (created as described above) and the SMART RACE cDNA Amplification Kit (Clontech). 5' and 3' RACE PCR products were amplified with Advantage II Polymerase and cloned and sequenced as above. These RACE sequences were assembled with the initial RT-PCR products using CodonCode Aligner (CodonCode corporation), and primers were designed to regions outside the predicted start and stop codons to obtain full length coding regions (for *per1*, *cry1a* and *tmt1*). These full-length coding regions were obtained by RT-PCR from cell lines of the different Astyanax populations; the fragments obtained were cloned and sequenced as described above. At least three different clones for each gene were sequenced. All sequence reads for each gene were assembled in CodonCode Aligner (CodonCode corporation) and the consensus of the three or more clones was taken as the true gene sequence. Alignments of predicted amino acid sequences from surface and cavefish populations were created using the ClustalW algorithm in MegAlign (DNASTAR). By this approach we obtained full-length sequences for 3 genes, *per1*, *cry1a* and *tmt1*, and partial sequences for *clk1*, *per2a*, *per2b*, *tef1*, *CPD phr* and *ddb2*.

Table 2.1: Primer sequences

Type of PCR	*Gene*	*Code*	*Sequence*
RT-PCR	*per1*	222-WPER1 5’	5’-GGAGACCACTGAGAGCAGCAAGAG-3’
		223-WPER1 3:	5’-CCACTGCTGGTAATATTCCTG-3’
	cry1a	306-MedCry1a5’	5’-CCCAGGCTTTTCAAGGAATGGAAC-3’
		307-MedCry1a3’	5’-GGTTCCCTTCCATTTTGTCAAAGC-3’
	per2	283-MedPer2F	5’-CAGTGTGTTCTACAGCTTCACCAC-3’
		284-MedPer2R	5’-AAGCTGGACCAGCTGGTGTC-3’
	clk1	1569-teleo clock1 fw1	5’-TGACAGCAGTATCWTTGATGGGTTAATGG-3’
		1572-Am clock1 rev1	5’-TAGTGTGTCTGAAGCCAAATCCACTGCT-3’
	tef1	1540-teleo tef1 fw1	5’-GAGTACATGGAYCTGGAGGAGTTYCTGATG-3’
		1542-teleo tef1 rev2	5’-GAGCGCTGAGTTTTCTCKCTCCAGRAAT-3’
	ddb2	1559-teleo ddb2 fw1	5’-TTTATTGGGGGDATGAAGTTCTGCCC-3’
		1557-teleo ddb2 rev1	5’-GGGTAACGGCCAGCCACAATGAGGTC-3’
	CPD phr	895-Zf CPD 5’1	5’-TTCAGGTTGATGCACATAATGTGG-3’
		898-Zf CPD 3’1	5’-AAAGATGGGTCGCTCTGCCCAGCC-3’
	tmt opsin 1	1086-TMT 1F1	5’-TAGTGCTCGTGYTKTTYTGYAARTTYAAGA-3’
		1087-TMT 1R2	5’-SMGGAAACACYTGTARAACTGYTTGTTCAT-3’
RACE-PCR	*per1*	851-Pach.RACE Per1 A	5’-GCATATAGTCTGCTGAGCGGCAGC-3’
		852-Pach.RACE Per1 B	5’-GCCGAAGATCCGGGTTCCTGCAG-3’
		853-Pach.RACE Per1 C	5’-CTTGTTTAACACAGTTAAGGGCG-3’
		854-Pach.RACE Per1 D	5’-CCTGCTCGGAGCTGCCGCTCAGC-3’
	cry1a	878-Asty Cry1a RACE2	5’-CGAATGGCTCGGAGTCGTACTCAT-3’
		879-Asty Cry1a RACE1	5’-CCATTGAGCTCTATGATCTTGTCC-3’
		880-Asty Cry1a RACE3	5’-GTTCGGCTGCCTCTCGTGTCAACT-3’
		881-Asty Cry1a RACE4	5’-TACGGGCAGCTGCTGTGGCGAGAG-3’
	per2	1388-Am per2y 5’ 2	5’-CCACCACTGATTCGGCAAAAGAAGG-3’
		1389-Am per2y 5’ 3	5’-TGCAGACCACCATCTTTCTCCTTACCA-3’
	CPD phr	1102-CPD 3’RACE U	5’-ATGCTGGTCAGCTGTCCGCT-3’
		1103-CPD 3’RACE N	5’-TGTACTGGGCCAAAAAGATTCTGG-3’
	ddb2	1588-Am ddb2 3’race1	5’-ACCACAGACCAAATGAATGAGATCAGGATTTA-3’
		1589-Am ddb2 3’race2	5’-AAGTGATTGGTCTAAGCCAGCTCAGGTTATTG-3’
	tmt opsin 1	1118-asty TMT race1n	5’-TACGATTCCGAAGCAGGAGT-3’
		1119-asty TMT race2	5’-GTCCGGGGCCTGTTTATTAT-3’
		1120-asty TMT race3	5’-ACCAGCTGTTCCGTTACCTG-3’
		1121-asty TMT race4n	5’-GTGCTATCTGGTGTGCTGGA-3’
Coding	*per1*	1077-AstyPer1-5’UTR	5’-ATCGCTGTGGAGCTGTTTTCATTT-3’
		1078-AstyPer1-3’UTR	5’-CAGAGCTCATTCCCATATAAAGGC-3’
	per2a	1306-asty Per2x 13	5’-GGAAAAGAGGAACAAGGGCAGCAAG-3’
		1307-asty Per2x 14	5’-CACTGGCTGGTTATGGACACCACAC-3’
	per2b	1575-Am per2y fw2	5’-GGTGTCTCTGATCACGGGTAAAATCGTGTA-3’
		1576-Am per2y rev2	5’-GTCTATCGTTGGGGTGAAGGTGGAGAAG-3’
	cry1a	1092-cry1a_sur_start	5’-GAACGAGATCCCTCAAAGCA-3’
		1093-cry1a_sur_stop	5’-TCAATGGGTCTATGACTACATGAAA-3’
	tef1	1555-Am Tef1 5’1	5’-ATCTGAAGAAGCCCAGGTAAAAGAGCTGAC-3’
		1556-Am Tef1 3’1	5’-CGTGAACGTTTAGCTGCAATATTGTTCTTC-3’
	CPD phr	1108-AstyCPD5’qPCR2	5’-GGCCTCTCCTAAGCTGGAGT-3’
		1626-Am cpd rev2	5’-GGACCTGAGATGAATCTTCTGGAAATAGAA-3’
	tmt opsin 1	1129-su TMT1 start1	5’-TCGTTCAGAGGGATCGTACC-3’
		1132-su TMT1 stop2	5’-GACGCCCATGAATGACTTCT-3’

2.6 Quantitative PCR

2.6.1 Assay

Quantitative PCR (qPCR) was used to measure gene expression by a SYBR® Green-based assay using an Eppendorf Mastercycler® ep *realplex²* Thermal Cycler.

2.6.2 Primer design and verification

Specific primers for qPCR were designed to *Astyanax* gene sequences using Primer3 Plus (Untergasser et al., 2007). Primers were selected based on their length (20 bp), melting temperature (~60°C), self-complementarity (within a primer and between primer pairs) and 3' self-complementarity. Primers were synthesised by MWG Eurofins Operon (Germany) or Sigma Aldrich (UK). Sequences of primers used are found in Table 1.2.

The efficiency of primer pairs was determined using the following protocol: six 5-fold serial dilutions of cDNA from *Astyanax* cell lines and a H_2O negative control were used as the templates for qPCR using SYBR® Green JumpStart™ *Taq* Readymix™ (Sigma) and each primer at a concentration of 500 nM. Each dilution of cDNA was analysed in triplicate. The efficiency of each primer pair was calculated from a plot of Ct value for each dilution against the log of the dilution. The efficiency was calculated from the linear regression as:

$$= 10^{\left(\frac{-1}{slope}\right)} - 1$$

Ct values at very low or very high dilutions were excluded if the correlation coefficient was <0.985. If this was the case, the primer pair had a narrower working range than if the points fit the regression line at all dilutions. Primer pairs with efficiencies of 90-105% within the working range of dilutions were used in gene expression analyses. The specificity of the primer pairs was verified by melt curve analysis. The identities of the PCR products were confirmed by subcloning into pGEM-T Easy (Promega) and sequencing as above.

2.6.3 ASSAY SETUP

For each gene, a master mix was prepared containing 500 nM of each forward and reverse primer, SYBR® Green JumpStart™ *Taq* Readymix™ (Sigma) and H_2O. 16 µl of the master mix was loaded into individual wells of a 96-well plate (TwinTec™ skirted, Eppendorf). cDNA was synthesised as described above and diluted 1:10 with molecular grade H_2O. 4 µl of this diluted cDNA was added to each well and mixed by pipetting up and down. Each sample was measured in triplicate including a no template control. Plates were sealed with clear PCR seals (Thermo Scientific) and centrifuged at 1000 rpm at 4°C for 5 min to collect all reagents to the bottom of the wells.

The qPCR reaction conditions were as follows: 95°C, 2 min and 40 cycles of 95°C, 15 s; 58°C, 15 s; 68°C, 20 s. A melting step was added to the end of the assay to verify the products by melt curve analysis (what does this mean?). Replicates were sometimes excluded if the standard deviation of the mean of the Ct of three replicates exceeded 0.33, and if analysis of the melt curve of each replicate was not uniform.

The reference genes used in this study were *rpl13α* and *ef1α*. These were selected based on the relative levels and lack of variability between timepoints and populations (a comparison of multiple reference genes in zebrafish is found in McCurley and Callard, 2008); the expression of *rpl13α* was very similar between cavefish and surface fish adults, as well as between timepoints and light treatments, allowing quantitative comparisons to be made between populations. *Rpl13α* expression was highly variable during embryonic development, whilst *ef1α* was less so and therefore *ef1α* was used at the reference during the examination of circadian clock gene expression during developmental stages.

Table 2.2: Primer sequences for quantitative PCR

Gene	*Code*	*Sequence*
per1	882-5'cfqPer1	5'-GAACAGTAGGGGCGTGGTCA-3'
	883-3'cfqPer1	5'-GAGTTGGAACTCTTGCTCTC-3'
cry1a	559-5'qcfcry1a	5'-GTCATGGGCTCCTGCACTAC-3'
	560-3'qcfcry1a	5'-GTCAAAACCAAGCTCCTCCA-3'
per2a	1069-5'qcfPer2b_2	5'-TGTCCCGTTGCTAGGCTACCTA-3'
	1071-3'qcfPer2b_2	5'-GCTGACCGGCATACTGCAGG-3'
per2b	714-5'cfqPer2	5'-AACACACACGCCCAACTGTA-3'
	715-3'cfqPer2	5'-GGTGAAGGTGGAGAAGGACA-3'
clk1	1580-Am qclock1 F1	5'-GCTCAGACCCTCGTTTGAAG-3'
	1581-Am qclock1 R1	5'-CTCTTCATTGGGTTCCTCCA-3'
CPD phr	1108-AstyCPD5'qPCR2	5'-GGCCTCTCCTAAGCTGGAGT-3'
	1109-AstyCPD5'qPCR2	5'-GTCCACAGGTGGGAATTCAG-3'
ddb2	1561-Am qddb2 F1	5'-AAGCTGCACAAAGCCAAAGT-3'
	1562-Am qddb2 R1	5'-AGACGATGTTGCCACTAGCC-3'
tef1	1545-Am qTef1 F2	5'-AGCCCAGGTAAAAGAGCTGA-3'
	1546-Am qTef1 R2	5'-GCTGGCTCTGTCTTCGAAAT-3'
tmt opsin 1	1173-5' cfqTMT1b (2)	5'-ATTCGTCAACTCCTGCTTCG-3'
	1174-3' cfqTMT1b (2)	5'-GTCCGGGGCCTGTTTATTAT-3'
rpl13α	658-L13a(F)	5'-TCTGGAGGACTGTAAGAGGTATGC-3'
	659-L13a(R)	5'-AGACGCACAATCTTGAGAGCAG-3'
ef1α	1459-q-elfa F1	5'-CAGCTGATCGTTGGAGTCAA-3'
	1460-q-elfa R1	5'-TGTATGCGCTGACTTCCTTG-3'

2.6.4 DATA ANALYSIS

Data was analysed in Microsoft Excel. Data are presented as the mean ± SEM (n > 3) and were analysed using a Student's *t* test or analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison post-test. Tests were performed in GraphPad Prism (GraphPad Software, UK).

2.7 BEHAVIOURAL STUDIES

Behavioural studies were performed by Dr Christophe Guibal. Single adult surface fish, Pachón, and Chica cavefish were maintained in the laboratory at 22˚±1˚C and entrained to a 12hr:12hr photoperiod for at least 7 days in separate tanks measuring 41 x 20 x 25 cm. Near-infrared cameras Sony CCTV CCD 420 TVL 3.6 mm wide angle lens were mounted in front of each tank to allow the recording of the fish in light and dark conditions. Analogue video signal was recorded and compressed with Xvid MPEG4 codec through a Steren USB external S-video card using Debut Video Capture software (NCH software) at 30 frames per second, with a resolution of 640 x 480 pixels. Fish were recorded for 2 days in LD before transfer into constant darkness and recorded for a further 2 days. Videos were analysed with the movement quantification module of videotrack software (Viewpoint), with a detection threshold of 15 and a threshold of analysis ("burst") of 500. For each fish, the time spent swimming faster than 5 cm/s in a 30 min bin was taken. Activity, binned over 30 min, was normalised to the mean activity level for each fish, and the mean of 4 or more normalised activity levels plotted. Data analysis and interpretation was performed with Dr Peter Krusche. Activity was analysed for significant rhythmicity by autocorrelation and spectral resampling in Matlab (Mathworks).

2.8 WHOLE MOUNT IN SITU HYBRIDISATION

Embryos were collected as described in Section 2.1.2 and maintained in constant darkness at 25˚C. At 8 hpf and 26 hpf after a 3 hour light pulse or 3 hours in the dark as a control.

Embryos were fixed in 4% PFA/PBS overnight at 4°C and on the next day washed 4 times with PBS before storage in 100% MeOH at -20°C. Embryos were rehydrated in a series of washes with 75% MeOH/PBT (PBT = PBS + 0.01% Tween-20), 50% MeOH/PBT, and 25% MeOH/PBT before two PBT washes. Embryos were then treated with 10 µg/ml proteinase K for 5 min, washed with PBT twice before refixing with 4% PFA/PBS for 20 min. After five PBT washes, embryos were washed with HYB+ solution and incubated in HYB+ for at least 2 hours at 65°C.

DIG-labelled probes (antisense and sense) were synthesised from 1 µg of linearised plasmid DNA containing a 559bp fragment of *per2b* using T7 or SP6 polymerase (Promega) and digoxigenin-labelled dUTP (Roche). DIG-labelled probes were prepared by denaturing in HYB+ at 80°C for 2 min before being diluted to 1 µg/ml in HYB+. Probes in HYB+ were applied to the embryos, which were then incubated at 65°C with gentle shaking overnight. The probes were removed the next day.

The embryos were washed at 65°C with HYB+, 50% HYB+/2X SSC, 2X SSC, and twice in 0.2X SSC before being cooled to room temperature. They were then washed three times with PBS before incubation with 2% Blocking Agent (Roche) in Maleic acid buffer (MAB) for at least 3 hours. The block was replaced with anti-DIG-AP (1:5000) in 2% Blocking Agent in MAB, and the embryos were incubated overnight at 4°C.

On the third day, embryos were washed four times in PBS, equilibrated in BM staining buffer, and incubated with BM purple in the dark at room temperature until the colour was sufficiently developed. To stop the reaction, embryos were washed twice with PBT and refixed with 4% PFA/PBS overnight at 4°C. For storage, the fixative was removed, and embryos were washed with PBS. They were then stored in PBS or 75% glycerol/PBS at 4°C.

2.9 DNA REPAIR ASSAYS

2.9.1 QUANTITATION OF REPAIR OF UV-INDUCED DNA DAMAGE BY ELISA

Adult fish were maintained on a 14:10 LD cycle as described above. At ZT23.5 both lobes of the caudal fin of surface, Pachón and Chica fish were collected under red light and placed in individual wells of a 6-well plate containing pre-warmed PBS with penicillin (200 U/ml) and streptomycin (200 µg/ml, Gibco). One lobe of each fin was exposed to 1 mJ/cm^2 of UV light for 2 s. The other lobe was kept in darkness as a non-damaged control and to control for different DNA damage amounts between individual fish. The PBS was replaced with L15 medium (Gibco) containing 15% FCS (Biochrom AG), penicillin (100 U/ml), streptomycin (100 µg/ml) and gentamycin (50 µg/ml, Gibco), and plates containing the fins were wrapped in foil and incubated in thermostatically controlled water baths at 22°C. Plates containing UV damaged fins and control non-damaged fins were collected from the water baths at 0.5 hrs and 24 hrs post UV pulse and immediately frozen at -80°C as recommended by the GeneJet Genomic DNA Purification kit (Fermentas). At the appropriate time, fins were thawed and genomic DNA was extracted using the GeneJet Genomic DNA Purification kit (Fermentas).

96-well polyvinylchloride flat-bottom plates, precoated with 0.003% protamine sulfate, were incubated overnight at 37°C with heat-denatured genomic DNA (diluted to 0.2 µg/ml in PBS, 10 ng/well). Plates were washed 5 times with PBS-Tween (0.05%). The monoclonal antibody to CPD photolesions (TDM-2, Cosmo Biosciences) was diluted in PBS (1:1000) and incubated with the immobilised DNA for 30 min at 37°C. After washing with PBS-Tween (0.05%), the bound antibody to the photolesions was detected by incubation with biotinylated $F(ab')_2$ fragment of goat anti-mouse IgG (1:2000; Zymed, Life Technologies) for 30 min with 37°C, washed with PBS-Tween (0.05%) and then incubated with Peroxidase-streptavidin (1:10000; Zymed, Life Technologies) for a further 30 min at 37°C. The plates were washed once with 150 µL/ well of Citrate-phosphate buffer (pH5.0)

(Citric acid monohydrate 5.10 g, Na2HPO4 7.30g, Distilled water 1000 ml). The plates were then incubated with 100 µl/well of substrate solution (8 mg o-Phenylene diamine, 4 µl H_2O_2 (35%), 20 ml Citrate-phosphate buffer (pH5.0)) for 30 min at 37°C to detect peroxidase activity; after stopping the reaction with 50 µl/well of 2 M H_2SO_4, absorbance was measured at 492 nm. DNA repair rate was taken as the number of CPD photoproducts (CPDpps) remaining at 24 hours and was quantified using the following formula:

$$CPDs\ remaining = 100\times\frac{OD_{24hr}}{OD_{initial}} = 100\times\frac{OD\ at\ 24hr\ after\ UV - OD\ at\ 24hr\ in\ the\ dark}{OD\ at\ 0.5hr\ after\ UV - OD\ at\ 0.5hr\ in\ the\ dark}$$

Data are presented as the mean ± SEM (n ≥ 3) and were analysed using a Student's analysis of variance (ANOVA) (http://www.physics.csbsju.edu/stats), followed by Newman-Keuls multiple comparison post-tests. Tests were performed in GraphPad Prism (GraphPad Software, UK).

2.10 Overexpression of *CPD PHR* in zebrafish cells

2.10.1 Tol2 system recombination (Kwan et al., 2007)

The coding region of *CPD photolyase* (zfCPDphr) was amplified by RT-PCR from zebrafish embryonic cell line RNA and subcloned into a Tol2 multiple cloning site middle vector containing a HA tag (pME-MCS-HA). A three-insert multisite Gateway LR reaction was performed, combining a 5' entry clone containing the CMV promoter (p5E-CMV), the middle clone containing the tagged zfCPDphr (pME-MCS-HA-zfCPDphr) and a 3' entry clone containing a polyA sequence (p3E-PolyA) with a puromycin resistant destination vector (pDestTol2PuroRpA2). The resultant plasmid is designated pDestTol2puroR-CMV-HA-zfCPDphr-polyA (#892). A second recombination was performed using pME-MCS-HA instead of pME-MCS-HA-zfCPDphr, creating an empty vector control plasmid pDestTol2puroR-CMV-HA-polyA (#893).

2.10.2 TRANSFECTION OF ZEBRAFISH CELLS

Zebrafish cells containing the *per1b*-luciferase reporter construct were grown to 80% confluence. These cells were trypsinised (0.05% solution with 0.5 mM EDTA), resuspended in media, washed in PBS and then resuspended at a concentration of 10,000,000 cells/ml in R buffer for electroporation using the Neon Transfection System (Invitrogen). 10 µl of the cell suspension was transfected with 0.5 µg of the puromycin-resistant destination vector (containing HA tagged zfCPDphr, #872, or the HA tag alone, #873) and 0.5 µg of a plasmid containing the Tol2 Transposase gene (#371 pCS-TP). These transfected cells were grown to confluence before selection with puromycin (0.25 µg/ml). The pooled populations of cells was then tested for the expression of the *per1b*-luciferase reporter on the Packard TopCount luminometer (PerkinElmer Inc.) as described in Section 2.1.4.

3 EXAMINATION OF THE CIRCADIAN SYSTEM OF *ASTYANAX MEXICANUS* IN THE LABORATORY

3.1 INTRODUCTION

The majority of animals and plants possess endogenous circadian clocks, which are considered an adaptation to life on a rotating planet. Indeed, these clocks are synchronized by rhythmic changes in the daily environmental cycle, with the major signal being the light-dark (LD) cycle. It is suggested that animals that have evolved in the absence of these cycles, for example in the deep sea or caves, have no need for a functional clock and will therefore no longer possess one. This makes apparent sense as studies investigating adaptive value of the circadian clock only show a benefit when the animals are exposed to light and dark cycles (DeCoursey et al., 2000; Green et al., 2002). However, there are relatively few studies that have examined clocks in obligate cave-dwelling or deep-sea animals.

The few studies to do so show a range of circadian clock phenotypes. Some studies show an apparent absence of the clock, for example, in cave amphipods (Blume et al., 1962) and cave salamanders (Hervant et al., 2001); some suggest clocks are retained, for example in cave crayfish (Brown, 1961; Jegla and Poulson, 1968), cave crickets (Reichle et al., 1965) and cave loaches (Pati, 2001); and some studies show animals that are highly variable from individual to individual, for example cave millipedes (Mead and Gilhodes, 1974; Koilraj et al., 2000). Though these studies offer some evidence for the state of circadian clocks in cave animals, results are mainly through behavioural or physiological data i.e. outputs of a clock and none look at the molecular clock, which is now well characterised in a number of organisms.

The blind Mexican cavefish, *Astyanax mexicanus*, is a useful model system for the study of the numerous changes that occur during adaptation to cave life. *Astyanax* is unique in that the ancestral surface population still exists in the neighbouring rivers to the caves in North East Mexico (Bradic et al., 2012), allowing direct comparison with the cavefish (Jeffery, 2001). Recent studies have exploited these facts and revealed that these changes are both

adaptive and regressive; these fish are blind and de-pigmented, but possess a highly tuned vibration sense and increased number of tastebuds (Jeffery, 2001; Yamamoto et al., 2009; Yoshizawa et al., 2010). These changes are seen in numerous cave species, showing an incredible level of convergence. Furthermore, there are over 30 caves harbouring *Astyanax* cavefish and good evidence to suggest independent origins for some populations (Bradic et al., 2012; Strecker et al., 2012). Because of the close relationship between the surface and cave forms, these comparisons can be made down to the molecular level, allowing us to determine how the circadian clock has evolved following several million years in constant darkness.

Much of what is known about teleost molecular clocks comes from studies in zebrafish. Self-sustaining, light responsive circadian clocks are found in nearly all zebrafish tissues, cells and early embryos (Whitmore et al., 1998; 2000; Dekens and Whitmore, 2008). Light has a very strong effect on zebrafish cell biology, not only on resetting the circadian clock (Vallone et al., 2004; Carr and Whitmore, 2005; Tamai et al., 2007), but also on other aspects, such as the activation of DNA repair processes (Tamai et al., 2004; Hirayama et al., 2009) and the regulation of cell cycle events (Dekens et al., 2003; Dickmeis et al., 2007; Idda et al., 2012; Tamai et al., 2012). In recent years, the components of the light input pathway have begun to be identified. D-box regulation, in particular by thyrotroph embryonic factor (tef), a member of the PAR (proline and acidic amino acid-rich) subfamily of basic region/leucine zipper (bZIP) transcription factors, is important not just for the circadian clock but also mediating light-induction of several other genes (Vatine et al., 2009; Gavriouchkina et al., 2010; Weger et al., 2011; Mracek et al., 2012). Evolving in a completely dark environment is therefore likely to impact fish physiology in a dramatic manner.

The highly decentralised nature of teleost clocks has allowed us to explore clock function using samples of the caudal fin of fish taken at different times in the day. *Period1* shows a high amplitude oscillation throughout tissues in all teleosts examined to date, and so

provides a suitable readout of the circadian clock (Vallone et al., 2004; Park et al., 2007; Velarde et al., 2009). In this way, this chapter aims to characterise how the circadian clock has evolved in *Astyanax* cavefish during life underground.

3.2 METHODS

3.2.1 BIOLOGICAL MATERIALS

Astyanax surface fish, Pachón, Chica and Tinaja cavefish, and F1 hybrids of surface and Pachón fish were maintained in the laboratory as described in Section 2.3.1. Samples were taken at the appropriate time and total RNA was extracted and cDNA synthesised as described in Section 2.4.

3.2.2 CLONING OF *ASTYANAX MEXICANUS* CLOCK GENES

A full description of the method and the primers used for cloning *Astyanax* genes can be found in Section 2.5. Nucleotide sequences for *period* (*per*) genes from multiple teleosts, mice and *Drosophila* were taken from ENSEMBL and Nucleotide and were aligned using the ClustalW2 algorithm in Seaview (Gouy et al., 2010). A Neighbour-Joining tree was created in Seaview (Gouy et al., 2010), with 2000 bootstrap replicates with *Drosophila per* as an outgroup. Gene IDs are found in Table 3.1. Alignments of the predicted amino acid sequences of Per1 and Cry1a from surface and cavefish populations, with zebrafish as a reference, were created using the ClustalW algorithm in MegAlign (DNASTAR). Sequences were annotated by hand.

Table 3.1: Period genes of species used in phylogenetic analysis. Information taken from Ensembl as of August 2012.

Species	*Gene name*	*ENSEMBL Gene ID*
Danio rerio	*per1a*	ENSDARG00000056885
	per1b	ENSDARG00000012499
	per2	ENSDARG00000034503
	per3	ENSDARG00000010519
Oryzias latipes	*per1*	ENSORLG00000006929
	per2a	ENSORLG00000016612
	per2b	ENSORLG00000015456
	per3	ENSORLG00000015952
Gasterosteus aculeatus	*per1*	ENSGACG00000019308
	per2a	ENSGACG00000013485
	per2b	ENSGACG00000005662
Tetraodon nigroviridis	*per1*	ENSTNIG00000007198
	per2a	ENSTNIG00000015238
	per2b	ENSTNIG00000014323
	per3	ENSTNIG00000007273
Takifugu rubripes	*per1*	ENSTRUG00000014420
	per2a	ENSTRUG00000007894
	per2b	ENSTRUG00000006817
	per3	ENSTRUG00000003219
Mus musculus	*Per1*	ENSMUSG00000020893
	Per2	ENSMUSG00000055866
	Per3	ENSMUSG00000028957
Drosophila melanogaster	*per*	FBgn0003068

3.2.3 QUANTITATIVE PCR

The principle method used in this chapter was quantitative PCR (qPCR). A detailed description of the method can be found in Section 2.6, and the primers used are shown in Section 2.6, Table 2.2.

3.2.4 BEHAVIOURAL STUDIES

Single adult surface fish, Pachón, and Chica cavefish were maintained in the laboratory at 22±1˚C. Experiments were performed as described in Section 2.7. Experimental set up and data collection was performed by Dr Christophe Guibal. Data analysis was performed by

Dr Peter Krusche. Activity was analysed for significant rhythmicity by autocorrelation and spectral resampling in Matlab (Mathworks).

3.3 RESULTS

3.3.1 ANALYSIS OF *ASTYANAX* SURFACE AND CAVEFISH CLOCK GENES

The first step in the examination of the molecular circadian clock of *Astyanax mexicanus* was to isolate and sequence clock genes by RT-PCR. *Per1* and *cry1a*, core components of the molecular clock of zebrafish (Vallone et al., 2004; Tamai et al., 2007), were isolated and sequenced as full length coding regions. Additionally, four genes were isolated as partial coding regions from surface, Pachón, Chica and Tinaja populations of *Astyanax* (initial isolation of short PCR fragments for *per1*, *per2a*, *per2b* and *cry1a* performed by Christophe Guibal). Identification of the genes was confirmed by phylogenetic and NCBI BLASTn analysis (Table 3.1 and Figure 3.1).

Table 3.2: Identity of cDNA sequences for *A. mexicanus* genes.

Gene name	*Fragment size (nt)*	Danio rerio *Identity (%)*	Danio rerio *accession number*
per1	4296	81	NM_212439.2
per2a	1657	81	NM_182857.1
per2b	481	78	NM_182857.1
cry1a	1878	86	NM_001077297.2
clk1	957	87	NM_130957.1
tef1	396	80	NM_131400.1

The predicted amino acid sequences of Per1 and Cry1a were compared between the three populations of *Astyanax* and zebrafish (Figure 3.2 and Figure 3.3). There are thirteen amino acid changes in Pachón Per1 compared to the surface sequence and twelve of those changes are identical between Pachón and Chica cave populations. Comparing the *Astyanax* protein to known Per1 protein crystal structures revealed that the F/Y and R/Q substitutions reside in the beta-sheets of the PAS-A domain, and the I/M substitution resides in the PAS-B domain (Hennig et al., 2009). The changes in the PAS-A domain in

cavefish may result in a more stable PAS-A structure (with a tyrosine), but with a less stable PAS-A-PAS-B interface (with a glutamine) (Eva Wolf, personal communication). These changes in the protein structure are likely to affect the binding ability of PER1, particularly when binding another PAS domain protein. A similar comparison of the light inducible Cry1a protein shows 5 amino acid differences, which are identical across three cave populations (Figure 3.3).

