Light and Clocks in Dark Places

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Thesis submitted for the fulfilment of the requirements
for the degree of Doctor of Philosophy

2021

I, Inga Angelica Frøland Steindal, 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 sun is the premise of our existence. Most animals and all plants use the predictability of the sunrise and sunset to drive a lot of their biological processes, which in turn gives these organisms a fitness advantage. However, there are a subset of animals that live in dark environments, hidden away from the light, yet we know very little about how these animals organise the biology that is normally light or clock driven in other animals. In this thesis, I explore how light impacts the biology of three very different species of fish and explore some aspects of how adaptation to a dark environment many have occurred.

1. The zebrafish, a well-developed model system that uses and depends heavily on sunlight for its survival. In this first data chapter I address how different wavelengths of light impact clock and light inducible genes in different ways. Furthermore, I present data that supports the hypothesis that the different organs of fish have different wavelength sensitivities.

2. I then move on to a relatively "recent" dark adapted animal, the Mexican blind cavefish, a species of fish that has been isolated in over 30 subterranean caves some million years ago, and show extreme adaptations to the dark, such as loss of pigment and eyes. In this second data chapter I show how all strains of cavefish show a delay in the onset of its clock in development, and how different cave populations show slightly different expression patterns in response to light/dark cycles, hinting at different evolutionary adaptations.

3. Finally, I dive deep, into the deep-sea, an environment that shows a strange duality of being pitch black but with animals that possess some of the most impressive visual systems on earth. In this last chapter I present work on two species of deep-sea hatchetfish that have evolved over the past 200 million years in the deep-sea. In this final chapter I present the first molecular study on light and circadian rhythms in deep-sea vertebrates. I explore how two species of hatchetfish, one rhythmic and one arrhythmic, have adapted to a light in the open ocean, and find surprising evidence of *in vitro* light-sensitivity.

Impact statement

This thesis addresses several research areas in circadian and light biology in fish. Research of this thesis is first and foremost is important for our continued development of understanding the circadian clock system and light biology in fish, which has resulted in 3 published data articles and one review, as well as another manuscript in preparation. I have highlighted several new findings in zebrafish monochromatic light sensitivity, identified the developmental clock in Mexican blind cavefish (*Astyanax mexicanus*), as well as making 4 new *Astyanax* cell lines that can be used in future circadian and other research, limiting research animal use. I have also presented the first circadian experiments of deep-sea vertebrates. As a largely unexplored field, particularly in terms of our molecular knowledge, I have presented new method relating to circadian field work on a research vessel, as well as identifying several issues and improvements that needs to be undertaken for future work on circadian and light related research in deep-sea teleost. I have also presented the first deep-sea clock genes, their ability to oscillate and shown how deep-sea animals still retain light sensitivity.

Personally, the work underpinning this thesis has prepared me to continue in academia, with a solid background in the fundamentals of light and circadian biology. As a result, I have been successful in my endeavours in securing postdoctoral funding, where I will continue my work on circadian clock and light sensitivity as well as broadening my scope into seasonality.

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Abbreviations

C

CT	Circadian Time

D

D1-n	Day 1-n
DD	Constant dark
DE	Differential expression
DIG-probe	Digoxigenin probe
DL	Dark – light cycle
DNA	Deoxyribonucleic acid
DVM	Daily vertical migration

E

E-box	Enhancer box

F

FBS/FCS	Foetal bovine/calf serum

G

G_1/G_2	Growth/Gap phase ½
G6PD	Glucose-6-phosphate dehydrogenase
G-Protein	Guanin Protein
GO	Gene onthology
GPCR	G-protein coupled receptor

H

h	hour(s)
hpf	Hours post fertilization

I

IR	infrared
IVF	in vitro fertilization

L

LED	Light emitting diode
LD	Light-Dark cycle
LL	Constant light
LP	Light pulse

M

MAB	maleic acid buffer
min	minute(s)

O

OMZ	Oxygen minimum zone
OVM	Ontogenetic vertical migration

P

PBS	Phosphate buffered saline
PBT	Phosphate buffered saline with tween
PFA	paraformaldehyde
PCR	Polymerase chain reaction

Q

QC	quality control

R

RNA	Ribonucleic acid
ROS	Reactive oxygen species

RPE	retinal pigment epithelium
RPL-13	ribosomal protein L13
rpm	revolutions per minute
RRE	REV-ERB/ROR-binding element
RT	Room temperature
RT-qPCR	Real Time quantitative polymerase chain reaction

S

S-phase	Synthesis-phase
SCN	Suprachiasmatic nucleus
Sec	Second(s)
SEM	Standard Error of the Mean
SSC	saline sodium citrate

U

UV	Ultra Violet light

Z

ZT	Zeitgeber time

Acknowledgements

I would like to dedicate this thesis to my grandparents; Kristin, Torbjørn, Atle, Ragnhild, Hans and Rita.

I wanted to do a PhD since I was a child, but it wasn't until I did my first biology course as a teenager, and started taking pleasure in nature that I understood that biology was for me. Although I have been a lone wolf for most of my PhD, I have a few key people to thank for training, guiding and helping me along the way:

Firstly, I would like to thank my PhD funding MRC, and for picking me up as a PhD candidate despite my research interest diverging from medical research. Also thank you to crew and scientists at the FS Sonne and GEOMAR for funding the expedition.

A huge thank you to Lucy Young for setting me up to become a competent lab scientist during my bachelor, and for recommending me the Whitmore lab. Thank you to the people at CEES for the help and friendly banter, especially Siv, William and Monica, and to Kjetill and Sissel for believing in my project, part funding and collaborating on the deep-sea project. Thank you, Charles, for being a constant and friendly face during the UCL years. A big thank you to Yama for the collaborations on cavefish. And, of course, thank you David for your gentle guidance, vigorous quality, endless conversations, generousness and for letting me explore and work on my own terms. I also owe a big thank you to my super special agent pack of dogs Freddie, Koko, Kobi, Baloo and Teebo, and to M^2, Stephanie, for all dinners, chats, wine and love, especially during the first wave of COVID.

Lastly, I would also like to extend my gratitude to my close family Jenny, Finn, Nina and Ketil, for always trusting my judgement and my choices.

1 General Introduction

1. Introduction

1.1 Let There Be Light

The premise of our whole existence, is the sun. Ever since life began, 3.5 billion years ago, the earth has revolved around the sun and turned on its own axis. Temperatures have risen and fallen, ice ages have come and gone, but there has always been day and night. As a result, the daylength has been one of the most predictable ques, with a day being extended with 1.8 milliseconds each century. Furthermore, the earth makes an elliptical journey around the sun taking 365.25 days. As the earth is tilted on its axis, it changes in day length in places away from the equator as the earth makes it way around the sun, giving rise to the seasons (Figure 1.1)

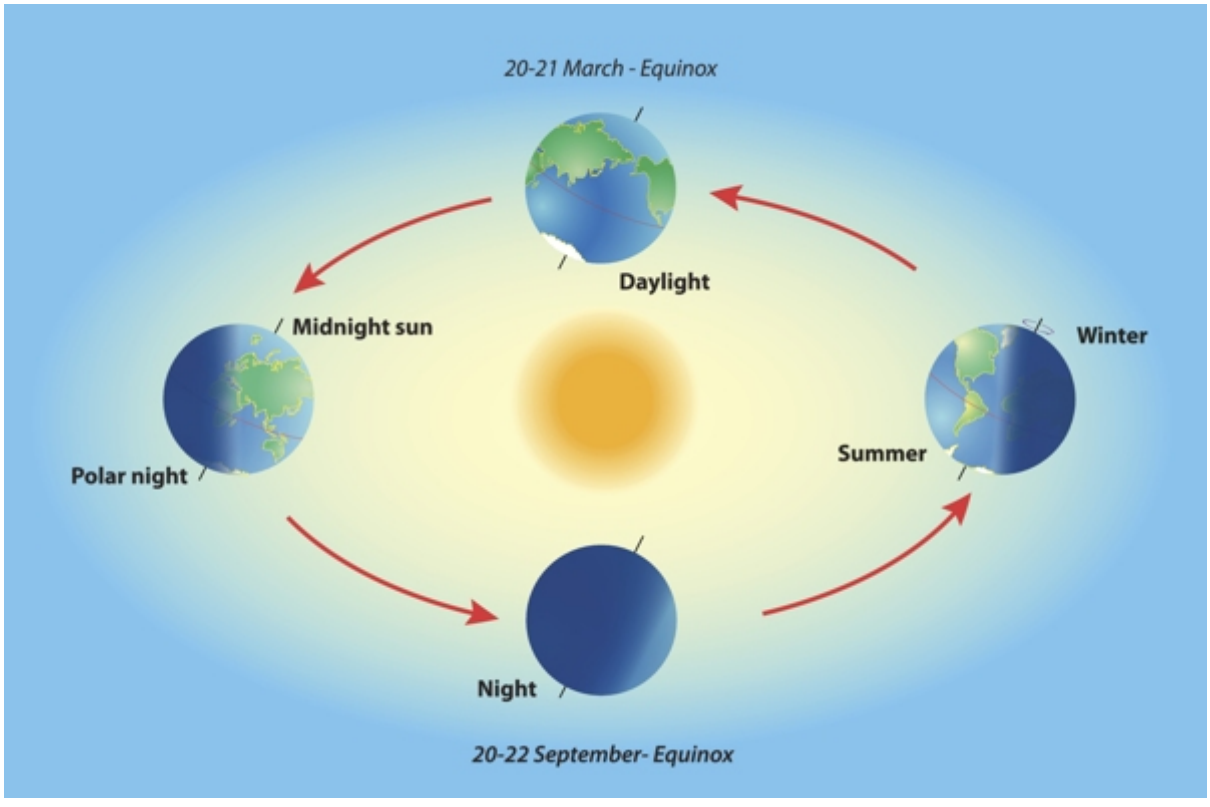


Figure 1.1 The earths journey around its own axis and the sun creates day-night differences and seasons away from equator.

As life has evolved, many organisms, from single celled prokaryotes to multi-celled complex life forms, like plants and animals, have evolved fundamental biology that utilises and depends on sun light. The periodicity that is imposed on the organisms of earth has resulted in a ubiquitous, endogenous timing system, known as the circadian clock, to allow temporal coordination of this biology (Pittendrigh 1993; Pittendrigh 1960; Sharma 2003). This is particularly evident as predictability of the onset of day/night as well as seasons, acts as inducers and repressors for behaviours such as sleep, mating, migration, feeding etc.

Light is the major, but far from only, environmental signal that sets the clock, and work on fish has revealed some unexpected aspects of non-visual photoreception. In this respect, various fish models offer unique tools with which to study the wide significance of light responsiveness, and this is no more true than in the use of naturally occurring cave populations of fish, in particular the blind Mexican cavefish (*Astyanax mexicanus*) and the Somalian cavefish (*Phreatichthys andruzzii*). These animals, which have evolved in complete darkness, can effectively be viewed as circadian/light responsive "mutants", and as such offer considerable potential for exploring the global importance of light and the mechanisms by which it is detected (Cavallari et al., 2011; Beale et al., 2013, 2016; Steindal et al., 2018).

1.2 Clocks everywhere

Still one of the most useful ways to view the circadian clock system is to employ the "Eskinogram" model, first proposed by Arnold Eskin (Eskin 1979), in which the clock is broken up into light detection, input pathway, core oscillator and then downstream, clock-regulated output events (Figure 1.2). In this context, it is probably fair to say that the fish models have not yet contributed significantly to our understanding of the core clock mechanism. However, it is in the areas of input and output events where most significant contributions have occurred from many research groups, along with emerging studies on the ecological adaptations that the clock undergoes in unusual environments (Beale et al., 2011; Foulkes et al., 2016; Frøland Steindal et al., 2018).

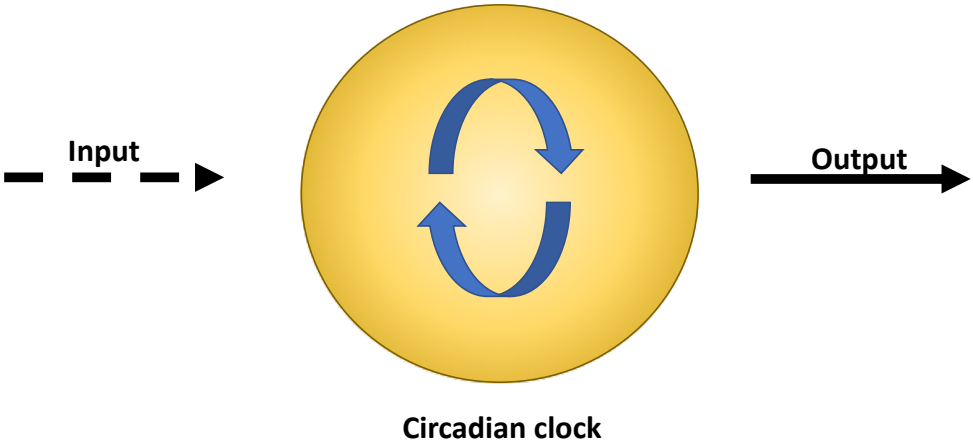


Figure 1.2 Eskinogram Inputs (zebitgebers) such as light serves as an input into the circadian clock. This entrains the clock which in turn regulates rhythmic outputs such as behaviour and basic cellular functions such as gene expression.

The late 1990's saw a relatively fast transition from the classical clock view that circadian pacemakers are restricted to specialized "clock-containing" structures, to the idea of clocks residing in most, if not all cells and tissues (Tosini and Mennaker, 1996; Whitmore et al., 1998; 2000; Balsalobre, 2002). These discoveries followed on from the initial isolation and cloning of clock genes, which then allowed for their expression patterns to be determined, and in mouse, these genes showed up in most tissues (King et al., 1997; Chang and Reppert, 2001; Panda et al., 2002). Then with the advent of mammalian cell line experiments, it was shown that mammalian cell lines could produce oscillations in clock gene expression, if the cells are synchronized in an appropriate pharmacological manner (Balsalobre et al., 1998). Work in zebrafish was occurring in parallel to these mammalian studies, and it was quickly shown, after the cloning of the zebrafish *clock* gene, that oscillations in this clock component occurred in all tissues examined, both *in vivo* and *in vitro* (Whitmore et al., 1998, 2000). There are clearly independent circadian pacemakers within all of the fish tissues that have been examined. These observations were then expanded to include zebrafish cell lines, which show high-amplitude, robust clock rhythms at the transcriptional level (Whitmore et al., 2000). Figure 1.3 is a simple diagrammatic summary of how we view zebrafish input and outputs.

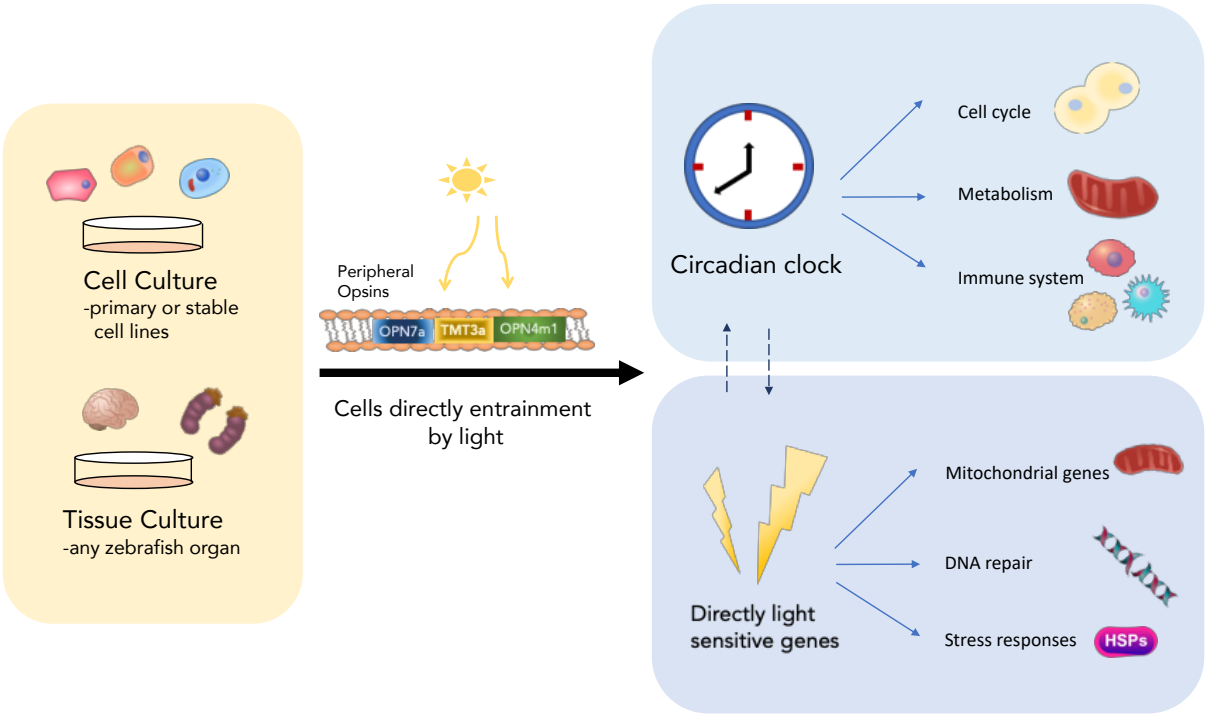


Figure 1.3 Zebrafish tissues are rhythmic and directly light-responsive All zebrafish cell types and tissue/organs examined to date, are directly light-responsive and do not require a centralised photosensitive structure to turn on light-induced transcription. Cells and organs can be entrained directly by light stimuli through the use of visual and non-visual peripheral opsins. The light signal starts transcription of light-sensitive genes, such as stress responses and DNA repair, as well as the clock genes *per2* and *cry1a,* which sets the circadian clock. The peripherally entrained clock in turn regulates a plethora of downstream cellular processes. (Frøland Steindal and Whitmore, 2019)

1.3 Global light sensitivity

At a very overt level, the big difference between mammalian cell/tissue clocks and those found in fish cells and tissues is, of course, the fact that fish cells are directly light responsive (Whitmore et al., 2000). The clock appears to be set by light directly, without any apparent need for eyes or pineal gland, the classical light responsive structures. It is clear that most of the studies that have addressed this issue have been performed with tissues or cells in culture, where light sensitivity is fully retained, but it is also apparent that peripheral light responsiveness is retained in zebrafish larval mutants, where either eyes or pineal are missing/defective (Tamai et al., 2004). This does not mean that the eyes/pineal might not contribute to peripheral tissue light sensitivity, via either neural or hormonal signals, as

modulators of this response, but there is no evidence at this time that this either occurs or is necessary for clock entrainment.

This highly decentralized model of fish circadian biology, with independent, light responsive circadian pacemakers in all tissues and most cells, does appear to hold true for most fish species that have been examined to date with the largest species being Senegalese sole (Beale et al., 2013; Blanco-Vives et al., 2012; Cuesta et al., 2014, Martín-Robles et al., 2011, 2012; Whitmore et al., 2000) . There is, however, a lack of data on larger marine species. In mammals, the environmental light signal is transmitted to the suprachiasmatic nucleus (SCN) to set a "central" clock, which then plays a role in coordinating the timing of peripheral pacemakers (Mohawk et al., 2012). This level of organization does not appear to be necessary in fish, but again this does not mean that there is not potential interaction between tissue clocks within the fish body. It is clear that there are separate clocks in the brain and pineal gland, as there are in the heart and liver. They can all be synchronized independently, but may interact. A whole variety of hormonal signals, including rhythmic melatonin cues, could also be influencing tissue specific, daily oscillations. It is interesting that the environmental light signal appears to set the clock to the same phase in all cells and tissues *in vitro*. Of course, what might occur *in vivo* is that light sets all of the body clocks to the same phase, and then various hormonal/neural cues apply subtle (or not so subtle) adjustments to this timing, generating tissue-specific phasing of rhythms. There is no doubt that the pineal pacemaker plays a key role in influencing sleep processes and rhythmic behaviour, just as the heart clock plays a role in rhythmic heart physiology (Chen et al., 2017; Gandhi et al., 2015; Livne et al., 2016; Tamai et al., 2004).

We know that the fish central nervous system contains many opsins, which are expressed in most, if not all brain regions (Davies et al., 2015). The whole cultured fish brain, or regions thereof, can directly respond to light at the transcriptional level (Moore and Whitmore 2014). But does this direct brain light detection translate into actual behavioural responses to light? Amazingly, the answer is yes. Eyeless, pineal-less zebrafish larva do indeed change their swimming behaviour in response to light exposure, and the presence of opsins expressed specifically within regions of their brain is responsible for this. Visually blind fish still swim towards light stimuli and perform simple light-seeking behaviour, triggered by loss of illumination (Fernandes et al., 2012).

1.4 Light and clock function is critical for survival in many species of fish

Vertebrate photoreception is often thought of as a process exclusively involving the visual system. Although visual light detection using rods and/or cones is obviously important in most vertebrates, non-visual photoreception and the use of non-visual opsins is also important in many critical biological processes, such as seasonality/photoperiodism, circadian entrainment and DNA repair (Daan, 2000; Goldman, 2001; Nishiwaki-Ohkawa and Yoshimura, 2016; Tamai et al., 2004; 2007). Historically, there was an assumption that there would be one key opsin for non-visual photoreception, underpinning, for example, clock entrainment. Thus, when melanopsin was discovered, many researchers believed that no more non-visual opsins would be discovered (at least not in mammals). However, this view was not to last for long, and as phylogenetic studies now show that teleost genomes encode 20 different classes of opsins, while reptiles, birds and amphibians also show a high genomic diversity of opsin classes, with 19, 17 and 18 classes respectively (Davies et al., 2015). The opsins are introduced in more detail in Chapter 3.

Light is essential for successful development in many teleost species, and the lack of light during development is associated with higher mortality rates and more deformities (Tamai et al., 2004). In some fish species, like the flatfish *Solea senegalesis*, there is a remarkable 100% mortality in embryos by Day 4 of development when animals are raised in constant darkness (Blanco-Vives et al., 2012). It is quite a common phenomenon in teleosts that light exposure is essential for early survival. Light-regimes and the clock also have an effect on the hatching of larvae. Entrained zebrafish and *S. senegalesis* embryos restrict hatching to a particular time window in the day. Constant light conditions disrupt this timed regulation, with resulting ultradian bouts for zebrafish and 24-hour delays or advances in DD (constant dark) and LL (constant light) respectively for flatfish hatching (Blanco-Vives et al., 2012). Such results have significant implications for commercial fisheries, which often employ constant light conditions to influence early larval growth rates.

Everybody that works with zebrafish is aware that spawning is tightly timed, and that the fish lay eggs just after dawn. This may sound somewhat illogical, as the embryo will undergo DNA replication and rapid cell division at the peak of diurnal UV light exposure, consequently increasing the chance of DNA damage dramatically at this sensitive early stage of development. As it turns out, exposure to light is actually beneficial and, in fact, essential

for survival in early embryos, with survival rates of embryos raised in the dark down at 20% compared to 85% in embryos raised on an LD cycle when exposed to a 5 second UV pulse (Tamai et al., 2004). This increase in survival rate in embryos raised on LD is due to the light-induced expression of DNA-repair enzymes, such as *6-4 photolyase (cry5)* and *CPD Phr*, which is expressed and transcriptionally light-regulated from 6 hpf (Tamai et al., 2004). During the first 6 hours of development however, zebrafish rely on maternally deposited *6-4 photolyase* transcript. The large quantities of maternally deposited *6-4 photolyase* is not only found in zebrafish, but also in cavefish embryos, and undoubtedly in many other species of fish (Tamai et al., 2004; Steindal et al., 2018)

Several light pulse experiments, followed by whole transcriptome analysis, have identified that around 20% of all light induced genes in zebrafish are involved in DNA-repair (Gavriouchkina et al., 2010; Weger et al., 2011). There is also an enrichment of genes involved in circadian clock entrainment, stress responses, as well as heme-metabolism, mitochondrial genes and retinoid binding genes. Furthermore, promoter analysis of these light-induced genes shows an enrichment of E- and D-box enhancers, suggesting these genes use similar signalling pathways as *cry1a* and *per2* (Buhr et al., 2015). Findings from light-pulsed zebrafish pineal gland transcriptomes were similar to those performed on other tissues, but in addition included transcript targets related to reactive oxygen species (ROS) (Ben-Moshe et al., 2014). Interestingly, the greatest L/D fold difference in light induced gene expression identified by the pineal transcriptome, is a metabolic gene, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (pfkfb4). *pfkfb4* is a target of Hypoxia-inducible factor 1-alpha (hif1a), which is also light induced (Minchenko et al., 2004; Ben-Moshe et al., 2014). An implication of this might be that light exposure can directly feed into cellular metabolic regulation.

1.5 Development of the clock and its relevance

One of the major advantages of using fish as a model system for circadian and light biology is to study the ontogeny or development of clock function and light sensitivity in the earliest stages of embryogenesis. Such experiments are difficult to perform in mammals for obvious reasons, mostly relating to the internal development of the foetus within the mother.
Zebrafish embryo development is also rapid, with the first 24-hours being equivalent to about 1 month of human development. Consequently, zebrafish also have a short generation

time. What also makes the zebrafish useful for circadian studies is that they mate just after daybreak, meaning that developmental stage and circadian time are well aligned. Despite these advantages, this whole story marrying development and circadian rhythms got off to a dismal start, with the publication of the idea that embryos inherit a sense of circadian phase from their mothers. This is not correct. Subsequent data in fact supports the idea that a circadian pacemaker is indeed present in the early stages of embryo development, with a peak in *per1* gene expression clearly present 27 hours post fertilization (hpf) when embryos are raised on a light-dark cycle. However, when raised in the dark, no such molecular clock rhythms are seen at the population level (in contrast to the initial reports). Considerable evidence backs up this theory, with multiple RT-qPCR experiments in zebrafish, as well as other teleosts, showing that the embryos require light as an entraining signal during the first day of development, to synchronize the temporally dispersed cellular oscillators (Dekens et al., 2003; Martin-Roblez et al., 2011; Steindal and Whitmore, 2018, Ziv and Gothilf, 2006). Embryos are certainly strongly light responsive by 9 hpf, when they are only just beginning the process of gastrulation, and long before any classical light responsive structures, such as eyes and pineal, have developed. In addition to light, temperature cycles can also entrain this embryonic clock, and similar light-dependent entrainment of a clock in the embryonic pineal gland is essential for early rhythms in NAT (*N-Acetyltransferase*) expression and melatonin release (Kaneko and Cahill, 2005; Ziv et al., 2005).

Light is not only important for the embryos in terms of regulating DNA repair as discussed above, but also to set the clock in larval development. However, there is a time when the clock "starts working" in larval development; from 72h onwards, i.e. after embryogenesis is finished, several genes become rhythmic, such as cell cycle regulators (e.g. *p21* and *p20*), and cell fate determining transcriptional regulators (e.g. *neurod* and *cdx1b*). Most of the zebrafish organs and general organisation is established by 72 hours. By delaying the clock regulation until this point in development, the rapid cell division is permitted, and cell fate genes such as *neurod* (early differentiation of neurons and pancreas), and *cdx1b* (early endoderm, digestive tract formation and intestine) are allowed to be expressed at relative high levels (Cheng et al., 2008). However, from 72 hpf onwards, these genes become highly rhytmic indicating that these genes come under clock regulation after their regulatory role in early development is complete. One possibility is that these developmental genes are now being used for a different purpose in later stage larvae and adults, than in the early stages of development.

This ability to decouple from the clock from its output is also seen in regeneration of limbs (Idda et al., 2012).

1.6 Light Input pathway

In zebrafish, although the precise nature of the signalling pathways is not clear, what has been well-defined is that light dramatically increases expression of the clock genes *period2* (*per2*) and *cryptochrome 1a* (*cry1a*) in all tissues and cell lines that have been examined (Ziv et al., 2005; Tamai et al., 2007). PER2 proteins contain a C-terminal CRY binding domain enabling dimerization of PER2 and CRY1a. In turn, the CRY1a protein interacts directly with core clock components, CLOCK and BMAL, blocking their ability to dimerize and thereby repressing transcription by CLOCK:BMAL, providing a likely mechanism for clock resetting (Tamai et al., 2007). Furthermore, light-induced CRY1a acts to "lock up" or "jam" the clock mechanism, and prevent the molecular core clock from oscillating. It does so in a phase-dependent manner, as CRY1a can only interact with CLOCK and BMAL proteins of course when they are present, which is typically after Zeitgeber Time (ZT) 12. In this way, constant light acts to "stop" the clock at almost precisely ZT12, and the clock oscillation will not continue again until the light stimulus is removed, and CRY1a protein most likely degrades. This represents a fascinating potential interaction between an hour-glass time measuring system and a circadian pacemaker.

How does light regulate the transcription of these two key genes? Promoter analysis of *per2* and *cry1a* has identified a 'Light Responsive Module' consisting of E- and D-box elements spaced close together and in proximity to the transcriptional start site (Tamai et al., 2007; Vatine et al., 2009). This module is also strongly conserved in other *per2* vertebrate genes, including species lacking directly light-sensitive clocks (Rollag et al., 2003; Hatori et al., 2010). The D-box confers light-driven expression through binding of the thyrotroph embryonic factor (TEF) zebrafish homologue, whilst the E-box directs circadian clock regulation by mediating CLOCK/BMAL activity (King et al., 1997; Balsalobre et al., 1998). In addition to TEF, zebrafish possess an additional eleven D-box binding factors, with nine of them enhanced in the pineal gland, further supporting the involvement of this pathway in the circadian clock mechanism (Martin-Roblez et al., 2012).

Although both *per2* and *cry1a* genes possess the light responsive module, their regulation is markedly different. Upon blocking protein synthesis with cycloheximide, the light response using the D-box enhancer is attenuated in *cry1a*, making light-induction of *cry1a* dependent on *de novo* protein synthesis. This is not the case for light-dependent expression of *per2*, which seems to utilise the E-box when protein synthesis is blocked (Hirayama et al., 2005; Mracek et al., 2012). Furthermore, AP-1 enhancer elements have also been implicated in *cry1a* light-driven expression (Hirayama et al., 2005). These results taken together suggests that both these core clock genes use D-boxes to drive their expression, but that multiple other enhancer and control elements ensure that light-driven expression is controlled in a gene promoter-specific manner. Light sensitivity is not constant over a 24-hour period, due to clock-feedback on to the input pathway. Light-pulsing experiments show that light sensitivity in zebrafish is time of day dependent, with more than twice the induction of *cry1a* at CT20 compared to CT8 (Tamai et al., 2007). In turn, the light intensity also impacts the size of the phase shift. Results from phase and intensity response curves demonstrate a strong correlation between light induction of the *cry1a* gene and clock resetting.

Zebrafish has one *per2* gene, in contrast to many other teleost species, such as *Astyanax mexicanus*, where there has been a clear duplication of the *per2* gene. The distinct roles of *per2a* and *per2b* are unclear, but in *A. mexicanus*, the expression patterns in LD and DD differ, indicating that they may have distinct roles (Frøland Steindal et al., 2018). The situation for the multiple *cryptochromes* in zebrafish is equally unclear, where there are at least six distinct *cry* genes. However, it is clear that some of these CRY proteins can act as transcriptional repressors, as in the mammalian clock system (Ishikawa et al., 2002). It has also been suggested that CRY4 might act as a photopigment, akin to the situation in Drosophila, but at the minute there is no compelling data to support this hypothesis.

1.7 The Clock Outputs

The clock regulates multiple outputs, with the most “famous” of them being sleep, which is largely conserved from teleosts to mammals (Appelbaum et al., 2009; Woods et al., 2014; Gandhi et al., 2015; Singh et al., 2015). But the clock and light regulate much more than just behaviour, with some of the cellular processes summarised in figure 1.3. One such output is the timing of the cell cycle in a wide range of animals and plants, ranging from cyanobacteria to human tissues. In the case of zebrafish cell lines, DNA replication (S-phase) typically

occurs in the late evening, and mitosis just before dawn (Tamai et al., 2012; Laranjeiro et al., 2013), and similar timing is found for the cell cycle in many healthy proliferative human tissues (Bjarnason et al., 1999, 2001). This includes the cell cycle regulator cyclin-dependent kinase inhibitor 1a (CDKN1A or *p21*) (controlling the entry of cells from G1 into S-phase), which shows a high amplitude rhythm, peaking around dawn and having a low point of expression in the early evening. Zebrafish also express a gene similar to *p21*, named *p20* (Laranjeiro et al., 2013). *p20* is also under strong clock control, but the timing of peak expression is shifted by about 6 hours relative to *p21* which is due to an RRE (REV-ERB/ROR-binding element) responsive element in *p20* in addition to the E-boxes found in the promoter of both genes. Deletion of this regulatory sequence in the *p20* promoter removes nearly 4 hours of the phase difference between the two cell cycle regulators (Laranjeiro et al., 2013; 2018).

The down or dysregulation of the clock in zebrafish can be detrimental, which is exemplified in cancers, something we also see in human studies (Filipski and Lévy, 2009; Filipski et al., 2006; Lahti et al., 2012). In zebrafish melanomas for example, the light responsive element of the clock (*cry1a* and *per2*) stops "working". This is particularly severe in zebrafish, as the light inducible DNA repair machinery is also blocked as a result, further accelerating the cancer progression (Hamilton et al., 2015). It should also come as no surprise that light and clock also play a key role in immune responses in zebrafish. Pro-inflammatory cytokine genes, Tumour necrosis factor-α, Interleukin-8 and Interferon-γ, and increased neutrophil and macrophage recruitment are observed during the light compared to the dark on an LD cycle, and remains high during constant light (Du et al., 2017). It has also been reported that several circadian genes show a change in amplitude in response to inflammation (Mosser et al., 2019). Another example of a circadian regulated process is metabolism, something that has gained interests from dieters and given rise to fads such as "circadian diets". Zebrafish do not follow diet fads, but do show extensive crosstalk between the mitochondria and clock (Huang et al., 2016; Kelu et al., 2020; Morbiato et al., 2019; Weger et al., 2016). The field of circadian/metablic crosstalk has seen an explosion in interest during the last 20 years with over 27,000 hits for the search terms "circadian metabolism" on PubMed restricted to the last 20 years, in contrast with search terms such as "circadian immune system" which only give around 1000 hits for the same time period.

1.8 Fishes of the dark – an alternative way of studying clocks

Much of the progress in understanding the circadian clock mechanism has come from the use of forward genetic mutant screens. The cavefish are in some ways "naturally occurring mutants" in terms of circadian genes, but instead of being classical "knock out" mutants, they are a result of high evolutionary pressure to a life in the dark. Two cavefish species, the Somalian Blind Cavefish (*Phreatichthys andruzzii*) and the Mexican Blind Cavefish (*Astyanax mexicanus*) have been exploited in this way to study light detection and clock function. The Somalian cavefish are thought to have been isolated in completely dark underground caves around 2 million year ago (Colli et al., 2009). They have typical troglomorphic phenotypes, such as loss of eyes and pigment. The Somalian cavefish have also lost their ability to entrain to an artificially provided light-dark cycle, which is possibly due in part to an aberrantly spliced variant of the *per2* transcript which lacks a C-terminal cryptochrome binding domain. This splice variant of PER2 protein is unable to dimerise with Cry and is consequently localised predominantly to the cytoplasm (Ceinos et al., 2018). *P. andruzzii* can, however, entrain to feeding cues, showing a clear anticipatory increase in activity around feeding time. This non-photic zeitgeber also entrains the molecular clock, showing *per1* mRNA rhythms in several organs (Cavallari et al., 2011). Presumably, unlike for light, *per2* is not required for food entrainment of the oscillator. The Somalian blind cavefish expresses two truncated non-visual opsins, melanopsin (*opn4m2*) and a teleost-multiple-tissue opsin (known as *TMT3a*) (Cavallari et al., 2011). These two opsins have been proposed as key photopigments for entrainment. However, as the genome of the Somali cavefish has yet to be sequenced, it is likely that there will be many other candidate opsins to explore. It will be interesting to see how many of the possible 20 classes of opsins are mutated in this species.

The Mexican blind cavefish, *Astyanax mexicanus*, also have a troglomorphic phenotype. In contrast to *P. andruzzii*, which represents one species and one strain, *A. mexicanus* is the name for over 30 unique Mexican cavefish populations, descending from the same ancestral river strain. The descendants of the founding river species of *A. mexicanus* are still swimming in the local Mexican rivers, making this cavefish a unique and powerful evolutionary and adaptational model. When studied in the wild, the Mexican blind cavefish show no molecular or behavioural circadian rhythms. However, in contrast to the Somalian cavefish, several of the Mexican blind Cavefish strains can entrain to LD cycles under lab conditions, although

the phase and timing of *per1* gene expression is altered (Beale et al., 2013). We will get to know this species of fish in more detail in Chapter 4.

From the introduction above, it is clear that zebrafish depend on light for its survival, and with its 42 photopigments, the zebrafish is akin to a "swimming photoreceptor". The clock and exposure to light is not only beneficial for the survival of the fish, but vital. This need for light and a clock, is also seen in other species of fish such as *Medaka* and sole. It is therefore interesting to study animals that have adapted to an environment that is so different from what we live in. Although the research on its own is interesting, this insight could also prove useful for animals that live in an increasingly constant and arrhythmic environment, such as humans and animals in and around cities, where social jetlag and light pollution is an increasing problem. This thesis broadly aims to study light sensitivity and the clock using a range of teleost species, ranging from zebrafish to others that live in a much darker world, where light signalling is limited.

Zebrafish remains an excellent model species for exploring biology like light sensitivity (Chapter 3). We still know very little about why they express so many photopigments, but its becoming increasingly clear that in fish, possessing a large amount of non-visual and visual opsins is not uncommon, with cavefish being an example. Early on in this PhD I blasted the zebrafish opsins against the cavefish (surface fish) genome and found around 30 non-visual candidates. Two years later Yoshizawas group published that they had found 33 opsin genes, 24 of which were expressed in cavefish extraocular tissues (Simon et al., 2019). The brain remained the highest expressing organ with the pineal being the most opsin dense. However, a pinealectomy of the fish did not change the cavefish light-responsive locomotor activity. This may suggest a repurposing of opsins from light-inducible GPCRs to something different, or that the pineal is not really that important in fish light-responsive behaviour. Regardless, this richness of photopigments are truly fascinating, and in my opinion, and the best way of studying the opsins is to use the zebrafish. It has the obvious benefits of being a model organism, with annotated genomes, transcriptomes and numerous well-developed protocols. With eggs being laid every other day, as opposed to every 3 weeks in cavefish, downstream knock-out experiments are also much easier in zebrafish. We also have the mammoth study of opsin expression patterns in each adult tissue (Davies et al., 2015). In Chapter 3, I investigate how different monochromatic light impacts the different zebrafish organs as well

as cells in culture. We know that each organ has a particular set of opsins expressed, how does that affect their sensitivity to monochromatic light?

In Chapter 4, I then move on to studying the onset of light sensitivity and clock in cavefish embryos. By using embryos of 3 different cave populations and the surface fish, we can compare circadian evolution in separate caves to the same environment, and how these fascinating animals repurpose their clock mechanism to fit a life in the dark. I also introduce the pilot data for adherent embryonic cell lines from 3 hypogean and 1 surface cave fish.

In the final chapter, we go to another dark place, namely the deep-sea. Circadian rhythms in deep-sea vertebrates have never been explored before, maybe because these animals are extremely hard to come by, or because scientists have believed that they have no clock, or a bit of both. As it turns out, there are many clues that the great depths are not as dismal as they initially seem. The first hint is that most fish living down to around 2000 metres have highly sophisticated eyes. Eyes are very metabolically demanding, so if you do not need them, you get rid of them like the cave animals. These deep-sea animals clearly use their eyes, but is it to see faint photons from the sun? Maybe. To see bioluminescence from other animals? Most likely. The second clue is that a lot of animals show daily vertical migrations, such rhythmic events are usually attributed to a functional clock, but is that the case for animals living in pitch black darkness? In Chapter 5, I introduce two species of deep-sea fish, and present data that support that they both have light sensitive transcriptomes. Furthermore, I show that both species of animals have the capability to entrain to LD cycles, but that only one of them retain an endogenous rhythm in the dark.

Aims and research questions

I start by examining how the zebrafish, an animal absolutely packed with photosensitive opsins in all organs, react to different wavelengths of light. In this chapter I ask the following questions:

1. Zebrafish cell lines are used extensively in circadian research, but we have never examined what opsins are present in these cell lines- which opsins are present? Do they cycle? Do they match a zebrafish tissue or organ in terms of expression?
2. How does this combination of opsins impact the monochromatic light sensitivity of the cells? Are they more red or blue shifted?
3. Zebrafish have several UV-sensitive opsins, but does that mean that UV/blue light is more potent at inducing gene expression? How do different wavelengths of light impact phase shifting of the clock?
4. As the different organs express different combination of opsins, does that mean that each organ has a separate and defined wavelength-sensitivity?

In the second data chapter I look at cavefish, *Astyanax mexicanus*, a species that is in many ways the opposite to zebrafish as it has to live and organise its biology without periodic or light stimuli. However, as this is a relatively "new" subspecies, it still retains a light entrainable clock and multiple opsins. My aims for this chapter are to establish when the clock "starts ticking" in the different populations of cavefish and compare their clock to the river dwelling, founding species of cave fish. As we have very few adult fish and the fish only mate every 3 weeks, I also want make stable cavefish cell lines and test if they were suitable for future circadian study. In the second chapter I ask the following:

1. When do the cavefish become light sensitive?
2. When does the clock start oscillating in the cavefish? Do we see any differences between the different cave populations?
3. Do cavefish have directly entrainable peripheral clocks in culture? Can we make a cave specific cell line to use as a tool for future experiments?

In the third data chapter of this thesis, I worked on two species of deep-sea hatchetfishes which were caught in the field on the FS Sonne. Many have assumed that deep-sea animals do not possess a circadian clock as they live in such dark conditions. However, many animals show daily vertical migrations over long distances relative to body size, but is it clock regulated? For these scientifically and geographically unchartered waters, my aims were:

1. Do deep-sea fish have a circadian clock?
2. If so, do they use light to set this clock, and what genes are up-regulated to light? Are they widely different to those of zebrafish?
3. What kind of photopigments do the deep-sea fish possess?
4. Do we see a difference in light sensitivity and clock function between vertical and non-vertical migrators?

2 Methods

2 Methods

2.1 Biological samples

2.1.1 Zebrafish husbandry

Adult wild type zebrafish of different ages (all less than 18 months) and sexes were kept at 28°C on a 14:10 light-dark (LD) cycle (lights on at 09:00). All fish were maintained by the staff of UCL Zebrafish facility in the "ultra clean" facility (free of *Mycobacterium haemophilum*), and fed a mix of brine shrimp and pellets twice a day. All animals were maintained in a Home Office approved facility and handled in accordance with the Animal Welfare Act of 2006.

2.1.2 Tissue and cell culture zebrafish

Adult fish were euthanised by the administration of tricaine (Ethyl 3-aminobenzoate methanesulfonate) (Sigma-Aldrich), prior to dissection. Gut, liver, eye, brain and heart were dissected at ZT10 and washed in PBS and subsequently transferred to Leibovitz -15 medium (Gibco) supplemented with 15% FBS (Biowest), 0.05mg/ml of gentamicin (Gibco) and 1x Penicillin-Streptomycin. Tissues were kept at 28°C in a water bath on a 12:12 LD cycle for one cycle, and during the subsequent cycle tissues were light-pulsed at ZT21. PAC2 and *clockDN* (clock "mutant" cells) cell lines were kept in culture media, as described above. Cells were seeded at 50,000 cells/ml and kept at 28°C in a water bath on a 12:12 LD cycle for 3 days before being light-pulsed at ZT21 for 3 hours. Both organs and cells were light pulsed with different monochromatic wavelengths of light: IR 850nm, red 650 nm, blue 450 nm, UV 350 nm and white 400-700 nm (LED Array Light source, Thorlabs) with an intensity of 200 $\mu W/cm^2$ for 3 hours (Figure 2.1 a,b). Intensity was measured using a Macam power meter. The lid was removed from the petri dish to avoid any light attenuation during the light pulse. Light pulsed and dark control tissues were harvested at ZT0 in 1 ml of TRIzol (Invitrogen). Tissues were homogenized using 3mm stainless steel beads and a TissueLyser II (Qiagen), whilst cells were washed with 1x PBS and homogenized in TRIzol with a cell scraper. Due to the small tissue size, organs from two fish were pooled to give n=1. The intensity of 200 $\mu W/cm^2$ was selected based on previous published and unpublished data as a value approximately half-maximum on the intensity response curve, so as not to saturate the light response (Tamai et al., 2007).

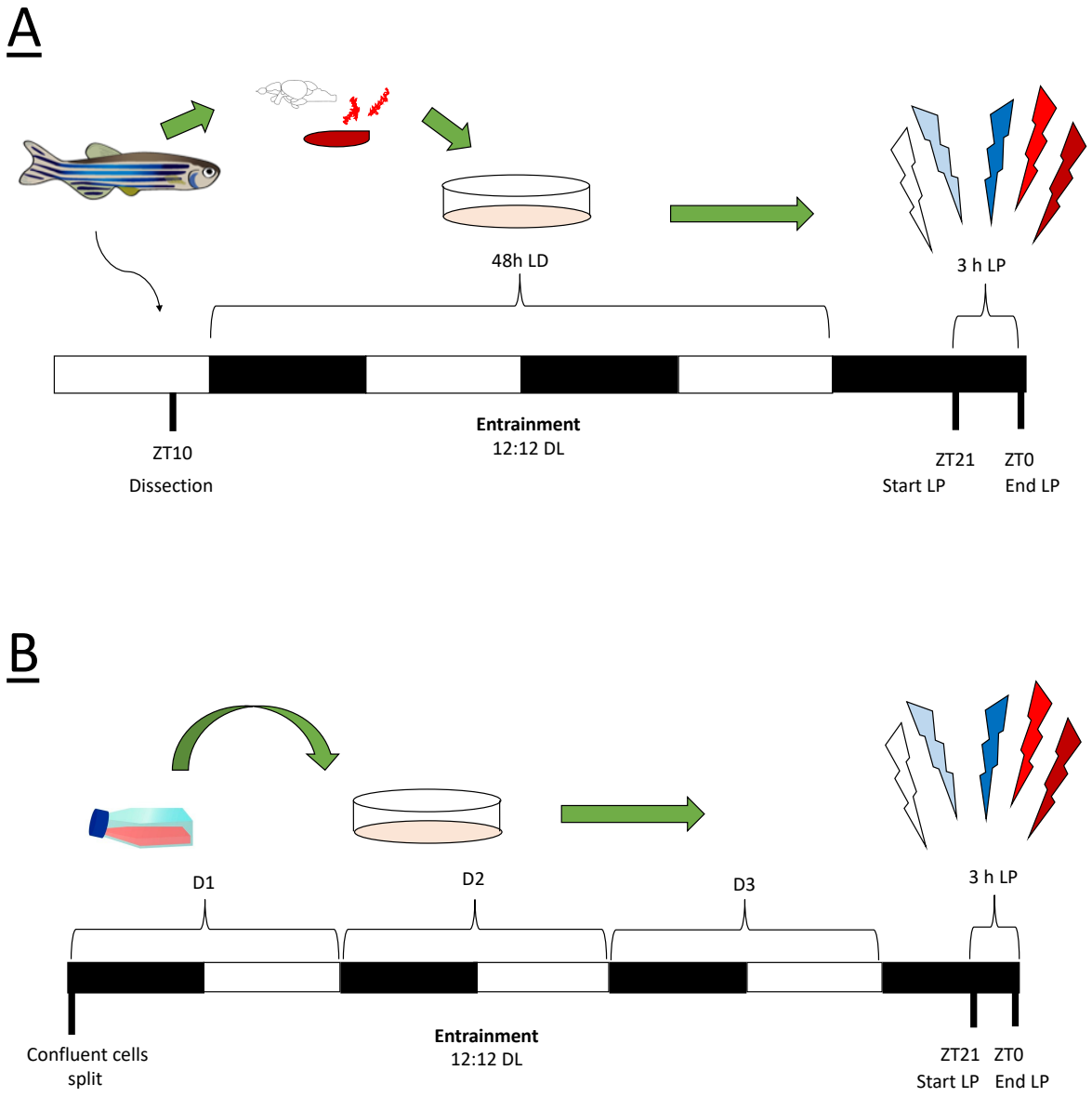


Figure 2.1 Monochromatic light pulse experimental design for a) Zebrafish adult organ culture b) PAC2 zebrafish cell culture. Black bars represent 12h of dark and white bars represent 12 h of light

2.1.3 Cavefish husbandry

Adult surface, Pachón, Tinaja and Chica cavefish were maintained at 22 °C on a 14:10-hour photoperiod. Fish were maintained by the lab of Dr Yoshiyuki Yamamoto in 45 litre tanks with 8 and 12 fish per tank and fed flake food once per day. Cave populations were originally captured in the 1990s from Cueva El Pachón, Cueva El Chica and Cueva de la Tinaja in North East Mexico by a team of cavers and scientists led by Dr William Jeffery (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). Some of the descendants from Prof Jeffery's lab (University of Maryland) has been has been maintained at University College London since 2004. All animals were maintained in a Home Office approved facility and handled in accordance with the 'Animals (Scientific Procedures) Act 1986'.

2.1.4 Embryo collection and maintenance

The cavefish were mated every 3 weeks, and 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 during the day. Spawning then takes place in the next two consecutive dark phases, approximately 1 hour after lights off for surface fish, and 3-8 hours after lights off depending on the population of cavefish. When the fish showed spawning behaviour, they were caught and eggs and sperms harvested for *in vitro* fertilization (IVF).

Embryo development in cavefish has been studied in parallel to zebrafish, and the mid blastula transition (MBT), when we see an increase in zygotic transcription, is seen at approximately 3.5 hpf (Hinaux et al., 2011, Kane and Kimmel 1993). Embryos were therefore sorted 1-2 hours post IVF and unfertilised eggs and embryos with abnormal cell division were discarded to avoid any effect from light contamination and general stress. Embryos were kept in petri dishes in 50% tank water and 50% E3 fish water (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, 0.00001% Methylene Blue) to make up one biological replicate. The embryos were kept at 25°C in a thermostatically controlled water bath and placed on a 12:12 DL cycle or in constant darkness for the duration of the experiment. From 9 hpf 12-20 embryos were harvested per biological replicate at 6-hour intervals in TRIzol Reagent (Invitrogen), homogenized and stored at −20 °C. For acute light pulse experiments, embryos were kept in constant darkness and given a 3-hour light pulse at

5hpf, 14 hpf and 23 hpf, or kept in the dark as a control (Figure 2.2). Embryos were then collected and stored as above.

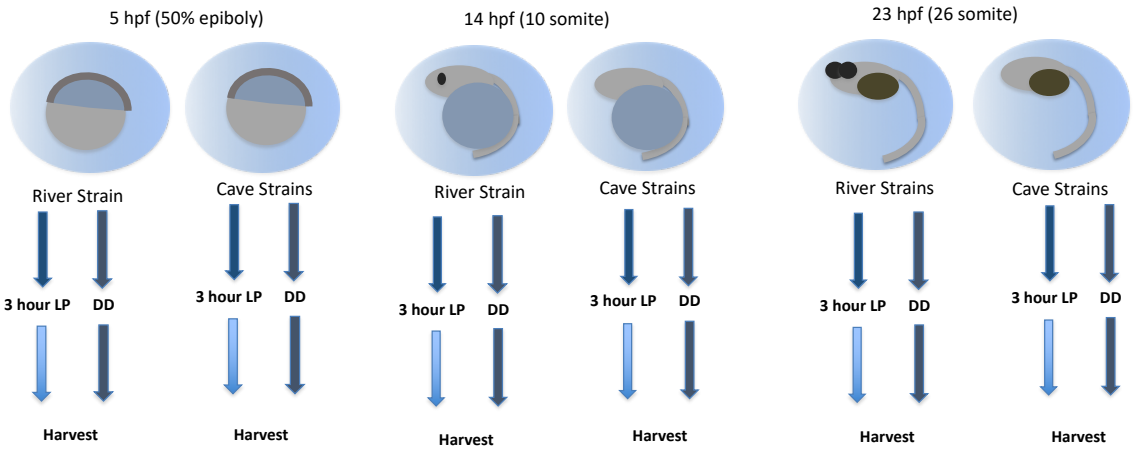


Figure 2.2 Light-pulse experiments in embryonic cavefish Cave fish of three different ages were used to assess the beginning of light sensitivity

2.1.5 Embryo dissociation and creation of *Astyanax mexicanus* cell lines

Embryos were obtained and kept as described above. In a tissue culture hood, around 50 newly hatched embryos of all 4 strains (24 hpf – 30) hpf were washed 5 times in a 2 ml Eppendorf tube with 1.5 ml 1x PBS per wash. A new tube was used per wash to minimise the chance of contamination. Embryos were then dissociated in 1 ml of 0.5% Trypsin- no phenol red (Gibco) for 20 minutes at room temperature (RT). Pipetting vigorously up and down with a P1000 and P200 was done every 2-5 minutes to help break up the embryos. Dissociated cells were transferred to a 25 cm^2 flask (Greiner) with 7 ml of fish cell culture media (constituents described in section 4.1) and kept in a cell culture incubator at 28 °C, no CO_2. The following day, most cells had adhered and flattened. Culture media was changed and any debris or cells that had not adhered to the plastic was discarded. It took about 2 weeks before the primary cultures were confluent. Upon reaching confluency, cells were washed with 1x PBS at RT. The *Astyanax* cell lines are very adherent, so 1-2 ml of 0.5% Trypsin for 10 minutes at RT would typically be used to dissociate cells from plastic in a small 25 cm^2 flask. Cells would be seeded at 1:7 and split every 5-7 days.

2.1.6 Field work and deep-sea fishing

All fieldwork was carried out between 07.06 - 09.07.2017 as part of Ingon258 expedition on the Research Vessel Sonne (RV/FV Sonne) owned and managed by Geomar (Kiel, Germany). The research expedition across the Indian Ocean was split 50:50 between geological and biological research and led by Prof Reinhard Werner (Geology) and Prof Emeritus Hans-Joachim Wagner (Biology). A new single, tethered 1 metric ton midwater rectangular tucker trawl net with an opening area of 45m^2, with an electronically operated opening-closing mechanism was used for fishing (Figure 2.3). The net was deployed over the stern of the vessel down to 1200 m, with the RV Sonne cruising at 1.5 knots during fishing. The net was raised in steps of 50m every 30 minutes. 25 tucker trawls were performed across the Indian Ocean (Figure 2.3), and all data concerning each trawl can be found in the Sonne Ingon258 report- Appendix IV Tucker Trawl Deployment (Werner et al., 2017).

Most of the trawling happened during the night, when most deep-sea animals are higher in the water column. The nets were mostly raised in the dark, ensuring that the catch would be exposed to a minimum of light. The catch collected from the end of the net and was subsequently brought in a Styrofoam box into the cold-room and then divided up between scientists.

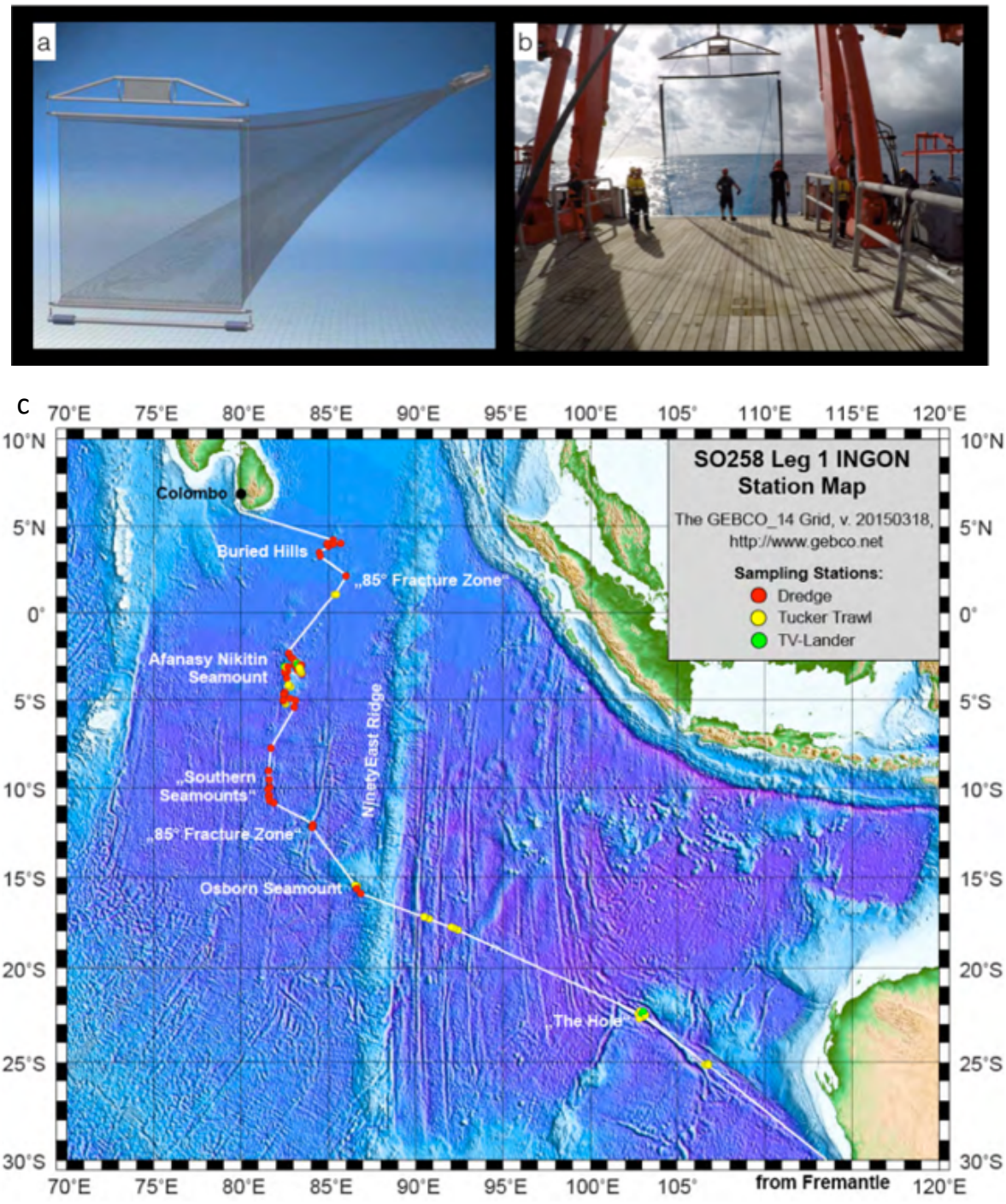


Figure 2.3 Tucker trawl design and Expedition transect for Sonne INGON

a) Design of tucker trawl net used on the fish (bucket at the end of net). b) Deployment of net off the RV Sonne c) Transect with yellow dots marking tucker trawls

(Photo: Dr WS Chun) (Map and figure a: Werner et al., 2017)

All 4 biological replicates for *Argyropelecus hemigymnus* and *Sternoptyx diaphna* for the 3 h light-pulse experiment and dark control was collected on 02.07.2017 from two different trawls undertaken at the Northern end of the Afanasy Nikitin seamount (Figure 2.3). There were also enough *S. diaphna* fish from these trawls to make up the whole basis for the LD and DD experiments. In order to make up the minimum of biological replicates and time points for LD and DD experiments for *A. hemigymnus,* samples were collected over a larger area (Exact collection times and can be found in Table 2.1 and Table 2.2).

Date	Sample	Replicates	Temp	Coordinates	Trawl depth
12.06	ZT3	n=2	7C	22.16,25S 102.26,132E	1200m, 1000m, 800m, 600m, 400m, 200m
	ZT9	n=2	6.5C	22.16,25S 102.26,132E	1200m, 1000m, 800m, 600m, 400m, 200m
	ZT15	n=2	6C	22.16,25S 102.26,132E	1200m, 1000m, 800m, 600m, 400m, 200m
	ZT21	n=2	6C	22.16,25S 102.26,132E	1200m, 1000m, 800m, 600m, 400m, 200m
26.06	CT3	n=3	6.2C	5.1,134S 82.17,844E	400m & 250m
	CT9	n=3	6.2C	5.1,134S 82.17,844E	400m & 250m
	CT15	n=3	6C	5.1,134S 82.17,844E	400m & 250m
	CT21	n=3	6.2C	5.1,134S 82.17,844E	400m & 250m
	CT3	n=3	5.7C	5.5.805S 82.21.498E	400m, 250m, 150m
	CT9	n=3	5.9C	5.5.805S 82.21.498E	400m, 250m, 150m
	CT15	n=3	5.6C	5.5.805S 82.21.498E	400m, 250m, 150m
	CT21	n=3	5.7C	5.5.805S 82.21.498E	400m, 250m, 150m
30.06	ZT3	n=3	5.9C	3.22,078S 83.14,561E	800m & 150m
	ZT9	n=3	6C	3.22,078S 83.14,561E	800m & 150m
	ZT15	n=3	5.3C	3.22,078S 83.14,561E	800m & 150m
	ZT21	n=3	5.6C	3.22,078S 83.14,561E	800m & 150m
2.07	LP/DD	n=3	5.9C	2,57,870S 83.3,683E	1000 & 300m

Table 2.1 ***Argyropelecus hemigymnus*** **sampling overview**: Date of catch, subsequent circadian time in culture, number of biological replicates, temperature of cold room at circadian times, start of trawl coordinates and depth(s) of trawl.

Date	Sample	Replicates	Temperature	Coordinates of Trawl	Trawl depth
2.07	CT3	n=5	5.9C	2,57,870S 83.3,683E	1000m & 300m
	CT9	n=5	6C	2,57,870S 83.3,683E	1000m & 300m
	CT15	n=5	5.3C	2.59,958S 83.2,964E	1200 m
	CT21	n=5	5.6C	2.59,958S 83.2,964E	1200 m
	ZT3	n=5	5.9C	2,57,870S 83.3,683E	1000m & 300m
	ZT9	n=5	6C	2,57,870S 83.3,683E	1000m & 300m
	ZT15	n=5	5.3C	2.59,958S 83.2,964E	1200 m
	ZT21	n=5	5.6C	2.59,958S 83.2,964E	1200 m
	LP/DD	n=3	5.9C	2.59,958S 83.2,964E	1200 m

Table 2.2 ***Sternoptyx diaphna*** **sampling overview**: Date of catch, subsequent circadian time in culture, number of biological replicates, temperature of cold room at circadian times, start of trawl coordinates and depth(s) of trawl.

2.1.7 Field set-up for deep-sea organ culture and lab set-up for zebrafish control culture

The RV/FS Sonne is a ship built for Geology and not biology, and consequently the labs were rooms with a fridge and a freezer. We had no culture hood or climate chambers/incubators, and the shipment of lab equipment from London consisted of a dissection microscope, dissecting tools, PBS and general consumables. Prior to departure, the net size, trawling times etc. was not clear. In addition, it is a complete unknown as to what fish we were likely to catch and all specimens would have to be shared. We had no means to perform any molecular analysis or test samples whilst on board. The experimental set up and design is therefore a compromise, driven by onboard circumstances and the vagaries of trawling. After a couple of trawls, we decided to focus on two species of hatchet fish for a couple of reasons: 1. They were sufficiently abundant to make up enough time points. 2. They were both species we could identify with some help from fellow, more experienced scientists. 3. In the limited literature one species reportedly undergoes vertical migration, while the other does not.

After we had split the catch with the other scientists onboard, organs from both species (2-5 cm long) were dissected in a cold room (6 ºC) under red light and washed in 1x PBS before being transferred to culture media (Leibovitz -15 medium (Gibco) supplemented with 15% FBS (Biowest), 0.1 mg/ml of gentamicin (Gibco) and 2x Penicillin-Streptomycin (Gibco) and 10mg/ml RedSea salt mix (RedSea) to adjust for the salinity). Organ cultures were maintained at 6ºC±1 ºC, as it was the closest temperature we could get to match the 4ºC that the fish would have been living in. The air temperature would fluctuate in the cold room by a maximum of 0.7ºC over a circadian cycle, which means there were likely also some smaller fluctuations in the culture media. However, temperature was measured at each circadian timepoint (Table 2.2 and Table 2.3). An overview of experimental set-up can be found in Figure 2.4.

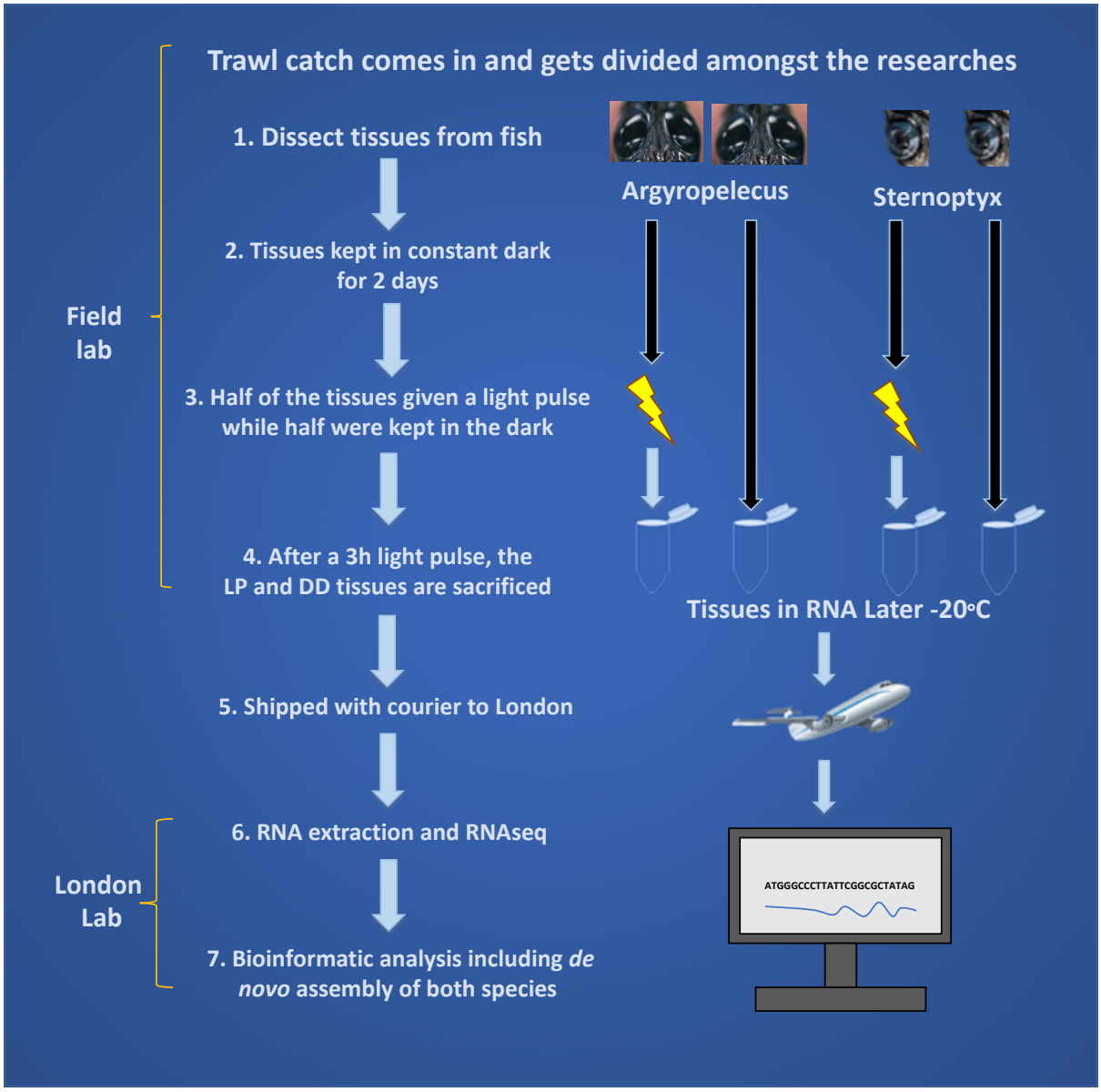


Figure 2.4 Experimental light pulse design on ship. An overview of experimental design in the field, shipment to London and subsequent work in the lab.

The light source used for entrainment and light-pulse was a Quad Tube Shape CFL Bulb, 40 W, 4000K, Cool White Colour Tone (Osram) placed 40 cm above the cultures, and kept on a 12:12 LD schedule which closely matched the day-night cycle of the sun on the day and location of sampling. Samples were foil wrapped during the dark phase to avoid light contamination if someone entered the cold room, and unwrapped during the light phase. Samples for LD and LD into DD experiments underwent 2 cycles of LD before being sampled every 6 hours at ZT/CT 3, 9,15 and 21 in RNA*later*. Light pulsed samples underwent 1 full cycle of LD in culture before being pulsed at ZT21 for 3-hours during the second LD cycle. Light-pulsed and control DD samples were collected at CT/ZT1 in

RNA*later* (Figure 2.5). In London, the light-pulse experiment was repeated in zebrafish to create a matching dataset. Zebrafish eyes were dissected, washed in 1x PBS and kept in culture media as described above at 28ºC in a thermostatically controlled water bath, for 2 day on an LD cycle before being light-pulsed at ZT21 for 3-hours. Light-pulsed and DD control samples were collected at CT/ZT1 in RNAeasy Lysis buffer (Qiagen).

2.1.8 Deep-sea sample handling

Samples were harvested in RNA*later* and left for 12 hours at 6 ºC before being transferred to the freezer. Upon disembarking in Colombo, Sri Lanka, the samples were refrigerated for 2 days before being picked up by courier, which supposedly kept them frozen for 14 days awaiting shipment on dry ice to the UK. Judging by the later QC report and the amount of degradation we had in the samples, it is likely that the samples were not handled as were instructed.

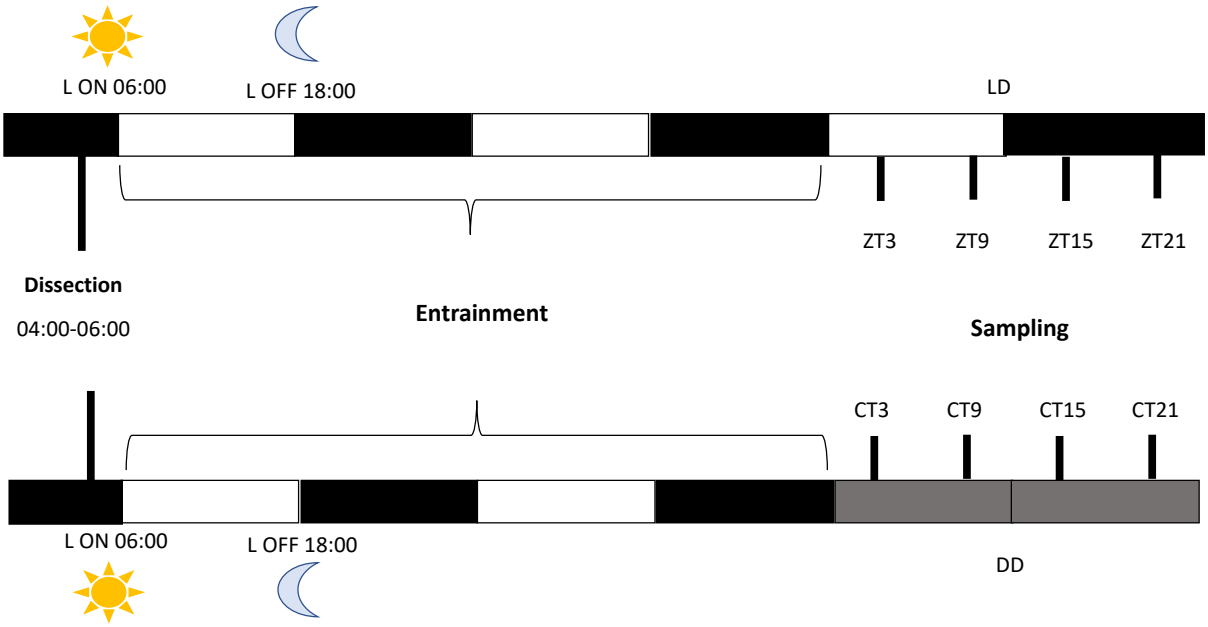


Figure 2.5 Light/dark and sampling regimen for deep-sea circadian samples
Black bars represent 12h of dark and white bars represent 12 h of light. Dark grey bars represent constant dark after entrainment.

2.2 Molecular analysis

2.2.1 RNA, cDNA and RT-qPCR

RNA was extracted from homogenised cells, tissues or embryos according to the manufacturer's guidelines (TRIzol, Invitrogen): 200 µl of chloroform was added to 1 ml of TRIzol and vortexed for 15 seconds, incubated at RT for 2 minutes and then spun for 15 minutes at 4°C at 13,000 rpm. 400 µl of aqueous phase was transferred to a clean 1.5 ml Eppendorf tube and 500 µl isoproanol (propan-2-ol) was added and mixed by a quick vortex, RNA was left to precipitate over night at -20°C. The following day, the tubes were spun for 20 minutes at 4°C at 13,000 rpm. RNA pellets were then washed with 1 ml 75% EtOH and spun again for 5 minutes at 4°C. As much EtOH was removed without disturbing the pellet and left to airdry. Pellets were resuspended in re-suspended in 20-50 µl of RNase free water depending on size of pellet (Ambion). Concentration was determined by NanoDrop2000 Spectrophotometer (Thermofisher) and integrity was determined by running 1 µl of RNA and 9 µl 11.1% glycerol on a EtBr 1.5% agarose gel in 1x TAE

2 µg of RNA was reverse transcribed to cDNA using Superscript II Reverse Transcriptase (Invitrogen), random hexamers (Invitrogen) and oligo dT primers (Invitrogen) as follows per reaction (all reagents Invitrogen):
1 µl OligodT
1 µl Random hexamers
1 µl dNTPs
2 µg of RNA
Adjust volume with ddH_2O to 10 µl
Samples were then heated to 65°C for 5 min and then chilled to 4°C.

A 10 µl master-mix was then added to the tubes:
4 µl 5X First-Strand Buffer
3 µL ddH_2O
2 µL 0.1 M DTT
0.5 µL RNaseOUT™ (40 units/µL)
0.5µL Superscript II Reverse Transcriptase

Contents were mixed with gentle pipetting, incubated at 25°C for 10 min, 42°C for 50 min and then inactivated by heating at 70°C for 15 min before being brought down to 4°C. cDNA was diluted 1:10 for RT-qPCR.

RT-qPCR was performed on a C1000 Touch™ Thermal Cycler with the CFX96™ Optical Reaction Module (Bio-Rad) using KAPA SYBR FAST qPCR mix (Kapa Biosystems) in technical triplicates with gene specific-primers (see Tables 2.3-2.5), following cycling temperatures and times as per manufacturers protocol.

Reaction constituents:
7.5 µL 2x SYBR FAST
3.9 µL ddH_2O
0.6 µL Forward & Reverse primer (100nM)
3 µL cDNA

ΔCt was determined using reference genes (for Zebrafish and Cavefish) and relative expression levels were plotted using the ΔΔCt method. Gene specific primers are listed in tables below.

2.2.2 Primer design

New primers (most opsin primers and all deep-sea primers) were designed using Primer3 from NCBI (Untergasser et al., 2007). For zebrafish, all genes of interest were annotated and we could simply extract FASTA files from NCBI to design primers. For the deep-sea primers, we did not have any annotated genes, so zebrafish clock opsin genes were blasted against the transcriptomes to obtain candidate genes. These candidate genes were aligned to multiple annotated clock genes from different species of fish using MEGA7 and muscle algorithm. Using MEGA one neighbour joining trees was made (Bootstrap, 500 replicates), to determine which type of copy/version of cryptochromes and period genes (Supplementary Figure 2.1- 2.4).

Primer efficiency was assessed by six 1:5 serial dilutions with technical duplicates (RT-qPCR protocol as above). Average C_t values were plotted against the log of the dilution factor. Using Excel, a linear trend line was fitted to the data to obtain the slope ($R^2 > 0.98$ was considered sufficient) and primers with efficiency 75-110% were used. The efficiency was calculated as:

$$Primer\ efficiency = (10^{-\frac{1}{slope}} - 1) \times 100$$

Melt curves were also examined to see that the primers only generated one PCR product across the dilutions. PCR products from effective primers were sequenced to confirm that the primers produce the correct PCR product.

Accession no.	Current name	Alt. Name	Forward 5' -> 3'	Reverse 5' -> 3'
KT008391	Exorhodopsin		GTA CGC TCC GCT ATC CCA TA	ACG TGT GAA AGC CCC TAC TG
KT008402	Valopa	Va1	ACT TCC ACG ACC ACA CCT TC	CGG ATG AGT TTG CAG TAG CA
KT008403	Valopb	Va2	GGC GAG GAT GGT CGT TGT AA	ATG CTG CAT AAG GCG TCC AT
KT008404	parapinopsin-1		CTG TGG TCG TTC ATC TGG AA	GGC CAG ATC TCT GCT GTA CC
KT008405	parapinopsin-2		GCA GCA CTG TAT ACA ACC CCT	ATA CGT CGT CCT CTG AAG GC
KT008406	parietopsin		TGT TGG CGT ATG AGC GTT AT	AGC CAT ACC AAC AGC AGA CC
KT008407	TMT1a	tmt6	TGT TAC AGT CGG CTC ATC TGT GCT	ATG TGG TAC TCT CTC CGT CTT GCT
KT008408	TMT1b	tmt9	TGT TGG TGT GTA TGT TCG GGACGA	AGG AGT TGA TGA AGC CGT ACC ACA
KT008409	TMT2a	tmt10	TTA GTA AGA AGC GGA GCA GAA CCT	ATC CCA TAG GGA TGC AGT GTT GTT
KT008410	TMT2b	tmt14	CGC AGA GGA GAG AGA ACC AC	TTA GTC CCG TTC TGC CAA AG
KT008411	TMT3a	tmt2	AGG TCG ATG CGA CCA ACT ACA AGA	AAA CAG AGG AGG CAG GGT CCA AAT
KT008412	TMT3b	tmt24	TGC GTG TGG TAC GGT TTC ATC AAT	ATC ATG GTG CAG TAA CGC TCG TAT
KT008413	encephalopsin (opn3)	panopsin	CCCTAT GCT GTG GTC TCC AT	TAG ATG ACG GGG TTG TAG GC
KT008414	neuropsin (opn5)	OPN5m1	ACA CCA TCT GTC GCT CCA TC	CTG CAA ATT GCC CAG TGT C
KT008415	OPN6a	novo3b	GTG GTC AAC ATC CCC TGG AG	ACA ACC AGC CGA GTA TGA GC
KT008416	OPN6b	novo3a	AAT CCA GCC AGG GAG GAG AT	AAG GCG GAC CAC ATG GAA AT
KT008417	OPN7a	novo1x	GTT TAA ACA CTA CCCGCG CC	GCTCTG GCTCCA ATT CAG GT
KT008418	OPN7b	novo1a	TGC TAT ATC GTG CCC TGC TG	CGTACC GTC ACC AGG ATG AG
KT008419	OPN7c	novo1b	GTG AAC CTG TCT GTG AGC GA	CTC CCC AAA CAA CCA CCT GT
KT008420	OPN7d	novo1y	CTG CCA CTT GGA ATC ATC CT	GCG ACA CAT GCT GCT GTA CT
KT008421	OPN8a	novo2b	TGA CTG ACA TTG GCA TGG CT	TGG TTG AAA GCA GAG GCG AT
KT008422	OPN8b	novo2a	TTC GCT TCA TCG TGT CTT TG	CAG TGG GAA AAT AGC CCA GA
KT008423	OPN8c	novo2x	TGG GCT TTA TCC TTG CCT GG	AGA TGAAGC CTT CTG GTG CC
KT008424	OPN9	OPN5m2	TCA GGG CTT TGT TTT CGG GA	GCA GCG GTC AAG GGA TAT GA
KT008425	Peropsin (RRH)		AGT GGT TGC CAT TGA CCG AT	ATG CGG CCA CAA TCA GAA GA
KT008426	RGR1		CCT GGC TTT CTA CGC CGC AG	GGA CTT GTT CTC AAT AGC AGG ACT CTC
KT008427	RGR2		GAG CAC GTC TAT CAC CAT CAG CT	ACA CCC CAG CCA ATG GCA GG
KT008428	OPN4m1		CGT CAT CAC CTC TGA GTC CA	GCT GGA TTT GTC CCA ACA GT
KT008429	OPN4m2		AGC AAT GCT AGT GGG CAG AA	CGT CTG CTG CAT CCG TTT CA
KT008430	OPN4m3		AAG GCC AAT GGT TCG GAT CC	CCA GGT ATG AGC CTG GAA GA
KT008431	OPN4x1		GCT ACA CCT TGA TGC TCT GC	CTG TTG GAT GAG GGT GGT CT
KT008432	OPN4x2		CTT TGT GAA GCA GCA GTC CA	TAT GGA GCC CAG GAC AAA AC
NM_001077297.2	Cry1a		AGG CTT ACA CAG CAG CAT CA	CTG CAC TGC CTC TGG ACT TT
NM_182857.2	Per2		TGG CTC TGG ACA GAA GTG AG	GGA TGT CTC GAG AAG GCA AC
NM_198143.1	L13		TCT GGA GGA CTG TAA GAG GTA TGC	AGA CGC ACA ATC TTG AGA GCA G
AB042254.1	6-4 photolyase	cry5	TGT GGA TCA TGA GGT TGT CC	TTG ATG GAT GGA CTC GCT TT
NM_001030183.1	Per1a	per1	ATC CAG ACC CCA ATA CAA C	GGG AGA CTC TGC TCC TTC T
AF057040.1	Beta actin		CGC AAA TAC TCC GTC TGG AT	TCC CTG GAG AAG AGC TAC GA

Table 2.3 Gene specific primers *Danio rerio*

Accession Number	Gene Name	Forward 5'-->3'	Reverse 5'-->3'
NM_001291258.1	*Per1*	ATC GCT GTG GAG CTG TTT TCA TTT	CAG AGC TCA TTC CCA TAT AAA GGC
-	*per2a*	TGT CCC GTT GCT AGG CTA CCT A	GCT GAC CGG CAT ACT GCA GG
KF737851.1	*per2b*	AAC ACA CAC GCC CAA CTG TA	GGT GAA GGT GGA GAA GGA CA
NM_001291262.1	*cry1a*	GTC ATG GGC TCC TGC ACT AC	GTC AAA ACC AAG CTC CTC CA
KF737857.1	*6-4'phr*	GGC CTC TCC TAA GCT GGA GT	GTC CAC AGG TGG GAA TTC AG
JF273743.1	ef1α	CAG CTG ATC GTT GGA GTC AA	TGT ATG CGC TGA CTT CCT TG
XM_007234224.3	rpl13α	TCT GGA GGA CTG TAA GAG GTA TGC	AGA CGC ACA ATC TTG AGA GCA G

Table 2.4 Gene specific primers *Astyanax mexicanus*

Gene name	Forward 5'-->3'	Reverse 5'-->3'
per1	AAC ACC ATC AGC AGT CCA GT	CTG AAG CGG CTG AGG AAC A
per2	GAC AGC ATC ACC TCC GAG TA	TAT AGA ACA GGC CGA CGT CC
per3	AGA AGA CGA AGA GAT GAG CAC T	CTG TCA GCA TCT CCT CTC TGT
cry1a	CGA ACT TGA CCG AGG ACC TG	CAA ACA CGT GTA TCC GCA CG
cry2	AGC CTG GAG TTC AGC TTT CG	ATC CTG GAT CCG TGG TTT GC
cry3a	GCA TCC AGA TCT TCG AGG CA	GTT AAG GGG GCA GAC ACA GT
cry3b	CAG GGA CAA GTA CGG GGT TC	GCT AGA GCT TCC ATC TCG CC
β-actin	CGC AAA TAC TCC GTC TGG AT	TCC CTG GAG AAG AGC TAC GA

Table 2.5 Gene specific primers *Sternoptyx diaphna*

Gene name	Forward 5'-->3'	Reverse 5'-->3’
per1a	AGC GCT GGT AGA GAC AGA GA	CTG ATC CCA AAG TCC CCA CC
per1b	CTG GAC CTG GAC ACC TTC AC	CCT TCC CCG ACA GGA AAG AC
per2x	GAA AGT CCC ACC AGG AGA GC	CAG CTC CAC AGA GAC TGA GC
per2y	AGC TCC AAT GCT TTC AGC CT	GCT CTT TCT GGG TCT TGG CT
per2z	CCA ACG CCA AGT TTG TGG AG	GGG CGT GGT GAA ACT GTA GA
cry1a	GGT AGG GAC TGA GGC CTG TA	TTG AGC GTC CCA GGA TGA AC
cry2	GAG TTC CTG ACT GCC GTC TC	TGA CTC TGA GCC ATA CGG GA
cry3a	*	
cry3b	TAT CCA GGG GTA TCC GGT CC	TAT CCA GGG GTA TCC GGT CC
G-6-P	TGA TAG GGA GGA CAG GTG GG	TAC TGT CCG AAC ACT GCG TC

Table 2.6 Gene specific primers *Argyropelecus hemigymnus*

* cry3a the gene exist in *A. hemigymnus* but none of the primers were efficient. Further testing was not prioritised. No *per3* was found in *A.hemigymnus* transcriptome.

