Clock genes and circadian rhythmicity in juvenile Atlantic salmon in response to light conditions

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DECLARATION OF ORIGINALITY

This thesis is the result of my own work and composed solely by myself except where specifically indicated in the text. It has not been previously submitted, in part or whole, to any university of institution for any degree, diploma, or other qualification.

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Contents

Abstract

The manipulation of biological timekeeping through the modification of light environment is widely used in aquaculture to stimulate desirable physiological responses in Atlantic salmon at key developmental stages. However, the understanding of the intricate interactions between light conditions, the circadian mechanism, and the control of downstream outputs in the species remains largely unknown.

In this research, the effects of light condition on the expression of clock genes in Atlantic salmon were investigated at key freshwater life stages, early development and parr. This was achieved by identifying the effect of whole genome duplication on clock genes. Family members were identified and classified, detailing the enhanced number of clock genes present in Atlantic salmon in relation to the latest common ancestors and other salmonids. Comparison of gene expression profiles across multiple light conditions identified evidence of altered clock gene expression under different photo cue, light intensity, and spectral composition. Photo cue was the most influential light condition.

Exploring the effect of developmental stage, the milestone of first feeding was key in the entrainment of the circadian clock. Displaying a significant increase in the number of clock genes which were rhythmically and cyclically expressed after the event, in keeping with the number of entrained genes identified in parr. Overall, this research provides fundamental insight into the significance of key early developmental stages and the effects of light conditions on clock gene expression in Atlantic salmon.

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Confirmation of ethical approval

All experiments were ethically reviewed and approved before work was undertaken. Experiments undertaken in the University of Bergen (UiB) followed local animal care guidelines and were subject to ethical review and approval was granted by the Norwegian Veterinary Authorities. All work conducted in these studies, both at UiB and at the Institute of Aquaculture (IoA) at the University of Stirling (UoS) was non-ASPA regulated research and was subjected to internal ethical reviews at the UoS and was carried out by the Animal Welfare and Ethical Review Body (AWERB), approval was confirmed before the studies were conducted (AWERB 19 20 097, AWERB 19 20 107).

Abbreviations and acronyms

ALAN	Artificial light at night
ANOVA	Analysis of variance
ARRIVE	Animal research: reporting of *in vivo* experiments
AWERB	Animal welfare and ethical review board
BLAST	Basic local alignment search tool
bp	Base pairs
C.N.	Copy number
cDNA	Complimentary deoxyribonucleic acid
CDS	Coding DNA sequence
CP	Crossing point, second derivative maximum value qPCR
DD	Continuous darkness
dd	Day degrees
DNA	Deoxyribonucleic acid
dpf	Days post fertilisation
e.g. (italicised)	Exempli gratia (for example)
EBI	European bioinformatics institute
ENA	European nucleotide archive
FCR	Feed conversion rate
FDR	False discovery rate
FF	First feeding
FPKM	Fragments per kilobase million
g (italicised)	Relative centrifugal force, 9.80665 m/s^2
HKG	Housekeeping gene
hpf	Hours post fertilisation
i.e. (italicised)	id est (that is)
In silico (italicised)	conducted or produced by computer modelling or simulation
in situ (italicised)	in the original place
in vivo (italicised)	within the living
K	Fulton's condition factor
kb	Kilobase
LB	Luria Bertani
LD	Light, dark
LDB	LD blue light

LDG	LD green light
LDH	LD high intensity
LDL	LD low intensity
LDM	LD medium intensity
LDR	LD red light
LDW	LD white light
LED	Light emitting diode
LL	Continuous light
ML	Maximum likelihood
mRNA	messenger RNA
MS222	Tricaine methanesulfonate
NBFRU	Niall Bromage freshwater research unit
nt	Nucleotide
p (italicised)	*p*-value
PCR	Polymerase Chain Reaction
Pers. Comm. (italicised)	Abbreviation. Personal Communication
pH	Potential of hydrogen, measure of alkalinity and acidity
PNES	Photoneuroendocrine system
qPCR	Quantitative Polymerase Chain Reaction
QTL	Quality trait loci
RAS	Recirculating aquaculture system
RIN	RNA integrity number
RNA	Ribonucleic acid
RT	Room temperature
S.E.	Standard error
SCN	Suprachiasmatic nucleus
SGR	Specific growth rate
Ss4R	Salmonid specific whole genome duplication
T_a	Annealing temperature
TPM	Transcripts per kilobase million
Ts3R	Teleost specific whole genome duplication
UiB	University of Bergen
UoS	University of Stirling
WGD	Whole genome duplication
x-Gal	5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside

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Chapter One
General introduction

1.1. General background

Atlantic salmon (*Salmo salar*) belong to the Teleostei infraclass, which represents the ray-fined fish (Actinopterygii), the most diverse group of vertebrates known to man and is comprised of 96 % of all identified living fish species. In comparison to terrestrial vertebrates, they are extraordinarily varied, each species possesses distinct characteristics and displays considerable anatomical and physiological plasticity (Bone, 2019). This is arguably the direct result of exposure to multiple, variable selection pressures and caused by the highly dynamic aquatic environments the have inhabited throughout evolutionary development. Teleosts are exposed to multiple naturally occurring rhythmic environmental cues (zeitgebers) such as light and temperature which display predictable cyclic variations (Reppert and Weaver, 2001). Daily cycles (~24 hours) are referred to as circadian and are regarded as the most influential factor in the regulation of biological rhythms. Organisms have evolved biological clocks to capitalise upon predictable rhythmic changes and display distinct endogenous oscillations which synchronise biochemical, physiological and behavioural responses with zeitgebers (Sánchez-Vázquez and López-Olmeda, 2018). Coordinating their existence with the external world by anticipating predictable daily and seasonal events (Foster and Kreitzman, 2009).

The aquatic environment can further influence light cues as the photic environment in water exhibits greater variance as water quality, dissolved solutes, suspended particles, and flow rate can all affect the absorbance properties of the water column (Smith, 1974). Advances in aquaculture technologies may help to minimise the effect of water quality as recirculating aquaculture systems (RAS) offer greater environmental control, reducing the effect of poor water quality in comparison to more traditional outdoor freshwater flow-through or sea cage systems, in which water quality parameters are influenced greatly by constantly changing environments (Naas, Huse and Iglesias, 1996). The environment in which temperate species inhabit varies greatly throughout the year, alongside circadian rhythms temperate species are also exposed to annual environmental cycles (circannual rhythms). Seasonal changes or annual cycling in the energy received from the sun is the result of the earth's spatial relationship with the sun and the intensity of sunlight or energy exposure at a given point on the Earth's surface at a given time of the year (Villamizar *et al.*, 2011). The degree of seasonal variation or extremity is dependent upon latitude, the further from the equator the greater the annual seasonal variation, this is due to the angle at the point which solar radiation meets the Earth's surface. Consequently, in addition to a circadian clock, organisms also display a biological calendar (circannual clock) to synchronise physiological and behavioural responses with seasonal changes to maximise the chances of survival and reproduction (Foster and Kreitzman, 2009).

However, understanding the intricate interactions between zeitgebers, circadian rhythmicity, seasonality, and control of biochemical, physiological, and behavioural rhythms of teleosts is complex (Migaud, Davie and Taylor, 2010), to date there has been limited research carried out in temperate species, leaving a gap for construction of a more appropriate model.

1.1.1. The seasonality of Atlantic salmon

At temperate latitudes, freshwater species experience predictable annual cycles in biotic and abiotic environmental factors. For ectotherms such as Atlantic salmon growth and food consumption are strongly dependent on seasonal changes, especially temperature in which there is a period where there is an optimum temperature for growth and development as well as upper and lower thermal limits (Finstad, Næsje and Forseth, 2004). Commercial salmonid production is governed by the seasonality and natural life cycle of the fish – which has evolved in response to the seasons. Increased growth in the summer when the temperature is higher and daylight hours are longer which slows with the shortening of the days and decrease in temperature (Jobling, 2002). The seasonal change in photoperiod length also provides a proximate environmental cue for physiological adaptations such as smoltification (Hoar, 1976), breeding and spawning (Bromage, Porter and Randall, 2001; Pankhurst and Porter, 2003) allowing a greater chance of survival (Sumpter, 1992). Based around the seasonality of the species management practices have been adapted to maximise production and profitability (Spillman and Hobday, 2014).

The magnitude of seasonal variance in thermal performance differs amongst Atlantic salmon populations – this suggests that the salmon have adapted based on geographical location due to the differing amplitude and magnitude of the seasonal environmental changes along a latitudinal gradient. As such considerable variation in growth is apparent between populations of salmonids (Finstad, Næsje and Forseth, 2004). Salmon from less favourable environments with lower temperatures and short growing seasons performed better at all temperatures when compared to other salmon populations from more favourable growing environments (Conover and Schultz, 1995). Atlantic salmon in the wild display a seasonally changing metabolism, in which periods of high specific growth rates (SGR) are concurrent with low feed conversion rates (FCR).

Photoperiod is also regarded as a major determinant of seasonal physiological patterns (Schultz and Kay, 2003), variations in performance unexplainable by temperature differences are likely generated by other zeitgebers such as photoperiod and food availability (Jensen, 1990, 2003; Jones *et al.*, 2002). Salmon exposed to continuous light (LL) in sea cages displayed a distinct shift in seasonal growth pattern of SGR and Fulton's condition factor (K). With those under LL performing better than those under natural light

(Oppedal *et al.*, 1997, 1999). Supporting the hypothesis that photoperiods alongside temperature are a major effector of circannual growth (Endal *et al.*, 2000). Further work identified that the removal of seasonal signalling by exposing Atlantic salmon to LL resulted in an increase in growth during the spring, which is typically when growth rates reduce (Nordgarden *et al.*, 2003). The focus of research on the role of seasonality should be widened to include early developmental stages to identify whether there are any key effects of seasonality at other commercially important time periods.

1.2. Ontogeny of the Atlantic salmon

Metamorphosis meaning 'changing form' is an inherently integrative concept, there is no clear consensus on its definition biologically. In its broadest terms it can be used to describe biological transformations. However, due to its polyphyletic nature metamorphosis is not homologous between species and should therefore be defined in the context of individual species (Bishop *et al.*, 2006). There has been a history of conflicting views and criticism surrounding the mechanism of development in teleosts and whether ontogeny is saltatory or whether it could be considered gradual. Ambiguity surrounding the definition of ontogenetic intervals in scientific literature led to the development by Balon (1975) of five widely recognised ontogenic intervals in which life stages were clearly defined: embryonic, larval, juvenile, adult and senescent. With regard to salmonids, it is accepted that the larval stage of development is absent (Balon, 1975). The alevin stage of development is referred to as the remnant of larval development in salmonids, therefore, salmonids progress directly from the embryonic stage straight to the juvenile stage, undergoing a species-specific development in which the juvenile developmental stage can be divided into multiple well-defined phases: alevin, fry, fingerling, parr and smolt (Gorodilov, 1987). Saltatory approaches to define fish ontogeny do not consider the change in morphological state throughout any given stage or phase, due to the variable rate at which the embryos develop they can often exhibit characteristics of two adjacent stages. Therefore, the transition between transitional stages is not always as clear cut (Kamler, 2002).

1.2.1. Early ontogeny of the Atlantic salmon

During the early developmental stages, the predominant ontogenic processes are growth and tissue differentiation, these are accompanied by distinct physiological changes. The process of embryogenesis and the time taken for development is greatly influenced by environmental factors, especially temperature as this has a direct effect on metabolism (McMenamin and Parichy, 2013). Previous methods of broadly defining Atlantic salmon ontogeny was often restrictive and dismissed more subtle changes which occurred in early ontogeny (Gorodilov, 1987).

Thus, proposing a compromise between saltatory and continuous developmental ideas by creating a new developmental scale in which to assess early ontogeny salmon. The sequence of morphological changes from egg insemination until completed yolk absorption (around the onset of first feeding) can be divided into more than 100 different morphologically discrete stages (Gorodilov, 1987). To date Gorodilov's is still regarded as one of the most detailed descriptions of embryonic development in Atlantic salmon (Table 1.1). Whilst there is a multitude of discrete stages associated with early ontogeny, the major events in the early developmental stages of yolk-feeding fish are: egg activation or fertilisation, hatching (eleutheroembryonic, free embryo), first exogenous feeding and intestinal (alevins) digestion and completion of yolk reabsorption (Kamler, 2002).

Table 1.1: Sub-periods and states of early ontogeny in Atlantic salmon and their relative age in T_s units as described by Gorodilov, 1996.

State	Key characteristic	Main events in subperiods and additional state characters	T_S
Fertilisation	State defined by the phase of the nuclear cycle	Blastodisc forms on the animal pole of the yolk. Fusion of pronuclei occurs followed by the preparation of the zygotic nucleus for first cleavage	0-2
Cleavage	State defined by the number of cells: 2,4,8, etc.	11 synchronous cleavages of equal duration. High blastodisc forms at the end of the subperiod, consisting of 2000 cells with two differentiated cell layers formed – epiblast and periblast	2-12
Blastulation	Subperiod is divided into early, mid, and late blastula	Rate of cell division decreases sharply; the nucleoli appear in the nuclei by the middle of the subperiod (20 T_S). Morphogenetic cell movements begin, blastodisc is flattening. The thickened rim of cells along its border, formation of the germ ring	12-33
Gastrulation	States are defined according to the shape and dimensions of the embryo	Subperiod lasts from the appearance of the germ node up to the formation of the axial organ complex. The outer layer of blastoderm cells is named periderma, a derivative of the epiblast. Epiboly begins	34-55
Somitogenesis	Up to 65 states can be defined by the number of somite pairs	All together 65 to 67 somite pairs are forming. At least 60 pairs require the same time to be formed. Only the last few pairs are forming at a declining rate.	56-123
Vascularisation of yolk sac	Five states dependent on the extent of coverage of the vitelline plexus	The formation of the blood vascular system below the yolk sac epidermis takes place.	126-180
Formation of cordal fin rays	States based on the number of lepidotrichia in the cordal fin	Lepidotrichia are formed in equal intervals, 20 rays should be formed by the end of the subperiod	185-320
Free embryos	Three states dependent on further fin development, presence of melaophores and yolk sac decrease	Subperiod begins when hatching is complete and ends with attempts of the embryos to swim-up	340-450
Alevin	Three states based on the location of appearance of parr marks	Begins at swim up when they begin to externally feed and ends once the yolk sac has been full absorbed 'button up'	450-600

Every state has a certain duration as morphological changes do not come about instantaneously. Therefore, embryonic states should be considered as intervals during embryogenesis. The identification of states in embryogenesis should be based on the following premises: development should be considered as a dynamic process and not a succession of arbitrarily separated morphological states. Discrete and meristic characters are best suited for identification markers, and they make up the chain of predictable states. Regardless of the number of morphological indices or characters used embryogenesis is never described in literature as a continuous process. Artificial intervals of development are created by scientists to aid the understanding of development – these artificial intervals may not necessarily represent natural intervals nor be related to any important functions. In order to reflect continuity a measure of time might be incorporated into descriptions of changing morphology by measuring incubation time at a constant temperature. The greater the number of key characters are involved in the description of successive states, the more adequate a picture of the continuous process of embryogenesis may be drawn (Gorodilov, 1987). In the wild, these early developmental stages from fertilisation to alevin are accomplished in the interstitial spaces between substrate in the gravel redds in which the female spawned. Alevins emerge from the substrate once they are ready to exogenously feed (Quinn, Groot and Margolis, 1992; Hart, 2006; Kihslinger and Nevitt, 2006). This early life history leaves lasting effect on morphology and physiology (Jonsson and Jonsson, 2014).

1.3. Current applications of light in salmonid aquaculture

Light profoundly influences the physiology and behaviour of many teleosts and is a powerful tool used in production of Atlantic salmon because of their high sensitivity to light (Migaud *et al.*, 2006). The zeitgeber is regularly used in the Atlantic salmon production as a management tool to manipulate the development of the fish at multiple major life stages to ensure year round production (Handeland and Stefansson, 2001). Photoperiod is the most commonly manipulated light condition, in which dynamic photoperiod regimes are applied to optimise production (Good and Davidson, 2016). In freshwater, exposure to a long photoperiod (summer signal) after short photoperiod (winter signal) for 5-7 weeks initiates smoltification (Sigholt *et al.*, 1995). Light intensity is positively correlated with growth, light intensities <43 lux have been shown to decrease smolt quality through increased skeletal abnormalities and reduced silvering (Handeland *et al.*, 2013).

During the sea water on growing phase, artificial lighting regimes are used to help delay sexual maturation (Leclercq *et al.*, 2011), facilitating rapid growth, increasing survival rates, improving production quality, and reducing production related costs (Migaud *et al.*, 2006; Taranger *et al.*, 2010).

Alongside the reduction in early maturation, multiple studies have shown the value of using continuous lighting in post-smolts during the winter and spring to counteract the effects of shortened photoperiods on growth (Oppedal *et al.*, 1997; Endal *et al.*, 2000; Nordgarden *et al.*, 2003; Nilsson, Stien and Oppedal, 2017). A trial run by Philips in collaboration with Leroy at Gildeskål Research Station, Norway, virtually eliminated variance in maturity previously attributed to as 'unavoidable background noise' by switching from metal halide lighting (2.58 %) to LED (0.13 %), resulting in a 14 % increase in mean weight and 4.7 % increase in SGR, further highlighting the short return on investments in LED technology (Orrego, 2015).

Additional research areas of interest regarding light include the environmental manipulation of increased swimming depth of salmon to reduce sea lice burdens (Frenzl *et al.*, 2014), and biomass density control (Oppedal, Dempster and Stien, 2011). The robustness of fish in freshwater stages is linked to productivity post-smolt, therefore it is of commercial interest to better understand the relationship between light exposure (e.g., photo cue, photoperiod, intensity, and spectral composition) and performance-based characteristics (e.g., growth, mortality, and smoltification success) throughout early developmental stages to further improve productivity during freshwater rearing stages (Migaud, Davie and Taylor, 2010). Continual development of LED technology has enabled an increased level of control over all light components, allowing light profiles to be easily adapted for specific requirements such as light regimes tailored for developmental stages through the ability to discretely manipulate the components of light. Whilst the technology exists, additional research is required to define the parameters which are the most beneficial for specific life stages - it is also important to identify detrimental lighting regimes and components so that these can be avoided to further maximise productivity.

1.4. Evolution and whole genome duplication events

Alongside environmental selection pressures, multiple rounds of whole genome duplication (WGD) have had a large influence on the evolution of lineages to date, specifically teleosts (Glasauer and Neuhauss, 2014). Duplication of a gene results in two daughter genes names paralogs, immediately post duplication the genes are identical and functionally redundant. The retention of resulting gene duplications has been largely biased with regard to gene function (Brunet *et al.*, 2006). The duplicated genes provide the genetic raw material for evolutionary innovation through the process of subfunctionalisation, the partitioning of ancestral function; or neofunctionalisation, the assignment of novel function not present pre-duplication (Ohno, 1970; Rastogi and Liberles, 2005), thus resulting in genome reshaping (Inoue *et al.*, 2015). In Atlantic salmon 80 -100 million years post the salmonid specific WGD event (Ss4R) analysis of duplicate retention identified that 20 % of duplicates from Ts3R and 55 % of duplicates from Ss4R were retained as functional copies.

The prominent mechanism for duplicate loss was pseudogenisation (Lien *et al.*, 2016). Whereas in rainbow trout 48 % of all of the pre-Ss4R ancestral genes were retained as duplicates. The remainder of the genes underwent fractionation and the duplicated protein-coding genes were lost (Berthelot *et al.*, 2014). With regard to the clock genes, identifying the functions of individuals within the mechanism has become increasingly hard in vertebrates such as salmonids, whom have undergone multiple rounds of WGD in their evolutionary history due to the resulting large compliment of individual genes, genomic analysis revealed an unusually large compliment of clock genes in Atlantic salmon, double that of the zebrafish, *Danio rerio* (West *et al.*, 2020).

1.5. Clock genes and the circadian mechanism

Biological timekeeping is regulated by the circadian mechanism. At a molecular level the circadian oscillations are generated by a transcriptional-translational feedback loop composed of core clock-genes which drive the rhythmic accumulation of downstream outputs, otherwise known as clock-controlled genes (CCGs) (Li *et al.*, 2015). This is an evolved adaptation to maintain synchrony between the organism and their environment, through the activation and repression of gene transcription networks, the full extent to which cells maintain the clock is still under investigation in mammals (Cox and Takahashi, 2019).

In its most basic form, the auto regulatory molecular clock is comprised of a positive feedback loop of *clock* and *arntl* (*bmal*), and a negative feedback loop of *period* and *cryptochrome* (Darlington *et al.*, 1998; Reppert and Weaver, 2001; Lien *et al.*, 2016). The complexity of gene interactions within the mammalian model to date can be appreciated in Figure 1.1.

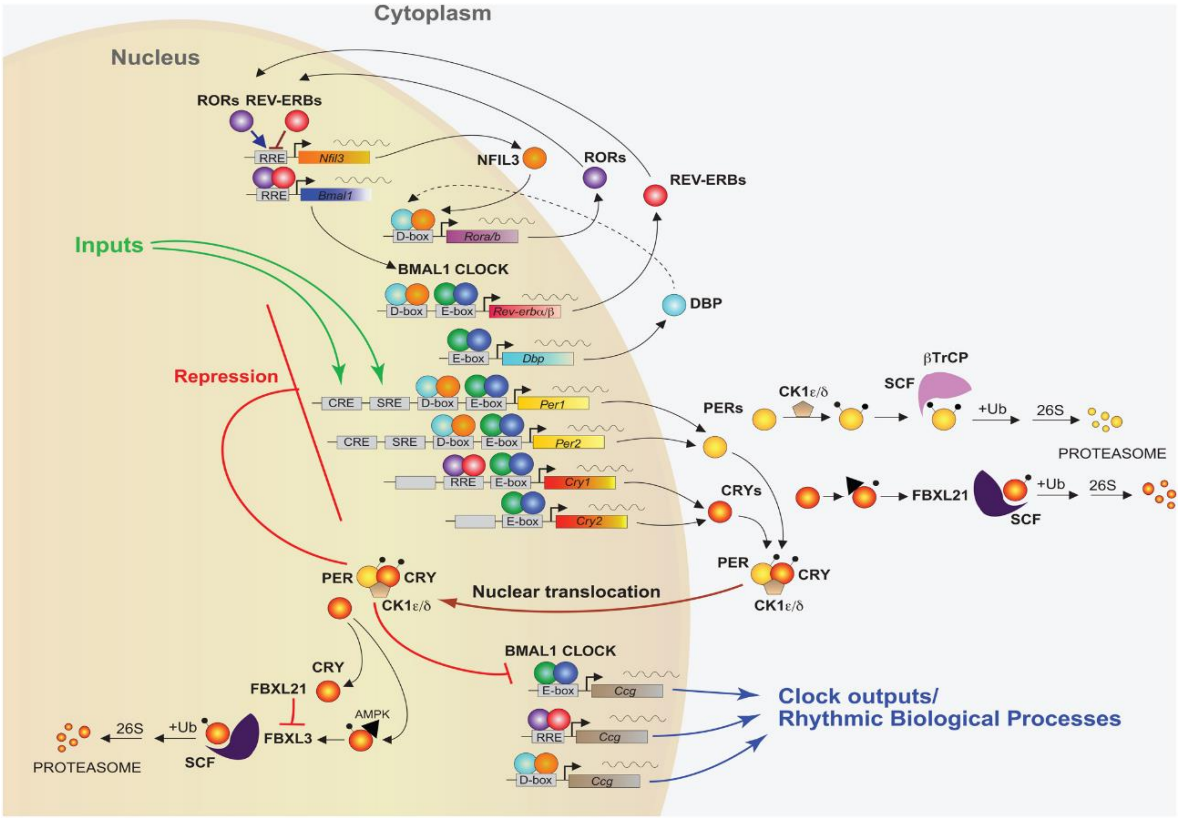


Figure 1.1: Core components of the mammalian circadian clock. In the core feedback loop, the transcription factors BMAL1 (green circles) and CLOCK (blue circles) bind to E-box domains on gene promoters, including the genes for Per1 and Per2 (yellow) and Cry1 and Cry2 (red/yellow). PERs (yellow circles) and CRYs (red/yellow circles) dimerize and translocate to the nucleus after binding with casein kinase δ (CK1δ) or CK1ϵ, where they repress their own transcription. The stability of PER and CRY is regulated both in the cytoplasm and within the nucleus by several proteins, including FBXL21 and FBXL3. In a second feedback loop, CLOCK and BMAL1 also regulate the transcription of genes for the nuclear receptors REV-ERBα and REV-ERBβ (red circles), which compete with the retinoic acid-related orphan receptors, RORα, RORβ, and RORγ (purple circles) for binding to RRE elements on the BMAL1 gene promoter, providing both positive (ROR) and negative (REV-ERB) regulation of BMAL1 transcription. A third feedback loop is mediated by CLOCK/BMAL1-mediated transcription of the gene Dbp (light blue) and the ROR/REV-ERB-mediated transcription of Nfil3 (orange). DBP (light blue circles) and NFIL3 (orange circles) dimerize and bind to D-box elements on the promoters of many of the core clock genes, providing additional layers of regulation. In addition, CLOCK/BMAL1, ROR/REV-ERB, and DBP/NFIL3 regulate the transcription of many other clock output genes (From Cox and Takahashi, 2019).

The rhythmic transcription of the *period* and *cryptochrome* genes is driven by the transcription factors *clock* and *arntl* (Ko and Takahashi, 2006; Layeghifard *et al.*, 2008). *Clock* and *Arntl* proteins form a heterodimer in the cell cytoplasm and are translocated to the nucleus where it binds to DNA E-boxes (CACGTG), regulatory elements which promote the transcription of down-stream target genes including the *period/cryptochrome* heterodimer which forms the negative arm of the feedback loop. The *period* and *cryptochrome* transcripts migrate from the nucleus, generating the resulting proteins.

Which accumulate, dimerise and translocate into the nucleus where they inhibit their own transcription by blocking the E-box binding of the *clock/arntl* heterodimer, before the proteins are inactivated through post-translational modification such as phosphorylation and degradation (Gallego and Virshup, 2007). This degradation marks the end of one complete cycle (~24 h) and once *period* and *cryptochrome* levels are sufficiently decreased in the nucleus, repression is relieved and a new cycle begins (Takahashi, 2017). In mammals the positive feedback loop is associated with the photophase, and the negative feedback loop the scotophase (Partch, Green and Takahashi, 2014).

Alongside the core feedback loops there are accessory loops in which secondary processes occur. Whilst they do not drive the circadian rhythm, they are fundamental in its regulation and the overall accuracy of the clock (Takahashi, 2016). The *clock/arntl* heterodimer also activates the transcription of *nuclear receptor subfamily1 group D* (*nr1d1* or *rev-erbα*) and *retinoic acid related orphan receptor response elements* (*ror α, β,* and *γ*) which both form a secondary loop to repress (*nr1d1*) or activate (*ror α, β,* and *γ*) *arntl* transcription. The subsequent heterodimer *clock/arntl* then controls the rhythmic expression of other genes and pathways via E-box and D-box elements (Ueda *et al.*, 2005; Ko and Takahashi, 2006). Numerous genes including *aanat2* are regulated by E-box elements in their promoter regions (Gothilf *et al.*, 2002; Zilberman-Peled *et al.*, 2007). These genes are up-regulated on a daily cycle as one of the main output connections of the body clock and are referred to as clock-controlled genes (Ko and Takahashi, 2006).

Underlying these loops, and fundamental to the accuracy of the circadian clock, are processes that control phosphorylation and ubiquitylation. *Casein kinases* (*CSNK1ε/δ*) have a fundamentally important role in the post-translational regulation of the molecular circadian clock through phosphorylation of key circadian proteins (*per1*) in the cytoplasm leading to their degradation (Yan *et al.*, 2008). They primarily but not exclusively act upon the *period/cryptochrome* heterodimer, through nuclear localisation (cytoplasmic accumulation) or the nuclear translocation of *period.* It is thought that the phosphorylation of *period* proteins at many *csnk1*ε/δ sites may be linked to the repression of transcription as phosphorylation of *period* proteins has been shown to be up regulated whilst *clock* and *arntl* are under the highest degree of suppression.

Crucially ubiquitylation is also important in regulation of the circadian molecular clock. This has been well described in the fruit fly, *Drosophila melanogaster* (Peschel and Helfrich-Förster, 2011). Phosphorylation of the *period* proteins also marks them for ubiquitin mediated degradation via the 26S proteasome (Yang and Sehgal, 2001; Eide *et al.*, 2005; Shirogane *et al.*, 2005). Allowing the *clock/arntl* heterodimer to restart the cycle (Ko and Takahashi, 2006; Gallego and Virshup, 2007).

1.5.1. Influence of clock genes upon physiology

Circadian clocks have a well-defined role in regulating physiological and behavioural events on a 24 h basis and have extended that role into seasonal timing and photoperiodism (Schultz and Kay, 2003). The disruption of clock gene regulation can lead to a number of pathological conditions inclusive of metabolic disorders and increased susceptibility to cancer (Sahar and Sassone-Corsi, 2009). The presence of peripheral oscillators suggests that tissue-specific regulatory pathways may be integrated with the clock machinery, in which clock genes and clock gene regulators may operate within given transcriptional pathways in addition to their circadian function (Schibler and Sassone-Corsi, 2002; Grimaldi *et al.*, 2010a). Moreover, clock regulators appear to be intimately implicated in cellular functions other than circadian control, thereby influencing cellular metabolism, cell cycle, and cell proliferation (Wijnen and Young, 2006). There is a strong correlation between myogenic genes and clock genes. It is suggested that there is a putative clock system in fast skeletal muscle which influences the regulation of muscle physiology through control of the expression of genes related to myogenesis (Schultz and Kay, 2003). It has been demonstrated in mice that PER2 directly and specifically represses the nuclear receptor PPARγ which is critical in adipogenesis, insulin sensitivity and inflammatory response. Mice deficient in PER2 displayed altered lipid metabolisms with greatly reduced levels of triacylglycerol and non-esterified fatty acids. Thus, identifying that PER 2 is necessary for normal lipid metabolism (Grimaldi *et al.*, 2010b).The ontogeny of clock-regulated processes has only been systematically studied in a handful of cases, and the precise onset of embryonic circadian regulation appears likely to occur at different times in different tissues and to depend on the light cycle to which it has been exposed (Schultz and Kay, 2003). Therefore, additional research must be undertaken to close the research gap within this area.

1.5.2. Entrainment

Multicellular cellular organisms with differentiated tissues possess the ability to partition clock function amongst cell types to coordinate tissue specific circadian rhythms and maintain precision whilst under the influence of multiple oscillators to produce gene expressions which are robustly entrained to a circadian rhythm (Bell-Pedersen *et al.*, 2005). In the complex architecture of biological timekeeping in mammals, peripheral circadian oscillators are controlled centrally by the circadian oscillators in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus (Reppert and Weaver, 2001). Which acts as a master clock, generating circadian rhythms (Klein, Moore and Reppert, 1991) and synchronising circadian rhythms in subsidiary clocks in nearly every cell in the body (Dibner, Schibler and Albrecht, 2009). However, in teleost species such as zebrafish it is apparent that there is the regulation of circadian oscillators is more devolved, with fish in general displaying an

impressive flexibility in their clock function in relation to their environment (Idda *et al.*, 2012). Research has displayed that circadian oscillators in cultured zebrafish peripheral tissues are not only rhythmically expressed but they are also directly entrainable by light, unlike peripheral oscillators in mammals which are not directly responsive to light (Kaneko, Hernandez-Borsetti and Cahill, 2006).

1.5.2.1. Photic entrainment

Photoentrainment is significant in the development of Atlantic salmon. Except from melatonin there is limited information on the photoneuroendocrine (PNES) organisation of teleosts. Melatonin influences growth, maturation, and reproductive processes in a number of teleost species, however, research to date has identified that species, sex, maturity, and photoperiod also influence physiological function (Falcón et al., 2007; Migaud et al., 2010b). It is suggested that melatonin acts directly upon the pineal due to the presence of melatonin binding sites within the tissue (Falcón et al., 2011). Research involving the removal of the pineal (pinealectomy) in Atlantic salmon has inferred that pineal melatonin may be involved in the timing and regulation of spawning (Migaud et al., 2010b).

In most species, the pineal is directly photoreceptive and is therefore entrainable by light (Migaud et al., 2007), remaining photoreceptive even in isolated organ culture (Iigo et al., 2007). The pineal is also the primary site for the endogenous production and circulation of melatonin. In zebrafish the pineal is considered to be the central pacemaker due to the rhythmic clock gene expression within the tissue (Vatine et al., 2011). Melatonin displays a seasonal pattern in temperate species. During the summer melatonin rhythms have a short duration and are high in amplitude, this is converse in winter periods in which the duration of rhythmic expression is long, and the amplitude lower. In the spring and autumn there are intermediary rhythms (Besseau et al., 2006). These rhythms persist in constant darkness in a number of species, demonstrating the endogenous nature of melatonin rhythms (Migaud et al., 2010b). With the exception of salmonids, as constant lighting conditions interfere with rhythmical melatonin production (Falcón, 1999). Whilst the synthesis of pineal melatonin is photo-entrainable it remains non-endogenous. Under constant darkness rhythmicity of pineal melatonin production is completely lost in all salmonid species investigated (Gern and Greenhouse, 1988; Max and Menaker, 1992; Iigo et al., 2007). Hypotheses that ancestral salmonids lost the ability of endogenous regulation of pineal melatonin synthesis when they diverged away from the osmerids, as they still possess the ability to endogenously produce pineal melatonin in constant darkness (Iigo et al., 2007).

There is suggestion that the lack of endogenous melatonin production in the salmonid pineal is an evolutionary adaptation due to the long migratory distances and extreme photoperiod variations salmonids are exposed to in order to return to their natal spawning grounds (Iigo

et al., 2007). In addition to the effects of melatonin on reproduction, molecular clocks and the PNES have been shown to influence reproduction physiology at differing levels of organisation. The endogenous production of melatonin in non-salmonid teleosts in the pineal is likely to be under control of molecular clock mechanisms (Iigo et al., 2007). Clock genes have also been associated with maturation and reproduction processes. clock has been directly mapped to quantitative trait loci (QTL) regions for spawning time in rainbow trout. Clock genes *cry2b* and *clock1b* have also been mapped to QLT regions got growth in Coho salmon (Oncorhynchus kisutch). The length of polymorphisms within *clock1b* polyglutaimine domains have been found to be associated with differential migratory run times in the Chinook salmon, Oncorhynchus tshawytscha (O'Malley and Banks, 2008). clock1b has also been implicated in the reproductive timing of a number of pacific salmon species (O'Malley et al., 2010). In pre-maturing males *bmal1* (also referred to as *arntl1*) was found to be up-regulated (Aubin-Horth et al., 2005). *clock1* (*clock1a*) *npas2* (*clock2*) and *clock3* (*clock1b*) were mapped to QLTs for life history regions in salmonids (Paibomesai et al., 2010).

Despite several papers linking clock genes to maturation, migration, and reproduction there is still a lack of clarity and research surrounding the mechanisms linking clock genes to seasonal processes. However, it is becoming clearer that the teleost PNES is more diverse than the mammalian model and further research is required to create more fitting models.

1.5.2.2. Non-photic entrainment

Light is considered to be the principal input in synchronisation of the clock to the environment. However, temperature fluctuations and food availability are also involved in the entrainment of the clock (Isorna *et al.*, 2017). Water temperature influences behaviour and physiology (López-Olmeda, 2017), with thermocycles of as little as ± 2 °C resulting in self-sustaining clock gene expression under complete darkness in zebrafish (Lahiri *et al.*, 2005, 2014). In a more recent study, both zebrafish and Nile tilapia displayed a thermal rhythm in temperature under LD lighting, indicative that it is coupled with the light driven circadian cycle (Vera *et al.*, 2023). Relationships between thermocycles and photoperiod are additive. When LD and thermocycles are combined, thermophase with photophase and cryophase with scotophase a stable phase and maximum amplitude can be observed in the resulting rhythms (López-Olmeda, 2017).

Feeding regimes and subsequent feeding entrainment are contributory factors of the expression pattern of clock genes in the liver, which is often found to be out of phase with that of the brain (Frøland Steindal and Whitmore, 2019). Studies in goldfish and gilthead sea bream identified the localisation of clocks, discovering phase shifts between clock gene expression in the liver when compared to the brain suggestive of the uncoupling of the light

entrainable oscillators and food-entrainable oscillators in the liver (Vera *et al.*, 2013). In goldfish, distinct differences were identified in the amplitude and phase of clock gene expression in liver and gut tissues was different to that of the retina, suggesting that feeding time was a contributory oscillator in the entrainment of circadian rhythmicity in both the liver and the gut (Velarde *et al.*, 2009). Further developments in the understanding of feeding time and clock gene rhythmicity revealed that the link between time of last meal and the phase of clock gene expression in the liver (Feliciano *et al.*, 2011). Similarly, peripheral circadian clock gene expression (*clock1* and *cry5*) was also influenced by feeding times and displayed a phase shift in comparison to that of the brain (Costa *et al.*, 2016).

1.6. Teleosts and the clock

Since the mid-1990s the understanding surrounding the biological clock and their control mechanisms has advanced considerably, largely due to the implementation of genetic analysis (Foulkes *et al.*, 2016). As such the underlying mechanism surrounding circadian rhythmicity has been characterised in several model species: cyanobacterium, neurospora, drosophila, mice (*Mus musculus*), and zebrafish.

1.6.1. Zebrafish as a model species for the circadian clock

Whilst the mechanisms of the circadian clock are relatively well described in mammals, this is not the case in teleosts. Evidence of endogenous clocks were first identified in Northern pike (*Esox lucius*) as it was discovered that a number of pineal hormones (including melatonin) displayed circadian expression profiles (Falcon, Geffard and Juillard, 1984; Falcón *et al.*, 1987; Prusik *et al.*, 2015). These rhythms were later found to be endogenous and regulated internally by the circadian clock (Bolliet *et al.*, 1996; Falcón, 1999; Prusik *et al.*, 2015). Since this discovery the majority of circadian rhythmicity in teleosts was undertaken in zebrafish after it was reported that they could be used as a model to study circadian rhythmicity in teleosts (Cahill and Besharse, 1995).They are a powerful and well-established model organism (Sacksteder and Kimmey, 2022) and have been fundamental in the unravelling of the circadian clock due to their fast development, ease of breeding and small adult size making them relatively inexpensive to keep in the lab (Vatine *et al.*, 2011).

The molecular feedback loops which instigate and regulate the endogenous circadian rhythmicity appear to have remained remarkably conserved throughout evolution (Paibomesai *et al.*, 2010). Whilst there are some distinct and important differences between the zebrafish and mammalian models the molecular mechanisms underpinning the zebrafish clock are remarkably similar to those of the mammalian clock (Cahill, 2002; Vatine *et al.*, 2011). When using the mammalian genes to identify clock genes in zebrafish (Whitmore *et al.*, 1998; Kobayashi *et al.*, 2000; Tamai, Young and Whitmore, 2007; Wang,

2008a, 2009), multiple copies were identified (Table 1.2), this is thought to be a direct result of the teleost specific whole genome duplication (Ts3R). Analysis of the genetic expression profiles has also shown that the additional copies may not be redundant (Vatine *et al.*, 2011).

Table 1.2: Summary of clock genes and accessory loop genes identified in zebrafish to date, using ZFIN to ensure up to date nomenclature.

Nomenclature	Previous Nomenclature	Accession
clock		
clock 1a	*clock 1*	NM_130957
clock 1b	*clock 3*	NM_178295
clock 2	*clock 2, npas 2*	NM_178299
arntl/ bmal		
arntl 1a	*arntl 1a, bmal 1a*	NM_131577
arntl 1b	*arntl 1b, bmal 1b*	NM_178300
arntl 2	*arntl 2, bmal 2*	NM_131578
period		
per 1a	*per 1*	NM_001030183
per 1b	*per 4*	NM_212439
per 2	*per 2*	NM_182857
per 3	*per 3*	NM_131584
cryptochrome		
cry 1a	*cry1aa*	NM_001077297
cry 1b	*cry1ab*	NM_131790
cry 2	*cry 3*	NM_131786
cry 3a	*cry1ba, cry2a, cry3a*	NM_131791
cry 3b	*cry1bb, cry2b, cry3b*	NM_131792
cry 4	*cry 3*	NM_131787
cry 5		NM_131788
csnk1e/d		
csnk1e	-	NM_212747
csnk1da	-	NM_199583
csnk1db	-	NM_213250
ror		
ror aa	*ror a, ror a2*	NM_001110167
ror ab	*ror a1*	NM_201067
ror b	-	NM_001082856
ror ca	*ror c*	NM_001082819
ror cb	-	NM_001277094
rev-erb/ nr1d		
nr1d1	*rev-erb a*	NM_205729
nr1d2a	*rev-erb b1*	NM_001130592
nr1d2b	*rev-erb b2*	NM_131065
nr1d4a	*rev-erb g1*	NM_001285536
nr1d4b	*rev-erb g2*	NM_001285533

Using the *cryptochrome* family as an example, *cry1a, cry1b, cry2a, cry2b, cry3,* and *cry4* all cycle rhythmically, however, they exhibit different acrophases. *cry1a* appears to be the only zebrafish *cryptochrome* which seems to be light induced, therefore it is reasonable to assume that the genes may all serve different roles within the circadian mechanism (Kobayashi *et al.*, 2000; Vatine *et al.*, 2011). With regards to the organisation of the clock in teleosts, it is suggested that due to the ubiquitous expression of clock genes that there may be multiple clock systems rather than one centrally controlled mechanism as is found in mammals which is controlled by the suprachiasmatic nucleus, SCN (Kumar and Sharma, 2018).

More recently there has been a drive to understand clock mechanisms and the effect of light upon fish biology in a number of commercially important temperate and tropical species including: zebrafish (Vuilleumier *et al.*, 2006; Dekens and Whitmore, 2008; Wang, 2008b; Vatine *et al.*, 2011; Di Rosa *et al.*, 2015); rainbow trout (Davie *et al.*, 2011); Senegalese sole, *Solea senegalensis* (Martín-Robles, Aliaga-Guerrero, *et al.*, 2012; Martín-Robles, Whitmore, *et al.*, 2012); gilthead sea bream, *Sparus aurata* (Mata-Sotres *et al.*, 2015; Yúfera *et al.*, 2017); European sea bass (Villamizar, García-Alcazar and Sánchez-Vázquez, 2009); goldfish, *Carassius auratus* (Velarde *et al.*, 2009; Feliciano *et al.*, 2011; Isorna *et al.*, 2017); Atlantic turbot, *Scophthalmus maximus* (Sierra-Flores *et al.*, 2016); and Atlantic cod, *Gadus morhua* (Migaud *et al.*, 2009; Sierra-Flores *et al.*, 2016).

Whilst zebrafish have been instrumental in the developmnt of knowledge surrounding clock genes and circadian rhythmicity, due to their tropical environment, they lack distinct seasonal organisation of physiology (Davie, Minghetti and Migaud, 2009). Therefore, they may not be the best comparative model for many commercially important aquaculture species, which are typically temperate and therefore also exposed to circannual rhythms and adjust accordingly. Dissecting the clock mechanism in Atlantic salmon may help to better understand the effects of seasonality and circadian rhythmicity.

1.6.2. Circadian studies in other teleosts

The effect of light and feeding entrainment upon circadian biology has been studied in several species by exposing fish during varied developmental stages to different lighting environments: zebrafish, *Danio rerio* (Vuilleumier *et al.*, 2006; Dekens and Whitmore, 2008; Vatine *et al.*, 2011; Di Rosa *et al.*, 2015); rainbow trout, (Davie *et al.*, 2011; Hernández-Pérez *et al.*, 2019); Senegalese sole (Martín-Robles, Whitmore, *et al.*, 2012); gilthead sea bream (Vera *et al.*, 2013; Paredes *et al.*, 2014; Mata-Sotres *et al.*, 2015); European sea bass (Villamizar, García-Alcazar and Sánchez-Vázquez, 2009; Rodríguez *et al.*, 2021); Atlantic turbot (Sierra-Flores *et al.*, 2016; Ceinos *et al.*, 2019); Atlantic cod (Migaud *et al.*, 2009; Sierra-Flores *et al.*, 2016); goldfish (Feliciano *et al.*, 2011; Isorna *et al.*, 2017; Sánchez-Bretaño *et al.*, 2017); and Atlantic bluefin Tuna (Betancor *et al.*, 2020).

Studies in sea bream have broadened the understanding of clock-driven transcription (Yúfera *et al.*, 2017), and clock gene entrainment by feeding activity and the localisation and uncoupling of central and peripheral clocks in relation to differing zeitgebers (Vera *et al.*, 2013; Gilannejad *et al.*, 2021). Highlighting the need to consider the timings of lighting and feeding regimes upon clock controlled gene outputs to ensure optimal protocols within aquaculture production (Paredes *et al.*, 2014). Research in cavefish have revealed that whilst circadian rhythms are supressed in their natural habitat, in the presence of zeitgebers

such as LD cycles and scheduled feeding events in lab environments the expression pattern of clock and clock controlled genes became circadian in rhythm (Pavlova and Krylov, 2023).

Amongst salmonid species there has been a limited number of studies, which cover various aspects of clock gene expression, typically focusing on the expression of single genes. The majority of studies have been undertaken in Atlantic salmon (Davie, Minghetti and Migaud, 2009; Huang, Ruoff and Fjelldal, 2010b; Betancor *et al.*, 2014; McStay *et al.*, 2014; Vera and Migaud, 2016). Studies have also been conducted in rainbow trout (Davie *et al.*, 2011) and Chinook salmon.

A number of circadian clock genes have additionally been associated with seasonal processes and behaviours involved in key life history events, including migratory runtime and maturation in a number of salmonid species (Aubin-Horth *et al.*, 2005; Leder, Danzmann and Ferguson, 2006; O'Malley, Camara and Banks, 2007; O'Malley and Banks, 2008; O'Malley, McClelland and Naish, 2010; Paibomesai *et al.*, 2010).

The major advancement of technology and the introduction of next-generation sequencing has dramatically expanded our ability to study the expression, processing, and stability of rhythmically expressed genes. This has enabled the identification of additional copies of clock genes which will aid the development of understanding surrounding the function of duplicates (West *et al.*, 2020).

Comparison of clock genes and circadian rhythmicity in a number of teleost species helps to further close the research gaps surrounding circadian rhythmicity and clock mechanisms in fish, further elucidating the involvement of clock genes in downstream pathways. Together this knowledge can be combined to continually improve the model for the circadian mechanism in teleosts. As such, based on current literature the model outlined in Figure 1.2. is proposed for salmonids. At present the effects of the salmonid specific whole genome duplication event upon the clock and the full complement of clock genes remains largely unknown and requires further investigation. Therefore, gene family interactions have been depicted removing the detail of individual gene and transcript until they can be confirmed in future research.