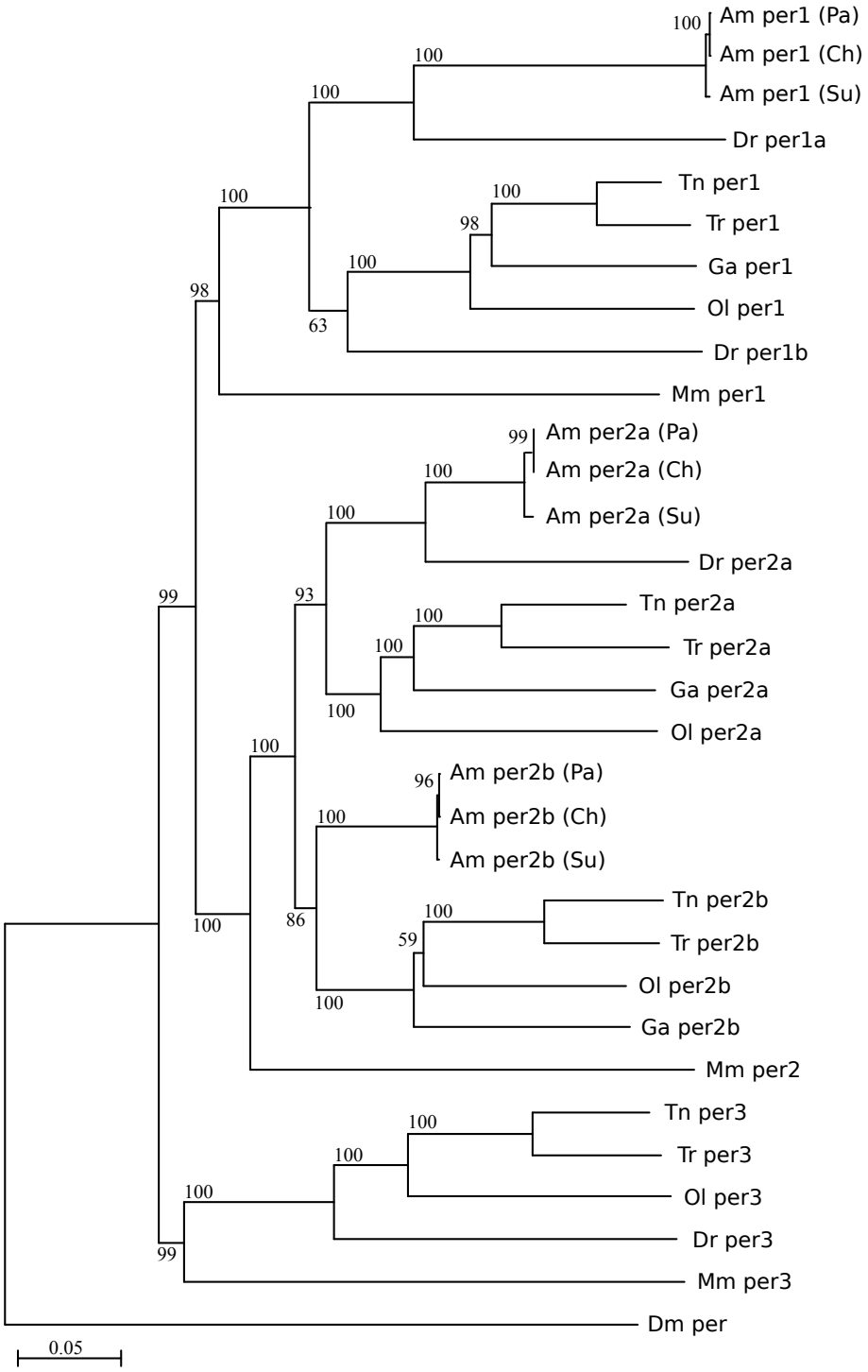

Figure 3.1: Phylogenetic tree showing the relationship of *Astyanax period* genes.
A consensus tree corresponding to 2,000 bootstrap replicates was obtained using the neighbour-joining method. *Drosophila melanogaster* (Dm) *per* is used as an outgroup. Numbers indicate percentage bootstrap support, with branch length proportional to phylogenetic distance (scale bar). Mm, *Mus musculus*; Dr, *Danio rerio*; Ol, *Oryzias latipes*; Tr, *Takifugu rubripes*; Tn, *Tetraodon nigroviridis*; Ga, *Gasterosteus, aculeatus*; Am, *Astyanax*

mexicanus; Su, surface; Pa, Pachón; Ch, Chica. Ensembl and NCBI Gene Ids are listed in the methods, section 3.2.

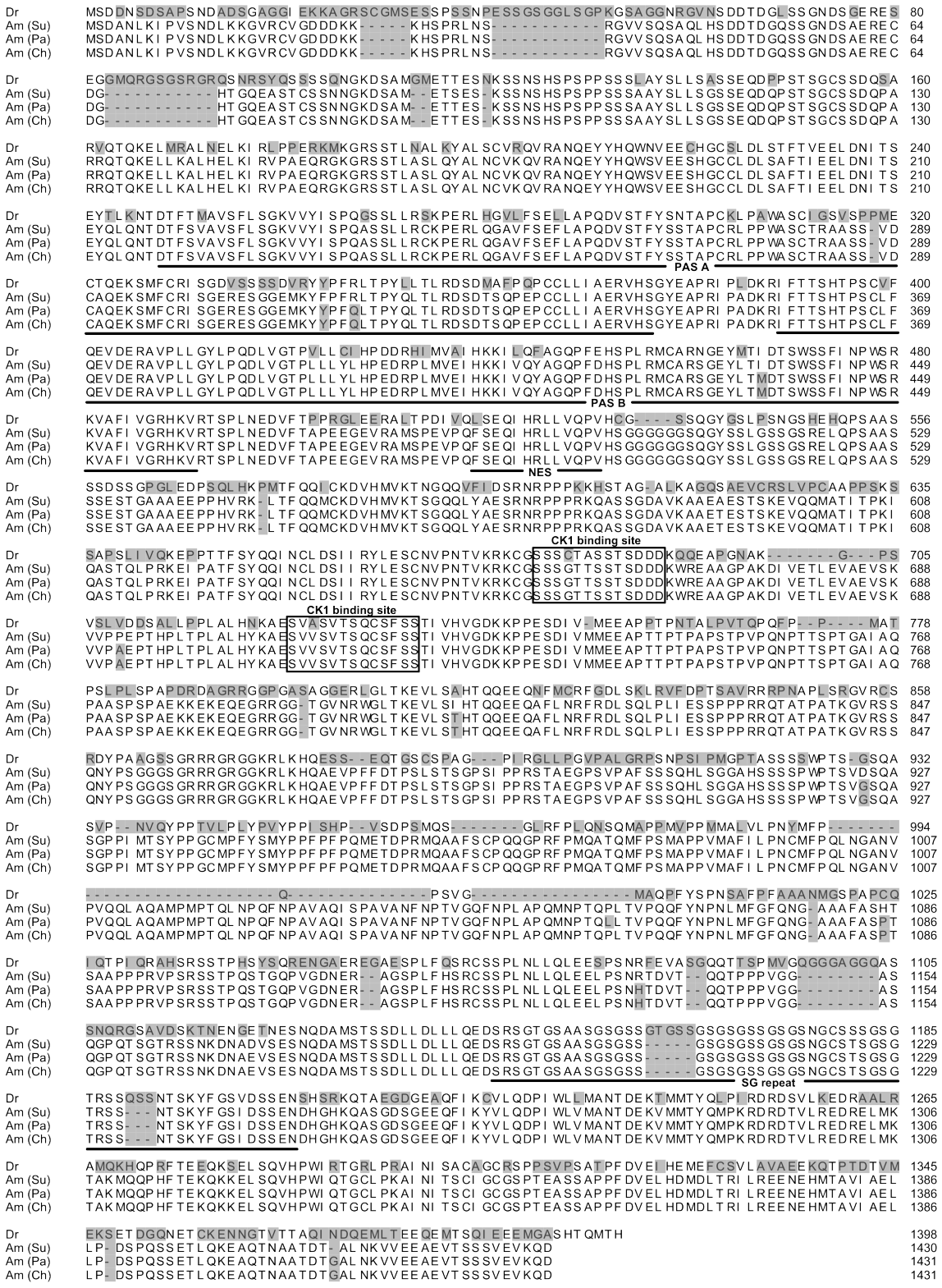

Figure 3.2: Per1 from different cave populations shows highly similar amino acid changes compared to the surface fish protein.

The predicted protein sequences of Per1 from *Astyanax mexicanus* (Am) and *Danio rerio* (Dr) as a reference were aligned using Clustal W. Thirteen amino acid differences were identified in Pachón Per1 compared to the surface protein and twelve of these changes

were identical in Chica Per1 (Changes compared to surface Per1 are highlighted grey). PAS A and PAS B domains are highlighted (Hennig et al., 2009), as well as a nuclear export signal (NES), potential casein kinase 1 (CK1) binding sites, and an SG repeat region. Su, Surface; Pa, Pachón; Ch, Chica.

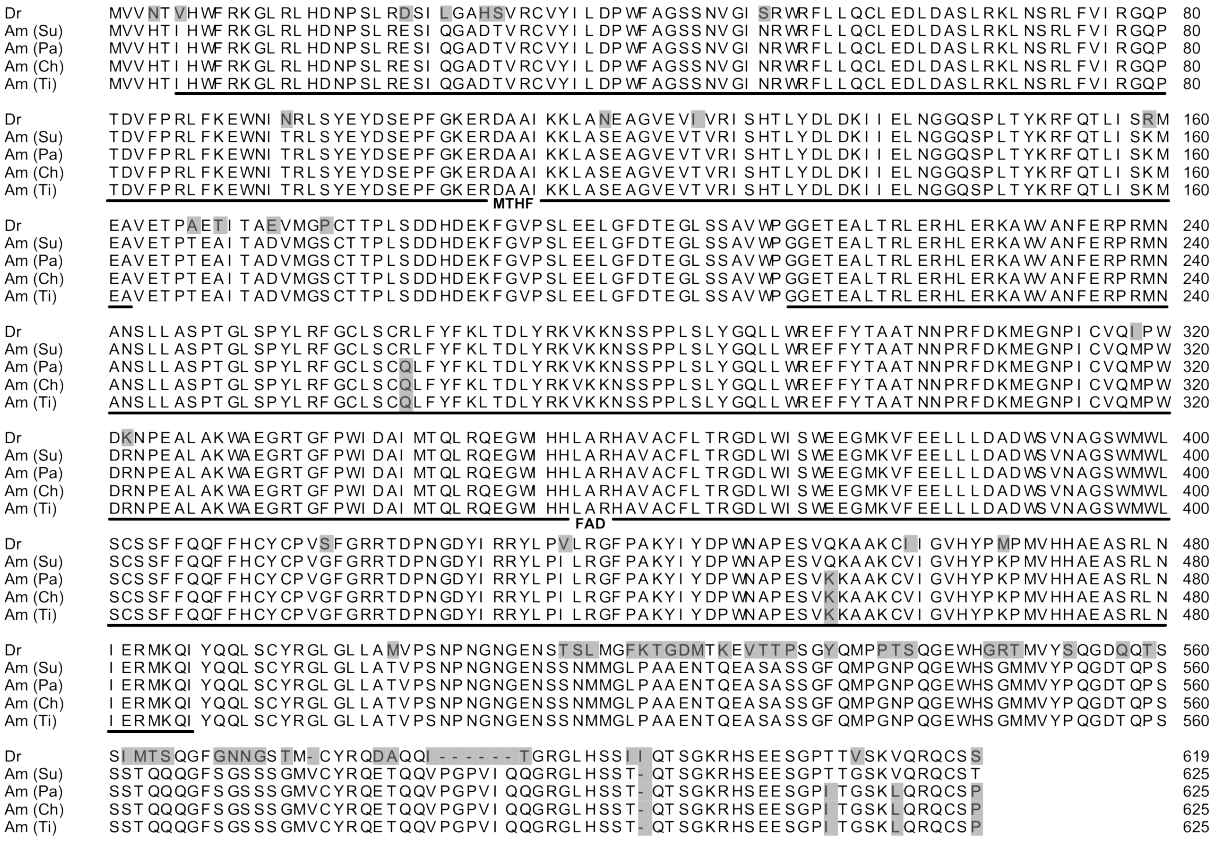

Figure 3.3: Cavefish Cry1a shows identical amino acid changes compared to the surface fish protein.

The predicted protein sequences of Cry1a from *Astyanax mexicanus* (Am) and *Danio rerio* (Dr) as a reference were aligned using Clustal W. Five amino acid differences were identified in Cry1a of three cavefish compared to the surface protein (Changes compared to surface Cry1a are highlighted grey). Known Cry functional domains are highlighted, including the chromophore-binding domains for methenyltetrahydrofolate (MTHF) and FAD. Su, Surface; Pa, Pachón; Ch, Chica; Ti, Tinaja.

The similarity in these protein sequences is reflected in the nucleotide sequences for all 6 genes isolated, showing a very high similarity between cave and surface populations and very little difference between different cave populations (Table 3.2). It was recently shown that the three populations of cavefish shown in Figure 3.3 were the result of three separate invasions of the underground from the same surface fish ancestor (Bradic et al., 2012). Therefore the similarity in sequence may reflect a remarkable convergence in molecular sequence amongst three independent caves.

Table 3.3: Comparison of *A. mexicanus* genes.

Gene name	*Fragment size (nt)*	*Cave of origin*	*Surface Identity (%)*	*Pachón Identity (%)*
per1	4296	Pachón	99.5	-
		Chica	99.5	99.9
per2a	1657	Pachón	99.3	-
		Chica	99.2	99.8
per2b	481	Pachón	99.8	-
		Chica	99.8	100
cry1a	1878	Pachón	99.3	-
		Chica	99.2	99.9
		Tinaja	99.3	100
clk1	957	Pachón	99.7	-
		Chica	99.9	99.8
tef1	396	Pachón	99.5	-
		Chica	99.5	100

However, there is debate about the identity of the surface fish which is the direct ancestor to these cave populations. Some studies suggest that the ancestor colonised the surrounding rivers in the region of 3 to 8 million years ago, but have subsequently been replaced by a 'newer' surface fish population, of which the surface fish in this study is a representative (Strecker et al., 2004; Ornelas-García et al., 2008; Bradic et al., 2012). Other studies report a relationship between the Pachón and Chica cavefish and the surrounding surface populations (Dowling et al., 2002; Strecker et al., 2003). It is therefore possible that these identical 'changes' could instead reflect the protein sequence present in the 'old'

surface fish ancestor (different to the 'new' surface fish stock) with conservation of that sequence during evolution in the separate caves.

3.3.2 *Astyanax* surface and cave populations show rhythms in clock gene expression following light entrainment in the laboratory

To determine if *Astyanax* cave populations still retain a molecular circadian clock, surface fish, Pachón and Chica cavefish were entrained to a 12hr:12hr LD cycle under laboratory conditions and fin clips were collected at 6 hour intervals. After 2 days of sampling on a LD cycle, the animals were allowed to free-run into constant darkness, with sampling continuing for a further 2 days. Samples were subsequently analysed by quantitative PCR (qPCR) to determine the expression levels of *per1*. Surface fish have a high amplitude rhythm in *per1* expression, with a peak in the late evening at zeitgeber time 21 (ZT21; Figure 3.4a). This rhythm continues robustly as the animals free-run into constant darkness. *Per1* expression also oscillates in the two cave populations, showing that these cave strains retain the ability to generate molecular circadian oscillations. However, there are clear and consistent differences between the rhythms seen in surface and cave populations. First, the cavefish *per1* rhythm is lower in amplitude, with a relative peak to trough expression in LD of 7.71- and 8.26-fold in Pachón and Chica cavefish, respectively, compared to 20.61-fold in surface fish. Secondly, the timing or entrained phase of the *per1* rhythm is clearly different, with the peak of expression occurring 6 hours later in both cave populations, though accurate measurement of phase and amplitude is difficult with 6 hour resolution. Furthermore, whilst the rhythm in cavefish appears to be lower in amplitude than surface fish, we do not observe faster dampening, even through the third day of constant darkness (Figure 3.5).

Clock (*clk1a*) was the first clock gene to be shown to be rhythmic throughout the tissues of the zebrafish, demonstrating the decentralised nature of the clock in teleosts (Whitmore et al., 1998). Zebrafish and teleosts in general have multiple clock genes, which activate expression through binding to E-boxes (Ishikawa et al., 2002). We examined the

expression of this transcriptional activator, a key component of the molecular clock in teleosts, to see its expression in *Astyanax*. *Clk1* is rhythmically expressed in *Astyanax* surface and cave forms, though the amplitude of this rhythm is much smaller than the *per1* rhythm and it quickly dampens in DD in both forms (Figure 3.4). Interestingly, *clk1* peaks in all fish at a similar time, ZT15, though upon transfer to DD the rhythm becomes broader and less precise in cave populations, with high expression seen at CT15 and CT21 (Figure 3.4b). Therefore, the delay seen in *per1* expression in cavefish does not appear to be due to differences in the expression of a transcriptional activator, *clk1*.

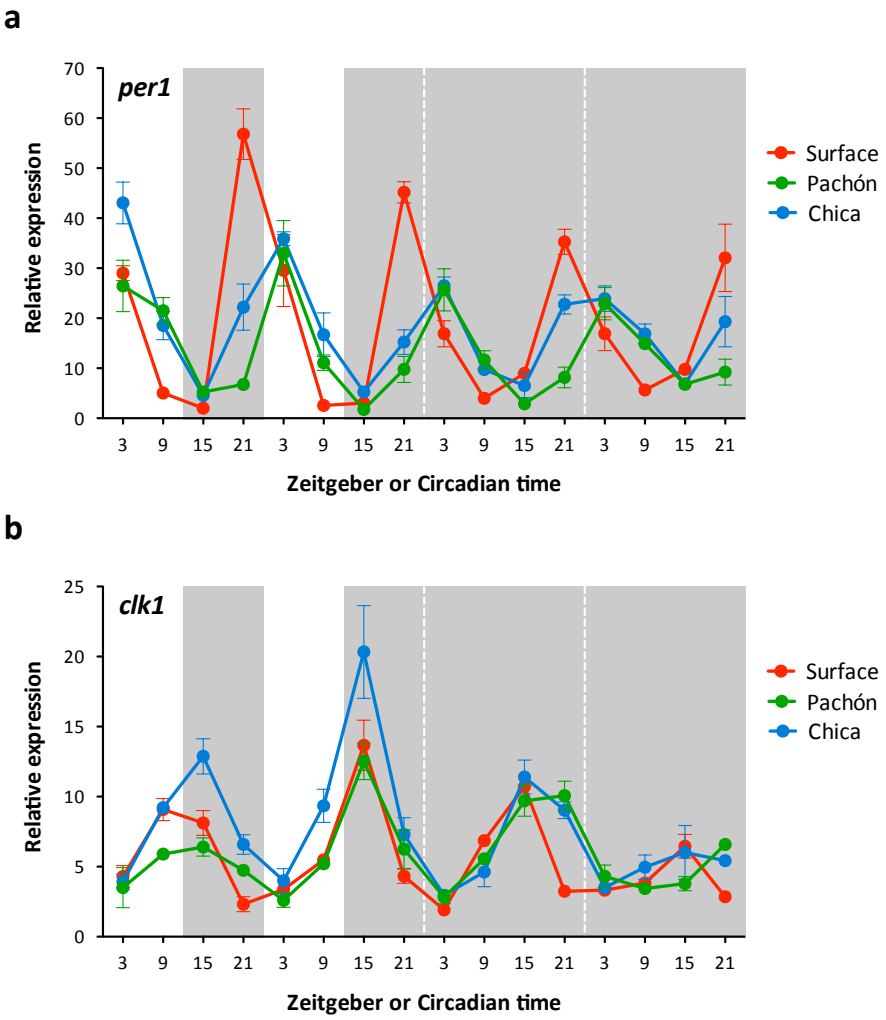

Figure 3.4: Clock gene expression in *Astyanax mexicanus*. Adult fish were entrained to a LD cycle for 7 days and transferred into constant darkness.

Fin samples were taken every 6 hours at the indicated zeitgeber or circadian time (ZT or CT), where ZT0 indicates 'lights on'. *Per1* (a) and *clk1* (b) mRNA levels were measured by qPCR and normalized to the reference gene *rpl13α*. The relative expression of each gene was calculated and plotted using the ΔΔCt method. (a) *Per1* shows a high amplitude oscillation that continues in constant darkness in all three populations. Cavefish exhibit a rhythm that is delayed and smaller in amplitude relative to surface fish. Rhythm amplitude for *per1* was estimated by averaging peak to trough values in LD, and was 20.61-fold for surface, 7.71-fold for Pachón and 8.26-fold for Chica (amplitudes were compared using an ANOVA followed by Newman-Keuls multiple comparison tests. ANOVA $p<0.001$, $n=44$; Surface vs Pachón, $p<0.001$; Surface vs Chica, $p<0.001$; Pachón vs Chica, $p>0.05$). (b) *Clk1* expression is rhythmic in *Astyanax* in LD but dampens quickly in DD. Surface and cavefish show rhythms of similar phase and amplitude. White and grey bars indicate light and dark periods, respectively. Data represent the mean ± SEM of at least 4 different fish.

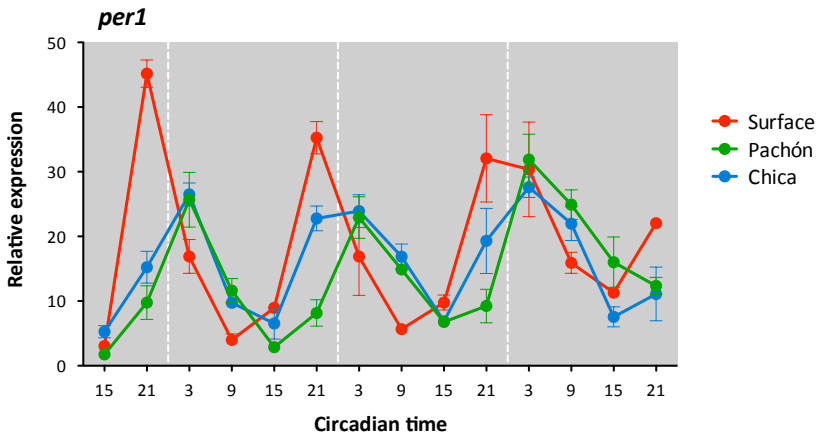

Figure 3.5: *Per1* oscillations continue in constant darkness.
Adult fish were entrained to a LD cycle for 7 days and transferred into constant darkness for 3 days. Fin samples were taken every 6 hours, and the relative expression of *per1* mRNA (RT-qPCR) was plotted using by the ΔΔCt method. The first two and a half days of data are reproduced from Figure 3.4a, and illustrate the free-running characteristics of the *per1* rhythm in *Astyanax*. Data represent the mean ± SEM of at least 4 different fish.

3.3.3 THE ACUTE LIGHT-INDUCTION OF GENES INVOLVED IN CLOCK ENTRAINMENT IS ALTERED IN CAVE POPULATIONS OF *ASTYANAX*

These differences in rhythm amplitude and *per1* phase angle between surface and cave strains could be a consequence of changes within the core clock mechanism that generates the oscillation, alterations in the light input pathway or, of course, both. From work in zebrafish, several light-induced genes, including *cry1a* and *per2*, are known to be critical for light resetting of the circadian pacemaker (Ziv et al., 2005; Tamai et al., 2007). As we have shown, *Astyanax* cave strains are able to entrain to some extent to laboratory light-dark conditions, but impairment or specific alterations of the input pathway could lead to a less robust rhythm through weaker cell synchronization, as well as changes in the phase angle between the clock and the LD cycle (Figure 3.4a). So is the light input pathway altered in cave populations of *Astyanax*?

To answer this question, we looked at the induction of both *cry1a* and *per2* mRNA in *Astyanax mexicanus*, following a 3 hour light pulse applied to whole fish in the early

evening at ZT16. Surface fish show a significant acute induction of *cry1a*, similar to that previously reported in zebrafish (Hirayama et al., 2005; Figure 3.6a and Tamai et al., 2007). This acute response is greatly reduced in Pachón cavefish, though still statistically significant, and does not occur in Chica or Tinaja cavefish. Interestingly, this reduction or absence of a light response is due to significantly raised basal levels of *cry1a* transcript in the dark control samples of cavefish compared to surface individuals, rather than a change in the actual light-induced levels. For Chica and Tinaja, *cry1a* is present at near maximal levels in the absence of any light stimulus (Figure 3.6a). Similar results are seen for *per2a* and *per2b* (Figure 3.6b and c), where surface fish show a strong acute induction of mRNA levels in response to light, but this is greatly reduced in cave strains, as a consequence of significantly raised starting levels in the dark control samples. Thus, there is a clear perturbation of the light input pathway components in cave populations of *Astyanax mexicanus*, with normally light responsive genes expressed at high levels.

Examination of *cry1a* and the *per2* genes across LD and DD cycles shows that all three genes are primarily light-regulated (Figure 3.6d-f). Upon transfer to constant darkness, *cry1a*, *per2a* and *per2b* are at best, weakly clock-controlled (Figure 3.6d-f), as reported for zebrafish (Pando et al., 2001; Tamai et al., 2007; Vatine et al., 2009). *Cry1a* is statistically more highly expressed in cavefish than surface fish at three of eight time points, but this is likely to be due to the pronounced phase shift of the *cry1a* rhythm in cavefish. However, more interestingly, *per2b* is present at significantly raised levels in cave strains compared to surface fish through the constant dark condition. As *per2* is a light-induced clock repressor and is thought to be key for clock entrainment in fish systems (Ziv et al., 2005; Vatine et al., 2009), this level of increased tonic expression could explain the reduced amplitude and altered phase of molecular clock rhythmicity in cave populations. We know that over-expression of *per2* to even relatively low levels in zebrafish cell lines has a significant dampening effect on clock gene rhythms, a fact that is also true for the other light-induced clock repressor, *cry1a* (Tamai et al., 2007).

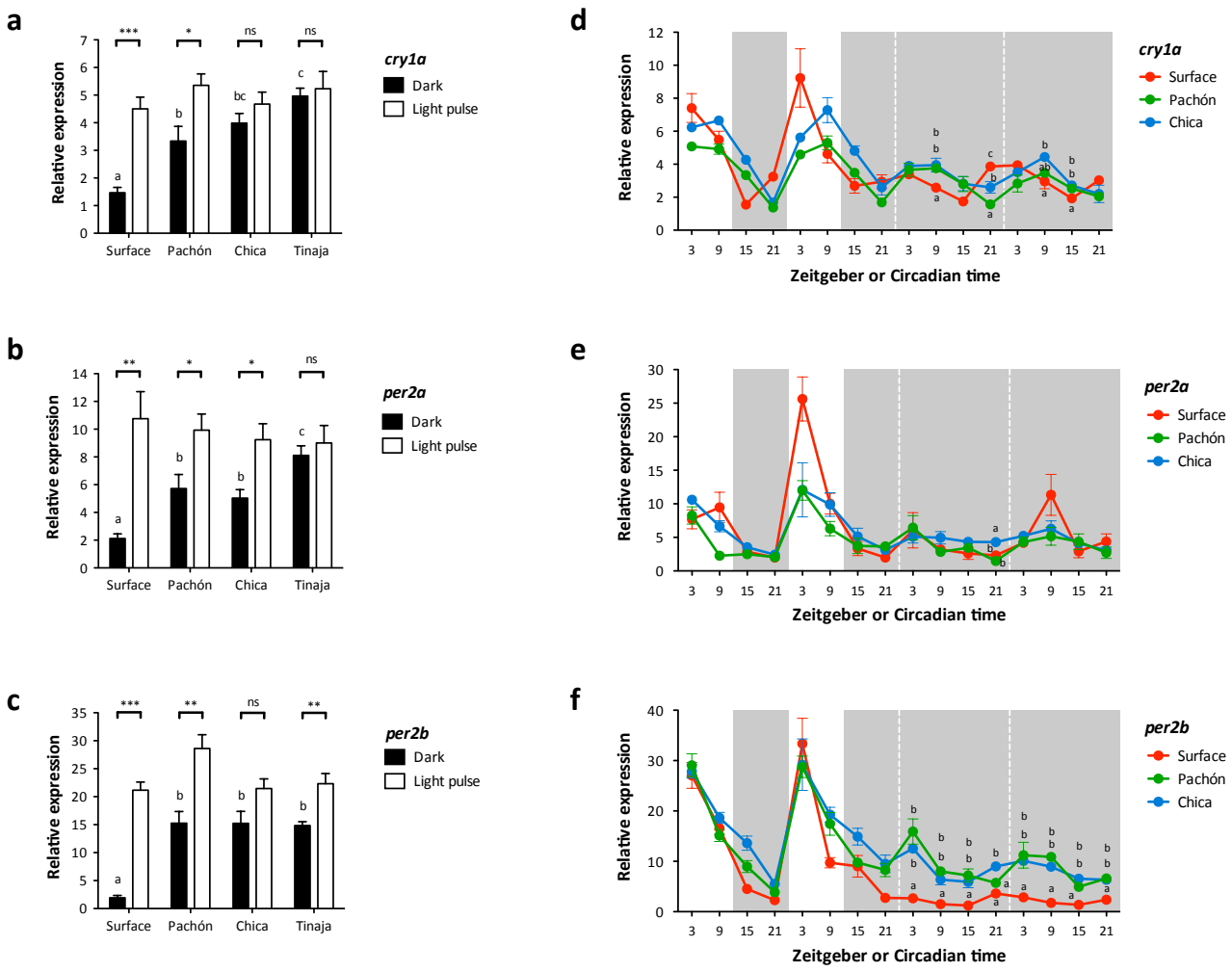

Figure 3.6: Acute light induction of *cry1a*, *per2a* and *per2b* is reduced in cavefish due to increased basal expression levels.

The relative expression of *cry1a*, *per2a* and *per2b* mRNA (RT-qPCR) was plotted using by the ΔΔCt method. (a-c) Adult fish were entrained on a LD cycle for 7 days and given a 3 hour light pulse at ZT16. Expression of *cry1a* (a), *per2a* (b) and *per2b* (c) was determined in light-pulsed and dark control fin samples by qPCR. Dark and light-induced levels of all genes within each population were compared using a Student's t-test (unpaired, two tailed; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Dark levels of all genes were compared between all populations using ANOVA followed by Newman-Keuls multiple comparison tests. Different lower case letters indicate significant differences between comparisons. Light induction (light pulse expression divided by dark control expression) was compared between all cavefish populations for each gene by ANOVA followed by Newman-Keuls multiple comparison tests. For all genes, surface fish had significantly higher induction

relative to cavefish, and no significant difference was seen between cavefish populations (all populations, n=39, $p<0.001$; Pa vs Ch vs Ti, n=29, $p>0.05$. *Cry1a*: Su, 3.07±0.29; Pa, 1.60±0.15; Ch, 1.17±0.09; Ti, 1.05±0.09. *Per2a*: Su, 5.06±0.84; Pa, 1.74±0.23; Ch, 1.84±0.20; Ti, 1.11±0.12. *Per2b*: Su, 10.91±1.28; Pa, 1.88±0.17; Ch, 1.41±0.13; Ti, 1.50±0.09). (d-f) The expression of *cry1a* (d), *per2a* (e) and *per2b* (f) was determined in the same samples as Figure 3.4. Expression levels of all genes (d-f) were compared between all populations at each time point in DD by ANOVA followed by Newman-Keuls multiple comparison tests. Different lower case letters indicate significant differences ($p<0.05$) between comparisons. *Per2b* is significantly more highly expressed at all DD time points in cavefish. *Cry1a* does not show the same expression pattern in DD as *per2b*, but does show a more pronounced and phase-shifted rhythm in cavefish. White and grey bars indicate light and dark periods, respectively. Data represent the mean ± SEM from at least 4 different fish. (a-c) Performed in collaboration with Christophe Guibal and Elodie Peyric.

