Photoperiod regulation of molecular clocks and seasonal physiology in the Atlantic salmon *(Salmo salar)*

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DECLARATION

This thesis has been composed in its entirety by the candidate. Except where specifically acknowledged, the work described in this thesis has been conducted independently and has not been submitted for any other degree.

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ABSTRACT

Recent years have seen considerable advances in the study of biological rhythms and the underlying molecular mechanisms that drive the daily and seasonal physiology of vertebrates. Amongst teleosts the majority of work in this field has focused on the model species the zebrafish to characterise clock genes and the molecular feedback loop that underpins circadian rhythms and physiology. Daily profiles of clock gene expression in a wide variety of tissues and cell types are now relatively well described. However the zebrafish is a tropical species that does not display distinct seasonality and therefore may not be the species of choice to investigate the entrainment of circannual physiology. In contrast, Atlantic salmon is a highly seasonal teleost that displays considerable temporal organisation of most physiological processes. In salmonids photoperiod is widely known to synchronise physiology to the environmental conditions and as such photoperiod manipulation is routinely used by the salmon industry throughout the production cycle to control and manipulate spawning, smoltification and puberty. Previous studies in salmonid species have already identified a set of clock genes that are linked to these seasonal physiological processes. However, to date, the molecular mechanisms regulating daily and seasonal physiology are largely unknown despite the strong commercial relevance in the Atlantic salmon.

In the Atlantic salmon, Davie et al (2009) was the first to report the photoperiod dependent circadian expression of clock genes (*Clock, Bmal* and *Per2* and *Cry2)* in the brain of the Atlantic salmon. In the same investigation the expression of clock genes was reported in a wide variety of peripheral tissues, however 24h profiles of expression in peripheral tissues were not characterised. In order to examine further the role of seasonal photoperiod on the circadian expression of clock genes, the present work first aimed to characterise diel profiles of *Clock, Per1* and *Per 2* expression in the brain together with plasma melatonin levels in

Atlantic salmon acclimated to either long day (LD), short day (SD), 12L:12D (referred to as experiment 1 throughout) and SNP (referred to as experiment 2 throughout). Photoperiod dependent clocks were also investigated in peripheral tissues, namely in the fin and liver. Results showed circadian profiles of melatonin under all photoperiods. In experiment 1 both *Clock* and *Per2* displayed significant circadian expression in fish exposed to LD. This is in contrast to previous results where rhythmic clock gene expression was observed under SD. In addition, clock gene expression differed in response to experimental photoperiod in the liver, and diel rhythm differed to that of the brain. No rhythmic expression was observed in the fin. Levels of plasma melatonin exhibited a circadian rhythm peaking during the nocturnal phase as expected. However the amplitude of nocturnal melatonin was significantly elevated under LD (experiment 1) and the SNP long day photoperiod and 2010 autumnal equinox samples (experiment 2). Overall results from these experiments suggested that the control of clock gene expression would be photoperiod dependent in the brain and the liver however photoperiod history is also likely to influence clock gene expression. Interestingly, the gradual seasonal changes in photoperiod under SNP did not elicit similar profiles of clock gene expression as compared to experimental seasonal photoperiods and clock gene expression differed between experimental photoperiod and SNP treatments. In experiment 2 significant seasonal differences were also observed in the amplitude of individual clock gene expression. The mechanisms underlying this and potential impact on seasonal physiology are unknown. Developmental changes such as the smoltification process or abiotic factors such as temperature or salinity should be further investigated.

In mammals previous work has focused on the molecular switch for photoperiod response and regulation of thyroid hormone bioactivity via deiodinase mediated conversion of T4 to the biologically active form T3. In mammals and birds expression of key seasonal molecular markers i.e. *Tshβ, Eya3* and *Dio2,* are up-regulated hours after exposure to the first LD and

persist under chronic LD conditions. In order to confirm the involvement of these genes in the seasonal photoperiodic response in salmon, a microarray study was first carried out. Results displayed transcriptome level differences in the seasonal expression of a wide variety of target genes including *Eya3* and *Dio1-3* in relation to LD and SD photoperiod suggesting that these genes may have a conserved role in salmon. qPCR validations of selected genes of interest were then performed (*Dio1*, *Dio2* and *Dio3*, *Eya3* and *Tshβ)* over diel cycles in fish exposed to LD and SD photoperiod (autumn acclimated fish). In addition an unrelated qPCR study was undertaken in salmon parr acclimated to LD, 12L12D and SD photoperiod (spring acclimated fish)(*Dio2*, *Eya3* and *Tshβ*). Consistent with findings obtained in other vertebrate species, circadian expression of *Dio2* was observed under LD. However expression of *Eya3* and *Tshβ* appeared to be dependent on photoperiod history prior to acclimation to the experimental photoperiods as already suggested for clock gene expression in this thesis. This is potentially a consequence of direct regulation by clock genes. To our knowledge, this is the first report on the expression of key molecular components that drive vertebrate seasonal rhythms in a salmonid species.

The thesis then focused on another key component of the photoneuroendocrine axis in fish, the pineal organ. In the Atlantic salmon, as in other teleosts the photoreceptive pineal organ is considered by many to be essential to the generation, synchronisation and maintenance of circadian and seasonal rhythms. This would be primarily achieved via the action of melatonin although direct evidence is still lacking in fish. In salmonids the production of pineal melatonin is regulated directly by light and levels are continually elevated under constant darkness. In non salmonid teleosts the rhythmic high at night/ low during day melatonin levels persists endogenously under constant conditions and is hypothesised to be governed by light and intra- pineal clocks. The aims of the present *in vitro* and *in vivo* trials were to determine if circadian clocks and *Aanat2* expression, the rate limiting enzyme for melatonin

production, are present in salmon, test the ability of the pineal to independently re-entrain itself to a different photoperiod and establish whether the candidate clock genes and *Aanat2* expression can be sustained under un-entrained conditions. Expression of clock genes was first studied *in vitro* with pineal organs exposed to either 12L:12D photoperiod, reversed 12D:12L photoperiod and 24D. Clock gene expression was also determined *in vivo*, in fish exposed to 12L:12D. Results were then contrasted with an *in vitro* (12L:12D) investigation in the European seabass, a species displaying endogenous melatonin synthesis. Results revealed no rhythmic clock gene (*Clock, per1* and *per2*) expression in isolated salmon pineals in culture under any of the culture conditions. In the seabass, *Clock* and *Per1* did not also display circadian expression *in vitro*. However rhythmic expression of *Cry2* and *Per1* was observed *in vivo* in the salmon pineal. This suggested some degree of extra-pineal regulation of clocks in the Atlantic salmon. In terms of *Aanat2* no rhythmic expression was observed in the Atlantic salmon under any experimental conditions while rhythmic expression of *Aanat2* mRNA was observed in seabass pineals. This is consistent with the hypothesis that in salmonids AANAT2 is regulated directly at the protein level by light while in other teleosts, such as seabass, AANAT2 is also regulated by clocks at a transcriptional level. *Post hoc in silico* analysis of the *Aanat 2* 5' regulatory region revealed the absence of a functional E-box element in the salmon in comparison to other teleosts, including the European seabass, confirming the absence of clock regulation of *Aanat2* mRNA in salmon.

Although it is crucial to first characterise the molecular mechanisms regulating daily and seasonal rhythms, understanding how these mechanisms impact on the animal's physiology is critical. One such aspect is the circadian regulation of fatty acid metabolism and cholesterol homeostasis ultimately impacting fat deposition in commercially produced Atlantic salmon. This is an area of considerable research interest both in terms of human health and improving the sustainability of commercial salmon feed. In mammals a number of genes involved in

liver lipid and cholesterol homeostasis are rhythmically expressed under the control of clock genes via *Rev-erb α*. The aim of the present work was to determine diel mRNA expression patterns of selected genes involved in cholesterol homeostasis (*Srebp 1, Srebp 2, Fas, Lxr, Elovl5, Hmgcr* and *D6 Fad*) together with circadian clocks (*Bmal1, Clock, Per* 1, *Per 2* and *Rev-erb* α) in the liver of the Atlantic salmon. Results demonstrated significant circadian expression of *Srebp 1* and *Bmal 1*, similar to previous results in mice, *Lxr* also exhibited significant circadian expression. Additionally the gene coding for the rate limiting enzyme in cholesterol synthesis, *Hmgcr*, was significantly elevated during the day. This is in contrast to mammals where mRNA expression and protein activity was elevated during the night. Also in contrast to results obtained in mammals, *Per1, Per2, Fas,* and *Reverb α* did not display significant circadian rhythmicity in salmon. This investigation represents the first attempt to correlate 24h profiles of clock gene expression to a functionally important process in peripheral tissues, lipid metabolism, which is an area of considerable interest for future research in this commercially important species.

This thesis has significantly advanced knowledge on the expression of clock and seasonal genes in response to photoperiod information in the Atlantic salmon. Moreover it has given an important insight into the expression of clock genes in multiple tissue types and how clocks can regulate important physiological processes. However research is still in the early days and much work is needed to understand such a complex network in this highly seasonal and commercially important species.

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SPECIES LIST

Anguilla anguilla	European Eel
Carassius auratus	Goldfish
Clarias gariepinus	African catfish
Coregonus lavaretus	Whitefish
Coturnix japonica	Japanese quail
Cricetus cricetus	European hamster
Danio rerio	Zebrafish
Dicentrarchus labrax	European seabass
Drosophila melanogaster	Fruit fly
Esox lucius	Pike
Fundulus heteroclitus	Mummichog
Hucho perryi	Japanese huchen
Hypomesus nipponensis	Japanese smelt
Mesocricetus auratus	Syrian hamster
Micropogonias undulatus	Atlantic Croaker
Mus musculus	Domestic mouse
Oncorhynchus keta	chum salmon
Oncorhynchus masou	Masu salmon
Oncorhynchus mykiss	Rainbow trout
Oncorhynchus tshawytscha	Chinook salmon
Oreochromis niloticus	Nile tilapia
Ovis aries	Sheep
Phodopus sungorus	Djungarian / Siberian hamster
Platichthys flesus	Flounder
Plecoglossus altivelis altivelis	Ayu
Rattus norvegicus	Brown rat
Salmo salar	Atlantic salmon
Salmo trutta	Brown trout
Salvelius fontinalis	Brook trout
Salvelius leucomaenis pluvius	Japanese charr
Siganus guttatus	Rabbitfish
Solea senegalensis	Senegalese sole
Thymallus thymallus	Grayling

LIST OF ABBREVIATIONS

AAAD	Aromatic amino-acid decarboxylase
Aanat	Arylalkylamine N-acetyltransferas
Bmal	Brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like
BPG	Brain-pituitary-gonadal
cAMP	Cyclic AMP
CCAATβ	CCAAT enhancer binding protein beta
CDS	Coding sequence
cDNA	Complementary DNA
CK1	*Casein kinase 1 epsilon*
Clock	Circadian Locomotor Output Cycles Kaput
Cry	Cryptochrome
D6 Fad	D6 fatty acid desaturase
Dio1	Type I iodothyronine deiodinase
Dio2	Type II iodothyronine deiodinase
Dio3	Type III iodothyronine deiodinase
DM-SCN	Dorsomedial SCN
DNA	Deoxyribonucleic acid
EC	Ependymal *cells*
Elovl5	Elongation of very long chain fatty acids protein 5
ERK	Extracellular signal- regulated kinase
EST	Expressed sequence tags
Eya3	Eyes Absent 3
FAO	Food and Agriculture Organization
Fas	Fatty acid synthase
FASPS	Familial advanced sleep phase syndrome
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
HCL	Hydrochloric acid
HIOMT	Hydroxyindol - O- methyltransferase
Hmgcr	HMG-CoA reductase
Kiss	Kisspeptin
LD	Long Day
LH	Luteinizing-hormone
Lxr	Liver X receptor
NCBI	National Centre for Biotechnology Information
PACAP	Pituitary adenylate cyclase-activating polypeptide
PCR	Polymerase chain reaction
PD	Pars distalis
Per	Period
PNES	Photoneuroendocrine system
PP	Protein phosphatases

PRDM	Pineal – restrictive down-stream module
pRGCs	Photoreceptive retinal ganglion cells
PT	Pars tuberalis
PVN	Paraventricular nuclei
qPCR	Quantitative PCR
Rev-erb α	Nuclear receptor subfamily 1, group D, member 1
RHT	Retino-hypothalamic tract
RIA	Radioimmunoassay
RNA	Ribonucleic acid
Ror	Related orphan nuclear receptors
RORE	Retinoic acid-related orphan receptor response elements
SCN	Suprachiasmatic nucleus
SD	Short day
SECIS	Selenocysteine insertion sequence
Six1	Sine oculis homeobox 1
SLMB	Supernumerary limbs
SNP	Simulated Natural Photoperiod
Srebp	Sterol regulatory element-binding proteins
T3	Triiodothyronine
T4	Thyroxine
Tef	Thyrotroph embryonic factor
TPOH	Tryptophan hydroxylase
Tshβ	Thyroid stimulating hormone Beta
UTR	Untranslated region
VL-SCN	Ventrolateral SCN

Chapter 1

Introduction

INTRODUCTION

1. THE ATLANTIC SALMON.

The Atlantic salmon *(Salmo salar)* is an iconic species in the rivers and marine waters of northern Europe and North America. It is a species of considerable importance to the aquaculture industry. Production has increased dramatically since the mid 1980s and now exceeds 1.4- 1.5 million tonnes worldwide (FAO 2010) (Figure 1). In the wild the Atlantic salmon's distribution ranges from the rivers of New England in the west and Portugal in the east to as far north as Greenland and the Barents Sea. Like many salmonid species the Atlantic salmon is anadromous. Juvenile Atlantic salmon spend the early part of their lives in freshwater prior to the smoltification process. Salmon go through considerable morphological and physiological changes enabling them to migrate and thrive in marine waters and return to natal freshwater breeding grounds in order to spawn. Unlike the pacific salmon the Atlantic salmon is iteroparous and capable of multiple reproduction events over the course of a life time. However as a result of the high energetic costs involved in salmonid reproduction most individuals will spawn only once or twice during their life (Oystein Aas et al. 2010). As is the case for a number of temperate species the Atlantic salmon displays a considerable degree of temporal organisation in terms of ecology, behaviour and physiology (Davie et al. 2009).

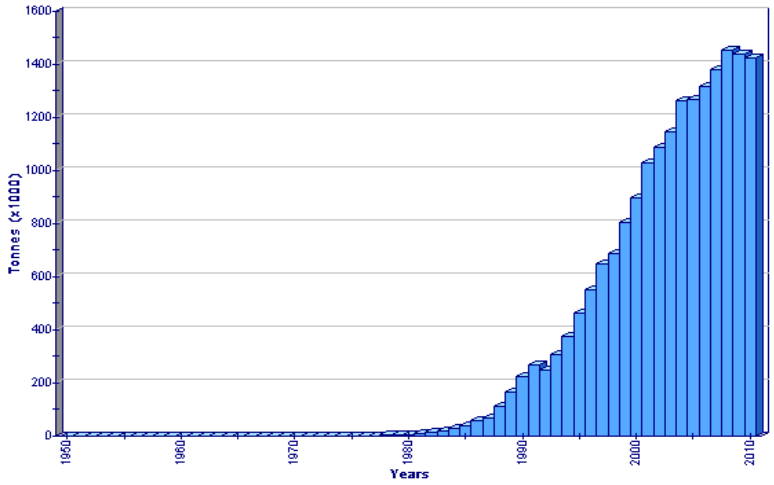


Figure 1: Global aquaculture production of Atlantic salmon (*Salmo salar*) from 1950 - 2010 (FAO 2010)

Fundamental biological processes such as smoltification, migration and reproduction are timed to coincide with optimal environmental conditions the precise mechanisms underpinning such timing are considered to be endogenous and internally driven by the organism enabling a timed reaction to predictable environmental changes (Davie et al. 2009). As will be presented further below a number of life history events including; spawning time and smolt migration are synchronised to coincide with optimal environmental conditions such as food availability and temperature (Oystein Aas et al. 2010). Moreover, population specific variance in the return of mature adults to spawning grounds has been attributed to an internal genetic component (Stewart et al. 2002; Stewart et al. 2006). Such innate regulation is commonly linked to the body clock however in teleosts and Salmonids in particular there is a lack of understanding of how this mechanism could drive the temporal control of physiology and behaviour.

2. WHAT ARE BIOLOGICAL CLOCKS?

Life on earth exits in 24 hour cycles synchronised to the daily light-dark cycle, a consequence of the earth spinning as it orbits the sun (Edery 2000; Maronde & Stehle 2007). The sun's

rays are consistent and concentrated at the equator where they are perpendicular to the tangent of the earth (Foster & Kreitzman 2009). As we move north or south of the equator this angle decreases yet the same amount of energy is dissipated over a larger surface area, generating latitudinal differences in climate and hours of daylight. Additionally as shown in Figure 2 over a period of 365.25 days the earth spins on its axis resulting in considerable annual variations in photoperiod and climactic conditions which generates the seasons. The degree of seasonal variation in the 24h light dark cycle over the course of the year is a direct result of latitude, the higher the latitude the more annual variations (Foster & Kreitzman 2009). Over the course of evolution organisms have synchronised a variety of biological processes to these predictable daily and seasonal cycles (Yoshimura 2010). These rhythms characterise life on our planet and are present in species as diverse as neurospora and drosophila to mice and humans ; (Reppert & Weaver 2002; Ko & Takahashi 2006; Dekens & Whitmore 2008).

Rhythmic behaviour and physiology represent ancient time keeping mechanisms enabling an organism to synchronise a variety of biological processes to the external environment. Biological rhythms occur on a wide variety of scales (Figure 3). Ultradian rhythms occur on a scale less than 24h such as 90 minutes cycles in rapid eye movement (Refinetti 2006) or in coastal marine ecosystems biological rhythms cycle to coincide with changing tidal conditions (Refinetti 2006). Circadian rhythms are the best characterised biological rhythm and occur on a near 24h basis (Refinetti 2006). Rhythms are also present on a lunar scale with organisms synchronising physiology and behaviour to the waxing and waning of the moon (Refinetti 2006). While on a larger time scale, infradian rhythms include cycles such as animal migrations or the human menstrual cycle (Refinetti 2006) and finally circannual rhythms occur on an annual basis and are characterised by endogenous persistence for several years (Refinetti 2006). This thesis focuses on the circadian rhythms and the common

molecular mechanisms that underpin circadian and seasonal physiology. The occurrence of cyclic physiology and consequent behaviour is not purely a reaction to external environmental conditions but an internal endogenous response, allowing organisms to anticipate and react to predictable environmental changes(Hazlerigg & Wagner 2006; Dardente et al. 2010).

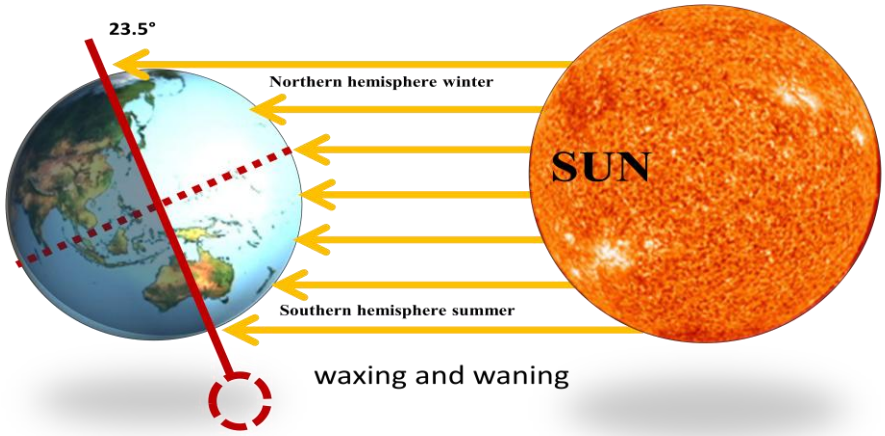


Figure 2: The earth is tilted on its axis at an angle of 23.5°. The earth spins on this axis over the duration of a year. Consequently outside of the tropics seasonal changes in photoperiod and climate occurs over the course of a year. Above displays the impact of the sun's rays during the southern hemisphere summer and northern hemisphere winter.

Circadian rhythms occur on a near 24h scale, however not all rhythms observed on a daily scale are circadian. The term daily has been reserved for rhythms with a period of 24h that have not been proven to be endogenous in nature (Aschoff 1981; Refinetti 2006). Jürgen Aschoff, one of the pioneers of circadian research, formalised the criteria for a 24h rhythm to be termed circadian(Aschoff 1981; Refinetti 2006). Firstly it must be endogenously generated i.e. it must be internally driven from within an organism as opposed to a reaction to external stimuli. Secondly the rhythm is required to free run with a period of approximately 24h (between 19 and 28 hours). Under constant conditions true circadian rhythms persist in near

24h cycles in the absence of environmental cues. There are however exceptions to this rule. Some circadian rhythms are inhibited by environmental signals, for example constant light or constant darkness.

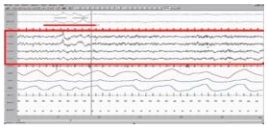
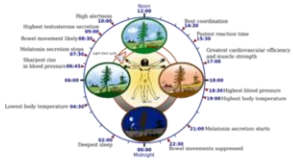
Ultradian (< 24h)	Biological rhythms on a scale of less than 24h E.g. the 90 minute REM cycle	
Tidal rhythms	Organisms adjust physiology to follow the12-hour transition from high to low tide and back	
Circadian rhythms	Near 24h rhythm E.g. the sleep wake cycle and body temperature.	
Lunar rhythms	Rhythms coinciding with the waxing and waning of the moon.	
Infradian rhythms	Biological rhythms occurring on a scale of greater than 24h but less that 1 year. E.g. annual migrations	
Circannual	Rhythms occurring on an annual basis	

Figure 3: Scales of rhythmic physiology and biological rhythms from ultradian to circannual rhythms.

Moreover circadian rhythms can be disrupted by drugs such as methamphetamine and deuterium oxide (Refinetti 2006). Finally circadian rhythms are flexible and can be synchronised by environmental cycles with 24 hour periods, such as the 24h light dark cycle. However, the base value or free running time of the rhythmicity is genetic and species specific. In mammals the most well known genes to impact the period of circadian

rhythmisity are *Casein kinase 1 epsilon* (*CK1ε)* in Syrian hamsters (*Mesocricetus auratus*) and *Circadian Locomotor Output Cycles* Kaput *(Clock)* in domestic mice (*Mus musculus*) (Refinetti 2006). Internally driven endogenous circadian rhythms are regulated at a molecular level by a number of clock genes in an auto-regulatory feedback loop commonly referred to as the clock gene system (McWatters et al. 1999). In summary, a number of clock genes including *Clock, Brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like* (Bmal), *Period* (*Per*) and *Cryptochrome* (*Cry*) are involved in the auto-regulatory feedback loop. In its simplest sense the CLOCK and BMAL proteins form the positive arm of the feedback loop while the negative arm of the feedback loop is comprised of CRY and PER proteins. Together these components make up the core oscillator of the molecular circadian clock (Ko & Takahashi 2006).

The components of the circadian feed back loop are synchronised to external environment, directly or indirectly receiving input from environmental signals called zeitgebers. A range of external signals have been implicated in the regulation of the circadian clock including 24h light dark cycle, temperature, food availability, social interactions and reward systems such as those associated with drugs. Of the variety of zeitgeber signals that have been described photoperiod appears to be the dominant signal entraining the circadian oscillator (McWatters; et al. 1999 Dibner et al. 2010; Golombek & Rosenstein 2010). In many organisms the duration of photoperiod acts as a reliable indicator of time of year. This is of particular importance in temperate and higher latitudes where other signals such as temperature or food availability can vary considerably on an inter-annual basis (Dardente. 2012; Ikegam and Yoshimura. 2012) Circadian molecular oscillators are also hypothesised to be involved in the regulation of seasonal rhythmic processes (Ikegam and Yoshimura. 2012).

Biological rhythms are present on a wide degree of biological scales from clock gene oscillations in individual cells to seasonal rhythms observed in population ecology. Moreover

the mechanisms of the circadian clock are highly conserved across an extraordinary degree of taxa from single cell organisms to humans and are present in the majority of tissue types. There are a diverse range of neural, endocrine, physiological and even behavioural processes that occur on a periodic basis. From cyclic melatonin production, core body temperature to the opening and closing of flowers in plants and sleep wake cycle. In humans a wide assortment of everyday processes are regulated in a circadian manner including mental alertness, reaction time, cardiovascular and muscular efficiency and even bowel movements.

In salmonids, in particular rainbow trout *(Oncorhynchus mykiss)*, rhythmic behaviour and physiology have been observed on a variety of temporal scales. Feeding, locomotor and hormonal rhythms and have been reported on a circadian scale (Sanchez-Vazquez & Tabata 1998; Zaunreiter et al. 1998; Zaunreiter et al. 1998). Over a longer time frame, seasonal and circannual rhythms in maturation of Atlantic salmon and reproduction in rainbow trout have previously been described (Bromage et al. 1988; Duston & Bromage 1988; Duston & Saunders 1992). The energetic costs of reproduction and related processes are high and it is necessary to time maturation and reproduction with optimal environmental conditions for offspring to have the best chance of survival. This is particularly important in temperate and Polar Regions (Ikegam and Yoshimura. 2012). While for mammalian seasonality the duration of the melatonin signal appears to be the messenger synchronising internal seasonal physiology to external photoperiod (Dardente. 2012), the link in teleosts between clocks, photoperiod and melatonin and seasonal physiology and behaviour is somewhat unclear (Davie et al. 2009; Migaud et al. 2010).

In salmonids circadian as well as seasonal rhythms in physiology and behaviour have been described. Daily locomotor activity and seasonal regulation of reproduction and immunity have previously been attributed to an internal, endogenous biological clock (Bromage et al.

2001; Morgan et al. 2008). Work by Davie et al (2009) has identified the presence and photoperiod specific rhythmic activity of a number of core clock genes. However the mechanisms of the salmonid clock are largely unknown. Before we consider the mechanisms of the Atlantic salmon biological clock we must first understand the basic model of the clock as derived from the mammalian system. It is later necessary to consider the fundamental differences between the mammalian and teleost clocks. Amongst teleosts, the majority of research has focused on the model organism the zebrafish (*Danio rerio*) and clockwork mechanisms have been relatively well described in the species (Vatine et al. 2011). However recent research in the field of the teleost clock has demonstrated an extraordinary degree of diversity (Kulczykowska and Popek 2010). Within the teleostei there appears to be considerably more variation than has previously been describe amongst mammals (Kulczykowska and Popek 2010). Consequently the Atlantic salmon clockwork system must be considered in relation to the teleost and mammalian clock work mechanisms, bearing in mind the diversity of the teleost group.

3. MOLECULAR CLOCKWORK MECHANISMS

The molecular basis of the biological clock

For hundreds of years scientists have observed the presence of biological rhythms yet the underlying molecular mechanisms driving endogenous rhythms remained elusive. However recent decades have seen major advances in this field initially through the study of circadian mutations that occur in many organisms. In 1971 the first circadian mutants were identified in the fruit fly, *Drosophila melanogaster*. A mutant screen on 2000 fruit flies revealed three separate circadian mutants that were either arrhythmic or that had a truncated or extended circadian period (19 or 28 hours) (Konopka & Benzer 1971). These circadian phenotypes in *D. melanogaster* were later attributed to mutations in a gene which was given the name

Period (*Per)* to indicate its importance in the circadian clock (Bargiello et al. 1984; Reddy et al. 1984). In 1988 the *Tau* mutants in the Syrian hamster were identified. In wild type animals a circadian period of ~24h was observed. This was shortened to 22 and 20h in *Tau* heterozygous and homozygous individuals respectively (Ralph & Menaker 1988). Meanwhile in mice the first clock mutant was characterized in 1994 (Vitaterna et al. 1994) and the core components *Clock* (Antoch et al. 1997; King et al. 1997), *Bmal* (Ikeda & Nomura 1997) and *Per* (Darlington et al. 1998) were identified and their role in auto-regulatory feedback loops were recognised in 1998 (Sangoram et al. 1998). This was then followed by subsequent discoveries in non mammalian vertebrates such as the Japanese quail (Yoshimura et al. 2000) and the zebrafish (Whitmore et al. 1998). However, overall our knowledge of the underlying molecular mechanisms of clocks is still largely based on the mammalian model.

Clock mechanisms are believed to be highly conserved at a molecular level across a wide variety of taxa, tissue and cell types (Dibner et al. 2010; Edery 2000). The molecular clock of vertebrates can be summarised as a pair of auto-regulatory feedback loops that cycle with a period of approximately 24 hours (Figure 4). These genes include: *Clock, Bmal, Per and Cry*. The auto-regulatory feedback loop is comprised of a positive arm (*Clock* and *Bmal*) and negative arm including the *Per* and *Cry* gene. In terms of the positive components both genes (*Clock* and *Bmal*) are members of the Basic helix-loop-helix (bHLH)-PAS (*Period-Arnt-Single-minded*) transcription factor family (Ko & Takahashi 2006; Layeghifard et al. 2008). The feed back loop begins with CLOCK and BMAL proteins which form a heterodimer in the cell cytoplasm and is then translocated to the nucleus where it binds to DNA regulatory elements called E-boxes (CACGTG). When bound to the E-box the BMAL/CLOCK dimer then promotes the transcription of down-stream target genes including the negative portion of the feedback loop (*Per and Cry*). A number of genes, including arylalkylamine N-acetyltransferase-2 *(Aanat2)* (Gothilf et al. 2002; Zilberman-Peled et al. 2007) are regulated

by E-box elements in their promoters. 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 & Takahashi 2006). *Per* and *Cry* transcripts migrate out of the nucleus to generate the resulting PER and CRY proteins. PER and CRY then accumulate dimerise and translocate to the nucleus where they inhibit their own transcription by blocking E-box binding of the CLOCK: BMAL heterodimer. The PER and CRY proteins are then inactivated or removed by post-translational modifications such as phosphorylation and by degradation (Gallego & Virshup 2007). This in essence then initiates the beginning of a new circadian cycle a process which takes around 24h to compete and is the core of the circadian clock.

There are however secondary loops/processes that play important roles in the molecular clock work. These are not the components that drive circadian rhythms but are fundamental to the regulation and accuracy of the clock. The CLOCK: BMAL hetrodimers also activate the transcription of *nuclear receptor subfamily 1, group D, member* (*Rev-erbα*) and *Retinoic acid related orphan nuclear receptors (Rorα),* which form a secondary loop to regulate the core clock component, *Bmal*. *REV-ERBα* and *RORα* compete to bind to Retinoic acid-related orphan receptor response elements (ROREs) in the gene promoter of *Bmal*. While RORs (α, β and γ) activate its transcription, REV-ERBα represses *Bmal's* transcription. The CLOCK:BMAL heterodimer then controls the rhythmic expression of other clock controlled genes and pathways and subsequent physiology and behaviour via E-box and D-elements (binding sites for PAR bZip factors) (Ueda et al. 2005; Ko & Takahashi 2006).

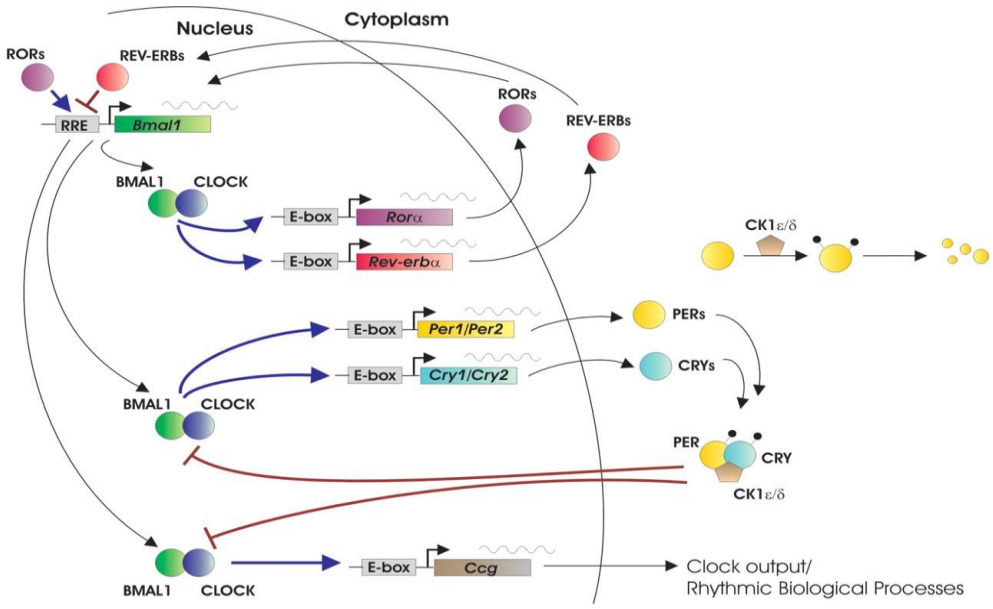


Figure 4: A network of transcriptional–translational feedback loops constitutes the mammalian circadian clock from Ko & Takahashi (2006). The feed back loop begins with CLOCK and BMAL proteins which form a hetrodimer in the cell cytoplasm which is translocated to the nucleus where it binds to DNA regulatory elements called E-boxes. The BMAL/CLOCK dimer then promotes the transcription of down-stream target genes including the negative portion of the feedback loop (*Per and Cry*). PER and CRY then accumulate before dimerising and then translocating into the nucleus to inhibit their own transcription by blocking the CLOCK: BMAL heterodimer. The PER and CRY proteins are then removed by post translational modifications such as phosphorylation and degradation (Gallego & Virshup 2007). This in essence then initiates the beginning of a new circadian cycle a process which takes around 24h to compete and is the core of the circadian clock.

Underlying these loops, and fundamental to the accuracy of the circadian clock, are processes that control phosphorylation and ubiquitytation (Gallego & Virshup 2007). *Casein kinases* (CK1ε and CK1δ) have a fundamentally important role in the regulation of the molecular circadian clock through phosphorylation. CK1ε and δ regulate the turnover of key circadian proteins. *CK1* regulates circadian timing and the molecular feedback loop via a number of routes and primarily, but not exclusively, acts on the PER: CRY complex. Firstly it regulates the nuclear localisation of PER. This is achieved in a number of ways. In some cell types cytoplasmic accumulation of PER1 occurs as a result of CK1 activity. In others this mediates

the nuclear translocation of *Per1* (Gallego & Virshup 2007). In another regulatory pathway it is thought that the phosphorylation of PER proteins at a number of CK1 sites may be linked to the repression of transcription. As such phosphorylation of the PER proteins has been shown to be up-regulated while the positive elements of the molecular clock (CLOCK & BMAL) are under the highest degree of suppression. The CK1 phosphorylation of PER also has a significant impact on protein stability, therefore providing another route by which CK1 exercise regulation over the clockwork system. CK1 phosphorylation of the PER1 and PER2 proteins marks the proteins for ubiquitin-mediated degradation (via 26S proteasome). This then allows the CLOCK: BMAL heterodimer to begin the cycle again. The CK1s also exerts control over BMAL1 transcription activity via phosphorylation (Gallego & Virshup 2007). Other kinases such as *Casein kinases 2* (CK2), Glycogen synthase kinase-3 (GSK3) have been implicated in the regulation of clocks in non-vertebrates and mammals, respectively. Moreover protein phosphatases (PP1 and PP2A) play a role in PER protein stability (Ko & Takahashi 2006; Gallego & Virshup 2007;). Crucially ubiquitylation is also important in regulation of the circadian molecular clock. This has been well described in *Drosophila* (Peschel and Helfrich-Foerster. 2011). Specific substrates are identified (mediated by proteins containing an F-box domain) by an E3 ubiquitin ligase which, in combination with an E2 ubiquitin-conjugating enzyme catalyses ubiquitinylation. F-box domain containing protein Supernumerary limbs (SLMB). has been shown to be involved in the circadian clockwork in *D. melongaster* , and in mammals PER protein ubiquitinylation is regulated by orthologues of SLMB (Ko & Takahashi 2006; Gallego & Virshup 2007). The underlying molecular mechanisms of circadian clock, although based around a negative feedback loop, are increasingly appearing to be reliant on a multitude of regulatory mechanisms.

Mutation effects on clock cycling

As described above the identification of mutations that have a profound effect on circadian functions has been instrumental in identifying the core molecular components of the circadian clock. Since the characterisation of the vertebrate circadian clock in the 1990's a wide variety of mutations have now been characterised. A number of relevant examples will be described in the following paragraphs to illustrate the importance of the genes and processes previously described.

In mammals the *Tau* mutant hamster was the first circadian mutant to be characterised (Ralph & Menaker 1988). The *Tau* mutant was later found to be a result of a mutation in *CK1ε* (Lowrey et al. 2000). In the *Tau CK1ε* allele there is a G to C mutation. This alteration induces a BstAP1 restriction site, consequently changing an arginine to a cysteine at amino acid residue 178 (Lowrey et al. 2000; Monecke et al. 2011). The change in amino acids increases *CK1ε* phosphorylation of PER proteins. It is hypothesised that the increased speed of PER phosphorylation and eradication from the circadian feedback loop is responsible for the shortened circadian rhythm observed in *Tau* mutants (Gallego et al. 2006; Meng et al. 2008). The consequence of the altered *Tau* CK1e in hamsters was a 22h circadian period in heterozygote animals (τ_{DD}) and a 20h period in homozygote animals (τ_{SS}). More recently the breeding of *Tau* homozygote hamsters has resulted in additional shorting of the circadian period to 17.8h in a circadian mutant named "duper" (Monecke et al. 2011). However the mechanisms of how this mutation alters the circadian clock remain elusive.

The *Tau* allele has also been observed in mice resulting in a 4h reduction in circadian period. A mutation in CK1δ, a homologue of CK1ε (Lowrey et al. 2000), additionally reduced the wild type circadian period by 30 minutes (Xu et al. 2005). Mutations have also been described in the clock genes of the primary feedback loop in mice and can have a dramatic

phenotypic effect (Ko & Takahashi 2006). An alteration to the mouse *Bmal* gene results in complete arrhythmicity (Bunger et al. 2005), as does the $Per2^{ldc}$ mutation (Bae et al. 2001). A mutation in the mouse clock gene ($Clock^{\Delta 19/\Delta 19}$) can additionally result in an arrhythmic or elongated (28h) circadian phenotype (Vitaterna et al. 1994). Outside of laboratory animals mutations in clock genes can have a profound effect on human pathologies.

The most well known effect of clock gene mutation in humans is familial advanced sleep phase syndrome (FASPS). The resulting phenotype is a circadian rhythm considerably advanced in comparison to the majority of the population. Individuals with FASPS exhibit the onset of sleep at approximately 19:30 and awake at 04:30 (Jones et al. 1999). This was found to be a result of two separate mutations in circadian clock components that have similar impact on the timing of the circadian clock. Initially a serine to glycine point mutation on the PER2 protein CK1 binding region was identified as the basis for FASPS. The disruption to the CK1 binding resulted in hypophosphorylation of the *Per2* gene in the auto-regulatory feedback loop (Toh et al. 2001). In 2005 another mutation, this time in *CK1*δ was identified as a cause of FASPS. On this occasion it was an A-to-G missense mutation inducing a threonine to alanine modification to the CK1δ protein that resulted in the hypophosphorylation of the *Per2* (Xu et al. 2005).

The above examples clearly demonstrate the effect mutation can have on clock genes and resulting phenotypes. The discovery of such circadian mutants has not only provided us with the tools to unveil the individual components of the circadian clock but it has enabled the detailed evaluation of the roles of the core mechanisms of the circadian clock.

Molecular clocks in fish.

In mammals the mechanisms of the circadian clock are relatively well described. This is not the cases in teleost fish. The first evidence for endogenous clocks in fish originated in the

study of the pineal. In the 1980's and 90's Jack Falcon was one of the first researchers to describe circadian rhythms in a number of pineal hormones in the Northern pike (*Esox lucius*), including the nocturnally synthesised hormone melatonin (Falcon et al. 1984; Falcon et al. 1987; Falcon et al. 1989; Falcon et al. 1994). Rhythms in pineal melatonin were later found to be endogenous and regulated by an internal circadian clock (Falcon et al. 1989; Bolliet et al. 1996; Falcon 1999;). Moreover enzymes involved in the biosynthesis of melatonin were also found to be regulated by the circadian clock in zebrafish and pike (Bolliet et al. 1997; Begay et al. 1998; Coon et al. 1998). However in more recent years the majority of studies concerning clocks in fish have utilised the zebrafish as a model.

The zebrafish is a well established model organism amongst teleost and has proven to be most useful in unravelling the circadian clockwork (Vatine et al. 2011). The zebrafish provides a number of key advantages in the study of circadian rhythms. Development is fast and easily observed in a non invasive manner. The species reaches sexual maturity at around 3 months and reproduction is easily achieved. Moreover the species is easy to maintain in a lab and relatively inexpensive (Vatine et al. 2011). The capacity to cultivate transgenic cell lines with fluorescent reporter genes and the capacity to carry out forward genetic screening has now been established (Vatine et al. 2011), atool that has also proved invaluable in the investigation of the circadian clock in mammals and drosophila.

In a 1995 Cahill and Besharse reported that zebrafish could be used as a model system for the study of circadian rhythms in teleost fishes. They hypothesised that if zebrafish expressed a strong circadian rhythm it would be an ideal candidate for genetic studies on the teleost clock because of the advantages described above. (Cahill & Besharse 1995). Results demonstrated robust circadian rhythms of melatonin production in the pineal organ and to some extent the retina (Cahill & Besharse 1995). Since this publication the *D. rerio* clock has been the subject of numerous investigations. A number of clock genes, homologous to mammalian clock

genes, have now been identified and expression characterised (Whitmore et al. 1998; Whitmore et al. 2000a; Whitmore et al. 2000b; Carr et al. 2006). Results have shown that the molecular mechanisms underpinning the zebrafish clock to be remarkably similar to those of the mammalian clock.

The molecular feedback loops that instigate and regulate endogenous rhythmicity have been remarkably conserved throughout vertebrate evolution (Figure 5) (Vatine et al. 2011). However there are some important differences at a molecular level between the zebrafish and the mouse clock model. As a result of the teleost genome duplication multiple copies of zebrafish clock genes have been identified in comparison to the mammalian and *D. melanogaster* circadian mechanisms (Cahill 2002). The most prominent example of this is the number of *Cry* genes identified in the zebrafish. In comparison to the two copies of *Cry* in mammals, there are six copies of *Cry* (*zCry*) in the zebrafish, and all 6 z*Cry* genes are rhythmically expressed in zebrafish tissues. Four of the z*Cry* genes (1a, 1b, 2a and 2b) have been shown, by phylogenetic analysis, to be homologues of the mammalian mouse *Cry1*. These have also been shown to inhibit transcriptional activation by mammalian CLOCK: BMAL1 dimers (Vatine et al. 2011). The circadian profiles of the z*Cry1s* and *2s* exhibited different acrophase or peak in rhythmic mRNA expression. z*Cry1a* and b peak during the day whilst z*Cry2a* and b peak later in the evening, suggesting the extra copies of the genes are not entirely redundant (Kobayashi et al. 2000; Cahill 2002;; Vatine et al. 2011). z*Cry3* and z*Cry4* also appear to be homologues of mouse *Cry1* and are rhythmically expressed with highest levels of mRNA expression in the morning. However unlike z*Cry1a/b* and z*Cry2a/b* neither inhibited the transcriptional activation of the mammalian CLOCK: BMAL1 dimmers (Cahill 2002; Vatine et al. 2011). z*Cry4*, which is not as closely related to mouse Cry1,is thought to have a role in photoreception, based the role of a similar Cry protein in *D. melanogaster* as a blue light photoreceptor (Cahill 2002; Vatine et al. 2011).

In the zebrafish clock mechanism both mammalian orthologs of *Bmal1* and *Bmal2* have been described. The 2 *Bmal (zBmal)* genes in Zebrafish are expressed rhythmically in a number of peripheral tissues, inferring their involvement in the circadian system (Cahill 2002). There are however some important differences form mammals in the activities and expression patterns of these two z*Bmal* genes and their proteins. zBMAL1 appears to bind more tightly to CLOCK than zBMAL2, while zBMAL2 is a more potent transcriptional activator, inferring the two copies have slight differences in function within the circadian system (Cahill 2002; Cermakian et al. 2000). In the zebrafish there also appears to be differences in the regulation of the *Per* genes in comparison to mammals. In the zebrafish *Per2* appears to be stimulated by light and has been proposed as a mode of circadian entrainment to light (Cahill 2002). This is comparable to *per2* regulation in the retina of the amphibian *Xenopus laevis* (Zhuang et al. 2000). Although, the clock mechanisms of vertebrates appear to be largely conserved throughout evolution, the zebrafish system does posses some important difference in comparison to mammals. Whether these differences extend to other teleost species is unclear.