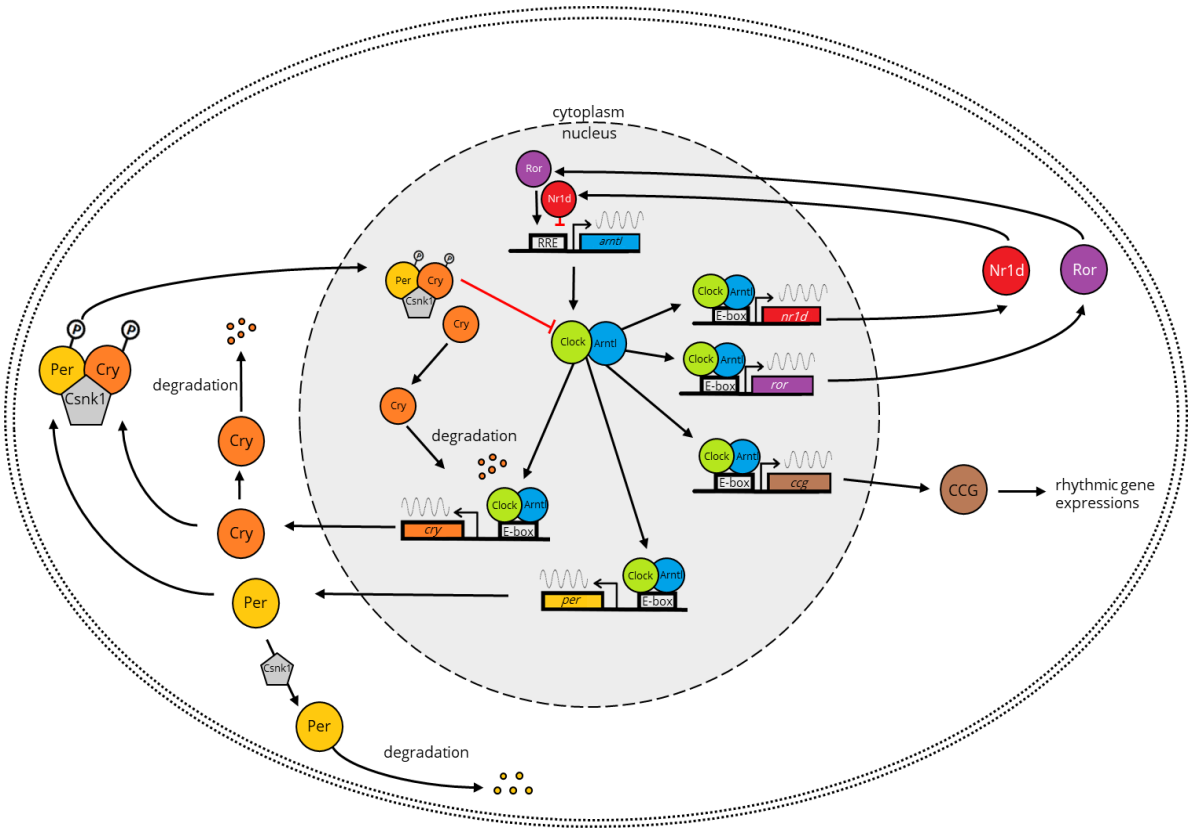


Figure 1.2: Proposed model of the molecular clock mechanism in Atlantic salmon based on literature. Red pathways represent inhibition by both the *period: cryptochrome* heterodimer and *nr1d* family which repress/ inhibit the formation of the *arntl: clock* heterodimer and the post-translational degradation of *period* and *cryptochrome* proteins. Thus, allowing the *arntl: clock* heterodimer to begin the cycle again. Adapted from: (Wang, 2008b; Li *et al.*, 2015; Isorna *et al.*, 2017; Takahashi, 2017; Cox and Takahashi, 2019).

1.7. Thesis objectives

Current salmonid aquaculture is the result of continual development and optimisation of production due to the increased understanding of developmental processes and the specific requirements associated with defined life stages. Atlantic salmon have historically been less problematic to rear commercially in early life stages unlike some marine aquaculture species. This is thought to be due to the large yolk sac fuelling development before first feeding, therefore there has been limited focus on their early developmental requirements.

To date there has been minimal investigation into the effects of lighting on the earliest developmental stages. The use of lighting in industry practice is highly variable and in freshwater is predominantly focused on improving growth and triggering smoltification. It is known that light influences cell cycle progression in teleosts (Frøland Steindal and Whitmore, 2019). It was reported in 2007 that lighting systems had not yet been tuned specifically for aquaculture or for specific fish sensitivities (Migaud *et al.*, 2007). To date there is still limited scientific information on the role of light and the effect of lighting regimes within this critical developmental period for Atlantic salmon.

Therefore, in order to improve the knowledge and understanding of clock genes and the circadian mechanism in Atlantic salmon. The specific aims of this thesis are to further the knowledge surrounding the clock mechanism and the effects of light condition on clock gene expression during early developmental stages.

a) To identify the full complement of clock genes in Atlantic salmon (*Salmo salar*) in comparison to other commercially important salmonid species and evaluate the expression patterns of the identified genes (Chapter Three).

b) To identify clock gene expression during early developmental stages in Atlantic salmon and investigate the influence of lighting conditions (photo cue, light intensity, spectral composition) upon clock gene expression (Chapter Four).

c) To identify the effect of feeding entrainment, during the switch from endogenous to exogenous feeding on the circadian entrainment of the clock. (Chapter Five).

Chapter Two
General materials and methods

2.1. Ethics statement

All experiments were ethically reviewed and approved before work was undertaken. Experiments undertaken at the University of Bergen (UiB) followed local animal care guidelines and were subject to ethical review and approval was granted by the Norwegian Veterinary Authorities. All work conducted in these studies, both at UiB and at the Institute of Aquaculture (IoA) at the University of Stirling (UoS) was non-ASPA regulated research and was subjected to internal ethical reviews at the UoS and was carried out by the Animal Welfare and Ethical Review Body (AWERB), approval was confirmed before the studies were conducted (AWERB 19 20 097, AWERB 19 20 107).

2.2. Experimental overview

The three experiments conducted were designed and conducted with the aim of fulfilling the objectives of the thesis. These experiments are outlined below in Table 2.1.

Table 2.1: Outline of experiments undertaken.

Experiment	Chapter	Location
Rhythmic Clock Gene Expression in Atlantic Salmon Parr Brain	Three	UoS, NBFRU, UK
Effects of light conditions on circadian clock gene rhythms in early development	Four	UiB Bergen, No
Clock gene expression and the influence of feeding entrainment	Five	UiB Bergen, No

2.2.1. Rhythmic clock gene expression in Atlantic salmon parr brain

The experiment in Chapter Three was undertaken at the University of Stirling's Niall Bromage Freshwater Research Unit (Buckieburn). The aim of the study was to confirm the presence of the clock genes identified in *in* silico work and identify the number of rhythmically expressed clock genes in Atlantic salmon parr brain. Figure 2.1 shows the basic experimental design.

Figure 2.1: Experimental setup for 12:12 LD clock gene expression trial at the Niall Bromage Freshwater Research Unit, University of Stirling, 2020.

2.2.2. Effects of light conditions on circadian clock gene rhythms in early development

The experiment in Chapter Four was undertaken at the University of Bergen's approved laboratory facilities in the High Technology Centre. The aim of the study was to identify clock gene expression during early developmental stages in Atlantic salmon and investigate the influence of lighting conditions (photo cue, light intensity, spectral composition) upon clock gene expression. Figure 2.2 shows the experimental set up and sampling points undertaken in this experiment.

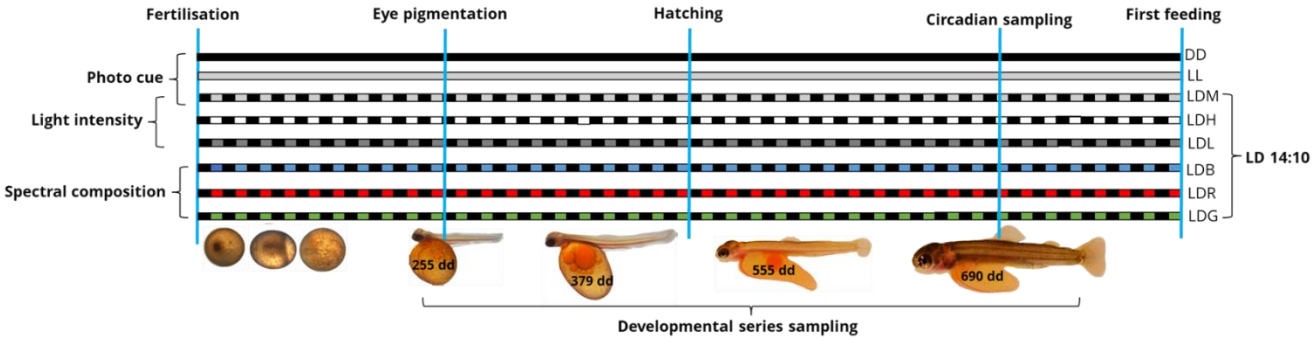


Figure 2.2: Experimental setup for light experiments, developmental series sampling was conducted on fish from the LDM treatment group only.

2.2.3. Clock gene expression and the influence of feeding entrainment

The experiment in Chapter Five was undertaken at the University of Bergen's approved laboratory facilities in the High Technology Centre. The aim of the study was to identify the effect of feeding entrainment, during the switch from endogenous to exogenous feeding on the circadian entrainment of the clock. Figure 2.3 shows the experimental set up and sampling points undertaken in this experiment.

Figure 2.3: Experimental set-up plan for endogenous vs exogenous clock gene expression trial at the University of Bergen, 2020.

2.3. RNA extraction

All tissue samples were snap frozen in liquid nitrogen vapour upon collection and stored in the freezer at -70 °C prior to ribonucleic acid (RNA) extraction. RNA was isolated from the samples using TRI Reagent® (Sigma Aldrich, St Louis, MO, USA). The standard manufacturers protocol was amended to suit the samples taken and is outlined below (Sigma Aldrich, 2014). Once extracted all the samples then underwent cDNA synthesis (see 2.3 cDNA synthesis).

Homogenisation

Add 1mL of TRI Reagent® per 100 mg of tissue (the brains sampled weighed ~140 mg, therefore were homogenised in 1.5 mL of TRI Reagent®, due to the pellet size from livers only 50 mg of tissue was homogenised in 0.5 mL of TRI Reagent®).

1. Cut tissue and add to TRI Reagent® and incubate on ice for 15-60 minutes.
2. Homogenise samples for 30-60 seconds (s) using the bead-beater, until tissue is significantly disrupted. Repeat this step if needed. Fully homogenised samples can be stored at -70 °C for up to one month before completing phase separation.

Phase separation

3. Incubate homogenised samples at room temperature (RT) for 5 minutes.
4. Transfer homogenate to microcentrifuge tube (transfer 1 mL or 0.5 mL depending on tissue).
5. Add 100 µL of 1-Bromo-3-chloropropane (BCP) (per mL of TRI Reagent used) and shake tube vigorously by hand.
6. Incubate at RT for 15 minutes.
7. Centrifuge at 20,000 *g* for 15 minutes at 4 °C.
8. Transfer aqueous upper phase to a new microcentrifuge tube, take two volumes of 150 µL, ensure that the pipette tip is placed just under the surface of the aqueous phase to ensure the interface isn't disturbed and contamination of supernatant is reduced.

RNA precipitation

9. Add ½ volume of RNA precipitation solution and ½ volume of isopropanol to the transferred upper phase. Gently invert samples 4-6 times.
10. Incubate for 10 minutes at RT.
11. Centrifuge at 20,000 *g* for 15 minutes at 4 °C. A pellet should form at the bottom of tube.

RNA wash

12. Remove the supernatant by pipetting.
13. Wash the pellet for 15 minutes at RT in 1 mL of 75 % ethanol. Flick the tube to lift the pellet from the bottom and place the microcentrifuge tube into the Cole-Parmer Tube Rotator 18 (Cole-Parmer, St. Neots, UK) to ensure that the whole pellet and tube are washed.
14. Centrifuge at 20,000 *g* for 5 minutes at RT.
15. Carefully remove the supernatant using a 1 mL pipette, re-spin samples (5 second pulse) and remove all remaining ethanol with a 20 µL pipette.

a. Repeat steps 15-17 for liver samples due to high levels of glycogen in liver tissue.

16. After final wash, air dry RNA pellets until all visible traces of ethanol are gone.

Resuspension of RNA

17. Resuspend in 70 µL nuclease-free water, incubate samples at RT for a minimum of 60 minutes, gently flicking the tubes every 10 minutes to aid resuspension.
18. Measure concentration of RNA using the Nanodrop 2000c spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA)* and Qubit 2.0 Fluorometer (ThermoFisher Scientific, Waltham, MA, USA).
19. Heat an aliquot of RNA (~150 ng/µL to avoid overloading the gel) with X6 loading dye and heat for 5 minutes at 75 °C using the Biometra TAdvanced 96G thermocycler (Analytik Jena, Jena, Thuringia, Germany), chill on ice and then run gel electrophoresis using a 1 % agarose gel to check the integrity of the 28S and 18S rRNA (ribosomal RNA).
20. Due to concentration of RNA ≤ 1000 ng/µL, dilute extracted RNA to 500 ng/µL and store at -70 °C in preparation for cDNA synthesis (see 2.2 cDNA synthesis).

Notes:

*****For RNA the A260/A280 and A260/A230 ratios should be ≥ 1.7, anything below this can indicate amount of sample not great enough, no rest period/ not long enough at RT post-homogenisation, contamination of aqueous phase with phenol from interphase or RNA pellet not fully resuspended. For samples with a low A260/280 and A260/A230 ratio the following protocol was followed.

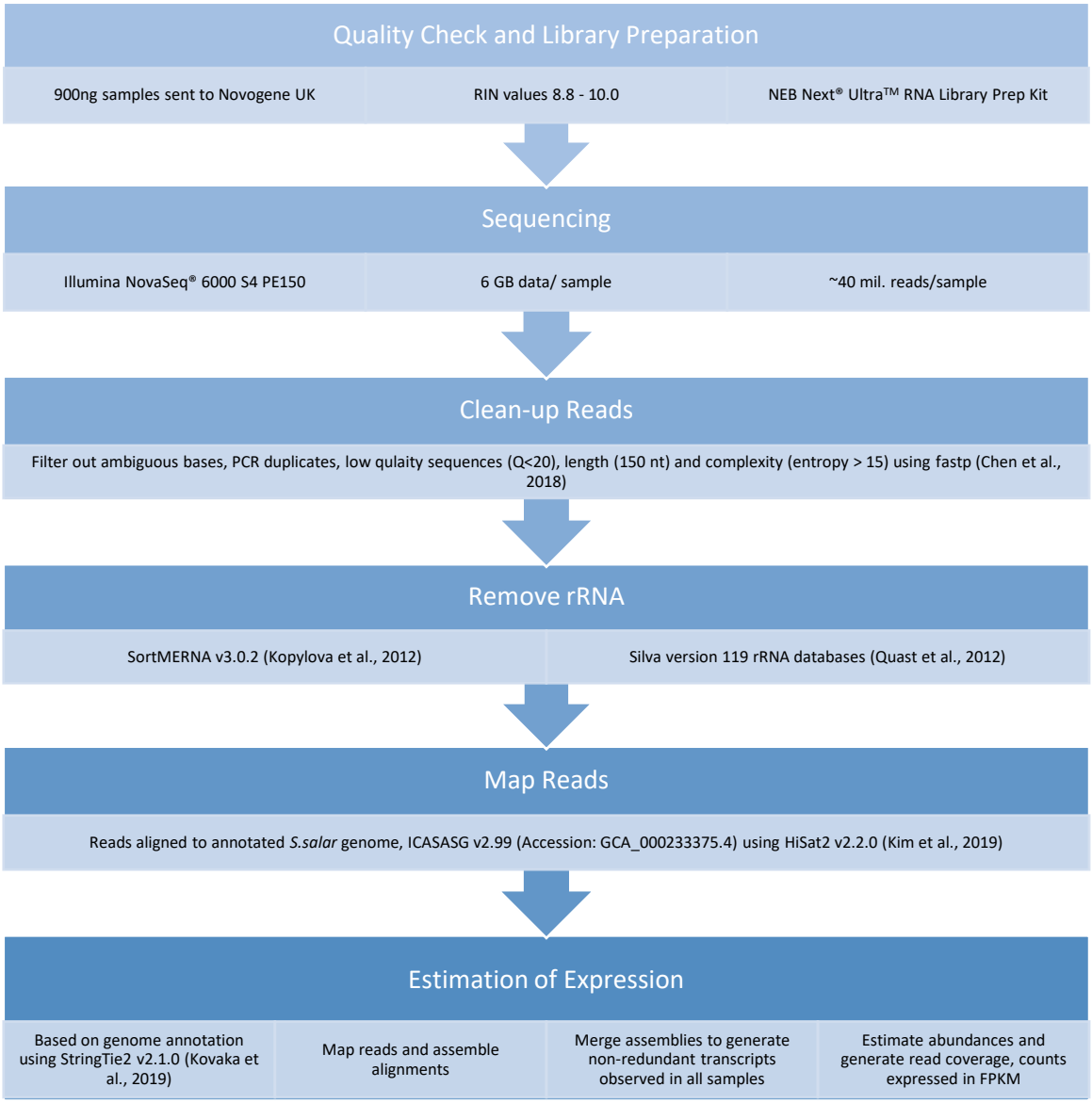
Reprecipitation of samples with a low A260/A280

1. Increase the volume of the RNA up to 500 µL with nuclease-free water.
2. Add 50 µL of 3M NaAc (pH 5.5).
3. Add 500 µL of RT isopropanol.
4. Mix well and leave at RT for 20 minutes.
5. Centrifuge at 20,000 *g* for 10 minutes at 4 °C to pellet RNA.
6. Wash the pellet for 15 minutes twice with 500 µL ice cold 75 % ethanol at RT. Flick the tube to lift the pellet from the bottom and place the microcentrifuge tube into the Cole-Parmer Tube Rotator (Cole-Parmer, St. Neots, UK) to ensure that the whole pellet and tube are washed.
7. Carefully remove the supernatant using a 1mL pipette, re-spin samples (5 s pulse) and remove all remaining ethanol with a 20 µL pipette.
8. After final wash, air dry RNA pellets until all visible traces of ethanol are gone.
9. Resuspend the pellet with 50 µL of nuclease-free water.

2.4. RNA sequencing

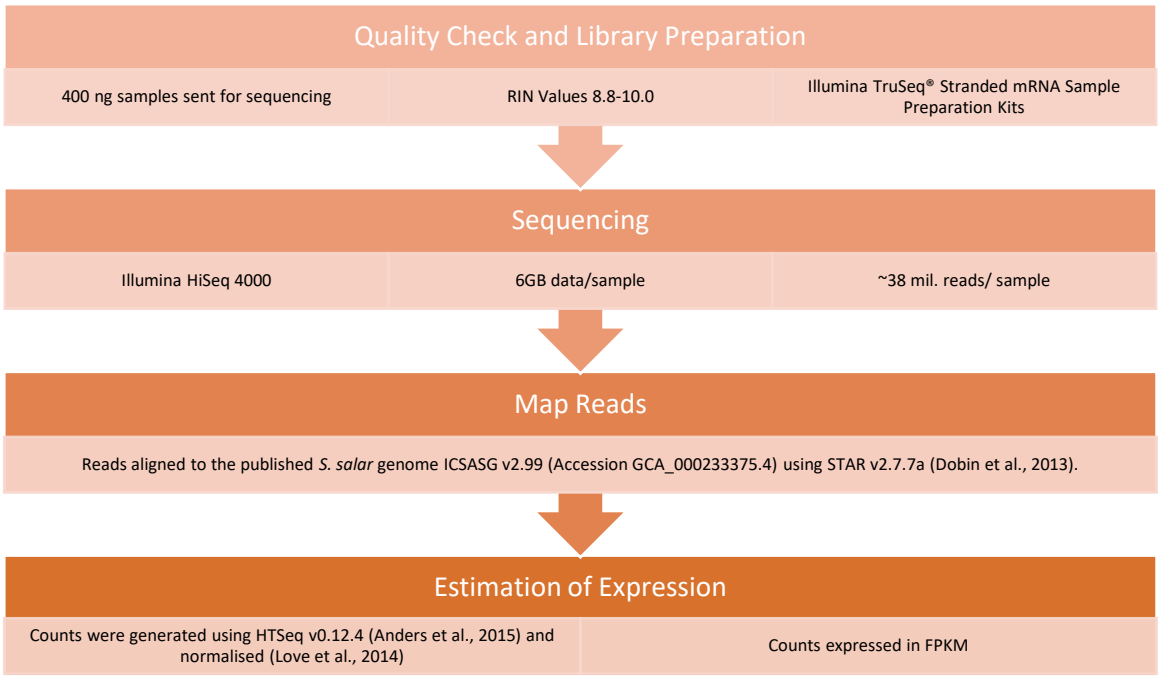
RNA was isolated from the samples using TRI reagent (Sigma, St Louis, MO, USA) and RNA concentration was tested using a Qubit 2.0 Fluorometer (ThermoFisher Scientific, Waltham, MA, USA). All samples collected from the experiments at the UoS and the UiB were submitted to quality control (Illumina BioAnalyzer®) revealing the RNA integrity number (RIN) of samples valued between 8.8-10.0. The workflows for each set of samples differed slightly due to the facility the samples were sent to for sequencing. Samples collected in Chapter Three as the University of Stirling were send to Novogen UK, Cambridge and were processed in accordance with Figure 2.4 below.

Figure 2.4: Workflows for RNA sequencing samples sent from the University of Stirling (Chapter Three) to Novogene UK Cambridge.



Samples collected as part of the study undertaken at the University of Bergen (Chapter Four) were submitted to the Genomics Core Facility at the University of Bergen for RNA sequencing and were processed as outlined by Figure 2.5 below.

Figure 2.5: Workflows for RNA sequencing samples sent from the University of Bergen (Chapter Four) to the Genomics Core Facility at the University of Bergen.



2.5. cDNA synthesis

cDNA was synthesised from the RNA extracted from samples in 2.2 RNA extraction using the QuantiTect® reverse transcription kit (Qiagen, Hilden, Germany). The standard manufacturers protocol was amended to suit the samples taken and is outlined below (Protocol, 2015).

1. Thaw template RNA on ice. Thaw gDNA wipeout buffer, Quantiscript reverse transcriptase, Quantiscript RT buffer and nuclease-free water at RT (15-25 °C). Mix the tubes by flicking the tubes – centrifuge in the bench top microfuge to collect residual liquid form the sides of the tube and then keep on ice. If precipitate forms in gDNA wipeout buffer vortex to dissolve any precipitates.
2. Prepare the genomic DNA elimination reaction on ice according to Table 2.1. Mix and keep on ice.

Table 2.2: Genomic DNA elimination reaction components

Component	**Volume**
Genomic DNA elimination	
gDNA Wipeout Buffer, X7	2 µL
Template RNA (up to 1 µg)	2 µL @ 500 ng/µL
Nuclease-free water	10 µL
Total Reaction Volume	**14 µL**
Reverse-transcription master mix	
Quantiscript reverse transcriptase	1 µL
Quantiscript RT buffer, 5x	4 µL
RT primer mix	1 µL
Template RNA	
Entire gDNA elimination reaction	14 µL
Total Reaction Volume	**20 µL**

3. Incubate for 2 minutes at 42 °C in the Biometra TAdvance 96G thermocycler (Analytik Jena, Jena, Thuringia, Germany), then immediately place on ice.
4. Prepare the reverse transcription master mix on ice according to Table 2.1.
5. Add 6 µL of reverse transcription master mix to the tubes containing the template RNA from the genomic DNA elimination reaction in step 3. Mix and then store on ice.
6. Incubate at 42 °C for 30 minutes in the Biometra TAdvance 96G thermocycler (Analytik Jena, Jena, Thuringia, Germany), then incubate for 3 minutes at 95 °C to inactivate the Quantiscript reverse transcriptase.
7. The reverse transcription reactions were then diluted to a 1/10 working solution in nuclease-free water to a total volume of 150 µL and placed into 96-well plates to ensure ease of plating up multiple 384-well plates in 2.8 qPCR.

2.6. Primer design and validation

From a review of literature, previous clock gene primer pairs and housekeeping genes (HKGs) were identified (Davie, Minghetti and Migaud, 2009; Huang, Ruoff and Fjelldal, 2010a; Betancor *et al.*, 2014; McStay *et al.*, 2014; Vera and Migaud, 2016) and were interrogated using NCBI Primer BLAST to identify potential PCR products *in silico* against the totality of the Atlantic salmon genome. From this investigation it was possible to ascertain that historic primer pairs were not specific. Some primer pairs picked up paralogs resulting from Ss4R as well as sister genes which were the result of the previous teleost specific WGD. In some instances, primer pairs also returned un-related genes *in silico*.

As a result of the *in-silico* investigation (Chapter Three) and interrogation of previous clock gene primers for PCR it was decided to create new primer sequences which would be more specific for this series of work. Sequences for the Atlantic salmon core clock genes were recovered and used to design new primers to distinguish between clock gene family members and between paralogs where possible. Due to the high level of similarity between paralogs > 82 % with single nucleotide substitutions across the gene (rather than conserved to specific regions) it was not possible to distinguish between many of the clock gene paralogs (Ss4R duplicates). Therefore, primers were created from consensus sequences of the paralogs to ensure that they were both picked up in the qPCR assay. Primer products were then checked using NCBI primer-BLAST to identify potential products *in silico*, from 10 potential primer pairs for each gene, one or two were selected per gene (provided they identified both paralogs without picking up sister genes) and ordered. Initially primers were tested using MyTAQ™ HS Mix (Meridian BioScience, Cincinnati, OH, USA) – however as seen in Figure 2.6 there were issues with multiple bands appearing on the gel, even when primers were redesigned.

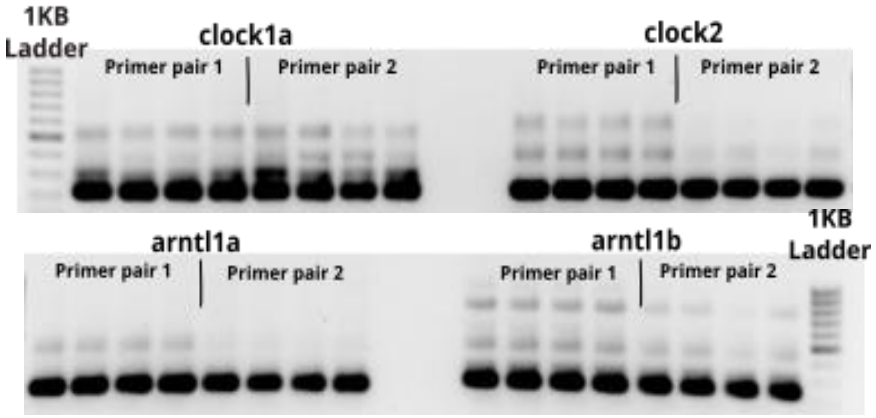


Figure 2.6: Gel electrophoresis of MyTAQ™ HS PCR product from clock gene primer testing for *clock1a, clock2, arntl1a, arntl1b* primer pairs (two per gene, tested in quadruplicate) run on a 1 % agarose gel, with a 1 kb ladder.

Therefore, primers were tested on the LightCycler 480 II® (F. Hoffmann-La Roche Ltd., Basel, Switzerland) using Luminaris ™ Color HiGreen qPCR Master Mix which revealed that there was only one product formed. Therefore, artifacts in Figure 2.1 are likely to be caused by technical error from the thermocycler used. Resulting primer pairs in Table 2.2 were selected based on their annealing temperature, due to the high volume of plates being run this enabled standardisation of the process, thus increasing efficiency with timekeeping. Housekeeping genes (HKGs) were also tested.

Table 2.3: Primer sequences of target clock genes and associated information used for qPCR gene expression assays in Atlantic salmon. T_a – annealing temperature was optimised to 60 °C across all genes.

Gene	ID	Amplicon (bp)	FWD Sequence 5'-3'	REV Sequence 5'-3'	NCBI Accession
Clock Genes					
clock 1a	*clock1a*	187	CGAGTCTGTTTC ATAGCAACTGT	ATCCAGATGTACC CAGAACCTC	XM_014122997 XM_014169004
clock 2	*clock2*	158	AGACGGTTTCC TCATAGCACTA	ATAGCAGCTTATA GACCTCCCC	XM_014137489 XM_014196792
arntl 1a	*arntl1a*	127	GGATACCCATA CTCCAACCAGT	GGAGACTCATGA TCACAGCCAT	XM_014125430 XM_014148046
arntl 1b	*arntl1b*	270	GAGTTGCTGGG TACATCGTTTT	GAGCTGAGGATA GATGCCATCA	XM_014126699 XM_014175326
arntl 2a	*arntl2a*	170	GATGAACAACC TGATCGACGAG	TCAGGGAGGAAT GAAGGCTTAT	XM_014125375
arntl 2b/c	*arntl2bc*	185	AGATGCCAAAA CAGGGTTACAG	AATGGACAGTGC AGTATCGTTG	XM_014153577 XM_014208404
period 1a	*per1a*	171	GTCAGCCAACT ACTCCTACCAG	GAGCCACATTTCC TCTTCACTG	XM_014196530 XM_014128896
period 1b	*per1b*	153	CAACGTCTCGG CATGTATGG	GTCACTTCCTGCT CCTCTGT	XM_014206471
period 2	*per2*	106	CAATGAGGAGT ACTACCAGCTG	GTACTCTGAGGT GATGCTGTCT	XM_014139595 XM_014190838
period 3	*per3*	145	AACTTTCCCAGC CAGAACTTTC	GGTAAGAGGGGT AGTTGGGTAG	XM_014166967
cryptochrome 1a	*cry1a*	203	GAGACTACATA CGGCGCTATCT	GCAGGAGAGCTG TTGGTAGAT	XM_014148221 XM_014125353
cryptochrome 2	*cry2*	265	GGGAGTCTGAA GCACTAGAGAG	GTTCCCGCTGCA TAGAAGAATT	XM_014125728
cryptochrome 3b	*cry3b*	175	CAGCTGACGTT TGAGTGTGAC	TACTTGTCCCTGT GGTTGTCTG	XM_014131850 XM_014167724
cryptochrome 4	*cry4*	287	ATACCCAAGCT GAAGGACTACC	GCGTGTTGTAGA CCTGTTTCAT	XM_014176427
cryptochrome 5	*cry5*	180	GCTGTTTGTGG TGAGAGGTAAG	AGTGTGTGGGAG ACTTTGTAGA	XM_014138346
House Keeping Genes					
beta-2 microglobulin	*b2m*	138	TCCCAGACGCC AAGCAG	TGTAGGTCTTCAG ATTCTTCAGG	XM_014188686 XM_014184299
elongation factor 1 alpha	*ef1a*	175	CTGCCCCTCCA GGACGTTTACA A	CACCGGGCATAG CCGATTCC	NM_001123629

2.7. Cloning and plasmid synthesis

Cloning was undertaken to incorporate a PCR product for each of the target genes identified in Table 2.2 into a plasmid to enable the absolute quantification of the complimentary gene using qPCR. Comprising of PCR and PCR clean-up prior to cloning (ligation, transformation, extraction, and quantification). PCR was carried out using MyTAQ™ HS Mix (Meridian BioScience, Cincinnati, OH, USA).

PCR purification

Prior to cloning the PCR reactions were cleaned up using GeneJET PCR Purification Kit (ThermoFisher Scientific, Waltham, MA, USA). The manufacturer protocol was amended as below (ThermoScientific, 2015).

1. Adjust the volume of the PCR product to 100 µL with nuclease-free water.
2. Add 1:1 volume of binding buffer (100 µL) to completed PCR mixture, mix thoroughly, and ensure colour of solution remains yellow – this indicates optimum pH for DNA binding.
3. Add a 1:2 volume of 100 % isopropanol (100 µL) to the binding buffer, PCR product mixture and mix thoroughly. This increases the target DNA yield for fragments ≤ 500bp.
4. Transfer the solution from step 2 to the GeneJET purification column and centrifuge at 12,000 *g* for 1 minute at RT and discard the flow-through. Place the purification column back into the collection tube.
5. Add 700 µL of wash buffer to the GeneJET (ensure this is diluted with ethanol as instructed) and centrifuge at 12,000 *g* for 1 minute at RT and discard the flow-through. Place the purification column back into the collection tube.
6. Centrifuge the empty GeneJET purification column at 12,000 *g* for a further 1 minute at RT to completely remove any residual wash buffer.
7. Transfer the GeneJET purification column to a clean 1.5ml microcentrifuge tube and 50 µL of elution buffer to the centre of the GeneJET purification column membrane and centrifuge at 12,000 *g* for one minute at RT.
8. Discard the GeneJET purification column and if not using the purified DNA immediately store at -20 °C.

Cloning

Cloning was undertaken using P-GEM T Easy vector with JM109 competent cells, high efficiency (Promega, Madison, WI, USA). Plates were impregnated with ampicillin 100 mg/mL, 0.2 micro filtered (ThermoFisher Scientific, Waltham, MA, USA) and X-Gal (ThermoFisher Scientific, Waltham, MA, USA) and IPTG (ThermoFisher Scientific, Waltham, MA, USA) for blue/white cell competency identification. Once cloned, six colonies per gene selected for on growing were then sent off for sanger sequencing (Eurofins Genomics, Ebersberg, Germany) and selected based on alignment to the target sequence before the serial dilution 10^8 – 10^1 was prepared for use in absolute quantification qPCR.

Ligation

1. Briefly centrifuge the pGEM-T Easy vector and control insert DNA tube to collect contents at the bottom of the tube after thawing.
2. Set up ligation reaction for each target as overleaf (Table 2.4) in clean 0.2 mL PCR tubes and incubate overnight at 4 °C. Vortex 2X Rapid Ligation Buffer vigorously before each use – ensure it is thawed on ice as it is temperature sensitive.

Table 2.4: Ligation reaction components.

Reagents	Standard reaction	Positive Control	Background Control
2X rapid ligation buffer	5 µL	5 µL	5 µL
pGEM-T Easy vector (50 ng)	1 µL	1 µL	1 µL
PCR product *****	n µL	-	-
Control insert DNA	-	2 µL	-
T4 DNA ligase	1 µL	1 µL	1 µL
Deionized nuclease-free water	-	1 µL	3 µL
Total volume	10 µL	10 µL	10 µL

*Molar ratio of PCR product: vector was calculated based on the below calculation (Equation 2.1) for the optimisation of insert: vector molar ratios based on a 3:1 insert: vector molar ratio, PCR product where n is variable.

$$\frac{ng\ of\ vector \bullet kb\ size\ of\ insert}{kb\ size\ of\ vector} \bullet insert{:}\ vector\ molar\ ratio = ng\ of\ insert$$

$$\frac{50\ ng\ of\ vector \bullet 0.187\ kb\ insert}{3.0\ kb\ vector} \bullet \frac{3}{1} = 9.35\ ng\ of\ insert$$

Equation 2.1: General formula for the optimisation of insert: vector ratio calculation for ligation reaction. Formula for the optimisation of *clock1a* gene insert: vector ratio calculation for ligation reaction.

Preparation of Luria-Bertani (LB) agar plates

LB plates were prepared using 500 mL of pre-made LB agar stock. This was gently warmed in the microwave until the agar was dissolved and 45 mL was transferred into 50 mL falcon tubes to enable to solution to cool quicker. Once the temperature was below 55 °C, 45 µL of ampicillin [100 mg/mL stock] was added to the 45 mL of liquid LB agar and inverted several times to ensure it was mixed well. 15 mL of LB agar and ampicillin mix was then poured into a petri dish under aseptic conditions and allowed to dry before being inverted and placed in the incubator at 37 °C prior to use. If plates were made in advance, rather than on the same day of use, plates were stored at 4 °C in the fridge for up to one week until use.

Transformation

1. Turn on the water bath to 42 °C and the shaking incubator to 37 °C and allow them to come up to temperature. Ensure LB agar plates are placed into incubator set to 37 °C to warm.
2. Take the JM109 high efficiency competent cells (200 µL aliquots) out of the -80 °C freezer and thaw the super-competent cells on ice.
3. Mark the clean microcentrifuge tubes (Target, positive control, background control).
4. Gently flick mix the cell via and add 50 µL of cells into clean 1.5 mL PCR tubes.
5. Add 5 µL of ligated DNA to one aliquot of cells, for the positive control only add 1 µL of the ligated DNA to the tube. Swirl the tubes gently, ensure the content of tubes are well mixed.
6. Incubate on ice for 30 minutes.
7. Heat shock the cells for 45 second at 42 °C.
8. Incubate the tubes on ice for 2 minutes.
9. Add 300 µL of LB broth (warmed to 37 °C) without ampicillin to each tube.
10. Incubate at 37 °C for one hour in a shaking incubator at 150 rpm.
11. During this time, prepare a master mix of 80 µL X-gal [20 mg/mL] (ThermoFisher Scientific, Waltham, MA, USA) and 40 µL IPTG [100 mM] (ThermoFisher Scientific, Waltham, MA, USA) per plate and spread 120 µL onto each plate under aseptic conditions (to allow for blue and white colony screening) and allow to dry.
12. Mark up two plates for each target and the positive and background controls and place back into the 37 °C incubator.
13. Spread the cell suspension onto each plate, 150 µL onto one and 200 µL onto the other. Spread gently until the cell suspension is dry. Make sure to place the cell spreader in ethanol and flame it in between each target to prevent contamination. Working under a flame to prevent contamination of the plates.

14. Close the plates with parafilm and incubate upside down to prevent condensation from dripping onto the agar overnight at 37 °C.
15. The next day observe the plates for bacterial colony growth and record observations. Background control should have no colonies growing and there should be colonies present on the positive control (control DNA insert).
16. Add 8 mL of LB and 8 µL of ampicillin to 12 mL bacterial culture tubes (six tubes per target) and label accordingly.
17. Pick one white colony per tube from the corresponding target using a sterile cocktail stick and inoculate in the corresponding tube. Incubate at 37 °C in the shaking incubator at 150 rpm overnight. Ensure that the lids on the bacterial culture tubes are on but not fully closed.
18. After overnight incubation in the shaking incubator at 37 °C check the tubes for turbidity.
19. To preserve the bacterial colonies, take 500 µL of the transformed bacteria from each culture tube and add it to 500 µL of glycerol-LB (50 % glycerol and 50 % LB broth – with no ampicillin) to act as glycerol stocks and store at -70°C.

Plasmid DNA Extraction

Plasmids were purified using the GenElute™ Plasmid MiniPrep Kit (Sigma Alrich, St Louis, MO, USA). Ensure all reagents are thoroughly mixed, if any of the reagents have precipitated ensure that they are warmed to 55-65 °C until the precipitate has dissolved, allow to cool to RT before use. Ensure the wash solution has been diluted with the appropriate amount of 95-100 % ethanol prior to use.

1. To harvest the transformed cells, transfer 5 mL of the transformed bacteria into a 15 mL falcon tube and centrifuge at 12,000 *g* for 1 minute, remove and discard the supernatant (ensure this is disposed of in accordance with cell culture waste protocols).
2. Completely resuspend the cells with 200 µL of resuspension solution, to resuspend the cells scrape the bottoms of the tubes over the bottom of a microcentrifuge tube rack back and forth 5 times, transfer the resuspended cells to a 1.5 mL microcentrifuge tube.
3. Lyse the resuspended cells by adding 200 µL of lysis solution, immediately mix the solution by gentle inversion 4-6 times until the mixture becomes clear and viscous. **DO NOT** vortex – harsh mixing will shear genomic DNA resulting in chromosomal DNA contamination of the plasmid.

   DO NOT allow the lysis reaction to exceed more than 5 minutes – prolonged alkaline lysis may permanently denature supercoiled plasmid DNA, rendering it unsuitable.
4. Precipitate the cell debris by adding 350 µL of the neutralisation/ binding solution. Gently invert the tube 4-6 times. Pellet the cell debris by centrifuging the solution at 12,000 *g* for 10 minutes at RT. If the supernatant is cloudy repeat this step before proceeding onto step 6.

5. Whilst the tubes are being centrifuged in step 4, prepare the columns. Insert a GenElute Miniprep Binding Column into a provided microcentrifuge tube and add 500 µL of the column preparation solution and centrifuge at 12,000 *g* for 1 minute to maximise the binding of DNA to the column membrane. Discard the flow-through liquid.
6. Load the supernatant from step 4 and centrifuge the columns at 12,000 *g* for 1 minute at RT. Discard the flow-through liquid.
7. Wash the column, add 750 µL of the diluted wash solution to the column and centrifuge at 12,000 *g* for 1 minute at RT. Discard the flow-through and re-centrifuge at 12,000 *g* for 2 minutes (without additional wash solution) to completely remove excess ethanol.
8. Transfer the column to a fresh collection tube. Add 100 µL of nuclease-free water to the centre of the membrane and centrifuge at 12,000 *g* for 1 minute. The DNA is now present in the eluate and is ready for immediate usage.
9. If the plasmid is for later use store in the freezer at -20 °C.

Quantification

1. Measure the extracted plasmid using the Nanodrop 2000c spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) to quantify the plasmid DNA.
2. Conduct a PCR on the extracted plasmid of the different targets at a 1/100 dilution to check the integrity of the plasmids, there should be 3 bands on the gel (1 for the product and 2 larger fainter bands from the plasmid).
3. Add 5 µL of plasmid to 5 µL of T7 primer to each 1.5 mL tube and add the corresponding sequencing label and then send the samples off for sequencing (Eurofins Genomics, Ebersberg, Germany).
4. Additional plasmid stocks should be stored in the freezer at -20 °C.
5. Once the sequencing results are returned check each sequence for the forward (+/+) and reverse (+/-) insertion of the PCR product into the plasmid. The plasmids with the highest concentration and complete PCR product sequence were selected (one per target gene) to progress to the serial dilution stage for the absolute qPCR plasmid standards.

Plasmid Standard Serial Dilutions

For ease of replicability and avoiding constantly reprogramming the template on the LightCycler 480 II® (F. Hoffmann-La Roche Ltd., Basel, Switzerland) the plasmids were all diluted in 200 µL of nuclease-free water in relation to the plasmid construct size and PCR product size. They were diluted into a final concentration of 10^8. From this initial dilution it was possible to set up a serial dilution from 10^8 to 10^1 to ensure that there were enough points on the standard curve to enable a greater efficiency and therefore more accurate measurement of the samples (Table 2.5).

Table 2.5: Initial plasmid dilution based on total plasmid construct size.

Gene Id	Insert (bp)	Vector (bp)	Plasmid Construct (bp)	Mass of 1 plasmid (ng)	Mass of 10^9 copies (ng)	Plasmid Conc. (ng/ µL)	Vol. of plasmid (µL)
clock1a	187	3015	3202	$3.49 \cdot 10^{-9}$	0.349	8.76	7.97
clock2	158	3015	3173	$3.46 \cdot 10^{-9}$	0.346	3.06	22.61
arntl1a	127	3015	3142	$3.42 \cdot 10^{-9}$	0.342	5.28	12.97
arntl1b	270	3015	3285	$3.58 \cdot 10^{-9}$	0.358	4.06	17.64
arntl2a	170	3015	3185	$3.47 \cdot 10^{-9}$	0.347	5.46	12.72
arntl2b/c	185	3015	3200	$3.49 \cdot 10^{-9}$	0.349	3.48	20.05
per1a	171	3015	3186	$3.47 \cdot 10^{-9}$	0.347	72.80	0.95
per1b	153	3015	3168	$3.45 \cdot 10^{-9}$	0.345	11.60	5.95
per2	106	3015	3121	$3.40 \cdot 10^{-9}$	0.340	51.60	1.32
per3	145	3015	3160	$3.44 \cdot 10^{-9}$	0.344	15.60	4.42
cry1a	203	3015	3218	$3.51 \cdot 10^{-9}$	0.351	16.20	4.33
cry2	265	3015	3280	$3.58 \cdot 10^{-9}$	0.358	70.00	1.02
cry3b	287	3015	3302	$3.60 \cdot 10^{-9}$	0.360	34.60	2.08
cry4	175	3015	3190	$3.48 \cdot 10^{-9}$	0.348	64.20	1.08
cry5	180	3015	3195	$3.48 \cdot 10^{-9}$	0.348	75.6	0.92
elf1a	175	3015	3190	$3.47 \cdot 10^{-9}$	0.347	11.60	5.95
b2m	138	3015	3153	$3.42 \cdot 10^{-9}$	0.342	14.34	4.79

2.8. qPCR

Absolute quantification qPCR assays were designed for genes described in Table 2.2, qPCR was performed in duplicate on individual samples using a LightCycler 480 II® (F. Hoffmann-La Roche Ltd., Basel, Switzerland) and Luminaris ™ Color HiGreen qPCR Master Mix (ThermoFisher Scientific, Waltham, MA, USA) in the reaction in Table 2.6 in 384-well plates in a standardised format for each experiment.

Table 2.6: qPCR Reaction components.

Component	**Volume**	**Concentration**
Luminaris ™ Color HiGreen qPCR Master Mix	5.0 µL	-
Forward Primer (10 pmol/ µL)	0.5 µL	5 pmol
Reverse Primer (10 pmol/ µL)	0.5 µL	5 pmol
Nuclease-free water	2.0 µL	-
cDNA	2.0 µL	-
Total Reaction Volume	**10.0 µL**	**-**

The thermocycling programme consists of a pre-incubation stage at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 s, 60 °C (annealing temperature – uniform across all genes) for 30 s and 72 °C for 30 s. Followed by a temperature ramp from 60 °C to 90 °C to enable melt curve analysis to verify the absence of any primer-dimer artefacts and the presence of only one product from each qPCR assay. Quantification of gene concentration was quantified using a parallel set of reactions containing standardised plasmids for each of the target genes ranging from 10^8 to 10^1 copies, in duplicate (see 2.5 Cloning and Plasmid Sequencing). qPCR results are expressing in copy number.

2.8.1. qPCR troubleshooting

In addition to the samples sent off for RNA sequencing in Chapter Three, further samples were taken from the liver with the aim of identifying the differences in clock gene expression between central (brain) and peripheral (liver) tissues to further our understanding of the central vs peripheral clock gene expression and their entrainment by light. To cross validate the qPCR results, the expression patterns of clock genes in the samples sent for sequencing were compared. The qPCR results revealed similarities in the gene expression patterns with regard to peaks and troughs of most singletons (*arntl2a, per1b, per3, cry2, cry4, cry5*) for which qPCR primers were able to be designed.

When looking at the expression of paralogs, the RNA expression profiles were combined to create an expression pattern closer to what we could reasonably expect, again there were some similarities. However, when statistically analysing the expression for rhythmicity (RAIN) and cyclicity (MetaCycle with JTK) there was a distinct lack of rhythmicity or cyclicity identified with only *arntl1a* appearing to be significantly cyclically expressed (p = 0.045). The level of significance was also much lower than that identified in the RNA sequencing results (Table 2.7).

Table 2.7: Cross-validation of absolute quantification qPCR against RNA sequencing results for rhythmical and cyclical core clock gene expression in Atlantic salmon parr brain under 12:12 LD light regime ($p < 0.05$).

		Rhythmicity/ Cyclicity			
Gene ID	**Single/ Duplicated**	**RNA sequencing**		**Absolute qPCR**	
		RAIN	**MetaCycle w/ JTK**	**RAIN**	**MetaCycle w/ JTK**
clock1a	duplicated	$1.85 \cdot 10^{-6}$	$1.80 \cdot 10^{-2}$	-	-
clock2	duplicated	$2.05 \cdot 10^{-4}$	$1.94 \cdot 10^{-2}$	-	-
arntl1a	duplicated	$2.15 \cdot 10^{-9}$	$1.14 \cdot 10^{-5}$	-	0.045
arntl1b	duplicated	$1.21 \cdot 10^{-9}$	$1.09 \cdot 10^{-8}$	-	-
arntl2a	single	$6.49 \cdot 10^{-4}$	$3.55 \cdot 10^{-3}$	-	-
arntl2bc	duplicated	$1.35 \cdot 10^{-5}$	$2.63 \cdot 10^{-4}$	-	-
per1a	duplicated	$1.54 \cdot 10^{-10}$	$1.13 \cdot 10^{-6}$	-	-
per1b	single	$7.10 \cdot 10^{-6}$	$4.22 \cdot 10^{-3}$	-	-
per2	duplicated	$2.92 \cdot 10^{-8}$	$3.46 \cdot 10^{-5}$	-	-
per3	single	-	-	-	-
cry1a	duplicated	-	-	-	-
cry2	single	-	-	-	-
cry3b	duplicated	$7.40 \cdot 10^{-4}$	-	-	-
cry4	single	$2.46 \cdot 10^{-2}$	$1.33 \cdot 10^{-2}$	-	-
cry5	single	$3.47 \cdot 10^{-2}$	-	-	-

Whilst there is questionability over the reliability of the data, results for the statistical analysis on the first and second sampling periods are shown for both tissue types under the continuous light and 12:12 LD (Table 6.3 and Table 6.4).

Table 2.8: First 24 h sampling, rhythmic and cyclically expressed core clock genes in the brain and liver under 12:12 LD or continuous light regimes ($p < 0.05$).

	LD (12:12)				LL (continuous light)			
	Brain		Liver		Brain		Liver	
Gene ID	**RAIN**	**JTK**	**RAIN**	**JTK**	**RAIN**	**JTK**	**RAIN**	**JTK**
clock1a	-	-	0.013	0.004	-	-	-	-
clock2	-	-	-	-	-	-	-	-
arntl1a	-	-	-	-	-	-	-	0.008
arntl1b	-	-	-	-	-	-	-	-
arntl2a	-	-	$7.45 \bullet 10^{-4}$	$1.56 \bullet 10^{-5}$	-	0.030	-	0.025
arntl2bc	-	-	-	$6.23 \bullet 10^{-5}$	-	-	-	0.003
per1a	-	-	$4.03 \bullet 10^{-5}$	-	-	0.006	-	-
per1b	-	-	0.016	-	-	-	-	-
per2	-	-	$7.45 \bullet 10^{-4}$	0.001	-	0.01	-	-
per3	-	-	0.013	0.002	-	-	-	-
cry1a	-	-	0.003	$6.62 \bullet 10^{-5}$	-	0.024	-	-
cry2	-	-	0.019	0.009	-	-	-	-
cry3b	-	-	$9.14 \bullet 10^{-5}$	-	-	0.040	-	-
cry4	-	-	-	0.003	-	-	-	0.011
cry5	-	-	-	0.004	-	-	-	0.018

Table 2.9: Second 24 h sampling, rhythmic and cyclically expressed core clock genes in the brain and liver under 12:12 LD or continuous light regimes ($p < 0.05$).