This upregulation of *cry1a*, *per2a* and *per2b* could be a consequence of alterations in their transcriptional activation. *Tef1*, via D-boxes, is believed to sit upstream of *cry* and *per* induction, regulating downstream light induction and is itself induced by light (Vatine et al., 2009). Therefore, to gain more insight into the changes in the light input pathway, *tef1* was isolated from *Astyanax* and its expression analysed (Table 3.1 and Figure 3.7). However, unlike in zebrafish, *tef1* is not light induced in *Astyanax*, even in surface fish populations (Figure 3.7a). In fact, *tef1* shows a strong circadian rhythm in *Astyanax*. Interestingly there is a higher amplitude rhythm in the cavefish than surface fish after transfer into constant conditions. *Tef1* is significantly more highly expressed in cavefish over surface fish in the subjective day but not in the subjective night (Figure 3.7b). The weak underlying day-night difference in *per2b* expression in cavefish raises the possibility that *tef1* may contribute to its expression. However, the lack of expression difference in *tef1* between surface fish and cavefish in the subjective night precludes it somewhat from mediating the increased expression levels of light induced genes in the absence of light.

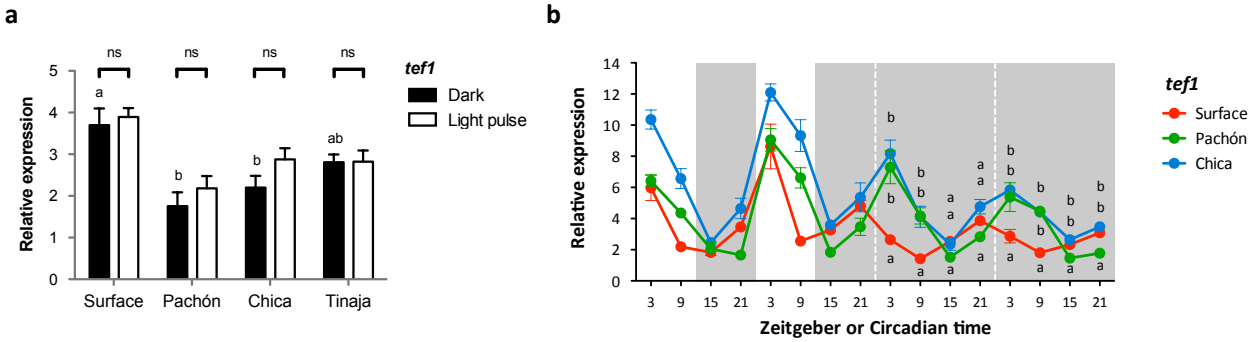

Figure 3.7: *Tef1* is not a light induced gene in *Astyanax mexicanus*, and shows high amplitude oscillations in cavefish in constant darkness.

The expression of *tef1* was determined in the same samples as Figure 3.6. (a) light-pulsed and dark control samples. (b) 6-hourly samples. Expression levels in (a) and (b) were compared using the same statistical tests as Figure 3.6. Rhythm amplitude for *tef1* in DD was estimated by averaging peak to trough values in DD, and was 2.71-fold for surface, 4.80-fold for Pachón and 3.48-fold for Chica on the first day and 1.71-fold for surface, 3.68-fold for Pachón and 2.21-fold for Chica (Amplitudes were compared using an ANOVA followed by Newman-Keuls multiple comparison tests. Day 1 DD: ANOVA $p<0.01$; Su vs Pa, $p<0.05$; Su vs Ch, and Pa vs Ch, $p>0.05$. Day 2 DD: ANOVA $p<0.001$; Su vs Pa, $p<0.001$; Su vs Ch, $p<0.01$; Pa vs Ch, $p>0.05$). White and grey bars indicate light and dark periods, respectively. Data represent the mean ± SEM from at least 4 different fish. Samples for (a) were collected in collaboration with Christophe Guibal.

3.3.4 LIGHT INPUT TO THE CIRCADIAN CLOCK IS RESTORED IN SURFACE/CAVE HYBRID FISH

Astyanax surface and cave strains belong to the same species and, as such, may be crossed within different populations to produce F1 hybrid offspring. We therefore generated F1 surface x Pachón hybrid fish and explored aspects of the circadian light input pathway in these adult animals. The acute induction of *cry1a*, *per2a* and *per2b* to a 3 hour light pulse was measured in these hybrid individuals and compared to both surface and Pachón responses (Figure 3.8a-c; surface and Pachón are reproduced from Figure 3.6a-c). Light-induction of all three genes is restored in hybrid animals with no significant differences between the level of induction in surface and hybrid fish (t-test, n=19. *Cry1a*: Su, 3.07±0.29; F1, 2.72±0.36. *Per2a*: Su, 5.06±0.84; F1, 4.42±0.50. *Per2b*: Su, 10.91±1.28; F1, 10.36±1.93), though the absolute induced levels of *cry1a* mRNA are slightly reduced, this is not significant (Newman-Keuls multiple comparison, $p>0.05$). The raised basal levels found in DD in Pachón fish are now suppressed (Figure 3.6a-c,e).

An examination of *per1* rhythmicity in the hybrid population shows that both the amplitude and entrained phase of the oscillation now match the surface fish population (Figure 3.8d. Surface and Pachón as reproduced from Figure 3.4a). Thus, the reduced amplitude, as well as the 6 hour delay in *per1* timing, observed in the Pachón strain has been reversed within one generation. When these hybrid animals are allowed to free-run into constant darkness for 2 days, both the amplitude and phase tend to be intermediate between surface and Pachón *per1* oscillations, though more cycles at higher resolution would ideally need to be analysed in order to make statements about oscillator period. These results suggest that alterations in the light input pathway are recessive and reversed within one cross or generation. However, changes in core oscillator function appear to be more complex and reside at some intermediate state in hybrid animals, but this may simply reflect the greater molecular complexity of the clock mechanism and consequently, the greater potential for molecular alterations.

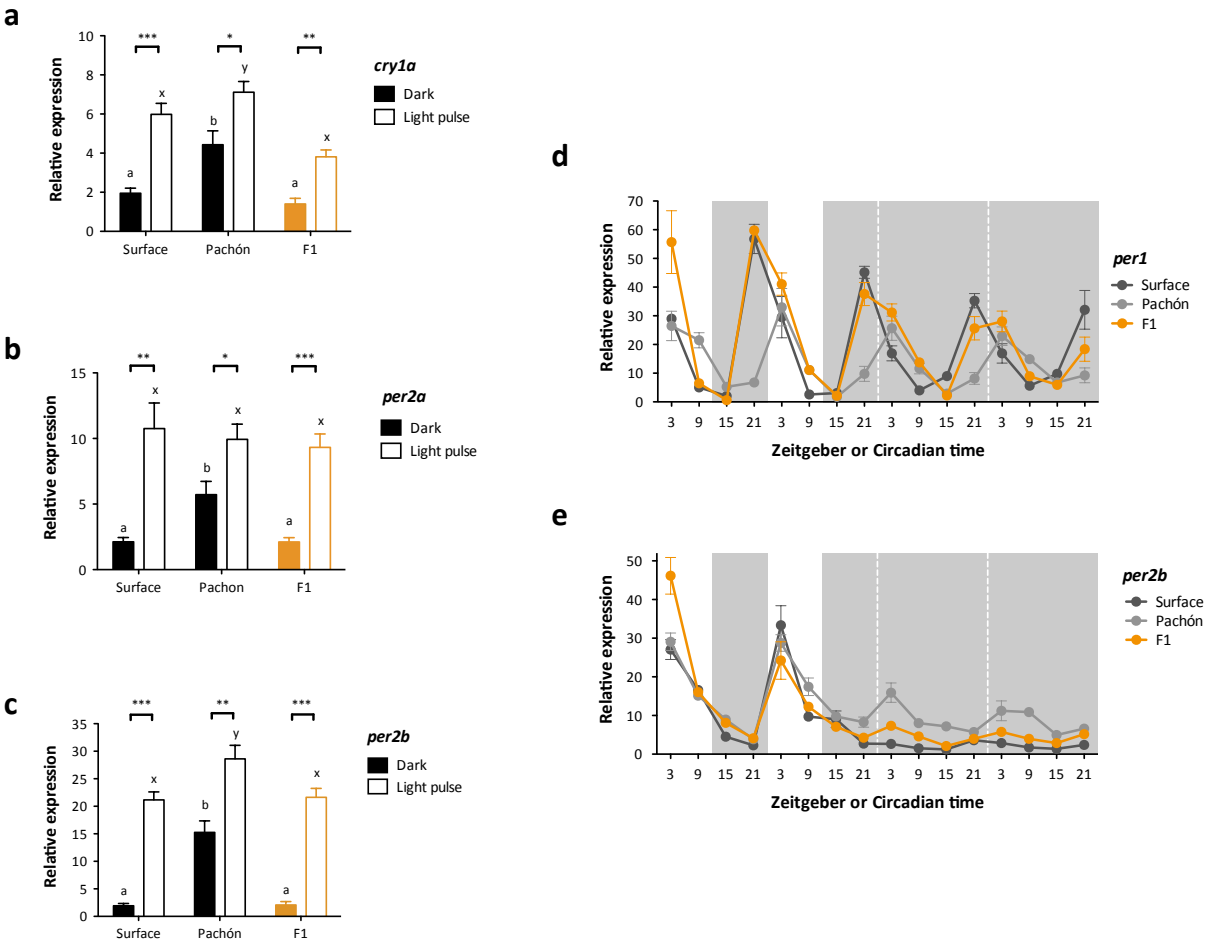

Figure 3.8: The acute light response is restored in F1 hybrid fish.

(a-c) Adult F1 surface x Pachón hybrid fish were entrained on a LD cycle for 7 days and given a 3 hour light pulse at ZT16. Expression of *cry1a* (a), *per2a* (b) and *per2b* (c) was determined in light-pulsed and dark control fin samples by qPCR. Dark and light-induced levels of all genes were compared within each population using a Student's t-test (unpaired, two tailed; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Dark and light induced levels of all genes were compared between all populations using ANOVA followed by Newman-Keuls multiple comparison tests. Different lower case letters indicate significant differences between comparisons (a vs b; x vs y). Light induction (light pulse expression divided by dark control expression) was compared between all populations for each gene by ANOVA followed by Newman-Keuls multiple comparison tests. For all genes no significant difference was seen between surface fish and F1 hybrid fish ($p>0.05$). (d-f) The expression of *per1* (d) and *per2b* (e) was determined by qPCR and compared to surface and Pachón cavefish (reproduced from Figure 3.4 and Figure 3.6 respectively). *Per1* amplitude was estimated by averaging peak to trough values in LD and was 35.24-fold in

F1 Hybrid fish. (Amplitudes were compared using an ANOVA followed by Newman-Keuls multiple comparison tests. ANOVA, $n=46$, $p<0.001$; Su vs F1, $p<0.05$; Pa vs F1, $p>0.001$). Data represent the mean ± SEM from at least 4 different fish. (a-c) Performed in collaboration with Christophe Guibal and Elodie Peyric.

3.3.5 CLOCK-CONTROLLED LOCOMOTOR RHYTHMS ARE ABSENT IN *ASTYANAX* CAVEFISH IN THE LABORATORY

Rhythms of locomotor activity have been demonstrated in *Astyanax* cavefish at juvenile and adult stages, with some suggestion that these rhythms are controlled by the circadian clock (Erckens and Martin, 1982b; Duboué et al., 2011). We took these data further to analyse locomotor rhythms of individual surface fish and Pachón and Chica cavefish (Figure 3.9), whose molecular clock is entrained by light. Fish were entrained to a 12hr:12hr LD cycle and recorded for 4 days. Analysis for burst activity (velocity > 5 cm/s) reveals alterations in rhythmic behaviour in addition to the molecular alterations described above (Figure 3.9a-c). Surface fish show a significant 24 hour rhythm in LD, and though the DD rhythm is not significant with a circadian period by autocorrelation over 4 days, there is an autocorrelation with a 12 hour offset. Furthermore, when the DD data is analysed alone, a significant autocorrelation is present. Both cavefish show significant 24 hour rhythms in LD by autocorrelation but after entry into constant darkness, both cavefish are behaviourally arrhythmic and show substantial variation between individuals (Figure 3.9b and c). Interestingly, upon transfer to DD cavefish activity remains at median to peak levels whereas surface fish activity drops to basal levels. These observations agree with the phenotype observed by Duboué et al., who show reduction in sleep and increased levels of activity throughout the day in *Astyanax* cavefish fry (Duboué et al., 2011).

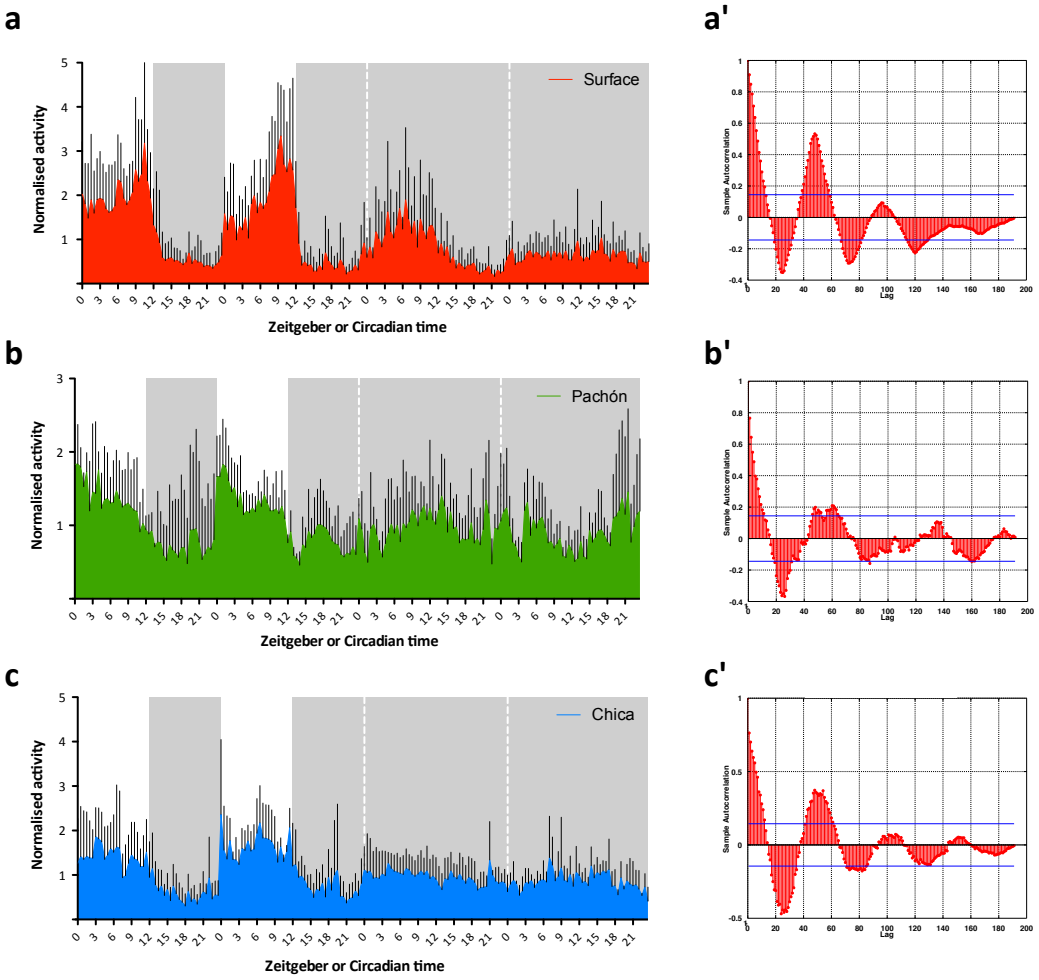

Figure 3.9: *Astyanax* cavefish are behaviourally arrhythmic in constant darkness. Adult fish were kept separately in individual tanks with an IR camera mounted above. Fish were entrained to a 12hr:12hr LD cycle for 7 days and transferred into constant darkness for 2 days. Filming took place over the final 2 days in LD and first 2 days in constant darkness. (a-c) Data plotted represent the average of normalized activity for 4 or more fish, error bars show standard deviation. (a) surface, (b) Pachón, (c) Chica. (a'-c') Data were analysed by autocorrelation over the 4 days of the experiment. (a') Surface, (b') Pachón, (c') Chica. Surface, Pachón and Chica show significant 24 hour rhythms in LD. In constant darkness no population shows clear autocorrelation with a circadian period, although negative autocorrelation with a 12 hour offset in surface fish is indicative of the presence of a behavioural rhythm, though this is absent in the second day of DD. Data for the figure was provided by Christophe Guibal and autocorrelation performed by Peter Krusche.

3.4 DISCUSSION

Astyanax mexicanus has established itself as an important evolutionary model system for the study of a wide variety of biological processes, not least because surface populations still live in the rivers alongside a series of isolated cave populations of the same species. This allows for direct molecular and physiological comparisons to be made between surface and cave individuals of the same species, and for statements to be made about how these processes have changed after millions of years in complete darkness. In this study, we have explored under laboratory conditions how the circadian clock has evolved in this species.

In the laboratory, surface fish showed a robust circadian oscillation in *per1* expression, similar to that previously reported in zebrafish and other teleost species (Vallone et al., 2004; Park et al., 2007; Velarde et al., 2009). When entrained to a light-dark cycle in the laboratory, cave strains of *Astyanax mexicanus* show clear circadian oscillations in *per1* expression, which free-run in constant darkness. They therefore still retain the molecular machinery necessary to generate a clock oscillation, as well as the capability to entrain to a light-dark cycle. This is the first documented light-entrainable molecular circadian clock in a cave animal and contrasts directly with the results from a recent study in another cavefish species, *Phreatichthys andruzzi* (Cavallari et al., 2011). This fact demonstrates that each cave species is likely to differ in its level of circadian clock function. *Astyanax* is unique in that it allows a deeper investigation into the specific nature of the clock changes during adaptation to the caves due to the fact that a direct comparison can be made with an ancestral form.

Whilst cavefish clearly retain a functional circadian clock, there are clear differences between surface and cave populations, the most striking of which is a delay in the phase of the *per1* rhythm relative to the light-dark cycle in cave animals. Additionally, the amplitude of the rhythm also appears reduced, and though it is not possible to obtain

reasonable estimates of clock period with such limited data sets, we can build a pattern of consistent changes that have occurred in the cave clocks during evolution and their possible causes.

3.4.1 Details of clock changes between surface and cavefish – phase difference

The phase difference between surface and cave populations in *per1* expression can also be seen in *cry1a* and *tef1* under free-run conditions. What could cause the phase delay of three clock-controlled genes? There are a number of potential mechanisms that lead to this phenotype, including alterations in the transcriptional control of those genes and alterations in the characteristics of the core oscillator.

Clock and Bmal control circadian transcriptional activation through binding to E-boxes in promoters. *Clock* and *bmal* are rhythmically expressed in zebrafish, a fact that led to the discovery of the decentralised organisation of the clock in fish (Whitmore et al., 1998; Cermakian et al., 2000). Since Clock is a transcriptional activator, a phase delay in *clock* expression in cavefish may drive E-box regulated expression later than in the surface fish. However, *clk1* expression rhythms peak at the same sampling time in cave and surface fish in LD, and only show the possibility of phase difference in DD (Figure 3.4). Differential expression of this transcriptional activator is not present. However, multiple *clock* and *bmal* genes are present in zebrafish and other teleosts (Wang, 2008b; 2009), so it is possible that other clock paralogues differentially regulate expression. The paralogous *bmal* genes are expressed differentially in zebrafish, indicating they may have a number of possible, as yet unexplored, roles in *Astyanax* (Cermakian et al., 2000; Ishikawa et al., 2002). However, as additional *clock* or *bmal* genes have not yet been discovered in *Astyanax*, we shall consider other explanations.

Differences in how the clock/bmal heterodimer activates downstream genes via promoter changes are another possible explanation. The context of the E-box elements in the promoter affects phase: a 6 hour phase delay is present in rhythms controlled by a 4xE-

box heterologous promoter compared to a *per1b* minimal promoter in zebrafish cells (Vallone et al., 2004), and a 47bp E-box containing fragment of the *Cry1* promoter is sufficient to drive rhythms with an appropriate 4 hour delay (i.e. similar to the endogenous *Cry1* rhythm) relative to *Per2* in mice (Fustin et al., 2009). It is possible that a similar alteration in the promoter environment in cavefish causes phase differences in expression. However, promoter changes would have to be present across multiple genes to see the phase differences between surface and cave populations.

The phase delays in *per1* and *cry1a* are present in *Astyanax* cavefish even in entraining conditions, which suggest a true change to the phase angle of the core clock. Phase of entrainment is a function of free-running period and the phase response curve (PRC) of entrainment for a given stimulus (Johnson et al., 2003); different period oscillators show different phase angles and one example is the earlier onset of activity shown by tau mutant hamsters (Ralph and Menaker, 1988). Altered free-running periods have been documented for other cave animals including the cave amphipod and the Somalian cavefish (Blume et al., 1962; Cavallari et al., 2011). Therefore, the presence of an oscillator with a reduced period and/or amplitude in cavefish is another explanation for the altered phase, though our data is not sufficient to confirm this. An extended study in *Astyanax* cavefish and surface fish with increased sampling frequency and cycle number is needed to test this prediction.

Interestingly, whilst F1 hybrid fish also have a phase delay of *per1* expression similar to Pachón cavefish in free-running conditions, this is not present in entraining conditions. F1 hybrid fish show a surface-like response to light, so the rescue seen in LD is likely to be masking. Masking of the rhythm by the light-dark cycle in F1 hybrid fish suggests that there are alterations in the light input pathway to the clock in cavefish population in addition to core clock changes, and each alteration exhibits differences in their dominant or recessive characteristics.

3.4.2 DETAILS OF CLOCK CHANGES BETWEEN SURFACE AND CAVEFISH – AMPLITUDE

It is clear from light pulse experiments performed in the lab that the circadian light input pathway has changed in cave strains of *Astyanax*. The acute light induction of *cry1a*, *per2a*, and *per2b*, genes known to be important for fish clock entrainment (Ziv et al., 2005; Tamai et al., 2007), is clearly altered in cavefish, with significantly raised basal levels of expression for all three genes. *Per2b* expression is consistently higher at all but one time points when samples are collected and compared over several days in continuous darkness. These results lead us to conclude that the light input pathway exists in a more activated state in cave populations, as if the fish were actually experiencing a constant light stimulus in the constant darkness.

It is a highly likely that these molecular changes in components of the light input pathway contribute to the phase differences seen between surface and cave populations in the lab, and especially to the reduced amplitude of the core clock oscillation. Constant light has been shown in zebrafish to "stop" the circadian oscillator, and over-expression of both *cry1a* and *per2* genes mimic this action of light (Tamai et al., 2007). Both Cry1a and Per2 act as strong repressors of CLOCK-BMAL protein function, reducing their transcriptional activity, and thus leading to a decrease in *per1* gene expression (Hirayama et al., 2003; Tamai et al., 2007).

What could cause an increase in the basal levels of light responsive genes? Zebrafish *cry1a* and *per2* possess D-boxes in their promoters suggesting the involvement of D-box binding factors in their regulation (Weger et al., 2011). One candidate is the PAR-bZIP transcription factor, Thyrotroph embryonic factor (Tef), a light-induced gene that directs light-driven expression of these genes, as well as a number of other light-regulated genes in zebrafish (Vatine et al., 2009; Gavriouchkina et al., 2010; Weger et al., 2011). However, *tef1* is not significantly light induced in our experiments and does not show higher expression in cavefish. *Tef1* also shows strong circadian clock regulation in contrast to its expression in zebrafish (Vatine et al., 2009; Gavriouchkina et al., 2010). These differences

together suggest it is unlikely to mediate the increased basal levels of *per2b*. However, 11 other D-box transcription factors have been identified in zebrafish, which may have roles in the circadian clock mechanism (Ben-Moshe et al., 2010). Of interest in this study are members of the *e4bp4* repressors, which could direct repression of the light-regulated genes in darkness (Doi et al., 2001). Reduced expression of the *e4bp4* genes in cavefish would potentially explain the increased expression of *per2b* in constant darkness, and needs to be tested.

Interestingly, as previously mentioned, the F1 hybrid of surface and Pachón cavefish shows a mixture of these two conditions. F1 fish show high amplitude oscillations in *per1* in LD, with a rescue of light induction of *cry1a*, *per2a* and *per2b* and corresponding reduction in their basal levels of expression. These results suggest that alterations in the light input pathway are recessive and reversed within one cross or generation, but core clock changes may be more complex.

3.4.3 THE INCREASED AMPLITUDE OF CLOCK OUTPUTS IN CAVEFISH

Finally, cavefish show an apparent increase in amplitude of rhythm of *tef1* expression. In the context of a reduced amplitude oscillator as shown by *per1* rhythms, how can a greater amplitude rhythm of a gene be generated in cavefish? Promoter context affects the strength of binding, as a specificity of Clock/Bmal for an 'A' in the +4 position of an E-box construct shows (Hogenesch et al., 1998). Therefore, promoter changes in this gene may contribute both to the altered phase and the increased amplitude.

3.4.4 CLOCKS IN CAVES

The current study shows that, in general, the cavefish molecular clock appears less robust than that found in equivalent surface individuals with modifications to the core clock and the light input pathway. Cavefish also show a significantly weaker locomotor rhythm than surface fish and in contrast to surface fish, neither cavefish population shows locomotor rhythms in DD and only weak rhythms in LD. However, previous studies suggest that

Astyanax cavefish (Pachón) do in fact possess clock-controlled locomotor rhythms, though they are much weaker and more variable than surface fish (Erckens and Martin, 1982a; 1982b). It is interesting to note that the few studies of circadian rhythms in cave animals have used locomotor activity to examine clock function. These previous studies have revealed a range of circadian phenotypes in other cave animals. Some retain at least partially functioning oscillators (Jegla and Poulson, 1968; Pati, 2001), whilst others appear to lose them (Blume et al., 1962; Hervant et al., 2001). However, circadian rhythms of locomotor activity are not always clear in the raw data in these studies and conclusions are often drawn from subsequent analysis (for example Mead and Gilhodes, 1974; Erckens and Martin, 1982b; Pati, 2001). Furthermore, the observation of clock controlled rhythms is also often sensitive to assay type, such as separately measuring components of total activity at the surface or bottom of the tank (Erckens and Martin, 1982a). Despite these caveats, it is possible to suggest for *Astyanax* that selection in the cave has weakened the strength of coupling between molecular and behavioural oscillators. Circadian rhythms of behaviour may not be important in the cave due to the absence of external signals, and loss of this circadian output may in fact be advantageous for the animal. Supportive of this is the fact that *Astyanax* cavefish show a general trend of increased activity throughout the day and reduced sleep compared to surface fish (Erckens and Martin, 1982b; Duboué et al., 2011), which is proposed to improve chances for foraging in a highly food restricted environment.

The persistence of a light-entrainable molecular oscillator in *Astyanax* cavefish is therefore intriguing. The only other study on molecular clocks in cave animals is a recent report for the Somalian cavefish, *Phreatichthys andruzzii* (Cavallari et al., 2011). The *Astyanax* phenotypes are less dramatic than those reported for *P. andruzzii*, which have completely lost the capacity to entrain to a light-dark cycle (Cavallari et al., 2011). *P. andruzzii* possess mutations in the candidate circadian photoreceptors, TMT opsin and melanopsin, a fact that potentially contributes to their 'blind' circadian phenotype (Cavallari et al., 2011).

However, the Somalian cavefish are able to entrain their molecular clock to a scheduled feeding regime, indicating that they also retain clock function. This fact demonstrates that each cave species is likely to differ in the precise details of its circadian clock function, though elements of the circadian light input pathway are likely 'targets' for evolutionary change. The persistence of molecular oscillators in these two cases provide support for the importance of the 'day within' hypothesis to the adaptive importance of circadian clocks (Pittendrigh, 1993; Sharma, 2003). It would be interesting to see if other cave animals retain molecular clock function, and to what extent it is responsive to light.

Astyanax cavefish retain a light-entrainable clock but it is clearly altered with respect to the light input pathway. Could there be any selective advantage for cavefish of 'perceiving' sustained light exposure? Light has a wide impact on gene expression and physiology in fish (Gavriouchkina et al., 2010; Weger et al., 2011). Consequently, one would predict that isolation in a cave would have an influence on a range of biological processes in these animals. This will be discussed further in Section 6.

3.4.5 THE EVOLUTIONARY RELATIONSHIPS OF CAVEFISH

Whilst the functional significance on the clock of the amino acid changes seen in multiple clock genes have not been analysed in detail in this study, their significance with regard to phylogenetic relationships of the caves can be discussed. Two competing hypotheses emerge. The first is that the changes reflect a remarkable convergence in molecular sequence in independent cave populations. This view is supported by Bradic et al. (2012), who present evidence to suggest the Pachón, Chica and Tinaja populations represent three independent invasions of the caves by a single surface fish ancestor. Alternatively, the very similar sequence changes reflect a true close relationship between these cave populations, which possibly extends to a single invasion into a cave environment and subsequent underground dispersal (Espinasa and Borowsky, 2001). However, this is unlikely and most recent studies support the multiple origin hypothesis, though there is some debate about the exact relationships between caves (Dowling et al., 2002; Strecker et al., 2003;

2004; Hausdorf et al., 2011; Bradic et al., 2012). The multiple origin hypothesis agrees with the geographical distribution of the caves, which are separated by drainage basins, mountain ranges, and vertical height (Mitchell et al., 1977).

A final note is of the identity of the surface fish used in this study. Firstly, the surface fish itself has been undergoing evolution in the rivers whilst the cavefish have in the caves. The cave 'changes' may therefore represent the ancestral form of the gene, whilst the surface fish has evolved. Alternatively, the surface fish may belong to a 'new' stock of surface fish that replaced the original surface fish stock, after it invaded Mexico around 1.8 million years ago (Strecker et al., 2004; Bradic et al., 2012). If this is the case, the true ancestor of the cave populations used in this study may therefore have gone extinct locally and we are therefore restricted in comparing two more distantly related populations of the species (it would also suggest that the cave colonisation events occurred at the upper estimate of the divergence times between the multiple surface and cave populations). This relationship between 'old' surface fish and cavefish is not universally accepted however, as studies based on mitochondrial DNA suggest close relationship between some caves (for example, Chica cave) and the surface fish in the surrounding rivers (Dowling et al., 2002; Hausdorf et al., 2011). Intriguingly, in all phylogenetic studies, Tinaja cavefish clusters with phylogenetically 'old' cavefish and separate from the surrounding surface fish (Dowling et al., 2002; Strecker et al., 2003; Bradic et al., 2012). Therefore, the very similar Cry1a sequence between Tinaja, Pachón and Chica cavefish adds support for the phylogenetically 'old' position of Pachón and Chica cavefish and agrees with the conclusions made by Bradic et al. (2012). A detailed examination of the genomes of the multiple populations of cavefish and surface fish would make a very interesting study indeed.

4 EXAMINATION OF THE CIRCADIAN SYSTEM OF *ASTYANAX MEXICANUS* IN THE FIELD

The results presented in this chapter were performed in collaboration with Christophe Guibal and David Whitmore.