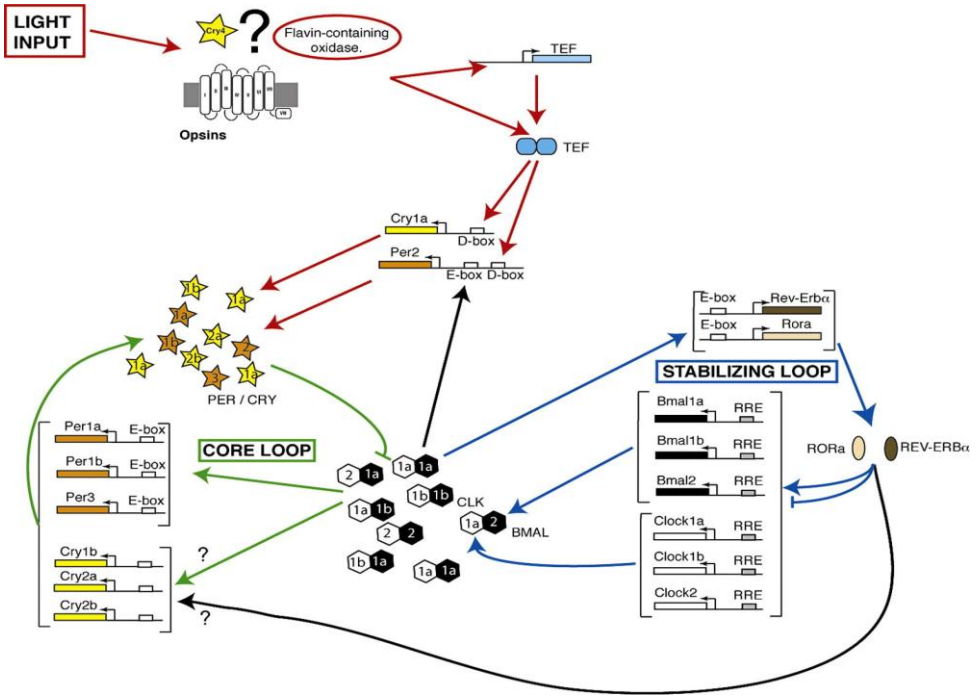


Figure 5: Current model of the core molecular components of the zebrafish circadian. As in mammals the CLOCK: BMAL hetrodimer drives the expression of the PER and CRY proteins. Additionally the regulatory loop involving Rev-Erbα and Rora is present. The primary differences between mammalian and zebrafish models include multiple copies of the clock, genes. Light-induced expression of the clock genes *Cry1a* and *Per2* . This provides a pathway for light to directly modulate the *Per* and *Cry* and resulting effects on the clock. From Vatine et al (2011).

In recent years there has been a drive to understand molecular clock mechanisms in a number of cultured and tropical fish species such as Senegalese sole (*Solea senegalensis*) (Martin-Robles et al. 2011), European seabass (Sanchez et al. 2010), goldfish (*Carassius auratus)* (Velarde et al. 2009; Feliciano et al. 2011) and the rabbitfish (*Siganus guttatus)* (Park et al. 2007). Amongst the salmonids there have been a limited number of studies on various aspects of clock gene expression and have mainly been concerned with the links between clocks and seasonality, including expression during early ontogeny in the rainbow trout (*Oncorhynchus mykiss).* In the Atlantic salmon clock gene expression in the brain and pineal of parr, smolts and postsmolts (Huang et al. 2010a; Huang et al. 2010b) and daylength dependent clock gene

expression in the brain has previously been observed in salmon parr (Davie et al. 2009). A number of clock genes have additionally been associated with seasonal processes involved in key life history events (including migratory runtime and maturation) in a number of salmonid species (Aubin-Horth et al. 2005; Leder et al. 2006; O'Malley et al. 2007; O'Malley & Banks 2008; O'Malley & Banks 2008; Davie et al. 2009;; O'Malley et al. 2010; O'Malley et al. 2010; Paibomesai et al. 2010).

Research to date in teleosts has primarily focused on the zebrafish. However it is clear that clock mechanisms are present in all other species investigated. There also appears to be a high degree of conservation in clock gene sequences between teleost species and mammals. Yet the teleostei are an incredibly diverse infraclass of species and it is unclear how the circadian clockwork may have adapted over the course of evolution to cope with the multitude of different habitats and ecological niches the group inhabits. Moreover investigations of clocks in the zebrafish are not useful for seasonal studies as the zebrafish is not a highly seasonally teleost. Considerably more work is necessary to reveal the specific molecular mechanisms of the teleost biological clock.

Central and peripheral clocks

Mammalian clocks

Clock genes appear to be present in almost every tissue and cell type. However in mammals there appears to be a clear distinction between central and peripheral tissues. It has previously been hypothesised that clock genes in the peripheral tissues are regulated by a central circadian pacemaker. Recent research now points towards a central pacemaker as a synchronising tissue as opposed to a master circadian pacemaker (Schibler & Sassone-Corsi 2002). In any case this brain region has been identified as the suprachiasmatic nucleus (SCN). The SCN is located in the anterior hypothalamus just above the optic chiasm and consists of

around 20,000 neural and glia cells (mice) divided into a paired structure, each cell with their own molecular clockwork (Meijer et al. 2010; Welsh et al. 2010; Mohawk & Takahashi 2011) . Previously, the SCN was believed to consist of two distinct regions, the shell (dorsomedial SCN) and the core (ventrolateral SCN) (Welsh et al. 2010). However it is now understood that the SCN structure is more heterogeneous than initially supposed and is thought to vary considerably across a number of mammalian species (Welsh et al. 2010; Mohawk & Takahashi 2011;). Despite the presence of the independent cellular oscillators that encompass the SCN it is now understood that the coupling between these cells is fundamental to the synchronising properties of the region and its status as the "master pacemaker of the circadian clockwork" (Mohawk & Takahashi 2011). The cells of the SCN are tightly coupled to form the SCN oscillators. Coupling between the cells involves coordination of electrical activity between the cells of the SCN and is thought to be achieved via electrical connections between cells via gap junction channels. This coordination enables fast entrainment of the SCN region to alterations in the external photoperiod (Dibner et al. 2010). Moreover communication between cells in the region enables coupled endogenous cycling of circadian oscillations in the absence of environmental cues (Dibner et al. 2010). The SCN then orchestrates rhythmicity in the periphery (Meijer et al. 2010). An array of experiments, primarily in rodents has demonstrated that damage and destruction of the SCN region result in the abolition of a number of rhythmic outputs (Stephan & Zucker 1972; Moore & Bernstein 1989; Welsh et al. 2010). Moreover rhythmicity can be restored to some extent when foetal SCN tissue is implanted to the desynchronised organisms (Lehman et al. 1987; Welsh et al. 2010). In mammals, at least, it is becoming clear that although most tissues investigated do possess the underling mechanisms driving circadian rhythmicity it is the SCN that synchronises many of these peripheral clocks to generate robust cyclic physiology.

In mammals the rhythmic activity of the SCN is synchronised to the environment via the input of photic information from the retina. Daily 24h photoperiod information is transmitted from the photoreceptors in the retina via the retinohypothalamic tract to the SCN coordinating the rhythmic electrical activity of the SCN. This in turn activates the nocturnal secretion of pineal melatonin which consequently regulates a wide variety of daily and seasonal physiology throughout the body. The retina, SCN and the nocturnal production of pineal melatonin encompass the initial synchronising components of the photoneuroendocrine system (PNES). Among vertebrates there is considerable diversity in the entrainment of the PNES (Migaud et al. 2010). The mechanisms of the mammalian and fish PNES will be discussed further below. Additionally the SCN is responsible for the synchronisation and coordination of circadian clock gene expression and rhythmic physiology in a variety of peripheral tissues.

The SCN coordinates rhythmicity in other brain regions and in the periphery in a number of ways, including hormonal pathways and the transfer of information from the central nervous system to peripheral clock via the autonomic nervous system (Balsalobre et al. 2000; Le Minh et al. 2001; Nakamura et al. 2008; Dibner et al. 2010; Welsh et al. 2010). In mammals the coupling of the SCN neurons and the consequent synchronising rhythmicity exerts considerable control over a number of peripheral tissues. Peripheral clocks have been observed in tissues including the heart, lung, liver, kidney and a number of cell types including fibroblast cells. A number of rhythmic outputs have been observed in these tissues for example heart rate, systolic blood pressure (maximum blood pressure between heartbeats), vasodilation (widening of blood vessels). Additionally in the liver various aspects of metabolism and detoxification have been observed on a circadian time scale (Bell-Pedersen et al. 2005). Indeed clocks are hypothesised to be present in the majority of tissue

and cell types consisting of systems similar to the basic molecular organisation of the SCN (Bell-Pedersen et al. 2005).

There are however a number of fundamental differences between the mechanisms of the SCN and those of the peripheral tissues. Firstly the SCN is entrainable by light. In mammals there is no consistent data to suggest the presence of a light entrainable clock in peripheral tissues (Bell-Pedersen et al. 2005). Moreover there is generally a 4 to 12h time lag between central and peripheral clock gene expression (Zylka et al. 1998). Importantly, the presence of an intact SCN has been shown to be fundamental to the persistence of rhythmic output. In isolated tissue cultures luciferase reporting has additionally shown circadian rhythms to persist endogenously for several weeks (Bell-Pedersen et al. 2005). In non SCN tissues circadian rhythms in gene expression dampen when isolated in tissue culture (Yamazaki et al. 2000). These observations are consistent with the assumption of a hierarchal structure of the mammalian circadian network (SCN regulating peripheral clocks (Bell-Pedersen et al. 2005). The SCN acts as an intermediate between photic input and circadian physiology. The phase delay observed between central and peripheral tissues indicates the time taken for photic information to travel from the retina to the SCN and on to the various peripheral oscillators (Bell-Pedersen et al. 2005). The role of peripheral clocks in regulation of tissue specific rhythmic outputs is largely unclear. Although in mammals it appears the SCN is necessary, rhythmic gene expression in non-SCN tissues is highly tissue specific (Panda et al. 2002; Storch et al. 2002; Duffield 2003; Bell-Pedersen et al. 2005).

In mammals it is now becoming increasingly evident that clocks in peripheral tissues, in particular the liver, can be entrained by a number of zeitgeber signals. The SCN was previously hypothesised to be essential to rhythmic output, however more recent evidence suggests that it may not be essential for the synchronisation of hepatic clocks (Welsh et al. 2010) . Of these factors, feeding time is thought to entrain tissues such as the liver, pancreas,

kidney, heart and skeletal muscles (Welsh et al. 2010). The timing of feeding is comparable with the organism's rest/ activity cycle under a natural photoperiod and clock gene oscillations in the peripheral organs reflect this. However under restricted feeding conditions (i.e. diurnal feeding in nocturnal rodents) the pattern of gene expression is inverted to mirror the feeding schedule and is decoupled from rhythms in the SCN (Damiola et al. 2000; Stokkan et al. 2001; Dibner et al. 2010). It is thought that occurrence of feeding entrainment and the presence of SCN entrainable clock mechanisms in tissues such as the liver facilitates the temporal separation of biologically incompatible processes such as those involved in metabolism (Schibler 2007; Dibner et al. 2010). The entrainment of hepatic clocks may occur via the SCN or direct to the liver. The pathways by which feeding entrainment may occur are still under debate but are thought to involve the redox state of the cell, hormonal pathways and the presence of food metabolites such as cholesterol (Dibner et al. 2010; Schibler et al. 2010). One area of particular interest is the relationship between peripheral clocks and metabolism. While outputs of cellular metabolism have been shown to affect the molecular clockwork, a number of genes involved in metabolism also display clear circadian profiles of expression (Dibner et al. 2010). Although many of the mechanisms and pathways involved in the expression and entrainment of clocks in peripheral tissues as well as the degree of involvement of the SCN are still not fully understood, it is clear that it is not only light and the SCN that regulate temporal processes outside of the brain in mammals.

Teleost clocks

In teleosts the hierarchical structure of clocks is not so clear and the presence of an SCN or SCN-like structure has yet to be identified. The majority of work has been carried out in the zebrafish. In comparison to mammals, zebrafish peripheral clock gene oscillations are not under the control of a SCN-like structure but are self sustaining and individually entrainable by light (Whitmore et al. 1998; Carr et al. 2006; Kaneko et al. 2006; Vatine et al. 2011).

Furthermore the presence of self sustaining peripheral circadian oscillators has been documented. Tissues such as the heart and kidney possess independent circadian oscillators persisting *in vitro* as do individual cells in cultured cell lines. Additionally under constant darkness circadian clock gene expression in peripheral tissues display considerable differences in free running period (circadian period when not entrained to 24 light dark cycle), demonstrating the importance of light of synchronising and stabilising rhythmisity not only to external conditions but throughout the body (Carr et al. 2006; Kaneko et al. 2006; Vatine et al. 2011). It has been hypothesised that each cell is photoreceptive with possible candidates including extra retinal opsins, flavin containing oxidase or photoreceptive cryptochrome genes (Vatine et al. 2011). In spite of the growing body of work on peripheral clocks in the zebrafish, rolesin the PNES are largely unknown.

In other teleosts very little information is available on the presence and expression of clocks in peripheral tissues outside of the liver. However clock gene expression in the liver has been described in a relatively large number of species. Yet no clear pattern of expression has been established. For example in the European seabass rhythmic *Per1* expression has been reported in the brain, heart and liver with the acrophase synchronised in all tissues occurring at similar times during the circadian cycle. Similarly in the Golden rabbit fish(*Siganus corallines*) (Sanchez et al. 2010) comparable patters of day/ night expression were observed between the brain, retina and the liver (Park et al. 2007). However in the gold fish *(Carassius auratus auratus*) differential expression was reported, with *Per3* expressed rhythmically in the liver while *Per2* and *Per3* displayed significant rhythmic expression in the gut and retina. Discrepancies in the expression of *Cry 1- 3* was also observed between central and peripheral tissues with all three cry genes rhythmically expressed in the retina while only *Cry2* and *Cry3* displayed circadian expression in the gut and the liver(Velarde et al. 2009). Moreover the

acrophase of the circadian cycles and levels of expression varied between the central (retina) and peripheral tissues (gut and liver) (Velarde et al. 2009). In the Senegalese sole *(Solea senegalensis)* it was *Per3* that displayed prominent rhythmic expression in liver tissue(Martin-Robles et al. 2011). Overall no clear pattern of clock gene expression in the liver can be established amongst teleosts.

As has been reported for mammals and reviewed for zebrafish above, feeding entrainment of clocks in the liver has been observed. A recent study has demonstrated the presence of a food entrainable circadian oscillator and robust regulation of clock gene expression in the goldfish liver (Feliciano et al. 2011). Moreover *Per1* expression in the liver has been linked to food availability in zebrafish (Lopez-Olmeda et al. 2010). Preliminary work in salmon has shown that clock genes are present in central and peripheral tissues ranging from the liver to the intestine and spleen (Davie et al. 2009). Daily patters of clock gene expression appear to differ under 12hL: 12hD between the brain, pineal and the liver for a number of clock genes (Huang et al. 2010). However the role of feeding entrainment or mechanisms controlling clock gene expression in the liver of the Atlantic salmon is not yet known.

Although clock machinery has been described in most tissues and cell types in mammals, rhythms in peripheral clocks are thought to be driven and synchronised to external environmental conditions, in the most part, by the SCN. This is not the case in the zebrafish as individual cells and tissues appear to contain independently light entrainable and self sustaining clocks (Vatine et al. 2011). Moreover to date no SCN, master clock like structure has been found in the teleost brain. Consequently in the zebrafish it is clear that regulation of peripheral clocks is considerably different to mammals. However the specific mechanisms in the regulation of clocks in the zebrafish remain elusive (Vatine et al. 2011). In other teleost species the situation is especially unclear. The expression of clock genes has not been investigated in a wide variety of peripheral tissues. Investigations have primarily focused on

the liver as a consequence of the importance in understanding metabolism, cholesterol homeostasis and ultimately fat deposition in commercially important species. Patterns of clock gene expression also appear to be dependent on species. Additionally in the absence of photic cues, feeding appears to act as a zeitgeber to entrain clocks in the liver (Kornmann et al. 2009). As a result of the interesting developments in the study of clock regulation of liver lipid metabolism in mammals, for example clock gene regulation of cholesterol and fatty acid metabolism in rodents (Le Martelot et al. 2009)it is important to investigate the role of clocks in the regulation of liver lipid metabolism in the Atlantic salmon. Within the aquaculture industry this is an area of particular interest, as regulation of processes associated with fat deposition has fundamental importance for feed formulation and product quality.

4. PHOTONEUROENDOCRINE AXIS

The mechanisms of the circadian clock described in detail in the sections above are directly involved in the regulation of a wide variety of molecular and physiological processes. However clocks, in particular central clocks, also act as a component of the vertebrate photoneuroendocrine axis (PNES). The PNES is the system that connects photic perception to the brain-pituitary-gonadal (BPG) axis (Migaud et al. 2010). As with aspects of the circadian clock, knowledge of the PNES is considerably more advanced in mammals and in invertebrates such as drosophila.

The mammalian PNES

In mammals, the retina is fundamental to the perception of non-visual photic information. However the rods and cones essential for visual perception are not required for the perception of non-visual photic information and entrainment of the circadian system (Freedman et al.

1999; Golombek & Rosenstein 2010). In recent years photoreceptive retinal ganglion cells (pRGCs) have been identified as playing a key role in the transduction of photic cues to the SCN, where photic information is deciphered (Berson et al. 2002; Hattar et al. 2002; Golombek & Rosenstein 2010). Melanopsin, a photo pigment present in pRGCs, is thought to be a primary component responsible for the transduction of photic information through the retino-hypothalamic tract (RHT) and the initiation of the PNES (Golombek & Rosenstein 2010). The RHT is the primary input pathway to the SCN. The importance of the RHT to the SCN has previously been demonstrated in lesion and stimulation experiments (Johnson et al. 1988; Shibata & Moore 1993; Golombek & Rosenstein 2010). The RHT utilises glutamate, asparate, pituitary adenylate cyclase-activating polypeptide (PACAP) as neurotransmitters to transmit photic information to the SCN (Ebling 1996; Chen et al. 1999; Hannibal 2002; Golombek et al. 2003; Fahrenkrug 2006; Hannibal 2006; Golombek & Rosenstein 2010). Substance P is additionally thought to be involved in the process (Hannibal 2002; Golombek & Rosenstein 2010). Of these neuropeptides glutaminergic SCN fibres are hypothesised to be the primary signal innervating the clock by making synaptic connections and transmitting electrical signals to SCN neurons (Golombek & Rosenstein 2010).

As described above the SCN is a heterogeneous paired structure, situated in the anterior hypothalamus and drives circadian rhythmicity. The structure is crudely divided into two primary elements. The ventrolateral SCN (VL-SCN i.e. the core) and the shell, the dorsomedial SCN (DM-SCN). The RHT transmits directly to VL-SCN neurons (Dibner et al. 2010; Golombek & Rosenstein 2010; Colwell 2011;). This then activates the expression of *Per* genes via the extracellular signal- regulated kinase (ERK) pathway and cAMP response elements in the promoter regions of the *Per1* and *Per2* genes (Dibner et al. 2010). The VL-SCN neurons then transmit this information to the DM–SCN where clock genes oscillate

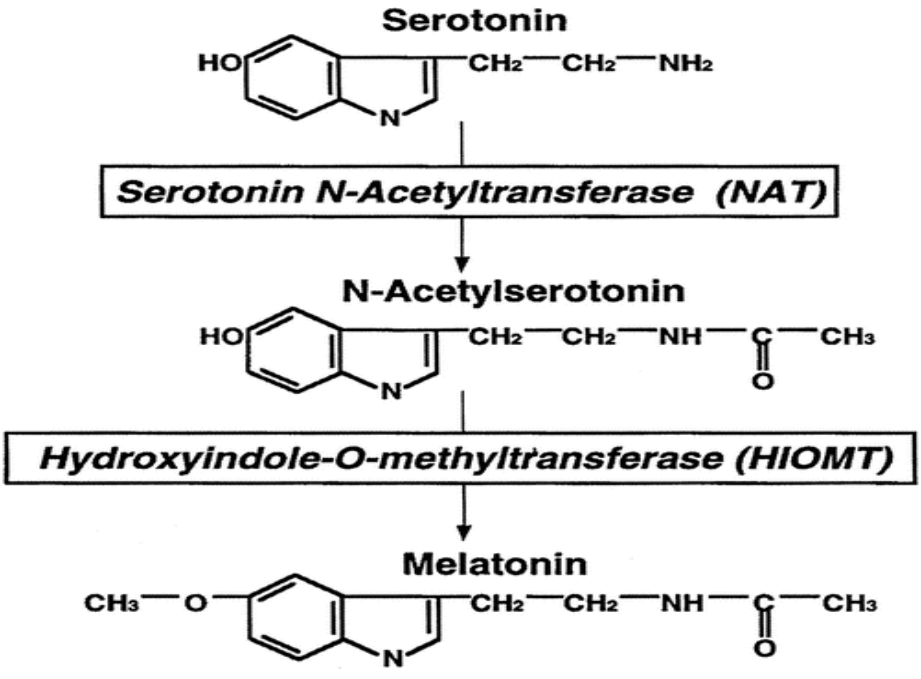
endogenously (Colwell 2011). This is the core mechanism entraining the endogenous clock of the SCN to environmental photoperiod

.**Melatonin**

Both photoperiod entrainment and the endogenous circadian clock come together to regulate the mammalian PNES (Migaud et al. 2010). This regulation is primarily achieved via SCN stimulation of the pineal melatonin biosynthesis. Photoperiod information is then transmitted to the pineal via a succession of processes involving: paraventricular nuclei or PVN of the hypothalamus, intermediolateral cell column of the spinal cord, the superior cervical ganglia (Klein 1985) to the pineal organ. At night SCN stimulates the release of norepinephrine into the pineal perivascular space. Norepinephrine then activates adenylyl cyclase, via β_1-adrenergic receptors, and increases intracellular Ca^{2+} and protein kinase C activity via α_{1b}-andrenergic receptors, consequently potentiating the β_1-adrenergic receptor activation of adenylyl cyclase. This process causes a large rapid increase in cAMP. In unglates and primates this is the only cellular mechanism known to control AANAT activity. Research in rodents has shown that cAMP also controls AANAT transcription but a number of other factors also appear to modulate its transcription. Including: -$[Ca^{2+}]_{I,}$ unidentified rapid turnover protein repressor and endogenous clock control of cAMP(Coon & Klein 2006; Klein 2007). The melatonin pathway (Tryptophan → Hydroxytryptophan →N-acetylserotonin → Melatonin) (Figure 6) and the daily rhythms in circulating melatonin levels are highly associated with the changes in AANAT activity, the penultimate enzyme involved in the production of melatonin (Coon & Klein 2006; Klein 2007). AANAT activity mirrors a 24h profile in the retina as well as the pineal which plays a unique role in vertebrate biology. The activity of AANAT has been observed increasing by 10-100 fold during the night and results

in an increase in the biosynthesis of melatonin (Coon & Klein 2006; Klein 2007). The mammalian melatonin biosynthesis pathway is dependent on light information being transmitted from the retina, as opposed to the photoreceptive pineal present in teleosts.

Figure 6: Melatonin biosynthesis pathway encompassing the conversion of serotonin to the nocturnally occurring hormone melatonin from Borjigin et al 1999.



Melatonin and seasonality

Pineal melatonin synthesis mirrors the dark period of the 24h light dark cycle. Changes in duration of melatonin signal provide information on seasonal changes over the course of the year. Melatonin is the primary output of the mammalian PNES from the SCN and pineal organ. The hormone binds to melatonin receptors at a variety of sites in order to stimulate reproduction (Simonneaux & Ribelayga 2003). In a number of seasonally breeding species melatonin binds to melatonin receptors in the pars tuberalis (PT) of the pituitary and stimulates the expression of clock gene expression and thyroid stimulating hormone (TSH) in

the region. Consequently, this activates the deiodinases 2 and 3 (DIO2 and DIO3) in the mediobasal hypothalamus and regulates the bioactivity of the thyroid hormones by mediating the conversion of T4 to T3 by DIO2 and vice versa DIO3 catalyses the conversion of biologically active T3 to an inactive form. The biologically active T3 in turn regulates reproductive processes (Yasuo et al. 2007). Melatonin additionally stimulates the hormone kisspeptin by the activation of the *Kiss-1* gene in the hypothalamus. This ultimately mediates sexual development via regulation of GnRH (Smith & Clarke 2007). In mammals these mechanisms of the PNES appear relatively conserved among a wide variety of species. Difference in the timing of reproductive initiation (i.e. short day breeding species such as sheep and long day breeders such as hamsters) have been hypothesised to occur at the junction where melatonin and brain pituitary gonadal axis (BPG axis) interact (Dardente. 2012). Clock genes have also been implicated in the regulation of reproduction (Kennaway 2005; Boden & Kennaway 2006), however the precise mechanisms mediating this are unclear. In mammals the pathways, mechanisms and components underpinning the mammalian PNES are relatively well understood in comparison to other vertebrates.

The teleost PNES

In teleosts there is very little information on the PNES organisation as a whole. One of the aspects of the teleost PNES that has received the most attention is melatonin. Yet studies on the direct role of melatonin in reproduction, as for the physiological functions, have given contrasting results depending on; species, gender, photoperiod and reproductive status (Falcon et al. 2007; Migaud et al. 2010). Melatonin has been shown to influence growth, maturation and reproductive processes in a number of teleost species. Low concentrations of melatonin have been shown to stimulate the release of luteinizing hormone (LH) from the pituitary and consequent elevation in the Atlantic Croaker *(Micropogonias undulatus)* while melatonin implants in the eel *(Anguilla anguilla)* resulted in a decrease LHβ and follicle-

stimulating hormone FSHβ (Khan & Thomas 1996; Sebert et al. 2008). This has also been demonstrated in the Pacific salmonid species, the masu salmon *(Oncorhynchus masou)* (Amano et al. 2003; Amano et al. 2004; Amano et al. 2006). The administration of melatonin reduced both gonadotropin-releasing hormone (GnRH) and LH within the pituitary however FSH was stimulated. It was speculated that melatonin acts directly on the pituitary as a number of melatonin binding sites are present in the tissue (Amano et al. 2003; Amano et al. 2004; Amano et al. 2006; Falcon et al. 2011) . In salmon removal of the pineal organ (pinealectomy) has suggested that pineal melatonin may be involved in the regulation of spawning time, however other mechanisms are likely to be involved as removal of the pineal does not fully abolish reproductive processes such as maturation (Mayer 2000; Migaud et al. 2010). In contrast to the mammalian PNES there are some fundamental differences in the teleost production of pineal melatonin and the role of the pineal.

In contrast to mammals, the pineal of the majority of teleost species studied to date, excluding tilapia *(Oreochromis niloticus)* and catfish (*Clarias gariepinus*), is directly photoreceptive (Migaud et al. 2007). It is entrainable by light and is the primary site for the endogenous production of circulating melatonin. In culture the mammalian pineal is unable to synthesise melatonin *in vitro*, the addition of nor-epinephrine to the culture medium is necessary for the hormone to be synthesised (Falcon 1999). Isolated in organ culture, the teleosts pineal remains directly photoreceptive and light entrainable (Bolliet et al. 1995; Iigo et al. 2007). In non salmonid teleosts the production of melatonin is endogenous. This is mediated in the pineal via clock control of AANAT2. As in peripheral tissues rhythmic clock gene expression is present in the zebrafish pineal. As a result the pineal is considered to be the central circadian pacemaker in this species (Vatine et al. 2011). However it is difficult to extrapolate these results and apply generalisations to the whole teleost group as a result of the diversity found amongst species in the group.

In general in non-mammalian vertebrates photoperiod dictates the rhythmic AANAT activity and melatonin synthesis. For example the presence of light during the night will inhibit the dark induced rise in levels of AANAT activity and subsequent melatonin production within the pineal (Falcon 1999). In a number of non-mammalian vertebrates melatonin rhythms appear to be driven by an internal endogenous clock, with photoperiod acting to entrain the clock to external environmental conditions (Falcon 1999). In fish, summer melatonin rhythms have a short duration and high amplitude, the situation is converse in winter photoperiods with rhythmic production of the hormone displaying a long duration and lower amplitude. Spring and autumn exhibit intermediate rhythms (Besseau et al. 2006). In most species these rhythms persist in constant darkness thus demonstrating the endogenous nature of these rhythms (Migaud et al. 2010), however there are exceptions (Falcon 1999; Iigo et al. 2007).

Among salmonid species no rhythmicity appears to exist in the synthesis of pineal melatonin under constant conditions inferring the lack of circadian regulation over pineal melatonin production within this group (Falcon 1999; Abe et al. 2002). Extensive studies in salmonids have demonstrated photo-entrainable non-endogenous synthesis of pineal melatonin. Under constant darkness rhythmic melatonin production is lost in all salmonid species investigated to date (Gern & Greenhouse 1988; Max & Menaker 1992; Iigo et al. 2007). A 2007 study (Iigo et al. 2007) set out to monitor the secretion of pineal melatonin in seven salmonids and their relatives, the osmerids (2 species), in order to determine the evolutionary history of the pineal organ. 24h profiles of melatonin synthesis from individual salmonid pineals, common whitefish (*Coregonus lavaretus*), grayling (*Thymallus thymallus*), Japanese huchen (*Hucho perryi*), Japanese charr (*Salvelius leucomaenis pluvius*), brook trout (*Salvelius fontinalis*), brown trout (*Salmo trutta*) and chum salmon (*Oncorhynchus keta*), and from individual osmerid pineals from ayu (*Plecoglossus altivelis altivelis*) and Japanese smelt (*Hypomesus nipponensis*) were determined under a light dark/cycle and under constant darkness. In

response to a light/dark cycle all species displayed rhythmic melatonin synthesis with elevated levels of melatonin produced during the night. Under constant darkness rhythmic production of melatonin persisted endogenously, displaying elevated melatonin during the previously entrained nocturnal period. However in all salmonid species investigated the synthesis of melatonin from the isolated pineal organ was not rhythmic and levels were continually elevated during the 24 hour period. This was not the case in the osmerid species studied. The authors hypothesised that ancestral salmonids lost the endogenous circadian regulation of pineal melatonin synthesis after divergence from the osmerids (Iigo et al. 2007).

The study by Iigo et al (2007) concentrated on the circadian control of melatonin in the pineal and not the retina. In another study in 2007 Migaud et al addressed this issue, demonstrating that the role of melatonin within the circadian system of teleost fishes varies to a far greater extent than initially realised. This report described three different ways in which light acts on the circadian axis of teleost fish. In the two salmonid species studied (Atlantic salmon and rainbow trout) circulating levels of melatonin were not affected by the removal of the eye, inferring that pineal photo-receptor cells are solely responsible for regulating circulating levels of melatonin. The situation was very different in seabass and cod. The removal of the eye resulted in a significant reduction in night time levels melatonin suggesting that both the eye and the pineal are responsible for regulating melatonin rhythms. It is possible that light perceived by the eye could regulate melatonin synthesis in the pineal via neural projections into the brain as is the case in mammals but it is also possible the system is dependent on deep brain receptors present in non-mammalian vertebrates (Migaud et al. 2007). The situation was different again in tilapia and catfish, suggesting a third kind of circadian control, whereby the pineal is not light sensitive or has significantly reduced sensitivity. This is similar to the situation in mammals, except with the lack of central circadian pacemaker

which would drive the production of melatonin in the absence of the eye. It was hypothesised that this system may also be dependent on deep brain photoreceptors (Migaud et al. 2007). Further studies into the circadian control of melatonin production in the Nile and Mozambique tilapia have shown there to be a combination of both direct light and circadian control (Martinez-Chavez et al. 2008; Nikaido et al. 2009). Nikaido et al additionally hypothesised that a circadian pacemaker may exist in the retina or perhaps that a transducing network relays photic information from the retina to the pineal organ. To date the mechanisms of the salmonid circadian axis and the role of clocks in melatonin synthesis are not fully understood in the Atlantic salmon. There has however been some speculation as to why a lack of endogenous melatonin production in the salmonid pineal may have evolved. One hypothesis is that salmonids migrate long distances in order to return to their natal grounds, often experiencing extreme variations in photoperiod. The loss of endogenous melatonin production may have evolved as an adaptation to suit such environmental conditions (Iigo et al. 2007). However further work is necessary to unravel the mechanisms behind the circadian light axis in salmonid species.

5. CLOCKS, PNES AND PHYSIOLOGY

Mammals

Amongst mammals the effect of the PNES and clocks on physiology is evident. The regulation of seasonal reproductive physiology by melatonin and clocks is relatively well described. The importance of melatonin and clocks to human physiology is a subject that has received considerable attention in recent years, in particular with regard to disease and health. Various aspects of clock and PNES dysfunction have been shown to result in pathologies such as cancer, metabolic syndrome, type 2 diabetes, hypertension and a number of mood and cognitive disorders (Hardeland et al. 2012). With regard to the auto-regulatory feed back loop

that drives the molecular mechanism of the clock disruption of various elements can have a substantial impact on human physiology. For example the CLOCK protein appears to play a fundamental role in stimulating the cell cycle and is hypothesised to act in a potential tumour promoting manner (Hardeland et al. 2012). Furthermore, it has been hypothesised that the hypermethylation of the CLOCK promoter region may suppress the growth of tumours (Hoffman et al. 2010; Hardeland et al. 2012). More over disruption of the circadian system has previously been associated with the formation of tumours and incidence of cancer. In addition to clocks disruption of melatonin, a key component of the vertebrate PNES system can have a profound effect on physiology and disease. For example polymorphisms in the melatonin receptor MT2 have been associated with type two diabetics and disruption of MT1 has previously been associated with prostate cancer (Hardeland et al. 2012). Interruption of the melatonin system has additionally been associated with breast cancer, obesity and various forms of depression and mood disorder (Hardeland et al. 2012). In humans outside of the reproductive axis it is becoming increasingly evident that clocks and the PNES system have a profound effect on human physiology and disruption can result in profound health problems. In other vertebrates much of the research has focused on reproduction.

Teleosts

In teleosts both the PNES and molecular clocks have been shown to impact physiology, in particular with respect to reproduction. In addition to the effects of melatonin on reproduction, molecular circadian clocks have been implicated in the teleost PNES at different levels of organisation. As described above in non-salmonid teleosts the endogenous production of pineal melatonin in the isolated pineal is strongly suggested to be under the control of molecular clock work (Iigo et al. 2007). Moreover clock genes have been associated with maturation and reproductive processes. *Clock* has been mapped to

quantitative trait loci (QTL) regions for spawning time in rainbow trout. Additionally *Cry2b* and *Clock1b* were mapped to (QTL) regions for growth in the Coho Salmon. Length polymorphisms in *Clock1b* polyglutamine domains were found to be associated with differential migratory run times in the Chinook salmon (O'Malley & Banks 2008; O'Malley & Banks 2008). The gene is also implicated in the reproductive timing of a number of pacific salmon species (O'Malley et al. 2010). In Atlantic salmon *Bmal1* was found to be up-regulated in prematurely maturing males (Aubin-Horth et al. 2005). Finally a 2010 publication mapped *Clock1*, *Npas2* and *Clock3* to QTL life history regions in salmonids (Paibomesai et al. 2010). The role of clocks and clock mechanisms in salmonid maturation and reproduction will additionally be discussed further in the section below. In spite of the numerous publications linking clock genes to maturation, migration and reproduction and the clear control photoperiod exerts on salmonid life history events, the mechanisms linking clocks to seasonal processes are largely un-described in teleosts.

Kisspeptin also is likely to be of importance to the teleost PNES. In mammals kisspeptin is an important regulator of reproductive processes (Pinilla et al. 2012). In teleost the role of kispeptin appears to be relatively conserved (Migaud et al. 2010). In fish, kisspeptin has been associated with regulation of GnRH and the onset of puberty (Filby et al. 2008; Martinez-Chavez et al. 2008; Elizur 2009). In fish two KISS proteins and two GPR54 receptor proteins are present in fish, possibly as a result of the teleost genome duplication event (Felip et al. 2009; Mechaly et al. 2009). However further work is necessary to fully determine the role of the Kiss system in the teleost PNES.

As yet the teleost PNES has not yet been fully described. In a 2010 review Migaud et al proposed a potential model for the teleost PNES (Figure 7), although it is becoming apparent that the teleost PNES is more diverse than in mammals (Figure 8). In fish the pineal is photoreceptive to at least some degree and has the capacity to produce melatonin when

isolated in culture. In most fish the pineal organ appears to harbour the molecular clock work necessary for the endogenous production of pineal melatonin. Interestingly in salmonids this does not appear to be the case. The production of pineal melatonin does not persist in the absence of photic cues and appears to be decoupled from the circadian clock work. However the implications for this in the salmon PNES and subsequent impacts on physiology are unclear and require further investigation.

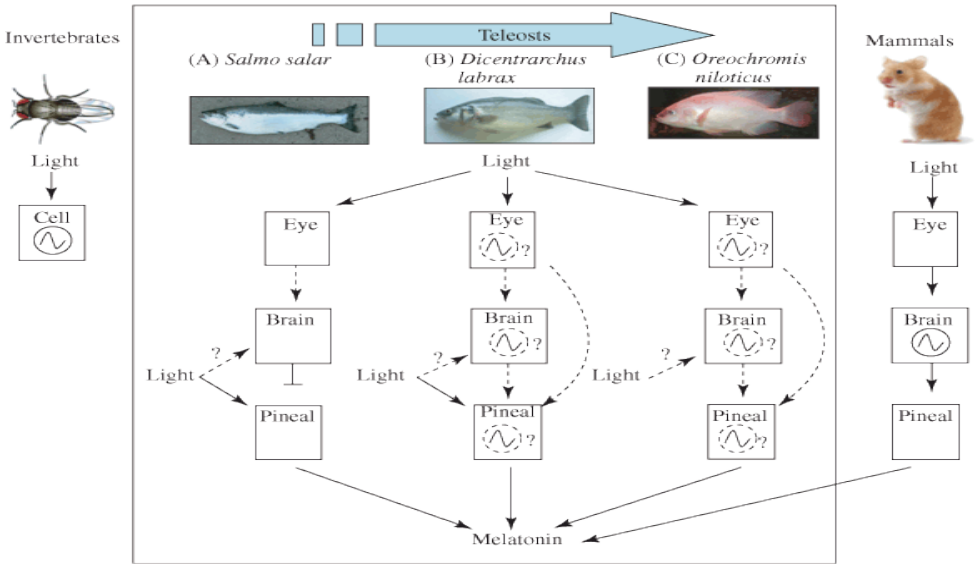


Figure 7. Suggested evolution of the regulation of pineal melatonin synthesis in teleosts. In addition to the two types of circadian organization already proposed in fishes (A and B), a third type could exist where pineal light sensitivity would be dramatically reduced (C). The regulation of pineal activity would have thus evolved from an independent light-sensitive pineal gland, without pacemaker activity, as seen in salmonids, *e.g. Salmo salar* (A), to an intermediary state where the pineal gland remains light sensitive and could possess a circadian pacemaker, but is also regulated by photic information perceived by the retina as seen in *Dicentrarchus labrax* (B) (and *Gadus morhua*), to reach a more advanced system closer to higher vertebrates where light sensitivity of the pineal gland would be significantly reduced and its melatonin synthesis activity primarily regulated by a circadian pacemaker (unknown location) entrained by photic information perceived by the retina, *e.g. Oreochromis niloticus* (C) from Migaud et al (2010).

6. PHOTOPERIOD AND SEASONAL REPRODUCTION

Mammals and Birds

In order to better understand the underling mechanisms driving the temporal control of seasonal process we must again look to mammalian and avian models, where the underlying mechanisms driving seasonal reproduction are becoming increasingly well understood. It is now evident that duration of photoperiod appears to be fundamental in the synchronisation of endogenous rhythms to external environmental conditions. Melatonin appears to be the link between clocks and seasonal reproduction in mammals (Yoshimura 2010; Dardente 2012).

Thyroid hormone metabolism in the brain is an integral part of the seasonal regulation of reproductive physiology (Figure 8) and is regulated by the seasonally changing melatonin signal in mammals (Dardente 2012). Melatonin binds to the high density of melatonin receptors present in the pars tuberalis (PT) of the pituitary and alters the expression of clock genes *per* and *cry*. However the removal of the thyroid in a number of mammals has been shown to block photoperiodic response; while response is reinstated by thyroxin administration (Yasuo et al. 2007). The production of thyroxin is induced by thyroid stimulating hormone (TSH) by cells in the PT and can be altered by seasonally shifting *Per* and *Cry* expression in these cells (Yasuo et al. 2007). TSH then acts on the ependymal cells in the hypothalamus to control the release of thyroxin. Circulating thyroxin T_4 displays very little biological activity (Dardente et al 2010). However when an iodine is removed, biologically active T_3 is formed via the activity of the deiodinase enzyme DIO2. Additionally, DIO3 catalyses the conversion of the active T_3 to the biologically inactive form of thyroxin (Lechan & Fekete 2005). DIO2 and DIO3 are under the control of external photoperiod via TSH, when external photoperiod is increasing the discharge of TSH from the PT cells of the pituitary is high and triggers conversion of T_4 to T_3 via the action of DIO2. Conversely under

short day photoperiod release of TSH is low and DIO3 is stimulated to convert T_3 to its inactive form (Lechan & Fekete 2005). The action of DIO2 and DIO3 on thyroxin pathway is highly conserved amongst vertebrates.

The role of deiodinases on the regulation is fundamental in the control of seasonal reproduction in vertebrates. Common to all the deiodinase genes a selenocysteine (sec) region is present. This changes the stop codons to a sec amino acid forming a selenocysteine interaction sequence (SECIS). This then functions as a binding site for a number of accessory proteins, this may have regulatory effects on the properties of the deiodinase genes (Arrojo & Bianco 2011). Moreover the deiodinases are homodimers and appear necessary for optimal catalytic activity (Arrojo & Bianco 2011). Importantly the DIO2 enzyme has a short half life. This is, in part, a result of the interaction with T_4 acceleration of ubiquitination and proteasome uptake, this in turn regulates the stability of T_3 (Arrojo & Bianco 2011). The residency of Diodinase 3 in the endoplasmic reticulum additionally contributes to the short half life of the enzyme (Arrojo & Bianco 2011). Consequently the properties of the deiodinase genes and enzyme regulate not only its own stability but also the stability of the thyroxin pathway.

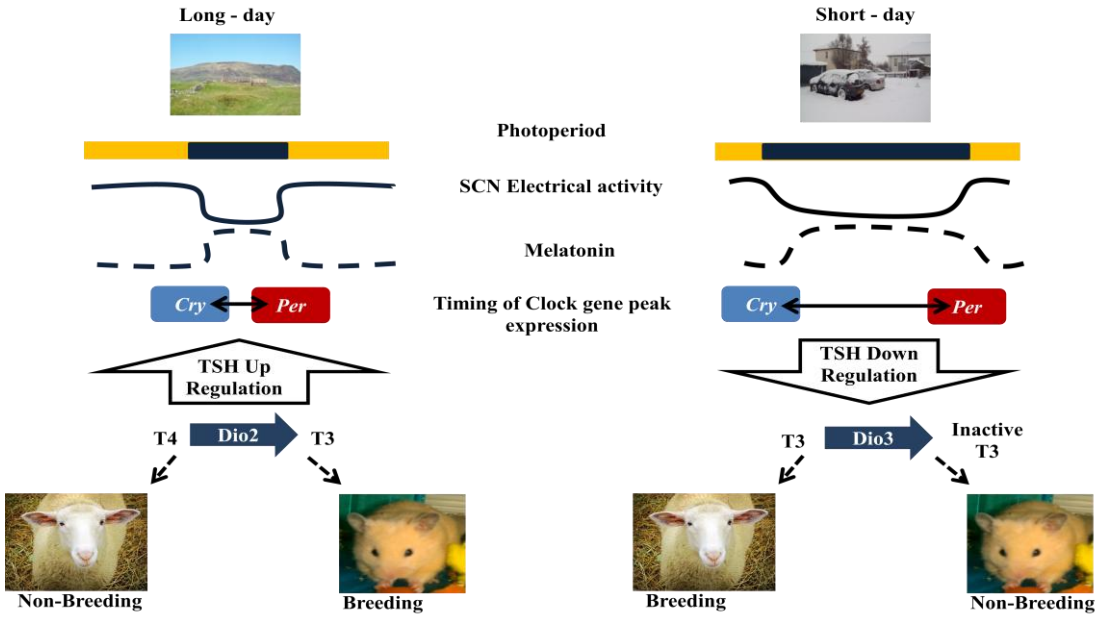


Figure 8: Schematic diagram of photoperiod regulation of seasonal reproduction in mammals adapted from Foster and Kreitzman et al 2009. Seasonal photoperiod information is perceived in the retina and transmitted to the SCN where electrical activity is adjusted to photoperiod. This regulates nocturnal melatonin synthesys so it reflects the nocturnal portion of the 24h cycle. The binding of melatonin to M1 melatonin receptors then alters the timing of clock gene expression in the pars tuberalis (PT) of the pituitary. While *Per* peaks around sunrise the expression of *Cry* follows the beginning of the nocturnal phase. The coincidence of *per/Cry* expression then regulates levels of thyroid stimulating hormone (TSH). Elevated TSH in turn stimulates *Dio2* expression and catalyses the conversion of T4 to the biologically active T3 resulting in breeding in long day breeders such as the hamster. Conversely the down-regulation of TSH results in the DIO3 mediated conversion of T3 to its biologically inactive for. While this suppresses breeding in long day breeders, in short day breeders such as the sheep breeding is stimulated.