	LD (12:12)				LL (continuous light)			
	Brain		Liver		Brain		Liver	
Gene ID	**RAIN**	**JTK**	**RAIN**	**JTK**	**RAIN**	**JTK**	**RAIN**	**JTK**
clock1a	-	-	-	-	-	-	-	-
clock2	-	-	-	-	-	-	-	-
arntl1a	-	0.045	-	-	-	-	-	-
arntl1b	-	-	-	-	-	-	0.043	-
arntl2a	-	-	-	-	-	-	-	-
arntl2bc	-	-	-	-	-	-	-	-
per1a	-	-	0.017	-	0.015	-	-	-
per1b	-	-	-	-	0.025	-	-	-
per2	-	-	-	-	-	-	-	-
per3	-	-	-	-	-	-	-	-
cry1a	-	-	-	-	-	-	-	-
cry2	-	-	-	-	-	-	-	-
cry3b	-	-	-	-	-	-	-	-
cry4	-	-	-	-	-	-	-	0.021
cry5	-	-	-	-	-	-	-	-

There appears to be more genes which are significantly rhythmic and cyclically expressed in the LD 12:12 liver samples from the first sampling point. However, it is important to note that comparatives should not be drawn from these results as there is clear disparity between the results from RNA sequencing and those identified by qPCR. Therefore, troubleshooting was conducted to elucidate the potential issues with the samples which were analysed by qPCR.

Records were kept detailing the dates and descriptions of all samples and processes undertaken with regard to this experimental chapter. To gain a better overview of the process and the delays faced due to COVID-19 these are outlined in Table 6.5. From this record, it was easy to identify the scope of delays faced and identify where to start in the troubleshooting process.

Table 2.10: Record of lab-based protocols relating to the samples collected.

Date	Sample Description	Description
19/08/2020	brains 1-84 and livers 1-84	Sampling
25/08/2020	brains 85-168 and livers 85-168	Sampling
17/09/2020	brains 85-90, 97-102	RNA extraction
18/09/2020	brains 109-114, 121-126, 133-138, 145-150, 157-162	RNA extraction
23/09/2020	brains 85-90, 97-102, 109-114, 121-126, 133-138, 145-150, 157-162	RNA sequencing samples sent
12/10/2020	brains 1-10	RNA extraction
13/10/2020	brains 11-60	RNA extraction
14/10/2020	brains 61-84, 91-96	RNA extraction
15/10/2020	brains 103-108, 127-132, 151-156, 163-168	RNA extraction
19/10/2020	livers 1-60	RNA extraction
21/10/2020	livers 61-120	RNA extraction
22/10/2020	livers 121-168	RNA extraction
02/02/2021	brains 1-10	cDNA synthesis
11/02/2021	brains 11-70	cDNA synthesis
12/02/2021	brains 71-168, livers 1-52	cDNA synthesis
15/02/2021	livers 53-168	cDNA synthesis
07/09/2021	*b2m* brain and liver	qPCR
08/09/2021	*clock1a* brain and liver	qPCR
09/09/2021	*ef1a* brain and liver, *clock1a* brain – REPEAT	qPCR
14/09/2021	*rpl2* brain and liver, *clock2* brain, *arntl1a* brain	qPCR
15/09/2021	*clock2* liver, *arntl1a* liver, *arntl1b* brain, *arntl2a* brain	qPCR
16/09/2021	*arntl1a* liver – REPEAT, *arntl1b* liver, *arntl2a* liver, *arntl2b/c* brain and liver	qPCR
18/11/2021	*per1a* brain, *per1b* brain, *per2* brain, *per3* brain	qPCR
26/11/2021	*per1a* liver, *per1b* liver, *per2* liver, *per3* liver	qPCR
02/12/2021	*cry1a* brain, *cry2* brain, *cry3b* brain, *cry4* brain	qPCR
03/12/2021	*cry1a* liver, *cry2* liver, *cry3b* liver, *cry4* liver	qPCR
20/12/2021	*cry1a* liver – REPEAT, *cry4* liver – REPEAT, *cry5* brain and liver	qPCR
10/02/2022	REPEAT cDNA synthesis for Brain LD 2nd sampling	cDNA synthesis
10/02/2022	*arntl2b/c* comparative qPCR to check cDNA synthesis, old vs new.	qPCR
10/02/2022	Re-check RNA integrity by running a gel electrophoresis on a 1% agarose gel	RNA QC

Working backwards, the first stage which was investigated was the protocol surrounding plating up and running of the 384-well qPCR plate. There were a few genes which were lowly expressed, especially in the liver. This was indicated by the high cycle number in which gene expression started to be picked up by the light cycler.Two genes which were re-run were *cry1a* and *cry4*, this verified in fact that the low expression levels were replicable, with highly similar CP values and resulting copy numbers.

From this point onward all troubleshooting was undertaken on the LD (12:12) brain samples collected during the second 24 h sampling period, as these samples were also sent off for RNA sequencing (2020-09-23). Thus, the expression pattern was able to be compared with the results from the qPCR as the results should cross-validate.

After checking to ensure that there was no human error introduced from the qPCR preparation. The next logical area for consideration was the cDNA synthesis process. To validate the cDNA synthesis process worked initially a quality control check was undertaken. This involved running a PCR to check for the presence of DNA of the correct amplicon size for a known primer pair when the PCR product was checked by running gel electrophoresis in a 1 % agarose gel. To validate the original cDNA synthesis process it was decided to repeat the step for the 42 samples which represent LD brain – 2nd sampling, using the excess RNA which was still stored at -70 °C. Once the cDNA synthesis was repeated, the new cDNA was tested alongside the old cDNA in a qPCR for the gene *arntl2b/c* to identify if the expression patterns were similar, and whether they matched the expression pattern identified from the original qPCR run on the old cDNA. These expression patterns were then compared to the combined expression pattern for *arntl2b* and *arntl2c* from the RNA sequencing results in Chapter Three (Figure 2.7).

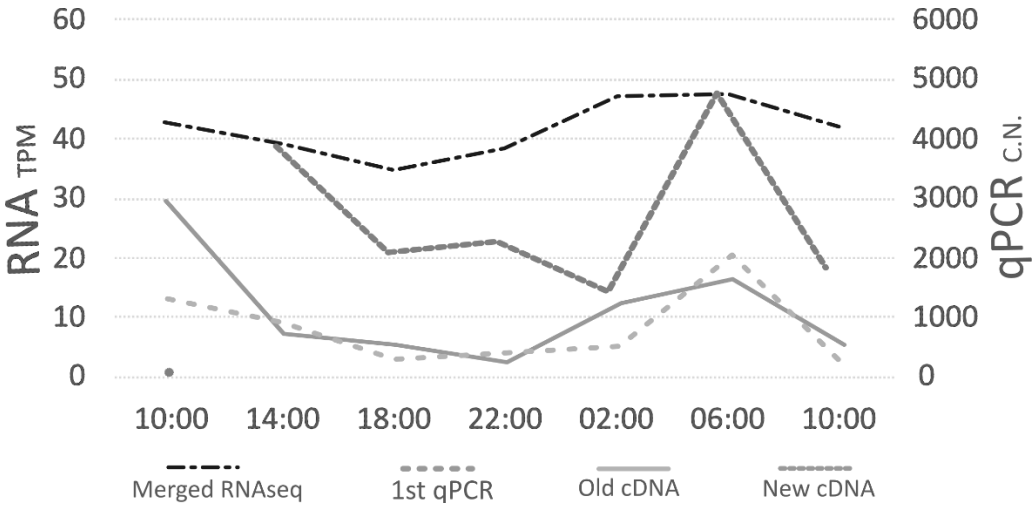


Figure 2.7: Comparison of expression patterns for *arntl2b/c*, from RNA sequencing, original qPCR assay and a repeated qPCR assay comparing old and new cDNA in attempt to troubleshoot strange qPCR results.

From the repeated cDNA synthesis and subsequent qPCR assay, it was identified that there was no quantifiable expression at the 10:00 [1] timepoint – indicative of RNA degradation. When removing the samples at this point the expression pattern for all three qPCR assays share key characteristics, peaks at 14:00 and 06:00, troughs around 18:00 02:00 (repeated assay comparing old and new cDNA only).

The trough at 18:00 shares similarities with the RNA sequencing but the major peak appears to be out of phase by 4 h. Therefore, it was concluded that due to the partial mismatch of expression patterns there was likely an issue with the RNA.

Due to the length of time between initial homogenisation and RNA extraction it was no longer possible to re-extract RNA from the homogenate which had been stored in in the freezer at -70 °C. As by the time troubleshooting was commenced the samples had long exceeded the manufacturer's recommended maximum storage in TRI-reagent time of one month by up to an additional 17-months. Therefore, samples were re-run on a gel electrophoresis for the 42 LD brain 2nd time point samples to compare to initial gels run when the RNA was first extracted.

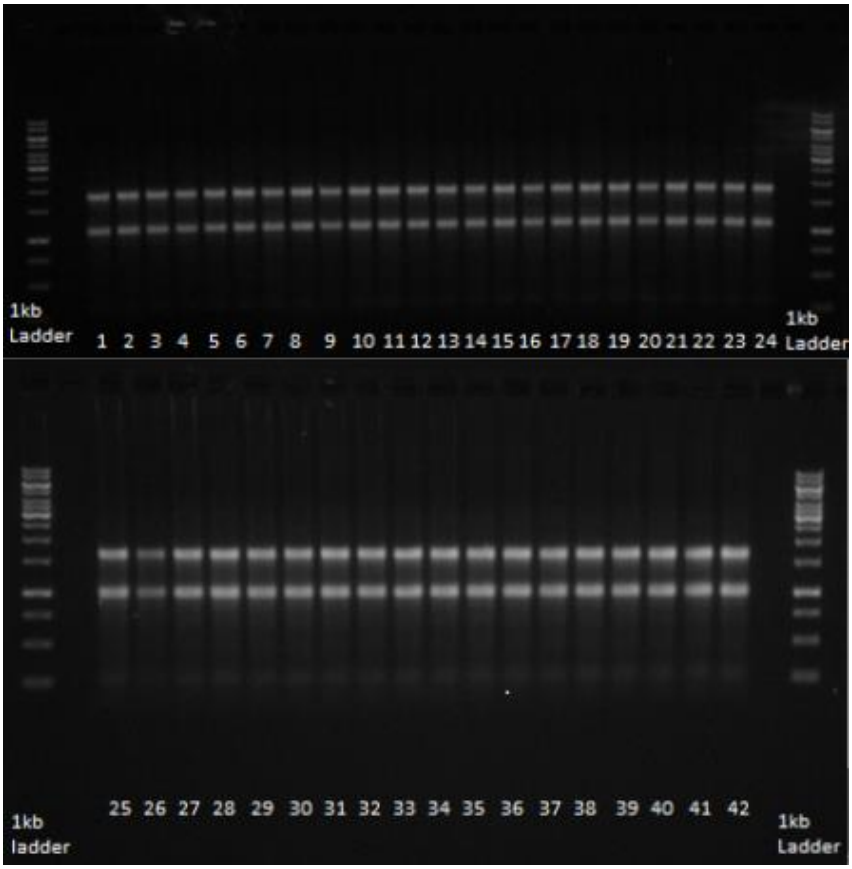


Figure 2.8: Gel images from trans illuminator at 80 ms exposure time from RNA (150 ng/µL) run on a 1 % agarose gel at 70 V for 45-minutes to quantify quality of RNA after RNA extraction prior to sending 42 LD brain samples from the second sampling point for RNA sequencing and cDNA synthesis.

RNA integrity numbers (RIN) reported by Novogene UK (Cambridge) for the 42 samples sent for RNA sequencing were reported within the range of 8.8 – 10.0 (Chapter Three). Therefore, it is certain that at the point of extraction the quality of the RNA extracted was more than adequate (Figure 2.8) and unlikely to influence the gene expression patterns to the extent that was seen in this experiment. As such the issues must have arisen whilst the samples were stored for an extended period of time in the -70 °C freezer.

RNA concentrations were measured prior to extraction and all samples were diluted down to 500 ng/µL using nuclease-free ultra-pure water for ease of use when progressing to the cDNA synthesis step. Samples were taken out of the freezer after being stored for 4-5 months at -70 °C prior to completing the cDNA synthesis process (Table 2.10). Quality control checks after cDNA synthesis showed the presence of a PCR product when a PCR was run, indicative of the presence of cDNA following successful cDNA synthesis from RNA. Following the repeated synthesis of cDNA from the RNA which was still stored at -70 °C, the 42 LD brain samples from the second sampling point were re-run on a 1 % agarose gel to check the quality of the RNA (Figure 2.9).

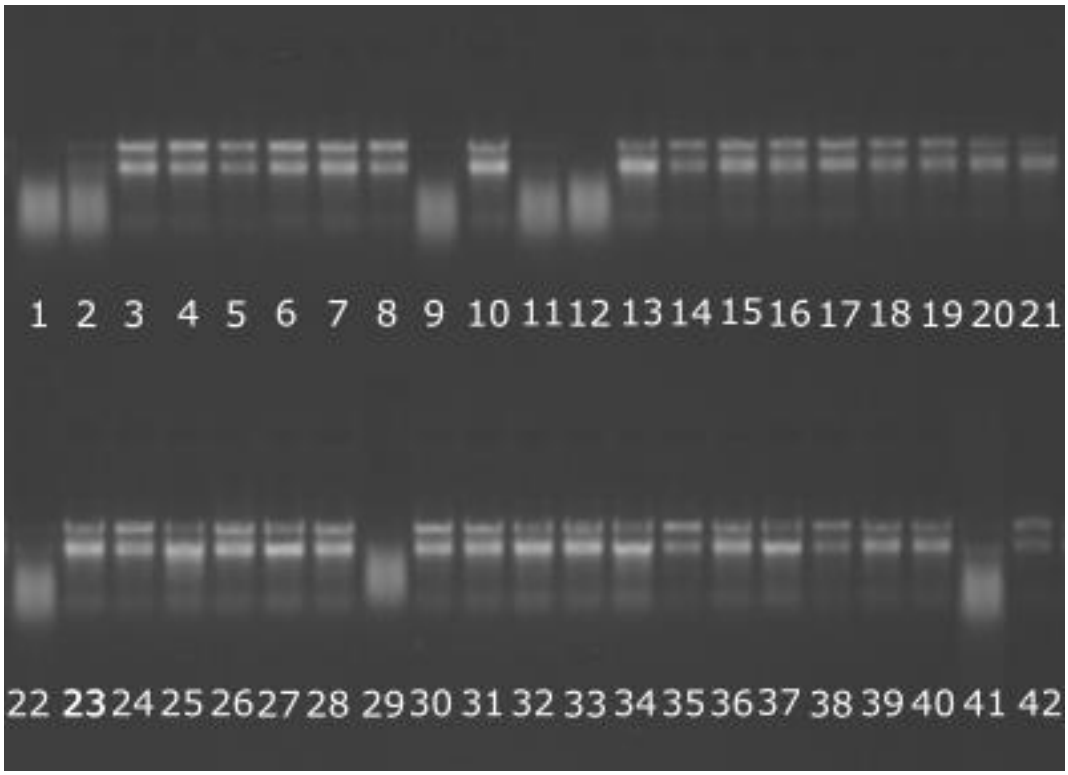


Figure 2.9: Gel images from trans illuminator at 80 ms exposure time from RNA (150 ng/µL) run on a 1 % agarose gel at 70 V for 45-minutes to quantify quality of RNA after long term storage at -70 °C.

From the repeated gel image of the 42 LD brain samples (2nd sampling) it is apparent that there is varied levels of degradation of the RNA in samples which has occurred between extraction and re-analysis. The gene expression patterns for all qPCR regardless of time of analysis and whether it was the first or repeated cDNA synthesis step were highly similar to one another and displayed large individual variance. Therefore, it is likely that the degradation of RNA occurred prior to the synthesis of cDNA when the samples remained in the freezer for 4-5 months due to COVID-19 legislation and the restriction of travel due to geographic location of residency meaning access to the laboratory facilities to complete the work was delayed until travel was permitted.

Core clock gene expression patterns un-normalised or normalised (using the geometric mean of HKGs *ef1a* and *b2m*) did not conform to what we reasonably expected to find from the results of the RNA sequencing (Chapter Three), even after combining the expression of paralogs with high similarity for which we were unable to distinguish by qPCR. The number of significantly rhythmically or cyclically expressed genes identified decreased upon normalisation of the gene expression values (copy number). There was no consistency between the first and second 24 h sampling periods, additionally there was also a lack of significantly rhythmically or cyclically expressed genes across the totality of treatments, regardless of tissue type or photoperiod. Whereas, from the results we identified in both Chapter Three and post-first feeding in Chapter Five we expected to find the majority genes in the corresponding brain samples to exhibit significantly rhythmic or significantly cyclical expression patterns, entrained over a period of 20, 24, or 28 h. Which was not the case. After rigorous troubleshooting of every step and tracing sample history, the likely explanation for the lack of significance identified is issues with temperature stability during the storage of the samples at -70 °C between the stages of RNA extraction and cDNA synthesis resulting in the varied level of degradation in the RNA and high level of individual variance identified from the qPCR assays. Samples were stored for extended periods of time beyond what was planned due to localised lockdowns restricting the ability to complete lab-based work because of COVID-19.

During this time there were issues with temperature control in the -70 °C freezer in which the samples were being stored, this was discovered when the alarm was set off by the freezer thawing to -50 °C. This resulted in them being transferred to another -70 °C freezer, in the external storage facilities. Once the fault with the original freezer was corrected samples were then moved back to the internal freezer in which they were originally stored.

These fluctuations in temperature as the result of mechanical issues with the freezer in which they were stored may have contributed to the varied level of degradation of RNA which was identified from the troubleshooting conducted in this chapter. At the time of RNA extraction all of the whole brain homogenate was used.

For the liver samples, there was still additional homogenate remaining in the freezer due to the reduced quantity needed to complete the extraction protocol. However, at the time of testing this had been stored in the -70 °C freezer in TRI reagent for over 18-months, long exceeding the recommended storage period by the manufacturer [Sigma] of 1-month. It was decided not to trouble shoot the liver samples by repeating the RNA extraction step as the effects of tissues being stored in TRI-reagent for extended periods of time was unknown. Also, there was no corresponding RNA sequencing data against which to cross-validate the results.

Unfortunately, due to the critical technical issues which resulted in the varied level of degradation of the RNA samples the qPCR data collected was not analysed fully due to the inability to assure an accurate conclusion. To further explore the effects of central and peripheral clock gene expression and the influence of light environment upon these expressions it is recommended that this experiment be repeated at a further date to further bridge the gap in our understanding of the influence of photo cues upon core clock gene oscillations in central and peripheral tissue types. To broaden the reach of the experiment it is suggested that there are several amendments to the original experimental plan. This includes conducting the study in a RAS system to counteract the effect of daily temperature fluctuations experienced in flow-through systems due to the increased ability to control water temperature. In addition to this, it is proposed that additional tissues are sampled for, to gain a broader insight into the dissociation and localisation of clocks in Atlantic salmon.

2.9. Statistical analysis

All statistical analysis for each of the three experimental chapters was performed using R v4.1 (RDC, 2010). An overview of the statistical analysis used within these chapters can be seen in Figure 2.10.

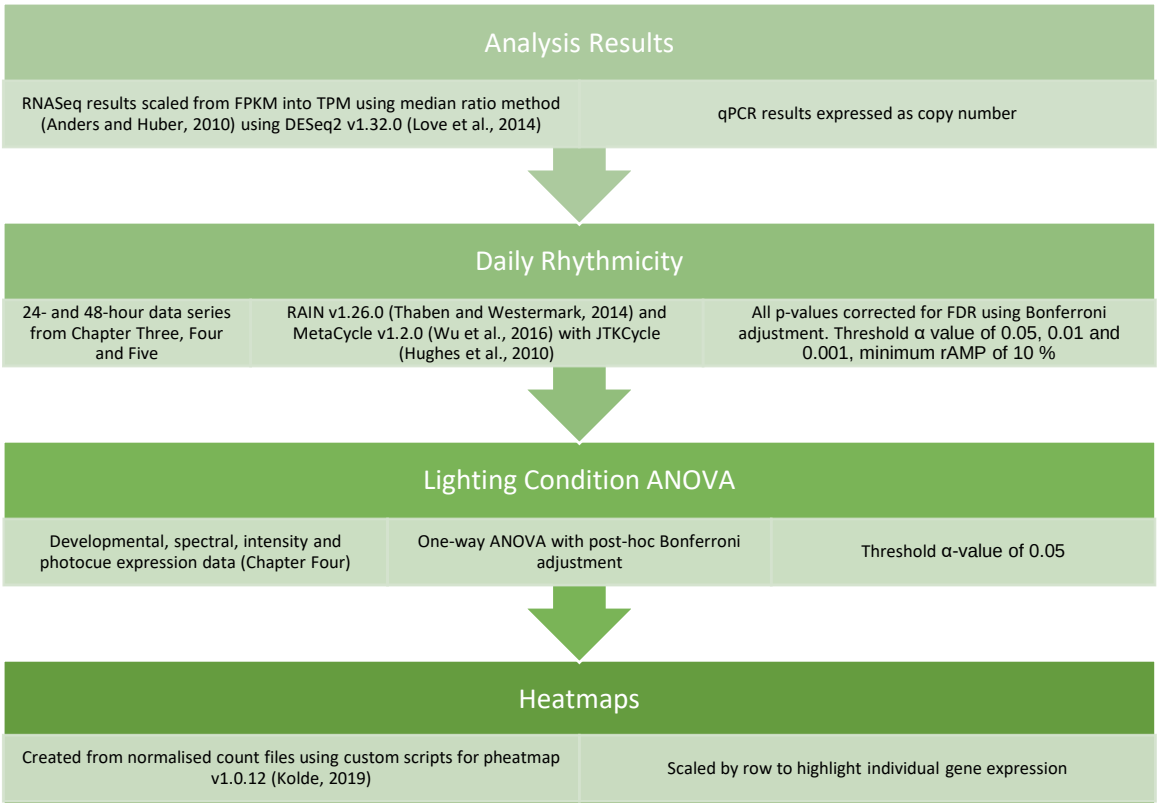


Figure 2.10: Workflows for statistical analysis of RNA sequencing and qPCR results

Chapter Three
Rhythmic clock gene expression in Atlantic salmon parr brain

Bolton, C. M., Bekaert, M., Eilertsen, M., Helvik, J. V., and Migaud, H. (2021). Rhythmic Clock Gene Expression in Atlantic Salmon Parr Brain. *Front. Physiol.* 12, 2173. doi:10.3389/fphys.2021.761109.

3.1. Abstract

To better understand the complexity of clock genes in salmonids, a taxon with an additional whole genome duplication, an analysis was performed to identify and classify gene family members (*clock*, *arntl*, *period*, *cryptochrome*, *nr1d*, *ror*, and *csnk*1). The majority of clock genes in zebrafish and Northern pike, appeared to be duplicated. In comparison to the 29 clock genes described in zebrafish, 48 clock genes were discovered in salmonid species. There was also evidence of species-specific reciprocal gene losses conserved to the *Oncorhynchus* sister clade. From the six *period* genes identified three were highly significantly rhythmic, and circadian in their expression patterns (*per1a.1, per1a.2, per1b*) and two was significantly rhythmically expressed (*per2a, per2b*). The transcriptomic study of juvenile Atlantic salmon (parr) brain tissues confirmed gene identification and revealed that there were 2,864 rhythmically expressed genes ($p < 0.001$), including 1,215 genes with a circadian expression pattern, of which 11 were clock genes. The majority of circadian expressed genes peaked two hours before and after daylight. These findings provide a foundation for further research into the function of clock genes circadian rhythmicity and the role of an enriched number of clock genes relating to seasonal driven life history in salmonids.

3.2. Introduction

The importance of biological time keeping is apparent across all organisms, from bacteria to humans (Wulund and Reddy, 2015). These time-dependent adaptations can last seconds or minutes and recur throughout the day (ultradian) or endure days or months (infradian). They are an evolutionary trait which enables all living organisms to maximise fitness in anticipation of endogenous (molecular and cellular) and external stimuli or zeitgebers (Andreani *et al.*, 2015; López-Olmeda, Sánchez-Vázquez and Fortes-Silva, 2021). Circadian rhythms are the oscillatory expression of genes with a periodicity of approximately 24-hours (Rijo-Ferreira and Takahashi, 2019). These rhythms can be result of expression of endogenous clocks which synchronise biochemical, physiological, and behavioural responses enabling organisms to respond to diel environmental changes (Li *et al.*, 2015). The transcription of 43% of all protein-coding genes in mice displayed circadian rhythms across numerous organs (Zhang *et al.*, 2014). Clock genes are of particular interest in salmonids due to their association with physiological traits such as reproduction migration and smoltification (Leder, Danzmann and Ferguson, 2006; O'Malley, Camara and Banks, 2007; O'Malley, McClelland and Naish, 2010; Paibomesai *et al.*, 2010). Allelic diversity and variation in length polymorphism of the *clock* PolyQ domain was reported in four Pacific salmon species (chinook, chum, coho and pink) with overlapping geographical ranges and diversity in spawning times.

This implicates clock gene duplicates may be involved in the seasonal and geographical variation in reproduction (O'Malley, Camara and Banks, 2007; O'Malley, McClelland and Naish, 2010).In addition, a copy of the gene *clock* has been localised to a quantitative trait locus (QTL) responsible for 20-50% of the variation in spawning dates in female rainbow trout (Leder, Danzmann and Ferguson, 2006).

The circadian clock consists of intracellular transcriptional-translational feedback loops (TTFL) composed of core clock genes and stabilising accessory loop genes, which drives the rhythmic accumulation of downstream outputs, or clock-controlled genes (Reppert and Weaver, 2001; Partch, Green and Takahashi, 2014). Circadian mechanism is highly conserved across animal species (Lowrey and Takahashi, 2011). However, deciphering the circadian clock mechanism in fish is complex. Clock and clock-related genes which are found in single copies in invertebrates such as *clock*, *period* and *cryptochrome* are duplicated in vertebrates (Tauber et al., 2004). In addition, salmonids were subjected to two rounds of whole-genome duplication (WGD) events (Ts3R, teleost-specific third whole-genome duplication, 320 million years ago, and Ss4R, salmonid-specific fourth whole-genome duplication, 80 million years ago) resulting in an abundance of circadian related genes (Lien *et al.*, 2016; Huang, 2018).

The molecular mechanisms underlying circadian rhythmicity have been characterised in several model animal species including the fruit fly (*Drosophila melanogaster*), mice (*Mus musclus*), and humans (*Homo sapiens*), with relatively limited work undertaken in teleosts (Wang, 2008b; Huang, 2018) predominantly centred on zebrafish (*Danio rerio*) (Cahill, 2002; Vallone, Santoriello, *et al.*, 2007). The circadian system comprises all the different components by which light is perceived by the organism and is transformed into a nervous or hormonal signal (Falcón *et al.*, 2010). Therefore, manipulations of photic inputs and cues impact rhythms, which can be commercially exploited for aquaculture production (Falcón *et al.*, 2010). Research based on zebrafish has been fundamental in describing and broadly characterising clock genes and circadian rhythmicity as many findings are applicable to numerous vertebrate species. However, fish models have not yet significantly contributed to our understanding of core clock mechanisms and circadian clock control of fish physiology (Frøland Steindal and Whitmore, 2019). Teleosts represent the largest and most diverse group of vertebrates, with over 30,000 species identified to date. Each species possesses distinct characteristics and displays a considerable amount of anatomical and physiological plasticity. This is arguably the direct result of exposure to multiple, variable selection pressures caused by the highly dynamic aquatic environments they have inhabited throughout their evolutionary development (Bone, 2019). Alongside selection pressures, multiple rounds of WGD have had a large impact upon the evolution of lineages

to date, with the retention of resultant gene duplicates being biased with regard to gene function (Brunet *et al.*, 2006).

Gene duplication through WGD led to redundant genes either lost, non-functionalisation, with a different function than the ancestral gene (sub-functionalisation) or which acquired new functions, neo-functionalisation, (Pasquier *et al.*, 2016), thus resulting in genome reshaping (Inoue *et al.*, 2015). An example of this is the salmonid specific WGD (Ss4R) which preceded the origin of anadromy in salmonids, an important milestone in the evolutionary development of salmonid migration (Alexandrou *et al.*, 2013). Whilst WGD events led to gene duplication, these duplicated genes typically resolved over time (Inoue *et al.*, 2015). In rainbow trout, 80 to 100 million years post Ss4R, 48% of the pre-duplication ancestral genes were retained as duplicates, the remainder of the genes underwent fractionation and the duplicated protein-coding genes were lost (Berthelot *et al.*, 2014; Lien *et al.*, 2016). Analysis of duplicate retention in Atlantic salmon identified that 20% of duplicates from Ts3R and 55% of duplicates from Ss4R were retained as functional copies, with the prominent mechanism for duplicate loss being pseudogenisation (Lien *et al.*, 2016). While there is a general lack of clarity surrounding the duplication and retention of functional genes post WGD, there is an unusually large complement of clock genes in salmonids (West *et al.*, 2020).

Salmonids are amongst the most widely studied groups of fish species both scientifically and commercially, as many species of salmonid are of significant economic, societal, and environmental importance (Thorgaard *et al.*, 2002). Many of the species within the 11 genera of the Salmonidae (Hubbs and Nelson, 1978) are of great commercial value and contribute significantly to both local and global economies through aquaculture, wild stock fisheries and recreational sport (Reppert and Weaver, 2001; Davidson *et al.*, 2010a; Frøland Steindal and Whitmore, 2019). Unlike zebrafish, salmonid species are highly seasonal in their physiology, including migration, smoltification and reproduction. Lighting and temperature manipulations are routinely used by industry to manipulate commercial broodstock ovulatory rhythm and smoltification, enabling year-round production (Migaud *et al.*, 2013). However, understanding the intricate interactions between zeitgebers, circadian rhythmicity, seasonality, and the control of biochemical, physiological, and behavioural rhythms is complex (Migaud, Davie and Taylor, 2010). The completion and publication of salmonid genomes (*Salmo salar, Salvelinus alpinus*, *Oncorhynchus mykiss*, *Oncorhynchus kisutch* and *Oncorhynchus tshawytscha* to date) as part of the Functional Annotation of All Salmonid Genomes (FAASG) project (Macqueen *et al.*, 2017), alongside RNAseq have provided great tools to study clock genes of salmonids.

The aim of this study was to identify the full complement of clock genes in Atlantic salmon (*Salmo salar*) in comparison to other commercially important salmonid species and evaluate the expression patterns of the identified genes.

To do so, phylogenetic analysis of clock gene ohnologues [functional product of WGD event (Ohno, 1970)] has been performed to classify and name salmonid clock genes based on published zebrafish references and nomenclature. This was confirmed by a transcriptomic approach looking into gene expression over 24-hours in freshwater salmon kept under a controlled lighting regime. This study provides a new nomenclature for salmon clock genes that will serve as a tool for further circadian research in salmon.

3.3. Materials and methods

3.3.1. Ethical statement

Animals were treated in accordance with the UK Animals (Scientific Procedures) Act 1986 Amended Regulations (SI 2012/3039) and the experiment was approved by the Animal Welfare and Ethical Review Body of the University of Stirling (AWERB/19 20/097/).

3.3.2. Identification of clock genes in salmonids with published genomes

The protein sequences of the 29 zebrafish (*D. rerio*) clock genes [*clock*, *arntl* (also referred to as *bmal*), *period*, *cryptochrome*, and *csnk1e/d* (Huang, 2018), *nr1d* (also referred to as *rev-erb*), and *ror* (Wang, 2009)] were recovered from GenBank and used as reference to interrogate the Northern pike (*Esox lucius*) [a closely related sister taxa which did not undergo the salmonid specific WGD (Rondeau *et al.*, 2014; Macqueen *et al.*, 2017; Varadharajan *et al.*, 2018)] and Atlantic salmon (*S. salar*) genomes. For the benefit of this study, the core clock genes (the heterodimers forming the positive and negative feedback arms of the TTFL, *clock:arntl* and *period:cryptochrome* respectively) and accessory loop genes (individual genes *ror, nr1d,* and *csnk1* which interact with the core clock loop to either promote or repress specific heterodimer interactions) are commonly referred to collectively as clock genes. Putative core clock gene sequences were also identified for several salmonid species (*S. alpinus*, *O. mykiss*, *O. kisutch* and *O. tshawytscha*) through a combination of literature searches, BLASTp and BLASTn searches of published salmonid genomes identified as part of the Functional Annotation of All Salmonid Genomes (FAASG) initiative (Macqueen *et al.*, 2017). For the benefit of this publication, they will be referred to collectively as salmonids. A BLASTp search using the default settings against the protein sequences were used for a first characterisation of the putative core clock genes. This was further refined using BLASTn using the coding sequence (CDS) against the RNA sequences (refseq_rna) database and was optimised for highly similar nucleotide sequences (megablast) with an E-value below 10^{-300} and ensuring a negligible probability

that the sequence was returned by chance. From the final BLASTn search, the gene and their transcriptomic isoform were aligned to the CDS of zebrafish reference genes using ClustalOmega v1.2.2 (Sievers *et al.*, 2011).

3.3.3. Phylogenetic alignment

Amino acid sequences were aligned using GramAlign v3.0 (Russell, 2014). A Maximum Likelihood (ML) tree was inferred under the GTR model with gamma-distributed rate variation (Γ) and a proportion of invariable sites (I) using a relaxed (uncorrelated lognormal) molecular clock in RAxML (Stamatakis, 2014) with 10,000 bootstrap replicates. Gaps were handled as undetermined characters (N).

3.3.4. Classification

Nomenclature of the putative salmonid clock genes was based on phylogenetic analyses using CDS and full-length sequences to the *D. rerio* and *E. lucius* core clock genes. Genes have been renamed after the zebrafish orthologues. As a result of the salmonid specific WGD Ss4R, salmonids often have two copies of a gene which is present as a single copy in zebrafish. In most instances, this involved renaming the gene from their given predicted name. Genes were re-classified based on the nomenclature of the zebrafish reference genes (Dunn and Knight, 2015). If a single representative was identified per species, the same name as the zebrafish reference was used. For groups with multiple representatives, the name of the orthologs were appended with numerical suffixes (*.1, .2*) or alphabetical suffixes (*a, b*) depending on the nomenclature of the zebrafish reference genes as a result of the previous ray fin fish WGD event, as some zebrafish genes were already denoted with alphabetical suffixes in relation to their duplication. Those with the highest percentage identity to the reference gene phylogenetically are labelled a. For example: the Atlantic salmon has two *period 1a* (*per 1a*) paralogs, *period 1a.1* (*per1a.1*) has the highest percentage identity when compared to the reference gene and is therefore closer to the zebrafish gene so is denoted by *.1*, and *period 1a.2* (*per1a.2*) is less identical to the ancestral form and is therefore denoted by *.2*.

3.3.5. Animal husbandry and sampling

All juvenile Atlantic salmon (*S. salar*) used in the experiment were kept at the Niall Bromage Freshwater Research Facilities at the University of Stirling. Fish (100 per tank – balanced biomass, mean weight of 40 g, Benchmark Genetics Iceland origin) were held in four 800 L tanks in a flow through system and maintained under a 12:12 Light:Darkness photoperiod (photophase from 08:00 to 20:00 using TMC AquaRay LED lamps, 2000 lumen, 6500 kelvin). From which 60 fish were sampled (mean weight of 130 g, Benchmark Genetics Iceland origin) from 14th April 2020 to 19th August 2020 (Figure 3.1).

Figure 3.1: Experimental setup for 12:12 LD clock gene expression trial at the Niall Bromage Freshwater Research Unit, University of Stirling, 2020.

The ambient temperature ranged from 8.1 °C to 15.2 °C during this period (Figure 3.2). In the month before sampling the temperature range was 14.6 °C ± 0.6 °C.

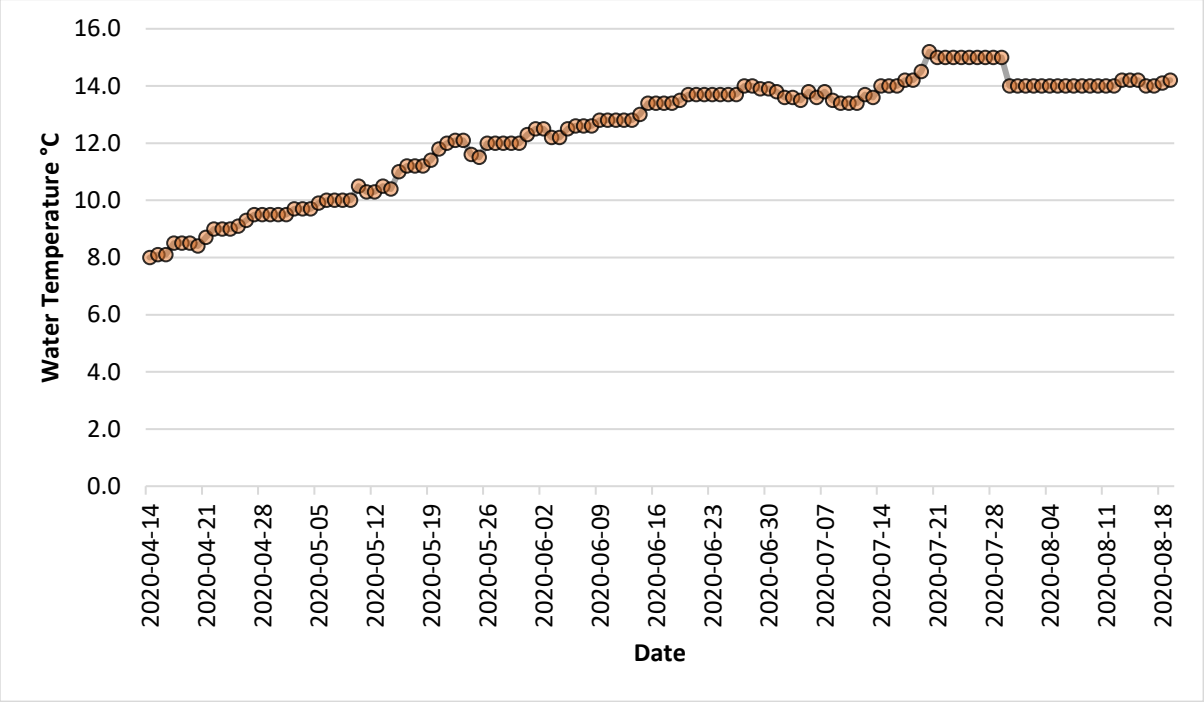


Figure 3.2: Graph displaying average daily inlet temperature variation across the duration of the lighting experiment 2020-04-14 to 2020-08-19.

Fish were fed daily with BioMar Orbit (2 and 3 mm pellet) using automatic feeders (Arvo-Tec, Sterner) which were programmed to feed at equally spaced intervals during the light period. Total amount fed was calculated by biomass (kg) using feed tables and growth models in FishTalk which were driven by temperature and mean weight (g). Fish were not fed during the light period on the day of sampling. Sampling was conducted every 4-hours over a 24-hour period, starting at 10:00 on the first day and ending at 10:00 the following day (10:00[1], 14:00, 18:00, 22:00, 02:00, 06:00, 10:00[2]). At each time-point, six fish were sampled (Table 3.1.). Following lethal anaesthesia (MS222, 250 mg/L, pH neutralised with sodium bicarbonate) and confirmatory severing of the spinal column (posterior to the opercula), brains were dissected out, pituitary glands removed and snap frozen directly in liquid nitrogen and stored at -80 °C until analysis.

Table 3.1: Samples and reads detail.

Sample	SampleID	BrainID	Sample EBI Accession	Raw Reads	Date	Time (ZT)	Timepoint	Mapping
A1	2/LD/T0/1	85	ERS5329244	47723702	2020-08-24T09:00:00Z	0	10:00	97.8%
A2	2/LD/T0/2	86	ERS5329245	41776194	2020-08-24T09:00:00Z	0	10:00	98.0%
A3	2/LD/T0/3	87	ERS5329246	48551618	2020-08-24T09:00:00Z	0	10:00	97.9%
A4	2/LD/T0/4	88	ERS5329247	40458840	2020-08-24T09:00:00Z	0	10:00	98.0%
A5	2/LD/T0/5	89	ERS5329248	39687614	2020-08-24T09:00:00Z	0	10:00	97.5%
A6	2/LD/T0/6	90	ERS5329249	47388666	2020-08-24T09:00:00Z	0	10:00	97.9%
A7	2/LD/T1/1	97	ERS5329250	48544056	2020-08-24T13:00:00Z	4	14:00	97.9%
A8	2/LD/T1/2	98	ERS5329251	42958682	2020-08-24T13:00:00Z	4	14:00	98.0%
A9	2/LD/T1/3	99	ERS5329252	48892118	2020-08-24T13:00:00Z	4	14:00	97.9%
A10	2/LD/T1/4	100	ERS5329253	57346024	2020-08-24T13:00:00Z	4	14:00	98.0%
A11	2/LD/T1/5	101	ERS5329254	44973308	2020-08-24T13:00:00Z	4	14:00	97.9%
A12	2/LD/T1/6	102	ERS5329255	46567214	2020-08-24T13:00:00Z	4	14:00	97.8%
A13	2/LD/T2/1	109	ERS5329256	46177414	2020-08-24T17:00:00Z	8	18:00	97.9%
A14	2/LD/T2/2	110	ERS5329257	49718214	2020-08-24T17:00:00Z	8	18:00	97.8%
A15	2/LD/T2/3	111	ERS5329258	40568918	2020-08-24T17:00:00Z	8	18:00	97.7%
A16	2/LD/T2/4	112	ERS5329259	47615560	2020-08-24T17:00:00Z	8	18:00	97.8%
A17	2/LD/T2/5	113	ERS5329260	49860608	2020-08-24T17:00:00Z	8	18:00	97.8%
A18	2/LD/T2/6	114	ERS5329261	45925090	2020-08-24T17:00:00Z	8	18:00	97.4%
A19	2/LD/T3/1	121	ERS5329262	49124232	2020-08-24T21:00:00Z	12	22:00	97.2%
A20	2/LD/T3/2	122	ERS5329263	46414190	2020-08-24T21:00:00Z	12	22:00	97.8%
A21	2/LD/T3/3	123	ERS5329264	53764366	2020-08-24T21:00:00Z	12	22:00	97.5%
A22	2/LD/T3/4	124	ERS5329265	46872910	2020-08-24T21:00:00Z	12	22:00	97.8%
A23	2/LD/T3/5	125	ERS5329266	41175448	2020-08-24T21:00:00Z	12	22:00	97.8%
A24	2/LD/T3/6	126	ERS5329267	39772946	2020-08-24T21:00:00Z	12	22:00	97.6%
A25	2/LD/T4/1	133	ERS5329268	56203190	2020-08-25T01:00:00Z	16	02:00	97.6%
A26	2/LD/T4/2	134	ERS5329269	46268272	2020-08-25T01:00:00Z	16	02:00	97.4%
A27	2/LD/T4/3	135	ERS5329270	68576730	2020-08-25T01:00:00Z	16	02:00	97.0%
A28	2/LD/T4/4	136	ERS5329271	48524028	2020-08-25T01:00:00Z	16	02:00	97.3%
A29	2/LD/T4/5	137	ERS5329272	45795270	2020-08-25T01:00:00Z	16	02:00	97.4%
A30	2/LD/T4/6	138	ERS5329273	49615352	2020-08-25T01:00:00Z	16	02:00	97.7%
A31	2/LD/T5/1	145	ERS5329274	49813802	2020-08-25T05:00:00Z	20	06:00	97.9%
A32	2/LD/T5/2	146	ERS5329275	50785920	2020-08-25T05:00:00Z	20	06:00	97.7%
A33	2/LD/T5/3	147	ERS5329276	40505336	2020-08-25T05:00:00Z	20	06:00	97.6%
A34	2/LD/T5/4	148	ERS5329277	42291530	2020-08-25T05:00:00Z	20	06:00	97.9%
A35	2/LD/T5/5	149	ERS5329278	49107588	2020-08-25T05:00:00Z	20	06:00	97.7%
A36	2/LD/T5/6	150	ERS5329279	43759616	2020-08-25T05:00:00Z	20	06:00	97.6%
A37	2/LD/T6/1	157	ERS5329280	40172870	2020-08-25T09:00:00Z	24	10:00+1	98.0%
A38	2/LD/T6/2	158	ERS5329281	43202190	2020-08-25T09:00:00Z	24	10:00+1	97.6%
A39	2/LD/T6/3	159	ERS5329282	45730066	2020-08-25T09:00:00Z	24	10:00+1	97.8%
A40	2/LD/T6/4	160	ERS5329283	46641334	2020-08-25T09:00:00Z	24	10:00+1	97.7%
A41	2/LD/T6/5	161	ERS5329284	49706180	2020-08-25T09:00:00Z	24	10:00+1	97.4%
A42	2/LD/T6/6	162	ERS5329285	44532910	2020-08-25T09:00:00Z	24	10:00+1	97.7%

3.3.6. RNA extraction and sequencing

RNA was isolated from the whole brain using TRI reagent (Sigma, St Louis, MO, USA) and RNA concentration was tested using a Qubit 2.0 Fluorometer (ThermoFisher Scientific, Waltham, MA, USA). The 42 RNA samples were submitted to Novogene UK (Cambridge) for RNA sequencing. Samples were submitted to quality control (Illumina BioAnalyzer®) revealing the RNA integrity number (RIN) of samples valued between 8.8-10.0. For each sample (900 ng), a library was prepared using NEB Next® Ultra™ RNA Library Prep Kit and processed and sequenced using Illumina NovaSeq® 6000 S4 PE150 (6 GB of data per sample, ~40 million reads).

3.3.7. RNA sequencing analysis

Clean reads were obtained from the raw reads by filtering ambiguous bases, PCR duplicates, low quality sequences (< Q20), length (150 nt), absence of primers/adaptors and complexity (entropy over 15) using fastp (Chen *et al.*, 2018). Ribosomal RNA was further removed using SortMeRNA v3.0.2 (Kopylova, Noé and Touzet, 2012) against the Silva version 119 rRNA databases (Quast *et al.*, 2013). The remaining reads were aligned to the annotated *S. salar* genome ICSASG v2.99 (Accession GCA_000233375.4) using HiSat2 v2.2.0 (Kim *et al.*, 2019). The expression levels were estimated based on the genome annotation using StringTie2 v2.1.0 (Kovaka *et al.*, 2019) following the workflow: (a) for each sample, map the reads to the genome with HiSat2 and assemble the read alignments with StringTie2; (b) merge the assemblies in order to generate a non-redundant set of transcripts observed in all the samples; (c) for each sample, estimate transcript abundances and generate read coverage tables expressed in the fragments per kilobase of exon per million mapped reads (FPKM).

3.3.8 Statistical analysis

All tests and analysis were performed using R v4.1 (RDC, 2010). The expression values were scaled in Transcripts Per Millions (TPM) before been normalised by size factor using the *median ratio method* described by Anders and Huber (2010) using DESeq2 v1.32.0 (Love, Huber and Anders, 2014). Differential expression was estimated using the function *lfcShrink* (Stephens, 2017). The heatmaps were created from the normalised count files and were scaled by row to highlight individual gene expression. Daily rhythms across the genome including identified clock genes were identified from the 24-hour dataset using RAIN v1.26.0 (Thaben and Westermark, 2014) and MetaCycle v1.2.0 (Wu *et al.*, 2016) implementation of JTKCycle (Hughes, Hogenesch and Kornacker, 2010) and a threshold *p*-value of 0.001 and a minimum relative oscillation amplitude of 10 %. All *p*-value reported were corrected for False Discovery Rate (FDR) using Bonferroni adjustment.

3.4 Results

3.4.1. Identification of clock genes in salmonids with published genomes

From the BLASTn, 143 clock gene variants were returned; a substantial number were highly similar predicted variants of the same gene and had identical EMBL accession numbers (LOC ID) and the CDS were over 90% identity. Most of the differences were found in the UTR. The CDS of variants sharing LOC ID were aligned to the CDS of *D. rerio* reference genes. Variants with the highest percentage identity compared to the reference for each locus were selected, leaving a total of 48 core clock genes identified in the *S. salar* across the 7 gene families explored (Table 3.2. and Table 3.3.).