4.1 INTRODUCTION

Cave animals have been used to investigate the adaptive and evolutionary significance of circadian clocks due to their permanent removal from environmental light and dark cycles. Many studies have taken these animals from caves and replicated cave conditions in the laboratory, which is comparatively easy due to the inherent stable nature of the cave environment. Cave forms from across a broad sample of the Animal Kingdom exist, and show a variation of circadian clock phenotypes. Investigation of cave clocks has primarily involved the observation of activity rhythms. Results have suggested an apparent absence of the clock in cave amphipods (Blume et al., 1962) and cave salamanders (Hervant et al., 2001), retention in cave crayfish (Brown, 1961; Jegla and Poulson, 1968), cave crickets (Reichle et al., 1965) and cave loaches (Pati, 2001), and highly variable phenotypes from animal to animal in cave millipedes (Mead and Gilhodes, 1974; Koilraj et al., 2000). Physiological studies have also revealed a varied range of phenotypes: circadian rhythms of retina pigment migration are preserved in *Astyanax* cavefish (Espinasa and Jeffery, 2006), but the molecular clock of the Somalian cavefish is degenerate (Cavallari et al., 2011).

However, as recently demonstrated in *Drosophila*, compared to the controlled environments of the laboratory, expression of the circadian clock is often different in the field (Vanin et al., 2012). For all previous cavefish studies in the lab, it has been assumed that the lack of a light-dark cycle in the only major environmental change. This is far from the truth, with alterations in pH, dissolved oxygen levels, food availability, and more, also changing between river and cave environments. Field studies for cave animals are therefore very important to both assess the true nature of the clock in these conditions and fully determine the adaptive significance of the circadian clock. Somewhat surprisingly then, circadian field studies in general are rare. One famous study to show the advantage of the circadian clock was the observation of the lower survival of SCN-lesioned

chipmunks compared to SCN-intact controls (DeCoursey et al., 2000). Conversely, other studies have focused on arrhythmic environments and the absence of circadian rhythms and by extension, the lack of advantage provided by the circadian clock in these environments. This is suggested to be the case in reindeer living above the Arctic Circle which display absence of circadian rhythms at certain times of year (van Oort et al., 2005). It appears, therefore, that synchronisation with the environment is only important in those environments where the cycles are regular, and so circadian clocks are lost. Though this explanation makes no consideration for the importance of internal 'temporal order', which is suggested as an alternative or additional adaptive function of the clock. There have been no reported field studies on circadian clock function in fish in rivers and certainly not within cave complexes.

We have already demonstrated that *Astyanax* cavefish have the capacity to entrain their molecular clock to cycles of light and darkness, but this doesn't appear to manifest in circadian rhythms of activity. Given the difference between lab and field studies, we extended our analysis of the *Astyanax* clock to its natural environment: the river and the cave.

4.2 METHODS

4.2.1 FIELD STUDIES

Field exercises were conducted annually in March between 2007 and 2010 by a team led by Professor David Whitmore and Dr Christophe Guibal. A description of the methods used in collecting tissue samples from the Micos River and Chica cave can be found in Section 2.2. Total RNA was extracted from individual samples, cDNA synthesised and qPCR carried out as described in Sections 2.4 and 2.6. Primers used in qPCR are shown in Section 2.6, Table 2.2**.** Data for the laboratory sampled surface and Chica cavefish is reproduced from Section 3.

4.3 RESULTS

4.3.1 THE CIRCADIAN CLOCK IS SUPPRESSED IN WILD CAVEFISH POPULATIONS

Studying cavefish clock biology in the laboratory, especially following light entrainment, is obviously an unnatural scenario and does not answer the more relevant question of what is happening in the actual caves themselves. We have therefore attempted, over several seasons of field trips to Mexico, to follow circadian clock function under the natural river and cave conditions. This is a far more challenging experimental situation than collecting samples in the laboratory and is prone to considerably more natural variation, with less control of environmental variables. Surface fish were fin clipped in the Micos River (+22° 6' 59.01" N, -99° 10' 16.8" W) at six time points per day over two days. Figure 1.1a shows the rhythm in the core clock gene *period1* (*per1*) expression in this wild river population. Although the light intensity at the water surface of a Mexican river is far greater than that achievable in the lab (Figure 1.2a), and this varies considerably both in intensity as well as spectral quality with time of day (Figure 1.2b-d), the relative timing of this oscillation matches that seen under laboratory conditions. *Per1* peaks roughly in the late night/early morning and reaches a low point of expression in the early evening, just after sunset.

The majority of our cave studies have focused to date on the Chica cave, 10 km south of Ciudad Valles in the Mexican state of San Luis Potosí (+21^0 51' 36.05" N, -98^0 56' 10.00" W). Of all the caves examined, we believe that this location has the highest probability of containing a rhythmic population of fish, mainly due to the large and rhythmic bat population (Figure 1.2e and Mitchell et al (1977)). However, as can be seen in Figure 1.1b, for samples collected every six hours over two days, there is no significant oscillation in the levels of *per1* expression (ANOVA, n=30, $p>0.05$). We also examined the expression of the transcriptional activator, *clock1* (*clk1*), in these cavefish. As expected, *clk1* is also arrhythmic in Chica cave (Figure 1.1c; ANOVA, n=30, $p>0.05$). *Astyanax* in this cave do not show an entrained molecular oscillation within the population.

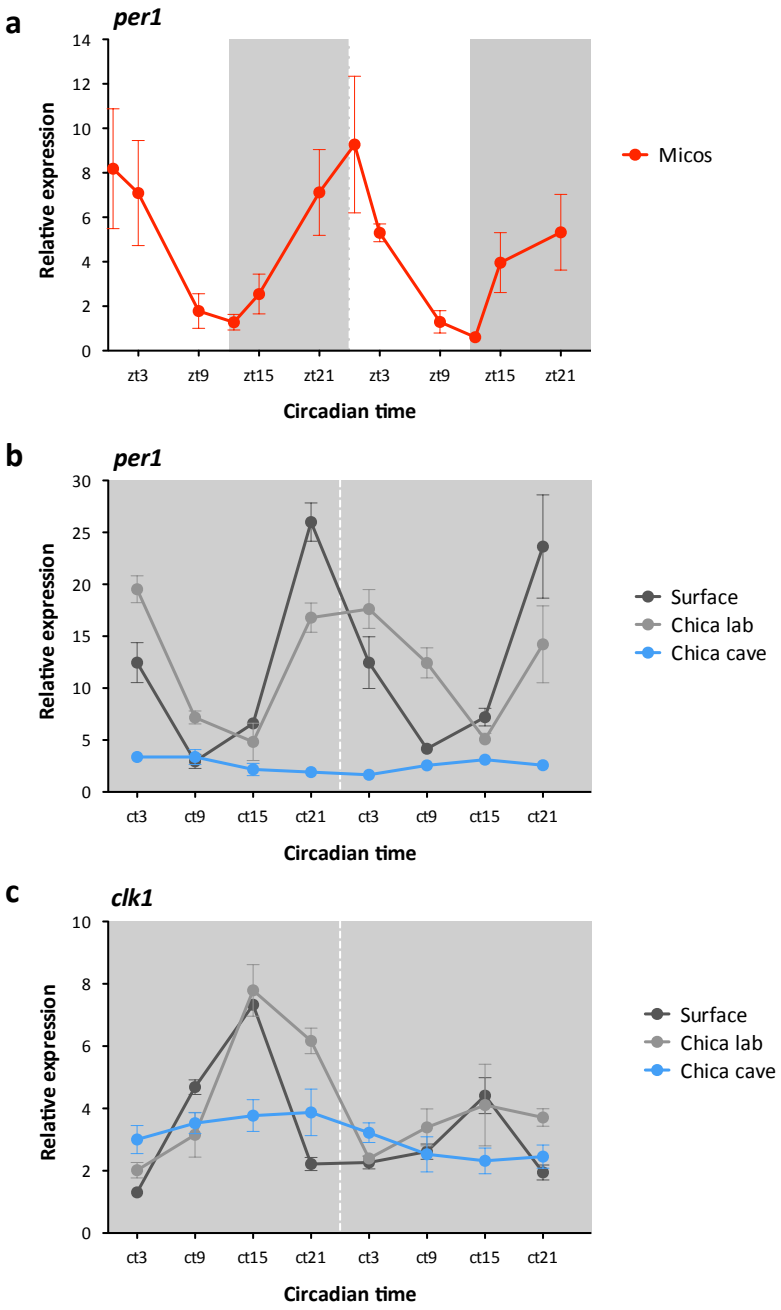

Figure 4.1: Expression of *per1* is rhythmic in surface fish but not in Chica cavefish in the wild.

(a) Fin samples from surface fish from the Micos River (+22° 6' 59.01" N, -99° 10' 16.8" W) were collected at six time points over two days. *Per1* expression was measured by qPCR and normalised to the reference gene *rpl13α*. The relative expression of each gene was calculated and plotted using the ΔΔCt method. Peak and trough values on each day are significantly different (ANOVA, n=64, $p<0.01$; t-test: day 1 ZT12.5 vs. ZT21, n=10, $p<0.05$; day2 ZT0.5 vs. ZT12.5, n=11, $p<0.05$). White and grey bars indicate light and dark periods, between sunrise and sunset, respectively. At this latitude in March, the photoperiod was almost exactly 12 hours light: 12 hours darkness. (b and c) Fin samples from four fish

were collected in the Chica cave every 6 hours over two days. As references for day times do not exist within the cave, samples from the Chica cave are plotted chronologically from the start of sampling (1pm) against entrained laboratory samples for comparison of relative expression. (b) *Per1* mRNA levels, measured by qPCR, are shown in blue and plotted relative to constant dark samples from surface and Chica cavefish entrained in the laboratory (reproduced in grey from Section 3). No significant difference exists between time points (ANOVA, n=30, $p>0.05$). (c) *Clk1* mRNA levels were measured by qPCR in the same samples as (b). No significant difference exists between time points (ANOVA, n=30, $p>0.05$). Data represent the mean ± SEM of at least 4 different fish. Samples for (a) and Chica cave samples in (b) and (c) were collected by David Whitmore and Christophe Guibal. qPCR and analysis for (a) was performed by Christophe Guibal.

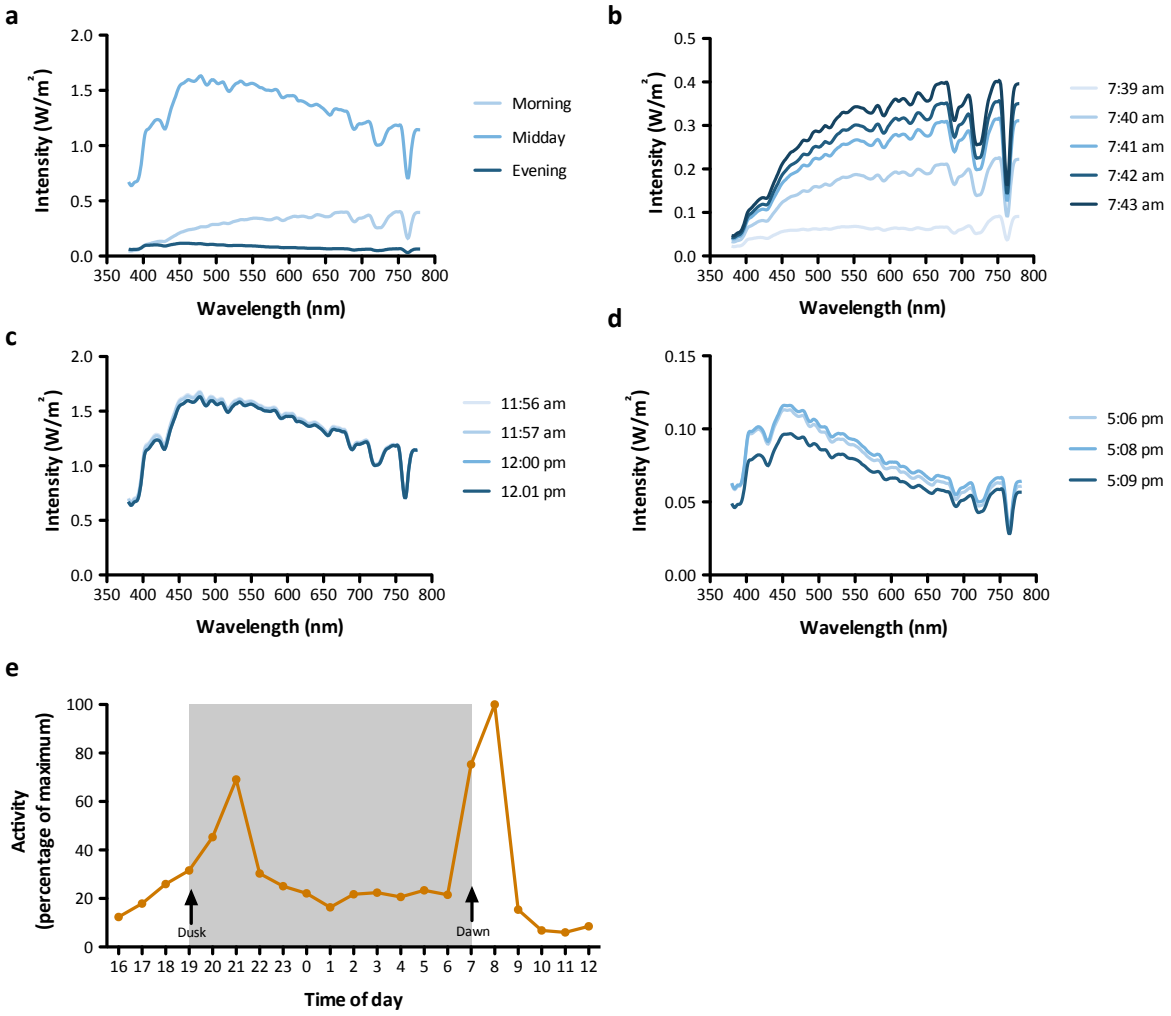

Figure 4.2: Environmental conditions at the Micos River and Chica cave.
(a-d) Intensity and spectral quality of light at the Micos River in Mexico. Light intensity and spectral quality was measured at the fish sampling point of the Micos river at the water surface. Intensity is plotted as Watts/m^2. The upper panel (a) provides an example of how light intensity varies over the day, during the experimental period, from sunrise, across midday until just prior to sunset. Panels (b), (c) and (d) track the light intensity change as the sun rises over the water surface over a short 3 to 5 minute period. The spectral quality of the light appears more "blue" biased in the evening, compared to the "red" bias of dawn. (e) Bat activity in the Chica cave in Mexico. Video cameras were placed within the cave tunnel, and bat activity was filmed over a 24 hour period. Filming started at 4pm (16:00) and the number of bats crossing the field of view is counted and binned into 1 hour windows. Dusk occurs at approximately 19:00, and corresponds to an evening increase in bat activity as individuals leave the cave. A second burst of activity is seen just prior to dawn as the bats return to their roost. Data provided by Christophe Guibal.

4.3.2 EXPRESSION OF LIGHT INDUCED GENES IS ALTERED IN WILD CAVEFISH

The general expression levels of *per1* are significantly lower in individual cave animals under natural conditions than those measured in surface fish, or equivalent cave strains within the lab. This is highly reminiscent of constant light or *cry1a* overexpression in zebrafish cells (Tamai et al., 2007). We have already demonstrated that the light-input pathway is altered in *Astyanax* cave populations in the laboratory, and seems to exist in a more activated state. Is this also the case in the natural conditions of the cave?

To answer this question, we examined the expression of *cry1a*, *per2a* and *per2b* in the samples from the Chica cave (Figure 1.3). Whilst the expression of *cry1a*, *per2a* are not significantly higher relative to laboratory surface and Chica cavefish samples, the expression of *per2b* in the cave field samples is at a raised level relative to laboratory surface fish (Figure 1.3c). This is very similar to that described for cave strains examined under lab conditions. We believe that the lack of rhythmicity seen in wild cave animals is unlikely to be due to the absence of sufficiently strong entraining signals within the cave, as the expression of *per1* is not at the mid-level one would predict from a rhythmic, but asynchronous population of individuals. Rather, it is clear that the core clock mechanism is tonically repressed or dampened in the cave, probably because of the basal activation of the light input pathway, especially *per2b*.

As a candidate regulator of *per2* expression in zebrafish (Vatine et al., 2009), we examined the expression of *tef1* in the cave samples (Figure 1.3d). However, this is not significantly raised in Chica fish from the cave and so, like observed in samples from the laboratory, is not likely to regulate upregulation of *per2b* in the manner proposed for zebrafish.

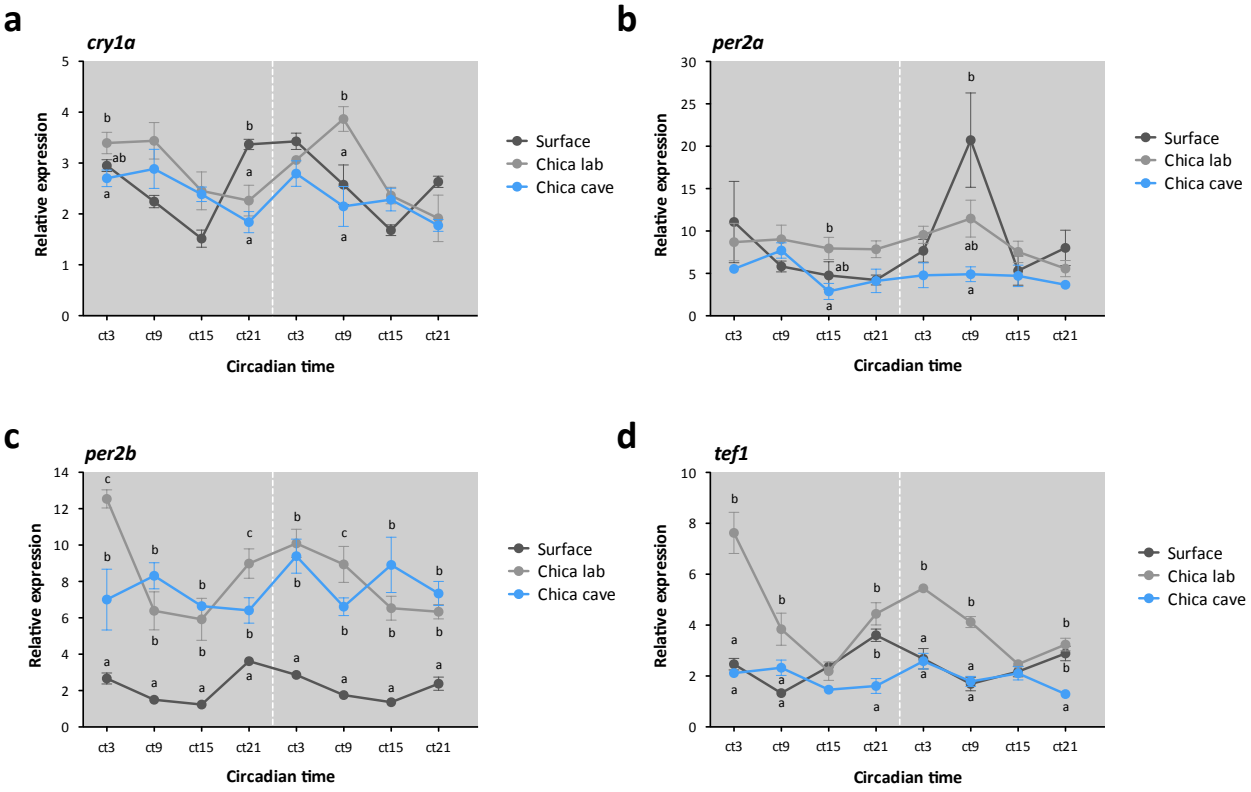

Figure 4.3: *Per2b* is expressed at increased levels in Chica cavefish under natural conditions.

Fin tissue samples were collected from Chica cavefish in the wild every 6 hours over two days. Expression of (a) *cry1a*, (b) *per2a*, (c) *per2b* and (d) *tef1* levels were measured by qPCR and normalized to the reference gene *rpl13α*. In the absence of clear zeitgebers in the cave, Chica cave samples are plotted chronologically from the start of sampling (1pm) relative to constant dark samples from laboratory-entrained surface and Chica cavefish (reproduced in grey from section 3) for ease of comparison. Expression levels were compared between all populations at each time point in DD by ANOVA followed by Newman-Keuls multiple comparison tests. Different lower case letters indicate significant differences ($p<0.05$) between comparisons. Time points with no significant difference ($p>0.05$) have no letters. *Per2b* is significantly more expressed in Chica samples from both the lab and field compared to surface fish. Samples for the Chica cave were collected by David Whitmore and Christophe Guibal. Data represent the mean ± SEM of 4 different fish per time point.

4.4 DISCUSSION

Investigations of circadian clocks in cave animals are relatively rare despite their huge potential for elucidating the adaptive significance of clocks and how they can evolve. Those few studies of cave clocks have exclusively focused on cave animals kept in the laboratory. How, then, is the circadian clock expressed in the wild?

We have previously demonstrated that robust *per1* expression rhythms exist in surface fish in laboratory conditions. A similar rhythm is apparent in surface fish under natural conditions in the Micos River. Fin samples were collected in March over several years, when the sunrise and sunset times create an almost perfect 12hr:12hr light-dark cycle, and no temperature fluctuations greater than 0.5°C were detected in the river. Though the fundamental timing of the oscillation is very similar, there is greater variation within the wild animal data set. There are several possible explanations for this, including greater genetic variation in the wild compared to the lab population (in contrast to the much smaller genetic diversity observed in cave populations (Bradic et al., 2012)), as well as differences in animal health and age. The light intensity at the water surface of a Mexican river is far greater than that achievable in the lab (Figure 1.2), and this varies considerably both in intensity as well as spectral quality with time of day (Figure 1.2). Together, these factors will have an impact on gene expression patterns under natural conditions.

For samples collected within Chica cave, the dampened *per1* expression seen in the laboratory is even more exaggerated, as no *per1* or *clk1* rhythm was detected over a two-day sampling period. Thus, there appears to be no detectable circadian rhythm within the native cavefish population. These data are representative of studies performed over several years of fieldwork in this cave between 2007 and 2010. At first thought, this lack of measureable rhythmicity could be due to the lack of an adequate entraining cue within the cave, with the obvious absence of a light-dark cycle. Monitoring water quality over several days in the cave showed no circadian variation in temperature, dissolved oxygen, pH, or nitrates. Chica cave does, however, have a large and highly rhythmic bat population, with

clear bursts of activity around dawn and dusk as bats enter and leave the cave and thus is considered to have the most rhythmic environment of all the caves in this area of Mexico (Figure 1.2e and Mitchell et al (1977)). The bats spiral over the underground ponds prior to exiting, and we hypothesised that this activity may provide an entraining signal to the fish in a variety of ways, including providing a feeding cue to the fish through rhythmic defecation (López-Olmeda et al., 2010; Feliciano et al., 2011). However, our *per1* expression analysis has failed to produce any evidence that this occurs. Rhythmic bat activity and a likely feeding cue does not appear to provide a strong enough zeitgeber to entrain the clock under natural conditions. A lack of overt clock function at this location makes it very unlikely that other cave populations, such as Pachón where there are even fewer possible entraining signals, will show any circadian rhythmicity.

A closer examination of *per1* expression in the cave field samples (Figure 1.1b) reveals that the actual levels are, in fact, much lower than those seen in surface fish or in cave strains in the lab. This very low transcript level is unlikely to be the consequence of simple asynchrony or lack of clock entrainment, but more reasonably reflects actual repression of core clock function. Taken together with the absence of clock oscillations in the actual cave environment, this supports the hypothesis that the oscillator is actually not "running" under these conditions.

Like the results from laboratory-kept fish, this situation draws parallels with constant light treatment of zebrafish cells, which represses *per1* expression through the raised expression of *cry1a* (Tamai et al., 2007). Unlike the results in zebrafish, *cry1a* is not significantly raised, but in wild cave animals *per2b* is. Although mechanistic data for the repression of clock driven expression by *per2* has not been as extensively explored for *per2* as *cry1a* in zebrafish, unpublished data in the lab has shown that overexpression of *per2* does lead to a repression of the *per1* rhythm (Tamai et al., personal communication). In addition, it is clear that Per2 has a key role in mediating light input to the clock downstream of a Tef and D-box mechanism (Ziv et al., 2005; Ziv and Gothilf, 2006; Vatine

et al., 2009). It is therefore highly likely that raised expression of *per2b* leads to a repression of the *per1* rhythm in cavefish, although this appears to be through a non-tef mechanism in *Astyanax*. However, it is clear that this level of expression of *per2b* cannot be wholly responsible for the very low level of *per1* in cave samples, as similar *per2b* expression levels exists between cave and lab Chica cavefish despite the much lower *per1* expression in the cave samples. It is clearly probable that the complete absence of light has further effects on the *per1* expression to lead it to this low level (possibly through additional unknown factors).

These results strengthen our conclusions that the light input pathway exists in a more activated state in cave populations, and suggest the fish in the cave actually experience something similar to a constant light stimulus rather than perpetual darkness. We examine possible consequences of this condition with relation to other light-regulated processes in Section 6.

5 DEVELOPMENT OF THE CIRCADIAN SYSTEM AND LIGHT RESPONSE IN *ASTYANAX* SURFACE AND CAVE POPULATIONS

5.1 INTRODUCTION

How and when the circadian clock starts oscillating are questions often asked in circadian biology. Teleosts, in particular zebrafish, are ideal for answering these questions due to their external development, the large number of embryos produced each morning and their direct light sensitivity. In zebrafish, the embryonic clock begins on the first day (Delaunay et al., 2000; Dekens and Whitmore, 2008), with clock controlled outputs such as locomotor activity, melatonin release and circadian rhythms of the cell cycle appearing later (Hurd et al., 1998; Kazimi and Cahill, 1999; Dekens et al., 2003). The first studies suggested that the embryonic clock is synchronous and oscillating even within oocytes, with circadian phase inherited from the mother (Delaunay et al., 2000), though subsequent reports have not reproduced this phenomenon (Dekens and Whitmore, 2008). It is now thought that though the embryonic clock commences on the first day, it oscillates asynchronously between cells in the embryo and requires an environmental transition or cycle to become synchronous across the whole animal (Ziv et al., 2005; Vuilleumier et al., 2006; Dekens and Whitmore, 2008).

These environmental cycles are able to set and influence the circadian clock very early in development, before the appearance of potential master clock or light sensitive structures or for that matter any clear cellular differentiation (Whitmore et al., 2000; Ziv and Gothilf, 2006; Dekens and Whitmore, 2008). Thus, the zebrafish embryo is able to detect light and pass on timing information through cell division and differentiation. As with the circadian clock as a whole, key regulators of the light response in the early embryonic clock are *cry1a* and *per2* which, when knocked down, eliminate the light-dependent entrainment of the core clock (Ziv et al., 2005; Ziv and Gothilf, 2006; Tamai et al., 2007). Upstream of these factors, in particular *per2*, is *tef*, which is able to potentiate the transcription of these genes in response to light (Gavriouchkina et al., 2010). Although knock-down does not completely eliminate light-dependent induction (Vatine et al., 2009; Gavriouchkina et al.,

2010; Weger et al., 2011), *tef* certainly plays a significant role in the early embryonic response to light.

Why does the zebrafish embryo perceive light so early in development? Apart from the aforementioned role in clock setting, light is able to influence a broad range of biological processes in zebrafish (Tamai et al., 2004; Gavriouchkina et al., 2010; Weger et al., 2011). It is crucial for the development of light-regulated DNA repair processes, such as the induction of photolyases, which enhance the early embryo's survival and protect against exposure to UV light during morning spawning (Tamai et al., 2004). The early embryonic detection of light also regulates the circadian timing of cell cycle events in the later embryo, and coordinates rhythms of locomotor activity and melatonin release (Kazimi and Cahill, 1999; Hurd and Cahill, 2002; Dekens et al., 2003). In these ways, light plays a key role in zebrafish development and survival.

We have already reported that the Mexican cavefish, *Astyanax mexicanus*, retains circadian clock function and the ability to detect light in the laboratory despite them being isolated underground for millions of years. *Astyanax* embryos retain the capacity to detect light as early as 1.5 days post fertilisation (dpf) with regards to pineal function (Yoshizawa and Jeffery, 2008); do early embryos have the capacity to detect light as early as zebrafish embryos and is this retained in cavefish? Differences in clock gene regulation have been reported between zebrafish embryos and adults, for example in the regulation of transcription of *clock* and *bmal* (Dekens and Whitmore, 2008). Are the differences seen in clock regulation between populations of adult *Astyanax* fish also seen during embryogenesis? How early is the characteristic phase and amplitude difference apparent? To answer these questions, we have examined clock gene expression in *Astyanax* embryos under light-dark cycles and constant darkness, and have examined the acute induction of genes in embryos exposed to light pulses throughout early development.

5.2 METHODS

5.2.1 BIOLOGICAL MATERIALS AND EMBRYO MAINTENANCE

Embryos were obtained as described in Section 2.1.2. Experiments were performed as described in Section 2.3.2, with Total RNA extraction and cDNA synthesis as described in Section 2.4.

5.2.2 QUANTITATIVE RT-PCR

Quantitative PCR was performed as described in Section 2.6 and the primers used are shown in Section 2.6, Table 2.2.

5.2.3 WHOLE MOUNT IN SITU HYBRIDISATION

Whole mount in situ hybridization was performed as described in Section 2.8. Light-pulsed and dark control embryos of surface and Pachón fish were developed for the same amount of time in order to assess relative expression levels across population and light treatment.

5.2.4 CLONING *ASTYANAX TELEOST MULTIPLE TISSUE OPSIN*

A full description of the method for cloning *Astyanax* genes can be found in Section 2.5. Briefly, a degenerate reverse transcriptase PCR (RT-PCR) approach was taken to initially isolate fragments of clock genes from *Astyanax mexicanus*. A fragment of *tmt1* was amplified from cDNA from *Astyanax* embryonic cell lines (creation described in Section 2.1.3) using degenerate primers for novel members of the *tmt1* class of opsin genes, Forward-1086-TMT1F1 (5'-TAGTGCTCGTGYTKTTYTGYAARTTYAAGA-3') and Reverse-1087-TMT1R2 (5'-SMGGAAACACYTGTARAACTGYTTGTTCAT-3'), which were designed by Wayne Davies. RACE PCR using 5' and 3' RACE libraries created from *Astyanax* embryonic cell lines was used for the subsequent extension of the initial PCR fragments (5' RACE: first round, 1119-asty TMT race2-5'-GTCCGGGGCCTGTTTATTAT-3'; nested round, 1118-asty TMT race1n-5'-TACGATTCCGAAGCAGGAGT-3'; 3'RACE: first round, 1120-asty TMT race3-

5'-ACCAGCTGTTCCGTTACCTG-3'; nested round, 1121-asty TMT race4n-5'-GTGCTATCTGGTGTGCTGGA-3'). The predicted coding region of *tmt1* was amplified from *Astyanax* embryonic cell lines using specific primers 1129-su TMT1 start1 (5'-TCGTTCAGAGGGATCGTACC-3') and 1132-su TMT1 stop2 (5'-GACGCCCATGAATGACTTCT-3'). The identity of isolated cDNA sequences was determined by the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/) and phylogenetic analysis performed in collaboration with Wayne Davies.