As a result of their action on the thyroid hormone the deiodinases have a fundamental role in a number of biological processes, including development and metabolic functions. In mammals the role of deiodinase appears to be conserved between long day and short day breeders. *Dio2* mRNA is up-regulated under the long day photoperiod and down-regulated under short days. Similarly *Dio3* mRNA is up-regulated under short day and down-regulated when exposed to the long day photoperiod in a number of rodent species including the European hamster (*Cricetus cricetus*)(Hanon et al. 2010), Djungarian / Siberian (*Phodopus sungorus*)(Watanabe et al. 2007) and Syrian hamsters (*Mesocricetus auratus*)(Revel et al. 2006; Yasuo et al. 2007) and the photoperiodic strain(the wistar rat) of brown rat (*Rattus norvegicus)* (Yasuo et al. 2007). This has also been observed in lager seasonal mammals such as the sheep (Wagner et al. 2008). The seasonal expression of the deiodinases is largely governed by the actions of melatonin (Revel et al. 2006; Yasuo et al. 2007). In Syrian hamster under long day photoperiod the administration of melatonin before sunset resulted in the suppression of *Dio2* mRNA expression. This effect lasted for a further 2 day after administration (Yasuo et al. 2007), consequently demonstrating the control of melatonin on DIO2 enzyme and resulting influence on the thyroid hormones and the reproductive system. Although melatonin is not thought to be essential for the seasonal control of reproduction in aves, the expression of deiodinases and their actions on thyroxin are largely conserved in relation to mammals. Work primarily conducted on the seasonally breeding Japanese quail has demonstrated similar long day and short day expression and actions of DIO2 and DIO3 conversion of T4 to T3 (Yoshimura et al. 2003; Nakao et al. 2008; Yoshimura 2010). However the mechanism linking the deep brain photoreceptors, the seasonal clock in the MBH and the control of deiodinases on the reproductive system is largely unknown (Dardente. 2012). Deiodinases have previously been identified in a number of teleost species; however the majority of work has focused on its role in developmental processes as opposed

to seasonal control of thyroxin and reproductive processes. It is thought functions of deiodinases are similar to those in other vertebrates (Johnson & Lema 2011).

In mammals the function of clocks and melatonin in transmitting seasonal photic information through the photo-neuroendocrine system is largely conserved between long day and short day breeders. The reproductive process will be initiated at different time in different species, as a consequence of the differential use of temporally available resources and gestation period. As a result seasonal mammals are generally categorised as either long day or short day breeders with reproduction triggered by increasing or decreasing daylength respectively(Hazelrigg and Loudon 2008; Hanon et al 2010). Although the exact mechanisms are unclear it is thought that T3 regulates the GnRH neurons in the hypothalamus. In short day breeders such as sheep there is a high pulse of GnRH under a short day photoperiod stimulating the activation of the reproductive system. When exposed to long day photoperiod a long duration GnRH pulse is observed. As a result the pituitary is not stimulated to release the reproductive hormones. In smaller seasonal mammals, such as the hamster, breeding is stimulated by increasing daylength, as the gestation period is considerably shorter than in larger mammals (Hazelrigg and Loudon 2008). The GnRH pulse is high in the spring/summer and low during the winter months. Resulting in reproductive initiation as daylength is increasing. In both the long and short day breeders GnRH is released in seasonal pulses that via the portal blood supply act on the pars distalis (PD) of the pituitary. Here GnRH instigates the release of LH and FSH, that travel through the circulatory system and trigger the release of the sex steroids testosterone and oestrogen and activate reproductive activity (Hazelrigg and Loudon. 2008)

In many vertebrates seasonal reproduction is not simply triggered by external photoperiod, melatonin and the SCN, it has been hypothesised that seasonal physiology is also regulated by a circannual oscillator (Lincoln et al. 2005; Lincoln et al. 2005; Lincoln 2006;). Under

constant photic conditions the seasonal occurrence of reproductive physiology has been shown to persist for a number of years. Sheep maintained under 12L: 12D will still continue to display seasonal reproduction for a number of years in the absence of annual variations in external photoperiod (Lincoln et al. 2005). Circannual rhythms in spawning have also been observed in a number of teleost species including the rainbow trout (Bromage et al. 1988; Duston & Bromage 1988; Duston & Bromage 1991; Bromage et al. 2001) and seabass (Bromage et al. 2001; Prat et al. 1999). Despite evidence supporting the endogenous annual control of reproduction and other physiological the location of such a circannual oscillator is not currently known (Ikegami and Yoshimura 2012).

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Although the molecular and neuroendocrine mechanisms underlying the seasonal control of reproduction are becoming increasing clear in mammals and to a lesser extent in birds the situation is still unclear in teleosts. However, photoperiod manipulation has a profound effect on seasonal physiology and reproduction in some salmonids has been observed to cycle endogenously (Bromage et al. 2001). Within the salmon aquaculture industry photoperiod manipulations are utilised in three primary areas of commercial production: firstly to control the timing of spawning in broodstock, secondly to regulate the timing of smoltification and finally to suppress early maturation (Bromage et al. 2001). Under natural conditions Atlantic salmon broodstock will spawn as the daylength is decreasing, however the maturation process is triggered almost a year earlier after the winter solstice as daylength begins to increase (Bromage et al. 2001). Photoperiod manipulation can advance spawning by exposing fish to long day followed by short day photoperiods. A delay in spawning can also be induced by delaying exposure to the long day photoperiod (Bromage et al. 2001). The elongation and condensing of photoperiod is widely used to regulate spawning. Under ambient conditions juvenile Atlantic salmon undergo the smoltification process and are transferred to sea water

in the spring. After on growing in marine environment fish are ready for harvest around 18 months later in the late summer. However as a consequence of higher year round demand photoperiod manipulation in combination with temperature is utilised to produce smolts out of season (in autumn) that are ready for harvest in the winter over a year later. A compressed short day followed by long day photoperiod is applied in order to achieve this. Smolts produced in this way are termed 0+ or S0 (Duston & Saunders 1990; Duston & Saunders 1992; Duston & Saunders 1995; Duston & Saunders 1995). The maturation process is initiated after the winter solstice as daylength increases under ambient conditions. However in order to inhibit maturation and maximise growth and flesh quality constant light is administered earlier than the long day signal under natural photoperiod (Bromage et al. 2001). The addition of constant light implies the early onset of summer and maturation is initiated, however as individuals do not yet have the energy resources necessary for the maturation process, they do not mature. The earlier constant light is applied, the fewer number of individuals will mature (Bromage et al. 2001; Migaud et al. 2010). The use of photoperiod in the aquaculture is now commonplace however the underlying mechanisms are as yet not fully described. The Atlantic salmon is one of the only farmed animals in which photoperiod manipulation is central to the industries profitability. Yet in spite of the capacity for photoperiod to regulate physiology the underlying mechanisms need considerable further investigation.

7. CONCLUSIONS AND AIMS

While many aspects of salmon physiology and behaviour have been well studied there remains a lack of understanding of the mechanisms that regulate the robust temporal organisation of its physiology and behaviour. Based on work in a diverse range of organisms,

from mammals to plants, it is evident that the circadian clockwork that drives both circadian and seasonal rhythmicity is of fundamental importance to an organisms fitness, enabling the timing of essential daily and seasonal events to coincide with optimal environmental conditions. The body clock of Atlantic salmon and in fact most teleosts remain largely un-described. The core molecular mechanisms that drive the clocks are highly conserved amongst vertebrates; however investigations in the tropical zebrafish have demonstrated some important differences between clocks in fish and clocks in other vertebrates. Moreover in fish the presence of a master circadian clock has yet to be identified.

In some fish species it is hypothesised that the pineal may be the site of central circadian control as, unlike in mammals, the salmon pineal is photoreceptive and contains all the molecular components of the circadian clock. It is also the primary site of synthesis of circulating melatonin, the so called "timekeeping hormone". However the role of the pineal and the production of melatonin appear to differ between teleosts. In any case the fundamental differences between the mammalian and teleost circadian light axes and PNES make teleosts an interesting group to investigate.

Due to the gap in the knowledge on circadian and seasonal clocks in temperate teleosts the aim of the present thesis has been to further investigate various aspects of clock gene expression in the Atlantic salmon primarily in relation to seasonality. This thesis sets out to investigate various components of the Atlantic salmon clockwork mechanisms and PNES. These include the seasonal expression of clock genes and genes involved in mammalian seasonal regulation. The role of clocks in the pineal organ of the Atlantic salmon in comparison to the European seabass and finally how do clocks regulate circadian function such as the liver lipid metabolism. In order to achieve this project set out with a number of aims.

Seasonal melatonin and clock gene expression in the brain fin and liver of the Atlantic salmon (Chapter 3)

This chapter aimed to investigate how diel clock gene expression varies as a result of exposure to artificial long day, short day and 12L: 12D photoperiod in the brain, specifically how does expression differ between central and peripheral tissues, and, how are clock genes expressed under natural seasonal photoperiods over the course of a year?

- **Photoperiod regulation of *Deiodinase, Eyes Absent 3* and *Thyroid stimulating hormone beta* in the Atlantic salmon** (Chapter 4)

This chapter aimed to determine seasonal gene expression and identify elements of the molecular switch for photoperiod response, based on known elements in mammals. Specifically how are expression of these elements related to photoperiod?

- ***In vitro* and *in vivo* expression of clock genes and the endogenous production of pineal melatonin in the Atlantic salmon and the European seabass** (Chapter 5)

This chapter aimed to characterise functional clocks in the pineal of the Atlantic salmon. Are clock genes expressed in the pineal organ of the Atlantic salmon in vivo and in vitro? If so are they endogenous and entrainable by light? How does the expression of pineal clocks compare with other teleost species such as the European seabass?

- **Circadian Expression of Clock Genes, *Sterol Regulatory Element-Binding Proteins* and SREBP Targets in the Liver of the Atlantic salmon.** (Chapter 6)

This chapter aimed to characterise clocks and examine rhythmic gene expression in the Liver. What initial functional links can be made with clocks in peripheral tissues? Is there a correlation between clocks in the liver and the liver lipid metabolism?

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

In order to investigate the mechanisms underpinning Atlantic salmon *(Salmo salar)* circadian and seasonal physiology this thesis has investigated the photoperiod regulation of molecular clocks and seasonal physiology in the species. This chapter is a summary of the general materials and methods employed during the course of this work.

1. FISH STOCK AND HOUSING

Fish origin and housing

Fish all originated from Howietoun fishery (56.0728, -3.9532) (for further details see Table 1). In all experiments fish were held in flow-though tank systems ranging from 1-2m in diameter in fresh water and aproximatly 3m in diamater in marine systems (7 m^3). Freshwater rearing was performed at the Niall Bromage Freshwater Research Facilities (Institute of Aquaculture, Stirling, UK, 56: 02N) while the marine stages were housed at Machrihanish Marine Environmental Research Laboratories (Institute of Aquaculture, Machrihanish, UK 55.4333333 N, -5.75 W) (Table 1). All fish were maintained at ambient temperatures under a simulated natural photoperiod (SNP) unless otherwise stated. In chapter 3 experiment 2 fish were held under constant light (LL) from first feeding (March 2009) prior to exposure to SNP photoperiods in June 2009. In chapter 5 "Comparative study of clock gene expression and melatonin in the Atlantic salmon and European seabass pineal" experiments were also performed on European Seabass. Fish were obtained from CULMAREX (Aguilas, Murcia, Spain). Fish were housed at the Department of Physiology, Faculty of Biology, University of Murcia, Chronobiology laboratory at the Algameca naval station (37.6 N, -0.98333 W) near Cartagena where they were maintained under ambient environmental conditions before experimentation (Table 1).

In all experiments fish were sacrificed via a schedule 1 killing method. Salmon were exposed to a lethal anaesthesia (2-phenoxyethanol 1ml/L sigma) and subsequently decapitated. Similarly in seabass fish were euthanized using clove oil (Eugenol, Guinama, Valencia, Spain) dissolved in 10 ml of ethanol at a final concentration of 50 µl/l, followed by decapitation. All experiments were performed following review by the local ethical review committees and in the UK, complied with the Home Office Animal (Scientific Procedures) act 1986, UK.

Blood sampling.

Where blood samples were required, up to 1ml of blood was removed with a 1ml sterile syringe previously heparinised (heparin ammonium salt, 4mg/ml Sigma-Aldrich (Gillingham, UK*).* Samples removed during the dark were done so with the aid of a dim red head torch with minimal light exposure. Blood samples were maintained at 4^{o}C until centrifugation. In order to separate blood plasma, samples were centrifuged at 2500rpm for 15 minutes. Plasma was removed, frozen over liquid nitrogen vapour and stored at -70^{o}C for further analysis.

24 hour sampling and tissue dissection

In all experiments, with the exception of the *in vitro* seabass and salmon pineal culture, sampling was carried out over 24 hours. To characterise a diel cycle of expression, tissue samples were removed every 4 hours at seven time points over the 24h period. Sampling was carried out rapidly and tissues removed during the nocturnal portion of the 24h cycle were carried out under a dim red light.

For the dissection of the brain a section of the skull cap was removed above the brain and the whole brain including the pituitary was removed dorsally. For peripheral tissues the whole left pectoral fin was removed and approximately 100 mg of liver tissue was removed. All

tissue were instantly frozen over liquid nitrogen vapour after removal and stored at -70 ^{o}C for later analysis. In order to remove the salmon pineal *in vivo* a section of cranial tissue encompassing the pineal situated in the pineal cavity was removed. Tissue was then stored in RNA stabilisation solution (RNAlater® ©2011 Applied Biosystems) for 24h at 4^{o}C. With the aid of a dissection microscope pineals were delicately removed from surrounding tissue and instantly frozen over liquid nitrogen vapour before being stored at - 70 ^{o}C for later analysis.

Table 1. Origin and housing for fish used for all experimental chapters

Species	Experimental chapter	Origin	Year class	Housing
Salmo salar	• Seasonal Melatonin and Clock Gene Expression in the Brain, Fin and Liver of the Atlantic Salmon (Experiment 1, Chapter 3) • Photoperiod Regulation of *Dio1-3*, *Eya3* and *Tshβ* in the Atlantic Salmon (Chapter 4) • Circadian Expression of Clock Genes, *Srebp* and SREBP Targets in the Liver of the Atlantic Salmon (Chapter 6)	Howietoun Fishery	Jan 08	• Niall Bromage freshwater research facilities (Institute of Aquaculture)
Salmo salar	• Seasonal Melatonin and Clock Gene Expression in the Brain, Fin and Liver of the Atlantic Salmon (Experiment 2, Chapter 3)	Howietoun Fishery	Jan 09	• Niall Bromage freshwater research facilities (Institute of Aquaculture) • Machrihanish Environmental Research Laboratories (Institute of Aquaculture)
Salmo salar	• Comparative study of clock gene expression and melatonin in the Atlantic salmon and European seabass pineal (*S. salar in vitro*)(Chapter 5)	Howietoun Fishery	Jan 09	• Niall Bromage freshwater research facilities (Institute of Aquaculture)
Salmo salar	• Comparative study of clock gene expression and melatonin in the Atlantic salmon and European seabass pineal (*S. salar in vivo*)(Chapter 5)	Howietoun Fishery	Jan 10	• Niall Bromage freshwater research facilities (Institute of Aquaculture)
Dicentrarchus labrax	• Comparative study of clock gene expression and melatonin in the Atlantic salmon and European seabass pineal (*D. labrax in vitro*) (Chapter 5)	CULMAREX, Aguilas, Murcia, Spain	2010	• Chronobiology Laboratory at Algameca naval station (Department of Physiology, Faculty of Biology, University of Murcia)

2. ORGAN CULTURE

In Chapter 5 "Comparative study of clock gene expression and melatonin in the Atlantic salmon and European seabass pineal." the *in vitro* performance of isolated pineal organs were studied as follows.

Collection of the pineal organs

Atlantic salmon parr housed at the Niall Bromage Freshwater Research Facilities under ambient temperature conditions (2.2 ± 0.1 ^{o}C) were acclimated to 12L:12D photoperiod with the light phase running from 07:00 to 19:00 daily. After 4 weeks ~70 salmon parr, for the 12L:12D pineal culture, were sacrificed using a schedule 1 killing method as described above. Pineal organs were dissected out of the pineal cavity from a section of skull and tissue removed from the head above the brain with the aid of a dissection microscope and light. Once dissected out, isolated pineals were maintained (35 pineals in 100 ml of culture medium (see below)) at 8^{o}C for a maximum of three hours until placed under culture conditions. The same protocol was used for the 12D:12L and 24hD pineal cultures however 140 fish were used for each of these cultures.

For a comparative study in European seabass approximately 70 seabass (169.9 ± 10.6 g) were acclimated for 2 weeks at the Department of Physiology, Faculty of Biology, University of Murcia Chronobiology laboratory at the Algameca naval station under ambient temperature conditions (16 ^{o}C) to 12L:12D (lights on 06:00, lights off 18:00). After acclamation fish were sacrificed using a schedule 1 killing method as described above. Pineals were removed dorsally by thinning the tissue and bone around the pineal window then carefully removing the whole pineal with the aid of a dissection microscope. Isolated pineal organs were then maintained in culture medium (35 per 100 ml) until culture conditions were established.

Culture medium

Both salmon and seabass pineal cultures were carried out in light and temperature controlled chamber using RPMI – 1640 without phenol red culture medium (Sigma ref R8755-10X1L, Gillingham, UK). Medium was supplemented with 4.77 g Hepes Sodium salt per litre, 10mg/L Penicillin-streptomycin (Sigma-aldrich, Gillingham, UK) solution and 5 mg/L Fungizone (amphotericn # B from Streptomyces Sp) (Sigma-aldrich, Gillingham, UK) to avoid fungal and bacterial growth. All medium was made with distilled water and pH was adjusted to 7.4. Media was sterile filtered to 0.4 µm with a pneumatic pump into a sterile bottle and stored at 4 ^{o}C for no more than 3 days to avoid contamination. Before use the required volume of medium were warmed to culture temperature before being introduced to the cultured organs.

Culture conditions (temperatures and three photoperiods LD, DL and DD)

All pineal culture experiments were preformed in static organ culture. Atlantic salmon culture experiments were carried out at 8 $^{\circ}$C. Seabass culture was conducted at 18 $^{\circ}$C. In both species the 12L:12D pineal cultures were carried out as follows. Seventy pineal were divided into seven (one per time point) glass vials, each containing 20 ml culture medium and 10 pineal organs under a sterile nylon fine mesh. For 12D:12L reversed photoperiod and 24h dark photoperiod 140 pineal organs were used for each as culture was extended from 48h to 96h with two 24h sample cycles (Figure 1).

Sampling and medium exchange

In all pineal culture experiments pineal organs were maintained in culture for 24 h prior to sampling. In the initial acclamation period 15 ml of the 20 ml total culture medium was exchanged every 6 h. During sampling periods pineals were instantly removed and frozen over liquid nitrogen vapour while culture medium was frozen for melatonin analysis and at

the remaining time points culture medium was exchanged every four hours. A 12L:12D photoperiod (the same period to which the experimental source fish were initially exposed) was used in the salmon and seabass 12L:12D experiments. (Figure 1a). For the 12D:12L salmon pineal culture the photoperiod was reversed during the first nocturnal phase of the culture (Figure 1b). Similarly in the 24h Dark experiment the photoperiod was switched to constant darkness during the first nocturnal phase of the 24h cycle and maintained for the duration of the experiment (Figure 1c). Once removed from culture pineal tissue and culture medium were frozen at -70^{o}C until RNA extraction and melatonin radioimmunoassay was performed.

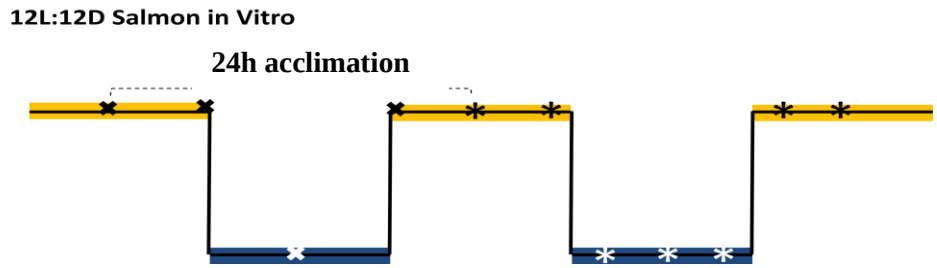
3. MELATONIN RADIOIMMUNOASSAY (RIA)

Levels of circulating melatonin in blood plasma (Chapters 3 and 5) as well as melatonin released by isolated pineal glands into culture media (Chapter 5) were measured by radioimmunoassay. The assay protocol has previously been validated by Migaud et al (2007) however in-order to ensure measured levels stayed within the functional range of the assay samples had to be differentially diluted. For blood plasma samples were diluted 1:2 with assay buffer (see below) while for culture media samples were diluted 1:10 with assay buffer.

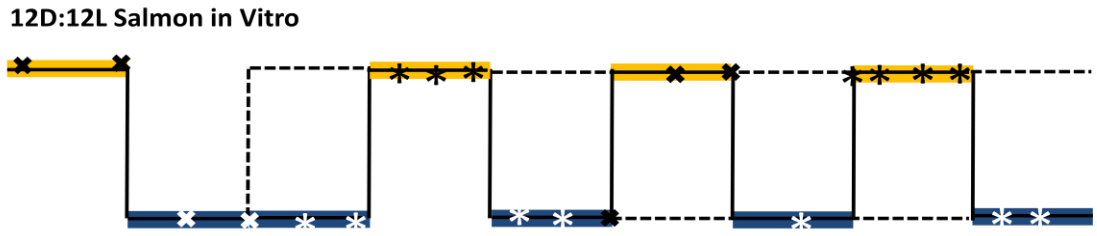
Buffer

The buffer for the melatonin RIA was used not only to dilute samples but also to dilute the ^{3}H (tritium) melatonin label and antibody. Buffer consisted of 2.688 g Tricine [N-Tris (hydroxymethyl)methylglycine] (Sigma-aldrich, Gillingham, UK), 1.350 g sodium chloride (NaCl) (Sigma-aldrich, Gillingham, UK) and 0.15 g of gelatine (Sigma-aldrich, Gillingham, UK). This was dissolved in 150 ml nanopure H_2O (DNA and RNA free sterile H_2O). Once all components were dissolved, buffer was stored at 4 $^{\circ}$C until use.

A.



B.



C.

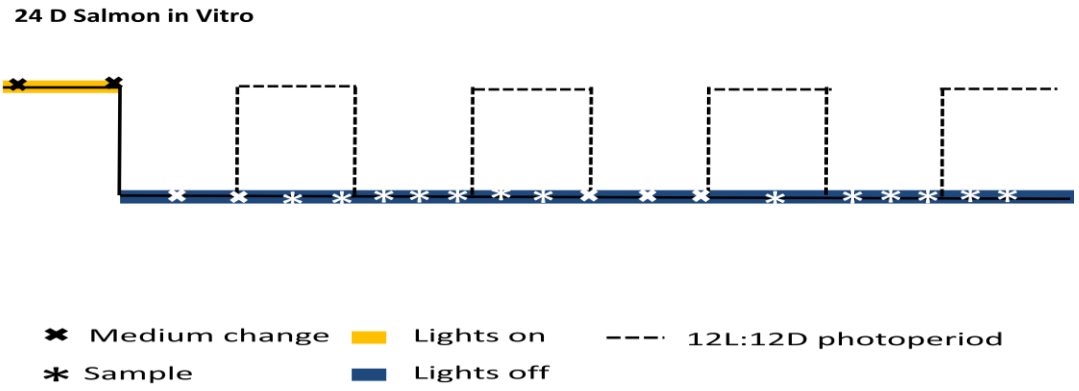


Figure 1: Experimental photoperiods of the salmon and seabass 12L:12D culture (A) reversed photoperiod 12D:12L salmon culture (B) and 24h dark salmon culture (C). The figure also shows times of medium changes and sampling times.

Antibody

A freeze dried sheep anti-melatonin antiserum (Stockgrand Ltd, Surrey, UK) was rehydrated in 2 ml of nanopure H_2O. This was then aliquoted into 100 µl samples and stored at -20 °C. One 100 µl was used per assay and diluted in 19.9 ml assay buffer.

Radiolabel

From an initial stock of tritated melatonin [*O*-methyl -^{3}H] melatonin (Amersham Pharmacia Biotech, UK Ltd, little Chalfort, UK) an intermediate stock consisting of 20 µl in 2 ml of ethanol was created and stored at -20 °C. For each assay preformed a new working dilution of the radiolabel were made from 16 µl ^{3}H intermediate stock in 10ml of buffer.

Standards

A 1mg/ml stock of melatonin standard solution (10 mg melatonin in 10 ml absolute ethanol) was used to generate 4 standards (A – D)

Composition of standards of melatonin RIA:

Standard		Standard concentration
A	100 µl (1 mg/ml) + 9.9 ml buffer	10 µg/ml
B	100 µl (10 µg/ml = A) + 9.9 ml buffer	100 ng/ml
C	100 µl (100 ng/ml = B) + 9.9 ml buffer	1 ng/ml
D	100 µl (100 ng/ml = B) + 4.9 ml buffer	2 ng/ml

A standard curve using standards D and a serial dilution from C was then generated in duplicate in 22 tubes (Table 2). Duplicate line standards were also used to calculate assay variability.

RIA protocol

The standard melatonin RIA assay is carried out over 2 days.

Day 1 –

- All samples and standards were diluted to 500 µl with the addition of 250 µl of buffer and vortexed to mix.
- 200 µl of antibody was added to all tubes except the non specific binding (NSB) tubes. Samples were mixed by vortexing and incubated at 20 $^{\circ}$C for 30 minutes.
- 100 µl of ^{3}H radiolabel was added to all tubes, vortex all samples and incubate at 4 $^{\circ}$C for 18 hours
- In addition to standards and samples, 100 µl of ^{3}H was added to the totals in to 2 scintillation vials with 4ml scintillation fluid and vortex to mix.

Day 2 –

- Dissolve 0.48 g dextran coated charcoal in 50ml buffer and stir on ice for 30 minutes
- 500 µl of charcoal solution was added to each tube. Tubes were vortexed to mix and incubated at 4 $^{\circ}$C for 15 minutes.
- Samples were then centrifuged at 2000 RPM at 4 ^{o}C for 15 minutes.
- 1ml of supernatant was then transferred to scintillation vials with 4ml of scintillation fluid including one blank.
- Samples were vortexed to mix and radio activity was counted for 10 minutes in a scintillation counter.

Table 2: Composition of Melatonin RIA standard curve.

Tube number	Standard conc	+ 250□l	+ buffer μl
1 & 2	500	Standard D	none
3 & 4	250	Standard C	none
5 & 6	125	Standard C	250
7 & 8	62.5	5 & 6	250
9 & 10	31.3	7 & 8	250
11 & 12	15.6	9 & 10	250
13 & 14	7.8	11 & 12	250
15 & 16	3.9	13 & 14	250
17 & 18	1.95	15 & 16	250(mix & remove 250)
19 & 20	0.0	None	250
21 & 22	None specific binding	None	450

Analysis

Results from the unknown samples were compared to the standard curve and concentration of melatonin in pico grams (pg) per tube was then calculated using Riasmart software (Perkin Elmer, Waltham, Massachusetts, USA). Results were then converted to pg per ml of blood plasma or culture media.

4. MOLECULAR EXPRESSION ANALYSIS

In Chapters 3, 4, 5 and 6 the mRNA expression of a number of different targets were measured using either Microarray (Chapter 4) or quantitative Real-time PCR (qPCR). The

analytical approaches used to isolate and identify targets of interest and thereafter quantify expression are summarised below.

RNA EXTRACTION, DNase TREATMENT AND cDNA SYNTHESIS

RNA Extraction

For all brain, fin and liver samples approximately 100 mg of tissue was homogenised in 1 ml in guanidinium/phenol extraction reagent (TRIzol®;Invitrogen UK), according to manufactures instructions. The homogenised samples were then centrifuged at 12,000 g at 4 ^{o}C for 10 minutes in order to separate tissue debris. The supernatant was removed into a fresh DNA and RNA free eppendorf tube and incubated at room temperature for 5 minutes. 100 µl of 1-Bromo-3-chloropropane (BCP) (Sigma-Aldrich, Gillingham, UK) was added to each sample and samples were vortexed to mix and incubated at room temperature for 10 minutes. RNA extractions were then centrifuged for 15 minutes at 4 ^{o}C at 12,000 g. The clear aqueous layer was removed into a fresh eppendorf and totRNA was precipitated from sample by adding 500µl of isopropanol and vortexed to mix. Extractions were then incubated at room temperature for 10 minutes to precipitate RNA. Extractions were centrifuged at 4 ^{o}C, 12,000 g for a further ten minutes (RNA pellet should be visible at this point). The remaining liquid was then removed and discarded while the RNA pellet was then washed in 75 % ice cold ethanol and pellet air dried. RNA pellets were then rehydrated in an appropriate volume of nanopure H_2O to achieve a concentration ≤ 1000 µg/µl.

In the case of the pineal RNA extractions individual tissues were homogenised in 500 µl of TRIzol®. The remainder of the extraction was carried out at half the reaction volume. However during RNA precipitation step samples were precipitated overnight at -20 ^{o}C. Reactions were then centrifuged for 30 minutes and RNA pellet was washed in 75% ice cold ethanol. Pellet was rehydrated in 12 µl nanopure H_2O.

RNA Quality control

Total RNA concentration and quality was determined using a ND-1000 Nanodrop spectrophotometer (Labtech Int., East Sussex, UK). When the 260/280 ratio was between 1.8 and 2.0 the RNA is considered to be pure. Furthermore in samples with a sufficient excess volume of total RNA 1μg was analysed on a 1 % agarose denaturing RNA gel electrophoresis (Figure 2).

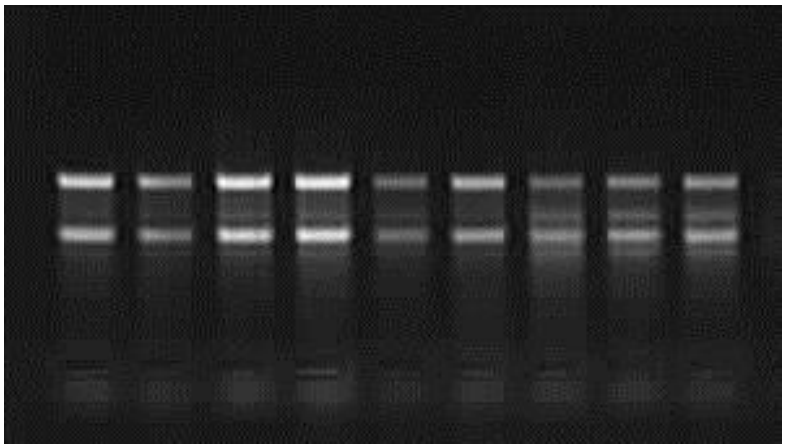


Figure 2: Typical example of mRNA quality assessment by visualising the quality of the ribosomal RNA bands in a 1 μg totRNA sample on 1 % agarose denaturing RNA gel.

DNase treatment

After the totRNA was extracted and the quality assessed, all samples were DNase treated in order to eliminate genomic contamination using a commercial kit (DNA-free™: Applied biosystems, UK). 5 μg Brain, Fin and Liver RNA in 19.5 μl was combined with 2.5 μl of reaction buffer and 0.5 μl of DNAfree enzyme in each reaction and gently mixed and samples were incubated at 37 ^{o}C for 27 minutes. After incubation reactions were immediately placed on ice. 2.5 μl of the resuspended DNase inactivation reagent was then added to each sample to give a total volume of 25 μl. Each reaction was mixed by vortex and incubated at room temperature for 2 minutes while continuously mixing. Reactions were then centrifuged for 1.5 minutes at maximum speed. The supernatant was then removed into fresh eppendorf.

Final DNase treated RNA was quality and concentration was analysed by nanodrop spectrophotometer and stored at -70 ^{o}C, just as totRNA.

In the case of the pineal the entire volume of totRNA was DNase treated. 1 µl of buffer and 0.5 µl DNAfree enzyme was added to each reaction. The reaction was carried out as above with using 2 µl of inactivation reagent.

cDNA synthesis

cDNA was then reverse transcribed from DNase treated RNA using a high capacity reverse transcription kit without RNase inhibiter (Applied biosystems, UK). RNA was diluted to 10 µl. 10 µl of master mix (see below) was then added to each reaction.

cDNA master mix

- 4.2 µl H_2O
- 2.0 µl 10 x Buffer
- 0.8 µl 25 x DNTP mix (100 mM)
- 2.0 µl 10 x RT Random primers
- 1.0 µl MultiScribe™ reverse transcriptase 50 U/µL

The 20 µl reaction is then incubated in a thermocycler at 25 ^{o}C for 10 minutes followed by 37 ^{o}C for 120 minutes and 85 ^{o}C for 5 minutes. Samples were then diluted as described in Table 3. In addition to cDNA to be synthesised for qPCR, cDNA was also generated to test primers and for general use. Reactions were carried out as described above and diluted 1:10 to a final volume of 200 µl. All cDNA samples were then stored at -20 ^{o}C.

Table 3: Concentration of totRNA used for cDNA synthesis for qPCR experiments

Tissue/ Experiment	**Concentration of RNA used**	**Dilution with H2O**	**Total volume (μl)**
Brain (Chapter 3, Experiment 1 & Chapter 4)	1 μg	1/10	200
Fin (Chapter 3, Experiment 1)	1 μg	1/10	200
Liver (Chapter 3, Experiment 1 & Chapter 5)	1 μg	1/10	200
Brain (Chapter 3, Experiment 2)	1 μg	1/5	100
Pineal (LD salmon *In vitro*)(Chapter 5)	500 ng	1/2.5	50
Pineal (DL and DD salmon *In vivo*) (Chapter 5)	1μg	1/5	100
Pineal (salmon *in vivo*) (Chapter 5)	1μg	1/5	100
Pineal (LD Seabass *in vitro*)(Chapter 5)	500ng	1/2.5	50

5. GENE DISCOVERY AND BIOINFORMATICS

For the majority of genes investigated sequences had previously been identified and reported. primers pairs were used (Table 4). However for Atlantic salmon it was necessary to identify, clone, sequence and design appropriate qPCR primers for *Aanat2*, *Dio2*, *Per1* and *Rev-erb* α (Table 5)

Table 5: All genes investigated with full name and sequence source.

Species	**Short name**	**Full name**	**GenBank accession number**
S.salar	*Aanat2*	*arylalkylamine N-acetyltransferase 2*	Unpublished
	β-Actin	*Beta - Actin*	AF012125
	Clock	*Circadian Locomotor Output Cycles Kaput*	CA038738
	Bmal	*Brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like*	DY735402
	Cry2	*Chryptochrome 2*	DY730105
	D6 Fad	*Delta 6 fatty acyl desaturase*	AY458652
	Dio1	*Type 1 Iodothyronine deiodinases*	EG868394
	Dio2	*Type 2 Iodothyronine deiodinases*	Unpublished
	Dio3	*Type 3 Iodothyronine deiodinases*	DW562425
	EF- α	*Elongation factor 1-alpha*	NM_001141909
	Eya 3	*Eyes Absent 3*	CU071998
	ElovL5	*Elongation of very long chain fatty acids protein 5*	AY170327
	Fas	*Fatty acid synthase*	DW551395
	Hmgcr	*HMG-CoA reductase*	DW561983
	Lxr	*Liver x receptor*	FJ470290
	Per1	*Period 1*	Unpublished
	Per2	*Period 2*	FM877775
	Rev-erb α	*nuclear receptor subfamily 1, group D, member 1*	Unpublished
	Srebp 1	*Sterol Regulatory Element-Binding Protein 1*	TC148424
	Srebp 2	*Sterol Regulatory Element-Binding Protein 2*	TC166313
	Tsh β	Thyroid stimulating hormone, beta	NM_001123528
D. labrax	*Β Actin*	*Beta - Actin*	AJ537421
	Aanat2	*arylalkylamine N-acetyltransferase 2*	European seabass genome project
	Clock	*Circadian Locomotor Output Cycles Kaput*	Provided by University of Murcia
	Per1	*Period 1*	GQ353293

Gene discovery

Although sequences were available and primers were previously designed for the majority of clock genes investigated (Davie et al 2009) some target genes had not been previously reported therefore it was necessary to identify *Aanat2*, *Dio2 per1 and Rev- erb* α in the Atlantic salmon. In order to identify new genes, first we had to perform *in silico* sequence analysis on the same gene in a number of teleost species. Sequence information from a number of teleosts were aligned and compared in BioEdit v7.1.3 (Ibis Biosciences). In BioEdit conserved regions between different teleost species were identified. BLAST searches were performed against salmon ESTs . In 2010 the first draft Atlantic salmon Genome sequence became available, although it was too late to help with initial gene discovery work however it did enhance subsequence *in silico* analysis (Davidson et al. 2010). From the available sequence information various primer pairs were designed in Primer Select as part of Lasergene® core suite applications (DNASTAR). Primers are then tested by PCR on appropriate cDNA. PCR products are then sequenced directly for smaller products or, for longer products the PCR reaction is cloned and the plasmid is consequently sequenced. TBlastX (NCBI http://blast.ncbi.nlm.nih.gov) was then used to confirm the identity of sequence. This process is fully described below in the description of the identification of individual genes.

Arylalkylamine N-acetyltransferase-2 (*Aanat 2*) (Chapter 5).

Aanat2 sequence information for a variety of teleosts was acquired from NCBI search (National Centre for Biotechnology Information http://www.ncbi.nlm.nih.gov/) (Table 6). Sequences information was compiled in BioEdit with the addition of a number of predicted salmon sequences generated from salmon expressed sequence tag (EST) database ASalBase (http://www.asalbase.org/sal-bin/index) (Table 6). A number of primer pairs were then designed to amplify the rainbow trout *Aanat2* mRNA sequence (accession no

NM_001124257.1), and a salmon *Aanat2* theoretical mRNA contig from a salmon EST contig cluster, and genomic sequence (Table 6). Primer locations were designed with regard to the additional teleost sequence information. Primers designed were then tested by PCR on pineal and brain cDNA. Amplicons were cloned and sequenced as described below. Gene identity was established by the alignment of *Aanat2* sequences in clustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The salmon cDNA fragments displayed an identity score of 96% with the rainbow trout mRNA. Salmon-specific qPCR primers for *Aanat2* were designed to amplify a sub-fragment using Primer Select (Lasergene® DNASTAR).

In order to establish sequence identify for Atlantic salmon, *Aanat2* primer pairs (Table 7) were tested by PCR. PCR products were then cloned and sequenced in order to generate standards for each qPCR assay. Partial cDNA sequences were generated by PCR using 0.5 µM of primers (Eurofins MWG Operon, Edersberg, Germany) (Table 7) one fortieth of the original cDNA synthesis reaction, Klear Taq polymerase with supplied buffer (Kbiosciences, UK), and 1 mM MgCl2 in a final volume of 20 µl using a routine PCR strategy: 15 min 95 °C followed by 30 cycles of 95 °C 20 s, X °C 20 s, 72 °C 1 min. The annealing temperature is denoted as X °C in the description as it varied with the different primer pairs (Table 7). All primer pairs generated a single PCR product and those products used for qPCR standards were cloned into a pGEM-T Easy vector (Promega, UK) and sequenced (CEQ-8800 Beckman Coulter Inc., Fullerton, USA). The identities of the cloned PCR products were then verified (100% overlapping) using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Sequencing was performed using a Beckman 8800 autosequencer. Lasergene SEQman software (DNASTAR, www.dnastar.com) was used to edit and assemble DNA sequences. ClustalW was used to generate multiple alignments of deduced protein sequences (Thompson

et al.. 2000) MEGA version 4 was used to deduce and bootstrap phylogenetic trees using the neighbour joining method (Saitou & Nei 1987; Tamura et al. 2007).

Table 6: Available teleost *Aanat2* sequences and Atlantic salmon ESTs utilised for the generation of a salmon *Aanat2* partial sequence.

Species (Latin)	**Species (Common)**	**Ascension number**	**Source**
Danio rerio	Zebrafish	NM_131411.1	NCBI
Carassius auratus	Gold fish	GU205782.1	NCBI
Oryzias latipes	Medaka	NM_001104846.1	NCBI
Solea senegalensis	Senegalese sole	GQ340973.1	NCBI
Paralichthys olivaceus	Olive flounder	HQ883478.1	NCBI
Oncorhynchus mykiss	Rainbow trout	NM_001124257.1	NCBI
Scophthalmus maximus	Turbot	EF033250.1	NCBI
Sparus aurata	Gilt head seabream	AY533403.2	NCBI
Esox lucius	Pike	AF034082.1	NCBI
Salmo salar	Atlantic Salmon	Cluster ID# 3912632	ASalBase
Salmo salar	Atlantic Salmon	Cluster ID# 3920741	ASalBase
Salmo salar	Atlantic Salmon	S0250N08SP6	ASalBase

Table 7: *Aanat2* primer pairs, sequences, and location on rainbow trout partial sequence.

Primer	**Region**	**Location on *O. mykiss***	**Primer sequence 5' - 3'**	**Annealing temperature °C**
***Aanat2* A**	ORF	372bp - 1040bp	F: AGGTCAGCCGCTCTCCGTTCC	60
			R: CCAGTGCTAGGGTTGATGTGATTATGA	
***Aanat2* B**	ORF + 3'	373bp - 1620bp	F: GGTCAGCCGCTCTCCGTTCCT	61
			R: TGGTGCTGCAGCTGAGATTGATGG	
***Aanat2* C**	ORF + 3'	373bp- past end of RT sequence	F: GGTCAGCCGCTCTCCGTTCCTC	62
			R: CTGCAGCGCCTCAATGACAAAGTG	
***Aanat2* D**	5'+ ORF (partial)	115bp- 796bp	F: AGACAGGCAGATAGAAAGCACAGAGCA	61
			R: CAGGTAGCGCCACAGCAGGATG	
***Aanat2* E**	ORF(partial)+ 3'	771bp - 1566bp	F: TCAGCCCAGTAAGTGACCATCATGACACAT	63
			R: GTTGCAACCTGGTCTGGACGGTCAAC	

Type II iodothyronine deiodinase *(Dio2)* (Chapter 4)

Identification of the Atlantic salmon *Dio2* was based on the published rainbow trout *Dio2* sequence (AF207900) (Sambroni et al. 2001). BLAST analysis identified two salmon expressed sequence tags (EST's) (GE782599 and DY713483) aligning to the published rainbow trout sequence. Specific Atlantic salmon *Dio2* primers were then designed on the salmon EST's with reference to the rainbow trout sequence (Primer Select Ver.6.1 DNASTAR Lasergene, www.dnastar.com) (Table 8).

Primers were tested on various Atlantic salmon cDNA samples using PCR with SuperTaq™ Plus (Ambion, Applied Biosystems, Warrington, UK) producing a product size of approximately 2Kb. Several PCR Atlantic Salmon *Dio2* reactions were cloned using illustra GFX PCR DNA and GelBand Purification Kit (GE Healthcare) according to manufactures instructions. The purified product was then added to a ligation reaction at ratio of 3:1 insert to vector using a pGEM®-T Easy vector system (promega). Colonies were grown on LB/ampicillin/IPTG/ X-gal plates. Plasmids were prepared using a GenElute™ Plasmid Miniprep Kit (Sigma Aldrich, Gillingham, UK) plates. Plasmids were sequenced Beckman 8800 auto sequencer. Sequence results were then analysed using SEQman as part of Lasergene software package (DNASTAR). Due to the size of the product further sequencing runs were required on the clones to obtain a complete sequence read. This was achieved using two additional primer pairs, (*Dio2*seqaF/R, *Dio2*seqbF/R)(Table 8), and aforementioned plasmid to gain 2kb salmon *Dio2* sequence. Percentage identities of the partial sequence with that of *Dio1*, *Dio2* or *Dio3* in other vertebrates were obtained by performing a BLAST analysis. The partial sequence was copied into BioEdit (http://www.mbio.ncsu.edu/), where ClustalW analysis was executed to generate multiple alignments with *Dio* genes of other vertebrates. MEGA Ver.4.1 (http://www.megasoftware.net/) was used to deduce a phylogenetic tree using the neighbour joining method. qPCR primers (Table 8) were designed

within this partial sequence using Primer select also as part of the Lasergene software package (DNASTAR). ClustalW was used to generate multiple alignments of deduced protein sequences (Thompson et al. 2000). MEGA version 4 was used to deduce and bootstrap phylogenetic trees using the neighbour joining method (Saitou & Nei 1987; Tamura et al. 2007).