2.2.3 Bioluminescent assays

Per-1 luciferase cells, described by (Vallone et al., 2004), were plated at 100,000 cell/ml in media (described above) in a white 96-well plate (Greiner) n=16. Cells settled over night at 28°C, and the following day the media was changed for media supplemented with 0.5 nM beetle luciferin (Promega). Plates were sealed with TopSeal clear adhesive from (Perkin Elmer). Bioluminescence was monitored on a TopCount NXT scintillation counter (Packard Instrument Company), in a temperature-controlled chamber (28 °C). Cells were entrained on a 12:12 LD cycle and given a light pulse (as described above) at ZT21 after 2 days. Cells were then kept in DD on the TopCount for two more days, at constant temperature, in order measure any phase shift in the gene expression rhythm. Luminescence from the cells was measured in counts per second (CPS) approximately every hour taking approximately 10 minutes for a 96-well plate to be read.

2.2.4 Whole mount *in situ* hybridisation

Whole mount *in situ* hybridisation was performed by Dr Andrew Beale.
At the assigned timepoints, embryos were fixed in 4% PFA/PBS overnight at 4°C. Early embryos were dechorionated. Embryos were washed 4 times with PBS before storage in 100% MeOH at -20°C. All further steps were conducted at room temperature unless otherwise stated. Embryos were rehydrated in a series of washes with 75% MeOH in PBT (PBS + 0.01% Tween-20), 50% MeOH in PBT, and 25% MeOH in PBT and twice in PBT. Embryos were then treated with 10 µg/ml proteinase K for 5 min, washed with PBT twice, before fixation 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. Digoxigenin-labeled (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+ (5xSSC, 0.1% Tween-20, 5 mg/ml torula (yeast) RNA, 50 µg/ml heparin) at 80°C for 2 min before being diluted to 1 µg/ml in HYB+. Embryos were incubated with DIG-labelled RNA sense or antisense probe in HYB+ overnight at 65°C with gentle shaking.

After hybridisation, the embryos were successively washed at 65°C with HYB+, 50% HYB+/2X SSC, 2X SSC, and twice in 0.2X SSC before being cooled to room temperature, and subject to a further three washes with PBS. After washing, embryos were incubated with 2% Blocking Agent (Roche) in maleic acid buffer (MAB) for at least 3 hours. The block was replaced with anti-DIG-alkaline phosphatase (1:5000) in 2% Blocking Agent in MAB, and the embryos were incubated overnight at 4°C. The embryos were subsequently 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. Finally, embryos were washed twice with PBT and refixed with 4% PFA/PBS overnight at 4°C.

In situ hybridization signal was quantified by densitometry in ImageJ (1.50i, Schneider, et al., (2012), "NIH Image to ImageJ: 25 years of image analysis", Nature methods). Images were converted to 8-bit grayscale and a rectangular encompassing the embryo was drawn using the Specify tool. Optical density was calculated using the “Analyze tool” from the peak of the profile plot of each sample after enclosing the peak and eliminating background noise.

2.2.5 Data and statistical Analysis

Data was analysed in Microsoft Excel. Statistics included T-tests, ANOVAs and post-tests for qPCRs and bioluminescent data were performed with the standard add-in software. A rhythmicity test was performed using BioDare2, using the classic JTK test method with cosine24h preset, *per1a* rhythms were determined "true" ($p<0.05$), so was *per1b* but only after a linear detrend of the dataset (Supplementary Table 2.1) (Zilenski et al., 2014).

Data analysis and statistics concerning the transcriptomic data are addressed in the script where appropriate.

2.2.6 RNA sequencing of deep-sea samples

RNA from deep-sea eyes and organs for sequencing was extracted using RNeasy Plus Micro Kit (Qiagen), using the manufacturers protocol. Zebrafish RNA from eye was extracted using RNeasy Mini Kit (Qiagen). The tissues selected for sequencing were n=3 light-pulsed eyes from each deep-sea species and n=3 eyes as dark controls for each species. A separate "mixed tissue" sample (liver, gill, muscle, brain, heart) was also submitted for each species to ensure a more complete transcriptome could be generated for both species. RNA samples were shipped on dry ice to the Norwegian Sequencing Centre NSC, which quality tested RNA using Qubit Fluorometric Quantification (Thermofisher) and prepared libraries using Illumina TruSeq mRNA stranded kit (Illumina). All samples were sequenced on one lane HiSeq4000 (Illumina) 150 bp paired-end reads. Deep-sea samples were later re-sequenced after initial analysis to increase sequencing depth. Reads per sample are summarised in Table 2.7 and quality control reports for the RNA are listed in Supplementary Figures 2.5-2.18.

2.3 Bioinformatics

2.3.1 Code for *de novo* assembly of deep-sea transcriptomes

Standard *de novo* protocol for assembly was followed with a few tweaks to fit the dataset. University of Oslo's computing services (Cod6, Cod7 and Abel) was initially used, and when these became redundant, the National Norwegian Saga and NIRD (National infrastructure for research data) nodes were used. FASTQC reports per base sequence quality for each sample is provided in Supplementary Figures 2.19-2.32.

Code below is written for command line (Mac). Lines starting with hash (#) is not code, but explanation for code that follows.

```
########################################
#Trimmomatic (Bolger et al., 2014)
########################################
#Removing Illumina adaptor and low-quality bases and short reads (below 36) from forward and reverse samples
#These were the options used:

Trimmomatic-0.38/trimmomatic-0.38.jar PE -threads 1 -phred33 -trimlog Sample_trimm.log <input 1> <input 2> <paired output 1> <unpaired output 1> <paired output 2> <unpaired output 2> ILLUMINACLIP:"Trimmomatic-0.38/adapters/TruSeq3-PE.fa":2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36

#Make trim_stats file
grep "Input" trimm/Sample*.err > trimmstats.txt

########################################
#END Trimmomatic –  Results Supplementary Table 2.2
########################################


########################################
#TRINITY (Grabgerr et al., 2011, Haas et al., 2013)
```

```
########################################

#Running Trinity v. 2.10.0

Trinity --seqType fq --max_memory 900G --CPU 32 --left SampleL --right SampleR \
--no_normalize_reads --SS_lib_type FR --bflyHeapSpaceMax 200G \
> trinity_no_norm_stern.out 2> trinity_no_norm_stern.err

########################################
####Quality control: ####
########################################

# Run assembly stats script from the trinity package (v. short time)

TrinityStats.pl trinity_out_dir/Trinity.fasta > assemblystats_x.out

#BUSCO evaluation (not part of Trinity) (Seppey et al., 2019)

busco.py -i /trinity_out_dir/Trinity.fasta -o Trinity.fasta.sternoptyx.BUSCO.out -l
eukaryota_odb9  -m tran -c 20

########################################
####Quality control END (Supplementary Table 2.3)
########################################

### SAMPLE-SPECIFIC MAPPING BACK TO ASSEMBLY ###

#Prepare the reference for alignment and abundance estimation using RSEM and bowtie2

ulimit -s unlimited
align_and_estimate_abundance.pl --transcripts trinity_out_dir/Trinity.fasta \
--est_method RSEM --aln_method bowtie --thread_count 10 --prep_reference --trinity_mode
\
--output_dir 1> bowtieprep.out 2> bowtieprep.err
```

```
####Mapping back as single (mapping paired gave no or few DE regulated genes) #####
#Forward and reverse samples are concatenated
# Map reads for each sample to the assembly and estimate abundance
#Example – this was repeated for each sample
#making directories for each sample

mkdir sampleLP1
mkdir sampleLP2
mkdir sampleLP3
mkdir sampleLDD1
mkdir sampleDD2
mkdir sampleDD3

cd  ~/SPECIFIC_DIRECTORY

align_and_estimate_abundance.pl --transcripts ~/Trinity.fasta --gene_trans_map
~/Trinity.fasta.gene_trans_map \
--seqType fq --single SampleN.fq.gz
--est_method RSEM --aln_method bowtie --thread_count 5 \
--output_dir 1> sampleN_s.out 2> sampleN_s.err

##############################################
#Trinity raw count extraction for DE analysis
##############################################

#make raw count matrix

abundance_estimates_to_matrix.pl \
--est_method RSEM --name_sample_by_basedir \
--out_prefix sample \
~/sampleLP1/RSEM.genes.results \
~/sampleLP2/RSEM.genes.results \
~/sampleLP3/RSEM.genes.results \
```

```
~/sampleDD1/RSEM.genes.results \
~/sampleDD2/RSEM.genes.results \
~/sampleDD3/RSEM.genes.results


#Make a sample file describing data

LP      sampleLP1
LP      sampleLP2
LP      sampleLP3
DD      sampleDD1
DD      sampleDD2
DD      sampleDD3
#save as sample_file

#make a contrast file describing the conditions

LP      DD
#save as contrast_file

#Run DE analysis

~/DifferentialExpression/run_DE_analysis.pl --matrix sample.counts.matrix \
--method edgeR --samples_file sample_file --contrasts contrast_file


#Change directory to edgeR folder and run DE analysis

~Analysis/DifferentialExpression/analyze_diff_expr.pl \
--matrix ~/sample.TMM.EXPR.matrix --samples ~/sample_file -P 0.05

################################################
#END TRINITY
################################################
```

```
##############################################
#Manually annotating significantly expressed genes:
##############################################

#Make a list of gene names of up-regulated genes save as .txt (taken from the log2.dat file)
#then obtain the sequences from the de novo transcriptome (.fas)

fastagrep.pl -f DE_genes.txt trinity_out_dir/Trinity.fasta > DE_genes.fas

#Blast these sequences to swiss or uniprot - I use a high cut-off value of e-20

BLAST+
blastx  -db ~/swissprot -query DE_genes.fas -out blast_swissprot_DE_genes_e20 \
-evalue 1e-20 -outfmt 6 -num_threads 5

#Genes are then manually addressed in a spreadsheet based on E-value and number of hits
etc.

##############################################
#END ANNOTATION
##############################################

##############################################
#Searching for genes of interest
##############################################

### Making a blast database from your transcriptome FASTA file from Trinity)
mkdir database

makeblastdb -in ~/trinity_out_dir/Trinity.fasta -dbtype nucl  -deep-sea_database.fasta

# The transcriptome can now be searched using other FASTA files
```

#Obtain mRNA FASTA files from e.g. NCBI - here the example is zebrafish opsins called OPSINS_DANIO_FASTA.fas

tblastn -db deep-sea_database.fasta -query OPSINS_DANIO_FASTA.fas -out blasout_opn -outfmt 0 -evalue 1e-20 -num_threads 5

#Manually assess and address which are good candidates from the list - get FASTA file for designing primers

fastagrep -f list_opsins ~/Trinity.fasta > transcripts_opsins.fas

2.3.2 Code for genome guided assembly of zebrafish transcriptome

Standard genome guided Trinity protocol for assembly was followed with a few tweaks to fit the dataset. University of Oslo's computing services (Cod6, Cod7 and Abel) was initially used, and when these became redundant, the National Norwegian Saga and NIRD (National infrastructure for research data) nodes were used. FASTQC reports per base sequence quality for each sample is provided in Supplementary Figures 2.33-2.40.

Code below is written for command line (Mac). Lines starting with hash (#) is not code, but explanation for code that follows.

```
##########################################
#### Prep for genome guided Trinity  ####
##########################################
```

####Download Zebrafish genome #####

#Download from ensmbl the full genome and support file that contains the annotation Danio_rerio.GRCz11.dna.toplevel

#Genome guided assembly using hisat2 (v2.1.0) for alignment and stringtie (v 1.3.1) (Kim et al., 2019; Kim et al., 2015; Pertea et al., 2015)

```
ln -s ~/zebrafish_genome_GRCz11/Danio_rerio.GRCz11.fa #softlink to genome

mkdir -p reads
zcat ~/Sample_*/*R1*fastq.gz > ./reads/read1.fastq
zcat ~/Sample_*/*R2*fastq.gz > ./reads/read2.fastq

hisat2-build Danio_rerio.GRCz11.fa zebrafish_index 2> hisat-build.err

hisat2 -p 32 --dta -x zebrafish_index -1 ./reads/read1.fastq -2 \
./reads/read2.fastq 2> hisat.err | samtools view -buS - | \
samtools sort -T tmp -O bam - > zebrafish_index_hisat2.sort.bam 2> samtools.err

stringtie zebrafish_index_hisat2.sort.bam > zebrafish_index_stringtie.gtf 2> stringtie.err

#########################################################################
#### Genome guided Trinity v 2.3.2 (Grabgerr et al., 2011, Haas et al., 2013) ####
#########################################################################

Trinity --genome_guided_bam zebrafish_index_hisat2.sort.bam \
        --genome_guided_max_intron 100000 \
        --max_memory 900G --CPU 32 > trinity2.3.2_gg.out 2> trinity2.3.2_gg.err

########################################
####Quality control: ####
########################################

# Run assembly stats script from the trinity package (v. short time)

~/TrinityStats.pl trinity_out_dir/Trinity-GG.fasta > assemblystats.out

#BUSCO evaluation (not part of Trinity) (Seppey et al., 2019)
```

```
busco.py -i /trinity_out_dir/Trinity.fasta -o Trinity.fasta.sternoptyx.BUSCO.out -l
eukaryota_odb9  -m tran -c 20

##################################################
####Quality control END (Supplementary Table 2.4)
##################################################

##################################################
#######StringTie v 1.2.2 (Pertea et al., 2016; 2015)######
##################################################

#assemble and quantitate full-length transcripts
## create file for input from

samtools view -o zebrafish_index_hisat2.sort.bam | samtools sort -o
zebrafish_index_hisat2.sort.bam.sorted

stringtie zebrafish_index_hisat2.sort.bam > zebrafish_index_stringtie.gtf 2> stringtie.err

#Run stringtie

stringtie ~/zebrafish_index_hisat2.sort.bam.read_coords.sort_by_readname -p 10 -G
~/zebrafish_genome_GRCz11/Danio_rerio.GRCz11.gff3 -B -e

stringtie ~/zebrafish_index_hisat2.sort.bam -p 10 -G ~/zebrafish_index_stringtie.gtf -B -e

stringtie zebrafish_index_hisat2.sort.bam -G
~/zebrafish_genome_GRCz11/Danio_rerio.GRCz11.gff3 -o 1> zebrafish_index_stringtie.gtf
2> stringtie.err

### SAMPLE-SPECIFIC MAPPING BACK TO ASSEMBLY ###
#making directories for each sample
```

```
#Prepare the reference for alignment and abundance estimation using RSEM and bowtie2

ulimit -s unlimited
align_and_estimate_abundance.pl --transcripts trinity_out_dir/Trinity-GG.fasta \
--est_method RSEM --aln_method bowtie --thread_count 10 \
--prep_reference --trinity_mode --output_dir 1> bowtieprep.out 2> bowtieprep.err

# Map reads for each sample to the assembly and estimate abundance
#making directories for each sample

mkdir sampleLP1
mkdir sampleLP2
mkdir sampleLP3
mkdir sampleLP4
mkdir sampleLDD2
mkdir sampleDD3
mkdir sampleDD4
mkdir sampleDD5


cd  ~/SPECIFIC_DIRECTORY

####MAPPING back as single to replicate deep-sea mapping protocol #####
#Forward and reverse samples are concatenated

#Example

align_and_estimate_abundance.pl --transcripts ~/Trinity-GG.fasta --gene_trans_map
~/Trinity.fasta.gene_trans_map \
--seqType fq --single SampleN.fq.gz
--est_method RSEM --aln_method bowtie --thread_count 5 \
--output_dir 1> sampleN_s.out 2> sampleN_s.err

##################################################
```

```
#Trinity raw count extraction for DE analysis
##############################################
#make raw count matrix

abundance_estimates_to_matrix.pl \
--est_method RSEM --name_sample_by_basedir \
--out_prefix sample \
~/sampleLP1/RSEM.genes.results \
~/sampleLP2/RSEM.genes.results \
~/sampleLP3/RSEM.genes.results \
~/sampleLP4/RSEM.genes.results \
~/sampleDD2/RSEM.genes.results \
~/sampleDD3/RSEM.genes.results \
~/sampleDD4/RSEM.genes.results
~/sampleDD5/RSEM.genes.results \

#Make a sample file describing data
LP      sampleLP1
LP      sampleLP2
LP      sampleLP3
LP      sampleLP4
DD      sampleDD2
DD      sampleDD3
DD      sampleDD4
DD      sampleDD5

#save as sample_file

#make a contrast file describing the conditions

LP      DD
#save as contrast_file

#Run DE analysis
```

```
~/DifferentialExpression/run_DE_analysis.pl --matrix sample.counts.matrix \
--method edgeR --samples_file sample_file --contrasts contrast_file

#Change directory to edgeR folder and run DE analysis

~Analysis/DifferentialExpression/analyze_diff_expr.pl \
--matrix ~/sample.TMM.EXPR.matrix --samples ~/sample_file -P 0.05

################################################
#END TRINITY
################################################

################################################
#Manually annotating significantly expressed genes #
################################################

#Make a list of gene names of up-regulated genes save as .txt (taken from the log2.dat file)
#then obtain the sequences transcriptome (.fas)

fastagrep.pl -f DE_genes.txt trinity_out_dir/Trinity-GG.fasta > DE_genes.fas

#Blast these sequences to zebrafish genome - I use a high cut-off value of e-20

BLAST+
blastn  -db ~/zfgenome -query DE_genes.fas -out blast_zfgenome_DE_genes_e20 \
-evalue 1e-20 -outfmt 6 -num_threads 5

#Genes are then manually addressed in a spreadsheet based on E-value and number of hits
etc.

################################################
#END DE ANNOTATION
################################################
```

Zebrafish	Fragments
H73C3BBXY_25-dd-2_TATCGCAC-ACACTAAG_L004_R1_001.fastq.gz	41,824,357
H73C3BBXY_25-dd-2_TATCGCAC-ACACTAAG_L004_R2_001.fastq.gz	41,824,357
H73C3BBXY_26-dd-3_CGCTATGT-GTGTCGGA_L004_R1_001.fastq.gz	41,047,483
H73C3BBXY_26-dd-3_CGCTATGT-GTGTCGGA_L004_R2_001.fastq.gz	41,047,483
H73C3BBXY_27-dd-4_GTATGTTC-TTCCTGTT_L004_R1_001.fastq.gz	33,674,717
H73C3BBXY_27-dd-4_GTATGTTC-TTCCTGTT_L004_R2_001.fastq.gz	33,674,717
H73C3BBXY_28-dd-5_ACGCACCT-CCTTCACC_L004_R1_001.fastq.gz	41,283,129
H73C3BBXY_28-dd-5_ACGCACCT-CCTTCACC_L004_R2_001.fastq.gz	41,283,129
H73C3BBXY_29-lp-1_TACTCATA-GCCACAGG_L004_R1_001.fastq.gz	40,472,553
H73C3BBXY_29-lp-1_TACTCATA-GCCACAGG_L004_R2_001.fastq.gz	40,472,553
H73C3BBXY_30-lp-2_CGTCTGCG-ATTGTGAA_L004_R1_001.fastq.gz	33,373,530
H73C3BBXY_30-lp-2_CGTCTGCG-ATTGTGAA_L004_R2_001.fastq.gz	33,373,530
H73C3BBXY_31-lp-3_TCGATATC-ACTCGTGT_L004_R1_001.fastq.gz	61,190,177
H73C3BBXY_31-lp-3_TCGATATC-ACTCGTGT_L004_R2_001.fastq.gz	61,190,177
H73C3BBXY_32-lp-4_CTAGCGCT-GTCTACAC_L004_R1_001.fastq.gz	44,101,323
H73C3BBXY_32-lp-4_CTAGCGCT-GTCTACAC_L004_R2_001.fastq.gz	44,101,323
Deep-sea Run1	
HNTLVBBXX_59-A_CGGCTATG-AGGATAGG_L005_R1_001.fastq.gz	41,080,254
HNTLVBBXX_59-A_CGGCTATG-AGGATAGG_L005_R2_001.fastq.gz	41,080,254
HNTLVBBXX_60-S_CGGCTATG-TCAGAGCC_L005_R1_001.fastq.gz	51,647,285
HNTLVBBXX_60-S_CGGCTATG-TCAGAGCC_L005_R2_001.fastq.gz	51,647,285
HNTLVBBXX_61-487_CGGCTATG-CTTCGCCT_L005_R1_001.fastq.gz	21,571,044
HNTLVBBXX_61-487_CGGCTATG-CTTCGCCT_L005_R2_001.fastq.gz	21,571,044
HNTLVBBXX_62-488_CGGCTATG-TAAGATTA_L005_R1_001.fastq.gz	21,091,987
HNTLVBBXX_62-488_CGGCTATG-TAAGATTA_L005_R2_001.fastq.gz	21,091,987
HNTLVBBXX_63-488-2_CGGCTATG-ACGTCCTG_L005_R1_001.fastq.gz	22,525,655
HNTLVBBXX_63-488-2_CGGCTATG-ACGTCCTG_L005_R2_001.fastq.gz	22,525,655
HNTLVBBXX_64-494_CGGCTATG-GTCAGTAC_L005_R1_001.fastq.gz	19,196,837
HNTLVBBXX_64-494_CGGCTATG-GTCAGTAC_L005_R2_001.fastq.gz	19,196,837
HNTLVBBXX_65-495_TCCGCGAA-AGGCTATA_L005_R1_001.fastq.gz	21,426,037
HNTLVBBXX_65-495_TCCGCGAA-AGGCTATA_L005_R2_001.fastq.gz	21,426,037
HNTLVBBXX_66-496_TCCGCGAA-GCCTCTAT_L005_R1_001.fastq.gz	26,886,648
HNTLVBBXX_66-496_TCCGCGAA-GCCTCTAT_L005_R2_001.fastq.gz	26,886,648
HNTLVBBXX_67-497_TCCGCGAA-AGGATAGG_L005_R1_001.fastq.gz	23,733,774
HNTLVBBXX_67-497_TCCGCGAA-AGGATAGG_L005_R2_001.fastq.gz	23,733,774
HNTLVBBXX_68-498_TCCGCGAA-TCAGAGCC_L005_R1_001.fastq.gz	22,526,932
HNTLVBBXX_68-498_TCCGCGAA-TCAGAGCC_L005_R2_001.fastq.gz	22,526,932
HNTLVBBXX_69-499_TCCGCGAA-CTTCGCCT_L005_R1_001.fastq.gz	25,376,396
HNTLVBBXX_69-499_TCCGCGAA-CTTCGCCT_L005_R2_001.fastq.gz	25,376,396
HNTLVBBXX_70-505_TCCGCGAA-TAAGATTA_L005_R1_001.fastq.gz	24,524,808
HNTLVBBXX_70-505_TCCGCGAA-TAAGATTA_L005_R2_001.fastq.gz	24,524,808
HNTLVBBXX_71-506_TCCGCGAA-ACGTCCTG_L005_R1_001.fastq.gz	26,116,341
HNTLVBBXX_71-506_TCCGCGAA-ACGTCCTG_L005_R2_001.fastq.gz	26,116,341
HNTLVBBXX_72-507_TCCGCGAA-GTCAGTAC_L005_R1_001.fastq.gz	27,058,554
HNTLVBBXX_72-507_TCCGCGAA-GTCAGTAC_L005_R2_001.fastq.gz	27,058,554
Deep-sea Re-run	
H33LGBBXY_59-A_CGGCTATG-AGGATAGG_L002_R1_001.fastq.gz	39,480,991
H33LGBBXY_59-A_CGGCTATG-AGGATAGG_L002_R2_001.fastq.gz	39,480,991
H33LGBBXY_60-S_CGGCTATG-TCAGAGCC_L002_R1_001.fastq.gz	26,776,602
H33LGBBXY_60-S_CGGCTATG-TCAGAGCC_L002_R2_001.fastq.gz	26,776,602
H33LGBBXY_61-487_CGGCTATG-CTTCGCCT_L002_R1_001.fastq.gz	19,463,060
H33LGBBXY_61-487_CGGCTATG-CTTCGCCT_L002_R2_001.fastq.gz	19,463,060
H33LGBBXY_62-488_CGGCTATG-TAAGATTA_L002_R1_001.fastq.gz	22,303,420
H33LGBBXY_62-488_CGGCTATG-TAAGATTA_L002_R2_001.fastq.gz	22,303,420
H33LGBBXY_63-488-2_CGGCTATG-ACGTCCTG_L002_R1_001.fastq.gz	18,287,803
H33LGBBXY_63-488-2_CGGCTATG-ACGTCCTG_L002_R2_001.fastq.gz	18,287,803
H33LGBBXY_64-494_CGGCTATG-GTCAGTAC_L002_R1_001.fastq.gz	23,920,223
H33LGBBXY_64-494_CGGCTATG-GTCAGTAC_L002_R2_001.fastq.gz	23,920,223
H33LGBBXY_65-495_TCCGCGAA-AGGCTATA_L002_R1_001.fastq.gz	22,027,629
H33LGBBXY_65-495_TCCGCGAA-AGGCTATA_L002_R2_001.fastq.gz	22,027,629
H33LGBBXY_66-496_TCCGCGAA-GCCTCTAT_L002_R1_001.fastq.gz	13,633,193
H33LGBBXY_66-496_TCCGCGAA-GCCTCTAT_L002_R2_001.fastq.gz	13,633,193
H33LGBBXY_67-497_TCCGCGAA-AGGATAGG_L002_R1_001.fastq.gz	18,721,747
H33LGBBXY_67-497_TCCGCGAA-AGGATAGG_L002_R2_001.fastq.gz	18,721,747
H33LGBBXY_68-498_TCCGCGAA-TCAGAGCC_L002_R1_001.fastq.gz	17,189,418
H33LGBBXY_68-498_TCCGCGAA-TCAGAGCC_L002_R2_001.fastq.gz	17,189,418
H33LGBBXY_69-499_TCCGCGAA-CTTCGCCT_L002_R1_001.fastq.gz	14,992,114
H33LGBBXY_69-499_TCCGCGAA-CTTCGCCT_L002_R2_001.fastq.gz	14,992,114
H33LGBBXY_70-505_TCCGCGAA-TAAGATTA_L002_R1_001.fastq.gz	16,157,391
H33LGBBXY_70-505_TCCGCGAA-TAAGATTA_L002_R2_001.fastq.gz	16,157,391
H33LGBBXY_71-506_TCCGCGAA-ACGTCCTG_L002_R1_001.fastq.gz	13,323,717
H33LGBBXY_71-506_TCCGCGAA-ACGTCCTG_L002_R2_001.fastq.gz	13,323,717
H33LGBBXY_72-507_TCCGCGAA-GTCAGTAC_L002_R1_001.fastq.gz	15,413,923
H33LGBBXY_72-507_TCCGCGAA-GTCAGTAC_L002_R2_001.fastq.gz	15,413,923

Table 2.7 Sequencing depth from paired end NGS.

59A= mixed tissue *A.hemigymnus* 60S= mixed tissue *S.diaphna.*

Sample 61-63 = LP & 64-66= DD *A.hemigymnus*

Sample 67-69=LP & 70-72= DD *S.diaphana.*

3 Spectral Sensitivity in Zebrafish Cell Lines and Tissues

3.1 Introduction

3.1.1 History of light sensing and opsins

Light sensing and entrainment were always thought to be a process associated exclusively with the eyes and the Suprachiasmatic Nucleus (SCN) in mammals, and the pineal gland in non-mammalian vertebrates (Cahill, 1996; Elliott, 1976; Ibuka and Kawamura, 1975; Suburo and Iraldi, 1969; Underwood and Groos, 1982). It therefore came as a surprise, some 20 years ago, that the process of non-visual photoreception is something that all tissues in the zebrafish are capable of (Whitmore et al., 1998). Although the zebrafish pineal has key functions (Ben-Moshe et al., 2014; Livne et al., 2016), teleost clock systems appear to be highly decentralized, with all tissues and the majority of cells possessing a directly light entrainable circadian pacemaker (Carr et al., 2004; Steindal and Whitmore 2019; Tamai et al., 2005; Whitmore et al., 1998, 2000).

Peripheral light sensitivity is not exclusive to zebrafish, as most non-mammalian vertebrates such as fish, reptiles and birds show high opsin diversity (Davies et al., 2015). Deep-brain photoreception has been researched in avian seasonal physiology for many years, as has similar hypothalamic responses in reptiles (Benoit, 1935a, 1935b; Takahashi and Menaker, 1979; Underwood and Groos, 1982; Underwood and Menaker, 1976; Wyse and Hazlerigg, 2009). So, perhaps it should not have come as such a surprise when this well-established direct brain light-sensitivity was expanded to include the majority of other tissues. Monotremes and mammals also express non-visual opsins that facilitate a range of biological processes, of which, melanopsin in mammalian clock entrainment is the most explored (Halford et al., 2001; Provencio et al., 1998; Tarttelin et al., 2003).

When peripheral photoreception was discovered in amniotes, the next obvious question concerns the nature of the photopigment responsible for this peripheral light detection and clock entrainment? Visual photopigments have been studied extensively since the 19th century (Arey, 1915; Norris, 1895). However, a whole century past before science turned its interest to the discovery of the non-visual photopigments, and several candidates appeared through the late 90's and early 2000s (Blackshaw and Snyder, 1997; Okano et al., 1994; Provencio et al., 1998; Soni and Foster, 1997; Sun et al., 1997). The number of opsins discovered since the early 1990s has increased to include 32 non-visual and 10 visual opsins

in zebrafish (Davies et al., 2015), and new opsins, splice variants and isoforms are discovered in new species on a regular basis.

3.1.2 Non-visual opsins signal through a range of G-coupled proteins

The non-visual opsins are all seven-transmembrane-domain proteins, like the visual opsins and function using similar mechanisms to those of the classical extra ocular photoreceptors. As opsins belong to the G protein-coupled receptor (GPCR) superfamily of proteins, it follows that opsins may signal and thereby activate light-induced and clock genes, using the classic, well-established downstream pathways. The non-visual and visual opsins are divided into 5 or 8 classes based on photoisomerase activity, molecular function and how they couple and signal through G-proteins (Davies et al., 2015, Perez et al., 2019). Phylogenetic studies show that teleost genomes encode 20 different opsin genes, while reptiles, birds and amphibians also show a high genomic diversity of opsin classes, with 19, 17 and 18 genes respectively (Davies et al., 2015). Zebrafish, is currently the animal with the highest reported diversity of opsins in any animal with other ray-finned fishes expressing 22-32 non-visual opsin genes (Beaudry et al., 2017; Braasch et al., 2016). However, an analysis studying visual opsins in deep-sea fishes found that species such as the spinyfins (Diretmidae), have a staggering 18-35 copies of RH1 rhodopsin (Musilova et al., 2019). This study looking at 101 fish genomes did not look for non-visual opsins, but it would not be surprising if there was a correspondingly high number of non-visual opsins in some of the deep-sea fish species.

The biochemistry and function of visual opsins and the visual cycle has been extensively researched in mammalian eyes. In short, a photon hits the bound 11-cis retinal in the visual opsins, causing a conformational change to all-trans retinal state and also setting off the phototransduction cascade. When the retinal is in its all-trans state, the opsin cannot absorb photons and signal, and in the eyes, the visual opsins are dependent on the visual cycle for the opsin to regenerate a 11-cis retinal. Photons only initiate chemical reactions when they are absorbed (first law of photochemistry). The visual cycle involves several enzymatic steps both in the rod/cone cell and in the neighbouring retinal pigment epithelial cells (RPE) where the all-trans is converted back into 11-cis, which can bind to its opsin and re-absorb a photon (Wright et al., 2015, Wald 1935). The rhodopsins and cone opsins are termed monostable, as they require the visual cycle to regenerate the retinal. In contrast, many of the non-visual

opsins are bistable which means that they do not require the enzymatic activity and the multiple cell layers of the visual cycle to regenerate its retinal, but simply use the energy of a photon to convert the retinal back (Tsukamoto 2014). Other non-visual opsins, such as some of the melanopsins (opn4m2 and opn4x1-2) and opn6b are however found to be monostable (Davies et al., 2011, 2015). This would suggest that monostable opsins like these, as well as the visual opsins found expressed throughout the zebrafish, would require the help of the visual cycle in order to regenerate the pigment. This is backed up by an *in vivo* study on melanopsin which showed no electrical current could be recorded if you express a monostable melanopsin (opn4x1) in a Neuro2a cell line with all-trans retinal, but you can indeed record a current from a bistable melanopsin (opn4m3). However, if you co-express the monostable opsin with bistable melanopsin you get a massively increased electrical current, suggesting that there is some form of dimerization or other interaction between different types of opsins which in turn increase cellular signalling (Davies et al., 2011). The study raises the possibility that some monostable opsins may use dimerization in extra-ocular tissues in order for the opsin to regenerate its bleached pigment back to an 11-cis conformation.

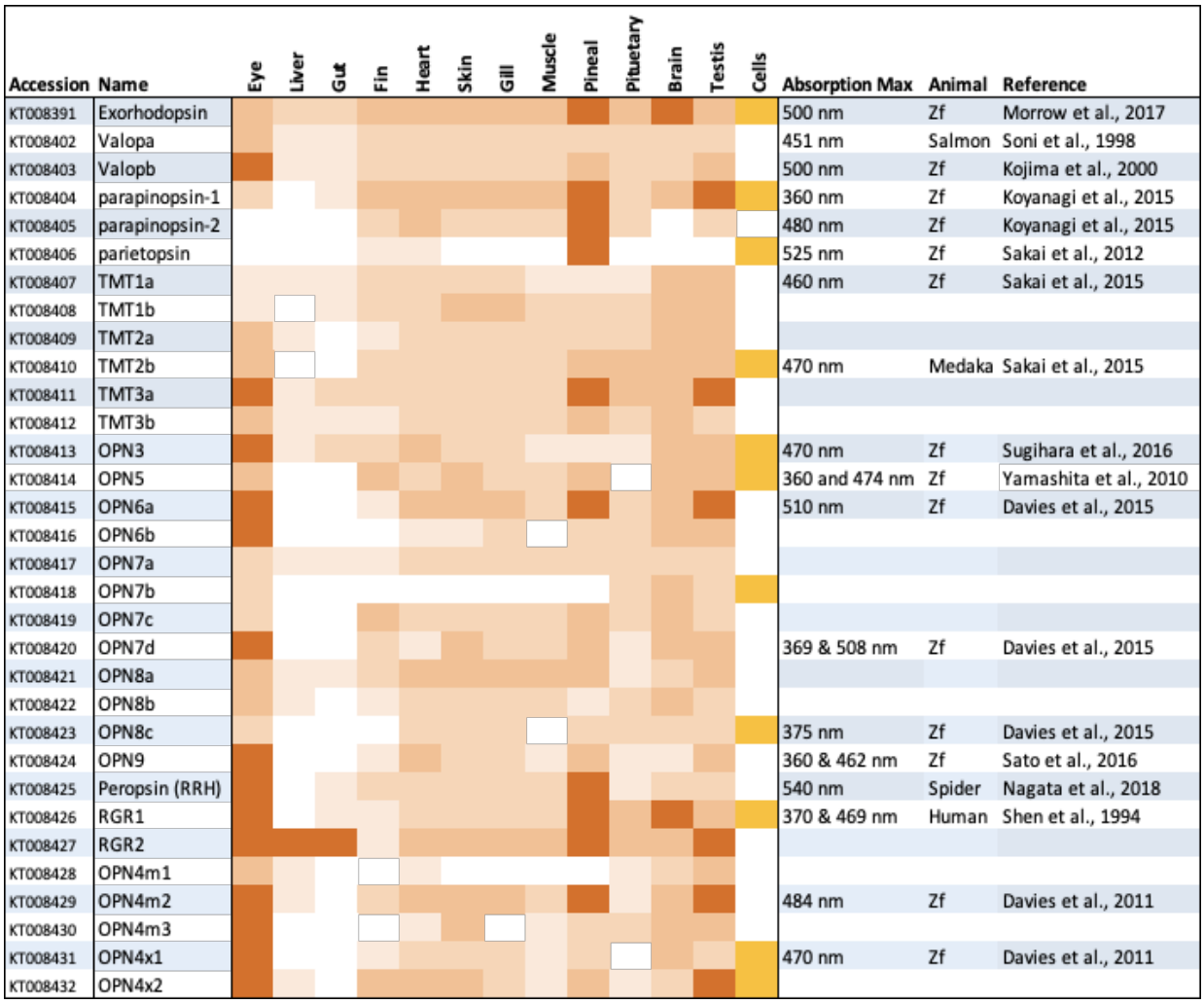
Another way the monostable opsins may regenerate their bleached pigments is through the use of photoisomerases. The retinal G-protein coupled receptors (RGR) are found ubiquitously expressed in all zebrafish tissues, and functional studies indicate that this opsin might actually act as a light dependent photoisomerase. Experiments on *in vivo* RPE mouse cells have shown that RGR inhibits lecithin:retinol acyltransferase and all-*trans*-retinyl ester hydrolase *in the dark,* and that this inhibition is released upon exposure to light (Radu et al., 2008). The study further suggests that RGR mediates light-dependent translocation of all-*trans*-retinyl esters from a storage pool in lipid droplets to an "isomerase pool" in membranes of the endoplasmic reticulum, and that such a translocation permits insoluble all-*trans*-retinyl esters to be utilized as substrate for the synthesis of a new visual chromophore (Radu et al., 2008). Whether this happens in extra ocular tissues has not yet been proven.

Of the opsins for which we know the absorption spectra, it is clear that most absorb in the UV to blue/green range (360-480nm) of the spectrum (Figure 3.1). This makes sense as the shorter wavelength blue light penetrates deeper into the turbid water in which zebrafish are

found in the wild. Although most opsins are sensitive in the same wavelength region, the different families of opsins do however use a range of signalling pathways through different G-protein coupling. Opn5, opn3 and the TMTs are thought to be Gi/o coupled opsins (Terakita et al., 2014, Yamashita et al., 2010). Go/i signal transduction may use many different effectors and pathways depending on the tissue and cell type. This ranges from inhibiting adenylyl cyclase to decrease cAMP, through to PI3-K (Phosphoinositide-3 pathway), B-Raf, phospholipase C pathway (PLC) and IP3 or DAG-kinase, CaMk Ca^{2+}/calmodulin-dependent protein kinase, Rho, and PI4-kinase, and not to mention their direct impact on Na+, Ca2+ and K+ ion conductances (Jiang & Bayapajee 2009). In other words, there are w wide range of potential signalling events. The novopsins (Opn6-9) are also predicted to be Go/i coupled (Pérez et al., 2019, Beaudry et al., 2017). The melanopsins are thought to be a Gq coupled opsins (Melyan et al., 2005, Panda et al., 2005, Pierson et al., 2007, Terakita et al., 2008, Qiu et al., 2005). Just like Go/i signalling, Gq is implicated in many different signalling pathways, some which overlap with Go/i signalling, such as PLC and Rho. Other Gq pathways effectors are PKC (protein kinase C), which increases the concentration of diacylglycerol (DAG) or calcium ions (Ca^{2+}), as well as several effectors feeding into MAPK (mitogen-activated protein kinase**)** pathway (Dorsam et al., 2007, Harden et al., 2011, Mizuno and Itoh 2009). The non-visual OPN1 genes (Va1/2, parietopsins and parapinopsins) are thought to use G protein-coupled cyclic nucleotide signalling (Baylor et al., 1979, Leskov et al., 2000, Pérez et al., 2019). The peropsin and RGRs are the only opsins that are not thought to signal through a classical G-protein pathway, but rather work as photoisomerases, although this has not yet been explored in fish (Terakita and Nagata 2014).

Very little work has been done in zebrafish on light dependent signalling through specific opsins, and the data on opsin GPCR signalling is from a range of models, from spiders to humans. There have, however, been several reports in zebrafish implicating the MAPK pathway with light-dependent, transient induction of phosphorylated ERK and MEK (Cermakian et al., 2002, Hirayama et al., 2009, Hirayama et al., 2007, Mracek et al., 2013). Furthermore, pharmacological assays have also pointed to signalling through the phosphoinositide pathway, which interacts with nitric oxide (NO) and the MAPK pathway (Ramos et al., 2014). The diversity in opsin signalling adds another layer of complexity to non-visual light induced signalling, and opens up another theory on why zebrafish have evolved and express so many different opsins; could it be that different combinations of

opsins in different tissues, allows the same wavelength of light to be turned into tissue specific cellular signalling?



Accession	Name	Eye	Liver	Gut	Fin	Heart	Skin	Gill	Muscle	Pineal	Pituetary	Brain	Testis	Cells	Absorption Max	Animal	Reference
KT008391	Exorhodopsin														500 nm	Zf	Morrow et al., 2017
KT008402	Valopa														451 nm	Salmon	Soni et al., 1998
KT008403	Valopb														500 nm	Zf	Kojima et al., 2000
KT008404	parapinopsin-1														360 nm	Zf	Koyanagi et al., 2015
KT008405	parapinopsin-2														480 nm	Zf	Koyanagi et al., 2015
KT008406	parietopsin														525 nm	Zf	Sakai et al., 2012
KT008407	TMT1a														460 nm	Zf	Sakai et al., 2015
KT008408	TMT1b																
KT008409	TMT2a																
KT008410	TMT2b														470 nm	Medaka	Sakai et al., 2015
KT008411	TMT3a																
KT008412	TMT3b																
KT008413	OPN3														470 nm	Zf	Sugihara et al., 2016
KT008414	OPN5														360 and 474 nm	Zf	Yamashita et al., 2010
KT008415	OPN6a														510 nm	Zf	Davies et al., 2015
KT008416	OPN6b																
KT008417	OPN7a																
KT008418	OPN7b																
KT008419	OPN7c																
KT008420	OPN7d														369 & 508 nm	Zf	Davies et al., 2015
KT008421	OPN8a																
KT008422	OPN8b																
KT008423	OPN8c														375 nm	Zf	Davies et al., 2015
KT008424	OPN9														360 & 462 nm	Zf	Sato et al., 2016
KT008425	Peropsin (RRH)														540 nm	Spider	Nagata et al., 2018
KT008426	RGR1														370 & 469 nm	Human	Shen et al., 1994
KT008427	RGR2																
KT008428	OPN4m1																
KT008429	OPN4m2														484 nm	Zf	Davies et al., 2011
KT008430	OPN4m3																
KT008431	OPN4x1														470 nm	Zf	Davies et al., 2011
KT008432	OPN4x2																

Figure 3.1 Collated key info on zebrafish opsins Heatmap indicate expression of opsins present in different zebrafish organs, where the darker the colour, the more highly expressed. (Davies et al., 2015), while light orange indicates presence/no presence in PAC2 cell lines (Frøland Steindal and Whitmore 2020). Absorption max are listed and referenced, and if no data for zebrafish (zf) is present, the closest related animal is listed instead.

3.1.3 Distribution and light-sensitivity of non-visual opsins

With such a large diversity of opsins, identifying key candidates in the fish for photoentrainment of the clock, or how these photopigments work synergistically together, is now more complicated than ever. Absorption spectra have been performed on many of these

zebrafish opsins. Most are monophasic, but seemingly with somewhat broad absorption peaks, with most opsins absorbing in the blue-green, while some absorb up in the red end of the spectrum (Su et al., 2006; Davies et al., 2011, 2015; Koyanagi et al., 2015; Morrow et al., 2016; Sato et al., 2016, 2018; Sugihara et al., 2016; Steindal and Whitmore 2019). Thus, the zebrafish has the theoretical capacity to detect light ranging from UV to red and even into IR.

To further complicate the matter, each organ expresses different combinations of opsins (Figure 3.1). It is also worth mentioning that the visual, image forming opsins, rods and cones, which are not summarised here, are found highly expressed in the eyes, but also at low levels throughout zebrafish tissues (Davies et al., 2015). Each tissue expresses at least one of the 6 rhodopsins, as well as the short-wave sensitive cone opsin 1 (sws1) and low wave sensitive cone opsin 1 (lws1). This raises the question, do zebrafish show such a diversity in opsins in order to be able to capture all photons of any wavelength, such that the system is simply designed to detect the presence or absence of light, regardless of wavelength? Or does this different opsin expression pattern mean that particular organs have specific wavelength sensitivities and therefore differing responses to the environmental light signal?

Non-visual opsins are found expressed in all zebrafish tissues explored to date (Figure 3.1). The internal organs, such as the heart and liver, possess the least, while the brain and retina express almost the "full set". Most organs do however, express one or more opsins from each family, with ExoRhd and Opn7a being the only variants found ubiquitously expressed in all tissues (Figure 3.1). In contrast, the opsin that seems to be the most restrictive in its expression, is parietopsin which is only highly expressed in the pineal (and small amounts in testis and fin). The exact function of all of these non-visual opsins remains largely unexplored, although there are some data showing knocking out single opsins such as tmt-opsin and melanopsin affect light mediated behaviours such as phototaxis (Fernandes et al., 2012; Fontinha et al., 2021; Horstick et al., 2017).

Most of our knowledge of non-visual opsins come from work on mice, but as mammals have such a reduced number of opsins compared to zebrafish, it is not clear if the opsins have similar roles in the different vertebrates. Melanopsin (OPN4m), is the most explored non-visual opsin to date and has been implicated in circadian clock entrainment, sleep, pupillary

constriction and several light driven behaviours such as negative masking of locomotion and photic aversion (Altimus et al., 2008, Freedman et al., 1999, Hatori and Panda 2010, Lucas et al., 2003, Lupi et al., 2008, Mrosovsky and Hattar 2003, Rollag et al., 2003, Semo et al., 2010). Alternative splicing of melanopsins have also been found to invoke different behavioural responses to light in the mouse eye (Jagannath et al., 2015). If this is also the case for teleost opsins, we are yet again into an expanding number of possible specialised roles for the opsins.

Neuropsin (OPN5) has also gained some recent interest, and initially was shown to play a role in photoentrainment in mouse retina (Buhr et al., 2015). OPN5 is UV sensitive and found expressed in extra ocular tissues such as skin and ears in humans and mice (Kojima et al., 2011). Recently it was also found that OPN5 give mammalian melanocytes direct light sensitivity like zebrafish, and that they can entrain in culture to a LD cycle and phase shift in response to short wavelength light (Buhr et al., 2019). The same study also found that $Opn4^{-/-}$; $Pde6b^{rd1/rd1}$ mutant mice that cannot behaviourally entrain to LD cycles, still have a synchronised clock in the skin. There are also some emerging results from rather unexpected tissues, including human adipocytes, suggesting a wide range of light-regulated biology may even exist in humans, indicating that peripheral light sensitivity is not restricted to non-mammalian vertebrates (Ondrusova et al., 2017).

In many respects, it is hard to understand the requirement for such a large number of photopigments, as one would imagine that the role of just detecting light could be performed adequately by far fewer. However, this diversity would certainly ensure a wide range of spectral sensitivity and that "no photon goes undetected". Presumably there must be some biological value to this. In reality, most of our functional knowledge comes from mouse studies, yet there is still relatively little examination of the role of these opsins in tissues other than the retina and brain.

In this chapter, we start to examine what different monochromatic wavelengths of light impact of clock gene expression. We show that the light response goes well beyond the visual wavelengths, with both UV and infrared (IR) light pulses having the ability to induce clock gene expression, but interestingly with IR not able to set phase shift the molecular clock, at least in cell lines. I also present some pilot data that explores if opsin diversity in tissues leads

to an organ specific response to light in terms of DNA repair and clock genes. We show that zebrafish cell lines, rather like the adult tissues (Davies et al., 2015), display a diversity of expressed opsins, a number of which are under clock-control and as such show robust daily rhythms in expression. Whether this transcriptional rhythm in specific opsins translates into matching protein changes is yet to be determined, but it opens up the possibility of a direct temporal regulation of light sensitivity, as well as the more conventional spatial aspects. In this regard, the clock is likely to be gating the process of its own entrainment by regulating expression of components of the light-input pathway; with a specific pathway acting as a zeitnehmer or "time taker" (McWatters et al., 2000). Part of this chapter is published as a paper titled "*Zebrafish Circadian Clock Entrainment and the Importance of Broad Spectral Light Sensitivity"* (Frøland Steindal and Whitmore 2020).

3.2 Results

3.2.1 Opsin expression in cell culture

As well as having directly light sensitive organs, zebrafish cell lines, typically generated from early stage larvae, such as the PAC2 cell line, are also directly light responsive (Whitmore et al., 2000). However, the photopigment content of these cells has never previously been explored. To examine which opsins are expressed in the cells, both PAC2 cells, and transformed cells expressing a *clock*-dominant negative construct (*clock*DN cells) were kept on a 12:12 light dark cycle at constant temperature, and cells were harvested at ZT3 and ZT15. Both cell lines express opsins from all classes of non-visual opsins, with a total of 11 out of 32 non-visual opsins expressed at a detectable level (C_t lower than 30) (Figure 3.2). There is no apparent difference between PAC2 and the *clockDN* lines in opsin expression pattern. By comparing the expression pattern at two different times of day, we also observed that half of the opsins show a day-night difference in expression pattern in PAC2, but not *clockDN* cells, which shows that some opsin expression is clock controlled (Figure 3.2). Interestingly, two forms of OPN4 are expressed in these zebrafish cell lines and one, OPN4X2, shows a strong day-night difference in expression. This is also the case for exo-rhodopsin, which is typically considered to be a pineal specific photopigment. RGR1, a

putative photoisomerase, also shows robust daily changes, and is the most abundant transcript.

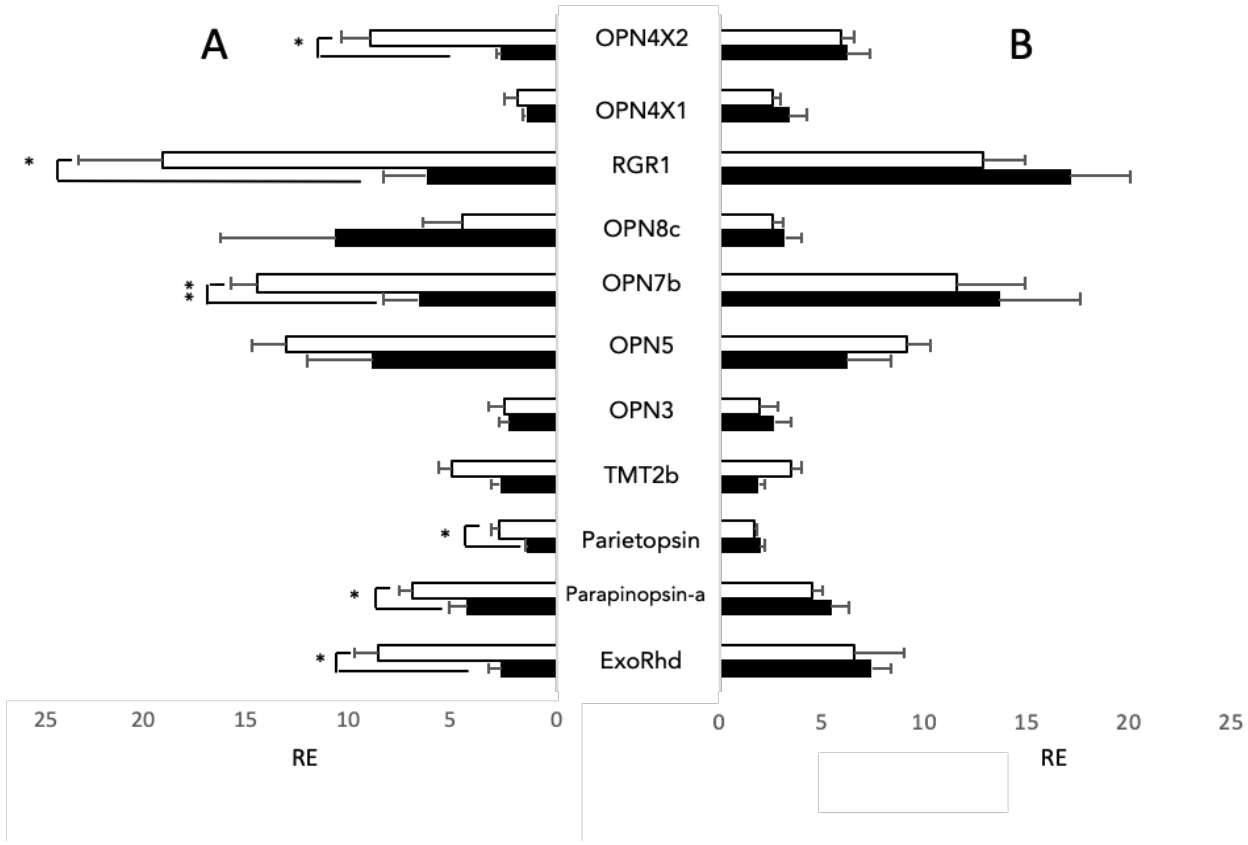


Figure 3.2 Non-visual opsins expressed in zebrafish cell lines

All 32 non-visual opsins were explored by RT-qPCR in entrained zebrafish cell lines at opposite times of day; ZT3 (white) and ZT15 (black). 11 opsins showed detectable expression levels, using a cut-off value of $< C_q$ 30. A) Opsins expressed in PAC2 cell lines. B) Opsins expressed in *clockDN* cell lines. Opsin expression is plotted relative to the lowest detectable opsin, with error bars depicting SEM. An unpaired students t-test was used to assess if the opsins expressed show a time of day specific expression pattern. $p < 0.05$ is marked with * and $p < 0.001$ with ** (n=4).

3.2.2 Impact of light on clock genes in cells

To explore how monochromatic light of selected wavelengths impacts gene expression in zebrafish cell lines, *ClockDN* and *PAC2* cells were entrained, like the organs, on a 12:12 LD cycle at constant temperature and given a monochromatic light pulse for 3-hours at ZT21, when cells are most light responsive (Tamai et al., 2005). Using RT-qPCR, we examined the effect of these light pulses on different, well-established light responsive clock genes, such as *cryptochrome1a (cry1a)* and *period2 (per2),* as well as the light induced DNA repair gene, *6-4 Photolyase* (*6-4 Ph*) (Tamai et al., 2007; Vatine et al., 2009). In *PAC2* cells, white, blue and UV light pulses of the same intensity give very similar induction in all genes explored, whilst red generates a slightly smaller, yet not statistically different induction (Figure 3.3a-c). IR pulses give the smallest induction of the genes explored. For *cry1a* we see a significant 1.6-fold induction, as opposed to ~4-fold induction by the other wave lengths (Figure 3.3a). For *per2* IR gives a ~5-fold induction, as opposed to 20-30-fold by the other wavelengths (Figure 3.3b). Finally, IR gives a 2.6-fold induction as opposed to up to 13-fold induction, by the other wavelengths (Figure 3.3c). IR does indeed induce significant induction of the light sensitive clock and DNA repair genes. However, compared to the other wavelengths, it is between 2.5- 6 times less potent, depending on the gene in question.

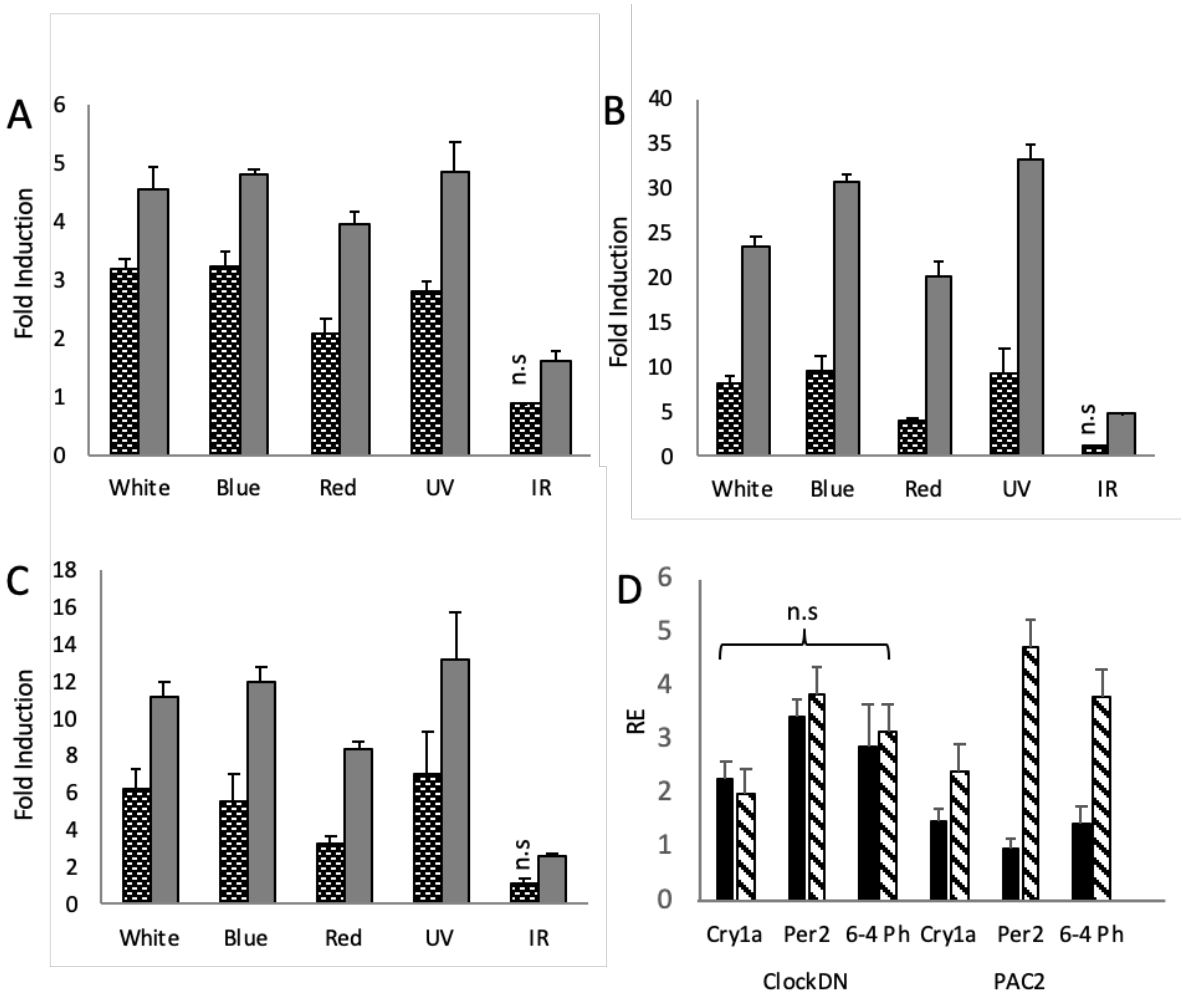


Figure 3.3 Light induction by monochromatic light-pulses in PAC2 and *clockDN* cell lines Zebrafish cell lines were maintained on a 12:12 light-dark cycle before being given a three-hour light pulse of varying wavelengths. *B*lack and white bars represent *clockDN* cell line expression, whilst solid grey represent PAC2 cell line expression. A) Light induction of *cry1a* . B) Light induction of *per2*. C) Light induction of 6-4 Photolyase. A-C is plotted as fold induction relative to dark control. D) Dark controls (black bars) vs IR monochromatic light pulse (striped bars) plotted relative to lowest expressed gene (PAC2 *per2* DD) in *clockDN* and PAC2 cells. Significance was addressed with an one-way ANOVA (alpha=0.05) for each light-pulse, cell line and gene, followed by a Bonferroni post-test. All light pulses give a significant increase of $p<0.05$ unless marked on the graph (n=3).

ClockDN cells show a reduced fold induction to all the wavelengths (Figure 3.3a-c). The raw C_t values seen in *clockDN* and PAC2 cells are however, very similar when given a light-pulse. These clock mutant cells show a higher basal DD expression of the clock and DNA repair genes, and thus the fold induction is subsequently lower (Supplementary Figure 3.1). This is particularly evident when giving an IR light pulse (Figure 3.3d).

3.2.3 Phase shift in cell culture

To explore how the monochromatic light phase shifts the molecular clock in cell culture, *per1-luciferase* luminescent reporter cells (Vallone et al., 2004) were entrained for 3 days at 28°C and light pulsed the same way as the cells described above (Figure 3.4a**)**. *Per1* luminescent traces were then monitored for 2 days post light pulse in DD using a Packard TopCount luminometer. UV, blue, red and white light are all capable of causing a phase advance in the cell culture clocks when light is applied at this particular time in the cycle (ZT21) (Figure 3.4b). Interestingly, a 3-hour light exposure of IR light does not give a phase shift regardless of the acute molecular response to this light signal, increasing both *cry1a* and *per2* expression, a result which is worthy of further discussion.

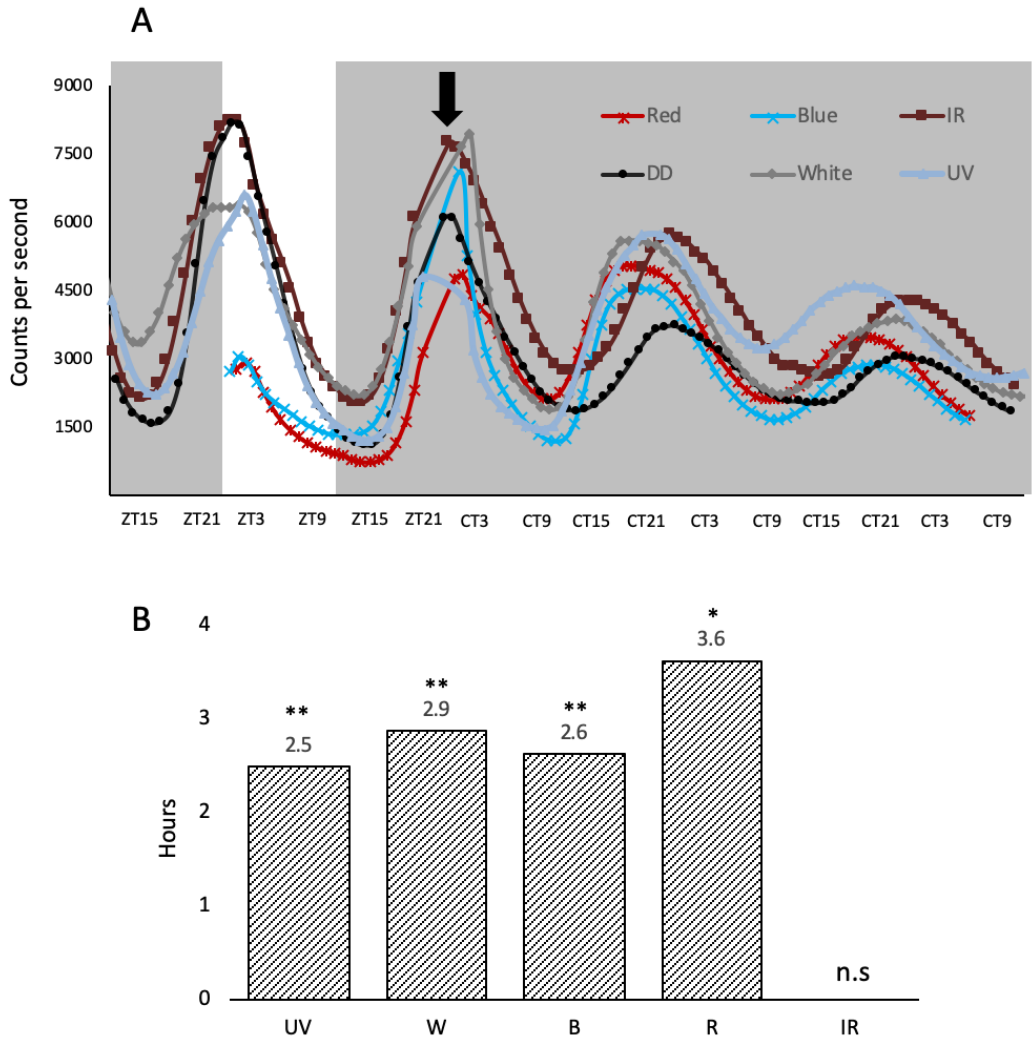


Figure 3.4 Light-induced phase shifts in response to monochromatic light-pulses in *per1-luciferase* zebrafish cells A) Cells were entrained for 3 days on a 12:12 LD cycle, before they were given a variety of monochromatic light pulses at ZT21, denoted by black arrow. The cells were kept in constant dark over two subsequent days. B) Light pulses cause a phase advance in hours, determined at the half maximum between peak and trough vs no LP controls. A two-way ANOVA (time, wavelength) was used to determine significant variation between samples. Amplitude and baseline were detrended using BioDare2 (Zielinski et al., 2004), and a Tukey post-test was used to determine significance in phase shift relative to DD control. * denotes $p < 0.01$ and ** denotes $p < 0.001$ (n=8).

3.2.4 The spectral response of adult tissues to monochromatic light

All zebrafish tissues that have been examined to date (including internal organs such as heart, gut and liver), express several of the non-visual opsins (Davies et al., 2015). As each tissue has different expression patterns, does this consequently mean that different organs have different spectral sensitivities? To examine this, we dissected a variety of adult tissues and entrained them on a LD cycle in culture conditions, before subjecting the organs to a 3-h monochromatic light pulses of UV, blue, red and IR, as well as full spectrum 400-700nm white light at ZT21. Tissues were subsequently harvested and RNA was extracted. Using RT-qPCR, we examined the effect of these light pulses on different, *cry1a, per2* and *6-4 Photolyase.*

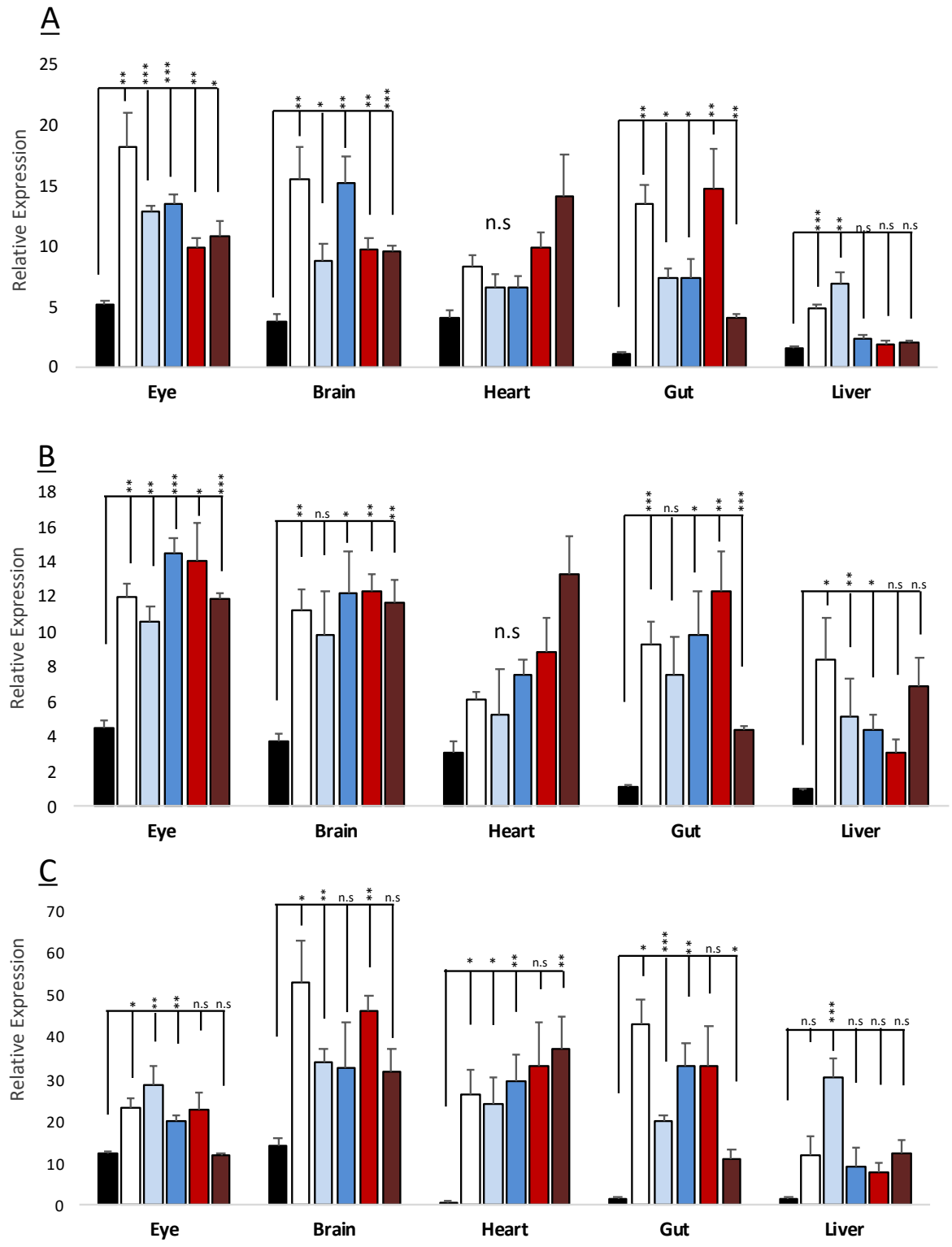


Figure 3.5 Monochromatic induction of light sensitive genes in 5 different organs

Zebrafish tissues were dissected and maintained in culture on a light-dark cycle before being given a 3-hour light pulse at ZT21. Black bar: DD Control white light (400-700nm): white bars, UV light (350nm): light blue bars, blue (450nm): dark blue bars, red (650nm): red bars

and IR light (850nm): dark red bars. The gene expression of three target genes was examined: A) *Cry1a* B) *Per2* C) *6-4 photolyase*. Data is normalised to housekeeping gene EF1a and plotted relative to the lowest expressed DD sample of all tissues. Significance was addressed with an ANOVA (alpha=0.05) for each organ and each gene, followed by a Bonferroni post-test with Holm correction. Significance is marked with asterix between DD and light pulse as $p < 0.05$ *, $p < 0.01$ ** and $p< 0.001$ *** (n=3).

Eyes and brain are the most opsin-dense tissues in any vertebrate including fish and most wavelengths induce a significant increase of *cry1a* and *per2* as well as a *6-4 Phr*, across all wavelengths **(**Figure 3.5). The internal organs show a somewhat more diverse response. We fail to see a statistically significant response to any of the monochromatic light-pulses in the heart for the two clock genes, but it is worth noting that the heart has a high basal expression in DD, thus more replicates could probably yield significance for some of the wavelengths. We do however know that light impacts the hearts clock as, we have recently showed that the zebrafish heartbeat frequency in culture is clock regulated (Fong et al., 2021), which is in line with previous findings in mice (Thomson et al., 2008). White, blue and IR give a significant response in *6-4 Phr* in zebrafish heart. The gut seemed to be slightly red shifted in induction of clock genes, but with low response to IR (Figure 3.5 a,b) with white and blue light creating the strongest expression of *6-4 Phr* (Figure 1.5c). The clock genes show a significant response to white and UV light in the liver, but only UV gives a significant increase in *6-4 Phr* transcript (Figure 3.5). An interesting difference between the organs in the observed amount of basal/DD transcript. The eye and brain show high basal expression in all three genes explored, while the heart shows high basal transcription of *cry1a* and *per2*, but low for 6-*4 Phr*. Gut and liver show a general very low basal expression across all three genes.

3.3 Discussion

3.3.1 Cells show a diversity in opsin expression patterns

Zebrafish cell cultures have long been used for clock studies because of their direct light sensitivity. However, the opsin composition of these cells lines has never previously been explored or published. We, therefore, performed RT-qPCR on PAC2 cell lines to explore what opsins are expressed. With RT-qPCR and setting a cut off value at C_q 30 as a measure of "no expression", we can identify the presence of 11 out of 32 non-visual opsins (Figure 3.2a). Interestingly, 6 of these opsins show a clear day-night difference in expression and appear to oscillate. All of these opsins show higher levels of expression during the day time-point compared to night. To explore this further, we therefore also examined expression in the *ClockDN* cell line, lacking a functional circadian clock, to manifest whether this difference is light-driven or clock-dependent. Interestingly, the *ClockDN* cells express the same specific opsins exactly, but they no longer oscillate (Figure 3.2b**)**, which supports the idea that expression of these opsins is directly clock controlled and not directly light-driven. Furthermore, averaging expression of ZT3 and ZT15, there is no significant difference in the amount of transcript produced in the two different cell lines. This is interesting as it supports the idea that basal expression is not dependent on a functional clock, which is seen for numerous other genes. The opsin expression profile in cells does not resemble any particular tissue type that we know of today. However, it is worth noting that the cell line express one opsin from all the opsin families, like most tissue types, and thus possess G_q, G_t, G_i and G_o coupled opsins, as well as a putative photoisomerase (RGR1). The cell line should, therefore, be able to signal through the same pathways in response to light as any other fish tissue.

3.3.2 Monochromatic light (350-650nm) pulses are potent inducers of light responsive genes in cell culture

Expression of the light responsive clock genes to controlled light pulses of various wavelengths of light in cell culture is rather flat with a broad response to white, blue and UV light. There is a slight but statistically significant drop in the response to red light in the cells, and a marked drop in the response to IR (Figure 3.3).

6-4 photolyase catalyses the photo-reversal of the (6-4) dipyrimidine photoproducts induced in DNA by ultraviolet light (Zhao et al., 1997). A simple prediction might be that UV/blue light should be more efficient at inducing expression of this DNA repair enzyme. However, this does not appear to be the case, with red light and even IR light able to increase transcript levels. Red light photons have lower energy than blue light photons, therefore, the same intensity of blue and red light will have different number of emitted photons. Consequently, one hypothesis is that the opsins simply 'count' photons, not the energy of the photons they absorb, meaning that the zebrafish cell simply wants to know whether there is light present or not. To address such issues, these experiments will need to be repeated considering aspects of photon flux over a wider range of light intensities. Of course, it may be biologically essential to activate expression of your DNA repair machinery in the presence of light, regardless of the subtleties of the specific wavelength, and of course the 6-4 photolyase protein itself absorbs light to perform its role in replacing cross-linked nucleotides. It is this aspect of light driven DNA repair that is most likely to be wavelength sensitive.

Comparing cells without a functional clock to "wildtype" cells, we also see that the fold induction of genes in response to light is lower, due to a higher basal transcription of these target genes in DD. *ClockDN* cells show a higher basal DD expression of the clock and DNA repair genes, and the fold induction is subsequently lower (Figure 3.3a-c). This is interesting, as it demonstrates the steady state expression levels that these genes reach in a non-rhythmic mutant background. The absolute expression remains the same (Supplementary Figure 3.1). Interestingly, the high basal level of transcript means that there is no induction of light responsive clock genes in the *clockDN* cells in response to IR.

3.3.3 UV- Red light can alter gene expression and phase-shift cell lines

The impact of "visible" wavelengths of light (380-740nm) on the zebrafish clock system has been described in numerous previous studies. However, exploring this phenomenon outside of the visual spectrum are rarely performed in fish. UV light of 350nm (UVA) has a clear impact on gene expression and can clearly phase shift the circadian clock in cell lines. Perhaps this is not so surprising from what we now know about zebrafish photobiology. After all, 350nm is only 50nm below the violet/blue wavelengths that can so robustly impact the clock in an aquatic organism. In future, it would be interesting to try wavelengths at the more extreme end of the UVA range and well away from the visual spectrum. This UV response

also fits well with the previously determined absorption spectra for purified opsin proteins, which reveals a wide sensitivity in the UV/blue wavelengths (Davies et al., 2015)

The impact of these monochromatic light pulses was explored using our luminescent reporter cell lines. At the phase (ZT21) and intensity used, each wavelength generated a very similar phase advance in the rhythm, including UV light pulses, but not IR at 850nm (Figure 3.4a). This similarity in size of phase advance correlates well with the similarity in molecular response, induction of *cry1a* and *per2*, seen in the cell lines (Figure 3.3). Furthermore, using a Tukey post-test, there is statistical difference in the size of phase shift generated by each of these light pulses (except between blue and UV) (Figure 3.4b**)**. Since this difference in shift is so small, it may be due to the sampling frequency (plate counted once an hour) rather than real difference, thus we do not speculate any further.

Compared to previous studies on phase shifting in zebrafish cell lines, in response to white light, the size of the phase shift is relatively small and is actually a phase advance rather than a large phase delay previously reported (Tamai et al., 2007). The reasons for this simply relate to the differences in light intensity used. Early studies applied light at 5000µW/cm^2, compared to the 200µW/cm$_2$ used in this study. Consequently, the Type 0 PRC previously reported switches to a more "standard" Type 1 PRC as the lower light intensity, as historically seen in many previous studies. Interestingly the switch in PRC amplitude, therefore, occurs between these two intensities, and strongly suggests that fish under natural conditions, as a diurnal animal, will be "working with" a Type 0 PRC. This kind of issue stresses the value of actually performing such experiments under natural, wild conditions where clock entrainment and even clock function itself may be very different to that seen in the lab.

3.3.4 External and internal organs respond differently to monochromatic light

We have known for over 20 years that the zebrafish tissues and cells are directly light sensitive and that their clock can be set without any input from the eyes or brain (Whitmore et al., 2000; Carr and Whitmore, 2005). Furthermore, we also know that different organs have different opsin expression patterns (Davies et al., 2015), though it was not previously known if this changes their responsiveness to light. Trying to tease this mystery apart, we dissected and subjected several zebrafish organs to monochromatic light-pulses. In addition to

exploring the impact of monochromatic light of the visual spectrum, we also wanted to explore the effects of light *outside* the visual spectrum, UV and IR. Some of the opsins have peak absorption in the ultraviolet light range. None of the non-visual opsins we know have peak absorption of light in the red or infrared range of the spectrum. However visual opsins, such as lws1 and lws2, which are found in all zebrafish tissues, have absorption spectra with peaks around 550 nm that tail off at 700nm (Chinen et al., 2003).

Across the light-inducible genes, brain and eye show a broad response to all wavelengths, which is to be expected as most opsins are expressed in these two organs. Heart and gut also show a broad response, yet show a trend to being longer wave-length shifted (Figure 3.5). To put it crudely; the inside of a fish is a relatively "red environment" due to the greater penetrance through tissue of long wavelength light, thus the subsequent discovery that these internal organs are red shifted might not be a surprise. The liver stands out as the organ that looks the most different from all others (Figure 3.5). The normalized amount of light-induced clock gene transcript is lower than the other organs, and the data so far would indicate that it is actually slightly blue-UV shifted rather than red-IR. These differences in the liver could possibly be explained by the idea that food rather than light is the dominant entraining signal. Such an idea, of course, fits well with mammalian ideas of clock organization and may in fact also be true in the teleost community, though this still remains pure speculation at this time.

These differences in organ wavelength sensitivity certainly make sense in the context of the different opsin expression profiles possessed by each tissue. Also, this tendency to "red-shifted" light responsiveness of deeper internal organs also fits with a simplistic logic. It is very tempting to speculate that these different light responses somehow lead to subtle adjustments of relative circadian phase between clocks in different tissues *in vivo* in the fish body. Data published so far, has argued that zebrafish organ clocks all reside at the same circadian phase on a light-dark cycle. In reality these experiments have never been performed with sufficient temporal resolution to actually detect possible, more subtle phase differences. Answering this question will require significant advances in *in vivo* imaging where clock rhythms can be followed in individual tissues in living animals. Such studies are infinitely possible using zebrafish larvae and optimized reporter gene constructs. The role of non-visual light detection goes beyond just setting the circadian clock. It is equally likely that specific tissues are using light, possibly of differing wavelengths, to control many other aspects of their cell biology, as shown here by the examination of DNA repair gene expression.

Stringent statistical tests show no significance in the internal organs, yet there are large individual variation and low sample size, thus trends themselves are interesting. A detailed comparison of tissue transcriptomes, following monochromatic light exposure, would go a long way to answering such questions.

Another reason why we see a high response to red or IR light could be down to the fact that we do not account for photon-flux. Spectral irradiance takes the photon flux into account and is a measure of watts per square metre per wavelength of light ($W \cdot m^{-2} \cdot nm^{-1}$). Light in the blue end of the spectrum has more energy per photon than the red end of the spectrum. A red monochromatic light pulse will actually have 1.4 times the photons per uW than a blue light pulse, so although the intensity is the same, the number of photons the tissues are bombarded with are less. If we compare either end of the spectrum we used for this experiment, UV (350nm vs IR 850 nm), IR will yield about 2.4 times the photons per uW compared to UV (Table 3.1). While most circadian experiments control for intensity, spectral irradiance is not generally considered, and I have not been able to find any literature studying the effect of intensity vs spectral irradiance. Provided that we do not actually know the amount of red sensitive vs blue sensitive opsins that are present in the different tissues, let alone which membranes they are embedded. If all the bi-stable opsins use similar wavelengths to convert back to 11-cis, it may mean that the higher the photon-flux, the more the signalling through bistable pigments, although this is just speculation on my part and needs further discussion.

Colour	**Wavelength** nm	**Photon flux** photons / s · cm^2
UV	350	3.52388e+14
Blue	450	4.53071e+14
Red	650	6.54435e+14
IR	850	8.55800e+14

Table 3.1 Photon-flux corresponding to experimental wave-lengths
Photon flux per cm^2 is calculated for each wave-length using Planck's equation (Calctool.org, 2021)

Furthermore, it may also be important to consider the penetrance of the tissues when trying to understand how the different wavelengths impact the different tissues. Due to the relationship between scattering, reflection and absorption in tissues, short wave visible light has a shallow penetration whilst the longer wavelengths are mostly scattered rather than absorbed, which means that it can penetrate further. A study looking at penetration of tissues found that IR light (850nm) would penetrate 4 times as far into the tissue as green-blue at 500nm (Douplik

et al., 2013). This is exploited in low-level light therapies, where a 500mW laser set to 1060 nm wavelength can penetrate as far as 10 cm into soft tissue. IR and red light would penetrate furthest into the organs, and taken together with the fact that there are twice the number of photons than that of blue light, it may account for the strong gene induction we see in many of the organs, but only see a moderate response in cells. An explanation like this does not contradict the high gene induction we generally also see to blue and UV light as these experiments are performed in a culture dish, meaning that light does not need to penetrate through skin and subcutaneous tissues to reach the internal organs.

That said, our data shows no clear statistical difference in response for the majority of wavelengths used. In part, this could be due to the variation between the three biological replicates that was used and a greater sample size may be required to detect subtle differences. Whilst preforming the experiments I identified a potential issue that might be the cause of the variation between biological replicates: The monochromatic light sources we used have a small area where the intensity is at the intended 200 $\mu W/cm^2$. There is however a sharp drop off to just half the intensity when just moving a few cm out from the centre of the light source. Over 15 organs were light pulsed simultaneously in a sectoral divided petri dish, so not all organs could be at the centre of the beam, which means that the organs the furthest away from the centre might have received a lower intensity light-pulse, thus accounting for some of the variation in expression between the biological replicates. It is clear that if the experiment were to be repeated, we need to improve the quality of the equipment used, benefiting from multiple monochromatic light sources and use of reflectors, allowing a larger area to maintain the same intensity. Improved monochromatic LED technology would also help. With the strict rules on animal use, repeating such an experiment that requires a minimum of 24 fish for n=4 or 35 fish for n=5 is not straight forward.

One of the more surprising findings from these data is that there is a difference in basal transcript levels between the organs. Interestingly, the most light-sensitive tissues show a much higher basal transcription in DD compared to liver and gut (Figure 3.5). This in effect means that the response in terms of fold-difference in gene expression is generally bigger in the internal organs than the eye and brain of the same light intensity. This can be exemplified with *per2* for example, where a white light pulse yields an approximate 3-fold difference in expression in eye and brain, but a whopping 9–fold difference in gut. In a way, this makes sense as the intensity of light reaching an internal organ would be much lower than the

intensity of light hitting the eye or brain of the fish. What the underlying mechanism is that allows for the difference in basal transcription is unclear, but if it is indeed true, it would ensure that lower intensities of light can maintain a robust, light-driven clock rhythm in the internal organs.