5.3 RESULTS

5.3.1 DEVELOPMENT OF THE CIRCADIAN CLOCK IN *ASTYANAX* SURFACE AND CAVEFISH

The circadian clock of zebrafish begins on the first day of development. Even before any anatomical features are present, light is able to set and synchronise the cellular clocks throughout the embryo (Ziv and Gothilf, 2006; Dekens and Whitmore, 2008). To see if an early embryonic clock is present in *Astyanax*, we analysed the expression of *per1* in early embryos maintained on a light-dark cycle. Development proceeds at the same rate between surface fish and cavefish embryos, so close comparisons of the origins of the circadian clock in relation to developmental stage and environmental conditions can be made. As for zebrafish, we observe rhythms of *per1* expression during the first days of development (Figure 5.1). Surface fish embryos show a *per1* expression rhythm very similar to that of adult fish, peaking at ZT21. Interestingly, the rhythm in Pachón embryos peaks at the same time as surface fish, unlike the expression pattern seen in adult fins. However, the characteristic difference in rhythm amplitude is present, even at this very early stage.

5.3.2 LIGHT IS NOT REQUIRED FOR ONSET OF *PER1* EXPRESSION AND RHYTHMICITY

Although cavefish spawning has never been observed in the wild, it is certain that cavefish embryos develop in the dark. To reflect this situation, we examined *per1* expression in embryos raised in constant darkness and constant temperature. Surprisingly, in the

absence of any environmental signal from fertilisation, rhythms of *per1* expression are still seen in both surface and Pachón cavefish (Figure 5.2). Similar to the situation in LD cycles, the amplitude of the rhythm in Pachón embryos is reduced compared to surface embryos. However, the difference in amplitude of the *per1* rhythm between surface and Pachón is smaller in DD than it is in LD conditions (Day 2 LD: surface, 24.38-fold; Pachón, 6.05-fold. Day 2 DD: surface, 5.06-fold; Pachón, 2.57-fold), mainly due to higher *per1* expression at 33 hpf and 39 hpf in surface, which greatly reduces the peak:trough amplitude. In general, Pachón embryos show higher levels of *per1* expression in DD compared to LD (for example at 21 hpf, *per1* is expressed 6.15-fold higher in embryos maintained in DD than embryos maintained in LD; Figure 5.2a'). Surface embryos show higher minimum or trough expression of *per1*, reflecting the importance of light in generating high amplitude rhythms, as suggested for zebrafish by Dekens and Whitmore (2008).

We next looked at components of the light input pathway to see if they have a role in the differences seen between surface and Pachón embryos.

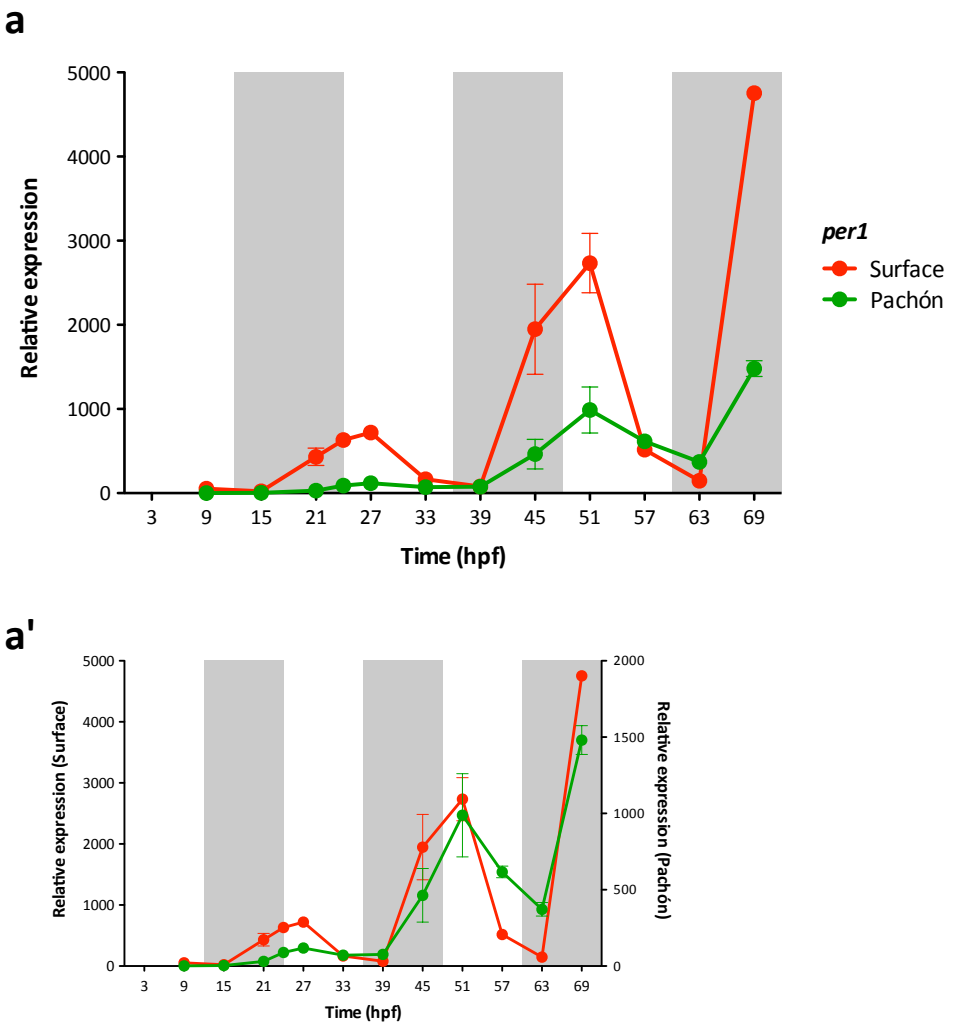

Figure 5.1: *Per1* is rhythmically expressed during development.
(a) Surface and Pachón embryos were collected, sorted into flasks, placed in a water bath and exposed to a 12hr:12hr LD cycle. Flasks were taken every 6 hours for 3 days from 9 hours post fertilisation (hpf), with an additional sample at 24 hpf (before lights on). *Per1* mRNA levels were measured by qPCR, normalised to the reference gene *ef1α* and relative expression was calculated using the ΔΔCt method. (a') *Per1* mRNA levels in Pachón from a) are plotted on a secondary axis for ease of comparison. White and grey bars indicate light and dark periods respectively. Data represent the mean ± SEM for 3 embryo samples.

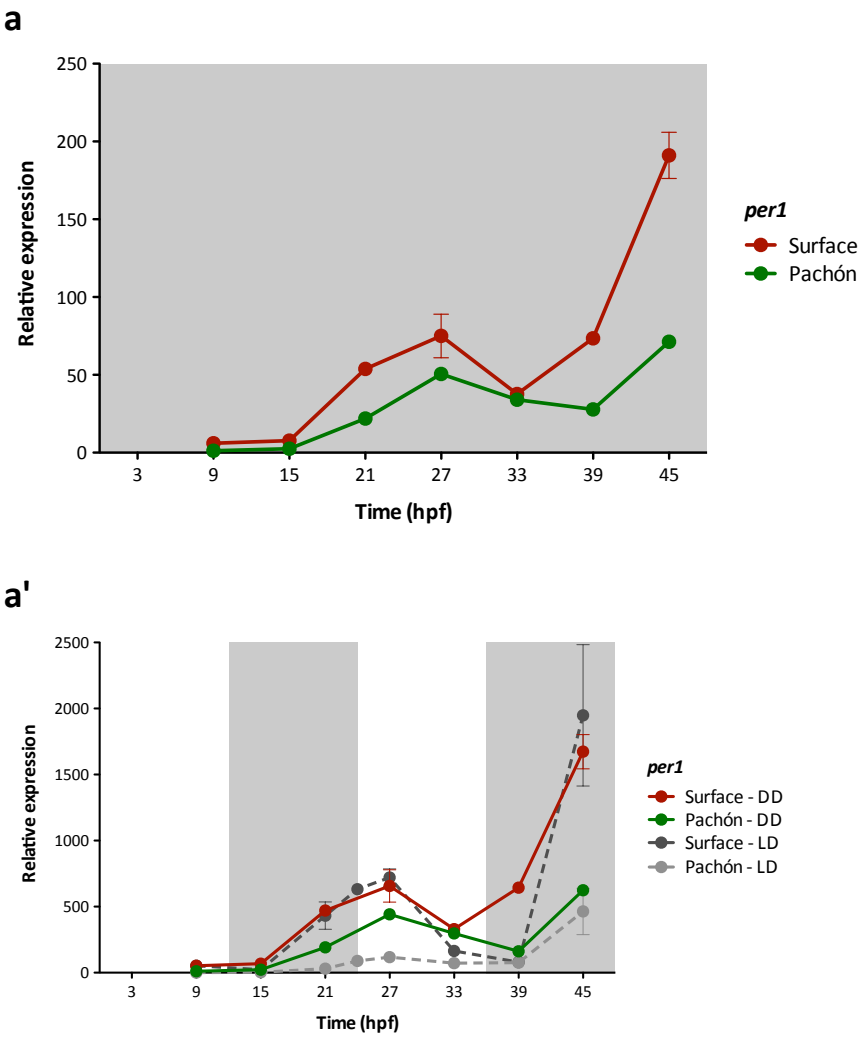

Figure 5.2: *Per1* is rhythmically expressed during development in the absence of environmental cycles.

(a) Surface and Pachón embryos were collected, sorted into flasks and placed in a water bath in constant darkness. Flasks were taken every 6 hours from 9 hpf. *Per1* mRNA levels were measured by qPCR, normalised to the reference gene *ef1α* and relative expression was calculated using the ΔΔCt method. Rhythm amplitude on the second day of development was estimated by dividing peak values by trough values and was 5.06 fold for surface and 2.57 fold for Pachón. (a') Expression of *per1* in DD was compared to expression in LD (reproduced in grey from Figure 5.1). Relative expression is plotted against a single sample using the ΔΔCt method. Rhythm amplitude on the second day of development in LD cycles was estimated by dividing peak values by trough values and was 24.38 fold for surface and 6.05 fold for Pachón. White and grey bars in a' indicate light and dark or subjective light and dark periods for LD and DD respectively. Data represent the mean ± SEM for 3 embryo samples.

5.3.3 PACHÓN CAVEFISH EMBRYOS ARE SLOWER TO DEVELOP LIGHT RESPONSE THAN SURFACE EMBRYOS AND SHOW RAISED DARK EXPRESSION DURING DEVELOPMENT

We have demonstrated that an increased expression of *per2b* and an altered light input pathway in adult *Astyanax* cavefish correlates with a reduction of *per1* rhythm amplitude. Work in zebrafish shows that *per2* and *cry1a* are involved in the entrainment of the clock to light and the maintenance of high amplitude rhythms of clock gene expression in LD cycles in the embryo (Ziv and Gothilf, 2006; Tamai et al., 2007; Dekens and Whitmore, 2008). Therefore, the reduced amplitude of the embryonic cavefish clock could, like adults, be a consequence of changes within the light input pathway. Is this mechanism present in the early embryo, and if so, how does it develop?

It is clear that light is able to influence the circadian clock in *Astyanax* within the first 12 hours of development as differences in the *per1* rhythm exist on the first day of development between embryos raised in LD and DD (Figure 5.2a'). Furthermore, light of day 2 of the LD cycle appears important in the *per1* rhythm, especially that of surface fish embryos. We therefore examined the induction of *per2a*, *per2b* and *cry1a* in response to light pulses throughout development.

The induction of expression by light is reduced in Pachón embryos and is slower to develop. *Per2a*, *per2b* and *cry1a* are not induced by light in Pachón embryos between 5 and 8 hpf, in contrast to the strong induction we see at this time point in surface fish embryos and zebrafish (Figure 5.3 and Tamai et al., 2004). Embryonic light detection in Pachón begins to appear at 14-17 hpf in the *per2* genes and by 23-26 hpf is apparent for *cry1a* (Figure 5.3 d, e and g-l). Light induction of clock genes is present at 50 hpf, though at this stage absolute fold induction for all genes is significantly lower in Pachón compared to surface fish embryos (Figure 5.4). Throughout development, the basal levels of the *per2* genes are raised in Pachón cavefish compared to surface fish (Figure 5.3 and Figure 5.4). This is similar to the condition in adult fish.

To see if the light-induced genes are raised throughout development and correlate with reduced *per1* amplitude, we examined the expression of *per2b* in embryos maintained in LD and DD. During development in a LD cycle, expression of *per2b* is significantly higher in Pachón than surface at all dark-phase time points during the first 3 days of development and at ZT9 on the second and third days (Figure 5.5a). The expression difference between surface and Pachón is independent of the conditions in which the embryo is raised: *per2b* is significantly more highly expressed in Pachón embryos than surface even when raised in constant darkness (Figure 5.5b). Thus, as in the adult, raised levels of *per2b* correlate with lower amplitude *per1* rhythms of Pachón relative to surface. Similarly, the lower levels of *per1* at 21 hpf in Pachón embryos raised in LD compared to DD correlate with significantly higher expression of *per2b* at 21 hpf in LD-raised embryos. Likewise, the expression of *per1* in surface embryos at 21 hpf is the same between LD and DD and it follows that there is no difference in *per2b* expression between the two conditions at this time.

These results are similar to observations in zebrafish development: the key difference between embryos maintained in LD and DD is the higher expression of per1 in DD at 21 hpf (Dekens and Whitmore, 2008). This can be replicated in LD conditions by injection of a *per2* morpholino, demonstrating *per2*'s key role in light signalling (Dekens and Whitmore, 2008). Therefore, it is apparent that *per2b* plays a very important role in the embryonic *Astyanax* clock. Differences in light-induction, such as the lack of embryonic light detection in Pachón embryos before 8hpf and the raised basal levels of *per2b* and other critical components of the light-resetting pathway, are likely to be responsible for the differences seen between surface and Pachón *per1* rhythms.

It is interesting to note that light induction in Pachón develops at different stages for *per2* genes and *cry1a*, and that the raised basal level appears later for *cry1a* than *per2* (contrast Figure 5.3c, f, i and Figure 5.4c with Figure 5.3b, e, and h). In addition, the absolute fold

induction for both *per2* genes increases with developmental age, possibly reflecting a maturation of the light signalling process, whereas *cry1a* induction stays constant at around 2-fold in surface embryos. This suggests that there may be differential mechanisms involved in *per2* and *cry1a*'s response to light.

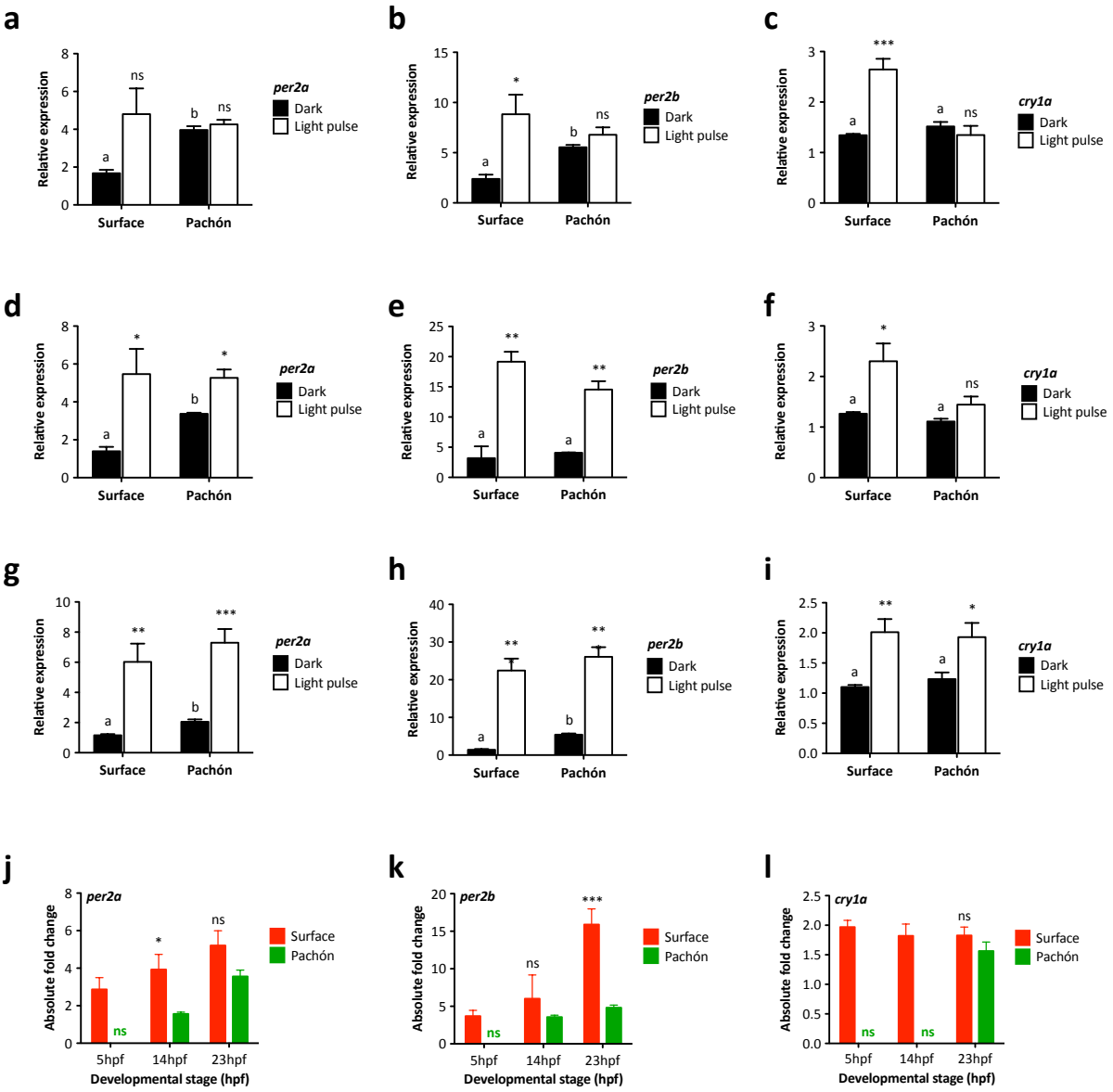

Figure 5.3: Acute light induction of clock genes is slower to develop in Pachón cavefish than surface fish during development.

Surface and Pachón embryos were kept in constant darkness until a 3 hour light pulse was given at different developmental stages. Expression of *per2a*, *per2b* and *cry1a* was determined by qPCR in light-pulsed and dark control samples and normalised to the reference gene *rpl13α*. Relative expression was calculated using the ΔΔCt method. (a-c) Light pulse given at 5 hpf, (d-f) light pulse given at 14 hpf, (g-i) light pulse given at 23 hpf. Dark and light-induced levels were compared using a Student's t-test (unpaired, two tailed; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; significant differences at $p<0.05$ in dark samples indicated by different lower case letters). (j-l) Absolute fold induction of the expression of each gene in response to light was determined in samples with significant difference between light-induced and dark samples. Fold induction was compared between surface and Pachón at each developmental time point using a Student's t-test (unpaired, two

tailed; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Data represent the mean ± SEM for between 3 and 5 embryo samples.

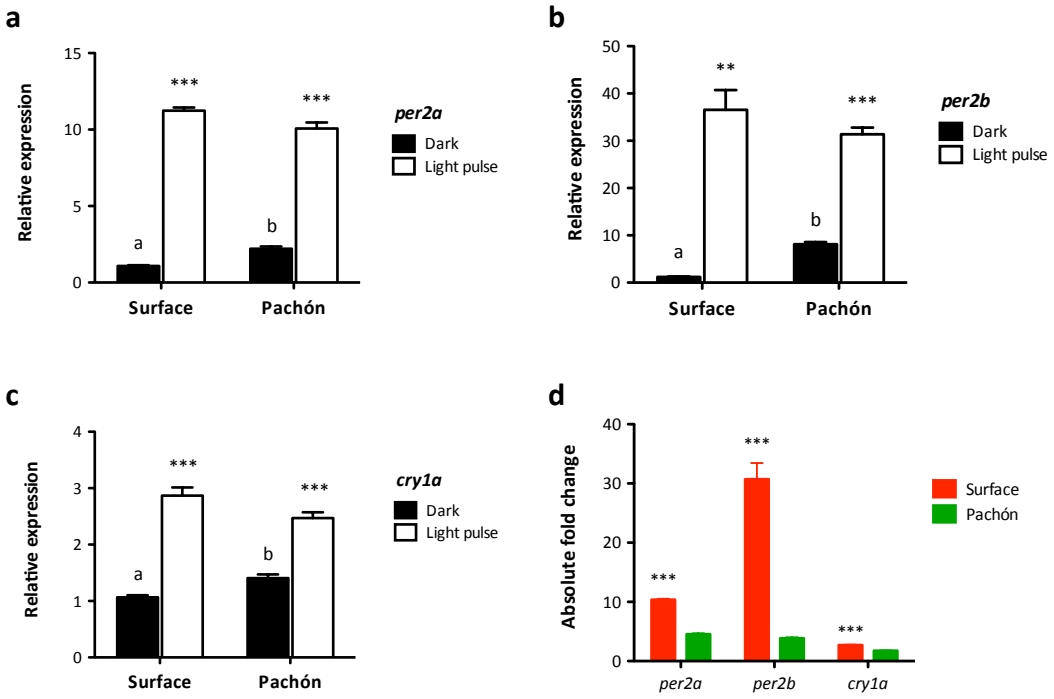

Figure 5.4: Acute light induction of clock genes is reduced in Pachón cavefish compared to surface fish at 50 hpf.

Surface and Pachón embryos were kept in constant darkness until a 3 hour light pulse was given at 50 hpf. Expression of *per2a*, *per2b* and *cry1a* was determined by qPCR in light-pulsed and dark control samples and normalised to the reference gene *rpl13α*. Relative expression was calculated using the ΔΔCt method. (a) *Per2a*, (b) *per2b*, (c) *cry1a*. Dark and light-induced levels were compared using a Student's t-test (unpaired, two tailed; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; significant differences at $p<0.05$ in dark samples indicated by different lower case letters). (d) Absolute fold induction of the expression of each gene in response to light was determined. Fold induction was compared between surface and Pachón using a Student's t-test (unpaired, two tailed; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Data represent the mean ± SEM for 3 embryo samples for surface and 5 samples for Pachón.

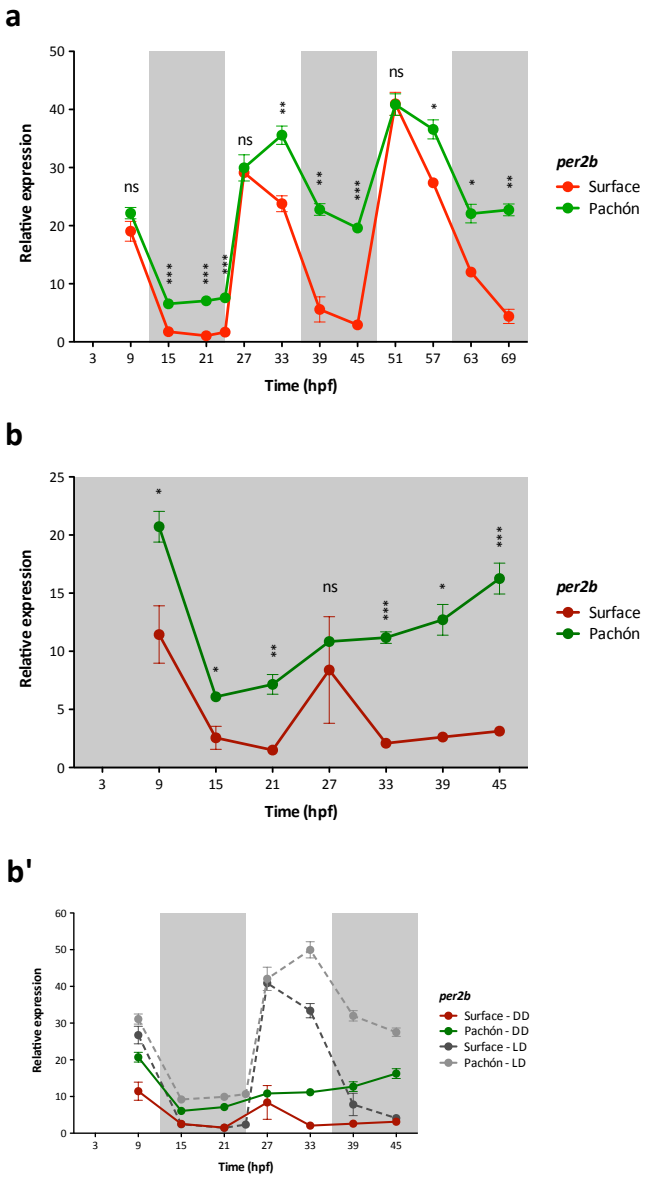

Figure 5.5: *Per2b* is significantly more highly expressed in Pachón embryos during development.

(a) *Per2b* mRNA levels during development in 12hr:12hr LD cycles were measured in surface and Pachón embryos by qPCR in the same samples as Figure 5.1. Ct values were normalised to the reference gene *ef1α* and relative expression was calculated using the ΔΔCt method. (b) *Per2b* mRNA levels during development in constant darkness were measured in surface and Pachón embryos by qPCR in the same samples as Figure 5.2. Ct values were normalised to the reference gene *ef1α* and relative expression was calculated using the ΔΔCt method. (b') Expression of *per2b* in DD was compared to expression in LD (reproduced in grey from a). Relative expression is plotted against a single sample using the ΔΔCt method. White and grey bars indicate light and dark periods respectively. Data represent the mean ± SEM for at least 3 embryo samples.

5.3.4 Is the pineal gland responsible for the later light response of Pachón?

Although it is clear that a light response exist in early Pachón embryos (from both the differences between *per1* expression rhythms in LD and DD and significant acute responses to light at 14-17 hpf), a light response almost as strong as surface is only present at 23-26 hpf. This maturation of the acute light response (when *per2a*, *per2b* and *cry1a* are all significantly induced) coincides with the development of a functional pineal gland in *Astyanax* (Yoshizawa and Jeffery, 2008). In addition, *per2* mRNA expression is rapidly induced in response to light in zebrafish, with high signal in the pineal gland (Ziv et al., 2005; Vatine et al., 2009). From this, we reasoned that the pineal gland might be important for Pachón's light response. Therefore, we analysed the expression of *per2b* mRNA using whole mount in situ hybridisation to examine whether the high induction gained at 23 hpf in Pachón embryos is due to pineal specific expression. *In situ* hybridisation confirmed the increased expression of *per2b* after light exposure at 5 hpf and 23 hpf in surface embryos (Figure 5.6 a and b, and f and g). This expression difference is only present in Pachón embryos when the light pulse is given at 23 hpf (Figure 5.6h and i), similar to results by qPCR. *Per2b* is expressed more highly in Pachón embryos than surface fish embryos in the dark controls, as seen by qPCR, which is apparent in these samples at both time points (Figure 5.6c and h). At 26 hpf, the expression in both surface and Pachón embryos is ubiquitous throughout the embryo, with only a slight increase in staining in the pineal gland. The ubiquitous expression of *per2b* at 26 hpf observed by *in situ* hybridisation and the clear light response present before 17 hpf, suggest that it is unlikely that the pineal gland mediates the development of the light-induction of clock genes in Pachón. A mechanism of light detection is present throughout the embryo and is not restricted to central photoreceptive structures.

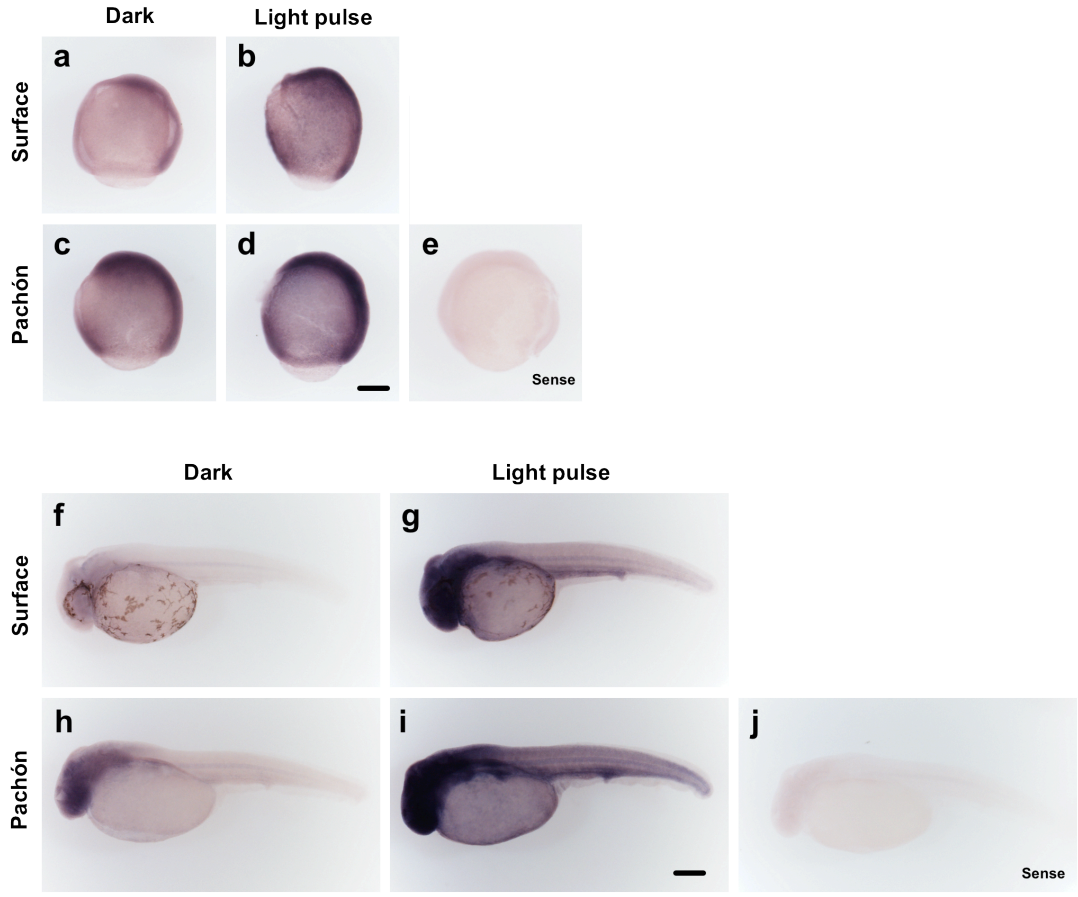

Figure 5.6: Acute light induction develops within the first day of development in Pachón cavefish.

Surface and Pachón embryos were kept in constant darkness until a 3 hour light pulse was given beginning at (a-d) 5 hpf and (f-i) 23 hpf. Expression of *per2b* mRNA was analysed by *in situ* hybridisation in light-pulsed and dark control samples, with the same detection time for all treatments. (e and j) *per2b* sense control for embryos at 8 hpf and 26 hpf respectively. Scale bar, 0.4 mm.