Table 3.2: Genomic structure of the gene families associated with the circadian clock of salmonids. Phylogenetic trees upon which the table was based on can be found in Figure 1 and Supplementary Figures S1-S7. Key: ● gene detected in genome; - gene not detected in genome; * gene not detected in genome but identify from transcriptomes data. DR *Danio rerio* (GCA_000002035.4), EL *Esox lucius* (GCA_011004845.1), SSA *Salmo salar* (GCA_000233375.4), SA *Salvelinus alpinus* (GCA_002910315.2), OM *Oncorhynchus mykiss* (GCA_002163495.1), OK *Oncorhynchus kisutch* (GCA_002021735.2), OT *Oncorhynchus tshawytscha* (GCA_002872995.1).

Family	Genes	DR	EL	SSA	SA	OM	OK	OT
clock	*clock1a*	●	●	●●	●●	●●	●●	●●
	clock1b	●	●	-	-	-	-	-
	clock2 (npas2)	●	●	●●	●●	●●	●●	●
arntl	*arntl1a*	●	●	●●	●●	●●	●●	●●
	arntl1b	●	●	●●	●	●●	●●	●
	arntl2	●	●●	●●●	●●	●●●	●●●	●●
period	*per1a*	●	●	●●	●●	●●	●●	●●
	per1b	●	●	●	●	●	●	●
	per2	●	●	●●	●●	●●	●●	●●
	per3	●	●	●	●	-	-	-
cryptochrome	*cry1a*	●	●	●●	●●	●●	●●	●●
	cry2	●	●	●	●	●	●	*
	cry3a	●	●	-	-	-	-	-
	cry3b	●	●	●●	●●	●	●	*
	cry4	●	●	●	-	-	-	-
	cry5	●	●	●	●	●	●	●
nr1d	*nr1d1*	●	●	●●	●●	●●	●●	●●
	nr1d2a	●	●	●●	●●	●●	●	●●
	nr1d2b	●	●	●●	●●	●●	●●	●●
	nr1d4a	●	●	●●	●●	●●	●●	●●
	nr1d4b	●	●	●●	●●	●●	●●	●●
ror	*roraa*	●●	●●	●●	●●	●●	●●	●●
	rorab	●	-	-	-	-	-	-
	rorb	●	●	●●	●●	●●	●●	●●
	rorca	●	●	●●	●●	●●	●●	●●
	rorcb	●	●	●●	●●	●●	●	●●
csnk1e/d	*csnk1e*	●	●	●●●●	●●●●	●●●●	●●	●●●●
	csnk1da	●	●	-	-	-	-	-
	csnk1db	●	●	●●	●	●●	●●	●●

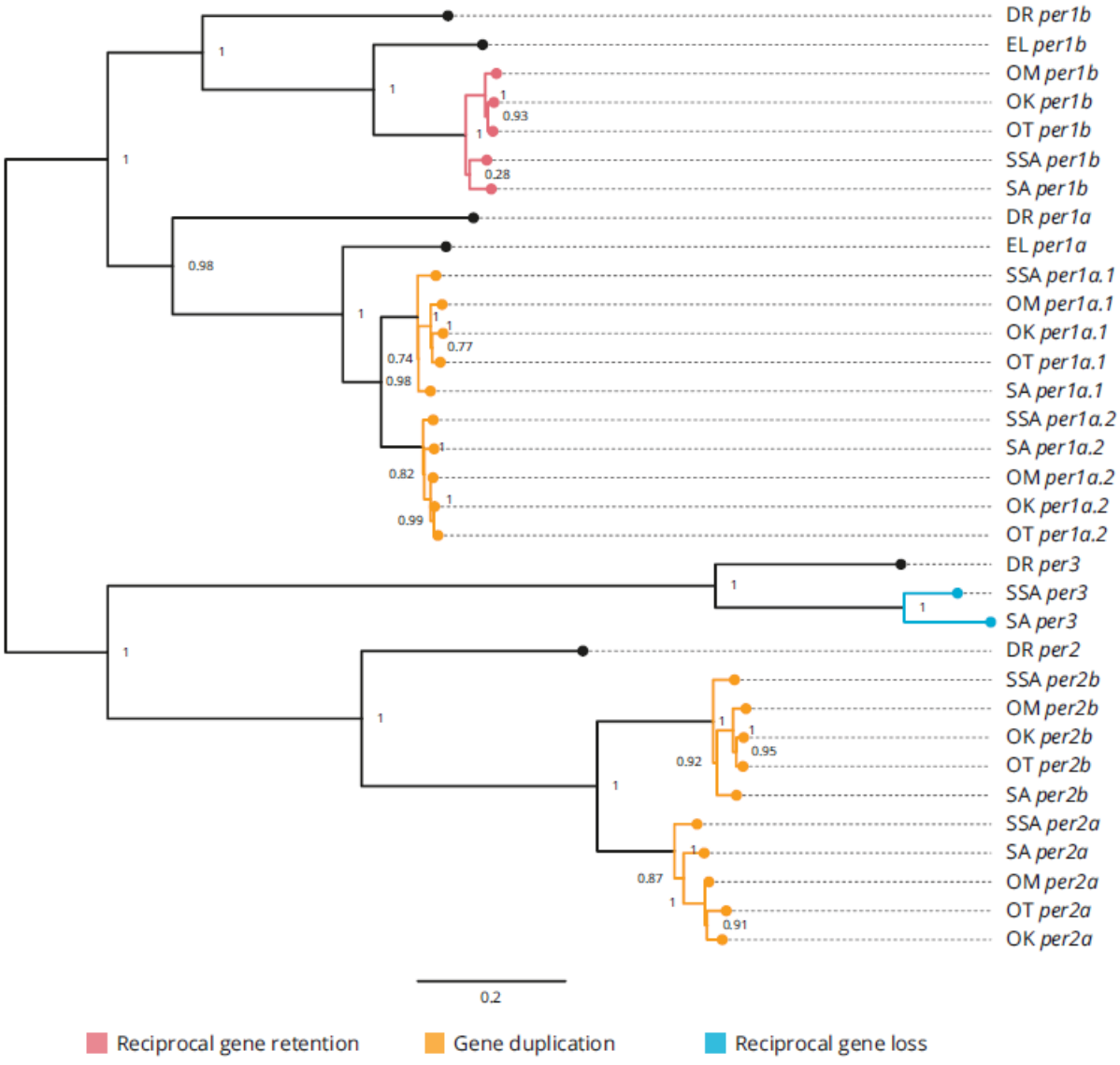
Table 3.3: *S. salar* clock gene accessions and locations

Clock gene	Ensembl LOC_ID	NCBI Accession	NCBI Gene_ID	Chromosome location
clock1a.1	ENSSSAG00000056165	XM_014169004.1	LOC106584123	ssa23:19987839-20018200
clock1a.2	ENSSSAG00000003979	XM_014122997.1	LOC106560272	ssa10:31345809-31398321
clock2a	ENSSSAG00000072526	XM_014137489.1	LOC106567770	ssa13:81971735-82044254
clock2b	ENSSSAG00000063571	XM_014196792.1	LOC106603298	ssa04:42639734-42675993
arntl1a.1	ENSSSAG00000055510	XM_014148046.1	LOC106573213	ssa16:12086304-12095636
arntl1a.2	ENSSSAG00000063495	XM_014125430.1	LOC106561455	ssa10:99088660-99104965
arntl1b.1	ENSSSAG00000073101	XM_014175326.1	LOC106587199	ssa26:18743473-18765117
arntl1b.2	ENSSSAG00000068292	XM_014126699.1	LOC106562106	ssa11:18402203-18411618
arntl2a	ENSSSAG00000059155	XM_014125375.1	LOC106561420	ssa10:94715917-94748435
arntl2b	ENSSSAG00000008440	XM_014153577.1	LOC106576418	ssa17:47744203-47759123
arntl2c	ENSSSAG00000009920	XM_014208404.1	LOC106609509	ssa07:45552387-45568717
per1a.1	ENSSSAG00000077896	XM_014196530.1	LOC106603198	ssa04:38522751-38532432
per1a.2	ENSSSAG00000063164	XM_014128896.1	LOC106563389	ssa11:73693186-73705207
per1b	ENSSSAG00000002804	XM_014206471.1	LOC106608520	ssa07:4731276-4739498
per2a	ENSSSAG00000076981	XM_014139595.1	LOC100301980	ssa14:16742620-16769326
per2b	ENSSSAG00000001704	XM_014190838.1	LOC106599551	ssa03:17486279-17517618
per3	ENSSSAG00000077674	XM_014166967.1	LOC106583133	ssa22:25358497-25382546
cry1a.1	ENSSSAG00000077590	XM_014148221.1	LOC106573292	ssa16:15906400-15976136
cry1a.2	ENSSSAG00000071693	XM_014125353.1	LOC106561407	ssa10:95086446-95106453
cry2	ENSSSAG00000073888	XM_014125728.1	LOC106561609	ssa10:105488616-105553942
cry3b.1	ENSSSAG00000081201	XM_014131850.1	LOC106565112	ssa12:46213293-46226192
cry3b.2	ENSSSAG00000081121	XM_014167724.1	LOC106583488	ssa22:40956787-40966869
cry4	ENSSSAG00000069768	XM_014176427.1	LOC106587781	ssa26:36623393-36647646
cry5	ENSSSAG00000079794	XM_014138346.1	LOC106568206	ssa13:86762607-86774549
nr1d1a	ENSSSAG00000003153	XM_014204497.1	LOC106607496	ssa06:40433770-40444554
nr1d1b	ENSSSAG00000007825	XM_014192527.1	LOC106600825	ssa03:57681841-57689097
nr1d2a.1	ENSSSAG00000037545	XM_014164862.1	LOC106582110	ssa02:25864163-25873212
nr1d2a.2	ENSSSAG00000005012	XM_014200712.1	LOC100136378	ssa05:54986045-54995902
nr1d2b.1	ENSSSAG00000056065	XM_014142438.1	LOC106570286	ssa14:68249965-68261020
nr1d2b.2	ENSSSAG00000054663	XM_014177878.1	LOC106588668	ssa27:19843632-19851666
nr1d4a.1	ENSSSAG00000065806	XM_014147027.1	LOC106572653	ssa15:88787769-88813331
nr1d4a.2	ENSSSAG00000077410	XM_014135912.1	LOC106567064	ssa13:35389895-35404997
nr1d4b.1	ENSSSAG00000081117	XM_014167340.1	LOC106583303	ssa22:32346247-32358789
nr1d4b.2	ENSSSAG00000067342	XM_014132672.1	LOC106565463	ssa12:54465885-54474575
roraa.1	ENSSSAG00000066827	XM_014148628.1	LOC106573506	ssa16:27588967-27706002
roraa.2	ENSSSAG00000074849	XM_014124804.1	LOC106561150	ssa10:82919138-82990987
rorb.1	ENSSSAG00000069458	XM_014160683.1	LOC106580068	ssa20:14747696-14776762
rorb.2	ENSSSAG00000065430	XM_014172220.1	LOC106585703	ssa24:30374236-30414448
rorca.1	ENSSSAG00000073620	XM_014169656.1	LOC106584392	ssa23:30486289-30543797
rorca.2	ENSSSAG00000068177	XM_014216453.1	LOC106613816	ssa10:21617008-21834653
rorcb.1	ENSSSAG00000079594	XM_014140953.1	LOC106569518	ssa14:38121993-38144650
rorcb.2	ENSSSAG00000080721	XM_014191359.1	LOC106600026	ssa03:37981172-38001032
csnk1db.1	ENSSSAG00000068205	XM_014179815.1	LOC106589616	ssa28:16447101-16461477
csnk1db.2	*ENSSSAG00000003495*	*XM_014209485.1*	*LOC106610228*	*ssa01:77662396-77680525*
csnk1ea	*ENSSSAG00000001530*	*XM_014193266.1*	*LOC106601228*	*ssa03:65302128-65311565*
csnk1eb	*ENSSSAG00000068400*	*NM_001140384.1*	*LOC100195355*	*ssa06:33906202-33916152*
csnk1ec	*ENSSSAG00000015336*	*XM_014179651.1*	*LOC106589542*	*ssa28:14144088-14151122*
csnk1ed	*ENSSSAG00000003438*	*XM_014208340.1*	*LOC106609470*	*ssa01:74533732-74548069*

3.4.2. Interpretation and classification

All the core clock and clock accessory loop genes were identified and classified (Table 3.2). Gene members of each family were aligned together in a gene-family approach to aid clarification of paralog nomenclature. The *period* (*per*) family is used as a typical example (Figure 3.3), showing the resulting gene tree from the identification and classification of *per* genes in salmonids indicating reciprocal gene retention (red), gene duplication (orange), and reciprocal gene loss (blue).

Figure 3.3: Phylogenetic relationship of the period clock gene family (*per*). Gene family members from *Danio rerio* (DR), *Esox Lucius* (EL), *Salmo salar* (SSA), *Salvelinus alpinus* (SA), *Oncorhynchus mykiss* (OM), *Oncorhynchus kisutch* (OK) and *Oncorhynchus tshawytscha* (OT) were re-annotated. Values on the relevant node depict the bootstrap values. Reciprocal gene retention (red), gene duplication (orange) and reciprocal gene loss (blue).



Period (Figure 3.3 overleaf, and Figure 3.4 below). *per1a* is duplicated across all salmonids (*per1a.1, per1a.2*). *per1b* is retained in all salmonids (*per1b.1, per1b.2*). *per2* appears to be duplicated after the latest common ancestor *E. lucius* (*per2a, per2b*). *per3* is retained in *S. salar* and *S. alpinus*, but appears to have been lost in *O. mykiss*, *O. kisutch,* and *O. tshawytscha*.

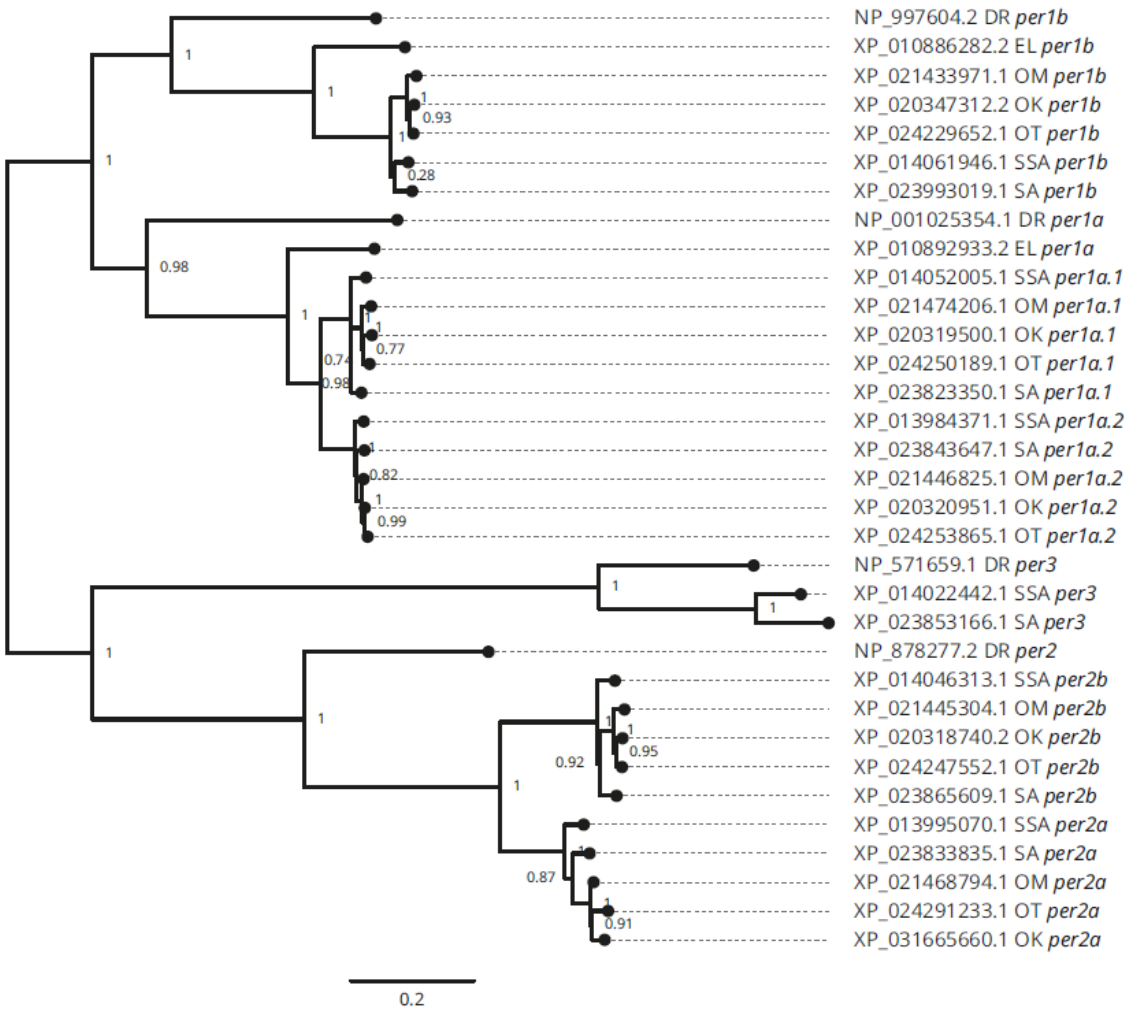


Figure 3.4: Phylogenetic relationship of the *period* gene family. Values on the relevant node depict the bootstrap values. Sequence ID used and identified are provided.

Clock (Figure 3.5). *clock1a* is duplicated in all salmonids (*clock1a.1, clock1a.2*). *clock1b* was lost compared to *D. rerio* and *E. lucius* and is not present in the salmonids. *clock2* (*npas2*) is duplicated across all salmonids analysed (*clock2a, clock2b*) with a differential ohnolog loss in *O. tshawytscha* (aligns to *clock2a*).

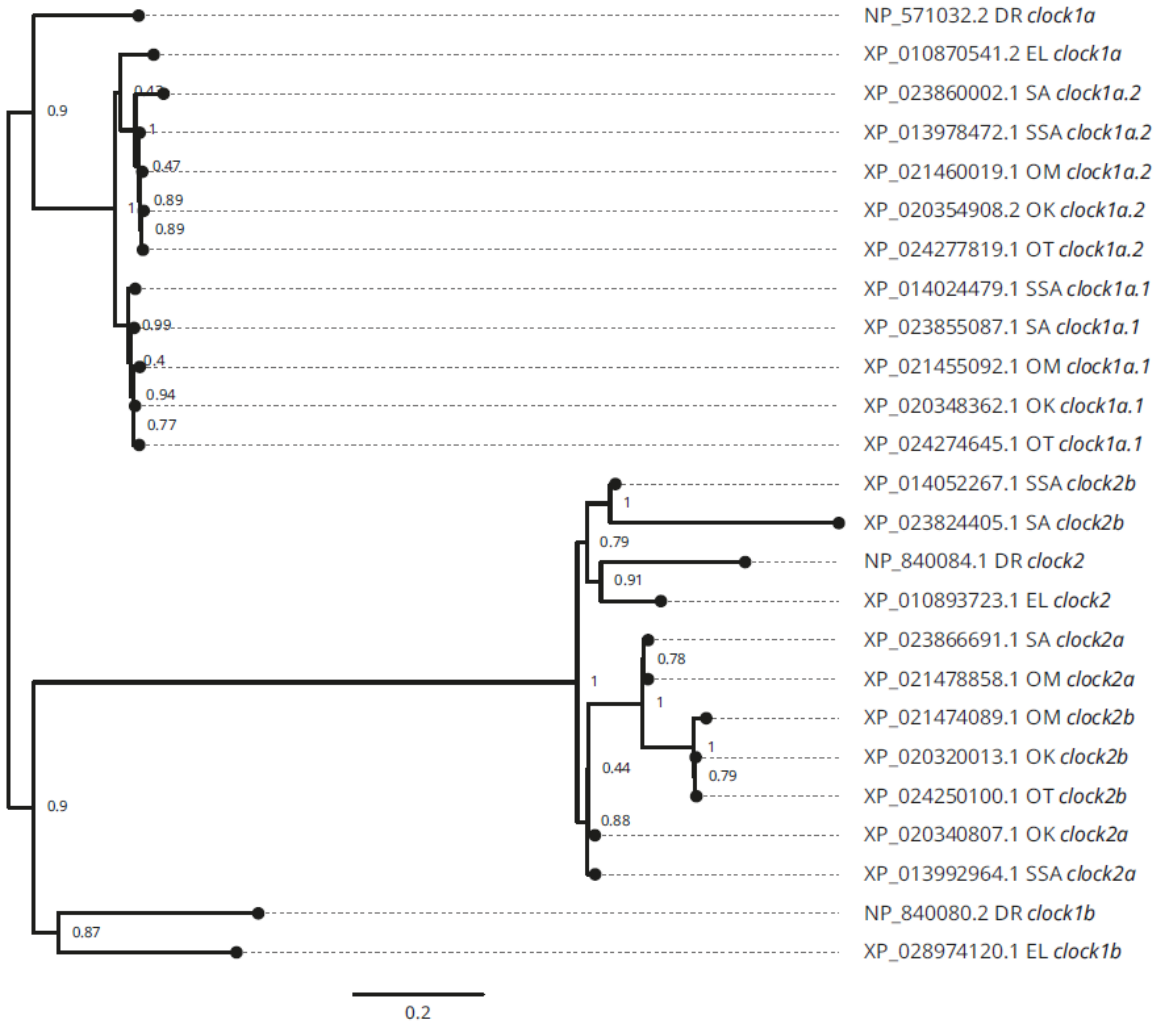


Figure 3.5: Phylogenetic relationship of the *clock* gene family. Values on the relevant node depict the bootstrap values. Sequence ID used and identified are provided.

Aryl hydrocarbon receptor nuclear translocator-like (Figure 3.6). *arntl1a* is duplicated in all salmonids (*arntl1a.1, arntl1a.2*). *arntl1b* is duplicated in all salmonids (*arntl1b.1, arntl1b.2*) with differential ohnolog loss in both *S. alpinus* (aligns to *arntl1b.2*) and *O. tshawytscha* (algins to *arntl1b.1*). There appears to be a duplication of *arntl2* in the latest common ancestor which is retained across all salmonids (*arntl2a, arntl2b*). There is a third copy identified in *S. salar*, *S. alpinus*, *O. mykiss* and *O. kisutch* (*arntl2c*).

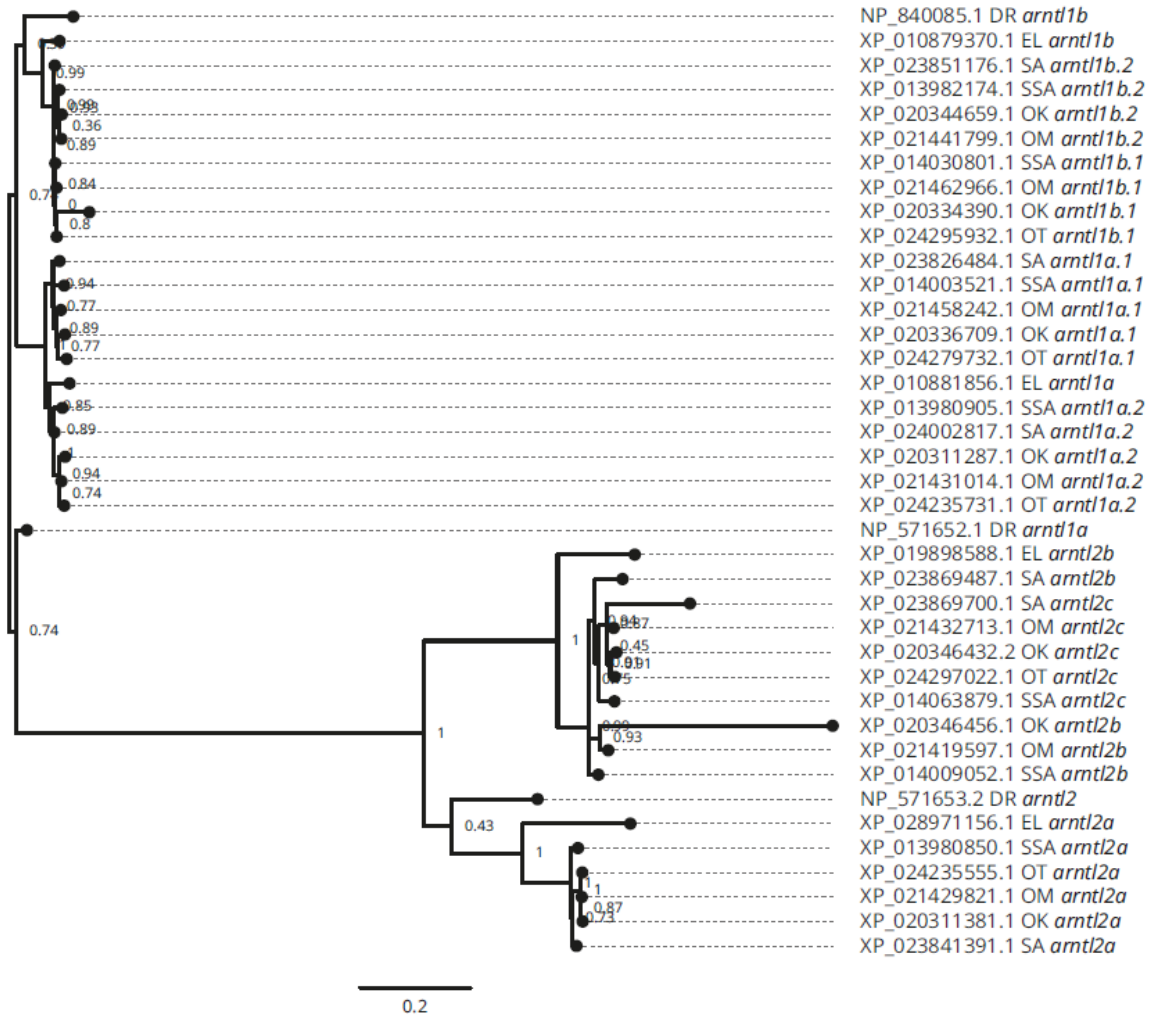


Figure 3.6: Phylogenetic relationship of the *aryl hydrocarbon receptor nuclear translocator-like* gene family (*arntl*). Values on the relevant node depict the bootstrap values. Sequence ID used and identified are provided.

Cryptochrome (Figure 3.7). *cry1a* is duplicated across all salmonids (*cry1a.1, cry1a.2*). *cry1b* appears to be lost after *D. rerio*. *cry2* is retained in all salmonids with the exception of *O. tshawytscha* as there is currently not enough information available regarding *cry2* in the species. There is an apparent gene loss of *cry3a* in salmonids after the most recent common ancestor, *E. lucius. cry3b* is duplicated in *S. salar* and *S. alpinus* (*cry3b.1, cry3b.2*) there is an apparent gene loss in *O. mykiss* and *O. kisutch* (aligns to *cry3b.1*), currently there is not enough information available regarding *cry3b* in *O. tshawtscha.* There is an apparent gene loss of *cry4* in all salmonids after the latest common ancestor *E. Lucius* in all species of salmonid except from *S. salar. cry5* is retained in all salmonids.

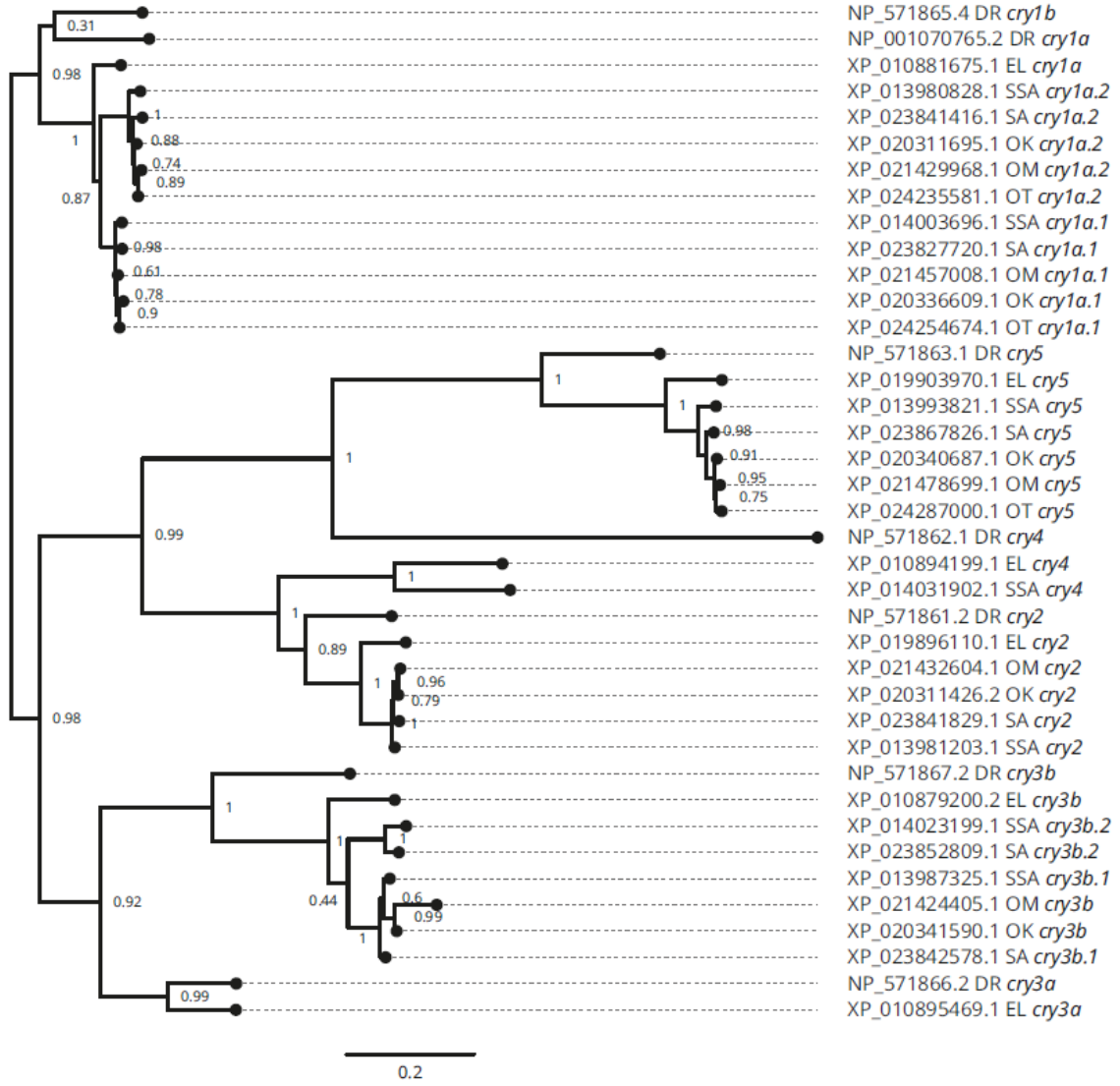


Figure 3.7: Phylogenetic relationship of the *cryptochrome* gene family (*cry*). Values on the relevant node depict the bootstrap values. Sequence ID used and identified are provided.

Nuclear receptor subfamily 1 group d (Figure 3.8). *nr1d1* is duplicated across all salmonids (*nr1d1a, nr1d1b*). *nr1d2a* is duplicated across all salmonids (*nr1d2a.1, nr1d2a.2*), with an apparent differential ohnolog loss in *O. kistuch* (aligns to *nr1d2a.2*). *nr1d2b* is duplicated across all salmonids (*nr1d2b.1, nr1d2b.2*). *nr1d4a* is duplicated across all salmonids (*nr1d4a.1, nr1d4a.2*). *nr1d4b* is duplicated across all salmonids (*nr1d4b.1, nr1d4b.2*).

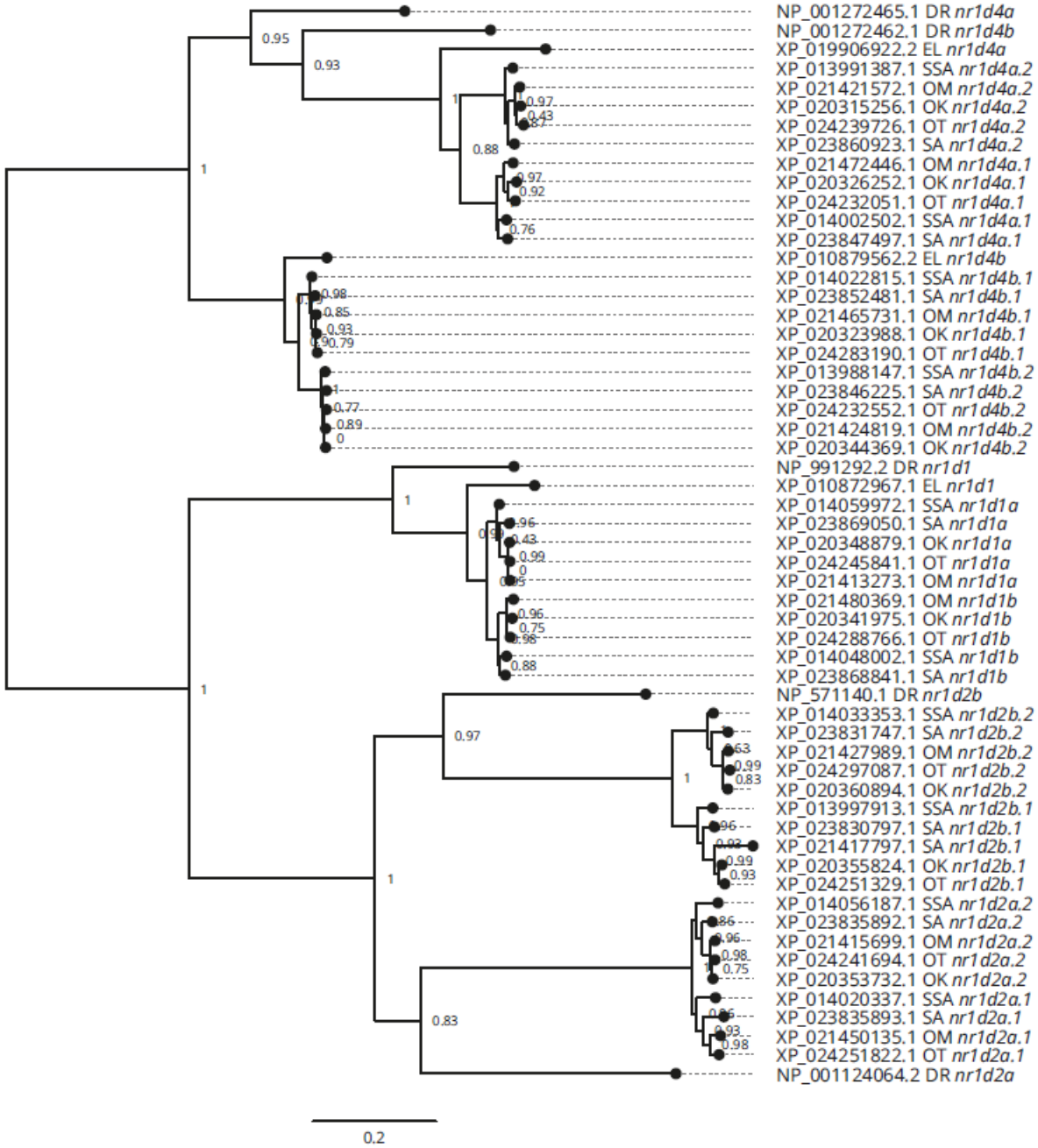


Figure 3.8: Phylogenetic relationship of the *nuclear receptor subfamily 1 group d* gene family (*nr1d*). Values on the relevant node depict the bootstrap values. Sequence ID used and identified are provided.

RAR-related orphan receptor (Figure 3.9). *roraa* is duplicated across all salmonids (*roraa.1, roraa.2*), there is an apparent differential gene loss in *O. tshawytscha* (aligns to *roraa.1*) and an apparent loss of both ohnologs in *O. kisutch*. There is an apparent loss of *rorab* after the latest common ancestor, *E. lucius*. *rorb* has been duplicated across all salmonids (*rorb.1, rorb.2*). *rorca* has been duplicated across all salmonids (*rorca.1, rorca.2*). *rorcb* has been duplicated across all salmonids (*rorcb.1, rorcb.2*) with an apparent differential ohnolog loss in *O. kisutch* (aligns to *rorcb.2*).

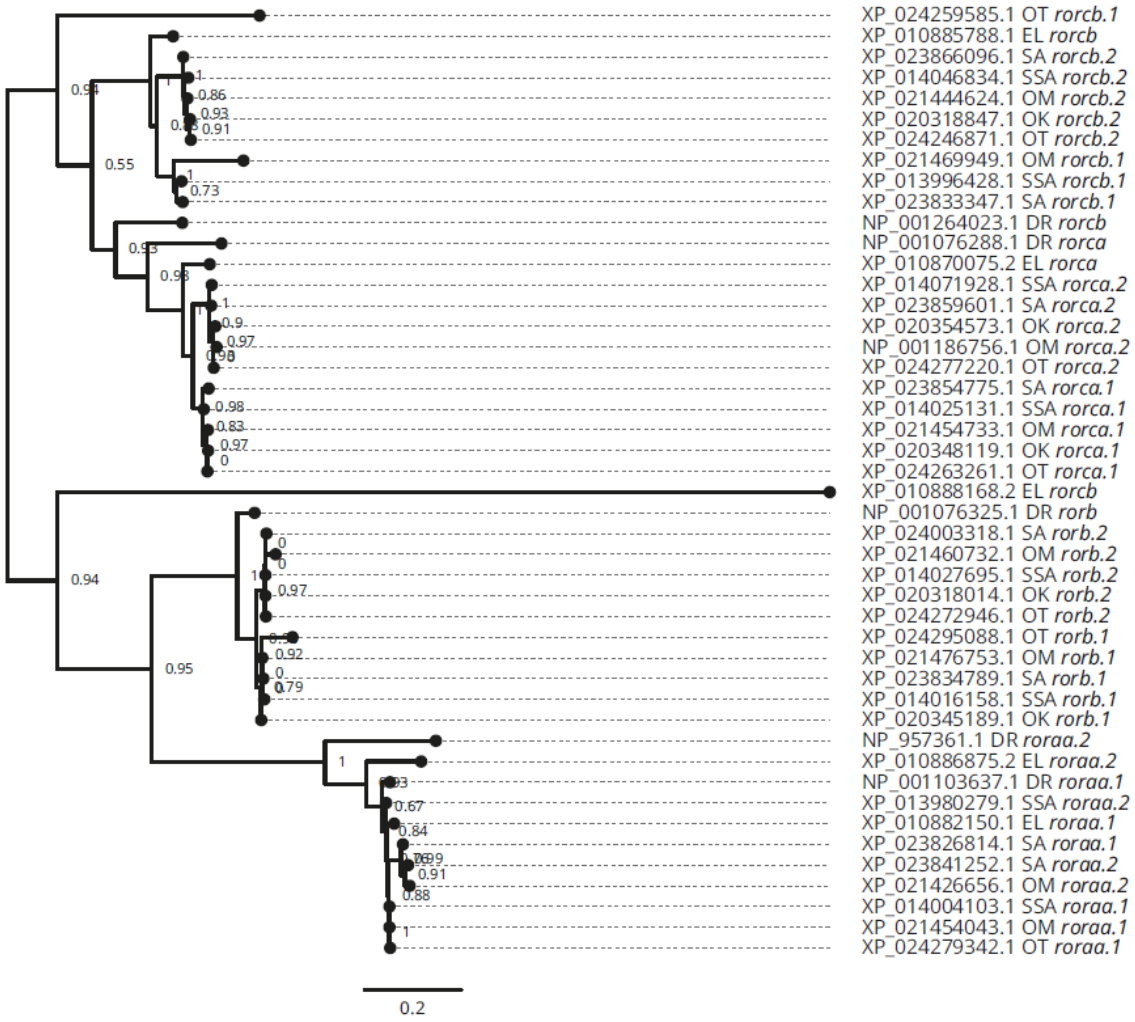


Figure 3.9: Phylogenetic relationship of the *RAR-reticulated orphan receptor* (*ror*). Values on the relevant node depict the bootstrap values. Sequence ID used and identified are provided.

Casein kinase 1 delta (Figure 3.10). *csnk1db* appears to be duplicated across all salmonids (*csnk1db.1, csnk1db.2*) apart from *S. alpinus* which displays an apparent differential ohnologs loss (aligns to *csnk1db.1*).

Casein kinase 1 epsilon (Figure 3.10). There appears to be multiple duplications of *csnk1e* across all the salmonids, resulting in four *csnk1e* paralogs (*csnk1ea, csnk1eb, csnk1ec, csnk1ed*). With *O. kisutch* displaying a potential differential ohnologs loss (aligns to *csnk1ea, csnk1eb*).

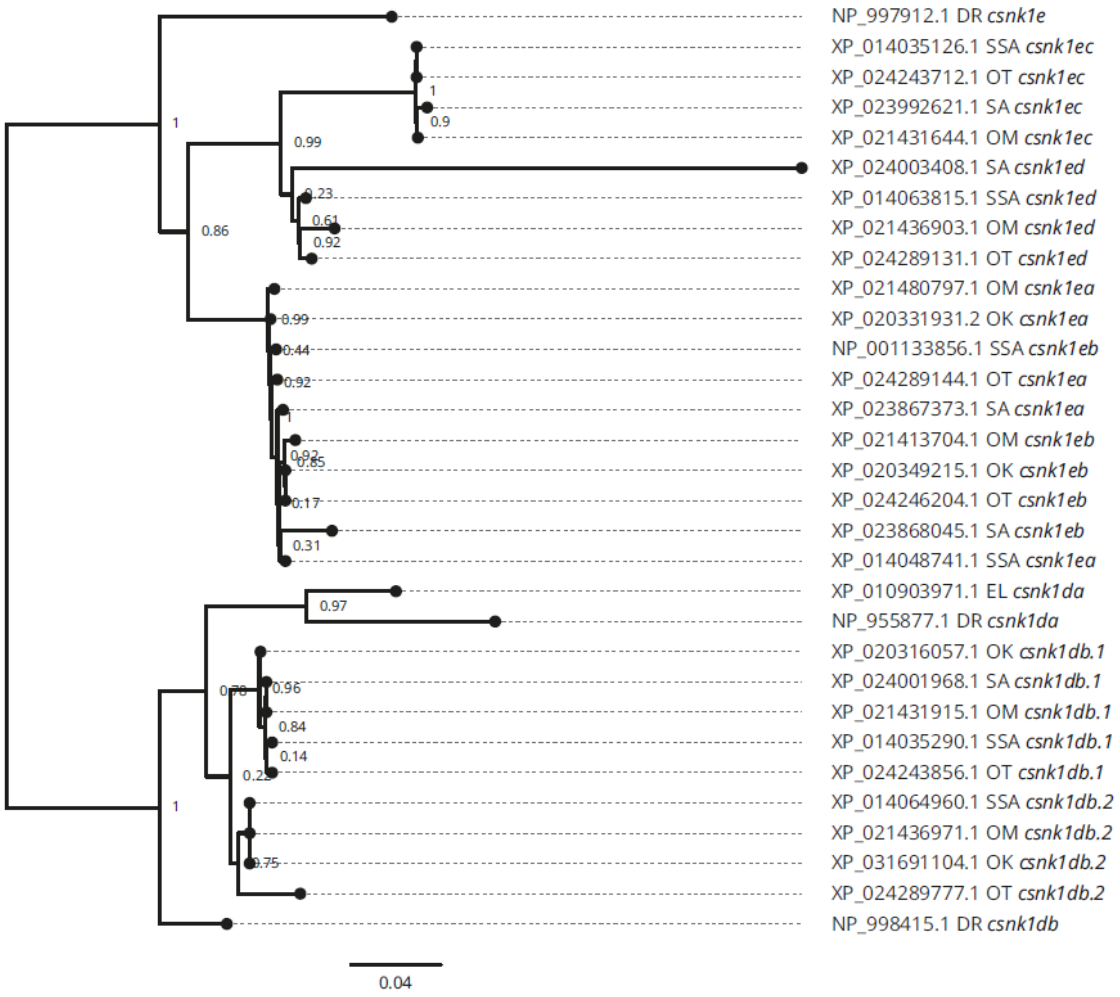


Figure 3.10: Phylogenetic relationship of the *casein kinase 1 delta* (*csnk1d*) and *epsilon* (*csnk1e)*. Values on the relevant node depict the bootstrap values. Sequence ID used and identified are provided.

3.4.3. Transcriptomic analysis

In total, 986,545,058 raw reads were sequenced for 42 samples (Table 3.1, see page 51). The reads were deposited in the European Bioinformatics Institute (EBI) European Nucleotide Archive (ENA) project ID PRJEB41327. After filtering, 975,284,560 clean reads (98.86%) passed the mRNA cleaning step and were used for the following process. Of the clean reads, 97.71% were aligned to the published *S. salar* genome ICSASG v2.99 (Accession GCA_000233375.4). A total of 55,819 distinct genes were recovered. All clock genes classified in the *in-silico* gene identification were recovered.

3.4.4. Rhythmic and circadian gene expression

Significantly rhythmically expressed genes were identified using RAIN and MetaCycle with JTK analysis. Various thresholds were evaluated (Table 3.4).

Table 3.4: Number of significant genes: Rhythmic (RAIN) or circadian (JTK) depending on the P-value (FDR adjusted) or relative Amplitude thresholds used. All dataset and clock genes only (in brackets).

	RAIN	**JTK**	**overlap**
adjP-value < 0.05			
rAmp ≥ 0	12,322 (32)	6,485 (22)	6,427 (22)
rAmp ≥ 5%	9,818 (25)	5,954 (21)	5,896 (21)
rAmp ≥ 10%	5,529 (20)	3,678 (18)	3,621 (18)
rAmp ≥ 15%	2,906 (8)	2,007 (8)	1,959 (8)
adjP-value < 0.01			
rAmp ≥ 0	9,064 (27)	3,786 (15)	3,760 (15)
rAmp ≥ 5%	7,413 (24)	3,627 (15)	3,602 (15)
rAmp ≥ 10%	4,234 (19)	2,374 (14)	2,349 (14)
rAmp ≥ 15%	2,206 (7)	1,264 (6)	1,241 (6)
adjP-value < 0.001			
rAmp ≥ 0	5,815 (22)	1,721 (11)	1,717 (11)
rAmp ≥ 5%	4,911 (21)	1,702 (11)	1,698 (11)
rAmp ≥ 10%	**2,864 (16)**	**1,215 (11)**	**1,211 (11)**
rAmp ≥ 15%	1,470 (6)	648 (5)	644 (5)

From the 48 genes clock genes, 16 were significantly rhythmically expressed (RAIN analysis, $p < 0.001$, relative amplitude ≥ 10%), of which 11 also had a significant circadian expression patten over a 24-hour period (JTK analysis, $p < 0.001$, relative amplitude ≥ 10%, and Figures 3.11. & 3.12.).