3.3.5 Infrared light has a considerable effect on clock gene expression in both tissues and cells

The response of light inducible genes to infrared light was not expected. As a stimulus, it is generally avoided in clock studies, due to the strong link with temperature effects/artefacts and the ability of temperature pulses to phase shift the circadian clock (Lahiri et al., 2005). It is a stimulus typically one aims to control against in circadian analysis. Yet the response to IR when controlling for temperature, of the zebrafish clock system is very interesting. IR is used for monitoring behaviour in several video systems of fish and fruit flies (such as the Noldus set-up). Any concerns that IR impacts the fish clock was rejected when Dekens et al., showed that zebrafish larvae cannot entrain on a 12:12 IR-D cycle, and that IR has minimum impact on *per2* genes or other downstream clock targets (Dekens et al., 2017). The findings I present here do not necessarily contradict these findings as the experiments are different, as well as the biological sample (cells and tissues vs larvae), and we also used higher intensity IR light. In the 2017 paper studying the impact of IR, I would however like to point out that is seems zebrafish larvae were kept in water in Greiner culture bottles, and both water and plastic absorb IR, which would mean that the intensity of the IR reaching the embryos would be much lower than the intensity measured outside the culture bottles. In contrast, the experiments presented here, had a minimum amount of media needed (clear, not with phenol red) so cells/organs would not dry out and any plastic lid was removed prior to the experiment. As we demonstrated that IR can induce clock genes across multiple organs and in a separate cell culture scenario, we are confident with the finding that IR at 200 uW/cm^2 can induce a change in what we think of as light responsive genes. However, to our surprise it does not induce a phase shift in cells, a finding that is in line with the study by Dekens et al., 2017. We had plans to replicate the phase shifting experiment in organs, using transgenic *per3*-luciferase zebrafish. However, our luminometer only takes 96-well plates and some of the organs, such as the brain of the adult fish proved too large. We decided to raise a new generation of *per3-luciferase* fish and use the much smaller organs of the juvenile fish, but as the fish larvae were growing, the first London Covid lockdown started. The comparison

between organs and cells would be indeed be interesting as although IR impacts clock gene expression of cells in culture, it is around 6 times less "potent" compared to other wavelengths, which may be why we do not see a downstream phase shift in these studies **(**Figure 3.2). In most of the organs however, the tissues respond much more strongly to IR (Figure 3.5), which may or may not impact a subsequent phase shift. This issue requires further experimental investigation.

3.3.6 Does IR use mitochondrial signalling to induce clock genes?

It is not clear that there are any opsins or photoreceptors that can absorb at 850nm. What we know is that IR and red light are absorbed by cytochrome c and mitochondrial bound water, and the mitochondria subsequently could signal using a change in mitochondrial membrane potential, reactive oxygen species (ROS), Ca^{2+} and/or nitric oxide (Hamblin 2018, Karu 2008, Passarella 2014, Sommer 2019). Furthermore, we also know that there is a lot of cross talk between the clock and the mitochondria with new pathways and interactions being added on a regular basis (Reinke and Asher 2019). Could the mitochondria themselves could be playing a direct role in the light-responsiveness of these cells and tissues? There is no evidence of which I am aware of that supports this idea. It is however interesting to note that tissues such as heart, where 40% of the cell volume is made up of mitochondria, shows a strong response of *cry1a, per2* and *6-4 Phr* to IR (Figure 3.5)(Page and McCallister, 1973). Another possibility is that some of the opsins in zebrafish are also acting as thermal sensors, along with or independent of their light detecting role. The idea of opsins having a thermal-sensing role is not new and stems in part from work performed in *Drosophila* (Leung and Montell, 2017). Certainly, from a pure light sensing role it does seem rather "overkill" to possess 32 non-visual opsins and so it is very likely that some of these photopigments have alternate sensory or cell regulatory functions. This could include an interesting, if unproven thermal sensing role.

In this study, we have shown that the spectral sensitivity of zebrafish cell lines extends beyond the classically perceived "visual" wavelengths of light and that supporting this wide spectral sensitivity, these cells express a large number of opsins. Furthermore, the clock itself regulates the temporal expression of these opsins, raising the interesting possibility that the clock itself controls light input to the pacemaker – the zeitnehmer concept that has so eloquently been described for plant clock systems.

4 The Development of Light Responses and Circadian Clock in Mexican Blind Cavefish, *Astyanax mexicanus*

4.1 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 non-visual light-sensitive biology comes from studies in zebrafish which is discussed in some detail in the general introduction (Chapter 1). 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). 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., 2012; Tamai et al., 2005; Tamai et al., 2004,). Not only is light necessary for the protein function of DNA repair enzymes, such as the photolyases, but 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.

4.1.1 The Mexican Blind Cavefish

In this context, as light detection impacts so many aspects of fish biology, 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 Micos and El Abra in Mexico and in Guatemala. As a result, we can today find over 29 distinct populations of *A. mexicanus* in numerous isolated caves (Gross, 2012), and due to the nature of the limestone geology, there might be more unexplored subterranean caves with other populations, the newest discovered being the Chiquitita Cave in 2018 (Espinasa et al., 2018). 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; Gross et al., 2009; Jaggard et al., 2018; Jeffery, 2009; Protas et al., 2007; Protas et al., 2006, 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 populations 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 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., 2004; Strecker et al., 2003).

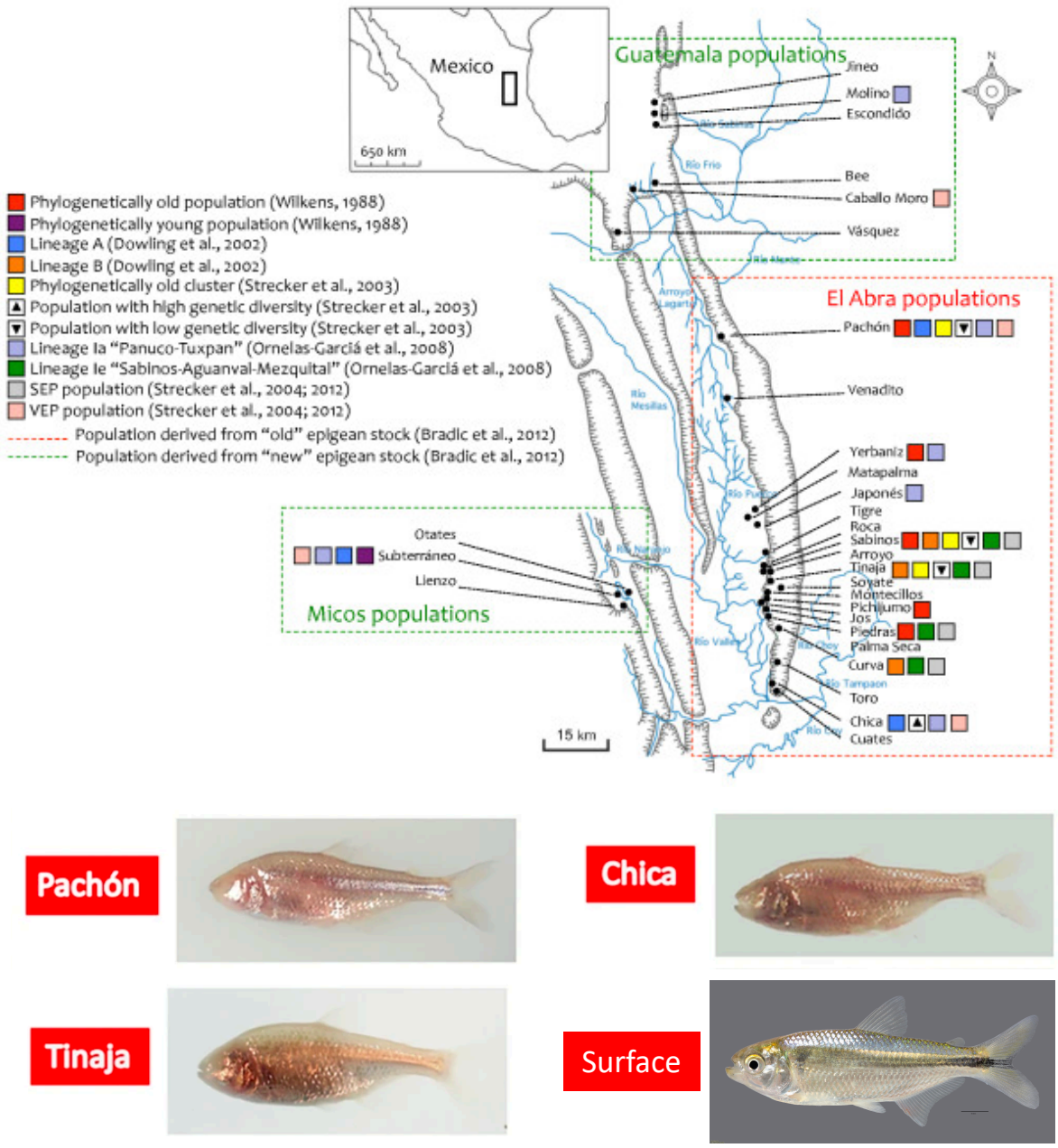


Figure 4.1 Map of the limestone cave systems with location and some key phylogeographic features Photos of 3 candidate cavefish and surface fish (Bradic et al., 2012) (Jeffrey, 2020)

4.1.2 Origins and features of candidate cavefish

The cave morphs described in this chapter are all originating from the El Abra limestone caves of north-eastern Mexico. The oldest estimation of surface fish invading the El Abra region is set to between ~8 million years ago, via an incipient land bridge, to ~3.1 million years ago post closure of the Panamanian-Colombian sea (Gross 2012). The most recent predicted colonisations of the caves are estimated to only 10 000 years ago, during a warming trend of Pleistocene inter-glacial events (Culver 1982; Barr 1965,1960). Newer whole/half-genome sequencing estimates that cave-surface population split only ~161,000- 191,000 generations ago (Herman et al., 2018). There are several easy to identify phenotypic differences between cavefish and surface fish. This ranges from behaviours such as feeding posture, lack of schooling and sleep-loss (Duboué et al., 2011, Schemmel 1980), to changes in organs such as heart, liver and brain (Moran et al., 2015, Xiong et al., 2018, Tang 2018). There are also a whole range of phenotypic differences between the different populations of cave fish which has led several groups to study the origins of the different cave populations since the 1940s. Several studies have tried to determine how old the different cavefish populations are, how many times the caves have been invaded by surface fish, and which cave populations are more closely related, using and quantifying phenotypic traits, mtDNA, micro satellite data and now more recently, short read sequenced genomes.

4.1.2.1 Pachón

Pachón is the northern most population in the El Abra region, and the most isolated from the other caves, it now sits high up from the river bed, making it an obvious perched pool which physically separates it from rivers in the vicinity. There are no regular bat roosts in the cave, but juveniles have been found to feed on micro-arthropods while adults feed on decomposing organic material (Espinansa et al., 2017). An analysis of three mitochondrial genes and 1 nuclear gene performed on the entire Astyanax genus in Mesoamerica, suggests that Pachón belongs to lineage 1a “Panuco-Tuxpan” which is one of the more ‘recent’ troglobitic forms (Ornelas-Garciá et al., 2008). Pachón is however also considered ‘phylogenetically old’ based on the amount/severity of troglomorphic traits (Wilkens et al., 1988), and of a ‘phylogenetically old cluster’ with low genetic diversity based on mtDNA analysis (Strecker et al., 2003). Another mtDNA analysis backs this finding, describing Pachón as belonging to “strongly eye- and pigment-reduced” (SEP) lineage, which is a from an “older” invasion of

the caves (Strecker et al., 2004, Strecker et al., 2012). Bradic et al., also treats Pachón as from an old epigean stock, but argues that the population was established independently of the other caves which they back up with geological findings paired with considerable degree of differentiation from the other populations (Bradic et al., 2012). A study basing lineage on analysis of NADH dehydrogenase 2 variance and number of rib-bearing thoracic vertebrae in their axial skeletons, place Pachón in "lineage A", which suggest that Pachón clusters with Chica and Subterráneo cavefish, but authors are not sure if this is an older or newer lineage (Dowling et al., 2002). In short, most of these studies agree on that Pachón is of an older lineage of fish, but as there are some variation in clustering, it might indicate that Pachón is indeed different to the other cave populations.

4.1.2.2 Chica

The Chica cave fish are different from the other caves in a couple of significant ways. The caves house a large, roosting and rhythmic bat population, which produce a large amount of bat droppings which in turn attracts insects, making it a rather biodiverse cave compared to Pachón. They have a high genetic variation (Strecker et al., 2003), frequent flooding which traps surface fish allows hybridisation. Several studies place the origins of Chica together with Pachón, belonging to the 'recent' "Panuco-Tuxpan" lineage (Ornelas-Garciá et al., 2008), the "Lineage A" (Dowling et al., 2002) as well as being a VEP - "variable eye- and pigment-reduced" (Strecker et al., 2004, Strecker et al., 2012), suggesting Chica originates from a younger stock of fish. Other studies suggest that Chica is in fact of an older stock, maybe even the first cave to be populated due to its geographical location, backed up by microsatellite data (Bradic et al., 2012).

4.1.2.3 Tinaja

The Tinaja cave sits geographically between Pachón and Chica, in an area close to many other separate caves. The cave is a typical roof collapse cave as opposed to the other caves, and it is thought to be the largest cave system in the region. The pools where the fish are located is some distance into the cave and requires some caving skills to get to the pools. The cave has some bats. There seem to be consensus that the Tinaja population belong to the more ancient invasion of the cave. Tinaja is placed in the phylogenetically old cluster with low genetic variation (Strecker et al., 2003) as well as the in the old lineage Ie, "Sabinos-

Aguanaval-Mezquital" (Ornelas-Garciá et al., 2008). This finding is supported by Tinaja belonging to the "strongly eye- and pigment-reduced populations" (SEP) population (Strecker et al., 2004, Strecker et al., 2012).

A more recent study using half the genome of several individuals from Tinaja, Pachón as well as Rascón, Molino and Rio Choy, support the theories that Pachón and Tinaja are both of the older invasions (Chica was not addressed in this study). The same study also hypothesises that the variations within cave populations are due to cave-cave hybridisation and subsequent geneflow *between* caves and cave/surface (Herman et al., 2018). The authors attribute the ability to mix between the caves to subterranean rivers, which can neither be confirmed nor rejected as we simply do not have the technology for such cave exploration. It should however be noted, and particularly in the case of Pachón, that the cave is elevated above a canyon, and that distance to the next known cave (Venadito cave) is considerable. If there are current intermixing between caves like Pachón, presumably these subterranean rivers can only be flooded in conjunction with heavy cyclones.

4.1.3 Cavefish as a model to study circadian adaptations to a dark environment

Today there are a series of distinct and independent cave populations, which may have experienced differing degrees of gene transfer over evolutionary time. As such, fish in the different caves have adapted to a similar environment, but more or less independently from each other in some caves and so represent a unique series of isolated populations in which to study the circadian clock. One would expect the fundamental aspects of light and clock biology to be very similar between surface populations and those described in zebrafish, if only because both live and have evolved in a rhythmic light-dark river environment in a tropical region. However, cave populations 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 populations 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 populations 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 populations. 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 appear 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 populations, perhaps the largest changes are seen in the response of these animals to light (Beale et al., 2013). In cave populations, light-inducible genes that are essential for clock entrainment are already highly transcribed in the dark. Cave populations 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 population 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 populations 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 (Tamai et al., 2004). 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 populations. What are the embryonic differences in early light sensitivity between populations? Does a molecular clock become established as early as detected in zebrafish, and is there a difference between surface and cave populations? Do cave populations develop a circadian clock in the same manner as surface fish, and are the differences reported in adult *Asytanax* present immediately in cave population 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 populations. We demonstrate that surface fish are acutely light responsive from the earliest stages of development, but that this light sensitivity appears to be developmentally delayed in cave populations. 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 populations, 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 appears to differ for classically light-regulated genes, such that the clock impacts these rhythms more strongly in cave populations than surface fish. As a result, one can detect more robust daily rhythms in the *period2* genes and *CPD*

photolyase in cave populations than surface embryos on a dark-light cycle. This may initially seem rather unexpected, but may reflect an evolutionary switch from light to clock gene regulation of critical genes in a constant dark environment.

The majority of these data were published in 2018, titled *"Development of the Astyanax mexicanus circadian clock and non-visual light responses"*. However, in that study we only examined clock and light responses in surface and Pachón. In this chapter, we also have added data from the first 81 hours of development in DL and DD for two other cave populations; Tinaja and Chica. The light-pulse experiments from D1 was not initially prioritised, and due to COVID-19 and a delay in obtaining a new licence from the Home Office there is still no complete data set for Tinaja and Chica. I have, however, made 4 new embryonic cavefish cell lines with some pilot data that is presented later in this chapter.

4.2 Results

4.2.1 Onset of light response in the developing embryo

The non-visual light response develops very early in small teleosts, well before the differentiation of the retina or pineal gland, and light exposure within the first few days of development has been shown to be crucial for embryonic development, survival and fitness in many species (Gavriouchkina et al., 2010; Martín-Robles et al., 2012; Tamai et al., 2004; Weger et al., 2011). What adaptations and changes in the light input pathway are found in cavefish embryos to compensate for developing in a dark environment?

Light regulates and sets the circadian clock through the transcriptional activation of light sensitive genes, which themselves are typically transcriptional repressors (Carr and Whitmore, 2005; Hirayama et al., 2005; Pando et al., 2001; Tamai et al., 2007; Vallone et al., 2004; Ziv et al., 2005). Work in zebrafish has shown that *per2* and *cry1a* are involved in the entrainment of the clock to light in the embryo and adult fish, which makes these light-inducible genes excellent markers for the onset of light-sensitivity (Dekens and Whitmore, 2008, Tamai et al., 2007, Ziv and Gothilf, 2006). The coding regions of the core clock and light-inducible genes are highly conserved between the different populations of *Astyanax*

mexicanus, which allows the use of the same gene-specific qPCR primers for all populations of fish (Beale et al., 2013).

To determine when cavefish become light responsive, Surface and Pachón embryos were raised in complete darkness and given a 3 h light-pulse at 3 different times during the first day of development (5 hpf, 14 hpf and 23 hpf). Light sensitivity is established very early in surface fish. Both *per* genes, as well as *cry1a* are induced by light as early as 5–8 hpf (Figure 4.2a-c). At 14–17 hpf and 23–26 hpf, we see further increases in light-inducible transcription of *per2a* and *per2b* mRNA transcript. In comparison, the Pachón embryo is much slower in developing a light response. There are no significant increases in any of the light sensitive genes in 5–8 h old Pachón embryos (Figure 4.2a,b,c). At 14–17 hpf, we see a robust light response in both the *per2* genes in Pachón embryos, but yet we do not see a *cry1a* response to light until the very end of Day 1 (4. i). Interestingly, throughout development, the basal levels (expression in DD) of the *per2* genes are raised in Pachón cavefish compared to surface fish (Figure 4.2), except *per2b* 14–17 hpf (4. e) where there is a non-significant expression difference between DD samples in Pachón and surface embryos. Presumably, it is these raised levels in the dark that prevent any additional measurable light-induction. We also observe this increased basal transcription of the *per2b* genes in adult Pachón fish (Beale et al., 2013). However, there is an even stronger *per2b* fold basal induction in Pachón embryos compared to adults. The basal levels of *cry1a* however, are the similar for cave and surface populations (Figure 4.2f,i).

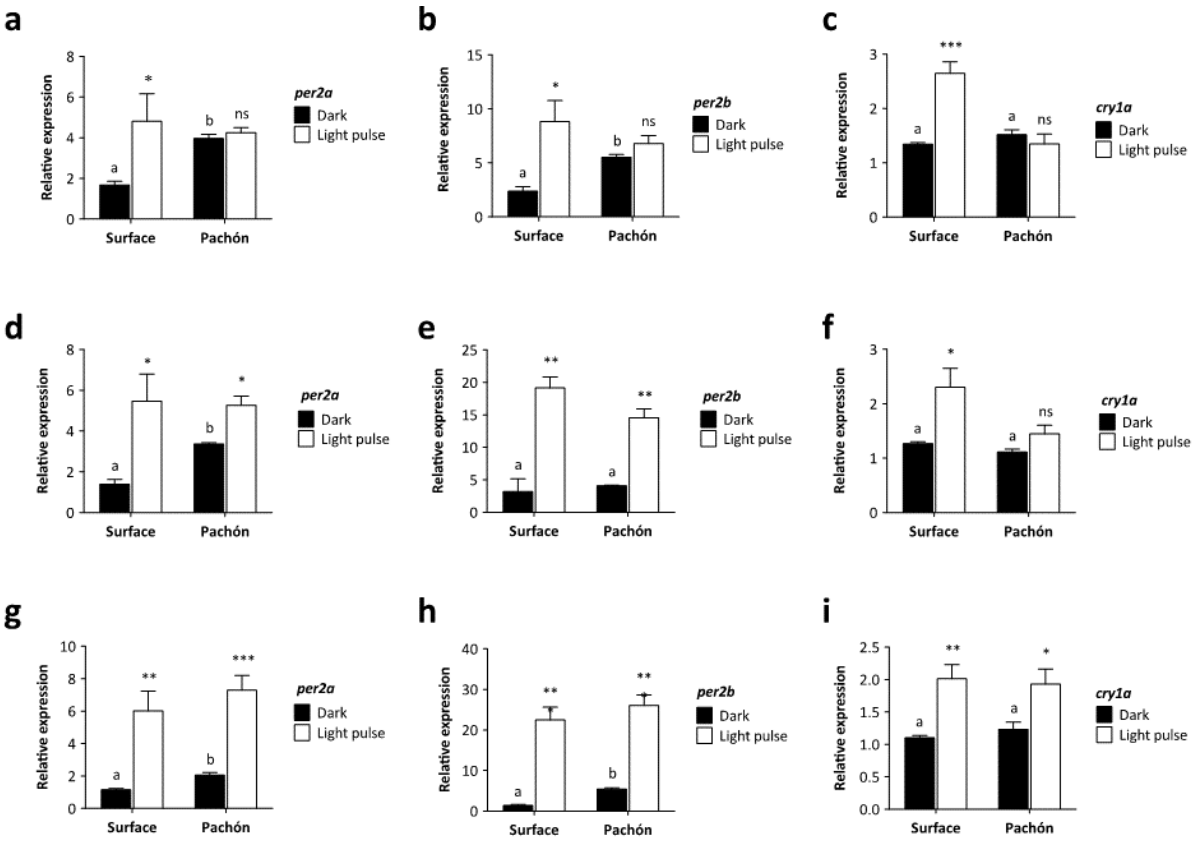


Figure 4.2: Acute light induction of clock genes is slower to develop in Pachón cavefish than surface fish during development. Surface and Pachón embryos were kept in constant darkness until a 3-hour light pulse was given at different developmental stages. Expression of *per2a*, *per2b* and *cry1a* was 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), Data represent the mean ± SEM for between 3 and 5 embryo samples. (Experiments in figure performed by Dr Andrew Beale)

Although it is clear that a light response exists in early Pachón embryos (from both the differences between *per1* expression rhythms in LD and DD (Figure 4.5) and significant acute responses to light at 14–17 hpf) (4. d,e), a light response similar to that seen in Surface fish is only present in Pachón embryos at 23–26 hpf (Figure 4.2g-i). 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 significant changes detected in the pineal gland (Vatine et al., 2009, Ziv et al., 2005). It could, therefore, be argued that much of this embryonic light response is pineal dependent. 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-enhanced expression. *In situ* hybridisation confirmed the increased expression of *per2b* after light exposure at 5 hpf and 23 hpf in surface embryos (Figure 4.3a 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 4.3h and i), similar to the results obtained by qPCR. *Per2b* is clearly expressed at raised levels in Pachón embryos compared to surface fish in the dark controls, as seen by qPCR, and which is apparent in these samples at both time points (Figure 4.3c and h). At 26 hpf, the expression in both surface and Pachón embryos is ubiquitous throughout the embryo, though clearly somewhat stronger in the head region of the larvae. The ubiquitous expression of *per2b* at 26 hpf observed by *in situ* hybridisation, and the clear light response present before 17 hpf, show that it is not the pineal gland alone that mediates the development of the light-induction of clock genes in Pachón. The mechanism of light detection is present throughout the embryo and is not restricted to central photoreceptive structures, in both surface and cave populations of Astyanax.

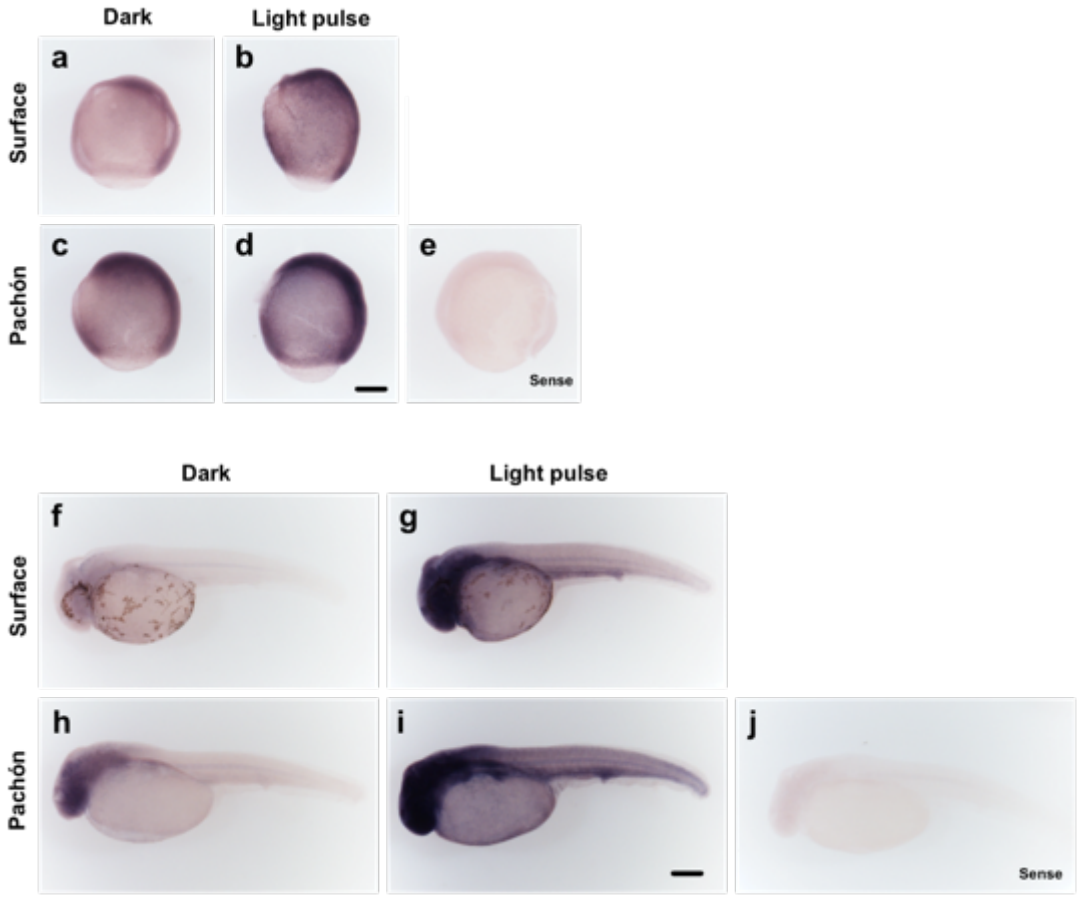


Figure 4.3: Acute light induction develops within the first day of development in Pachón cavefish. Surface and Pachón embryos were kept in constant darkness until a 3-hour light pulse was given beginning at (a-d) 5 hpf and (f-i) 23 hpf. Expression of *per2b* mRNA was analysed by *in situ* hybridisation 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. (Experiments in figure performed by Dr Andrew Beale)

4.2.2 Cave-cave hybrid fish do not show a rescued light-response in epiboly-stage embryos

Cave populations of *Astyanax mexicanus* 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 still be crossed to examine cave phenotypes by complementation tests. It is clear that development of the light response in Pachón embryos is delayed compared to surface fish. Can this delay in light responsiveness be rescued in a F1 generation created by mating two cave populations?

In order to test whether early light sensitivity can be rescued by another cave population, we examined the induction by light of multiple clock genes in Pachón, Chica and Pachón-Chica hybrid embryos by raising the embryos in the dark and giving a 3-h light-pulse at 5 hpf. *Per2a* show a very small (1.23 fold), but significant induction in Chica embryos, yet there is no induction of *per2b* or *cry1a* (Figure 4.4). Furthermore, Chica embryos also show high expression of both *per* genes in DD, similar to that described for Pachón (Figure 4.4a, b). Interestingly, we do not see any rescue of light responses in the "cave-cave" hybrid. The small *per2a* increase seen in Chica is no longer present, yet we still see an increased amount of *per2* transcript in the "cave-cave" hybrid animals (Figure 4.4a, b).

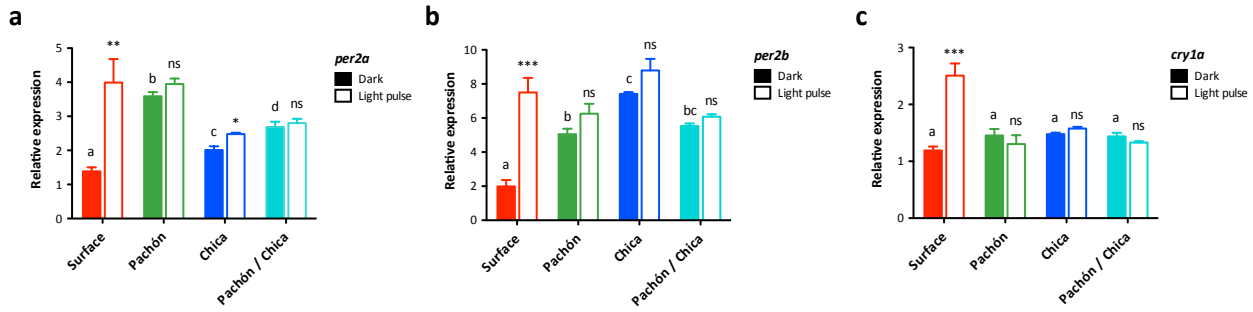


Figure 4.4: Acute light-induction at 5 hpf is not rescued in a cave-cave hybrid. Surface, Pachón, Chica and Pachón/Chica hybrid embryos were kept in constant darkness until a 3 hour light pulse was given at 5 hpf. Expression of (a) *per2a*, (b) *per2b* and (c) *cry1a* was determined by qPCR in light-pulsed and dark control samples and normalised to the reference gene *rpl13α*. 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. (Experiments in figure performed by Dr Andrew Beale)

4.2.3 Clock entrainment differs in cave and surface embryos

The circadian clock of zebrafish begins on the first day of development (Dekens and Whitmore, 2008; Ziv and Gothilf, 2006). The core circadian clock mechanism is generated by a transcriptional-translational negative feedback-loop, which is highly conserved in all vertebrates (Harmer et al., 2001; Takahashi, 2004; Wilsbacher and Takahashi, 1998). *Per1* is one of the key genes of the core clock, and shows high-amplitude circadian oscillations in entrained adult *Astyanax mexicanus*, making *per1* an excellent marker of clock function (Beale et al., 2013; Martín-Robles et al., 2012; Park et al., 2007, Tamai et al., 2007; Velarde et al., 2009).

To determine when the *Astyanax* circadian clock starts, Surface, Pachón, Chica and Tinaja embryos were entrained to a 12–12 h dark-light (DL) cycle and embryos were harvested at 6-h intervals for the first 3.5 days of development. It is worth noting that we employed a

reverse light-dark cycle for these experiments, compared to most studies in the literature, to match with the natural spawning times of the cave populations, which occur primarily during the night. Zebrafish, for example, spawn just after dawn. Samples were analysed by RT-qPCR to determine the levels of *per1* mRNA. During the first two days of development, surface embryo *per1* shows a low amplitude, non-significant rhythm that peaks 3 h after the onset of light (ZT3) (Figure 4.5a). However, late second/early third day of development, we start to observe a higher amplitude rhythm, were the peak has now shifted to ZT21, with significant expression differences between the peak and troughs, by 81hpf. In comparison, this is not an expression pattern followed by the three cave populations. Pachón show a non-significant, shallow amplitude change during the first day of development, and is the only cave embryo to undergo one trough-peak-trough cycle by 81 hpf, with a 6-hour delay in peak expression compared to surface embryos (Figure 4.5b). The expression pattern in Chica matches more that of the surface population, with two shallow, non-significant cycles over first 48 hours of development, which increases in amplitude during Day 3 giving us a significant trough and peak at ZT21 (Figure 4.5c). Tinaja on the other hand, looks different in its expression pattern, with no indication of shallow amplitude rhythms early in development, and only one significant peak at ZT15 or ZT21 late in the third day of development (Figure 4.5d).

When we examine *per1* expression in constant dark, none of the populations show a rhythm. The expression is erratic with a large spread in the expression between the biological replicates (Figure 4.5e). When we compare the populations, some of the time points show a significant difference in expression between the different populations. However, there is no clear pattern or differences, like we see in the light-inducible genes as shown below.

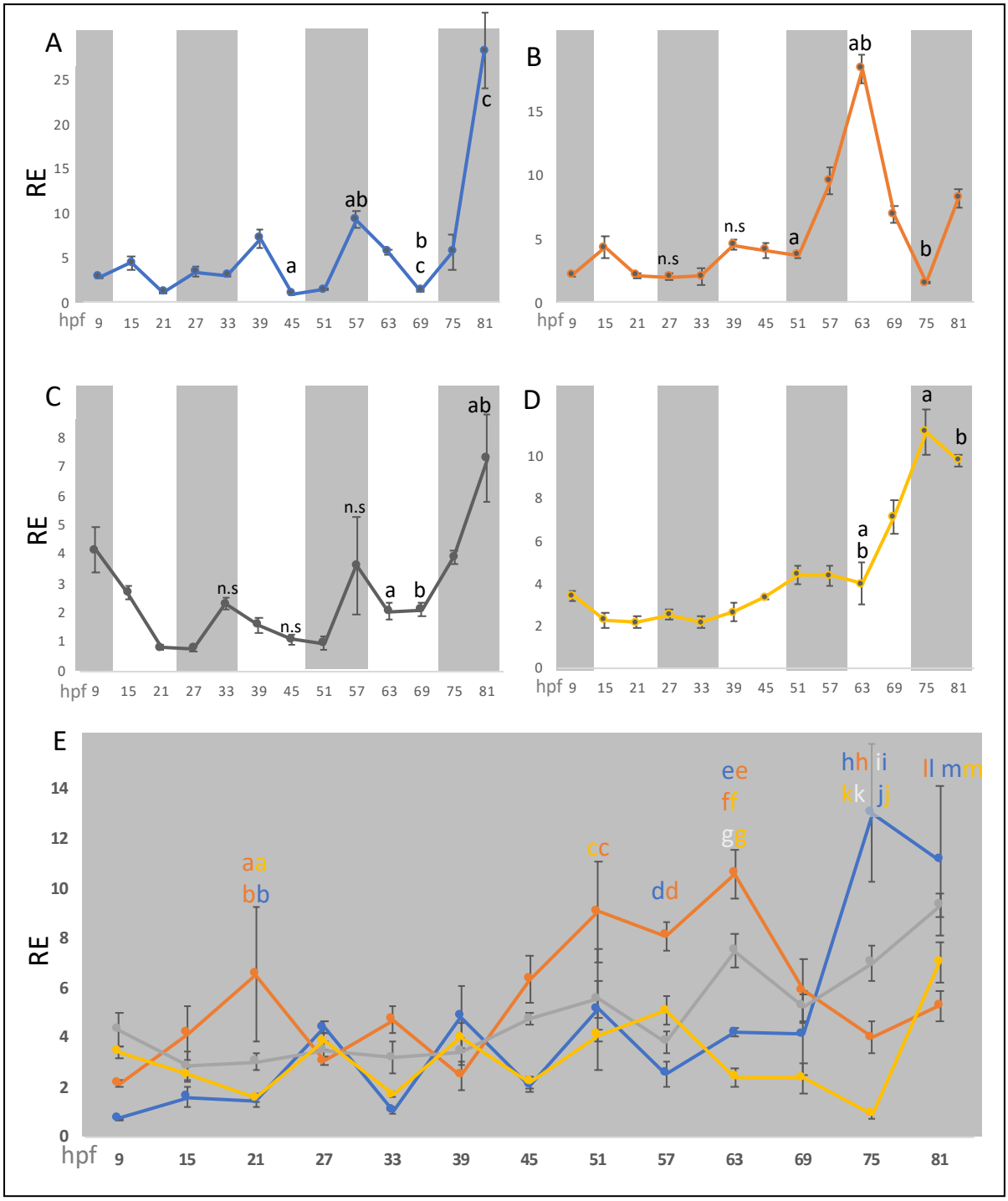


Figure 4.5: *Period1* expression over the first 3.5 days post fertilization in 4 different populations of cavefish A) Surface B) Pachón C) Chica D) Tinaja expression of *per1* under a 12:12 DL regime, where grey panels denote dark and white panels denote light. E) Shows expression of cavefish larvae raised in DD, where blue= surface, orange= Pachón, grey= Chica and yellow= Tinaja. Data is plotted as relative expression (RE) to the lowest expressed gene and normalised to the reference gene *rpl13α*. The X-axis shows hours post fertilization (hpf). An ANOVA was performed (α 0.05), with a Tukey Post-Test. For panel A-D, lower-

case letters indicate significance in expression between different timepoints. For panel E where different colours of the lower-case letters, indicate the significance of expression between the different strains for that timepoint i.e. orange and yellow letter "a" means significance in expression between Pachón and Tinaja for that timepoint. Non-significant expression is not marked, except timepoints of interest which are marked "n.s". Data represent the mean ± SEM (n=3-4).

4.2.4 Alterations in rhythmic expression of *per2a* and *per2b* transcriptional repressors in developing cave populations

We have previously hypothesised that the differences in rhythm amplitude and the phase angle of *per1* expression between cave and surface populations is likely to be due to changes within the core clock mechanism that generates the oscillation, as well as alterations in the light input pathway. So, what changes do we see in the expression of the *per2* light-induced transcriptional repressors in the developing embryo?

Both surface and Pachón populations show rhythmic and light induced expression of *per2a* from the first day of development, whilst Tinaja and Chica start on the second day (Figure4.6a-d). *Per2a* transcripts peaks clearly at ZT3 with an average 18-fold difference in expression between peak and trough expression in surface fish (Figure4.6a). In comparison, Pachón *Per2a* peaks 6 h later at ZT9, but with high expression also at ZT3, and how an average 3.4-fold change between peak and trough values (Figure4.6b). Chica also has cycling *per2a* transcripts, with an amplitude of less than a 2-fold change between day and night, with no clear differences between ZT3 and ZT9 (Figure4.6c). *Per2a* expression in Tinaja peaks ZT3 during the second day of development, and then at ZT9 during the third day of development, showing the same 6-hour delay as Pachón, with an average 2-fold amplitude between light and dark phase in Tinaja embryos (Figure4.6d). It is also worth noting that the relative abundance of transcripts of Chica and Tinaja, are considerably higher than that of Surface and Pachón, both when looking at maternally deposited RNA at 9hpf, and throughout the first 3 days of development (Figure4.6a-d).

The increased amount of basal transcript is also observed in constant darkness in the cavefish (Figure 4.6e). This is particularly evident, if we average the *per2a* expression during development in DD, where Chica embryos show an average 8.4-fold higher expression than that of surface embryos (Figure 4.9), while Pachón and Tinaja show an average of 2.6- and 4.5-fold increase respectively. This finding supports the theory that some light

inducible/clock genes show an increased basal expression in DD possibly to compensate for the lack of light exposure.

At first glance, the two *period* gene expression profiles look very similar, but there are some interesting differences. *Per2b* shows similar rhythmic expression to *per2a* in surface fish with peak expression at ZT3 (Figure 4.7a). However, *per2b* transcript does not start oscillating until the second day of development, with an average of just 4-fold difference between peak and trough, compared with a 18-fold difference in *per2a* (Figure4.6a). Pachón *per2b* amplitude, however, averages at 8-fold difference between peak and trough (Figure 4.7b), which is twice as high as observed for Pachón *per2a* (Figure 4.6b). Furthermore, in Pachón, we see *per2b* expression peak at ZT3 (15 hpf and 39 hpf), but then delay to ZT9 at 69 hpf, shifting 6h to match the phase of *per2a*. This shift is also seen in Tinaja for both period genes. We observe an average 1.7-fold amplitude between peak and trough phase in Tinaja embryos (Figure4.6d and Figure4.7d). Tinaja is by far the population with the highest relative expression of transcript, with basal DD expression being 10-50 times as high as that of surface embryos (Figure 4.7a,d). In contrast to the other populations, Chica does not show any statistically significant rhythm, which is most likely due differences in expression levels between biological replicates (Figure 4.7c).

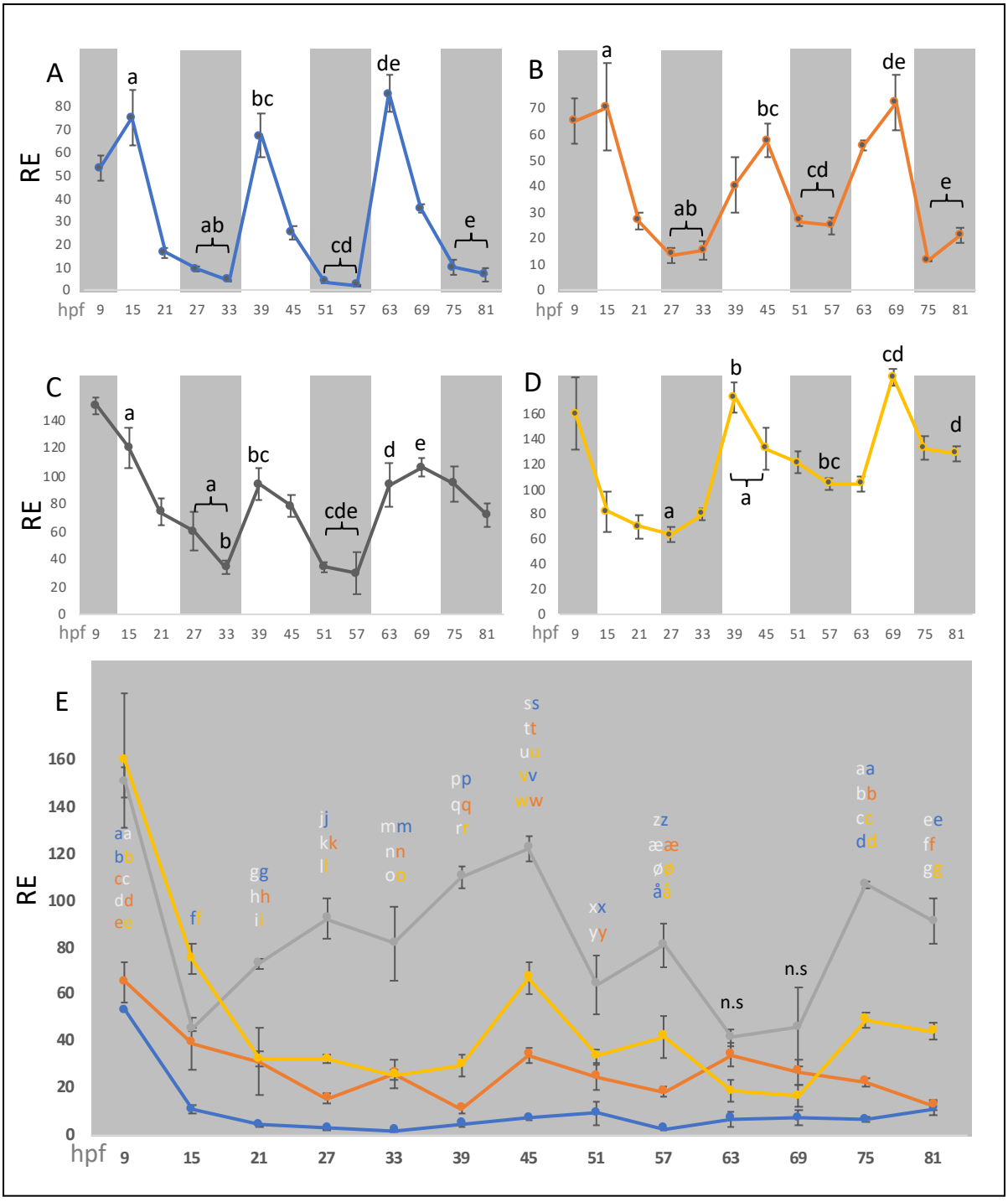


Figure 4.6: *Period2a* expression over the first 3.5 days post fertilization in 4 different populations of cavefish A) Surface B) Pachón C) Chica D) Tinaja expression of *per2a* under a 12:12 DL regime, where grey panels denote dark and white panels denote light. E) Shows expression of cavefish larvae raised in DD, where blue= surface, orange= Pachón, grey= Chica and yellow= Tinaja. Data is plotted as relative expression (RE) to the lowest expressed gene and normalised to the reference gene *rpl13α*. The X-axis shows hours post fertilization (hpf). An ANOVA was performed (α 0.05), with a Tukey Post-Test. For panel A-D, lower-

case letters indicate significance in expression between different timepoints. For panel E where different colours of the lower-case letters, indicate the significance of expression between the different strains for that timepoint i.e. orange and yellow letter “a” means significance in expression between Pachón and Tinaja for that timepoint. Non-significant expression is not marked, except timepoints of interest which are marked “n.s”. Data represent the mean ± SEM (n=3-4).

One of the more striking differences in *per2a* and *per2b* expression is seen in DD, whereas in the case of *per2a,* there is a consistent higher basal expression in the cave populations compared to surface (Figure 4.6e). In *per2b* however, we only see Chica having raised basal transcript levels (Figure 4.7e). This is also evident when we average the expression in DD, where we observe overall 4.5-fold increase in transcript in Chica embryos compared to surface, which is only half the average of *per2a* (Figure 4.9). Interestingly, there are no differences in expression between the other cave populations and surface fish (except at 9 hpf), like there was with *per2a*. There also seem to be differences in maternally deposited *per2a* and *per2b* mRNA. Chica show high amount of maternally deposited *per2a*/b (Figure 4.6c,e/6c,e). Tinaja show a similarly high *per2a* expression at 9phf, but only half the amount of *per2b* is present at the same time of development (Figure 4.6d,e/6d,e). Pachón and surface embryos also has a high amount of maternally deposited *per2a* RNA, although its 4.5 times smaller than that of Tinaja or Chica (Figure 4.6 a,b,e). Pachón and Tinaja embryos do not however, express a high level of *per2b* at 9hpf (Figure 4.7 a,b,e).

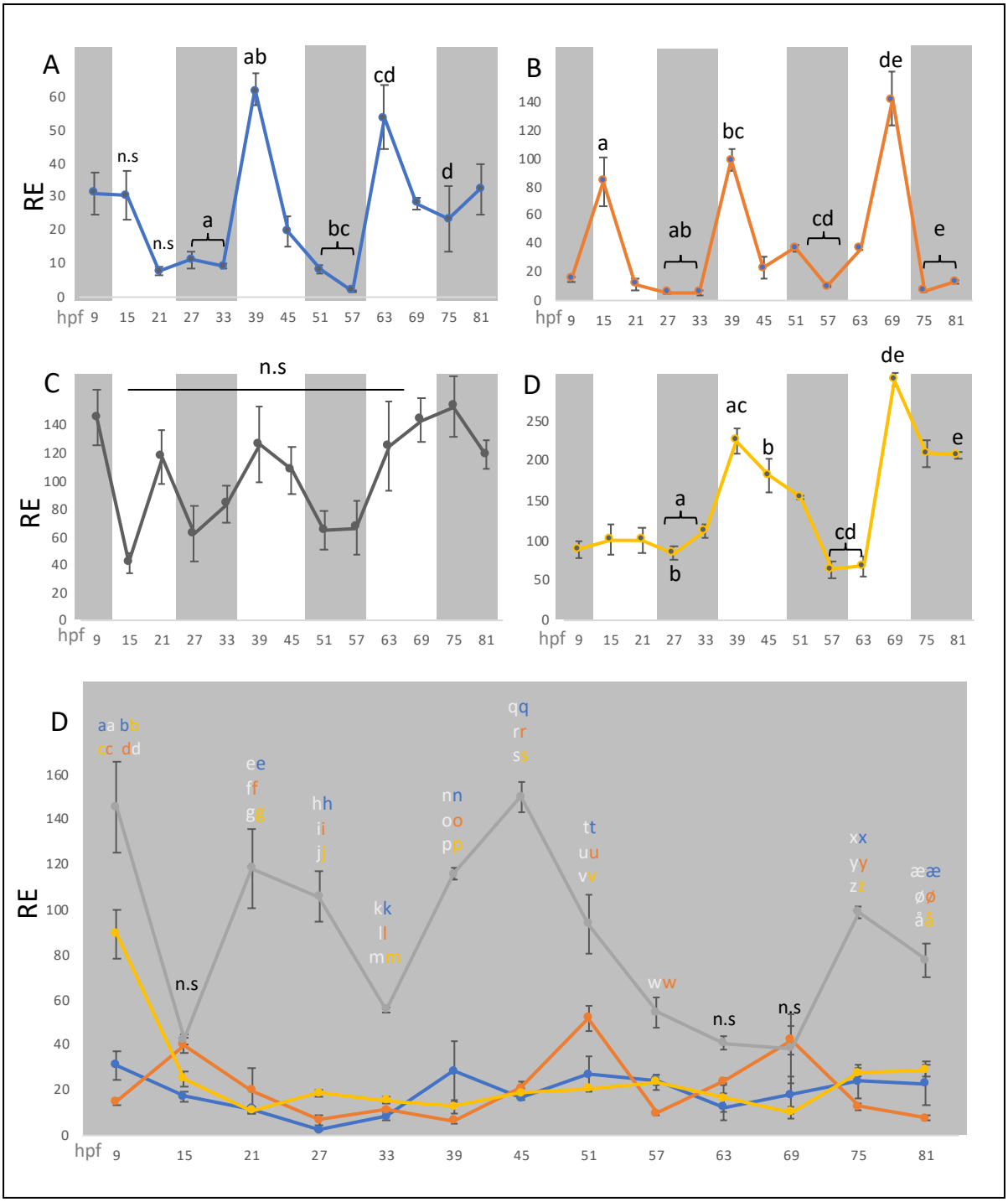


Figure 4.7: *Period2b* expression over the first 3.5 days post fertilization in 4 different populations of cavefish A) Surface B) Pachón C) Chica D) Tinaja expression of *per2b* under a 12:12 DL regime, where grey panels denote dark and white panels denote light. E) Shows expression of cavefish larvae raised in DD, where blue= surface, orange= Pachón, grey= Chica and yellow= Tinaja. Data is plotted as relative expression (RE) to the lowest expressed gene and normalised to the reference gene *rpl13α*. The X-axis shows hours post fertilization (hpf). An ANOVA was performed (α 0.05), with a Tukey Post-Test. For panel A-D, lower-

case letters indicate significance in expression between different timepoints. For panel E where different colours of the lower-case letters, indicate the significance of expression between the different strains for that timepoint i.e. orange and yellow letter "a" means significance in expression between Pachón and Tinaja for that timepoint. Non-significant expression is not marked, except timepoints of interest which are marked "n.s". Data represent the mean ± SEM (n=3-4).

4.2.5 DNA repair gene expression is altered in cave populations

DNA repair, using enzymes such as *CPD* photolyase (*CPD phr*), is one of several important processes that are light induced in small teleosts (Tamai et al., 2004, Gavriouchkina et al., 2010, Weger et al., 2011). Are the changes in light sensitivity that we describe for the clock also impacting the regulation of DNA repair gene transcription in the developing embryo? Entrained surface embryos show high amplitude rhythms of *CPD phr* expression with peaks at ZT3 from day 2, with very little transcript present at ZT9, ZT15 and ZT21 (Figure 4.8a). In Pachón, the *CPD phr* peaks 6 h later at ZT9, and both populations show an average 4-fold difference in transcript between peak and trough (Figure 4.8b). Whereas surface show a sharp peak at ZT3, Pachón express a high amount of *CPD* transcript expressed at ZT3 and ZT9, which means there is about twice the amount of *CPD* transcript present during the light phase in the Pachón embryo (Figure 4.8b). Chica peaks at ZT9 at 45 hpf, but rhythm dampens out by the next cycle (Figure 4.8c). In contrast to the other embryos, Tinaja show no light response to *CPD phr* (Figure 4.8d).

When looking at the *CPD* expression in DD during development, we see that the cavefish generally express higher amounts of *CPD phr* than surface (Figure 4.8e). If we average this expression, we actually see that the cave embryos overall transcribe twice as much photolyase as surface embryos (Figure 4.9), something that has also been observed in adult fish (Beale et al., 2013).

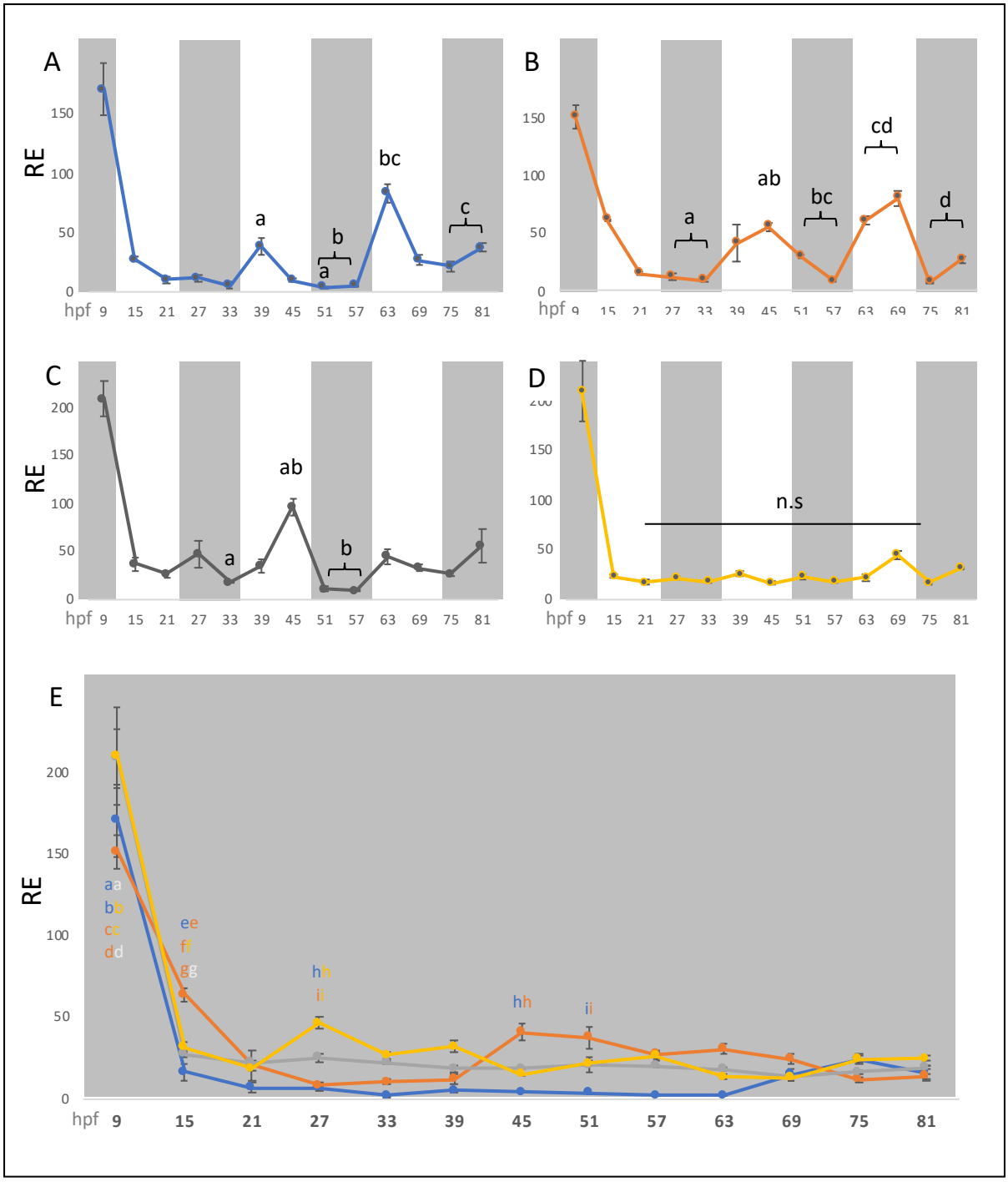


Figure 4.8: *CPD Phr* expression over the first 3.5 days post fertilization in 4 different populations of cavefish A) Surface B) Pachón C) Chica D) Tinaja expression of *CPD Phr* under a 12:12 DL regime, where grey panels denote dark and white panels denote light. E) Shows expression of cavefish larvae raised in DD, where blue= surface, orange= Pachón, grey= Chica and yellow= Tinaja. Data is plotted as relative expression (RE) to the lowest expressed gene and normalised to the reference gene *rpl13α*. The X-axis shows hours post fertilization (hpf). An ANOVA was performed (α 0.05), with a Tukey Post-Test. For panel A-

D, lower-case letters indicate significance in expression between different timepoints. For panel E where different colours of the lower-case letters, indicate the significance of expression between the different strains for that timepoint i.e. orange and yellow letter "a" means significance in expression between Pachón and Tinaja for that timepoint. Non-significant expression is not marked, except timepoints of interest which are marked "n.s". Data represent the mean ± SEM (n=3-4).

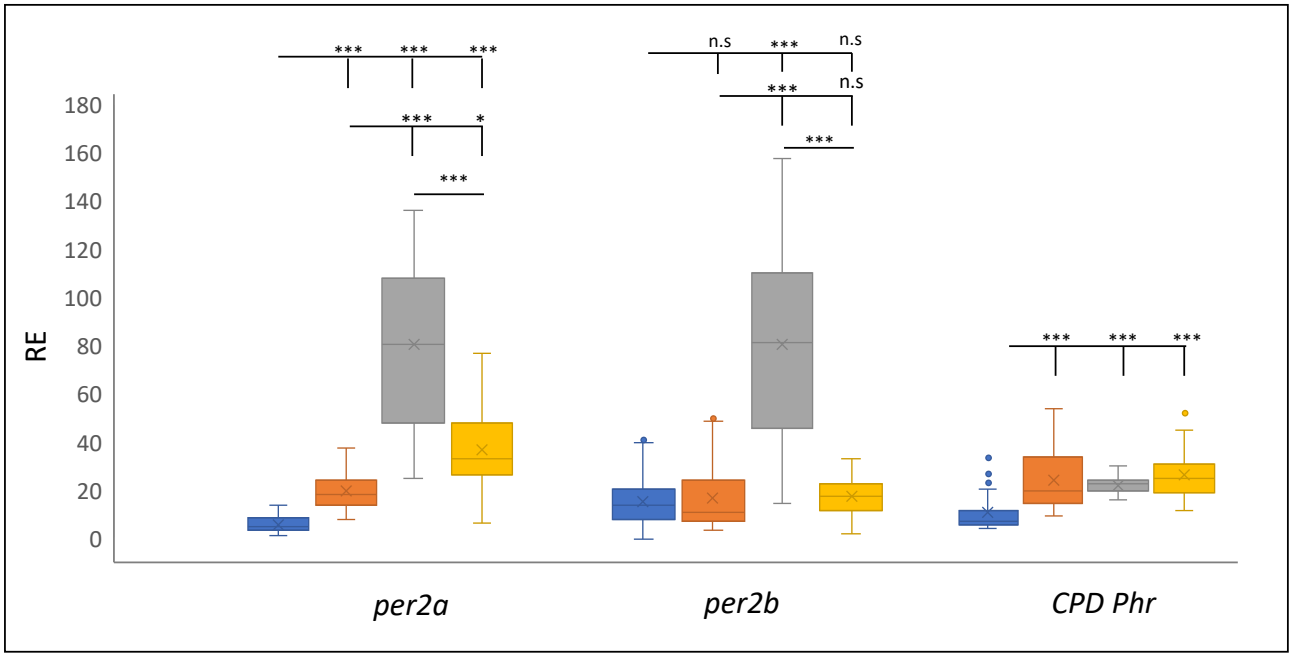


Figure 4.9: Box and whisker plot showing the expression of the three light inducible genes; *per2a*, *per2b* and *CPD Phr* over 13 time-points in DD

Blue= surface, orange= Pachón, grey= Chica and yellow= Tinaja. Graphs are created from re-plotting the data shown in Figure 4.6d, 4.7.d and 4.8d**.** The whiskers indicate highest and lowest expression, the line in the middle indicate the median expression, whereas the cross indicate the mean value. Dots represent outliers. Variation was assessed with an ANOVA, and samples were compared using a Student's t-test (unpaired, two tailed; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$) with Bonferroni correction (α 0.05).

4.2.6 Developing novel *Astyanax mexicanus* cell lines

The Mexican blind cavefish are a brilliant species to study the evolution and adaptation to a dark environment, with its founding surface variant and multiple cave morphs isolated over the past few million years. It has also proven to be a hardy fish which is easy to maintain both as a lab species and as an aquarium species. There are however not as easy and quick to work with as the zebrafish, as they sexually mature much slower and can only be mated 1-2 times a month as opposed to every other day in zebrafish. The cavefish are also considerably larger than the zebrafish, so fewer fish can be housed together, thus taking up considerably more space. When it comes to mating, the cavefish fish require a change of temperature to induce spawning, and it takes about 2-3 weeks for a fish to be able to mate again successfully. Although cavefish have a life expectancy of 10 years or more in captivity, older fish or fish that are not mated often, maybe not suitable for mating as they often yield embryos with early abnormal cell division or a high frequency of larvae with what looks like pericardial oedema, resulting in a high death-rate. Even when using younger fish, the cavefish require a much more 'hands on approach' when it comes to spawning, as in vitro fertilization is often required to get successful fertilisation and healthy embryos. The different populations time their spawning differently and usually over two days, with the Chica and Tinaja cave populations timing being the most unpredictable.

Circadian experiments require many samples, and with unpredictability in spawning, as well as waiting for new individuals to reach sexual maturity, experiments can become time-consuming. In addition, over the last few years, there has been a push for using as few animals as possible in research, and as a vertebrate, work on teleosts fall under the "3Rs", I decided that cavefish cell lines would be a beneficial tool. I made cultures out of the different populations of cavefish using 24-hour old embryos, and after passaging cells over 10 times, I performed some initial circadian experiments, much like the ones described above, to explore whether cavefish cell cultures are a good tool for circadian experiments, or not.

As the cavefish embryos have shown that they are light responsive early on in development, prior to the development of "classical" light sensitive structures such as eyes and pineal, we have *assumed* that the cells are themselves directly light responsive, as has been previously and extensively shown in zebrafish. This has however never been proven in the case of

Astyanax. Direct light sensitivity is the largest advantage of fish cell cultures over traditional mammalian cell cultures, where serum shocks, or other pharmacological treatments are required to reset the clock in the cells. It is therefore key to establish if these cavefish cells are indeed light sensitive. At this stage of passage (about 10) it is worth noting that all cultures look like morphologically, there are at least two types of cells present, and that cells, although they stick down, have a tendency of growing on top of each other as well (Supplementary Figure 4.2). Unlike with zebrafish, cavefish cells have not yet been FACS cell sorted to produce clonal populations.

To demonstrate that the cavefish cells are light responsive, cells were seeded and grown to confluency on a 12:12 LD cycles for 3 days, and then sampled at 6-hour intervals (ZT3,9,15,21). Controls were also maintained on a 12:12 LD cycle and sampled in DD every 6-hours (CT3, 9, 15, 21) the following day. The same light inducible genes that were examined during embryonic development; *per2a*, *per2b* and *CPD phr,* was also examined in the cell lines.

4.2.7 Cavefish cells are light sensitive in culture

Per2a expression shows the most robust rhythm in expression patterns in the developing embryo (Figure 4.7), something we also see in the cell line (Figure 4.10). Surface cells show the strongest cycling amplitude in *per2a* expression during LD, peaking at ZT3 (13.8-fold difference), and a low basal expression in DD (Figure 4.10a). The cavefish cell populations show similar *per2a* expression patterns (Figure 4.10 b-d), where the light inducible genes are highly expressed throughout the light phase peaking at ZT9, but with an almost equally high amount of transcript at ZT3. Pachón, Chica and Tinaja, show a 2-, 2.5- and 2.8-fold difference between ZT9 and ZT15 respectively, with Tinaja expressing the least amount of *per2a* transcript over the 4 time-points (Figure 4.10 b-d). In DD we see no rhythm, but observe a raised overall basal expression in the cave cells compared to the surface cells when looking at expression over the 24-h period (Figure 4.13). Furthermore, it is worth to note that *per2a* expression in DD is significantly higher in Pachón and Chica compared to Tinaja.

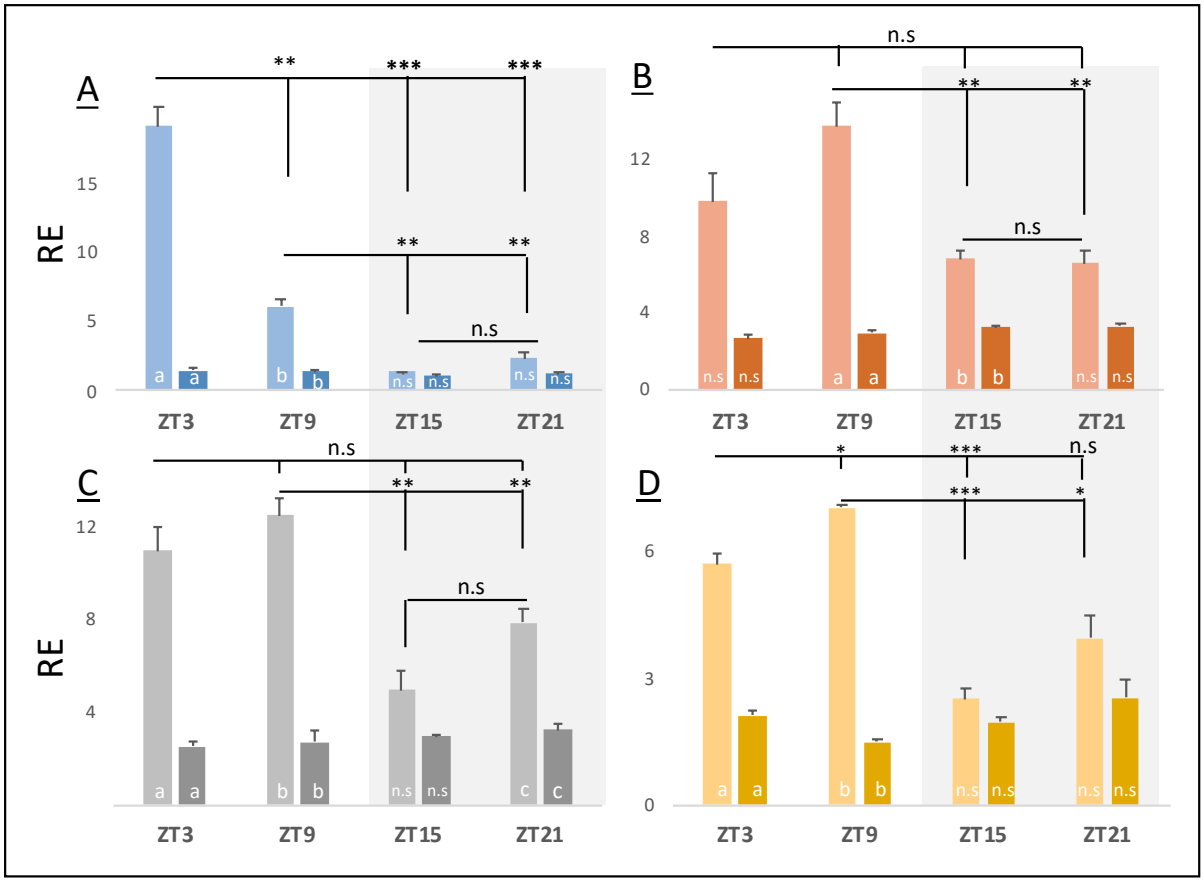


Figure 4.10: *Period2a* expression in cell culture of 4 different populations of cavefish
A) Surface B) Pachón C) Chica D) Tinaja *per2a* expression pattern under LD (light coloured graphs) and DD (darker coloured graphs). Data is plotted as relative expression (RE) to the lowest expressed time-point and normalised to the reference gene *rpl13α*. An ANOVA was performed (α 0.05), and significance was assessed with a Bonferroni post-test with Holm correction (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). For panel A-D, lowercase letters on the bars indicate a significant expression ($p < 0.05$) between LD samples and DD samples. For Panel E, n.s on the bars, suggests no significance within sample over the 4 time points. Data represent the mean ± SEM (n=4).

In surface cells, *per2b* expression pattern is robust (Figure 4.11a) and looks much the same to *per2a* expression (Figure 4.10a), with a clear ZT3 peak. The relative amount of transcript is reduced by about a third compared to *per2a,* something that is also reflected in a reduced amplitude of just 4-fold for *per2b* surface cells. In cavefish cell lines, *per2b* is highly expressed at both ZT3 and ZT9 (Figure 4.11 b-d) just like *per2a* (Figure 4.10 b-d). In Pachón,

Chica and Tinaja the relative amount of *per2b* over the LD cycle is also reduced as in surface cells. However, both Pachón and Chica show similar amplitude expression to *per2a* with 1.8-, 2.5- and 1.6-fold difference between ZT9 and ZT15 for Pachón and Chica (Figure4.8b-c), and ZT3 and ZT21 in Tinaja. In complete darkness, expression of *per2b* remains flat and similar for all populations of cavefish as well as surface fish (Figure 4.13), which is a key difference from *per2a* expression.

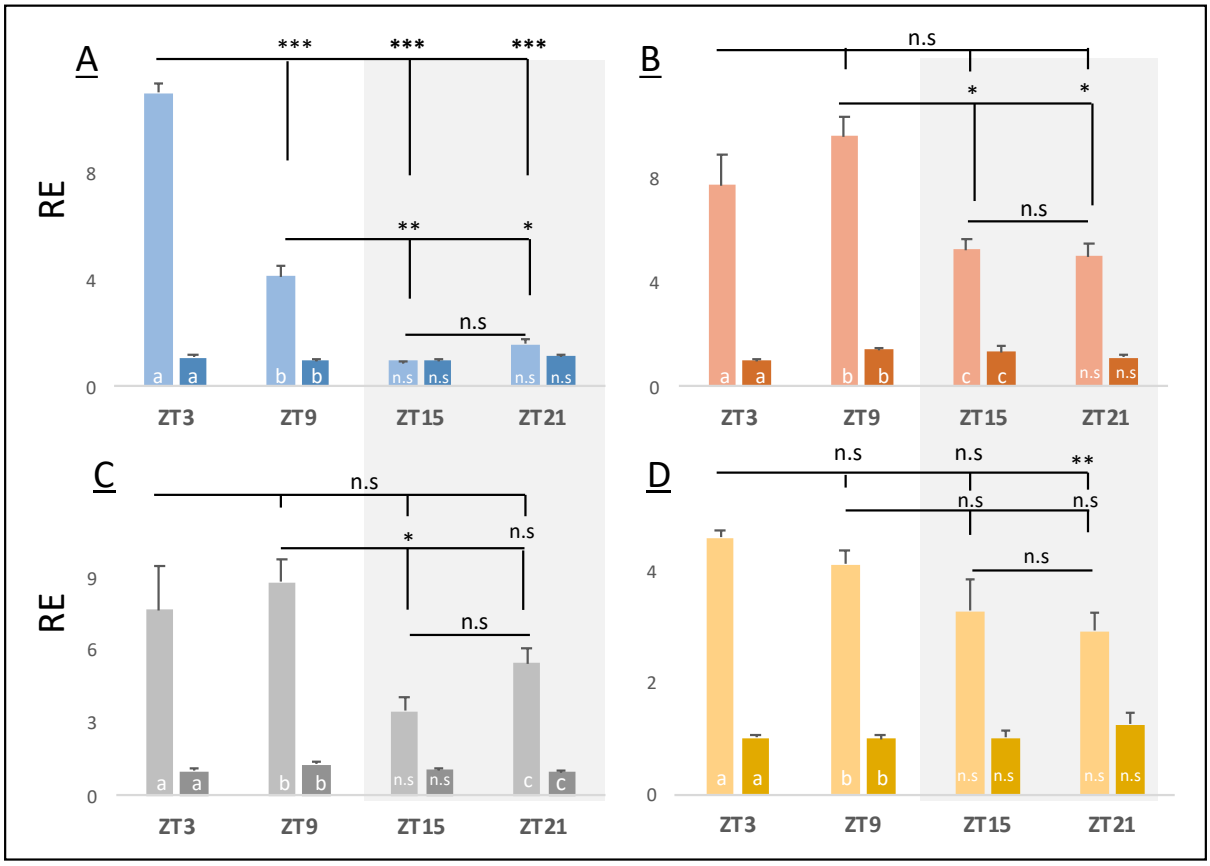


Figure 4.11: *Period2b* expression in cell culture of 4 different populations of cavefish
A) Surface B) Pachón C) Chica D) Tinaja *per2b*expression pattern under LD (light coloured graphs) and DD (darker coloured graphs). Data is plotted as relative expression (RE) to the lowest expressed time-point and normalised to the reference gene *rpl13α*. An ANOVA was performed (α 0.05), and significance was assessed with a Bonferroni post-test with Holm correction (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). For panel A-D, lowercase letters on the bars indicate a significant expression ($p < 0.05$) between LD samples and DD samples. For Panel E, n.s on the bars, suggests no significance within sample over the 4 time points. Data represent the mean ± SEM (n=4).

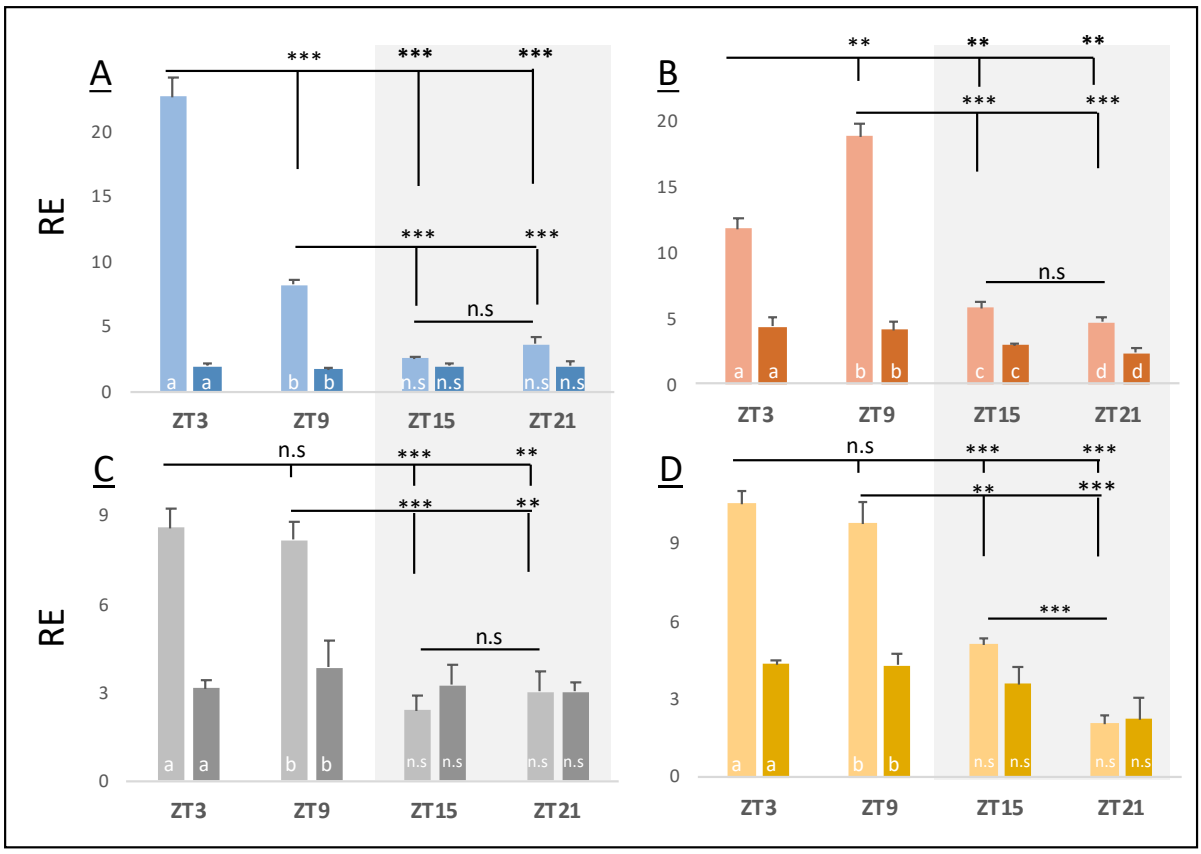


Figure 4.12: *CPD phr* expression in cell culture of 4 different populations of cavefish
A) Surface B) Pachón C) Chica D) Tinaja *CPD* expression pattern under LD (light coloured graphs) and DD (darker coloured graphs). Data is plotted as relative expression (RE) to the lowest expressed time-point and normalised to the reference gene *rpl13α*. An ANOVA was performed (α 0.05), and significance was assessed with a Bonferroni post-test with Holm correction (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). For panel A-D, lowercase letters on the bars indicate a significant expression ($p < 0.05$) between LD samples and DD samples. For Panel E, n.s on the bars, suggests no significance within sample over the 4 time points. Data represent the mean ± SEM (n=4).

4.2.8 DNA repair is increased in complete darkness in cave cells

CPD phr also follows the same pattern as *per2a* and *per2b* in surface cells with clear peak at ZT3 as well as a 9-fold difference between peak and trough (Figure 4.12a). Pachón displays the same ZT9 peak as for the *per2s* and a 3.5-fold difference in peak-trough (Figure 4.12b). While Chica and Tinaja has no clear peak, with ZT3 and ZT9 both significantly increased compared to ZT15 and ZT21, averaging both at an average 3-fold difference between peaks

and troughs (Figure 4.12 c-d). There are no cycling *CPD* transcript in DD for either of the 4 cell lines (Figure 4.12 a-d), but there is an increased basal expression in the cave cells compared to the surface cells (Figure 4.13). Interestingly, this gene shows much more variation in expression over the 24-hour period than the *per2* genes.

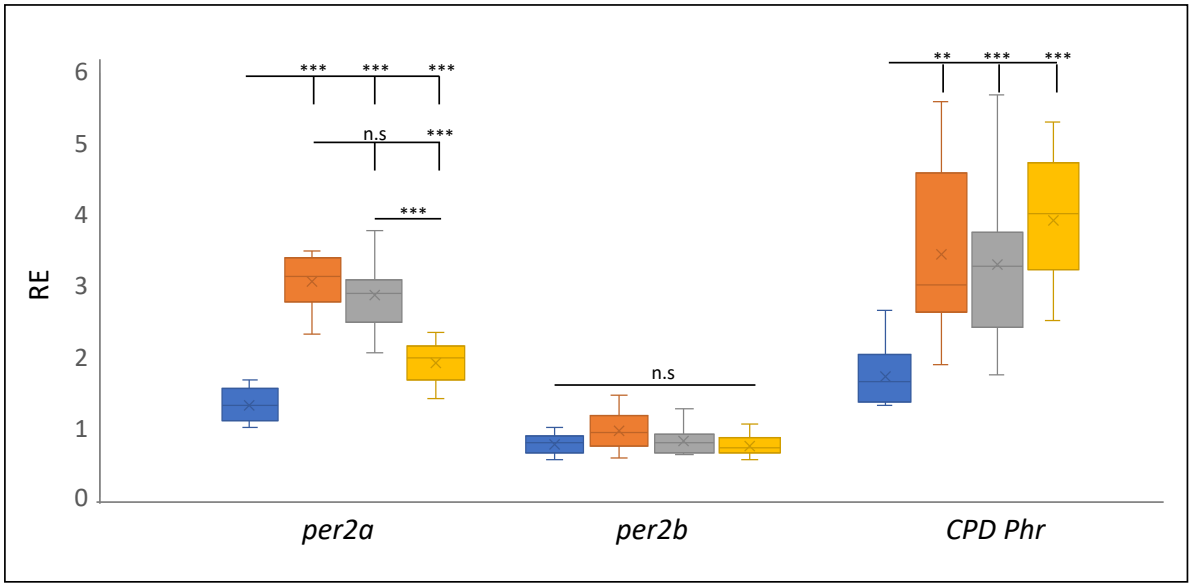


Figure 4.13: Box and whisker plot showing the expression of the three light inducible genes; *per2a*, *per2b* and *CPD Phr* over 4 time-points in DD after 3 LD cycles
Blue= surface, orange= Pachón, grey= Chica and yellow= Tinaja. Graphs are created from re-plotting the DD data shown in Figure 4.10-4.12**.** The whiskers indicate highest and lowest expression, the line in the middle indicate the median expression, whereas the cross indicate the mean value. Samples were compared using a Student's t-test (unpaired, two tailed; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$) with Bonferroni correction (α 0.05).

4.2.9 Circadian clock in cell cultures

Cell density and cell contact play a role in the establishment of robust molecular rhythms and in particular in the establishment of rhythmic clock outputs (unpublished results). As the cavefish cultures are not yet monoclonal, and grow both upwards and sideways, assessing confluency can be tricky and inaccurate. It was however worth examining the cells at this stage to in order to get an idea of whether the cells have a clock and if they made sufficient contact in order to observe rhythms.

Cells from surface embryos show a robust rhythm (21.5-fold difference) peaking at ZT3, but with high amounts of transcripts also observed at ZT21 and a subsequent trough at ZT9 (Figure 4.14a). Unfortunately, when the cells are subjected to 24-hours of dark after 3 LD cycles, we see a complete dampening of rhythm, which can either be explained by a lack of endogenous circadian rhythm or that the cells are not making sufficient amount of contact. In Pachón, *per1* cycles with a possible peak at ZT3, but due to the variation in the biological replicates, we only see significance between ZT15 and ZT9/ZT21 (Figure 4.14b). In contrast to surface cells, Pachón cells maintain the rhythm between CT9 and CT15/CT21. In Chica, *per1* appears to cycle both in a LD environment and in subsequent DD, yet the variation between the biological replicates is too great to give a statistically significant peak and trough when subjected to a Bonferroni correction (Figure 4.14c). Tinaja *per1* expression looks similar to that of the other two cavefish, with a peak at ZT3, but with high amount of transcript also present at ZT9, and a subsequent cycle observed in DD (Figure 4.14d). It is again worth noting that common for all cavefish, is that they display a much lower amplitude rhythm than surface fish. Pachón show a 5.2-fold difference in LD and a dampened 2.4-fold difference in DD. Tinaja peak-trough gives a 2.6-fold and 2.3-fold in LD and DD respectively.

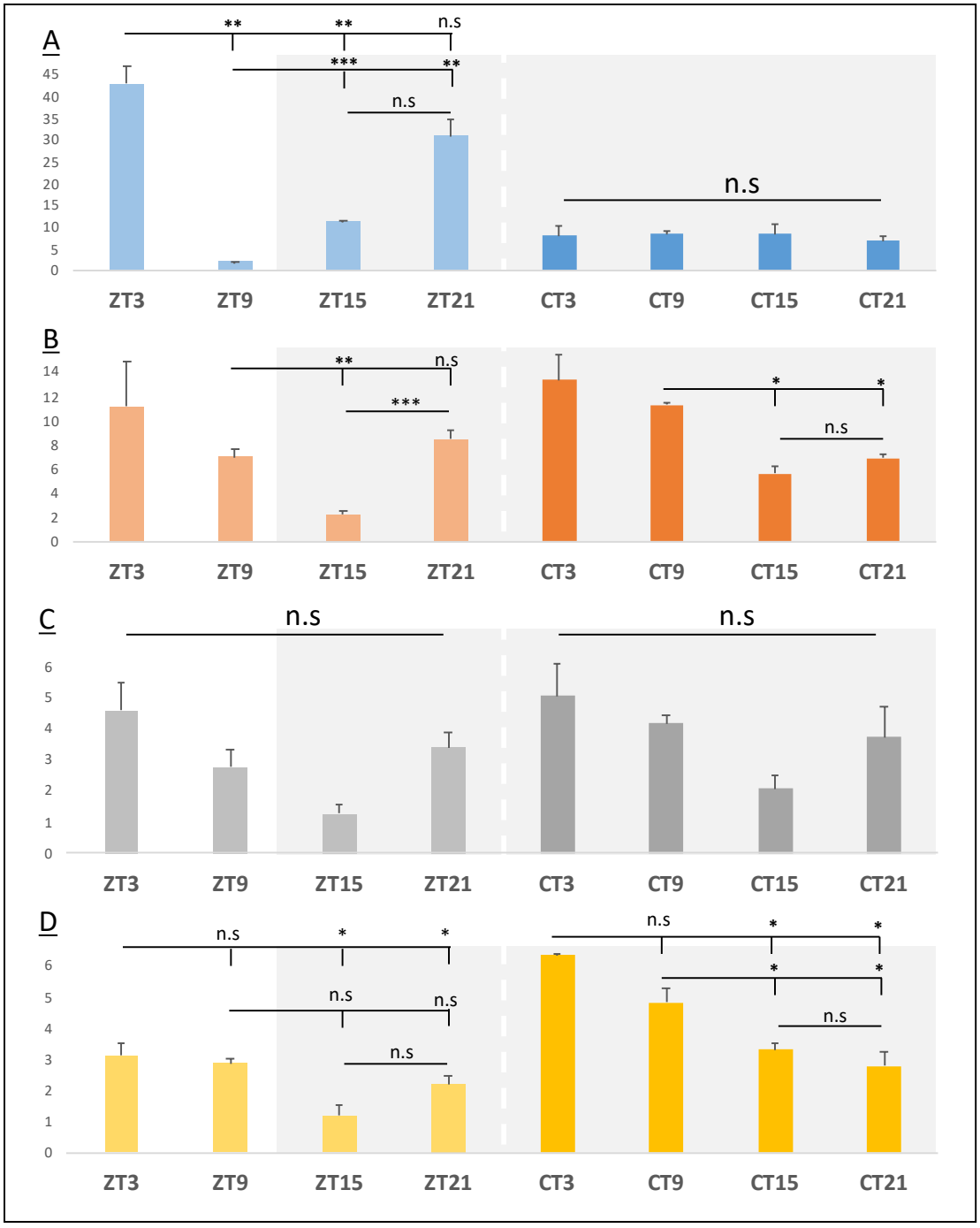


Figure 4.14: *per1* expression in cavefish cell lines in LD and LD into DD

A) Surface B) Pachón C) Chica D) Tinaja *per1* expression pattern under LD (light coloured graphs) and DD (darker coloured graphs). Data is plotted as relative expression (RE) to the lowest expressed timepoint and normalised to the reference gene *rpl13α*. X-axis is given as zeitgeber time (ZT) and circadian time (CT). An ANOVA was performed (α 0.05), and significance was assessed with a Bonferroni post-test with Holm correction (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Data represent the mean ± SEM (n=4). (Note difference in Y-axis).

