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Development of the *Astyanax mexicanus* circadian clock and non-visual light responses

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ABSTRACT

Most animals and plants live on the planet exposed to periods of rhythmic light and dark. As such, they have evolved endogenous circadian clocks to regulate their physiology rhythmically, and non-visual light detection mechanisms to set the clock to the environmental light-dark cycle. In the case of fish, circadian pacemakers are not only present in the majority of tissues and cells, but these tissues are themselves directly light-sensitive, expressing a wide range of opsin photopigments. This broad non-visual light sensitivity exists to set the clock, but also impacts a wide range of fundamental cell biological processes, such as DNA repair regulation. In this context, *Astyanax mexicanus* is a very intriguing model system with which to explore non-visual light detection and circadian clock function. Previous work has shown that surface fish possess the same directly light entrainable circadian clocks, described above. The same is true for cave strains of *Astyanax* in the laboratory, though no daily rhythms have been observed under natural dark conditions in Mexico. There are, however, clear alterations in the cave strain light response and changes to the circadian clock, with a difference in phase of peak gene expression and a reduction in amplitude. In this study, we expand these early observations by exploring the development of non-visual light sensitivity and clock function between surface and cave populations. When does the circadian pacemaker begin to oscillate during development, and are there differences between the various strains? Is the difference in acute light sensitivity, seen in adults, apparent from the earliest stages of development? Our results show that both cave and surface populations must experience daily light exposure to establish a larval gene expression rhythm. These oscillations begin early, around the third day of development in all strains, but gene expression rhythms show a significantly higher amplitude in surface fish larvae. In addition, the light induction of clock genes is developmentally delayed in cave populations. Zebrafish embryonic light sensitivity has been shown to be critical not only for clock entrainment, but also for transcriptional activation of DNA repair processes. Similar downstream transcriptional responses to light also occur in *Astyanax*. Interestingly, the establishment of the adult timing profile of clock gene expression takes several days to become apparent. This fact may provide mechanistic insight into the key differences between the cave and surface fish clock mechanisms.

1. Introduction

Most animals and plants live on a rhythmic planet, with regular and predictable periods of light and dark. As a result, they possess an endogenous circadian clock that synchronizes their physiology and behaviour with the environmental light-dark cycle. Light is the most significant signal for setting the clock, and animals possess a variety of non-visual light detection mechanisms to achieve this. Most of what we know about teleost clocks and light-sensitive biology comes from studies in zebrafish (Tamai et al., 2005., Tamai et al., 2007; Vallone et al., 2004; Weger et al., 2011; Whitmore et al., 1998; Whitmore et al., 2000). All zebrafish tissues are directly light-sensitive and contain a circadian pacemaker, which means that all tissues can detect light and set the circadian clock without the need for eyes or a centralized neural clock (Whitmore et al., 2000). Clocks contained within specific tissues control the rhythmic physiology of those tissues, though the requirement for whole body coordination of these daily oscillators in fish is not yet clear. As a consequence of this direct cellular light senstitivity, the tissues and cells of the fish body must contain the relevant photopigments or opsins, and the intracellular signalling pathways necessary to

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set this clock. This is, in fact, the case with fish expressing up to thirty-two non-visual opsins (Davies et al., 2003). Different tissues express various combinations of these opsins, the functional consequence of which is not yet completely clear, but it certainly provides fish tissues with a remarkable potential to absorb and respond to light of various intensities and wavelengths (Davies et al., 2011, 2003).

With such a remarkable whole body, light sensitivity, it is not surprising that setting the clock is not the only function of environmental light detection. It is clear that zebrafish light sensitivity activates numerous cell signalling events, which impact a variety of fundamental cell processes, including cell cycle regulation through the clock, metabolic processes and cell communication, but perhaps the most strongly light-regulated events are those relating to DNA repair (Dekens et al., 2003; Dickmeis et al., 2007; Hirayama et al., 2009; Tamai et al., 2004, 2005, 2012). Not only is light necessary for the protein function of DNA repair enzymes, such as the photolyases, but it is also for their transcriptional activation. If a fish is not exposed to light, then it is unable to turn on a wide range of pathways essential for DNA repair. It is clear, therefore, that light responsiveness and the presence of a clock are fundamental aspects of fish physiology.

In this context, the study of non-visual light detection and clock biology is extremely intriguing in species such as *Astyanax mexicanus* (Beale et al., 2016). Over the past few million years, groups of *Astyanax mexicanus*, has been isolated from neighbouring rivers in underground caves in the North East of Mexico (Gross, 2012). As a result, we can today find over 30 distinct populations of *A. mexicanus* in numerous isolated caves. All of these populations have evolved and adapted to a life in complete darkness. Adaptations to the dark include the loss of eyes and pigment, as well as changes in metabolic rates, activity and the loss of sleep activity/circadian rhythms to varying degrees (Beale et al., 2013; Jeffery, 2009; Gross et al., 2009; Jaggard et al., 2018; Protas et al., 2006, 2007; Yoshizawa et al., 2015). What makes the *A. mexicanus* such an excellent model for studying not only adaptive and regressive evolution, but also adaptations of light and clock biology to a dark environment, is that the founding species of river fish are still found in abundance in the rivers of Mexico. The surface fish and the cave strains of *A. mexicanus* have not fully speciated, and can therefore be crossed in the laboratory to produce F1 hybrids. It is therefore possible to determine molecular adaptations to the constant darkness, by directly comparing the founding river fish with the isolated cave populations (Bradic et al., 2012; Dowling et al., 2002; Strecker et al., 2003; Strecker et al., 2004).