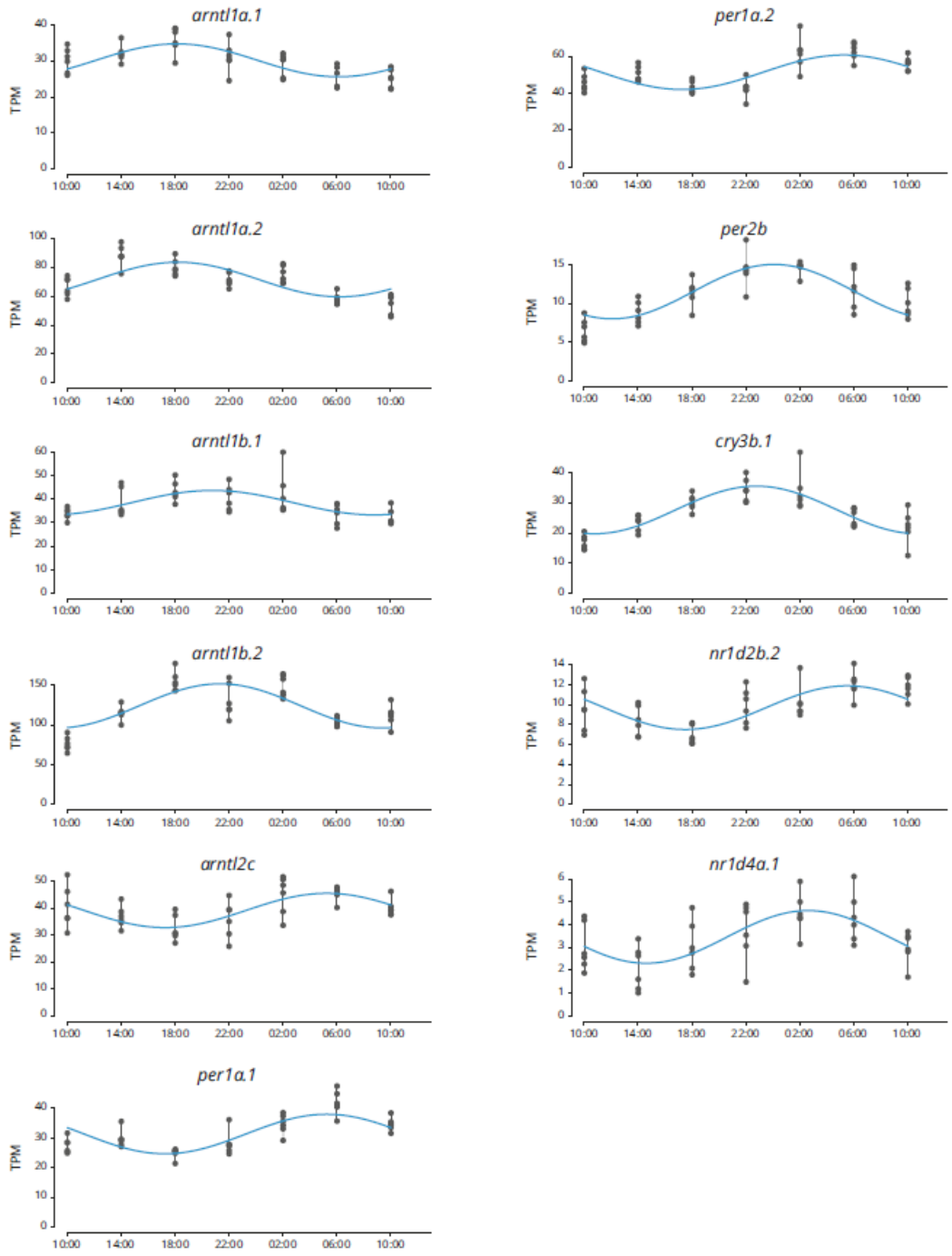


Figure 3.11: Significantly cyclical gene expression. Parameters of the cyclic sin- cosine function calculated by MetaCycle with JTK ($P < 0.001$) for diel expression of clock genes in brains collected from Atlantic salmon smolt exposed to an LD 12:12 photoperiod.

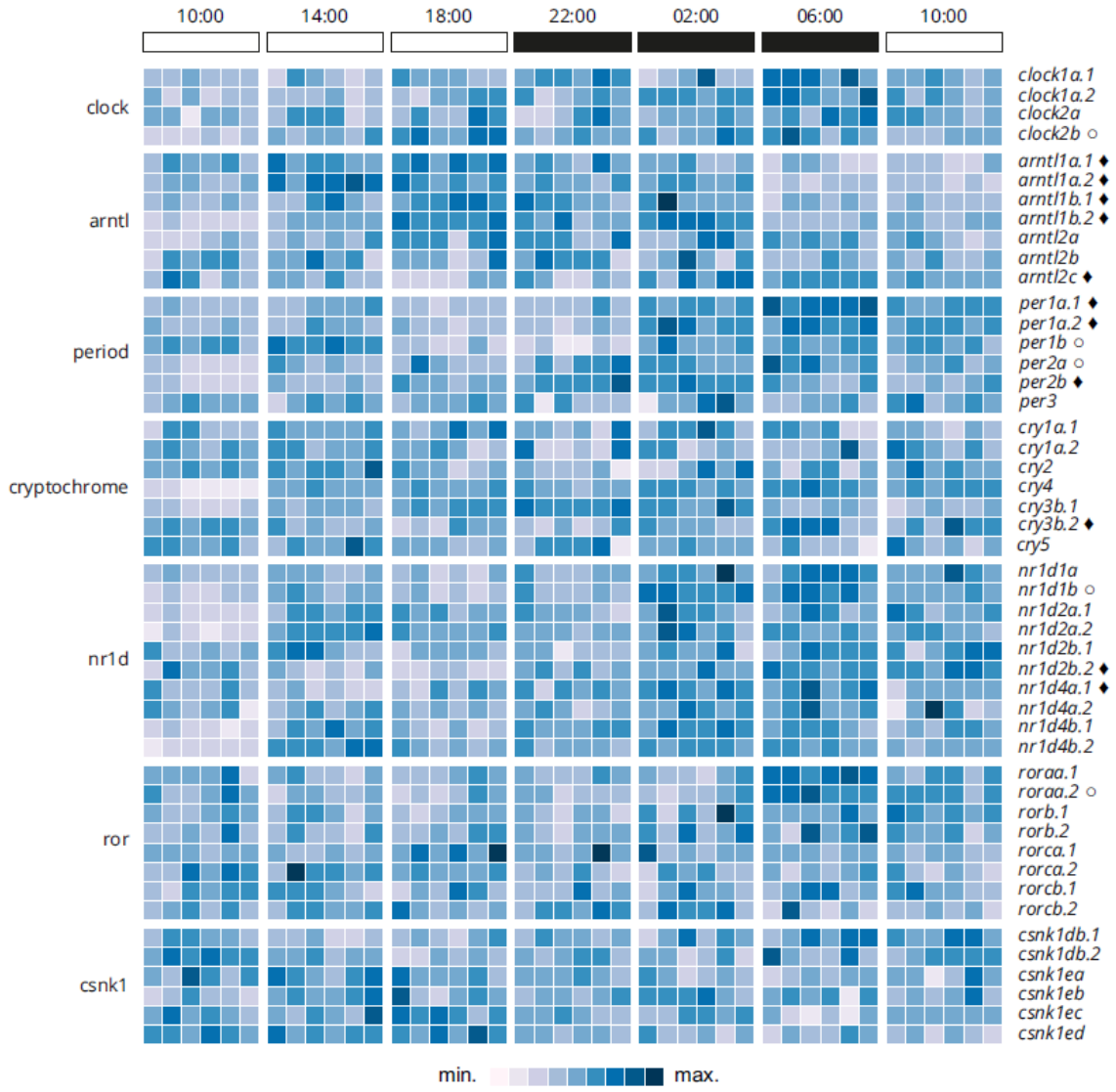


Figure 3.12: Heatmap displaying individual diel expression of identified clock genes under constant LD (12:12, n = 6 per time point). The heatmap of the relative expression of each individual gene [scaled from lowest expression to highest expression]. Black diamond indicates significantly cyclic gene ($p < 0.001$) [JTK and RAIN analysis], White circles denote rhythmic genes ($p < 0.001$) [RAIN analysis].

Details of the gene family *period* are plotted in Figure 3.13.A and 3.13.B, to enable direct comparison of all family members irrespective of significance of rhythmic and cyclical expression.

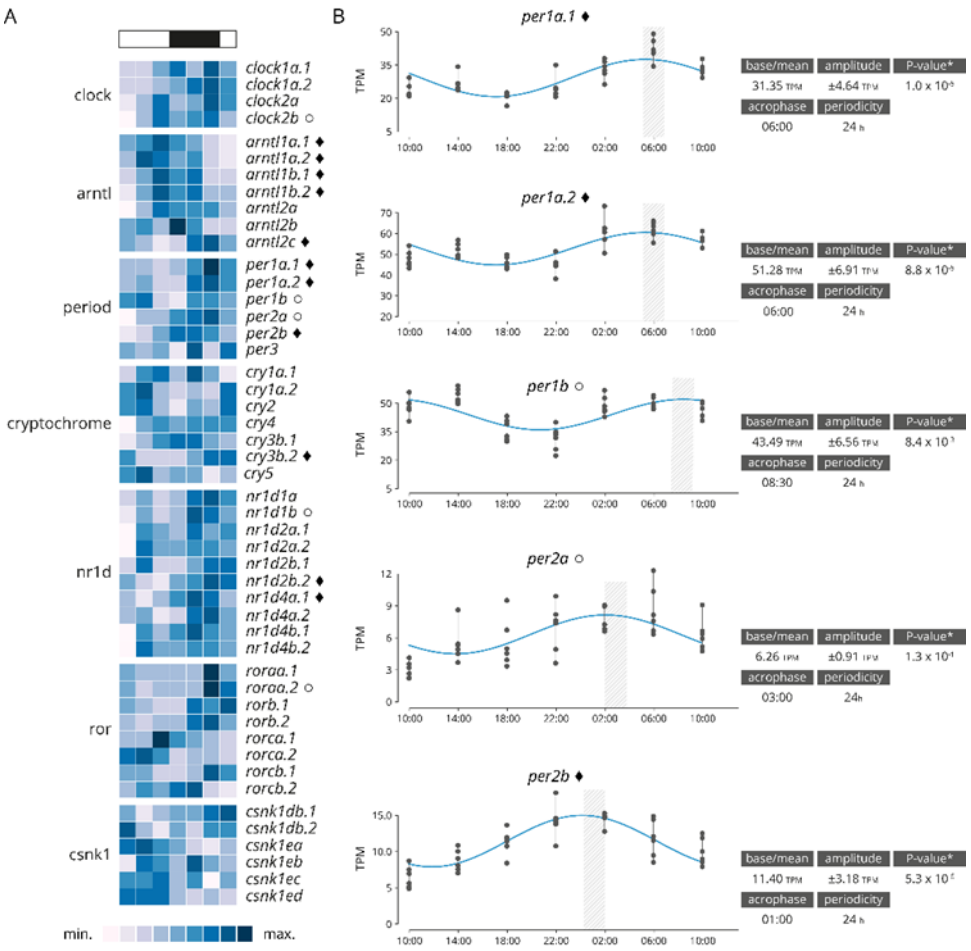
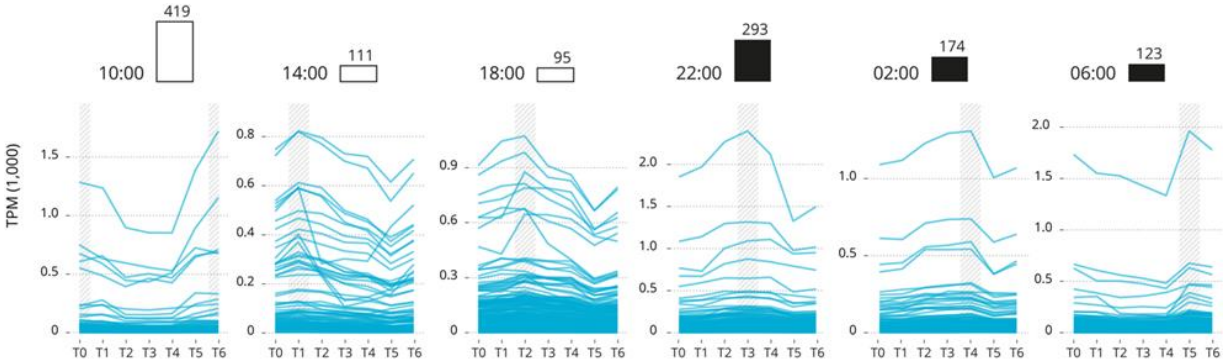


Figure 3.13: (A) Heatmap displaying average diel expression of identified clock genes under constant LD (12:12, n = 6 per time point). The heatmap of the relative expression of each individual gene [scaled lowest expression to highest expression]. Black diamond indicates significantly cyclic gene ($p < 0.001$) [JTK and RAIN analysis], White circles denote rhythmic genes ($p < 0.001$) [RAIN analysis]. **(B)** Significantly clock gene expression. Parameters of the cyclic sin-cosine function calculated by MetaCycle with JTK for diel expression of clock genes in brains collected from Atlantic salmon smolt exposed to an LD 12:12 photoperiod.

From the six *period* genes identified five were highly significantly rhythmically expressed (*per1a.1, per1a.2, per1b, per2a, per2b*), including three that also exhibited circadian expression pattern (*per1a.1, per1a.2, per2b*). The acrophase for *per1a.1* and *per1a.2* are in phase at 06:00, *per1b* has a negative phase shift in comparison to *per1a* paralogs at 08:30. *per2a* and *per2b* are out of phase by two hours, with *per2a* peaking at 03:00 and *per2b* at 01:00.

Overall, 2,864 genes exhibited a rhythmic expression pattern (rhythmic peak; RAIN analysis, $p < 0.001$; Supplementary Data S2), and of which 1,215 genes showed a significance circadian expression patten (peak and trough; JTK analysis; $p < 0.001$). Rhythmic gene were clustered by acrophase (expression peak) synchronicity (Figure 3.14).

Figure 3.14: Genes groups based on their acrophase (expression peak) synchronicity. The number of gene is reported over the barplot (white and black boxes denote light or darkness conditions respectively). Sub-figures exclude three highly expressed genes that bias the scale of the graphic: Ependymin-1 (> 10,000 TPM) Ependymin-2 (> 5,000 TPM) and calcium voltage-gated channel auxiliary subunit alpha 2 delta 4 (> 2,000 TPM).



The number of circadian expressed genes with the same acrophase range oscillate between 95 and 419. The majority of genes are expressed in one of two peaks at 10:00 [first sampling in the light] and 22:00 [first sampling in the darkness]. The highly significantly rhythmically expressed clock genes are distributed similarly to the rest of those which are significantly expressed in a circadian pattern (p = 0.15). The majority are peaking at 22:00. Sub-figures exclude 3 highly expressed genes that bias the scale of the graphic: Ependymin-1 (> 10,000 TPM) Ependymin-2 (> 5,000 TPM) and calcium voltage-gated channel auxiliary subunit alpha 2 delta 4 (> 2,000 TPM); all three genes are related with central nervous system plasticity and memory formation.

3.5 Discussion

In the present study, we have identified and characterised 48 core clock and accessory loop genes *in silico* in several salmonid species with published genomes using the latest common ancestors as reference points. For each of the salmonid species with published genomes, the core clock and accessory loop genes were identified and characterised *in silico*. There is a differential retention of genes originating in the latest common ancestors, *E. lucius* and *D. rerio*. In addition to this, there are numerous ohnologs (Ohno, 1970) which are consistent with the salmonid specific whole genome duplication event Ss4R (Lien *et al.*, 2016). There is also evidence of differential gene loss in salmonids based on the current gene annotations (Table 1). Findings from this study further highlight the rich complexity of core clock genes previously outlined in *S. salar* (West *et al.*, 2020), and is displayed across a wider complement of salmonid species. The gene families identified differ slightly to those explored by West et al. (West *et al.*, 2020) as the focus of this study was to identify all members of gene families *clock, arntl, period, cryptochrome, ror, nr1d* and *csnk1*. Identified genes common to both studies coincide with one another and paralog pairs identified using ML phylogenetic alignments are also identified as paralogs (West *et al.*, 2020).

In comparison to *D. rerio* with 29 reference clock genes (Wang, 2008b; Huang, Ruoff and Fjelldal, 2010a; Toloza-Villalobos, Arroyo and Opazo, 2015), most clock genes identified in salmonids appear to have been duplicated. In addition to these lineage-specific duplications there is also evidence of differential ohnolog loss in some species of salmonids based on the current genome annotations, *per1b*, *cry2*, and *cry5* appear to have been retained across all species from the latest common ancestors, *per3* appears to only be retained in *S. salar* and *S. alpinus* and *cry4* appears to only be retained in *S. salar.* Whereas *clock1b, cry1b, cry3a, csnk1da* all appear to have been lost in salmonids after the latest common ancestor *E. lucius.* Some species of salmonids have better genome annotations than others, and currently it is not possible to identify the effects of the WGD for every core clock gene family in *O. tshawytscha* at present. As expected across most of the gene families except where there is limited information, the *Oncorhynchus spp*. tend to appear together on the same clade with *S. salar* and *S. alpinus* commonly grouped together in a sister clade. A typical example of the clock gene families identified is the *cryptochrome* family. *cry1a* is one of the major clades in the cryptochrome family, as a result of duplication there are three main paralogous subfamilies *cry1a, cry1b* and *cry1c. cry1a and cry1b* are phylogenetically related to tetrapods with *cry1a* being the most conserved of the two subfamilies (Mei, Sadovy and Dvornyk, 2015). Zebrafish possess both *cry1a* and *cry1b* however, all the salmonid species only appear to have inherited *cry1a* which has been duplicated as a result of the WGD, resulting in two paralogs *cry1a.1* and *cry1a.2.* Except for *O. tshawytscha* (for which there is not currently enough information in the genome annotation) *cry3b* is duplicated in all salmonid species with an apparent gene loss in both *O. kisutch and O. mykiss*. This is suggestive of the non-functualisation and subsequent loss of *cry3b.2* in the *Oncorhynchus spp.* As a result of the Ss4R WGD clock most genes appear to be duplicated in salmonids with some exceptions that appear to have been lost in several of the salmonids. As annotations improve, it is thought that additional gene losses will become more apparent. This study gives insight into the post duplication effects on clock gene family members and provides a fundamental tool for further circadian work in salmonids.

Clock genes interact with each other, generating oscillations in gene expression. Their underlying principle is to create successive gene activation in the form of a cycle, forming an autoregulatory feedback loop which perpetually cycles approximately every 24 hours (Ripperger and Albrecht, 2008). This in turn influences downstream targets, whose time-of-day specific expression is determined by the central circadian mechanism (Ripperger and Albrecht, 2008).This study confirmed the expression of the identified clock genes in the brain of *S. salar* smolts over a 24-hour period and showed that 11 out of the 48 core clock genes were highly significantly ($p < 0.001$) rhythmically expressed.

We used a two complementary approach, RAIN which allowed us to detect accurately rhythms in time series irrespectively of the shape of the expression pattern, and JTK with a focus on circadian expression patterns. The vast majority of the JTK findings are included in the RAIN results (Table 3.4). This allowed us to distinguish between rhythmically expressed genes and genes with a circadian expression pattern.

The period family is particularly interesting, as except for one gene (*per3*) all the genes were significantly rhythmically expressed. A difference in acrophase between paralogs was also identified. There was a clear difference in expression pattern observed between *per1a* paralogs (*per1a.1, per1a.2*) and *per1b,* with *per1a* peaking at 06:00 displaying a positive phase shift in comparison to *per1b* which peaks at 08:30. This positive phase shift coincides with results observed in zebrafish, it was reported that *per1a* and *per1b* paralogs displayed a shifted phase of gene expression in zebrafish with *per1a* peaking 4 hours prior to *per1b* under a 12:12 LD lighting regime (Amaral and Johnston, 2012). In zebrafish, *per1* paralogs were shown to display a distinct difference in spatial and temporal expression in the brain, providing strong evidence for the duplicate pair to have undergone sub- or neo-functionalisation (Wang, 2008a). The duplicated Atlantic salmon *per1a* paralogs (*per1a.1* and *per1a.2*) share the same periodicity and acrophase, thus indicating that both genes may share a similar functionality. This indicates that the paralogs may have undergone sub-functionalisation – in which the ancestral functions have become partitioned, and each paralog potentially has particular adaptations for different tissues, developmental stages or environmental conditions (Innan, 2009). In mammalian literature, it is widely reported that *Cry1/2* form heterodimers with *Per1/2/3* (Rosensweig *et al.*, 2018). However, in this study, the only highly significantly rhythmically entrained *cryptochrome* genes was the *cry3b.2* paralog which peaked two hours before the closest *period* family member (*per2b*) at 20:30, in agreement with the pattern of *cry2* expression identified in zebrafish ~12-hours after light onset (Hirayama *et al.*, 2019). In mammals, *Per2* is said to repress *Nr1d2* transcription thus upregulating *arntl1* transcription (Chiou *et al.*, 2016), in keeping with these findings in this study *nr1d2b.2* peaks at 08:30, around 10 hours before peak expression of *arntl1a.1/1a.2/1b.1* (18:00) and *per2b* peaks at 01:00. This suggests that the relationship between clock gene paralogs in salmonids may be similar to that of those in mammalian species.

How salmonids fit into the widely accepted mechanism for circadian rhythmicity is difficult to evaluate due to the limited understanding surrounding circadian mechanisms in fish in general and the complexity are yet to be exploited to the full potential (Frøland Steindal *et al.*, 2018). So far, the expression of individual clock genes has been previously investigated in salmonids (Davie, Minghetti and Migaud, 2009; Huang, Ruoff and Fjelldal, 2010a; West *et al.*, 2020).

Clock gene member identification has previously been hampered by paralogs with high sequence similarity, which does not easily allow for individual identification by qPCR. Individual gene identification using RNA sequencing has allowed for paralogs to be better classified and individual gene expression patterns ascertained. Although, single gene duplication events in mammalian species have enabled the evolution of specialisation and regulatory sophistication in the temporal regulation of local physiology (Looby and Loudon, 2005) the core clock genes associated with circadian rhythmicity remains largely conserved across a diverse range of organisms spanning vast evolutionary time periods (Bell-Pedersen *et al.*, 2005; Cox and Takahashi, 2019). This study supports that whilst the compliment of clock genes is far richer in salmonids, the function of core clock genes remains conserved and are therefore likely function similarly to other more studied organisms. Some salmonid species display reciprocal gene losses post Ss4R, but many of clock genes identified appear to be duplicated in the salmonid species investigated. The existence of such paralogs whilst increasing genetic complexity may enable gene family members to specialise and extend their ancestral role, which can lead to a shift in the identity of components of the molecular clock (Looby and Loudon, 2005; West *et al.*, 2020). Ambient temperature affects gene expression and physiology in ectotherms; in zebrafish, the clock seems to be temperature-compensated, changing the amplitude of some critical clock genes (Lahiri *et al.*, 2005). However, the influence of temperature on the clock system in salmonids remains poorly studied and should be investigated further.

Whilst this study has furthered the identification of clock gene family members in central salmonids, additional work is required to further elucidate the complexity of the circadian mechanism and how the complement of clock genes identified individually function as components of this mechanism. It is important to note that alongside individual variability, the whole brain being analysed will have influenced gene expression levels and rhythmicity due to the highly decentralised organisation of clock genes in teleosts and localisation of clock gene expression in specific regions of the brain (Moore and Whitmore, 2014). This study provides a fundamental tool to explore the role of the enriched number of clock genes related to seasonal driven life history transition in salmonids.

Chapter Four

Effects of light conditions during early development of Atlantic salmon (*Salmo salar*) on circadian clock gene rhythms from transcriptomic data

The work in this chapter was undertaken as part of the collaborative project funded by the Research Council of Norway "*The effect of narrow banded LED light on development and growth performance"* (grant number 254894) and the University of Stirling PhD match funding scheme. Since initial submission this work has been collated along with targeted experimental research from the wider project and published.

Eilertsen M, Dolan DWP, Bolton CM, Karlsen R, Davies WIL, Edvardsen RB, et al. (2022) Photoreception and transcriptomic response to light during early development of a teleost with a life cycle tightly controlled by seasonal changes in photoperiod. *PLoS Genet* 18(12): e1010529. doi: 10.1371/journal.pgen.1010529.

4.1. Abstract

Light is known to play a significant role in synchronising an organism's biological clock with its environment, and it has been observed to affect the growth and development of commercially important aquaculture species. Clock genes have also been associated with the development of multiple species of teleost, influencing critical developmental stages like hatching. A comprehensive approach was taken to investigate the potential link between clock genes, light and development in Atlantic salmon by identifying the expression of clock genes during early developmental stages and analysing their response to different lighting conditions. The transcriptomes of whole embryos and alevins of Atlantic salmon were analysed under varying photo cues, light intensity, and spectral composition, with a focus on developmental and circadian profiles. The findings revealed that clock genes were expressed from eye pigmentation, with two genes (arntl2b and nr1d4b.2) showing significant cyclical expression ($p < 0.05$) before first-feeding. Moreover, several clock genes displayed significant differential expression between day and night within a light condition, indicating that the light environment impacts the expression of clock genes during early development. These results suggest a direct influence of light on clock gene expression, with some genes exhibiting cyclic patterns prior to first-feeding.

4.2. Introduction

Light is a complex environmental signal that provides photic cues to organisms defined by the period, intensity, and spectral composition. Knowledge surrounding an organism's ability to perceive, process and integrate light cues to entrain and regulate biological rhythms and physiological events is essential to better understand the impacts of lighting environments upon an individual (Gaston *et al.*, 2017). In non-mammalian vertebrates, the perception of light takes place in the retina, pineal organ, and in the deep brain (Peirson, Haiford and Foster, 2009; Davies, Hankins and Foster, 2010; Pérez *et al.*, 2019). The effect of light upon fish biology has been studied in several species by exposing fish during varied developmental stages to different lighting environments: zebrafish, *Danio rerio* (Vuilleumier *et al.*, 2006; Dekens and Whitmore, 2008; Vatine *et al.*, 2011; Di Rosa *et al.*, 2015); rainbow trout, (Davie *et al.*, 2011); Senegalese sole (Martín-Robles, Whitmore, *et al.*, 2012); gilthead sea bream (Paredes *et al.*, 2014; Mata-Sotres *et al.*, 2015); European sea bass (Villamizar, García-Alcazar and Sánchez-Vázquez, 2009); Atlantic turbot (Sierra-Flores *et al.*, 2016; Ceinos *et al.*, 2019); and Atlantic cod (Migaud *et al.*, 2009; Sierra-Flores *et al.*, 2016). Artificial light blurs the boundary between day and night, and therefore interferes with the physiological functions and behaviour of fish. It is already known that artificial lighting at night affects the growth and development of fish and can even disrupt the spawning

migration of diadromous (migratory) fish (O'Connor *et al.*, 2019), altering cues and the timing of biological activities (Gaston *et al.*, 2017).

Embryogenesis in fish has been considered a continuous progression of developmental stages whose pace is set by temperature: a specific embryonic stage is reached faster at higher temperatures (Guerreiro *et al.*, 2012). However, the biological clock has also been suggested to control embryo development and hatching rhythms (Gorodilov, 2010). A comparative study between zebrafish, Senegalese sole, and Somalian cavefish (*Phreatichthys andruzzii*), revealed that embryo development does not occur at a constant pace throughout day and night, leading to daily rhythms in developmental rate. These rhythms were synchronized to the light dark (LD) cycle, influenced by temperature and with acrophases determined by the daily type of behaviour of the species (Villamizar *et al.*, 2014). Zebrafish are a diurnal species and so embryos develop faster during daytime than during night-time, while in Senegalese sole (a nocturnal fish at juvenile and adult stages) embryos advanced their development at night and slowed down during the day. Thus, in these two fish species the pace of somitogenesis appears to be different in embryos raised under LD regardless of the temperature regime. This temperature-independence occurs because the circadian clock must be temperature-compensated to ensure reliability and the sustainability of stable circadian oscillations over time in a wide range of temperatures (López-Olmeda and Sánchez-Vázquez, 2011).

The visual capabilities of fish is likely to change during development, as well as in response to daily and seasonal fluctuations (Carleton *et al.*, 2020). Whilst the effect of light on the hatching of Atlantic salmon is not well studied, in zebrafish, larvae raised under varied lighting conditions displayed the highest hatching rate when exposed to blue or violet light conditions. However, those exposed to constant lighting displayed a higher percentage of malformations (Villamizar *et al.*, 2014). Atlantic cod and Atlantic turbot exposed to blue and green spectra showed significantly enhanced growth compared to those exposed to red light, even though the survivability appeared reduced under green light (Sierra-Flores *et al.*, 2016). The light dependent phenotypic responses reported in these studies are a result of the expression of many genes directly or indirectly influenced by light, however the underlying mechanisms and pathways have not yet been described or fully understood. Recently published microarray analysis of circadian gene expression in fish looking at the effects of light shifts provide interesting findings. A circadian feeding trial in gilthead sea bream revealed repeated daily activation of pathways associated with photo transduction, intermediary metabolism, development, chromatin remodelling and cell cycle regulation (Yúfera *et al.*, 2017). The shift in light environment (dark to light) in zebrafish revealed enrichment of genes involved in circadian rhythmicity, stress response and DNA repair (Weger *et al.*, 2011). The majority of light stimulation studies in marine fish larvae were

performed around first-feeding (Villamizar, García-Alcazar and Sánchez-Vázquez, 2009; Villamizar *et al.*, 2014; Sierra-Flores *et al.*, 2016; Yúfera *et al.*, 2017), with an applied focus to improve performances and hatchery output given the very low survival rates reported in these species.

The present study was undertaken in Atlantic salmon given its many advantages for circadian research due to its biology [eg: large demersal eggs, very high survival, long developmental window, absence of metamorphosis, and hatching of large alevins (Eilertsen *et al.*, 2022)], and the availability of genomic and transcriptomic data. Our light stimulation study is independent of exogenous feeding, the numerous robust nature of Atlantic salmon eggs enabled the use of ova from one female and milt from one male to reduce the effect of individual variance as all fish are full siblings. The collaborative project took a global transcriptomic approach, analysing gene expression in whole developing Atlantic salmon eggs and alevins exposed to different lighting conditions (photo cue, intensity, and spectral composition) from fertilisation to FF over multiple key developmental stages and 24-h to identify the effect of light environment on the early development of Atlantic salmon.

The aims of this study were to identify clock gene expression during early developmental stages in Atlantic salmon and investigate the influence of lighting conditions upon expression. To do so, the clock gene toolkit created in the Chapter Three was used to interrogate the transcriptome in early developmental Atlantic salmon kept under controlled lighting regimes from fertilisation at several key developmental stages and a 24 h period prior to FF. This study provides new insight into the ontogeny of the clock and the impact of early lighting regimes on the number of rhythmically and cyclically expressed genes in the totality of the transcriptome in Atlantic salmon.

4.3. Materials and method

4.3.1. Ethical statement

All experiments followed local animal care guidelines and were given ethical approval by the Norwegian Veterinary Authorities and University of Stirling (AWERB 19 20 097). The ARRIVE guidelines have been complied in this study (Percie du Sert *et al.*, 2020).

4.3.2. Animal husbandry

Eggs and sperm were obtained from one male and one female from Mowi (formerly Marine Harvest), Tveitevågen, Norway (2017-10-19) and were fertilised in an approved laboratory facility at the High Technology Centre, University of Bergen, Norway. Eggs were under experimentation from fertilisation until the end sampling point, prior to FF. The eggs were incubated in triplicated hatching trays (300-450 eggs per tray) in incubators at 6 °C under different light regimes (see details in 4.2.3 Light experiment). As temperature has a major impact upon development the experimental setup was designed to ensure that the temperature was equal in all tanks. The average inlet temperature from fertilisation to FF at 121 days was 6.1 °C ± 0.04 S.E. (Figure 4.1), therefore at the last sampling point just prior to FF the alevins across the trial were approximately 690 ± 6 degree-days (dd).

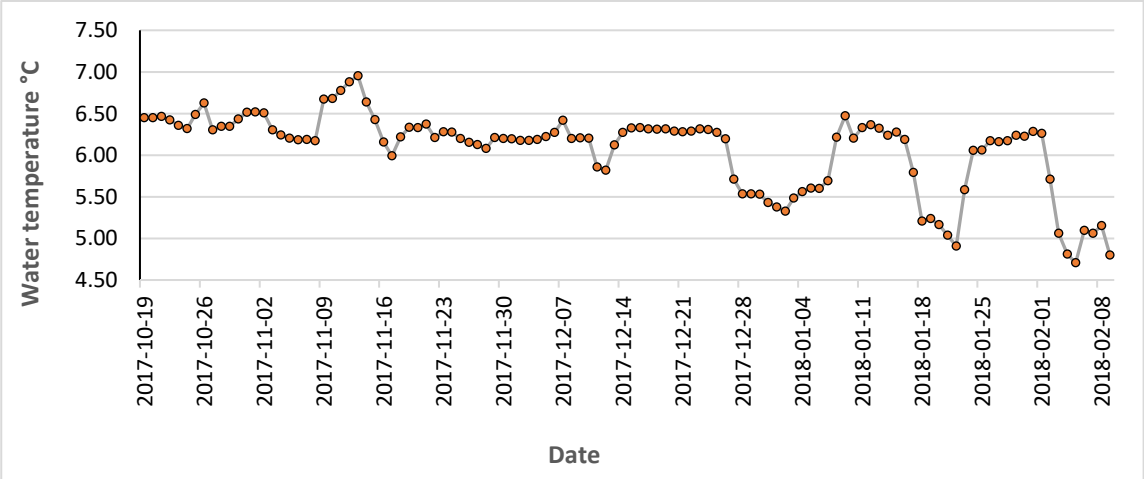


Figure 4.1: Graph displaying average daily inlet temperature variation across the duration of the lighting experiment. 2017-10-19 – 2018-02-09, average temperature 6.1 °C.

4.3.3. Light experiment

Eggs and alevins were incubated under different light conditions using state of the art light-emitting diode (LED) technology supplied by Signify (formerly Philips Lighting, The Netherlands). The experimental set up is shown in Figure 4.2.

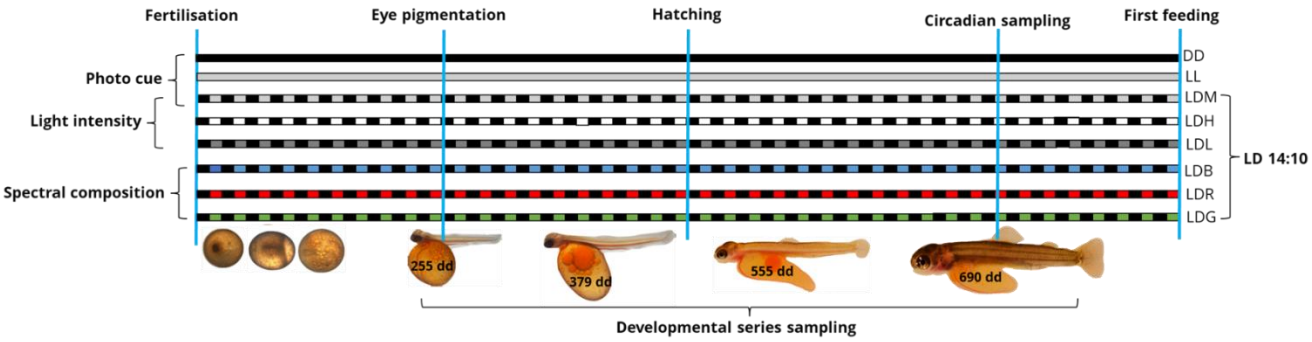


Figure 4.2: Experimental setup for light experiments, developmental series sampling was conducted on fish from the LDM treatment group only.

Eight different light conditions were applied from fertilisation to FF using state of the art light emitting diode (LED) technology (Signify, The Netherlands). Treatments included: continuous light (LL), continuous darkness (DD), three intensities of white light with a 14:10 photoperiod (low, 0.01 W/m^2 - LDL, medium, 0.1 W/m^2 - LDM, and high, 1 W/m^2 - LDH), and three different spectra of light, green, blue, and red (LDG, LDB, LDR) at the medium intensity 0.1 W/m^2. The luminaires were adjusted across colours using a spectroradiometer to equalise the photon flux between treatments (Ramses ACC-VIS, TriOS, Germany), setting the LL, LD, LDG, LDB, and LDR treatments to an intensity of 0.1 W/m^2 (Table 4.1). Except for LL and DD all light treatments were on a 14:10 LD cycle from 8am until 10pm with the first and last 30-minutes of the light simulating crepuscular periods to minimise stress on the fish during lighting transitions.

Table 4.1: Properties of light-emitting diodes (LEDs).

LED	Periodicity	Colour	Intensity	µE m^{-2} s^{-1}	W/m^2
1	24 Continuous Light (LL)	White	Medium	0.4925	0.1004
2	14:10, Light: Dark (LD)	White	High	4.9035	1.0002
3	14:10, Light: Dark (LD)	White	Medium	0.4932	0.1004
4	14:10, Light: Dark (LD)	White	Low	0.0507	0.0103
5	14:10, Light: Dark (LD)	Blue	Medium	0.4504	0.1199
6	14:10, Light: Dark (LD)	Green	Medium	0.4509	0.1009
7	14:10, Light: Dark (LD)	Red	Medium	0.4505	0.0824
8	24 Continuous Dark (DD)	-	-	-	-

4.3.4. RNA extraction and sequencing

Samples were sent for sequencing as outlined in Table 4.2. For the developmental series sampling, samples (n = 6, except n = 4 for 690 dd) were taken at 255 dd, 379 dd, 555 dd and 690 dd from the LDM treatment, these timepoints approximately coincide with the key developmental stages of eye pigmentation, pre-hatch, post hatch and pre-FF, respectively. For the remaining light treatments and circadian study, samples (n=4) were taken starting at 10:00 at 690dd every four hours over a period of 24-hours. Due to the cost of RNA sequencing, a limited number of samples were sent away for sequencing from the light condition series. These represent the middle of the light period and the middle of the dark period.

Table 4.2: Samples sent away for RNA sequencing based on treatment. Samples from the LDM 24 h circadian series were also used for 690 dd in the developmental series and LDM in the light condition series.

Treatment	Number of Samples	Description
Developmental Series		
255 dd	6	n=6
379 dd	6	n=6
555 dd	6	n=6
690 dd	4	n=4
Circadian series starting at 690 dd		
LDM	28	n=4/ timepoint, 10:00, 14:00, 18:00, 22:00, 02:00, 06:00 & 10:00
Light conditions series at 690 dd		
DD	8	n=4/ timepoint, 18:00 & 02:00
LDM	8	n=4/ timepoint, 18:00 & 02:00
LL	8	n=4/ timepoint, 18:00 & 02:00
LDL	8	n=4/ timepoint, 18:00 & 02:00
LDH	8	n=4/ timepoint, 18:00 & 02:00
LDB	8	n=4/ timepoint, 18:00 & 02:00
LDG	8	n=4/ timepoint, 18:00 & 02:00
LDR	8	n=4/ timepoint, 18:00 & 02:00
Total	**102**	

During sampling alevins were snap frozen in liquid nitrogen and stored at -80 °C, RNAlaterICE (ThermoFisher Scientific, Waltham, MA, USA) was added and the alevins were immersed for a minimum of 48 h at -20 °C before RNA was isolated using TRI reagent (Sigma, ST Louis, MO, USA) following the method in Chapter Two (2.2 RNA extraction). RNA integrity was tested using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) revealing the RNA integrity number (RIN) of samples valued between 8.8-10.0. The RNA samples were submitted to the Genomics Core Facility at the University of Bergen for RNA sequencing. Each sample (400 ng) was processed and sequenced using Illumina TruSeq® Stranded mRNA Sample Preparation Kits according to Illumina TruSeq® Stranded Preparation Guide on Illumina HiSeq 4000 (Illumina Inc., San Diego, CA, USA).

Poly (A)+ RNA was purified, fragmented, and converted to first strand and second cDNAs. The second strand cDNA was amplified using PCR (15 cycles) to create the final cDNA library. Sequencing generated and average of 38 million 75 bp paired end reads per sample.

4.3.5. RNA sequencing analysis

The 102 samples (see Table 4.2) were aligned to the published *S. salar* genome ICSASG v2.99 (Accession GCA_000233375.4) using STAR v2.7.7a (Dobin *et al.*, 2013). Counts were generated using HTSeq v0.12.4 (Anders, Pyl and Huber, 2015) and DESeq2 v1.30.1 (Love, Huber and Anders, 2014) was used to generate normalised counts and perform differential expression analyses. All count values were expressed in Fragments per kilo base per million mapped reads (FPKM). The mapped reads converted to counts per sample, represented a total of 42,853 genes for the developmental series and 45,292 genes for the 690 dd circadian sampling.

4.3.6. Statistical analysis

All tests and analysis were performed using R v4.0.2 (RDC, 2010). The expression values were converted from FPKM to Transcripts per Kilobase Million (TPM) to scale the data based on gene length followed by sequencing depth. Thus, allowing an easier comparison of the proportion of reads which mapped to a gene in each sample. Daily rhythms across the genome including identified clock genes were identified from the 24-hour dataset using custom scripts for RAIN v1.26.0 (Thaben and Westermark, 2014) and MetaCycle v1.2.0 (Wu *et al.*, 2016) using JTKCycle (Hughes, Hogenesch and Kornacker, 2010) and a threshold α-value of 0.05, 0.01, and 0.001. All *p*-value reported were corrected for False Discovery Rate (FDR) using Bonferroni adjustment. One-way ANOVAs with post-hoc Bonferroni adjustment with a threshold α-value of 0.05 were used to test the effect of lighting condition on clock gene expression and comparison between treatments for developmental, spectral composition, light intensity, and photo cue datasets. The heatmaps were created from the normalised count files for the identified clock genes using customised scripts for pheatmap v1.0.12 (Kolde, 2012). The heatmaps were scaled by row to highlight individual gene expression.

4.4. Results

All clock genes identified from *in silico* gene identification and RNA sequencing in parr stage Atlantic salmon brains (Chapter Three) were recovered from the RNA sequencing data.

4.4.1. Clock gene expression throughout early development

Excluding *clock1a.2* and *arntl2b,* all clock genes investigated were significantly ($\alpha = 0.05$) differentially expressed between all developmental stages (Figure 4.3). Most (37) displayed a highly significant ($\alpha < 0.001$) difference in expression between developmental stages [4 genes $\alpha < 0.01$, 5 genes $\alpha < 0.05$]. Whilst there appears to be a general increase in overall clock gene expression from eye pigmentation (255 dd) to pre-FF (690 dd). Interestingly there also appears to be a functional divergence of paralogs in relation to developmental stage (Figure 4.3). Using the *period* gene family as an example, both *per2* paralogs (*per2a & per2b*) appear to be more highly expressed pre-hatch compared to post-hatch. It is important to note that due to the extraction of RNA from the whole embryo that genes which are constantly expressed may be seen as decreasing in expression value due to the mRNA being diluted by larger organisms post-hatching in comparison to pre-hatching. The *arntl* gene family appear to be a good example of this as the majority of genes appear to be most highly expressed during the sampling which coincides with eye pigmentation (255 dd). Whereas *per1a* paralogs appear to be specialised, indicative of subfunctionalisation, with *per1a.1* being more highly expressed pre-hatch and *per1a.2* post-hatch.

One-way ANOVA ($p < 0.05$), with Post-hoc Bonferroni adjustment to identify significant difference in clock gene expression between developmental stages identified 41 genes which displayed significant differences ($p < 0.05$) in expression between timepoints. The developmental stages in which there was the greatest number of significant differences between gene expression occurred between pre-hatching timepoints and post-hatching timepoints. Timepoints which displayed the least amount of significant difference between one another were the two post-hatching timepoints (555 dd and 690 dd).

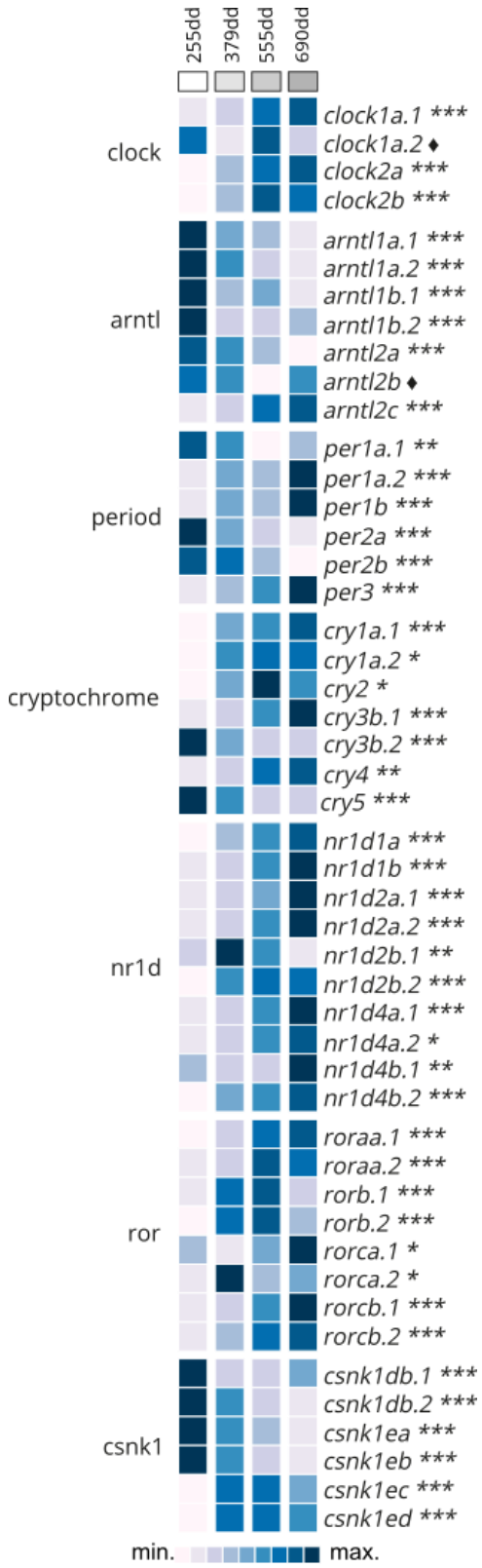


Figure 4.3: Heatmap displaying mean expression of identified clock genes under LD (14:10) across multiple developmental points (255 dd, 379 dd, 555 dd, and 690 dd, n = 6 except for 690 dd where n = 4). The heatmap of the relative expression of each individual gene [0, lowest expression, to 1, highest expression]. Asterisks represent level of significance ['*' = $p < 0.05$, '**' = $p < 0.01$, and '***' = $p < 0.001$] and black diamonds indicates lack of significant difference in gene expression between treatment groups [One-way ANOVA ($p < 0.05$), with Post-hoc Bonferroni adjustment to distinguish significantly different gene expression between developmental stages].

4.4.2. Clock gene expression over 24-hours

Significantly rhythmically and cyclically expressed genes were identified using RAIN (p < 0.05) and MetaCycle (p < 0.05, rAMP ≥ 10 %) over a period of 24 h (Table 4.3, Figure 4.4). There were 1,996 significantly rhythmically expressed genes entrained to a period of 24 h (p < 0.05), this decreased to 612 (p < 0.01) and 104 (p < 0.001) as the level of significance increased. Of these rhythmically expressed genes, 287 (p < 0.05), 66 (p < 0.01), and 12 (p < 0.001) of them were significantly cyclically entrained to 24 h. Within the strict parameters set for periodicity of 24 h, whilst six of the clock genes were significantly rhythmically expressed (p < 0.05, *clock1a.2, arntl2b, nr1d2a.1, nr1d2b.2, nr1d4b.1,* and *nr1d4b.2*), none of them were significantly cyclically expressed. Exploration of shortened (20 h) and extended (28 h) circadian periods resulted in one gene (*arntl2a*) being identified as significantly rhythmically expressed over 20 h and 11 genes (*clock1a.2, arntl2a, arntl2b, per1a.1, per1a.2, cry1b.1, nr1d2a.1, nr1d2a.2, nr1d4a.1, nr1d4b.1*, and *nr1d4b.2*) displaying a significantly rhythmic expression pattern (p < 0.05) over 28 h, of which two (*arntl2b* and *nr1d4b.2*) were significantly cyclically entrained (p < 0.05). In total, 12 out of the 48 clock genes investigated were reported to be significantly rhythmically expressed over a period of 20, 24, or 28 h.

Table 4.3: Number of significantly expressed genes: Rhythmic (RAIN) or circadian (MetaCycle with JTK) depending on the *p*-value (FDR adjusted) or period used. All rhythmic genes in the genome and clock genes only (in brackets). Unique genes represent the single genes from the concatenation and filtering out of duplicated significant genes form 20, 24 and 28 h periods.