4.2.10 Circadian clock targets cycles in cell culture

In other vertebrates, cyclin-dependent kinase inhibitor 1 (*p21*) is one of several circadian regulated genes involved in the cell cycle (Laranjeiro et al., 2013). . *p21* and the related *p20* gene appear to be essential for the clock regulation of DNA replication, or S phase timing. *p21* is a major target for *p53*, which in turn can arrest cell cycle at the G1/S point and thereby minimize the amount of DNA damage (Laranjeiro et al., 2013; Tamai et al., 2012). It is generally thought that by restricting the DNA replication event, when DNA is at its most vulnerable to UV damage, to the dark period of the day, rather than the light, UV induced DNA damage is minimized. As the cavefish live in complete darkness, it is indeed interesting to study such basic cell biology to see if *p21* has been uncoupled from *per1* regulation. To determine if *p21* is still regulated by the circadian clock, expression of *p21* was examined in all 4 different populations of cavefish, as above. Surface fish shows a clear peak at ZT21 and trough at ZT9 (11.8-fold difference), but no cycling in DD (Figure 4.15a). Pachón also shows a ZT21 peak, but a trough 6 – hours later at ZT15 and which is also present in DD, with a 6.5- and 1.7-fold difference respectively (Figure 4.15b). Neither Chica nor Tinaja show a rhythm (Figure 4.15c,d), however, there are hints that there may be a rhythm in LD, but more biological replicates would be required

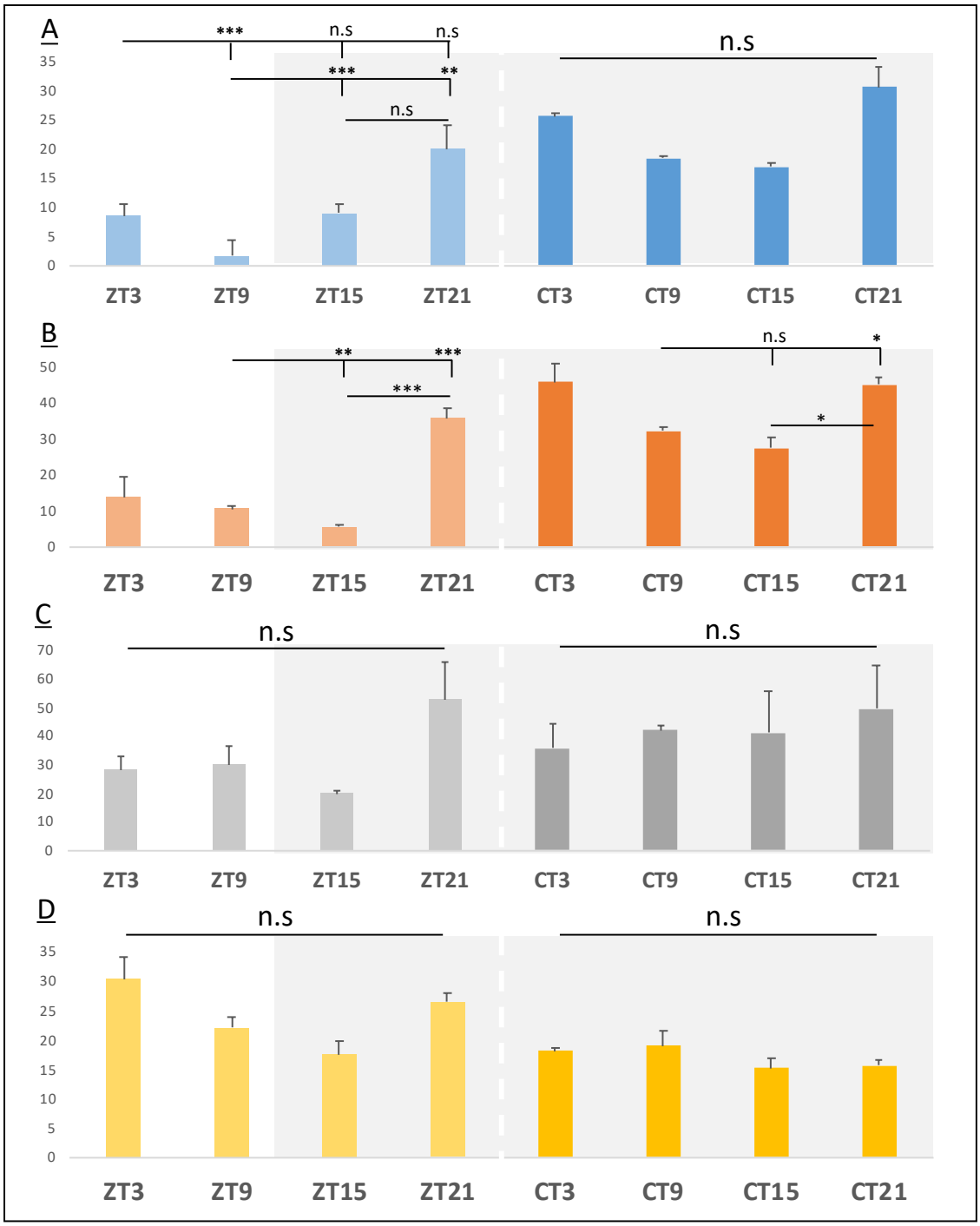


Figure 4.15: *p21* expression in cavefish cell lines in LD and LD into DD A) Surface B) Pachón C) Chica D) Tinaja *p21* expression pattern under LD (light coloured graphs) and DD (darker coloured graphs). Data is plotted as relative expression (RE) to the lowest expressed timepoint and normalised to the reference gene *rpl13α*. X-axis is given as zeitgeber time (ZT) and circadian time (CT). An ANOVA was performed (α 0.05), and significance was assessed with a Bonferroni post-test with Holm correction (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Data represent the mean ± SEM (n=4).

4.3 Discussion

4.3.1 Advent of light induction

Astyanax mexicanus has established itself not only as a major model system with which to study evolution, but also to examine circadian clock function and light regulated biology (Beale et al., 2013, Bradic et al., 2012, Strecker et al., 2003). How the circadian clock develops or "begins to tick" is a fundamental question in circadian biology. Clock function and light-dependent biology is absolutely crucial for healthy development in most teleost species, so what happens in an animal that develops in darkness and in fact never experiences light? Are there differences in the development of the clock mechanism in cave strains, which might shed light (no pun intended) on how the circadian system has altered following evolution in a dark environment?

In this study, we have explored clock and light responsive biology during the early developmental stages of *Astyanax mexicanus.* We have shown that there are differences in the development of the light response of surface and cave strains, as well as differences between the amplitude and phase of light-inducible genes under rhythmic conditions. Surface fish become light responsive during the first 5–8 h of development (Figure 4.1 a-c), similar to that reported for zebrafish, and before the development of any tissue or light responsive organs, such as eyes or the pineal gland. This early light sensitivity is similar to that described in zebrafish (Tamai et al, 2004). In contrast, the fold induction of light-inducible genes is reduced in Pachón embryos and is slower to develop. The *per2* genes do not show a light induction until 14–17 h (Figure 4.1d, e). In the case of the *per2* genes, this lack of induction is most likely the consequence of the fact that basal expression levels are raised in early Pachón embryos, leaving little range for a further light-driven increase. One argument is that this indicates that there may not be a delay in the development of actual light sensitivity. However, the data collected for *cry1a* expression does not suffer from the same issues, with basal levels being very similar between surface and Pachón, yet there is a clear developmental delay with *cry1a* induction not showing any light response until 23–26 hpf (Figure 4.1i). As such, it is interesting to note that light induction in Pachón develops at different stages for *per2* genes and *cry1a*, and that the increase of light induced transcript appears later for *cry1a* than *per2*. 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 (Figure 4.1). These results strongly suggest that there is a different mechanism involved in *per2* and *cry1a*'s response to light in Pachón. This might not be unexpected as the transcriptional regulation of *per2* and *cry1a* in zebrafish has also previously been shown to differ (Mracek et al., 2012). Although there are no matching light pulsing data for Tinaja and Chica embryos, *per2a* and *per2b* data from the LD cycles (Figure 4.5 and 4.6) as well as the LP data for Chica (Figure 4.4) suggest that there is a delay in the development of light response in these cave populations as well.

The underlying mechanism for this delayed development in light sensitivity is not yet clear, but could reflect alterations in any aspects of the signalling pathway, including the expression of the relevant opsins. Future studies could explore opsin expression in early embryo stages and determine if there are developmental timing expression differences between strains. What is clear is that *per2b* expression, when examined by *in situ* hybridisation, shows that there is a global response to light in both in surface and Pachón larvae (Figure 4.3). 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. We also observe a clear light response in embryos present before 17 hpf, which shows that it is not the pineal gland nor a delay in its development that mediates the light-induction of clock genes in *Astyanax* (Figure 4.3f-i). It is worth mentioning that there are no readily apparent morphological differences in the developmental rates between cavefish strains. This is in line with and expected from observations in zebrafish, where the mechanism of light detection is present throughout the embryo and is not restricted to central photoreceptive structures. This does not imply that the light responsiveness of the pineal gland is irrelevant, just that light sensitivity is a global fish-tissue phenomenon.

4.3.2 Absence of 'rescue'

Cave-cave hybrids are able to 'rescue' a number of degenerate features of cave animals, such as Pachón/Tinaja and Tinaja/Molino, which produce embryos with larger eyes than either parent, and Molino/Curva hybrids which are extensively pigmented (Borowsky, 2008, Jeffery, 2009). On the contrary, crosses of Pachón/Molino and Pachón/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).

Using a similar experimental paradigm, we examined the genetic basis of the absence of light-response in early embryos. Hybrid F1 embryos of Pachón and Chica are not light-responsive between 5 hpf and 8 hpf, just like the Pachón and Chica F0 embryos themselves (Fig. 3). 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 have been geographically isolated from each other for several million years, and so this result means a similar alteration in the light input pathway has evolved convergently, in the same way as albinism (Bradic et al., 2012, Protas et al., 2006). In the future, it would be interesting to expand this number of crosses between many different cave populations, firstly to determine if the surface-like response is recovered, and secondly to see if the alterations of the light input pathway in the different cavefish strains are due to selection or drift.

4.3.3 Cave strains show differences in light inducible genes during development

The light input and regulation of the circadian clock is one of the fundamental aspects of circadian biology. Studying and comparing the different expression patterns of light inducible genes in cave strains can give us some insight into what kind of evolutionary changes and adaptations to a life in the dark.

When examining the cavefish light response during development, we discovered that there is a great variation in expression patterns and maturation of light response across the different populations. The thing the cavefish have in common, is that they show an overall increased level of *per2a* and *CPD phr* transcript when raised in DD compared to surface fish (Figure 4.9), and the amplitude is shallow compared to surface embryos. This supports the hypothesis that cavefish has a set of light inducible genes (in this case *per2a* and *CPD*) tonically turned on, as if they lived in constant light, but also that other light inducible genes like *per2b* remains expressed at a low level. Similar findings were made by Beale et al. (2013) using

caudal fin clips from adult fish, where it is clear that certain, but not all light-inducible genes are turned on tonically in the dark.

Surface fish become light responsive during the first day of development (Figure 4.1), and *per2* and *CPD phr* start cycling with the same phase, ZT3 from the first (*per2a*) and second day (*per2b* and *CPD phr*) of development on a light-dark cycle (Figure 4.6a, 4.7a and 4.8a). In contrast, the different strains of cavefish, there is no common phase or even periodicity between the cave strains or between the genes. Chica embryos, show a great variation between biological replicates, with only *per2a* cycling with a 24-hour period during the first 3 days of development (Figure 4.6c, 4.7c, 4.8c). Tinaja on the other hand, has a long 30 h period for both *per2a* and *per2b*, with the first peak at ZT3 (39 hpf) and the second peak at ZT9 (69hpf) (Figure 4.6d and 4.7d), and no rhythm for CPD (Figure 4.8d). Pachón show robust *per2a* expression peaking at ZT9 from 15 hpf (Figure 4.6b), but the share *per2b* expression pattern with Tinaja (Figure 4.7 b,d).

What is the possible explanation of this rather complicated looking set of expression data? These data represent quite a complicated set of regulatory pathways, where we are integrating light input, clock regulation, and developmental differences in expression all acting on a set of target genes. There is clearly a difference between the expression patterns between *per2a* and *per2b*, which is especially apparent in constant darkness. In all cave strains, *per2a* shows significantly raised levels of transcript whilst *per2b* basal expression is the same as surface except for Chica which show increased amount of transcript (Figure 4.7 and 4.9). Taken together with the difference of expression patterns in DL, this clearly shows the two genes are regulated differently in the cavefish. This might indicate that the two different variants of *per2* are likely to play different roles in the clock and light-input pathway, although the precise mechanism is not yet clear (other model species like zebrafish only has one *per2* gene). It seems apparent that both *per2* genes in surface fish are primarily light-regulated. This is shown by the low expression in constant dark, and the nature/waveform of the rhythm on a light-dark cycle. However, in adult Pachón and Chica, the light inducible genes *per2a* and *CPD* continues to oscillate in the dark after being exposed to a LD – cycles (experiments of *per2b* were not performed at the time) (Beale et al., 2013). Furthermore, the phase difference between surface and cave strain suggests that the *per2* genes are now much more under the control of the circadian clock itself, in addition to an acute light input. This hypothesis also fits with the development of the clear 6-h phase difference, seen on day 2 or 3 of development, when a robust clock is beginning to function

in *Astyanax*. At first this would seem to make little sense, as exposing cave strains to a light-dark cycle is obviously an anomalous situation, and one they would not normally experience in nature. However, in cave populations clearly light-regulated genes would never normally be induced, due to the total darkness. So, by evolving additional regulation by clock-components, even in the dark, expression of these genes will be turned on, though they will not show daily rhythms in the cave. Perhaps these cave strains are using clock regulatory factors not to generate a rhythm, but to increase tonically the expression of genes that would normally be turned on by the light. Many of the core circadian clock components are rather fundamental transcriptional regulators and so it is not surprising if their function is co-opted into different regulatory roles after a million years or so of evolution. The phase differences we report are, in fact, effectively an artefact of "seeing" a rhythmic light-dark cycle. The proof of this, of course, would be to find a change in the promoter/regulatory regions of the *per2* genes that gives them this circadian "gain of function". What is clear is that there are individual cave strain alterations in each population, with differences in light sensitivity, clock function and possibly developmental events as well.

4.3.4 Development of the clock

In zebrafish, the molecular clock becomes functional from the first day of development (Dekens and Whitmore, 2008). In cavefish, however, we do not observe a robust daily oscillation in *per1* gene expression in either surface or cave populations until the third day of development (Figure 4.5a-c). This is an even more surprising result considering the fact that *Astyanax* embryos develop significantly faster than zebrafish larvae, under the same conditions. *Astyanax* larvae hatch after 24 hours, whereas zebrafish can take 3-4 days, yet they are "clockless" for those first few days of independent life. Just like we see a variation in light inducible genes, we see variation in cavefish *per1* expression, but it is worth noting that the entrained molecular period is about 24-hours long. The surface embryos show shallow *per1* oscillations from the first day of development that peaks at ZT3. However, during day three, we see a change in phase angle and amplitude similar to what we see in adults. This change in clock phase during development to an adult timing condition is quite an unexpected and unusual observation. Though we do not yet know the precise mechanism underlying this phase shift, it suggests that the clock mechanism itself undergoes a developmental maturation over the first three days of development, with perhaps not all of the components to form a robust clock being present until day three onwards. Though the

zebrafish clock appears to cycle earlier than the *Astyanax* pacemaker, there is also evidence that it too might not become fully functional until day 3–4 of development, as many clock output genes do not become rhythmic until this considerably later developmental stage (Laranjeiro and Whitmore, 2014).

Chica gives one significant peak at ZT21, after what looks like several non-significant "peaks" at ZT21. Tinaja has no apparent significant peak until 75/81 hpf (ZT15 or ZT21) and taken together with delay in expression of light inducible genes, we are reluctant to conclude that this is the peak of an actual circadian cycle in Tinaja embryos. Further cycles are required for later developmental stages to determine this. Pachón is the only cavefish that shows significance beyond a full 24 h cycle, with two matching troughs, with *per1* peaking at ZT3 (63hpf), the same phase as in adult Pachón (Figure 4.5b).

Summarising these findings, it is safe to say that there are clear differences in both light and clock components between surface and cavefish, but more interestingly, there are also differences and similarities within the different populations of cavefish. Tinaja is the population that is the slowest to develop a light response, and significantly it is also unclear if the embryos even have a clock after 3.5 days of development. In contrast, Chica seems to display some very low *per1* rhythm from very early on in development, with corresponding low amplitude *per2a* rhythm, but no rhythm in *per2b* or *CPD phr*. Pachón shows the highest over-all robust amplitude for both clock and light inducible genes, and arguably also the earliest of the 3 cave strains to develop a full light response for all genes explored.

The data presented here supports the original working hypothesis, which suggests that as an adaptation to living in the dark arrhythmic environment, some light inducible genes are tonically increased in the dark. Previous work in zebrafish has shown that constant light 'stops' the circadian oscillator. *Per2* and *cry1a* are involved in the entrainment of the clock to light and the maintenance of high amplitude rhythms, while overexpression of both these genes mimics constant light conditions (Dekens and Whitmore, 2008, Tamai et al., 2007, Ziv and Gothilf, 2006). Therefore, the reduced amplitude and timing of the embryonic cavefish clock could, like adults, be a consequence of changes within the light input pathway. Analysis of the coding sequence has shown a high conservation between the different cave strains (Beale et al., 2013). Therefore, again we hypothesize that the changes in the promoter regions, enhancing the expression of *per2a, per2b* (in Chica) and *CPD* in the absence of light.

It is clear that a comparative genomic, as well as full transcriptomic analysis is long overdue for the different types of cavefish. A future full transcriptomic analysis of cave strains, both rhythmically and in response to light, will demonstrate the full extent of these evolutionary changes. As a full comparison of genomic sequences will allow us to demonstrate that there are clear alterations in the enhancer/regulatory regions of these central clock and light responsive genes.

4.3.5 Cell cultures have the potential to be a great circadian tool

Cell cultures are a much-loved tool across biological disciplines. Previous attempts have been made at making stable *Astyanax mexicanus* cell lines, but as of March 2021 there are no publications relating or mentioning the creation of stable cell cultures. Here, I describe some circadian characteristics of 4 different cultures that have been passaged for several months prior to experimental examination.

The data presented here (Figure 4.10-11) clearly demonstrates that *A. mexicanus* cell cultures are light responsive, but is the expression pattern similar to what we see in adult fin clip data or the developing embryo? Table 4.1 summarises the key circadian features for each gene and cave population in adult fin clips, cell culture and developing embryo. In surface cells the light inducible genes show a near identical expression pattern to the embryos and adults, with expression peaking at ZT3, and low basal expression in the dark phase and complete darkness (Table 4.1). The only discrepancy between surface cells in culture, adult fin clips and developing embryos is the amplitude of the rhythm, still it is however generally much higher than the cave strains. In the different cave populations, there are much more variation, not only in amplitude, but also in peak/trough expression time.

	Surface			Pachón			Chica			Tinaja		
	Dev	Cell	AdF	Dev	Cell	AdF	Dev	Cell	AdF	Dev	Cell	AdF
Peak (*per2a*)	ZT3	ZT3	ZT3	ZT9	ZT9	ZT3	(ZT3) (ZT9)	(ZT3) (ZT9)	ZT3	(ZT3) (ZT9)	ZT9	N.D
Trough (*per2a*)	ZT15 ZT21	ZT15 ZT21	ZT15 ZT21	ZT15 ZT21	ZT15 ZT21	ZT21	ZT15 ZT21	ZT15 ZT21	ZT21	ZT15 ZT21	ZT15 ZT21	N.D
Fold diff (*per2a*)	~18	13.8	~10	~4.1	2	~7.5	3.6	2.5	~7	1.5	2.8	N.D
Raised basal DD	n/a	n/a	n/a	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	N.D
Peak (*per2b*)	ZT3	ZT3	N.D	(ZT3) ZT9	ZT9	N.D	N.R*	(ZT3) (ZT9)	N.D	(ZT3) ZT9	ZT9	N.D
Trough (*per2b*)	ZT15 ZT21	ZT15 ZT21	N.D	ZT15 ZT21	ZT15 ZT21	N.D	N.R*	ZT15	N.D	ZT15 ZT21	ZT21	N.D
Fold diff (*per2b*)	~5.4	4	N.D	23.7	1.8	N.D	N.R*	2.5	N.D	~2.2	1.6	N.D
Raised basal DD	n/a	n/a	n/a	No	No	N.D	Yes	No	N.D	No	No	N.D
Peak (*CPD*)	ZT3	ZT3	ZT3	(ZT3) ZT9	ZT9	ZT3 (ZT9)	(ZT9)	(ZT3) (ZT9)	ZT3 (ZT9)	N.R*	(ZT3) (ZT9)	N.D
Trough (*CPD*)	ZT15 ZT21	ZT15 ZT21	ZT15	ZT15 ZT21	ZT15 ZT21	ZT15 ZT21	ZT15 ZT21	ZT15 ZT21	ZT15 ZT21	N.R*	ZT15 ZT21	N.D
Fold diff (*CPD*)	~4.7	9	~7.5	~4.6	3.5	~11	6.4	3.1	~8	N.R*	2.9	N.D
Raised basal DD	n/a	n/a	n/a	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	N.D
Peak (*per1*)	ZT21	ZT3 (ZT21)	ZT21	ZT3	ZT3 (ZT9)	ZT3	ZT21	N.R*	ZT3 (ZT21)	N.R	ZT3	N.D
Trough (*per1*)	ZT9	ZT9	ZT9 ZT15	ZT15	ZT15	ZT15	ZT3 ZT9	N.R*	ZT15	N.R	ZT15	N.D
Fold diff (*per1*)	~16	21.5	~30	12	5.2	~6	3.7	N.R*	~6.5	~2.5	2.6	N.D
Rhythm LD->DD (*per1*)	N.D	N.R*	Yes	N.D	Yes 2.4	Yes	N.D	N.R*	Yes	N.D	Yes 2.3	N.D

Table 4.1 A comparative summary for most of the circadian data on 4 different populations of cavefish

Brackets around zeitgeber time indicate uncertainty of exact timing

Dev - Developing embryo

Cell - Cell line

AdF - Adult Fish

N.R - no rhythm

n/a - not applicable

N.D - no data

* - indicates that there is a likely explanation for why we do not observe a rhythm

~ - indicates that fold difference has been measured over several peak-trough time points.

Cave cells are also light inducible, but there is some variation in timing between embryo, cells and fin clips. In Pachón, *per2a* and *per2b* expression patterns are relatively similar between embryo and cell, whereas we the fin show a peak at ZT3 rather than ZT9 for *per2a*. *CPD phr* timing is the same across Pachón cells, fin clip and embryo (Table 4.1). Chica shows some variation in expression patterns between embryo, cell and adult fin clips. *Per2a, per2b* and *CPD phr* expression are high both at ZT3 and ZT9 in embryo and cell, but shows a peak at ZT3 in adult fin. *Per2b* expression show no significant rhythmic expression pattern in the developing embryo, though with more replicates there might be a rhythm matching that of the Chica cells (Table 4.1). Chica also showed a significantly upregulated amount of *per2b* during embryonic development in DD, something that we do not observe in cell lines, but it is worth noting that in contrast to the cell lines, the embryos in DD had never been exposed to light. Tinaja embryo and cell expression patterns match pretty well for *per2a* and *per2b* peaking at both ZT3 and ZT9, there is no adult fin clip data to compare to. The same rhythm is seen in *CPD phr* in Tinaja cells, in contrast there is no *CPD* rhythm observed in Tinaja by 81 hpf (Table 4.1), though that does not mean that it is not present later on in development. The low levels of *CPD phr* expression are quite striking in Tinaja, assuming that these expression levels are turned into corresponding low levels of protein. It raises interesting questions of how these cells repair damaged DNA and whether they are much more susceptible to DNA disruption. The biggest discrepancy in timing in cave populations, is between adult and cell/embryo, where all light inducible genes, across all populations peak at ZT3, and the amplitude is also generally higher, while we see high expression at both ZT3

and ZT9 in cave population (Table 4.1). So how do we account for these differences and are they relevant to keep in mind when we decide on the suitability for the cell line?

First of all, a direct comparison may not be fair or relevant for several reasons. Adult fish were maintained in aquariums, and cells and embryos in temperature-controlled water-baths, the lighting conditions were not replicated. The adult fish would have been subjected to a 14:10 LD cycle and the embryos and cells to a 12:12 LD cycle with higher intensity light. which may account for the high expression through-out the light period in cells and embryo. Secondly, tissues may have different amplitudes and timings, although this has not been examined in cavefish, but the idea of different light sensitivities in different tissues has been explored in Chapter 1 in zebrafish in this thesis. The depth of background research in *Astyanax* lags significantly behind that in zebrafish, in many areas not only circadian. Consequently, we have had to make a series of assumptions about the circadian system in cavefish, based logically on prior zebrafish studies, but lacking detailed examination in *Astyanax*. This includes detailed studies on the roles of specific clock genes, as well as the presence and timing of peripheral tissue clocks.

The cavefish cell lines are light sensitive, but what about clock function? Adult surface fish fin clips and embryos show peak expression of *per1* at ZT21, whereas surface cells peak at ZT3, but with high amount of transcript also seen at ZT21, this is also the case for *p21* (Figure 4.15a). Cells in DD do not show a *per1* rhythm, and neither does *p21*, but the expression pattern match the LD pattern, which may indicate that significance is a question of replicates. However, this lack of free-running DD rhythm is certainly unexpected and concerning. All zebrafish cell lines produced to date show robust circadian oscillations in gene expression. The above result suggests that cellular clocks might not be such a widespread phenomenon in *Astyanax* larvae as in zebrafish. This issue needs serious further investigation. From the data shown in this chapter, we know that light sensitivity is widespread, from *in situ* hybridization data. This fits well with the cell data we have obtained. So one possible explanation for the surface fish cell line data is that all cells in the developing embryo may well not be rhythmic. Pachón cells show the same ZT3 peak across embryo, fin clip and cell cultures, and also show the same *per1*cycling in the dark (Table 4.1). The same applies to *p21* expression in LD and DD (Figure 4.15b). Chica does not show a *per1* or *p21* rhythm in cell culture. This could be an issue of repeats and with more replicates, it is likely that we would see a clock rhythm in LD and DD, peaking at ZT3, which match the rhythm in

adult fin clips. Tinaja also show a rhythm peaking at ZT3 in LD and DD. In summary, we have data indicating that the three cave derived cell lines are light responsive with a functional clock, but that the surface derived cells are light responsive only, rhythmic on a LD cycle, but with no endogenous *per1* molecular clock. Is it really the case that the cavefish cell lines have a clock, but there is no clock in surface cells and that this clock is also out of synch with the clock we see in embryos and adult fish?

As mentioned above, it is possible that cells in tissues in this species either have differently timed/phase rhythms or possibly in some cases none at all. Equally, there could be technical issues with the selection and type of cell that simply survives and proliferates optimally in cell culture. If we examine the expression of Micos river fish in the field, an experiment with more time-points, we actually see that the peak expression is not at ZT21 or ZT3, but at ZT0. As there have never been any experiments examining clock and light induction with high resolution time-points in different organs, we simply do not know if the different organs in cavefish, and therefore different cell types may show different phases. When it comes to addressing the lack of cycling in DD, we believe that this could also be due to issues of cell confluency. Data on zebrafish PAC2 cells have shown that cells that are too sparsely seeded, and thereby do not make contact, result in poor synchronisation in endogenous rhythms and thereby no visible *per1* expression pattern in DD. To confirm these speculations, the experiments should be replicated, ensuring that cells are confluent at the time of the experiment. If these experiments were however to replicate the findings presented here, more investigations will have to be made, such as single cell sequencing, to confirm that the cells in culture do not have a clock, or lack expression of key clock components. This result emphasises the basic issue with cell lines, that they are an excellent tool for research, but may not reflect accurately the biology that actually occurs with an organism.

In conclusion, I believe that the cell lines have shown that they have potential, but that they would benefit from more passages and selections, to attempt making monoclonal, single layer cell lines, which would be easier to work with. Subsequent sequencing to identify possible cell types could also be beneficial. With some additional experiments and tweaks, we believe that these cell lines can be a useful future tool, both for circadian studies, but also for as a tool studying other basic cell biological adaptations to a cave environment, especially in a species where adult animals are rare and cannot easily be sacrificed.

5 Clock and light sensitivity in deep-sea fish

5.1 Introduction

"We know less about the deep sea than we know about the surface of the moon"

Animals that live in the absence of sunlight can either be found in subterranean caves or the deep-sea. For fish that live in caves, their habitat, interactions and genetic mixing is very limited and controlled. We can identify where they move, what they eat, mating activity, life cycles, what external factors affect them etc., in other words, it's a quite isolated and controlled environment. In the open ocean however, we enter a system that is huge, has many variables, high predation pressures, currents, mixing of world oceans etc. Other than the absence of sunlight, these environments are completely different.

Typical troglomorphic traits include loss of pigment and loss of eyes. However, this is not the norm for the animals of the deep sea. Rather, large eyes and sophisticated silvering and pigmented bodies are common despite the lack of sunlight. This is generally explained by two factors: the presence of bioluminescence in many species, where animals use light organs on their bodies to communicate, avoid predation or to attract prey, meaning that the dark world is not necessarily quite so dark. The other reason is that many animals also live on the boarders of the mesopelagic and bathypelagic zone, often migrating up during the night to feed, possibly catching some photons of the sun. But can such incredibly dim light set the clock of deep-sea fish, do they respond to light and do they have a classical circadian clock? To answer these questions, we choose to examine two different species of hatchet-fishes, one predicted vertical migrator, *Argyropelecus hemigymnus* and one that supposedly prefers deeper waters, *Sternoptyx diaphana*. To say that not very much is known about these fish is an understatement, with the exception a few papers describing their eye morphology. In general, the data is very thin on deep-sea animals, especially vertebrates, so in order to summarise what we do know about the habitat, behaviour and physical attributes of our species of interest, we have to examine data ranging from fish to zooplankton, which is hugely unspecific, but represents the only available data. Hopefully, it will provide a stage for which we can understand the rhythmic or non-rhythmic behaviour of our hatchetfish and underlying molecular biology driving this biology.

5.1.1 The Vertical Dimension

The open ocean is in essence ocean that is not coastal, and although it all looks the same from the surface, it can be incredibly diverse and is generally divided into 5 different aquatic layers (Figure 5.1) (Kingsford 2018). The top 50 metres of the epipelagic zone is classified as the photic zone (also known as epipelagic, euphotic and sunlight zone), and is the only zone where light is penetrating sufficiently to facilitate photosynthesis. Light is rapidly attenuated (Figure 5.2), and below 200m, less than 1% of light penetrates, before it is completely attenuated at 1000m, giving rise to the twilight/mesopelagic zone (Haltrin 1998). Below 1000m there is no sunlight, but the dark deep is subdivided into 3 further regions. Between 1000m – 4000m we find the bathypelagic zone a place where there is still quite a lot of biomass, but it is also usually at these depths that we find the oxygen minimum zone (OMZ). It is over the mesopelagic and down into the bathypelagic zone that we find our hatchetfish of interest. Then there is the abyssopelagic zone from 4000m- 6000m, which is much less dense in biomass. Finally, with even less biomass, there is the hadal zones which are the deep-sea trenches which are associated with the subduction of tectonic plates. There are only 20 major trenches and most are found in the Pacific, the deepest being the Mariana trench at 11 034 m (comparatively Mt Everest is 8 848m tall) (Britannica 2020).

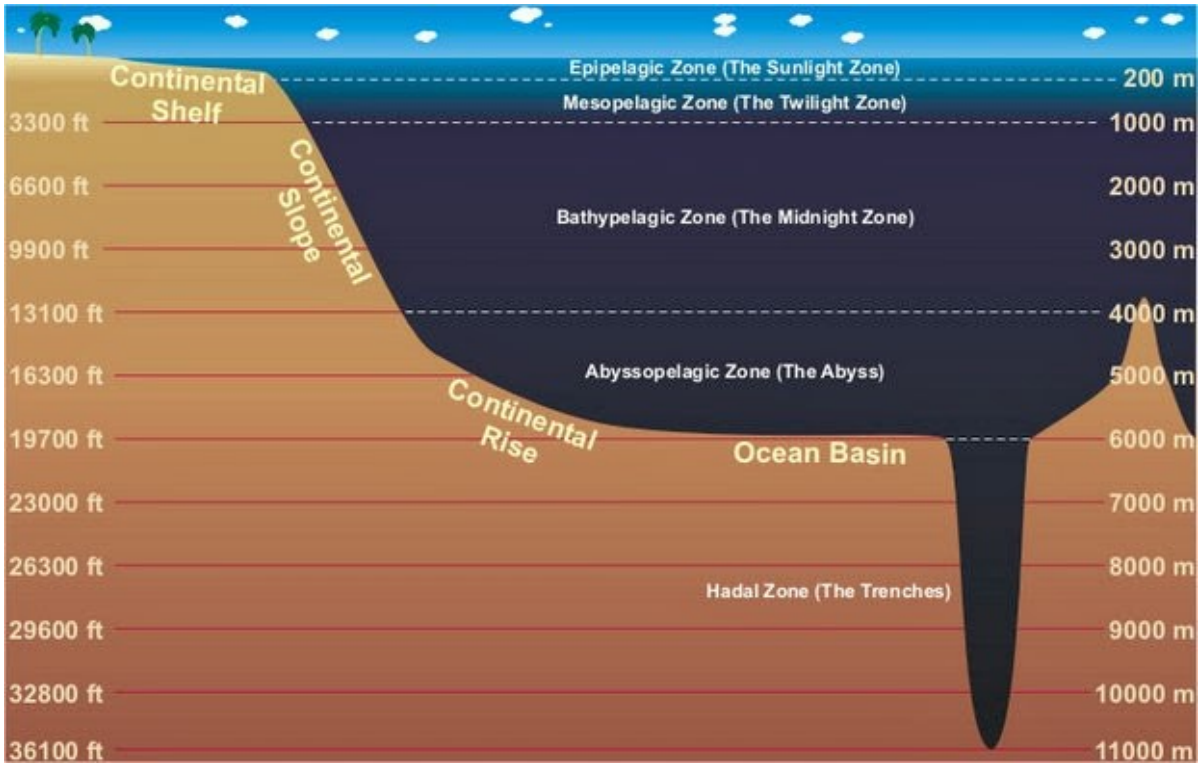


Figure 5.1 The Oceanic Zones. The open ocean is divided up into 5, vertical oceanic zones depending on light penetration (epipelagic ,mesopelagic and bathypelagic) or proximity to seafloor- abyssopelagic or trench – hadal.

5.1.2 Distribution of food in the open ocean

Phytoplankton (photoautotroph plankton) are the main, primary producers of the open oceans. The range of the phytoplankton is actually quite limited, due to most of light being reflected and rapidly absorbed, meaning that photosynthesis can only take place in the very top 50 metres of the ocean, or the top ~1% of the ocean (Uitz et al., 2006). Red light is attenuated first (Figure 5.2), which means plankton that rely on chlorophyll which absorbs red and blue, but reflects green, is likely to be found in the top metres of the ocean. In contrast, species using carotenoids absorb in the green-blue light, and may live deeper down. This in effect means that much of the micronekton and nekton can live up many thousands of metres away from the primary producers in their food web!

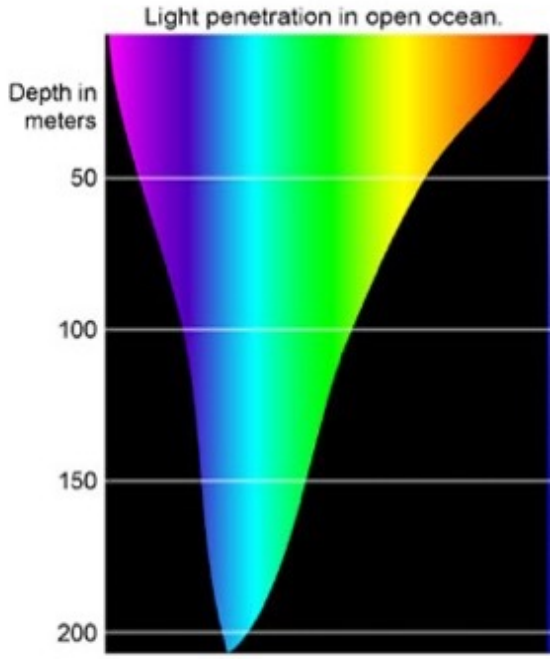


Figure 5.2 Open Ocean light penetration Red and orange light is attenuated first, whereas blue penetrates furthest

As a lot of phytoplankton does not have the ability to swim against currents, they will sink, if not eaten. It is estimated that between 5-20% of the primary production is exported out of the euphotic zone without being eaten, but only 1% reaches the abyssal floor, although this number can be markedly different in different regions depending on factors such as depths, currents and seasons (Doney 1997; Lampitt and Antia 1997; Welschmeyer and Lorenzen 1985). Because the food is so restricted to one part of the ocean, most of the open ocean ecosystems form food webs rather than food chains, with marine snow, a heterogeneous mix of debris from detritus, gelatinous material and faecal matter being a key food source for a lot of small prey animals that do not make the journey up the euphotic/epipelagic zone (Silver and Shanks 1978).

5.1.3 Diel Vertical Migration – why?

As the location of the food is so very limited to the surface, animals either have to wait for the marine snow or phytoplankton to sink down to them, or they have to relocate to where the food is, - this is called diel vertical migration (DVM). Most will be familiar with migrations of whales, sea turtles or salmon, where the animals relocate to a different part of the sea, in order to mate, feed or avoid predation. However, migrating in the vertical plane in response to the seasons or the day/night, are far more common among marine species, than geographic relocation (Angel and Baker 1982). The term diel vertical migration covers daily, vertical movements of any organism in the water column. Although this phenomenon has been known for a long time, it wasn't until the advent of the acoustic mapping technology (developed during WWII to detect submarines) that the extent of this migration was truly appreciated. DVM is actually the largest movement of biomass on the planet, and the small migrating fish alone is estimated to make up 1000 million tonnes (Irigoien et al 2014).

Why undergo DVM? The theories behind DVM revolves around food availability, predation but also respiratory demands. As a rule of thumb, the biomass is at its highest closest to the surface, which in turn generally, means that upward migration correlates with higher food availability (Angel and Baker 1982). The main consumers of phytoplankton are herbivore zooplankton. However, most predators consuming zooplankton are visual predators, thus keeping close to the surface during daylight hours means a much higher chance of being eaten. DVM permits organisms that feed on animals higher up in the water column to use the cover of night to feed, and animals that feed on these animals again, also migrate up. Predation avoidance, at least in copepods (small crustaceans and types of zooplankton), is the strongest drive for DVM- the so called "better underfed than dead" hypothesis (Bollens and Frost 1989; Frost 1988; Vourinen 1987). Several experiments undertaken in copepods support this hypothesis. One study on fjord copepods found that if visual predation is low, no DVM takes place (Ohman 1990). Another study with copepods performed in mesocosm, DVM was observed when free swimming sticklebacks were present, but when these were caged DVM was not observed. If the copepods were exposed visually to stickleback mimics during the day, then DVM was observed suggesting that DVM, at least in some copepods, seems to be a plastic behaviour as a direct response to predation (Verity and Smetacek 1996).

Furthermore, for some animals there is an ontogenetic component in DVM, which is exemplified in copepods where egg bearing females are prefer deeper waters, and show weaker DVM than females without eggs. Predators such as Pacific herring prefer egg bearing females, so although being in colder waters means slower egg development, the reduction of predation is significant (Bollens and Frost 1991).

5.1.4 Diel vertical migration – how deep?

When does DVM takes place and how far down into the sea do the different animals travel? The depth is generally thought to be limited by how far an animal can swim and how much dissolved oxygen is available. Most of the animals undertaking DVM are tiny, usually from less than 0.2 mm to 5 cm long, traveling several hundred metres is therefore a major feat. About 20 years ago it was generally believed that DVM only took place down to 700m, and the vertical distances travelled for migrators were generally of the order of 10^4 to $5x10^4$ body lengths, which translates to 50-250 m for a copepod and 500-700m for a small lantern fish (Herring 2002, Maynard et al. 1975). These findings were extrapolated from echograms such as seen in Figure 5.3, where most of the biomass move from just below 400-500 m below the surface, and then also what appears to be a more "stable" population of biomass around 800m. However, more recent studies in other parts of the sea such as the Gulf of Mexico show that DVM takes place even down to1600m (Ochoa de la Torre et al., 2013, van Haren and Compton 2013).

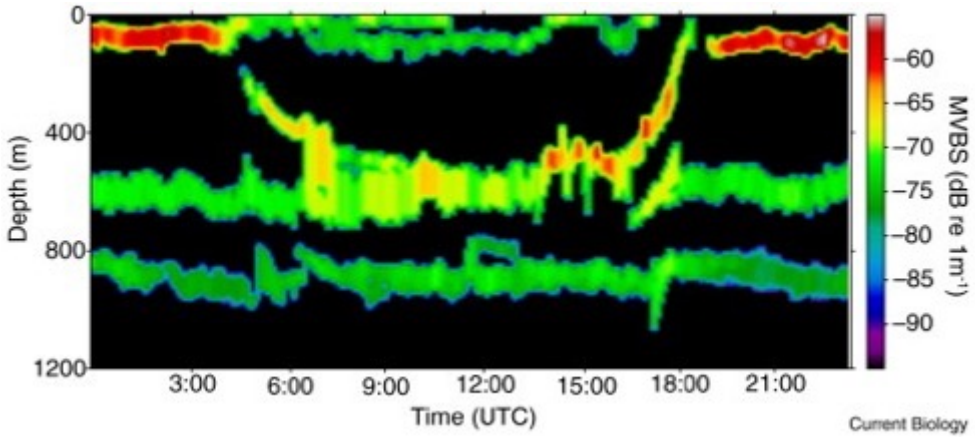


Figure 5.3: Synthetic echogram over 24 hours from the southwest Indian Ocean in December 2011. (Brierley 2014)

Echograms show that DVM timing is set to night-up-day-down for the majority of the migrating animals, probably due to the high number of visual predators. Although echograms are great for monitoring a large amount of biomass, it has low resolution, but it should be noted that it does not give us any data on individual movement. For an individual, are migrations indeed daily? How well timed is this event in a single species, what are the distance between the first and last to start migrating? How far do most species migrate? These questions are all interesting, but difficult to answer. The only way to determine if single species vertically migrate or not is through mapping depths a single species have been caught at. As no cruises have done vertical migration mapping as their sole purpose, and there have been no communal attempts collect and record catches, our best maps are still from 1971. Figure 5.4 and Figure 5.5 depicts the depth mapping of *A.hemigymnus* and *S.diaphana*, our species of interest. Perhaps in the future, when telemetry is small enough to fit on a 5 cm long fish, and we can catch the fish without killing it, we may be able to obtain individual data.

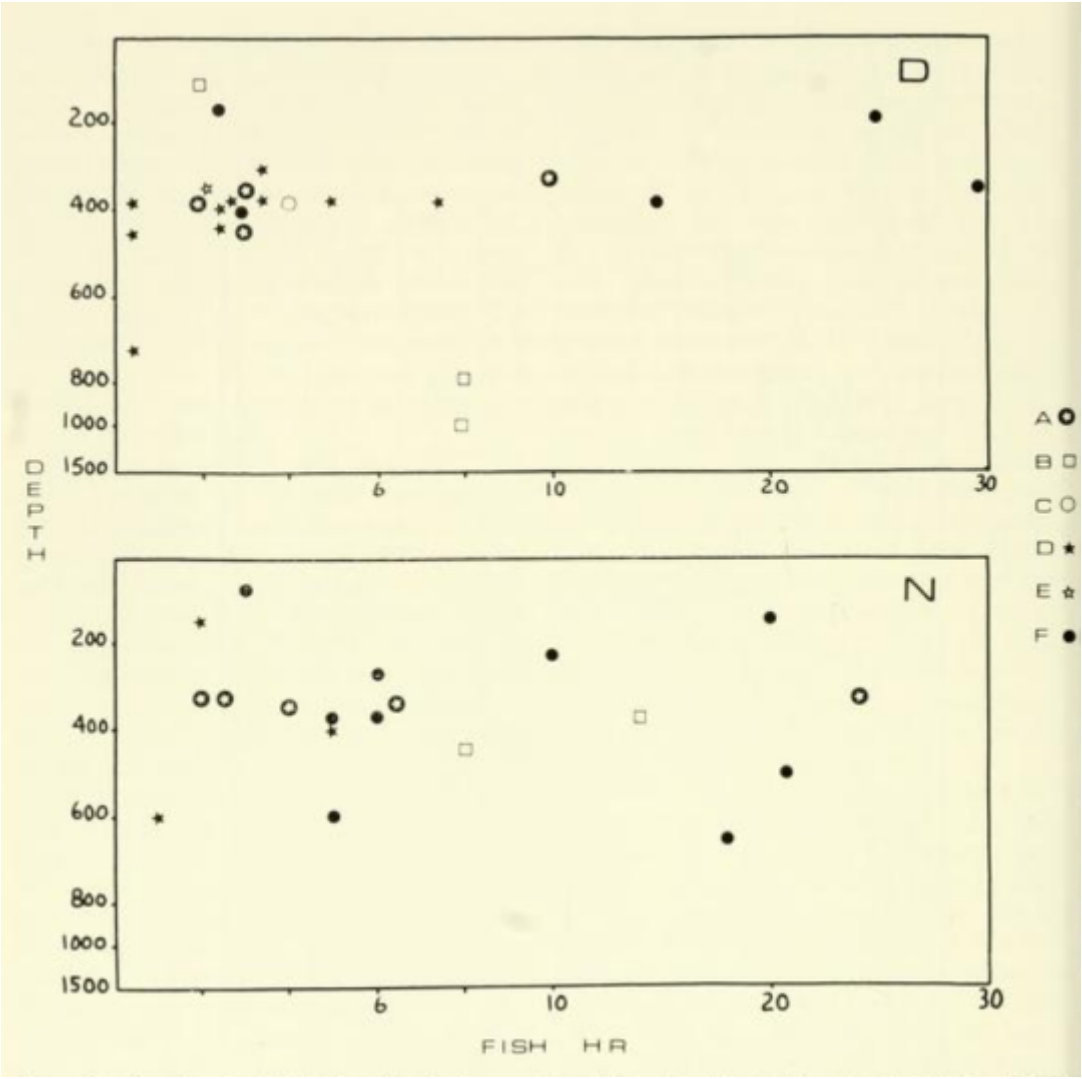


Figure 5.4 ***Argyropelecus hemigymnus*** **vertical distribution** during day (D) and night (N) from 6 separate places: A = Pacfic (California); B= Southern Ocean; C= South West Atlantic; D= Gulf of Mexico and Caribbean; E= North Atlantic; F= North East Atlantic (Baird 1971)

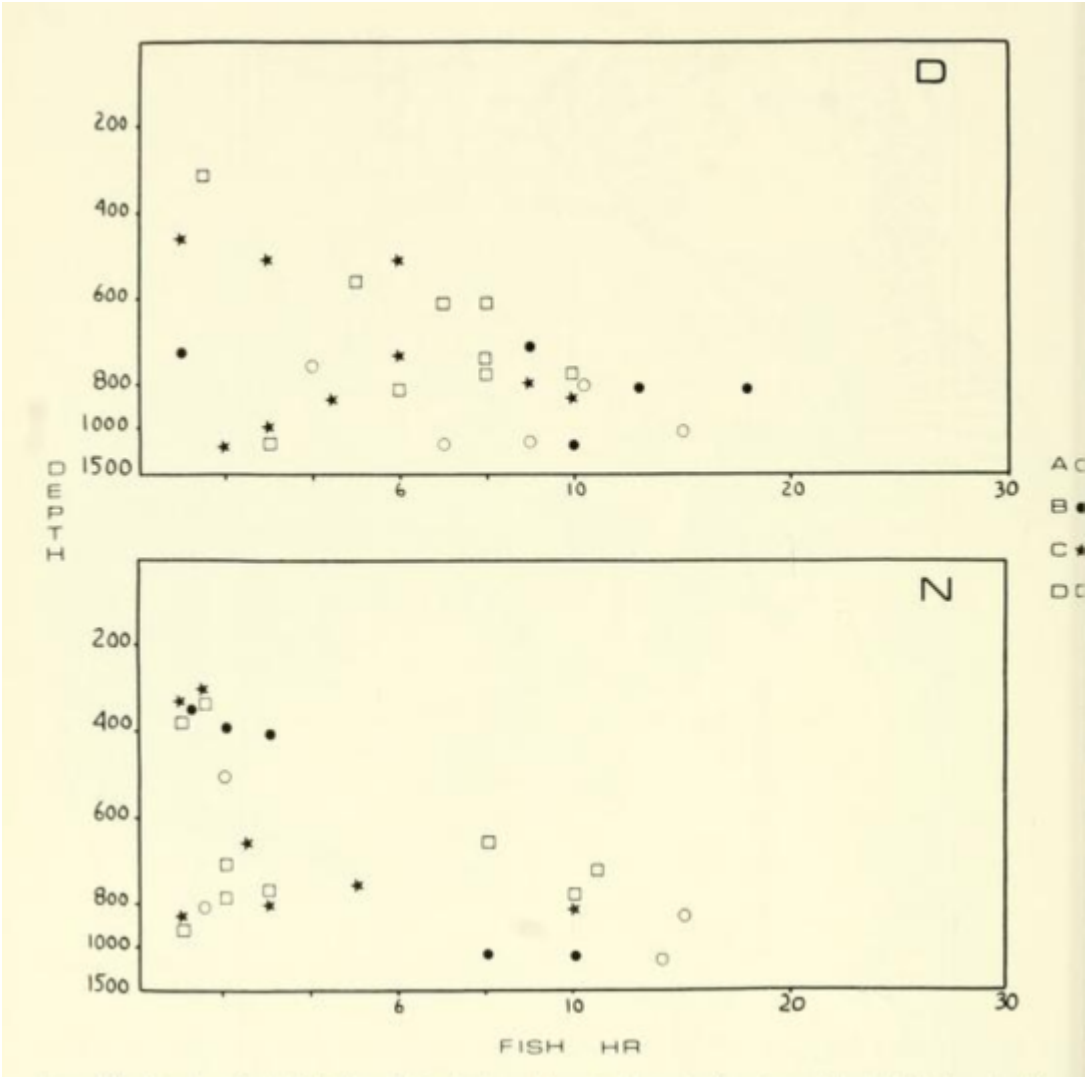


Figure 5.5 ***Sternoptyx diaphana*** **vertical distribution** during day (D) and night (N) from 6 separate places: A = Southern Ocean; B= Gulf of Guinea; C= Gulf of Mexico and Caribbean; D= North East Atlantic (Baird 1971)

5.1.5 Diel vertical migration – light or clock driven?

The mechanism or cue for DVM is a debated but not a well researched one, and has had close to no input from circadian biologists. The consensus in the field seems to be that the migrating biomass follow an absolute or a relative intensity (Ringelberg and van Gool 2003, Ringelberg 1999). It is particularly observed that animals in the epipelagic zone migrate further down if it is a particularly bright day or the moonlight is bright (Berge et al., 2009, Brierley 2014, Herring 2002, Kaartvedt *et al.* 1996). This should not come as a surprise,

considering the research on DVM shows that the cover of darkness leads to less predation. The light aversion response to higher intensities is therefore interpreted in that animals are using a set intensity as a cue for migration, or rather position in the water column. With these organisms regulating depth by maintaining exposure to a fixed light level. Equally, it could just be a standard negative phototaxis response to ensure that they are not eaten. There is no doubt that these animals are highly tuned to the light intensity, but this does not mean that they use it as a cue for DVM. If these animals are exposed to effectively a constant level of illumination how would they set a circadian pacemaker, assuming that they even possess one? It is more likely that organisms use a measure of light intensity to set the range of their migration, but an internal clock to regulate the time at which this migration will occur. In this case light is used in two ways, to set the clock and as a measure of position in the vertical water column. What many has failed to appreciate in the deep-sea community is that the circadian clock is not a rigid dominant cue for behaviour, but rather a guide for timing which can be altered by other pressures such as light intensity. It wasn't until 2017 that is was shown that epipelagic zooplankton has a molecular clock under LD and DD conditions (Häfker et al., 2017). It should be noted that these animals are living high up in the water column, with significant sun exposure.

The "constant light hypothesis" is problematic for many other reasons. Firstly, many animals are simply too small and too slow to be able to follow a constant light level, and due to the amount of clouds that may pass in a day it would also prove a very energetically demanding job to swim up and down. Secondly, we also know form a range of collated catch-data over several geographical locations that individuals of a species are not perfectly vertically distributed in the water column (Baird 1971), and due to the rapid light attenuation, different individuals will be subjected to different light intensities. The third point to why light intensity probably is not a global trigger for DVM is probably the most convincing. Echograms show that a considerable amount of biomass migrate from depths below a 1000m, i.e. depths where photons from the sun absent (Ochoa de la Torre et al., 2013, van Haren and Compton 2013), and even single species of lantern fish has been shown to have DVM from 1500 m to 20 m (Catul et al., 2011).

Behavioural rhythms observed in terrestrial and shallow coastal animals are attributed to an underlying circadian clock set by sun light. In deep-sea animals, sun light is absent or very weak, so if these animals have a clock, how do they set it? One candidate is indeed the sun but a common proposed candidate is also moonlight, especially for animals that migrate from below 1000m. Lunar cycles affect amplitude of DVM in lanternfish *Hygophum* (Linkowski 1996), while full moons has shown to delay DVM from below 1000m (Ochoa de la Torre et al., 2013). As these fish usually have eyes that are made for catching photons, rather than complex image formation, it is however possible that very dim light can set the clock (Section 5.1.7). Furthermore, it is possible that deep-sea fish are functioning more akin to nocturnal rodents, where they might only receive light exposure at dusk as they rise through the water column, or at dawn as they begin to sink to greater depths. Clock entrainment could then occur in this light pulse-type manner.

In short, with the evidence we have today, we cannot determine with certainty what mechanism that drives DVM. It may not be the same for all animals that undertake DVM, but the "constant light intensity" hypothesis does not hold up for the deeper species undergoing DVM. With the publication on molecular *per1* rhythms in epipelagic zooplankton, it has at least been demonstrated that some of these animals have a clock, whether it is driving behaviours such as DVM is currently not known.

5.1.6 Seasonality Vertical Migrations in the deep

Seasonal changes are another factor that affect patterns of DVM. Seasonality in terrestrial animals are usually triggered by changes in temperature, day length or a change of weather. However, in the great depths of the ocean, the temperature stays relatively the same, the lunar cycles are the same, the currents can be unpredictable and there is very little sunlight. It was therefore long believed that the deep did not experience any seasonal changes (Orton 1920; Thorson 1936; Thorson 1950). It wasn't until the 80's, where a series of time-lapse images from the Porcupine Seabight southwest of Ireland showed that the deep zones are subject to seasonal cues. At 2000m, cameras took pictures every few hours over a year, and revealed large changes in the early summer, where fluffy aggregates of plankton and marine snow appeared on a large scale and were subsequently eaten or dispersed over the summer (Billet *et al*., 1983). This change in food availability has later also been documented in other places

such as the Norwegian Sea, Greenland Sea and Barents Sea (von Bodungen *et al.,* 1995). Now that satellite images are readily available, we know that algal blooms are patchy and seasonal, thus distribution of grazers and their predators are also patchy and seasonal (Herring 2002). This "rain storm" of food may act as a seasonal trigger for deep-sea animals that show seasonal reproductive patterns such as several species of deep-sea molluscs, sponges, echinoderms and bivalves, as well as seasonal growth in rattails (Tyler 1988; Witte 1996). In most places, the rate of marine snow will vary greatly over the seasons, and depend greatly on the blooms of phytoplankton. The vertical and horizontal distributions of animals is therefore not stable or homogenous across the seasons, for example benthic animals might move higher up in the water column, forcing the animals above to migrate higher up to avoid predation. The food availability might also stop vertical migration in some species as the food availability is higher deeper down. As discussed above, avoiding predations is one of the most critical factors in DVM of the plankton and micronekton.

There are also examples where animals use different depths at different times of year. This is often the case in places with extreme differences in seasons, where primary production and subsequent food availability may come to a halt for long parts of the year. It is particularly common in copepods such as *Calanus finmarchicus* in the north east Atlantic, which overwinters in a pre-adult stage at around 1000-2000m, before moulting in spring and rising to the surface (Kaartvedt 1996).

5.1.7 Marine hatchetfish – a great candidate for studying underlying molecular mechanisms of DVM?

In order to study the molecular mechanisms underlying DVM in fish, we need a vertical migrator that is abundant, and that would undertake long vertical migrators. The order of Stomiiformes offers several deep-sea candidate fishes such as mycophids (laternfishes), cyclothone (genus of bristlemouths) and hatchetfishes as potential candidates. Cyclothone is an interesting candidate as it is thought to be the most abundant genus in the world with 10^{15} individuals distributed across the open ocean (Sutton et al., 2010), and compared to many of the other mesopelagic fish, their eyes are quite tiny. The lantern fishes are probably some of the best "known" vertical migrators, with huge spherical eyes, and they also have the benefit that there are several draft genomes available for lanternfishes such as *Betostema glaciale*

(Malmstrøm et al., 2017). We chose however to go for two different species of hatchetfishes for a couple of reasons: they seemed to be among the most common in our catch region, so we could get a reliable source of tissue for sufficient replicates and time points. They were also chosen as the two species showed different eye morphology and also different reported DVM, providing a comparative element to this project.

The marine hatchetfish are not related to the freshwater hatchetfish (common ancestor ~250 mya), but share name due to their hatchet-like appearance. The marine hatchetfishes belong to the Euteleostei clade, and it is estimated that *Sternoptyx* and *Argyropelecus* split into separate genus about 35 million years ago/early Oligocene which has been confirmed from the fossil record, making them two of the oldest genus of its Stomiiform order (Kenaley et al., 2014). *Sternoptyx diaphana* was the first marine hatchet fish to be described in 1781 by Hermann. He noticed the bioluminescent organs as well as the elaborate silvering and named the species *Sternoptyx diaphana* (sternon = chest , ptyx= plate diaphana= transparent/reflective in Greek) . The genus *Argyropelecus* was described 50 years later by Coco in 1829 (argos= silver, pelekys=hatchet/star axe hemigymnos=semi naked. The fish are found throughout the tropics and temperate regions, and both are smaller than 6 cm, which is a fairly common size for mesopelagic and bathypelagic fish (Riede 2004). While *Sternoptyx* diaphna prefers deeper waters (Baird 1971, Herring 2002) and are not vertically migrating (Figure 5.5), *Argyropelecus hemigymnus* are reported to be vertically migrating (Figure 5.4). These findings are mainly backed up by a compiled and reviewed catch data from midwater trawls (Baird 1971) and by a study by Roe and Badcock, which were specifically studying DVM by midwater trawling in the North East Atlantic at 300-600 m (Roe and Badcock 1984). They found that the majority of *A.hemigymnus* fed mainly during the afternoon and early part of the night, i.e. migrating up before dusk, although previous papers have reported on night time feeding being more common in other geographic locations, with some seasonal impact on DVM, and some individuals not migrating at all (Badcock, 1970; Badcock and Merrett, 1976, 1977; Gibbs and Roper, 1970). These studies have however been limited to the top 600 meters of the water column, and later trawls have reported *A.hemigymnus* down to 1000 m (Mytilineou et al., 2005). It has been noted that this species has two types of body pigmentation, A) which is darker and B) which is more diffuse. The pigmentation does not correlate to sex or size, but does somewhat seem to correlate in geographical and vertical distribution, with the more pigmented form being found slightly higher up in the water column (Badcock 1969). The more pigmented variation is the predominant form found in the

tropics and Indian Ocean. A summary of key features for both fish are summarised in Table 5.1

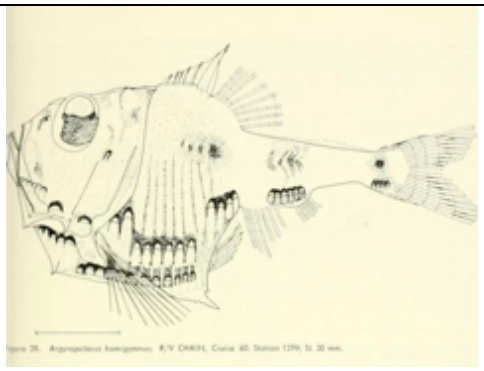
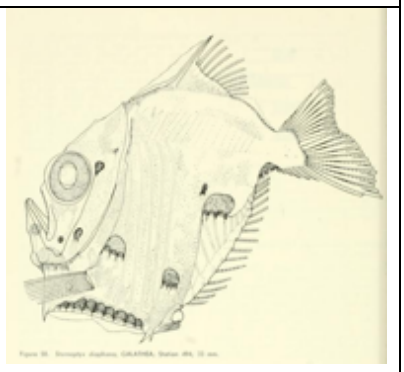
	Argyropelecus hemigymnus	*Sternoptyx diaphana*
Vertically migrating	Yes	No
Depths	50-1000m	400-3600m
"Preferred" depths	100-800m	500-1200 m
Distribution	Temperate-Tropic	Temperate-Tropic
Size	Adult ~35mm	Adult ~55 mm
Eye type	Tubular	Spherical
		

Table 5.1 Key information on the hatchetfish of interest. Drawings from Baird 1971.

5.1.8 Dual purpose vision

Terrestrial animals from insects to humans utilize vision as one of several sensory inputs, and it is not much different for many deep-sea organisms. Just like terrestrial organisms, marine life experiences a downward illumination, however in the open ocean there is nothing (except some marine snow or the animals that happen to be there) to reflect the light back up again. In contrast on land, a beach, field, trees etc will reflect light back (Denny 1993). Furthermore, due to the refraction of the water, it doesn't matter much where in the sky the sun is for the directionality of the light for animals that live in the mesopelagic zone. With blue light penetrating the furthest (Figure 5.2), the mesopelagic zone is relatively constant in its colour and light directionality, but with a dramatic fall in intensity.

So how dark is it really? At the surface of the sea, a human eye would detect about half a billion photons per second depending on the weather (Yu 2016). At 1000 m, the same human eye would detect about 1 photon every 1.5 minutes (Herring 2002). Despite this darkness, most animals living in the deep have a visual system. When comparing optic nerves and connections in shallow dwelling catfish to deep-sea rattails, it looks like the visual system is just as important in the deep as it is in shallow water (Douglas 1993). The visual system for many species of mesopelagic animals are both evolved to hunt at dawn/dusk as well as detecting and distinguishing bioluminescence signals. Both vertebrates and invertebrates of the mesopelagic zone tend to use bioluminescence in a range of ways, and is found in at least 12 animal phyla in the sea (Herring 2002). Many fish including the hatchetfishes, use bioluminescence for camouflage tougher with elaborate silvering. The ventral photophores on the belly and tail is used to produce similar wavelength as the downwelling light which camouflages the fish from predators below. Other fish such as dragon fish and angler fish use bioluminescence to attract prey. It is also thought that mesopelagic fish use it to advertise for mating or general communication, however, observations in the wild over time remains challenging. As a matter of fact, bioluminescence is often conferred if an animal possesses photophores, although it may never have been observed.

In most animals, bioluminescence can be genetically encoded using a luciferin/luciferase reaction. In some animals the energy can be transferred to an accessory fluorophore which will emit a different wavelength of light. The simplest form of a light emitting cell is called a photocyte, but in many animals the photocytes are included in specialised organs called photophores. These structures can be quite complex with lenses, colour filters and reflectors (Herring, 2002). Some animals use bioluminescent bacteria rather than photocytes, the most well-known example being the classic lure of the anglerfish (Freed et al., 2019). The majority of bioluminescence is thought to be blue, or blue/green, but there are several examples of fish that have other coloured bioluminescence such as red (Widder et al., 1984)

5.1.8 Deep-sea eyes

Fish eyes work simplistically in the same way as human eyes, with photoreceptor cells capturing photons. Deep-sea eye organisation is arguably one of the most diverse forms of eye organisations of any vertebrate or invertebrates. The range of different types of eyes and ocular modifications is immense, ranging from over 30 copies of rhodopsin genes being expressed in a single retina of a fish (Musilova et al., 2019), to many genera having multibank retina, where rows of rod stacked on top of each other (up to 40 rows), increasing the light path for photon absorption (Locket 1977). Spatial organisation of retinal ganglion cells, parallel-wiring of receptor cells, tapetums and very long rods are also common, some deep-sea fish like *Dolichopteryx longipes* and *Rhynchohyalus natalensis* use both lenses and mirrors to focus the image (Partridge et al., 2014).

The available literature on visual systems in deep-sea fishes make it clear; there are no candidate model species, and even closely related species within the same genus can show a range of adaptations and changes to their visual structure and biology. Luckily, the eyes of both *Sternoptyx diaphana* and *Argyropelecus hemigymnus* have been described. Rather unfortunately, the published study of the eye of *A. hemigymnus* is in German (Contino 1939), but best efforts have been made to extract some relevant information.

5.1.8.1 *Argyropelecus hemigymnus* eye structure

In mesopelagic and bathypelagic fishes, the eyes are generally either spherical or barrel shaped. Barrel or tubular eyes have evolved in 14 genera of fishes (Marshall 1971), and the genus *Argyropelecus* all have them. The tube eye is a great example of how the eye design makes the most out of dim light from the sun (which is only coming from above), with a large aperture lens focusing light on a small retina at the end of the tube. This design is perfect when stalking prey from below, but it also means that the peripheral vision is highly compromised. *A.hemigymnus* also have several other adaptations also thought to help *A.hemigymnus* detect its prey. Older and larger *Argyropelecus* have yellow lenses, but not red sensitive visual pigments. Many animals just like the hatchetfishes use bioluminescent counter-illumination as camouflage from predators below, however, bioluminescence and downwelling light have different wavelengths, and it is thought that the yellow filters short wavelengths of both, increasing contrast (Herring 2002, McFall-Ngai et al., 1986). Other than

that, *A.hemigymnus* has a fairly simple eye structure with one layer of long rods in its retina (Contino 1939). It is unclear from the Cortinos paper whether *A.hemigymnus* has a rod only retina, but the drawn transect show rods only (Figure 5.6 A). There is quite the variation in the photoreceptors found in different *Argyropelecus* species, for example *A.sladeni* has two distinct rods and one type of cone, *A. affinis* species have both rods and cones, while *A. aculeratus* has two types of rods (Biagioni et al., 2016).

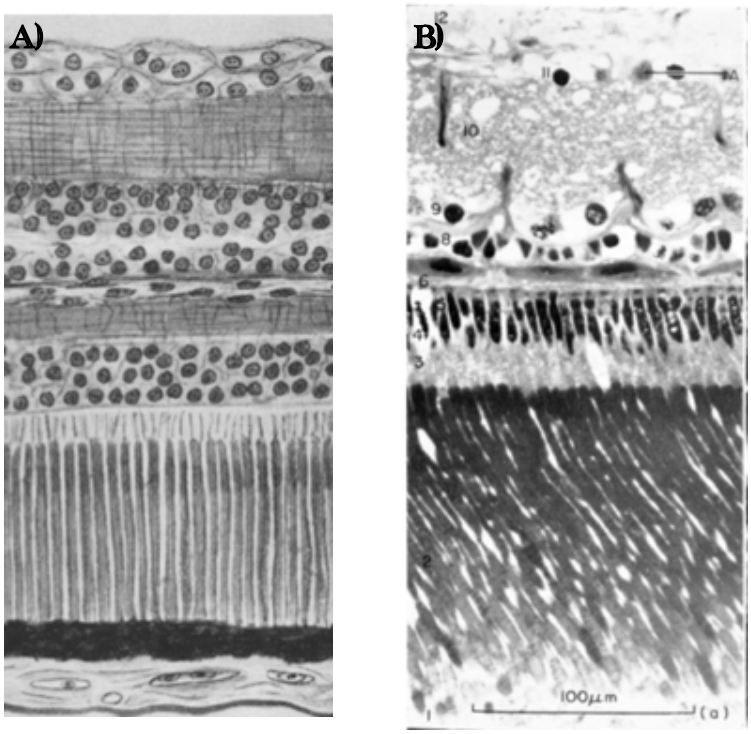


Figure 5.6 Radial retinal cross-section of hatchetfish eyes A) *A.hemigymnus* (Contino 1939) B) *S.diaphana* (Lockett 1970). The cross-sections are not drawn/photographed to the same scale. RPE is located at the bottom of the figure.

A.hemigymus larvae and young juveniles do not have tubular eyes. This is because *Argyropelecus*, and likely most other deep-sea species of fish, follow an ontogenetic vertical migration (OVM) pattern. OVM means that one species will use different depths at different stages of its' lifecycle. The eggs usually float up and many fish larvae feed in the sunny, epipelagic zone, where tubular eyes would be a big disadvantage. To get around this, the eyes

undergo a rearrangement as the juvenile becomes an adult, where the lens migrates up, and the retina migrates down to the ventral side of the eye, creating a tubular eye (Contino 1939).

5.1.8.2 *Sternoptyx diaphana* eye structure

Sternoptyx diaphana have regular spherical eyes, a single layer of long rods like *A.hemigymnus* (Figure 5.6 B). If you compare the two hatchetfish in terms retinal ganglion cells (RGCs), *A.hemigymnus* have more total RGCs (3.22 $x10^5$ cells per mm^2) and show a higher peak density of RGCs (area retinae) (33$x10^4$ cells per mm^2) than *S.diaphana*, which has a total of 2.43 $x10^5$ RGC cells per mm^2 and peak of 21.6 $x10^3$ cells per mm^2 (Wagner et al., 1998, Collin et al., 1997). Although *S.diaphana* has less peak density of RGC than any of the *Argyropelecus* species, the areae retinae, the area where photoreceptors distribution are at its densest, is located in the lower part of its retina, viewing upwards towards the surface (Figure 5.7). This arrangement is thought to give similar benefits as the tubular eyes when it comes to capturing the photons from above. What is also most curious is that *S.diaphana* also has two optic nerve heads/nerve discs per eye (Figure 5.7). In 1908, Brauer reported that the optic nerve of *Sternoptyx* left the retina by two branches, and as this was one of the very first deep-sea eyes to be described, it was thought normal amongst other fishes (Brauer 1908). This is however not common, and in the three articles that mention it (Brauer 1908; Locket 1970; Wagner et al., 1998) they never speculate what exact function it may have.

As mentioned above, *A.hemigymnus* can have yellow lenses, but that is not the case for *S. diaphana* which has no pigment in its lenses (McFall-Ngai et al., 1986). When compared to other deep-sea fishes and molluscs it was found that the lenses of *Sternoptyx diaphana* and *Argyropelecus aculeatus* (*A.hemigymnus* was not addressed) were far superior to the other deep-sea animals in focusing light, which may help the fish detect the counterillumination of its prey (Gagnon et al., 2015).

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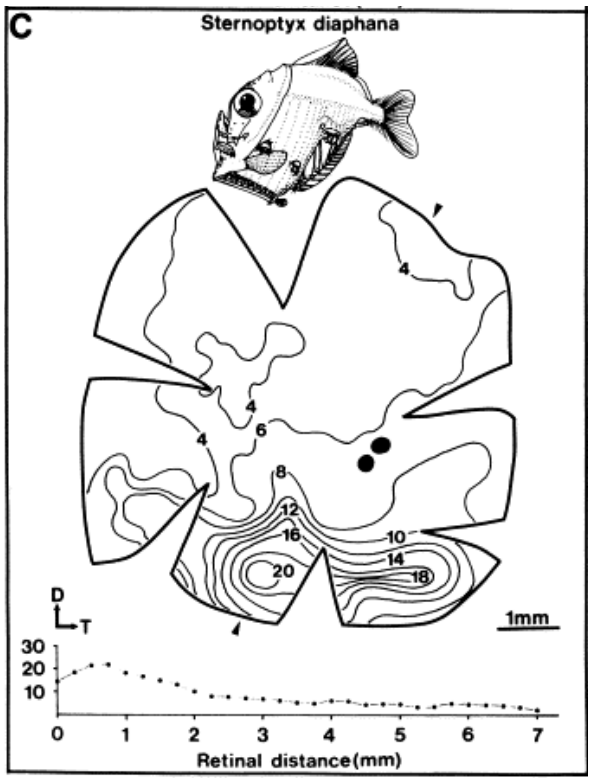

Figure 5.7 Retinal map of Sternoptyx diaphana
Iso-density contour map of whole-mount retina showing the density of neurons of the ganglion cell layer. Black dots depict two optic discs. The density profile shows the changes in density along a temporo-nasal transect (located between the arrows), revealing a pronounced ventral area retinae. (Wagner et al., 1998)

In this chapter we explore the unchartered waters of circadian rhythms and light-sensitivity in the sea, and we finally address the question many of the people in the circadian community has wondered – do animals that live in the deep-sea have a clock? By using the two species of hatchetfish that share many common traits and ancestry, but show differences such as distinct rhythmic behavioural differences, one vertical migrator and one non-vertical migrator, and different ways to structure the eyes (tubular and regular), we show interesting common and diverging adaptations to life in the deep.

5.2 Results

5.2.1 Light pulsing deep-sea retina

In order to explore light-induced gene expression in these two species, we decided to do a transcriptomic analysis of light pulsed eyes. We also decided to do a matching zebrafish experiment, an animal that we know has a lot of light regulated biology, and for which we know numerous positive controls. This would allow us to compare if there is any overlap at all in up-regulated genes in response to light across fish species. The deep-sea fish species do not have sequenced genomes. We therefore took a *de novo* approach with the deep-sea species and a genome guided approach with the zebrafish. The experimental light conditions were the same, but there were some differences in experimental set-up, such as temperature (28°C zebrafish and 6°C deep-sea fish) and few other factors due to the compromises we had to make in the field, which is discussed extensively in section **5.3.5 Experimental limitations and challenges of sampling.**

Eyes were dissected from the deep-sea fish and placed in culture media and after two days in the dark, light-pulsed with white light for 3 hours before being sacrificed (Figure Experimental layout). A standard *de novo* Trinity protocol was followed to assemble two transcriptomes for both species of deep-sea fish, whilst a genome guided Trinity protocol was used for the zebrafish transcriptomes. Sample specific transcripts were mapped back to the transcriptome with the appropriate Trinity packages, and edgeR was used for differential expression analysis ($p<0.05$). After the first round of *de novo* transcriptomic analysis, it was clear that we could benefit from a higher sequencing depth, as many of the genes were fragmented. We also made two different types of transcriptomes: one consisting of eye transcripts only, and one that included a mix of tissues to give a more representative transcriptome of the species. The new transcriptome with higher sequence depth is still being analysed for *Argyropelecus*, delays due to Covid, so here I show the data from the first *de novo* run.

Zebrafish show the largest amount of differentially expressed genes with 169 annotated genes (Table 5.4), with about ¼ being down regulated in response to light and ¾ up-regulated (Supplementary Figure 5.3). Around 70 genes gave no protein, mRNA or gene hits. We see up-regulation of several metabolic genes (cytochrome C genes, glycogenin1a, glucose

transporter X and more), immune genes (interleukin b, lymphocyte-specific protein 1, MHC1 and MHC two genes, to mention a few). We find many of the circadian genes such as *Cry1a* and *per2* and other classic light inducible genes like the DNA photolyases, such as 6-4 Phr (cry5) and CPD Phr, which act as our positive controls in this data set. Although we do not see all the classical circadian genes up-regulated after the initial analysis, we see upregulation of other circadian implicated genes such as *ryanodine receptor 3-like*, a gene implicated in the release of intracellular Ca2+ in the light-induced phase delay of the circadian clock restricted to the early night in the mammalian SCN (Ding et al., 1998; Pfeffer et al., 2009).

Zebrafish differentially express 5 different crystallin genes, which are the main overlapping genes across the three species. *Sternoptyx diaphana* differentially express probably 4 or 5 different targets, compared to *Argyropelecus hemigymnus* with 2. *A.hemigymnus* also show differential regulation of other genes associated with the lens, such as *lengsin* (l*gsn*) and lens fibre major intrinsic protein. Curiously, *Argyropelecus* show 28 up-regulated genes in response to light (Table 5.2), but no down-regulated genes (Supplementary Figure 5.1). This may change as we expand the transcriptome with the higher coverage. This small set of up-regulated genes echo the same biological processes as we see in zebrafish, with mitochondrial gene regulation (e.g. *Gamma-glutamylcyclotransferase* and *Acidic leucine-rich nuclear phosphoprotein 32 family member A*) and immune genes being (e.g. CD276 antigen-like). We also see more curious hits, such as *c-fos* an interesting up-regulated gene, with particular patterns in zebrafish brain, and rodent SCN, but as an early immediate gene, it is also used as a marker for neuronal activity (Dragunow and Faull 1989).