Table 8: Primers sequences used (5'-3')and annealing temperatures. For PCR and qPCR assays, including primer pairs for *Dio2* identification.

Primer Name	**Primer sequence Forward 5'-3'**	**Primer sequence reverse 5'-3'**	**Anneal °C**
Dio2	GGCAGCGCATGCTGACCTCG	ACCAGCCCCGTCTCGACCCA	62
Dio2seqa	CCATGGGCCCGTGCTCCTT	CATGTGGCGTAAGTCTGGGTTGCT	65
Dio2seqb	AACGTGGGCCTACGGCGTGT	TGCTGTGCCTTGCTCTACGGCT	65
Dio2qPCR	GGACGAGTGCCGCCTGCTGGACTT	GAAGGCGGGCAGGTGGCTGATGA	68

Period 1 *Per1* (Chapter 3, 5 and 6)

The Atlantic salmon *Per1* assay was designed as follows: sequence information from a variety of teleost *Per1* sequences were assembled in BioEdit and sequences were aligned using Clustal W 4. Primers were designed in conserved regions of the coding sequence of medaka (NM_001136520), seabass (GQ353293), partial rainbow trout CDS(AF228695), and construct of salmon ESTs (DW576689, DW584143, DY698298) from NCBI (National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov). PCR primer pairs (Table 9) were designed in the coding sequence. The product generated was cleaned using illustra GFX PCR DNA and GelBand Purification Kit (GE Healthcare) according to

manufactures instructions and then cloned into the pGEM®-T Easy vector system (Promega, Southampton, UK). Plasmids were harvested from discrete colonies using a GenElute™ Plasmid Miniprep Kit (Sigma Aldrich, Gillingham, *UK)* and the presence of an insert checked by enzymatic digestion (ECoR1, Invitrogen Paisely, UK). Plasmids with the correct sized insert were sequenced through a Beckman 8800 auto sequencer and sequence results were then analysed using SEQman as part of Lasergene software package (DNASTAR, Madison, Wisconsin, USA). qPCR primers were designed within this partial sequence using Primer select also as part of the Lasergene software package (DNASTAR Madison, Wisconsin, USA). ClustalW was used to generate multiple alignments of deduced protein sequences (Thompson et al. 2000). MEGA version 4 was used to deduce and bootstrap phylogenetic trees using the neighbour joining method (Saitou & Nei 1987; Tamura et al. 2007).

Table 9: PCR primer names and sequences used for Per1 identification.

Primer Name	Forward	Reverse
RTper1 pub	AGAGCCCATCCCCACCCAGCAGTT	TCGGCCCGTCAGGAAGGAGA
1Per1	CTGCTGTCGACCAGCTCGGAGCACGAC	GGCGCCAAAAGCTCCGAAAACATGGTG
2Per1	CTGCGCTGCAAGCCAGAGCGTCTCC	GCAGCTGGGAGTGTGACTGGTGGTGAAGAT
3Per1	GCCCTGCCGCCTGCCCAACTG	GCGCCCGACGATAAACGCCACCTTC

Nuclear receptor subfamily 1, group D, member 1 *Rev-erb1α* (Chapter 4)

Rev-erb1α was identified as follows: two Atlantic salmon expressed sequence tag clones (Genbank ID: DY724083 and DY731913) were identified by BLAST analysis of published

vertebrate *rev-erb1α* sequences. 5' and 3' ends from the constructed contig were amplified using Rapid Amplification of cDNA Ends (RACE)-PCR with the RACE cDNAs generated from 1 μg of salmon whole brain total RNA as described in the manual using the SMART™ RACE kit (Clontech, USA). The 5' and 3' RACE amplicons were generated by two rounds of PCR using *Rev-erb* 5'R1 and *Rev-erb* 5'R2 primers or *Rev-erb* 3'F1 and *Rev-erb* 3'F2 respectively (Table 10). The final full-length sequence was confirmed by two rounds of PCR using nested primers designed to amplify end to end full length cDNAs (Rev-erb_full_F1: Rev-erb_full_R1 & Rev-erb_full_F2: Rev-erb_full_R2) (Table 10). All PCRs were run at an annealing temperature as listed in Table 10 and the extension time was 1 min/Kb of predicted PCR product, and 3 min were applied for unpredictable RACE PCR products. All primers were designed using Primer Select Ver. 6.1 program (DNASTAR, www.dnastar.com).

Table 10: Primer pairs and sequences for *Rev-erb α* identification including primer name, purpose, sequence and annealing temperature.

Name	**Purpose**	**Sequence 5'-3'**	**Anneal Temperature**
Rev-erb 5'R1	RACE-PCR	GCCCCAGTTGTCCACCTCTCCGTTATGT	60 °C
Rev-erb 5'R2	RACE-PCR	AATGGCGGGCTTTGGGTGGATG	60 °C
Rev-erb 3'F1	RACE-PCR	TACCCCCAAGACGAACCCAACA	60 °C
Rev-erb 3'F2	RACE-PCR	GGGAGGCTTGCTAGACACCAT	60 °C
Rev-erb_full_F1	Full length outer PCR	AGGCCGACTTGGAAACTGC	57 °C
Reverb_full_R1		GTCTATTGGCCTTACCCCTATCA	
Rev-erb_full_F2	Full length inner PCR	GTTCAGACCTGCACCGATAGAGC	62 °C
Rev-erb_full_R2		TAGCCGCCCAACCACCACTGTC	

6. MOLECULAR TECHNIQUES

PCR

Routine PCR was used to test all of the primers for qPCR, cloning and sequencing. For the majority of PCR reactions, general use such as test PCR and cloning reactions) cDNA was used as part of a 20µl reaction with Klear Taq Hot Start DNA polymerase (KBiosciences Hoddesdon, Herts, UK). For a typical PCR reaction primers were reconstituted to a stock concentration of 100 pmol/µl. Stock primers were then diluted 1/10 and 1.6 µl of each forward and reverse primer were used per 20 µl reaction. 2.5 – 5 µl cDNA is used in each reaction with 15 – 17.5 µl of master mix.

Routine PCR Master Mix for 20µl reaction:

- 1.6 µl Forward primer
- 1.6 µl Reverse primer
- 2 µl 10x Klear taq buffer no MgCl2 no detergent.
- 1.8 µl MgCl2 50mM
- 0.5 µl dNTP
- 0.08 µl Klear taq
- DNA/ RNA free H_20 to 20 ml (7.42 – 9.92 µl)

Each PCR reaction is mixed and briefly centrifuged to collect all of the reaction components at the bottom of the tube. All reactions are then placed in a themocycler. The cycle begins with 15minutes at 95^{o}C followed by 35 cycles of 95 ^{o}C for 30 seconds, anneal for 30 seconds (anneal temperatures were generally first tested at mean TM of primer pair – 4 ^{o}C) followed by 72 ^{o}C extension (1 minute per Kb of expected product) this is followed by a final extension step of 72 ^{o}C for 7 minutes. Individual reaction samples were then visualised using gel electrophoresis on a 1 % agarose gel.

Cloning and sequencing

Cloning and plasmid preparation

PCR products for cloning were initially purified either directly from reaction or from agarose gel using an Illustra GFX PCR DNA and GelBand Purification Kit (GE Healthcare) according to manufactures instructions. The purified product was then added to a ligation reaction at ratio of 3:1 insert to vector using a pGEM®-T Easy vector system (Promega). Concentration of the purified PCR product was determined by nanodrop spectrophotometer. Amount of insert was calculated by the following equation.

[(ng of Vector x kb size of insert)/ (Kb size of vector)] * insert:vector molar ratio = ng of insert.

Volume of inset was then determined from known insert concentration ligation reactions were then set up as follows.

- 2.5 µl 2X rapid Ligation buffer, T4 DNA ligase
- 0.5 µl pGEM®-T Easy vector (50 ng)
- < 1.5 µl PCR product
- 0.5 µl T4 DNA ligase (3 weiss units/µl)
- Nuclease free H_2O to 5 µl

The ligation reaction is then mixed by pipetting gently and incubated overnight at 4 ^{o}C to obtain the maximum number of transformations. After the ligation is complete 2 µl of reaction is added to 50 µl just defrosted JM109 High Efficiency Competent cells and mix by flicking gently. Incubate on Ice for 20 minutes then heat shock for 45 seconds at 42 ^{o}C. Tubes were immediately returned to ice for 2 minutes.

Transformed cells were then added to 950ml of room temperature LB broth and incubated at 37 ^{o}C for 90 minutes. Cells were then gently centrifuged to collect cells at the bottom of the

tube half of the LB broth was removed in order to concentrate cells. 100 µl was then plated on two LB/ampicillin/IPTG/ X-gal plates. Plates were incubated overnight for 16 h at 37 ^{o}C. Plates were then stored at 4 ^{o}C until colonies were picked and grown in LB broth for 16h. Plasmids were then harvested using a GenElute™ *Plasmid Miniprep Kit* (sigma Aldrich). Each tube containing individual colonies grown overnight was then centrifuged at 4500RPM for 18 minutes to pellet cells and remove remaining liquid. Cells are then resuspended in 200 µl resuspension solution. 200 µl lysis solution is then added and tubes are rested for 5 minutes at room temperature. 350 µl neutralisation is added and tubes are centrifuged for a further 10 minutes at maximum speed. Supernatant is then place on a prepped GenElute Miniprep Binding Column and centrifuged in a desk top centrifuge for 1 minute at 13,000 g and liquid is discarded. 750 ml of was buffer is then added to the column and centrifuged for 1 minute. The liquid was then discarded and column was placed in a fresh DNA and RNA free tube and eluted in 50 µl nanopure H_2O Plasmids are then digested using ecoR1 to determine the presence of the insert as the vector consist of an EcoR1 site either side of the inserted sequence (Figure 3). Insert was then sequence using M13 forward and reverse primers. EcoR1 Digest reactions were maintained at 37 ^{o}C for 2 hours. Reactions were then run on a 1 % agarose gel to visualise results.

- 5 µl Plasmid
- 2 µl Enzyme buffer
- 0.2 µl EcoR1 Enzyme
- 2.8 µl H_2O

LB Broth and Agar Plates

LB Broth 500 ml

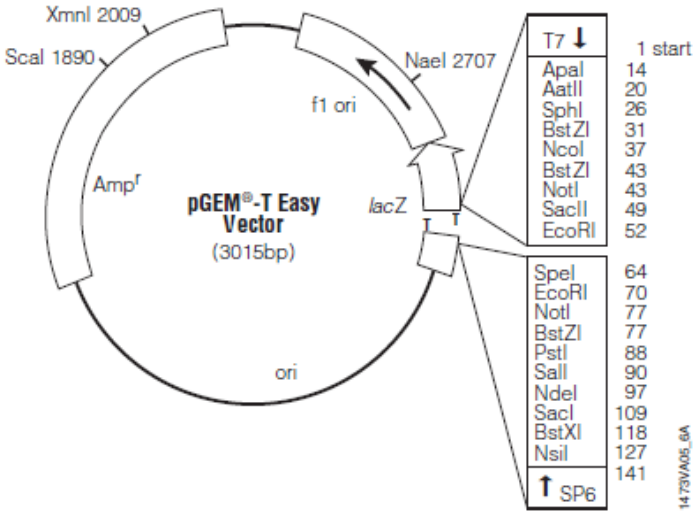
- 5 g tryptone
- 5 g NaCl
- 2.5 g yeast extract

- 500 ml dH_2O
- 500 µl Ampicillin (added after Autoclave)

LB Agar 250 ml

- 2.5 g tryptone
- 2.5 g NaCl
- 1.25 yeast extract
- 3.75 g Agar
- 250 ml dH_2O
- 250 µl ampicillin 100 mg/ml
- 500 µl 20 mg xgal 5-bromo-4-chloro-indolyl-β-D-galactopyranoside
- 1250 µl IPTG Isopropyl-β-D-thio-galactoside 0.1

5.D. pGEM®-T Easy Vector Map and Sequence Reference Points



pGEM®-T Easy Vector sequence reference points:

T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–128
SP6 RNA polymerase promoter (–17 to +3)	139–158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176–197
*lac*Z start codon	180
lac operator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
lac operon sequences	2836–2996, 166–395
pUC/M13 Forward Sequencing Primer binding site	2949–2972
T7 RNA polymerase promoter (–17 to +3)	2999–3

-

Figure 3: pGEM-T easy vector map.

Sequencing

Sequencing was carried out using a Beckman 8800 auto sequencer. Sequence results were then analysed using SEQman as part of Lasergene software package (DNASTAR). Amount of DNA was selected according to product size (Table 10). Between 25 and 100 fmol are used per reaction according to size of template, number of reactions and volume of template available.

Sequencing was prepared in a 200 µl DNA/RNA free PCR tube and all reagents and reactions were stored on ice. For each template DNA four sequencing reactions were prepared, two reactions using the forward M13 primer and 2 using the reverse M13 primer. For PCR reactions forward or reverse primers for the specific sequence (PCR primers) were used. Each reaction was prepared in 5 µl reaction volume as follows.

- Up to 2.5 µl Nanopure H_20
- <2.5 µl DNA template (Table 11)
- 0.5 µl Primer(m13F or R or PCR F or R)
- 2.0 µl DTCS Quick start Master Mix

Reaction components were mixed by vortexing and briefly centrifuged to collect all the components at the bottom of the PCR tube and run in a thermo cycling program of 30 cycles of 96 ^{o}C for 20 seconds, 50 ^{o}C for 20 seconds and 60 ^{o}C for 4minutes.

Table 11: Quantity of DNA template for sequencing from Beckman 8800 auto sequencer protocol dependent on product size.

Size (Kb)	ng for 25fmol	ng for 50fmol	Ng for 100fmol
0.2	3.3	6.5	13
0.3	4.9	9.8	20
0.4	6.5	13	26
0.5	8.1	16	33
1.0	16	33	65
2.0	33	65	130
3.0	50	100	195
4.0	65	130	260
5.0	80	165	325

The sequencing reaction was then precipitated with ethanol. All samples were diluted to 20 µl and 5 µl of STOP solution was added.

STOP solution per reaction:

- 2 µl of 3 M Sodium Acetate (pH 5.2)
- 2 µl of 100 mM Na_2- EDTA (pH 8.0)
- 1 µl of 20 mg/mg of glycogen

Mix STOP solution well with each reaction before adding 60 µl ice cold (from -20 ^{o}C freezer) 95 % ethanol/ nanopure H_2O and vortex to mix. Centrifuge at 14,000 rpm for 15 minutes at 4 ^{o}C. After centrifugation a pellet should be visible. Supernatant was then removed and pellet was washed a further 2 times in 200µl 70 % ethanol (14,000 rpm 4 ^{o}C for

2minutes) Samples are air dried and reconstituted in 30µl sample loading solution. Samples were loaded to appropriate wells of the CEQ sample plate. One drop of light mineral oil is added to each sample to prevent evaporation. Loading buffer is then added to the corresponding wells on the buffer plate and all samples are loaded into Beckman 8800 auto sequencer and sequencing procedure was initiated.

Sequences were then assembled using SEQman as part of Lasergene software package (DNASTAR) and alignments were performed using BioEdit (Ibis Biosciences) and clustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Sequencing of PCR reactions and plasmids was used for a number of purposes including gene discovery and generation of plasmid standards for use in qPCR.

qPCR

In order to quantify gene expression, absolute quantification qPCR assays were designed and validated for all genes shown in Table 12. Each qPCR assay was carried out in a 96 well plate (Figure 4). Where possible all qPCR analysis was performed in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al. 2009). Each qPCR reaction consisted of primer pairs (Table 12) at a concentration of 0.5 µM, 5 µl of cDNA (diluted either at 1/20 or 1/10 depending on the experiment), 3 µl DNA/RNA free H_2O and 10 µl ABsolute™QPCR SYBR Green master mix, (Thermo scientific, Leon-Rot, Germany) in a total reaction volume of 20 µl. The ABsolute™QPCR SYBR Green Mix was made up of Thermo-Start™ DNA polymerase, a proprietary reaction buffer, dNTP's and SYBR Green I with Mg++ at a concentration of 3 mM in the final 1× reaction. All qPCR assays were carried out in a Techne Quantica thermocycler (Techne, Quantica, Cambridge, UK) in a thermo cycling programme consisting

of a 15 minute initiation stage at 95 °C this is followed by 45 cycles of 3 temperature steps; 95 ^{o}C for 15 s anneal x°C (See Table 12 for target specific annealing temperatures) for 15 s and 72 ^{o}C for 30 s. This was followed by a temperature ramp from 70 – 90 °C for melt-curve analysis to verify that no primer–dimer artefacts were present and only one product was generated from each qPCR assay. Quantification was achieved by a parallel set of reactions containing standards consisting of serial dilution of spectrophotometrically determined, linearised plasmid containing target cDNA sequences. All samples were run in duplicate and each assay contained no-template controls.

qPCR normalisation and statistical analysis

Results generated from brain and fin and pineal samples in the Atlantic salmon and European seabass were normalised to expression levels of a*β-actin* reference gene as described in Davie et al. (2009) and Herrera- perez (2011). For liver samples, geNorm analysis was carried out on three potential house keeping genes tested on the long day liver samples to determine the most suitable reference gene including *β-Actin, ELF-α* and *GAPDH*. In the Atlantic salmon liver samples, *ELF-α* displayed the least variation and was therefore used for normalisation in this liver (Figure 5). Results were then converted to external time in accordance with Daan et al. (2002) whereby the external time 0 (ExT 0) is the middle of the dark phase for chapter 3 and 4. For example ExT 0 of experiment 1 long day treatment occurs at 04:00 when lights off occurs at 00:00 and lights on occurs at 08:00.Gene expression data are expressed as copy number per μg total RNA. Results for chapters 5 and 6 were converted to zeitgeber (ZT), whereby 00:00 is the onset of light as no comparison between photoperiods were made.

Table 12: All qPCR primer pairs and annealing temperatures

Species	Primer Name	Sequence (5'-3')	Anneal
Salmo salar	*β Actin* - Forward	ATCCTGACAGAGCGCGGTTACAGT	61°C
Reference genes	*β Actin* - Reverse	TGCCCATCTCCTGCTCAAAGTCCA	61°C
	EF - α - Forward	TCTGGAGACGCTGCTATTGTTG	61°C
	EF - α - Reverse	GACTTTGTGACCTTGCCGCTTGAG	61°C
Clock genes	*Aanat2* - Forward	GCTCTCCCTGGGCTGGTTTGAAG	62°C
	Aanat2 - Reverse	CATGGATGTGCACTGCCGAGGTT	62°C
	Bmal - Forward	GCCTACTTGCAACGCTATGTCC	64 °C
	Bmal - Reverse	GCTGCGCCTCGTAATGTCTTCA	64 °C
	Cry2 - Forward	GAGGGCATGAAGGTGTTTGAGGAG	59 °C
	Cry2 - Reverse	GTGGAAGAACTGCTGGAAGAAGGA	59 °C
	Clock - Forward	AGAAATGCCTGCACAGTCGGAGTC	64°C
	Clock - Reverse	CCACCAGGTCAGAAGGAAGATGTT	64°C
	Per1 - Forward	AGGGGGTCATGCGGAAGGGGAAGT	66°C
	Per1 - Reverse	TGGGCCACCTGCATGGGCTCTGT	66°C
	Per2 - Forward	GCTCCCAGAATTCCTAGTGACAAG	60°C
	Per2 - Reverse	GAACAGCCCTCTCGTCCACATC	60°C
	Rev-erb α – Forward	CCCCCAAGACGAACCCAACAAGAC	61°C
	Rev-erb α – Reverse	AGAGGGAGGCAAAGCGCACCATTA	61°C
Seasonal genes	*Dio1* - Forward	GACAACAGACCACTGGTGTTGACT	62°C
	Dio1 - Reverse	GCCTGCGCAATGTAGACCACC	62°C
	Dio2 - Forward	GGACGAGTGCCGCCTGCTGGACTT	68°C
	Dio2 - Reverse	GAAGGCGGGCAGGTGGCTGATGA	68°C
	Dio3 - Forward	CCTGGCTGCGTTTCAGCGCGT	64°C
	Dio3 - Reverse	ATCTGGTAAGGCGCGTCGGAG	64°C
	Eya3 - Forward	GGGCATCACGGACGGACGCTT	64°C

	Eya3 - Reverse	CCCAACCCCAATCAATGCTGCCTC	64^{o}C
	Tshβ - Forward	GAGCTCGCCGGACCACGTTTCCT	66^{o}C
	Tshβ-Reverse	AGTGGCAGCTGAGGGCTACGGG	66^{o}C
Liver lipid genes	*D6 Fad*- Forward	GTGAATGGGGATCCATAGCA	60^{o}C
	D6 Fad - Reverse	AAACGAACGGACAACCAGAC	60^{o}C
	Elovl5-Forward	ACAAGACAGGAATCTCTTTCAGATTAA	58 ^{o}C
	Elovl5-Reverse	TCTGGGGTTACTGTGCTATAGTGTAC	58 ^{o}C
	Fas-Forward	ACCGCCAAGCTCAGTGTGC	60^{o}C
	Fas-Reverse	CAGGCCCCAAAGGAGTAGC	60^{o}C
	Hmgcr - Forward	CCTTCAGCCATGAACTGGAT	60^{o}C
	Hmgcr - Reverse	TCCTGTCCACAGGCAATGTA	60^{o}C
	Lxr - Forward	GCCGCCGCTATCTGAAATCTG	58 ^{o}C
	Lxr - Reverse	CAATCCGGCAACCAATCTGTAGG	58 ^{o}C
	Srebp1 - Forward	GCCATGCGCAGGTTGTTTCTTCA	63 ^{o}C
	Srebp1 - Reverse	TCTGGCCAGGACGCATCTCACACT	63^{o}C
	Srebp2 - Forward	TCGCGGCCTCCTGATGATT	63 ^{o}C
	Srebp2 - Reverse	AGGGCTAGGTGACTGTTCTGG	63^{o}C
Dicentrarchus	*β Actin* - Forward	TGGCCGCGACCTCACAGAC	59 ^{o}C
labrax	*β Actin* - Reverse	TCCAGGGCGACATAGCACAGTTT	59 ^{o}C
	Aanat2 - Forward	ACGCCGCAGGATGCCATCAGTGTA	62 ^{o}C
	Aanat2 - Reverse	TCCTTGTCCCAGCCAGAGCCAATG	62 ^{o}C
	Clock - Forward	CAGACAAGTGCCAGGATTCAG	55 ^{o}C
	Clock - Reverse	CAGCGGTGTGCGAGGATTT	55^{o}C
	Per1 - Forward	CGGACAGCAGGTTTTTATCGA	54 ^{o}C
	Per1 - Reverse	GAAAAAACACCAGCACAGGC	54 ^{o}C

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	ITC1	1	5	9	13	17	21	25	29	33	37
B	NTC	ITC1	1	5	9	13	17	21	25	29	33	37
C	10^x	ITC2	2	6	10	14	18	22	26	30	34	38
D	10^x	ITC2	3	6	10	14	18	22	26	30	34	38
E	10^x	ITC3	3	7	11	15	19	23	27	31	35	39
F	10^x	ITC3	3	7	11	15	19	23	27	31	35	39
G	10^x	ITC4	4	8	12	16	20	24	28	32	36	40
H	10^x	ITC4	4	8	12	16	20	24	28	32	36	40

Figure 4: Typical layout of a qPCR 96 well plate lay out including non template controls (NTC) Internal template controls (ITC) and linerised quantified plasmid standards (10^x).

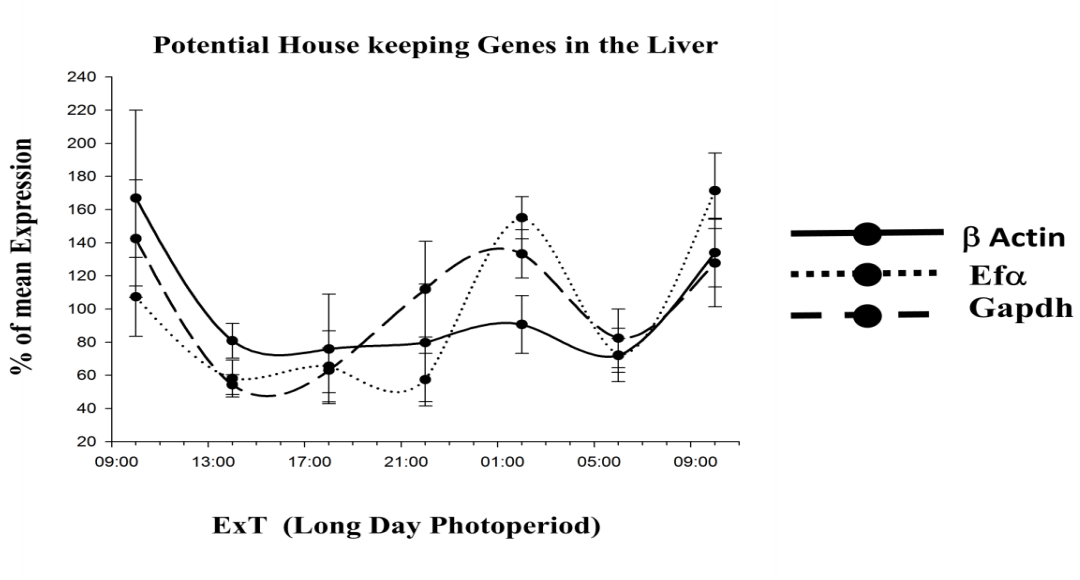


Figure 5: 24h expression of βActin, EFα and Gapdh in the liver of Atlantic salmon parr acclimated to experimental long day photoperiod for potential use as a housekeeping gene in the liver. While βActin was used in all other tissue types investigated EFα was used as a reference gene in the liver.

Microarray (Chapter 4)

In order to identify novel genes expressed on a daylength dependent basis, a pilot global gene expression analysis of brain tissue was undertaken. A custom-designed Atlantic salmon oligoarray with 44k features per array on a four-array-per-slide format (Agilent Technologies, Cheshire, U.K.), with each feature printed singly was utilized (http://www.ebi.ac.uk/arrayexpress/arrays/A-MEXP-2106). Each biological replicate (Cy3 – labelled) was co-hybridized in a dual dye experiment with a single pooled reference sample (Cy5 labelled). The pooled reference sample comprised equal amounts of amplified RNA from each of the 16 experimental fish. The study comprised 16 hybridisations: 2 states (long day / short day) × 2 time-points (midday/midnight) × 4 biological replicates (Individual

Atlantic salmon parr 26.0 ± 4.0 g (as described above). Amplified RNA amplification, dye labelling and hybridisations were performed as detailed in Morais et al. (2012). Each replicate was competitively hybridised with a pooled reference sample. An indirect labelling methodology was utilised to prepare microarray targets. For each sample 500 ng of purified total RNA was used to generate antisense amplified RNA (aRNA) (Amino Allyl MessageAmpTM II aRNA amplification kit, Ambion Applied Biosystems). Samples were then subject to Cy3 or Cy5 fluor incorporation mediated by a dye coupling reaction. All experimental reactions were labelled with Cy3 dye and pooled reference was labelled with Cy5 dye. The incorporation of the dye and aRNA yield was quantified by spectrophotometry (NanoDrop ND-1000). The quality of the assay was further controlled by the separation of 0.4 μl sample through a mini agarose gel. Products were displayed on a Typhoon trio florescence scanner (GE Healthcare). Hybridisation of microarray was carried out in a Lucidea semi-automated system (GE Healthcare) with no pre-hybridisation step.

In the hybridisation of each array, the sample and pooled reference sample, consisting of 40 pmol dye and 150 ng aRNA, were pooled and combined to the hybridisation solution (185 μl 0.7X UltraHyb buffer from Ambion, 20 μl Poly(A), 10 μl herring sperm, 10 ml ultra pure BSA all at a concentration of 10 mg/ml and from Sigma-Aldrich, Dorset UK)(Morais et al. 2011). Prior to scanning microarray, hybridisations were subjected to two post-hybridisation automatic and six manual washes to a stringency of 0.1 xSSC (EasyDipTM Slide staining system; Canemco Inc., Quebec Canada). The scanning was carried out at a resolution of 10 μl in an Axon GenePix 4200AL scanner (MDS analytical technologies, Wokingham, Brekshire U.K) with laser power constant (80 %) and “auto PMT” enabled to adjust PMT for each channel so that less than 0.1 % of features were saturated and mean intensity ratio of Cy3 and 5 signals was close to 1, as described in Morais et al 2011.This was followed by a manual spot removal procedure and the duplication of spot data (BlueFuse proprietary algorithm)

Florescence intensity values were extracted from TIF images of microarray slide with the use of BlueFuse software (BlueGenome, Cambridge, UK). Data was exported to GeneSpring GX version 10.0.2 (Agilent Technologies, Wokingham, Berkshire, U.K) after block Lowess normalisation. The data was then transformed and subject to quality filtering and all control features. Consequently 5893 genes were subject to statistical analysis.

7. STATISTICAL ANALYSIS

The majority of data presented in this thesis was taken over a 24h period. Consequently the statistical analysis implemented was largely concerned with the analysis of circadian rhythms. Analysis of Variance (ANOVA) was used to determine a significant effect of time and Turkey's test was used to determine the significance of differences between sample time points (Minitab16 Statistical Software, Minitab inc 2011). Data from each tissue/ photoperiod was then fitted to a cosine wave in order to determine the presence of a significant circadian rhythm. Raw data was analysed using acro circadian analysis programs (Refennetti R., University of South Carolina, USA; http://www.circadian.org/softwar.html). Acro analysis determines both the significance, acrophase (peak in expression) mean and amplitude of raw data using the equation **Y= A + B * cos (C *X –D)** whereby Y is level of gene expression as a percentage of the mean A is the baseline, C is the frequency multiplier and D is the acrophase of the data set (Davie et al. 2009; Refinetti 2006). Microarray data was analysed by two-way ANOVA with the use of GeneSpring GX version 10.0.2 (Agilent Technologies, Wokingham, Berkshire, UK). Data is presented with respect to P value and fold change. Minimum P value and fold change was determined for photoperiod (LD vs. SD) and day/night (day vs. night) differences. In addition to photoperiod/ day night interaction differences were analysed with regards to minimum p value and maximum fold change

across all interactions. A significant circadian rhythm was deemed present when p value was less than 0.05 in for all statistical analysis.

CHAPTER 3

SEASONAL MELATONIN AND CLOCK GENE EXPRESSION IN THE BRAIN, FIN AND LIVER OF THE ATLANTIC SALMON

Chapter 3

SEASONAL MELATONIN AND CLOCK GENE EXPRESSION IN THE BRAIN, FIN AND LIVER OF THE ATLANTIC SALMON.

1. ABSTRACT

The Atlantic salmon is a highly seasonal teleost species, displaying distinct temporal organisation of his physiology and behaviour. Amongst vertebrates temporal organisation is endogenously regulated by the molecular circadian clock and synchronised to the environment by photoperiod. In salmonids photoperiod is important in the synchronisation of physiology and photoperiod manipulation is commonly used to regulate maturation and reproduction in the aquaculture industry. More recently daylength dependent expression of clocks has been described in the salmon brain. In order to better understand the role of photoperiod in the seasonal regulation of clocks, the current study examined 24h profiles of clock gene expression and levels of plasma melatonin in Atlantic salmon acclimated to long day, short day, 12L:12D (experiment 1) and simulated natural photoperiods (experiment 2). Photoperiod dependent clocks were also investigated in the fin and the liver. Results show circadian profiles of melatonin under all photoperiods. In experiment 1 both *Clock* and *Per2* displayed significant circadian expression under the long day treatment. This is in contrast to previous results where rhythmic clocks were observed under a short day photoperiod. Comparisons between experiment 1 and 2 reveal no clear pattern of long day, short day or intermediate photoperiod expression. Additionally photoperiod dependent clock gene expression was observed in the liver, at a different phase to the rhythms in the brain. No rhythmic expression was observed in the fin. Results infer photoperiod dependent control of clocks in the brain however other factors such as temperature, salinity, food availability and life history may also be of importance. Moreover results differed form previous expression results in other vertebrate and fish species.

2. INTRODUCTION

In temperate regions photoperiod provides a robust indicator of external seasonality when other environmental signals such as temperature and food availability may vary from year to year. As such, photoperiod is believed to be the key proximate factor in temperate fish species used to entrain and synchronise most physiological processes (Bromage et al. 2001; Golombek & Rosenstein 2010). However additional environmental signals, such as temperature, are also likely to be of importance in synchronising an organism to its environment (Golombek & Rosenstein 2010). Amongst teleosts, salmonids display some of the clearest seasonal phenotypes with the timing of most life history events such as migration, smoltification and spawning being synchronised by seasonal environmental cues, in particular photoperiod (Bromage et al. 1988; Randall et al. 1998; Stefanson et al. 2008). The pivotal role that photoperiod plays in the regulation and synchronisation of seasonal processes to the environment is evident from the widespread use of photoperiod manipulation in the aquaculture industry to manipulate broodstock spawning and produce eggs out-of-season, produce out-of-season smolts for year round stocking to sea cages and suppress puberty/early maturation during seawater ongrowing (Bjornsson et al. 1994; Duncan et al. 1998; Porter et al. 1998). However, while the principle of photoperiod control of physiology is widely accepted, the actual mechanisms by which photoperiod information is perceived and then integrated to regulate these processes is poorly understood.

It is believed that photoperiod regulates seasonal physiology via the photoneuroendocrine system (PNES) (Migaud et al. 2010). The PNES has not been accurately defined yet in teleosts, however, in its simplest conceptual form it is believed to consist of three main elements (see review by Falcon et al. 2010). Firstly light is perceived via a number of non visual photoreceptors. This information is then relayed to the clock mechanism which cycles

with a period of approximately 24 hours and can maintain its rhythm in the lack of external cues. This clock mechanism then relays synchronised timing information to the final element of the PNES which include various possible neuroendocrine output messengers that relay this time information throughout the animal's physiology (see review by Migaud et al. 2010). To date the majority of research into the PNES has focused on the first and last elements of the system. Studies of the clock mechanisms are lacking in most teleost species with the exception of zebrafish (Whitmore et al. 1998; Whitmore et al. 2000; Whitmore et al. 2000). From this body of work it is evident the underlying molecular mechanisms bear a clear similarity to the mammalian models e.g. mouse which is indicative that the circadian clock mechanisms may be highly conserved amongst vertebrates (Ko & Takahashi 2006). The core clock cycle consists of a positive and negative arm which combine to form a self-sustaining feedback loop which takes approximately 24 hours to cycle and drives the circadian rhythms in the expression of core genes involved (Ko & Takahashi 2006). CLOCK and BMAL proteins form a heterodimer that makes up the positive arm of the auto-regulatory feedback loop. The CLOCK:BMAL heterodimer then initiates the accumulation of PER and CRY proteins that comprise the negative arm of the molecular feedback loop. The PER: CRY complex then translocates back into the nucleus, inhibiting its own transcription by acting on the CLOCK:BMAL heterodimer. This process takes around 24h to complete and is synchronised to the daily light dark cycle. Consequently, the expression of corresponding mRNAs follows a circadian profile over a 24h period and the phase expression is dependent of photoperiod. The CLOCK:BMAL heterodimer additionally acts on E-box and D-elements in target genes, which in turn regulate circadian physiology via clock controlled genes (CCG)(Crane 2012).

While clock genes and the molecular feedback loop are well known to regulate daily cycles there is also increasing evidence that clock genes can also reflect seasonal information. In mammals, clock gene expression localised in the pars tuberalis (PT) of the pituitary have been shown to exhibit seasonal patterns of expression over and above their diel rhythms. These patterns, in *per* and *Cry* in particular, have been reported in a number of mammalian species and are expressed as amplitudinal differences, phase shifts in gene expression or a combination of both (Lincoln et al 2003; Wagner et al 2008). Comparable work in teleosts is lacking despite the fact that that temperate species such as the Atlantic salmon display robust seasonal organisation of a number of biological processes. In salmonids a number of clock genes have previously been associated with reproduction and migration. Two core genes of the circadian feedback loop, *Clock* and *Per 1,* have been linked to spawning time in rainbow trout (Leder et al. 2006). In Atlantic salmon, *Bmal1* has been linked to male reproductive strategy as expression of the circadian gene was found to be up-regulated in early maturing "sneaker" males in comparison to immature males of the same age (Aubin-Horth et al. 2005). Furthermore, variation in the length of PolyQ region of *Clock* in the Pacific Chinook salmon have been associated with differences in migratory run times (O'Malley & Banks 2008; O'Malley et al. 2007). Davie et al (2009) was the first study to suggest that daylength dependent patterns of clock gene expression existed within Atlantic salmon brain. In their study, *Clock, Per2*, *Bmal* and *Cry2* all displayed a circadian pattern of expression under a short day/winter seasonal photoperiod (8h light: 16h dark) while only *Cry 2* was rhythmically expressed under the long day/summer seasonal photoperiod (16h light: 8h dark).

Outwith the brain, clock genes are apparently present in the majority of vertebrate tissues and cell types (Dibner et al. 2010). In mammals the expression of clock genes in peripheral tissues is relatively hierarchical. The majority of peripheral oscillators are synchronised to

external photoperiod via retinal input and clock gene expression in the suprachiasmatic nuclei (SCN), the master oscillator in the mammalian circadian organisation (Dibner et al. 2010). In fish the situation is not as clear as a master circadian oscillator has not yet been identified though its existence has been hypothesised (Ali 1992). In the zebrafish clock genes are constitutively expressed and reactive to light throughout a number of central and peripheral tissues *in vitro* (Whitmore et al. 1998; Whitmore et al. 2000). However it is unclear whether these independently entrainable and self-sustaining peripheral oscillators are synchronised to any potential master circadian clock. Davie et al. (2009) demonstrated in Atlantic salmon parr that clock genes are expressed in both central and peripheral tissues ranging from the liver to the intestine and spleen however this work did not characterise diel expression profiles in these tissues. Huang et al 2010 (a and b) thereafter demonstrated that, in contrast to zebrafish, there were different circadian patterns of clock gene expression in the brain, pineal and liver in salmon parr.

To build on this work and further our understanding of seasonal clock gene expression in salmonids, the aim of the present study was two fold. The first aim was to compare daylength dependent circadian expression of core circadian clock genes (*Clock, Per1 and Per2*) in the brain and two peripheral tissues (fin and liver) of Atlantic salmon parr. The second aim was to compare circadian clock gene expression (*Clock*, *Cry2*, *Per1* and *Per2*) in the brain over the course of a year at the natural equinox and solstices. The expression of *Per1* was analysed in both experiments in addition to *Per 2* which has known importance in the seasonal control of physiology in mammals (Dardente 2012).

3. MATERIALS AND METHODS

Animals and tissue Sampling

Both experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. Experimental animals were sacrificed via a lethal anaesthesia (phenoxyethanol 2ml/L) followed by decapitation.

Experiment 1- Effect of photoperiod on circadian clock gene expression and melatonin.

Atlantic salmon parr (*n*=100 per treatment, mean 24.9 ± 5.4 g, 140.6 ± 7.8 mm) were acclimated during one month (from 02/03/2009) to either long day (16h light: 08h dark, LD), short day (08h light: 16h Dark, SD) or 12h light: 12h dark photoperiod (12/12). Fish were stocked into a $1m^3$ light proofed tank (one tank per treatment) at the Niall Bromage Freshwater Research Facilities (Institute of aquaculture, Stirling, UK, 56: 02N). The tanks were illuminated using a 28W, fluorescent light with a spectral content comparable to a 3700 °K black-body radiator (IP65 prismatic 2D round bulkhead 28W HF, RS Components Ltd, Glasgow, UK) connected to an automatic timer to regulate photoperiod. In all treatments lights were switched on at 08:00h and switched off at 16:00h (SD), 20:00h (12/12) and 00:00h (LD). Over the course of the study water temperature averaged 4.6 ± 0.73 ^{o}C. Fish were sampled on the 6^{th} and 7^{th} of April 2009. Brain (including pituitary), left pectoral fin and liver tissue samples were removed (n = 6 individuals per sample point) every four hours over a 24h period. Blood was withdrawn from n = 6 individuals per sample using a 1 ml sterile heparinized syringe with 21G gauge needle. Tissue and plasma samples (previously separated by centrifugation at 2000 g for 15 mins) were instantly frozen over liquid nitrogen vapour and then stored at -70 °C until use. Samples retrieved during the nocturnal phase of each 24 h light dark cycle were done so with the use of a dim red light.

Experiment 2 - Seasonal/Life history changes in circadian clock gene expression and melatonin.

In June 2009, approximately 700 salmon parr (approximately 7 months post-hatch) were stocked into a 2 m^3 light proofed tank illuminated by a 28W, fluorescent light with a spectral content comparable to a 3700 °K black-body radiator (IP65 prismatic 2D round bulkhead 28W HF, RS Components Ltd, Glasgow, UK) which was then regulated by digital electronic timer. Fish were maintained on a simulated natural photoperiod that was adjusted on a weekly basis to local changes in photoperiod (max. daylength 17h39 min, min. daylength 07h30min). Prior to acclamation to SNP fish were held under constant light from first feeding (March 2009) A total of 5 diel profiles were sampled coinciding with the autumn (6^{th} and 7^{th} of October 2009 and 7^{th} and the 8^{th} of October 2010) and spring (29^{th} and 30^{th} of March 2010) equinoxes and the summer (01^{st} and 2^{nd} of July 2010) and winter (12^{th} and the 13^{th} of January 2010) solstices (Table 1, Figure 1). At each time point, following euthanasia, blood was withdrawn via a 1 ml heparinised syringe with 21G gauge needle by superficial venepuncture (5 per time point) before the whole brain (including pituitary) was dissected out (6 per sample point). Tissue and plasma samples (previously separated by centrifugation at 2000 g for 15 mins) were instantly frozen over liquid nitrogen vapour and then stored at -70 °C until use. Samples retrieved during the nocturnal phase of each 24 h light dark cycle were done so with the use of a dim red light.

Table 1: Experiment 2 seasonal sample points; photoperiod, date, sample location, mean weight and length of salmon and mean water temperature. NBFRF: Niall Bromage Freshwater Research facilities (IoA); MERL: Marine Environmental Research Laboratory, Machrihanish. (IoA).

Sample	**Photoperiod**	**Date**	**Sample points**	**Location**	**Mean Length and Weight**	**Water Temp °C**
1	Sunrise 7:30 Sunset 18:04	06/10/09 – 07/10/09	09:00, 13:00, 17:00, 21:00, 01:00, 05:00, 09:00	NBFRF	135 ± 4 mm 31.1 ± 0.9 g	11.4
2	Sunrise 08:40 Sunset 16:10	12/01/10 – 13/01/10	14:00, 18:00, 22:00, 02:00, 06:00, 10:00, 14:00	NBFRF	154 ± 2 mm 41.3 ± 1.4 g	1.5
3	Sunrise 06:50 Sunset 19:50	29/03/10 – 30/03/10	13:00, 17:00, 21:00, 01:00, 05:00, 09:00 *	NBFRF	162 ± 3 mm 42.2 ± 1.6 g	3.8
4	Sunrise 04:34 Sunset 22:05	01/07/10 – 02/07/10	15:00, 19:00, 23:00, 03:00, 07:00, 11:00, 15:00.	MERL	197 ± 4 mm 77.9 ± 4.6 g	13.4
5	Sunrise 07:31 Sunset 18:39	07/10/10 – 08/10/10	13:30, 17:30, 21:30, 01:30, 05:30, 09:30, 13:30.	MERL	260 ± 7 mm 188 ± 15.3 g	13

* Last sample point was unable to be repeated due to adverse weather conditions.