	RAIN	**JTK**	**Overlap**
adjP-value < 0.05			
20 h	1,988 (1)	327 (-)	293 (-)
24 h	1,996 (6)	287 (-)	264 (-)
28 h	2,740 (11)	262 (2)	250 (2)
adjP-value < 0.01			
20 h	648 (-)	81 (-)	73 (-)
24 h	612 (2)	66 (-)	60 (-)
28 h	912 (4)	49 (-)	48(-)
adjP-value < 0.001			
20 h	117 (-)	12 (-)	9 (-)
24 h	104 (-)	12 (-)	12 (-)
28 h	165 (1)	4 (-)	4 (-)

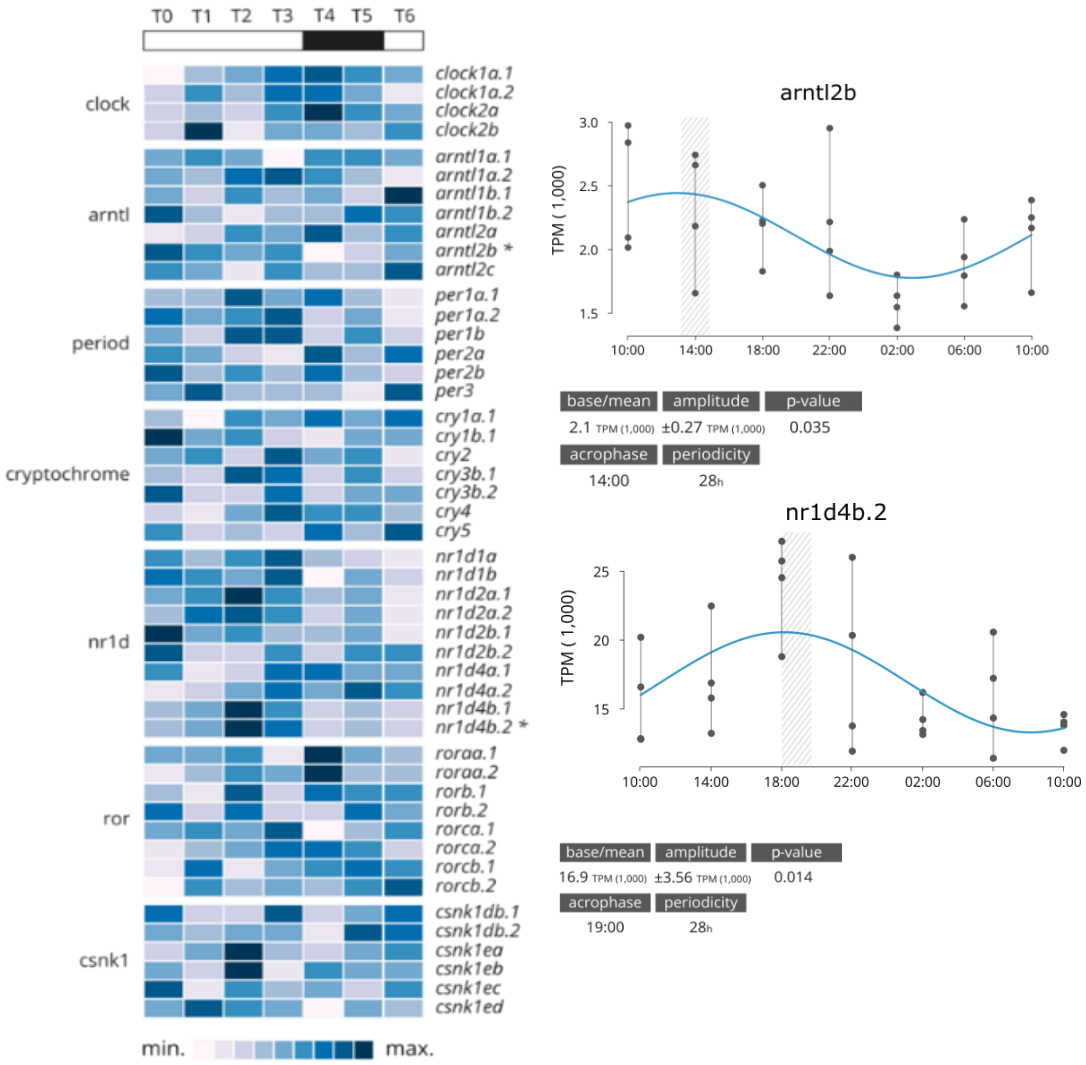


Figure 4.4: (A) Heatmap displaying average diel expression of identified clock genes under constant LD (14:10, n = 4 per time point). The heatmap of the relative expression of each individual gene [scaled from lowest expression to highest expression]. Asterisks indicate significantly rhythmic genes (p <0.05) [RAIN analysis, period 20, 24, 28 h]. **(B)** Significantly cyclical (p < 0.05, MetaCycle with JTK, period = 28 h) clock gene expression, parameters of the cyclic in-cosine function calculated by MetaCycle with JTK for diel expression of clock genes (*arntl2b* and *nr1d4b.2*) in whole Atlantic salmon alevins exposed to a constant 14:10 LD photoperiod.

Overall, 1996 genes exhibited a rhythmic expression pattern (rhythmic peak; RAIN analysis, $p < 0.05$), of which 261 genes showed a significantly circadian expression pattern (peak and trough; MetaCycle with JTK analysis; $p < 0.05$ and rAMP ≥ 10 %). Rhythmic genes were clustered by acrophase (expression peak) synchronicity (Figure 4.5). The number of genes with the same acrophase range between 71 and 26. The highest number of genes peak at 20:00, the last sampling prior to the lights turning off at 22:00. Only one gene was excluded from the subfigure (Figure 4.5A) as it biases the scale of the graphic: acyl-CoA dehydrogenase very long chain (> 100,000 TPM) it involved in acyl-CoA dehydrogenase activity and flavin adenine dinucleotide binding (Bateman *et al.*, 2021).

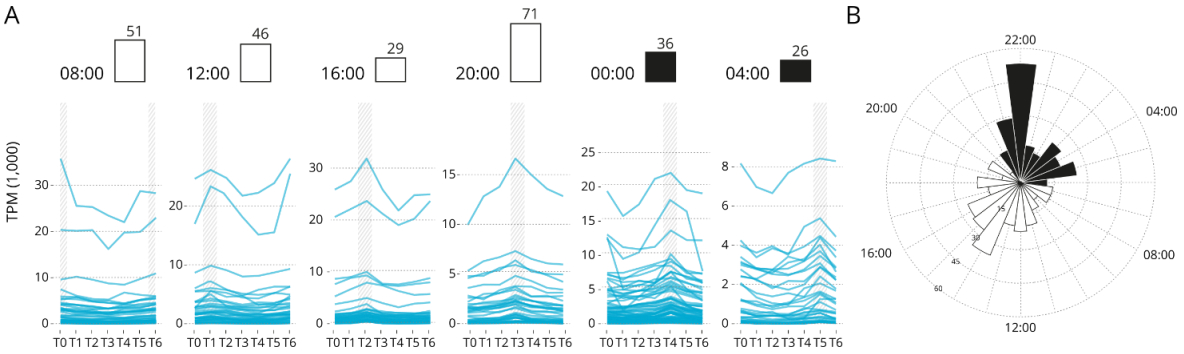


Figure 4.5: (A) Genes grouped based on their acrophase (expression peak) synchronicity. Based on the average recorded TPM values. The number of genes is reported over the bar plot (white and white boxes denote light or darkness conditions, respectively). Subfigures exclude one highly expressed gene that biases the scale of the graphic: acyl-CoA dehydrogenase very long chain (> 100,000 TPM, ENSSSAG00000049619). **(B)** Rose plot of the phase distribution of significantly circadian gene expression ($p < 0.05$, rAMP ≥ 10 %, JTK analysis, period 24h). Colour coding indicates whether the phase occurs during the light (white) or dark (black) periods of the LD 14:10.

4.4.3. Clock gene expression under different light Conditions

To investigate the effects of light conditions (photo cue, light intensity, and spectral composition) on the differential expression of clock genes between the photo- and scotophase one-way ANOVAs ($p < 0.05$) were conducted to identify the significantly differentially expressed clock genes within each light condition (Figure 4.6). Out of the 48 clock genes investigated over half (n = 26) displayed significantly differentially expressed clock genes between treatments and time points. Light intensity and photo cue displayed the highest number of significantly differentially expressed genes ($p < 0.05$, n = 16) out of the three light conditions, in comparison to spectral composition ($p < 0.05$, n = 11). There were a number of genes which were identified as significantly differentially expressed across a number of the three lighting conditions investigated. However, *arntl2a, nr1d1b, nr1d2a.2,* and *nr1d4b.1* were all significantly differentially expressed between treatments in all three different light condition sub-experiments. Coincidentally, these were also some of the most highly significantly differentially expressed genes. To further understand the influence of light condition upon clock gene expression post-hoc Bonferroni adjustments were used to test the effect of light condition on clock gene expression within treatments (timepoint comparison) and between treatments.

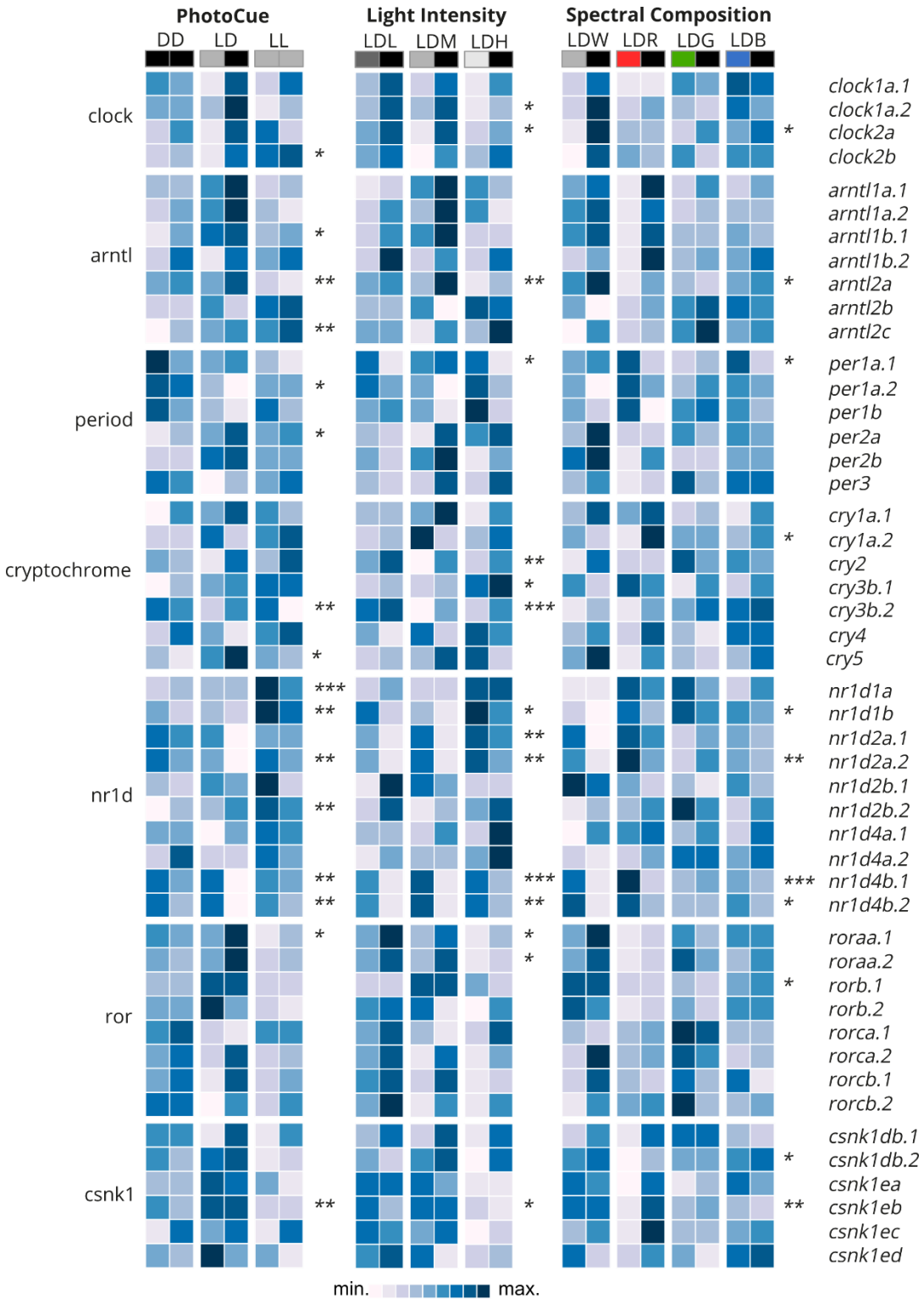


Figure 4.6: Heatmap displaying mean expression of identified clock genes under three light conditions: photo cue (n = 4, DD – absence of light and cues, LD – 14:10 photoperiod, LL – continuous light, absence of cues), light intensity (n = 4, LD Low – 0.01 W/m^2, LD Medium – 0.10 W/m^2, and LD High – 1.00 W/m^2, white light on a 14:10 LD photoperiod), and spectral composition (n = 4, LD White, LD Red, LD Green, and LD Blue on a 14:10 LD photoperiod). The heatmap of the relative expression of each individual gene [0, lower expression, to 1, highest expression]. Asterisks represent level of significance ['*' = $p < 0.05$, '**' = $p < 0.01$, and '***' = $p < 0.001$] in gene expression between treatment groups [One-way ANOVA ($p < 0.05$), with Post-hoc Bonferroni adjustment to distinguish significantly different gene expression between light treatments].

4.4.3.1. Clock gene expression under different photo cues

Of the 16 clock genes which displayed a significantly differential gene expression (α = 0.05) under different photo cues (DD, 14:10 LD, LL), post-hoc Bonferroni adjustment ($p < 0.05$) reveals six genes (*arntl2a, cry3b.2, cry5, nr1d1a, nr1d2b.2, csnk1eb*) which are significantly differentially expressed between treatment within each timepoint sampled, 18:00 or 02:00 (Table 4.4).

Table 4.4: Significantly different (ADJ.p < 0.05, One-way ANOVA with Post-hoc Bonferroni adjustment, rounded to 3 d.p.) clock gene expression in relation to Photo cue (DD, LD 14:10, & LL) in Atlantic salmon at 690dd under white light between treatment at 18:00 or 02:00 [↑ = significantly increased, ↓ = significantly decreased].

Gene	Treatment difference	Time point	P-value (3 d.p.)	Directionality
arntl2a	LL & LD	02:00	0.001	↑ LL, DD, LD ↓
cry3b.2	LL & DD	18:00	0.019	↑ LL, LD, DD ↓
cry5	LD & DD	02:00	0.033	↑ DD, LL, LD ↓
nr1d1a	DD & LL LD & LL	18:00	< 0.001 0.002	↑ LL, DD ↓, LD ↓
nr1d2b.2	DD & LL	18:00	0.006	↑ LL, LD, DD ↓
csnk1eb	LD & LL	18:00 02:00	0.012 0.020	↑ LD, DD, LL ↓ ↑ LD, DD, LL ↓

In the absence of a scotophase, there appears to be a significantly increased ($p = 0.001$) expression of *arntl2a* at 02:00 in comparison to LD. Under 14:10 LD and DD there is a clear increase in expression overnight. Whilst not significant those under LD display a greater difference in expression values for *arntl2a* between 18:00 and 02:00 than those under DD, in which expression values appear to be fairly static. Expression of *cry3b.2* and *nr1d2b.2* are significantly increased ($p = 0.019$, $p = 0.006$, respectively) during the photophase at 18:00 in comparison to individuals under DD. During the scotophase *cry5* expression is significantly increased ($p = 0.033$) in comparison to individuals under LD. The absence of a photophase appears to be more influential on gene expression than the absence of a scotophase. The expression of *nr1d1a* is significantly increased under LL than LD and DD ($p < 0.001$, $p = 0.002$, respectively) at 18:00, the absence of a scotophase has a greater impact on gene expression than the absence of a photo cue. Under LD conditions expression of *csnk1eb* is significantly increased in comparison to LL in both the photophase and scotophase ($p = 0.012$, $p = 0.020$, respectively).

4.4.3.2. Clock gene expression under different light Intensities

Of the 15 clock genes which displayed a significantly differential gene expression (α = 0.05) under different photo cues (LDL – 0.01 W/m^2, LDM – 0.10 W/m^2, and LDH – 1.00 W/m^2), post-hoc Bonferroni adjustment (p < 0.05) reveals just one gene which is significantly differentially expressed (p < 0.05) between treatment within each timepoint sampled, 18:00 and 02:00 (Table 4.5). Along with two paralogs which are significantly differentially expressed between timepoints within the same treatment (Table 4.6).

Table 4.5: Significantly different (ADJ.p < 0.05, One-way ANOVA with Post-hoc Bonferroni adjustment, rounded to 3 d.p.) clock gene expression in relation to light intensity [low (0.01 W/m^2), medium (0.10 W/m^2) and high (1.00 W/m^2) white light under LD 14:10 photoperiod] in Atlantic salmon at 690 dd, between treatment at 18:00 or 02:00 [↑ = significant increase, ↓ = significant decrease].

Gene	Treatment interaction	Time point	P-value (3 d.p.)	Directionality
cry3b.2	LDL & LDH	18:00	0.044	↑ LDH, LDM, LDL ↓
	LDL & LDH	02:00	0.015	↑ LDH, LDM ↓, LDL ↓
	LDM & LDH	02:00	0.033	↑ LDH, LDM ↓, LDL ↓

Table 4.6: Significantly different (ADJ.p < 0.05, One-way ANOVA with Post-hoc Bonferroni adjustment, rounded to 3 d.p.) clock gene expression in relation to light intensity and time of day [low (0.01 W/m^2), medium (0.10 W/m^2) and high (1.00 W/m^2) white light under LD 14:10 photoperiod] in Atlantic salmon at 690 dd, within treatment between 18:00 and 02:00 [↑ = significantly increased, ↓ = significantly decreased].

Gene	Time point interaction	Treatment	P-value (3 d.p.)	Directionality
cry2	18:00 & 02:00	LDM	0.040	↑ 02:00, 18:00 ↓

Expression of *cry3b.2* is significantly higher (p < 0.05) in individuals under the high intensity (1.00 W/m^2) LD light treatment in comparison to the low intensity (0.01 W/m^2) LD light treatment at 18:00 (p = 0.045) and significantly higher than individuals under LDL and LDM at 02:00 (p = 0.015, p = 0.033, respectively). Implying that there is a positive trend between light intensity and *cry3b.2* expression in this study. There is only one gene which appears significantly upregulated at 02:00 in comparison to 18:00 which is *cry2* (p = 0.040).

4.4.3.3. Clock gene expression under different spectral compositions

Of the 11 clock genes which displayed a significantly differential gene expression (α = 0.05) under different spectral compositions (LDW, LDG, LDB and LDR), post-hoc Bonferroni adjustment (p < 0.05) reveals five genes which are significantly differentially expressed (p < 0.05) between treatment within each timepoint sampled, 18:00 and 02:00 (Table 4.7). Along with four genes (including two paralogs) which are significantly differentially expressed between timepoints within the same treatment (Table 4.8).

Table 4.7: Significantly different (ADJ.p < 0.05, One-way ANOVA with Post-hoc Bonferroni adjustment, rounded to 3 d.p.) clock gene expression in relation to spectral composition [white, red, blue, and green coloured light under LD 14:10 photoperiod] in Atlantic salmon at 690 dd, between treatment at 18:00 or 02:00 [↑ = significantly increased, ↓ = significantly decreased].

Gene	Treatment interaction	Time point	ADJ.P-value (3 d.p.)	Directionality
per2a	LDW &LDR	02:00	0.028	↑ LDW, LDB, LDG, LDR ↓
nr1d2a.2	LDR & LDB LDR & LDG	18:00 18:00	0.014 0.005	↑ LDR, LDW, LDB ↓, LDG ↓
nr1d4b.1	LDR & LDB LDR & LDG	18:00 18:00	0.028 0.008	↑ LDR, LDW, LDB ↓, LDG ↓
rorb.1	LDW & LDR	18:00	0.025	↑ LDW, LDB, LDG, LDR ↓
csnk1eb	LDR & LDB	02:00	0.034	↑ LDR, LDW, LDG, LDB ↓

Table 4.8: Significantly different (ADJ.p < 0.05, One-way ANOVA with Post-hoc Bonferroni adjustment, rounded to 3 d.p.) clock gene expression in relation to spectral composition [white, red, blue, and green coloured light under LD 14:10 photoperiod] in Atlantic salmon at 690 dd, within treatment between 18:00 and 02:00 [↑ = significant increase, ↓ = significant decrease].

Gene	Time point interaction	Treatment	P-value (3 d.p.)	Directionality
cry1a.2	18:00 & 02:00	LDR	0.022	↑ 02:00, 18:00 ↓
nr1d4b.1	18:00 & 02:00	LDW	0.001	↑ 18:00, 02:00 ↓
		LDR	$2.1 \bullet 10^{-4}$	↑ 18:00, 02:00 ↓
nr1d4b.2	18:00 & 02:00	LDW	0.041	↑ 18:00, 02:00 ↓
csnk1eb	18:00 & 02:00	LDR	0.032	↑ 02:00, 18:00 ↓

There is a significant decrease ($p = 0.028$) in the expression of *per2a* during the scotophase at 02:00 in fish under LDR in comparison to LDW. The expression of *per2a* appears to be static between 18:00 and 02:00, whereas, under all the other treatments there is a difference in expression between the two timepoints. In the nuclear receptors *nr1d2a.2* and *nr1d4b.1* there is a significant decrease in the expression of both blue ($p = 0.014$, $p = 0.028$, respectively) and green ($p = 0.005$, $p = 0.008$, respectively) light in comparison to red light during the photophase at 18:00. The expression of *rorb.1* is significantly decreased ($p = 0.025$) under the longer wavelength red light in comparison to those under white light at 18:00. Exposure to blue light significantly decreased ($p = 0.034$) expression of *csnk1eb* during the scotophase (02:00) in comparison to red light. This significant ($p < 0.05$) decrease or increase in expression of specific genes in relation to the spectra composition of light individuals were exposed to indicates that the wavelength of light may influence the expression of clock genes differently depending upon the gene of interest. Both *nr1d4b* paralogs were significantly ($p = 0.001$, $p = 0.041$, respectively) upregulated under white light during the photophase in comparison to the scotophase. Paralog *nr1d4b.1* is also show significantly ($p = 2.1 \bullet 10^{-4}$) increased expression under red light during the photophase.

Both *cry1a.2* and *csnk1eb* were found to be significantly ($p = 0.022$, $p = 0.0.32$, respectively) increased during the scotophase after having been exposed to red light during the photophase. The majority of differences in clock gene expression under different spectral compositions occurs during the photophase. Interestingly the influence of red light appears to influence the expression of both *cry1a.2* and *csnk1eb* in both the photophase and scotophase.

4.5. Discussion

This study has explored the effect of light on the expression of clock genes during the early development of Atlantic salmon. Similar to previous studies in Senegalese sole (Martín-Robles, Aliaga-Guerrero, *et al.*, 2012) and gilthead sea bream (Yúfera *et al.*, 2017), the use of whole embryos and fish in this study is suggested to be a limiting factor, as tissue specific effects may be diluted by overall gene expression (Mazurais *et al.*, 2011; Yúfera *et al.*, 2017). Despite this, this study provides additional insight into clock gene expression in Atlantic salmon and identifies candidates in which future studies may focus upon. It is important to recognise that due to the diurnal and seasonal variation in temperature, its effects as a zeitgeber cannot be ruled out during this study as temperature fluctuations are also involved in the entrainment of the clock (Isorna *et al.*, 2017; Vera *et al.*, 2023). However, based on literature it is likely that the coinciding thermophase with the photophase and the acrophase with the scotophase had an additive effect upon the amplitude of the cyclically entrained genes (López-Olmeda, 2017). Findings can be classified in one of three categories: Developmental clock gene expression, Circadian clock gene expression, and the effect of light conditions on clock gene expression.

4.5.1. Developmental clock gene expression

Unsurprisingly, during early development, most clock genes were significantly differently expressed between developmental stages. This is indicative of the continuum of morphological stages all vertebrates undergo during early developmental stages (Kaitetzidou *et al.*, 2015). Embryos were sampled close to key developmental stages, the sampling at 255 dd corresponded with eye pigmentation stage which occurs around 250 dd post-fertilisation (Gorodilov, 1987; Webb *et al.*, 2007). Atlantic salmon embryos begin to hatch around 510 dd (Webb *et al.*, 2007), all embryos had hatched prior to the sampling at 555 dd. The final sampling occurred at 690 dd, approximately 60 dd before FF (at 750 dd). In this experiment it was identified that all clock genes were expressed during in early development, including the first sampling point coinciding with eye pigmentation at 255 dd. Across the four key developmental stages investigated, 95.8 % of the clock genes were significantly differently expressed, with 85.4 % of genes displaying an identifiable significant differences between one or more developmental stages (Bonferroni adjustment, $p < 0.05$).

The findings in this study coincide with those in mummichog (*Fundulus heteroclitus*), in which the greatest number of differentially expressed genes were identified between pre- and post-hatching timepoints, encompassing tissue specialisation and organ differentiation (Bozinovic *et al.*, 2011). Throughout this period the salmon embryos are undergoing sequential morphological changes (Gorodilov, 1987).

There are a number of clock genes which appear to be sub-functionalised from the *period* and *cryptochrome* families in this study. including *per1a* and *cry3b* paralogs, which appear to be specialised, with *per1a.1* and *cry3b.1* being more highly expressed pre-hatch (255 dd) and *per1a.2* and *cry3b.2* post-hatch (690 dd).

Clock genes are implicated in numerous metabolic pathways in a whole host of species which are especially important during early developmental stages including fatty acid (FA) oxidation, lipid synthesis and energy metabolism (McCarthy *et al.*, 2007; Gooley, 2016), glucose metabolism (Rudic *et al.*, 2004; Mazzoccoli, Pazienza and Vinciguerra, 2012), and cell cycling and sexual maturation (Paibomesai *et al.*, 2010). In peripheral tissues, hundreds of genes involved in lipid metabolism are rhythmically activated or repressed by clock genes (*arntl:clock, nr1d*), coordinating lipid synthesis, FA oxidation and the absorption of dietary lipids with daily feeding cycles in feeding behaviour (Gooley, 2016). In the skeletal muscle of mice it has been shown that the majority of circadian regulated transcripts are involved in lipid pathways (McCarthy *et al.*, 2007). The expression of *arntl* (BMAL1) in salmonids was found to be significantly circadian entrained in the liver of Atlantic salmon and thus potentially instrumental to the rhythmic control of hepatic lipid metabolism (Betancor *et al.*, 2014). Facilitation of development and minimising disruption at these early stages has beneficial impacts upon key production indicators such as growth and level of deformity (Villamizar *et al.*, 2011).

It is important to note that the whole embryo homogenates were used for RNA sequencing. Whilst this eliminated bias against tissue or organ selection, there may be a potential dilution effect upon the samples especially when comparing embryos at different developmental stages especially as cells differentiate and tissues become specialised. In addition, there is potential of maternal influence through inclusion of the yolk sac in the homogenised sample. Studies in zebrafish indicate that transcripts for some clock gene family members (*clock, arntl, period*) are maternally inherited. However, there is uncertainty over the functional significance of these maternally inherited transcripts (Delaunay *et al.*, 2003; Dekens and Whitmore, 2008; Vatine *et al.*, 2011). It has been displayed that maternally inherited *per1* genes are only present for around 4 h post fertilisation (hpf), post mid-blastula transition until zygotic gene transcription begins (Dekens and Whitmore, 2008). If this is similar in Atlantic salmon, then it is arguable that the potential maternal influence on clock genes is minimal to non-existent during the early developmental stages.

We observed (255 dd – 690 dd) as mid-blastulation occurs before the eyed egg stage (255 dd) at which the first samples were taken. It is important to note that there is limited understanding of the ontogeny of clock genes and circadian entrainment in early developmental Atlantic salmon, and this is a valuable starting point for the continuation of investigation.

4.5.2. Circadian clock gene expression

Advances in 'omics' technologies such as genomics and transcriptomics alongside the development of the high-quality genome assembly for Atlantic salmon (Davidson *et al.*, 2010b; Lien *et al.*, 2016) has led to the availability of a vast resource which has the potential to unravel the underlying circadian mechanism in salmonids through a suite of tools and approaches (Beale, Karpe and Ahmed, 2016). Whilst this technology alone cannot capture the entire biological complexity, it is an approach to provide a more comprehensive overview (Karczewski and Snyder, 2018). This study builds on the clock gene toolkit in Chapter Three and provides valuable insights into the clock genes and the ontogeny of the clock in Atlantic salmon.

In this study all investigated clock gene family members identified were expressed during early developmental stages, these findings are similar to those in Atlantic salmon parr (Chapter Three). The noticeable difference in results from the two studies are between the number of significantly rhythmic and cyclically entrained genes. During early development there appear to be fewer genes with a circadian expression pattern; 1,996 significantly rhythmically expressed genes and 287 significantly cyclically expressed ($p < 0.05$) genes with a periodicity of 24 h, this decreased to 104 and 12 respectively when increasing the *p*-value threshold to $p < 0.001$. In comparison to 2,864 significantly rhythmically expressed ($p < 0.001$) genes and 1,215 significantly cyclically expressed ($p < 0.001$) genes identified over a period of 24 h in Atlantic salmon parr (Chapter Three). The number of rhythmically and cyclically entrained genes over a period of 24-hours appears to be much lower in early developmental fish. However this may be result of using the whole embryos or fish as tissue specific rhythms may be diluted by overall gene expression (Mazurais *et al.*, 2011; Yúfera *et al.*, 2017). Clock gene expression in fish is known to be localised in different tissue types (Whitmore *et al.*, 1998). Localised gene expression in central and peripheral tissues often appear out of phase with one another due to the method of entrainment, light entrainable (brain, retina, hypothalamus) versus food entrainable (liver) (Isorna *et al.*, 2017; Ceinos *et al.*, 2019). The potential phase shifts in clock gene expression between different tissue types may further contribute to the apparent lack of rhythmicity due to peaks in gene expression in one tissue lining up with the trough in another tissue.

Inclusion of the yolk sac may have also contributed to the further dilution of circadian expression within the overall gene expression. At 690 dd, the Atlantic salmon are ~60 dd away from FF, whereas zebrafish are commonly FF at 5 dpf (Hernandez *et al.*, 2018), and therefore are not yet reliant upon exogenous feeding due to their large yolk sac, so comparatives between these two studies are limited. Future studies should consider these distinctions in species development to enable better comparatives to be drawn.

For the purpose of this experiment periods interrogated were expanded to include both 20 h and 28 h periods in attempt to identify additional significantly cyclically or rhythmically expressed clock genes due to the unknown level of entrainment in the clock and the nature of artificial environments, self-sustaining clock-generated rhythms may not be precisely 24 h (Vallone, Lahiri, *et al.*, 2007). Under normal day-night conditions, the phase of the clock is reset on a daily basis through entraining zeitgebers such as light or temperature. Thus ensuring that the clock does not free-run and progressively drift out of phase with respect to their surrounding environment (Roenneberg, Daan and Merrow, 2003).

Whilst there do not appear to be many significantly cyclically entrained genes expressed during early development in whole larvae it is important to note that this study is the first of its kind and provides valuable teachings to help develop our understanding and further the characterisation of clock genes and circadian rhythmicity in Atlantic salmon. In zebrafish it is known that some clock genes start to be rhythmically expressed from 0 days post fertilisation (*arntl1/bmal1*) whilst others take longer to establish a rhythmic expression pattern [3 days - *per1b* and *per2*, 7 days – *clock1*] (Di Rosa *et al.*, 2015). A microarray study exploring the analysis of gene regulatory cascades and the mediation of circadian rhythms revealed 2,856 significantly cyclical genes in zebrafish larvae 5 days post fertilisation (dpf) (Li *et al.*, 2013). Thus, suggesting that the accelerated development of zebrafish in comparison to Atlantic salmon is also reflected in the time taken for the clock system to become fully functional.

4.5.3. The effect of light conditions on clock gene expression

Photo cue is arguably the most influential of all the light conditions regarding clock gene expression and the circadian mechanism, with light cycle referred to as the primary zeitgeber for circadian entrainment (Davidson and Menaker, 2003). Findings in this study display a significant effect of photo cue on the number of genes which are significantly differentially expressed between 18:00 and 02:00, with 16 genes being identified as significantly differentially expressed as a result of photo cue.

There are several genes in which expression is either increased or decreased under DD or LL when compared to LD. However, to assess the full impact of photo cue, further investigation is required to assess the impact upon the gene expression profile.

This coincides with findings in Senegalese sole, in which there was a marked effect on the expression of clock genes, with those exposed to continuous light or darkness displaying a loss of rhythmic oscillations in *period* and *clock* genes. Which was directly linked to key performance indicators such as mortality, through genetic factors such impaired DNA repair mechanisms (Martín-Robles, Whitmore, *et al.*, 2012). In the zebrafish, whilst the expression of individual genes in embryos may be independent of light exposure (Dekens and Whitmore, 2008), the entrainment of the clock in the pineal is initiated my minimal photic cues, both transitions from dark to light and light to dark have been found to initiate the cycling of the clock (Vuilleumier *et al.*, 2006). Daily light signals are responsible for resetting the clock and correcting discrepancies between the free-running period and 24-hours. The absence (continuous darkness and continuous light) of lighting cues results in the free-running of the clock, in which the organisms circadian mechanism still oscillates but the perception of time and the associated biological responses and behaviours drift out of phase with the real world (Ceinos *et al.*, 2019).

Light intensity appeared to be equally as important in terms of influence upon the number of significantly differentially expressed clock genes. However, there was only one gene which displayed a significantly different expression under increasing light intensity which was *cry3b.2*. There is a limited understanding surrounding the impact of light intensity on the expression of clock genes in fish (Frøland Steindal and Whitmore, 2019). There is an increasing focus of the effect of artificial lighting at night (ALAN) and how the intensity of light pollution from ALAN sources influences or disrupts fish physiology and behaviour in their natural environments (Riley *et al.*, 2013; Gaston *et al.*, 2017; O'Connor *et al.*, 2019), less so in cultured fish species. Studies in the cyanobacterium *Synechococcous elongatus* have shown a significant effect of natural light fluctuations upon the dynamics of circadian gene expression. Whilst many light-responsive genes are activated under diminished lighting conditions, gene expression patterns were influenced by light intensity (Piechura, Amarnath and O'Shea, 2017).

Genes from the *cryptochrome* family are expected to be elevated during the dark period (02:00) due to their role within the feedback loop, they form a heterodimer with period and is the negative arm of the auto regulatory feedback loop of the circadian mechanism, repressing *arntl* translation (Cox and Takahashi, 2019). Findings in this experiment display the increase in *cryptochrome* expression at night in two genes *cry2* and *cry3b.* However, upon further statistical analysis only paralog *cry3b.2* is significantly differentially expressed between treatments, with the gene expression displaying a positive trend with light intensity both during the photophase and scotophase.

To further close the research gap surrounding the effects of light intensity upon clock genes additional work needs to be undertaken to further our understanding on the function of clock genes and the influence of light intensity on Atlantic salmon clock gene expression profiles, including identifying the optimum light intensity for early developmental culture.

Species specific spectral sensitivities are believed to be an adaptation to the species typical environment, predisposing them to better performance under specific light environments (Sierra-Flores *et al.*, 2016). Literature predominantly focuses upon the effect of spectral composition on key production-based traits such as growth performance, survivability, and morphological issues. Studies in European sea bass (Villamizar, García-Alcazar and Sánchez-Vázquez, 2009) and Atlantic cod and Turbot (Sierra-Flores *et al.*, 2016), reveal better performance of fish under shorter wavelength, blue light (435-500 nm) in comparison to longer wavelength, red light (641-718 nm). Additional experiments in zebrafish under a wider range of wavelengths of light similarly concluded that early developmental zebrafish also performed best under shorter wavelengths of light in comparison to longer wavelength (red) light and continuous darkness (Villamizar *et al.*, 2014). Comparison of findings from this experiment to literature on the influence of spectral composition on clock gene expression in zebrafish (Di Rosa *et al.*, 2015) was limited due to only two timepoints being measured. Expression of *nr1d2a.2* and *nr1d4b.1* were significantly increased at 18:00 under LDR compared to LDG and LDB, with no difference seen compared to LDW, *csnk1eb* was significantly increased under LDR compared to LDB.

This suggests that the expression of these genes is increased by exposure to long wavelength light. Expression of *per2a* at 02:00 and *rorb.1* at 18:00 was significantly increased under LDW was significantly increased compared to LDR, indicating that long wavelength light decreased the expression of both genes at each respective timepoint. It would be interesting to follow the ontogeny of the clock to see if findings were comparable to those published in zebrafish and other commercially important marine species. As the ontogeny of the clock in response to spectral composition is still scarcely understood on a wider scale.

4.6. Conclusion

This study has explored the effect of light conditions on the expression of clock genes during the early development of Atlantic salmon. Whilst there were only two clock genes which were significantly cyclically entrained in the whole embryo pre-FF. It is evident that clock genes play a role in the early development of Atlantic salmon and that light conditions significantly influence clock gene expression. Understanding the gene expression of genes of interest in relation to the presence or absence of light and dark cues alongside light intensity can help to build a picture of genes which are directly entrainable by light. Altered expression of specific genes in relation to light intensity and spectral composition indicates that intensity and wavelength influences the expression of clock genes, depending on the target gene. *In vivo* light stimulation experiments during early development are integral in characterising the 'early clock' and elucidating the ontogeny of the circadian mechanism, specifically the development and entrainment of clock genes alongside the perception of light. Further studies should be conducted to identify the influence of the FF event on the expression patterns of clock genes in Atlantic salmon.

Chapter Five

Clock gene expression and the influence of feeding entrainment during early development of Atlantic salmon (*Salmo salar*)

The work in this chapter was undertaken as part of the collaborative project funded by the Research Council of Norway "*The effect of narrow banded LED light on development and growth performance"* (grant number 254894) and the University of Stirling PhD match funding scheme.

5.1. Abstract

The influence of first-feeding upon the clock gene expression in Atlantic salmon is relatively unknown in comparison to zebrafish and other commercially important marine aquaculture species. There is a known influence of feeding activity on the expression of clock genes in a number of teleost species, however there is limited information surrounding the influence of the switch from endogenous to exogenous feeding on the expression of clock genes, and the role of the clock within this important life stage. This study was designed to investigate the link between the key developmental stage of first-feeding and the switch from endogenous to exogenous feeding in Atlantic salmon by identifying and comparing the expression of clock genes in the brain in pre- and post-first-feeding alevins kept under LD light conditions over a 48 h period. Findings revealed an increase in the number of clock genes significantly rhythmically and cyclically expressed at 920 dd when compared with samples taken immediately prior to first-feeding at 730 dd. Significantly cyclical expression of members of the *period* clock gene family within the brain in fish under an LD cycle aligns with findings from *in situ* hybridisation studies in zebrafish and goldfish. Results from this study indicate that the point of first-feeding and the switch from endogenous to exogenous feeding is influential in the initiation of the clock within the brain of Atlantic salmon alevins and warrants further investigation.

5.2. Introduction

Circadian rhythmicity is known to be influenced by several factors including light, temperature and feeding cycles. These external cues together, ultimately entrain the expression of clock genes (Vera *et al.*, 2013), with light being the most widely studied entraining oscillator (Feliciano *et al.*, 2011). Commercially early developmental Atlantic salmon are subjected to artificial lighting regimes around the point of first feeding (FF), with continuous light regimes often used widely during this timepoint to enhance feeding and growth rates (Stefansson *et al.*, 1990). Despite exposure to artificial lighting regimes during one of the most critical events in the life cycle of the Atlantic salmon (Stefansson *et al.*, 1990), the initiation of clock function in early development and the influence FF can have upon the clock mechanism remains largely unknown in salmonids. Something which has previously been investigated in the teleost model zebrafish (Gothilf *et al.*, 1999; Dekens and Whitmore, 2008; Idda *et al.*, 2012).

The currently accepted view regarding the entrainment and synchronisation of the core molecular clock in early development is the requirement of light for synchronisation, an important development to note as most eggs are reared in dark incubators (Frøland Steindal and Whitmore, 2019).

Studies on several teleost species report a favourable link between increased lighting periods and the growth and development of the respective species. Thus, suggesting that lighting conditions and feeding have a synergistic effect upon growth performance, through enhanced feeding or improved food conversion efficiencies (Villamizar *et al.*, 2011). This synergistic effect has been reported to have an impact on fish species prior to exogenous or FF. European Sea bass five days post hatch exposed to constant light (LL) or Light: Dark (LD) 12:12 reported increased total length in comparison to those reared under constant darkness (DD). Fish under LL conditions also exhausted their yolk sac reserves two days earlier than those exposed to DD or LD red wavelength light at nine days post-hatch (Villamizar, García-Alcazar and Sánchez-Vázquez, 2009). Senegal sole exposed to LD 12:12 also reported increased growth in comparison to those under LL and DD conditions and depleted their yolk sac reserves 4 days earlier than those under DD at seven days post-hatch (Blanco-Vives *et al.*, 2010). However, the link between improved growth and development characteristics and the entrainment of the circadian mechanism remains vastly unexplored in teleost species. In the zebrafish, the circadian clock begins autonomously within 12 hours post fertilisation and is marked by the upregulation of *per1b.* At the end of the first day the expression of *per1b* is robustly circadian in nature, peaking after sunrise (Dekens and Whitmore, 2008).

A study undertaken in rainbow trout (Davie *et al.*, 2011), identified that *per1* and *clock* were actively expressed in unfertilised eggs and throughout embryonic development (up to 57 days post-fertilisation – 420 dd), and their expression pattern was influenced by light conditions prior to the reported development of known photoreceptive structures (pineal and retina). The gene *per1* was significantly rhythmically expressed at 8-9 dpf (60 dd) under both LD 12:12 and LL conditions whilst *clock* was found to be rhythmically expressed initially at 0-1 days post-fertilisation but lacking in rhythmicity until 42-43 days post-fertilisation (300 dd) under LD 12:12 only. The study (Davie *et al.*, 2011), also highlighted the need for re-examination of the development of salmonid embryos regarding the onset of environmental entrainment, especially as they have a much longer incubation period than zebrafish (Idda *et al.*, 2012). Whilst there is some literature regarding the expression of clock genes during embryogenesis (Villamizar, García-Alcazar and Sánchez-Vázquez, 2009; Davie *et al.*, 2011; Villamizar *et al.*, 2011), there is a significant lack of research regarding the expression of clock genes and the circadian mechanism during later stages of early development such as the critical point at FF, in which fish transition from endogenous to exogenous feeding.

In the previous chapters (Chapter Three and Chapter Four) there are distinct difference in the total number of clock genes which are significantly expressed between the two different life stages (pre-FF and parr). Results of a 20-28 h interrogation of the RNA sequencing data revealed 12 clock genes which were significantly rhythmically expressed ($p < 0.05$).

Of which two (*clock1a.2* and *nr1d2b.2*) were significantly rhythmically expressed ($p < 0.05$, rAMP ≥ 10%) in whole Atlantic salmon embryos at 690 dd (Chapter 4). Of which over 24 h there were only six genes which were significantly rhythmic. In comparison, over a 24 h period there were 32 clock genes which were significantly rhythmically expressed ($p < 0.05$), and 19 which were significantly cyclically entrained ($p < 0.05$, rAMP ≥ 10%) in Atlantic salmon parr brain (Chapter Three).

The main aim of this study was to characterise clock gene expression during fundamental early developmental stages in Atlantic salmon and to identify the effect of feeding entrainment, during the switch from endogenous to exogenous feeding on the expression of clock genes.

5.3. Materials and method

5.3.1. Ethical statement

All experiments followed local animal care guidelines and were given ethical approval by the Norwegian Veterinary Authorities. The ARRIVE guidelines have been complied in this study (Percie du Sert *et al.*, 2020).

5.3.2. Animal husbandry

All Atlantic salmon (*S. salar*) eggs and sperm were obtained from two males and two females from Mowi (Marine Harvest), Tveitevågen, Norway and were fertilised on the 2020-01-29 and kept in an approved laboratory facility at High Technology Centre, University of Bergen, Norway. Eggs were under experimentation from fertilisation until the end sampling point on the 2020-06-25. Fertilisation rate and mortality was not measured in this trial, a pilot study was conducted in 2016 using 20,000 eggs revealed a fertilisation rate of 85 % from a late egg group where fertilisation rate is low. The subsequent mortality of eggs once the unfertilised, white eggs were removed was ~1 %, post-hatch the mortality rate was minimal, but not recorded. Therefore, it was decided not to count this in subsequent studies and unfertilised or dead eggs and alevins were removed (M. Eilertsen, 2022, pers. comm., 2022-04-20).

From fertilisation to FF fish were incubated in triplicated hatching trays (350-400 eggs per tray) in two tanks (separated by family until mixed post-FF) at an average temperature of 5.7°C ± 0.05°C (S.E.), after transferring the fish to triplicated tanks on the 2020-06-05 this then rose to an average temperature of 11.5°C ± 0.02°C (S.E.) over the period of a week from the 2020-06-06 (Figure 5.1). Due to a technical issue the temperature was raised more suddenly that planned, however the alevins responded well, with nominal mortality over the transfer period. From FF (2020-06-08) the alevins were fed continuously using belt feeders. The alevins were fed with EWOS® Micro starter diet (0.6 mm) at 4 % of mean weight (g) plus 25 % excess during the light period.

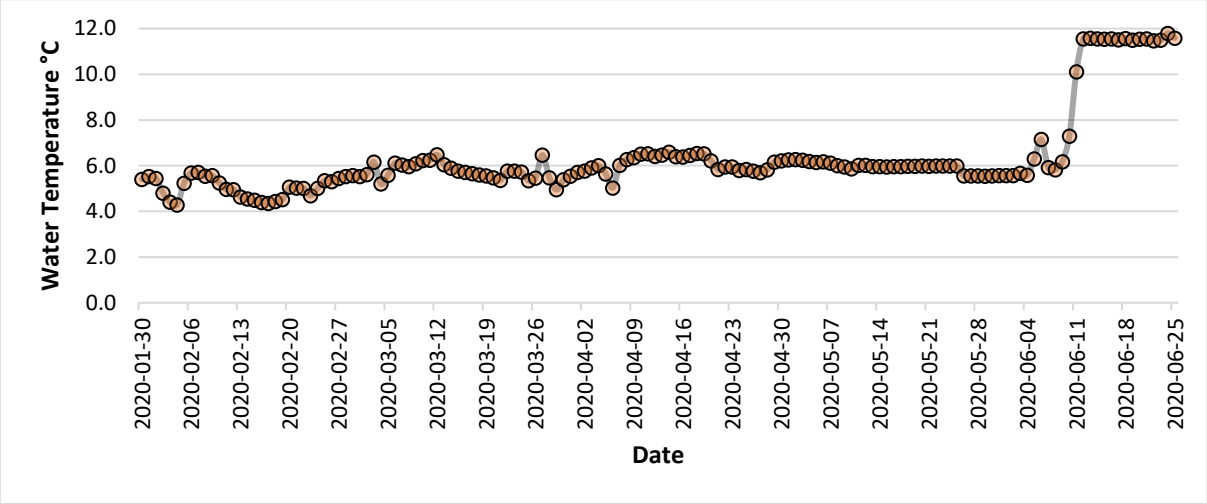


Figure 5.1: Graph displaying average daily inlet temperature variation across the duration of the lighting experiment 2020-01-30 to 2020-06-25, average temperature pre-FF 5.7 °C, average temperature post-FF 11.5 °C.

5.3.3. Light experiment

The eggs (350-400 per rack) were exposed to a day, night lighting condition (LD 14:10, 0.1 W/m^2) using state of the art light emitting diode (LED) technology (Signify, The Netherlands). Lighting was dimmed on and off over a 30-minute period within the 14 h light period (08:00 – 22:00). Fish were sampled at two points capturing the key milestone, transferring from endogenous to exogenous feeding. From fertilisation, the light intensity was then increased to 1.0W/m^2 from the 2020-06-05 when alevins were moved into tanks from the hatching racks until the end of the experiment on the 2020-06-25 (Figure 5.2) to compensate for the increased water depth to maintain the same perception of light (M. Eilertsen, 2022, *pers. comm.*, 2022-04-20).

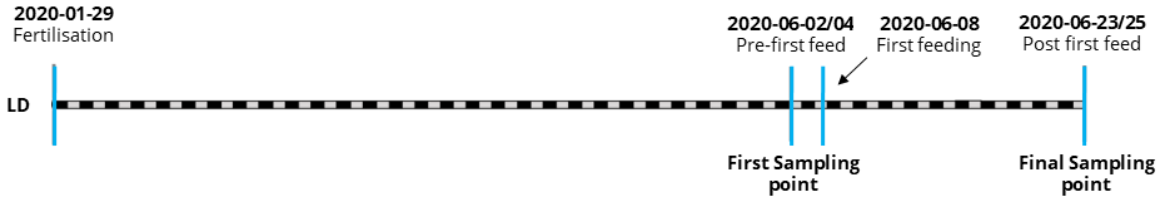


Figure 5.2: Experimental set-up plan for endogenous vs exogenous clock gene expression trial at the University of Bergen, 2020.

Sampling was conducted every 4 h over a 48 h period, starting at 08:00 on day one and ending at 08:00 on day three (08:00, 12:00, 16:00, 20:00, 00:00 [2], 04:00 [2], 08:00 [2], 12:00 [2], 16:00 [2], 20:00 [2], 00:00 [3], 04:00 [3], 08:00 [3]). The sampling teams were made up of six people who worked in teams of three (due to COVID-19 working restrictions in Norway) and the same person from each team was responsible for the removal of the head to reduce subjectivity. Samples were taken as outlined in (Table 5.1).

Table 5.1: Sampling plan and description.