In *Sternoptyx*, we see that about half of the genes are upregulated in response to light, and the other half is downregulated (Supplementary Figure 5.2). Just like for the other transcriptomes, we find a range of transcriptional/translational genes (e.g. *nucleolin*, *prefoldin*, *Transgelin-3*, Eukaryotic translation initiation factor 3, *EF-2*). There is also several metabolic and mitochondrial genes (e.g. *ALOXE3*, *Vigilin*, *Mitochondrial carrier homolog 2*, *NADH dehydrogenase 1 alpha subcomplex subunit 1*, *Ornithine transcarbamylase*, outer *mitochondrial membrane protein porin 2*) (Table 5.3). In contrast to the other two transcriptomes, there seem to be no obvious immune genes upregulate, with the most likely candidate being *Immunoglobulin superfamily member 4*, but this gene also has many other GO terms associated. We do however see many differentially regulated cell cycle genes such as *Protein SET*, *cyclin-dependent kinase 8*, Mitotic interactor and substrate of *PLK1*, *Protein*

*phosphatase methyleste*rase, *protein reprimo A*. Other than the 5 crystallin genes, the only other gene implicated in vision that we find differentially regulated is -*lactase-phlorizin hydrolase-related protein*. Another interesting gene that is down-regulated in response to light is *homeobox protein otx5*, which is associated with circadian expression in zebrafish pineal, where depletion of *otx5* inhibits circadian gene expression (Gamse et al., 2002)

No hits: 5	**No hits: 20**
***Argyropelecus* Eye Only**	***Argyropelecus***
Acidic leucine-rich nuclear phosphoprotein 32 family member A	60S ribosomal protein L23
Developmentally-regulated GTP-binding protein 2	Acidic Nuclear phosphoprotein 32 familymember B (anp32b)
Echinoderm microtubule-associated protein-like 1	c-fos
Eukaryotic translation initiation factor 5A-1	CD276 antigen-like
Gamma-crystallin M2	Cript
	Gamma crystallin M3
	Gamma glutamylcyclotransferase
	Lens fibre major intrinsic protein
	Lengsin (lgsn)
	Leucine-rich repeat-containing protein 3-like
	Matrix metallopeptidase 15 (mmp15)
	Mycotrophin
	Myosin XVB (myo15b)
	Non-histone chromosomal protein HMG -like
	NSFL1 cofactor p47
	pancreatic progenitor cell differentiation and proliferation factor (ppdpf)
	Polymeric immunoglobulin receptor-like
	Proteasome subunit alpha (psma2)
	Ribosomal protein RPL30
	ssal rgf pre splicing factor
	Syndecan-4
	TNF alpha-induced protein 8 2B
	Tubulin alpha-1A chain-like

Table 5.2 *Argyropelecus hemigymnus* upregulated genes. Listed are genes found differentially expressed in eye transcriptome, or full transcriptome. No hits indicates how many trinity genes that gave no hits in the blast search

No hits: 70	No hits: 200
Sternoptyx eye only	**Sternoptyx**
Beta-crystallin B1	26S proteasome non-ATPase regulatory subunit 3
Eukaryotic translation initiation factor 3 subunit B eIF3b	39S ribosomal protein L42 mitochondrial
Eukaryotic translation initiation factor 3 subunit C	Protein kinase A-anchoring protein 2
G protein pathway suppressor 2	Activated RNA polymerase II transcriptional coactivator p15
Gamma-crystallin M3 or M2	Beta-crystallin A3-2
Heat shock 70 kDa protein	Beta-crystallin A1
Hydroperoxide isomerase ALOXE3	Cell adhesion molecule 1/Immunoglobulin superfamily member 4
LINE-1 reverse transcriptase homolog	Cyclin-dependent kinase 8/Cell division protein kinase 8
Vigilin	Cysteine-rich secretory protein 3
	DNA mismatch repair protein Msh2
	DNA-binding protein inhibitor ID-3-A
	Elongation factor 2(EF-2)
	Endoplasmin/94 kDa glucose-regulated protein
	Epithelial membrane protein 3/Hematopoietic neural membrane protein 1
	Eukaryotic peptide chain release factor subunit 1
	Eukaryotic translation initiation factor 3 subunit
	Gamma-crystallin M2
	Gamma-crystallin M3
	Gamma-crystallin S/Beta-crystallin S/Gamma-S-crystallin
	Guanine nucleotide-binding protein subunit beta-5a
	Heat shock 70 kDa protein
	Heat shock protein HSP 90-beta
	Haemoglobin subunit alpha-1
	High mobility group-T protein
	Histone chaperone asf1b-B/Anti-silencing function protein 1 homolog Bb
	Histone H5
	Homeobox protein otx5
	Lactase-like protein/Klotho-lactase-phlorizin hydrolase-related protein
	Leucine-rich repeat-containing protein 47
	Methylthioribulose-1-phosphate dehydratase/APAF1-interacting protein homolog
	Microtubule-associated protein RP-EB family member 3
	Mitochondrial carrier homolog 2
	Mitotic interactor and substrate of PLK1

	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 1
	Desmoyokin
	Nucleolin/Protein C23
	Ornithine transcarbamylase mitochondrial
	Polyadenylate-binding protein 1A/Poly(A)-binding protein 1A
	Polypeptide N-acetylgalactosaminyltransferase 12/Polypeptide GalNAc transferase 12
	Prefoldin subunit 5/Myc modulator 1/c-Myc-binding protein Mm-1
	Proteasome activator complex subunit 2/Proteasome activator 28 subunit beta
	Protein AHNAK2
	Protein phosphatase methylesterase 1
	Protein reprimo A
	Protein SET/Phosphatase 2A inhibitor I2PP2A/Template-activating factor I
	Ran-specific GTPase-activating protein/Ran-binding protein 1
	Small integral membrane protein 8
	Sorting nexin-2
	Osteonectin
	Spectrin beta chain non-erythrocytic 2
	Sterile alpha motif domain-containing protein 3
	Transgelin-3
	Glycoprotein 25L2
	Transmembrane protein 269
	Outer mitochondrial membrane protein porin 2

Table 5.3 *Sternoptyx diaphana* up and down regulated genes Listed are genes found differentially expressed in eye transcriptome, or full transcriptome. No hits indicates how many trinity genes that gave no hits in the blast search

No hits: 74
Zebrafish
1-acylglycerol-3-phosphate O-acyltransferase 3 (agpat3)
2-epi-5-epi-valiolone synthase (eevs)
2'3'-cyclic nucleotide 3' phosphodiesterase (cnp)
6-phosphofructo-2-kinase/fructose-26-bisphosphatase 3 isoform X3 []
ABI family member 3 binding protein (abi3bp)
acyl-CoA synthetase short chain family member 2 (acss2)
adaptor related protein complex 1 subunit mu 3 (ap1m3)
ADP ribosylation factor like GTPase 3a (arl3a) transcript variant 1
ankyrin repeat and protein kinase domain-containing protein 1-like
ATPase aminophospholipid transporter class I type 8B member 1 (atp8b1)
autophagy-related protein 2 homolog A-like (LOC113113759)
B-cell scaffold protein with ankyrin repeats [Anabarilius grahami]
baculoviral IAP repeat containing 6 (birc6) 6
beta-2-microglobulin (b2m) transcript variant 2
C-C motif chemokine 2-like (LOC100538242)
C-factor-like (LOC107554061)
calpain 12 (capn12)
cAMP responsive element modulator b (cremb)
cartilage acidic protein 1a (crtac1a)
CDC28 protein kinase regulatory subunit 2 (cks2)
chitin synthase gene
claudin b (cldnb)
coagulation factor VII coagulation factor VIIi and coagulation factor X genes
cocaine- and amphetamine-regulated transcript protein 2b (CART2b)
cortexin 3 (ctxn3)
cryptochrome 1a
cryptochrome 5
crystallin beta A1 like 2 (cryba1l2)
crystallin beta B1 like 1 (crybb1l1)
crystallin gamma M3 (crygm3)
crystallin gamma MX like 2 (crygmxl2)
crystallin gamma S1 (crygs1)
cytochrome c oxidase subunit I (COXI) ; mitochondrial
cytochrome P450 family 26 subfamily A polypeptide 1 (cyp26a1)
Deoxyribodipyrimidine photo-lyase (CPD Phr)
deoxyribonuclease 1 like 4 tandem duplicate 2 (dnase1l4.2)
discoidin domain receptor tyrosine kinase 1 (ddr1)

dual specificity phosphatase 11 (RNA/RNP complex 1-interacting) (dusp11)
E3 ubiquitin-protein ligase RNF38-like (LOC113044194)
ECRG4 augurin precursor a (ecrg4a)
ELOVL fatty acid elongase 5 (elovl5)
eukaryotic translation elongation factor 1 alpha 1 (EEF1A1)
F-box and WD repeat domain containing 7 (fbxw7)
ferrochelatase (fech)
fibroblast growth factor binding protein 2b (fgfbp2b)
forkhead box J1b (foxj1b)
furry homolog b (Drosophila) (fryb) transcript variant X9
glucose transporter X (glutX)
glutathione S-transferase pi 2 (gstp2)
glycogenin 1a (gyg1a)
golgin A7 family member Ba (golga7ba)
GTP binding protein 2b (gtpbp2b)
guanine nucleotide binding protein (G protein) gamma transducing activity polypeptide 2b (gngt2b)
H/ACA ribonucleoprotein complex subunit 1-like (LOC107746431)
heme binding protein 2 (hebp2)
heme-binding protein soul5 (soul5)
heparan sulfate proteoglycan 2 (hspg2) 8
heparin-binding EGF-like growth factor a (hbegfa)
high mobility group AT-hook 1b (hmga1b)
histidine-isoleucine receptor
interleukin 1 beta
integrin beta 2 (itgb2)
interferon-induced protein with tetratricopeptide repeats 14 (ifit14)
interferon-induced protein with tetratricopeptide repeats 16 (ifit16)
isocitrate dehydrogenase (NADP(+)) 1 (idh1)
junction mediating and regulatory protein p53 cofactor (jmy)
kinase insert domain receptor like (kdrl)
laminin beta 2-like (lamb2l)
leucine rich repeat and fibronectin type III domain containing 4b (lrfn4b)
leucine rich repeat containing 28 (lrrc28)
leucine-rich repeats and calponin homology (CH) domain containing 3 (lrch3)
LIM domain kinase 1b (limk1b)
lipoic acid synthetase (lias)
LON peptidase N-terminal domain and ring finger 1 like (lonrf1l)
lymphocyte-specific protein 1 (lsp1)
lysyl oxidase-like 2a (loxl2a)

major histocompatibility complex class I LDA (mhc1lda)
major histocompatibility complex class II DCB gene (mhc2dcb)
matrix-remodelling associated 8b (mxra8b)
MAX dimerization protein 4 (mxd4)
mediator complex subunit 19b (med19b)
mediator of RNA polymerase II transcription subunit 19-B-like (LOC109050521)
melanophilin a (mlpha)
mesothelin-like protein (LOC113079444)
MID1 interacting protein 1b (mid1ip1b)
mitochondrial ribosomal protein S36 (mrps36)
multiple C2 domains transmembrane 2b (mctp2b)
muscleblind-like protein 1 (LOC109110048)
N-myristoyltransferase 1a (nmt1a)
NACHT LRR and PYD domains-containing (LOC100334861) misc_RNA
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4-like 2a (ndufa4l2a)
NADH:ubiquinone oxidoreductase subunit B7 (ndufb7)
NADP-dependent mannitol dehydrogenase
NADPH oxidase activator 1-like (LOC109081638)
neurofibromin 1b (nf1b)
neurolysin mitochondrial
neuron navigator 1a (nav1a)
neuropilin 1a (nrp1a)
neurotensin gene
neurotrophic tyrosine kinase receptor type 2a (ntrk2a)
nipsnap homolog 3A (C. elegans) (nipsnap3a)
NLR family CARD domain-containing protein 3 (LOC100332612) ncRNA
NLR family pyrin domain containing 1
noncompact myelin-associated protein-like (LOC107668511)
NOTCH regulated ankyrin repeat protein a (nrarpa)
nucleolar protein 6 (RNA-associated) (nol6)
olfactory receptor C family d3 (olfcd3)
ORF1p ORF2p genes for ORF1-encoded protein ORF2-encoded protein
organic cation transporter protein-like (LOC107704351)
p53 and DNA-damage regulated 1 (pdrg1)
pan-epithelial glycoprotein
pantothenate kinase 2 (pank2)
phosphatidic acid phosphatase type 2D (ppap2d)
phosphodiesterase 4D cAMP-specific (pde4d)
phospholipid phosphatase 3 (plpp3)

Pitx3 gene
platelet-derived growth factor receptor-like (pdgfrl)
pleckstrin homology domain containing family M (with RUN domain) member 1 (plekhm1)
polycystic kidney and hepatic disease 1 -like
polypyrimidine tract binding protein 1b (ptbp1b)
potassium channel tetramerization domain containing 10 (kctd10)
prefoldin 5 (pfdn5)
period2
premelanosome protein a (pmela)
profilin 2 (pfn2)
prostaglandin E synthase-like (LOC107587481)
protein GPR108-like (LOC108187081)
protein SNORC-like (LOC113113664)
RAP1B member of RAS oncogene family (rap1b)
ras association domain-containing protein 8-like (LOC100003291)
reverse transcriptase
ribophorin II (rpn2)
ribosomal protein S16 (rps16)
RNA binding motif single stranded interacting protein 2b (rbms2b)
RUN and FYVE domain containing 2 (rufy2) transcript variant 2
ryanodine receptor 3-like
sema domain immunoglobulin domain (Ig) transmembrane domain (TM) and short cytoplasmic domain (semaphorin) 4Ga (sema4ga)
sentrin-specific protease 1-like (LOC107668340)
serpin peptidase inhibitor clade B (ovalbumin) member 14 (serpinb14)
SH3 and multiple ankyrin repeat domains 3b (shank3b) 0
solute carrier family 22 member 6-A-like (LOC107670234)
solute carrier family 46 member 3-like (LOC109048722)
solute carrier family 6 member 11a (slc6a11a)
SOUL1 (soul1)
SRY-box containing gene 9b (cDNA clone MGC:76805 IMAGE:6964230)
stonustoxin subunit alpha-like (LOC109099094)
taspase threonine aspartase 1 (tasp1)
TATA-binding protein-associated factor 2N-like (LOC113076755)
tetratricopeptide repeat domain 22 (ttc22)
TIMP metallopeptidase inhibitor 2b (timp2b)
TraB domain containing 2B (trabd2b)
transcription factor Sox9b (sox9b)

transmembrane protein 154 (tmem154)
transmembrane protein 185 (tmem185)
transmembrane protein 203 (tmem203)
transposon Tf2-1 polyprotein (LOC108190699)
troponin C type 1 (slow) (cDNA clone MGC:103465 IMAGE:7236936)
tubulin alpha 8 like 4 (cDNA clone MGC:171443 IMAGE:5916489)
tumor protein translationally-controlled 1 (tpt1)
U2 small nuclear RNA auxiliary factor 2b (u2af2b)
UAP56-interacting factor-like (LOC107596211)
uncharacterized protein C2orf82 homolog (LOC107728388)
uromodulin-like 1 (LOC113115095)
ventral neural cadherin
WD repeat domain 26a (wdr26a)
WD repeat domain 76 (wdr76)
Wolf-Hirschhorn syndrome candidate 1-like 1 (whsc1l1)
zinc finger and BTB domain containing 1 (zbtb1)
zinc finger NFX1-type containing 1 (znfx1)

Table 5.4 Zebrafish up and down regulated genes Listed are genes found differentially expressed in eye transcriptome. No hits indicates how many trinity genes that gave no hits in the blast search

5.2.2 Deep-sea opsins

Deep-sea eye research is usually all about visual opsins. The largest study to date on deep-sea rhodopsin shows a large variation in how many copies different species express from 1 copy to over 30 and usually also one cone type (RH2, SWS or LWS) (Musilova et al., 2019). The non-visual opsins have not been studied or identified at all in deep-sea fish, until now. To identify if the deep-sea express any, we blasted all 42 annotated opsin genes found in zebrafish to the *de novo* transcriptomes. In *Sternoptyx*, we found ~18 non-visual opsins expressed, and in *Argyropelecus* we found 14 expressed (Table 5.5). These observations are based on low E-values ($<1e^{-80}$), which is a blast value which gives us the number of expected hits of similar quality that could be found by chance. For some genes such as rhodopsin that yielded multiple hits, but generally gave E-values of $1e^{-170}$, alignment and neighbour joining trees would be required for us to determine if we are looking at several copies of a gene, a

fragmented gene or the same gene that just has different identifiers. We also identified several potential rhodopsin genes (rh1) as well as one cone opsin gene, *rhodopsin like 2* (*rh2.3*) is both species (green sensitive in zebrafish), and an additional cone opsin in *Sternoptyx*, *short wavelength sensitive* (*sws1*) (blue in zebrafish).

Both species of deep-sea fish show quite a similar opsin expression pattern. Until further analysis is done, it unclear if both species express e*xorhodopsin* (*ExoRhd*), as the sequence is highly similar to visual *rh1* genes. *Retinal pigment epithelium-derived rhodopsin homolog/peropsin* (*RRH*) an important GPCR in retinal pigment epithelium (RPE) is also expressed in both species, but two *Argyropelecus de novo* genes map to RRH. Furthermore, both species express one variant of *vertebrate ancient opsin*, and both species express the photo-isomerases *RGR1* and *RGR2* (Retinal G protein coupled receptors), as well as a combination of 4 melanopsins (zebrafish have 5). *Encephalopsin* (*opn3*) is also found in both species, with two separate *opn3* hits in *Sternoptyx*. Two or three teleost multiple tissue opsins (*tmt1b* and *2b/a*) are also expressed in both species. We also see that both species express "novepsins", a new family of opsins that were discovered only 6 years ago" (Davies et al., 2015). Two variants of *opn6* are found in both species, while one variant of *opn7,8* and *9* are found in *Sternoptyx* as well.

Interestingly, it is worth noting that if we extract sequences from the "eye only" transcriptomes, we only identify a couple of transcripts. When we look at the transcriptome that includes extra ocular tissues however, we get a longer list of candidate opsins! That means that extra ocular opsins are something that exist also in deep-sea fish. It does not necessarily mean that the eyes do not have expressed non-visual opsins, but simply that the sequence may have been too fragmented or too rare to make up an opsin transcript in the eye.

Argyropelecus (old)	Sternoptyx
rh1 (maybe 2)	rh1 (maybe 1-6)
rh2.3	rh2.3
-	sws1
ExoRhd?	ExoRhd?
rrh x2	rrh
Valop	Valop
opn4m1 or 3	opn4m3
opn4m2	opn4m1 and/or 2
opn4x1	opn4x1
opn4x2	-
opn3	opn3 x2
RGR1	RGR1
RGR2	RGR2
tmt1b	tmt1b
tmt2b	tmt2b (and/or tmt2a)
opn6a x2	opn6a x2
-	opn7c/b
-	opn8c
-	opn9/opn5m2

Table 5.5 Opsins expressed in deep-sea transcriptomes

- indicates no finds, "and/or" "maybe" "?" indicates that more analysis is required to determine if this is the correct gene due to high sequence similarities and hits with very similar E-value.

5.2.3 Vertebrate deep-sea clocks

The light-inducible transcriptomes show that these animals have the ability to use light for a range of biological processes, and the opsin data make it clear that these animals have the full set of opsins capable of detecting light of different wave-lengths, but does this mean that they possess a circadian clock? In order to determine whether these deep-sea fish have a molecular clock or not, we first had to look if there were any clock genes expressed in the transcriptomes. Zebrafish *period* and *cryptochrome* genes were used to blast the transcriptome to pull out candidate sequences. The top hits were aligned with 2-4 *period* or *cryptochrome* genes from zebrafish and other species of teleosts. Depending on how

fragmented the deep-sea genes were, one or more neighbor joining trees (Supplementary Figures 2.1-2.4) were made for the genes and each species. *Cry1a*, *Cry2*, *Cry3a* and *Cry3b* was found expressed in *Argyropelecus hemigymnus*, as well as *per1a*, *per1b* as well as three "different" *per2s*. In *Sternoptyx diaphana*, the same cryptochromes where found, but only one copy of *per1* and *per2*, as well as *per3*.

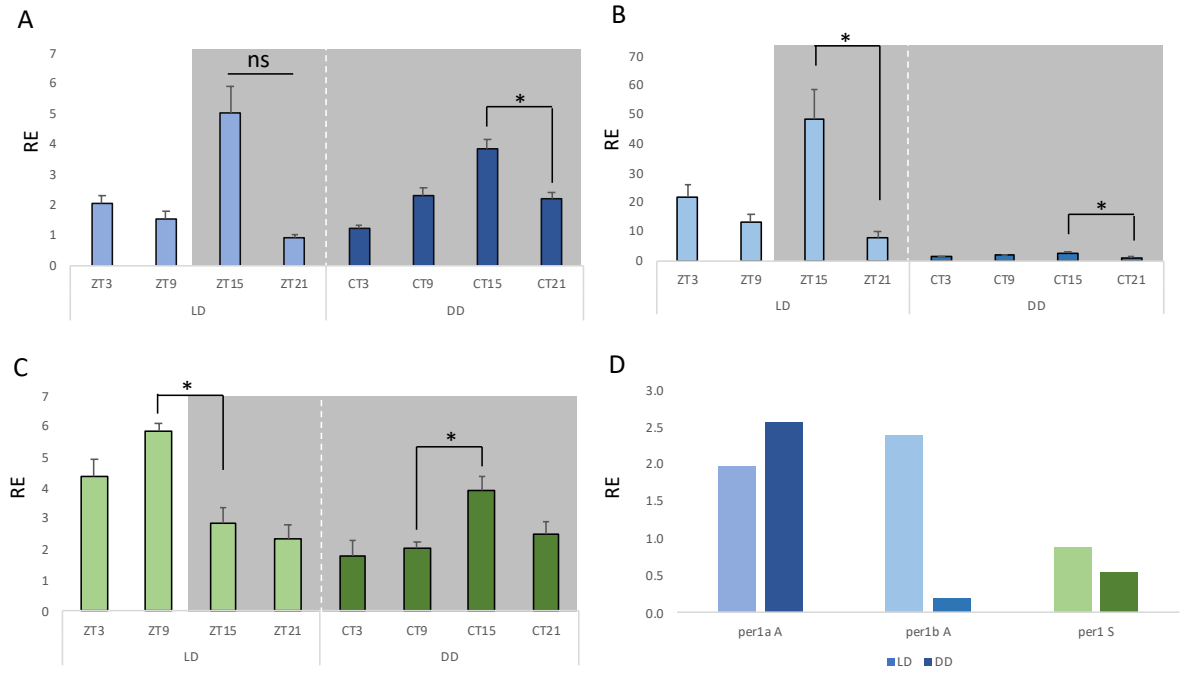

Figure 5.8 *per1* expression in *Argyropelecus* and *Sternoptyx*

Deep-sea eyes were dissected and maintained on a 12:12 light-dark cycle for 1 day before being sampled every 6 hours over the next 24-hour period in LD and then a following dark period. ZT denotes zeitgeber time and CT denotes circadian time. Dark -grey panels indicate dark period. A) *per1a* expression in *A. hemigymnus* B) *per1b* expression in *A. hemigymnus* C) *per1* expression in *S. diaphana* D) total transcript of *per1* over the LD and DD period (blue = *A. hemigymnus,* green= *S. diaphana*). Data is plotted relative to the lowest expressed gene. Significance was addressed with a two-way ANOVA, followed by a Tukey post-test. Significance * $p<0.05$ (n=3-6).

Primers were subsequently designed and tested, for RT-qPCR. Several housekeeping genes were also designed and tested, but none seemed to be stable across the timepoints, so the graphs here are plotted without normalization to housekeeping genes. Data plotted relative to the housekeeping genes can be found in Supplementary Figures 5.4-5.9. The data retains the

same trends, but are not significant statistically, which shows that it is prudent to test new housekeeping genes. Furhermore, there is also a section in supplementary material with box and whisker plots for all genes explored to give an idea of how much gene expression varies across the different genes (Supplementary Figures 5.10-5.14).

As *per1* is by far the most robustly cycling clock gene in zebrafish, it was also the main candidate for the mesopelagic hatchetfishes. Using eyes cultured in LD and LD into DD, I examined clock gene expression. In the *Argyropelecus*, both *per1a* and *per1b* seem to be cycling in LD and LD into DD (Figure 5.8 a,b). Both genes peaks at ZT15, with significance between CT15 and 21 for *per1a* and ZT/CT15 and 21 for *per1b*, with the amplitude being slightly dampened in DD. While the absolute amount of *per1a* and 1b is approximately the same for LD, *per1b* has a dramatically reduced expression in DD compared to LD (Figure 5.8 d), although there is still a significant difference between CT15 and CT21 (Figure 5.8 b). To further confirm that the cycling of *per1* genes were actually rhythmic, the averaged dataset (LD and LD into DD) was uploaded into BioDare2. The program has a rhythmicity test tab that are intended for sparse and small datasets (fewer than 5 days), which fits our deep-sea data. Using the classic JTK test method with cosine24h preset, *per1a* rhythms were determined "true" ($p<0.05$), so was *per1b* but only after a linear detrend of the dataset (Supplementary Table 5.1).

Sternoptyx, only express one copy of the *per1* gene which show significant expression between ZT9 and ZT15/21 in LD (Figure 5.8 c). In DD, there is still a significant difference between CT9 and CT 15, but rather with CT15 as peak, rather than CT9. Though these data show a significant rhythm, which may represent a long or unstable clock period, ideally additional cycles would be required to confirm this. It is however worth noting that when we compare the two hatchetfish, *Argyropelecus* show a 5-6 fold difference in amplitude in LD while *Sternoptyx* only show a 2-3 fold difference in LD (Figure 5.8 a-c). When comparing the total *per1* transcripts from RT-qPCR, *Sternoptyx* has considerably lower *per1* transcript levels (Figure 5.8 d).

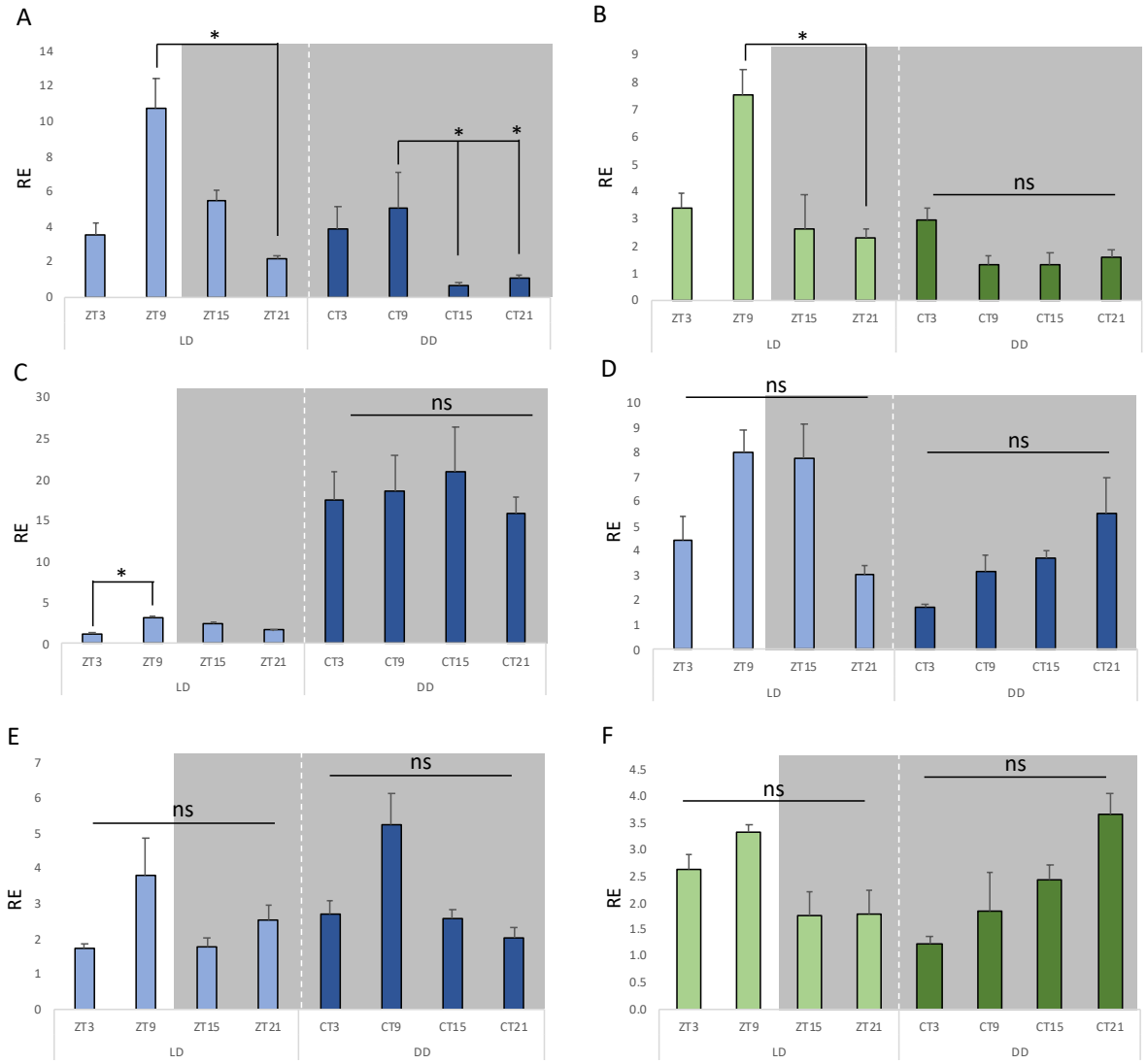

Figure 5.9 Classic light inducible genes in *Argyropelecus* and *Sternoptyx*

Deep-sea eyes were dissected and maintained on a 12:12 light-dark cycle for 1 day before being sampled every 6 hours over the next 24-hour period in LD and then a following dark period. ZT denotes zeitgeber time and CT denotes circadian time. Dark -grey panels indicate dark period. Blue = *A. hemigymnus,* green= *S. diaphana*. A) *cry1a* expression in *A. hemigymnus* B) *cry1a* expression in *S. diaphana* C) *per2x* expression in *A. hemigymnus* D) *per2y* expression in *A. hemigymnus* E) *per2z* expression in *A. hemigymnus* F) *per2* expression in *S. diaphana*. Data is plotted relative to the lowest expressed gene. Significance was addressed with a two-way ANOVA, followed by a Tukey post-test. Significance * $p<0.05$, non-significance = n.s (n=3-6).

5.2.4 Light-sensitive clock gene candidates

The next step was to examine light inducible genes. *Cry1* and *per2* are the most studied two candidate genes for circadian light input in zebrafish and cavefish. Is it the case that these genes are also induced in a deep-sea eye? Only one *cryptochrome1* gene was found in the transcriptome for each fish. In *Argyropelecus* we see an induction at ZT9, and a matching, but lower amplitude expression pattern in DD (Figure 5.9 a), an expression pattern that matches that of cave dwelling *A.mexicanus* (Beale et al., 2011). In *Sternoptyx* we also see an induction in cry1 at ZT9, but flat expression in DD. Three *per2* genes have been identified in *Argyropelecus*, and they all seem to have different expression patterns. The most unexpected expression pattern may belong to *per2x* which has one small significant peak at ZT9 compared to ZT3, but then 7 times as high expression in DD (Figure 5.9 c). *Per2y* has no significant expression between the time points in either LD or DD (Figure d). *Per2z* has no significant expression between the different timepoints, either, but the expression profile is similar to that of *cry1* (Figure 5.9 e). *Sternoptyx* only express one *per2* gene, the amplitude is low compared to *per2* in *Argyropelecus*, and we see no significant induction during the light phase (Figure 5.9 f).

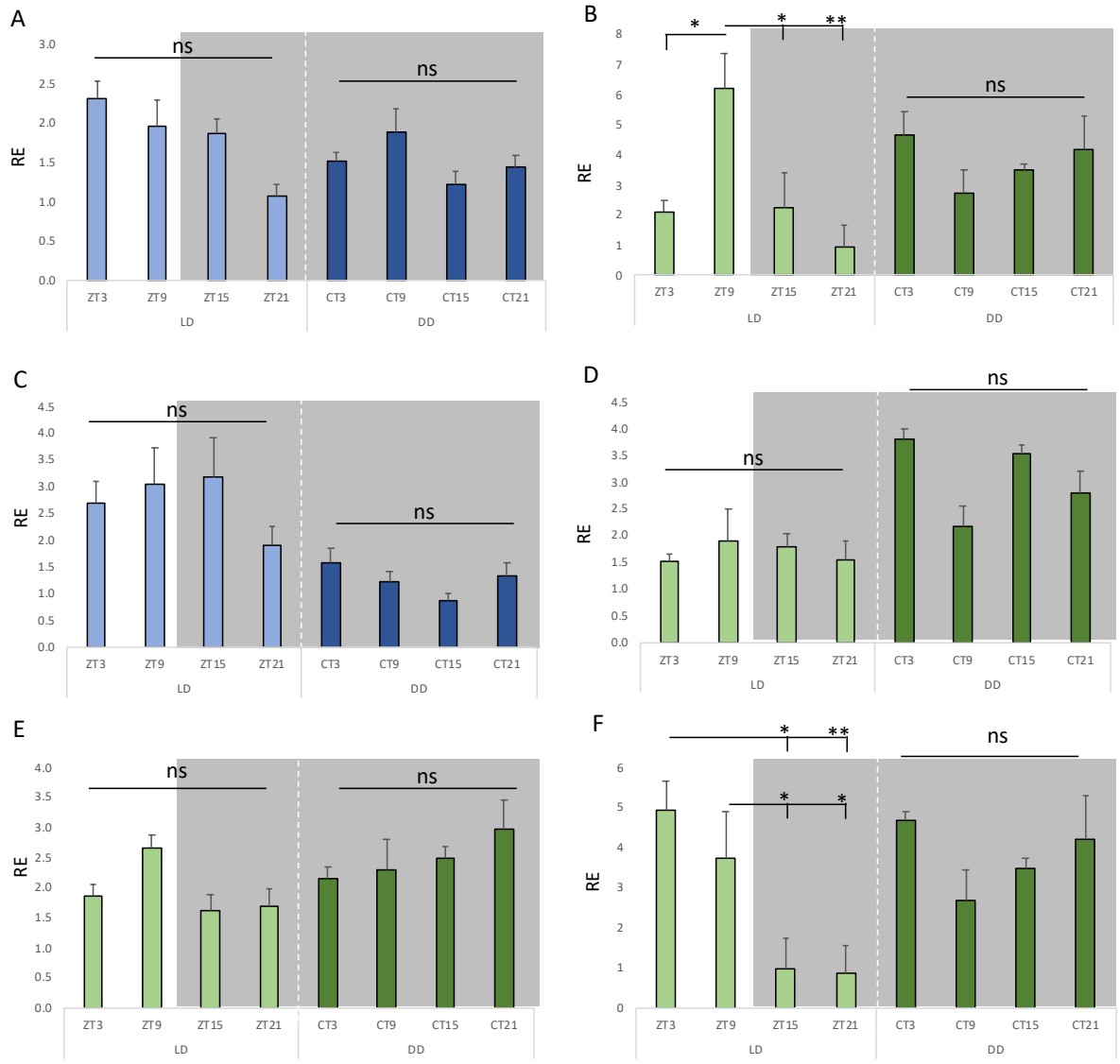

Figure 5.10 Potential light inducible and clock genes in *Argyropelecus* and *Sternoptyx*
Deep-sea eyes were dissected and maintained on a 12:12 light-dark cycle for 1 day before being sampled every 6 hours over the next 24-hour period in LD and then a following dark period. ZT denotes zeitgeber time and CT denotes circadian time. Dark -grey panels indicate dark period. Blue = *A. hemigymnus,* green= *S. diaphana*. A) *cry2* expression in *A. hemigymnus* B) *cry2* expression in *S. diaphana* C) *cry3b* expression in *A. hemigymnus* D) *cry3b* expression in *S. diaphana* E) *cry3a* expression in *S. diaphana* F) *per3* expression in *S. diaphana*. Data is plotted relative to the lowest expressed gene. Significance was addressed with a two-way ANOVA, followed by a Tukey post-test. Significance * $p<0.05$, ** $p<0.01$. Non-significance = n.s (n=3-6).

Due to the atypical *per2* expression and the shallow cry1 response, we also examined the expression over a LD cycle of the other *cryptochrome* and *period* genes to see if any of these genes were candidates for directly light responsive clock components. Both species have the same *cryptochrome* genes (*cry1,2,3a* and *3b*) expressed in both transcriptomes. While *Cry2* expression is flat and non-significant in *Argyropelecus* (Figure 5.10 a), *Cry2* show a very similar expression to cry1 in *Sternoptyx* (Figure 5.10 b). *Cry2* also peak at ZT9, and interestingly, the amplitude is quite high, a 6 -fold difference between ZT9 and ZT21, but in the following dark phase, there is not even a hint of a rhythm. *Cry3b* expression is flat under LD and DD in both species, which is also the case for *Cry3a* in *Sternoptyx* (efficient *Cry3a* primers are still to be designed for *Argyropelecus*) (Figure 5.10 c,d). From the current transcriptomic dataset there is no gene that is identified as *per3* in *Argyropelecus hemigymnus*, *Sternoptyx* however express *per3*, and interestingly it also has a very different expression pattern from the other *per*-genes and the *cryptochromes*. While *cry1* and *cry2* shows a peak at ZT9, *per3* show high expression, during the light phase, whilst in the following DD it is low, but in the subsequent dark phase, it is back highly expressed again over the next 4 time points (Figure 5.10 f).

5.3 Discussion

In this chapter I present the first two light inducible transcriptomes ever performed on mesopelagic, deep-sea fish. We have shown that these deep-sea animals have the capability to use light to up-regulate genes, and in the case of the vertical migrator, they also show endogenous rhythmic *per1* expression *in vitro*. We have also isolated several non-visual opsin genes, revealing a great variation of opsins, potentially matching that of zebrafish.

5.3.1 Light inducible transcriptomes

The light-inducible transcriptomes show that in theory, the deep-sea fish can induce and repress several genes in response to light (Table 5.2 & 5.3). *Sternoptyx diaphana* show the highest number of upregulated genes of the two deep-sea species, with 64 upregulated genes (270 genes gave no hits). In contrast the *Argyropelecus* transcriptome shows only 28

upregulated genes (25 genes gave no hits), but this analysis has not considered the newest resequencing data with may increase and refine the number of identified genes. It may very well be that the deeper dwelling, non-migrating *Sternoptyx* is more responsive to light than *Argyropelecus*, but we cannot say for certain, especially when the latter transcriptome is slightly more fragmented, meaning that we may "lose" genes due to poor overlap of transcripts. If in the future, we can obtain samples of higher-quality DNA for genomic sequencing, it might answer the question if the different animals operate with different light-sensitivities. The high level of *c-fos* induction in the *Argyropelecus* transcriptome, but the absence of *c-fos* in the other species could well support this idea. *c-fos* is an immediate early gene which is induced in the rodent SCN by a photic stimulus, but only during a window when light is capable of phase-shifting the circadian clock (Rusak et al., 1990). In the zf brain, *c-fos* is induced after a 30 min light-pulse, but then drops to lower levels after 3 hours (Moore and Whitmore 2014), in *Sternoptyx* and zebrafish we do not observe any significant upregulation *c-fos* after a 3-hour, but we do in *Argyropelecus* which may indicate that the eye either requires more light stimulus or that the kinetics of induction are slower, or even that our light pulse may have aligned with the circadian window when *c-fos* is light inducible in *Argyropelecus*, but not in zebrafish or *Sternoptyx*.

When analysing the initial transcriptomes, the differentially regulated genes were relatively few, so a different approach was taken creating a separate *de novo* transcriptome for the eye samples only, excluding the tissue samples. This increased somewhat the total number of genes upregulated, but only marginally. Further analysis using different differential expression packages such as DEseq as well as edgeR and easing the parameter stringency, might reveal more genes. It is also important to remember that when comparing zebrafish and deep-sea fish that not only do they live in widely different habitats, where it's only natural for the zebrafish to use light to a larger degree, the RNA submitted for sequencing for zebrafish was also of high, optimal quality due to the ease with which the samples can be collected. Several other technical reasons, such as the zebrafish used were of the same age and genetically closely related will usually mean that the expression profiles are more similar, and the transcriptomes were also mapped to the zebrafish genome, which means that shorter non-overlapping transcripts would not be discarded. These limitations of the deep-sea sampling and its consequences are discussed below.

At this time, these data are rather an initial examination of the transcriptomes. More and different data analysis would clearly improve these datasets, and "tweaks" done in the Trinity pipeline may also increase the number of genes expressed. The analysis of the zebrafish transcriptome also needs much more work, and should be compared to previous zebrafish light-induced transcriptomes, such as the pineal transcriptome by Ben-Moshe and colleagues (Ben-Moshe et al., 2014). The lists of light-inducible genes are long, especially for zebrafish and would benefit from a gene ontology (GO) analysis (Table 5.4). There are obviously no GO annotations for our deep-sea species, and GO annotation in fish is generally quite poor, even for zebrafish. Using human homologues/paralogues/orthologues to create a map of biological function would create a more complete picture of the genes expressed along a GO network based on zebrafish GO annotations. These GO terms should often be taken with a large pinch of salt, this is exemplified by for example by *AHNAK2* which is upregulated in *S.diaphana*, which has one GO term associated: RNA splicing. If we look at other genes from the same transcriptome such as *26s proteasome non-atpase regulatory subunit 3* and *proteasome activator complex subunit 2* they yield over 10 GO terms, which ranges from mRNA stability to mitotic transition to MAPK to MHC class I.

Although we observe that many of the DE genes across all 3 species have the similar GO terms associated with them, we do not see any great overlap in specific genes other than the crystallins. This could be due to a number of reasons, the first one being that there is not any great overlap. The deep-sea animals and zebrafish have different purposes for their vision, so a light signal could result in different responses in the different eyes. However, one would expect that there would be more overlap between the two deep-sea species and also imagine that there is some basic cell biology that is common for all eyes. It is more likely due to fact that there is high genetic variation in the deep-sea samples, so the DE does not reach significance. To confirm this, we can blast the upregulated genes in zebrafish back to the deep-sea transcriptomes. Another reason why we do not see a great overlap, and far fewer DE genes, which is confirmed by the transcriptomic quality control (Supplementary Table 2.3, 2.4), is that there is much more fragmentation in the deep-sea transcriptomes, so we may also lose some genes due to this.

5.3.2 Opsins

Here we show for the first time, that deep-sea species not only have a range of visual opsins, but also at least 15-20 non-visual opsins (Table 5.5). Considering that these animals are creatures that use their visual system to both detect bioluminescence and dim downwelling sunlight and moonlight, it must be very important to be able to be able to differentiate between the different "types" of light. We do not know the absorption peak of these opsins, and single amino acids can change peak sensitivity, but if we compare it to the ones we do know in zebrafish, the list of non-visual opsins would mean a wavelengths sensitivity from 360 nm to 540 nm: opn8c (375nm), bi-phasic opn9 (360 and 462 nm), tmt1a (460nm), tmt2b (470nm), opn3 (470 nm), opn4m2 (484nm), opn4x1 (470nm) are UV-blue sensitive. Valopb (500nm), opn6a (510 nm), exorhodopsin (500nm) and rrh (540nm- spider) are more sensitive in the green spectrum (Chapter 1 Figure 1.1). The wavelengths of bioluminescence and downwelling light are different, and we know that these animals possess several retinal adaptations to be able to tell these two light sources apart, such as the yellow lenses in the case of *Argyropelecus* (McFall-Ngai et al., 1986). Perhaps the multitude of opsins also help to distinguish these two sources. Furthermore, these predatory animals are usually also the prey of larger fish, and must therefore be able to fine tune their elaborate counter illuminating ventral photophores (i.e. their camouflage) as they are moving up and down the water column. Furthermore, we also know that at least the vertical migrator has a theoretical light-entrainable molecular *per1* clock, which means that some of these opsins must also be used to "feed into"/entrain the circadian pacemaker.

Considering that different sources of light impact the behaviour of these animals in such profound ways, would suggest that a wavelength sensitive eye is key for these animals to be able to tell different light sources apart and how to behave accordingly. It is therefore tempting to speculate that the non-visual opsins serve key roles in telling apart light that serve as potential circadian cues and/or photic avoidance (sun and moonlight) from bioluminescence of different sources, whether it be a mate or food. The list of opsins provided here is probably not even the extensive list. Rare transcripts can be lost in the sequencing, and although most opsins are expressed in the zebrafish eye, some are brain specific (parietopsin and parapinopsin b), and some are more lowly expressed than others (Davies et al., 2015).

It is curious, as we see the same diversity in expression of opsins in zebrafish and deep -sea fish. In zebrafish, it seems "downright overkill" to have so many photopigments that detect in the same predicted spectral region, however this makes a lot of simplistic sense in the case of the deep-sea eye. The zebrafish and the hatchet fishes shared a common ancestor ~250 million years ago, and a whole genome duplication (WGD) event. It is estimated that the zebrafish has retained up to 20% of its duplicated genome, compared to pufferfish that has only retained 1-5% (Aparicio et al., 2002; Jaillon et al., 2004; Postlethwait et al., 2000; Postlethwait et al., 2004; Woods et al., 2005). It is possible of course that the opsins in zebrafish are part if this retainment, and that they are indeed somewhat complimentary. In the deep-sea fish we do not know how much of the WGD is retained, but they are arguably under a higher selective pressure than zebrafish, which may mean that these animals have retained their opsins as they provide an evolutionary advantage. This is of course pure speculation, and it may of course be that some of the opsins are repurposed for other biological roles, as discussed in this first data-chapter. However, the lack of functional data on fish non-visual opsins leaves us with only speculations at this time.

5.3.3 Clock genes in deep-sea fish

Here, we have shown that the vertically migrating fish *A.hemigymnus* have a molecular circadian rhythm that persists in DD, which is the first demonstration of a molecular clock in a vertebrate that lives that deep in the ocean. *Per1a* and *per1b* show rhythmic expression patterns in LD and LD into DD, but they are generally quite shallow, if compared to zebrafish or even cavefish (Figure 5.8 a,b). The two copies of *per1* show the same peak expression, where *per1a* keeps a similar amplitude in the first 24 h in DD, but *per1b* although cycling in DD with a significant peak at ZT15, is much lower. This would perhaps suggest that the two genes, although both rhythmic, serve different roles. We also have matching (n=3) samples in LL, which will be processed and examined post-pandemic travel restrictions. If expression is "flat" and the clock stops, it would be a complimentary piece of data showing that *per1a* and *per1b* are indeed clock components. It is also worth considering, that these were *in vitro* experiments, and it is tempting to speculate that *in vivo* experiments may have yielded stronger responses to light and more robust rhythms in DD (as is always the case). When these animals undergo DVM, they would also experience a large difference in day and night temperature, oxygen availability, not to mention pressure etc., which might impact on

amplitude and period of the molecular rhythm, as well as representing additional, unexplored entrainment cues. The fact that feeding for these animals is very restricted to the night, also means that this further metabolic cross talk to the clock may strengthen period and amplitude, or again represent a food entrainment signal itself. The difference in pressure would be difficult to replicate in a field lab or any lab, but with more sophisticated thermostatically controlled incubators on board, exploring temperature cycles to see how it would impact the circadian clock and its outputs in deep-sea cultures would indeed be interesting to explore.

Another thing to consider, is that most deep-sea species, including the hatchetfishes, are epipelagic during their larval stage, keeping to the top 100 metres of the water. As they transform into adults, they descend into the deep and change behaviour. Such ontogenetic behavioural changes are also seen in flatfishes such as *Solea senegalesis,* which switch from diurnal to nocturnal during embryo development and as they move to the seabed (Martin-Robles et al., 2012). As juvenile *A. hemigymnus* are epipelagic, they would experience strong LD cycles from the sun, and might also show shallower DVM. From these experiments we know that the eyes have the ability to entrain, but the experimental light cycle we subjected them to would be much stronger possibly than what they experience in the wild, so is this clock rhythm basically a remnant from the past life stages of the fish? Most likely not, as we would then probably also have seen a robust rhythm in *Sternoptyx*. However, to truly confirm that the adult fish use a molecular clock we would need a matching set of *in vivo* samples, i.e. take samples from trawls every 2-6 hours, and also trawl at different depths to try and track DVM. Sadly, this was not a possibility on our expedition.

5.3.4 Light input

The light input pathway into the deep-sea clock is more obscure. *Cry1a* is a definite candidate, it has a delayed *Cry1a* peak at ZT9 much like cavefish, and it also cycles in the dark (Figure 5.9 a). *Argyropelecus* seems to have three copies of *per2* and they are all widely different in their expression patterns. *Per2z* resembles the *per2* that we know in zebrafish. Although shallow and not significant, it does show the same pattern as *Cry1a* which makes it a likely light input candidate. The delay in the kinetics of its induction is interesting and suggests some lack of sensitivity in the clock input pathway. What I have here called *per2x*, is highly upregulated and flat across the 4 time points in the dark, but looks like it is

repressed in LD but with similar expression pattern in LD to *per2z* and *Cry1a*. Curiously, we also see a similar phenomenon in cavefish, that show high *per2*a expression across the cave types in DD, but not for *per2*b (Frøland Steindal et al., 2018). We hypothesised for the cavefish that this higher basal *per2*a expression compared to surface fish is helping the fish drive typical clock outputs by tonically turning them on. Perhaps this *per2x* acts in a similar way, but it can also be downregulated in response to light and act like *per2z*. This would make sense if the animals are not strictly rhythmic, but adapt their behaviours for example to fit seasons and food availability, but then the clock driven outputs would still be transcribed due to this *per2x*. Genomic sequencing and promoter analysis would allow us to identify if these different *per2*s are regulated in different ways, but for now, it remains as an interesting speculation.

When it comes to *Sternoptyx*, the gene expression patterns are actually very similar to that of *Argyropelecus* in LD, but do not persists in DD, exemplified by *Cry1a*, *per1* and *per2*/*per2z*. *Sternoptyx* also show cycling of *Cry2* in LD (Figure 5.10). *Per3* also has a curious expression pattern, which at this time is hard to interpret (Figure5.10 f). It is interesting to speculate that perhaps as the animal grows into an adult, moves deeper into the ocean and does not require a clock anymore, it disconnects its endogenous clock from light inputs. This could potentially explain why we see a significant induction in LD, but none in LD to DD. To answer such questions, we would need to start by examining rhythms in *Sternoptyx* larvae from epipelagic planktonic trawls. It remains none the less an exciting possibility that these animals might be able to turn their clock on and off depending on their life stage.

In zebrafish, *per1* and *Cry1a*/*per2* peak at ZT0 (Tamai et al., 2012). In *Argyropelecus*, *Cry1a* and *per2* peak at ZT9 while *per1* peaks at ZT15. This is an unusual delay, but the kinetics of transcription is probably different in the deep-sea at such cold temperatures. A recent study in clownfish (*Amphiprion ocellaris*), show that the diurnal fish, the phase of the molecular rhythm is different to that of zebrafish, with *per1* peaking at ZT21, and *per2*, *per3* and *Cry1a* peaking at ZT3, and *BMAL* at ZT9 (Schlam et al., 2021). This may indicate that peak amplitude between the different clock genes may be indicative for its subsequent behaviour, and that the delay between *Cry1a*/*per2* and *per1* can still yield a functional behavioural clock.

Most echograms show that fish start migrating up in the afternoon, and down before dawn, suggesting that they would perhaps receive a light pulse at dusk (peak *Cry1a* and *per2*), and

maybe one also at dawn. It is still unclear whether the fish use sun-light as a zeitgeber or if they use moonlight, or neither. For any future cruise, it would therefore be interesting to explore a skeleton entrainment protocol, with light-pulses placed at dawn and dusk, something that is required for zebrafish to entrain optimally to a skeleton regime (Tamai et al., 2007). A moonlight experiment could also be devised, with low intensity lighting placed between the skeleton light pulses. It should be noted that although moonlight is absorbed and scattered in the same way to sunlight, the sun hitting the surface of the ocean has an intensity of 0.1 watts per cm^2, whereas the moon has an intensity of 0.1 microwatt per cm^2, i.e. one millionth of the intensity of the sun (NASA Goddard Space Flight Center 1969). Still, we see that DVM is generally deeper during full-moons (Ochoa-de-la-Torre et al., 2013).

If truly light is the primary zeitgeber for clock function in vertically migrating fish is still unclear. The fish may use a range of zeitgebers to strengthen its amplitude and period, as well as the light signal. However, our data showing that the non-vertical migrator lacks endogenous *per1* rhythm in response to an LD cycle, but the vertical migrator does possess such a rhythm, supports the hypothesis that light can entrain the clock of these deep-sea vertical migrators.

5.3.5 Experimental limitations and challenges of sampling

Designing and setting up experiments on a ship that is not made for biologist and definitely not for circadian or molecular biology means that there are a range of compromises that had to be made when designing experiments. As it was the first research expedition for the Whitmore lab, we also had no preconception about what kind of fish we would catch, the facilities on board or how much or little trawl time we had. It was a steep learning curve in all ways, from learning to identify the different species of fish and "snatch it" with a ladle before any other researcher could, to designing experiments to fit the constantly variable number of samples we obtained rather than the other way around.

Sampling is still is one of the great limitations in deep-sea biology, something I can personally vouch for. It is a relatively barren environment, and most animals do not form any form of shoal or groups, unless feeding on larger carcasses on the abyssal or hadal plains. For catching fish in the water column trawling is the most common tool used. There are a range

of net designs available for trawling, but regardless of your net, you are limited by the amount of wire that is coiled up on the trawl winch drum or how fast you want to cruise. Due to the drag of the net, and the desire to trawl at say 3000m, you will require considerably more wire than 3km. The force exerted on the ship due to the drag of the net, means that the ships has to be of a large size and speeds limited. The FS Sonne's largest drum with the most amount of wire was out for repair, so we had to make do with the second largest drum, which took us down to ~1200m. Initially we were hoping for bathypelagic and abyssopelagic animals that live in complete darkness, but knowing what we know now, even if we could trawl or use landers to catch fish on the bottom, we would never get enough sample to perform biological replicates.

Another thing to consider when sampling is the net. The mesh size will determine the size of the catch, but will also limit the speed of trawling due to the rate at which water can flow through. If there is a reduction in the filtration of the net, water will back up creating a pressure wave in front of the mouth of the net, keeping any catch out. Each net therefore has to be a compromise between mouth area, length and mesh size. Several net designs are available, and the best candidate for potential future research cruises would be a set-up, where multiple nets are attached to the wire, which mean sampling at different depths can be done simultaneously.

Furthermore, due to the change of depth, oxygen and thermocline as well as the forces the fish experience when trawled, most fish come up either dead or at least the worst for wear. Experiments with overtly active, "live" animals were not possible from trawl catches. Even if we could get hold and maintain free swimming deep-sea fish, there are no institution that will make a 1000m deep tank that would allow behavioural studies of vertical migration with the requisite water pressure. The best current compromise would be to devise a circadian trawling schedule, with multiple nets. This would allow us to identify which animals are likely to be vertical migrators as well as providing a time course series of tissue samples. Trawling in such a circadian manner was not agreed with other scientists and crew on the ship, so organ culture was therefore the only possible way to assess light sensitivity and circadian rhythmicity.

In retrospect it seems naive, but we were initially baffled to find that nothing "survived" a trawl in good shape, except a few copepods. Although we had brought culturing media and

plastic, we didn't expect that we had to rely solely on tissue culture, we therefore had to improvise considerably. The tissue culture conditions are an example: Most tissue culture conditions are warm rather than cold, which is based on the core temperature of the animals' tissue like mice or human, or its environment for poikilothermic like teleosts. We know very little about the fish we sampled, but we know that they would both experience temperatures down to 4-8C based on an average thermocline, but the vertical migrator may experience temperature up to 3-4 times that of its daytime depth when it migrates up at night to feed. We do not know exactly how far up either of the species go, but as we had no incubator that would keep temperatures under room temperatures, we decided to culture the organs in the cold room which we kept at 6C. The decision to keep the tissues so cold were also backed up by the fact that we didn't have any culture hood that would allow us to work in sterile conditions. Bacterial growth in the media were likely to be lower at 6C compared to say 12 or 15C.

The light cycles and light pulses are also something that these fish would never experience in the wild, so we had to start with the simplest scenario, so this study is as much a proof of principle rather than a mimic of their natural environmental conditions. Future experiments should however consider and test the deep-sea spectral qualities and intensity, sensitivity and temperature cycles. Sadly, the second cruise onboard RS Sonne never came to fruition and so we have been unable to extend this data set with a second expedition.

Here, I have presented an initial analysis of the deep-sea data to date. It is clear that more analysis is needed and perhaps also a few more experiments required, and this is currently being worked on. It is rather typical that a set of experiments raises more questions than they answer. I do however believe that the data we have gathered here has enable us to to answer some of these questions and prepared the way for future cruises. We now have a much clearer idea of what to expect and what to ask for in terms of trawling times, the species we can expect at different depths and the conditions on board. The biggest challenge now is to get funding for another deep-sea cruise.

Whoever stated that *there is light in the darkest of places* was certainly not wrong when it comes to the deep-sea.

6 Concluding remarks

6. Concluding Remarks

In this PhD I have presented data on how light impacts different species of fish in very different environments.

In the first data chapter, I have explored how the zebrafish, a "swimming photoreceptor", responds to different wavelengths of light. The zebrafish express 42 different opsin genes, with each organ expressing a specific combination. Why the different organs express different combination of opsins, we do not know, but we hypothesised that it may give each organ a separate and defined wavelength-sensitivity. In this chapter I tested this hypothesis, and show that indeed, different organs do respond differently to different monochromatic wavelengths, from UV to IR, when examining *cry1a*, *per2* and *6-4 Phr* expression *in vitro*. While eye and brain show a similar response to all monochromatic wave-lengths, organs like the heart have a red-light "preference". Using zebrafish PAC2 cell lines, we showed that a monochromatic light pulse from 350-850nm can induce light responsive clock genes, such as *cry1a* and *per2*, but only light 350-700 nm can phase-shift the clock. Upon examining what opsins are present in zebrafish cell lines, we found that they express 11 out of 32 non-visual opsins, and that about half of these are even showing a day-night difference. Their expression pattern does not mimic any tissues we have examined to date, but show that some of the opsins are under direct clock regulation themselves, which may impact the light responsiveness of the cell in a circadian manner.

With light being such an important input into zebrafish biology, the Mexican blind cavefish, *Astyanax mexicanus* and its rapid evolution to a life in the dark is fascinating to study in parallel. In the second data chapter, I established that there is a big difference to when the clock "starts ticking" in the different populations of cavefish, and that the light inducible genes such as *per2a* and *per2b* have very different expression patterns, not only compared to the river dwelling surface fish, but also across the different cave populations. The same kinds of expression patterns were also seen in the embryonic cell lines that I established. This would suggest that the adaptations to the dark in terms of clock and light inducible genes are slightly different in the different caves, and a comparative genome study examining the circadian/light/opsin genes and regulatory sequences would be a very interesting future study.

Finally, I presented data on two species of deep-sea hatchetfishes. We have long wondered if deep-sea fish possess a clock and how they respond to light. Many animals show daily vertical migrations over long distances relative to body size, and historically this has been attributed to the animals following a certain, fixed light intensity. This hypothesis is problematic, and there is no conclusive evidence that backs the theory. In this final chapter I have presented evidence that the vertically migrating hatchetfish *Argyropelecus hemigymnus* have an endogenous clock *in vitro*. In contrast, the non-migrating fish *Sternoptyx diaphana* has the ability to entrain, but the period is unstable in DD. Interestingly, when we examine their response to light, we see that the non-vertical migrator shows a more than twice the amount of differentially expressed genes to that of the vertical migrator. Whether this is down to issues with bioinformatic analysis, or the fact of the matter, is something I will continue to explore.

Although working on wild animals, like deep-sea fish and cavefish, are hugely exciting, there are still some "perks" on working with a model organism, such as the zebrafish. This is particularly evident when we come to design light and circadian experiments for our non-model organisms, when do we light pulse? How long? How many time points are needed? Many assumptions have to be made, based on previous zebrafish studies. For zebrafish, we have almost 30 years of experimental evidence to help design the best circadian experiments, not to mention that we have over 100 000 fish in the basement in London. The zebrafish remains, therefore, a key model species to work with when it comes to questions, such as the function of the different opsins – not least because Crispr-CAS approaches have now been established in this model animal.

Cave animals and deep-sea animals both live in the dark, but their habitats are actually quite different, and the more I have worked with this project, the more it has become clear to me that these two habitats only have darkness as their common denominator. This is exemplified in the lack of cues that are present in the caves, especially the caves that have no bat populations. Some caves, like the Chica cave, have a bat population, and thereby a daily cue driven by rhythmic bat behaviour. However, bat populations are not stable over time, and although they might have been roosting in the same cave for a few hundred years, their ancestors are likely to have roosted in other caves. As such, a stable entrainment cue has therefore not been present over the time that the cavefish have evolved in the caves. In contrast, the mesopelagic zone is home to many animals which undergo daily vertical

migrations. Although light is incredibly dim below 200 metres, it's likely that it is enough to entrain circadian clocks in animals, and having a clock in such an environment is highly likely to give a fitness benefit. There are however more dark places to explore. The abyssal floor in the deep-sea for example, which is probably more similar to a cave environment being barren, dark and bioluminescence being rare. The animals that live there have very reduced or no eyes for example, but seeing as these animals are caught once every few decades, it doesn't exactly serve as a candidate for gene expression work, so we can only speculate.

In this thesis I have touched upon three very different fish species, and demonstrated how light and clock impacts biology in very different ways. I started with the zebrafish, which is highly light sensitive and clock-regulated. I continued with the cavefish, that do not use light or clock in any way in the wild, and across all cave populations, show adaptations such as raised expression levels of clock and light inducible genes in the dark. I ended with the deep-sea hatchet fish, *A. hemigymnus,* which in some strange way is an intermediate of the two, as it has a molecular clock that they may use to determine vertical migration, but has few up-regulated genes in response to light, - indicating that some biology may be clock, but not light driven. Taking an evolutionary, comparative approach to studying clocks and light sensitivity has been interesting and productive, but raises many new questions.

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Figure/Picture reference 2.1:

Map: (Bradic et al. 2012)

Cave pictures: (Jefferey, 2020)

Surface picture: https://gallery.nanfa.org/ Astyanax mexicanus Mexican Tetra 2245WS (Link last accessed Dec 2020)

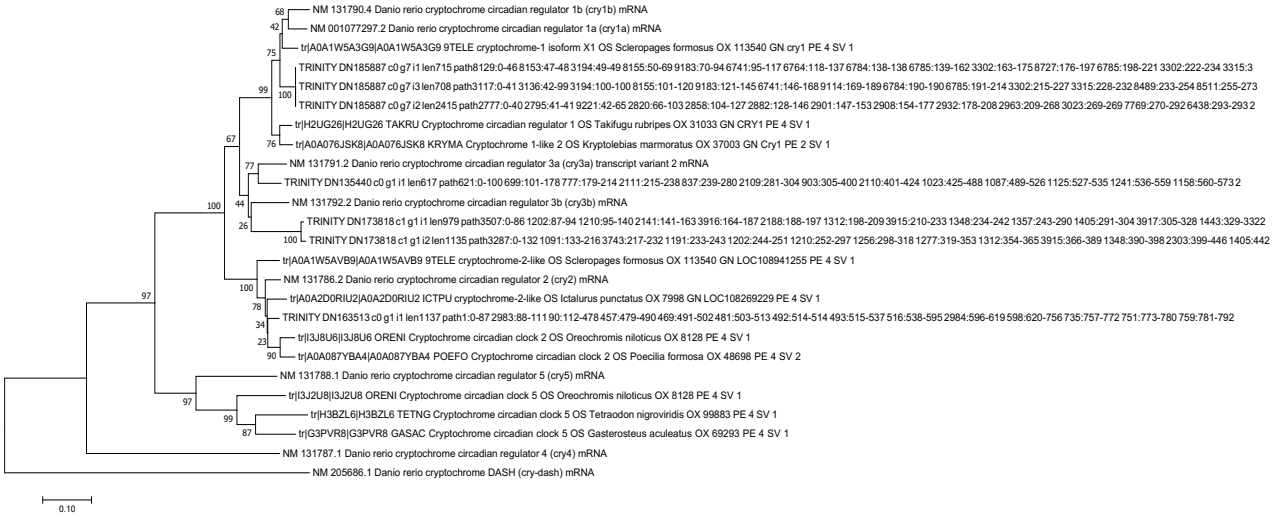
Figure 3.1 Reference:

Oceanic Zones:
https://www.nps.gov/subjects/oceans/open-ocean.htm (last accessed Nov 2020)

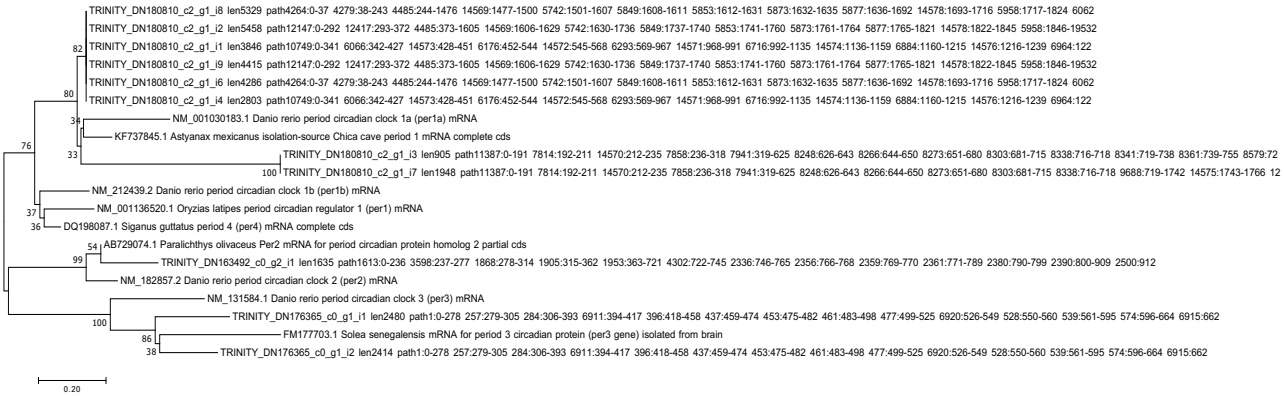
Figure 3.2 Reference:

https://oceanexplorer.noaa.gov/explorations/04deepscope/background/deeplight/media/diagram3.html (Link last accessed December 2020) (source *Image courtesy of Kyle Carothers, NOAA-OE*)

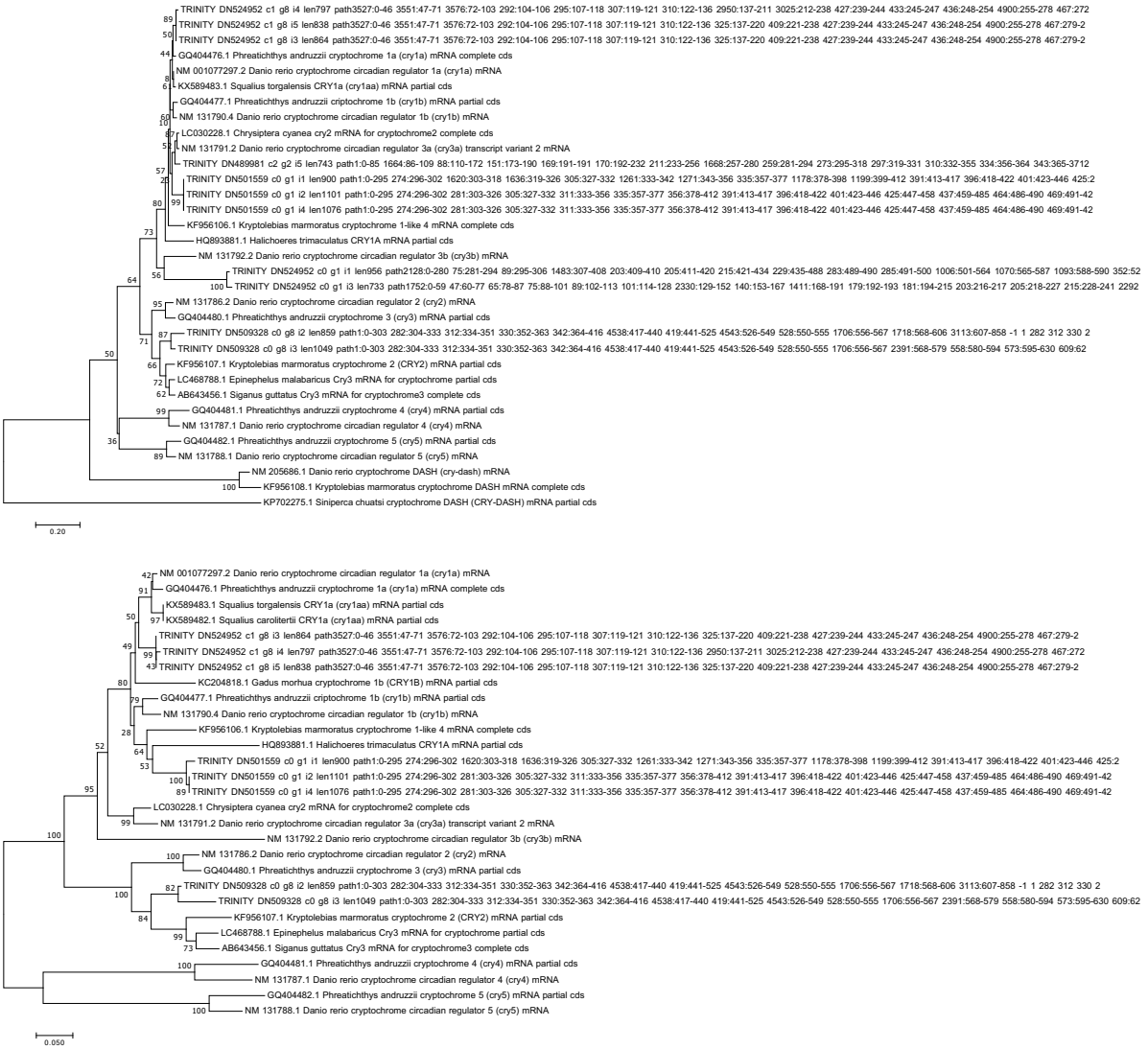
Appendix 1
Supplementary figures and tables



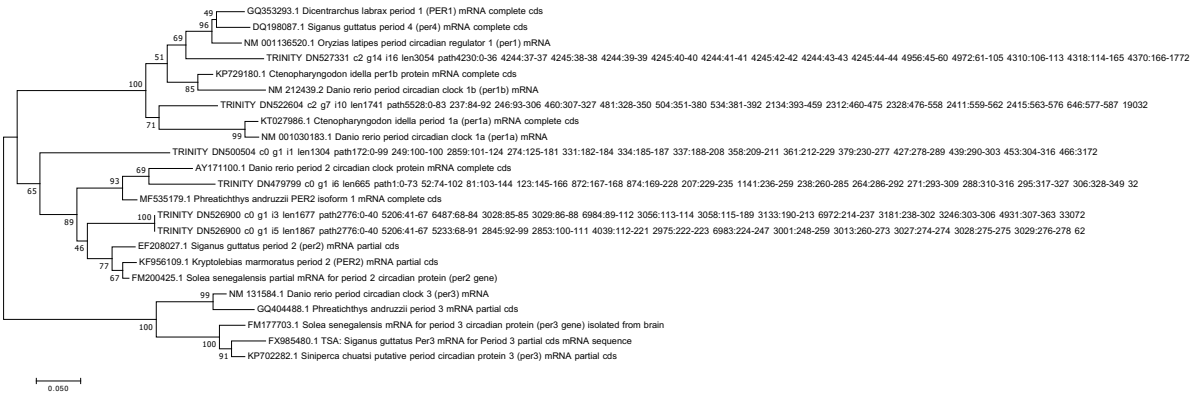
Supplementary Figure 2.1 Neighbour-joining tree for *Sternoptyx* Cryptochromes



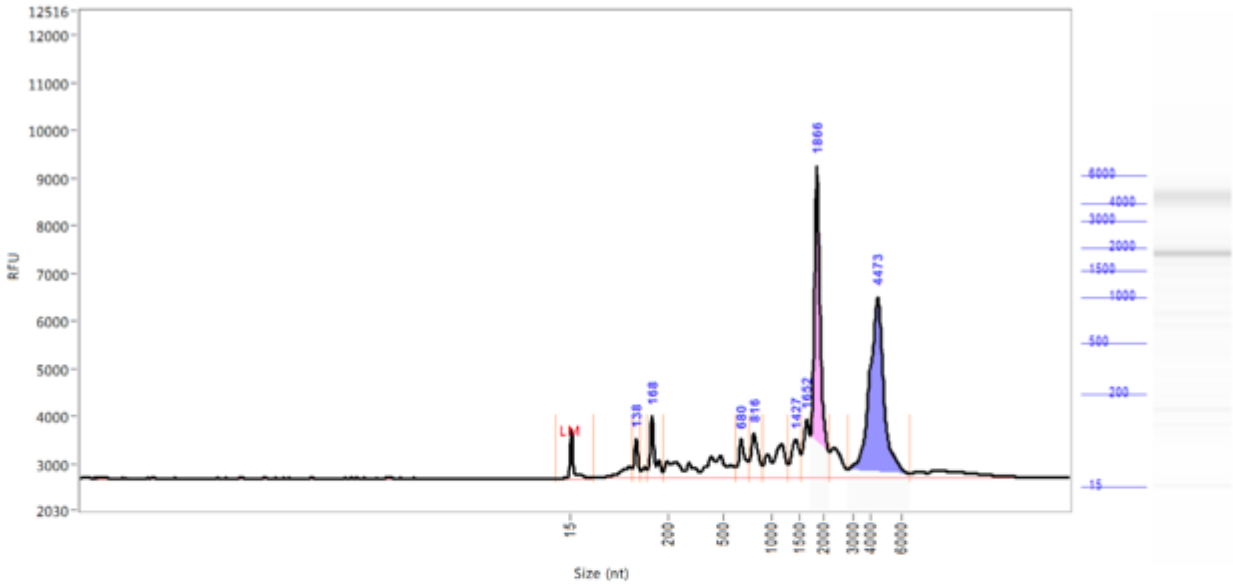
Supplementary Figure 2.2 Neighbour-joining tree for *Sternoptyx* Period Genes



Supplementary Figure 2.3 Neighbour-joining trees (trinity genes did not overlap) for *Argyropelecus* Cryptochrome Genes



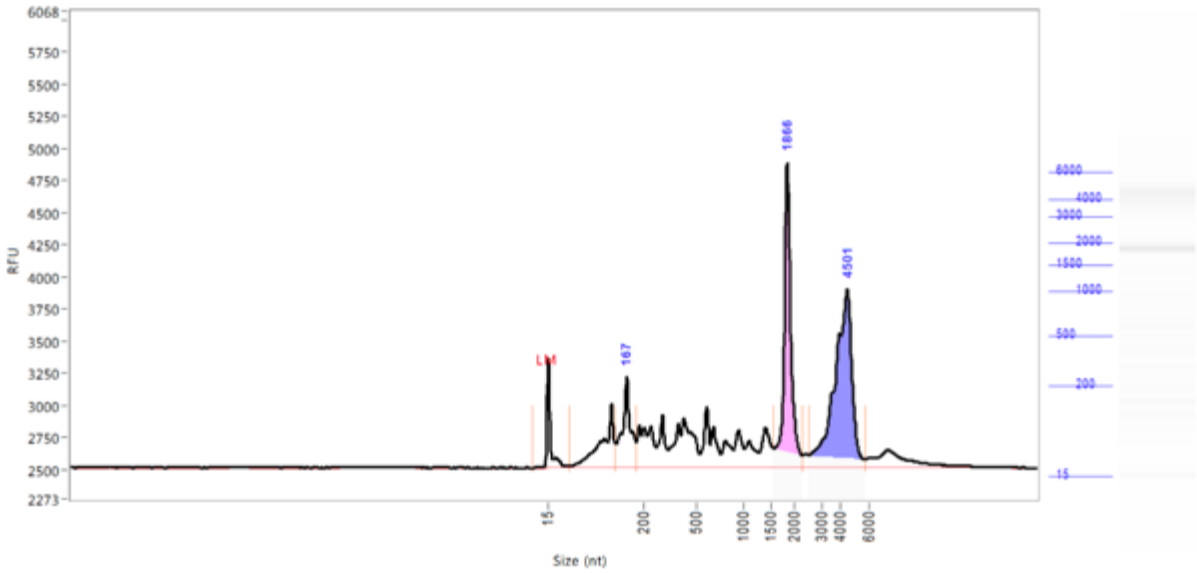
Supplementary Figure 2.4 Neighbour-joining tree for *Argyropelecus* Per genes



Conc. (ng/uL)	Molarity (nmole/L)	From (nt)	To (nt)	Rel. Conc. % (ng/uL)
1.0546	177.8941	0	57	
10.5268	238.8782	130	147	3.1
20.1698	369.1130	159	188	6.0
14.5071	65.2691	628	764	4.3
18.2365	68.7618	764	908	5.4
17.1953	37.7694	1295	1528	5.1
18.1818	34.6652	1528	1724	5.4
106.9865	176.6667	1724	2204	31.6
133.0844	94.9881	2855	6577	39.3

TIC:	338.8880	ng/uL
TIM:	1086.1116	nmole/L
Total Conc.:	456.8482	ng/uL
28S/18S:	1.5	
RQN	7.0	

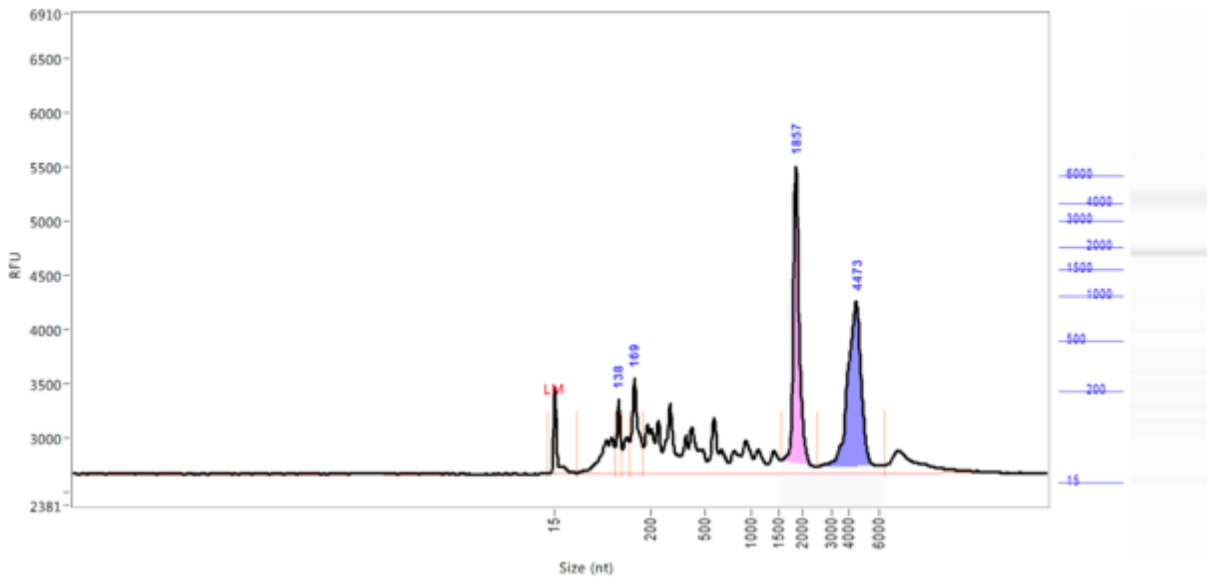
Supplementary Figure 2.5 RNA quality assessment of *Argyropelecus* mixed tissues (59-A)



Conc. (ng/uL)	Molarity (nmole/L)	From (nt)	To (nt)	Rel. Conc. % (ng/uL)
1.0546	177.8942	0	57	
22.1558	415.1973	146	186	16.2
47.9851	79.9572	1563	2320	35.1
66.5993	50.7134	2551	5750	48.7

TIC:	136.7401	ng/uL
TIM:	545.8679	nmole/L
Total Conc.:	258.8138	ng/uL
28S/18S:	1.4	
RQN	6.2	

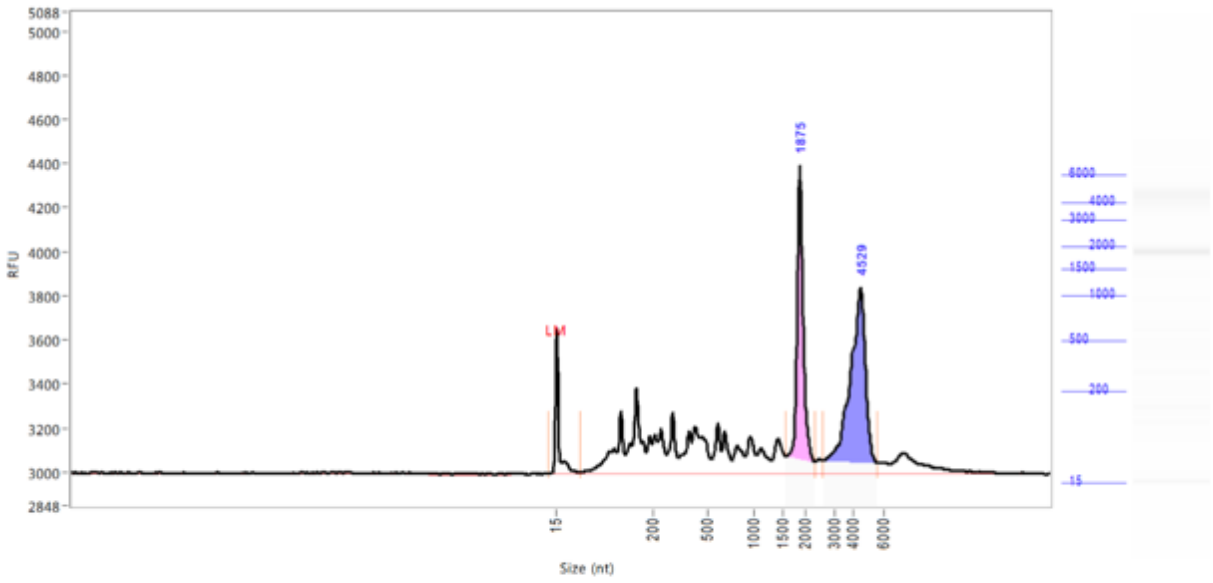
Supplementary Figure 2.6 RNA quality assessment of *Argyropelecus* LP Sample (61-487)



Conc. (ng/uL)	Molarity (nmole/L)	From (nt)	To (nt)	Rel. Conc. % (ng/uL)
1.0546	177.8942	1	57	
11.2050	252.4336	131	145	6.8
23.2853	423.6429	159	186	14.2
60.1442	99.6325	1546	2537	36.6
69.7447	50.7669	2537	6384	42.4

TIC:	164.3792	ng/uL
TIM:	826.4760	nmole/L
Total Conc.:	309.0534	ng/uL
28S/18S:	1.2	
RQN	5.9	

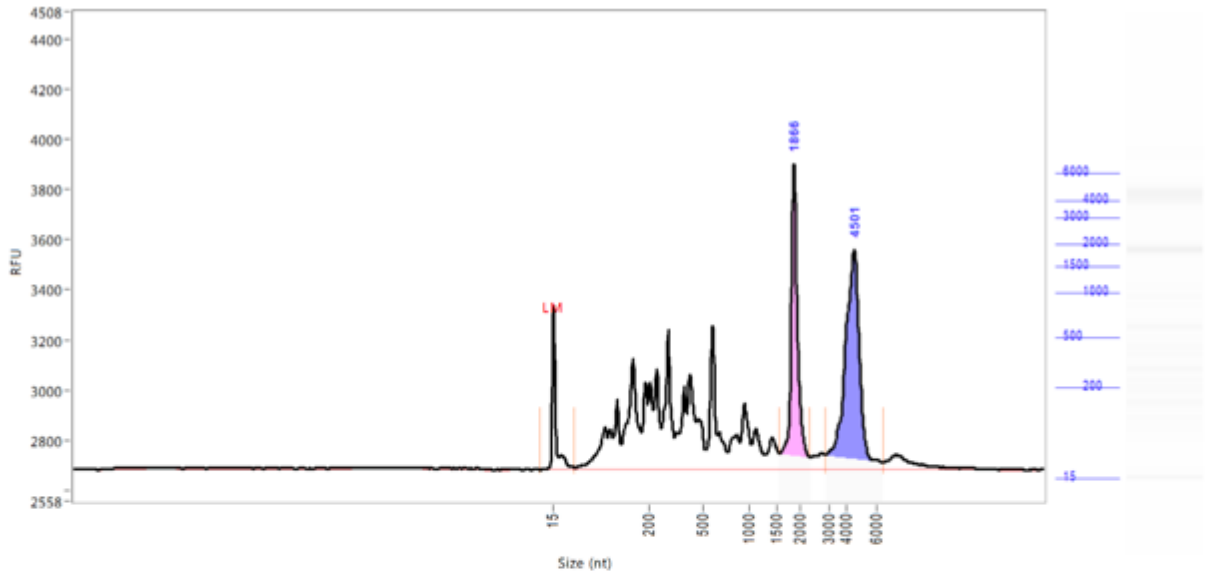
Supplementary Figure 2.7 RNA quality assessment of *Argyropelecus* LP Sample (62-488)



Conc. (ng/uL)	Molarity (nmole/L)	From (nt)	To (nt)	Rel. Conc. % (ng/uL)
1.0546	177.8942	0	60	
36.1686	59.9155	1572	2320	41.9
50.1325	37.5513	2624	5667	58.1

TIC:	86.3011	ng/uL
TIM:	97.4667	nmole/L
Total Conc.:	191.6291	ng/uL
28S/18S:	1.4	
RQN	6.3	

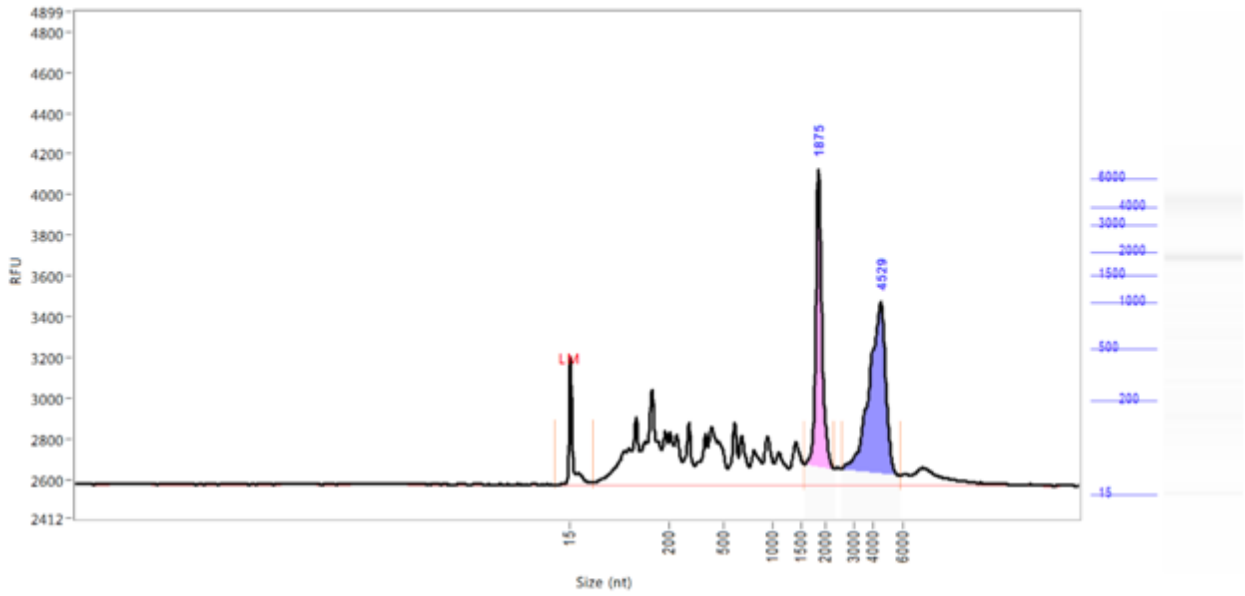
Supplementary Figure 2.8 RNA quality assessment of *Argyropelecus* LP Sample (63-488-2)



Conc. (ng/uL)	Molarity (nmole/L)	From (nt)	To (nt)	Rel. Conc. % (ng/uL)
1.0546	177.8942	0	56	
32.9926	54.6543	1554	2348	41.0
47.5620	34.1816	2913	6522	59.0

TIC:	80.5547	ng/uL
TIM:	88.8359	nmole/L
Total Conc.:	226.7233	ng/uL
28S/18S:	1.5	
RQN	5.0	

Supplementary Figure 2.9 RNA quality assessment of *Argyropelecus* LP (Sample 64-494)

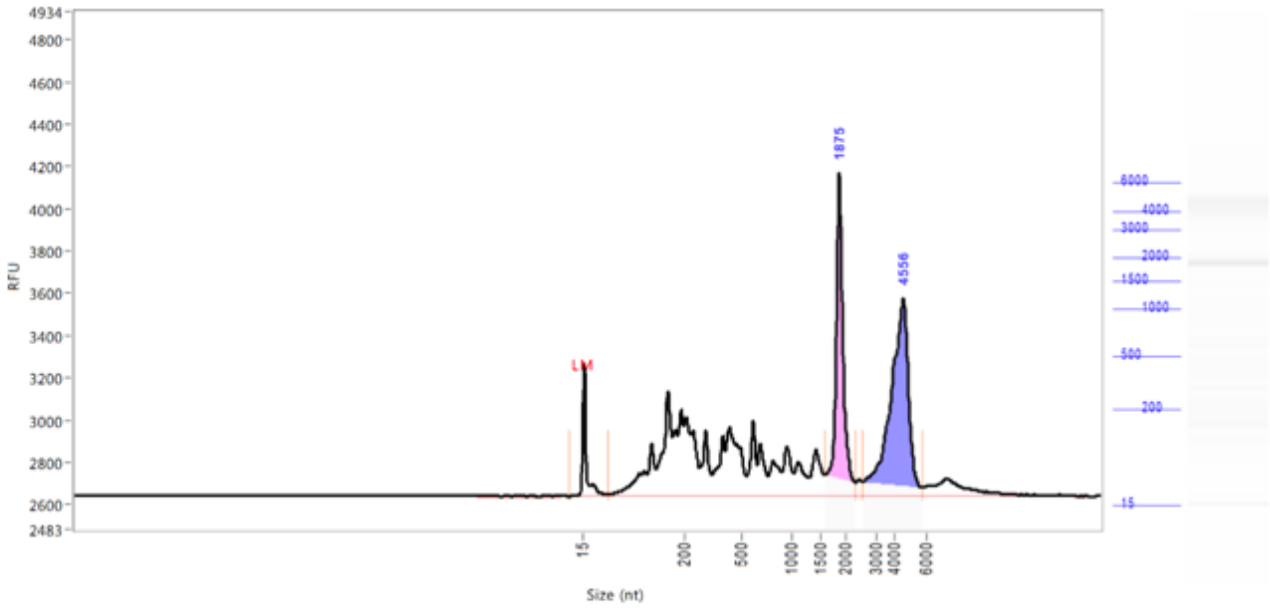


Conc. (ng/uL)	Molarity (nmole/L)	From (nt)	To (nt)	Rel. Conc. % (ng/uL)
1.0546	177.8941	0	57	
43.7049	72.3230	1572	2334	41.8
60.8356	45.9433	2580	5833	58.2