5.3.5 EXPRESSION OF CANDIDATE GENES UPSTREAM OF LIGHT INDUCTION

Thyrotroph embryonic factor (tef) and opsin based photopigments are suggested to play key roles in early light detection of the circadian clock in zebrafish, and have been especially examined in *per2*'s response to light (Vatine et al., 2009; Gavriouchkina et al., 2010; Weger et al., 2011). Although tef does not seem to be involved in the alterations seen in the light-input pathway of the adult clock of *Astyanax*, it is possible that differential mechanisms are involved between adult and embryo. Therefore we examined the expression of *tef1* throughout development in response to light pulses.

The light-dependent expression of *tef1* throughout development closely resembles that of the critical components of the resetting pathway, *per2a*, *per2b* and *cry1a*. *Tef1* is induced by light in surface embryos by 14-17 hpf, but only later does it become light sensitive in Pachón embryos, at 23-26 hpf (Figure 5.7). Therefore, in contrast to adult fins and similar to zebrafish, *tef1* is acutely induced by light in the embryo. Tef1 plays a key role in mediating the light response and regulating *per2* expression in zebrafish (Vatine et al., 2009). Is this also true in *Astyanax* embryos?

Unlike other genes that show acute light induction in *Astyanax*, *tef1* is significantly less expressed in dark control samples of Pachón embryos relative to surface fish until the beginning of the second day of development. In addition, although the fold induction of the gene is the same between Pachón and surface by 23-26 hpf (Figure 5.7e), light-induced levels are lower in Pachón embryos at all developmental time points (expression of *tef1* at 8 hpf, 17 hpf and 53 hpf is significantly lower. Expression at 26 hpf is lower, but not significant by t-test, p=0.053). Therefore, in contrast to the other acutely light induced genes we have observed, *tef1* is downregulated in Pachón cavefish embryos. Thus, the raised level of expression of *per2b* in Pachón in the dark is unlikely to be mediated by *tef1* driven expression. However, we cannot rule out that *tef1* mediates light-induction of *cry1a* (and potentially other light-responsive genes) in *Astyanax* embryos like zebrafish genes

(Weger et al., 2011), as appearance of the light-induction of *cry1a* coincides with that of *tef1* in Pachón. It is clear that differential regulation of this gene occurs between embryo and adult stages. Upregulation of light-induced genes in the darkness in Pachón may be due to other D-box binding factors. This will be discussed below.

Tmt opsin is a candidate photoreceptor for the circadian clock, and is widely expressed throughout the photoresponsive zebrafish tissues and embryonic cell line (Moutsaki et al., 2003). In order to test whether this gene may have a role in embryonic light detection, we cloned one gene of this class, *tmt opsin 1*, and measured its expression. *Astyanax* Tmt1 contains all the amino acid motifs characteristic to opsin photopigments, including the ERY motif at position 145-147 (bovine rhodopsin equivalent, 134-136) and the lysine at position 302 (bovine rhodopsin equivalent, 296) residue important for the retinal attachment (Palczewski et al., 2000). Three amino acid differences are seen between surface and Pachón, but all lie outside of the features conserved across opsin photopigments (Figure 5.8a). As a candidate photoreceptor to the circadian clock of zebrafish it is suggested that this opsin would lie upstream of genes critical for clock resetting such as *per2* and *cry1a*. As such, we hypothesised that expression differences between the two *Astyanax* populations may mediate the differential light-induction observed. However, this is not what we observed. The relative expression is very similar between surface and Pachón throughout development, with no obvious correlation of light-induction response with difference in *tmt1* expression (Figure 5.3 vs. Figure 5.8b). Therefore, this opsin is not likely to be responsible for the differential light response in surface and Pachón embryos. However, as zebrafish have many other extra-retinal opsins including multiple *tmt opsin* genes (W. Davies, personal communication), some of which may signal to the circadian clock, differential regulation of another opsin photopigment cannot be ruled out. Multiple *tmt opsin* genes exist in *Astyanax*: a partial fragment of another *tmt opsin* gene was isolated in the initial RT-PCR and has been cloned from

Astyanax surface and cave populations (W. Davies, personal communication) but its expression was not examined in this study.

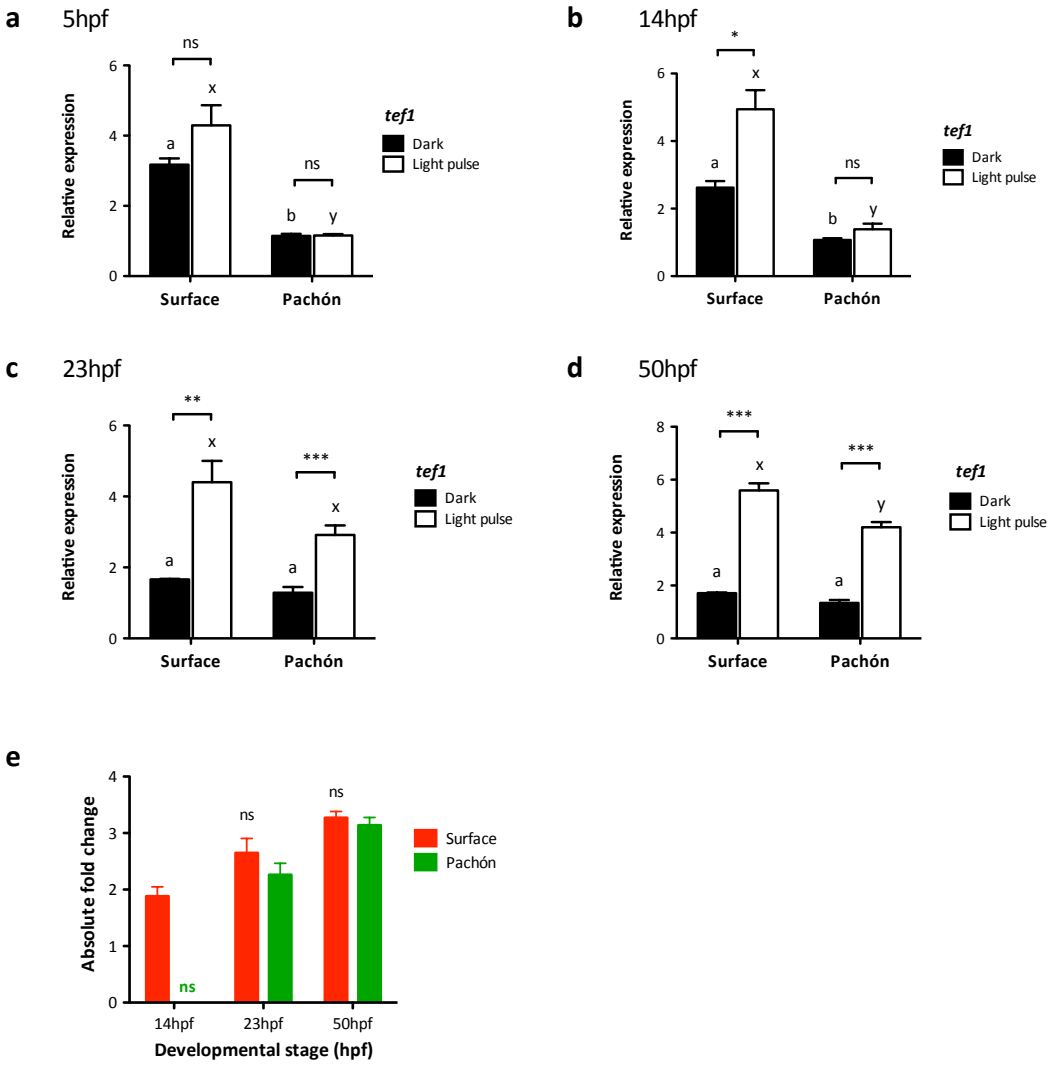

Figure 5.7: *Tef1* expression becomes responsive to light at different stages in surface and Pachón embryos.

Expression of *tef1* was determined by qPCR in the same samples as Figure 5.3 and Figure 5.4. Light pulses were given at (a) 5 hpf, (b) 14 hpf, (c) 23 hpf, (d) 50 hpf. Dark and light-induced levels were compared using a Student's t-test (unpaired, two tailed; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; significant differences at $p<0.05$ in dark and light samples indicated by different lower case letters, a vs. b; x vs. y). (e) Absolute fold induction of the expression of each gene in response to light was determined. Fold induction was compared between surface and Pachón using a Student's t-test (unpaired, two tailed; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Data represent the mean ± SEM for 3 to 5 embryo samples.

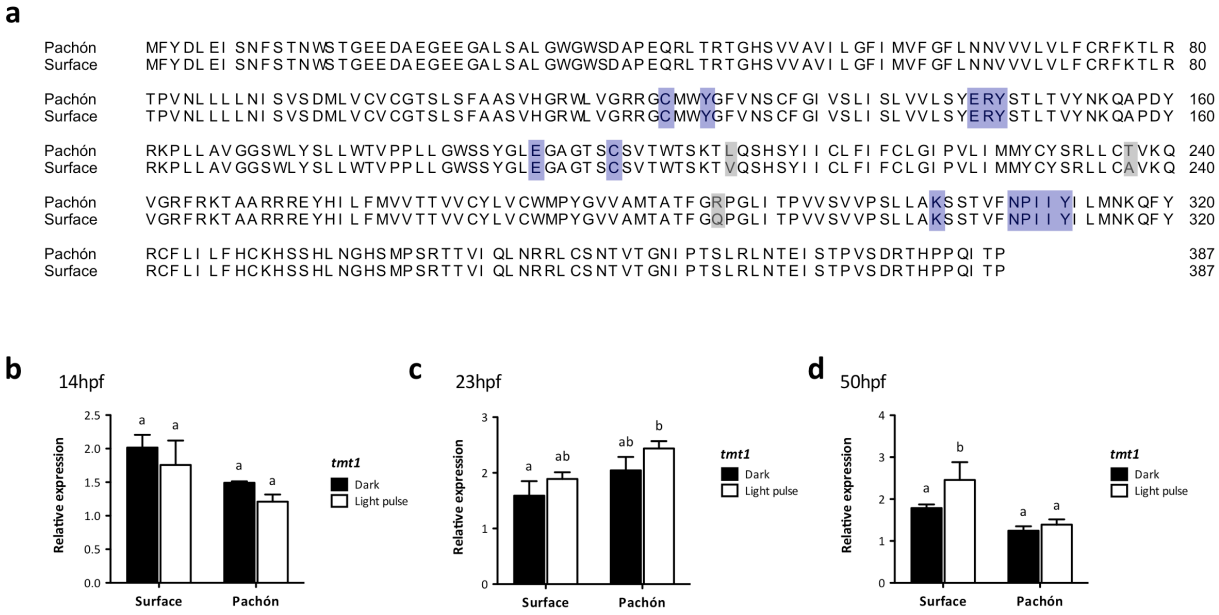

Figure 5.8: A candidate circadian photoreceptor, *tmt opsin 1*, is similarly expressed in surface and Pachón embryos.

(a) Alignment of the predicted amino acid sequences from surface and Pachón. Residues identified as critical for opsin protein structure and function (Davies et al., 2010) are shaded (blue). These include a conserved lysine at position 302 important for chromophore binding; a ERY motif (145-147) conserved within GPCRs; a NPIIY motif (308-313) important for conformational change; two conserved cysteine residues (121 and 197) predicted to from a disulphide bridge; and conserved tyrosine (144) and glutamate (191) residues, similar to the zebrafish melanopsin photopigments (Bellingham et al., 2002). Residues differing between Pachón and surface are shaded in grey. (b-d) Expression of *tmt1* was determined by qPCR in the same samples as Figure 5.3 and Figure 5.4. (b) 3 hour light pulse given at 14 hpf, (b) 3 hour light pulse given at 23 hpf, (b) 3 hour light pulse given at 50 hpf. Expression was compared by ANOVA and Newman-Keuls multiple comparison test (significant differences at $p<0.05$ are indicated by different lower case letters). Data represent the mean ± SEM for 3 to 5 embryo samples.

5.3.6 CAVE-CAVE HYBRID FISH DO NOT RESCUE LIGHT-RESPONSE IN EPIBOLY-STAGE EMBRYOS

Whilst the relationships between populations of *Astyanax* cavefish are still the subject of much debate, there is evidence that cave related phenotypes in *Astyanax* cavefish are the result of convergent evolution. Cave populations have arisen at least five times independently (Bradic et al., 2012) and show remarkable convergence in characteristics such as eye loss and pigmentation. Furthermore, a unique and valuable feature of *Astyanax* is that cavefish from different caves can be crossed to examine cave phenotypes by complementation tests. In order to test whether the absence of light response in early Pachón embryos is present in all cave embryos and gain more insight into the regulation of the light response, we examined the induction by light of multiple clock genes in Pachón, Chica and Pachón-Chica hybrid embryos at 5 hpf.

As for embryos from Pachón cavefish (Figure 5.3), Chica cavefish embryos do not show a strong response to light at this early stage, with *per2a* the only gene to show a significant induction (though this is small, 1.23 fold, Figure 5.9). Chica embryos also show high expression of the genes during darkness, similar to Pachón (Figure 5.9). There are three possibilities to explain these results: the two cave populations could have the same mutation or alteration in the same gene or pathway, different mutations or alterations in the same gene or pathway, or mutations or alterations in distinct genes or pathways. In order to distinguish between these possibilities, we gave a light pulse to embryos of a cross between Pachón and Chica cavefish. Pachón and Chica cavefish are from independent caves within the El Abra geographical region (Mitchell et al., 1977; Bradic et al., 2012), though we have already demonstrated that a high degree of similarity exists within the coding region of multiple clock genes. Embryos of this cross do not show a response to light, similar to their parent cavefish populations (Figure 5.9). This suggests that the early light insensitivity phenotype in Pachón and Chica cavefish embryos may

have the same genetic basis, as is the case for the pigmentation defect of Pachón and Molino cavefish (Protas et al., 2006).

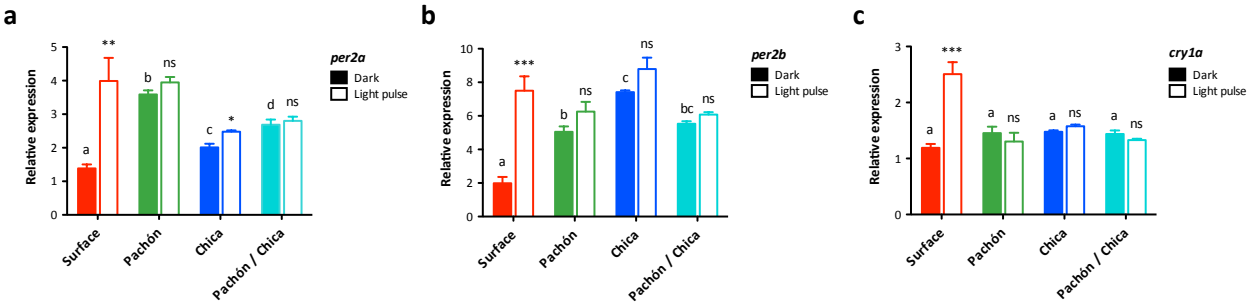

Figure 5.9: Acute light-induction at 5 hpf is not rescued in a cave-cave hybrid.
Surface, Pachón, Chica and Pachón/Chica hybrid embryos were kept in constant darkness until a 3 hour light pulse was given at 5 hpf. Expression of (a) *per2a*, (b) *per2b* and (c) *cry1a* was determined by qPCR in light-pulsed and dark control samples and normalised to the reference gene *ef1α*. Dark and light-induced levels were compared using a Student's t-test (unpaired, two tailed; *, p<0.05; **, p<0.01; ***, p<0.001) and dark levels were compared by ANOVA and Newman-Keuls multiple comparison test (significant differences at p<0.05 in dark samples indicated by different lower case letters). Data represent the mean ± SEM for 3 to 5 embryo samples.

5.4 DISCUSSION

How the circadian clock develops or "begins to tick" is a fundamental question in circadian biology. As an externally developing organism with light-entrainable cellular oscillators, zebrafish has proved a good model to examine this question and has been used to show that light-responsive molecular oscillators are present in the very early embryo (Ziv and Gothilf, 2006; Dekens and Whitmore, 2008). This makes apparent sense for a fish that begins its life at dawn (Tamai et al., 2004). What happens in fish that begin development in the night, or more extremely, never experience light? How does the clock mechanism develop?

We examined the expression of *per1* in LD and DD conditions and acutely light induced genes in response to short light pulses during development. To summarise, we have shown that embryos of *Astyanax* surface and cavefish are light-responsive from very early in development and possess entrainable circadian clocks. In contrast to zebrafish, the embryonic circadian clock is synchronous even in the absence of an environmental stimulus. Two candidate genes for the regulation of light-induction of clock genes were explored and results suggest a possible differential role for *tef1* between the embryo and the adult, although there is not strong evidence for a role for *tef1* or *tmt1* in the regulation of high basal levels of light-induced genes in cavefish in the absence of light. Interestingly the absence of light induction in the gastrulating embryo, and the raised basal levels at this stage, persist within a cave-cave hybrid. This is highly suggestive of a similar genetic basis for the alterations in the light input pathway of the early embryo in two independent caves.

5.4.1 THE BEGINNING OF THE CIRCADIAN CLOCK

The debate about how the circadian clock starts in zebrafish was originally between two camps: one proposed clocks began early and were synchronous throughout the embryo due to maternal factors (Delaunay et al., 2000); the other proposed that clocks began

autonomously, but were asynchronous without an environmental stimulus (Ziv and Gothilf, 2006; Dekens and Whitmore, 2008). Evidence now lies firmly with the latter. Zebrafish are exposed to light from the first moments of development, and so it makes apparent sense that they adapt to the exposure both by using it to synchronise the embryonic circadian clock and control the expression of photolyases to protect from the harmful UV wavelengths (Tamai et al., 2004). In contrast to zebrafish, *Astyanax* surface fish spawn in the dark and cavefish never experience light in their lifetimes, and so we might expect different mechanisms in the early stages of development. Whilst we observe rhythms of *per1* expression when raised in LD, we also observe rhythms (though of lower peak:trough amplitude) when raised in constant darkness, in direct contrast to the situation in zebrafish. It could be that handling or the temperature change going from flasks in air to flasks in a water bath cause the synchronisation of the rhythms (similar to that proposed in Hurd and Cahill, 2002), though unlike Hurd and Cahill, all handling was done prior to the MBT and so a non-transcriptional mechanism would have to be involved.

As observed in adult fish, the embryonic *per1* rhythm in LD has a lower amplitude in Pachón than surface embryos. In contrast to the adult, the rhythm is of the same phase, so the amplitude difference may not be due to clock period and entrainment differences. Therefore, we propose that alterations seen during development lie in the light input pathway: the late development of light sensitivity and the high expression of light-induced genes. Both of these are likely to cause the lower amplitude rhythms of the embryonic circadian clock. Supporting the latter view is observation of differences in rhythms in constant darkness – the amplitude of the *per1* rhythm is lower in Pachón than surface in both LD and DD conditions. Although *per2b* is a light induced gene and shows induction to light on an LD cycle and in response to light pulses, it is significantly more highly expressed in Pachón embryos than surface embryos even in the constant darkness. In the absence of any environmental cycle (and thus any difference of direct light sensitivity between Pachón and surface fish), *per1* rhythms are lower in amplitude. As the clock is

free running in these conditions, it is highly suggestive that difference in amplitude is due to the innate upregulation of the clock-repressor, *per2b* (or possibly additional unknown factors). As discussed below, the regulation of *per2b* seems to be altered in cavefish relative to surface fish, and it follows that this regulation difference is independent of environmental conditions.

5.4.2 THE LIGHT-RESPONSIVE *ASTYANAX* EMBRYO AND ROLE OF D-BOX BINDING FACTORS

Our study did not reveal strong evidence for the role of *tef1* or *tmt1* in the raised expression of *per2a*, *per2b* and *cry1a* in cavefish in the absence of a stimulus. This is contrary to the expectation made from *tef*'s role in zebrafish. *Tef*, through D-box elements, contributes to the light-driven transcription of *per2* (Vatine et al., 2009). When this gene is knocked down, by antisense morpholino, the response of many genes, including *per2*, *cry1a*, *6-4 photolyase*, *cpd photolyase* and *ddb2* is attenuated (Vatine et al., 2009; Gavriouchkina et al., 2010; Weger et al., 2011); likewise, overexpression of *tef* imitates the light induced expression even in darkness (Gavriouchkina et al., 2010). We therefore hypothesised that upregulation of *tef* may be responsible in Pachón cavefish for the higher levels of predicted *tef* targets. This is not observed, which rejects this hypothesis, although *tef1* may still mediate the light response in the embryo.

What other factors may be involved? D-boxes are also the target of a set of repressive factors, the *e4bp4* family, which have multiple homologues in zebrafish (Doi et al., 2001; Ben-Moshe et al., 2010). Although the expression of these genes has not been examined thus far in *Astyanax*, it is possible that downregulation of these genes in cavefish may lead to the high expression in the target genes. This prediction is reinforced by close examination of the data presented in this study. Careful observation reveals that not only is the level of gene expression high in the darkness, it also approaches maximal levels. This is especially clear in the gastrulating embryo (5-8 hpf) and through the dark periods of the second and third days of development. Furthermore, comparison of the light-induced

levels in Pachón and surface shows they are not significantly higher; this is also clear during development in LD at ZT3 (Figure 5.3a-c and Figure 5.5a). Hence, rather than the upregulation of an activator in the dark, downregulation of a repressor in the dark may achieve the same effect. Consistent with this hypothesis, treatment of zebrafish cells with a protein synthesis inhibitor, cyclohexamide, results in a super induction of *per2* expression which shows raised levels of the gene in the dark control sample suggesting the absence of a suspected transcriptional repressor (Hirayama et al., 2005). Although the results in the present study do not exactly match this data as there is no 'superinduction' of expression in the light, it is possible that an antagonistic relationship between repressors (bound in the dark) and activators (bound in the light) exists, as suggested in mouse and chick (Doi et al., 2001; Mitsui et al., 2001), which differentially mediates the light response in *Astyanax*. In Pachón, light exposure and signalling through an activator, possibly Tef, would have to be unaffected by the absence of repressive function in order to maintain similar light-induced levels as surface.

The differential timing of the development of the light response between *per2a* and *per2b*, and *cry1a* in Pachón embryos, and the difference in fold induction as development proceeds, suggest that these two genes may have different regulation or regulatory pathways. This is supported by the observation in adult *Astyanax*, where *cry1a* shows a strong oscillation in cavefish unlike either of the *per2* genes. Intriguingly, differential mechanisms for light induction may exist between *per2* and *cry1a* in zebrafish: in contrast to *per2*, tef morpholinos do not eliminate the light response of *cry1a* (Weger et al., 2011), and its induction is protein synthesis sensitive (Hirayama et al., 2005).

5.4.3 THE ABSENCE OF 'RESCUE'

Cave-cave hybrids are able to 'rescue' a number of degenerate features of cave animals. Crosses of Pachón with Tinaja and Tinaja with Molino produce embryos with larger eyes than either parent, and crosses of Molino and Curva cavefish produce extensively

pigmented offspring (Borowsky, 2008; Jeffery, 2009). On the contrary, crosses of Pachón and Molino, and Pachón and Japonés cavefish are albino, like their parents (Protas et al., 2006). These complementation tests reveal that, in addition to the independent evolutionary origin, eye regression in Pachón, Tinaja and Molino is predicted to be due to separate genetic mechanisms. Conversely, the genetic basis for albinism in Pachón, Molino and Japonés is the same: a mutated form of *oca2* (Protas et al., 2006). In a similar experimental procedure, we examined the genetic basis of the absence of light-response in early embryos.

Hybrid embryos of Pachón and Chica are not light-responsive between 5 hpf and 8 hpf, like the pure Pachón and Chica embryos themselves. Whilst we have not been able to identify the nature of the mechanism that is responsible for this phenotype yet, it does suggest that Pachón and Chica have alterations in the same gene or pathway. This is a remarkable result as Pachón and Chica cavefish are predicted to have separate evolutionary origins and so this result means a similar alteration in the light input pathway has evolved convergently in the same way as albinism (Protas et al., 2006; Bradic et al., 2012). Why might this be the case? It is possible that the mechanism is under strong selective pressure in the cave environment, or is a common target of evolution. Evidence from QTL analyses argues against selection's role in pigment loss as cave alleles at each QTL do not specifically reduce pigmentation (Protas et al., 2007). Protas et al. suggest that *oca2* may be a common target of evolutionary change due to its chromosomal position or large genomic size (Protas et al., 2006), which may allow this gene to be subject to many deleterious mutations. As discussed above, the cause of the absence of the light response may be due to alterations in the function of D-box binding factors and opsin expression (as has been suggested for the Somalian cavefish). The alteration (or alterations) may result in the expression of many other light-induced genes being upregulated in the dark. This includes DNA repair genes such as *CPD photolyase* and *ddb2*, which may offer an advantage in the cave environment. This is examined in further detail in Section 6. It

would be interesting to cross many different cave populations, firstly to see if rescue of surface like behaviour occurs, and secondly to see if the alterations of the light input pathway in Pachón and Chica are due to selection or drift.

6 FURTHER MODIFICATIONS OF LIGHT-DEPENDENT BIOLOGY IN *ASTYANAX* CAVEFISH

6.1 INTRODUCTION

One of the many fundamental environmental conditions that most organisms are exposed to is the daily cycle of light and dark with the majority, if not all, organisms adapting to this by evolving an internal or endogenous circadian clock. Circadian clocks coordinate the timing information obtained from the light-dark cycle with appropriate downstream physiological processes. Many animals, including *Drosophila* and zebrafish, possess cellular circadian clocks that are directly light responsive, containing dedicated light responsive genes to transmit the light signal to the core clock, which in turn coordinates downstream physiology (Plautz et al., 1997; Whitmore et al., 1998). Indeed, each cell of the zebrafish contains a light-entrainable molecular clock and the photoreceptive elements that permit this light perception (Whitmore et al., 2000; Carr and Whitmore, 2005; Tamai et al., 2007; Vatine et al., 2009).

Nevertheless, light does not just have indirect effects on physiology via the circadian clock. Light can affect physiology directly, extending to many other aspects of cell biology beyond the molecular circadian clock. The global light sensitivity of zebrafish (and other teleosts) provides a very good basis for investigation of these direct effects of light in vertebrate cell biology. Recently it has been shown that transcriptional regulation by light is a more common level of control in zebrafish biology than previously thought. For example, light is able to induce the expression of genes that mediate stress responses and promote DNA repair amongst others (Gavriouchkina et al., 2010; Weger et al., 2011). Many promoters of the induced genes contain D-boxes, which have led to suggestions that D-box binding factors, such as *thyrotroph embryonic factor* (*tef*), regulate light signalling in zebrafish (Gavriouchkina et al., 2010; Weger et al., 2011).

The close relationship between the circadian clock, light and DNA repair in particular has been the subject of many studies, and suggest that the two systems are inherently linked (Pittendrigh, 1993; Sancar et al., 2010). Indeed, the 'Escape from Light' hypothesis

maintains that the circadian clock evolved as a mechanism to avoid the DNA damaging effects of UV light (Pittendrigh, 1993). The close relationship between the two mechanisms is highlighted in the evolutionary relationship between Cryptochromes, as core members of the circadian clock, and Photolyases, as DNA repair proteins (Lin and Todo, 2005). In addition to the close sequence similarity, cryptochromes and photolyases share upstream signalling pathways that regulate their expression, which in zebrafish may include the MAPK pathway and signalling via reactive oxygen species (ROS) (Lin and Todo, 2005; Hirayama et al., 2009; Uchida et al., 2010). Interestingly, downstream crosstalk between the circadian clock and DNA repair exists. Some DNA repair genes, such as *Xeroderma Pigmentosum A* (*XPA*), are under the control of the circadian clock and result in DNA repair that is enhanced at different times of the day (Kang et al., 2009; Sancar et al., 2010). Furthermore, not only is ROS implicated in the signalling of light to the circadian clock and DNA repair genes (Hirayama et al., 2009; Uchida et al., 2010), the cellular production of ROS itself is a common source of DNA damage (Cooke et al., 2003). This oxidative damage is predominantly repaired by the base excision repair pathway with some evidence of contribution by the nucleotide excision repair pathway (Cooke et al., 2003). Finally, potential feedback from the DNA damage and DNA repair genes on the circadian clock has been demonstrated in mice. DNA damage caused by ionizing radiation is able to affect the phase of the circadian clock (Oklejewicz et al., 2008). DNA repair genes can also interact with the circadian clock, as the expression of marsupial *CPD photolyase* in mice not only increases DNA repair but can rescue a *Cryptochrome* deficient clock when driven by a *Cry1* promoter (Chaves et al., 2011a). This study may indicate the significance of the core structure of the protein, rather than simply its primary amino acid sequence in clock function, and although it is a cross-species study with a photolyase that does not exist in mice, it demonstrates the potential for these pathways to interact. Taken together, DNA repair and the circadian clock are closely related, both in upstream regulation by light and downstream crosstalk of interlinked pathways.

We have already demonstrated that *Astyanax mexicanus* cavefish have an altered light-input pathway and core clock with respect to surface populations of the same species. It is therefore highly likely that cavefish possess alterations in other closely related aspects of light-regulated biology, such as DNA repair. In this chapter, we will examine the effect of the loss of light on DNA repair gene expression and activity in *Astyanax*.

6.2 METHODS

6.2.1 ADULT FISH

Adult fish were entrained and samples of the caudal fin were taken at 6-hourly intervals and after a 3 hour light pulse as described in Section 2.3.1.

6.2.2 EMBRYOS

A full explanation of the methods for embryo experiments can be found in Section 2.3.2.

6.2.3 CLONING *ASTYANAX* DNA REPAIR GENES

A full description of the method for cloning *Astyanax* genes can be found in Section 2.5. Fragments of *CPD photolyase* (*CPD phr*) and *damage-specific DNA binding protein 2* (*ddb2*) were amplified from cDNA from *Astyanax* embryonic cell lines (creation described in Section 2.1.3) using specific primers designed to zebrafish and other teleost homologues of the genes. *CPD phr*: Forward-895-Zf CPD 5'1 (5'-TTCAGGTTGATGCACATAATGTGG-3') and Reverse-898-Zf CPD 3'1 (5'-AAAGATGGGTCGCTCTGCCCAGCC-3'); *ddb2*: Forward-1559-teleo ddb2 fw1 (5'-TTTATTGGGGGDATGAAGTTCTGCCC-3') and Reverse-1557-teleo ddb2 rev1 (5'-GGGTAACGGCCAGCCACAATGAGGTC-3'). Sophie Cowen was responsible for the initial PCR of a small fragment of *CPD phr*. RACE PCR using 5' and 3' RACE libraries created from *Astyanax* embryonic cell lines was used for the subsequent extension of the initial PCR fragments (*CPD phr* 3' RACE: first round, 1102-CPD 3'RACE U-5'-ATGCTGGTCAGCTGTCCGCT-3'; nested round, 1103-CPD 3'RACE N-5'-TGTACTGGGCCAAAAAGATTCTGG-3'; *ddb2* 3'RACE: first round, 1588-Am ddb2 3'race1-5'-

ACCACAGACCAAATGAATGAGATCAGGATTTA-3'; nested round, 1589-Am ddb2 3'race2-5'-AAGTGATTGGTCTAAGCCAGCTCAGGTTATTG-3'). A partial fragment of the coding region of *CPD phr* was amplified from *Astyanax* adult fin cDNA using specific primers 1108-AstyCPD5'qPCR2 (5'-GGCCTCTCCTAAGCTGGAGT-3') and 1626-Am cpd rev2 (5'GGACCTGAGATGAATCTTCTGGAAATAGAA-3'). Primers for PCR are also listed in Table 2.1. The identities of isolated cDNA sequences were determined by the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/) and phylogenetic analysis.