Time point	Date	Fish	Description
Pre-FF (~730 dd)	2020-06-02/04	78	n = 6 • 13 sampling points
Post FF (~920 dd)	2020-06-23/25	117	n = 9 (3 fish/tank, triplicated) • 13 sampling points

Following terminal anaesthesia in tricaine methanesulfonate (MS222, 250 mg/L, pH neutralised with sodium bicarbonate), the spinal column was severed by cutting the head anterior to the yolk sac (exact location depending on fish age, see Figure 5.3) and snap frozen in liquid nitrogen in cryovials. Samples were stored at -80 °C until processing.

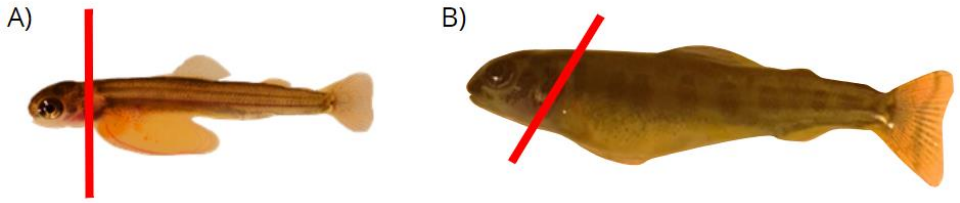


Figure 5.3: Dissection guideline for A) pre-FF and B) post-FF sampling points. The red line in the picture represents the point at which the head was removed, anterior to the yolk sac.

5.3.4. RNA extraction, cDNA synthesis and qPCR

Prior to dissection heads stored at -80 °C were soaked in RNAlater™ ICE (ThermoFisher Scientific, Waltham, MA, USA), and allowed to thaw to -20 °C for a minimum of 48 h (maximum of one week) prior to the whole brain minus the pituitary gland (to minimise previously identified potential dilution effect from whole embryos) being dissected out in RNAlater™ ICE. RNA was extracted from the brains and cDNA synthesised in the methods outlined in the Chapter Two – 2.2 RNA Extraction from Tissue and 2.3 cDNA Synthesis.

Due to COVID-19 this was undertaken at the University in Bergen by the technician and a fellow PhD student, before cDNA samples were shipped on dry ice to the University of Stirling for analysis by qPCR. To identify core clock genes from the *clock, arntl, period,* and *cryptochrome* families identified in Chapter Three, qPCR primers (Chapter Two – 2.8 qPCR) were designed and used to interrogate brain cDNA from the early developmental Atlantic salmon kept under LD (14:10) controlled lighting regimes from fertilisation over an extended 48 h circadian sampling period immediately prior to and after the first-feeding life event.

5.3.5. Statistical analysis

All tests and analysis were performed using R v4.1 (RDC, 2010). Daily rhythmicity across the four gene families interrogated by absolute quantification qPCR across the 48-hour sampling period was identified using RAIN v1.26.0 (Thaben and Westermark, 2014) and MetaCycle v1.2.0 (Wu *et al.*, 2016) with the implementation of JTKCycle (Hughes, Hogenesch and Kornacker, 2010).All *p*-values were corrected for False Discovery Rate (FDR) using Bonferroni adjustment. Due to the questionable stability and reliability of the housekeeping genes (HKGs) *ef1a,* and *b2m* regarding circadian sampling as both genes being significantly rhythmically and cyclically significantly expressed pre and post FF, despite being identified as not significantly or rhythmically expressed from the RNA sequencing results in the previous chapter. Whilst customary to normalise qPCR readings to further minimise the influence of RNA quality and the efficacy of cDNA synthesis (Bustin *et al.*, 2009), it was decided not to use these HKGs to normalise copy numbers for this experiment.

5.4. Results

Significantly rhythmically expressed genes were identified using RAIN and MetaCycle with JTK analysis ($p < 0.05$) over a 48 h circadian sampling at two different time points reflective of pre-FF and post-FF to identify the effects of exogenous feeding entrainment on the expression of clock gene paralogs. Various periods reflecting a circadian nature (20, 24, and 28 h) were evaluated, in keeping with the methodology of Chapter Four due to the great plasticity and adaptive potential of clock mechanisms in teleosts.

5.4.1. Effects of normalisation

Two HKGs (*ef1a* and *b2m*) were chosen based on their reported previous use to normalise qPCR results in circadian studies in literature to date. However, upon comparison to un-normalised results there was a significant difference in the number of genes which were significantly rhythmically or cyclically express and in the periodicity which they most aligned to, especially in the result from the pre-FF sampling point (Table 5.2).

Table 5.2: Normalised, significantly rhythmic (RAIN) or cyclical (MetaCycle with JTK) clock gene expression, depending on periodicity analysed in early developmental Atlantic salmon prior to FF (~730 dd) over a 48 h circadian sampling period. Values are expressed as copy number. Blue fill (light blue - RAIN) indicates the periodicity with the highest significance for results which are significant for more than one interrogated periodicity.

	Rhythmic			**Circadian**		
Gene	20 h	24 h	28 h	20 h	24 h	28 h
clock1a	0.019	-	0.029	-	-	-
clock2	$4.74 \bullet 10^{-4}$	0.039	-	0.005	-	-
arntl1a	$3.47 \bullet 10^{-4}$	0.036	-	-	-	-
arntl1b	$1.23 \bullet 10^{-3}$	0.049	-	0.005	-	-
arntl2a	$1.82 \bullet 10^{-4}$	-	-	$3.61 \bullet 10^{-4}$	-	-
arntl2b/c	-	-	0.038	-	-	-
per1a	-	0.046	0.047	0.015	-	-
per1b	-	-	-	-	-	-
per2	-	-	-	-	-	-
per3	$3.6 \bullet 10^{-3}$	0.019	-	0.001	-	-
cry1a	0.041	-	-	0.044	-	-
cry2	0.031	0.012	0.032	0.029	-	-
cry3b	-	-	-	-	-	-
cry4	-	-	-	-	-	-
cry5	-	-	-	-	-	-

Upon further investigation into the possible reasons behind the significant increase in the number of significantly rhythmically and cyclically expressed genes, especially those appearing to be entrained to a 20 h periodicity. It was discovered when graphing the gene expression pattern (Figure 5.4) that there was clear peaks and troughs in the expression pattern of both HKGs.

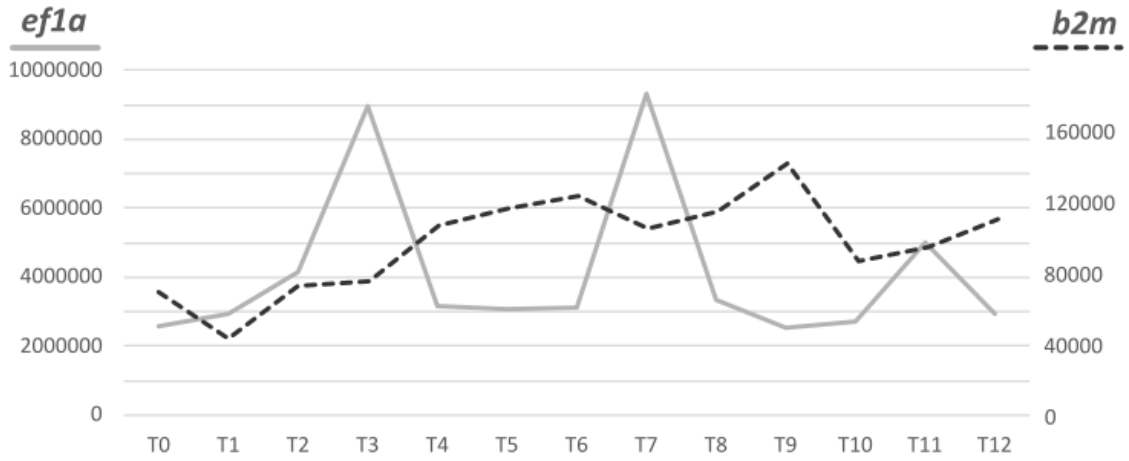


Figure 5.4: Gene expression patterns of housekeeping genes *ef1a* and *b2m* pre-FF in copy number.

Upon interrogation of the HKGs using RAIN and MetaCycle with JTK it was identified that both *ef1a* and *b2m* are significantly ($p < 0.05$) rhythmically and cyclically expressed over a period of 20 h which would explain why there are more clock genes which are appearing to be significantly expressed over 20 h when compared to the unnormalised results (Table 5.3).

Table 5.3: Significantly rhythmic (RAIN) or cyclical (MetaCycle with JTK) housekeeping gene expression, depending on periodicity analysed in early developmental Atlantic salmon pre-FF (~730 dd) over a 48 h circadian sampling period.

	20 h *p*-value		**24 h *p*-value**		**28 h *p*-value**	
Gene	**RAIN**	**MetaCycle with JTK**	**RAIN**	**MetaCycle with JTK**	**RAIN**	**MetaCycle with JTK**
ef1a	0.002	0.001	0.663	0.486	1.000	1.000
b2m	0.025	0.040	0.350	0.336	0.239	0.392

Similarly, to the effect of normalising the data pre-FF, there was an increase in the overall number of significantly rhythmically or cyclically expressed clock genes between the unnormalised and normalised results post-FF after normalisation (Table 5.4). There was a distinct difference in the periodicity which had the highest level of significance. Upon graphing it was apparent that both HKGs were also rhythmically expressed post-FF (Figure 5.5).

Table 5.4: Normalised, Significantly rhythmic (RAIN) or cyclical (MetaCycle with JTK) clock gene expression, depending on periodicity analysed in early developmental Atlantic salmon post FF (~920 dd) over a 48 h circadian sampling period. Blue fill (light blue - RAIN, dark blue - MetaCycle with JTK) indicates the periodicity with the highest significance for results which are significant for more than one interrogated periodicity.

	Rhythmic			Circadian		
Gene	20 h	24 h	28 h	20 h	24 h	28 h
clock1a	-	-	-	-	-	-
clock2	-	$3.59 \cdot 10^{-5}$	$1.52 \cdot 10^{-6}$	-	-	$3.11 \cdot 10^{-4}$
arntl1a	5.85×10^{-3}	$3.77 \cdot 10^{-3}$	$2.14 \cdot 10^{-2}$	-	$2.02 \cdot 10^{-2}$	-
arntl1b	-	-	-	-	-	-
arntl2a	$6.57 \cdot 10^{-3}$	$4.50 \cdot 10^{-2}$	$5.02 \cdot 10^{-4}$	$8.62 \cdot 10^{-3}$	-	$1.67 \cdot 10^{-2}$
arntl2b/c	$2.51 \cdot 10^{-4}$	$4.59 \cdot 10^{-4}$	-	$7.93 \cdot 10^{-3}$	$1.12 \cdot 10^{-3}$	-
per1a	$1.32 \cdot 10^{-6}$	$3.40 \cdot 10^{-5}$	-	$6.32 \cdot 10^{-4}$	$3.65 \cdot 10^{-4}$	-
per1b	$2.34 \cdot 10^{-5}$	$6.34 \cdot 10^{-6}$	$1.77 \cdot 10^{-2}$	$5.39 \cdot 10^{-3}$	$1.60 \cdot 10^{-5}$	$4.31 \cdot 10^{-2}$
per2	-	-	$1.13 \cdot 10^{-2}$	-	-	-
per3	$1.87 \cdot 10^{-4}$	$3.21 \cdot 10^{-10}$	$2.55 \cdot 10^{-2}$	$1.34 \cdot 10^{-2}$	$1.28 \cdot 10^{-7}$	$1.53 \cdot 10^{-2}$
cry1a	$4.17 \cdot 10^{-3}$	-	-	$4.92 \cdot 10^{-2}$	$4.39 \cdot 10^{-2}$	-
cry2	$9.52 \cdot 10^{-4}$	$2.35 \cdot 10^{-4}$	-	$2.42 \cdot 10^{-2}$	$1.06 \cdot 10^{-2}$	-
cry3b	-	$6.51 \cdot 10^{-3}$	$5.11 \cdot 10^{-3}$	-	-	-
cry4	$1.07 \cdot 10^{-4}$	$5.62 \cdot 10^{-3}$	-	$9.20 \cdot 10^{-3}$	$1.44 \cdot 10^{-3}$	-
cry5	$2.74 \cdot 10^{-4}$	$2.81 \cdot 10^{-2}$	-	$2.39 \cdot 10^{-2}$	$2.29 \cdot 10^{-2}$	-

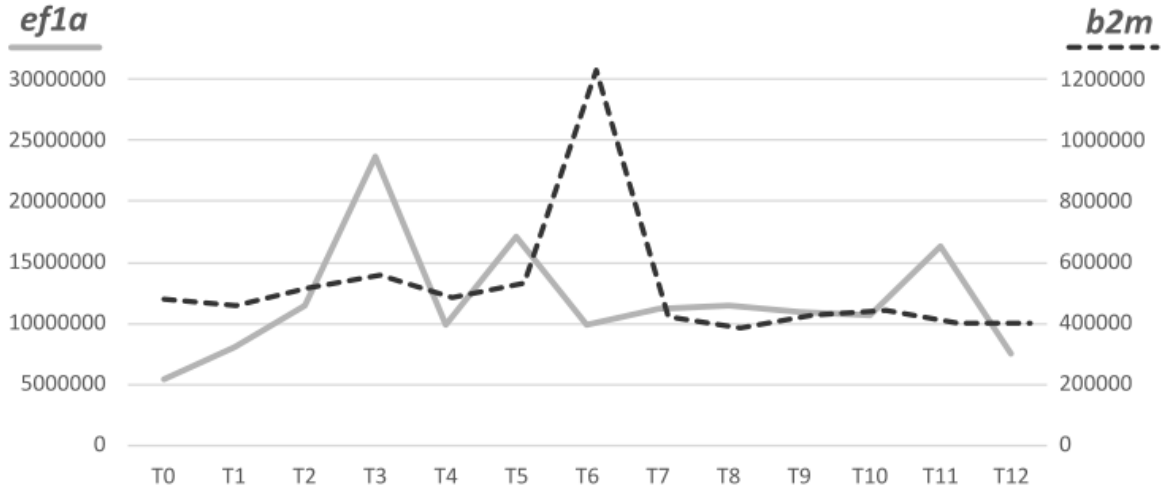


Figure 5.5: Gene expression patterns of housekeeping genes *ef1a* and *b2m* post-FF in copy number.

Table 5.5: Significantly rhythmic (RAIN) or cyclical (MetaCycle with JTK) housekeeping gene expression, depending on periodicity analysed in early developmental Atlantic salmon post FF (~920 dd) over a 48 h circadian sampling period. Blue fill (light blue - RAIN, dark blue - MetaCycle with JTK) indicates the periodicity with the highest significance for results which are significant for more than one interrogated periodicity.

	20 h *p*-value		**24 h *p*-value**		**28 h *p*-value**	
Gene	**RAIN**	**MetaCycle with JTK**	**RAIN**	**MetaCycle with JTK**	**RAIN**	**MetaCycle with JTK**
ef1a	0.206	0.836	$1.21 \bullet 10^{-6}$	$9.61 \bullet 10^{-4}$	$9.42 \bullet 10^{-5}$	$1.81 \bullet 10^{-3}$
b2m	0.048	0.169	0.092	0.148	0.113	0.334

Both *ef1a* and *b2m* are significantly ($p < 0.05$) rhythmically expressed *ef1a* over a 24 h period and *b2m* over a 20 h period. Only *ef1a* is significantly ($p < 0.05$) cyclically expressed, this is most significant over a period of 24-hours. Whilst the number of significantly cyclical genes increases by one in comparison to the un-normalised data, there is a distinct difference in the genes which appear as significant between the two analyses. With notably more significant results in the normalised dataset over all three periods in comparison to the non-normalised dataset.

5.4.2. Pre-first feeding

There are distinct differences in the number of analysed genes which are significantly ($p < 0.05$) rhythmically and cyclically expressed pre- and post-FF. At the pre-FF sampling point ~730 dd (Table 5.6) whilst there are not any genes which are either rhythmically (RAIN) or cyclically (MetaCycle with JTK) expressed over 24 h. There are three genes (*arntl1a, per1a,* and *per3*) which are significantly rhythmically expressed over a period of 28 h, and a further two genes (*per1a* and *per1b*) which are significantly cyclically expressed over a period of 20 h.

Table 5.6: Significantly rhythmic (RAIN) or cyclical (MetaCycle with JTK) clock gene expression, depending on periodicity analysed in early developmental Atlantic salmon prior to FF (~730 dd) over a 48 h circadian sampling period. Values are expressed as copy number.

	Rhythmic			Circadian		
Gene	20 h	24 h	28 h	20 h	24 h	28 h
clock1a	-	-	-	-	-	-
clock2	-	-	-	-	-	-
arntl1a	-	-	0.006	-	-	-
arntl1b	-	-	-	-	-	-
arntl2a	-	-	-	-	-	-
arntl2b/c	-	-	-	-	-	-
per1a	-	-	0.034	0.024	-	-
per1b	-	-	-	0.024	-	-
per2	-	-	-	-	-	-
per3	-	-	0.025	-	-	-
cry1a	-	-	-	-	-	-
cry2	-	-	-	-	-	-
cry3b	-	-	-	-	-	-
cry4	-	-	-	-	-	-
cry5	-	-	-	-	-	-

It is important to note that for the periodicity of 20 h the *p*-values of *per1a* and *per1b* for the RAIN analysis whilst not significant ($p < 0.05$) they are almost significant at $p = 0.073$ and $p = 0.072$, respectively. The *per1* paralogs *per1a* and *per1b* are out of phase with one another, at 20:00 and 22:00 respectively, with *per1a* displaying a negative phase shift, peaking 2 hours after *per1b* (Figure 5.6).

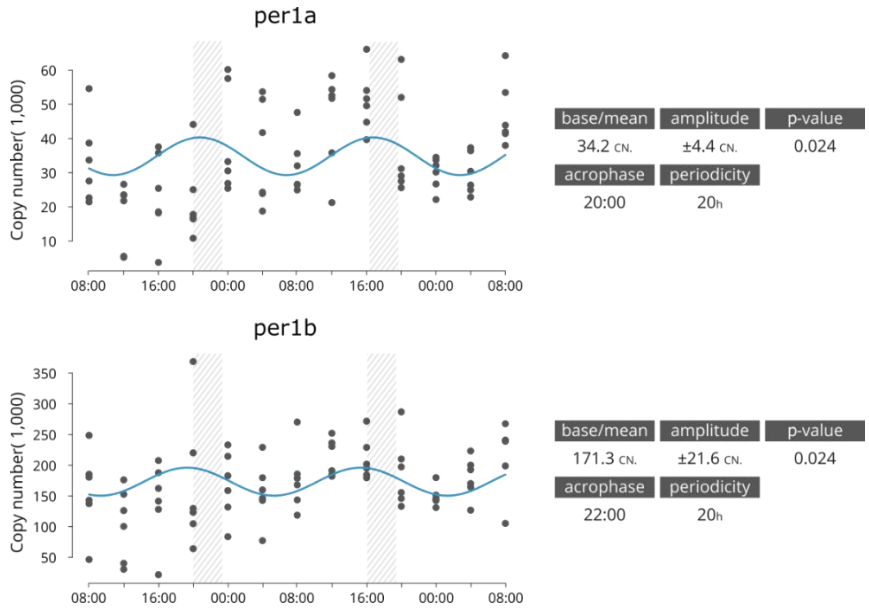


Figure 5.6: Significantly cyclically ($p < 0.05$) expressed clock genes *per1a* and *per1b*. Parameters of the sin-cosine function calculated by MetaCycle with JTK for diel expression of clock genes in whole brain cDNA collected from early developmental Atlantic salmon prior to first feeding (~730 dd), exposed to a LD 14:10 photoperiod.

5.4.3. Post-first feeding

There is a clear increase in the number of genes which are significantly ($p < 0.05$) rhythmically (RAIN) or cyclically (MetaCycle with JTK) expressed post-FF (Table 5.7). With six genes (*per1a, per1b, per2, per3, cry4,* and *cry5*) significantly rhythmically expressed over a period of 20 h, eight genes (*clock1a, clock2, arntl1b, arntl2b/c, per1a, per1b, per2, per3,* and *cry4*) significantly rhythmically expressed over a period of 24 h, and 12 genes (*clock1a, clock2, arntl1a, arntl1b, arntl2b/c, per1a, per1b, per2, cry1a, cry2, cry3b,* and *cry5*) significantly rhythmically expressed over a period of 28 h. With *arntl2a* being the only gene not rhythmically expressed across any of the periods interrogated, despite being significantly cyclically expressed ($p = 0.043$) over a 20 h period (20 h RAIN, $p = 0.244$). There are two genes (*arntl2a* and *per1a*) which are significantly cyclically expressed over 20 h, six genes (*clock2, arntl2b/c, per1a, per1b, per2,* and *cry4*) which are significantly cyclically expressed over 24 h and seven genes (*clock2, arntl1b, per1a, per1b, per2, cry1a,* and *cry3b*) which are significantly cyclically expressed over 28 h.

Table 5.7: Significantly rhythmic (RAIN) or cyclical (MetaCycle with JTK) clock gene expression, depending on periodicity analysed in early developmental Atlantic salmon post FF (~920 dd) over a 48 h circadian sampling period. Blue fill (light blue - RAIN, dark blue - MetaCycle with JTK) indicates the periodicity with the highest significance for results which are significant for more than one interrogated periodicity. Values are expressed in copy number.

	Rhythmic			**Circadian**		
Gene	20 h	24 h	28 h	20 h	24 h	28 h
clock1a	-	0.018	0.011	-	-	-
clock2	-	0.002	$8.46 \cdot 10^{-4}$	-	0.010	0.004
arntl1a	-	-	0.012	-	-	-
arntl1b	-	0.009	0.001	-	-	0.009
arntl2a	-	-	-	0.043	-	-
arntl2b/c	-	0.026	0.003	-	0.035	-
per1a	$1.36 \cdot 10^{-4}$	$4.42 \cdot 10^{-6}$	$1.31 \cdot 10^{-5}$	0.004	$5.59 \cdot 10^{-5}$	$6.69 \cdot 10^{-4}$
per1b	0.023	$1.69 \cdot 10^{-6}$	0.001	-	0.002	0.004
per2	0.040	$1.52 \cdot 10^{-6}$	$1.36 \cdot 10^{-8}$	-	0.001	$7.44 \cdot 10^{-6}$
per3	0.011	0.047	-	-	-	-
cry1a	-	-	$1.81 \cdot 10^{-5}$	-	-	0.044
cry2	-	-	0.001	-	-	-
cry3b	-	-	0.024	-	-	0.046
cry4	0.004	0.018	-	-	0.046	-
cry5	0.020	-	$4.58 \cdot 10^{-4}$		-	-

Selecting periodicity using the lowest *p*-value, there is one gene significantly cyclically entrained to a 20 h periodicity (*arntl2a*), four genes which are significantly cyclically entrained to a 24 h periodicity (*arntl2b/c, per1a, per1b,* and *cry4*), and five genes which are significantly cyclically entrained to a 28 h periodicity (*clock2, arntl1b, per2, cry1a,* and *cry3b*) which can be seen overleaf in (Table 5.8).

Table 5.8: Significantly cyclically (MetaCycle with JTK, $p < 0.05$) expressed clock gene parameters. Absolute quantification qPCR values are expressed as copy number.

Gene	Periodicity (h)	*p*-value	Base/ Mean	Amplitude	Acrophase
clock2	28	0.004	262592.00	88073.92	18:00
arntl1b	28	0.009	198752.40	41529.19	18:00
arntl2a	20	0.043	125356.40	4391.71	08:00
arntl2b/c	24	0.035	141909.30	19768.38	02:00
per1a	24	> 0.001	79381.04	18541.40	02:00
per1b	24	0.002	196189.70	20953.95	04:00
per2	28	> 0.001	85438.87	19729.81	18:00
cry1a	28	0.044	42858.30	5307.91	18:00
cry3b	28	0.046	11989.29	2446.84	18:00
cry4	24	0.046	2670.71	500.52	06:00

It is important to note the differences in periodicity. However, there is a clear difference in expression patterns within individual clock gene families, *clock2* and *arntl1b* are in phase with one another with expression peaking at 18:00, ten hours after the lights were first turned on. Whereas *arntl2a* appears to peak as the lights are turned on at 08:00 and *artnl2b/c* peaks around 02:00 in the middle of the dark period. The *period* family genes are also out of phase with one another, *per1a* and *per1b* paralogs are two-hours of phase with one another, with *per1a* peaking two hours before *per1b* at 02:00 during the middle of the dark period in comparison to 04:00 (Figure 5.7). Additionally, *per2* expression is out of phase with the two *per1* paralogs, peaking at 18:00 in phase with *clock2* and *arntl1b.* Which are also in phase with *cry1a* and *cry3b*. All significantly expressed genes over a 28 h periodicity appear to be in phase with one another. The expression of *cry4* peaks at 06:00, two hours before the lights come on, and does not appear to be in phase with any of the other genes investigated.

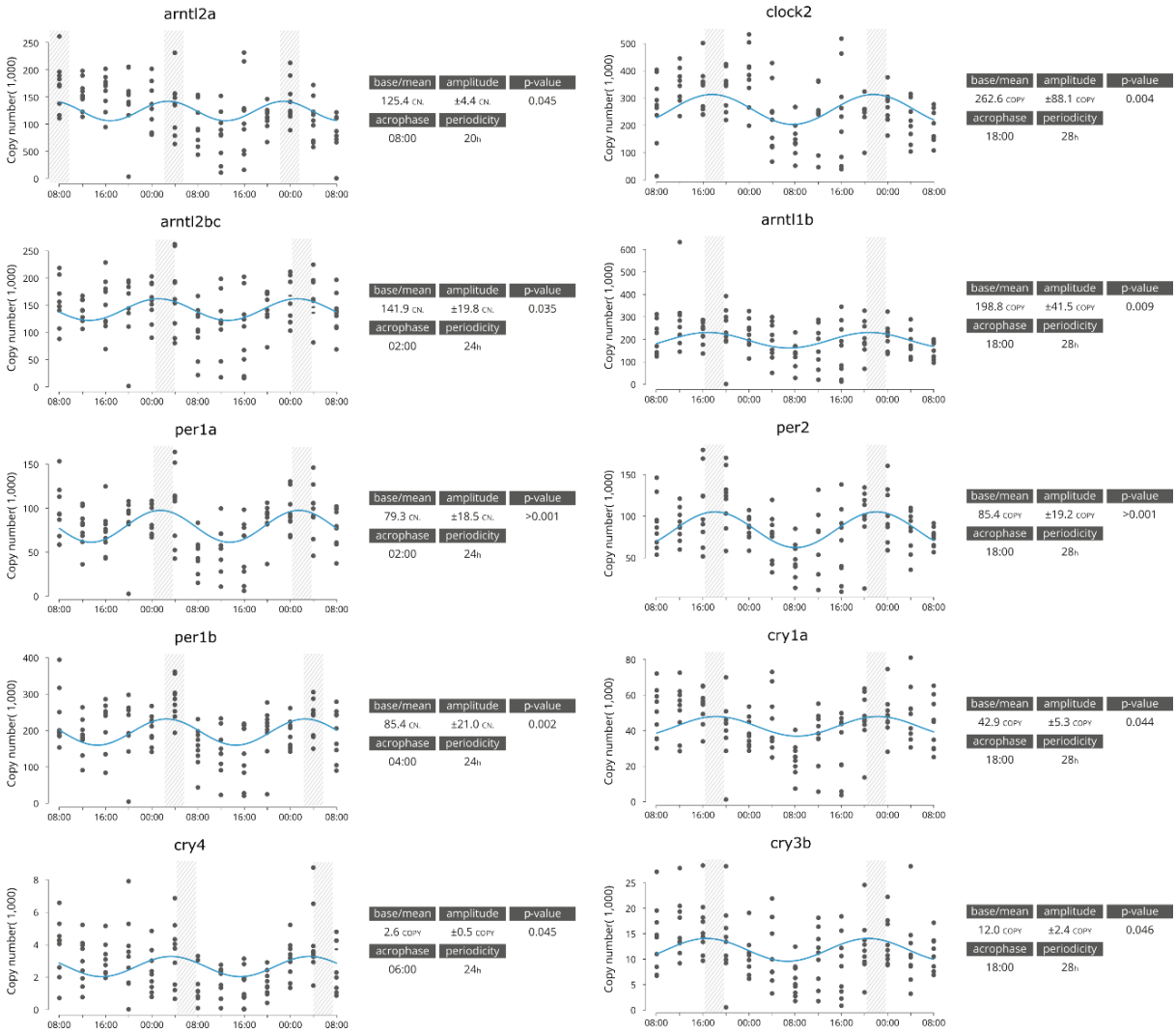


Figure 5.7: Significantly cyclically ($p < 0.05$) expressed clock genes *clock2, arntl1b, arntl2a, arntl2b/c, per1a, per1b, per2, cry1a, cry3b,* and *cry4.* Parameters of the sin cosine function calculated by MetaCycle with JTK for diel expression of clock genes in whole brain cDNA collected from early developmental Atlantic salmon post first feeding (~920 dd), exposed to a LD 14:10 photoperiod.

5.5. Discussion

This study has explored the effect of the key milestone of FF on the expression of clock genes during early development of the Atlantic salmon. Results from this work indicate that there is a large proliferation in the number of clock genes which become significantly rhythmically or cyclically entrained post-FF in comparison to individuals sampled pre-FF. From these findings, it is reasonable to assume that the key life event of switching from endogenous to exogenous feeding is important for the entrainment of circadian clocks in Atlantic salmon during early development.

It is important to note that due to the feeding of alevins in the light period and the diurnal temperature fluctuations, the effects of temperature and feed entrainment cannot be fully separated from the significant cyclicity of clock gene expression resulting from light as both temperature and food availability are also involved in the entrainment of the clock (Isorna *et al.*, 2017). Due to clock genes in the brain predominantly being entrained by light, the likely effect of temperature fluctuations and feeding entrainment in which the photophase coincides with the thermophase and feeding activity is additive and may have amplified the cyclical expression of clock genes under the LD cycle (López-Olmeda and Sánchez-Vázquez, 2009; Vera *et al.*, 2013, 2023; Costa *et al.*, 2016; Steindal and Whitmore, 2020).

The lack of significantly rhythmically or cyclically expressed clock genes pre-FF at ~730 dd aligns with earlier findings in Atlantic salmon at 690 dd in Chapter Four. However, it was not possible to cross validate significantly expressed genes from either study (*arntl2b* – Chapter Four, *per1a* and *per1b* – Chapter Five). Due to the close age of the individuals on both studies (40 dd), differences in the individual genes which were returned as significantly cyclically expressed is likely due to the adaptation of the methodology surrounding sample collection to reduce the potential dilution effects of whole embryo sampling. The extraction and analysis of the whole fish including the yolk sac in Chapter Four will have undoubtedly influenced the overall gene expression and rhythmicity due to the increased localisation of clock gene expression in specific tissues and regions in teleosts (Moore and Whitmore, 2014). In a number of species, peripheral tissues such as the liver, in which oscillations of the clock are independent to those in the brain and largely entrained by feeding (Feliciano *et al.*, 2011; Vera *et al.*, 2013; Costa *et al.*, 2016; Ceinos *et al.*, 2019). Due to the lack of feeding entrainment pre-FF the expression of clock genes in the liver may have either minimised the amplitude, cancelled out the rhythmicity through out of phase expression from the different tissue types or had an additive effect from phases aligning. Which would explain the differences in the genes which were significantly cyclically expressed between the two studies.

This experiment builds upon the previous chapter by removing the effect of multiple tissue types on the expression of clock genes. Nonetheless, it is important to note the potential contribution from the uncoupling of clock gene expression within regions of the brain. Studies in zebrafish (Moore and Whitmore, 2014) and goldfish (Sánchez-Bretaño *et al.*, 2015) identified similarities in the localisation of *period* gene expression within the forebrain and midbrain regions (*per2* in zebrafish and *per1b* in goldfish). Clock gene expression was also noted to be limited to specific brain nuclei within some regions of the brain (Moore and Whitmore, 2014). It is reasonable to assume that the expression of the *period* genes within the brain of Atlantic salmon would be similar to that of zebrafish and goldfish, therefore, there is likely still an influence of conflicting clock gene expression in specific regions of the brain upon whole brain clock gene expression. For those clock genes which are directly influenced by light (light entrainable) it could be assumed that the affect upon the resulting clock gene expression pattern would be minimal due to the gene expression being in phase across the different nuclei in the brain regions.

Whilst the individual function of the clock genes and the regulation of clock gene expression within the circadian mechanism remains unknown (Yúfera *et al.*, 2017), it is important to compare the expression pattern of clock genes in relation to light environment in Atlantic salmon to other species which have been more widely investigated. The significant expression of *per1* paralogs as a whole during early developmental stages aligns to research undertaken in rainbow trout which identified *per1b* as rhythmically expressed over a 24 h period in rainbow trout embryos from as early as 8-9 dpf under LD 12:12 lighting conditions (Davie *et al.*, 2011). Studies regarding the ontogeny of the clock in zebrafish also revealed once established peak gene expression for *per1* occurs at the beginning of the scotophase (Dekens and Whitmore, 2008). Interestingly the peak expression of *per1b* in early development (420 dd) in rainbow trout under a 12:12 LD light regime appears to occur around 21:50, towards the end of the photophase (Davie *et al.*, 2011). This draws parallels with this study in which 22:00 is peak expression of the *per1b* gene identified in Atlantic salmon, which also coincides with the end of the photophase and beginning of the scotophase.

Significantly rhythmically expressed clock genes identified in the whole brain post-FF were consistent with findings reported from the optic tectum of Atlantic salmon smolt (West *et al.*, 2020) and *per1b* in goldfish (Sánchez-Bretaño *et al.*, 2015), for genes which were targeted in both studies. However, in this study, all *per1* genes [*per1a.1, per1a.2* and *per1b*] (Chapter Three) appeared to peak at the same time towards the end of the scotophase, 21 h after the lights were turned on (6:18 LD). Whereas in this study *per1a* and *per1b* were out of phase by 2 h and peaked and 18 and 20 h respectively after the lights were turned.

The differences in expression pattern and phase are likely due to the differences in the tissue samples and the photoperiod. The phase shift in the expression of the two *per1* paralogs, *per1a* and *per1b* is also consistent with the phase shift of the paralogs in Chapter Three, with both studies displaying around a 2 h negative phase shift in *per1b* when compared with *per1a*. The difference in acrophase time may be a direct result of a combination of factors including differences in experimental set up, life stage and most likely the difference in photoperiod (LD 12:12 in Chapter Three, LD 14:10 in this experiment). Expression patterns of *clock2* and *arntl1b* coincides with findings in gilthead sea bream, which reports the clustered peaking of *clock* and *bmal* (*arntl*) genes at 18:00 (Yúfera *et al.*, 2017). However, in this study the other two significantly cyclically expressed *arntl* genes *arntl2a* and *arntl2b/c* display a negative phase shift of 14 and 8 h respectively in comparison to *arntl1b*. Findings from the post-FF sampling point are comparable to that of findings in Chapter Three. There is an increased number of both rhythmically and cyclically entrained clock genes across a range of periodicities.

For those which were identified to be significantly cyclically entrained ($p < 0.05$) to a 24 h periodicity (*arntl2b/c, per1a, per1b,* and *cry4*), all genes were also found to be significantly cyclically expressed in the parr, except for *cry4* which was found not to be significantly cyclically expressed ($p < 0.05$), however it was significantly rhythmically expressed over 24 h ($p = 0.025$).

For the genes which were significantly rhythmically expressed over 24 h ($p < 0.05$) there were a few more discrepancies between the results with *arntl1a, arntl2a, cry3b,* and *cry5* all being rhythmically expressed during the later developmental stage. In addition to this *per3* was found to be significantly rhythmically expressed over a 24 h period in alevins, but not in parr. These differences in clock gene expression could be the result subfunctionalisation or neofunctionalisation of clock genes from multiple whole genome duplication events. Due to the differences in clock gene regulation in early development from that of adults (Dekens and Whitmore, 2008), duplicated clock genes may even display developmental stage specific clock gene expression patterns in addition to tissue specific clock gene expression (Moore and Whitmore, 2014).

5.6. Conclusion

This study provides fundamental information about the entrainment of the clock during the early ontogeny of Atlantic salmon regarding the change from endogenous to exogenous feeding. There is a clear difference in the number of clock genes which are significantly rhythmically and cyclically expressed around this major key life event in the ontogeny of Atlantic salmon. Findings suggest a lack of entrainment of clock genes in the brain prior to the first feeding event, highlighting the need for additional research within this critical life stage to further elucidate the role of first feeding in the entrainment of the clock mechanism.

It is also interesting to note the difference between the clock gene families which were significantly entrained pre-FF in qPCR assays conducted using RNA isolated from the brain in comparison to the previous RNA sequencing experiment (Chapter Four) in which RNA was isolated from the whole fish including the yolk sac. Indicating a potential dilution effect upon clock gene expression from extracting RNA from multiple tissue types, in comparison to one isolated tissue type. Therefore, additional studies should also be conducted to explore the effect of central vs peripheral entrainment of the clock mechanism and assess the impact upon the number of significantly rhythmic or cyclically expressed clock genes by tissue type.

Overall, the major life event of first feeding is important in kick-starting the clock mechanism in Atlantic salmon.

Chapter Six
General discussion

How do salmonid clock genes fit into the widely accepted mechanism for circadian rhythmicity? Whilst the expression of individual clock genes has been investigated in salmonids and clock gene members previously identified (Davie, Minghetti and Migaud, 2009; Huang, Ruoff and Fjelldal, 2010b, 2010a; West *et al.*, 2020). The role of fish in the understanding of circadian mechanisms has yet to be fully exploited. Individual gene expressions and protein-protein interactions have yet to be collectively analysed regarding their interactions in the mechanism as a whole (Frøland Steindal *et al.*, 2018). There are still many unknowns surrounding the mechanism in salmonids as they have been less researched than model species (fruit fly, mice, humans, and zebrafish) and other of teleosts (gilthead sea bream, European sea bass, Senegalese sole, and goldfish) (Frøland Steindal *et al.*, 2018). Core clock genes identified in this thesis complement those identified by West *et al.* (2020) and further highlight the complexity of the clock within *S. salar*.

There are an unusually large compliment of clock genes in salmonids, with minimal ancestral gene losses from *D. rerio* and *E. lucius* in the salmonids (Chapter Three). The existence of clock gene paralogs increases genetic complexity, thus enabling gene family members to specialise and extend their ancestral role, which may lead to a shift in the identity of components of the molecular clock (Looby and Loudon, 2005; West *et al.*, 2020). Whilst *clock1b, cry1b, cry3a,* and *csnk1da* appear to have been lost after the *E. lucius*, in comparison to the zebrafish, there are an additional 11 core clock genes identified, they appear to have been duplicated, likely in the Ss4R WGD event.

Despite gene duplication events in mammalian species introducing specialisation and regulatory sophistication, the function of core genes associated with circadian rhythmicity remains largely conserved across a diverse range of organisms, spanning vast evolutionary time periods (Looby and Loudon, 2005). Due to the highly conserved nature of clock genes in other species, the mechanism proposed for salmonids is likely to be highly similar to the proposed model by Takahasi (Cox and Takahashi, 2019). The additional compliment of clock gene family members brought about by the salmonid specific WGD SsR4 creates a gap surrounding the knowledge of individual gene interactions suggest within the mechanism and specific clock gene interactions to date remains unknown. Additional work is required to further elucidate the complexity the circadian mechanism in salmonids and how the complement of clock genes identified individually function as components of this mechanism.

The *in-silico* study in Chapter Three furthered the identification and classification of clock gene family members in salmonids, contributing to the creation of a toolkit for use in further circadian studies and was fundamental to subsequent work in this study. As well as providing insight into the number of clock genes which are significantly cyclically and rhythmically in parr. The phase shifts observed in gene expression in the *period,*

cryptochrome and *nr1d* families coincided with findings in zebrafish and mammals (Wang, 2008a; Amaral and Johnston, 2012; Chiou *et al.*, 2016; Hirayama *et al.*, 2019).

Comparative analyses between two life stages revealed a significant decrease in the number of clock genes which were reported to be significantly rhythmically and cyclically entrained during early development compared to parr (Chapter Four). Whilst not rhythmically entrained in whole embryo RNA, clock gene expression was detectable as early as eye pigmentation at 255 dd. Of the three light conditions explored, somewhat unsurprisingly, due to its reference as a primary zeitgeber (Davidson and Menaker, 2003), photo cue was the most influential of all light conditions. The absence of light and dark cues are reported to leave the clock free-running and out of phase with the real world (Ceinos *et al.*, 2019). Whilst the influence of light intensity upon clock genes expression was minimal, there was a profound influence upon the expression of *cry3b.2*. The elevated expression of *cry3b.2* at night compared to during the day coincides with findings in zebrafish (Liu *et al.*, 2015). With increasing light intensity, there was a significant increase in *cry3b.2* expression during the photophase (LDH vs LDL) and the scotophase (LDH vs LDL, LDH vs LDM).

With regard to spectral composition, it is clear that exposure to long wavelength light in comparison to either white light or shorter wavelength light displayed the most significant differences in clock gene expression. The influence of coloured light appears to be more significant during the photophase, fewer gene expression patterns were significantly altered during the scotophase. In other marine species and zebrafish exposure to longer wavelength light was reported to be detrimental to development and survivability, with shorter wavelength blue light described as optimum (Villamizar, García-Alcazar and Sánchez-Vázquez, 2009; Villamizar *et al.*, 2014; Sierra-Flores *et al.*, 2016).

The common use of light as an effective tool in commercial aquaculture practice and its use to increase growth and to delay or expedite key life stages such as smoltification and spawning (Stefansson *et al.*, 1990). Very little is known about the effect of light conditions upon the circadian mechanism. A mechanism which is implemented in a number of these important life stages through the regulation of downstream output genes, such as: FA oxidation, lipid synthesis and energy metabolism (Gooley, 2016). It would be interesting to identify whether the same is true for Atlantic salmon and determine through RNA sequencing if the detrimental effects of long wavelength light are linked to disturbances of the clock mechanism. Early ontogeny of the Atlantic salmon occurs in the interstitial spaces of gravel redds (Gorodilov, 1987), whereas zebrafish spawn in silty, vegetated environments, and hatch within 48-72 hpf (Kimmel *et al.*, 1995). Ecological consideration of the Atlantic salmon's natural environment may explain the tolerance of the fish to increased levels of longer wavelength light in comparison to zebrafish.

Expanding upon the initial broad experiment in early developmental stages, due to the lack of rhythmicity in whole pre-FF alevins it was decided to expand the time period to encompass pre- and post-FF, dissecting out the brain to minimise the potential dilution effect on the expression patterns of the clock genes. There was a clear difference in the number of clock genes which were significantly rhythmically and cyclically expressed around the major life event on FF in Atlantic salmon. Findings suggest a lack of entrainment of clock genes in the brain prior to the FF event, highlighting the need for additional research within this critical life stage to further elucidate the role of first feeding in the entrainment of the clock mechanism. This lack of significantly rhythmically or cyclically expressed clock genes pre-FF at ~730 dd (Chapter Five) aligned with earlier findings in Atlantic salmon at 690 dd in Chapter Four. The extraction and analysis of the whole fish including the yolk sac in Chapter Four will have undoubtedly influenced the overall gene expression and rhythmicity due to the increased localisation of clock gene expression in specific tissues and regions in teleosts (Feliciano *et al.*, 2011; Vera *et al.*, 2013; Moore and Whitmore, 2014; Costa *et al.*, 2016; Ceinos *et al.*, 2019).

6.1. Limitations

The experiments conducted within this thesis are novel in early developmental Atlantic salmon and as such are a good pilot for future research, providing groundwork and highlighting areas which require an increased focus going forward. It is important to recognise and understand the limitations within the present studies as an acknowledgement for the need for future research to further develop our understanding of clock genes and the circadian mechanism in Atlantic salmon.

Due to the availability of experimental facilities, the studies at UiB and UoS were undertaken in flow-through systems. Therefore, it is important to acknowledge the presence of diurnal temperature fluctuations in the incoming water as a potential limitation. In addition to this all fish were fed their ration during the light period using automatic feeders. Consequently, the effect of diurnal temperature fluctuation and feeding during the lit period cannot be fully separated from the significant cyclicity of clock gene expression from light, as both temperature and food are involved in the entrainment of the clock (Isorna *et al.*, 2017). In the respective studies, artificial lighting times coincide with the timing of natural daylight. Therefore, due to the brain being predominantly entrained by light, the influence of diurnal temperature fluctuations and feeding during the photophase is likely to be additive(López-Olmeda, 2017); this may have amplified the cyclical expression of clock genes under the LD cycle (López-Olmeda and Sánchez-Vázquez, 2009; Vera *et al.*, 2013, 2023; Costa *et al.*, 2016; Steindal and Whitmore, 2020). To remove the influence of feeding and temperature on clock gene expression in future studies it is recommended that trials are

completed in recirculating aquaculture systems with full temperature control to maintain a constant temperature and that fish are fed either continuously or in randomly allocated meals to prevent food anticipatory activities.

Fish which underwent circadian sampling periods were subject to LD cycles only (either 14:10 or 12:12), initial plans were to compare these fish to those which were reared in continuous light or continuous darkness (pre-feeding alevins only due to welfare implications). Due to COVID-19 restrictions, it was not possible to complete this within the scope of this thesis. This would have enabled the direct comparison of genes which were significantly cyclical and rhythmic under both diurnal and constant lighting conditions; indicating which of the clock genes were able to oscillate in the absence of external stimuli within the brain and therefore likely endogenous in nature and not the direct response to diurnal zeitgebers (Andreani *et al.*, 2015; López-Olmeda, Sánchez-Vázquez and Fortes-Silva, 2021).

Similar to previous studies in Senegalese sole (Martín-Robles, Aliaga-Guerrero, *et al.*, 2012) and gilthead sea bream (Yúfera *et al.*, 2017), whole embryos were initially sampled in the very early developmental studies, which is suggested to be a limiting factor. Clock gene expression in fish is known to be localised in different tissue types (Whitmore *et al.*, 1998). Localised gene expression in central and peripheral tissues often appear out of phase with one another due to the method of entrainment, light entrainable (brain, retina, hypothalamus) versus food entrainable (liver) (Isorna *et al.*, 2017; Ceinos *et al.*, 2019). As tissue specific effects may be diluted by overall gene expression (Mazurais *et al.*, 2011; Yúfera *et al.*, 2017). However, the effect of food entrainable oscillators is thought to be minimal prior to the onset of exogenous feeding (Martín-Robles, Aliaga-Guerrero, *et al.*, 2012). This dilution effect may also be present within the whole brain samples due to the differentiation of cell types within brain regions and the uncoupling of clock gene expression within these regions (Moore and Whitmore, 2014; Sánchez-Bretaño *et al.*, 2015).

6.2. Conclusion

Studies conducted in this thesis have contributed to the fundamental research surrounding the effect of lighting conditions on the expression of clock genes during early development in Atlantic salmon. Contributions to narrowing the research gap include:

- **Identified and classified clock genes in salmonids:** Across seven clock gene families, 48 clock genes were identified in Atlantic salmon and compared against four other salmonids, standardising naming based on zebrafish nomenclature. Thus, creating a fundamental resource for future clock gene studies in salmonids.

- **Displayed that lighting characteristics affect clock gene expression of Atlantic salmon during early development:** something which has previously been overlooked in commercial aquaculture practice. highlighting the need for further studies to identify the optimal lighting conditions for early development and help to tailor hatchery best practice guidelines.
- **Proliferation of rhythmic and cyclical clock genes post-FF:** a greatly increased number of clock genes which were significantly rhythmically and cyclically expressed in the brain were identified in comparison to pre-FF. This highlights the need for further research into the role of first-feeding to elucidate the role of the key life stage in relation to the clock.
- **Identified clock genes with circadian expression patterns in whole brain:** clock genes which were significantly rhythmically and cyclically expressed in the brain under LD cycles can be used as targets in future work to determine endogenous entrainment or response to external stimuli.

6.3. Future perspectives

There are several avenues arising from the results and discussion in the experimental chapters of this thesis in which future research will further the development of knowledge and understanding surrounding the circadian mechanism in Atlantic salmon. Thus, helping to categorise the impact of lighting regimes upon the mechanism and downstream outputs. These include but are not limited to:

- **Isolation of differing lighting conditions from the influence of other zeitgebers:** to further close the research gap surrounding the circadian mechanism in relation to light conditions, it is recommended that studies are conducted in RAS systems as they provide a greater degree of control over the environment, specifically, minimising the effect of daily temperature fluctuations which are known to influence circadian entrainment.
- **Development of HKGs suitable for circadian studies for qPCR:** There is a need to identify HKGs which are suitable for normalising qPCR results from circadian studies (*i.e*., stable, and not rhythmically or cyclically expressed over 24 h) to ensure that the effects of RNA quality from extraction and efficacy of cDNA synthesis are minimised (Bustin *et al.*, 2009). *In silico* analysis of 24 h RNA sequencing databases will help to identify suitable candidates which are the most stable and not rhythmically or cyclically expressed over 24 h that are suitable for use to normalise gene expression values using a geometric mean in circadian experiments.