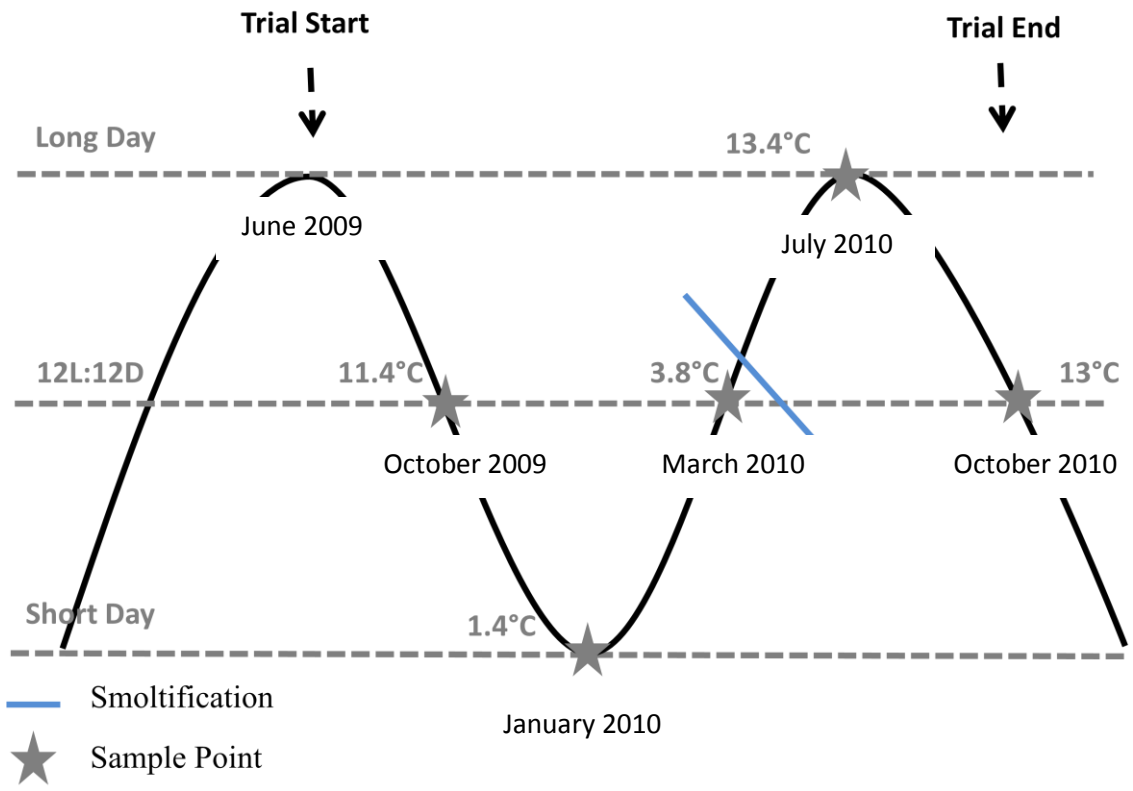


Figure 1: Photoperiod and temperature variations over the course of the experiment showing times of sampling for experiment 2.

RNA Extraction, DNase Treatment and cDNA synthesis

Whole brain, fin and liver samples were individually homogenised in TRIzol® (Invitrogen UK) in accordance with the manufacturer's protocol at a ratio of 100 mg of tissue per 1 ml of reagent. RNA extraction was carried according to manufacturer's instructions. RNA pellets were rehydrated in MilliQ water in varying volumes to achieve a final RNA concentration of approximately 1000 ng/ul. Total RNA concentration was calculated using a ND-1000 Nanodrop spectrophotometer (labtech Int., East Sussex, UK). In order to eliminate any DNA contamination 5 µg of totRNA was treated with a DNase enzyme following DNA-free™ kit guidelines (Applied biosystems, UK). cDNA was then synthesised using 1 µg of DNase treated totRNA in a 20 µl reaction volume using random primers according to manufacturer's protocol (High capacity reverse transcription kit without RNase inhibiter, Applied

biosystems, UK). Final reactions were then diluted with MilliQ water to a final volume of 200 µl (experiment 1) and 100 µl (experiment 2). Brain, fin and liver cDNA reactions were then stored at -20 ^{o}C.

***In Silico* Atlantic salmon *Per1* partial Identification.**

All qPCR assays used were previously established and verified by Davie *et al.* (2009) with the exception of Period 1. This assay was designed as follows: sequence information from a variety of teleost *Per1* sequences were assembled in BioEdit and sequences were aligned using Clustal W 4. Primers were designed in conserved regions of the coding sequence of medaka (NM_001136520), seabass (GQ353293), partial rainbow trout CDS(AF228695), and construct of salmon ESTs (DW576689, DW584143, DY698298) from NCBI (National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov). PCR primer pairs (Table 2) were designed in the coding sequence. The product generated was cleaned using illustra GFX PCR DNA and GelBand Purification Kit (GE Healthcare) according to manufacturer's instructions and then cloned into the pGEM®-T Easy vector system (Promega, Southampton, UK). Plasmids were harvested from discrete colonies using a GenElute™ Plasmid Miniprep Kit (sigma Aldrich, Gillingham, UK) and the presence of an insert checked by enzymatic digestion (ECoR1, Invitrogen Paisely, UK). Plasmids with the correct sized insert were sequenced through a Beckman 8800 auto sequencer and sequence results were then analysed using SEQman as part of Lasergene software package (DNASTAR, Madison, Wisconsin, USA). qPCR primers were designed within this partial sequence using Primer select also as part of the Lasergene software package (DNASTAR Madison, Wisconsin, USA).

Table2: PCR primer names and sequences used for Per1 identification.

Primer	**Forward**	**Reverse**
RTper1 pub	AGAGCCCATCCCCACCCAGCAGTT	TCGGCCCGTCAGGAAGGAGA
1Per1	CTGCTGTCGACCAGCTCGGAGCACGAC	GGCGCCAAAAGCTCCGAAAACATGGTG
2Per1	CTGCGCTGCAAGCCAGAGCGTCTCC	GCAGCTGGGAGTGTGACTGGTGGTGAAGAT
3Per1	GCCCTGCCGCCTGCCCAACTG	GCGCCCGACGATAAACGCCACCTTC

Quantitative PCR

In order to determine diel patterns of clock gene mRNA expression, absolute quantification qPCR assays were established for *Clock*, *Cry2*, *Per1*, and *Per2*. *β-Actin* was used as a housekeeping reference gene for all analysis or brain and fin samples while *Elongation factor 1α* (*ELF-1α*) was used for liver samples (justification of the selection of housekeeping genes is explained further below). Each qPCR reaction consisted of primer pairs (Table 3) at a concentration of 0.5 μM, 5 μl of cDNA (1/20 for experement1 and 1/10 for experiment 2), 3 μl DNA/RNA free H_2O and 10 μl ABsolute™QPCR SYBR Green master mix (Thermo scientific, Leon-Rot, Germany) in a total reaction volume of 20 μl. The ABsolute™QPCR SYBR Green Mix was made up of Thermo-Start™ DNA polymerase, a proprietary reaction buffer, dNTP's and SYBR Green I with Mg++ at a concentration of 3 mM in the final 1× reaction. All qPCR assays were carried out in a Techne Quantica thermocycler (Techne, Quantica, Cambridge, UK) in a thermo cycling programme consisting of a 15 minute initiation stage at 95 °C this is followed by 45 cycles of 3 temperature steps; 95 ^{o}C for 15 s anneal x°C (see Table 3 for target specific annealing temperatures) for 15 s and 72 ^{o}C for 30s. This was followed by a temperature ramp from 70 - 90°C for melt-curve analysis to verify

that no primer-dimer artefacts were present and only one product was generated from each qPCR assay. Quantification was achieved by a parallel set of reactions containing standards consisting of serial dilution of spectrophotometrically determined, linearised plasmid containing partial cDNA sequences generated. All samples were run in duplicate and each assay contained non-template controls.

Table 3: Names of qPCR primer, sequence information and annealing temperature for primer pairs used in experiments 1 and 2.

Primer Name	**Sequence (5'-3')**	**Anneal**
β Actin - Forward	ATCCTGACAGAGCGCGGTTACAGT	61^{o}C
β Actin - Reverse	TGCCCATCTCCTGCTCAAAGTCCA	61^{o}C
Clock - Forward	AGAAATGCCTGCACAGTCGGAGTC	64^{o}C
Clock - Reverse	CCACCAGGTCAGAAGGAAGATGTT	64^{o}C
Cry2 - Forward	GAGGGCATGAAGGTGTTTGAGGAG	61^{o}C
Cry2 - Reverse	GTGGAAGAACTGCTGGAAGAAGGA	61^{o}C
EF - α - Forward	TCTGGAGACGCTGCTATTGTTG	61^{o}C
EF - α - Reverse	GAC TTGTGACC TTGCCGCTTGAG	61^{o}C
Per1 - Forward	AGGGGGTCATGCGGAAGGGGAAGT	66^{o}C
Per1 - Reverse	TGGGCCACCTGCATGGGCTCTGT	66^{o}C
Per2 - Forward	GCTCCCAGAATTCCTAGTGACAAG	60^{o}C
Per2 - Reverse	GAACAGCCCTCTCGTCCACATC	60^{o}C

qPCR normalisation and statistics

Results generated from brain and fin samples were normalised using *β-Actin* as described in Davie et al. (2009). For liver samples, geNorm analysis was carried out on three potential house keeping genes tested on the long day liver samples to determine the most suitable

reference gene including *β-Actin, ELF-α* and *GAPDH*. In the Atlantic salmon liver samples, *ELF-α* displayed the least variation and was therefore used for normalisation in this liver (Figure 2). Results were then converted to external time in accordance with Daan et al. (2002) whereby the external time 0 (ExT 0) is the middle of the dark phase. For example ExT 0 of experiment 1 long day treatment occurs at 04:00 when lights off occurs at 00:00 and lights on occurs at 08:00. Gene expression data are expressed as copy number of the target gene per μg total RNA (normalised to the appropriate reference gene).

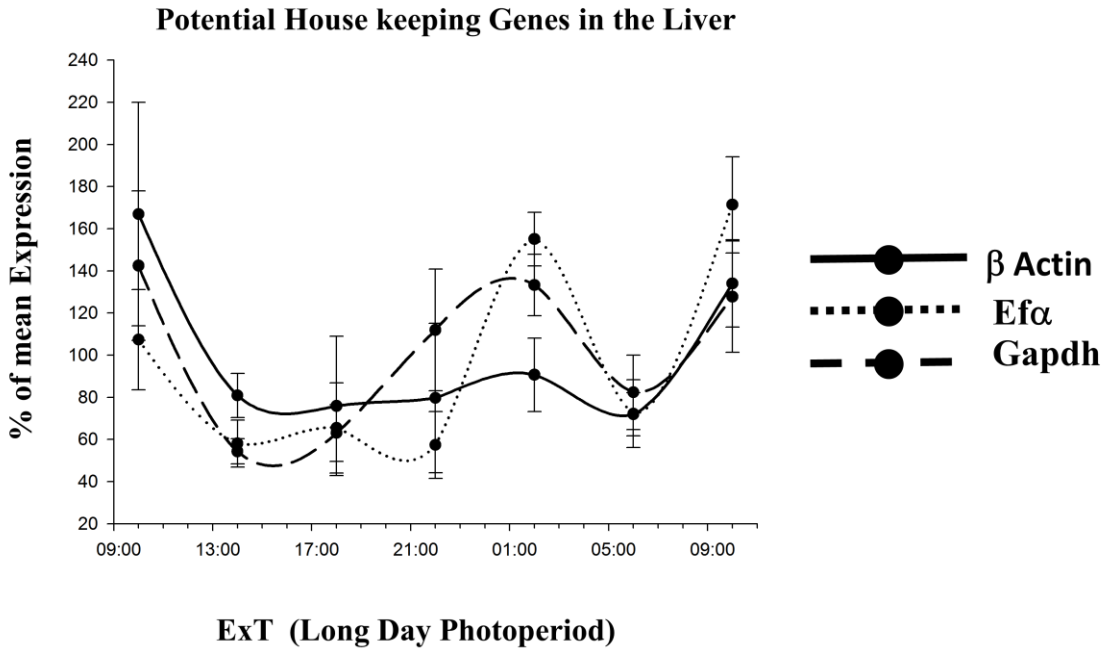


Figure 2: 24h expression of *β–Actin*, EFα and Gapdh in the liver of Atlantic salmon parr acclimated to experimental long day photoperiod for potential use as a housekeeping gene in the liver.

Melatonin Radioimmunoassay

Relative levels of circulating melatonin from blood plasma were determined by melatonin radioimmunoassay (RIA) according to Randall *et al.* (1995). In experiments 1 and 2 blood

plasma was pooled in order to achieve a volume of 250 µl and a total of n = between 3 and 6 pools were analysed per time point. In all cases, plasma (250 µl) was diluted to 500 µl with assay buffer. For further details of melatonin RIA see chapter 2.

Statistical analyses

Analysis of Variance (ANOVA) was used to determine a significant effect of time and Turkey's test was used to determine significant differences between sample time points and mean of different sample sets (InStat® 3.1, Graphpad software inc). Data from each tissue/ photoperiod was then fitted to a cosine wave in order to determine the presence of a significant circadian rhythm. Raw data was analysed using the acro circadian analysis program (Refennetti R., University of South Carolina, USA; http://www.circadian.org/softwar.html). Acro analysis also determines both the significance, acrophase (peak in expression) mean and amplitude of raw data using the equation **Y= A + B * cos (C *X –D)** whereby Y is level of gene expression as a percentage of the mean A is the baseline, C is the frequency multiplier and D is the acrophase of the data set (Refinetti 2006; Davie et al. 2009). Significance was determined by a p value less than 0.05.

4. RESULTS

Per1

An 899 bp partial sequence was isolated which contained an 844 bp coding sequence and a 55bp 3'untranslated region (UTR) (Figure 3). Within the coding sequence the Period circadian-like C terminal domain, also referred to as the CRY binding domain can be seen (Figures 3 and 4). Phylogenetic analysis of the deduced a sequence for *Period 1* in relation to other vertebrate Period sequences shows the transcript grouped within the teleost Period 1

cluster >80% identity with other teleost *Per1's* and 60-70% identity with mammalian *Per1* sequences (Figure 5).

```
         10        20        30        40        50        60        70        80        90
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CCGCCTGCCTGGCCGAGTGCACGGGGACAGGGTCGCTCCAGCACCCTGGACGCCCTCCGTTACGCCCTGAACTGTGTGAAGCAGGTGCGA
 P  P  A  W  P  S  A  R  G  Q  G  R  S  S  T  L  D  A  L  R  Y  A  L  N  C  V  K  Q  V  R

        100       110       120       130       140       150       160       170       180
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GXCCAGGATGGTGAATCCACCCCCAGTGACCTACTGGACCTGCTCCTGCAAGAGGACTCCCGCTCAGGCTCCGGCTCAGCTGCCTCTGGG
 X  Q  D  G  E  S  T  P  S  D  L  L  D  L  L  L  Q  E  D  S  R  S  G  S  G  S  A  A  S  G

        190       200       210       220       230       240       250       260       270
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TCAGGGTCCTCTGGGTCAGGGTCCTCTGGGTCAGGGTCCTCAGGGTCGGGTTCTGTCTCCTTGGGCACTGTGTCCAACGGCTGCAGCTCC
 S  G  S  S  G  S  G  S  S  G  S  G  S  S  G  S  G  S  V  S  L  G  T  V  S  N  G  C  S  S

        280       290       300       310       320       330       340       350       360
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TCAGGCAGTGGAACTAGTCGCAGTCGGAGTAGCCACACCAGCAAGTACTTTGGCAGCATCGACTCGTCAGAGAACGACCACTCCCGCAAG
 S  G  S  G  T  S  R  S  R  S  S  H  T  S  K  Y  F  G  S  I  D  S  S  E  N  D  H  S  R  K

        370       380       390       400       410       420       430       440       450
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CAGCCAGCAGGTGGCAGCAGAGCCAGAGGAGACGGGGAGGAGCAGTTGATTAAGTGTGTCCTGCAGGATCCCATCTGGCTCCTCATGGCC
 Q  P  A  G  G  S  R  A  R  G  D  G  E  E  Q  L  I  K  C  V  L  Q  D  P  I  W  L  L  M  A

        460       470       480       490       500       510       520       530       540
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AACACCGACCACAAGGTGATGATGACCTACCAGCTGCCGACCAAGGACAGGGAGACGGTGCTGCGGCAGGACCGCGAGGCCCTGCGGGCC
 N  T  D  H  K  V  M  M  T  Y  Q  L  P  T  K  D  R  E  T  V  L  R  Q  D  R  E  A  L  R  A

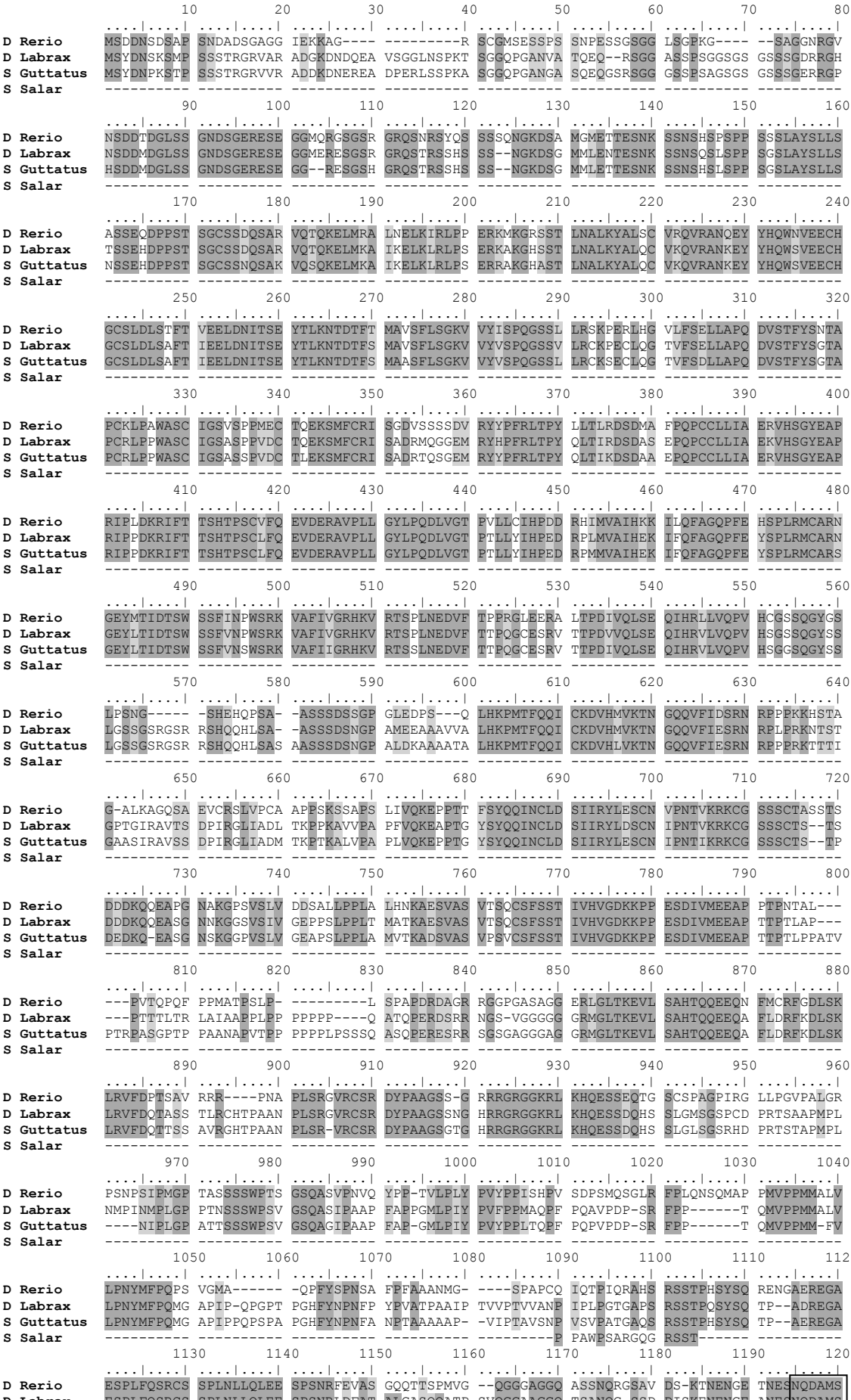
        550       560       570       580       590       600       610       620       630
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ATGCACAAACACCAGCCTCGCTTCACTGAGGACCAGAAGAGAGAACTGAGCCAGGTCCACCCCTGGATACGGACCGGGCGCCTGCCCCGT
 M  H  K  H  Q  P  R  F  T  E  D  Q  K  R  E  L  S  Q  V  H  P  W  I  R  T  G  R  L  P  R

        640       650       660       670       680       690       700       710       720
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GCCATCAACGTCTCGGCATGTATGGGGTGCAAGTCTTCCCCCTTACCAGAGAGCACCATGGACCAGGGAGGAGAGGCTTGCAGAGAAGAG
 A  I  N  V  S  A  C  M  G  C  K  S  S  P  L  P  E  S  T  M  D  Q  G  G  E  A  C  R  E  E

        730       740       750       760       770       780       790       800       810
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GGGCATGCGGCGCACTCCGAAACACAAGGCAACGACCAGGAAATGACAACAGAGGAGCAGGAAGTGACCTGGCCAACGGAGGAGGAGCAA
 G  H  A  A  H  S  E  T  Q  G  N  D  Q  E  M  T  T  E  E  Q  E  V  T  W  P  T  E  E  E  Q

        820       830       840       850       860       870       880       890
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|...
GCAAGGGCTGCCGATACCGACATGACCCACTGAAAGGTTGAGAATGGATCAAGAACACTTAAGTTATTGTGAATTTTTCACCTGACAG
 A  R  A  A  D  T  D  M  T  H  *
```

Figure 3: Partial Atlantic salmon *Per1* sequence including 844 bp coding sequence.


D Rerio
D Labrax
S Guttatus
S Salar

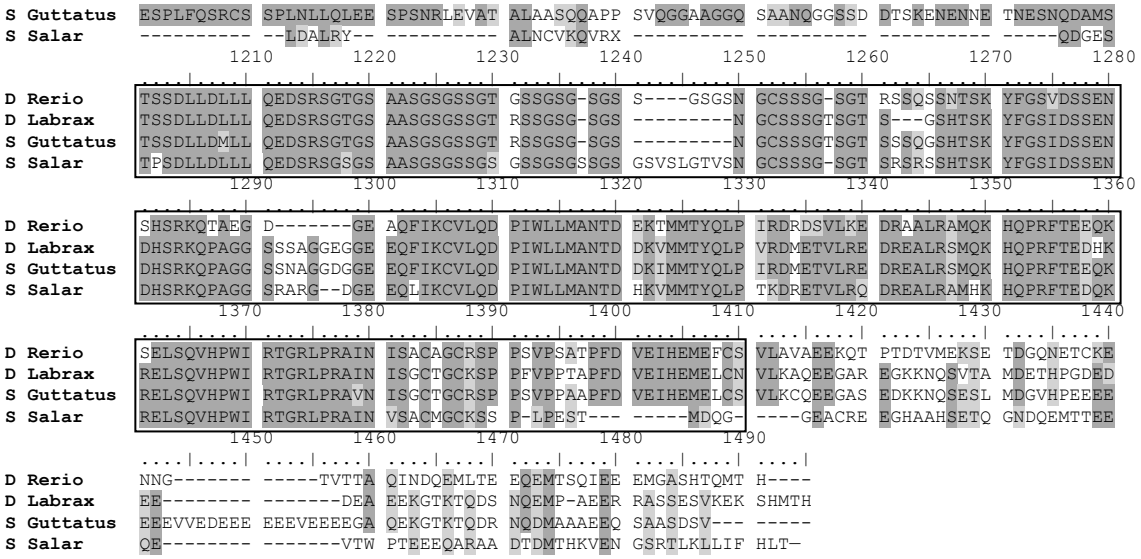


Figure 4: Partial Atlantic salmon *Per1* protein alignment with other available teleost sequence information.

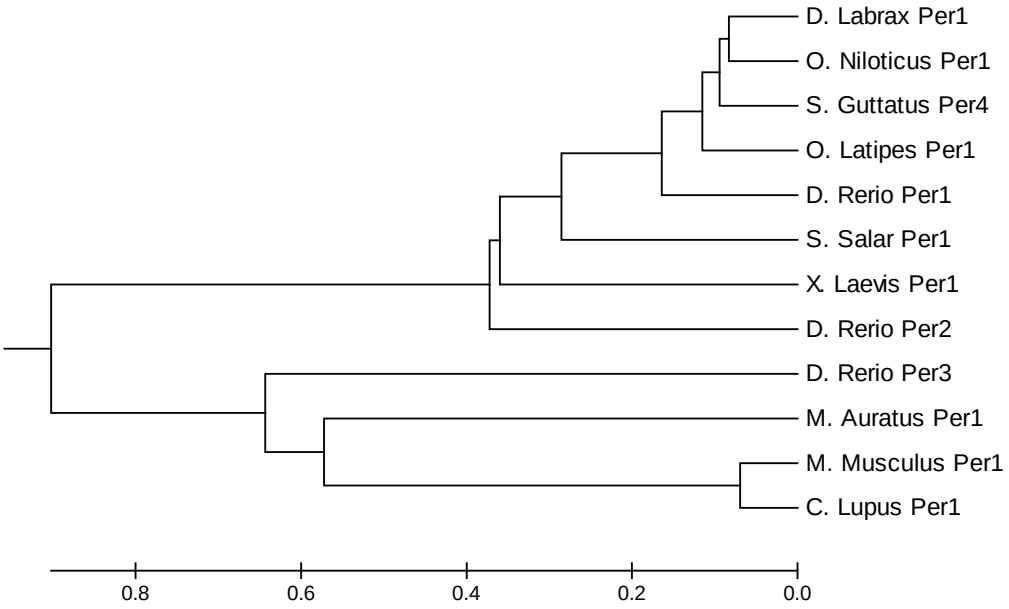


Figure5: Phylogenetic tree of teleost Period genes. The partial sequence of Atlantic salmon *Per1* shows highest similarity to other teleost Per1 genes . The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2007) and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007).

Experiment 1 – Effect of photoperiod on circadian clock gene expression and melatonin.

Plasma melatonin levels showed a significant circadian rhythm in fish exposed to all experimental photoperiods (Figure 6). In all cases levels were significantly elevated during the night in comparison to during the day. Under all experimental treatments the acrophase or peak of levels of plasma melatonin occurred during (SD and 12:12) or just following (LD) the nocturnal phase of the 24h cycle (Figure 6, Table 4). A significant difference was only observed between the acrophase of the SD and 12:12 in comparison to the LD treatments. No significant difference was observed in mean diurnal melatonin levels between photoperiodic treatments. However, mean nocturnal melatonin levels were significantly elevated in fish reared under LD in comparison to SD and 12:12 (Figure 6).

All clock genes investigated were expressed under all treatments and in totRNA extracted from all three tissues studied. In the brain samples, in smolts acclimated to LD, both *Clock* and *Per2* displayed a significant circadian expression rhythm (Figure 7). The peak (acrophase) of *Clock* and *Per2* expression occurred at the onset of the diurnal phase at ExT 05:00 h (Table 4). In fish exposed to SD and 12:12, none of the clock genes investigated displayed a significant circadian rhythm of expression in the brain. In addition, no *Per1* rhythmic expression was observed under any of the experimental photoperiods tested (Figure 7). In the fin samples, a significant circadian rhythm was only observed for *Clock* in salmon acclimated to SD (Figure 8). However, ANOVA analyses showed no significant difference between time points ($P = 0.143$). No other genes displayed significant circadian rhythms however *Clock* under 12:12 and *Per 2* under all three photic conditions did have significant differences in expression during the 24 hr period. In the liver *Clock* expression profile displayed a significant circadian rhythm under 12:12 treatment only while *Per1* displayed significant rhythmic expression in salmon parr exposed to SD (Figure 9). The acrophase of

Clock and *Per1* occurred at ExT 15:00 ± 2.33 h and 13:00 ± 2.64 h, respectively (Table 4). Irrespective of circadian rhythms, significant differences in expression over the 24 hour period were observed in *Clock* 12:12 and LD; *Per1* SD and 12:12 and *Per 2* in all three photic conditions (Figure 9).

Mean expression (copy number per μg of totRNA normalised using β-Actin) over the 24h profiles showed significant differences both in relation to the photoperiod treatments and the tissue types (Figure 10). *Clock* in Brain samples were 2.5 and 2.3 fold higher in fish acclimated to LD and 12:12 in comparison to SD photoperiod. *Clock* in fin and liver showed no response to photoperiod and had low expression levels being statistically comparable to the brain SD pool. For *Per1* expression both brain and liver samples showed a significant response to photoperiod. In the brain mean SD levels were 1.5 fold higher compared to 12:12 with LD levels being intermediate to these. In the liver, levels were 3 and 4 fold elevated under LD compared to SD or 12:12 respectively. *Per1* levels in the fin were low and showed no response to photoperiod (Figure 10). In the brain and fin mean expression levels of *Per2* expression were not significantly different between the three experimental photoperiods levels were significantly lower however in the fin in comparison to the brain. In the liver there was a significant effect of photoperiod on mean expression levels with 3.7 fold higher mean expression level of *Per2* being observed under SD in comparison to 12:12 (Figure 10).

Table 4: Significance of 24h profiles of clock gene expression and levels of plasma melatonin, as defined by Acro and ANOVA analysis, in Atlantic salmon parr acclimated to long day, Short day and 12L12D treatments (Experiment 1).

Gene / Photoperiod/ tissue	**Acro**	**analysis**	**ANOVA**	**Significant Circadian Rhythm**
Brain	**P Value**	**Acrophase (ExT±SEM)**	**P Value**	**Sig/NS**
Clock SD	>0.05	-	<0.05	NS
Clock 12:12	>0.05	-	>0.05	NS
Clock LD	**<0.05**	**05:00 ± 2.68**	**<0.05**	**Sig**
Per1 SD	>0.05	-	<0.05	NS
Per1 12:12	>0.05	-	>0.05	NS
Per1 LD	>0.05	-	<0.05	NS
Per2 SD	>0.05	-	<0.05	NS
Per2 12:12	>0.05	-	>0.05	NS
Per2 LD	**<0.05**	**05:00 ± 2.45**	**<0.05**	**Sig**
Fin				
Clock SD	**<0.05**	**21±2.2**	>0.05	NS
Clock 12:12	>0.05	-	<0.05	NS
Clock LD	>0.05	-	>0.05	NS
Per1 SD	>0.05	-	>0.05	NS
Per1 12:12	>0.05	-	>0.05	NS
Per1 LD	>0.05	-	>0.05	NS
Per2 SD	>0.05	-	<0.05	NS
Per2 12:12	>0.05	-	<0.05	NS
Per2 LD	>0.05	-	>0.05	NS
Liver				

Clock SD	>0.05	-	>0.05	NS
Clock 12:12	**<0.05**	**15:00 ± 2.33**	**<0.05**	**Sig**
Clock LD	>0.05	-	<0.05	NS
Per1 SD	**<0.05**	**13:00 ± 2.64**	**<0.05**	**Sig**
Per1 12:12	>0.05	-	<0.05	NS
Per1 LD	>0.05	-	=0.05	NS
Per2 SD	>0.05	-	<0.05	NS
Per2 12:12	>0.05	-	<0.05	NS
Per2 LD	>0.05	-	<0.05	NS
Melatonin				
SD	**<0.05**	**01:00 ± 1.33**	**<0.05**	**Sig**
12:12	**<0.05**	**23:00 ± 0.95**	**<0.05**	**Sig**
LD	**<0.05**	**05:00 ± 1.56**	**<0.05**	**Sig**

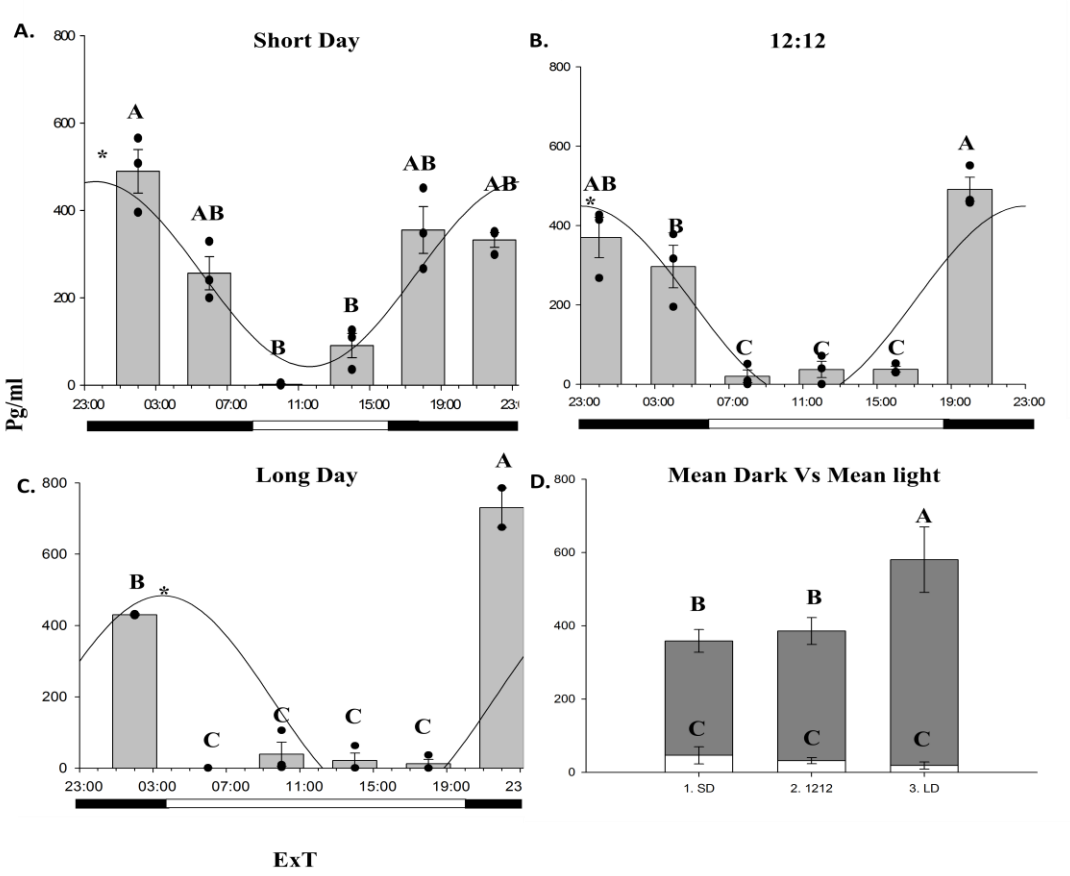


Figure 6: 24h profiles and mean plasma melatonin from experimental seasonal photoperiods with spread of data in addition to SEM (experiment 1). Panels A to C display 24h profiles of melatonin in relation to external time (whereby ExT 0 is the midpoint of the dark phase). The presence of a cosine wave denotes a significant circadian rhythm by acro analysis and * represents the acrophase (peak) of the 24h cycle. D shows mean nocturnal (dark grey) and diurnal (white) levels of melatonin with SEM. In all graphs the presence of different letters represents statistically significant difference between samples by way of ANOVA and Turkeys test where by $P<0.05$

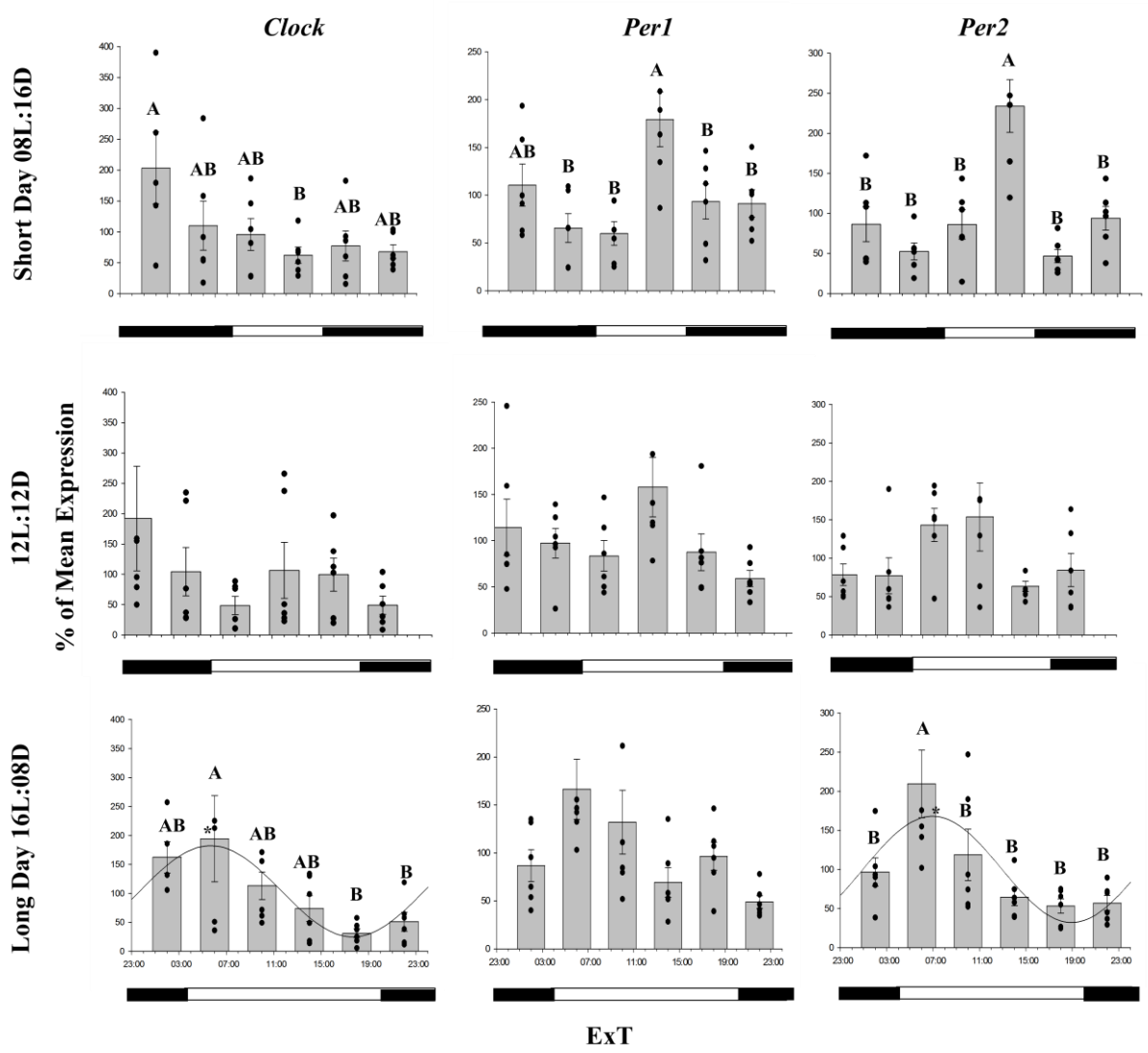


Figure 7: Daily profiles of clock (*Clock, Per1 and Per2*) gene expression in the Brain of Atlantic salmon parr acclimated to experimental long day short day and 12L: 12D photoperiod experiment 1. Results are displayed in relation to external time, where by ExT 0 is the mid point of the nocturnal phase. Gene expression data is displayed as the percentage of the mean ± the SEM and includes the spread of the data.The presence of a cosine wave denotes a significant circadian rhythm by acro analysis and * represents the acrophase (peak) of the 24h cycle. The presence of different letters represents statistically significant difference between samples by way of ANOVA and Turkeys test where by $P<0.05$

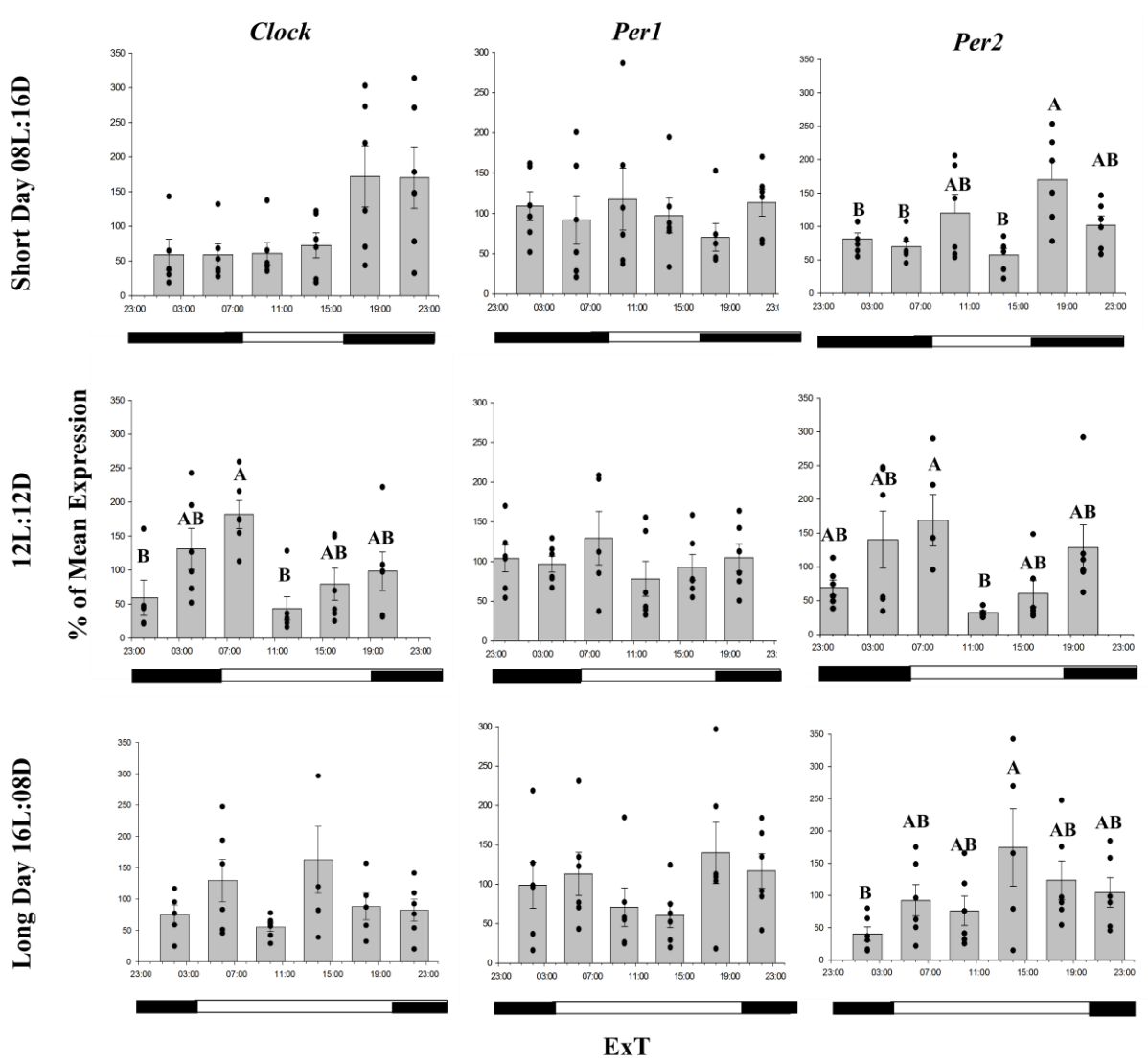


Figure 8: Daily profiles of clock (*Clock, Per1 and Per2*) gene expression in the fin of Atlantic salmon parr acclimated to experimental long day short day and 12L: 12D photoperiod (Experiment 1. Results are displayed in relation to external time, where by ExT 0 is the mid point of the nocturnal phase. Gene expression data is displayed as the percentage of the mean ± the SEM and includes the spread of the data.The presence of a cosine wave denotes a significant circadian rhythm by acro analysis and * represents the acrophase (peak) of the 24h cycle. The presence of different letters represents statistically significant difference between samples by way of ANOVA and Turkeys test where by $P<0.05$