TIC:	104.5405	ng/uL
TIM:	118.2663	nmole/L
Total Conc.:	248.7735	ng/uL

28S/18S:	1.4
RQN	5.9

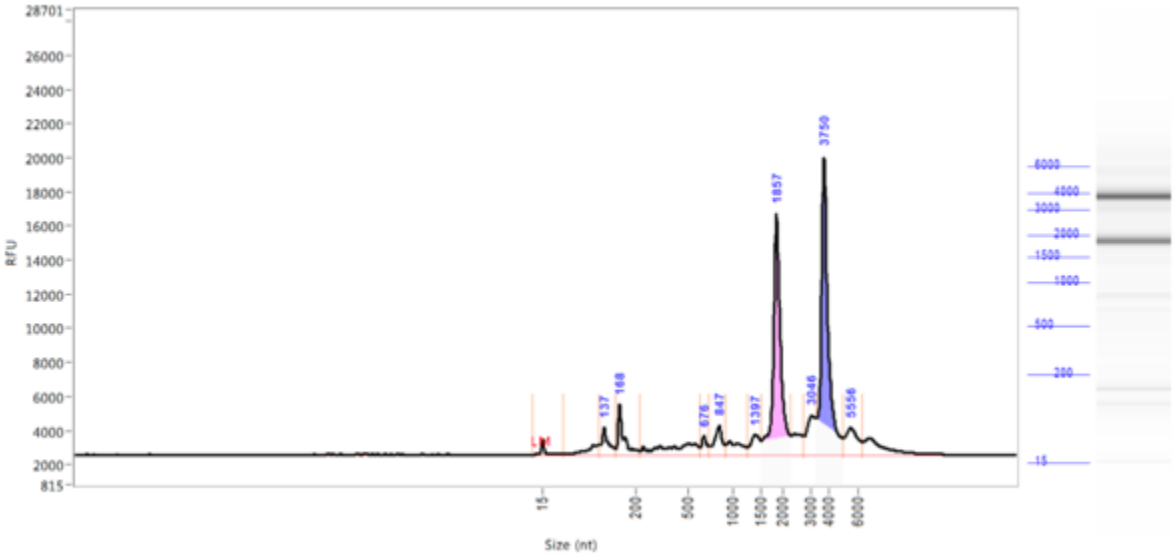
Supplementary Figure 2.10 RNA quality assessment of *Argyropelecus* DD (Sample 65-495)



Conc. (ng/uL)	Molarity (nmole/L)	From (nt)	To (nt)	Rel. Conc. % (ng/uL)
1.0546	168.7696	0	61	
41.9354	69.3581	1581	2334	41.4
59.4719	44.6004	2595	5778	58.6

TIC:	101.4073	ng/uL
TIM:	113.9584	nmole/L
Total Conc.:	253.5183	ng/uL
28S/18S:	1.5	
RQN	5.5	

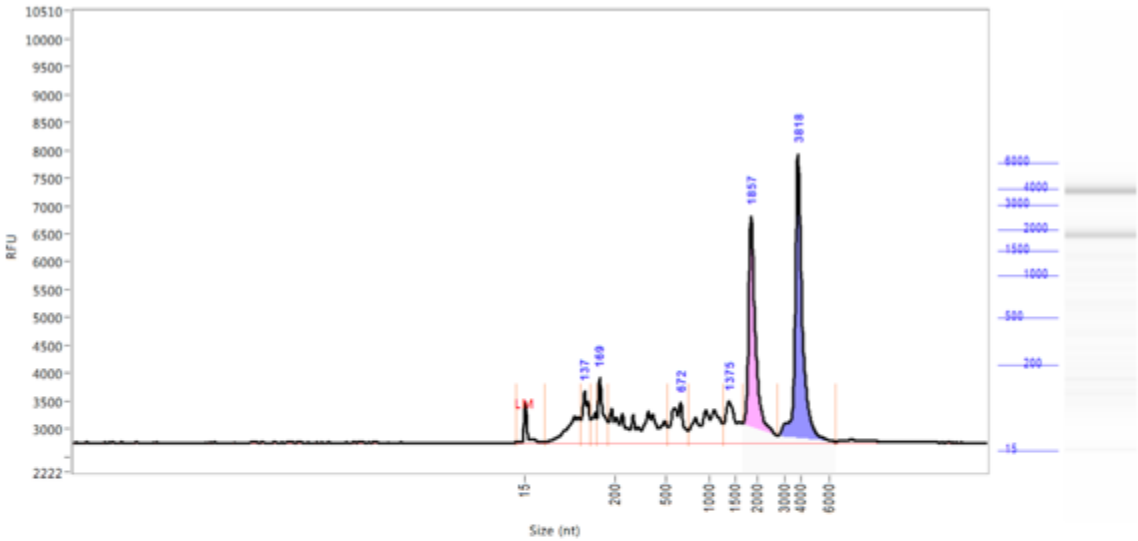
Supplementary Figure 2.11 RNA quality assessment of *Argyropelecus* DD (Sample 66-496)



Conc. (ng/uL)	Molarity (nmole/L)	From (nt)	To (nt)	Rel. Conc. % (ng/uL)
1.0546	168.7696	0	57	
38.7094	853.5788	128	160	4.2
60.0061	1060.7974	160	224	6.5
18.1837	83.3736	632	733	2.0
41.5603	156.1396	733	917	4.5
32.0416	71.6916	1243	1510	3.5
300.2722	500.8760	1510	2291	32.4
55.1432	56.7741	2768	3296	6.0
328.5695	265.5560	3296	5028	35.5
51.0147	28.1397	5028	6384	5.5

TIC:	925.5006	ng/uL
TIM:	3076.9268	nmole/L
Total Conc.:	1138.3527	ng/uL
28S/18S:	1.0	
RQN	8.3	

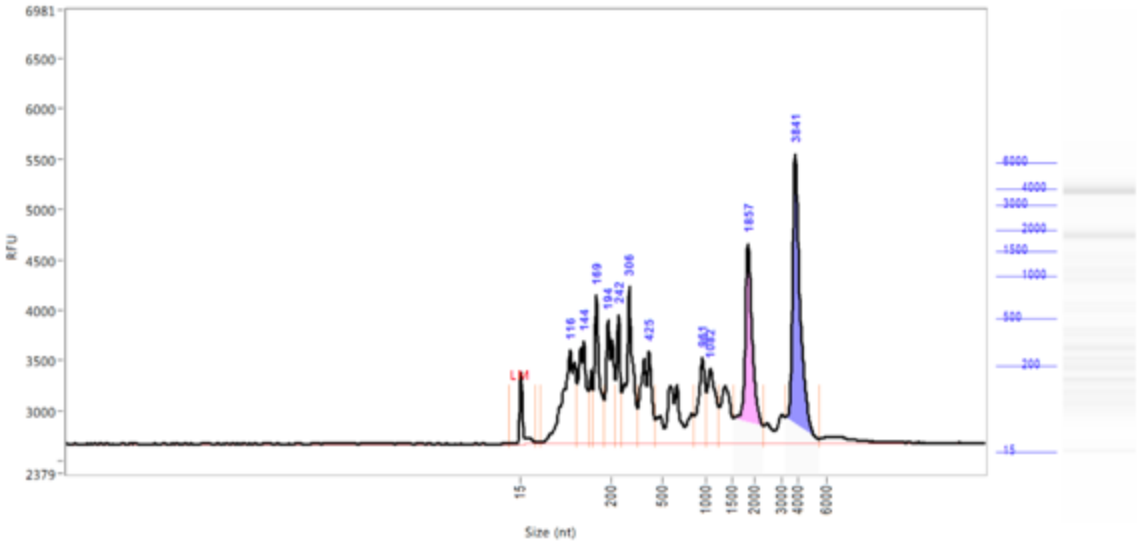
Supplementary Figure 2.12 RNA quality assessment of *Sternoptyx* mixed tissue sample (60-S)



Conc. (ng/uL)	Molarity (nmole/L)	From (nt)	To (nt)	Rel. Conc. % (ng/uL)
1.0546	168.7696	0	57	
26.5880	594.6962	129	149	7.1
30.5203	552.0541	162	187	8.2
31.9539	157.1327	527	759	8.6
30.6933	66.6671	1265	1661	8.2
116.5528	187.0199	1661	2768	31.2
137.2255	109.6303	2768	6467	36.7

TIC:	373.5339	ng/uL
TIM:	1667.2004	nmole/L
Total Conc.:	548.4821	ng/uL
28S/18S:	1.4	
RQN	6.0	

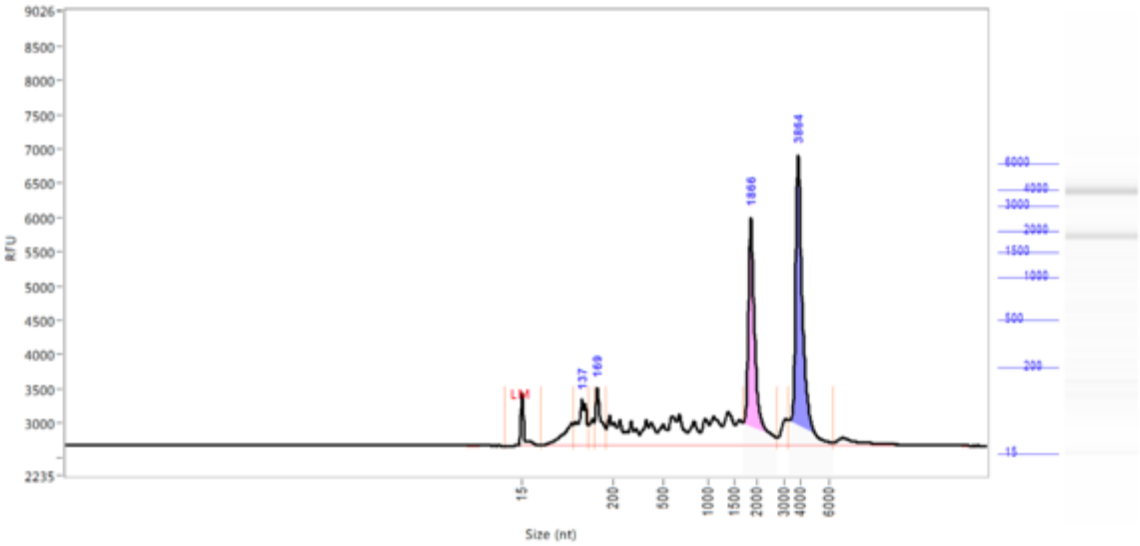
Supplementary Figure 2.13 RNA quality assessment of *Sternoptyx* LP (Sample 67-497)



Conc. (ng/uL)	Molarity (nmole/L)	From (nt)	To (nt)	Rel. Conc. % (ng/uL)
1.0546	177.8941	0	45	
61.3757	1748.9188	57	130	12.3
39.0110	860.2299	130	154	7.8
37.9758	686.9099	163	185	7.6
41.5732	650.2060	185	224	8.3
24.0056	308.8731	224	263	4.8
47.9169	486.2072	263	354	9.6
42.4709	325.9920	354	463	8.5
24.7065	81.3591	864	1008	5.0
25.3351	70.7372	1008	1250	5.1
67.7993	113.0338	1519	2334	13.6
86.3305	68.0809	3228	5472	17.3

TIC:	498.5005	ng/uL
TIM:	5400.5479	nmole/L
Total Conc.:	589.4081	ng/uL
28S/18S:	1.5	
RQN	4.2	

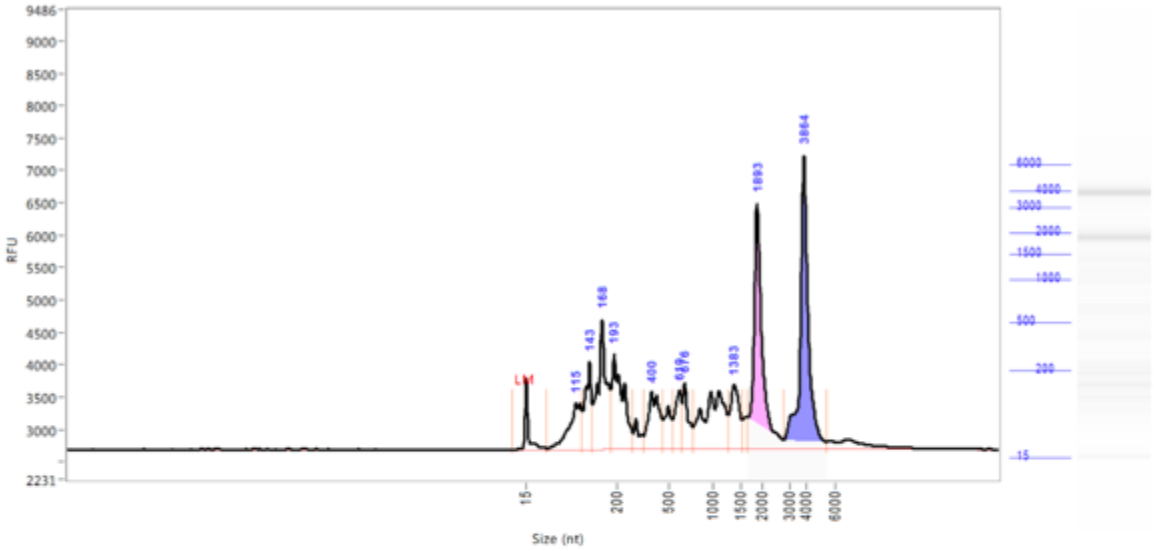
Supplementary Figure 2.14 RNA quality assessment of *Sternoptyx* LP (Sample 68-498)



Conc. (ng/uL)	Molarity (nmole/L)	From (nt)	To (nt)	Rel. Conc. % (ng/uL)
1.0546	177.8942	0	55	
26.2883	600.9156	119	150	10.9
21.4140	387.3382	162	186	8.8
86.9942	139.3038	1688	2768	35.9
107.5469	83.6495	3296	6302	44.4

TIC:	242.2433	ng/uL
TIM:	1211.2072	nmole/L
Total Conc.:	421.5766	ng/uL
28S/18S:	1.3	
RQN	6.2	

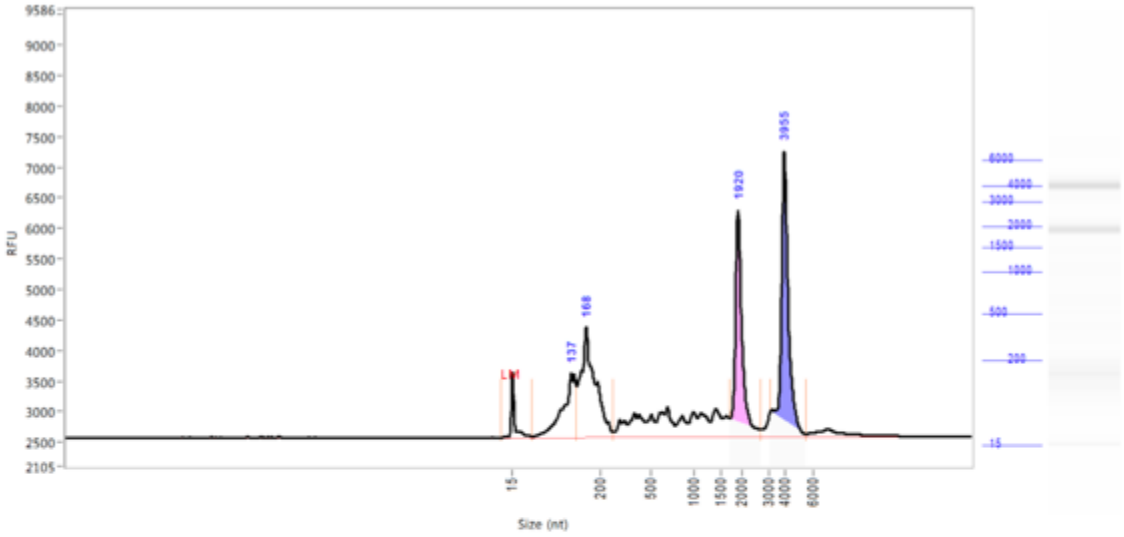
Supplementary Figure 2.15 RNA quality assessment of *Sternoptyx* LP (Sample 69-499)



Conc. (ng/uL)	Molarity (nmole/L)	From (nt)	To (nt)	Rel. Conc. % (ng/uL)
1.0546	177.8941	0	57	
29.3209	843.2099	57	129	7.4
24.3845	545.4110	129	148	6.2
52.7060	975.9820	148	186	13.4
48.4221	697.8553	186	290	12.3
27.4817	207.8722	352	466	7.0
16.0460	83.9325	536	645	4.1
15.2832	68.6620	645	768	3.9
21.8677	48.7532	1273	1537	5.6
75.6077	120.3294	1670	2797	19.2
82.8259	66.6996	2797	5389	21.0

TIC:	393.9457	ng/uL
TIM:	3658.7063	nmole/L
Total Conc.:	471.0270	ng/uL
28S/18S:	1.4	
RQN	4.7	

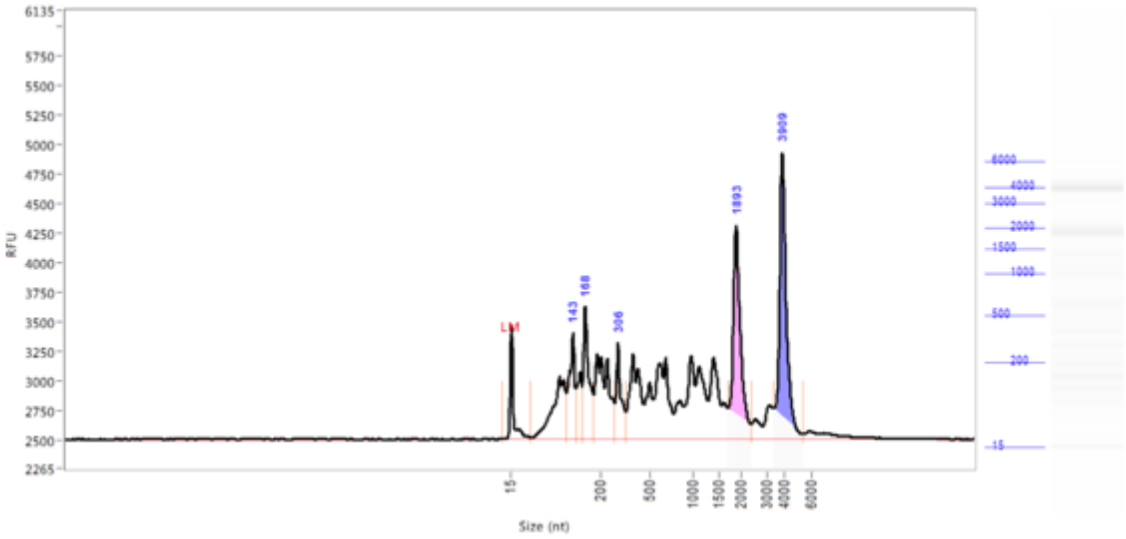
Supplementary Figure 2.16 RNA quality assessment of *Sternoptyx* DD (Sample 70-505)



Conc. (ng/uL)	Molarity (nmole/L)	From (nt)	To (nt)	Rel. Conc. % (ng/uL)
1.0546	168.7696	0	57	
44.8487	1115.0430	57	148	16.6
81.5301	1433.1805	148	272	30.2
63.8201	101.1567	1715	2710	23.6
80.2125	62.4512	3137	5528	29.7

TIC:	270.4113	ng/uL
TIM:	2711.8315	nmole/L
Total Conc.:	365.3610	ng/uL
28S/18S:	1.3	
RQN	5.5	

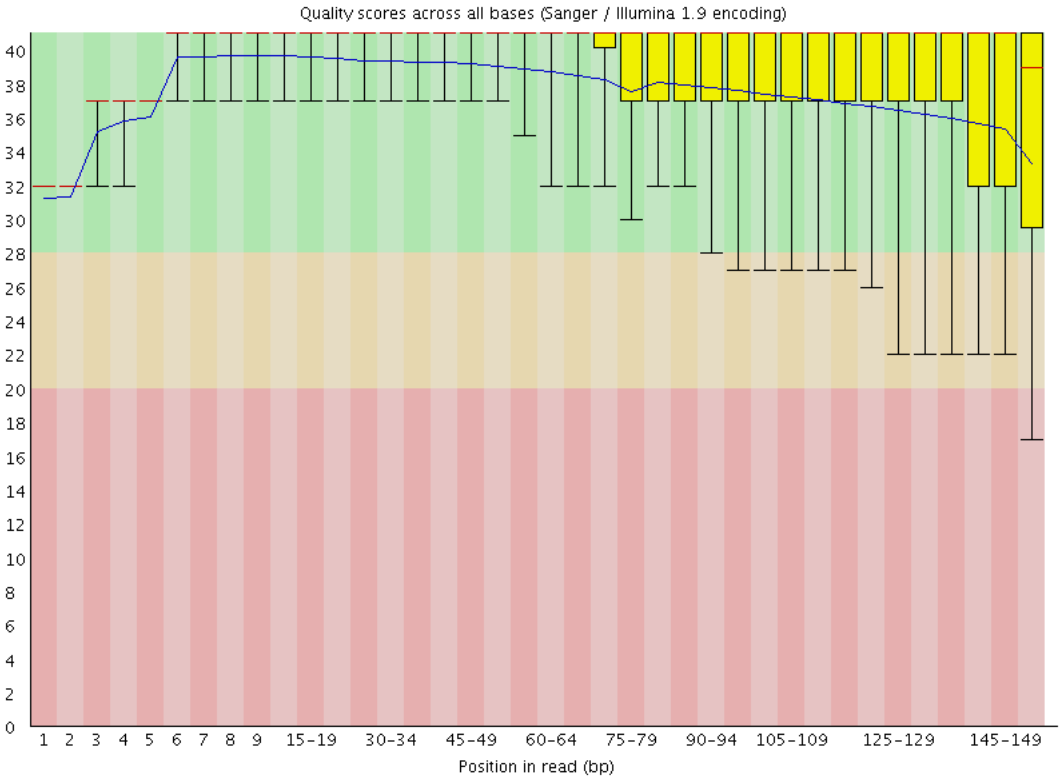
Supplementary Figure 2.17 RNA quality assessment of *Sternoptyx* DD (Sample 71-506)



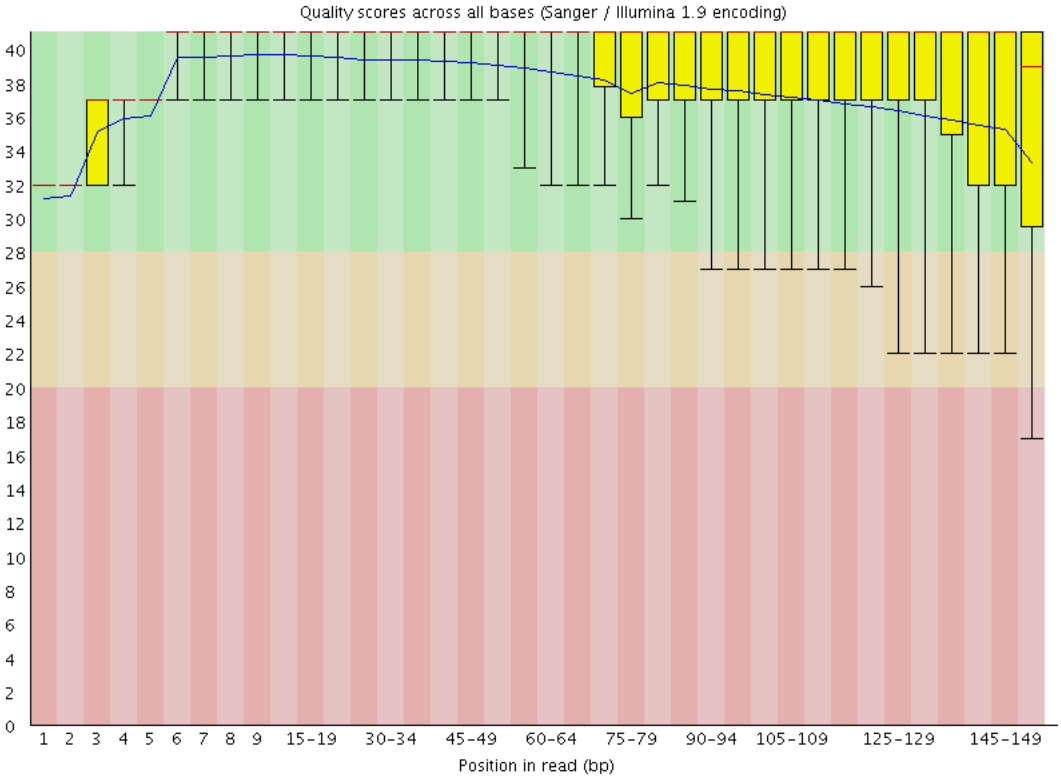
Conc. (ng/uL)	Molarity (nmole/L)	From (nt)	To (nt)	Rel. Conc. % (ng/uL)
1.0546	177.8941	0	56	
18.4433	412.5235	129	149	13.3
22.9734	415.5453	162	185	16.6
13.7606	136.0866	288	354	10.0
38.7159	62.6711	1697	2377	28.0
44.3495	34.7460	3364	5389	32.1

TIC:	138.2427	ng/uL
TIM:	1061.5725	nmole/L
Total Conc.:	334.2388	ng/uL
28S/18S:	1.2	
RQN	4.0	

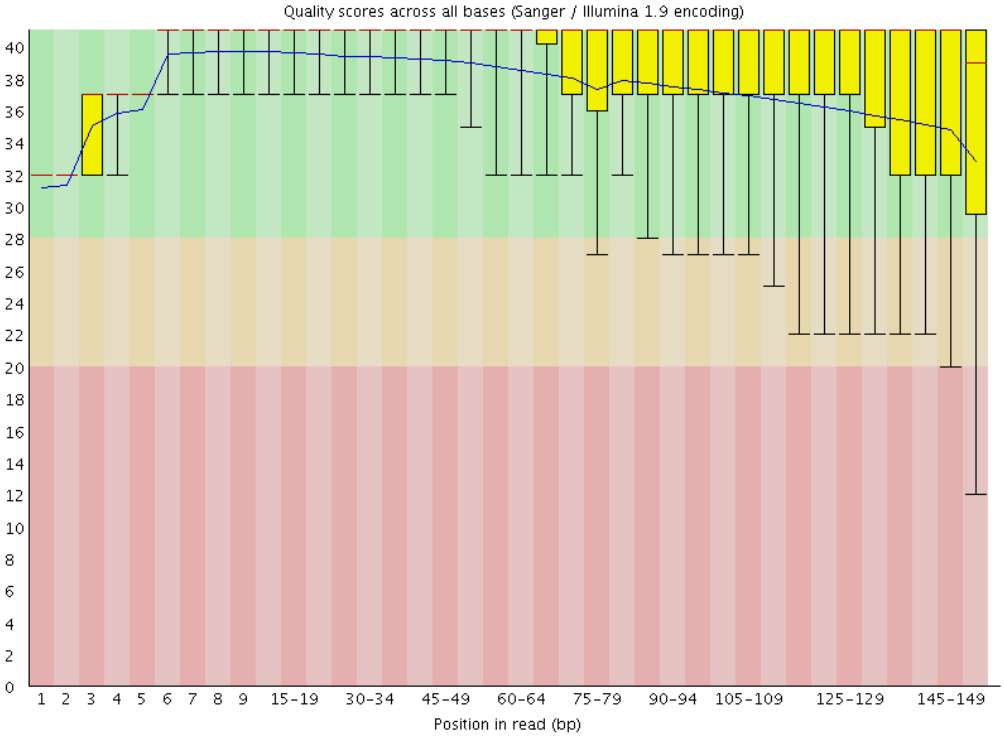
Supplementary Figure 2.18 RNA quality assessment of *Sternoptyx* DD (Sample 72-507)



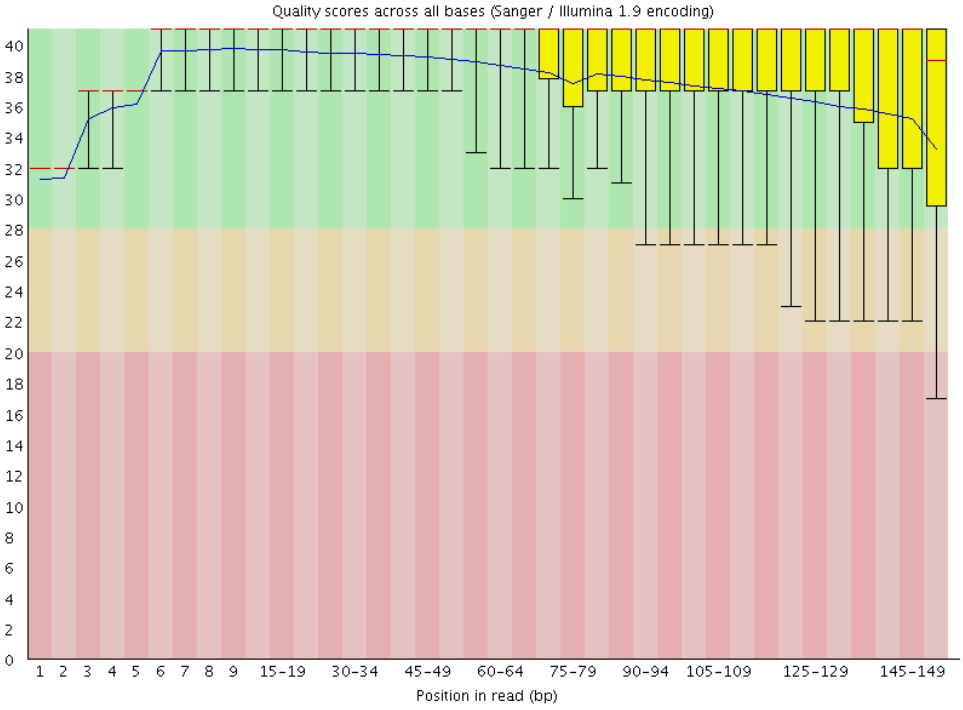
Supplementary Figure 2.19 FastQC report showing per base sequence quality for *Argyropelecus* mixed tissues (Sample 59-A)



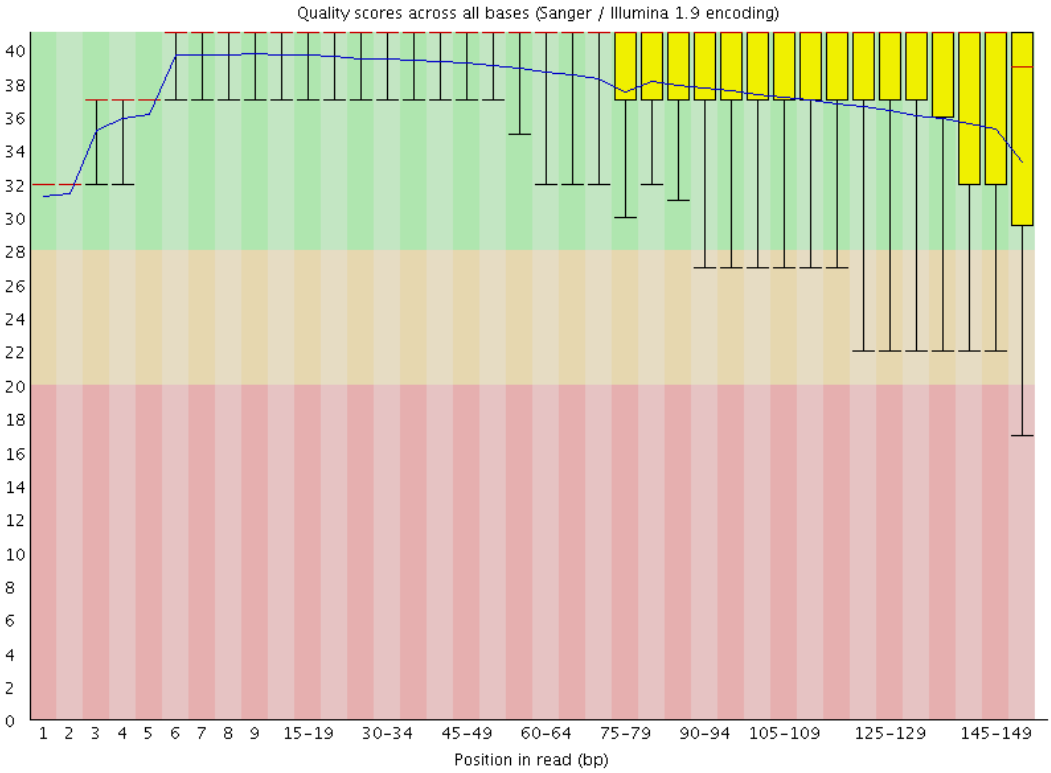
Supplementary Figure 2.20 FastQC report showing per base sequence quality for *Argyropelecus* LP (Sample 61-487)



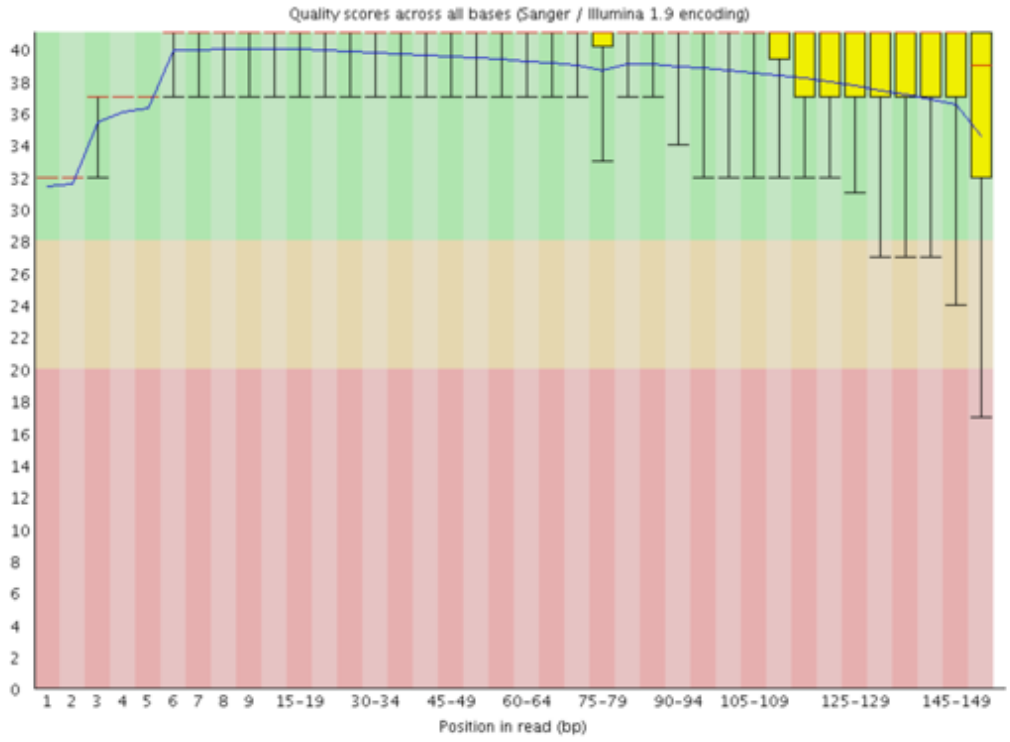
Supplementary Figure 2.21 FastQC report showing per base sequence quality for *Argyropelecus* LP (Sample 62-488)



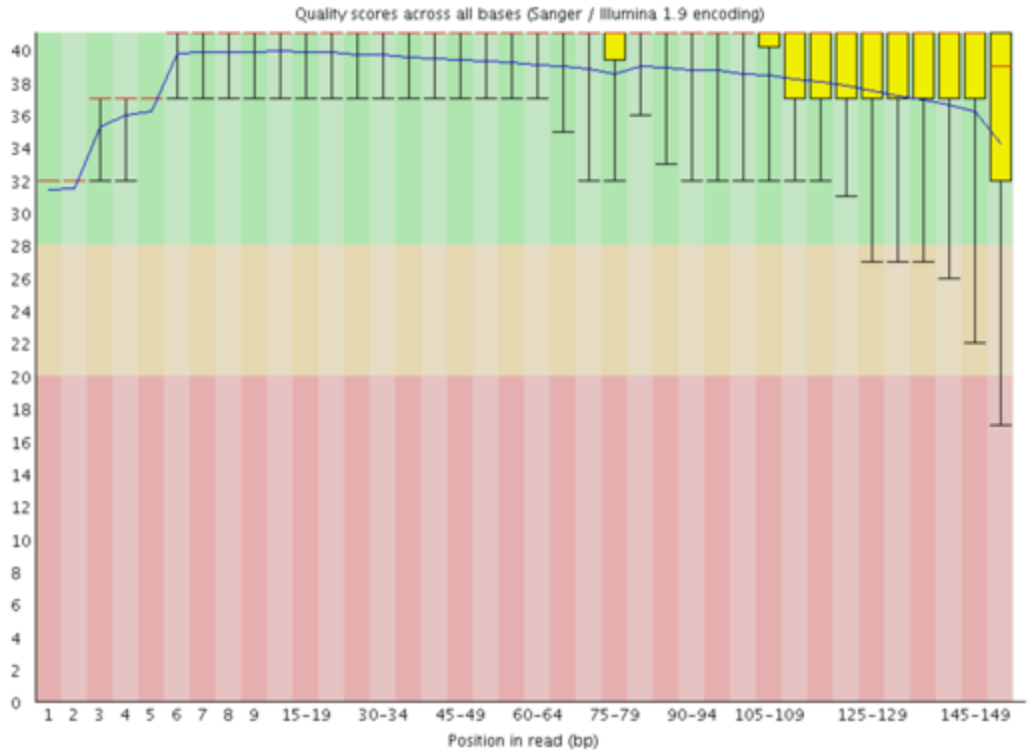
Supplementary Figure 2.22 FastQC report showing per base sequence quality for *Argyropelecus* LP (Sample 63-488-2)



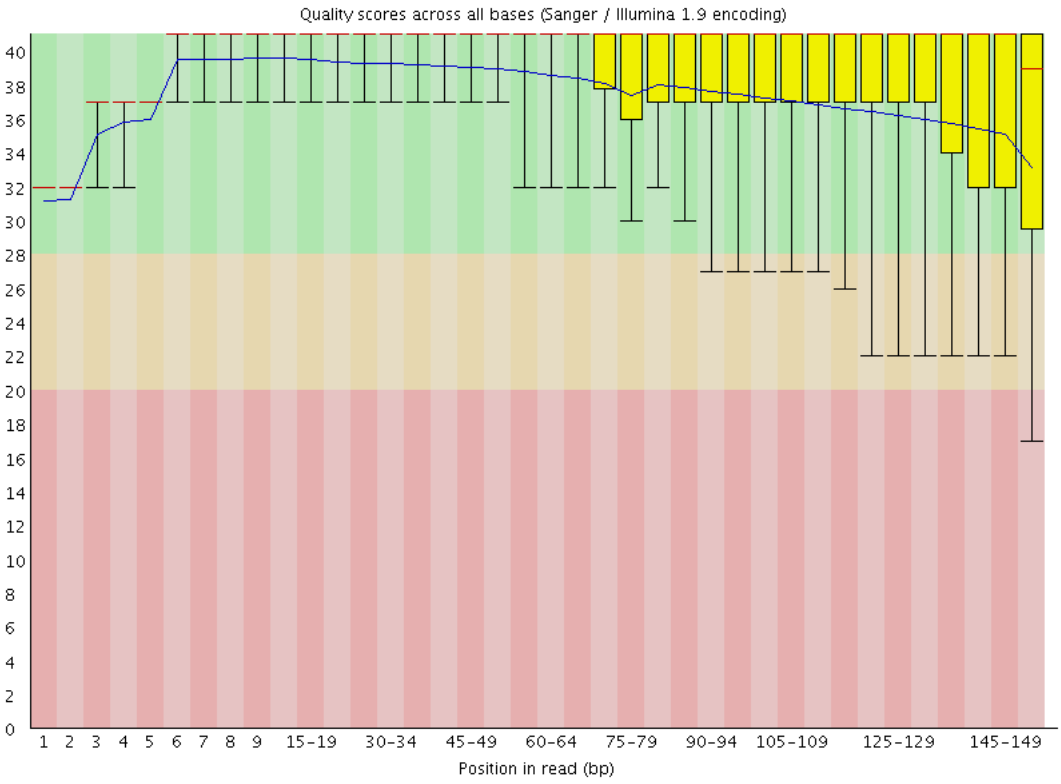
Supplementary Figure 2.23 FastQC report showing per base sequence quality for *Argyropelecus* DD (Sample 64-494)



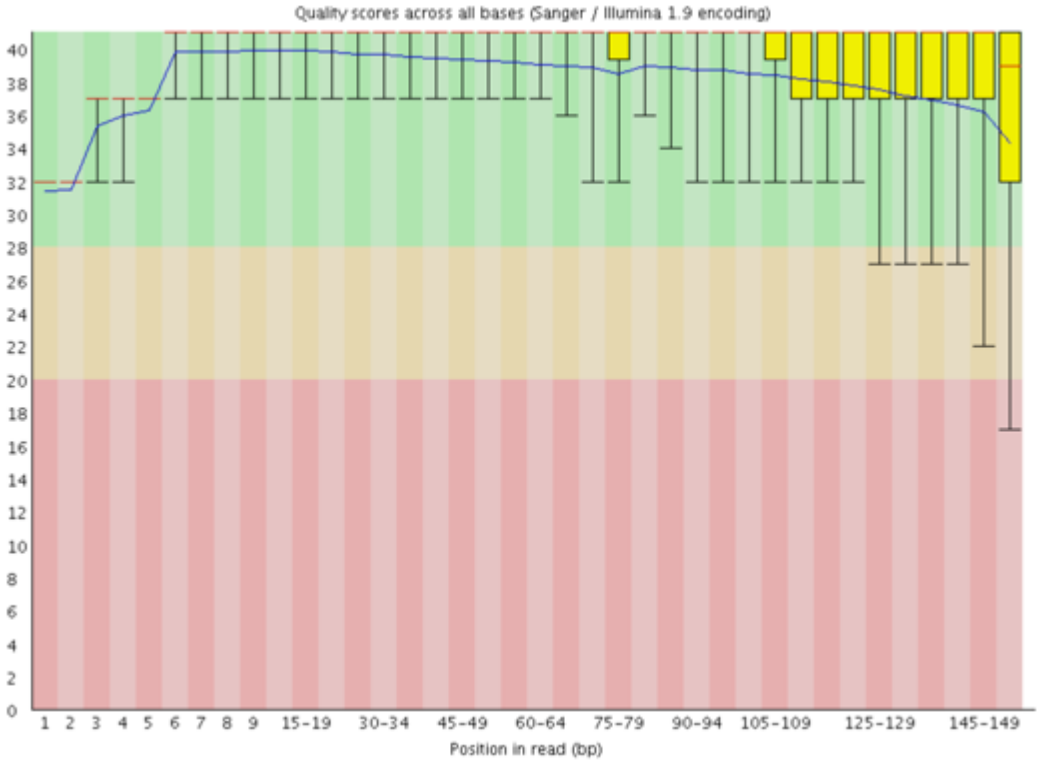
Supplementary Figure 2.24 FastQC report showing per base sequence quality for *Argyropelecus* DD (Sample 65-495)



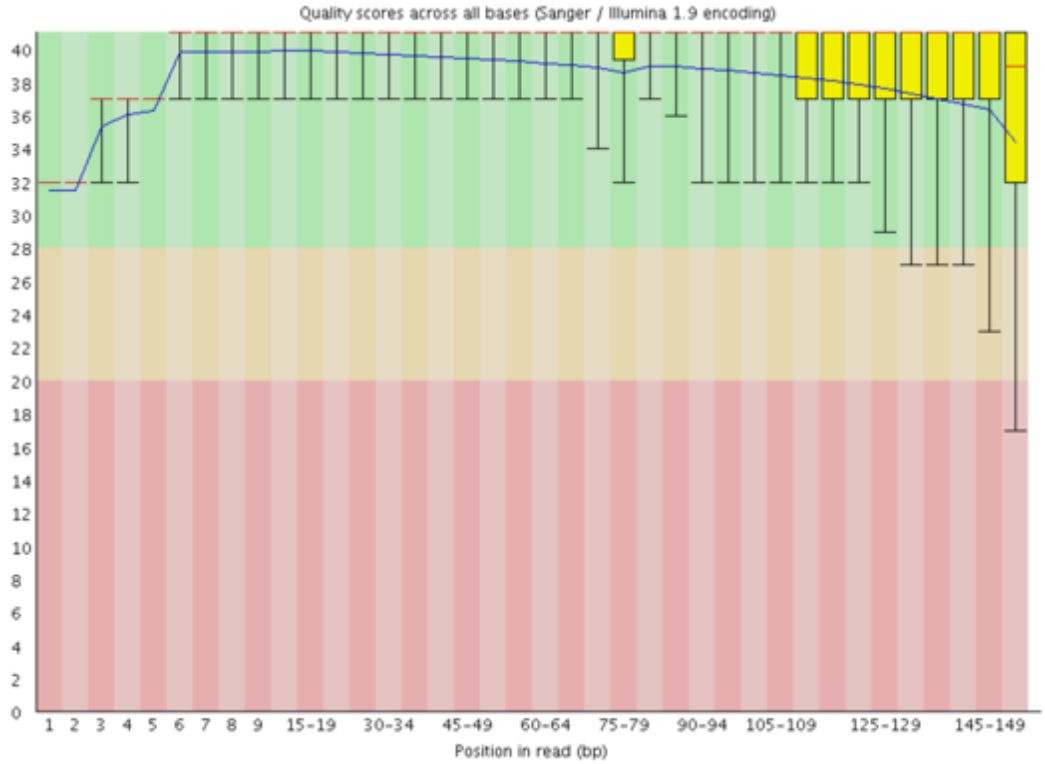
Supplementary Figure 2.25 FastQC report showing per base sequence quality for *Argyropelecus* DD (Sample 66-496)



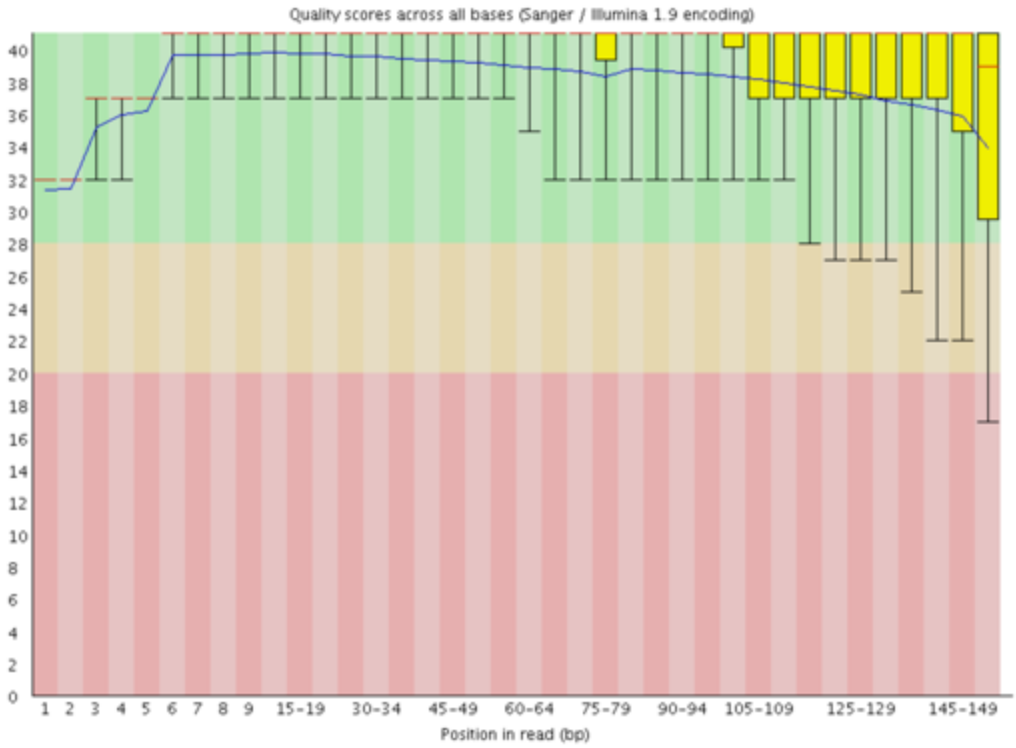
Supplementary Figure 2.26 FastQC report showing per base sequence quality for *Sternoptyx* mixed tissues (Sample 60-S)



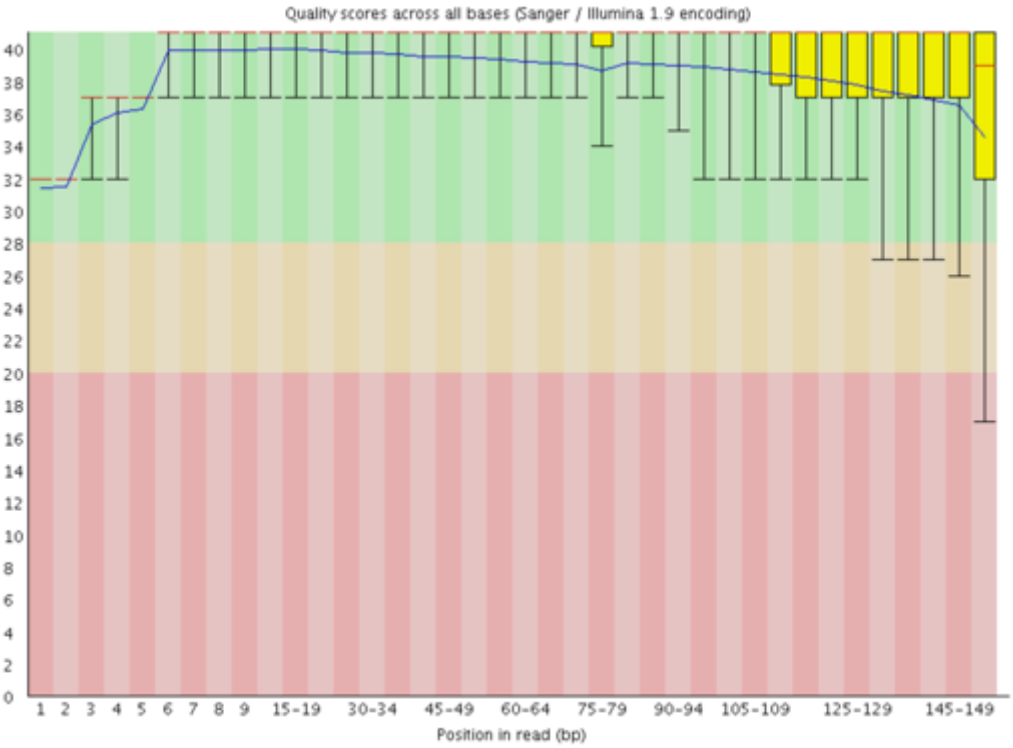
Supplementary Figure 2.27 FastQC report showing per base sequence quality for *Sternoptyx* LP (Sample 67-497)



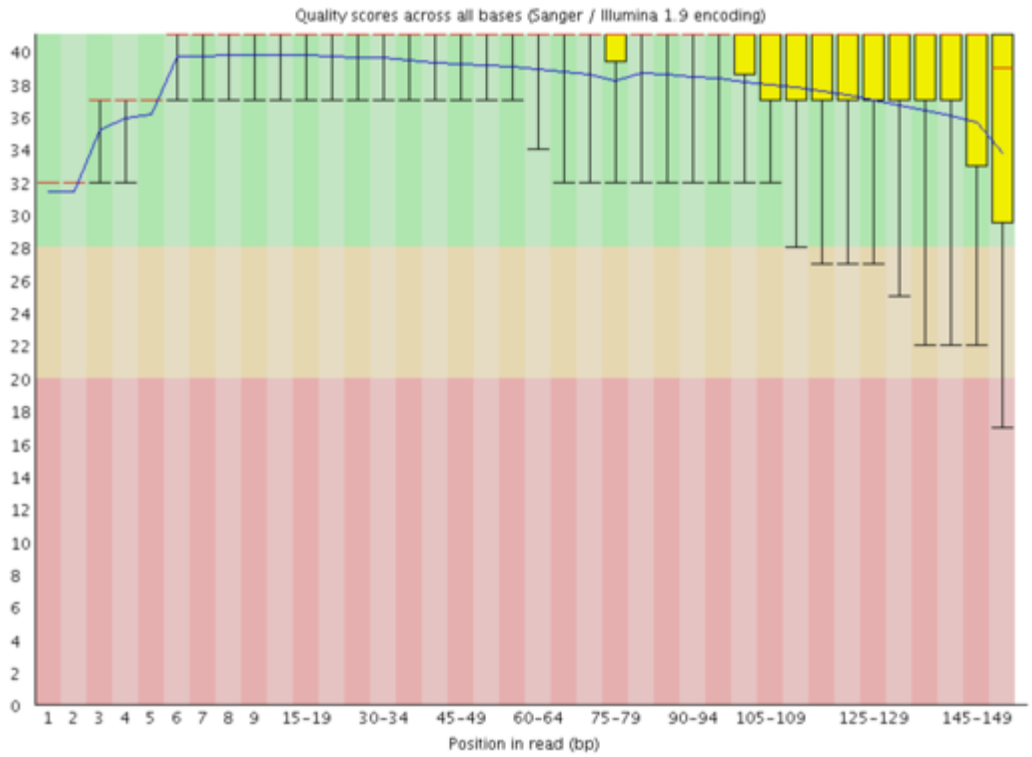
Supplementary Figure 2.28 FastQC report showing per base sequence quality for *Sternoptyx* LP (Sample 68-498)



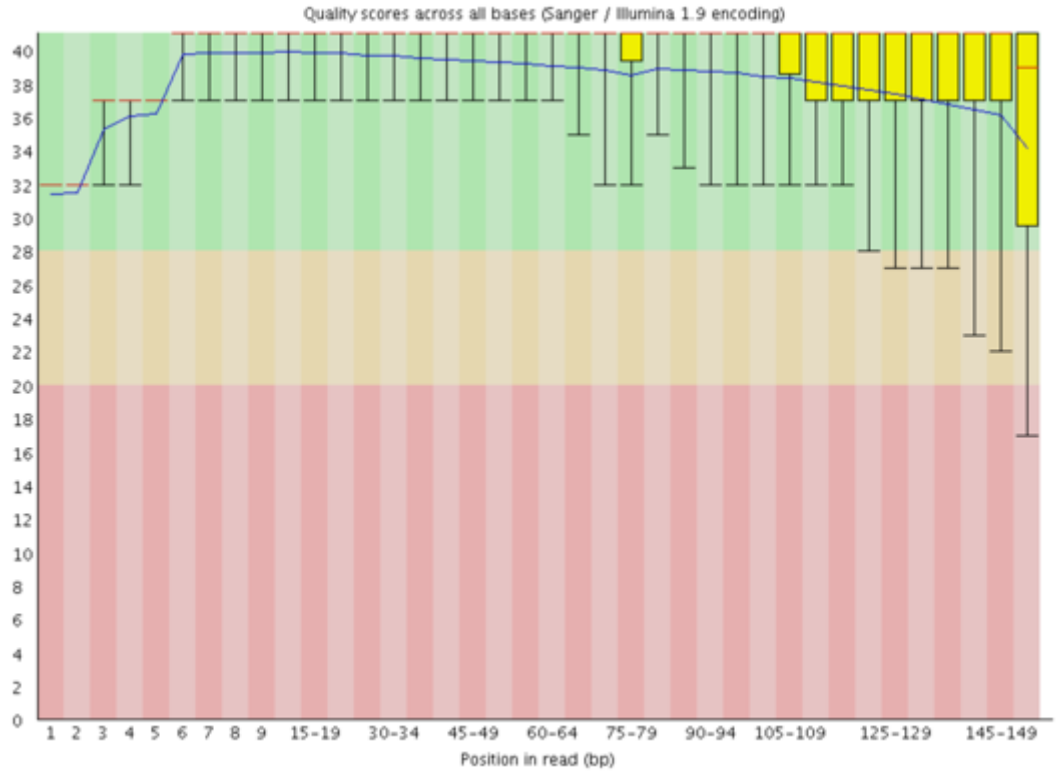
Supplementary Figure 2.29 FastQC report showing per base sequence quality for *Sternoptyx* LP (Sample 69-499)



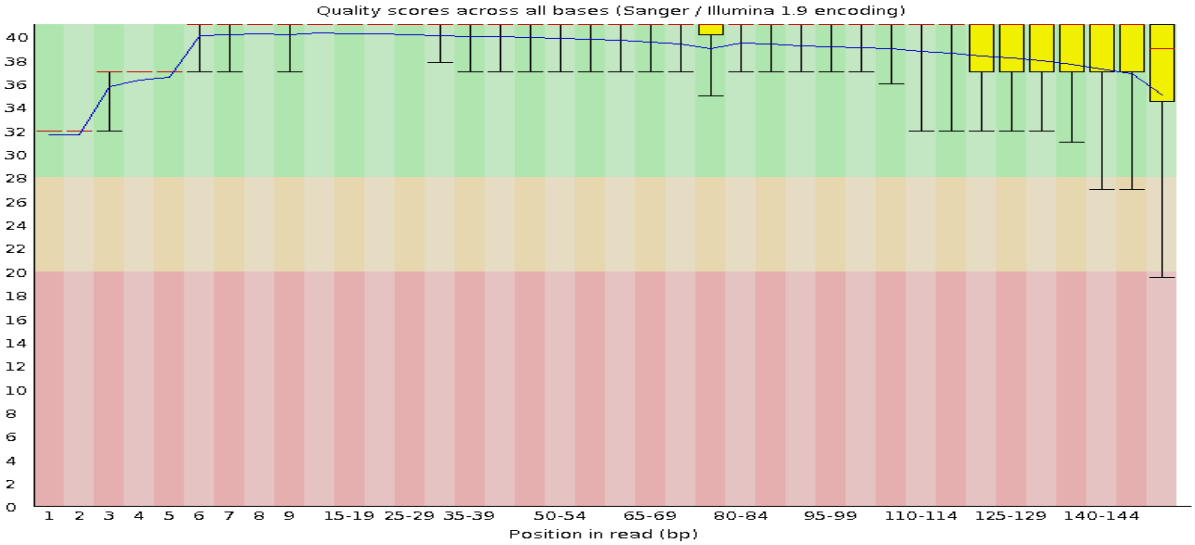
Supplementary Figure 2.30 FastQC report showing per base sequence quality for *Sternoptyx* DD (Sample 70-505)



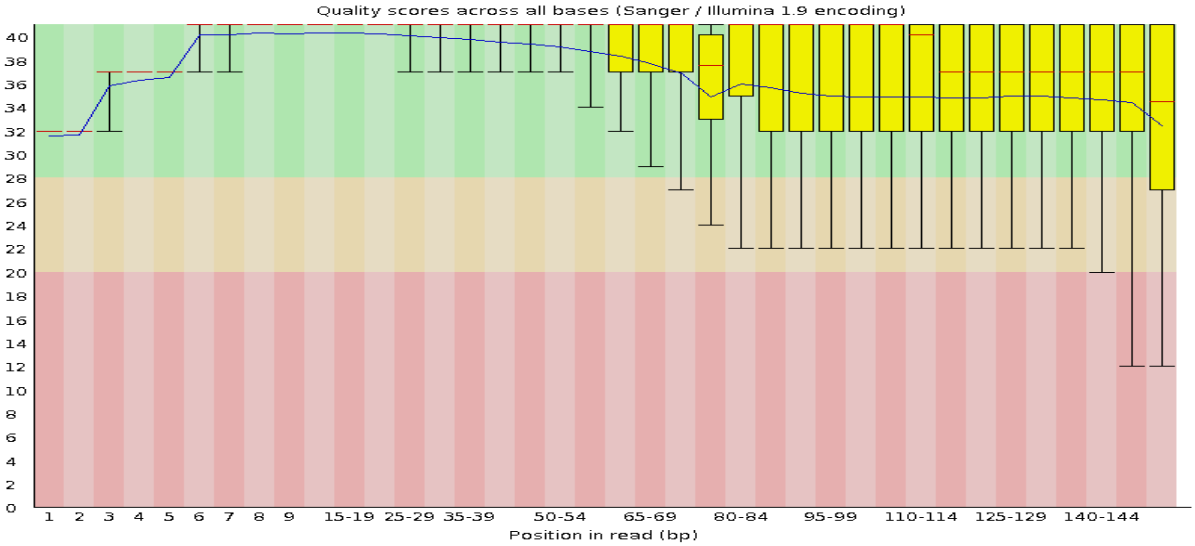
Supplementary Figure 2.31 FastQC report showing per base sequence quality for *Sternoptyx* DD (Sample 71-506)



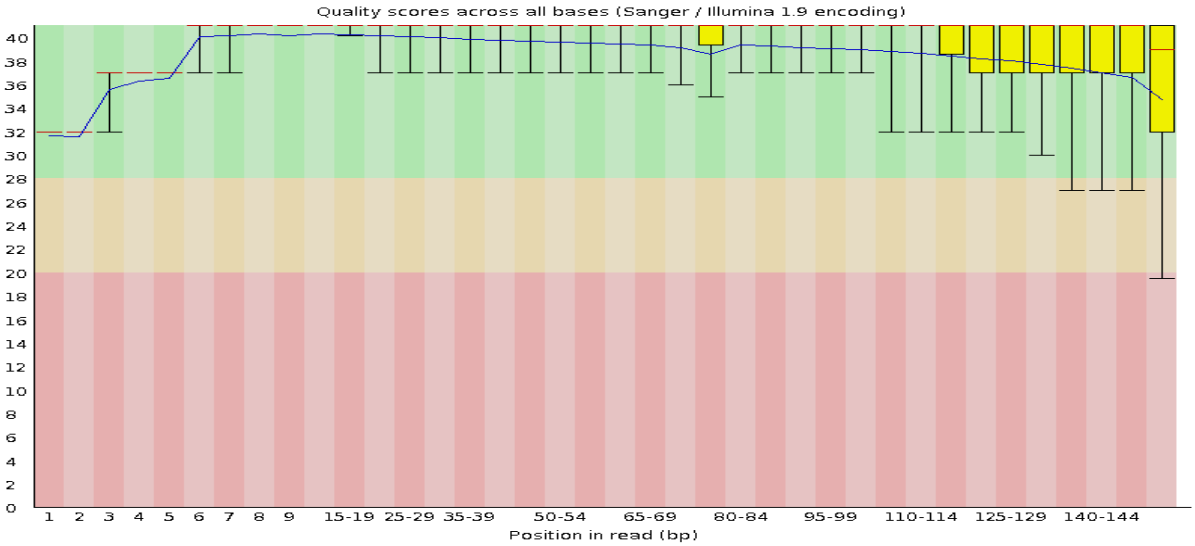
Supplementary Figure 2.32 FastQC report showing per base sequence quality for *Sternoptyx* DD (Sample 72-507)



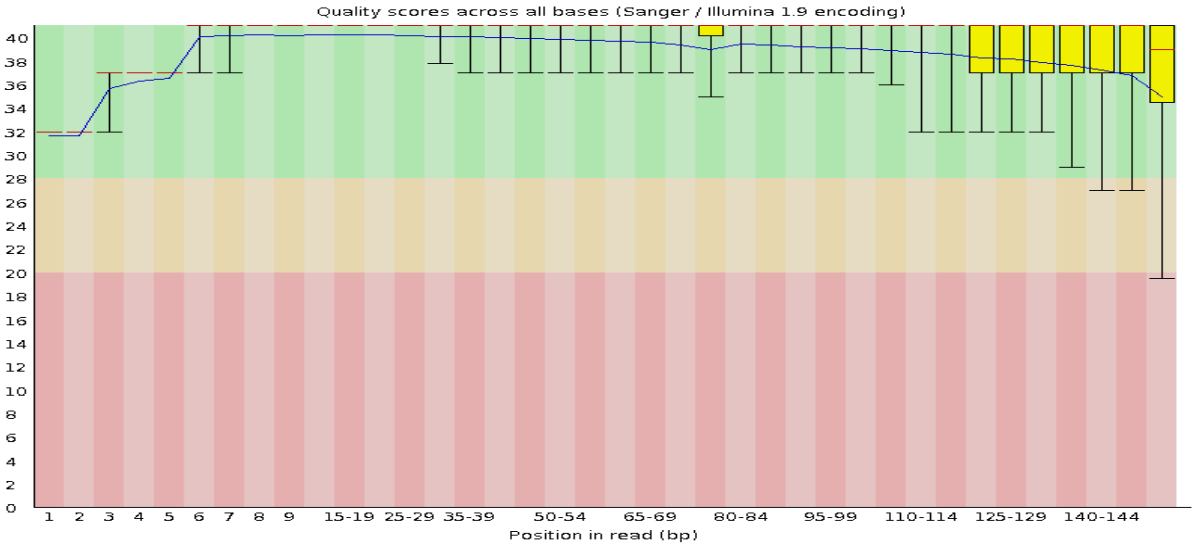
Supplementary Figure 2.33 FastQC report showing per base sequence quality for zebrafish LP-1



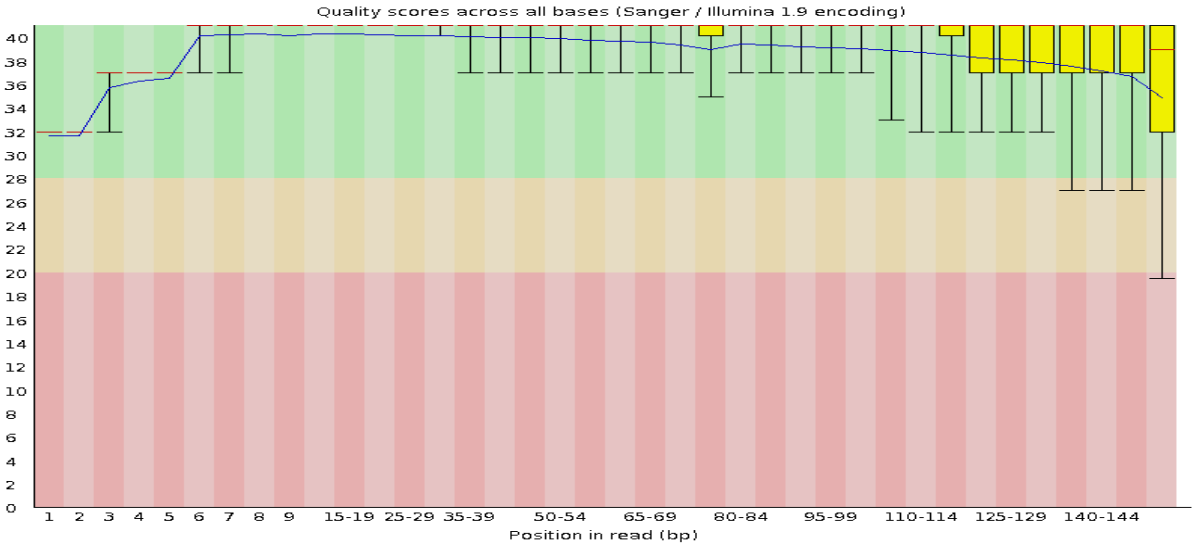
Supplementary Figure 2.34 FastQC report showing per base sequence quality for zebrafish LP-2



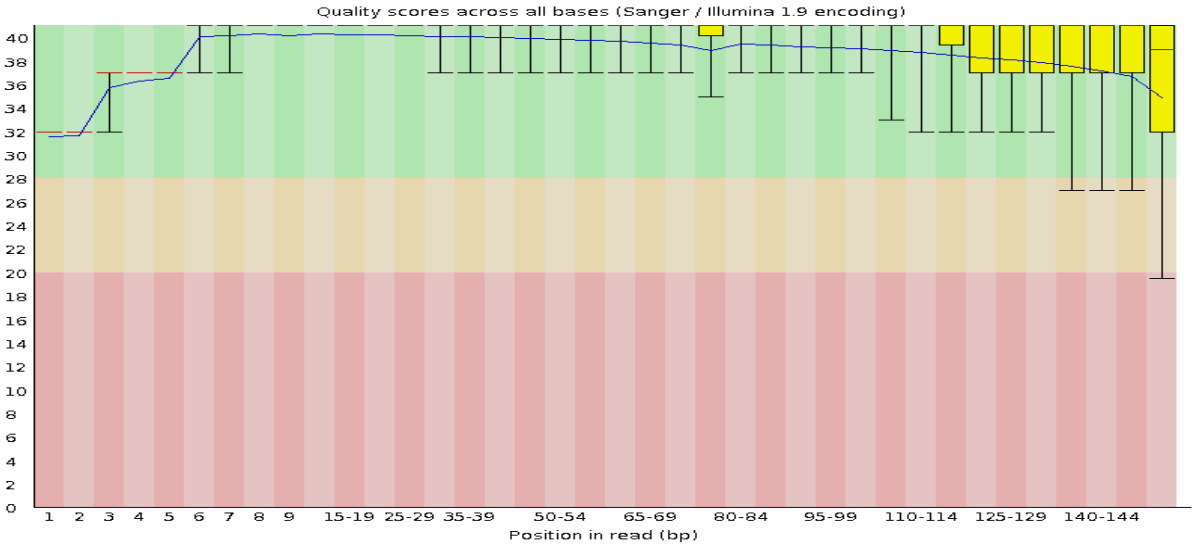
Supplementary Figure 2.35 FastQC report showing per base sequence quality for zebrafish LP-3



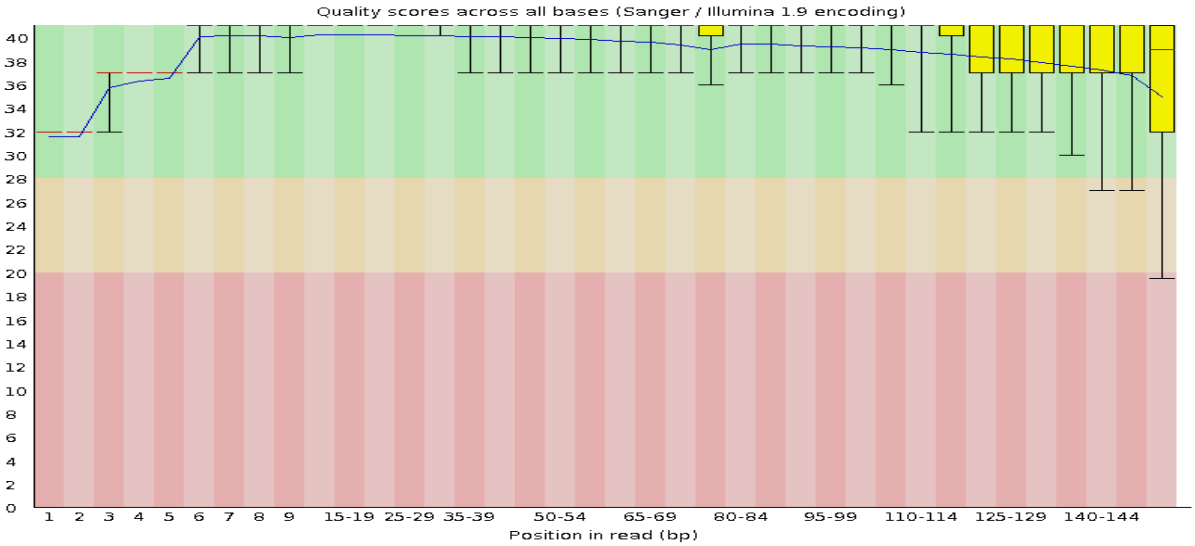
Supplementary Figure 2.36 FastQC report showing per base sequence quality for zebrafish LP-4



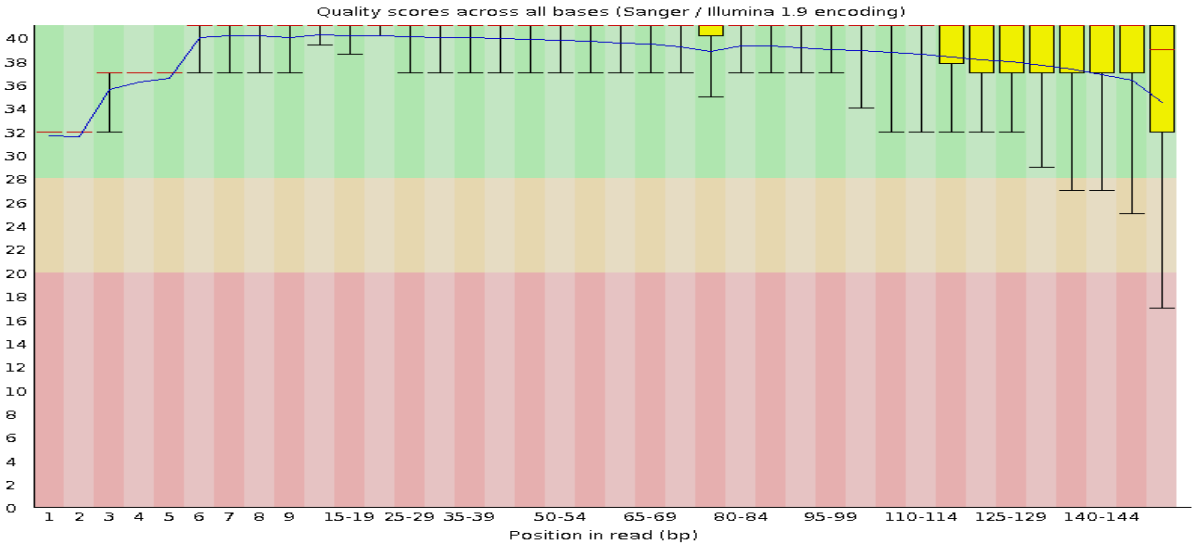
Supplementary Figure 2.37 FastQC report showing per base sequence quality for zebrafish DD-2



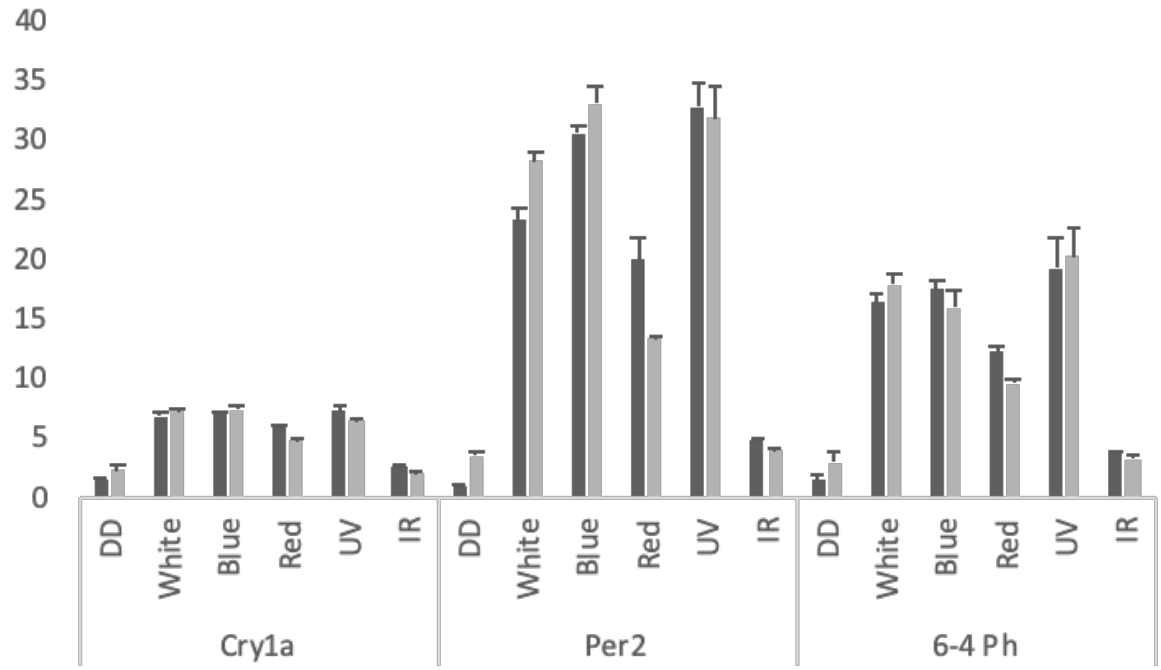
Supplementary Figure 2.38 FastQC report showing per base sequence quality for zebrafish DD-3



Supplementary Figure 2.39 FastQC report showing per base sequence quality for zebrafish DD-4



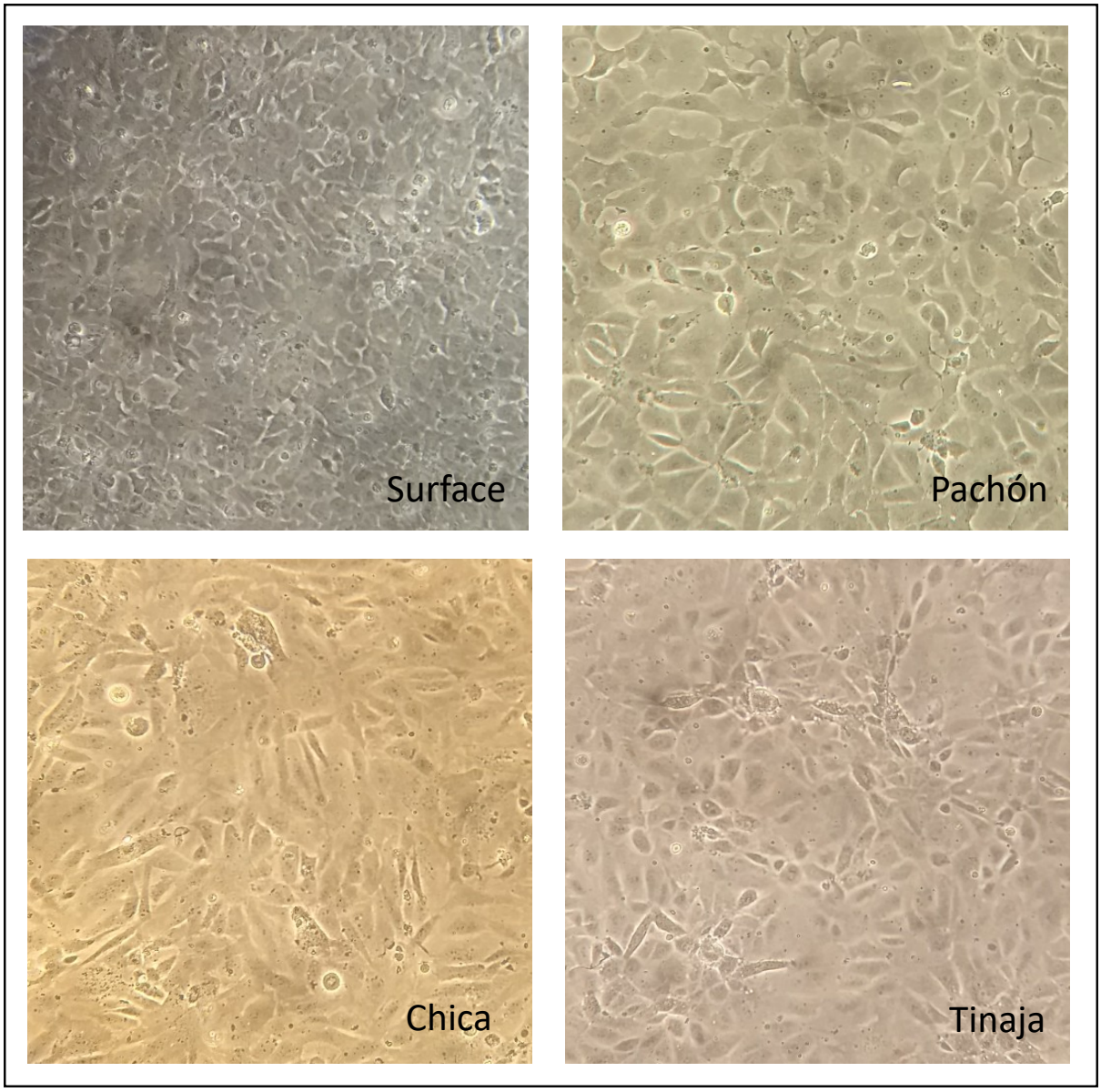
Supplementary Figure 2.40 FastQC report showing per base sequence quality for zebrafish DD-5



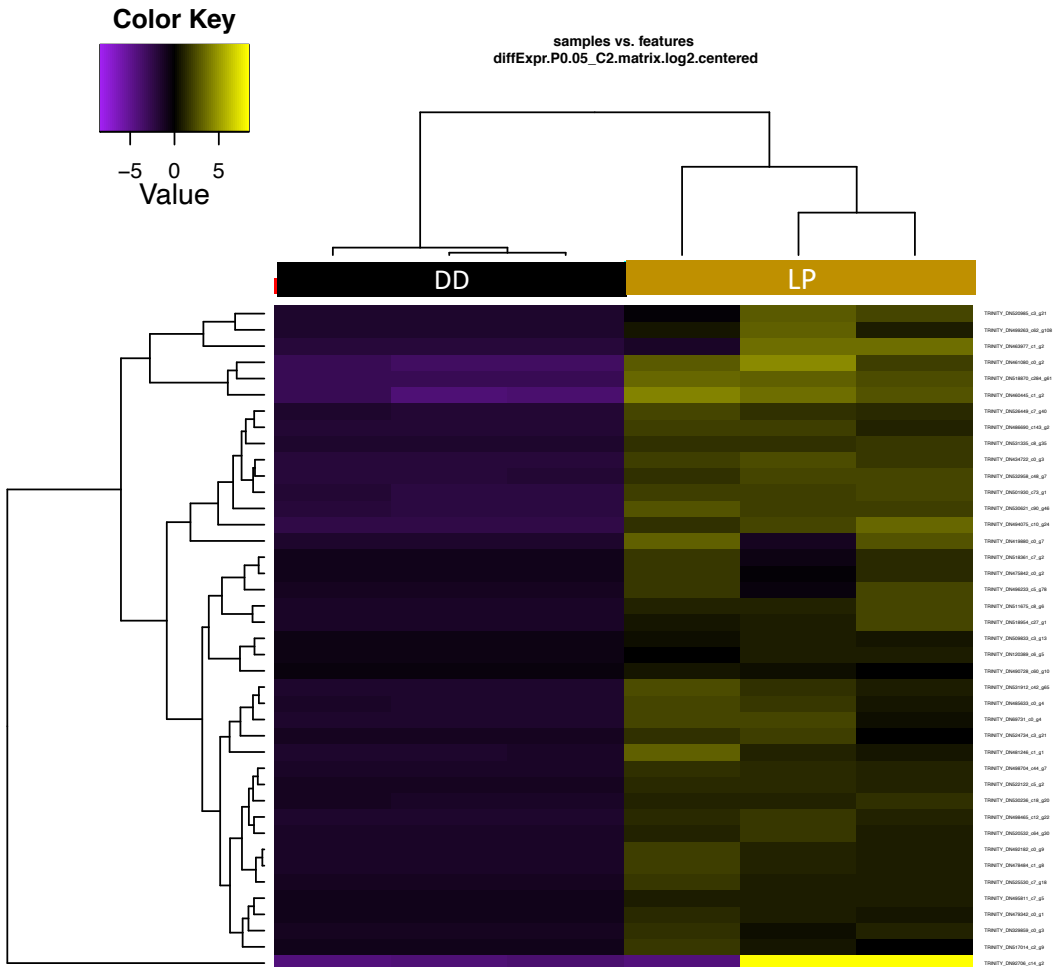
Supplementary Figure 5.1 Monochromatic light pulses in PAC2 vs *clockDN* cell lines

ClockDN cell lines (light grey) show increased basal expression of all genes explored in the dark control compared to PAC2 (dark grey).