- **Identification of endogenously oscillating clock genes:** studies comparing the expression of clock genes under both LL and LD environments should be undertaken to compare the resulting rhythmic and cyclically expressed clock genes to distinguish which are endogenous in nature and continue to oscillate in the absence of a light cue versus those which are dependent upon daily external stimuli for their circadian expression pattern.
- **Central vs peripheral clock gene expression:** It is suggested that a large-scale RNA sequencing experiment is conducted in adults to expand the understanding of central vs peripheral clock gene expression, in which numerous tissue types can be analysed to gain a broader insight into the dissociation and localisation of clocks in Atlantic salmon under lighting regimes which are widely used commercially (LL and LD of varying photoperiod). RNA sequencing would also enable the expression of clock-controlled genes to be identified in the different tissue types.
- **Influence of spectral composition and light intensity on circadian clock gene expression:** expanding on the differentially expressed genes identified in Chapter Four, completing a full circadian sampling to identify the full influence of lighting conditions upon clock gene expression.
- ***In situ* hybridisation to identify areas of clock gene expression within regions of the brain:** *in situ* hybridisation can be used to map out clock gene expression in specific tissues such as the brain. Once localisations of the clock are mapped, they can be targeted in subsequent studies and regions of the brain can be dissected out and sampled for RNAseq or qPCR assays. Mapped clock gene expression can also be used to identify co-localisations with other genes such as visual opsins to determine light entrainable oscillators or genes which are known to be influenced by clock genes in other species to identify clock-controlled genes.

There is a need for the development of a model for the circadian mechanism in salmonids to aid the identification of gene function and the subsequent gene interactions. As a response of the variable expression identified in early developmental stages which may by further complicated by experimental design. It is suggested that initial work on this model is developed in adults. Creation of a baseline model in older individuals can then be used to investigate the effects of developmental stage upon clock gene expression in juveniles. Due to the vast array of environments that salmonids inhabit, it is proposed that this model should be expanded to include additional species of salmonid such as the rainbow trout. From this foundation a more robust picture of the biological clock and circadian entrainment can be formed in salmonids.

Once this foundation based on fundamental research has been created, targeted experiments can be conducted to identify and classify the effects of light conditions at varying life stages. The long-term goal would be to identify optimal lighting conditions for Atlantic salmon based on their developmental stage which can then be used to underpin industry best practice guidelines promoting the welfare and health of farmed salmonids. The introduction of targeted lighting regimes during early development may also help to pre-programme fish to lighting regimes and can be monitored to identify the effects upon industry key performance indicators.

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Publications

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Rhythmic Clock Gene Expression in Atlantic Salmon Parr Brain

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To better understand the complexity of clock genes in salmonids, a taxon with an additional whole genome duplication, an analysis was performed to identify and classify gene family members (*clock*, *arntl*, *period*, *cryptochrome*, *nr1d*, *ror*, and *csnk1*). The majority of clock genes, in zebrafish and Northern pike, appeared to be duplicated. In comparison to the 29 clock genes described in zebrafish, 48 clock genes were discovered in salmonid species. There was also evidence of species-specific reciprocal gene losses conserved to the *Oncorhynchus* sister clade. From the six period genes identified three were highly significantly rhythmic, and circadian in their expression patterns (*per1a.1*, *per1a.2*, *per1b*) and two was significantly rhythmically expressed (*per2a*, *per2b*). The transcriptomic study of juvenile Atlantic salmon (parr) brain tissues confirmed gene identification and revealed that there were 2,864 rhythmically expressed genes ($p < 0.001$), including 1,215 genes with a circadian expression pattern, of which 11 were clock genes. The majority of circadian expressed genes peaked 2 h before and after daylight. These findings provide a foundation for further research into the function of clock genes circadian rhythmicity and the role of an enriched number of clock genes relating to seasonal driven life history in salmonids.

Keywords: clock genes, ohnologs, circadian, whole-genome duplication, rhythmic expression

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1. INTRODUCTION

The importance of biological time keeping is apparent across all organisms, from bacteria to humans (Wulund and Reddy, 2015). These time-dependent adaptations can last seconds or minutes and recur throughout the day (ultradian) or endure days or months (infradian). They are an evolutionary trait which enables all living organisms to maximise fitness in anticipation of endogenous (molecular and cellular) and external stimuli or zeitgebers (Andreani et al., 2015; Sánchez-Vázquez et al., 2021). Circadian rhythms are endogenous oscillatory expression of genes with a periodicity of approximately 24-h. These rhythms are the expression of endogenous clocks which synchronises biochemical, physiological, and behavioural responses enabling organisms to respond to diel environmental changes (Li et al., 2015). The transcription of 43% of all protein-coding genes in mice displayed circadian rhythms across numerous organs (Zhang et al., 2014). Clock genes are of particular interest in salmonids due to their association with physiological traits such as reproduction migration and smoltification (Leder et al., 2006; O'Malley et al., 2007, 2010; Paibomesai et al., 2010). Allelic diversity and variation in length polymorphism of the clock PolyQ domain was reported in four Pacific salmon species (chinook, chum, coho, and pink) with overlapping geographical ranges and diversity in spawning times. This implicates clock gene

duplicate may be involved in the seasonal and geographical variation in reproduction (O'Malley et al., 2007, 2010). In addition, a copy of the gene *clock* has been localised to a quantitative trait locus (QTL) responsible for 20–50% of the variation in spawning dates in female rainbow trout (Leder et al., 2006).

The circadian clock consists of intracellular transcriptional-translational feedback loops (TTFL) composed of core clock genes and stabilising accessory loop genes, which drives the rhythmic accumulation of downstream outputs, or clock-controlled genes (Reppert and Weaver, 2001; Partch et al., 2014). Circadian mechanism is highly conserved across animal species (Lowrey and Takahashi, 2011). However, deciphering the circadian clock mechanism in fish is complex. Clock and clock-related genes which are found in single copies in invertebrates such as clock, period and cryptochrome are duplicated in vertebrates (Tauber et al., 2004). In addition, salmonids were subjected to two rounds of whole-genome duplication (WGD) events (Ts3R, teleost-specific third whole-genome duplication, 320 million years ago, and Ss4R, salmonid-specific fourth whole-genome duplication, 80 million years ago) resulting in an abundance of circadian related genes (Lien et al., 2016; Huang, 2018).

The molecular mechanisms underlying circadian rhythmicity have been characterised in several model animal species including the fruit fly (*Drosophila melanogaster*), mice (*Mus musclus*), and humans (*Homo sapiens*), with relatively limited work undertaken in teleosts (Wang, 2008b; Huang, 2018) predominantly centred on zebrafish (*Danio rerio*) (Cahill, 2002; Vallone et al., 2007). The circadian system comprises all the different components by which light is perceived by the organism and is transformed into a nervous or hormonal signal (Migaud et al., 2010a). Therefore, manipulations of photic inputs and cues impact rhythms, which can be commercially exploited for aquaculture production (Migaud et al., 2010a). Research based on zebrafish has been fundamental in describing and broadly characterising clock genes and circadian rhythmicity as many findings are applicable to numerous vertebrate species. However, fish models have not yet significantly contributed to our understanding of core clock mechanisms and circadian clock control of fish physiology (Frøland Steindal and Whitmore, 2019). Teleosts represent the largest and most diverse group of vertebrates, with over 30,000 species identified to date. Each species possesses distinct characteristics and displays a considerable amount of anatomical and physiological plasticity. This is arguably the direct result of exposure to multiple, variable selection pressures caused by the highly dynamic aquatic environments they have inhabited throughout their evolutionary development (Bone, 2019). Alongside selection pressures, multiple rounds of WGD have had a large impact upon the evolution of lineages to date, with the retention of resultant gene duplicates being biased with regard to gene function (Brunet et al., 2006). Gene duplication through WGD led to redundant genes either los, non-functionalisation, with a different function than the ancestral gene (sub-functionalisation) or which acquired new functions, neo-functionalisation, (Pasquier et al., 2016), thus resulting in genome reshaping (Inoue et al., 2015). An example of this is the salmonid specific WGD (Ss4R) which preceded the origin of anadromy in salmonids, an important milestone in the evolutionary development of salmonid migration (Alexandrou et al., 2013). Whilst WGD events led to gene duplication, these duplicated genes typically resolved over time (Inoue et al., 2015). In rainbow trout, 80–100 million years post Ss4R, 48% of the pre-duplication ancestral genes were retained as duplicates, the remainder of the genes underwent fractionation and the duplicated protein-coding genes were lost (Berthelot et al., 2014; Lien et al., 2016). Analysis of duplicate retention in Atlantic salmon identified that 20% of duplicates from Ts3R and 55% of duplicates from Ss4R were retained as functional copies, with the prominent mechanism for duplicate loss being pseudogenisation (Lien et al., 2016). While there is a general lack of clarity surrounding the duplication and retention of functional genes post WGD, there is an unusually large complement of clock genes in salmonids (West et al., 2020).

Salmonids are amongst the most widely studied groups of fish species both scientifically and commercially, as many species of salmonid are of significant economic, societal, and environmental importance (Thorgaard et al., 2002). Many of the species within the 11 genera of the Salmonidae (Nelson et al., 2016) are of great commercial value and contribute significantly to both local and global economies through aquaculture, wild stock fisheries and recreational sport (Reppert and Weaver, 2001; Davidson et al., 2010; Frøland Steindal and Whitmore, 2019). Unlike zebrafish, salmonid species are highly seasonal in their physiology, including migration, smoltification and reproduction. Lighting and temperature manipulations are routinely used by industry to manipulate commercial broodstock ovulatory rhythm and smoltification, enabling year-round production (Migaud et al., 2013). However, understanding the intricate interactions between zeitgebers, circadian rhythmicity, seasonality, and the control of biochemical, physiological, and behavioural rhythms is complex (Migaud et al., 2010b). The completion and publication of salmonid genomes (*Salmo salar*, *Salvelinus alpinus*, *Oncorhynchus mykiss*, *Oncorhynchus kisutch*, and *Oncorhynchus tshawytscha* to date) as part of the Functional Annotation of All Salmonid Genomes (FAASG) project (Macqueen et al., 2017), alongside RNAseq have provided great tools to study clock genes of salmonids.

The aim of this study was to identify the full complement of clock genes in Atlantic salmon (*Salmo salar*) in comparison to other commercially important salmonid species and evaluate the expression patterns of the identified genes. To do so, phylogenetic analysis of clock gene ohnologs [functional product of WGD event (Ohno, 1970)] has been performed to classify and name salmonid clock genes based on published zebrafish references and nomenclature. This was confirmed by a transcriptomic approach looking into gene expression over 24-h in freshwater salmon kept under a controlled lighting regime. This study provides a new nomenclature for salmon clock genes that will serve as a tool for further circadian research in salmon.

2. MATERIALS AND METHODS

2.1. Identification of Clock Genes in Salmonids With Published Genomes

The protein sequences of the 29 zebrafish (*D. rerio*) clock genes [*clock*, *arntl* (also referred to as *bmal*), *period*, *cryptochrome*, and *csnk1e/d* (Huang, 2018), *nr1d* (also referred to as *rev-erb*), and *ror* (Wang, 2009)] were recovered from GenBank and used as reference to interrogate the Northern pike (*Esox lucius*) [a closely related sister taxa which did not undergo the salmonid specific WGD (Rondeau et al., 2014; Macqueen et al., 2017; Varadharajan et al., 2018)] and Atlantic salmon (*S. salar*) genomes. For the benefit of this study, the core clock genes (the heterodimers forming the positive and negative feedback arms of the TTFL, *clock:arntl* and *period:cryptochrome* respectively) and accessory loop genes (individual genes *ror*, *nr1d*, and *csnk1* which interact with the core clock loop to either promote or repress specific heterodimer interactions) are commonly referred to collectively as clock genes. Putative core clock gene sequences were also identified for several salmonid species (*S. alpinus*, *O. mykiss*, *O. kisutch*, and *O. tshawytscha*) through a combination of literature searches, BLASTp and BLASTn searches of published salmonid genomes identified as part of the Functional Annotation of All Salmonid Genomes (FAASG) initiative (Amaral and Johnston, 2012). For the benefit of this publication, they will be referred to collectively as salmonids. A BLASTp search using the default settings against the protein sequences were used for a first characterisation of the putative core clock genes. This was further refined using BLASTn using the coding sequence (CDS) against the RNA sequences (refseq_rna) database and was optimised for highly similar nucleotide sequences (megablast) with an *E*-value below 10^{-300} and ensuring a negligible probability that the sequence was returned by chance. From the final BLASTn search, the gene and their transcriptomic isoform were aligned to the CDS of zebrafish reference genes using ClustalOmega v1.2.2 (Sievers et al., 2011).

2.2. Phylogenetic Alignment

Amino acid sequences were aligned using GramAlign v3.0 (Russell, 2014). A Maximum Likelihood (ML) tree was inferred under the GTR model with gamma-distributed rate variation (Γ) and a proportion of invariable sites (I) using a relaxed (uncorrelated lognormal) molecular clock in RAxML (Stamatakis, 2014) with 10,000 bootstrap replicates. Gaps were handled as undetermined characters (N).

2.3. Classification

Nomenclature of the putative salmonid clock genes was based on phylogenetic analyses using CDS and full-length sequences to the *D. rerio* and *E. lucius* core clock genes. Genes have been renamed after the zebrafish orthologues. As a result of the salmonid specific WGD Ss4R, salmonids often have two copies of a gene which is present as a single copy in zebrafish. In most instances, this involved renaming the gene from their given predicted name. Genes were re-classified based on the nomenclature of the zebrafish reference genes (ZFIN, 2019). If a single representative was identified per species, the same name as the zebrafish reference was used. For groups with multiple representatives, the name of the orthologs were appended with numerical suffixes (*0.1*, *0.2*) or alphabetical suffixes (*a*, *b*) depending on the nomenclature of the zebrafish reference genes as a result of the previous ray fin fish WGD event, as some zebrafish genes were already denoted with alphabetical suffixes in relation to their duplication. Those with the highest percentage identity to the reference gene phylogenetically are labelled *a*. For example: the Atlantic salmon has two period 1a (per 1a) paralogs, *period 1a.1* (*per1a.1*) has the highest percentage identity when compared to the reference gene and is therefore closer to the zebrafish gene so is denoted by *0.1*, and *period 1a.2* (*per1a.2*) is less identical to the ancestral form and is therefore denoted by *0.2*.

2.4. Animal Husbandry and Sampling

All juvenile Atlantic salmon (*S. salar*) used in the experiment were kept at the Niall Bromage Freshwater Research Facilities at the University of Stirling. Fish (60, mean weight of 130 g, Benchmark Genetics Iceland origin) were held in an 800 L tank in a flow through system and maintained under a 12:12 Light:Darkness photoperiod (photophase from 08:00 to 20:00 using TMC AquaRay LED lamps) from 14th April 2020 to 19th August 2020 when sampling ended, ambient temperature ranged from 8.1°C to 15.2°C during this period. In the month before sampling the temperature range was 14.6°C ± 0.6°C. Fish were fed daily to satiation with BioMar Orbit (2 and 3 mm pellet) during the light period using automatic feeders (Arvo-Tec, Sterner). Fish were not fed during the light period on the day of sampling. Sampling was conducted every 4 h over a 24 h period, starting at 10:00 on the first day and ending at 10:00 the following day (10:00[1], 14:00, 18:00, 22:00, 02:00, 06:00, 10:00[2]). At each time-point, six fish were sampled (**Supplementary Table 1**). Following lethal anaesthesia (MS222), brains were dissected out and snap frozen directly in liquid nitrogen and stored at −80°C until analysis.

2.5. RNA Extraction and Sequencing

RNA was isolated from the whole brain using TRI reagent (Sigma, St Louis, MO, USA) and RNA concentration was tested using a Qubit 2.0 Fluorometer (ThermoFisher Scientific, Waltham, MA, USA). The 42 RNA samples were submitted to Novogene UK (Cambridge) for RNA sequencing. Samples were submitted to quality control (Illumina BioAnalyzer®) revealing the RNA integrity number (RIN) of samples valued between 8.8 and 10.0. For each sample (900 ng), a library was prepared using NEB Next® UltraTM RNA Library Prep Kit and processed and sequenced using Illumina NovaSeq® 6000 S4 PE150 (6 GB of data per sample, ca. 40 million reads).

2.6. RNA Sequencing Analysis

Clean reads were obtained from the raw reads by filtering ambiguous bases, PCR duplicates, low quality sequences (< Q20), length (150 nt), absence of primers/adaptors and complexity (entropy over 15) using fastp (Chen et al., 2018). Ribosomal RNA was further removed using SortMeRNA v3.0.2 (Kopylova et al., 2012) against the Silva version 119 rRNA

databases Quast et al. (2012). The remaining reads were aligned to the annotated *S. salar* genome ICSASG v2.99 (Accession GCA_000233375.4) using HiSat2 v2.2.0 (Kim et al., 2019). The expression levels were estimated based on the genome annotation using StringTie2 v2.1.0 (Kovaka et al., 2019) following the workflow: (a) for each sample, map the reads to the genome with HiSat2 and assemble the read alignments with StringTie2; (b) merge the assemblies in order to generate a non-redundant set of transcripts observed in all the samples; (c) for each sample, estimate transcript abundances and generate read coverage tables expressed in the fragments per kilobase of exon per million mapped reads (FRKM).

2.7. Statistical Analysis

All tests and analysis were performed using R v4.1 (R Core Team, 2020). The expression values were scaled in Transcripts Per Millions (TPM) before been normalised by size factor using the median ratio method described by Anders and Huber (2010) using DESeq2 v1.32.0 (Love et al., 2014). Differential expression was estimated using the function *lfcShrink* (Stephens, 2017). The heatmaps were created from the normalised count files and scaled by row to highlight individual gene expression. Daily rhythms across the genome including identified clock genes were identified from the 24-h dataset using RAIN v1.26.0 (Thaben and Westermark, 2014) and MetaCycle v1.2.0 (Wu et al., 2016) implementation of JTKCycle (Hughes et al., 2010) and a threshold p-value of 0.001 and a minimum relative oscillation amplitude of 10%. All p-value reported were corrected for False Discovery Rate (FDR) using Bonferroni adjustment.

3. RESULTS

3.1. Identification of Clock Genes in Salmonids With Published Genomes

From the BLASTn, 143 clock gene variants were returned; a substantial number were highly similar predicted variants of the same gene and had identical EMBL accession numbers (LOC ID) and the CDS were over 90% identity. Most of the differences were found in the UTR. The CDS of variants sharing LOC ID were aligned to the CDS of *D. rerio* reference genes. Variants with the highest percentage identity compared to the reference for each locus were selected, leaving a total of 48 core clock genes identified in the *S. salar* across the 7 gene families explored (**Table 1** and **Supplementary Table 2**).

3.2. Interpretation and Classification

All the core clock and clock accessory loop genes were identified and classified (**Table 1**). Gene members of each family were aligned together in a gene-family approach to aid clarification of paralog nomenclature. The *period* (*per*) family is used as a typical example (**Figure 1**), showing the resulting gene tree from the identification and classification of per genes in salmonids indicating reciprocal gene retention (red), gene duplication (orange), and reciprocal gene loss (blue).

TABLE 1 | Genomic structure of the gene families associated with the circadian clock of salmonids.

Family	Genes	DR	EL	SSA	SA	OM	OK	OT
clock	*clock1a*	•	•	••	••	••	••	••
	clock1b	•	•	–	–	–	–	–
	clock2 (npas2)	•	•	••	••	••	••	•
arntl	*arntl1a*	•	•	••	••	••	••	••
	arntl1b	•	•	••	•	••	••	•
	arntl2	•	••	•••	••	•••	•••	••
period	*per1a*	•	•	••	••	••	••	••
	per1b	•	•	•	•	•	•	•
	per2	•	•	••	••	••	••	••
	per3	•	•	•	•	–	–	–
cryptochrome	*cry1a*	•	•	••	••	••	••	••
	cry2	•	•	•	•	•	•	*
	cry3a	•	•	–	–	–	–	–
	cry3b	•	•	••	••	•	•	*
	cry4	•	•	•	–	–	–	–
	cry5	•	•	•	•	•	•	•
nr1d	*nr1d1*	•	•	••	••	••	••	••
	nr1d2a	•	•	••	••	••	•	••
	nr1d2b	•	•	••	••	••	••	••
	nr1d4a	•	•	••	••	••	••	••
	nr1d4b	•	•	••	••	••	••	••
ror	*roraa*	••	••	••	••	••	••	••
	rorab	•	–	–	–	–	–	–
	rorb	•	•	••	••	••	••	••
	rorca	•	•	••	••	••	••	••
	rorcb	•	•	••	••	••	•	••
csnk1e/d	*csnk1e*	•	•	••••	••••T	••••	••	••••
	csnk1da	•	•	–	–	–	–	–
	csnk1db	•	•	••	•	••	••	••

Phylogenetic trees upon which the table was based on can be found in ***Figure 1*** *and* ***Supplementary Figures 1–7****. • gene detected in genome; - gene not detected in genome; * gene not detected in genome but identify from transcriptomes data. DR Danio rerio (GCA_000002035.4), EL Esox lucius (GCA_011004845.1), SSA Salmo salar (GCA_000233375.4), SA Salvelinus alpinus (GCA_002910315.2), OM Oncorhynchus mykiss (GCA_002163495.1), OK Oncorhynchus kisutch (GCA_002021735.2), OT Oncorhynchus tshawytscha (GCA_002872995.1).*

3.2.1. *Period* (Figure 1 and Supplementary Figure 1)

per1a is duplicated across all salmonids (*per1a.1*, *per1a.2*). *per1b* is retained in all salmonids (*per1b.1*, *per1b.2*). *per2* appears to be duplicated after the latest common ancestor *E. lucius* (*per2a*, *per2b*). *per3* is retained in *S. salar* and *S. alpinus*, but appears to have been lost in *O. mykiss*, *O. kisutch*, and *O. tshawytscha*.

3.2.2. *Clock* (Supplementary Figure 2)

clock1a is duplicated in all salmonids (*clock1a.1*, *clock1a.2*). *clock1b* was lost compared to *D. rerio* and *E. lucius* and is not present in the salmonids. *clock2* (*npas2*) is duplicated across all salmonids analysed (*clock2a*, *clock2b*) with a differential ohnolog loss in *O. tshawytscha* (aligns to *clock2a*).

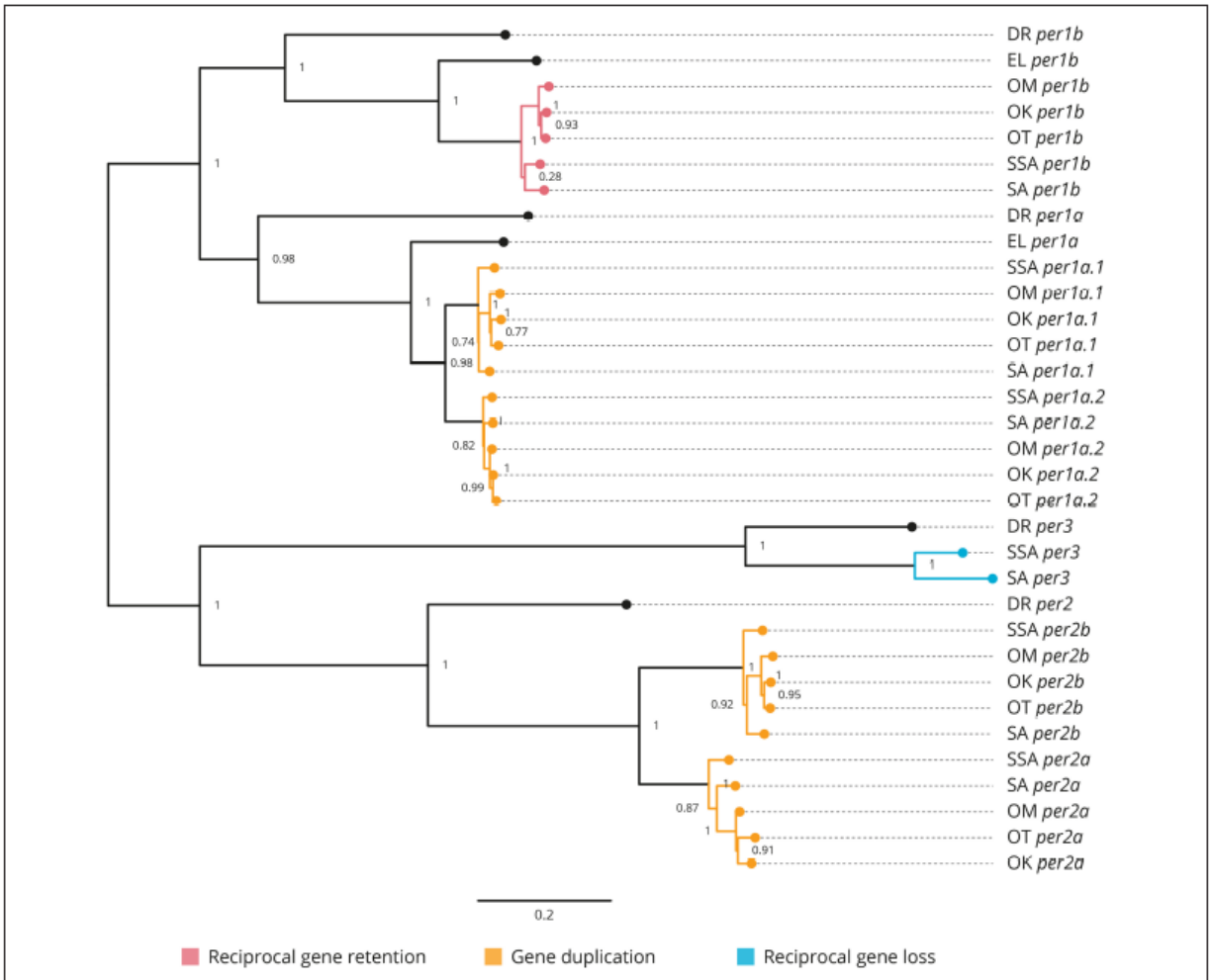

FIGURE 1 | Phylogenetic relationship of the period clock gene family (*per*). Gene family members from *Danio rerio* (DR), *Esox Lucius* (EL), *Salmo salar* (SSA), *Salvelinus alpinus* (SA), *Oncorhynchus mykiss* (OM), *Oncorhynchus kisutch* (OK), and *Oncorhynchus tshawytscha* (OT) were re-annotated. Values on the relevant node depict the bootstrap values. Reciprocal gene retention (red), gene duplication (orange), and reciprocal gene loss (blue).

3.2.3. *Aryl Hydrocarbon Receptor Nuclear Translocator-Like* (Supplementary Figure 3)

arntl1a is duplicated in all salmonids (*arntl1a.1*, *arntl1a.2*). *arntl1b* is duplicated in all salmonids (*arntl1b.1*, *arntl1b.2*) with differential ohnolog loss in both *S. alpinus* (aligns to *arntl1b.2*) and *O. tshawytscha* (algins to *arntl1b.1*). There appears to be a duplication of *arntl2* in the latest common ancestor which is retained across all salmonids (*arntl2a*, *arntl2b*). There is a third copy identified in *S. salar*, *S. alpinus*, *O. mykiss* and *O. kisutch* (*arntl2c*).

3.2.4. *Cryptochrome* (Supplementary Figure 4)

cry1a is duplicated across all salmonids (*cry1a.1*, *cry1a.2*). *cry1b* appears to be lost after *D. rerio*. *cry2* is retained in all salmonids with the exception of *O. tshawytscha* as there is currently not enough information available regarding *cry2* in the species. There is an apparent gene loss of *cry3a* in salmonids after the most recent common ancestor, *E. lucius*. *cry3b* is duplicated in *S. salar* and *S. alpinus* (*cry3b.1*, *cry3b.2*) there is an apparent gene loss in *O. mykiss* and *O. kisutch* (aligns to *cry3b.1*), currently there is not enough information available regarding *cry3b* in *O. tshawtscha*. There is an apparent gene loss of *cry4* in all salmonids after the latest common ancestor *E. Lucius* in all species of salmonid except from *S. salar*. *cry5* is retained in all salmonids.

3.2.5. *Nuclear Receptor Subfamily 1 Group d* (Supplementary Figure 5)

nr1d1 is duplicated across all salmonids (*nr1d1a*, *nr1d1b*). *nr1d2a* is duplicated across all salmonids (*nr1d2a.1*, *nr1d2a.2*), with an apparent differential ohnolog loss in

O. kistuch (aligns to *nr1d2a.2*). *nr1d2b* is duplicated across all salmonids (*nr1d2b.1*, *nr1d2b.2*). *nr1d4a* is duplicated across all salmonids (*nr1d4a.1*, *nr1d4a.2*). *nr1d4b* is duplicated across all salmonids (*nr1d4b.1*, *nr1d4b.2*).

3.2.6. *RAR-Related Orphan Receptor* (Supplementary Figure 6)

roraa is duplicated across all salmonids (*roraa.*1, *roraa.2*), there is an apparent differential gene loss in *O. tshawytscha* (aligns to *roraa.1*) and an apparent loss of both ohnologs in *O. kisutch*. There is an apparent loss of *rorab* after the latest common ancestor, *E. lucius*. *rorb* has been duplicated across all salmonids (*rorb.1*, *rorb.2*). *rorca* has been duplicated across all salmonids (*rorca.1*, *rorca.2*). *rorcb* has been duplicated across all salmonids (*rorcb.1*, *rorcb.2*) with an apparent differential ohnolog loss in *O. kisutch* (aligns to *rorcb.2*).

3.2.7. *Casein Kinase 1 Delta* (Supplementary Figure 7)

csnk1db appears to be duplicated across all salmonids (*csnk1db.1*, *csnk1db.2*) apart from *S. alpinus* which displays an apparent differential ohnologs loss (aligns to *csnk1db.1*).

3.2.8. *Casein Kinase 1 Epsilon* (Supplementary Figure 7)

There appears to be multiple duplications of csnk1e across all the salmonids, resulting in four csnk1e paralogs (*csnk1ea*, *csnk1eb*, *csnk1ec*, *csnk1ed*). With *O. kisutch* displaying a potential differential ohnologs loss (aligns to *csnk1ea*, *csnk1eb*).

3.3. Transcriptomic Analysis

In total, 986,545,058 raw reads were sequenced for 42 samples (**Supplementary Table 2**). The reads were deposited in the European Bioinformatics Institute (EBI) European Nucleotide Archive (ENA) project ID PRJEB41327. After filtering, 975,284,560 clean reads (98.86%) passed the mRNA cleaning step and were used for the following process. Of the clean reads, 97.71% were aligned to the published *S. salar* genome ICSASG v2.99 (Accession GCA_000233375.4). A total of 55,819 distinct genes were recovered (**Supplementary Data 1**). All clock genes classified in the *in silico* gene identification were recovered.

3.4. Rhythmic and Circadian Gene Expression

Significantly rhythmically expressed genes were identified using RAIN and MetaCycle with JTK analysis (**Supplementary Data 2**). Various thresholds were evaluated (**Supplementary Table 3**). From the 48 genes clock genes, 16 were significantly rhythmically expressed (RAIN analysis, $p < 0.001$, relative amplitude $\geq$ 10%), of which 11 also had a significant circadian expression patten over a 24-h period (JTK analysis, $p < 0.001$, relative amplitude $\geq$ 10%, **Figure 2A** and **Supplementary Figures 8**, **9**). Details of the gene family are plotted in **Figure 2B**. From the six period genes identified five were highly significantly rhythmically expressed (*per1a.1*, *per1a.2*, *per1b*, *per2a*, *per2b*), including three that also exhibited circadian expression pattern (*per1a.1*, *per1a.2*, *per2b*). The acrophase for *per1a.1* and *per1a.2* are in phase at 06:00, *per1b* has a negative phase shift in comparison to *per1a* paralogs at 08:30. *per2a* and *per2b* are out of phase by 2 h, with *per2a* peaking at 03:00 and *per2b* at 01:00.

Overall, 2,864 genes exhibited a rhythmic expression pattern (rhythmic peak; RAIN analysis, $p < 0.001$; **Supplementary Data 2**), and of which 1,215 genes showed a significance circadian expression patten (peak and trough; JTK analysis; $p < 0.001$). Rhythmic gene were clustered by acrophase (expression peak) synchronicity (**Figure 3**). The number of circadian expressed genes with the same acrophase range oscillate between 95 and 419. The majority of genes are expressed in one of two peaks at 10:00 [first sampling in the light] and 22:00 [first sampling in the darkness]. The highly significantly rhythmically expressed clock genes are distributed similarly to the rest of those which are significantly expressed in a circadian pattern ($p = 0.15$). The majority are peaking at 22:00. Sub-figures exclude 3 highly expressed genes that bias the scale of the graphic: Ependymin-1 (> 10,000 TPM) Ependymin-2 (> 5,000 TPM) and calcium voltage-gated channel auxiliary subunit alpha 2 delta 4 (> 2,000 TPM); all three genes are related with central nervous system plasticity and memory formation.

4. DISCUSSION

In the present study, we have identified and characterised 48 core clock and accessory loop genes *in silico* in several salmonid species with published genomes using the latest common ancestors as reference points. For each of the salmonid species with published genomes, the core clock and accessory loop genes were identified and characterised *in silico*. There is a differential retention of genes originating in the latest common ancestors, *E. lucius* and *D. rerio*. In addition to this, there are numerous ohnologs (Ohno, 1970) which are consistent with the salmonid specific whole genome duplication event Ss4R (Lien et al., 2016). There is also evidence of differential gene loss in salmonids based on the current gene annotations (**Table 1**). Findings from this study further highlight the rich complexity of core clock genes previously outlined in *S. salar* (West et al., 2020), and is displayed across a wider complement of salmonid species. The gene families identified differ slightly to those explored by West et al. (2020) as the focus of this study was to identify all members of gene families *clock*, *arntl*, *period*, *cryptochrome*, *ror*, *nr1d*, and *csnk1*. Identified genes common to both studies coincide with one another and paralog pairs identified using ML phylogenetic alignments are also identified as paralogs (West et al., 2020).

In comparison to *D. rerio* with 29 reference clock genes (Wang, 2008b; Huang et al., 2010; Toloza-Villalobos et al., 2015), most clock genes identified in salmonids appear to have been duplicated. In addition to these lineage-specific duplications there is also evidence of differential ohnolog loss in some species of salmonids based on the current genome annotations, *per1b*, *cry2*, and *cry5* appear to have been retained across all species from the latest common ancestors, *per3* appears to only be retained in *S. salar* and *S. alpinus* and *cry4* appears to only be retained in *S. salar*. Whereas, *clock1b*, *cry1b*, *cry3a*, *csnk1da* all appear to have been lost in salmonids after the latest common ancestor *E. lucius*.

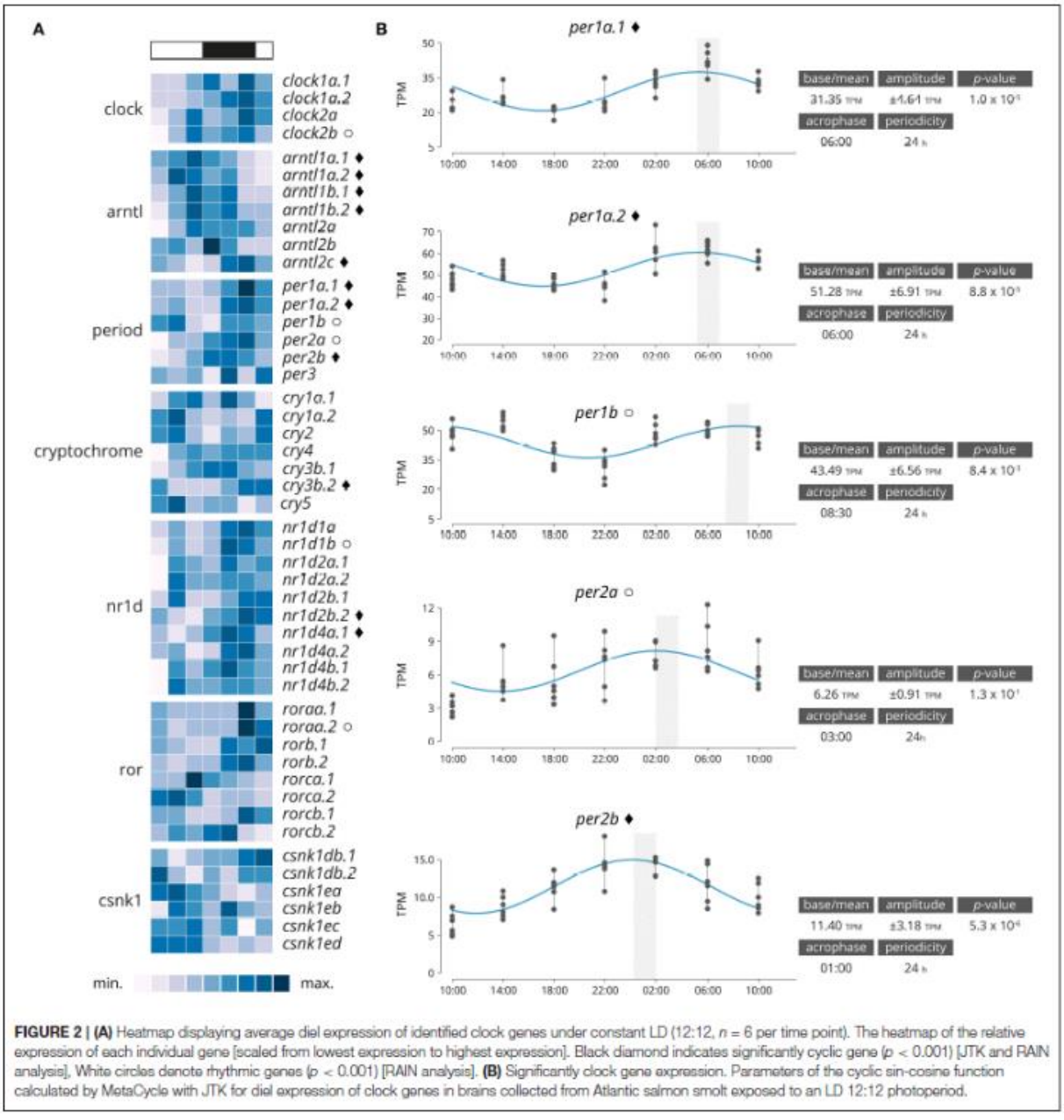

FIGURE 2 | (A) Heatmap displaying average diel expression of identified clock genes under constant LD (12:12, $n = 6$ per time point). The heatmap of the relative expression of each individual gene [scaled from lowest expression to highest expression]. Black diamond indicates significantly cyclic gene ($p < 0.001$) [JTK and RAIN analysis]. White circles denote rhythmic genes ($p < 0.001$) [RAIN analysis]. **(B)** Significantly clock gene expression. Parameters of the cyclic sin-cosine function calculated by MetaCycle with JTK for diel expression of clock genes in brains collected from Atlantic salmon smolt exposed to an LD 12:12 photoperiod.

Some species of salmonids have better genome annotations than others, and currently it is not possible to identify the effects of the WGD for every core clock gene family in *O. tshawytscha* at present. As expected across most of the gene families except where there is limited information, the *Oncorhynchus* spp. tend to appear together on the same clade with *S. salar* and *S. alpinus* commonly grouped together in a sister clade. A typical example of the clock gene families identified is the cryptochrome family. *cry1a* is one of the major clades in the cryptochrome family, as a result of duplication there are three main paralogous subfamilies *cry1a*, *cry1b* and *cry1c*. *cry1a* and *cry1b* are phylogenetically related to tetrapods, with *cry1a* being the most conserved of the two subfamilies (Mei et al., 2015). Zebrafish possess both cry1a and *cry1b* however, all the salmonid species only appear to have inherited *cry1a* which has been duplicated as a result of the WGD, resulting in two paralogs *cry1a.1* and *cry1a.2*. Except

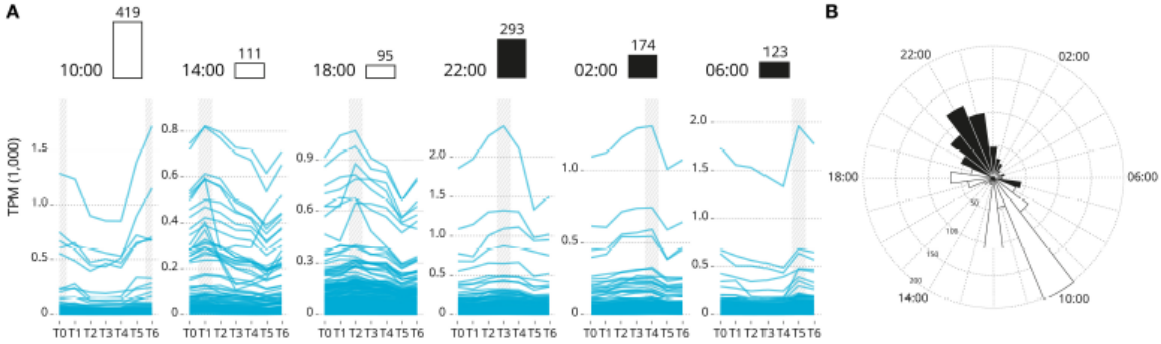

FIGURE 3 | Genes groups based on their acrophase (expression peak) synchronicity. **(A)** Based on the average recorded TPM values. The number of gene is reported over the barplot (white and black boxes denote light or darkness conditions, respectively). Sub-figures exclude three highly expressed genes that bias the scale of the graphic: Ependymin-1 (> 10,000 TPM) Ependymin-2 (> 5,000 TPM) and calcium voltage-gated channel auxiliary subunit alpha 2 delta 4 (> 2,000 TPM). **(B)** Rose plot of the phase distribution of highly significantly circadian gene expression ($p < 0.001$, JTK analysis). Color coding indicates whether the phase occurs during the light (white) or dark (black) (12:12 LD).

for *O. tshawytscha* (for which there is not currently enough information in the genome annotation) *cry3b* is duplicated in all salmonid species with an apparent gene loss in both *O. kisutch* and *O. mykiss*. This is suggestive of the non-functualisation and subsequent loss of *cry3b.2* in the *Oncorhynchus* spp. As a result of the Ss4R WGD clock most genes appear to be duplicated in salmonids with some exceptions that appear to have been lost in several of the salmonids. As annotations improve, it is thought that additional gene losses will become more apparent. This study gives insight into the post duplication effects on clock gene family members and provides a fundamental tool for further circadian work in salmonids.

Clock genes interact with each other, generating oscillations in gene expression. Their underlying principle is to create successive gene activation in the form of a cycle, forming an autoregulatory feedback loop which perpetually cycles approximately every 24 h (Ripperger and Albrecht, 2009). This in turn influences downstream targets, whose time-of-day specific expression is determined by the central circadian mechanism (Ripperger and Albrecht, 2009). This study confirmed the expression of the identified clock genes in the brain of *S. salar* smolts over a 24-h period and showed that 11 out of the 48 core clock genes were highly significantly ($p < 0.001$) rhythmically expressed. We used a two complementary approach, RAIN which allowed us to detect accurately rhythms in time series irrespectively of the shape of the expression pattern, and JTK with a focus on circadian expression patterns. The vast majority of the JTK findings are included in the RAIN results (**Supplementary Table 3**). This allowed us to distinguish between rhythmic expressed genes and circadian pattern expressed genes.

The period family is particularly interesting, as except for one gene (*per3*) all the genes were significantly rhythmically expressed. A difference in acrophase between paralogs was also identified. There was a clear difference in expression pattern observed between *per1a* paralogs (*per1a.1*, *per1a.2*) and *per1b*, with *per1a* peaking at 06:00 displaying a positive phase shift in comparison to *per1b* which peaks at 08:30. This positive phase shift coincides with results observed in zebrafish, it was reported that *per1a* and *per1b* paralogs displayed a shifted phase of gene expression in zebrafish with *per1a* peaking 4 h prior to *per1b* under a 12:12 LD lighting regime (Amaral and Johnston, 2012). In zebrafish, *per1* paralogs were shown to display a distinct difference in spatial and temporal expression in the brain, providing strong evidence for the duplicate pair to have undergone sub- or neo-functionalisation (Wang, 2008a). The duplicated Atlantic salmon *per1a* paralogs (*per1a.1* and *per1a.2*) share the same periodicity and acrophase, thus indicating that both genes may share a similar functionality. This indicates that the paralogs may have undergone sub-functionalisation—in which the ancestral functions have become partitioned and each paralog potentially has particular adaptations for different tissues, developmental stages or environmental conditions (Innan, 2009). In mammalian literature, it is widely reported that *Cry1/2* form heterodimers with *Per1/2/3* (Rosensweig et al., 2018). However, in this study, the only highly significantly rhythmically entrained *cryptochrome* genes was the *cry3b.2* paralog which peaked two h before the closest *period* family member (*per2b*) at 20:30, in agreement with the pattern of *cry2* expression identified in zebrafish 12-h after light onset (Hirayama et al., 2019). In mammals, *Per2* is said to repress *Nr1d2* transcription thus upregulating *arntl1* transcription (Chiou et al., 2016), in keeping with these findings in this study *nr1d2b.2* peaks at 08:30, around 10 h before peak expression of *arntl1a.1/1a.2/1b.1* (18:00) and *per2b* peaks at 01:00. This suggests that the relationship between clock gene paralogs in salmonids may be similar to that of those in mammalian species.

How salmonids fit into the widely accepted mechanism for circadian rhythmicity is difficult to evaluate due to the limited understanding surrounding circadian mechanisms in fish in general and the complexity are yet to be exploited to the full potential (Frøland Steindal et al., 2018), there are still many unknowns surrounding the mechanism in

salmonids as it has been far less explored than other species of teleost. In comparison to species such as *D. melanogaster*, *M. musclus*, *H. sapiens*, and even *D. rerio* there has been limited work undertaken in salmonids to further identify clock genes and their respective roles within the circadian system (Frøland Steindal et al., 2018). So far, the expression of individual clock genes has been previously investigated in salmonids (Davie et al., 2009; Huang et al., 2010; West et al., 2020). Clock gene member identification has previously been hampered by paralogs with high sequence similarity, which does not easily allow for individual identification by qPCR. Individual gene identification using RNA sequencing has allowed for paralogs to be better classified and individual gene expression patterns ascertained. Although, single gene duplication events in mammalian species have enabled the evolution of specialisation and regulatory sophistication in the temporal regulation of local physiology (Looby and Loudon, 2005); the core clock genes associated with circadian rhythmicity remains largely conserved across a diverse range of organisms spanning vast evolutionary time periods (Bell-Pedersen et al., 2005; Cox and Takahashi, 2019). This study supports that whilst the compliment of clock genes is far richer in salmonids, the function of core clock genes remains conserved and are therefore likely function similarly to other more studied organisms. Some salmonid species display reciprocal gene losses post Ss4R, but many of clock genes identified appear to be duplicated in the salmonid species investigated. The existence of such paralogs whilst increasing genetic complexity may enable gene family members to specialise and extend their ancestral role, which can lead to a shift in the identity of components of the molecular clock (Looby and Loudon, 2005; West et al., 2020). Ambient temperature affects gene expression and physiology in ectotherms; in zebrafish, the clock seems to be temperature-compensated, changing the amplitude of some critical clock genes (Lahiri et al., 2005). However, the influence of temperature on the clock system in salmonids remains poorly studied and should be investigated further.

Whilst this study has furthered the identification of clock gene family members in central salmonids, additional work is required to further elucidate the complexity of the circadian mechanism and how the complement of clock genes identified individually function as components of this mechanism. It is important to note that alongside individual variability, the whole brain being analysed will have influenced gene expression levels and rhythmicity due to the highly decentralised organisation of clock genes in teleosts and localisation of clock gene expression in specific regions of the brain (Moore and Whitmore, 2014). This study provides a fundamental tool to explore the role of the enriched number of clock genes related to seasonal driven life history transition in salmonids.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena, PRJEB41327.

ETHICS STATEMENT

The animal study was reviewed and approved by UK Animals (Scientific Procedures) Act 1986 Amended Regulations (SI 2012/3039) Animal Welfare and Ethical Review Body of the University of Stirling (AWERB/19 20/097/).

AUTHOR CONTRIBUTIONS

CB, JH, MB, and HM: study conception and design. CB and HM: sample and data acquisition. CB, MB, and ME: data analysis. HM and JH: funding acquisition. CB, HM, ME, and MB: drafting and editing of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.761109/full#supplementary-material

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