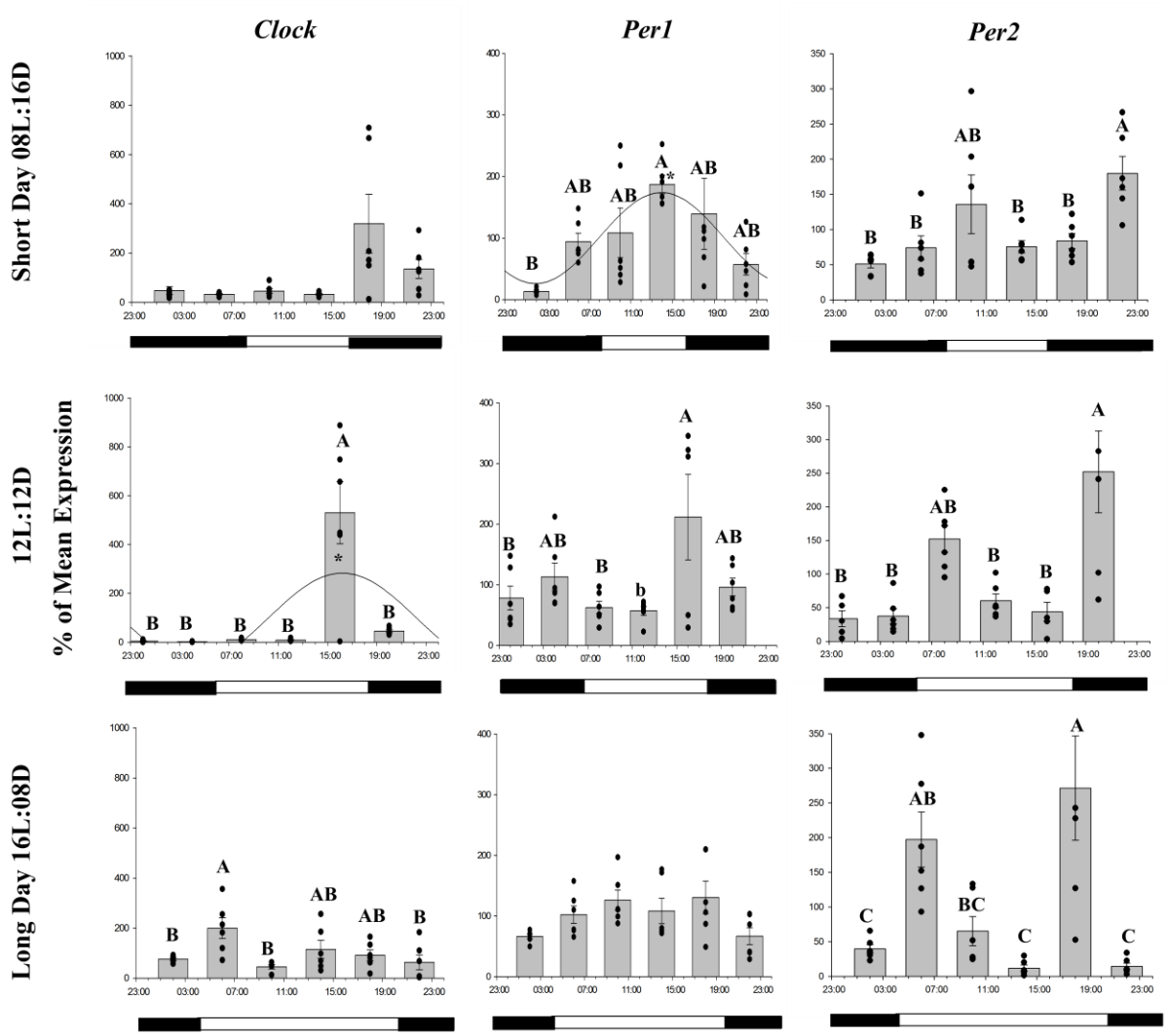


Figure 9: Daily profiles of clock (*Clock, Per1 and Per2*) gene expression in the liver of Atlantic salmon parr acclimated to experimental long day short day and 12L: 12D photoperiod (Experiment 1). . Results are displayed in relation to external time, where by ExT 0 is the mid point of the nocturnal phase. Gene expression data is displayed as the percentage of the mean ± the SEM and includes the spread of the data.The presence of a cosine wave denotes a significant circadian rhythm by acro analysis and * represents the acrophase (peak) of the 24h cycle. The presence of different letters represents statistically significant difference between samples by way of ANOVA and Turkeys test where by $P<0.05$

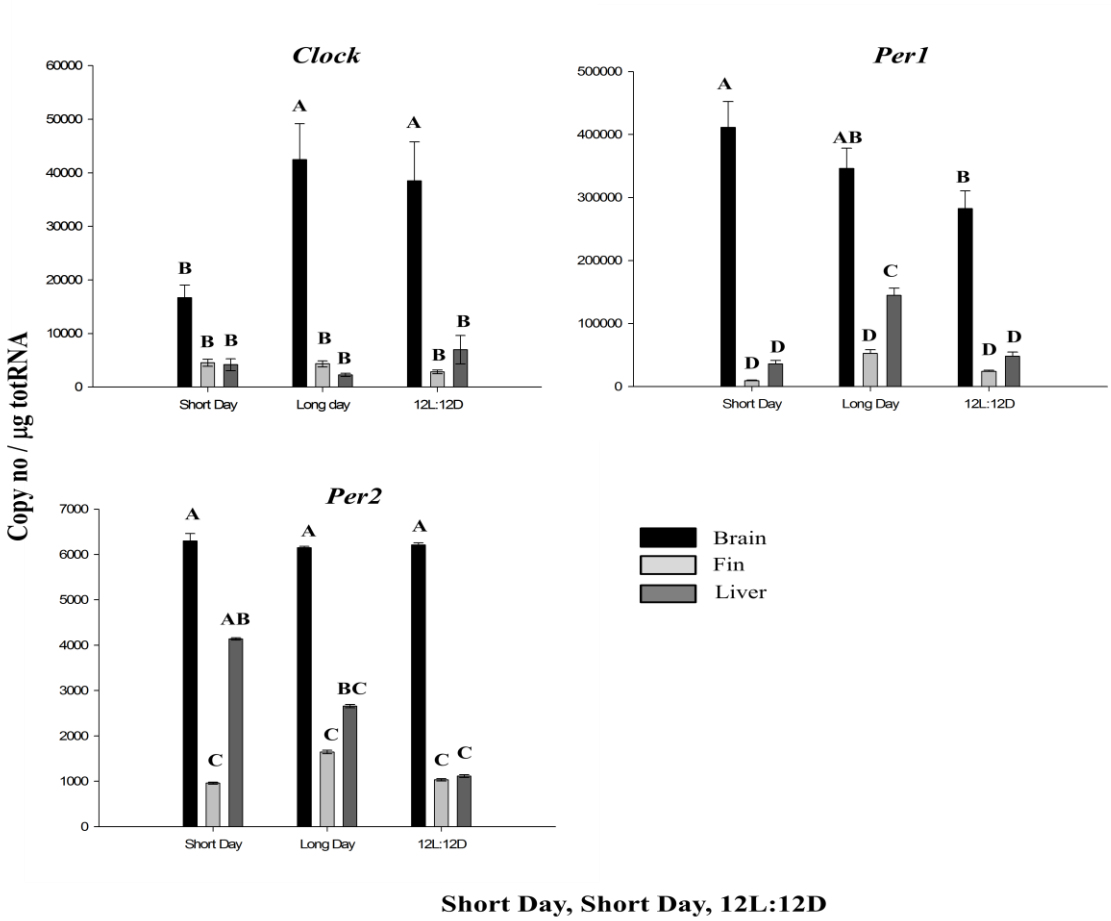


Figure 10: Mean levels of clock gene (*Clock, Per1 and Per2*) expression over 24h in the Brain Fin and the Liver from experiment 1. Gene expression data is displayed as the copy number per µg totRNA ± the SEM. The presence of different letters represents statistically significant difference between samples by way of ANOVA and Turkeys test where by $P<0.05$

Experiment 2 – Seasonal/Life history changes in circadian clock gene expression and melatonin.

Melatonin displayed circadian rhythms under each seasonal photoperiod and the acrophase of levels of plasma melatonin occurred during the nocturnal phase of the circadian cycle in all treatments (Table 5 and Figure 11). In comparison to all other seasonal sample sets, the acrophase of the June 2010 sample was significantly later in the circadian cycle at approximately one hour after the mid dark, as opposed to one hour prior to mid dark as was observed in all other sample sets. Nocturnal levels of plasma melatonin appear to be suppressed during the winter and early spring and appear elevated after transfer to seawater with mean nocturnal levels being highest in the October 2010 samples (Figure 11).

In the autumn and spring equinox, in the freshwater phase, no significant circadian rhythm for clock gene expression (*Clock, Cry2, Per1* & *Per2*) were present (Figure 12 and Table 5). Only in the winter solstice sample was a circadian rhythm observed in *Cry2* with expression peaking at sunrise (06:00 ± 2.75). Following seawater transfer, in the summer solstice sample, a significant circadian rhythm in expression of *Clock* and *Cry2* was observed with peak expression at 01:00 ± 2.3 and 05:00 ± 2.0 respectively. In the autumn equinox sample, a significant circadian rhythm was only observed in *Per2* with expression peaking at 23:00 ± 2.44. *Per1* did not display any significant circadian profile of expression in any of the photoperiods investigated however in the freshwater phase winter solstice sample there were significant variation in expression levels over the 24 hour period.

All four clock genes studied showed significant differences in mean expression level with respect to seasonal time (Figure 13). *Clock* expression levels were significantly higher in samples collected in the freshwater phase autumn equinox (October 2009) and winter solstice

(January 2010) samples compared to all others. In contrast *Cry2* expression was lowest in the Autumn equinox samples both in the freshwater and seawater phase (October 2009 and 2010) with the freshwater winter solstice (January 2010) and sea water summer solstice (June 2010) being comparable and significantly higher to these. Freshwater spring equinox sample (March 2010) were significantly higher than all others. Similarly *Per1* expression was statistically higher in January and March 2010 sample sets. Finally, *Per2* displayed the highest levels of mRNA expression in the summer solstice sample (seawater phase, June 2010) and lowest in the autumn equinox samples (both the freshwater and seawater phase in October 2009 and 2010 respectively).

Table 5: Significance of 24h profiles of clock gene expression and levels of plasma melatonin, as defined by Acro and ANOVA analysis, in Atlantic salmon acclimated to SNP (Experiment 2).

Sample Set	**Arco**	**analysis**	**ANOVA**	**Significant Circadian Rhythm**
Clock	**P value**	**Acrophase**	**P Value**	**Sig/NS**
October 09	>0.05	NS	>0.05	NS
January 10	>0.05	NS	>0.05	NS
March 10	>0.05	NS	>0.05	NS
July 10	**<0.05**	**01±02:30**	**<0.05**	**Sig**
Oct 10	>0.05	NS	<0.05	NS
Cry2				
October 09	>0.05	NS	>0.05	NS
January 10	**<0.05**	**06±02.75**	**<0.05**	**Sig**
March 10	>0.05	NS	<0.05	NS
July10	**<0.05**	**05±02.00**	**<0.05**	**Sig**
Oct 10	>0.05	NS	<0.05	NS
Per1				
October 09	>0.05	NS	>0.05	NS
January 10	>0.05	NS	<0.05	NS
March 10	>0.05	NS	>0.05	NS
July 10	>0.05	NS	>0.05	NS
Oct 10	>0.05	NS	>0.05	NS
Per2				
October 09	>0.05	NS	<0.05	NS
January 10	>0.05	NS	>0.05	NS
March10	>0.05	NS	>0.05	NS
July 10	>0.05	NS	>0.05	NS
Oct 10	**<0.05**	**23±2.44**	**<0.05**	**Sig**
Melatonin				
October 09	**< 0.05**	**23.00±2.80**	**<0.05**	**Sig**
January 10	**< 0.05**	**22.00±2.18**	**<0.05**	**Sig**
March 10	**< 0.05**	**23.00±2.15**	**<0.05**	**Sig**
July 10	**< 0.05**	**01.00±2.12**	**<0.05**	**Sig**
Oct 10	**< 0.05**	**23:00±1.61**	**<0.05**	**Sig**

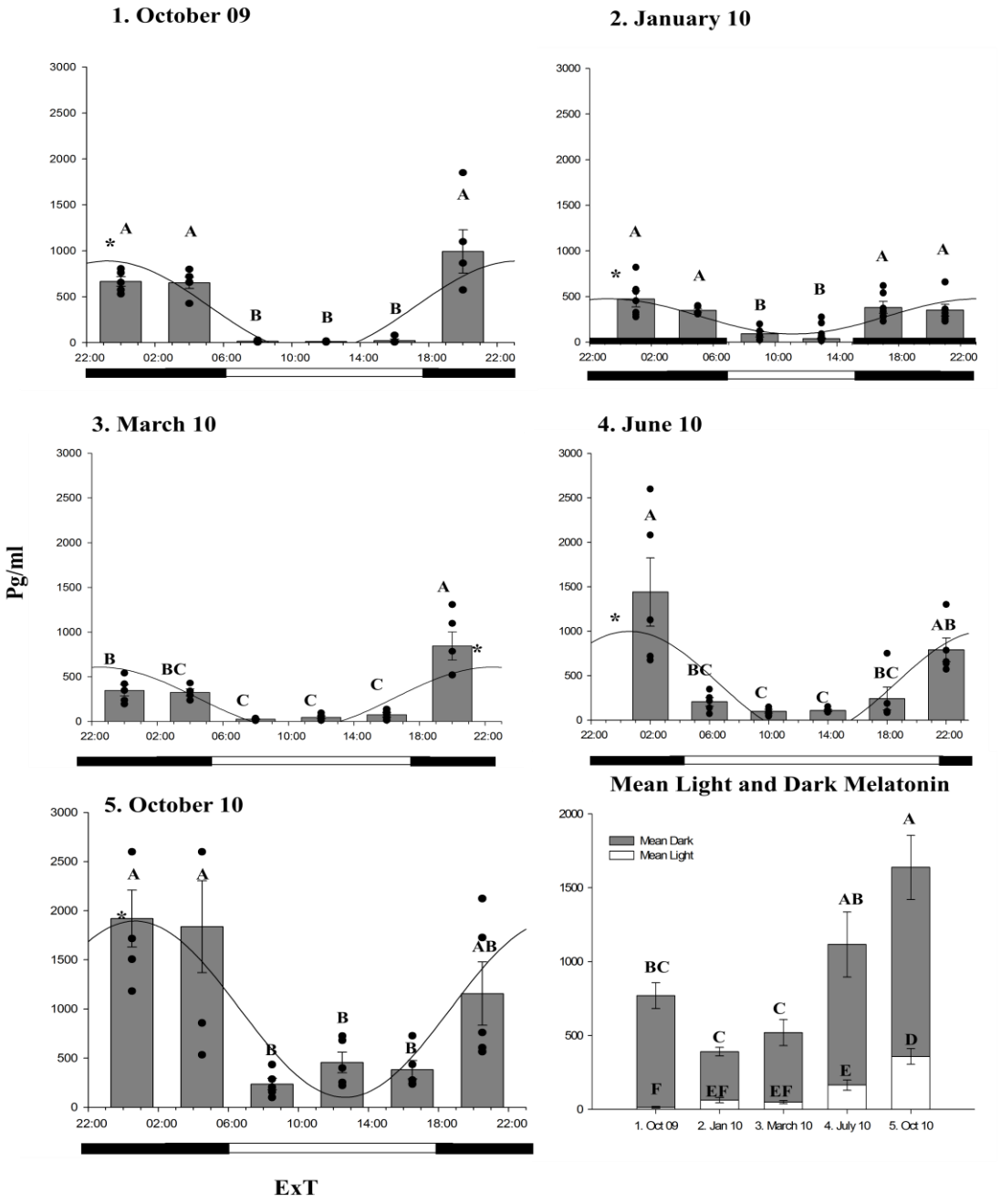


Figure 11: Five daily profiles of melatonin in Atlantic salmon parr acclimated to SNP and mean nocturnal and diurnal levels of melatonin from experiment 2 Panels 1 to 5 display 24h profiles of melatonin in relation to external time (whereby ExT 0 is the midpoint of the dark phase). The presence of a cosine wave denotes a significant circadian rhythm by acro analysis and * represents the acrophase (peak) of the 24h cycle. D shows mean nocturnal (dark grey) and diurnal (white) levels of melatonin with SEM. In all graphs the presence of different letters represents statistically significant difference between samples by way of ANOVA and Turkeys test where by $P<0.05$

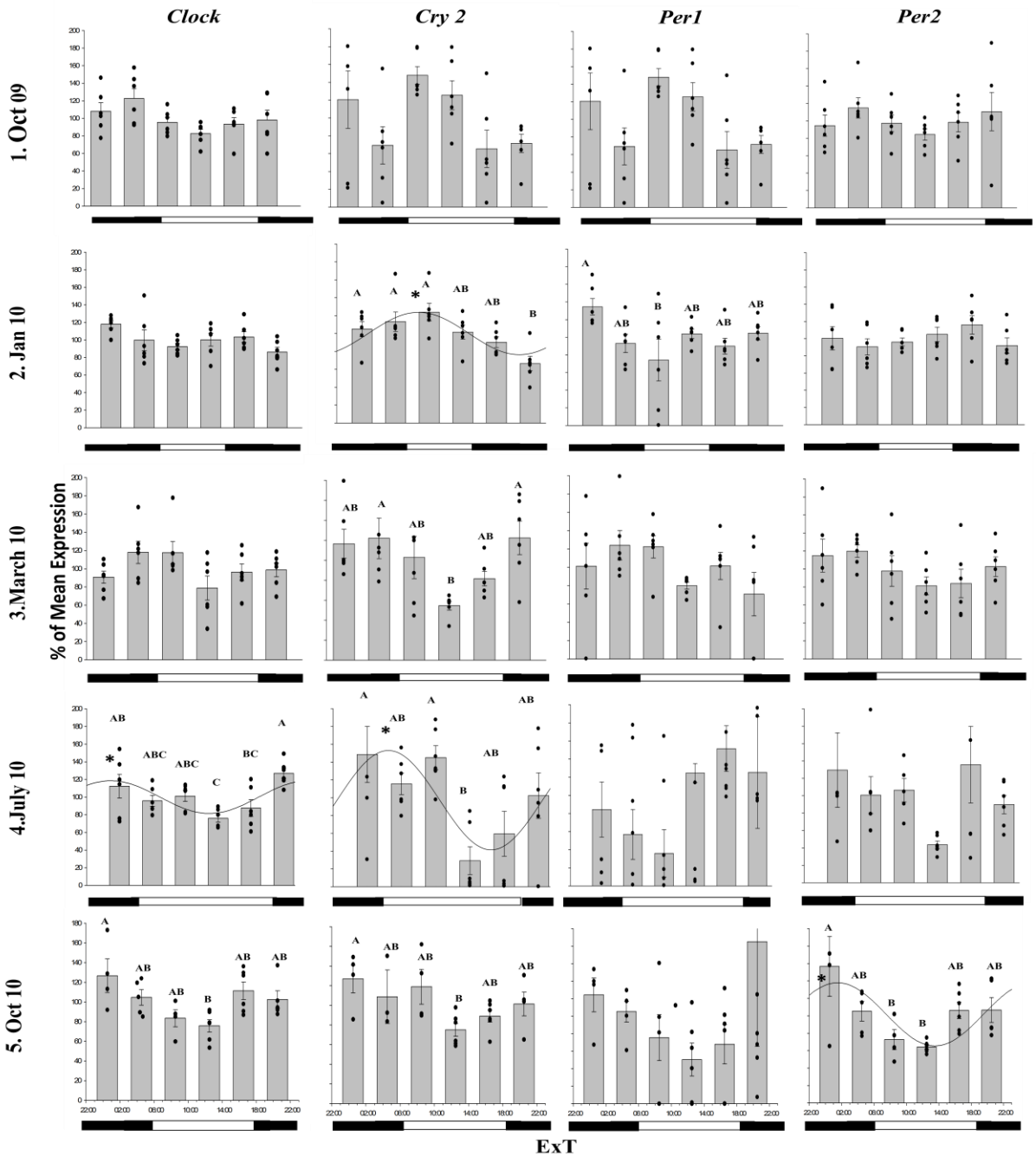


Figure 12: Daily clock gene (*Clock, Cry2, Per1 and Per2*) expression in the brain of Atlantic salmon parr acclimated to an SNP and sampled at 5 seasonal sample points (Experiment 2). Results are displayed in relation to external time, where by ExT 0 is the mid point of the nocturnal phase. Gene expression data is displayed as the percentage of the mean ± the SEM and includes the spread of the data.The presence of a cosine wave denotes a significant circadian rhythm by acro analysis and * represents the acrophase (peak) of the 24h cycle. The presence of different letters represents statistically significant difference between samples by way of ANOVA and Turkeys test where by $P<0.05$

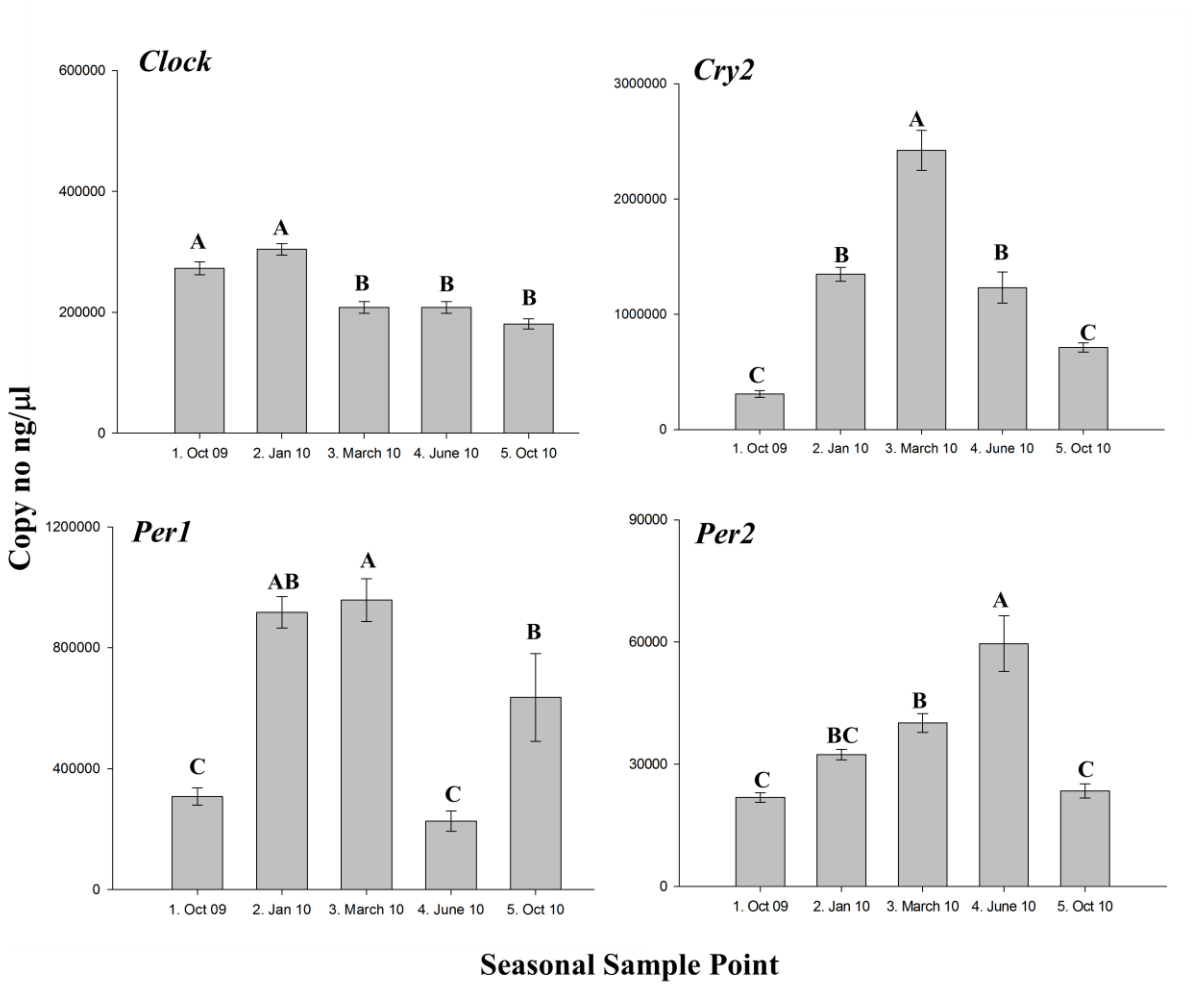


Figure 13: Mean clock gene (*Clock, Cry2, Per1 and Per2*) expression in the Brain of Atlantic salmon parr acclimated to an SNP and sampled at 5 seasonal sample points (Experiment 2). Gene expression data is displayed as the copy number per µg totRNA ± the SEM. The presence of different letters represents statistically significant difference between samples by way of ANOVA and Turkeys test where by $P<0.05$

5. DISCUSSION

The seasonal control of Atlantic salmon physiology is primarily entrained by environmental cues (e.g. photoperiod and temperature; refs).It is believed that this is achieved via the transmission of neuroendocrine messengers, such as the hormone melatonin, SCN electrical activity and the expression of clock and clock controlled genes, and these are entrained to

endogenous biological clocks.(Davie et al. 2009). To investigate this hypothesis, daily profiles of plasma melatonin and clock gene expression in the brain, fin and liver of salmon parr acclimated to different seasonal photoperiod treatments, were measured. Thereafter diel profiles of brain clock gene expression and plasma melatonin concentrations in salmon reared under a simulated natural photoperiod for one year, during which time they transitioned from freshwater parr to seawater post-smolt, were investigated. The specific aims of these experiments were to explore further seasonal dependent clock gene cycling; to determine if rhythmic expression in the brain was reflected in peripheral tissues and to determine if the effects observed following short term acclimation were replicated during normal development. Results displayed a clear correlation between photoperiodic conditions and clock gene cycling and melatonin. Moreover daylength dependent expression of clock genes was observed in the brain, however results appear to be dependent on photoperiod history as rhythmic clock gene expression is observed under photic conditions that best follow the natural seasonal progression. Moreover amplitude differences in seasonal clock gene expression appear to be dependent on photoperiod alterations and expression of clock genes in peripheral tissues differs considerably to the brain. Overall results in salmon clock gene expression differed from previous results in mammals and other teleosts previously investigated. Potentially highlighting differences clock mechanisms amongst cerebrates and in particular amongst the diverse teleost group.

As was confirmed in both experiments 1 & 2 melatonin synthesis and release in salmon, like all vertebrates, accurately reflects external photoperiod (Falcon et al. 2010; Falcon et al. 2011). Daily melatonin profiles closely mirror the external photoperiod expanding and contracting to adjust to seasonally changing ratio of light and dark. Interestingly, in experiment 1, mean levels of nocturnal melatonin were significantly higher in fish acclimated

to long day treatment irrespective of the equal temperature under all treatments (4.6 ± 0.7 °C). In experiment 2 the phase of melatonin rhythm mirrors photoperiod. However melatonin amplitude varied considerably over trial duration even under similar photic conditions. This could potentially be the result of annual variations in temperature. Indeed, water temperature in October 2010 was approximately 1.5 °C higher than October 2009 (Figure 1 and Table 1). Teleost fish are ectotherms consequently their internal body temperature and physiology, including melatonin, is regulated by external temperature (Falcon et al. 2010). In a number of teleosts temperature has been shown to regulate the amplitude of nocturnal melatonin (Randall et al. 1995; Bromage et al. 2001; Falcon et al. 2010; Falcon et al. 2011). Randall *et al* (1995) reported this effect in the Atlantic salmon under natural conditions and a 6 month out of phase photoperiod. The authors observed that melatonin was elevated during the summer irrespective of photic conditions, moreover, melatonin was highest in June and August coinciding with the natural elevation in water temperature (Randall et al. 1995). Additionally a correlation was observed between melatonin amplitude and temperature in rainbow trout (Tabata & Meissl 1993; Tabata et al. 1993). Temperature effect on melatonin amplitude has also been documented in seabass (Garcia-Allegue et al. 2001) and goldfish (Iigo et al. 1995) *in vivo* and the rainbow trout (Tabata & Meissl 1993; Tabata et al. 1993), white sucker (Zachmann et al. 1991), lamprey (Bolliet et al. 1993) and pike (Falcon et al. 1994) *in vitro*. *In vitro* the species specific temperature dependent regulation of AANAT2, the rate limiting enzyme in the synthesis of pineal melatonin has been studied (Klein 2007). Peak AANAT2 activity coincides with the optimal physiological temperature of the species (Falcon et al. 2010), 12 °C in rainbow trout, 25 °C in pike, 27 °C in seabream and 30 °C in zebrafish (Falcon et al. 1996; Coon et al. 1998; Benyassi et al. 2000; Zilberman-Peled et al. 2004; Falcon et al. 2010). This is in contrast to AANAT1, where optimum enzymatic activity occurs at 37 °C in the majority of the teleosts investigated (Kulczykowska et al. 2010).

However the precise mechanisms underpinning the temperature dependent regulation of AANAT2 are largely unclear.

There are however other potential influences of melatonin amplitude. In euryhaline fish for example, the amplitude of melatonin is altered by salinity. In salmonids such as the Coho salmon and rainbow trout, increased melatonin was observed after seawater transfer (Gern et al. 1984). In seabass and seabream, elevated melatonin was correlated with decreasing salinities, independent of photoperiod or temperature (Kleszczynska et al. 2006; Lopez-Olmeda et al. 2009). It was hypothesised by Lopez-Olmeda et al, 2009 that the inverted relationship between melatonin and salinity is a consequence of reversed migratory behaviours; salmon migrate to freshwater to breed while seabass spawn at sea and migrate to lower salinities to feed. Consequently elevated melatonin occurs with low salinity in seabass/bream and at high salinity in salmonids (Lopez-Olmeda et al. 2009). This is consistent with results in the current investigation where highest melatonin was observed in samples after salt water transfer. Animal size/stage of development has also been shown to influence melatonin release in a range of vertebrates. The fish studied in experiment 2 were reared over the duration of a year, during this period the size of the fish increased from 31.1 to 188.0 g. In sheep, increased amplitude of melatonin in the blood plasma has previously been attributed to pineal size (Coon et al. 1999). However the size of the pineal was determined to be a consequence of genetic influence. In salmon the pineal organ increases in size as the fish grows in size. The presence of genetic variations in melatonin amplitude is unknown. While work in rats correlated increased weight gain to low levels of melatonin (Rios-Lugo et al. 2010). However the effect of growth and nutritional status on melatonin amplitude remains unknown in teleosts. Despite the many potential regulators of amplitude, melatonin displays an extraordinarily close affiliation with external photic conditions and

closely mirrors the seasonally expanding and contracting photoperiod. Consequently melatonin has regularly been implicated in the involvement of seasonal regulation in salmonids (Bromage et al. 2001). Although the exact mechanisms and pathways are largely unknown in fish melatonin is thought to be involved in the main annual physiological processes such as smoltification, growth and reproduction (Falcon et al. 2007; Ebbesson et al. 2008; Falcon et al. 2010). Clock genes are additionally considered to play a role in the regulation of seasonal physiology and behaviour. In order to investigate the expression of clock genes in brain and peripheral tissues of the Atlantic salmon it was necessary to clone and sequence *Per1* gene. Per1 has previously been shown to be of importance to seasonal processes in seasonal mammals and birds (Foster & Kreitzman 2009).

In experiment one, daylength dependent (DLD) circadian expression of *Clock* and *Per2* was observed in brains sampled from fish exposed to LD. *Per1* did not display significant circadian expression under any photoperiod. This is in contrast to previous results by Davie et al. (2009) who described DLD expression of *Clock, Bmal* and *Per2* in the brain of salmon parr under SD photoperiod. Yet the phase and shape of the *Clock* and *Per2* expression between the two contrasting results share important characteristics. Results from Davie et al. (2009) and the present the acrophase of *Clock* and *Per2* expression occurs at similar times during the circadian cycle. Peak expression of *Clock* was ExT 01:00 ± 1:45 h (Davie et al. 2009) and displayed an overlapping SEM with current study 05:00 ± 02.38 h. Similarly *Per2* peak expression occurred at 05:00 ± 02.38 h and 05:00 ± 2:45 h in the previous and current investigations respectively. Differential DLD expression of *Clock* and *Per2* may be a result of different photoperiod history prior to acclimation to experimental photoperiods. In the current investigation salmon parr were acclimated to long day, 12L: 12D and short day photoperiods in March 2009 (11L: 13D) as daylength was increasing towards the summer solstice. Results

showed circadian expression of *Clock* and *Per2* in LD brain, the experimental photoperiod that best follows the natural seasonal progression. In the previous study acclimation was carried out during early October (10.5L; 13.5D) and DLD clocks were shown in SD salmon (Davie et al. 2009). Consequently it is hypothesised that circadian clock genes expression was therefore observed in the experimental photoperiods that provided the best fit to the natural seasonal progression at the time of acclimation. Such a theory requires further investigations. In mammals results in the Soay sheep have shown that the expression of clock genes expression in the SCN follows photoperiod for a period of time. Subsequently the animal become refractory to photoperiod and physiology begins to change after prolonged exposure to a particular photoperiod (Lincoln et al. 2005). However this study was concerned with photorefractoriness as opposed to photoperiod history.

The impact of photoperiod history on salmonid physiology has previously been documented and photoperiod manipulation is commonplace in commercial fish farm production. Photoperiod history is fundamental to physiology in species such as rainbow trout. Randall and Bromage (1998) demonstrated that it is not a specific daylength that triggers seasonal process but daylength in relation to previous photoperiod experienced. Photoperiods usually considered to represent long days (18L: 06D) are recognised as short if fish have been previously exposed to extreme long days (22L: 02D). Similarly spawning can be advanced under conventional short day photoperiods (e.g. 06L: 18D) if fish have previously been acclimated to extreme Short Days (02L: 22D) (Randall & Bromage 1998; Randall et al. 1998; Bromage et al. 2001). Investigation of clocks over a natural seasonal cycle will enable the hypothesis of photoperiod history to be put into context Data from experiment 1 demonstrated robust clock gene oscillations under long day photoperiod where results were generated from fish acclimated to rigid artificial photoperiods. Under natural conditions in

temperate latitudes the light dark ratio will not remain stationary for periods of up to one month. In central Scotland daylength expands and contract by 4 – 5 minutes per day, 30 minutes per week, around the autumnal and vernal equinoxes. Therefore a simulated natural photoperiod (SNP), adjusted on a weekly basis, would not provide as rigid entrainment signal as an artificial SD, LD or 12L: 12D photoperiod.

In experiment two fish were held under a SNP photoperiod for a period of a year encompassing the transfer from fresh water to marine environment. Expression of clock genes in the brain was analysed over five 24h periods over the trial period. As in experiment 1, *Per1* was not rhythmically expressed in the brain under any photoperiod (see above). Seasonal specific circadian profiles of clock genes were observed for *Clock*, *Cry2* and *Per2*. In agreement with experiment 1 *Clock* displayed a significant circadian profile of expression under LD (July 2010). This is in contrast to Davie et al. (2009) where clock appeared to be cycling under SD. In accordance with previous results significant circadian expression of *Cry2* was present in samples taken during the natural SD (January 2010) and LD photoperiods (June 2010). However the peak of *Cry2* expression occurred around the onset of the photophase in contrast to a nocturnal peak in previous work (Davie et al. 2009). *Per2* was rhythmically expressed in October 2010 (seawater). This is in contrast to previous reports using experimental photoperiods where *Per2* was rhythmic in fish acclimated to LD (experiment 1) and SD (Davie et al. 2009) treatments. Moreover *Per2* did not display circadian expression in the October 2009 samples. Rhythmic *Per2* has previously been reported in salmon post-smolts (marine) acclimated to 12L: 12D photoperiod (Huang et al. 2010). Mean levels of clock gene expression in the brain were additionally compared between SD, 12L: 12D and LD photoperiods and between the 5 natural sample points. However no clear pattern was observed between genes and sample sets.

Significant difference in the amplitude of clock gene expression, up to 7.9 fold change over the course of the year (*Cry2* march 2010 *vs.*, October 09) (Figure 13), were observed in fish acclimated to SNP conditions. These changes can not be explained by photoperiod variations alone as results differed between fish acclimated to SNP and experimental photoperiods (Figure 10 and 13). Moreover amplitudinal changes were not consistent across all clock genes. Consequently clock mechanisms as a whole are not changing with varying seasonality. Under SNP fish were subject to a number of seasonally changing non photic potential zeitgeber signals including temperature and salinity. Importantly, over the duration of this trial Atlantic salmon parr underwent the smoltification process in transfer from freshwater to marine environment. It is unknown what effect such physiological events may have on the expression of clock genes. On the other hand the expression of a number of clock genes have been identified as quantitative trait loci (QTLs) for a number of life history events in salmonids (Leder et al. 2006; O'Malley et al. 2010). However it is unclear how amplitude changes in gene expression may impact on physiology. Determining how clock gene expression regulates seasonal physiology and the location of seasonality specific sites of clock gene expression would additionally enable a better understanding of how amplitude changes in clock gene expression effect physiology.

Regarding the 24h profiles of clock gene expression in the brain some important similarities were observed between experiments 1 and 2, such as the expression of *Clock* in the brain. Differences in the profile and mean expression between experimental and stimulated natural photoperiods may be a consequence of the more rigid experimental photoperiod regime in comparison to the weekly adjusted SNP. Moreover in experiment 2 the time frame of the study encompassed the transfer from freshwater to seawater. The smoltification process includes a wide variety of physiological and morphological changes and may result in differential molecular responses to photoperiod. In spite of this results from the current study

infer some degree of photoperiod history may be involved in seasonal clock gene expression. In experiments 1 and 2 photoperiod and seasonal dependent rhythmic clock gene expression has been demonstrated in the brain. However the photoperiod dependent expression of clock genes outside of the brain has yet to be described.

In the Atlantic salmon clock gene expression has been described in a wide variety of central and peripheral tissues. Significant day night variations have been observed in clock gene expression in peripheral tissues such as muscle (*Clock*), Intestine and spleen (*Per2*) (Davie et al. 2009). However the photoperiod dependent 24h profiles of molecular clocks are unknown in peripheral tissues. In the present study, expression levels of all clock genes investigated were higher in the brain than in the fin or the liver. In the Liver the mean expression of *Per1* and *Per2* was elevated in comparison with *Clock* in the liver and *Clock Per1* and *Per2* in the fin. In the fin no significant circadian rhythm was observed when expression of *Clock*, *Per1* and *Per2* was analysed by ANOVA, turkey's test and Acro analysis for all treatments. This is in contrast to reports in the zebrafish where constitutive expression of a number of clock genes has been described *in vitro* (Whitmore et al. 2000; Whitmore et al. 1998). However *in vivo* tissue specific differences have been observed in response to light and temperature in the zebrafish (Kaneko et al. 2006). In Atlantic salmon liver, the profile of clock gene expression was very different to both the brain and the fin. A significant circadian rhythm for *clock* was observed under the 12L: 12D treatment as opposed to LD (present results) or SD (Davie et al. 2009) in the brain. Moreover the acrophase was considerably later in the day (ExT 16:11) in comparison to the brain. Additionally *Per1* as opposed to *Per2* was significantly rhythmic under the SD photoperiod. Differences in clock gene expression have previously been described between the brain and the liver in Atlantic salmon post-smolts acclimated to a 12L: 12D photoperiod (Huang et al. 2010b). Both *Clock* and *Per1* show significant circadian

expression in the liver, with peak expression several hours prior to the brain (Huang et al. 2010b). However it is difficult to make comparisons with the present study due to the different environmental conditions tested and the physiological status of experimental animals (freshwater *vs.* marine stages). Moreover fish had previously been subjected to artificial photoperiods in order to induce smoltification (Huang et al. 2010b). As discussed above there is evidence to suggest that photoperiod history can have a significant influence on clock gene expression. In contrast to other peripheral tissues, clock gene expression has been described in the liver of a wide variety of teleost species. Nevertheless a clear pattern of clock gene expression is yet to be established. In the European seabass similar profiles of *Per1* expression have been reported across the brain, heart and liver under 12:12 photo treatment (Sanchez et al. 2010). Similarly, comparable expression of clocks was shown in the Golden rabbitfish brain, retina and the liver (Park et al. 2007). However in the goldfish differential expression of *Per1-3* and *Cry1-3* was reported between retina, liver and the gut tissues (Velarde et al. 2009). Amongst teleosts clock gene expression appears species and tissue specific. Differences in expression may be influenced by the presence of non photic zeitgebers such as temperature and food availability or differences in circadian axis organisation. The majority of studies conducted on clocks in teleost have focused on light as the primary mode of entrainment via the retina or pineal while other zeitgebers are present. Temperature has been shown to differentially regulate peripheral oscillations of *Per3* in zebrafish (Kaneko et al. 2006); moreover, in the absence of photic cues *Per1* expression in the liver is speculated to be dependent on feeding time in zebrafish (Sanchez & Sanchez-Vazquez 2009; Lopez-Olmeda et al. 2010;). In the goldfish food entrainable oscillators (FEOs) have been implicated in the regulation of clock gene oscillations and locomoter activity (Vera et al. 2007; Feliciano et al. 2011;). In mammals restricted feeding in both mice and rats has resulted in altered clock gene expression between the brain and the liver

(Damiola et al. 2000; Stokkan et al. 2001). Atlantic salmon feeding time could not explain differential expression between the brain and the liver and between photoperiod as fish were fed in excess throughout the day. Moreover feeding and food availability was equal across all experimental treatments..Results highlight considerable differences in the expression of clock genes in central and peripheral tissues in salmon in comparison to other teleosts, potentially inferring the presence of divergent clock mechanisms within the diverse teleost group. Moreover results illustrate the need for further research into the presence and entrainment of peripheral clocks in teleosts as no clear generalisations can be made between species or tissue type.

Findings from the current investigation illustrate that under experimental photoperiods the circadian expression of clock genes is daylength dependent in the brain and expression in peripheral tissues was considerably different to the brain. This supports the conclusion that clock mechanisms in different tissues are subject to differential entrainment signals. The effect of this in central and peripheral tissues is unknown in fish. Under SNP amplitude of clock gene expression varied with season between genes. Variations could not be explained by photoperiod alone. Clock genes have previously been associated with seasonal physiology however the effects of seasonal amplitude alterations are unclear on seasonal physiology. Moreover expression results in salmon differ considerably from previous investigations in other fish species. Further comparative investigations should be carried out in order to investigate the difference in clock gene expression between the Atlantic salmon and other teleosts and better understand the potential presence of deferential clock mechanism and pathways amongst vertebrates and fish in particular.

6. CONCLUSIONS

The present study investigated the expression of clock genes and levels of circulating melatonin in Atlantic salmon parr acclimated to either fixed experimental photoperiods (experiment 1) or SNP (experiment 2) treatments. In experiments 1 and 2 levels of circulating melatonin mirrors the external light dark cycle and follows a significant circadian profile in the blood. There is evidence to suggest that the amplitude of the melatonin rhythm changes in response to daylength and to temperature, salinity and size/stage of development. In terms of clock gene expression, results from experiment 1 show clear daylength dependent expression of *Clock* and *Per2* in the brain of long day fish. Differences in DLD expression of clocks in experiment1 and previous results are proposed to be a consequence of differential photoperiod history. In order to investigate this, experiment 2 characterised expression of clock genes over the duration of a year (SNP). Comparison between clock genes in experiment1 and experiment 2 revealed no consistent pattern of long day, short day or intermediate photoperiod expression. However mean amplitude of clock genes across seasonal photoperiods varied considerably, up to 7.9 fold difference. Such amplitude changes could not be explained by photoperiod alone as differences were observed between experiment1 and experiment 2 results. Moreover amplitude changes differed between clock genes, indicating the clock mechanism is not shifting as a whole. The effect of clock gene amplitude on seasonal physiology is unclear. However with respect to the identification of a number of clock genes as QTLs for important life history processes and the role of clock genes in mammalian physiology, seasonal amplitudinal changes in clock gene expression is an interesting area of future research. In addition to characterising clock gene expression in the brain, photoperiod dependent clock gene expression was observed in the liver, but no rhythmic expression was observed in the fin. Moreover photoperiod dependent cycling of liver clock genes was observed under four different photoperiods and at a different phase to

the rhythms in the brain. This research is the first characterisation 24h profiles of clock gene expression in central and peripheral tissues and under natural seasonal photoperiods. Future research in this field would benefit from long term trials in other salmonid species, such as the rainbow trout, that don't migrate from freshwater to seawater. Moreover the effect of restricted feeding and temperature should be investigated on clocks in the liver and the brain.

CHAPTER 4

PHOTOPERIOD REGULATION OF *DEIODINASE*, *EYES ABSENT 3* AND *THYROID STIMULATING HORMONE BETA* GENES IN THE ATLANTIC SALMON.

PHOTOPERIOD REGULATION OF *DEIODINASE, EYES ABSENT 3* AND *THYROID STIMULATING HORMONE BETA* GENES IN THE ATLANTIC SALMON.

1. ABSTRACT

The Atlantic salmon (*Salmo salar*) is a highly seasonal teleost species. Within the aquaculture industry photoperiod manipulation is widely utilised in order to maximise growth and control reproduction in Atlantic salmon. Yet the molecular mechanisms underlying photoperiodic regulation of seasonality are unknown. In mammals and birds expression of key components of the molecular switch for photoperiod response such as *Eya3, Tshβ* and deiodinase genes *(Dio1, 2 and 3),* are initiated hours after exposure to the first long day and persist under chronic long day conditions. This pathway regulates thyroid hormone bioactivity and reproductive physiology. In order to understand the molecular mechanisms underpinning seasonal photoperiodic response in salmon, a microarray study was first carried out. Results showed differences in the seasonal expression of a wide variety of target genes including *Eya3* and *Dio1-3* in relation to photoperiodic conditions. In order to further investigate the presence of conserved molecular mechanisms for photoperiod responsiveness in salmon, daily expression of key seasonal genes (*Dio1-3, Eya3* and *Tshβ)* was analysed by qPCR in 2 sample sets (microarray validation and qPCR study sets). Results showed photoperiod dependent up-regulation and circadian mRNA expression of *Eya3, Tshβ* and *Dio2*. Dio2 was up-regulated and subjected to circadian expression under long day photoperiod in both sample sets, while *Eya3* and Tshβ were responsive to short day in the microarray validation set and long day in the second sample set, as has been previously reported for clock genes. This is consistent with previous reports in mammals describing clock dependent regulation of Eya 3 and photoperiod regulation of deiodinase genes. This is the first analysis of highly conserved vertebrate seasonal molecular mechanisms in salmonid species.