6.2.4 Quantitative RT-PCR

RNA extraction and cDNA synthesis was performed as described in Sections 2.4 and 2.6.

6.2.5 DNA repair assay

DNA repair was quantified in adult *Astyanax* fish using ELISA. A full description of the method can be found in Section 2.9.

6.3 Results

6.3.1 Surface and cave populations of *Astyanax mexicanus* possess *CPD photolyase* and *damage-specific DNA binding protein 2* genes

We have demonstrated the effect of the absence of light during evolution on the circadian clock in *Astyanax*. However, a large part of an animal's biology is regulated by light and the circadian clock, so it is likely that many other molecular mechanisms have adapted during evolution in the darkness. One such mechanism that is likely to have substantially changed is DNA repair. Genes involved in DNA repair comprise one of the largest functional clusters of light-induced genes in zebrafish (Gavriouchkina et al., 2010; Weger et al., 2011). In addition to transcriptional regulation by light, photolyases such as *CPD photolyase* and *6-4 photolyase* use light energy directly for repair of damage caused by UV light (Brettel and Byrdin, 2010). Without light-dependent activation, animals have reduced tolerance to environmental stress and increased mortality (Yasuhira and Yasui,

1992; Schul et al., 2002; Tamai et al., 2004). The absence of light, therefore, will have a significant impact on many aspects of DNA repair mechanisms.

Therefore, we isolated two of the most highly upregulated genes from two screens of the light-responsive transcriptome of zebrafish (Gavriouchkina et al., 2010; Weger et al., 2011), *CPD photolyase* (*CPD phr*) and *damage-specific DNA binding protein 2* (*ddb2*). These two genes are involved in separate DNA repair pathways: CPD phr specifically repairs cyclo-butane pyrimidine dimers and Ddb2 is a component of the nucleotide excision repair (NER) pathway, which repairs bulky distortions of the DNA by removal of a short piece of the DNA (de Laat et al., 1999). Partial fragments of *CPD phr* and *ddb2* were amplified from RNA extracted from embryonic cell lines and fins of *Astyanax* surface fish. Initial sequence analysis confirmed that these fragments were homologous to zebrafish and goldfish sequences for *CPD photolyase* and *ddb2* (Table 6.1). Subsequent RACE PCR to extend these initial fragments was successful in the 3' direction for both genes, but not in the 5' direction. For *CPD phr*, 3'RACE extended the initial PCR fragment a further 700bp to beyond the stop codon. Primers were then designed to amplify a 969bp product including the stop codon from RNA extracted from fins of *Astyanax* surface fish, Pachón and Chica cavefish. This fragment contained a partial open reading frame of 890bp. Similarly, 3'RACE PCR extended the initial *ddb2* fragment a further 650bp in the 3' direction, and subsequent sequence analysis was performed on the composite PCR fragment as generated by CodonCode Aligner. As observed for other genes examined in *Astyanax*, the predicted protein sequences show a high degree of similarity between the different populations and to zebrafish. No equivalent *6-4 photolyase* to that of zebrafish has been identified to date in *Astyanax*.

Table 6.1: Identity of cDNA sequences for *A. mexicanus* DNA repair genes

Gene name	*Fragment size (nt)*	Danio rerio *Identity (%)*	Danio rerio *accession number*
CPD phr	969	73	NM_201064.1
ddb2	1080	73	NM_001083061.1

6.3.2 THE EXPRESSION OF THE DNA REPAIR GENES, *CPD PHR* AND *DDB2*, IS LIGHT RESPONSIVE IN *ASTYANAX* SURFACE FISH AND SHOWS CHARACTERISTIC ALTERATIONS IN CAVEFISH

One of the most significant molecular changes between surface and cave populations of *Astyanax* is at the level of the clock input pathway and, in particular, acute light induction. Given that DNA repair is another critical light-dependent process in teleosts, we examined the expression of *CPD phr* and *ddb2* in *Astyanax* populations.

As expected from previous studies in zebrafish (Gavriouchkina et al., 2010; Weger et al., 2011), *CPD phr* and *ddb2* are strongly induced by light in surface fish. Both genes are also induced by light in Pachón cavefish, though the magnitude of the induction of *CPD phr* is significantly reduced compared to surface fish (t-test, two-tailed, $p<0.001$, n=19: Surface, 9.61±1.41, Pachón 2.00±0.24). As for the light induction of the clock genes, this is due to significantly raised expression in the dark (Figure 6.1). In contrast to the other cavefish populations studied, *CPD phr* is not light induced in Tinaja cavefish, reflecting the general reduction in light sensitivity in this population.

The expression of *ddb2* is unlike other light-induced genes in Astyanax: Pachón cavefish show a similar level of fold-induction to surface fish, and Chica cavefish do not show a significant induction. In addition, the basal expression of *ddb2* is not significantly raised in cavefish compared to surface fish in this light pulse experiment when compared by ANOVA and Newman-Keuls (Figure 6.1b). In order to gain a clearer picture of how the expression of these DNA repair genes compares over a broader time window, we examined their expression in adult fish sampled over four days (Figure 6.1c and d). Cavefish show raised levels of expression of both genes, in particular showing significantly higher expression of *CPD phr* over surface fish in the subjective day, and for Chica cavefish, of *ddb2* at seven of eight time points in the dark. Interestingly, *CPD phr* shows strong oscillatory expression after transfer into constant darkness, indicating a level of clock

regulation of the transcription of this gene. The oscillation appears more robust in Pachón and Chica cavefish, with greater amplitude rhythms. A closer examination of the oscillations by plotting expression data from each fish relative to its own trough value reveals that the rhythms are indeed larger in amplitude in cavefish, though statistical comparison by ANOVA of rhythm amplitude (peak/trough) shows this is not significant (Figure 6.1c', p=0.093, n=22).

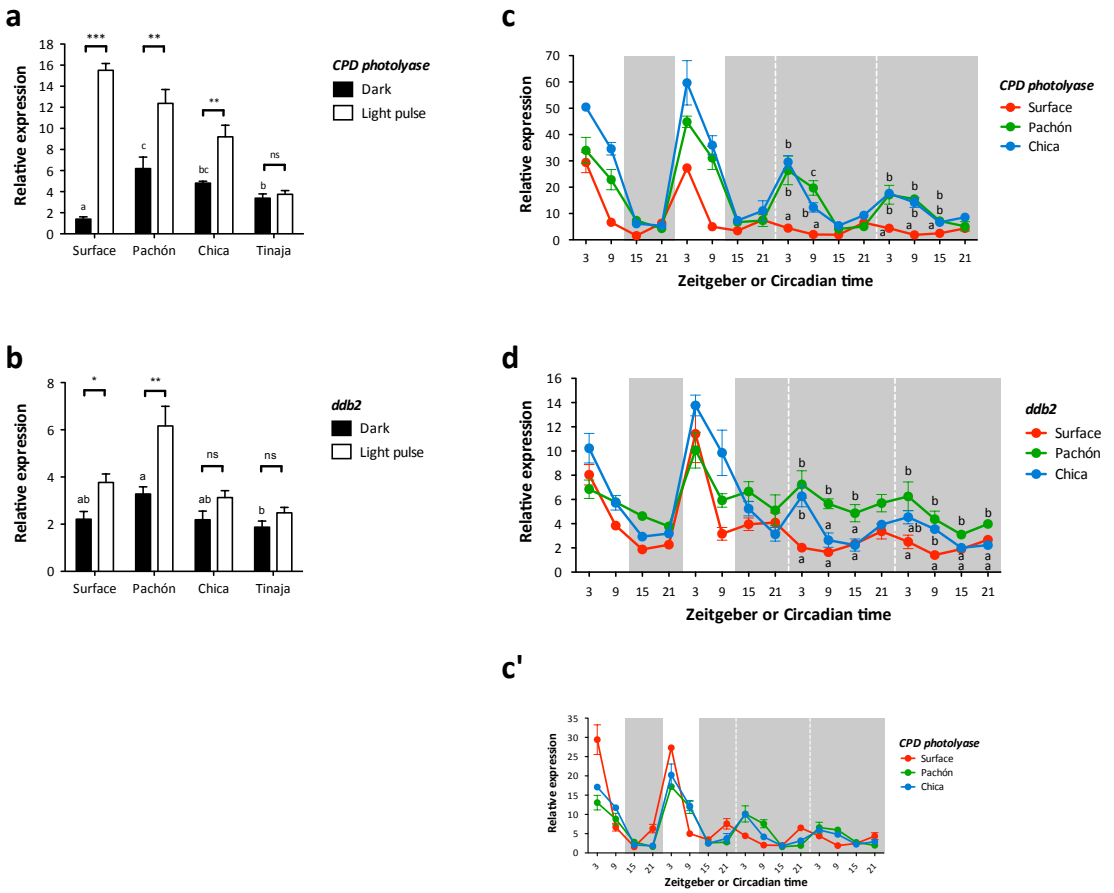

Figure 6.1: DNA repair genes are light responsive in adult *Astyanax* and upregulated in the dark in cavefish.

The relative expression of *CPD phr* and *ddb2* mRNA (RT-qPCR) was plotted using the ΔΔCt method. (a and b) Adult fish were entrained on a LD cycle for 7 days and given a 3 hour light pulse at ZT16. Expression of *CPD phr* (a) and *ddb2* (b) was determined in light-pulsed and dark control fin samples by qPCR. Dark and light-induced levels of *CPD phr* and *ddb2* were compared using a Student's t-test (unpaired, two tailed; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Dark levels were compared between all populations using ANOVA followed by Newman-Keuls multiple comparison tests. Different lower case letters indicate significant differences between comparisons. (c and d) the expression of *CPD phr* (c) and *ddb2* (d). Expression levels were compared between all populations at each time point in DD by ANOVA followed by Newman-Keuls multiple comparison tests. Different lower case letters indicate significant differences ($p<0.05$) between comparisons. (c') Data from (c) is replotted relative to each individual population's minimum expression to better view phase and amplitude differences. Data represent the mean ± SEM from at least four different fish. (a and b) Performed in collaboration with Christophe Guibal and Elodie Peyric.

6.3.3 DNA REPAIR GENES SHOW CHARACTERISTIC DEVELOPMENT OF REGULATION BY LIGHT IN *ASTYANAX* EMBRYOS

Differences in the development of transcriptional control by light are apparent in *Astyanax* embryos. We therefore examined the expression of *CPD phr* and *ddb2* throughout *Astyanax* embryonic development to see if they are subject to the same development of the light response.

We gave light pulses at various stages of development to embryos of surface and Pachón fish raised in constant darkness. The expression of *CPD phr* and *ddb2* was measured in light-pulsed and dark control samples (Figure 6.2a-h). *CPD phr* is significantly light inducible from 23 hpf in both surface and Pachón embryos, though Pachón embryos show a very small but significant induction at 5 hpf (1.18±0.097; t-test, two-tailed, $p<0.01$, n=10). *Ddb2* is light inducible from the earliest time point in surface embryos, but only significantly inducible from 23 hpf in Pachón embryos. By 50hpf light induction of both genes is significant in both populations, though the induction seen in surface is significantly larger than Pachón due in part to the raised basal levels seen in Pachón at this stage (Figure 6.2g-j). Over 3 days of development in a light dark cycle, Pachón show a significantly raised expression of *CPD phr* in both light and dark on the third day of development, very similar to the expression pattern observed for *per2b* (Figure 6.2k).

In summary, *CPD phr* and *ddb2* are transcriptionally induced by light during *Astyanax* development and in Pachón embryos are subject to the same developmental restrictions as observed for light-responsive clock genes.

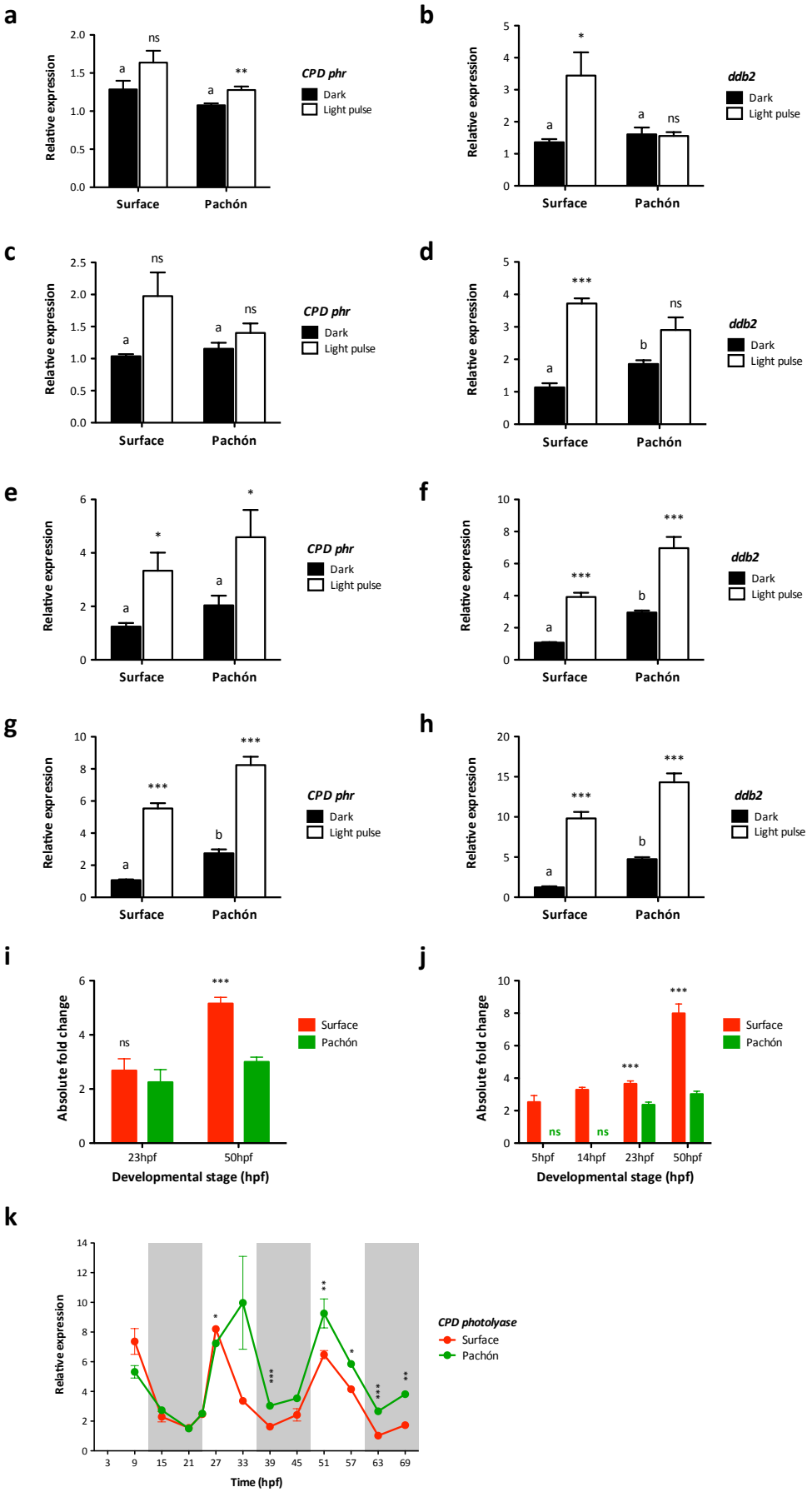
a
Relative expression
2.0
1.5
1.0
0.5
0.0
ns
**
a
a
Surface
Pachón
CPD phr
Dark
Light pulse
b
5
4
3
2
1
0
*
ns
ddb2
c
2.5
d
***
e
6
f
10
g
h
20
i
Absolute fold change
23hpf
50hpf
Developmental stage (hpf)
Surface
Pachón
j
5hpf
14hpf
k
CPD photolyase
Time (hpf)
3
9
15
21
27
33
39
45
51
57
63
69

Figure 6.2: DNA repair genes are regulated by light during development, and show characteristic differences between surface and Pachón embryos.

(a-h) The relative expression of *CPD phr* (a, c, e, g) and *ddb2* (b, d, f, h) was compared at different time points in response to a 3 hour light pulse. Relative expression was calculated using the ΔΔCt method, normalised to the reference gene *rpl13α*. Light pulses began at (a and b) 5 hpf, (c and d) 14 hpf, (e and f) 23 hpf and (g and h) 50hpf. Dark and light-induced levels of *CPD phr* and *ddb2* were compared using a Student's t-test (unpaired, two tailed; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Dark levels were compared between all populations using ANOVA followed by Newman-Keuls multiple comparison tests. Different lower case letters indicate significant differences between comparisons. (i and j) Absolute fold induction of the expression of each gene in response to light was determined in samples with significant difference between light-induced and dark samples. Fold induction was compared between surface and Pachón at each developmental time point using a Student's t-test (unpaired, two tailed; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). (k) Surface and Pachón embryos were subject to a 12hr:12hr LD cycle during development. Flasks were taken every 6 hours for 3 days from 9 hpf. *CPD phr* mRNA levels were measured by qPCR, normalised to the reference gene *ef1α* and relative expression was calculated using the ΔΔCt method. Expression of *CPD phr* in surface and Pachón at each time point was compared using a Student's t-test (unpaired, two tailed; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). White and grey bars indicate light and dark periods respectively. Data represent the mean ± SEM for at least 3 embryo samples.

6.3.4 IN THE CAVE ENVIRONMENT, DNA REPAIR GENES ARE HIGHLY UPREGULATED

In general, the expression of *CPD phr* and *ddb2* is higher in cavefish, and reflects the expression differences seen in acutely light-responsive clock genes. As for cavefish maintained in the lab, *per2b* is very highly expressed in cavefish sampled in the field. Are *CPD phr* and *ddb2*, as light-induced genes in *Astyanax*, also highly expressed in cavefish sampled from the caves themselves?

We measured the expression of *CPD phr* and *ddb2* in fin samples taken from fish caught in the caves. As expected, both genes are highly expressed in the fish from the Chica cave like *per2b*. Unexpected however, is the level of expression, which is significantly raised compared to either surface fish or cavefish within the laboratory (Figure 6.3). This unexpected result begs the question: how and why is the expression of DNA repair genes upregulated in the cave? Is it an adaptive response to the cave habitat to increase DNA repair activity or some direct influence of the environmental conditions of the cave on the expression of these genes beyond the absence of light? Light induced DNA repair gene expression has been shown to improve embryonic survival in response to a UV pulse (Tamai et al., 2004). It is likely this is due to the transcriptional induction by light of the many DNA repair and also due to direct effects of light on protein function (for photolyase). To see if the increased expression of *CPD phr* and *ddb2* in cavefish in the dark actually leads to increased DNA repair, we devised an approach to examine DNA repair activity in the dark in cavefish.

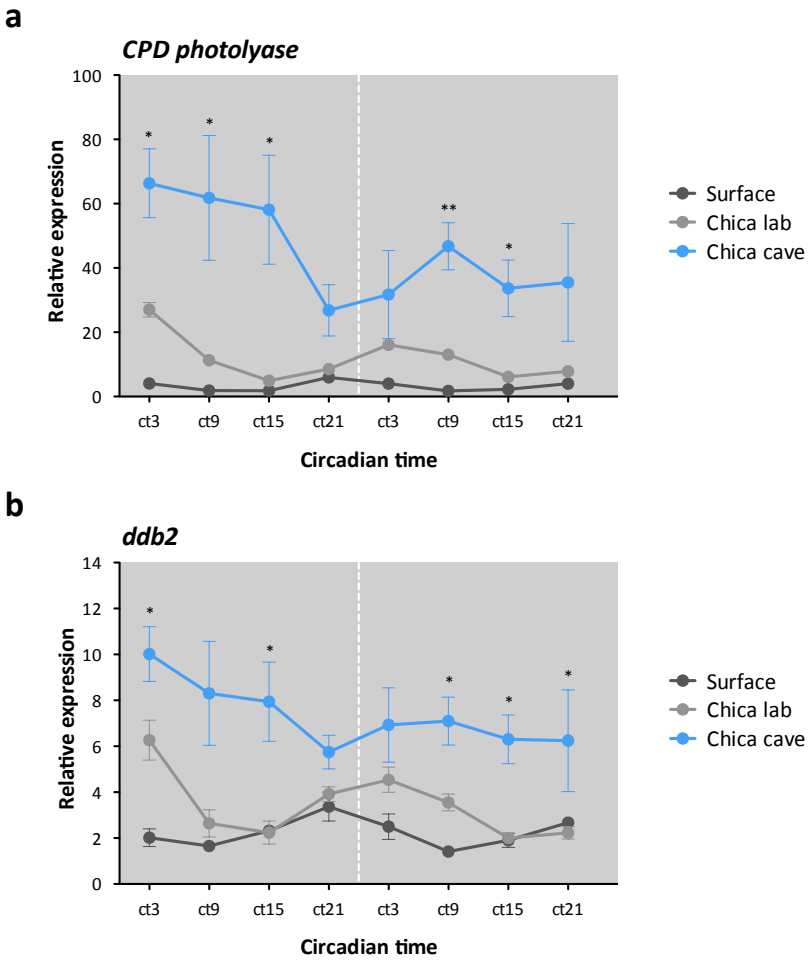

Figure 6.3: DNA repair genes are highly upregulated in the cave environment.
Chica fin samples from were collected in the wild (Chica cave) every 6 hours over two days. Expression of (a) *CPD phr* and (b) *ddb2* was measured by qPCR and normalized to the reference gene *rpl13α*. In the absence of clear zeitgebers in the cave, Chica cave samples are plotted chronologically from the start of sampling (1pm) relative to samples taken in constant darkness from laboratory-entrained surface and Chica cavefish ("surface" and "Chica lab"; reproduced in grey from Figure 6.1) for ease of comparison. Expression of *CPD phr* and *ddb2* in samples of Chica cavefish from the Chica cave and Chica cavefish from the lab at each time point was compared using a Student's t-test (unpaired, two tailed; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Expression of CPD phr and ddb2 in Chica cavefish across all time points was compared using ANOVA, but there was no significant difference. Data represent the mean ± SEM for at least 3 embryo samples. Chica cave samples were collected by David Whitmore and Christophe Guibal.

6.3.5 HIGH EXPRESSION OF *CPD PHR* AND *DDB2* CORRELATES WITH ENHANCED REPAIR ACTIVITY IN *ASTYANAX* CAVEFISH IN THE DARK

Cells are able to repair DNA damage caused by exposure to UV light, removing the photoproducts of UV exposure over time by multiple DNA repair pathways. Photoreactivation is one such pathway that in zebrafish increases survival and reduces mortality after exposure to UV light, but zebrafish also are able to repair DNA damage in the darkness by NER pathways (Ozer et al., 1995; Tamai et al., 2004; Zeng et al., 2009). We employed an in vitro assay to determine if the observed increases in DNA repair gene expression in the dark of *CPD photolyase*, involved in photoreactivation, and *ddb2*, part of the NER pathway, have an effect on DNA repair activity. Samples of the fin of different populations of adult *Astyanax* fish were exposed to UV light and incubated in the dark for 24 hours. DNA damage was quantified by ELISA on DNA extracted from the fins taken 24 hours and immediately after UV exposure using a monoclonal antibody to CPD photoproducts (Mori et al., 1991). The degree of repair was calculated relative to the initial damage and compared between surface fish and Pachón and Chica cavefish (Figure 6.4). DNA damage is significantly lower in Pachón and Chica cavefish than surface fish after 24 hours (Figure 6.4). This strongly suggests that the raised basal dark levels of DNA repair genes in cavefish contribute to an improved ability to repair DNA damage.

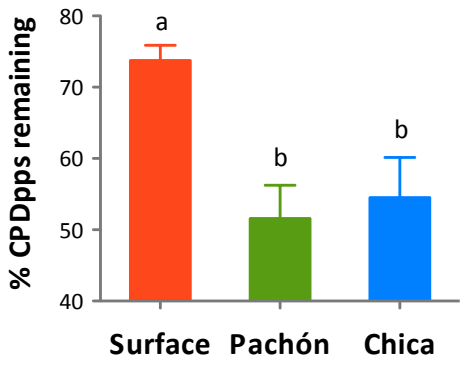

Figure 6.4: DNA repair is more efficient in cavefish
Samples of the caudal fin of surface, Pachón and Chica populations of *Astyanax mexicanus* were exposed to UV light or kept in the dark as a control. DNA was extracted from fins at 0.5hrs and 24hrs after the UV pulse. DNA damage 24 hours and immediately after UV exposure was quantified by ELISA using the monoclonal antibody TDM-2 to CPD photoproducts. The amount of DNA damage remaining was calculated by dividing the corrected OD value (UV damaged OD – dark control OD) at 24hrs by the corrected OD value at 0.5hrs. The percentage of CPDpps remaining at 24hrs was compared between all populations by ANOVA followed by Newman-Keuls multiple comparison test ($p<0.05$, $n=11$).

6.4 Discussion

In this thesis, I have presented evidence that the circadian clock in cavefish shows characteristic changes compared to surface fish that are suggestive of an increased activation of the light input pathway. This raises a fundamental question: why might animals in the dark possess an over-active light input pathway instead of it regressing, as do eyes and pigmentation?

To answer this question we looked at other aspects of light-responsive biology in teleosts. Light has a wide impact on gene expression and physiology in fish (Gavriouchkina et al.,

2010; Weger et al., 2011). Consequently, one would predict that isolation in a cave would have a large influence on a range of biological processes in these animals, not just the circadian clock. DNA repair is one such group of processes that is influenced by light in zebrafish (Yasuhira and Yasui, 1992; Tamai et al., 2004) and two recent studies showed that one of the largest clusters of genes to be upregulated by light in zebrafish are those involved in DNA repair (Gavriouchkina et al., 2010; Weger et al., 2011). In some cases the enzymes require light for catalytic function, further demonstrating the importance of light in this process (Tamai et al., 2004; Brettel and Byrdin, 2010). Therefore, genes encoding DNA repair proteins are good candidates for modified regulation in *Astyanax* cavefish, which have been devoid of light for most of their evolutionary history. To see if this process is altered in cavefish, we isolated and examined the expression of two well characterized DNA repair genes, *CPD photolyase* (*CPD phr*), and a member of the nucleotide excision repair pathway, *damage-specific DNA binding protein 2* (*ddb2*).

In *Astyanax* cave populations, *CPD photolyase* and *ddb2* show broadly raised basal levels of expression throughout all life stages. This is similar to the other light-regulated genes we have examined, thus confirming our initial prediction that light-dependent regulation is altered in cavefish. Surprisingly, this increase in *CPD photolyase* and *ddb2* expression is even more dramatic in the Chica cave field samples, with a significant increase over not only surface fish, but also cavefish kept in the lab. We tested whether this level of upregulation of DNA repair genes observed in cavefish in the dark translates to DNA repair activity by quantifying the level of DNA repair in cultured fins in the darkness. The results showed that, within animals kept in the lab, cavefish are more efficient at repairing their DNA. Thus, in a paralogous situation to the enhanced DNA repair activity observed in transgenic mice overexpressing marsupial *CPD photolyase* (Schul et al., 2002), *Astyanax* cavefish are a natural overexpressing condition with enhanced DNA repair activity. It is reasonable to suggest that the even greater expression of DNA repair genes in the wild cave populations would also lead to further enhanced DNA repair activity. Presumably,

such a large change provides some selective advantage to those animals living under these conditions.

At first, these results might seem rather counterintuitive. Firstly, photolyase proteins such as CPD photolyase require light energy in the catalytic activity, a key step in the DNA repair process (Brettel and Byrdin, 2010). Our experiment was to test DNA repair in the dark, mirroring the natural conditions of the cave. However, CPD photolyase is known to bind to sites of DNA damage in the absence of light and aid additional DNA repair proteins, such as those involved in nucleotide excision repair, to repair damaged DNA (Ozer et al., 1995; Thoma, 1999). It is reasonable, therefore, that *CPD photolyase* in wild cave populations will have a functional role in DNA repair, even within the complete darkness of the cave.

Secondly, what is the purpose of increased DNA repair activity in the dark? Many of the physiological and behavioural changes seen in cavefish have obvious benefits in the cave: an increased number of taste buds (Varatharasan et al., 2009; Yamamoto et al., 2009), reduction in sleep (Duboué et al., 2011), enhanced vibration attractive behaviour (Yoshizawa et al., 2010) all have clear benefits to fish living in the dark. Even so, it is less clear how enhanced DNA repair activity is advantageous in the dark especially since UV light (a major DNA damage agent and one that we have used to demonstrate the DNA repair activity) is not present in the dark cave. What may be responsible for DNA damage in the cave? Endogenous levels of DNA damage are significant in cells even without UV exposure, including the hydrolysis, oxidation and alkylation of DNA bases (reviewed in Barnes and Lindahl, 2004). These processes are enhanced by certain environmental factors. We can speculate that the conditions of the cave pools, which are hypoxic (dissolved oxygen below 0.5mg/l) and slightly acidic, are harsh enough environments to increase instances of DNA damage. Consistent with this are interesting studies that have found DNA damage and oxidative stress are caused by the hypoxic conditions in fish via

indirect generation of reactive oxygen species (Lushchak and Bagnyukova, 2007; Mustafa et al., 2011). Though photolyases and NER are important for repair of bulky lesions caused by UV light (Thoma, 2005; Herrlich et al., 2008), other DNA repair pathways, such as base excision repair (BER), account for the majority of the processing of lesions associated with oxidative damage (Cooke et al., 2003; Zharkov, 2008). Intriguingly, genes associated with the BER pathway, such as *neil1* and *xrcc1*, are also upregulated by light in zebrafish (Gavriouchkina et al., 2010; Weger et al., 2011). Although these genes are yet to be examined in cavefish, it is possible the results presented represent a global increase in activation and expression of DNA repair pathway components, which may have a protective function in the hypoxic caves. This link between DNA damage repair and hypoxia is an interesting area of research in cancer biology, with findings that show decreased DNA repair in the hypoxic tumour environment and therefore more unrepaired DNA lesions (Yuan et al., 2000; Bristow and Hill, 2008). This area may prove an interesting future research avenue in *Astyanax* cavefish.