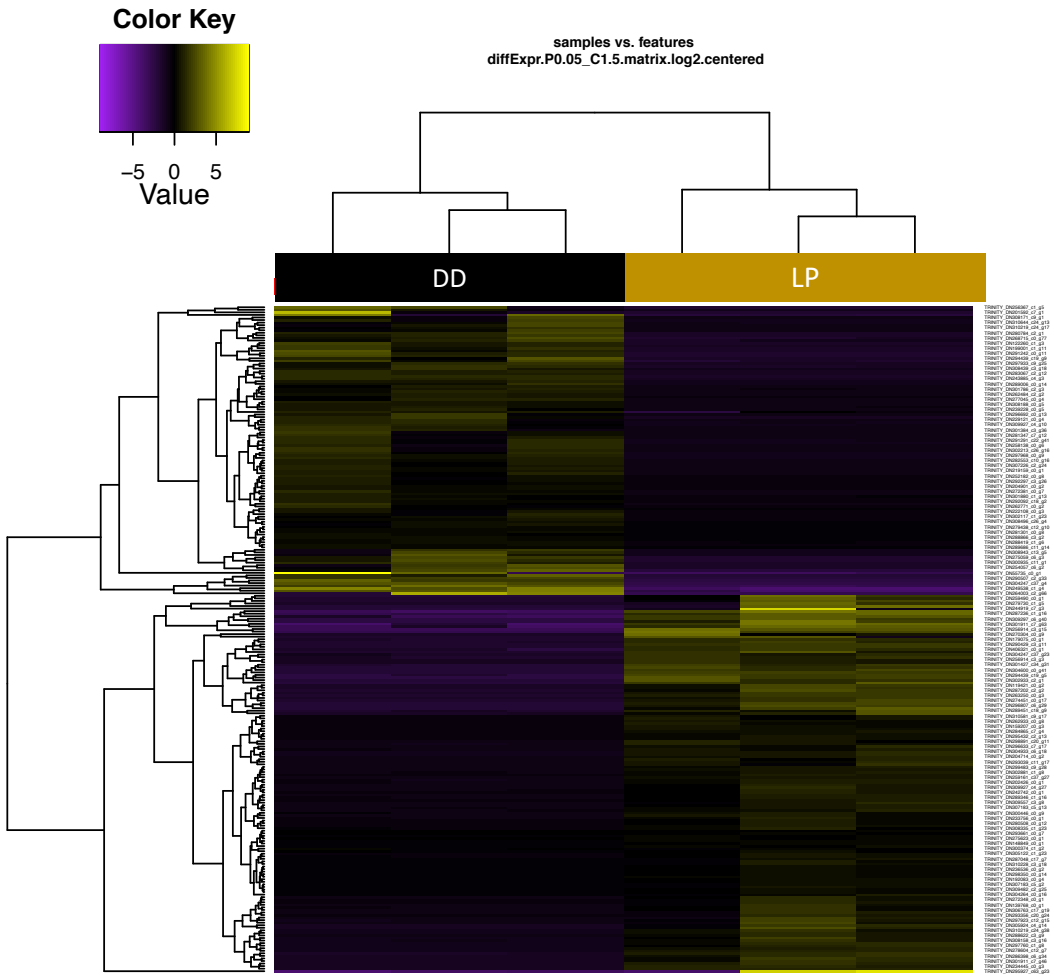
Significance was addressed with a one-way ANOVA (alpha=0.05) for each light-pulse, cell line and gene, followed by a Bonferroni post-test. All light pulses give a significant increase of $p<0.05$, except *clockDN* DD vs IR (n=3).



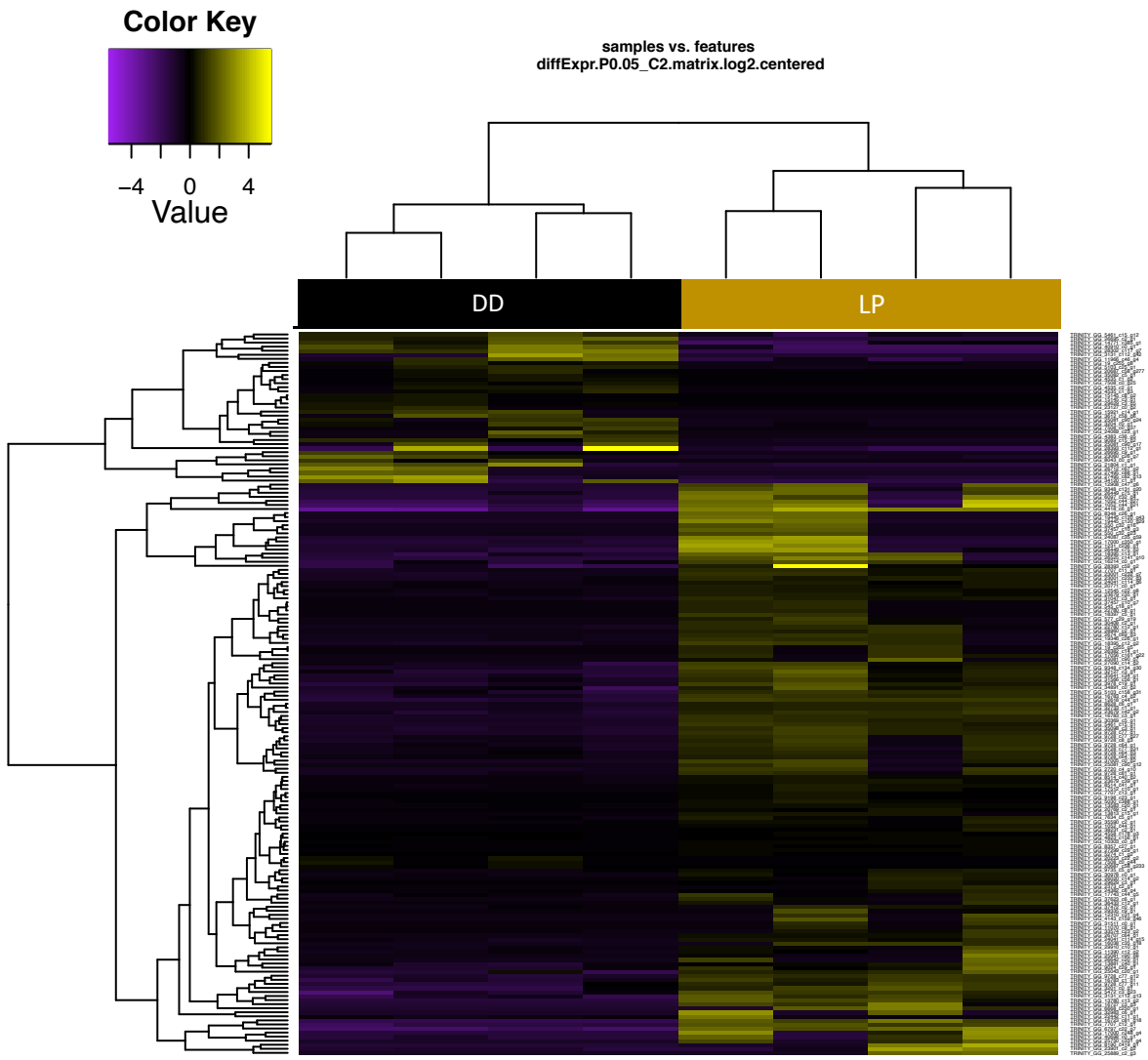
Supplementary Figure 4.1: Photos of cell cultures made from *Astyanax mexicanus* embryos



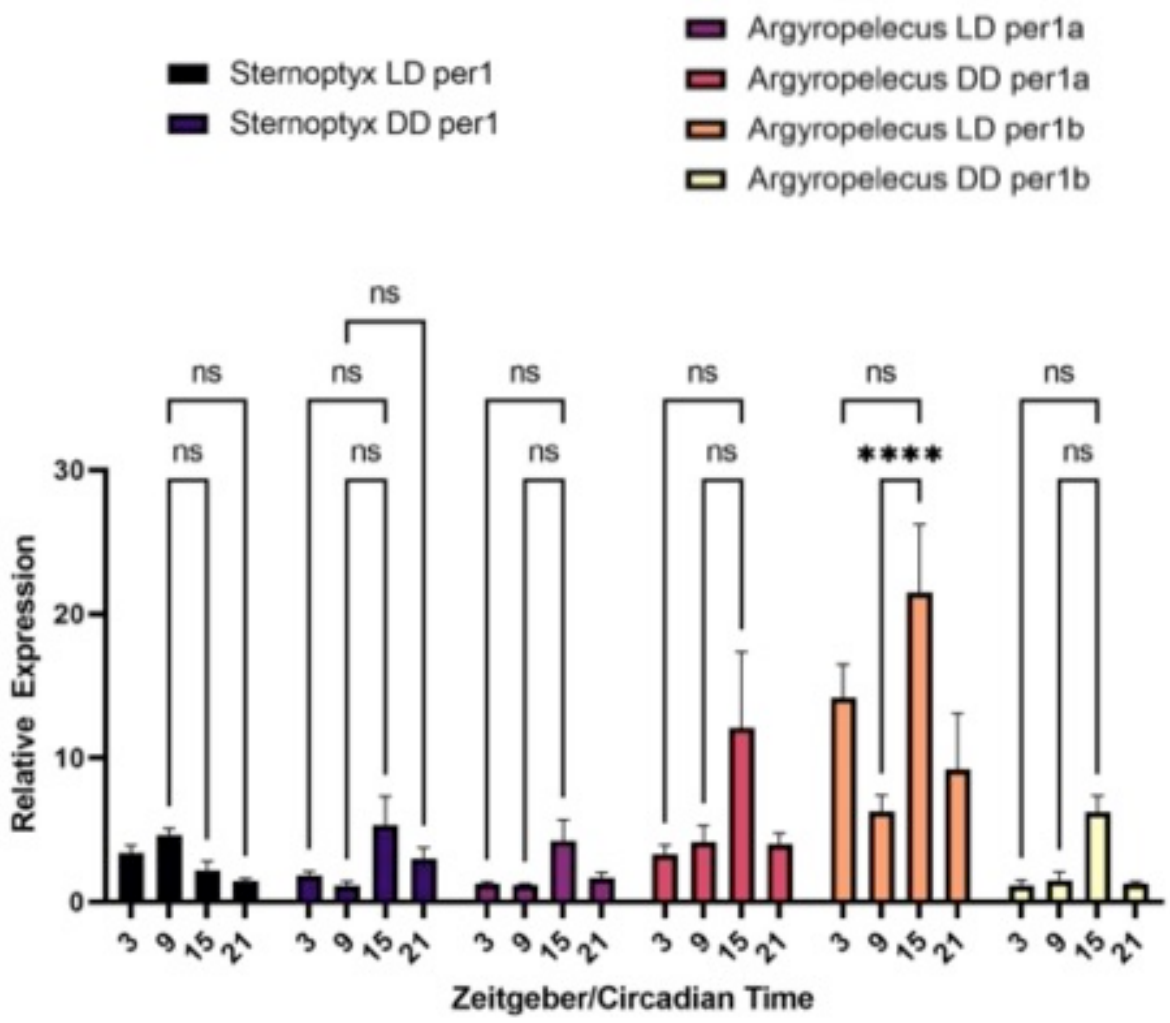
Supplementary Figure 5.1 ***Argyropelecus hemigymnus*** **DE genes** EdgeR heatmap with Trinity gene identifiers



Supplementary Figure 5.2 DE genes *Sternoptyx diaphana* EdgeR heatmap with Trinity gene identifiers

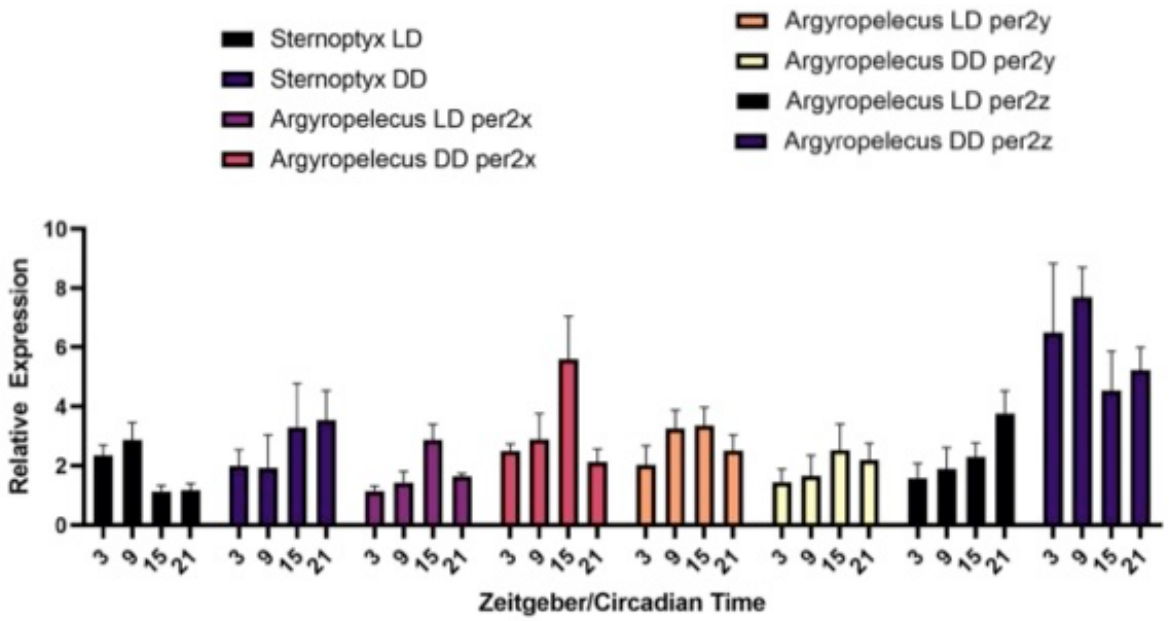


Supplementary Figure 5.3 DE expressed Zebrafish genes EdgeR heatmap with Trinity gene identifiers



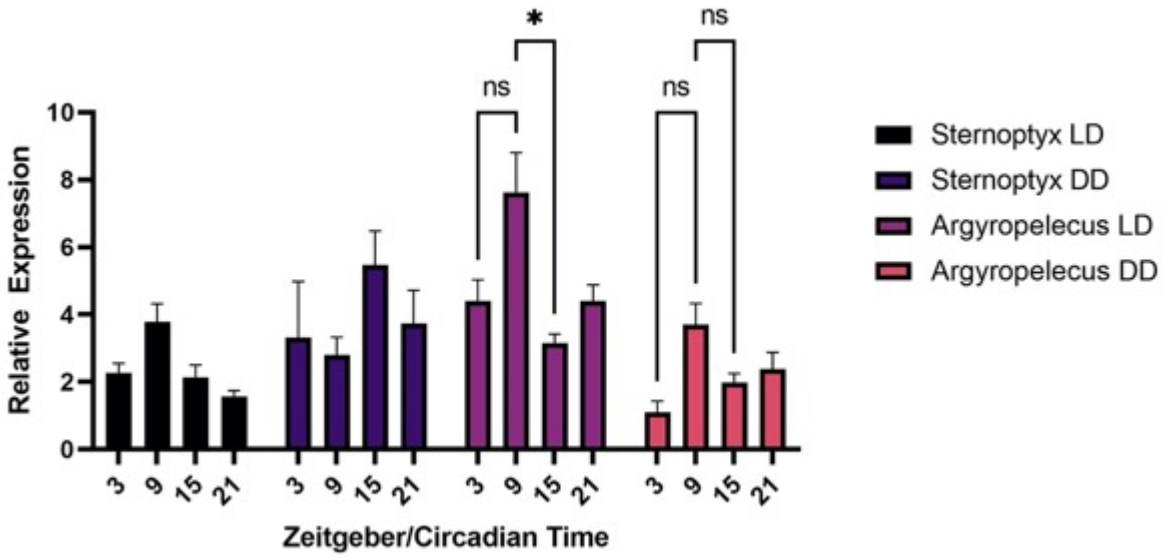
Supplementary Figure 5.4 *per1, per1a and per1b* expression in *Argyropelecus* and *Sternoptyx* normalised to housekeeping genes

Deep-sea eyes were dissected and maintained on a 12:12 light-dark cycle for 1 day before being sampled every 6 hours over the next 24-hour period in LD and then a following dark period. ZT denotes zeitgeber time and CT denotes circadian time. Data is normalised to the geometric mean of housekeeping-genes β-actin and ribosomal protein L13 (RPL-13α) for *Sternoptyx* and Glucose-6-phosphate dehydrogenase (G6PD) for *Argyropelecus*. Data is plotted relative to the lowest expressed gene. Significance ($\alpha = 0.05$) was addressed with a two-way ANOVA, followed by a Tukey post-test. Significance **** $p<0.0001$, ns indicate non-significant (n=3-6).



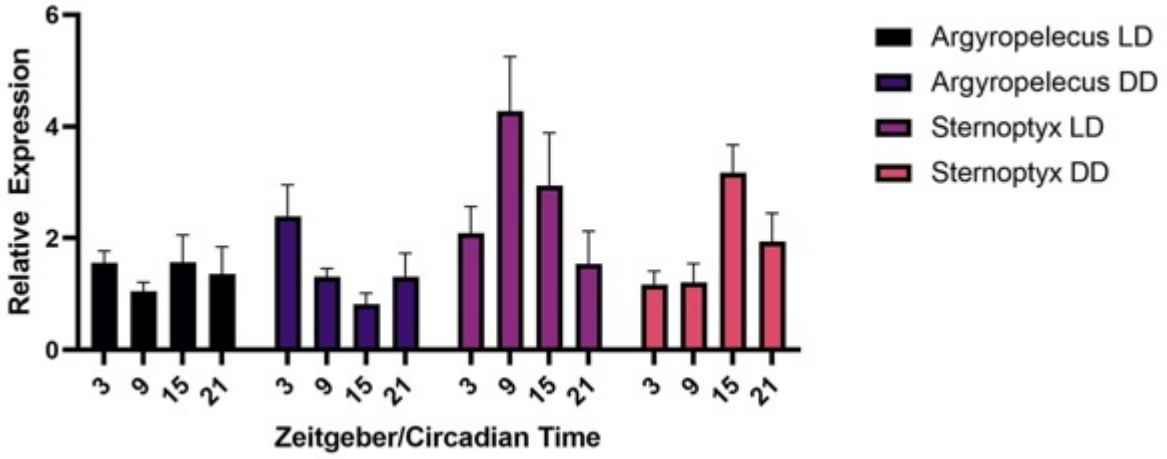
Supplementary Figure 5.5 ***per2, per2x, per2y and per2z*** **expression in** ***Argyropelecus*** **and** ***Sternoptyx*** **normalised to housekeeping genes**

Deep-sea eyes were dissected and maintained on a 12:12 light-dark cycle for 1 day before being sampled every 6 hours over the next 24-hour period in LD and then a following dark period. ZT denotes zeitgeber time and CT denotes circadian time. Data is normalised to the geometric mean of housekeeping-genes β-actin and RPL-13α for *Sternoptyx* and G6PD for *Argyropelecus*. Data is plotted relative to the lowest expressed gene. Significance (α =0.05) was addressed with a two-way ANOVA, followed by a Tukey post-test. No data was significant (n=3-6).



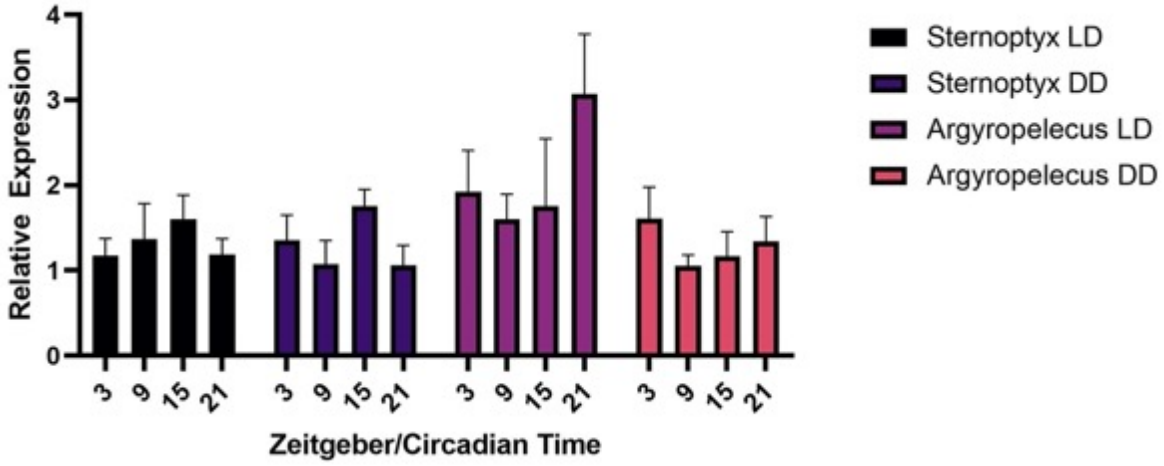
Supplementary Figure 5.6 *cry1a* expression in *Argyropelecus* and *Sternoptyx* normalised to housekeeping genes

Deep-sea eyes were dissected and maintained on a 12:12 light-dark cycle for 1 day before being sampled every 6 hours over the next 24-hour period in LD and then a following dark period. ZT denotes zeitgeber time and CT denotes circadian time. Data is normalised to the geometric mean of housekeeping-genes β-actin and RPL-13α for *Sternoptyx* and G6PD for *Argyropelecus*. Data is plotted relative to the lowest expressed gene. Significance (α =0.05) was addressed with a two-way ANOVA, followed by a Tukey post-test. No data was significant (n=3-6).



Supplementary Figure 5.7 *cry2* expression in *Argyropelecus* and *Sternoptyx* normalised to housekeeping genes

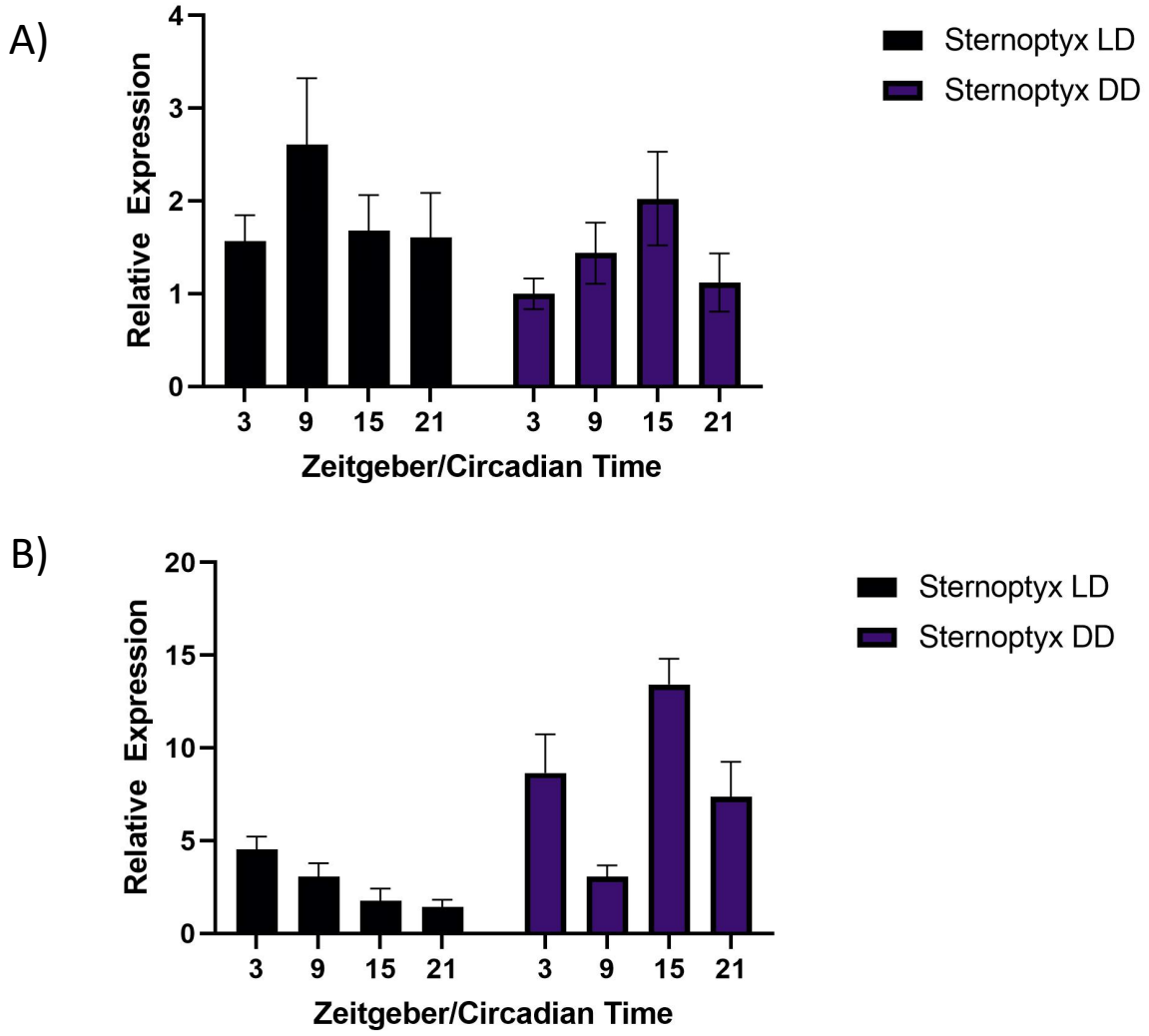
Deep-sea eyes were dissected and maintained on a 12:12 light-dark cycle for 1 day before being sampled every 6 hours over the next 24-hour period in LD and then a following dark period. ZT denotes zeitgeber time and CT denotes circadian time. Data is normalised to the geometric mean of housekeeping-genes β-actin and RPL-13α for *Sternoptyx* and G6PD for *Argyropelecus*. Data is plotted relative to the lowest expressed gene. Significance (α =0.05) was addressed with a two-way ANOVA, followed by a Tukey post-test. No data was significant (n=3-6).



Supplementary Figure 5.8 *cry3b* expression in *Argyropelecus* and *Sternoptyx* normalised to housekeeping genes

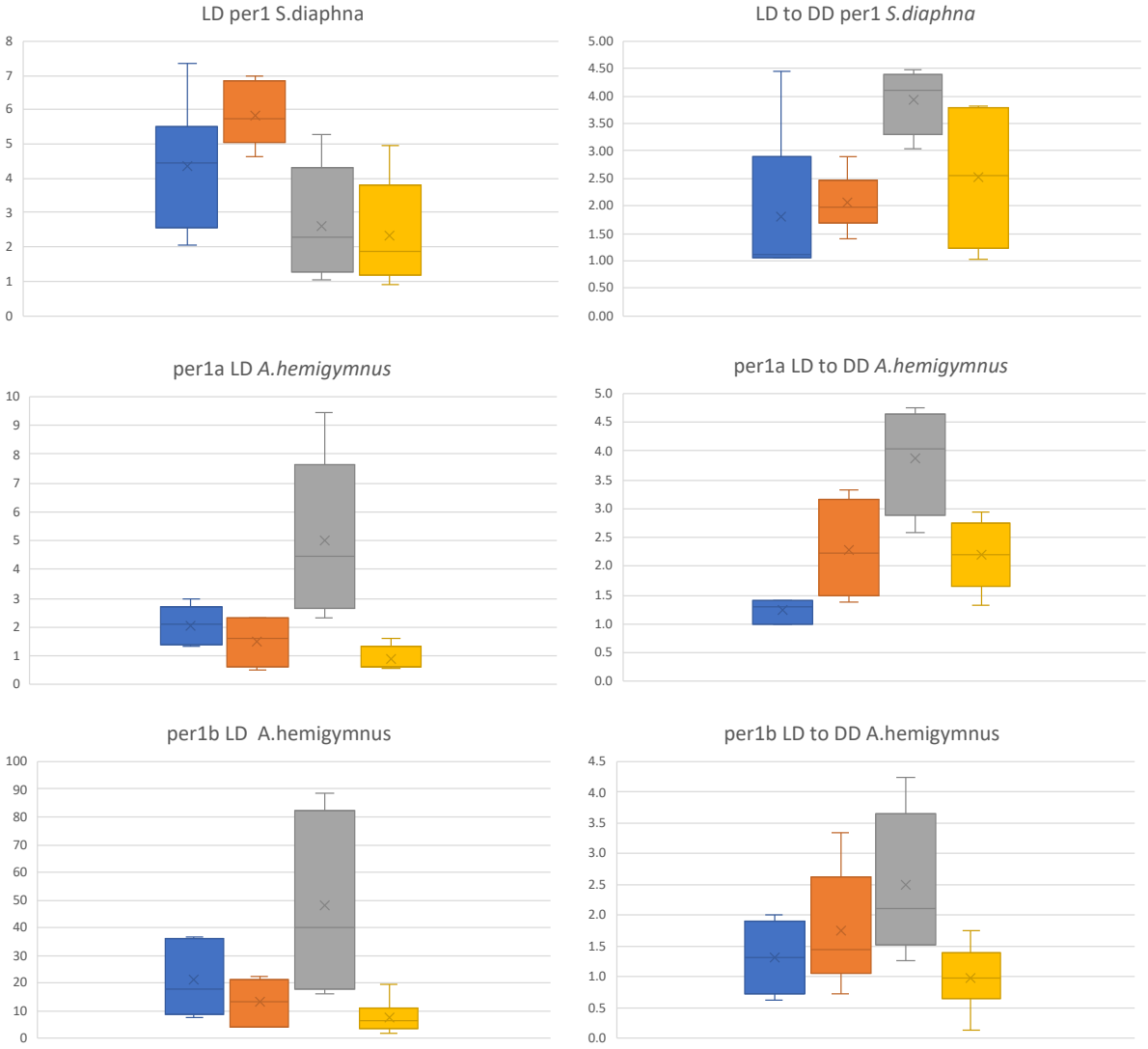
Deep-sea eyes were dissected and maintained on a 12:12 light-dark cycle for 1 day before being sampled every 6 hours over the next 24-hour period in LD and then a following dark period. ZT denotes zeitgeber time and CT denotes circadian time. Data is normalised to the geometric mean of housekeeping-genes β-actin and RPL-13α for *Sternoptyx* and G6PD for *Argyropelecus*. Data is plotted relative to the lowest expressed gene. Significance (α =0.05)

was addressed with a two-way ANOVA, followed by a Tukey post-test.
No data was significant (n=3-6).

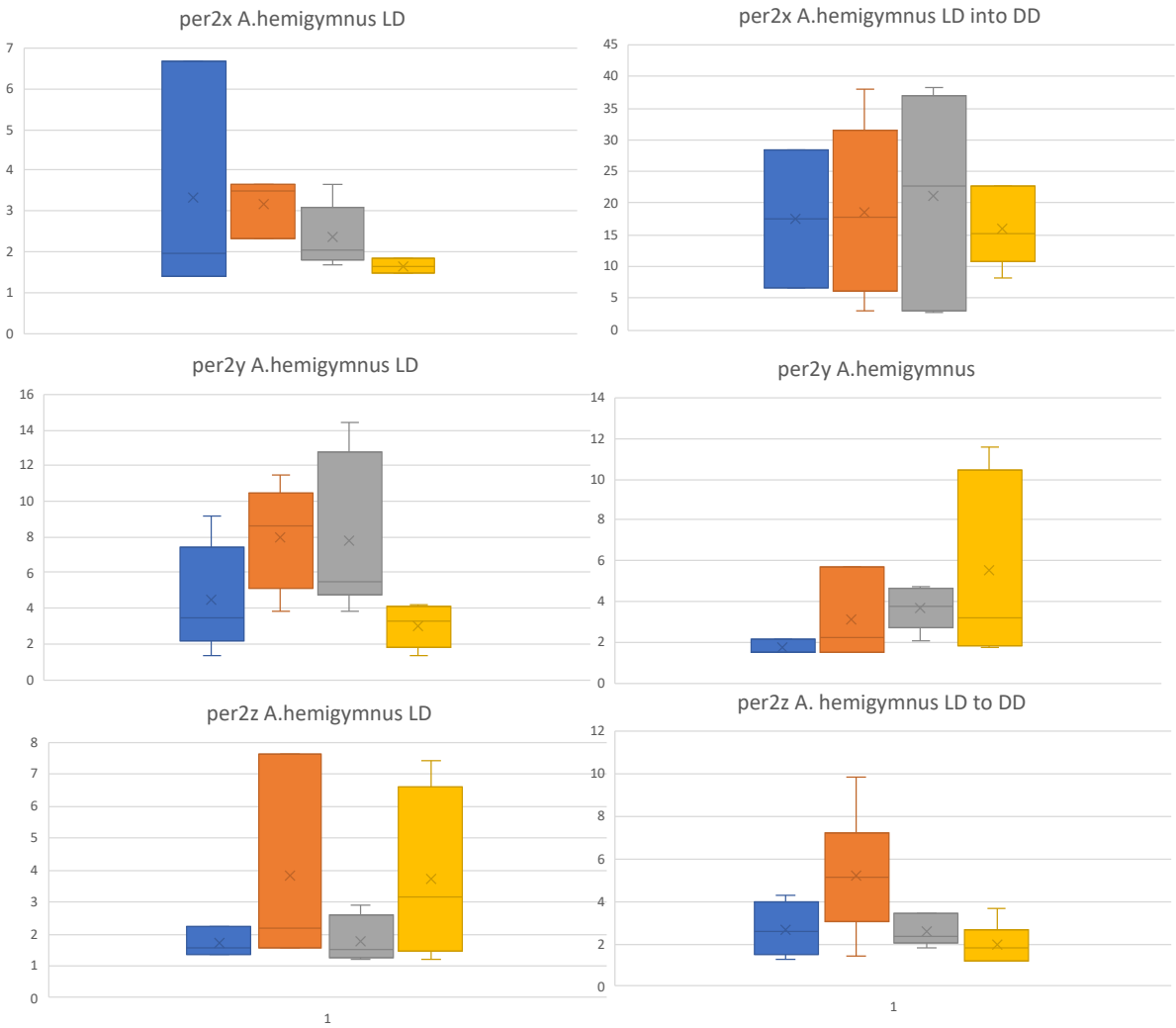


Supplementary Figure 5.9 Per3 and Cry3a expression in *Sternoptyx* normalised to housekeeping genes

Deep-sea eyes were dissected and maintained on a 12:12 light-dark cycle for 1 day before being sampled every 6 hours over the next 24-hour period in LD and then a following dark period. ZT denotes zeitgeber time and CT denotes circadian time. A) Per3 B) Cry3a. Data is normalised to the geometric mean of housekeeping-genes β-actin and RPL-13α. Data is plotted relative to the lowest expressed gene. Significance (α =0.05) was addressed with a two-way ANOVA, followed by a Tukey post-test.
No data was significant (n=3-6).

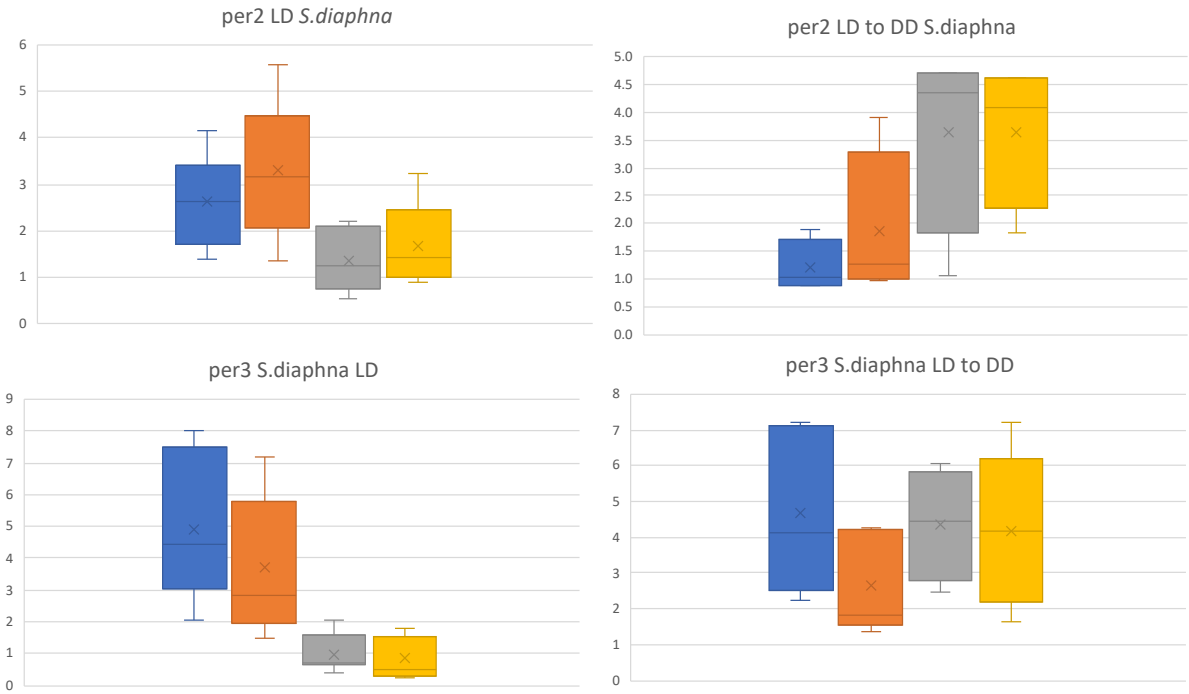


Supplementary Figure 5.10 Box and whisker plot of *per1* genes Blue= ZT/CT 3, Orange = ZT/CT9, grey = ZT/CT15, Yellow = ZT/CT21



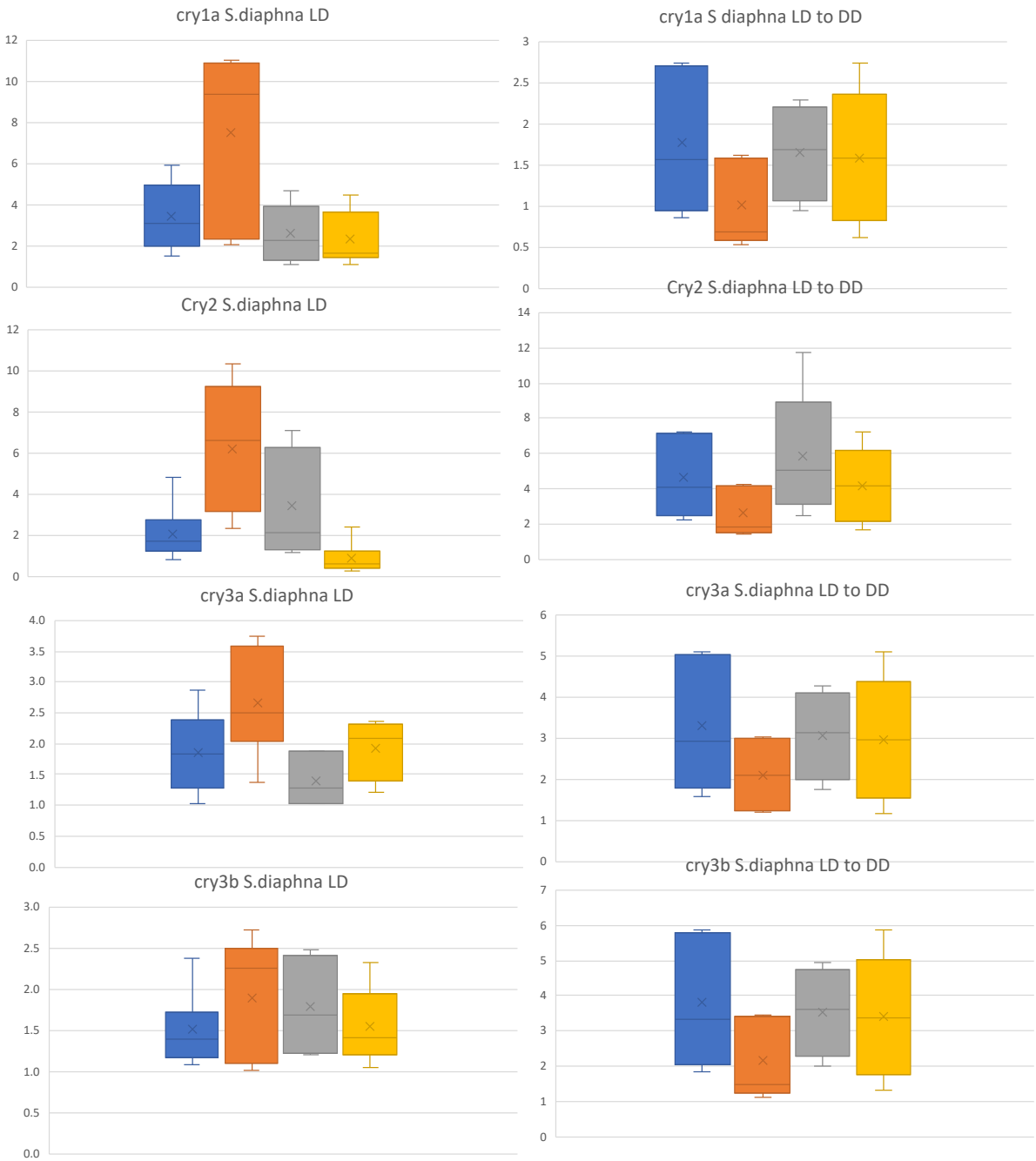
Supplementary Figure 5.11 Box and whisker plot of *per2* genes *Argyropelecus*

Blue= ZT/CT 3, Orange = ZT/CT9, grey = ZT/CT15, Yellow = ZT/CT21



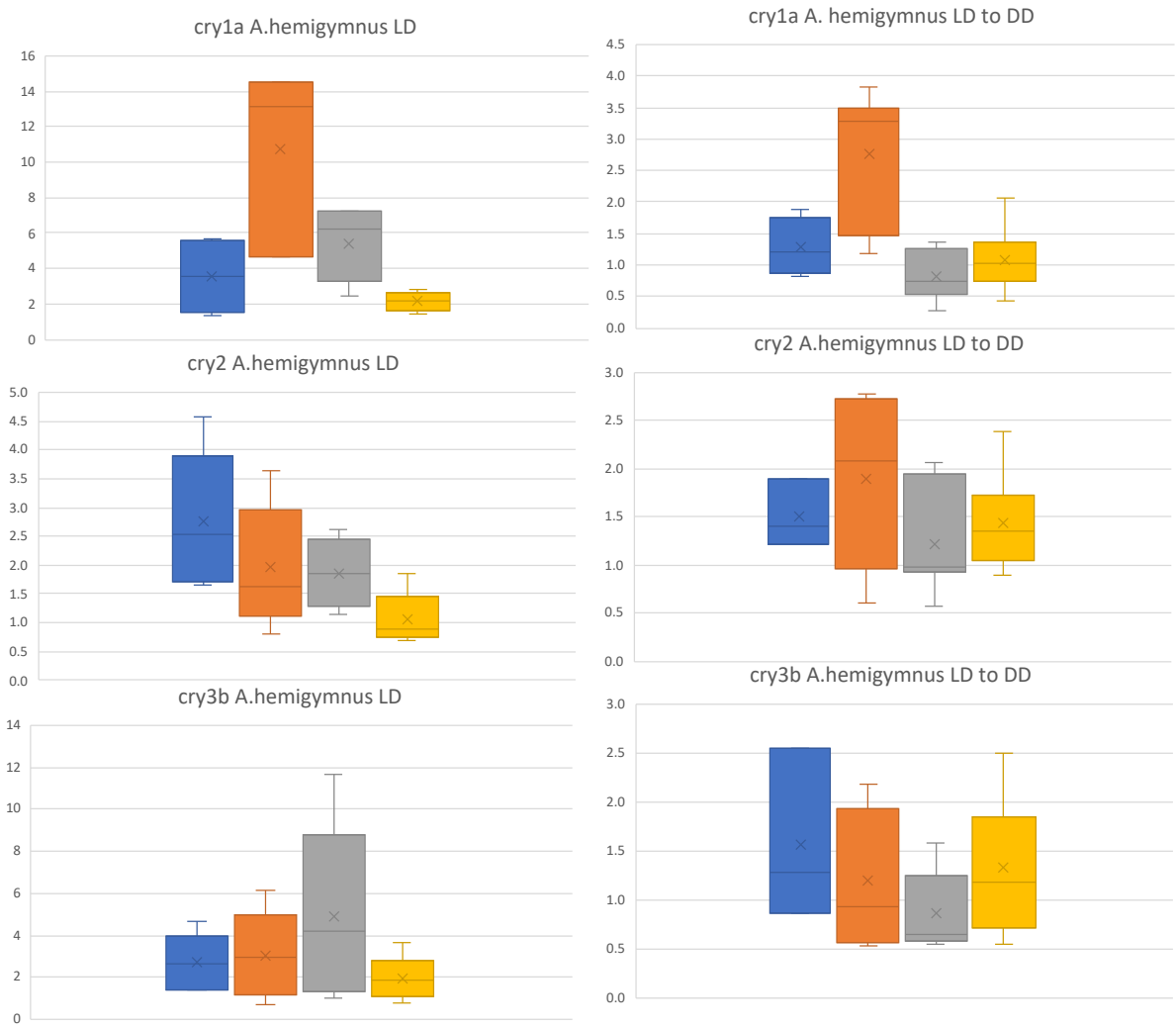
Supplementary Figure 5.12 Box and whisker plot of *per2* and *per3* genes *Sternoptyx*

Blue= ZT/CT 3, Orange = ZT/CT9, grey = ZT/CT15, Yellow = ZT/CT21



Supplementary Figure 5.13 Box and whisker plot of *cry* genes *Sternoptyx*

Blue= ZT/CT 3, Orange = ZT/CT9, grey = ZT/CT15, Yellow = ZT/CT21



Supplementary Figure 5.14 Box and whisker plot of *cry* genes *Argyropelecus*

Blue= ZT/CT 3, Orange = ZT/CT9, grey = ZT/CT15, Yellow = ZT/CT21

Data Id	tau	emp p	emp p BH Corrected	Pattern Shape	Pattern Period	Pattern Peak	P. Trough	P. Circ. Peak	P. Circ. Trough	P. Width	P. Asym	JTK p from tau	JTK p BH Corrected	bf corrected JTK p	BH bf corrected p
per1a	0.5	0	0	COS	24	16	4	16	4	24	0.5	0.041632262	0.041632262	0.24979358	0.249793575
per1b	0.5	0	0	COS	24	16	4	16	4	24	0.5	0.041632262	0.041632262	0.24979357	0.249793575

Supplementary Table 2.1 BioDare2 rhythmicity analysis

Trim Stats Trimmomatic A. hemigymnus
Sample_59-A_trimm.err:Input Read Pairs: 41080254 Both Surviving: 34159576 (83.15%) Forward Only Surviving: 6774982 (16.49%) Reverse Only Surviving: 72926 (0.18%) Dropped: 72770 (0.18%)
Sample_61-487_trimm.err:Input Read Pairs: 21571044 Both Surviving: 19187314 (88.95%) Forward Only Surviving: 2313566 (10.73%) Reverse Only Surviving: 39846 (0.18%) Dropped: 30318 (0.14%)
Sample_62-488_trimm.err:Input Read Pairs: 21091987 Both Surviving: 18582774 (88.10%) Forward Only Surviving: 2446318 (11.60%) Reverse Only Surviving: 37396 (0.18%) Dropped: 25499 (0.12%)
Sample_63-488-2_trimm.err:Input Read Pairs: 22525655 Both Surviving: 20063500 (89.07%) Forward Only Surviving: 2395939 (10.64%) Reverse Only Surviving: 39584 (0.18%) Dropped: 26632 (0.12%)
Sample_64-494_trimm.err:Input Read Pairs: 19196837 Both Surviving: 16429664 (85.59%) Forward Only Surviving: 2683898 (13.98%) Reverse Only Surviving: 42859 (0.22%) Dropped: 40416 (0.21%)
Sample_65-495_trimm.err:Input Read Pairs: 21426037 Both Surviving: 18870391 (88.07%) Forward Only Surviving: 2496827 (11.65%) Reverse Only Surviving: 39717 (0.19%) Dropped: 19102 (0.09%)
Sample_66-496_trimm.err:Input Read Pairs: 26886648 Both Surviving: 23691244 (88.12%) Forward Only Surviving: 3126002 (11.63%) Reverse Only Surviving: 44207 (0.16%) Dropped: 25195 (0.09%)
Trim Stats Trimmomatic S.diaphana
Sample_60-S_trimm.err:Input Read Pairs: 51647285 Both Surviving: 44720123 (86.59%) Forward Only Surviving: 6761676 (13.09%) Reverse Only Surviving: 83979 (0.16%) Dropped: 81507 (0.16%)
Sample_67-497_trimm.err:Input Read Pairs: 23733774 Both Surviving: 21021363 (88.57%) Forward Only Surviving: 2647202 (11.15%) Reverse Only Surviving: 40126 (0.17%) Dropped: 25083 (0.11%)
Sample_68-498_trimm.err:Input Read Pairs: 22526932 Both Surviving: 20250323 (89.89%) Forward Only Surviving: 2211613 (9.82%) Reverse Only Surviving: 41137 (0.18%) Dropped: 23859 (0.11%)
Sample_69-499_trimm.err:Input Read Pairs: 25376396 Both Surviving: 22417071 (88.34%) Forward Only Surviving: 2886755 (11.38%) Reverse Only Surviving: 44291 (0.17%) Dropped: 28279 (0.11%)
Sample_70-505_trimm.err:Input Read Pairs: 24524808 Both Surviving: 21896093 (89.28%) Forward Only Surviving: 2566192 (10.46%) Reverse Only Surviving: 39400 (0.16%) Dropped: 23123 (0.09%)
Sample_71-506_trimm.err:Input Read Pairs: 26116341 Both Surviving: 23576965 (90.28%) Forward Only Surviving: 2461571 (9.43%) Reverse Only Surviving: 49037 (0.19%) Dropped: 28768 (0.11%)
Sample_72-507_trimm.err:Input Read Pairs: 27058554 Both Surviving: 24169844 (89.32%) Forward Only Surviving: 2817293 (10.41%) Reverse Only Surviving: 44407 (0.16%) Dropped: 27010 (0.10%)

Supplementary Table 2.2 Trimmomatic statistics

	Sternoptyx	Sternoptyx Eye	Argyropelecus	Argyropelecus Eye
Total Trinity 'genes'	1,265,376	439,454	1,304,250	828,005
Total Trinity transcripts	1,510,873	779,283	1,570,775	1,378,313
%GC	44.42	45.02	44.59	44.87
ALL transcript contigs:				
Contig N10	1451	2711	1210	2059
Contig N20	817	1751	745	1281
Contig N30	544	1209	532	873
Contig N40	414	855	414	637
Contig N50	341	625	354	492
Median Contig length	**270**	**340**	**275**	**319**
Average contig	**362.61**	**528.28**	**360.74**	**460.17**
Total assembeled bases	547,857,376	411,681,624	566,641,812	634,262,191
Longest isoform per 'gene':				
Contig N10	1055	2246	907	1537
Contig N20	581	1364	552	902
Contig N30	425	908	418	631
Contig N40	350	650	349	489
Contig N50	306	496	307	401
Median Contig length	**262**	**313**	**265**	**298**
Average contig	**330.01**	**460.95**	**327.62**	**401.32**
Total assembeled bases	417,581,701	202,566,088	427,298,210	332,295,220
Complete BUSCOs (C)	263	234	259	232
Complete and single-copy BUSCO (S)	143	98	97	23
Complete and duplicated BUSCOs (D)	120	136	162	209
Fragmented BUSCOs (F)	**38**	**16**	**43**	**15**
Missing BUSCOs (M)	2	5	1	8
Summary	C:86.8%[S:47.2%,D:39.6%], F:12.5%,M:0.7%	C:91.7%[S:38.4%,D:53.3%] ,F:6.3%,M:2.0%	C:85.5%[S:32.0%,D:53.5%], F:14.2%,M:0.3%	C:91.0%[S:9.0%,D:82.0%], F:5.9%,M:3.1%

Supplementary Table 2.3 Transcriptome assessment for deep-sea transcriptomes. Method: TRINITY= green, BUSCO=blue results

Total Trinity 'genes'	697568
Total Trinity transcripts	790431
%GC	39.46
ALL transcript contigs:	
Contig N10	5330
Contig N20	3617
Contig N30	2546
Contig N40	1763
Contig N50	1173
Median Contig length	372
Average contig	715.92
Total assembeled bases	565881549
Longest isoform per 'gene':	
Contig N10	4283
Contig N20	2549
Contig N30	1596
Contig N40	1043
Contig N50	728
Median Contig length	347
Average contig	581.48
Total assembeled bases	405620437
Complete BUSCOs (C)	300
Complete and single-copy BUSCO (S)	168
Complete and duplicated BUSCOs (D)	132
Fragmented BUSCOs (F)	1
Missing BUSCOs (M)	2
Summary	C:99.0%[S:55.4%,D:43.6%],F:0.3%,M:0.7%

Supplementary Table 2.4 Transcriptome assessment for zebrafish transcriptome. Method: TRINITY= green, BUSCO=blue results

Appendix 2
Published Papers

frontiers in Physiology

ORIGINAL RESEARCH
published: 14 August 2020
doi: 10.3389/fphys.2020.01002

Zebrafish Circadian Clock Entrainment and the Importance of Broad Spectral Light Sensitivity

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One of the key defining features of an endogenous circadian clock is that it can be entrained or set to local time. Though a number of cues can perform this role, light is the predominant environmental signal that acts to entrain circadian pacemakers in most species. For the past 20 years, a great deal of work has been performed on the light input pathway in mammals and the role of intrinsically photosensitive retinal ganglion cells (ipRGCs)/melanopsin in detecting and sending light information to the suprachiasmatic nucleus (SCN). In teleost fishes, reptiles and birds, the biology of light sensitivity is more complicated as cells and tissues can be directly light responsive. Non-visual light signalling was described many years ago in the context of seasonal, photoperiodic responses in birds and lizards. In the case of teleosts, in particular the zebrafish model system, not only do peripheral tissues have a circadian pacemaker, but possess clear, direct light sensitivity. A surprisingly wide number of opsin photopigments have been described within these tissues, which may underpin this fundamental ability to respond to light, though no specific functional link for any given opsin yet exists. In this study, we show that zebrafish cells show wide spectral sensitivities, as well as express a number of opsin photopigments – several of which are under direct clock control. Furthermore, we also show that light outside the visual range, both ultraviolet and infrared light, can induce clock genes in zebrafish cells. These same wavelengths can phase shift the clock, except infrared light, which generates no shift even though genes such as *per2* and *cry1a* are induced.

Keywords: zebrafish, entrainment, opsin, non-visual photopigment, circadian clock, phase shift, monochromatic light

OPEN ACCESS

Edited by:
Charalambos P. Kyriacou, University of Leicester, United Kingdom

Reviewed by:
Cristiano Bertolucci, University of Ferrara, Italy
Rachel Ben-Shlomo, University of Haifa, Israel

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Specialty section:
This article was submitted to Chronobiology, a section of the journal Frontiers in Physiology

Received: *04 April 2020*
Accepted: *23 July 2020*
Published: *14 August 2020*

Citation:
Steindal IAF and Whitmore D (2020) Zebrafish Circadian Clock Entrainment and the Importance of Broad Spectral Light Sensitivity. Front. Physiol. 11:1002. doi: 10.3389/fphys.2020.01002

INTRODUCTION

The most ancient and predictable environmental cue for life on Earth is the onset of sunrise and sunset. In fact, it is hard to imagine any other environmental stimulus that an animal or plant experiences that lacks any biologically significant noise. As a consequence, most life under the Sun, from bacteria to plants to humans have evolved a circadian clock which internally represents this highly predictable change in day and night. A critical aspect of having such a circa 24-hour

pacemaker is that it needs to be entrained or set each day to the environmental light-dark cycle. Natural selection acts on correct phase relationships rather than clock period, such that it is essential both internal and external oscillations are appropriately phase aligned. Consequently, entrainment is an essential and defining feature of the circadian clock and the topic addressed in this study.

Light sensing and entrainment were always thought to be a process associated exclusively with the eyes and the Suprachiasmatic Nucleus (SCN) in mammals, and the pineal gland in non-mammalian vertebrates (Suburo and de Iraldi, 1969; Ibuka and Kawamura, 1975; Elliott, 1976; Underwood and Groos, 1982; Cahill, 1996). It therefore came as a surprise, some 20 years ago, that the process of non-visual photoreception is something that all tissues in the zebrafish are capable of Whitmore et al. (1998). Although the zebrafish pineal has key functions (Ben-Moshe et al., 2014; Livne et al., 2016), teleost clock systems appear to be highly decentralised, with all tissues and the majority of cells possessing a directly light entrainable circadian pacemaker (Whitmore et al., 1998, 2000; Carr and Whitmore, 2005; Tamai et al., 2005; Steindal and Whitmore, 2019).

Peripheral light sensitivity is not exclusive to zebrafish, as most non-mammalian vertebrates such as fish, reptiles and birds show high opsin diversity (Davies et al., 2015). Deep-brain photoreception has been researched in avian seasonal physiology for many years, as has similar hypothalamic responses in reptiles (Benoit, 1935a,b; Underwood and Menaker, 1976; Takahashi and Menaker, 1979; Underwood and Groos, 1982; Wyse and Hazlerigg, 2009). So, perhaps it should not have come as such a surprise when this well-established direct brain light-sensitivity was expanded to include the majority of other tissues. Monotremes and mammals also express non-visual opsins that facilitate a range of biological processes, of which, melanopsin in mammalian clock entrainment is the most explored (Provencio et al., 1998; Halford et al., 2001; Tarttelin et al., 2003).

When peripheral photoreception was discovered in anamniotes, the next obvious question concerns the nature of the photopigment responsible for this peripheral light detection and clock entrainment? Visual photopigments have been studied extensively since the 19th century (Norris, 1895; Arey, 1915). However, a whole century past before science turned its interest to the discovery of the non-visual photopigments, and several candidates appeared through the late 1990s and early 2000s (Okano et al., 1994; Blackshaw and Snyder, 1997; Soni and Foster, 1997; Sun et al., 1997; Provencio et al., 1998). The number of opsins discovered since the early 1990s has increased to include 32 non-visual and 10 visual opsins in zebrafish (Davies et al., 2015), and new opsins, splice variants and isoforms are discovered in new species on a regular basis (Musilova et al., 2019). The non-visual and visual opsins are divided into 8 classes based on photoisomerase activity, molecular function and how they couple and signal though G-proteins. What sets several of the non-visual opsins apart from the visual opsins, is that they are bistable, meaning that instead of bleaching like the visual opsins, the photoproduct can convert between photoproduct and photopigment without releasing the chromophore (Tsukamoto, 2014). Such a process makes sense in the context of a tissue that lacks the more sophisticated pigment-regeneration mechanisms found in the retina.

With such a large diversity of opsins, identifying key candidates in the fish for photoentrainment of the clock, or how these photopigments work synergistically together is now more complicated than ever. Absorption spectra has been performed on many of these zebrafish opsins. Most are monophasic, but seemingly with somewhat broad absorption peaks, with most opsins absorbing in the blue-green, while some absorb up in the red end of the spectrum (Su et al., 2006; Davies et al., 2011, 2015; Koyanagi et al., 2015; Morrow et al., 2016; Sato et al., 2016, 2018; Sugihara et al., 2016; Steindal and Whitmore, 2019). Thus, the zebrafish has the theoretical capacity to detect light ranging from UV to IR and across the visual spectrum. Zebrafish cell lines and other teleost cell lines have been used for years in clock studies, yet we do not actually know what opsins are expressed in these cultures.

This raises the question, do zebrafish show such a diversity in opsins in order to be able to capture all photons of any wavelength, such that the system is simply designed to detect the presence or absence of light, regardless of wavelength? Or does this different opsin expression pattern mean that particular organs have specific wavelength sensitivities and therefore differing responses to the environmental light signal?

In this paper, we demonstrate that the light response goes well beyond the visual wavelengths, with both UV and infrared (IR) light pulses having the ability to induce clock gene expression, but interestingly with IR not able to phase shift the molecular clock, at least in cell lines. Furthermore, we show that zebrafish cell lines, rather like the adult tissues (Davies et al., 2015), display a diversity of expressed opsins, a number of which are under clock-control and as such show robust daily rhythms in expression. Whether this transcriptional rhythm in specific opsins translates into matching protein changes is yet to be determined, but it opens up the possibility of a direct temporal regulation of light sensitivity, as well as the more conventional spatial aspects. In this regard, the clock is likely to be gating the process of its own entrainment by regulating expression of components of the light-input pathway; with a specific pathway acting as a zeitnehmer or "time taker" (McWatters et al., 2000).

MATERIALS AND METHODS

Cell Culture

PAC2 and *clockDN* (clock "mutant" cells) cell lines were kept in Leibovitz -15 medium (Gibco) supplemented with 15% FBS (Biowest), 0.05 mg/ml of gentamicin (Gibco) and 1x Penicillin-Streptomycin (Dekens and Whitmore, 2008). Cells were seeded at 50 000 cells/ml and kept at 28°C in a water bath on a 12:12 LD cycle for 3 days before receiving a light-pulsed with different wavelengths (IR 850 nm, red 650 nm, blue 450 nm, UV 350 nm and white 400-700 nm) with an intensity of 200 $\mu W/cm^2$ for 3 h starting light pulse at ZT21 (LED Array Light source, Thorlabs). After a 3-hour light pulse, cells were washed with PBS and homogenised in TRIzol with a cell scraper.

RNA, cDNA and RT-qPCR

RNA was extracted according to manufacturer's guidelines (TRIzol, Invitrogen) and the RNA pellet re-suspended in 30 µl of RNase free water (Ambion). 2 µg of RNA was reverse transcribed to cDNA using Superscript II Reverse Transcriptase (Invitrogen), random hexamers (Invitrogen) and oligo dT primers (Invitrogen) according to manufacturer's protocol. RT-qPCR was performed on a C1000 Touch™ Thermal Cycler with the CFX96™ Optical Reaction Module (Bio-Rad) using KAPA SYBR FAST qPCR mix (Kapa Biosystems) in technical triplicates with gene specific-primers at a concentration of 500 nM. ΔCt was determined using β-actin as a reference gene and relative expression levels were plotted using the ΔΔCt method. Gene specific primers are listed in **Table 1**.

Bioluminescent Assays

Per-1 luciferase cells, described by Vallone et al. (2004), were plated at 100,000 cell/ml in media (described above) in a white 96-well plate (Greiner) $n = 16$. Cells settled over night at 28°C, and the following day the media was changed for media supplemented with 0.5 nM beetle luciferin (Promega). Plates were sealed with TopSeal clear adhesive from (Perkin Elmer). Bioluminescence was monitored on a TopCount NXT scintillation counter (Packard Instrument Company), in a temperature-controlled chamber (28°C). Cells were entrained on a 12:12 LD cycle and given a light pulse (as described

TABLE 1 | Gene specific primers used for qPCR.

Accession no.	Current name	Alt. Name	Forward 5′ -> 3′	Reverse 5′ -> 3′
KT008391	Exorhodopsin		GTA CGC TCC GCT ATC CCA TA	ACG TGT GAA AGC CCC TAC TG
KT008402	Valopa	Val	ACT TCC ACG ACC ACA CCT TC	CGG ATG AGT TTG CAG TAG CA
KT008403	Valopb	Va2	GGC GAG GAT GGT CGT TGT AA	ATG CTG CAT AAG GCG TCC AT
KT008404	parapinopsin-1		CTG TGG TCG TTC ATC TGG AA	GGC CAG ATC TCT GCT GTA CC
KT008405	parapinopsin-2		GCA GCA CTG TAT ACA ACC CCT	ATA CGT CGT CCT CTG AAG GC
KT008406	parietopsin		TGT TGG CGT ATG AGC GTT AT	AGC CAT ACC AAC AGC AGA CC
KT008407	TMTla	tmt6	TGT TAC AGT CGG CTC ATC TGT GCT	ATG TGG TAC TCT CTC CGT CTT GCT
KT008408	TMTlb	tmt9	TGT TGG TGT GTA TGT TCG GGACGA	AGG AGT TGA TGA AGC CGT ACC ACA
KT008409	TMT2a	tmtlO	TTA GTA AGA AGC GGA GCA GAA CCT	ATC CCA TAG GGA TGC AGT GTT GTT
KT008410	TMT2b	tmtl4	CGC AGA GGA GAG AGA ACC AC	TTA GTC CCG TTC TGC CAA AG
KT008411	TMT3a	tmt2	AGG TCG ATG CGA CCA ACT ACA AGA	AAA CAG AGG AGG CAG GGT CCA AAT
KT008412	TMT3b	tmt24	TGC GTG TGG TAC GGT TTC ATC AAT	ATC ATG GTG CAG TAA CGC TCG TAT
KT008413	encephalopsin (opn3)	panopsin	CCCTAT GCT GTG GTC TCC AT	TAG ATG ACG GGG TTG TAG GC
KT008414	neuropsin (opn5)	OPN5ml	ACA CCA TCT GTC GCT CCA TC	CTG CAA ATT GCC CAG TGT C
KT008415	0PN6a	novo3b	GTG GTC AAC ATC CCC TGG AG	ACA ACC AGC CGA GTA TGA GC
KT008416	0PN6b	novo3a	AAT CCA GCC AGG GAG GAG AT	AAG GCG GAC CAC ATG GAA AT
KT008417	0PN7a	novolx	GTT TAA ACA CTA CCCGCG CC	GCTCTG GCTCCA ATT CAG GT
KT008418	0PN7b	novola	TGC TAT ATC GTG CCC TG C TG	CGTACC GTC ACC AGG ATG AG
KT008419	0PN7c	novolb	GTG AAC CTG TCT GTG AGC GA	CTC CCC AAA CAA CCA CCT GT
KT008420	0PN7d	novoly	CTG CCA CTT GGA ATC ATC CT	GCG ACA CAT GCT GCT GTA CT
KT008421	0PN8a	novo2b	TGA CTG ACA TTG GCA TGG CT	TGG TTG AAA GCA GAG GCG AT
KT008422	0PN8b	novo2a	TTC GCT TCA TCG TGT CTT TG	CAG TGG GAA AAT AGC CCA GA
KT008423	0PN8c	novo2x	TGG GCT TTA TCC TTG CCT GG	AGA TGAAGC CTT CTG GTG CC
KT008424	0PN9	OPN5m2	TCA G GG CTT TG T TTT CGG G A	GCA GCG GTC AAG GGA TAT GA
KT008425	Peropsin (RRH)		AGT GGT TGC CAT TGA CCG AT	ATG CGG CCA CAA TCA GAA GA
KT008426	RGR1		CCT GGC TTT CTA CGC CGC AG	GGA CTT GTT CTC AAT AGC AGG ACT CTC
KT008427	RGR2		GAG CAC GTC TAT CAC CAT CAG CT	ACA CCC CAG CCA ATG GCA GG
KT008428	OPN4ml		CGT CAT CAC CTC TGA GTC CA	GCT GGA TTT GTC CCA ACA GT
KT008429	OPN4m2		AGC AAT GCT AGT GGG CAG AA	CGT CTG CTG CAT CCG TTT CA
KT008430	OPN4m3		AAG GCC AAT GGT TCG GAT CC	CCA GGT ATG AGC CTG GAA GA
KT008431	OPN4xl		GCT ACA CCT TGA TGC TCT GC	CTG TTG GAT GAG GGT GGT CT
KT008432	OPN4 × 2		CTT TGT GAA GCA GCA GTC CA	TAT GGA GCC CAG GAC AAA AC
NM_001077297.2	Cry 1a		AGG CTT ACA CAG CAG CAT CA	CTG CAC TGC CTC TGG ACT TT
NM 182857.2	Per2		TGG CTC TGG ACA GAA GTG AG	GGA TGT CTC GAG AAG GCA AC
NM 198143.1	L13		TCT GGA GGA CTG TAA GAG GTA TGC	AG A CGC ACA ATC TTG AG A GCA G
AB042254.1	6–4 photolyase	cry5	TGT GGA TCA TGA GGT TGT CC	TTG ATG GAT GGA CTC GCT TT
NM_001030183.1	Perla	perl	ATC CAG ACC CCA ATA CAA C	GGG AGA CTC TGC TCC TTC T
AF057040.1	Beta a ctin		CGC AAA TAC TCC GTC TGG AT	TCC CTG GAG AAG AGC TAC GA

above) at ZT21 after 2 days. Cells were then kept in DD on the TopCount for two more days, at constant temperature, in order measure any phase shift in the gene expression rhythm. Luminescence from the cells was measured in counts per second approximately every hour taking approximately 10 min for a 96-well plate to be read.

Statistical Analysis

T-tests, ANOVAs and post-test were performed with the standard add-in software in Excel. Alpha was set at 0.5. Tukey numbers were calculated using values from a standard Tukey table.

RESULTS

Opsin Expression in Cell Culture

As well as having directly light sensitive organs, zebrafish cell lines, typically generated from early stage larvae such as the PAC2 cell line, are also directly light responsive (Whitmore et al., 2000). To examine which opsins are present in the cells, both PAC2 cells, and transformed cells expressing a *clock*-dominant negative construct (*clock*DN cells) were kept on a 12:12 light dark cycle at constant temperature, and cells were harvested at ZT3 and ZT15. Both cell lines express opsins from all classes of non-visual opsins, with a total of 11 out of 32 non-visual opsins expressed at a detectable level (C_t lower than 30) (**Figure 1**). There is no apparent difference between PAC2 and the *clockDN* lines in opsin expression pattern. By comparing the expression pattern at two different times of day, we also observed that half of the opsins show a day-night difference in expression pattern in PAC2, but not *clockDN* cells, which shows that some opsin expression is clock controlled (**Figure 1**). Interestingly, two forms of OPN4 are expressed in these zebrafish cell lines and one, OPN4 × 2, shows a strong day-night difference in expression. This is also the case for exo-rhodopsin, which is typically considered to be a pineal specific photopigment. RGR1, a putative photoisomerase, also shows robust daily changes, and is the most abundant transcript.

Impact of Light on Clock Genes in Cells

To explore how monochromatic light of selected wavelengths impacts gene expression in zebrafish cell lines, *ClockDN* and *PAC2* cells were entrained, like the organs, on a 12:12 LD cycle at constant temperature and given a monochromatic light pulse for 3-hour at ZT21, when cells are most light responsive (Tamai et al., 2005). Using RT-qPCR, we examined the effect of these light pulses on different, well-established light responsive clock genes, such as *cryptochrome1a* (*cry1a*) and *period2* (*per2*), as well as the light induced DNA repair gene, *6-4 Photolyase* (*6-4 Ph*) (Tamai et al., 2007; Vatine et al., 2009). In *PAC2* cells, white, blue and UV light pulses of the same intensity give very similar induction in all genes explored, whilst red generates a slightly smaller, yet not statistically different induction (**Figures 2A–C**). IR pulses give the smallest induction of the genes explored. In *cry1a* we see a significant 1.6-fold induction, as opposed to ∼4-fold induction by the other wave lengths (**Figure 2A**). For *per2* IR give a ∼5-fold induction as opposed to 20–30-fold by the other wavelengths (**Figure 2B**). Finally, IR gives a 2.6-fold induction as opposed to up to 13-fold induction, by the other wavelengths (**Figure 2C**). IR does indeed induce significant induction of the light sensitive clock and a DNA repair gene. However, compared to the other wavelengths, it is between 2.5 and 6 times less potent, depending on the gene in question.

ClockDN cells show a reduced fold induction to all the wavelengths (**Figures 2A–C**). The raw C_t values seen in *clockDN* and PAC2 cells are, however, very similar when given a light-pulse. These clock mutant cells show a higher basal DD expression of the clock and DNA repair genes, and thus the fold induction is subsequently lower (**Supplementary Figure S1**). This is particularly evident when giving an IR light pulse (**Figure 2D**).

Phase Shift in Cell Culture

To explore how the monochromatic light phase shifts the molecular clock in cell culture, *per1-luciferase* luminescent reporter cells (Vallone et al., 2004) were entrained for 3 days at 28°C and pulsed the same way as the cells described above (**Figure 3A**). *Per1* luminescent traces were then monitored for 2 days post light pulse in DD using the Packard TopCount. UV, blue, red and white light are all capable of causing a phase advance in the cell culture clocks when light is applied at this particular time in the cycle (ZT21) (**Figure 3B**). Interestingly, a 3-hour light exposure of IR light does not give a phase shift regardless of the acute molecular response to this light signal, increasing both *cry1a* and *per2* expression, a result which is worthy of further discussion.

DISCUSSION

Cells Show a Diversity in Opsin Expression Patterns

Zebrafish cell cultures have long been used because of their direct light sensitivity. However, the opsin composition of these cells lines has never previously been published. We, therefore, performed RT-qPCR on PAC2 cell lines to explore what opsins are expressed. With RT-qPCR and setting a cut off value at C_q 30 as a measure of "no expression," we can identify the presence of 11 out of 32 non-visual opsins (**Figure 1A**). Interestingly, 6 of these opsins show a clear day-night difference in expression and appear to oscillate. All of these opsins show higher levels of expression during the day time-point compared to night. To explore this further, we therefore also examined expression in the *ClockDN* cell line, lacking a functional circadian clock, to manifest whether this difference is light-driven or clock-dependent. Interestingly, the *ClockDN* cells express the same specific opsins exactly, but they no longer oscillate (**Figure 1B**), which supports the idea that expression of these opsins is directly clock controlled and not directly light-driven. Furthermore, averaging expression of ZT3 and ZT15, there is no significant difference in the amount of transcript produced in the two different cell lines. The opsin expression profile in cells does

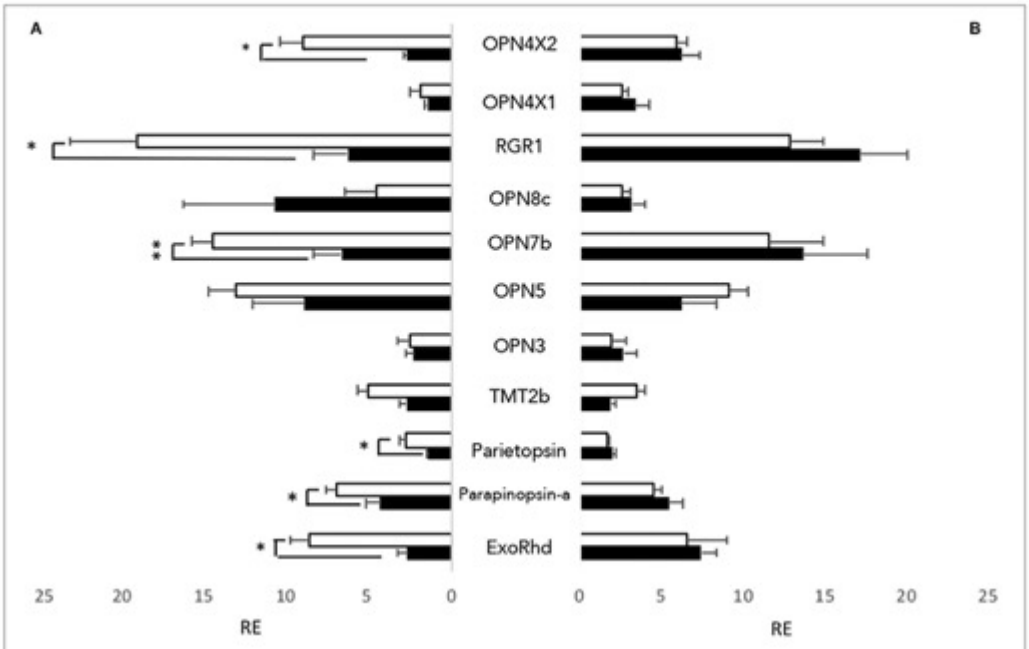

FIGURE 1 | Non-visual opsins expressed in zebrafish cell lines. All 32 non-visual opsins were explored by RT-qPCR in entrained zebrafish cell lines at opposite times of day; ZT3 (white) and ZT15 (black). 11 opsins showed detectable expression levels, using a cut-off value of $<C_q$ 30. **(A)** Opsins expressed in PAC2 cell lines. **(B)** Opsins expressed in *clockDN* cell lines. Opsin expression is plotted relative to the lowest detectable opsin, with error bars depicting SEM. An unpaired students *t*-test was used to assess if the opsins expressed show a time of day specific expression pattern. $*p < 0.05$ and $**p < 0.001$ ($n = 4$).

not resemble any particular tissue type that we know of today. However, it is worth noting that the cell line express one opsin from all the opsin families, like most tissue types, and thus possess G_q, G_t, G_i, and G_o coupled opsins, as well as a putative photoisomerase (RGR1). The cell line should, therefore, be able to signal through the same pathways in response to light as any other fish tissue.

Monochromatic Light (350–650 nm) Are Potent Inducers of Light Responsive Genes in Cell Culture

Expression of the light responsive clock genes in cell culture is rather flat with a broad response to white, blue and UV light. There is a slight but statistically significant drop in the response to red light in the cells, and a marked drop in the response to IR (**Figure 2**).

6–4 photolyase catalyses the photo-reversal of the (6–4) dipyrimidine photoproducts induced in DNA by ultraviolet light (Zhao et al., 1997). A simple prediction might be that UV/blue light should be more efficient at inducing expression of this DNA repair enzyme. However, this does not appear to be the case, with red light and even IR light able to increase transcript levels. Red light photons have lower energy than blue light photons, therefore, the same intensity of blue and red light will have different number of emitted photons. Consequently, one hypothesis is that the opsins simply "count" photons, not the energy of the photons they absorb, meaning that the zebrafish cell simply wants to know whether there is light present or not. To address such issues, these experiments will need to be repeated considering aspects of photon flux over a wider range of light intensities. Of course, it may be biologically essential to activate expression of your DNA repair machinery in the presence of light, regardless of the subtleties of the specific wavelength, and of course the 6–4 photolyase protein itself absorbs light to perform its role in replacing cross-linked nucleotides. It is this aspect of light driven DNA repair that is most likely to be wavelength sensitive.

Comparing cells without a functional clock to "wild type" cells, we also see that the fold induction of genes in response to light is lower, due to a higher basal transcription of these target genes in DD. *ClockDN* cells show a higher basal DD expression of the clock and DNA repair genes, and the fold induction is subsequently lower (**Figures 2A–C**). This is interesting, as it demonstrates the steady state expression levels that these

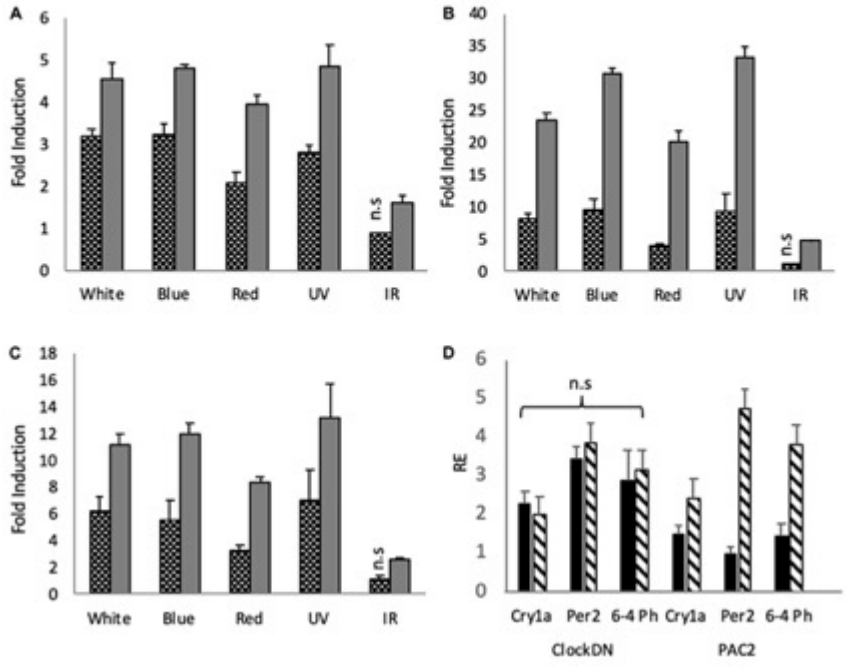

FIGURE 2 | Light induction by monochromatic light-pulses in PAC2 and *clockDN* cell lines. Zebrafish cell lines were maintained on a 12:12 light-dark cycle before being given a 3-hour light pulse of varying wavelengths. Black and white bars represent *clockDN* cell line expression, whilst solid grey represent PAC2 cell line expression. **(A)** Light induction of *cry1a*. **(B)** Light induction of *per2*. **(C)** Light induction of 6–4 Photolyase. **(A–C)** is plotted as fold induction relative to dark control. **(D)** Dark controls (black bars) vs. IR monochromatic light pulse (striped bars) plotted relative to lowest expressed gene (PAC2 *per2* DD) in *clockDN* and PAC2 cells. Significance was addressed with an one-way ANOVA ($\alpha = 0.05$) for each light-pulse, cell line and gene, followed by a Bonferroni post-test. All light pulses give a significant increase of $p < 0.05$ unless marked on the graph ($n = 3$).

genes reach in a non-rhythmic mutant background. The absolute expression remains the same (**Supplementary Figure S1**). Interestingly, the high basal level of transcript means that there is no induction of light responsive clock genes in the *clockDN* cells in response to IR.

UV- Red Light Can Alter Gene Expression and Phase-Shift Cell Lines

The impact of "visible" wavelengths of light (380–740 nm) on the zebrafish clock system has been described in numerous previous studies. However, exploring this phenomenon outside of the visual spectrum are rarely performed in fish. UV light of 350 nm (UVA) has a clear impact on gene expression and can clearly phase shift the circadian clock in cell lines. Perhaps this is not so surprising from what we now know about zebrafish photobiology. After all, 350 nm is only 50 nm below the violet/blue wavelengths that can so robustly impact the clock in an aquatic organism. In future, it would be interesting to try wavelengths at the more extreme end of the UVA range and well away from the visual spectrum. This UV response also fits well with the previously determined absorption spectra for purified opsin proteins, which reveals a wide sensitivity in the UV/blue wavelengths (Davies et al., 2015).

The impact of these monochromatic light pulses was explored using our luminescent reporter cell lines. At the phase (ZT21) and intensity used, each wavelength generated a very similar phase advance in the rhythm, including UV light pulses, but not IR at 850 nm (**Figure 3A**). This similarity in size of phase advance correlates well with the similarity in molecular response, induction of *cry1a* and *per2*, seen in the cell lines (**Figure 2**). Furthermore, using a Tukey post-test, there is statistical difference in the size of phase shift generated by each of these light pulses (except between blue and UV) (**Figure 3B**). Since this difference in shift is so small, it may be due to the sampling frequency (plate counted once an hour) rather than real difference, thus we do not speculate any further.

Compared to previous studies on phase shifting in zebrafish cell lines, in response to white light, the size of the phase shift

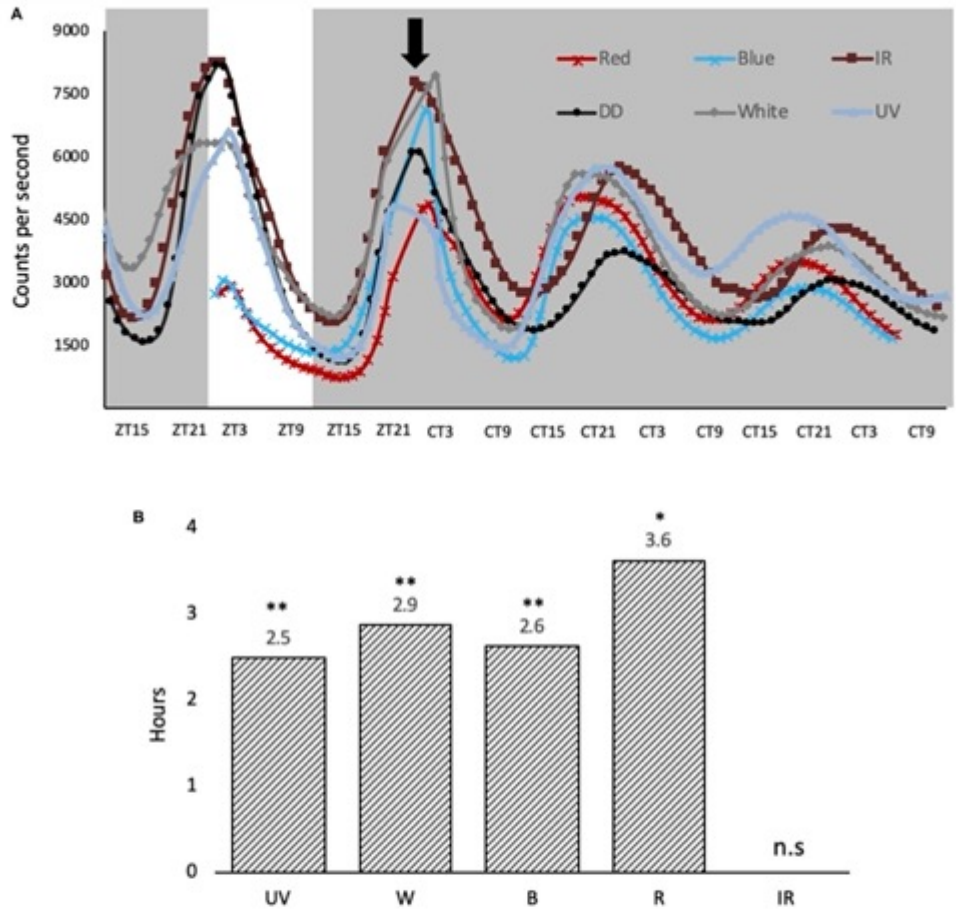

FIGURE 3 | Light-induced phase shifts in response to monochromatic light-pulses in *per1-luciferase* zebrafish cells. **(A)** Cells were entrained for 3 days on a 12:12 LD cycle, before they were given a variety of monochromatic light pulses at ZT21, denoted by black arrow. The cells were kept in constant dark over two subsequent days. **(B)** Light pulses cause a phase advance in hours, determined at the half maximum between peak and trough vs. no LP controls. A two-way ANOVA (time, wavelength) was used to determine significant variation between samples. Amplitude and baseline were detrended using BioDare2 (Zielinski et al., 2014), and a Tukey post-test was used to determine significance in phase shift relative to DD control. $^{*}p < 0.01$ and $^{**}p < 0.001$ ($n = 8$).

is relatively small and is actually a phase advance rather than a large phase delay previously reported (Tamai et al., 2007). The reasons for this simply relate to the differences in light intensity used. Early studies applied light at 5000 $\mu W/cm^2$, compared to the 200 $\mu W/cm_2$ used in this study. Consequently, the Type 0 PRC previously reported switches to a more "standard" Type 1 PRC as the lower light intensity, as historically seen in many previous studies. Interestingly the switch in PRC amplitude, therefore, occurs between these two intensities, and strongly suggests that fish under natural conditions, as a diurnal animal, will be "working with" a Type 0 PRC.