2. INTRODUCTION

Vertebrates display considerable temporal organisation in their biological processes (Foster & Kreitzman 2009) which represents an evolutionary adaptation to the changes in environmental conditions that occur over the course of a year. Temperate organisms use seasonal changes in daylength and temperature to synchronise their biological processes to the predictable environmental changes (Dardente et al. 2010; Dupre 2011). Within the teleosts, Atlantic salmon (*Salmo salar*) is an excellent example of a highly seasonal species where the seasonal changes in environmental conditions entrain and ultimately regulate most of its physiology including general growth, behaviour and developmental processes such as smoltification and reproduction (Hemre & Sandnes 2008; Morgan et al. 2008; Davie et al. 2009). To date research has mainly focused on the seasonal control of reproduction and smoltification. Moreover photoperiodic manipulation is widely used commercially to control puberty and manipulate broodstock reproduction (Bromage et al. 2001).

However, in the absence of photoperiodic cues seasonal reproduction persists endogenously in salmonids (Bromage et al. 1988; Duston & Bromage 1988; Duston & Bromage 1991). It has been proposed by Randall *et al.* (1998) that this is under the control of an endogenous clock mechanisms which was latterly supported by a number of studies which showed a link between clock genes and seasonal reproduction (Aubin-Horth et al. 2005; Leder et al. 2006; O'Malley et al. 2007; O'Malley & Banks 2008; O'Malley et al. 2010). Yet the underlying photoneuroendocrine mechanisms linking photoperiod and clocks to the regulation of reproduction remain unclear in teleosts.

In mammals and birds the molecular mechanisms underpinning photoperiodic regulation of reproduction are better described. In mammals photoperiod synchronises clock gene expression and the circadian feedback loop within the suprachiasmatic nuclei (SCN). Signalling from the SCN entrains the nocturnal production of pineal melatonin. Consequently

the synthesis of pineal melatonin mirrors the external photoperiod. This melatonin then binds to the high density of melatonin receptors (MT1) in the pars tuberalis (PT) of the anterior pituitary (Dardente 2012). The action of the MT1 binding then alters the expression profiles of clock genes such as *Cryptochrome (Cry) and Period (Per)* in the PT. Expression of *Cry* peaks early in the evening with rising melatonin while *Per* follows the decline in melatonin at the end of the nocturnal phase (Dardente 2012). Consequently the interval between the *Cry* and *Per* acrophase expands and contracts to accurately reflect the seasonally changing photoperiod (Dardente 2012) As a result of the changing profile of clock genes in the PT *Eyes absent homologue three* (*Eya3)* is up-regulated in response to long day photoperiod. This is achieved via clock gene regulation of 3 E-box elements in the promoter region of the *Eya3*. *Eya3* then forms a dimer complex with *sine oculis homeobox 1* (*Six1)* potentiating *thyrotroph embryonic factor* (*Tef).* The EYA3/SIX1/TEF complex then initiates long day *Tshβ* up-regulation in the PT. TEF regulates TSHβ expression, via conserved D-elements close to the transcriptional start site in the promoter region of the gene. Leaving the PT TSHβ binds to TshR receptors in the *ependymal cell* layer (EC) and leads to the subsequent up-regulation of *Dio2* and down-regulation of *Dio3* mRNA. *Dio2* then catalyses the conversion of biologically inactive T4 to biologically active T3 regulating thyroid hormone function and long day regulation of seasonal physiology (Dardente et al. 2010). While clock mechanisms and the seasonal signalling pathway (described further below) appear to be conserved in birds a key difference exits in that the disruption of melatonin synthesis has no effect on bird seasonal reproductive physiology (Yasuo & Koff 2011).. It has been suggested that the system is reliant on deep brain photoreceptors in the mediobasal hypothalamus (MBH), as opposed to melatonin, for the transmission of seasonal photoperiodic information (Follett et al. 1985; Nakane & Yoshimura 2010).

In recent years there have been considerable advances in the understanding of the molecular mechanisms involved in the photoperiodic regulation of seasonal reproduction. Analysis of seasonal gene expression in birds (Nakao 2009), sheep (Dardente et al. 2010) and rodents (Scherbarth & Steinlechner 2010) has identified a number of genes fundamental in the response to changing photoperiod. In 2008, Nakao et al. reported two successive waves of elevated gene expression in Japanese quail *(Coturnix japonica).* The first wave was observed 14 hours after dawn on the first long day and consisted of two genes: *Tsh β* and *Eya 3* (Nakao et al. 2008; Nakao et al. 2008). In the second wave *Dio2* was elevated approximately 4 hours later in the ependymal cells (EC) of the third ventricle and the infundibular nucleus of the MBH. The mRNA expression of another deiodinase, *Dio3* was significantly inhibited in birds exposed to long day photoperiod in comparison to short day photoperiod (Figure 1). (Yasuo et al. 2006; Nakao et al. 2008)

The DIO2 enzyme functions as the rate limiting factor in the conversion of the biologically inactive thyroxine (T4) to the biologically active form triiodothyronine (T3) (Dardente et al. 2010; Arrojo E Drigo & Bianco 2011). Additionally Type I iodothyronine deiodinase protein (DIO1) is also involved in the conversion of T4 to T3 (Lechan & Fekete 2005) (Figure 2). In mammals and birds DIO1 is predominantly found in the circulatory system while DIO2 action is localised to the brain (Walpita et al. 2009). Conversely DIO3 catalyses the conversion of T3 to biologically inactive forms including inactive reverse T3 (rT3) and 3', 3'-diiodothyronine (T2) (Figure 2) (Bianco et al. 2002; Bianco 2011;). All three deiodinase enzymes have also been identified in teleosts including rainbow trout, a close relative of the Atlantic salmon (Power et al. 2001). However, their role in salmonid seasonal physiology has yet to be described (Power et al. 2001).

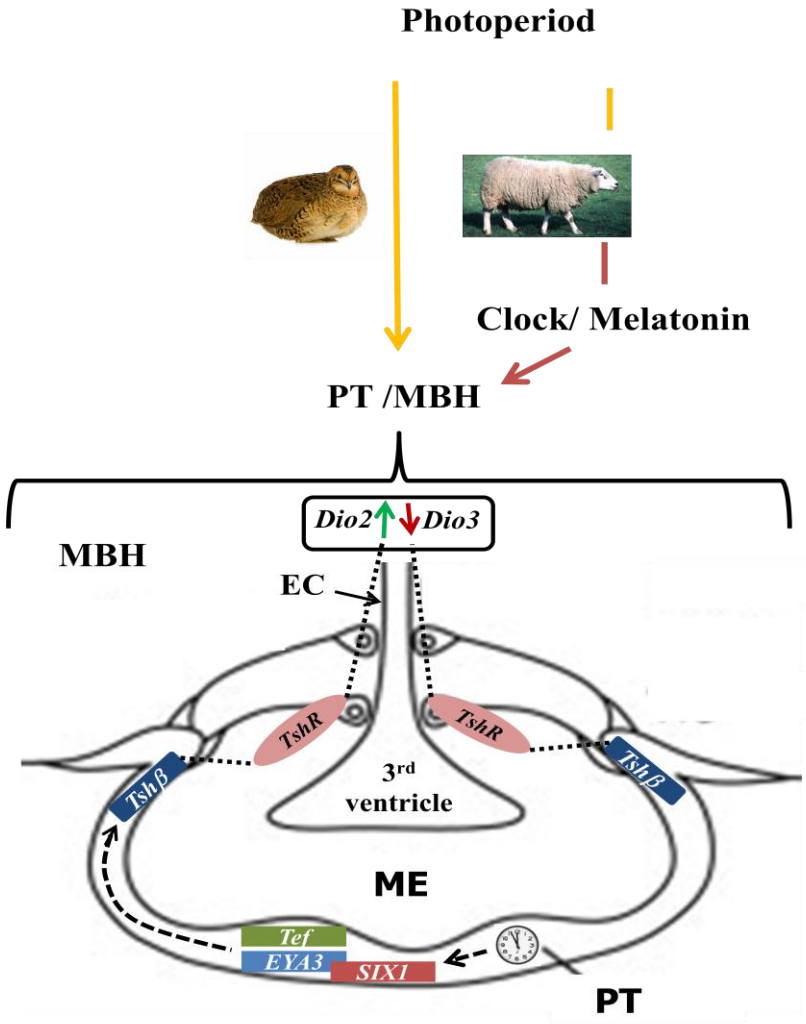


Figure 1: Molecular mechanisms underpinning photoperiod responsiveness in birds and mammals (adapted from Yasuo et al. 2009). In birds photoperiod information is perceived by deep brain photoreceptors present in the mediobasal hypothalamus (MBH) and the Pars tuberalis (PT) of the pituitary. In mammals photoperiod information is perceived by the retina and transmitted via the SCN to the pineal. The consequent melatonin signal then binds receptors in the PT. In the PT of both mammals and birds the phase of clock gene expression is altered. This in turn mediates the expression of *Eya3* via three conserved E-boxes in the promoter of the gene. *Eya3* forms a dimmer complex with *Six1* potentiating *Tef*. EYA3/SIX1/TEF initiates long day *Tshβ* up-regulation in the PT. TSHβ binds to TshR receptors in the *ependymal cell* layer (EC) and leads to the subsequent up-regulation of *Dio2* and down-regulation of *Dio3* mRNA.

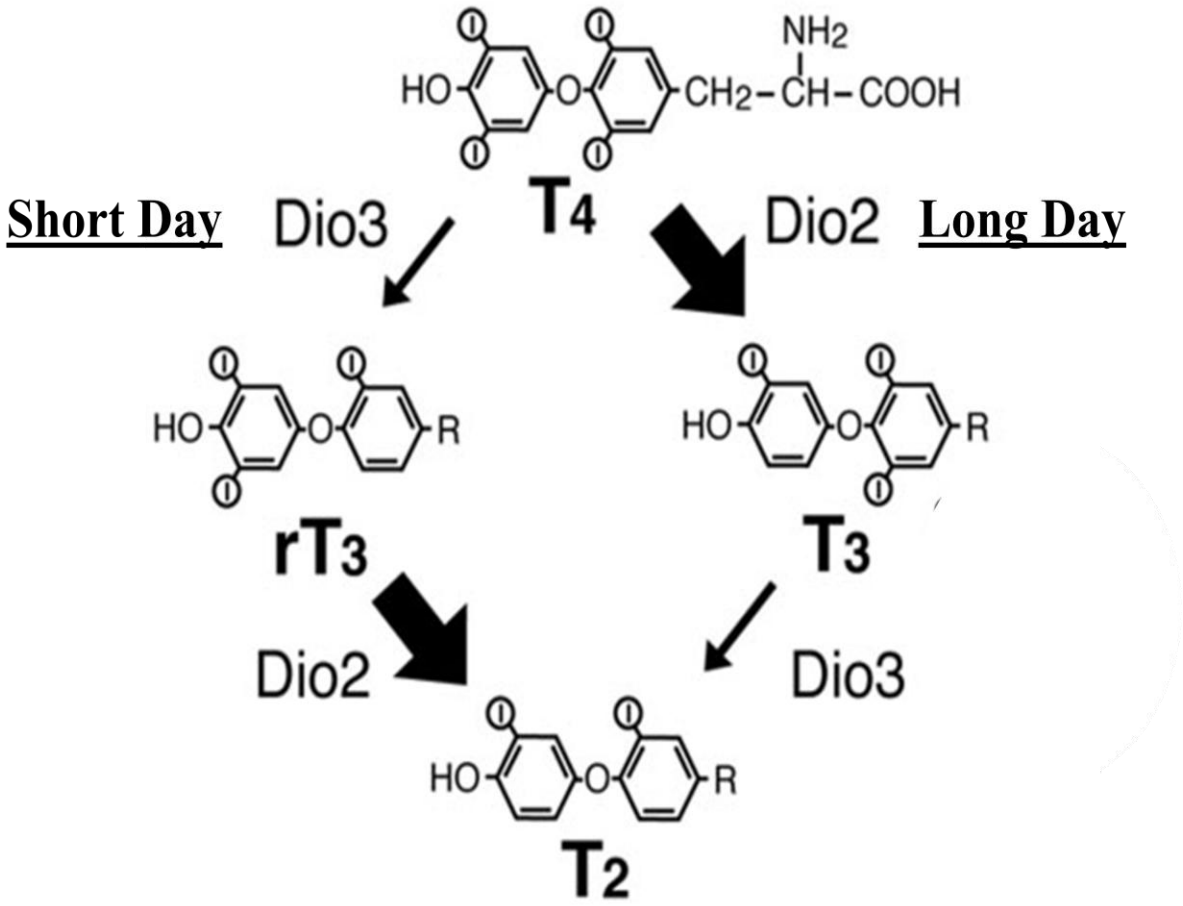


Figure 2: The *DIO2* mediated conversion of biologically inactive T4 to biologically active T3 under long day photoperiod and DIO3 mediated conversion of T3 to an inactive form by DIO3 under short day photoperiod (adapted from).

Amongst vertebrates photoperiodic initiation of thyroid hormone bioactivity is essential to the stimulation of reproductive physiology. In the Japanese quail the administration of T3 in the MBH has been shown to initiate testicular growth (Yasuo et al. 2009). Moreover T3 administration resulted in the reduced encasement of gonadotropin - releasing hormone (GnRH) nerve terminals; an effect similar to that of long day photoperiod conditions (Yasuo et al. 2009). The release of GnRH then stimulates the secretion of luteinizing hormone (LH). In the Japanese quail the two waves of long day gene expression were subsequently followed by an increase in plasma LH. The long day surge in LH subsequently initiates the quail reproductive access. Similar pathways have also been described in sheep and rodents

(Unfried et al. 2009; Yasuo & Yoshimura 2009;Dardente et al. 2010; Dupre et al. 2010;Scherbarth & Steinlechner 2010; Dupre 2011).

A better understanding of the molecular components of the salmonid photoneuroendocrine system (PNES) is fundamental to unravel the complex biological mechanisms driving photoperiodic regulation of seasonal reproduction. The aim of this study was to better understand the molecular mechanisms underpinning seasonal photoperiodic response in salmon. To do so, the first phase of the work employed microarray analysis to identify photoperiod (long day *vs.* short day) and day *vs.* night variation in the brain transcriptome. This was carried out in brain cDNA from Atlantic salmon parr previously acclimated to experimental long day and short day photoperiod obtained from Davie et al (2009) (referred to throughout as microarray validation study). Results were subsequently verified by qPCR for seasonal genes *Dio1*, *Dio2*, *Dio3* and *Eya3* with the addition of *Tsh*β not present in the microarray. Further investigation was subsequently carried utilising a qPCR expression study to determine 24h patterns of *Dio2*, *Eya3* and *Tsh*β mRNA expression in salmon parr acclimated to short day, 12L:12D and long day photoperiod (referred to as qPCR study). This was carried out with samples previously obtained in chapter 3. This work is the first attempt to characterise components of the molecular switch for photoperiod response in mammals in a commercially important species of teleost fish.

3. MATERIALS AND METHODS

Animals and tissue sampling

Samples obtained for microarray, microarray validation and qPCR studies were previously utilised to determine 24h of seasonal clock gene expression in Davie et al 2009 (microarray and microarray validation) and chapter 3 (qPCR study). Source and housing of both sample

sets was carried out in the same manner. Atlantic salmon parr of a farmed stock origin (mixed sex) were reared under an ambient photo-thermal cycle at the Niall Bromage Freshwater Research Facilities (microarray study: 26.0 ± 4.0 g; qPCR study: 24.9 ± 5.4 g) (Institute of Aquaculture, Stirling, Scotland, 56: 02 N). Fish were stocked into a $1m^3$ tank light proofed tank (n=100 fish/tank, one tank per treatment). The tanks were illuminated using a 28W, fluorescent light with a spectral content comparable to a 3700 °K black-body radiator (IP65 prismatic 2D round bulkhead 28W HF, RS Components Ltd, Glasgow, UK) connected to an automatic timer to regulate photoperiod. Samples for microarray investigation were previously used to determine clock gene expression in a previous study by Davie et al (2009). In early October, Atlantic salmon parr were transferred from ambient photoperiod (10.5L: 13.5D) to either a long (LD: 16L: 8D) or short day (SD: 8L: 16D) photoperiod at ambient temperature (10.4 ± 0.4 °C). For the qPCR study, salmon parr (*n*=100 per treatment, mean 24.9 ± 5.4 g, 140.6 ± 7.8 mm) were acclimated during one month (from 02/03/2009) to either long day (16h light: 08h dark, LD), short day (08h light: 16h Dark, SD) or 12h light: 12h dark photoperiod (12L:12D). Over the course of the study water temperature averaged 4.6 ± 0.7 °C. Food was offered in excess throughout the day and night. Experimental animals were sacrificed via a lethal anaesthesia (1mL/L, 2-phenoxyethanol, Sigma- Aldrich Co. Ltd, Poole, UK) and rapid decapitation. After 1 month acclimation to experimental photoperiods, brains (including pituitary and excluding pineal organ) were removed every four hours over a 24h period (n= 6 per sample point/ per photo treatment). Tissue samples for microarray study were homogenized in 1 ml TRIzol Reagent (Invitrogen, UK) per 100 mg of tissue over ice, rapidly frozen, and stored at -70 °C. For qPCR study tissue was instantly frozen over liquid nitrogen vapour and stored at -70 °C until use. Dim red light was used for night sampling. Experiments were carried out in accordance with accordance with the UK Animals (Scientific Procedures) Act 1986.

RNA extraction, DNase treatment and cDNA synthesis

All samples (approximately 100 mg) were individually homogenised in 1 ml of TRIzol® (Invitrogen UK). RNA extraction was carried according to manufacturers' instructions. RNA pellets were rehydrated in MilliQ DNA and RNA free water in varying volumes to achieve a final RNA concentration of approximately 1000 ng/ul. Total RNA concentration was assessed using ND-1000 Nanodrop spectrophotometer (Labtech Int., East Sussex, UK). In order to eliminate any DNA contamination 5 µg of RNA was treated with DNase enzyme following DNA-free™ kit guidelines (Applied biosystems, UK). cDNA was then synthesised using 1 µg of DNase treated total RNA in 20 µl reaction and random primers according to manufacturer protocol. High capacity reverse transcription kit without RNase inhibiter was used (Applied biosystems, UK). Final reactions were then diluted with DNA/RNA free H^2O to a final volume of 200 µl (experiment 1) and 100 µl (experiment 2). Brain cDNA reactions were then stored at -20 ^oC.

Microarray

In order to identify novel genes expressed on a daylength dependent basis, a pilot global gene expression analysis of brain tissue was undertaken. A custom-designed Atlantic salmon oligoarray with 44k features per array on a four-array-per-slide format (Agilent Technologies, Cheshire, U.K.), with each feature printed singly was utilized (http://www.ebi.ac.uk/arrayexpress/arrays/A-MEXP-2106). Each biological replicate (Cy3 – labelled) was co-hybridized in a dual dye experiment with a single pooled reference sample (Cy5 labelled). The pooled reference sample comprised equal amounts of amplified RNA from each of the 16 experimental fish. The study comprised 16 hybridisations: 2 states (long day / short day) × 2 time-points (midday/midnight) × 4 biological replicates (Individual Atlantic salmon parr 26.0 ± 4.0g as described above). Amplified RNA amplification, dye

labelling and hybridisations were performed as detailed in Morais et al. (2012). Each replicate was competitively hybridised with a pooled reference sample. An indirect labelling methodology was utilised to prepare microarray targets. For each sample 500 ng of purified total RNA was used to generate antisense amplified RNA (aRNA) (Amino Allyl MessageAmpTM II aRNA amplification kit, Ambion Applied Biosystems). Samples were then subject to Cy3 or Cy5 fluor incorporation mediated by a dye coupling reaction. All experimental reactions were labelled with Cy3 dye and pooled reference was labelled with Cy5 dye. The incorporation of the dye and aRNA yield was quantified by spectrophotometry (NanoDrop ND-1000). The quality of the assay was further controlled by the separation of 0.4 μl sample through a mini agarose gel. Products were displayed on a Typhoon trio florescence scanner (GE Healthcare). Hybridisation of microarray was carried out in a Lucidea semi-automated system (GE Healthcare) with no pre-hybridisation step.

In the hybridisation of each array, the sample and pooled reference sample, consisting of 40 pmol dye and 150 ng aRNA, were pooled and combined to the hybridisation solution (185 μl 0.7X UltraHyb buffer from Ambion, 20 μl Poly(A), 10 μl herring sperm, 10 ml ultra pure BSA all at a concentration of 10 mg/ml and from Sigma-Aldrich, Dorset UK) (Morais et al 2011). Prior to scanning microarray, hybridisations were subjected to two post-hybridisation automatic and six manual washes to a stringency of 0.1 xSSC (EasyDipTM Slide staining system; Canemco Inc., Quebec Canada). The scanning was carried out at a resolution of 10 μl in an Axon GenePix 4200AL scanner (MDS analytical technologies, Wokingham, Brekshire U.K) with laser power constant (80 %) and “auto PMT” enabled to adjust PMT for each channel so that less than 0.1 % of features were saturated and mean intensity ratio of Cy3 and 5 signals was close to 1, as described in Morais et al 2011.This was followed by inspection to remove fluorescent features which were obvious artifacts, before fusing of duplicate spot

intensity data (BlueFuse proprietary algorithm) Florescence intensity values were extracted from TIF images of microarray slide with the use of BlueFuse software (BlueGenome, Cambridge, UK). Data was exported to GeneSpring GX version 10.0.2 (Agilent Technologies, Wokingham, Berkshire, U.K) after block Lowess normalisation. The data was then transformed and subject to quality filtering and removal of all microarray slide control features. Data were normalised using a lowess transform with feature intensities <1.0 being set to 1.0. The normalised data were quality filtered by removal of saturated features, those showing non-uniform features, those representing population outliers and those features not significantly different from background. Consequently 5893 genes were subject to statistical analysis.

Isolation and identification of Atlantic Salmon *Dio*1-3*, Eya3* and *TSHβ* partial mRNA sequences

Transcriptomic analysis identified elements of the mammalian and avian seasonal signalling mechanism thus it was decided to identify salmon specific partial sequences for *Dio1, Dio2, Dio3, Eya3* and then verify expression using qPCR. In addition qPCR primers and standards were designed, cloned and sequenced based on a published Atlantic salmon *Tshβ* sequence (NM_001123528). For *Dio1 - 3* and *Eya3* were designed on Atlantic salmon expressed sequence tags (ESTs)(EG868394, DW562425 and DW551395) Identification of the Atlantic salmon *Dio2* was based on the published rainbow trout *Dio2* sequence (AF207900) (Sambroni et al. 2001). BLAST analysis identified two salmon expressed sequence tags (EST's) (GE782599 and DY713483) aligning to the published rainbow trout sequence. All primer pairs were then designed on the salmon EST's and published Tshβ sequence using Primer Select Ver.6.1 (DNASTAR Lasergene, www.dnastar.com). See Table 1 for all primer sequence information.

Partial fragments were produced from salmon brain cDNA by PCR using Klear Taq Hot Start DNA polymerase (KBiosciences, Hoddesdon, Herts, UK) for fragments <1 kb (*Dio1, Dio3, Eya3* and *Tshβ*) or SuperTaq™ Plus (Ambion, Applied Biosystems, Warrington, UK) for products > 1kb (*Dio2*). Following visualisation on a 1 % agarose gel PCR products were excised and purified using illustra GFX PCR DNA and GelBand Purification Kit (GE Healthcare) according to manufacturer's instructions. The purified products were then cloned by adding to a ligation reaction at ratio of 3:1 insert to vector using a pGEM®-T Easy vector system (promega Madison, WI, USA). Transformed competent cells were grown overnight on LB/ampicillin/IPTG/ X-gal plates with positive colonies (identified by colour reaction to selective agar) being selected and bulk produced in isolation overnight in LB media. Plasmids were then harvested using a GenElute™ *Plasmid Miniprep Kit* (sigma Aldrich, Gillingham, UK). Plasmids were sequenced using a Beckman 8800 auto sequencer. Sequence results were then analysed using SEQman as part of Lasergene software package (DNASTAR). In the case of *Dio2* the size of the product required further sequencing runs to obtain a complete sequence read which was achieved using two additional primer pairs (Dio2seqaF/R, Dio2seqbF/R) (Table 1). All sequences were assembled and edited on Lasergene SeqMan (DNASTAR, www.dnastar.com). Identity of salmon partial sequences was identified *in silico* by performing a BLAST analysis. MEGA Ver.4.1 (http://www.megasoftware.net/) was used to deduce a phylogenetic tree using the neighbour joining method. Plasmids produced were subsequently utilised as standards for qPCR assays.

Quantitative PCR

In order to determine diel patterns of clock gene mRNA expression, qPCR assays capable of absolute quantification were established for *Dio1-3, Eya3, Tshβ. β-actin* was used as a housekeeping reference gene for all analysis as described by Davie et al (2009). Each qPCR

reaction consisted of primer pairs (Table 1) at a concentration of 0.5 μM, 5 μl of cDNA (1/20 for sample set 1 and 1/10 for sample set 2), 3 μl DNA/RNA free H_2O and 10 μl ABsolute™QPCR SYBR Green master mix (Thermo scientific, Leon-Rot, Germany) in a total reaction volume of 20 μl. The ABsolute™QPCR SYBR Green Mix was made up of Thermo-Start™ DNA polymerase, a proprietary reaction buffer, dNTP's and SYBR Green I with Mg++ at a concentration of 3 mM in the final 1× reaction. All qPCR assays were carried out in a Techne Quantica thermocycler (Techne, Quantica, Cambridge, UK) in a thermo cycling programme consisting of a 15 minute initiation stage at 95 °C followed by 45 cycles of 3 temperature steps; 95 ^{o}C for 15 s anneal x°C (See Table 1 for target specific annealing temperatures) and 72 ^{o}C for 30s. This was followed by a temperature ramp from 70 - 90 °C for melt-curve analysis to verify that no primer–dimer artefacts were present and only one product was generated from each qPCR assay. Quantification was achieved by a parallel set of reactions containing standards consisting of serial dilution of spectrophotometrically determined, linearised plasmid containing partial cDNA sequences generated. All samples were run in duplicate and each assay contained non-template controls.

Statistical analysis and qPCR normalisation.

Microarray data was analysed by two-way ANOVA with the use of GeneSpring GX version 10.0.2 (Agilent Technologies, Wokingham, Berkshire, UK). False discovery correction was off for all analyses as it was considered to be overly conservative in its performance. Data is presented with respect to P value and fold change. Minimum P value and fold change was determined for photoperiod (LD vs. SD) and day/night (day vs. night) differences. In addition to photoperiod/ day night interaction differences were analysed with regards to minimum p value and maximum fold change across all interactions.

qPCR results are presented with respect to external time in accordance with Daan et al 2002 whereby the external time 0 (ExT 0) is the central point in the dark phase(Daan et al. 2002). Analysis of Variance (ANOVA) was used to determine significant time effects and Turkey's test was used to determine differences between the 6 sample points over the 24h sample period and mean differences between photoperiods (InStat® 3.1, Graphpad software inc). Data from each tissue/ photoperiod was then fitted to a cosine wave in order to determine the presence of a significant ($p<0.05$) circadian rhythm. Raw data was analysed using acrophase circadian analysis programs (Refennetti R., University of South Carolina, USA; http://www.circadian.org/softwar.html). Acro analysis also determined both the significance, acrophase (peak in expression), mean and amplitude of raw data using the equation **Y= A + B * cos (C *X –D)** whereby Y is level of gene expression as a percentage of the mean, A is the baseline, C is the frequency multiplier and D is the acrophase of the data set A significant circadian rhythm was deemed present when p value was less than 0.05 in for all statistical analysis.

Table1 Primers sequences used (5'-3') and annealing temperatures for PCR and qPCR assays, including primer pairs for *Dio2* identification.

Primer Name	**Primer sequence Forward 5'-3'**	**Primer sequence reverse 5'-3'**	**Anneal °C**
Dio2	GGCAGCGCATGCTGACCTCG	ACCAGCCCCGTCTCGACCCA	62
Dio2seqa	CCATGGGCCCGTGCTCCTT	CATGTGGCGTAAGTCTGGGTTGCT	65
Dio2seqb	AACGTGGGCCTACGGCGTGT	TGCTGTGCCTTGCTCTACGGCT	65
Actin qPCR	ATCCTGACAGAGCGCGGTTACAGT	TGCCCATCTCCTGCTCAAAGTCCA	61
Dio1qPCR	GACAACAGACCACTGGTGTTGACT	GCCTGCGCAATGTAGACCACC	62
Dio2qPCR	GGACGAGTGCCGCCTGCTGGACTT	GAAGGCGGGCAGGTGGCTGATGA	68

Dio3qPCR	CCTGGCTGCGTTTCAGCGCGT	ATCTGGTAAGGCGCGTCGGAG	64
Eya3qPCR	GGGCATCACGGACGGACGCTT	CCCAACCCCAATCAATGCTGCCTC	64
Thsb qPCR	GAGCTCGCCGGACCACGTTTCCT	AGTGGCAGCTGAGGGCTACGGG	66

4. RESULTS

Microarray

In order to identify genes that showed differential expression both with respect to photoperiod (LD *vs.* SD) and photophase (day *vs.* night) the single factor as well as the interaction ANOVA lists were examined from the microarray study. There were 2989, 2832 and 957 features that displayed significantly different expression with respect to photoperiod, day *vs.* night and the photoperiod/photophase interaction (Table 3). Of these features 2301, 2161 and 620 were unique to their respective specific condition (Figure 3). When p value was set at <0.001, 120, 128 and 10 features were significantly differentially expressed between photoperiods (SD *vs.* LD), time of the day (day *vs.* night) and photoperiod/time of the day interaction, respectively (Table 3, Appendix 1a-c). When microarray results were analysed in terms of fold change, close to 6000 probes displayed a fold change greater than 1 for any of the conditions (Table 4). This was reduced to 30, 13 and 111 probes when the fold change was increased to a threshold of 5 for photoperiod, time of day and photoperiod/time of the day interaction, respectively (Table 4, Appendix 2 a-c). Within the dataset as a whole, 13 features related to published results in the molecular switch for photoperiod responsiveness in mammals (Figure1 and Table 2). These included *Eya3*, *Dio1*, *Dio2* and *Dio3* which all displayed a significant difference in expression with relation to photoperiod (Table 5).

However, only *Eya3* expression was significantly different between day and night. Other genes of interest include circadian clock genes i.e. Cryptochrome 1 and 2 (*Cry1* and *Cry2*) and the period 1 (*Per1*) gene in addition to CCAAT enhancer binding protein beta (CCAAT beta) and CCAAT enhancer binding protein beta 2 (CCAAT beta 2) (Table 5). Both *Cry1* and *Per1* expression were significantly different between day and night (<0.001 and <0.05 respectively). Two CCAAT β (SSA#CL285CTG1 and SSA#STIR07904) and two CCAAT β2 (SSA#CL344CTG1 and SSA#535694434) displayed differential expression in relation to seasonal photoperiod and day night differences. All four CCAATs displayed a significant difference, all down-regulated, in relation to day *vs.* night, all with a minimum P value <0.01. In terms of photoperiod (LD vs. SD) differences, one CCAAT β (SSA#STIR07904) and both CCAAT β2 2s were up-regulated under LD photoperiod treatment. However, there was no significant difference between photoperiod / day *vs.* night interactions. The fold change observed for *Dio* genes and *Eya3* ranged from 1.2 to 1.6 fold between photoperiod conditions with *Dio1* and *Eya3* being down-regulated and *Dio2* and *Dio3* up-regulated under LD (Table 5). In addition, these genes were all down-regulated during the day except *Eya3*.

Table 2 Key genes involved in the mammalian and avian molecular switch for photoperiod response.

Gene	**Full name**	**References**
Eya3	Eyes absent homolog 3	(Dardente et al. 2010)
Six1	SIX-family protein 1	(Dardente et al. 2010)
Tef	Thyrotroph embryonic factor	(Dardente et al. 2010)
Tshb	Thyroid stimulating hormone beta	(Hanon et al. 2008)
Tshr	Thyroid stimulating hormone receptor	(Dardente et al. 2010)
Dio1	Type I iodothyronine deiodinase	(Lechan & Fekete 2005)
Dio2	Type II iodothyronine deiodinase	(Lechan & Fekete 2005)
Dio3	Type III iodothyronine deiodinase	(Lechan & Fekete 2005)

Table 3: Numbers of microarray probes displaying significant differences between experimental conditions.

P value	Photoperiod SD vs. LD	Day vs. Night	Photoperiod/Day night interaction
<0.05	2989	2832	957
<0.02	1449	1259	338
<0.01	802	707	174
<0.005	453	308	71
<0.001	120	128	10

Table 4 Numbers of microarray probes displaying different fold changes between experimental conditions.

Fold Change	Photoperiod SD vs. LD	Day vs. Night	Photoperiod/Day night interaction
>1	5892	5892	5892
>1.5	532	494	1418
>2	192	175	598
>3	79	54	250
>5	30	13	111

Table 5 Microarray results for known seasonally important genes including: probe name, blast results, P value and fold change subject to photoperiod, day/ night, and photoperiod/ day night interaction.

Description				**P value**			**Fold change**		
Probe	**Blast type**	**Blast result**	**Short name**	**Photo**	**Day / Night**	**Photo/ Day night**	**Photo (LD /SD)**	**Day /Night**	**Photo/ Day night**
Ssa#STIR18614	RefSeq_Hit Def	Salmo salar selenocysteine lyase (scly), mRNA >gi|223648997|gb|BT059544.1| Salmo salar clone ssal-rgf-511-328 Selenocysteine lyase putative mRNA, complete cds	Cry1	0.9946	0.0001	0.0038	-1.0008	-1.9871	2.9787
Ssa#DW579347	RefSeq_Hit Def	PREDICTED: Oreochromis niloticus cryptochrome-1-like (LOC100694774), mRNA	Cry1	0.1112	0.0005	0.0050	-1.1818	-1.6146	2.1461
Omy#S27583073	RefSeq_Hit Def	Oncorhynchus mykiss selenoprotein Ja (selja), mRNA	Cry2	0.0034	0.0613	0.2778	1.5029	1.2602	-1.8940
Ssa#S30289725	RefSeq_Hit Def	PREDICTED: Cavia porcellus period circadian protein homolog 2-like (LOC100713579), mRNA	Per1	0.0735	0.0322	0.9397	-1.1302	-1.1632	1.3147
Ssa#CL285Ctg1	RefSeq_Hit Def	Salmo salar CCAAT/enhancer binding protein (C/EBP), beta (cebpb), mRNA >gi|209152840|gb|BT044870.1| Salmo salar clone ssal-rgf-506-052 CCAAT/enhancer-binding protein beta putative mRNA, complete cds	CCAAT β	0.0586	0.0012	0.4554	1.1194	-1.3055	1.2585
Ssa#STIR07904	RefSeq_Hit Def	Salmo salar CCAAT/enhancer binding protein (C/EBP), beta (cebpb), mRNA >gi|209152840|gb|BT044870.1| Salmo salar clone ssal-rgf-506-052 CCAAT/enhancer-binding protein beta putative mRNA, complete cds	CCAAT β	0.0013	0.0028	0.8080	1.2139	-1.1905	1.2044
Ssa#CL344Ctg1	RefSeq_Hit Def	Oncorhynchus mykiss CCAAT/enhancer binding protein beta2 (LOC100379112), mRNA >gi|90019517|gb|DQ423470.1| Oncorhynchus mykiss CCAAT/enhancer binding protein beta2 mRNA, complete cds	CCAAT β2	0.0017	0.0131	0.3282	1.3225	-1.2239	-1.4194

Ssa#S35697434	RefSeq_HitDef	Oncorhynchus mykiss CCAAT/enhancer-binding protein beta (LOC100136165), mRNA >gi|33304537|gb|AY144611.1| Oncorhynchus mykiss CCAAT/enhancer-binding protein beta mRNA, complete cds	CCAAT β2	0.0404	0.0029	0.9566	1.1808	-1.3101	1.3049
Ssa#S35582016	RefSeq_HitDef	PREDICTED: Oreochromis niloticus Type I iodothyronine deiodinase (dio1), mRNA	DIO 1	0.0322	0.9269	0.0183	-1.2021	-1.0231	1.5336
Ssa#STIR15458	RefSeq_HitDef	Oncorhynchus mykiss deiodinase, iodothyronine, type II (dio2), mRNA >gi|16550969|gb|AF207900.1|AF207900 Oncorhynchus mykiss type II iodothyronine deiodinase mRNA, complete cds	DIO 2	0.0076	0.5060	0.8359	1.6111	-1.1074	-1.5612
Omy#TC151869	B2GO_BlastxHit	iodothyronine deiodinase type III	DIO 3	0.0068	0.5118	0.1950	1.2026	-1.0389	-1.0389
Ssa#DW562425	B2GO_BlastxHit	IOD3_SPAAURecName: Full=Type III iodothyronine deiodinase; AltName: Full=Type-III 5'-deiodinase; AltName: Full=Type 3 DI; AltName: Full=DIOIII; AltName: Full=5DIII	DIO 3	0.0443	0.2534	0.8150	1.2068	-1.0202	-1.3343
Omy#S34424874	RefSeq_HitDef	PREDICTED: Oreochromis niloticus eyes absent homolog 3-like (LOC100705151), mRNA	EYA3	0.0041	0.0131	0.9030	-1.3561	1.2844	1.2844

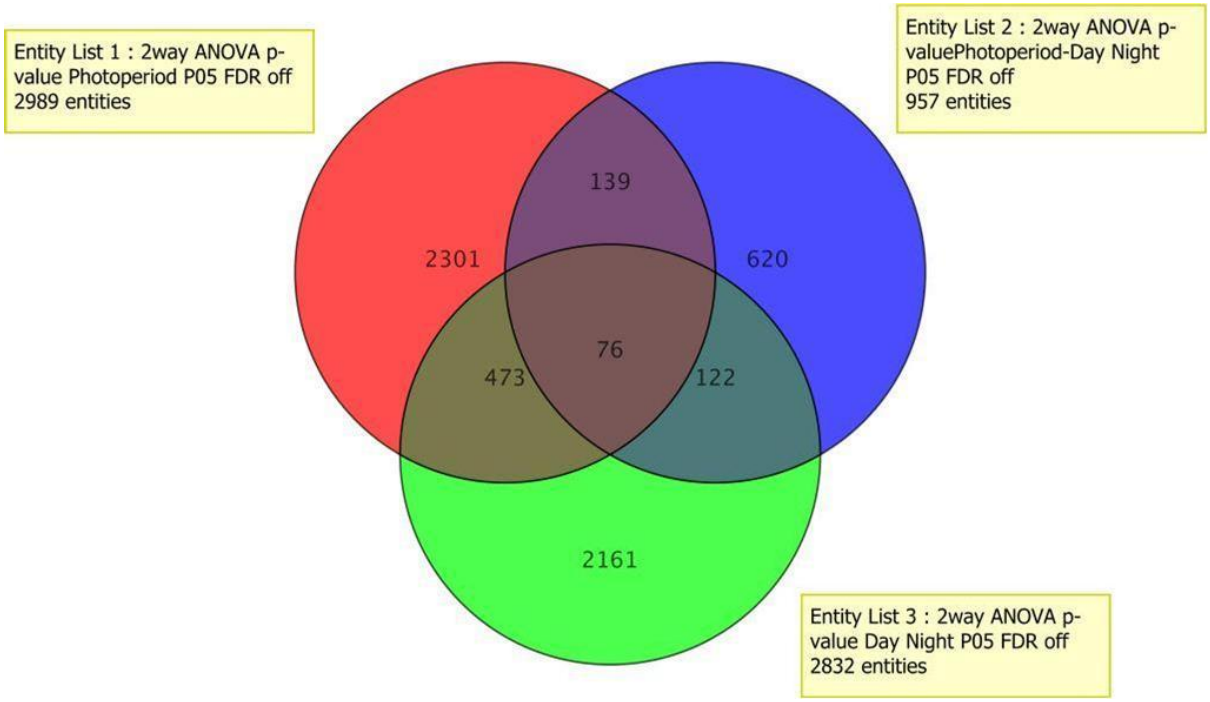


Figure 3: Venn diagram detailing the number of features from the microarray which showed significant differences in expression (P<0.05) with regard to Photoperiod (SD *vs.* LD), Day vs. Night and photoperiod/day night interaction and all possible combinations of conditions.

Seasonal gene sequence analysis

In order to set up qPCR assays for seasonal genes *Dio1*, *Dio2*, *Dio3*, *Eya3* and *Tshβ* it was necessary to clone and sequence partial fragments of each gene. *Dio2* sequence information will be discussed in further detail below. For *Dio1, Dio3 Eya3* and *Tshβ* 133 - 172bp fragments were isolated (data not shown) bearing a high identity to desired product when subjected to NCBI TblastX (http://www.ncbi.nlm.nih.gov/). *Dio1* (134bp) displayed 93% identity with Scorpion fish (*Sebastiscus marmoratus*) *Dio1* (JX135096). *Dio3* (172bp) displayed 98 % identity with gold fish *Dio3* (EF190704). *Eya3* (164bp) displayed 93 % identity with AGENAE Rainbow trout multi-tissues library (tcce) (CU071998) the sequence

the salmon *Eya3* was designed against. *Tshβ* (133bp) displayed 100 % identity with the published *Dio2* sequence (AY819642).

From the cloning of the *Dio2* fragment in the Atlantic salmon a 2027 bp fragment was successfully sequenced (Figure 4) and displayed 96 % identity with the rainbow trout sequence (AAL25715) and 71 % and 66 % sequence identity with the Japanese quail (ACB59241)and sheep (ACX31206). The sequenced product contained the majority of the coding sequence (CDS) and the 3'untraslated region (UTR). Interestingly the salmon *Dio2* CDS contains TGA sequence that codes for a selinocysteine as opposed to the usual stop codon. The function of the stop codon is suppressed by a selenocysteine insertion sequence SECIS element present in the 3'UTR. In the Atlantic salmon sequence the isolated 3'UTR contains four AU-rich elements (ARE's) and a region aligning with the beginning of the SECIS element found in the rainbow trout *Dio2* sequence. The presence of selinocysteine residue is fundamental to the enzymatic properties of the DIO2 enzyme. In silico analysis of the partial Atlantic salmon *Dio2* sequence confirmed a high level of sequence identity with the rainbow trout *Dio2* sequence (95 % tBLASTX). The sequence also shared close identity with other teleost species, mammalian and avian *Dio2* sequences (Figures 4-6). The salmon sequence was shown to be firmly grouped within the teleost D2 node of the Deiodinase phylogenetic tree (Figure 6).