One would expect the fundamental aspects of light and clock biology to be very similar between surface strains and those described in zebrafish, if only because both live and have evolved in a rhythmic light-dark river environment. However, cave strains offer a much more interesting scenario, where the existence and role of light and clock biology is obviously far from clear, considering the long period of evolution in a completely dark environment. Several previous studies have addressed this issue to some extent in adult animals, though not to date during embryo development. From an activity perspective, cave strains of *Astyanax* lack any robust day-night rhythms in activity that are seen in surface populations, being both effectively continuously active and not showing signs of classical sleep behaviour (Duboue et al., 2011). At the molecular clock level, cave strains in the laboratory are still capable of showing rhythmic, daily oscillations in gene expression (Beale et al., 2013). However, these clock rhythms show certain, specific alterations between surface and cave strains. Cave populations possess molecular clock rhythms with lower amplitude than surface fish, and the phase or daily timing of these rhythms is clearly delayed by up to 6 h. However, in the caves themselves, in North eastern Mexico, to date there is no evidence of any molecular clock rhythms, and in fact the expression levels of several clock components appears to be repressed. Under natural conditions, there is no evidence to date that they employ a rhythmic molecular clock to control timed aspects of their physiology.

Though there are clear mutations in the circadian clock mechanism in cave strains, perhaps the largest changes are seen in the response of these animals to light (Beale et al., 2013). In cave strains, light-inducible genes that are essential for clock entrainment are already highly transcribed in the dark. Cave strains look “molecularly” as if they are living under constant light conditions when in fact living in constant darkness. Consequently, the degree of apparent light activation is greatly reduced. As these genes, such as the light-inducible *period* genes and *cryptochrome 1a* (*cry1a*), are transcriptional repressors, one hypothesis is that their basally raised expression levels are in part the reason for the reduced amplitude of the cave strain clock, as well as the delayed phase seen in the molecular mechanism. This basal activation of light responsive genes is not only restricted to clock genes, but genes that encode the light responsive DNA repair genes, photolyases, also show increased levels of expression in the dark. As DNA repair is a highly light-dependent process in fish, this change in the regulation of these genes to being expressed at high levels in the dark in cave strains is probably a very critical adaptation for these animals to survive in the cave environment.

The above changes in clock and light biology have been explored in adult *Astyanax mexicanus*, but never during the early stages of embryo development. Yet in zebrafish, it has been shown that both the clock and light have a major impact on the process of embryo development, and the regulatory genes involved in embryogenesis (Dekens and Whitmore, 2008; Laranjeiro and Whitmore, 2014). The molecular clock appears to begin to oscillate early in zebrafish development with the first peak in *period1* gene expression seen at 27 h post fertilization. Acute non-visual light sensitivity can be detected even earlier by between 6 and 9 h post fertilization and before the differentiation of any classical light responsive structures in the embryo (Tamai et al., 2004; Dekens and Whitmore, 2008). Photolyases involved in DNA repair become transcriptionally activated at this developmental stage also, and a lack of light exposure during embryo development leads to a dramatic increase in larval mortality when these dark raised embryos are exposed to environmentally stressing conditions, such as UV light exposure. The clock controls the expression of many genes known to be important in the process of embryo development, including the regulation of genes critical in the regulation and timing of the cell cycle, such as *p20/p21* (Laranjeiro et al., 2013). Interestingly, the rhythmic regulation of these downstream/output genes often does not occur until day 3–4 of development, and raises the possibility that a fully functional circadian clock system is not present until these later stages of embryo development.

Considering the relevance of non-visual light detection and circadian rhythmicity to development in zebrafish, the obvious question arises about how these processes function during the development of *Astyanax mexicanus* comparing both surface and cave strains. What are the embryonic differences in early light sensitivity between strains? Does a molecular clock become established as early as detected in zebrafish, and is there a difference between surface and cave populations? Do cave strains develop a circadian clock in the same manner as surface fish, and are the differences reported in adult *Asytanax* present immediately in cave strain embryo development? Furthermore, how does this impact the critical regulation of DNA repair activation during development? In this study, we will address each of these issues in *Astyanax mexicanus*, exploring the differences between surface and cave strains. We demonstrate that surface fish are acutely light responsive from the earliest stages of development, but that this light sensitivity appears to be delayed in cave strains. This difference is not dependent upon alterations in pineal physiology, as this light response occurs globally in most cells in *Asytanax mexicanus*, as previously described for zebrafish. A very shallow circadian oscillation can be detected in surface embryos during the first two days of development, with no rhythm present in cave strains, but in both cases a more robust circadian clock begins to function on the third day of development. Interestingly, the balance of light *versus* circadian clock regulation