Our experiments do not assess the effect of hypoxia on DNA damage or on the efficiency of DNA repair (Mustafa et al., 2011). However, our results strongly suggest that DNA repair is more efficient in cavefish, and is likely to be due to an increase in expression of DNA repair genes, which is greatly exaggerated in the wild. We suggest due to the evidence showing a link between the light induction mechanisms of light-induced clock genes and DNA repair genes in zebrafish (Hirayama et al., 2009; Gavriouchkina et al., 2010; Weger et al., 2011), that a common alteration may lead to the upregulation of both sets of genes. Our hypothesis is that, by tonically activating light-dependent signalling pathways and therefore increasing DNA repair gene expression (and resultant DNA repair activity), individuals in the cave would reduce the frequency of deleterious mutational events. It is crucial for this hypothesis that the common change be found, though there is evidence in *Astyanax* cavefish evolution of other linked evolutionary changes (Jeffery, 2008; 2010): QTL analysis reveals overlapping QTL governing eye and taste bud traits (Protas et al.,

2008), which experimental data confirms through a reciprocal relationship, or pleiotropy, between eye size and taste bud number (Yamamoto et al., 2009). DNA repair and circadian activity may be another example of pleiotropy, where cavefish have developed a process that would provide a clear advantage to those animals possessing this trait, even at the expense of possessing a dampened or disrupted circadian pacemaker.

7 CONCLUDING REMARKS AND GENERAL DISCUSSION

Though the true fitness advantage of circadian clocks has not been fully established, their ubiquitous presence in most, if not all, organisms implies an adaptive nature. It is generally held that the adaptive nature relates to the environmental cycles on a rotating planet (for example Pittendrigh, 1993). Logically, this leads to the suggestion that in constant environments the advantages to an animal of possessing a clock are no longer present and so it is subject to regressive evolution. This thesis aimed to explore and challenge this statement by investigating the circadian clock of a cave-dwelling fish, *Astyanax mexicanus*.

Our work was predominantly focused on the core clock components as identified in a closely-related fish, the zebrafish, *Danio rerio*. The focus on these core components remained throughout the whole of this thesis, though the work expanded to consider broader aspects of cell biology that are controlled by light, including DNA repair pathways.

In summary, the main findings of this thesis are:

- *Astyanax mexicanus* surface fish possess an entrainable molecular circadian clock similar to that described for other teleosts.
- *Astyanax* cavefish express clock genes very similar in sequence to surface with a high degree of conservation between cave populations. Analysis of clock gene expression under LD cycles indicates that molecular clock function is retained in cavefish despite millions of years of evolution in darkness. However, in the wild, the molecular circadian clock is suppressed.
- The cavefish circadian clock, represented by *per1* expression rhythms, has acquired characteristic changes during evolution, including phase and amplitude differences. These differences are also present in the expression of *cry1a*, *tef1* and *CPD phr* and together indicate alterations of the core clock and light input pathway.

- A significant alteration of the light input pathway in cavefish is the raised expression of acutely light-induced genes in the dark, especially *per2b*, which may act to dampen the amplitude of the core clock oscillation.
- Transcriptional induction by light appears later in development in cavefish compared to surface fish, which suggests differential expression of upstream light-detecting genes such as opsins, though the expression of one candidate opsin, *tmt opsin 1*, did not correlate with the differences observed in light induction.
- We hypothesised that the other aspects of light-regulated biology are altered in the cave, including the regulation of DNA repair genes. Relative to surface fish, two genes involved in separate DNA repair pathways, *CPD phr* and *ddb2*, are highly expressed in cavefish in the darkness and are induced by light later in development in developing cavefish embryos compared to surface fish embryos. These genes are both very highly expressed in wild cavefish. These changes in expression are very similar to the changes in the expression of light induced genes of the molecular clock in cavefish relative to surface fish.
- We tested the DNA repair ability of cavefish, and showed that it is enhanced relative to surface fish. This correlates with the higher levels of expression observed in cavefish.

Collectively, these findings address the following issues, which will be discussed below:

- The presence of circadian clock mechanisms in caves
- Evolution and regression of the circadian clock
- Pleiotropy or trade-off as a mechanism for evolution
- *Astyanax mexicanus* cavefish relationships

7.1 CLOCKS IN CAVES

We have extended molecular circadian clock studies to another 'new' model organism that, in addition to showing the conservation of the circadian clock components and mechanism across teleosts, is a fascinating model for the study of circadian clocks in extreme environments and progresses the understanding of circadian clock function in cave-adapted animals.

Until very recently, circadian clock studies in cave animals examined behavioural rhythms, and showed a range of phenotypes from no circadian rhythms to pronounced circadian rhythms. However, as we show in this thesis, behavioural rhythms do not always reflect the underlying molecular oscillator; the rhythms can become uncoupled. This situation is similar to that observed in the blind mole rat, *Spalax ehrenberg*, in which there is a large variability in the presence of free-running, and therefore clock-controlled, activity rhythms despite evidence for a complete and functional molecular clock (Tobler et al., 1998; Avivi et al., 2002). One explanation for this uncoupling is that selection has relaxed the control of activity by the circadian clock in environments where there is little or no rhythmicity, perhaps to increase survival by improving chances for foraging in a food-restricted environment such as a cave, a suggestion that agrees with data proposing a reduction in sleep in *Astyanax* cavefish (Duboué et al., 2011). Therefore, analysis of the core molecular clock of cave animals is very important, as certain and specific clock outputs may no longer be under clock control.

A recent study by Cavallari and colleagues (Cavallari et al., 2011), which studied the molecular clock and activity rhythms of another cave-dwelling fish, the Somalian cave fish, *Panderichthys andruzzii*, provided a very interesting comparison to our studies in *Astyanax*. These fish are behaviourally and molecularly arrhythmic when exposed to light-dark cycles, but exhibit behavioural entrainment and weak molecular entrainment to regular feeding. Cavallari and colleagues suggest mutations in *melanopsin* and *tmt opsin*

are responsible for the 'blind' nature of the circadian clock, but do not fully explore the other features of the *P. andruzzi* molecular clock that they present, such as its reduced ability for temperature compensation and apparent long period, which indicate some significant changes within the core clock. However, it is possible that this cave fish retains at least partial clock function.

The data presented in this thesis, together with data from *P. andruzzii* and circadian activity data in other cave animals (including cave crayfish (Jegla and Poulson, 1968), cave cricket (Reichle et al., 1965) and cave loach (Pati, 2001)), suggests that the core clock mechanism is not fully lost in cave-dwelling animals despite the constant darkness of their environments. A possible explanation is that there simply has not been enough time or selective pressure to lose the clock mechanism in caves, and the redundancy in the network (especially in teleosts with multiple copies of genes) is a possible buffer to complete loss. However, this explanation is less favoured for at least two reasons. Firstly, characteristics such as pigmentation are lost due to neutral mutation and genetic drift, suggesting that relaxed selection for this amount of time is able to result in significant trait loss. The clock appears to be significantly repressed in cavefish in the wild (Chapter 4), and so it is remarkable that the mechanism remains largely intact. Secondly, circadian rhythms are in some form retained across many cave species, despite vast differences in their evolutionary histories and environmental pressures. These data suggest that the clock may have some intrinsic adaptive value even in the absence of environmental cycles, which prevents it from being completely lost. This may come from clock control of the cell cycle, demonstrated recently in zebrafish (Tamai et al., 2012), or other physiological processes.

7.2 How the circadian clock has evolved in *Astyanax* cavefish

In this thesis, we examined the expression of multiple genes during entrainment to light and dark cycles and upon transfer into constant darkness. Analysis of the *per1* expression

rhythms of *Astyanax* cavefish showed characteristic changes during evolution, including phase and amplitude differences. These differences are also especially clear in the expression of *cry1a*, *tef1* and *CPD phr* in constant darkness, and together suggest the presence of alterations to the core clock and light input pathway.

We have discussed the possible explanations for the phase delay of expression of rhythmic genes in Section 3.4, which include differences in free-running period and the phase response curve. However, these explanations were not pursued experimentally in this study and the focus of this thesis turned to the light input pathway to the circadian clock. We hypothesised that alterations in this pathway may explain the reduced amplitude of the cavefish clock as well as the changes in phase. We observed differences in the light input pathway, represented by the known clock-resetting genes *per2a/b* and *cry1a*, which show upregulation in cavefish in the dark. Again, we can contrast these results with those of *P. andruzzii* (Cavallari et al., 2011). As discussed above, *P. andruzzii* is 'blind' – its circadian clock fails to respond to light. By contrast, *Astyanax* cavefish are still able to 'see' light and in fact experience an overactivation of the light input pathway in the dark, for which possible reasons are discussed below. However, though the retention of some molecular clock function within the fish may be beneficial in terms of internal temporal order, one might question why *Astyanax* cavefish retain any ability to entrain to light. Interestingly, both cavefish retain a photophobic response: *Astyanax* larvae, which is linked to a functional pineal gland (Yoshizawa and Jeffery, 2008), and *P. andruzzii* adults, whose response is intriguingly linked to functional rhodopsin and exo-rhodopsin photopigments (Tarttelin et al., 2012). In the case of *Astyanax* cavefish, Yoshizawa and Jeffery suggest that retention of a light detecting pineal gland is a developmental constraint related to the pineal gland's role in neurosecretion (Yoshizawa and Jeffery, 2008).

P. andruzzii have been isolated from the day-night cycle for between 1.4 and 2.6 million years, a similar period of time to estimates of *Astyanax* cavefish divergence. Perhaps selection against components of the light input pathway has been stronger in the Somalian caves, which have led to their more degenerate phenotype, though this is purely speculation. If the circadian clock is retained in cave animals on the most part due to its benefit for internal synchronisation, then the retention of light detection in *Astyanax* cavefish may be a functional constraint of the clock mechanism, and the light input pathway be linked to the core clock itself. This link is found in *Drosophila* where, although *cry* is a major photopigment (Stanewsky et al., 1998; Emery et al., 2000), it also functions in the core clock in the periphery (Krishnan et al., 2001; Collins et al., 2006). In this way the photopigment may be retained during evolution due to its role in the core clock.

In summary, it is clear that the two cavefish have divergent evolutionary histories relating to their circadian clocks.

7.3 EVOLUTIONARY TRADE OFF

A benefit of *Astyanax* as a model system is the ability to make direct contrasts with a closely related surface-dwelling fish to find direct changes in the clock mechanism during evolution in the dark. These comparisons are not possible to perform in *P. andruzzii* because there is no surface fish ancestor to compare it with. In general, we observed increased dark activity of the light input pathway in *Astyanax* cavefish, with *per2b* upregulated in the majority of constant dark timepoints. This result was intriguing, and we therefore sought possible explanations for why the light input pathway is upregulated.

Research in zebrafish shows that light is important for the transcriptional control of a broad array of genes (Gavriouchkina et al., 2010; Weger et al., 2011). We hypothesised that some of these various functional classes of genes may be upregulated in cavefish. Genes involved in DNA repair were one of the largest classes of genes upregulated by light,

so we examined the expression of two DNA repair genes, *CPD phr* and *ddb2*. As predicted, in general these genes show similar expression patterns to the light-induced clock genes. This, and evidence that DNA repair and the circadian clock share upstream signalling pathways (Hirayama et al., 2009; Weger et al., 2011), suggested that the increased activation of the light input pathway may have a pleiotropic effect with selection acting to raise the expression and activity of the DNA repair pathways at the expense of some circadian clock function. A pleiotropic mechanism is suggested for other aspects of the evolution of *Astyanax*'s physiology (Protas et al., 2007; 2008), which suggests it may also be involved here. We can only speculate about the conditions that gave this selective pressure, but hypoxia has been linked to oxidative damage in other teleosts (Lushchak and Bagnyukova, 2007; Mustafa et al., 2011), and is a characteristic of many caves harbouring *Astyanax* cavefish. We predict that DNA repair pathways involved in repairing oxidative damage, such as BER, will be similarly upregulated in *Astyanax* cavefish.

7.4 EVOLUTION OF *ASTYANAX* CAVEFISH

In total, nine genes were cloned (either partially or in full) from populations of *Astyanax* surface fish and multiple cavefish. This data allowed us to add to conclusions on the evolutionary history of *Astyanax*. We have already discussed the problem of the identity of the surface fish ancestor to the cavefish in Section 3.4. However, despite this complication, from this data we can conclude that the coding regions of genes from multiple populations of *Astyanax* cavefish are very similar. This agrees with a statement made by Jeffery in a review on regressive evolution in the cavefish (Jeffery, 2009), who states that, "*Astyanax* cavefish populations show minimal genetic variation in the coding regions". Jeffery makes this statement to suggest that this is a reason why the more divergent microsatellite sequences and mitochondrial DNA are more suitable for inference on phylogenetic relationships of the cavefish. However, it is nonetheless remarkable that such a conservation of sequence is observed. There are no reliable estimates for the divergence

time of cavefish, but evidence does suggest very little if any gene flow between cave populations (Bradic et al., 2012; Strecker et al., 2012), and so the coding sequences reflect either:

- Conservation of the ancestral sequence, which is different to the current surface fish population;
- Molecular convergence during adaptation to the individual caves.

We attempted to assess this by performing light pulse experiments on cave-cave hybrids. This genetic complementation assay was designed to reveal whether the absence of light induction at 5-8 hpf in Pachón and Chica cavefish (independent populations according to Bradic et al. (2012)) is due to the same or different mutations or alterations in the light input pathway. This experiment was similar to published experiments that show non-complementation in pigmentation and complementation in eye size (Protas et al., 2006; Borowsky, 2008). Hybrid embryos did not show a light response, and therefore we concluded that the defect lies in the same gene or pathway. Given that surface fish embryos are able to respond to light at this stage (similar to zebrafish (Tamai et al., 2004; Ziv and Gothilf, 2006)), and these two populations are independent (Bradic et al., 2012), this result suggest that similar pathways have mutated during evolution in the darkness.

7.5 FUTURE DIRECTIONS

Astyanax mexicanus is a fascinating model system to examine the effect of the lack of light on the evolution of many physiological features. This thesis has focused on the circadian clock of *Astyanax* cavefish and has examined specific alterations in light-regulated gene expression. However, many other avenues for research have arisen from the data presented here.

7.5.1 DETAILED ANALYSIS OF THE PHASE DELAY

- Whilst we focused on the light input pathway to explain why the cavefish clock is altered, it is also a possibility that changes in the core clock are responsible for the phase delay. Alterations at the level of promoters have been shown to change phase in many studies, and are a possible mechanism here. Promoter alterations would have to be present in at least the *per1*, *cry1a*, *CPD phr*, and *tef1* genes and not be operating in the early embryos as no phase delay is seen in their *per1* rhythm at that stage. In order to carry out this analysis, we would require a higher resolution sampling method to accurately assess phase difference and the promoter environments. In the lab we have established an automated luciferase reporter system, which has been very successful in measuring promoter activity in cell lines (for example Tamai et al., 2007). Cell lines have been developed from *Astyanax* (See Appendix), which, although not exactly the same phenotype as adults or embryos, exhibit similar phase differences during entrainment to LD cycles. This system could be useful in examining promoter environments and also be used for the accurate calculation of free-running period during long experiments in constant darkness.

7.5.2 FURTHER EXAMINATION OF THE LIGHT RESPONSE

- The altered phase suggested for the cavefish clock may contribute to the reduced light response seen in acute light pulse experiments in adults. Extending this analysis to pulses throughout the circadian cycle may reveal a more accurate representation of the acute light response of the adult cavefish circadian clock.
- In embryos, we do not observe the phase delay in LD or DD, which would preclude an effect of phase difference between cavefish and surface fish as an explanation for the smaller light induction in cavefish. We suggest instead that the difference in light induction may be due to the differential expression of upstream light input pathway components such as opsins and D-box binding factors. A comparison of

embryos at 5-8hpf to see why cavefish are 'blind' at this stage and examination of opsin gene expression may reveal the genes responsible.

- As there is strong evidence of D-box regulation of the light-response in zebrafish, with evidence for a role for the D-box binding factor *tef*, we examined the expression of *tef* in cavefish. Our data does not support *tef* having an important function in the raised basal levels of light-induced genes. However, another unexplored candidate D-box binding factor is the repressive factor, E4bp4. Six *e4bp4* genes have been examined in zebrafish, and *e4bp4-6* is suggested as a good candidate for a role in light-signalling in the pineal gland (Ben-Moshe et al., 2010). An examination of the expression and function of this and other members of the E4bp4 family in *Astyanax* would be useful to see if these are the cause of the increase basal levels in DD and the reduced amplitude clock.

7.5.3 DNA REPAIR

- It is important to note that endogenous DNA damage occurs in all living cells at quite a remarkable rate (Barnes and Lindahl, 2004). This level of damage is increased by specific environmental factors, or stressors. In the absence of UV damage in the cave, we speculate that hypoxia may be the principle DNA damaging agent. We would like to test the effect of hypoxia on DNA in cavefish, and whether it induces DNA damage. This would include an examination of expression of DNA repair genes in more cave-like conditions, including water with low dissolved oxygen content.
- The BER pathway is the principle pathway responsible for the repair of oxidative damage. If hypoxia were the cause of DNA damage in the cave, we would expect members of this pathway to be amongst those DNA repair genes that are upregulated. BER pathway members including *neil1* and *xrcc1* were identified in screens of the light responsive transcriptome in zebrafish (Gavriouchkina et al., 2010; Weger et al., 2011). This makes them possible candidates for upregulation in

cavefish and an examination of their expression would enhance our DNA repair gene data.

- For a pleiotropic mechanism of enhancement of DNA repair and reduction in clock function to be true, there would need to be linked changes between DNA repair and circadian clock. We have suggested that this might lie in the upstream signalling pathways that are proposed to contribute to the regulation of genes in both pathways, such as D-box regulation and MAPK signalling (Hirayama et al., 2009; Gavriouchkina et al., 2010; Weger et al., 2011). As suggested above, an analysis of additional D-box binding factors such as *e4bp4* would be useful. A QTL analysis similar to Protas et al. (2007) would be able to extend this hypothesis to see if changes in the light input pathway to the circadian clock and DNA repair are linked traits in cavefish.
- Are DNA repair genes similarly upregulated in the Somalian cavefish, *P. andruzzii*? It would be difficult to say whether *P. andruzzii* has an *increased* expression of DNA repair genes in the same way as we present for *Astyanax* cavefish, as there is no closely related ancestral fish to act as a baseline for expression levels. However, DNA repair gene expression and activity could be examined in this species to see if they have a tolerance to DNA damaging agents similar to *Astyanax* cavefish, and determine whether DNA repair systems are a common target of change amongst cave animals.

Together, the work presented in this thesis demonstrates the power of comparative molecular biology techniques to isolate and study novel genes in novel organisms. We have used these techniques successfully to uncover circadian clock function in a cavefish species, *Astyanax mexicanus*, in the laboratory and the field, and present evidence to suggest possible adaptive explanations. Further functional studies on the regulation of light induced and DNA repair genes are needed to strengthen the suggestion that these pathways are altered in the cave and will go some way to test the hypothesis of

antagonistic pleiotropy as the evolutionary force leading to the cavefish circadian clock phenotype.

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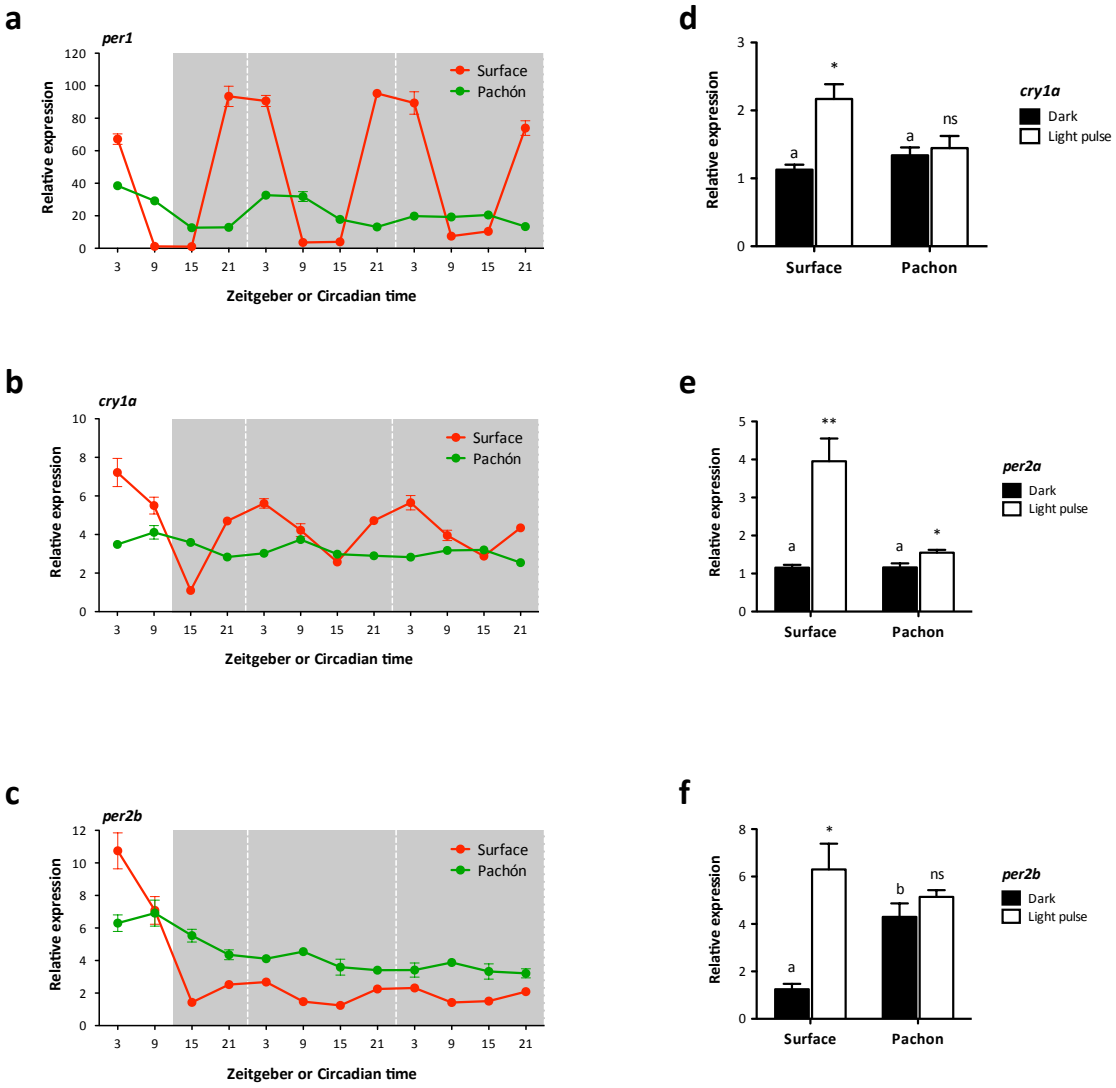
APPENDIX A



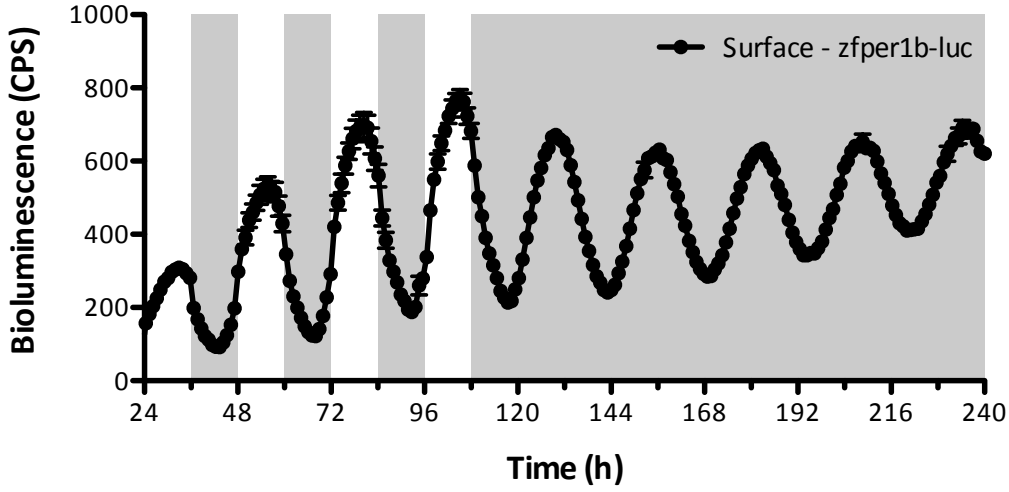
Clock gene expression in *Astyanax mexicanus* cell lines.

Cell lines of *Astyanax* surface fish and Pachón cavefish, created as described in Section 2.1.3, were cultured in 6-well plates in a thermostatically controlled waterbath at 28°C and entrained to a 12hr:12hr light-dark cycle. One 6-well culture plate was used per timepoint for each *Astyanax* population, with the cells of 2 wells being pooled together during harvesting. The cells were harvested in TRIzol, total RNA extracted and cDNA synthesised as described. (a-c) Entrainment continued for 7 days before transfer into constant darkness. Sampling began at ZT3 of the 7th day of entrainment and continued every 6 hours for 3 days. (d-e) At ZT16 of the 7th day of entrainment, cells were given a 3-hour light pulse or kept in the dark as a control. 3 plates of cells of each population were given the pulse. The expression of (a) *per1*, (b and d) *cry1a*, (c and f) *per2b* and (e) *per2a*

mRNA was measured by qPCR, normalised to the reference gene *rpl13α*. Relative expression was calculated using the ΔΔCt method. Light and dark samples in (d-f) were compared using a Student's t-test (unpaired, two tailed, n=6; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Dark and light induced levels of all genes were compared using a Student's t-test (unpaired, two tailed, n=6) with different lower case letters indicating significant differences ($p<0.05$).

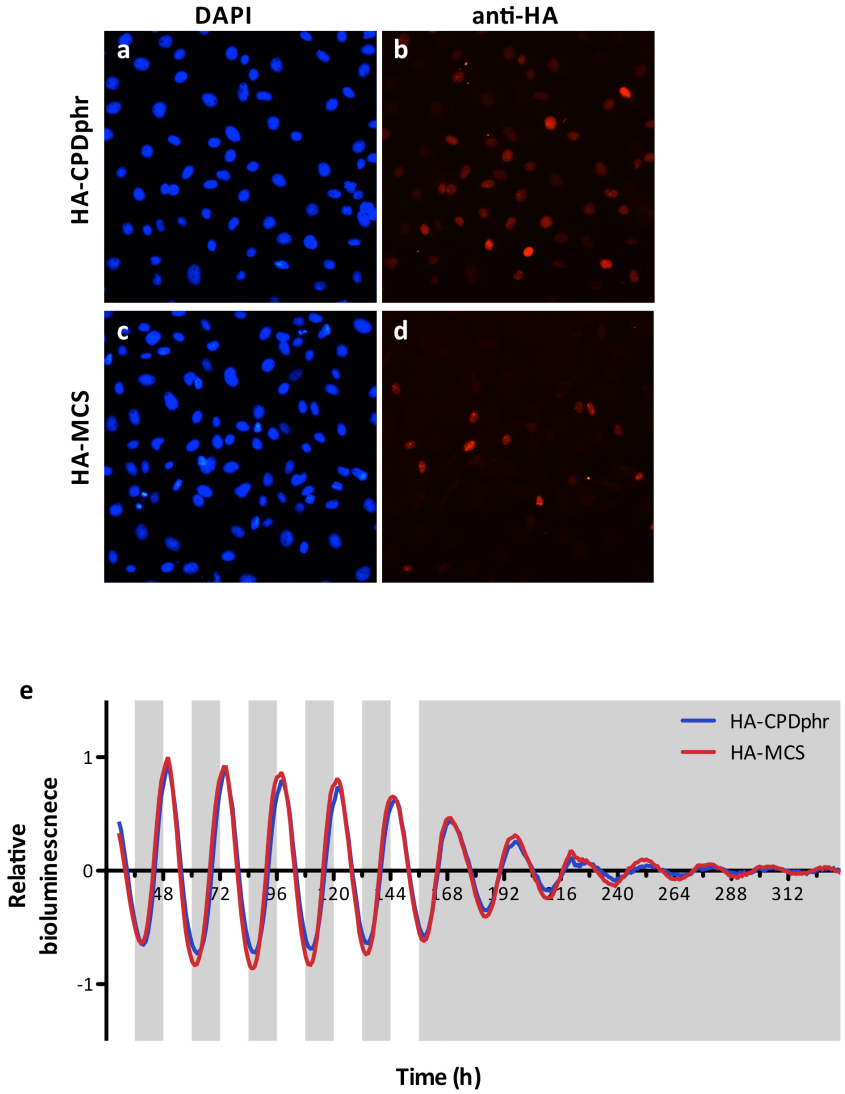
Pachón cavefish cell lines show a highly reduced circadian clock relative to surface fish cell lines and embryos or adults of the Pachón cave population. *Per2b* expression is higher in Pachón cells in the dark as seen in embryos and adults. Light pulse experiments show that the acute light response is almost lost in the cell lines, and together with the significantly raised levels of *per2b*, will lead to the very low amplitude circadian clock in Pachón cell lines under a light-dark cycle.

APPENDIX B



Zf*per1b* reporter expression in a surface fish cell line.
Surface cells were transfected with a zfper1b-luc reporter construct as described in Section 2.1.4. A clonal cell line, H9, was isolated and the expression of the reporter construct in this cell line was measured as described in Section 2.1.4. The cells were plated at a concentration of 100,000 cells/ml in individual wells of a 96-well plate with media containing luciferin, placed in a Packard TopCount luminometer in a 12hr:12hr light-dark cycle and bioluminescence (count per second, CPS) was recorded. The first 24 hours of data are not included in this graph. After 5 days, the light cycle was turned off and the cells were allowed to free-run. The zebrafish construct reports a peak of expression at ZT7-8 and a trough at ZT19, both appropriate delays relative to the endogenous expression of *per1* in the surface fish cell line. White and grey bars represent periods of light and dark respectively.

APPENDIX C



Overexpression of *CPD photolyase* in zebrafish cells

Photolyases, relatives of the cryptochromes, have been shown to interact with the circadian clock in overexpression studies in mice using marsupial and bacterial photolyase constructs (Chaves et al., 2011a; Biernat et al., 2012). As *Astyanax* cavefish express more *CPDphr* than their surface fish counterparts, we decided to investigate the interaction of a teleost photolyase on a teleost clock using zebrafish cell culture. Zebrafish zfper1b-luc reporter cells were transfected with an overexpression vector containing HA-tagged CPDphr (HA-CPDphr) or the multiple cloning site (HA-MCS) as a control as described in Section 2.10. (a-d) Expression of the construct was tested by immunohistochemistry for the HA tag (b and d) and the nuclei were stained with DAPI (a and c). (e) The expression of the *per1b*-luc reporter construct in both cell lines was measured as described in Section

2.1.4. The cells were plated at a concentration of 100,000 cells/ml in individual wells of a 96-well plate with media containing luciferin, placed in a Packard TopCount luminometer in a 12hr:12hr light-dark cycle and bioluminescence (count per second, CPS) was recorded. After 6 days, the light cycle was turned off and the cells were allowed to free-run. Bioluminescence counts for each cell line were detrended using a moving average of 24 hours and plotted relative to the peak bioluminescence of the empty vector cell line. White and grey bars represent periods of light and dark respectively. No significant difference in amplitude, phase or period is observed between the two populations of cells.