The response to infrared light was not expected. As a stimulus, it is generally avoided in clock studies, due to the strong link with temperature effects/artefacts and the ability of temperature pulses to phase shift the circadian clock. It is a stimulus typically one aims to control against in circadian analysis. Yet the response to IR when controlling for temperature, of the zebrafish clock system is very interesting. IR of 850 nm can clearly cause

specific transcriptional changes in zebrafish cells. Obviously, in our experiments we aimed to avoid the thermal heating effect of IR exposure, and none was detected in our cultures. Equally, the IR light pulse did not phase shift the clock, which has been shown to be robustly phase-shifted by temperature pulses (Lahiri et al., 2005). For cells in culture, IR causes a small, yet significant induction of *cry1a* and *per2*, however, there is no subsequent phase shift. As mentioned earlier, IR is up to 6 times less potent in cell lines, and the reduced induction of *cry1a* and *per2* compared the other wavelengths may not be sufficient to cause a downstream phase shift in these studies. Of course, it could also be the fact that *cry1a* and *per2* are not as central to phase shifting the teleost clock as has been previously proposed.

How IR signals to cells in a meaningful way is a fascinating question. It could be through mitochondrial-driven processes or it could be through the "re-purposing" of the mass of opsin in fish to perform other key sensory roles. If fish opsins are acting as "thermal" or IR sensors, as has been proposed in Drosophila, then this opens up a whole new world of interesting (fish) biology.

In this study, we have shown that the spectral sensitivity of zebrafish cell lines extends beyond the classically perceived "visual" wavelengths of light and that supporting this wide spectral sensitivity, these cells express a large number of opsins. Furthermore, the clock itself regulates the temporal expression of these opsins, raising the interesting possibility that the clock itself controls light input to the pacemaker – the zeitnehmer concept that has so eloquently been described for plant clock systems.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

Both authors performed the experiments, analysed the data, wrote the manuscript and contributed to the article and approved the submitted version.

FUNDING

MRC supported Ph.D. for IAF Steindal and consumables through the MRC DTP programme. Leverhulme Trust grant RPG-2017-299 used for consumables.

ACKNOWLEDGMENTS

We would like to thank past and present lab members of the Whitmore for discussions and historical input. Especially Kathy Tamai, Lucy Young, and Amanda Carr for their involvement. We would also like to thank the Leverhulme Trust for funding to DW and the MRC-DTP for funding to IAFS.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.01002/full#supplementary-material

FIGURE S1 | Monochromatic light pulses in PAC2 vs. *clockDN* cell lines. *ClockDN* cell lines (light grey) show increased basal expression of all genes explored in the dark control compared to PAC2 (dark grey). Significance was addressed with a one-way ANOVA ($\alpha = 0.05$) for each light-pulse, cell line and gene, followed by a Bonferroni post-test. All light pulses give a significant increase of $p < 0.05$, except *clockDN* DD vs. IR ($n = 3$).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Review

Circadian Clocks in Fish—What Have We Learned so far?

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Received: 7 December 2018; Accepted: 9 March 2019; Published: 19 March 2019

Abstract: Zebrafish represent the one alternative vertebrate, genetic model system to mice that can be easily manipulated in a laboratory setting. With the teleost Medaka (*Oryzias latipes*), which now has a significant following, and over 30,000 other fish species worldwide, there is great potential to study the biology of environmental adaptation using teleosts. Zebrafish are primarily used for research on developmental biology, for obvious reasons. However, fish in general have also contributed to our understanding of circadian clock biology in the broadest sense. In this review, we will discuss selected areas where this contribution seems most unique. This will include a discussion of the issue of central versus peripheral clocks, in which zebrafish played an early role; the global nature of light sensitivity; and the critical role played by light in regulating cell biology. In addition, we also discuss the importance of the clock in controlling the timing of fundamental aspects of cell biology, such as the temporal control of the cell cycle. Many of these findings are applicable to the majority of vertebrate species. However, some reflect the unique manner in which "fish" can solve biological problems, in an evolutionary context. Genome duplication events simply mean that many fish species have more gene copies to "throw at a problem", and evolution seems to have taken advantage of this "gene abundance". How this relates to their poor cousins, the mammals, remains to be seen.

Keywords: zebrafish; circadian clock; development; DNA repair; non-visual light detection; cell cycle

1. Introduction

As we have all noticed by now, the sun comes up in the morning and sets in the evening with some predictability. For those living in London, New York or Tokyo where the light is more or less constant—you are more likely to suffer from some horrible clock-disrupted illness. There is no doubt that possessing a functional clock is key to optimal health, and entrainment of that clock is an essential process. Light is the typical, but far from only, environmental signal that sets the clock, and work on fish has revealed some unexpected aspects of non-visual photoreception, as we will discuss below. In this respect, various fish models offer unique tools with which to study the wide significance of light responsiveness, and this is no more true than in the use of naturally occurring cave populations of fish, in particular the blind Mexican cavefish (*Astyanax mexicanus*) and the Somalian cavefish (*Phreatichthys andruzzii*). These animals, which have evolved in complete darkness, can effectively be viewed as circadian/light responsive "mutants", and as such offer considerable potential for exploring the global importance of light [1–4].

Still one of the most useful ways to view the circadian clock system is to employ the "Eskinogram" model, first proposed by Arnold Eskin, in which the clock is broken up into light detection, input pathway, core oscillator and then downstream, clock-regulated output events. In this context, it is probably fair to say that the fish models have not yet contributed significantly to our understanding of the core clock mechanism. However, it is in the areas of input and output events where most significant

contributions have occurred from many research groups, along with emerging studies on the ecological adaptations that the clock undergoes in unusual environments [5]. Some of these findings, though far from all, will be discussed in the following sections of this review.

2. Peripheral Clocks and the Emergence of Cell Lines

2.1. Clocks Everywhere

The late 1990s saw a relatively fast transition from the classical clock view that circadian pacemakers are restricted to specialized "clock-containing" structures, to the idea of clocks residing in most, if not all cells and tissues [6–9]. These discoveries, of course, followed on from the initial isolation and cloning of clock genes, which then allowed for their expression patterns to be determined. In mouse, these genes are expressed in most tissues [10–12]. Then, with the advent of mammalian cell line experiments, it was shown that mammalian cell lines could produce oscillations in clock gene expression if the cells are synchronized in an appropriate pharmacological manner [13]. Work in zebrafish was occurring in parallel to these mammalian studies, and it was quickly shown, after the cloning of the zebrafish *clock* gene, that oscillations in this clock component occurred in all tissues examined, both in vivo and in vitro [7,8]. There are clearly independent circadian pacemakers within all of the fish tissues that have been examined. These observations were then expanded to include zebrafish cell lines, which show high-amplitude, robust clock rhythms at the transcriptional level [8]. Figure 1 is a simple diagrammatic summary of how we view zebrafish clock organization.

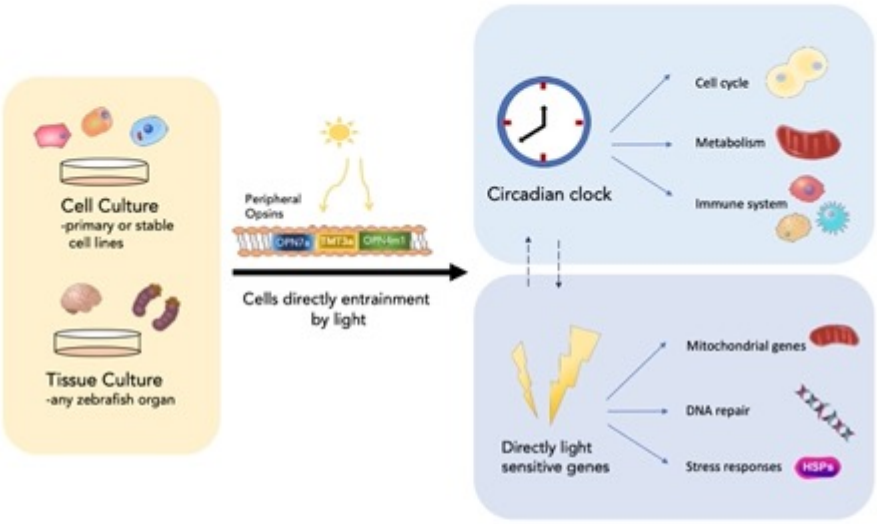

Figure 1. Zebrafish tissues are rhythmic and directly light-responsive. All zebrafish cell types and tissue/organs examined to date are directly light-responsive and do not require a centralised photosensitive structure to turn on light-induced transcription. Cells and organs can be entrained directly by light stimuli through the use of visual and non-visual peripheral opsins. The light signal starts transcription of light-sensitive genes, such as stress responses and DNA repair, as well as the clock genes *per2* and *cry1a*, which sets the circadian clock. The peripherally entrained clock in turn regulates a plethora of downstream cellular processes [14].

2.2. Global Light Sensitivity

At a very overt level, the big difference between mammalian cell/tissue clocks and those found in fish cells and tissues is that fish cells are directly light responsive [8]. The clock appears to be set by light directly, without any apparent need for eyes or pineal gland, the classical light responsive structures. Now it is clear that most of the studies that have addressed this issue have been performed with tissues or cells in culture, where light sensitivity is fully retained. But it is also apparent that peripheral light responsiveness is retained in zebrafish larval mutants, where either eyes or pineal are missing/defective. This does not mean that the eyes/pineal might not contribute to peripheral tissue light sensitivity, via either neural or hormonal signals, as modulators of this response, but there is no evidence at this time that this either occurs or is necessary.

This highly decentralized model of fish circadian biology, with independent, light-responsive circadian pacemakers in all tissues and most cells, does appear to hold true for most fish species that have been examined to date [15–17]. Although there is a lack of data on some of the really large marine species, which would represent an entertaining project if nothing else. In mammals, of course, the environmental light signal is transmitted to the suprachiasmatic nucleus (SCN) to set a "central" clock, which then plays a role in coordinating the timing of peripheral pacemakers [18]. This level of organization does not appear to be necessary in fish, but again this does not mean that there is not potential interaction between tissue clocks within the fish body. Obviously, a whole variety of hormonal signals, including rhythmic melatonin cues, could also be influencing tissue-specific, daily oscillations. It is interesting that the environmental light signal appears to set the clock to the same phase in all cells and tissues in vitro. Of course, what might occur in vivo is that light sets all of the body clocks to the same phase, and then various hormonal/neural cues apply subtle (or not so subtle) adjustments to this timing, generating tissue-specific phasing of rhythms. In the end, answering this question will require quite sophisticated imaging of organ rhythms in vivo, combined with a clever use of fish mutants, in order to determine the precise phase of various tissue clocks in response to numerous potential entraining signals. Such experiments may well be feasible in zebrafish, due to the small size and relative transparency of the larval body. It is not clear if fish do possess a "central" clock, but equally this idea might be rather redundant and something of a "red herring". It is clear that there are separate clocks in the brain and pineal gland, as there are in the heart and liver. They can all be synchronized independently, but may interact. There is no doubt that the pineal pacemaker plays a key role in influencing sleep processes and rhythmic behaviour, just as the heart clock plays a role in rhythmic heart physiology [19–21]. The ideas of "central" or "master" clock are no longer necessary or useful concepts. The importance of whole-body light sensitivity, however, is clearly important for fish physiology, and will be explored in more detail in Section 3.

2.3. Development of the Clock and Its Relevance

It is clear that the major advantage of using zebrafish as a model system is to study the ontogeny or development of clock function in the earliest stages of embryogenesis. Such experiments are difficult to perform in mammals for obvious reasons, mostly relating to the internal development of the foetus within the mother. The humble zebrafish is robust, cheap, well sequenced and shares similar genetic and organ structures to humans. Furthermore, the zebrafish is a well-established model for studying vertebrate development with transparent eggs being laid in their hundreds every other morning. Zebrafish embryo development is also rapid, with the first 24 h being equivalent to about 1 month of human development. Consequently, zebrafish also have a short generation time. What also makes the zebrafish useful for circadian studies is that they mate just after daybreak, meaning that developmental stage and circadian time are well aligned. Despite these advantages, this whole story marrying development and circadian rhythms got off to a dismal start with the publication of the idea that embryos inherit a sense of circadian phase from their mothers. This is not correct. Subsequent data in fact supports the idea that a circadian pacemaker is indeed present in the early stages of embryo development, with a peak in *per1* gene expression clearly present 27 h post fertilization (hpf) when

embryos are raised on a light-dark cycle. However, when raised in the dark, no such molecular clock rhythms are seen at the population level (in contrast to the initial reports). Considerable evidence backs up this theory, with multiple reverse-transcription quantitative polymerase chain reaction (RT-qPCR) experiments in zebrafish, as well as other teleosts, showing that the embryos require light as an entraining signal during the first day of development, to synchronize the temporally dispersed cellular oscillators [22,23]. Embryos are certainly strongly light responsive by 9 hpf, when they are only just beginning the process of gastrulation, and long before any classical light-responsive structures, such as eyes and pineal, have developed. In addition to light, temperature cycles can also entrain this embryonic clock, and similar light-dependent entrainment of a clock in the embryonic pineal gland is essential for early rhythms in *N*-acetyltransferase (NAT)expression and melatonin release [24,25].

The currently accepted view is that the core molecular clock starts to oscillate very early on in development, probably in all cells of the embryo, but that light exposure is necessary to synchronize each of these randomly phased cellular pacemakers. This is something of an important note to all of those developmental biology labs who still raise their larvae in dark incubators! But what is the clock actually regulating in embryo development and is this story as simple as it seems? To explore this question, larval samples were collected every 6 h from 72–168 h post-fertilization, and then the temporal expression pattern of nearly 100 specifically selected, developmentally critical genes was measured [26]. From this sample, a significant number of regulatory genes showed robust, high-amplitude circadian changes in expression levels. Figure 2 shows some of this embryonic gene expression data under light-dark conditions, and when the circadian pacemaker is stopped using constant light exposure. These downstream targets include numerous cell cycle regulators, discussed below, but also central, cell fate-determining transcriptional regulators, such as *neurod* and *cdx1b*. Both of these proteins play key roles in cell differentiation during development, with *neurod* regulating the early differentiation of neurons and pancreas, and *cdx1b* regulating early endoderm and digestive tract formation, as well as cell fate in the intestine [27]. These results suggest that the clock could play a major role in determining the timing of cell differentiation during development. However, this point remains to be proven and the situation may be more complex, especially in the context of *neurod*. Several aspects of its temporal and spatial regulation raise questions about its specific, clock-regulated role. Firstly, most of these developmental, output genes only begin to show robust daily rhythms from 72–96 hpf onwards. In zebrafish, this is quite late in development and after their recognized major developmental role, which would normally occur within the first 48–72 h. These results indicate that these genes come under clock regulation after their regulatory role in early development is complete. One possibility is that these developmental genes are now being used for a different purpose in later stage larvae and adults than in the early stages of development. In the case of *neurod*, the strongest daily rhythms occur in the retina. Retinal photoreceptor genesis requires precise regulation of cell cycle exit and differentiation, which is aided by *neurod* through the Notch signalling pathway. If one were to look, we might find that adult photoreceptor genesis is clock-regulated in zebrafish. Such results could have interesting implications for the regulation of stem cell niches within adult tissues of many species.

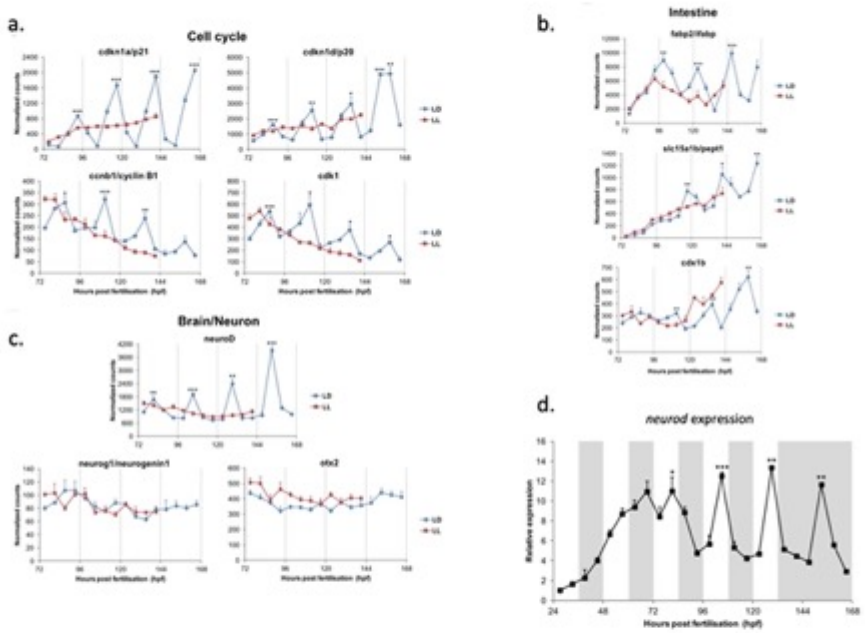

Figure 2. A selection of rhythmic clock-target genes regulated in the early stages of zebrafish larval development. A Nanostring-based gene expression analysis of a wide selection of genes examined between 72–168 h post-fertilization. The selected data shown in panels a, b and c reveals a wide range of genes that show robust oscillations during embryo development, when larvae are raised on a light-dark cycle. Constant light (in red) stops the circadian pacemaker in the embryo, as well as the rhythmic expression in downstream, clock-regulated genes. (**a**) shows that numerous cell cycle regulators have robust transcriptional daily rhythms. (**b**) shows changes in three genes involved in neuro-development and differentiation, with *neuroD* showing very high amplitude rhtyhms. (**c**) shows rhythms in three genes involved in cell fate decisions in the intestine. (**d**) shows how *neuroD* only begins to show robust oscillations from day 4–5 of development onwards. (Taken from Laranjeiro and Whitmore, 2014) [26].

3. The Importance of Light

3.1. The Photopigments

Vertebrate photoreception is often thought of as a process exclusively involving the visual system. Although visual light detection using rods and/or cones is obviously important in most vertebrates, non-visual photoreception and the use of non-visual opsins is also important in many critical biological processes, such as seasonality/photoperiodism, circadian entrainment and DNA repair [28–32]. Historically, there was an assumption that there would be one key opsin for non-visual photoreception, underpinning, for example, clock entrainment. Thus, when melanopsin was discovered, many researchers believed that no more non-visual opsins would be discovered (at least not in mammals). However, this view was not to last for long, and as of 2018, due to a considerable improvement in the quality of genome sequencing, we now know there is an immense diversity of non-visual opsins [33]. The non-visual opsins are all seven-transmembrane-domain proteins, like the visual opsins and function using similar mechanisms to those of the classical extra ocular photoreceptors. As opsins belong to the G protein-coupled receptor (GPCR) superfamily of proteins, it follows that opsins may signal and thereby activate light-induced and clock genes, using the classic, well-established downstream pathways. In zebrafish, several reports have implicated the

MAPK pathway with light-dependent, transient induction of phosphorylated ERK and MEK [34–38]. Furthermore, pharmacological assays have also pointed to signalling through the phosphoinositide pathway, which interacts with nitric oxide (NO) and the MAPK pathway [39]. Though there are concerns about the reproducibility of these findings, and their validity in general, the nature of this signalling pathway is likely to be very complex, and may include numerous downstream signalling events.

Phylogenetic studies show that teleost genomes encode 20 different classes of opsins, while reptiles, birds and amphibians also show a high genomic diversity of opsin classes, with 19, 17 and 18 classes respectively [33]. In zebrafish, we find 42 different opsin genes (10 visual and 32 non-visual), currently the highest reported number of opsins in any animal. The expression pattern of these opsins is quite interesting, with usually multiple non-visual opsins expressed in every organ [33]. The internal organs, such as the heart and liver, possess the least, but the brain and retina express almost the "full set". Non-visual opsins and non-visual light detection is, of course, not exclusive to the lower vertebrates. All three classes of mammals, including eutherians, encode several classes of non-visual opsins [33].

The exact function of all of these non-visual opsins remains largely unexplored. In fish, there has been little work to date on the biological relevance of this great diversity of light-detecting molecules, but absorption spectra and tissue-specific expression data have been published [33]. The majority of these opsins have been shown to form functional opsins when expressed in in vitro situations, such as neuro 2A cell lines. Whether this is their primary role in vivo is of course another matter.

In many respects, it is hard to understand the requirement for such a large number of photopigments, as one would imagine that the role of just detecting light could be performed adequately by far fewer. However, this diversity would certainly ensure a wide range of spectral sensitivity and that "no photon goes undetected". Presumably there must be some biological value to this. In reality, most of our functional knowledge comes from mouse studies, yet there is still relatively little examination of the role of these opsins in tissues other than the retina. There are some emerging results from rather unexpected tissues, including human adipocytes, suggesting a wide range of light-regulated biology may even exist in humans [40]. Melanopsin (OPN4m), is the most explored non-visual opsin to date and has been implicated in circadian clock entrainment and pupillary constriction in mammals [41,42]. Whether it performs the same circadian role in fish is currently under investigation. Neuropsin (OPN5) has also gained some recent interest, and in 2015, Van Gelder and colleagues showed that neuropsin is critical for photoentrainment in mouse retina [43]. The ultraviolet (UV) light sensitivity of OPN5, and its curious expression pattern in all species where it has been examined, suggest that it may play an interesting role in certain fundamental aspects of cell biology [44].

3.2. Light Input Pathway

In zebrafish, the precise nature of the signalling pathways is not yet clear. What is clear however, and has been well-defined, is that light dramatically increases expression of the clock genes *period2* (*per2*) and *cryptochrome 1a* (*cry1a*) in all tissues and cell lines that have been examined [24,28]. PER2 proteins contain a C-terminal CRY binding domain enabling dimerization of PER2 and CRY1a. In turn, the CRY1a protein interacts directly with core clock components, CLOCK and BMAL, blocking their ability to dimerize and thereby repressing transcription by CLOCK:BMAL, providing a likely mechanism for clock resetting [28]. Furthermore, light-induced CRY1a acts to "lock up" or "jam" the clock mechanism, and prevent the molecular core clock from oscillating. It does so in a phase-dependent manner, as CRY1a can only interact with CLOCK and BMAL proteins of course when they are present, which is typically after Zeitgeber Time (ZT) 12. In this way, constant light acts to "stop" the clock at ZT12, and the clock oscillation will not continue again until the light stimulus is removed, and CRY1a protein most likely degrades. This represents a fascinating potential interaction between an hour-glass time measuring system and a circadian pacemaker.

How does light regulate the transcription of these two key genes? Promoter analysis of *per2* and *cry1a* has identified a 'Light Responsive Module' consisting of E- and D-box elements spaced close together and in proximity to the transcriptional start site [28,45,46]. This module is also strongly conserved in other *per2* vertebrate genes, including species lacking directly light-sensitive clocks [41,42]. The D-box confers light-driven expression through binding of the thyrotroph embryonic factor (TEF) zebrafish homologue, whilst the E-box directs circadian clock regulation by mediating CLOCK/BMAL activity [11,13]. In addition to TEF, the zebrafish possess an additional 11 D-box binding factors, with nine of them enhanced in the pineal gland, further supporting the involvement of this pathway in the circadian clock mechanism [15].

Although both *per2* and *cry1a* genes possess the light responsive module, their regulation is markedly different. Upon blocking protein synthesis with cycloheximide, the light response using the D-box enhancer is attenuated in *cry1a*, making light-induction of *cry1a* dependent on *de novo* protein synthesis. This is not the case for light-dependent expression of *per2*, which seems to utilise the E-box when protein synthesis is blocked [47,48]. Furthermore, AP-1 enhancer elements have also been implicated in *cry1a* light-driven expression [47]. These results taken together suggests that both these core clock genes use D-boxes to drive their expression, but that multiple other enhancer and control elements ensure that light-driven expression is controlled in a gene promoter-specific manner.

Light sensitivity is not constant over a 24-h period, due to clock-feedback on to the input pathway. Light-pulsing experiments show that light sensitivity in zebrafish is time-of-day dependent, with more than twice the induction of *cry1a* at CT20 compared to CT8 [28]. In turn, the light intensity also impacts the size of the phase shift. Results from phase and intensity response curves demonstrate a strong correlation between light induction of the *cry1a* gene and clock resetting.

Zebrafish has one *per2* gene, in contrast to many other teleost species, such as *Astyanax mexicanus*, where there has been a clear duplication of the *per2* gene. The distinct roles of *per2a* and *per2b* are unclear, but in *A. mexicanus*, the expression patterns in light-dark (LD) and constant dark (DD) differ, indicating that they may have distinct roles [1]. The situation for the multiple *cryptochromes* in zebrafish is equally unclear, where there are at least six distinct *cry* genes. However, it is clear that some of these Cry proteins can act as transcriptional repressors, as in the mammalian clock system [48]. It has also been suggested that Cry4 might act as a photopigment, akin to the situation in Drosophila, but at the minute there is no compelling data to support this hypothesis.

3.3. Light Sensitivity in Fish—Light Detection Is Everywhere

Zebrafish and other teleosts, such as the Mexican blind cavefish (*Astyanax mexicanus*) and Medaka (*Oryzias latipes*), have directly light-entrainable tissues [2,49]. For zebrafish, both organs and cells in culture light can entrain the cell endogenous clock, but does this broad light sensitivity impact on other important aspects of cell and tissue biology?

Light is essential for successful development in many teleost species, and the lack of light during development is associated with higher mortality rates and more developmental deformities [32]. In some fish species, like the flatfish *Solea senegalesis*, there is a remarkable 100% mortality in embryos by Day 4 of development when animals are raised in constant darkness [50]. It is quite a common phenomenon in teleosts that light exposure is essential for early survival. Light-regimes and the clock also have an effect on the hatching of larvae. Entrained zebrafish and *S. senegalesis* embryos restrict hatching to a particular time window in the day. Constant light conditions disrupt this timed regulation, with resulting ultradian bouts for zebrafish and 24-h delays or advances in DD and LL respectively for flatfish hatching [1]. Such results have significant implications for commercial fisheries, which often employ constant light conditions to influence early larval growth rates.

Everybody that works with zebrafish is aware that spawning is tightly timed, and that the fish lay eggs just after dawn. This may sound somewhat illogical, as the embryo will undergo DNA replication and rapid cell division at the peak of diurnal UV light exposure, consequently increasing the chance of DNA damage dramatically at this sensitive early stage of development. Perhaps,

therefore, it is not surprising that early exposure to light is actually beneficial and, in fact, essential for survival in early embryos. Embryos on the first day of development, raised in DD, show only a 20% survival rate when exposed to a 5 s UV-pulse compared to 85% survival for sibling embryos raised on LD cycles [32]. This increase in survival rate in embryos raised on LD is due to the light-induced expression of DNA-repair enzymes, such as, but not exclusively, *6-4 photolyase*, which is expressed and transcriptionally light-regulated from 6 hpf. During the first 6 h of development, however, zebrafish rely on maternally deposited *6-4 photolyase* transcript. The large quantities of maternally deposited *6-4 photolyase* is not only found in zebrafish, but also in cavefish embryos, and undoubtedly in many other species of fish [1,32].

Several light pulse experiments, followed by whole transcriptome analysis, have identified that around 20% of all light-induced genes in zebrafish are involved in DNA-repair [51,52]. There is also an enrichment of genes involved in circadian clock entrainment, stress responses, as well as heme metabolism, mitochondrial genes and retinoid binding genes. Furthermore, promoter analysis of these light-induced genes shows an enrichment of E- and D-box enhancers, suggesting these genes use similar signalling pathways as *cry1a* and *per2* [43].

Findings from light-pulsed zebrafish pineal gland transcriptomes were similar to those performed on other tissues, but in addition included transcript targets related to reactive oxygen species (ROS) [50]. Interestingly, the greatest L/D fold difference in light-induced gene expression identified by the pineal transcriptome, is a metabolic gene, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (pfkfb4). *pfkfb4* is a target of Hypoxia-inducible factor 1-alpha (hif1a), which is also light induced [50,53]. An implication of this might be that light exposure can directly feed into cellular metabolic regulation.

Most of the results described above relate to directly light-driven transcriptional changes, a fact that extends to the fish brain itself. We know that the fish central nervous system contains many opsins, which are expressed in most, if not all brain regions. The whole cultured fish brain, or regions thereof, can directly respond to light at the transcriptional level. But does this direct brain light detection translate into actual behavioural responses to light? Amazingly, the answer is yes. Eyeless, pineal-less zebrafish larva do indeed change their swimming behaviour in response to light exposure, and the presence of opsins expressed specifically within regions of their brain is responsible for this. Visually blind fish still swim towards light stimuli and perform simple light-seeking behaviour, triggered by loss of illumination [54].

3.4. Alternative Fish Models—Cavefish

An alternative to exploring the clock and its downstream biology by "knocking out" key circadian or light-regulated genes, or performing forward genetic mutant screens, is to study "naturally occurring mutants"—especially animals that have adapted to a life in the dark. Two cavefish species, the Somalian blind cavefish (*Phreatichthys andruzzii*) and the Mexican blind cavefish (*Astyanax mexicanus*) have been exploited in this way to study light detection and clock function.

The Somalian cavefish are thought to have been isolated in completely dark underground caves around 2 million years ago [55]. They have typical troglomorphic phenotypes, such as loss of eyes and pigment. The Somalian cavefish have also lost their ability to entrain to artificially provided light-dark cycles, which is possibly due in part to an aberrantly spliced variant of the *per2* transcript which lacks a C-terminal cryptochrome binding domain. This splice variant of PER2 protein is unable to dimerise with CRY and is consequently localised predominantly to the cytoplasm [56]. *P. andruzzii* can, however, entrain to feeding cues, showing a clear anticipatory increase in activity around feeding time. This non-photic zeitgeber also entrains the molecular clock, showing *per1* mRNA rhythms in several organs [4]. Presumably, unlike for light, *per2* is not required for food entrainment of the oscillator. The Somalian blind cavefish expresses two truncated non-visual opsins, melanopsin (*opn4m2*) and a teleost-multiple-tissue opsin (known as *TMT3a*) [4]. These two opsins have been proposed as key photopigments for entrainment. However, as the genome of this cavefish has yet to be sequenced, it is

likely that there will be many other candidate opsins to explore. It will be interesting to see how many of the numerous classes of opsins are mutated in this species.

The Mexican blind cavefish, *Astyanax mexicanus*, also have a troglomorphic phenotype. In contrast to *P. andruzzii*, which represents one species and one strain, *A. mexicanus* is the name for 29 unique Mexican cavefish populations, descending from the same ancestral river strain [57,58]. The colonisation of caves is proposed to have happened in two waves in geographically distinct regions, with the oldest isolation event estimated at 2 million years ago [57,59]. The descendants of the founding river species of *A. mexicanus* are still swimming in the local Mexican rivers, making this cavefish a unique and powerful evolutionary and adaptational model. Furthermore, the cave strains and the river strain of *A.mexicanus* have not fully speciated from each other and can still be crossed in the laboratory to produce multiple cave/cave or cave/river hybrid F1 generations.

When studied in the wild, the Mexican blind cavefish show no molecular or behavioural circadian rhythms. However, in contrast to the Somalian cavefish, several of the Mexican blind cavefish strains can entrain to LD cycles under lab conditions, although the phase and timing of *per1* gene expression is altered [2]. Furthermore, light sensitivity is altered in cavefish, with higher basal levels of *cry1a* and *per2a* compared to the surface strains in both adult and embryonic cavefish [1,2]. Interestingly, there are also marked differences between the cave strains, meaning that although they have adapted to similar niches, the specific changes in the remaining clock mechanism is likely to be different. It is possible, therefore, that each cave strain could effectively represent a unique circadian clock and light-responsive mutant, making these animals a very powerful tool for future clock studies.

4. The Outputs

4.1. Sleep and Rhythmic Behaviour

The topic of sleep regulation is well beyond the scope of this manuscript, and there is a recent, excellent zebrafish sleep review by Oikonomou and Prober [60]. What is already apparent about sleep regulation in zebrafish is that there is a great deal of conservation in the process between teleosts and mammals, with important roles played by the hypocretin system, galanin-containing neurons and pineal melatonin in regulating sleep and activity rhythms [20,61–63]. Sleep regulation is always viewed as a two-process model, where a circadian arousal drive is integrated with a homeostatic sleep pressure. With the clock regulating the timing or phase of sleep, and the homeostat regulating the required duration or amount. Of course, this has been classically viewed as there being distinct sleep and circadian centres within the brain and, therefore, much of the effort in sleep research requires the identification of the relevant "sleep circuits". What has not been taken into account in the context of zebrafish (and possibly also somewhat in mammals) is the fact that circadian pacemakers are now viewed as being highly dispersed within the central nervous system. It is certainly clear in zebrafish that the majority, although possibly not all, neurons have a clock and show widespread direct light-sensitivity [64]. In addition, non-visual photopigments are present throughout most regions of the brain [33]. What this may mean is that the "homeostatic" sleep centres are inherently rhythmic and light responsive themselves, removing the need for any separate "clock circuitry" linking these two brain processes. The sense of time is inherent to the majority of neurons in the brain, and so that aspect of sleep drive is distributed throughout the nervous system. How this may all play out in terms of sleep regulation is for future studies to determine.

4.2. The Cell Cycle

One advantage of studying fundamental aspects of cell biology, over perhaps behaviour or physiology, is that the molecular basis of much cell biology is highly conserved across a wide range of species. For example, the basic mechanisms of mitochondrial function, transcriptional regulation and the cell cycle/cell division are very similar whether one is studying human or zebrafish cell cultures. On the whole, the same genes and regulatory proteins appear to be involved, if with some interesting

modifications. Studying the cell cycle in an animal like zebrafish should be relatively straightforward due to our molecular understanding from other model systems. Equally, new findings in fish should also be relevant to mammals by the same argument.

It has been well-established for many years that the clock controls the timing of the cell cycle in a wide range of animals and plants, ranging from cyanobacteria to human tissues. In the case of zebrafish cell lines, DNA replication (S-phase) typically occurs in the late evening, and mitosis just before dawn [65,66]. Similar timing is found for the cell cycle in many healthy proliferative human tissues [67,68]. Loosely speaking, there are two conceptual ways in which this might occur. One is that the clock establishes a "gate" or "window" at a specific time of day when cells will pass through a certain stage of the cell cycle. Such a mechanism appears to occur in cyanobacteria and possibly other situations where the cell cycle length is quite short, i.e. is significantly less than 24 h [69]. Another possibility is that the circadian clock entrains the cell cycle by changing its speed, or angular velocity, such that the period of the two oscillators becomes equal. This mechanism appears to occur in at least some mammalian cell lines in culture, where there is a direct coupling of period length between clock and cell cycle [70,71]. This perhaps makes sense in the context of mammalian/human cell division where the cell cycle length is typically close to 24-h.

So, what does zebrafish as a model bring to this particular party? In this case, two aspects of the zebrafish cell system prove to be very useful tools for the study of clock-cell cycle coupling: the direct light entrainment ability of each cell and a very wide "range of entrainment" (Type 0 PRC), allowing for entrainment to a wide range of differing driving light-dark or T-cycles [28,72,73]. Consequently, it is possible to synchronize the cell cycle in zebrafish simply by changing the lighting conditions, a fact that removes the need for pharmacological manipulations to the system [22]. In addition, one can apply a wide range of T-cycles from less than 16 h (8 h light:8 h dark) to 32-h days (16 light:16 dark). The circadian pacemaker can be set to this wide range of periods, and so by imaging cell cycle progression in individual cells, using the FUCCI cell cycle reporter system, it is possible to directly test two hypotheses: (a) the length of the cell cycle changes to match the length of the driving cycle, or (b) the length of the cell cycle remains unaltered, but the timing of a circadian-cell cycle gate changes [73]. Results clearly show that the cell cycle length remains invariant with T-cycle, at a relatively long 45 h. Entrainment of the zebrafish cell cycle, therefore, occurs through a gating process, as has been proposed for cyanobacteria. However, the cell cycle length in zebrafish is much longer. The phase or window of S-phase timing can be clearly shown to alter its phase angle of entrainment to the differing T-cycles. The clock entrains the mechanism that controls the cell cycle gate, allowing cell cycle progression to occur at specific times of day. This gate is not deterministic, but leads to an increased probability that cells will undergo a given cell cycle event at that time, i.e. it is stochastic. In cell culture, cells may "choose" to miss the next window, and a certain percentage will ignore it altogether, but the clock provides a bias in the population, such that cells are more likely to replicate their DNA, and divide at certain times of day.

What is this gating mechanism? A global analysis of well-established cell cycle regulators identified a number of key genes that are under robust circadian clock control. This includes the cell cycle regulator *cyclin-dependent kinase inhibitor 1a* (*CDKN1A* or *p21*), which shows a high amplitude rhythm, peaking around dawn and having a low point of expression in the early evening. *p21* regulates the entry of cells from G1 into S-phase, with high levels of holding cells in G1. The low level of expression at the end of the day corresponds to the time that more cells enter the S-phase from G1. The clock-controlled fall in *p21* levels is the circadian gate or window, which biases the population to proceed with DNA-replication during the dark phase of the day. The promoter, or upstream regulatory region of the *p21* gene contains a number of E-box elements, deletion of which abolishes the circadian rhythm in *p21* expression. Therefore, it does appear that the clock directly controls *p21* as an output gene to set this cell cycle window. Interestingly, correlative data supporting this hypothesis comes from the T-cycle experiments, where it is clear that the phase of the *p21* rhythm changes relative to the entraining light-dark cycle. Importantly, the actual timing of the S-phase also changes under T-cycle

conditions, and always matches the low point of *p21* expression—the circadian-cell cycle window for DNA replication [73].

An issue with the evolutionary "design" of the zebrafish clock system is that it is hard to see how one can simply generate internal phase differences between cells and organs, when cells are "all" directly light responsive, and presumably possess the same circadian pacemaker. It is possible that the plethora of photopigments could play some role in this. But how do zebrafish place different processes into different relative times of the daily light-dark cycle? Work on S-phase regulation demonstrates the mechanism that fish probably use, at least in part, to achieve this. A bioinformatic analysis of the zebrafish genome revealed a new, previously unexplored gene that had a high degree of similarity to *p21*, which we subsequently named *p20* [65]. *p20* is also under strong clock control, but the timing of peak expression is shifted by about 6 h relative to *p21*. A promoter analysis reveals that, as for *p21*, *p20* contains critical E-box elements that drive its rhythmic expression. However, inserted amongst these regulatory elements is also an RRE or Rev-erb responsive element. Deletion of this regulatory sequence in the *p20* promoter removes nearly 4 h of the phase difference between the two cell cycle regulators. Interestingly, the spatial expression pattern of these two genes also differs in the zebrafish larvae and adult tissue. *p20* is primarily expressed in the brain, whereas *p21* is primarily expressed in other tissues. An analysis of S-phase timing between brain and intestine reveals a 6-h difference in peak S-phase timing and of course this difference correlates perfectly with the expression patterns of *p20* and *p21*, respectively. Zebrafish generate a difference in the timing of output processes, in this case the S-phase, but employing a) a different but highly related gene and b) by "adding" additional regulatory components to the promoter region of that gene so as to modify phase of expression.

4.3. The Clock and Cancer

This strong link between the clock and cell cycle regulation raises the obvious question of what role the clock may play in cancer progression. This issue has been explored extensively in mammalian systems, where clock disruption (either through SCN ablation or "jet-lag" protocols) clearly accelerates tumour growth rates [74,75]. There is also an abundance of epidemiological data showing a link between shift work and cancer incidence in humans [76]. Zebrafish have also added to this area of investigation [77]. Using luminescent report animals, containing the *per3-luciferase* gene, it is possible to monitor clock function in a developing melanoma, versus that in neighbouring healthy tissue. Figure 3 summarises some of the data collected using this approach. In a zebrafish melanoma model, it is absolutely clear that the amplitude or robustness of the *per3* rhythm is dramatically reduced—the circadian pacemaker is simply not working as well in the tumour. A consequence of this is that the daily rhythm in mitosis within the melanoma is lost, such that cells now divide randomly across the day-night cycle. This corresponds to a loss in the circadian regulation of the underlying *cyclin B1* transcriptional oscillation, which underpins this mitotic rhythm. Interestingly, in the context of zebrafish melanoma, the first detectable changes in clock regulation all relate to the direct light responsiveness of the tumour. The acute light induction of all light-responsive genes appears to be lost or at least greatly reduced. Genes, like *cry1a* and *per2*, that are key to fish clock entrainment are barely light activated, which may well explain why the amplitude of oscillations in the melanoma are so low. Interestingly, the light activation of DNA repair genes is also inhibited within the melanoma. This fact may also be a key aspect as to why circadian disruption leads to accelerated tumour growth. Not only do cells in the tumour lose their daily, time restricted rhythms in mitosis, dividing at all times of the day, but they also lose the ability to repair additional (possibly light) induced mutations. Cells undergo active mitosis during the day, and then can not repair the additional DNA damage that might occur due to UV exposure, accelerating tumour progression even further. It will be very interesting to see how this scenario plays out in human melanoma development, when such studies are performed.

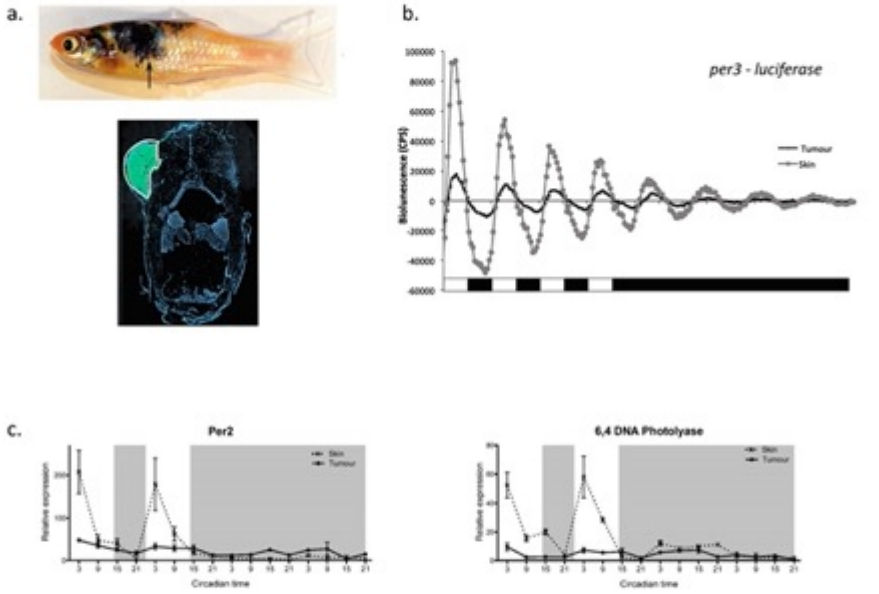

Figure 3. This figure shows specific circadian data collected from a zebrafish melanoma model. (**a**) Transgenic fish were generated, which develop a melanoma in which cells are both GFP-tagged and contain a *per3-luciferase* reporter gene. The lower panel shows a cross-section through a fish revealing the extend of the GFP-positive tumour. (**b**) Luminenescent gene expression recordings from both a developing melanoma and neighbouring skin reveal that circadian gene expression rhythms are significantly lower amplitude in the tumour and damp faster than in healthy skin. (**c**) Two strongly light responsive genes, *Per2* and *6-4 photolyase*, so no transcriptional light induction or rhythmicity in the developing tumour compared to adjacent, healthy skin. (Taken from Hamilton, Diaz-de-Cerio and Whitmore, 2015) [77].

5. Conclusions

In this short review we hope we have discussed the more novel contributions that zebrafish and certain other teleosts have made to our understanding of circadian biology, particularly focusing on unique aspects of clock entrainment as well as downstream, output regulation. This is, of course, only a small part of the teleost circadian field and has missed out excellent studies from a wide range of other species of fish and research groups. The strength of teleosts lies very much in the wide variety of species, over 30,000, of fish that exist. They have solved the evolutionary problem of how to live on a rhythmic planet in a mass of varied ways, and as such offer a remarkable resource for studying environmental adaptation at a molecular/genetic level. Moreover, many fish species are of considerable commercial significance, and the importance of fisheries/fish farming and fish stocks is of major international interest. Laboratory mice have not quite yet found their way to our supermarket shelves, and so studies of clock biology in "large" fish is not only of interest, but of significant economic value. There is some excellent work in this area that this review does not have space to discuss, but clearly this is a topic of major future importance. It is also worthy of note that most fish species are diurnal, although in fact many can switch between nocturnal and diurnal as environmental situations and seasons change. As a diurnal vertebrate model, as opposed to a nocturnal rodent, there may be much to learn from zebrafish clocks and their entrainment that relates to human clock function. The core clock mechanism of fish is still relatively unexplored, but there may yet be interesting surprises to uncover in that area as "time" progresses.

Funding: We would like to thank the Leverhulme Trust and BBSRC for funding (BB/I003592/1) much of the research presented in this review.

Acknowledgments: We would like to thank all previous members of the Whitmore lab for their contributions to this research over the years. This includes Kathy Tamai and Ricardo Laranjeiro for their work on cell cycle and many other areas, as well as Andrew Beale for the cavefish studies.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of these studies; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Developmental Biology 441 (2018) 345–354

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Original research article

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

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ARTICLE INFO

Keywords:
Astyanax mexicanus
Circadian clock
Development
DNA repair
Non-visual light detection

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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https://doi.org/10.1016/j.ydbio.2018.06.008
Received 15 February 2018; Received in revised form 6 June 2018; Accepted 13 June 2018
Available online 23 June 2018

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

appears to differ for classically light-regulated genes, such that the clock impacts these rhythms more strongly in cave strains than surface fish. As a result, one can detect more robust daily rhythms in the *period2* genes and *CPD photolyase* in cave populations than surface embryos on a dark-light cycle. This may initially seem rather unexpected, but may reflect an evolutionary switch from light to clock gene regulation of critical genes in a constant dark environment.

2. Materials and methods

2.1. Biological materials and embryo maintenance

Adult surface, Pachón and Chica cavefish maintained at 22–25 °C were exposed to a 14:10-h photoperiod (light intensity 300–500 μW/cm^2). The fish were mated every 3 weeks by *in vitro* fertilization (IVF) and 12–20 embryos were collected in E3 fish water (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, 0.00001% Methylene Blue) in 25 ml flasks. The embryos were kept at 25 °C in a thermostatically controlled water bath and placed on a 12:12 DL cycle or in constant darkness within 1–2 h of fertilization. 12–20 embryos were harvested at 6-h intervals in TRIzol Reagent (Invitrogen), homogenized and stored at − 20 °C. Total RNA was extracted following the manufacturer's instructions. For acute light pulse experiments, embryos were kept in constant darkness and given a 3-h light pulse at 5hpf, 14 hpf and 23 hpf or kept in the dark as a control. Embryos were then collected, homogenized and total RNA extracted as above. All animals were maintained in a Home Office approved facility and handled in accordance with the Animal Welfare Act of 2006.

2.2. Real-Time Quantitative PCR (RT-qPCR)

cDNA was synthesised from 2 μg of RNA using Superscript II Reverse Transcriptase (Invitrogen) with random hexamers and oligo dT primers. RT-qPCR was performed on a C1000 Touch™ Thermal Cycler with the CFX96™ Optical Reaction Module (Bio-Rad) using KAPA SYBR FAST qPCR mix (Kapa Biosystems) in technical triplicates with gene specific-primers (see Supplementary information) at a concentration of 500 nM. ΔCt was determined using *rpl13a* or *EF1a* as a reference gene and relative expression levels between surface and cavefish were compared directly using the ΔΔCt method

2.3. Whole mount in situ hybridisation

At the assigned timepoints, embryos were fixed in 4% PFA/PBS overnight at 4 °C. Early embryos were dechoreonated. Embryos were washed 4 times with PBS before storage in 100% MeOH at − 20 °C. All further steps were conducted at room temperature unless otherwise stated. Embryos were rehydrated in a series of washes with 75% MeOH in PBT (PBS + 0.01% Tween-20), 50% MeOH in PBT, and 25% MeOH in PBT and twice in PBT. Embryos were then treated with 10 μg/ml proteinase K for 5 min, washed with PBT twice, before fixation 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 h at 65 °C. Digoxigenin-labelled (DIG)-labelled probes (antisense and sense) were synthesised from 1 μg of linearised plasmid DNA containing a 559 bp fragment of *per2b* using T7 or SP6 polymerase (Promega) and digoxigenin-labelled dUTP (Roche). DIG-labelled probes were prepared by denaturing in HYB+ (5xSSC, 0.1% Tween-20, 5 mg/ml torula (yeast) RNA, 50 μg/ml heparin) at 80 °C for 2 min before being diluted to 1 μg/ml in HYB+ . Embryos were incubated with DIG-labelled RNA sense or antisense probe in HYB+ overnight at 65 °C with gentle shaking.

After hybridisation, the embryos were successively washed at 65 °C with HYB+ , 50% HYB+ /2 × SSC, 2 × SSC, and twice in 0.2 × SSC before being cooled to room temperature, and subject to a further three washes with PBS. After washing, embryos were incubated with 2% Blocking Agent (Roche) in maleic acid buffer (MAB) for at least 3 h. The block was replaced with anti-DIG-alkaline phosphatase (1:5000) in 2% Blocking Agent in MAB, and the embryos were incubated overnight at 4 °C.

The embryos were subsequently 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. Finally, embryos were washed twice with PBT and refixed with 4% PFA/PBS overnight at 4 °C.

The *In situ* hybridisation signal was quantified by densitometry in ImageJ (1.50i, Schneider et al. (2012). Images were converted to 8-bit grayscale and a region of interest, encompassing the embryo, was drawn using the Specify tool. Optical density was calculated using the "Analyze tool" from the peak of the profile plot of each sample after enclosing the peak and eliminating background noise.

2.4. Statistical analysis

The data in this study are presented as the mean ± s.e.m. ($n > 3$) and were analysed using a Student's *t*-test or analysis of variance (http://www.physics.csbsju.edu/stats). $P < 0.05$ was considered significant.

3. Results

3.1. Light responsiveness in early embryos

The non-visual light response develops very early in small teleosts, well before the differentiation of the retina or pineal gland, and light exposure within the first few days of development has been shown to be crucial for embryonic development, survival and fitness in many species (Gavriouchkina et al., 2010; Martín-Robles et al., 2012; Tamai et al., 2004; Weger et al., 2011). What adaptations and changes in the light input pathway are found in cavefish embryos to compensate for developing in a dark environment?

Light regulates and sets the circadian clock through the transcriptional activation of light sensitive genes, which themselves are typically transcriptional repressors (Carr and Whitmore, 2005; Hirayama et al., 2005; Pando et al., 2001; Tamai et al., 2007; Vallone et al., 2004; Ziv et al., 2005). Work in zebrafish has shown that the *per2* and *cry1a* are involved in the entrainment of the clock to light in the embryo and adult fish, which makes these three light-inducible genes excellent markers for the onset of light-sensitivity (Dekens and Whitmore, 2008; Tamai et al., 2007; Ziv and Gothilf, 2006). The coding regions of the core clock and light-inducible genes are highly conserved between the different populations of *Astyanax mexicanus*, which allows the use of the same gene-specific qPCR primers for all populations of fish.

To determine when cavefish become light responsive, Surface and Pachón embryos were raised in complete darkness and given a 3 h light-pulse at 3 different times during the first day of development (5 hpf, 14 hpf and 23 hpf). Light sensitivity is established very early in surface fish. Both *per* genes, as well as *cry1a* are induced by light as early as 5–8 hpf (Fig. 1a-c). At 14–17 hpf and 23–26 hpf, we see further increases in light-inducible transcription of *per2a* and *per2b* mRNA transcript. In comparison, the Pachón embryo is much slower in developing a light response. There are no significant increases in any of the light sensitive genes in 5–8 h old Pachón embryos (Fig. 1a,b,c). At 14–17 hpf, we see a robust light response in both the *per2* genes in Pachón embryos, but yet we do not see a *cry1a* response to light until the very end of Day 1 (Fig. 1. i). Interestingly, throughout development, the basal levels (expression in DD) of the *per2* genes are raised in Pachón cavefish compared to surface fish (Fig. 1), except *per2b* 14–17 hpf (Fig. 1. e) where there is a non-significant expression difference between DD samples in Pachón and surface embryos. Presumably, it is these raised levels in the dark that prevent any additional measureable light-induction. We also observe this increased basal transcription of

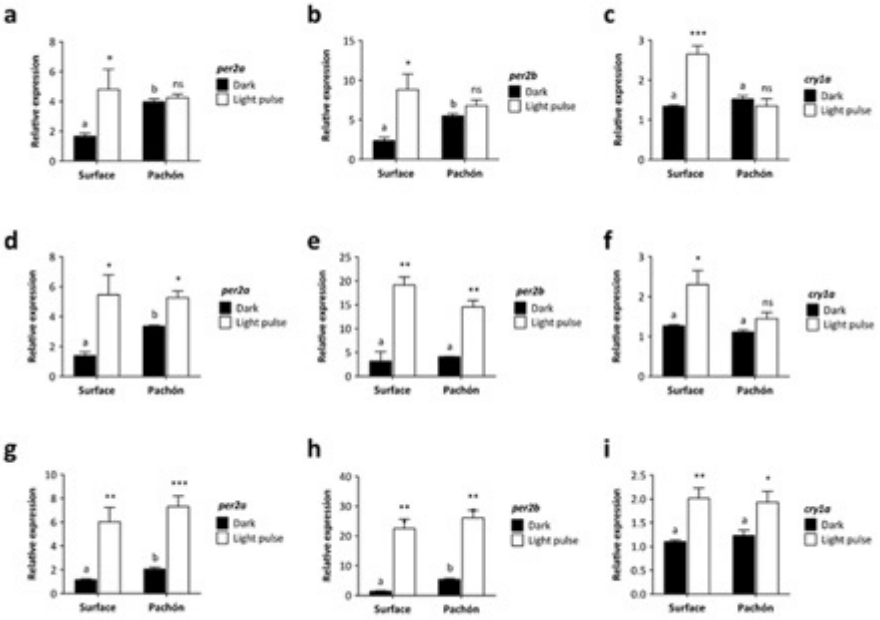

Fig. 1. Acute light induction of clock genes is slower to develop in Pachón cavefish than surface fish. Surface and Pachón embryos were maintained in constant darkness until a 3-h light pulse was given at different developmental stages. Expression of *per2a*, *per2b* and *cry1a* was determined by qPCR in light-pulsed and dark control samples and normalised to the reference gene *rpl13a*. 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), Data represent the mean ± SEM for between 3 and 5 embryo samples.

the *per2b* genes in adult Pachón fish (Beale et al., 2013). However, there is an even stronger *per2b* fold basal induction in Pachón embryos compared to adults. The basal levels of *cry1a* however, are the similar for cave and surface strains (Fig. 1f,i).

Although it is clear that a light response exists in early Pachón embryos (from both the differences between *per1* expression rhythms in LD and DD (Fig. 4) and significant acute responses to light at 14–17 hpf) (Fig. 1. d,e), a light response similar to that seen in Surface fish is only present in Pachón embryos at 23–26 hpf (Fig. 1g-i). 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 significant changes detected in the pineal gland (Vatine et al., 2009; Ziv et al., 2005). It could, therefore, be argued that much of this embryonic light response is pineal dependent. 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-enhanced expression. *In situ* hybridisation confirmed the increased expression of *per2b* after light exposure at 5 hpf and 23 hpf in surface embryos (Fig. 2a and b, and f and g). This expression difference is only present in Pachón embryos when the light pulse is given at 23 hpf (Fig. 2h and i), similar to the results obtained by qPCR. *Per2b* is clearly expressed at raised levels in Pachón embryos compared to surface fish in the dark controls, as seen by qPCR, and which is apparent in these samples at both time points (Fig. 2c and h). At 26 hpf, the expression in both surface and Pachón embryos is ubiquitous throughout the embryo, though clearly somewhat stronger in the head region of the larvae. The ubiquitous expression of *per2b* at 26 hpf observed by *in situ* hybridisation, and the clear light response present before 17 hpf, show that it is not the pineal gland alone that mediates the development of the light-induction of clock genes in Pachón. The mechanism of light detection is present throughout the embryo and is not restricted to central photoreceptive structures, in both surface and cave strains of *Astyanax*.

3.2. Cave-cave hybrid fish do not show a rescued light-response in epiboly-stage embryos

Cave populations of *Astyanax mexicanus* 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 still be crossed to examine cave phenotypes by complementation tests. It is clear that development of the light response in Pachón embryos is delayed compared to surface fish. Can this delay in light responsiveness be rescued in a F1 generation created by mating two cave strains?

In order to test whether early light sensitivity can be rescued by another cave strain, we examined the induction by light of multiple clock genes in Pachón, Chica and Pachón-Chica hybrid embryos by raising the embryos in the dark and giving a 3-h light-pulse at 5 hpf. *Per2a* show a very small (1.23 fold), but significant induction in Chica embryos, yet there is no induction of *per2b* or *cry1a* (Fig. 3). Furthermore, Chica embryos also show high expression of both *per* genes in DD, similar to that described for Pachón (Fig. 3a, b). Interestingly, we do not see any rescue of light responses in the "cave-cave" hybrid. The small *per2a* increase seen in Chica is no longer present, yet we still see an increased amount of *per2* transcript in the "cave-cave" hybrid animals (Fig. 3a, b).

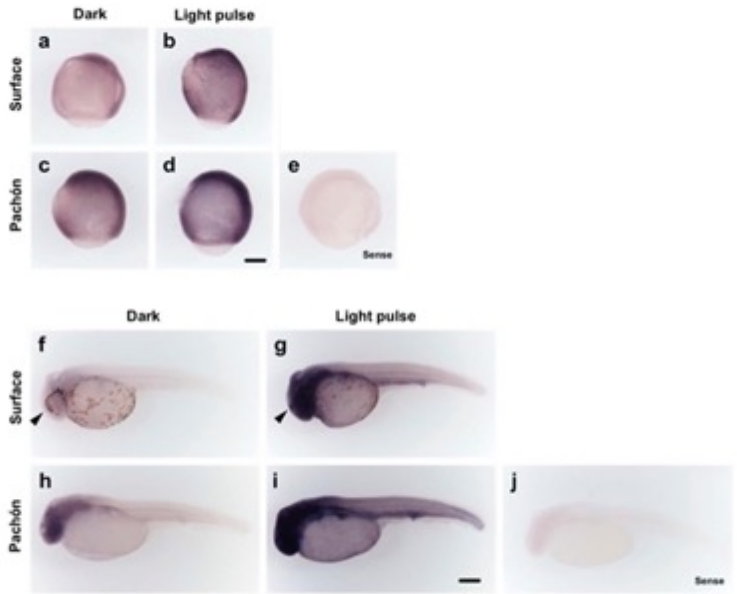

Fig. 2. Acute light induction develops within the first day of development in Pachón cavefish. Surface and Pachón embryos were kept in constant darkness until a 3-h light pulse was given beginning at (a-d) 5 hpf and (f-i) 23 hpf. Expression of *per2b* mRNA was analysed by *in situ* hybridisation in light-pulsed 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. Black arrow indicates the position of the pineal gland. Scale bar, 0.4 mm.

3.3. Clock entrainment

The circadian clock of zebrafish begins on the first day of development (Dekens and Whitmore, 2008; Ziv and Gothilf, 2006). The core circadian clock mechanism is generated by a transcriptional-translational negative feedback-loop, which is highly conserved in all vertebrates (Harmer et al., 2001; Takahashi, 2004; Wilsbacher and Takahashi, 1998). *Per1* is one of the key genes of the core clock, and shows high-amplitude circadian oscillations in entrained adult *Astyanax mexicanus*, making *per1* an excellent marker of clock function (Tamai et al., 2007; Park et al., 2007; Velarde et al., 2009; Martín-Robles et al., 2012; Beale et al., 2013).

To determine when the *Astyanax* circadian clock starts, Surface and Pachón embryos were entrained to a 12–12 h dark-light (DL) cycle and embryos were harvested at 6-h intervals for the first 3–4 days of development. It is worth noting that we employed a reverse light-dark cycle for these experiments, compared to most studies in the literature, to match with the natural spawning times of the cave populations, which occur primarily during the night. Samples were analysed by RT-qPCR to determine the levels of *per1* mRNA. During the first two days of development, surface fish *per1* show a low amplitude rhythm that peaks 3 h after the onset of light (ZT3) (Fig. 4a). However, from the third day of development, we start to observe a higher amplitude rhythm, were the peak has now shifted to ZT21. There is no apparent *per1* rhythm in surface embryos raised in constant dark (Fig. 4b). In comparison, we see an almost flat expression of *per1* for the first two days in Pachón embryos, and no robust *per1* rhythm until is observed until 51 hpf, with a peak at ZT3 (Fig. 4a). There is also no apparent *per1* rhythm in Pachón embryos maintained in DD (Fig. 4b).

There are clear and consistent differences between the rhythms seen in surface and Pachón embryo populations, which we also see in adult cavefish. *Per1* peak expression occurs 6 h later in Pachón compared to surface embryos, which means the timing and entrained phase of the *per1* rhythm is the same in adult and embryo for both strains.

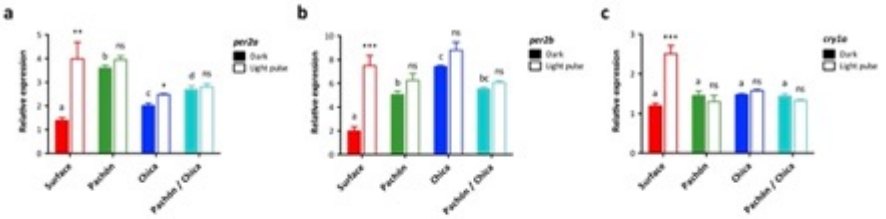

Fig. 3. Acute light-induction at 5 hpf is not rescued in a cave-cave hybrid. Surface, Pachón, Chica and Pachón/Chica hybrid embryos were kept in constant darkness until a 3-h light pulse was given at 5 hpf. Expression of (a) *per2a*, (b) *per2b* and (c) *cry1a* was determined by qPCR in light-pulsed and dark control samples and normalised to the reference gene *ef1a*. 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 3–5 embryo samples.

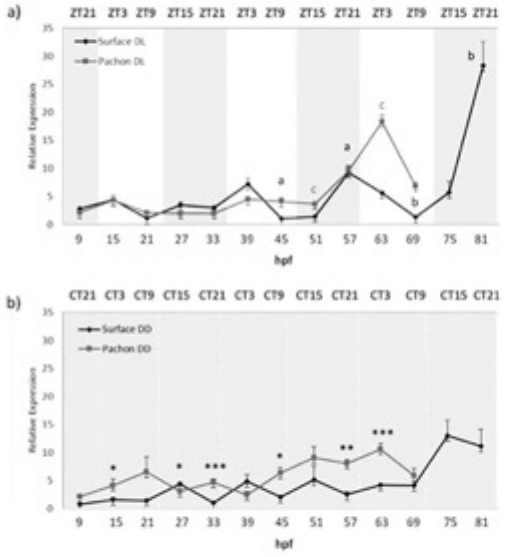

Fig. 4. *Per1* is rhythmically expressed during development. Embryos were sacrificed every 6 h for 3–4 days from 9 h post fertilization (hpf). *Per1* mRNA levels were measured by qPCR, normalised to the reference gene *RLP 13a* and relative expression was calculated using the ΔΔCt method. a) Surface and Pachón were entrained to a 12:12 DL cycle. Grey and white shaded areas indicate dark and light periods respectively. Zeitgeber time (ZT) on the upper x-axis gives hours after the light has come on. $P < 0.05$ significance between peak and trough is indicated by lower case letters in black for Surface and dark grey for Pachón. b) Surface and Pachón raised in the dark. Upper x-axis denotes circadian time (CT) for the dark samples. Levels of *per1* mRNA under constant dark were compared at each timepoint using a Student's *t*-test (unpaired, two tailed; *$P < 0.05$; < 0.01; ***$P < 0.001$). Data represent the mean ± SEM for 3 biological replicates.

3.4. Alterations in rhythmic expression of per2a and per2b transcriptional repressors in developing cave strains

We have previously hypothesised that the differences in rhythm amplitude and the phase angle of *per1* expression between cave and surface strains is likely to be due to changes within the core clock mechanism that generates the oscillation, as well as alterations in the light input pathway. So, what changes do we see in the expression of the *per2* light-induced transcriptional repressors in the developing embryo?

Both cave and surface strains show highly rhythmic and light induced expression of *per2a* from the first day of development (Fig. 5a). *Per2a* transcripts peak at ZT3 with an average 21.9-fold difference in expression between peak and trough expression in surface fish. In comparison, Pachón *Per2a* peaks 6 h later, at ZT9 and only shows an average 3.5-fold change between peak and trough values (Fig. 5a). This dramatic difference in day-night levels is once again due to raised basal levels of *per2a* transcripts in the cave population. It is also interesting to note that in DD, the amount of *per2a* transcript is on average 2.9 times as abundant in Pachón as in surface embryos (Fig. 5b). We also note that there are similar high amounts of maternally deposited RNA for *per2a* in both populations of cavefish.

Per2b shows similar rhythmic expression to *per2a* in surface fish, however, we do not see a marked increase in *per2b* transcript during the first light phase in these animals (Fig. 5c). In contrast, Pachón show a higher amplitude of *per2b* expression at ZT3 (15 hpf and 39 hpf), but at 69 hpf, *per2b* expression shifts 6 h and peaks at ZT9. In complete darkness, the expression of *per2b* is flat, but not raised in the cave strain (Fig. 5d). Furthermore, it is also interesting to note that there are barely any maternal deposits of *per2b* mRNA transcript in both strains, compared to *per2a*.

3.5. DNA repair gene expression is altered in cave strains

DNA repair, using enzymes such as CPD photolyase (*CPD phr*), is one of several important processes that are light induced in small teleosts (Tamai et al., 2004; Gavriouchkina et al., 2010; Weger et al., 2011). Are the changes in light sensitivity that we describe for the clock also impacting the regulation of DNA repair gene transcription in the developing embryo?

Entrained surface embryos show high amplitude rhythms of *CPD phr* expression with peaks at ZT3 from day 2, with very little transcript present at ZT9, ZT15 and ZT21 (Fig. 6a). In Pachón, the *CPD phr* peaks 6 h later at ZT9. Interestingly, there is also a high amount of *CPD* transcript expressed at ZT3, which means there is much more CPD transcript present at any time in the Pachón embryo, compared to the surface embryo (Fig. 6a). It is also clear that in the dark, there is a higher basal expression of *CPD phr* in Pachón embryos, compared to surface (Fig. 6b). In order to explore the maternal deposition levels of *CPD* transcript, and other clock genes, we examined the amounts of mRNA present in unfertilized oocytes for Pachón and surface strains (Supplementary Fig. 1). There is a considerable amount of *CPD phr* deposited maternally in both strains. This remains high in both strains even after the maternal RNA for the *period* genes has dropped, at the time that would typically correspond to the transcriptional activation of the zygotic genome. Interestingly, in both DL and DD, there is a raised level of *CPD phr* in 15 h old Pachón embryos, while in surface embryos the amount is considerably lower (Fig. 6). This might suggest that either the maternal *CPD* is more stable in the cave strains and remains present for longer, or that the zygotic transcriptional activation of *CPD* is more sustained in the early cave populations. Interestingly, there is considerably higher amounts of maternal RNA for the *period* genes in Pachón than surface fish. The biological significance of this is far from clear and will require further examination.

4. Discussion

4.1. Advent of light induction

Astyanax mexicanus has established itself not only as a major model system with which to study evolution, but also to examine circadian clock function and light regulated biology (Beale et al., 2013; Bradic et al., 2012; Strecker et al., 2003). How the circadian clock develops or "begins to tick" is a fundamental question in circadian biology. Clock function and light dependent biology is absolutely crucial for healthy development in most teleost species, so what happens in an animal that develops in darkness and in fact never experiences light? Are there differences in the development of the clock mechanism in cave strains, which might shed light on how the circadian system has altered following evolution in a dark environment?

In this study, we have explored clock and light responsive biology during the early developmental stages of *Astyanax mexicanus*. We have shown that there are differences in the development of the light response of surface and cavefish, as well as differences between the amplitude and of phase of light-inducible genes under rhythmic conditions. Surface fish become light responsive during the first 5–8 h of development (Fig. 1a-c), similar to that reported for zebrafish, and before the development of any tissue or light responsive organs, such as eyes or the pineal gland. In contrast, the fold induction of light-inducible genes is reduced in Pachón embryos and is slower to develop. The *per2* genes do not show a light induction until 14–17 h (Fig. 1d, e). In the case of the *per2* genes, this lack of induction is most likely the consequence of the fact that basal expression levels are raised in early

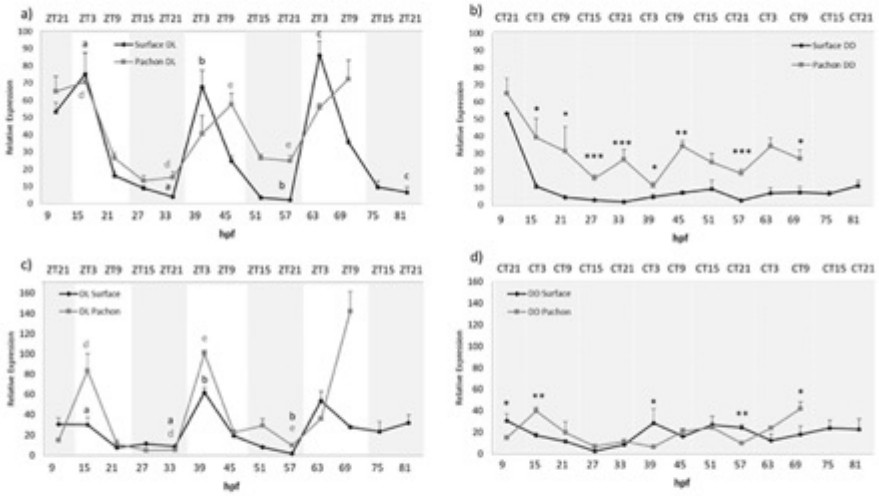

Fig. 5. Light inducible genes show altered expression patterns in Pachón embryos. Surface and Pachón embryos were sacrificed every 6 h for 3–4 days from 9 h post fertilization (hpf). *Per2a* and *per2b* mRNA levels were measured by qPCR, normalised to the reference gene *RLP 13a* and relative expression was calculated using the ΔΔCt method. a, b) *Per2a* expression in surface and Pachón embryos raised on a 12:12 DL cycle and DD, respectively. c, d) *Per2b* expression in surface and Pachón embryos raised on a 12:12 DL cycle and DD, respectively. $P < 0.05$ significance between peak and trough on a DL cycle is indicated by lower case letters in black for Surface and dark grey for Pachón. Expression of both *per2* genes was compared between surface and Pachón cavefish at each time point by using a Student's *t*-test (unpaired, two tailed; *$P < 0.05$; < 0.01; ***$P < 0.001$). Data represent the mean ± SEM for 3 biological replicates.

Pachón embryos, leaving little range for a further light-driven increase. One argument is that this indicates that there may not be a delay in the development of actual light sensitivity. However, the data collected for *cry1a* expression does not suffer from the same issues, with basal levels being very similar between surface and Pachón, yet there is a clear developmental delay with *cry1a* induction not showing any light response until 23–26 hpf (Fig. 1i). As such, it is interesting to note that light induction in Pachón develops at different stages for *per2* genes and *cry1a*, and that the increase of light induced transcript appears later for *cry1a* than *per2*. 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 (Fig. 1). These results strongly suggest that there is a different mechanism involved in *per2* and *cry1a*'s response to light in Pachón. This might not be unexpected as the transcriptional regulation of *per2* and *cry1a* in zebrafish has also previously been shown to differ (Mracek et al., 2012).

The underlying mechanism for this delayed development in light sensitivity is not yet clear, but could reflect alterations in any aspects of the signalling pathway, including the expression of the relevant opsins. What is clear is that *per2b* expression, when examined by *in situ* hybridisation, shows that there is a global response to light in both in surface and Pachón larvae (Fig. 2). 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. We also observe a clear light response in embryos present before 17 hpf, which shows that it is not the pineal gland nor a delay in its development that mediates the light-induction of clock genes in *Astyanax* (Fig. 2f-i). This is in line with and expected from observations in zebrafish, where the mechanism of light detection is present throughout the embryo and is not restricted to central photoreceptive structures. This does not imply that the light responsiveness of the pineal gland is irrelevant, just that light sensitivity is a global fish-tissue phenomenon.

4.2. Absence of 'rescue'

Cave-cave hybrids are able to 'rescue' a number of degenerate features of cave animals, such as Pachón/Tinaja and Tinaja/Molino, which produce embryos with larger eyes than either parent, and Molino/Curva hybrids which are extensively pigmented (Borowsky, 2008; Jeffery, 2009). On the contrary, crosses of Pachón/Molino and Pachón/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).

Using a similar experimental paradigm, we examined the genetic basis of the absence of light-response in early embryos. Hybrid F1 embryos of Pachón and Chica are not light-responsive between 5 hpf and 8 hpf, just like the Pachón and Chica F0 embryos themselves (Fig. 3). 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 have been geographically isolated from each other for several million years, and so this result means a similar alteration in the light input pathway has evolved convergently, in the same way as albinism (Bradic et al., 2012; Protas et al., 2006). In the future, it would be interesting to expand this number of crosses between many different cave populations, firstly to determine if the surface-like response is recovered, and secondly to see if the alterations of the light input pathway in the different cavefish strains are due to selection or drift.

4.3. Per2 gene rhythmic expression

Both the *per2* genes show different, but altered expression patterns during Pachón development, which may indicate that these two similar genes act in different ways in the clock mechanism. The *per2a*

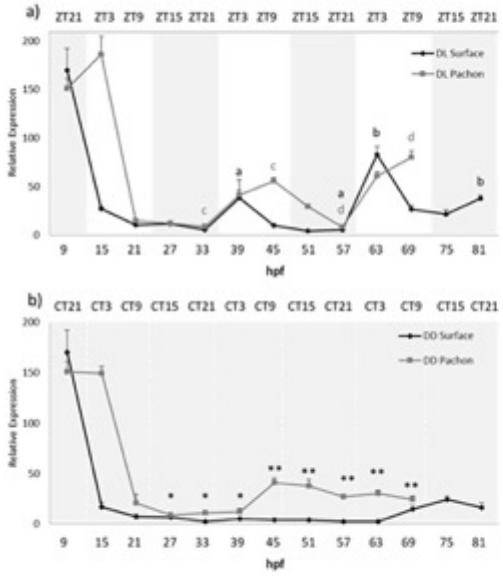

Fig. 6. Expression of the DNA repair enzyme CPD is differentially expressed in Pachón embryos raised both on a DL cycle and in DD. Surface and Pachón embryos were sacrificed every 6 h for 3–4 days from 9 h post fertilization (hpf). *CPD* mRNA levels were measured by qPCR, normalised to the reference gene RLP 13a and relative expression was calculated using the ΔΔCt method. a) *CPD phr* expression in embryos raised on a 12:12 DL cycle. $P < 0.05$ significance between peak and through is indicated by lower case letters in black for Surface and dark grey for Pachón. b) *CPD phr* expression in embryos raised in constant darkness. Expression levels of *CPD photolyase* were compared between surface and Pachón in DD at each time point by using a Student's *t*-test (unpaired, two tailed; $^{*}P < 0.05$; $^{**}P < 0.001$). Data represent the mean ± SEM for 3 biological replicates.

expression pattern is very different in Pachón compared to surface fish, both in the entrained state and in a constant dark environment (Fig. 5a, b). The basal levels of *per2a* expression are clearly raised in constant darkness in Pachón, as well as on a DL cycle. (Fig. 4a, b). On a light-dark cycle, surface fish *per2a* shows a strong acute light-driven rhythm, which is absent in the dark. This is in contrast to the entrained rhythm in Pachón, which shows a "smoother" expression pattern, rather than an acute and immediate response to light seen in surface fish, and with a clear 6-h delay in the timing of peak levels (Fig. 5a, b). Effectively, this means that there is more *per2a* transcript produced in the embryo on average during a day for Pachón. In the case of *per2b* expression, the daily rhythm in expression appears, if anything, to be more robust in Pachón than surface fish larvae, though this will require further statistical confirmation. A 6-h change in phase between the strains is not detected until day 3 of development, one day later than for *per2a* (Fig. 5c, d).

What is the possible explanation of this rather complicated looking set of expression data? There is clearly a difference between the expression patterns between *per2a* and *per2b*, which is especially apparent in constant darkness. *Per2a* shows significantly raised levels of transcript whilst *per2b* basal expression is the same as surface (Fig. 5). Taken together with the difference of expression patterns in DL, this clearly shows the two genes are regulated differently in Pachón. This might indicate that the two different isoforms of *per2* are likely to play different roles in the clock and light-input pathway, although the precise mechanism is not clear. It seems apparent that both *per2* genes in surface fish are primarily light-regulated. This is shown by the low expression in constant dark, and the nature/wave-form of the rhythm on a light-dark cycle. However, in adult Pachón, the light inducible genes *per2b* and *CPD* continues to oscillate in the dark after being exposed to a LD – cycles (experiments of *per2a* were not performed at the time) (Beale et al., 2013). Furthermore, the phase difference between surface and cave strain suggests that the *per2* genes are now much more under the control of the circadian clock itself, in addition to an acute light input. This hypothesis also fits with the development of the clear 6-h phase difference, seen on day 2 or 3 of development, when a robust clock is beginning to function in *Astyanax*. At first this would seem to make little sense, as exposing cave strains to a light-dark cycle is obviously an anomalous situation, and one they would not normally experience in nature. However, in cave populations clearly light-regulated genes would never normally be induced, due to the total darkness. So, by evolving additional regulation by clock-components, even in the dark, expression of these genes will be turned on, though presumably they will not show daily rhythms in the cave. These cave strains are using clock regulatory factors not to generate a rhythm, but to increase tonically the expression of genes that would normally be turned on by the light. The phase differences we report are, in fact, effectively an artefact of "seeing" a rhythmic light-dark cycle. The proof of this, of course, would be to find a change in the promoter/regulatory regions of the *per2* genes that gives them this circadian "gain of function".

4.4. Development of the clock

In zebrafish, the molecular clock becomes functional from the first day of development (Dekens and Whitmore, 2008). In cavefish, however, we do not observe a robust daily oscillation in *per1* gene expression in either of the strains until the third day of development. The surface strains show shallow *per1* oscillations from the first day of development that peaks at ZT3. However, during day three, we see a change in phase angle and amplitude similar to what we see in adults. This change in clock phase during development to an adult timing condition is quite an unexpected and unusual observation. Though we do not yet know the precise mechanism underlying this phase shift, it suggests that the clock mechanism itself undergoes a developmental maturation over the first three days of development, with perhaps not all of the components to form a robust clock being present until day three onwards. Though the zebrafish clock appears to develop earlier than the *Astyanax* pacemaker, there is also evidence that it too might not become fully functional until day 3–4 of development, as many clock output genes do not become rhythmic until this considerably later developmental stage (Laranjeiro and Whitmore, 2014). It is, however, clear that light has an impact on *per1* expression during the first day of development, as we can observe an increase in *per1* transcript in entrained embryos as opposed to those in constant dark. Pachón do not show any prominent *per1* peaks during the first two days of development, although light must have some impact, as *per1* expression in DD is different and much "noisier". It is not until day 3 that we see oscillations starting at 63 hpf, peaking at ZT3, the same phase as in adult Pachón.

In the wild, the expression of *per1* in adult cavefish is very low. This is likely due to the increased expression of CRY1a and PER2 proteins that act as strong repressors of CLOCK-BMAL transcriptional activity, which in turn dampen *per1* gene expression rhythms, reducing amplitude and slowing circadian oscillations. In contrast to adult cavefish, Pachón embryos show noisy expression of *per1*, looking as if the clock is running asynchronously. This correlates with *per2b* also showing "noisy/erratic" expression in DD. Previous work in zebrafish has shown that constant light 'stops' the circadian oscillator. *Per2* and *cry1a* are involved in the entrainment of the clock to light and the maintenance of high amplitude rhythms, while overexpression of both these genes mimics constant light conditions (Dekens and Whitmore, 2008; Tamai et al., 2007; Ziv and Gothilf, 2006). Therefore, the reduced amplitude and timing of the embryonic cavefish clock could,

like adults, be a consequence of changes within the light input pathway. Analysis of the coding sequence has shown a high conservation between the different cave strains (Beale et al., 2013). Therefore, again we hypothesize that the changes in *per2* transcript that we see in Pachón are likely to be due to changes in the promoter regions, enhancing the expression of per2a in the absence of light.

Our working hypothesis is that evolution has acted to convert the regulation of purely light responsive genes in surface fish over to effectively clock-regulated genes in cave strains. Even though these animals do not live in a rhythmic environment this adaptation enables the positive transcriptional regulation of clock elements to tonically increase the gene expression of genes that would otherwise be "switched off" in the transition from life in the river to life in the cave. This is certainly reflected in expression of the typically light-inducible *per* genes, but what about other teleost light-regulated processes, such as DNA repair. From our results, examining CPD photolyase expression, it is clear that the expression profile in Pachón closely resembles that of *per2a*. In the case of *CPD phr* there is also a large amount of maternally deposited transcript in both strains, which actually remains raised in Pachón for six hours longer. In surface fish the transcription of this gene is solely light-dependent, and so levels are very low under constant dark conditions. In Pachón the timing of the *CPD phr* rhythm is delayed by 6 h, and the expression levels on a light-dark cycle are consequently significantly greater. Again, this supports the idea of the circadian clock now playing a significant role in regulating the expression of this gene. The result of this, as discussed above, mean that under natural constant dark conditions the levels of *CPD phr* are raised. This fact is undoubtedly very important during embryo development in the darkness of the cave, as if this evolutionary change had not occurred then the ability of these animals to repair damaged DNA would be greatly impaired. These results fit with our previous data collected in adult *Astyanax mexicanus*, and show that this important biological adaptation occurs during the earliest stages of embryo development. A future full transcriptomic analysis of cave strains, both rhythmically and in response to light, will demonstrate the full extent of these evolutionary changes. As a full comparison of genomic sequences will allow us to demonstrate that there are clear alterations in the enhancer/regulatory regions of these central clock and light esponsive genes.

Acknowledgements

We would like to thank T. Katherine Tamai, as well as past members of the Whitmore lab for support with experiments and suggestions. Big thanks to Vilde Wiig Hanssen for helpful comments and reading through the manuscript. This work was supported by the BBSRC - BB/D522346/1 (D.W. and Y.Y.), the Wellcome Trust Developmental and Stem Cell Biology Programme (A.B.) and the MRC DTP (I.A.F.S).

Contributions

D.W, A.B and I.A.F.S designed the experiments. D.W, A.B, I.A.F.S and Y.Y performed the experiments. I.A.F.S and D.W wrote the paper with help from A.B.Competing interests

The authors declare no competing financial interests.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2018.06.008.

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