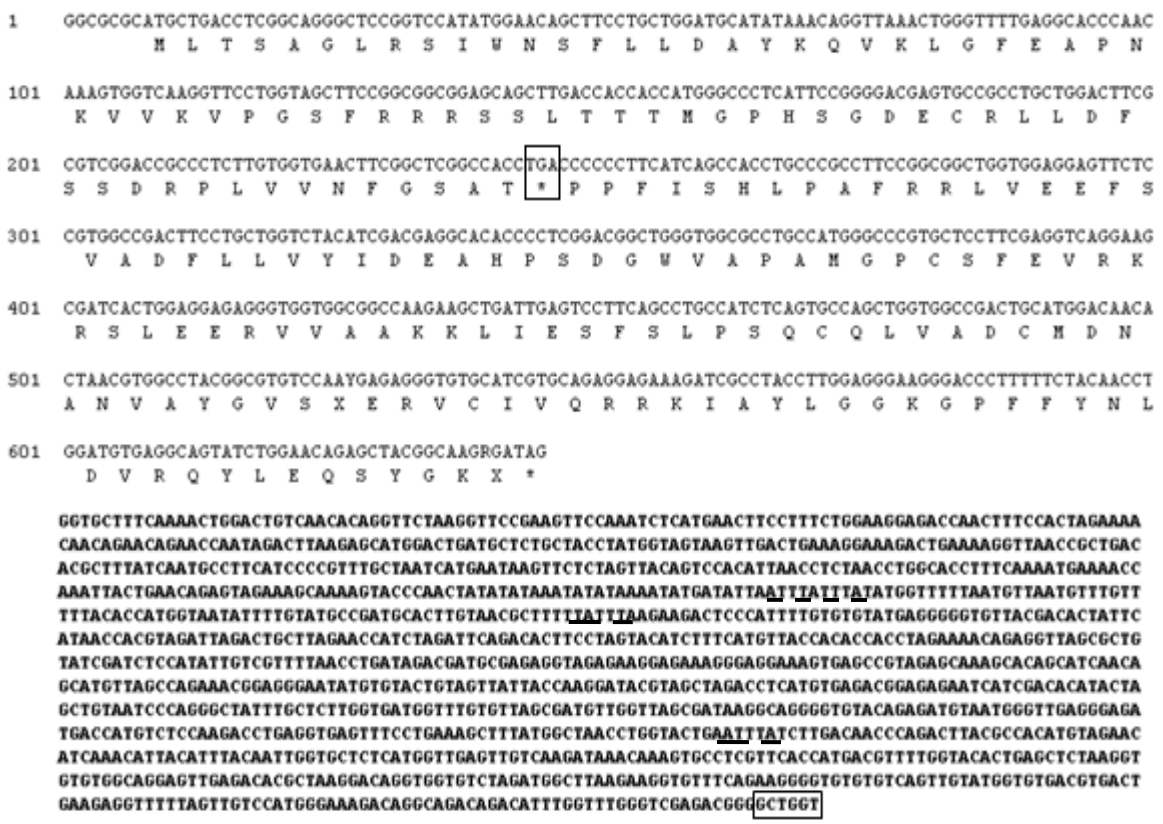


Figure 4: Partial sequence of Atlantic salmon *Dio2*. CDS is shown with a numbered margin, together with the amino acid residues. The selenocysteine codon (TGA) is indicated by a box. 5'UTR is shown in boldface, indicating part of the SECIS region by a box. The broken lines indicate the ARE regions.

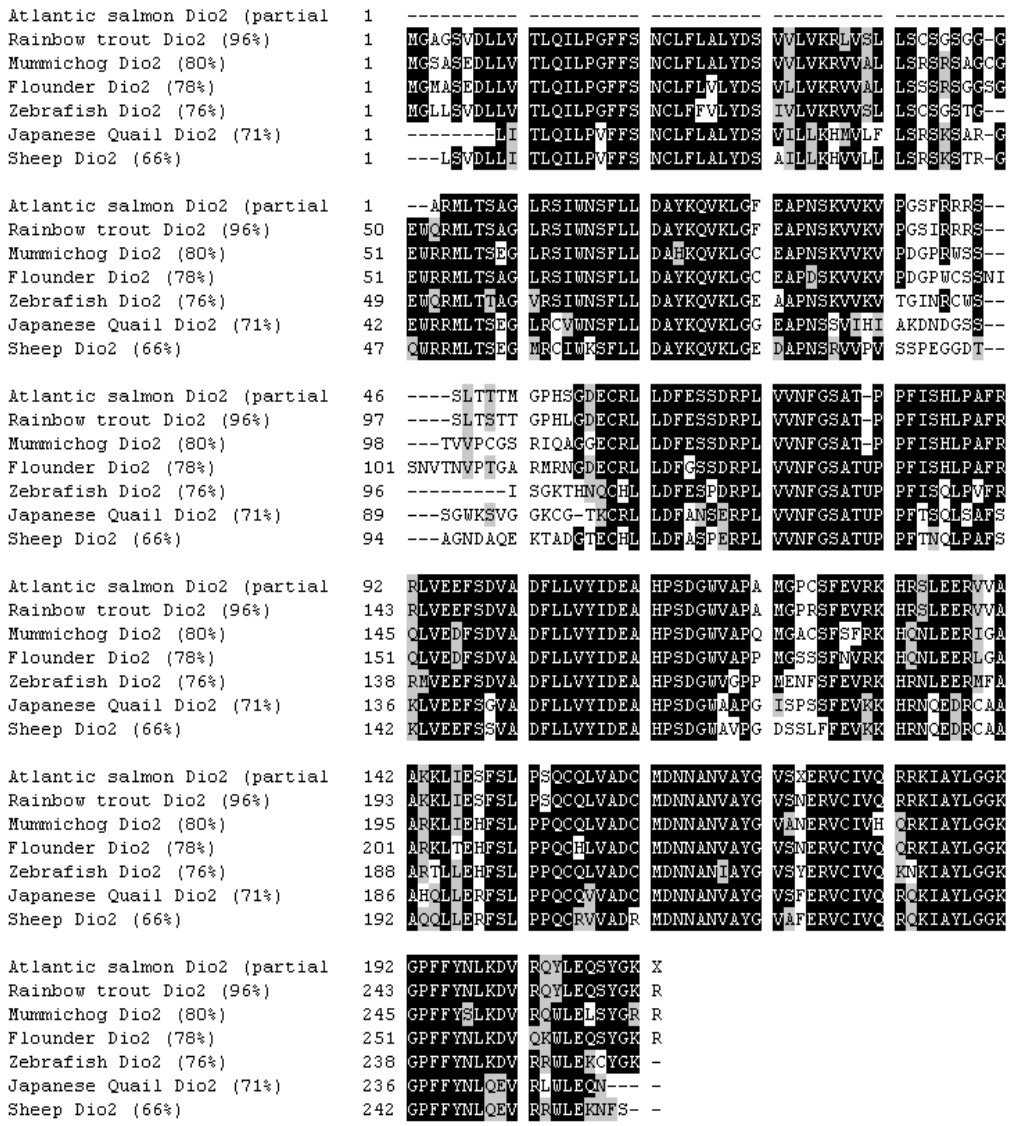


Figure 5 The partial amino acid sequence of Atlantic salmon compared with *Dio2* genes in other vertebrate species. The salmon gene shows highest identity with rainbow trout *Dio2*, followed by that in other teleost species. The percentage values shown in parentheses are the identities of the respective protein with that of the Atlantic salmon partial sequence, obtained through protein BLAST. The shading across the different amino acid sequences indicates 50% or more similarity. Accession numbers: Rainbow trout *Dio2* (AAL25715), Mummichog *Dio2* (AAL62449), Flounder *Dio2* (BAG15907), Zebrafish *Dio2* (AAH59608), Japanese quail *Dio2* (ACB59241), Sheep *Dio2* (ACX31206).

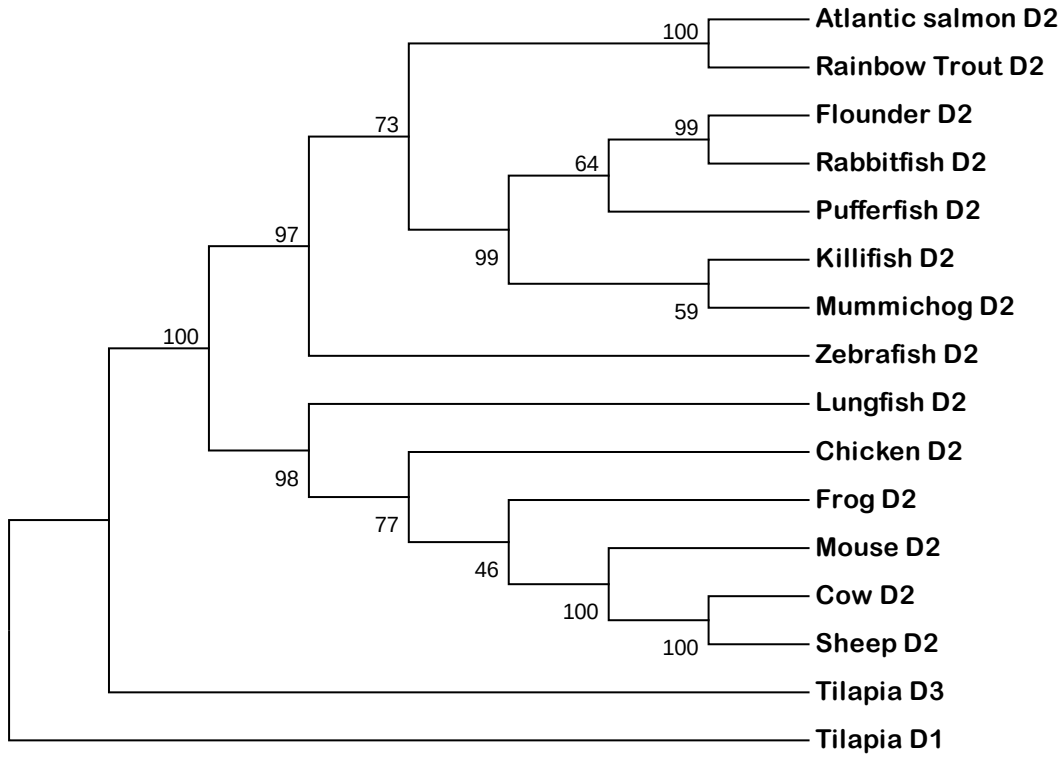


Figure 6: Phylogenetic tree of the Atlantic salmon partial *Dio2* sequence in relation to other species. The partial sequence of Atlantic salmon shows highest similarity to the rainbow trout *Dio2*. The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2007) and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007). Accession numbers: rainbow trout *Dio2* (AF207900), Japanese flounder *Dio2* (AB362422), golden rabbitfish *Dio2* (GU372962), pufferfish *Dio2* (AB360768), Japanese killifish *Dio2* (AB383147), mummichog *Dio2* (U70869), zebrafish *Dio2* (BC059608), Australian lungfish *Dio2* (AF327438), chicken *Dio2* (NM_204114), frog *Dio2* (L42815), mouse *Dio2* (NM_010050), cow *Dio2* (NM_001010992), sheep *Dio2* (GQ468498), Nile tilapia *Dio1* (Y11109), Nile tilapia *Dio3* (Y11111).

Diel expression of seasonal genes

Microarray validation study: long day (LD), short day (SD)

The diel expression profiles of three *Dio* genes (*Dio1-3*), *Eya3* and *Tshβ* was confirmed in brains sampled from fish acclimated to either LD or SD which had previously been used as part for the microarray study (Figure 7). For all genes, excluding *Dio2*, levels of mRNA expression over a 24h period were significantly elevated under SD. No significant difference in mean *Dio2* mRNA expression levels was found between LD and SD. With respect to the diel expression profiles *Dio2* and *Tshβ* displayed a significant circadian profile of gene expression under LD and SD respectively. *Dio2* expression peaked at ExT 05:00 ± 2.13 while the acrophase of *Tshβ* occurred at ExT 13:00 ± 2.34 when subjected to acro analysis.

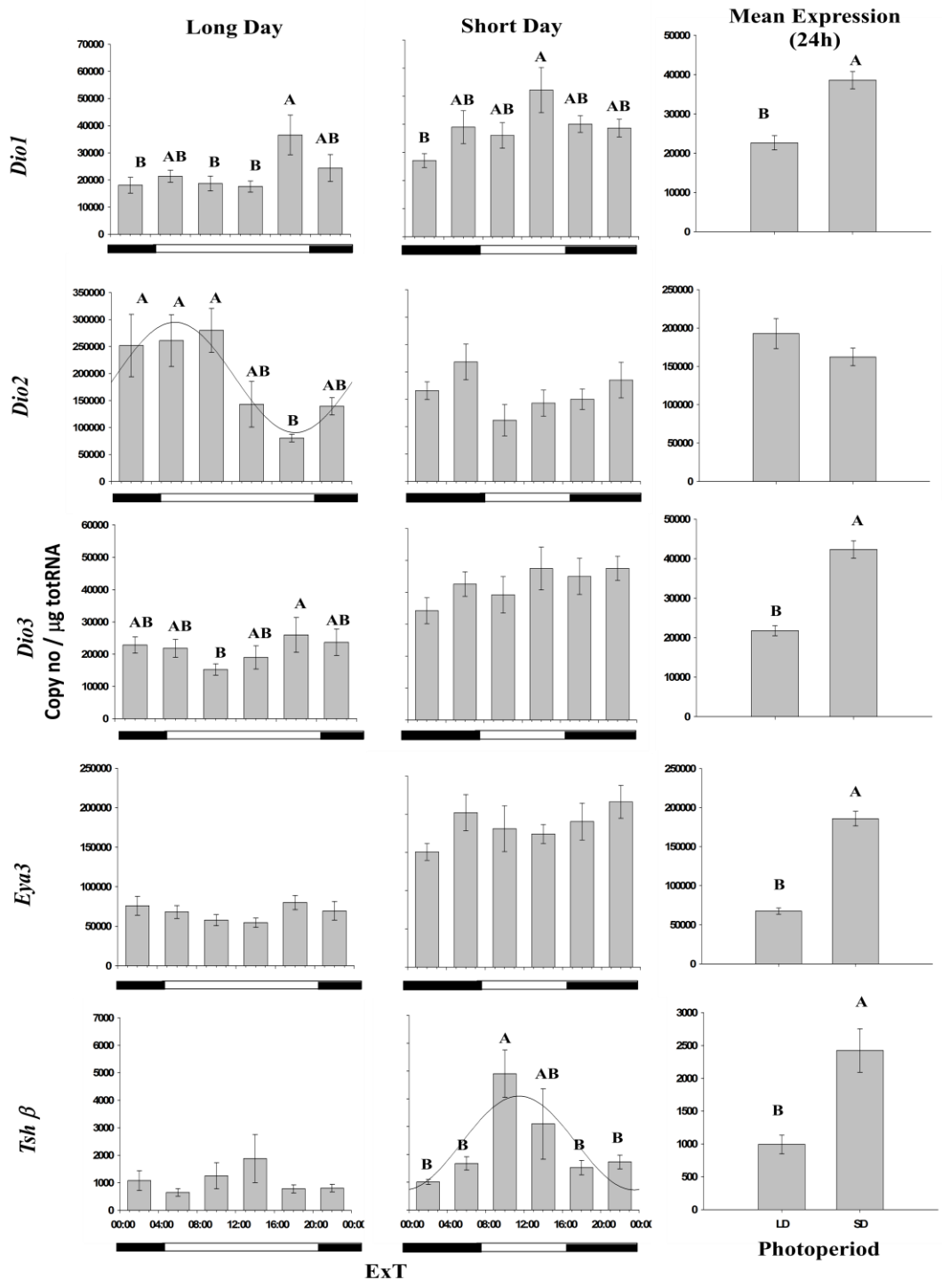


Figure 7 Diel and mean expression of *Eya3*, *Tshβ* and *Dio1-3* under long day and short day photoperiod for microarray validation. Expression is displayed as copy no per µg totRNA with SEM and are displayed in relation to external time, where by ExT 0 is the mid point of the of the nocturnal phase. The presence of a cosine wave denotes a significant circadian rhythm by acro. The presence of different letters represents statistically significant difference between samples by way of ANOVA and Turkeys test where by $P<0.05$

qPCR study: Long day (LD), short day (SD) and 12L:12D

In sample set 2, the expression of *Dio2*, *Eya3* and *Tshβ* was analysed over 24h in LD, SD and 12L:12D photoperiod (Figure 8). The expression of *Dio2*, *Eya3* and *Tshβ* displayed significant circadian rhythms under LD only. Acrophase over the 24h profile were ExT 05:00 ± 2.61, 05:00 ± 2.61 and 01:00 ± 3.19 respectively. Mean expression of *Dio2* over the 24h period was significantly higher under LD as opposed to SD and 12L:12D photoperiods. Mean expression of *Eya3* and *Tshβ* over the 24h period was significantly higher under LD in comparison to 12L:12D only.

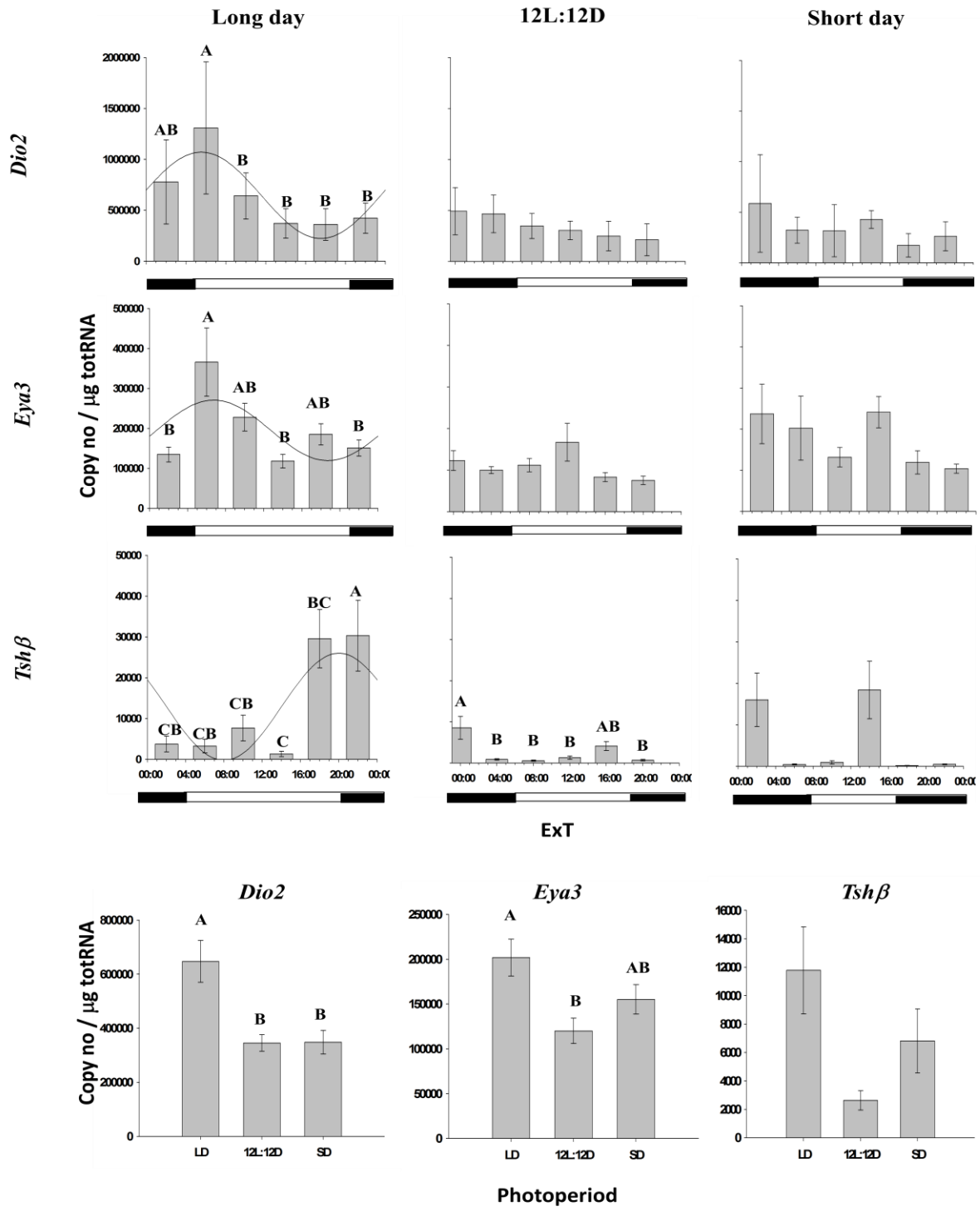


Figure 8 Diel and mean expression of *Eya3, Tshβ* and *Dio2* under long day, 12L:12D and short day photoperiod in qPCR study Expression is displayed as copy no per µg totRNA with SEM and are displayed in relation to external time, where by ExT 0 is the mid point of the of the nocturnal phase. The presence of a cosine wave denotes a significant circadian rhythm by acro. The presence of different letters represents statistically significant difference between samples by way of ANOVA and Turkeys test where by $P<0.05$

5. DISCUSSION

This study is the first investigation attempting to unravel temporal organisation mechanisms and identify elements of the molecular switch for photoperiod response in a commercially important teleost. Microarray results revealed photoperiod dependent expression of deiodinase genes and *Eya3*. These results were subsequently validated by qPCR for *Dio2*, Eya3 and Tshβ which provided evidence for the long day regulation of *Dio2* and the photoperiod dependent regulation of *Eya3* and *Tshβ* dependent on sample set.

In order to better understand the molecular mechanisms underpinning seasonal physiology in Atlantic salmon, a microarray study was first carried out to determine gene expression changes at the transcriptome level in response to photoperiodic conditions (seasonal cue) and time of the day (circadian cue). Significant differences between conditions were observed for three different clock genes of interest (*Cry1*, *Cry2* and *Per1*). Significant differences in the expression of *Cry 1* and *Per1* were shown between day and night while *Cry2* differences were shown between photoperiods. This is consistent with results in chapter 3 whereby the amplitude of *Cry2* expression varied considerably with photoperiod. Two CCAATβ and two CCAATβ2s were also identified with significant differences in expression shown between photoperiods and time of the day independently (no significant interaction difference). CCAAT-enhancer-binding-proteins interact with CCAAT box promoters present in a number of genes and act as co-activators promoting the expression of particular genes (Ramji & Foka 2002). Nakao et al (2008b) previously identified CCAATβ as one of nine genes present in the second wave of gene expression, with *Dio2*, after exposure to first LD photoperiod in the Japanese quail and is suggested to be regulated by first wave genes including *Tshβ*. Genes previously implicated in the mammalian molecular seasonal photoperiod switch i.e. *Dio 1-3* and *Eya3* and CCAATβs displayed significant differences in LD in comparison to SD

photoperiods. *Dio2* was up-regulated in LD in comparison to SD as previously been reported in mammals. However, in contrast to mammals, *Eya3* was down-regulated under LD conditions.

In order to confirm and expand upon the microarray results, expression of a series of genes known to be involved in photoperiod regulation of seasonal physiology in mammals (i.e. *Dio1, Dio2* and *Dio3, Eya3* and *Tshβ)* were analysed by qPCR firstly in the data set used to generate the microarray results (microarray validation) to confirm observations and then in a second unrelated dataset (qPCR study) to test the robustness of the expression patterns observed.

Results from both qPCR investigations showed significant differences in gene expression patterns between photoperiods however profiles of gene expression were notably different between sample sets. In the microarray validation study results confirmed the long day up-regulation of *Dio2* and down-regulation of *Dio1* and *Eya 3* as previously described in the microarray results. However *Dio3* expression displayed contrasting results between microarray (up-regulated under LD) and microarray validation (up-regulated under SD). While microarray validation results are consistent with observations in mammals and in birds the up-regulation of this gene in the microarray may be an artefact of investigating expression at mid night and mid day as opposed to over a complete 24h period. In addition, a significant circadian rhythm in the expression of Tshβ mRNA was observed under SD. In both microarray validation and qPCR studies, diel *Dio2* mRNA expression displayed a significant circadian profile under LD with acrophase at comparable times of day (i.e. ExT 05:00 ± 2.13 and 05:00 ± 2.61 in microarray validation and qPCR studies respectively). This is consistent with reports in mammals and birds according to which *Dio2* is up-regulated under LD and displays a significant circadian profile of expression (Nakao et al. 2008a; Nakao et al. 2008b;

Dardente et al. 2010). While Dio2 results are in agreement between microarray, microarray validation and qPCR study, contrasting patters of *Eya 3* and *Tshβ* were observed. In the qPCR study *Eya3* and *Tshβ* displayed a significant circadian profile of expression under LD. However results in the current investigation provide contradictory evidence for the role of *Eya 3* and *Tshβ* in the long day seasonal response and regulation of *Dio2* in the reproductive axis which will be discussed further below.

In birds and mammals the expression and function of the genes involved in the regulation of the long day photoperiod response are better understood. Despite differences in the role of melatonin (mammals) and deep brain photoreceptors (birds) in the transmission of seasonal photoperiodic cues to the MBH and the PT, the molecular mechanisms regulating the photoperiodic control of reproduction are remarkably conserved (Dardente et al. 2010). In birds, studies suggested photoperiod information is perceived by deep brain photoreceptors present in the MBH and the PT of the pituitary (Nakao et al. 2008a; Nakao et al. 2008b). In mammals, photoperiod information is perceived by the retina and transmitted via the SCN to the pineal (Dardente et al. 2010). In the PT of both mammals and birds the phase of clock gene expression is altered. This, in turn, mediates the expression of *Eya3* via three conserved E-box elements in the promoter region of the gene. *Eya3* forms a dimer complex with *Six1* potentiating *Tef*. In response to the EYA3/SIX1/TEF complex is *Tshβ* is up-regulated under long day photoperiod. TSHβ then binds to *Tsh* receptors (*TshR*) in the ependymal cell layer (EC) and leads to the subsequent up-regulation of *Dio2* and down-regulation of *Dio3* mRNA (Figure1). Therefore, in birds and mammals, two waves of gene expression, prior to LH secretion, have been described after exposure to the first long day photoperiod (Nakao et al. 2008a & b; Nakao 2009). At around 14:00 after dawn on the first long day a significant increase in the expression of both *Eya3* and *Tshβ* was observed. This was then followed by a second wave of gene expression approximately four hours later. The second wave included

the up-regulation of the *Dio2* gene and down-regulation of *Dio3*. Conversely under SD photoperiod *Dio2* was suppressed while *Dio3* was up-regulated. In the Japanese quail the two waves of gene expression occur during the photoinducible phase when the quail is responsive to light at a particular time of the circadian cycle (Nakao et al. 2008 a & b). It is the alternating seasonal profile of DIO2 and DIO3 that regulates the seasonal control of reproduction in both birds and mammals. In the current investigation results confirm the presence of similar molecular mechanisms in the Atlantic salmon in comparison to mammals and birds and elude towards the highly conserved nature of the molecular seasonal photoperiod switch in vertebrates. However the precise mechanisms connecting the various elements of this pathway are yet to be investigated in the Atlantic salmon.

In vertebrates *Tshβ* in particular, has been shown to be instrumental in the photoperiodic control of *Dio2* (Unfried et al. 2009). The administration of TSH resulted in the up-regulation of *Dio2* expression and other second wave genes. This is achieved by regulation of the thyroid hormones. DIO2 regulates the conversion of T4 into the bioactive form T3. Conversely increased concentrations of DIO3 convert T4 to a biologically inactive form rT3 (Figure 2). Seasonal differences in thyroid hormones then control the seasonal regulation of reproduction via GnRH and LH stimulation (Arrojo E Drigo & Bianco 2011). Accordingly the administration of an anti-TSHβ antibody suppressed *Dio2* expression under LD conditions. *Tshβ* is hypothesised to regulate the expression of the second wave of genes via TSHR-Gsα-cAMP signalling pathway (Dardente et al. 2010). Moreover the promoter sequence of DIO2 and other second wave genes were shown to contain highly conserved cAMP response elements (Unfried et al. 2009). In the current study, microarray validation study showed *Eya3* and *Tshβ* were up-regulated under SD photoperiod while qPCR study displayed a significant circadian profile under LD photoperiod. However in both the qPCR and microarray investigations *Dio2* was consistently up-regulated under LD conditions as

previously reported in mammals and birds. This eludes towards the differential regulation of the first and second wave genes in the Atlantic salmon. Moreover this pattern of SD regulation of genes in the microarray validation set and LD in qPCR study has previously been reported for the expression of clock genes.

The expression of a number of the core clock components have previously been investigated in microarray validation (Davie et al. 2009) and qPCR study (Chapter 3). Similar to the seasonal genes (i.e. *Eya3* and *Tshβ*), clock genes (i.e. *Clock* and *Per2*) displayed a significant rhythm of expression under SD photoperiod in the microarray validation and LD in the qPCR study. It has been hypothesised that this may be a consequence of the different photoperiodic history between the two sample sets. Atlantic salmon parr used for microarray validation were acclimated to experimental LD and SD photoperiods when the natural daylength was decreasing from the autumnal equinox towards the winter solstice. Conversely the salmon parr utilised for qPCR study were acclimated to LD, SD and 12L:12D photoperiods around the vernal equinox when the ambient daylength was increasing towards the summer solstice. It was therefore suggested that circadian profiles of clock gene expression in the Atlantic salmon brain were present in the photoperiod that best represented the natural photoperiod seasonal progression. Accordingly in microarray validation and qPCR studies significant circadian expression was observed in fish exposed to SD photoperiod when the natural daylength was decreasing and LD when natural daylength was increasing (Chapter 3). However, as yet, no link has been identified between clocks and the regulation of *Eya3* and Tshβ in fish.

In mammals regulation of *Eya3* and subsequent *Tshβ* expression has been shown to be regulated by the circadian clock (Dardente et al. 2010). The promoter region contains three conserved E-box elements sensitive to CLOCK and BMAL1 accounting for the rhythmic

expression of the gene (Dardente et al. 2010). Results reported previously in the thesis (Chapter 3) from qPCR study showed rhythmic clock gene expression in the brain under LD photoperiod while as previously reported by Davie et al (2009) clock gene expression in the microarray validation set was present under SD treatment. This supports the argument that *Eya3* may be regulated by clocks in an Atlantic salmon molecular photoperiod switch. In mammals *Tshβ* and subsequent deiodinase regulation has also been shown to be directly under the control of clocks in addition to melatonin and regulation by *Eya3* and *Tef* via D-elements. For example *Per1* knock out mice displayed an inverted pattern of *Tshβ* expression in comparison to wild type mice (Unfried et al. 2009). It has therefore been hypothesised that *Per1* suppresses *Tshβ* during the day and counteracts the suppressive effects of melatonin during the night (Unfried et al. 2009). The 5' up-stream region of mouse *Tshβ* contains 11 E-box like elements and has the capacity to be regulated by clock genes. Consequently the CLOCK and BMAL1 proteins together have been shown to induce an up to 71 fold increase in *Tshβ* activity via action on the genes promoter (Unfried et al. 2009). Certainly in mice these results demonstrate the importance of clock components in the regulation of *Tshβ* and consequent regulation of deiodinase and thyroid hormone metabolism. However in the mammalian PT the expression of clock genes is regulated by melatonin binding. (Unfried et al. 2009). In teleosts the regulatory mechanisms linking both melatonin and clocks to thyroid hormone metabolism remain to be elucidated however results from this study clearly demonstrate the presence of highly conserved seasonal mechanisms in the Atlantic salmon with "first wave genes" potentially regulated by photoperiod dependent clock gene expression in the brain.

Previous studies in mammals and birds have focused on the expression of the molecular mechanisms responsible for photoperiod response in the PT of the pituitary while this study investigates the expression of the genes involved in this pathway in the whole brain including

the pituitary. It is possible that a number of the seasonal genes may be expressed differently throughout the brain. For example *Eya 3* is a member of a developmental regulatory network thought to have a role in a number of other seasonal processes including the development of the eyes, pineal organ and pituitary gland (Jemc & Rebay 2007; Dardente et al. 2010). Consequently photoperiod specific expression may be biased in whole brain samples in comparison to isolated brain regions. As yet in teleosts neither the presence of circadian (SCN- like structure) or seasonal control centres have been identified. Considerable differences are present in the way in which photoperiodic information is perceived in teleosts, in particular in comparison to mammals. Future research in the Atlantic salmon would benefit from determining localised expression of both clock and seasonal genes within the brain. Moreover determining the mechanisms regulating individual seasonal genes, such as the presence of E-boxes and D-elements in gene promoters would be of further benefit. Also of interest may be the investigation of such conserved mechanisms in more ancient vertebrate species such as hag fish or lampreys. In any case this study has been the first investigation describing the presence of the highly conserved molecular switch for photoperiod response amongst commercially important teleost species.

6. CONCLUSIONS

The current study is the first to investigate 24h profile of expression of seasonal genes in Atlantic salmon, one of the more clearly seasonal fish species. An investigatory microarray was initially carried out to investigate transcriptome level alterations in seasonal gene expression. Results highlighted the photoperiod dependent expression of elements of the mammalian and avian molecular switch for photoperiod response (*Dio1-3* and *Eya3)* in the Atlantic salmon. Results were subsequently verified by qPCR where the expression of *Dio1-3*, *Eya3* and *Tshβ* was analysed over 24h in response to LD and SD photoperiods. The

expression of three deiodinase *Dio1-3*, *Eya3* and *Tshβ* was then investigated in the brain in an unrelated qPCR in an attempt to identify potentially conserved molecular components involved in vertebrate seasonal physiology. Results demonstrate photoperiod effect on the mean levels and 24h profiles of mRNA expression in the brain. *Dio2* was consistently up-regulated under LD photoperiod or circadian in expression in microarray, microarray validation and qPCR study as has been reported in other vertebrates. However *Eya3* and *Tshβ* were responsive to SD in microarray validation and LD in qPCR study. A pattern previously observed for clock genes in these samples. Contrasting results between microarray verification and qPCR study may be a consequence of photoperiodic history as is hypothesised for clock genes in chapter 3 or direct regulation of *Eya3* and *Tshβ* by clock genes as has been reported in mammals. Interrogation of the promoter regions of *Dio2* would enable a better understanding of what regulates its expression and why differences were observed in the expression of its potential regulator *Tshβ* in both sample sets. Future work would additionally benefit from the localisation of the expression of both clock and seasonal genes within the brain and the pituitary. The identification of an SCN-like structure and a seasonally centre within the brain would enable considerable advances in the understanding of biological rhythms in fish. Current results represent the first attempt to identify the expression of a number of seasonally important genes in the Atlantic salmon. Understanding the way in which the teleost PNES functions is not only essential for the commercialisation of cultured species, it also highlights the incredible level of diversity amongst teleost physiology in comparison to other vertebrate species.

CHAPTER 5

COMPARATIVE STUDY OF CLOCK GENE EXPRESSION AND MELATONIN IN THE ATLANTIC SALMON AND EUROPEAN SEABASS PINEAL.

Chapter 5

COMPARATIVE STUDY OF CLOCK GENE EXPRESSION AND MELATONIN IN THE ATLANTIC SALMON AND EUROPEAN SEABASS PINEAL.

1. ABSTRACT

The photoreceptive pineal organ of teleost fish is considered by many to be essential to the generation, synchronisation and maintenance of biological rhythms, primarily via the action of melatonin. Amongst salmonids the production of pineal melatonin is regulated directly by light and levels are elevated under constant darkness. In non salmonid teleosts the rhythmic high at night / low at day melatonin profile persist endogenously under constant darkness and are hypothesised to be governed by light and clock genes in the pineal. In order to better understand the role of clocks in the Atlantic salmon pineal this study aimed to characterise the expression of clock genes *in vitro* under different photoperiodic conditions: 12L:12D, reversed 12D:12L and 24D. Clock gene expression was also determined *in vivo* in salmon acclimatised to a 12L:12D photoperiod. Results were then compared with an *in vitro* (12L:12D) investigation in the European seabass, a species displaying endogenous melatonin synthesis. Results revealed no rhythmic clock gene (*Clock, per1* and *per2*) expression in salmon pineals *in vitro* under any culture conditions. In seabass, *Clock* and *Per1* did not display circadian expression *in vitro*. However rhythmic expression of *Cry2* and *Per1* in the salmon pineal was observed *in vivo*. This infers some degree of extra pineal regulation of clocks in the Atlantic salmon. With regard to *Aanat2* no rhythmic expression was observed in the Atlantic salmon under any experimental conditions. In the seabass rhythmic expression of *Aanat2* mRNA under 12L:12D treatment was observed in the pineal. This is consistent with the hypothesis that in salmonids AANAT2 is regulated directly at the protein level by light while in other teleosts AANAT2 is regulated by clocks at the of mRNA level and then light at the protein level. *In silico* analysis of the *Aanat2* 5'region revealed the absence of a functional E-box element in the promoter region of the salmon gene in comparison to other

teleosts, including the European seabass. This would explain the differences seen in *Aanat2* mRNA expression between species with no clock regulation of *Aanat2* mRNA in salmon.

2. INTRODUCTION

The pineal is considered by many to be fundamental in the making and maintenance of biological rhythms. This is particularly true in non-mammalian vertebrates where the pineal organ is directly photosensitive and is the primary source of circulating melatonin (Falcon et al. 2010; Migaud et al. 2010). In teleosts, the pineal organ is a vesicle of differing size, colour and transparency depending on the species. In adult Atlantic salmon, rainbow trout and pike the pineal organ is large and covers the telencephalon while in other species such as Nile tilapia, Atlantic cod and seabass the pineal is considerably smaller (Ekstrom and Meissl 1997; Migaud et al. 2007; Herrera-Perez et al. 2011). The vesicle itself is located above the telencephalon and below an area of thinned skull and tissue which has the greatest optical transparency (pineal window) and is connected to the brain (diencephalon) via the pineal stalk (Ekstrom & Meissl 1997; Falcon 1999; Falcon et al. 2010; Vera et al. 2010).

In teleosts the pineal shares a number of common features with the retina (Ekstrom & Meissl 1997; Falcon et al. 2010). In most species studied, both tissues are directly photosensitive. In fact the epithelium of the teleost pineal is made up of photoreceptor cells that structurally and functionally resemble retinal cones (Ekstrom & Meissl 1997; Falcon et al. 2010). Additionally pineal photoreceptors have similar lipids to the retina and similar proteins that make up the photic transduction cascade such as opsin, arrestin and cyclic nucleotide gated channel. The pineal also resembles the retinal cones with respect to response to light. As a result of exposure to light cells become hyperpolarized, inhibiting the excitatory neurotransmitters asparatate and/or glutamate (Falcon et al. 2010). In the pineal the neurotransmitters extend to the ganglion cells, this in turn relays information to the brain (Falcon et al. 2010). These similarities are explained by the fact that during development both the pineal and the retina are formed from invaginations of the primary forebrain (Ekstrom & Meissl 1997). However, the pineal, unlike the retina, does not have the capacity to

discriminate between rapid and more gradual changes in light (Ekstrom & Meissl 1997). This is thought to be a consequence of a slower time course of photoreceptor response in the pineal in comparison to the retina. For example time from onset to peak potential is up to 300 ms under saturating light flashes in the rainbow trout pineal (Meissl & Ekstrom 1988). This is between 5 and 6 fold less than the retina of other vertebrates (Baylor & Hodgkin 1974; Cervetto et al. 1977). Moreover, in the pineal organ response time of up to 60 s have been observed for membrane recovery (Meissl and Ekstrom. 1988). As a result of the relatively slow reactivity of pineal photoreceptors, rapid changes in photic stimuli cannot be detected. This is consistent with the pineal organs proposed role as a photoreceptive tissue capable of deciphering of daylength information as opposed to more complex "visual" photic information (Meissl and Ekstrom. 1988).

In the pineal, as in the retina, photic transduction is initiated as a response to light. Photoreceptor cells become hyperpolarised which in turn inhibits neural signalling by excitatory neurotransmitters and release of aspartate or glutamate as well as hormonal signalling by melatonin (Meissl & Ekstrom 1988; Meissl & Ekstrom 1988; Ekstrom & Meissl 1997; Falcon 1999; Falcon et al. 2010 . The two signalling pathways are believed to target different locations with neurotransmitters stimulating ganglion cells whose axons extend directly into the brain while the hormonal signal conveys photic information to the rest of the central nervous system and peripheral tissues via the cerebrospinal fluid and blood circulation (Ekstrom & Meissl 1997; Falcon 1999; Forsell et al. 2001). It is the hormonal signalling pathway which has received greater research focus and forms the basis of our understanding of light perception and entrainment in teleosts.

Melatonin biosynthesis begins with the uptake of tryptophan by the pineal and subsequent hydroxylation catalysed by tryptophan hydroxylase (TPOH) to create 5 - hydroxytryptophan.

Serotonin is then produced via decarboxylation by aromatic amino-acid decarboxylase (AAAD) (Falcon et al. 2010; Falcon et al. 2011). Interestingly serotonin displays an inverse pattern of synthesis in comparison to melatonin with elevated levels during the day and suppression at night (Falcon et al. 2011). Serotonin is then converted, via the catalytic action of the light mediated arylalkylamine N-acetyltransferase (AANAT) to N-acetyltransferase. N-acetyltransferase is then converted into melatonin by Hydroxyindol - O- methyltransferase (HIOMT)(Falcon et al. 2011). Of the two enzymes involved in the conversion of serotonin into melatonin the expression of the aanat genes and action of AANAT enzymes in the pineal is closely mirrored by the profile of circulating melatonin and is commonly described as the rate limiting enzyme for melatonin synthesis (Falcon et al. 2010). In comparison to mammals, that have one form of AANAT, teleosts have at least two forms following the teleost wide genome duplication. AANAT1 is preferentially expressed in the retina while AANAT2 in the pineal. However expression of both is not limited to the pineal and the retina and is observed in other tissues such as the brain. Furthermore in some teleost species two forms of AANAT1 (a & b) have been described in the retina. This was first identified in puffer fishes including fugu (*Takifugu rubripes*) however this was not observed in the Zebrafish (*Danio rerio*) (Coon & Klein 2006; Zilberman-Peled et al. 2011).

Rhythmic melatonin synthesis in teleosts is driven by the daily cycling in AANAT2 activity and is up-regulated in the dark in most teleost species. In non salmonid teleosts *Aanat2* mRNA transcription mirrors enzymatic activity (Klein et al. 1997; Ganguly et al. 2002; Falcon et al. 2003; Appelbaum et al. 2004; Falcon et al. 2011;). This rhythmic abundance/activity is regulated in two ways. The activity of the AANAT2 enzyme is regulated, in most teleosts, directly via the 24h light/dark cycle and endogenous circadian clocks. In darkness conditions, photoreceptors become depolarised, intracellular calcium (Ca^{2+}) is accumulated which then regulates the light dependent action of AANAT2 by

increasing the efficiency of β 1-adrenergic receptor activation of adenylyl cyclase (Klein 2007). This consequently results in an elevation of cAMP which in turn results in the formation of the AANAT/14-3-3 complex (Klein 2007). When light is present Ca^{2+} is depleted, AANAT2 is degraded and melatonin synthesis ceases (Falcon et al. 2011). Direct regulation by light occurs at the protein level through the AANAT2 enzyme however regulation by the circadian clock occurs at a transcriptomic level. The CLOCK:BMAL heterodimer, a component of the core molecular clock up, regulates *Aanat2* mRNA transcription by binding to E-box elements in the promoter region of the *Aanat2* gene (Appelbaum et al. 2004; Appelbaum & Gothilf 2006; Appelbaum et al. 2006). In fact it is hypothesised that it is the circadian clock that drives the rhythmic activity and expression of AANAT2 in the pineal, while phosphorylation induced by Ca^{2+} (direct regulation by light) protects AANAT2 against degradation. Melatonin is highly lipophilic and therefore released continuously into circulation when synthesised. With the onset of light both AANAT activity and melatonin are rapidly degraded (halving time of approximately 3.5 minutes) (Falcon 2007; Klein 2007). Melatonin has therefore a low residency time within the plasma and levels of circulating melatonin are directly reflective of AANAT2 activity and melatonin synthesis. Melatonin acts as an accurate internal chemical signal of external daylength (Iigo et al. 2007).

Endogenous regulation of AANAT2 and melatonin by clocks is evident in the majority of teleost species. *In vivo* and *in vitro* studies have shown that melatonin day/night cycling can persist under constant darkness in most teleosts studied. Moreover pineal melatonin synthesis is independently entrainable by light. When exposed to alter Light: Dark cycles the pineal can re-entrain the melatonin rhythm to the external conditions (Iigo et al. 2007; Migaud et al. 2007). However, amongst teleosts it appears that the salmonids are exceptions. Under continuous darkness melatonin production does not follow an endogenous circadian profile, instead levels of melatonin are consistently high throughout as shown in both *in vivo* and *in*

vitro conditions (Amano et al. 2004; Iigo et al. 2007; Iigo et al. 2007; Migaud et al. 2007; Falcon et al. 2010; Migaud et al. 2010). Following a comparison of salmonids and closely related Osmerids and Pike species, Iigo *et al.* (2007) proposed that the pineal organ of ancestral protacanthopterygians harbour the circadian clock but ancestral salmonids lost the circadian regulation of melatonin production in the pineal organ during evolution after the divergence from osmeriformes / esociformes. It is widely recognised that the salmonid pineal has lost endogenous clock regulation of melatonin synthesis (Iigo et al. 2007; Migaud et al. 2007). However it remains unclear whether clock gene cycling has become decoupled from melatonin synthesis or if in fact functional circadian clocks are no longer present in the salmonid pineal.

In order to better understand the role of clock genes in the pineal the current study aimed to study the expression of clock genes in the Atlantic salmon and seabass pineal. To do so, mRNA expression of a suite of clock genes and *Aanat2* as well as melatonin concentrations were first analysed in isolated Atlantic salmon pineal organs exposed to standard photoperiod (12L:12D), reversed photoperiod (12L:12D – 12D:12L) and 24 hours darkness (DD). The aims of these *in vitro* trials were to determine if circadian clocks and *Aanat2* expression are present in salmon, test the ability of the pineal to independently re-entrain itself to a different photoperiod and establish whether the candidate clock genes and *Aanat2* expression can be sustained under un-entrained conditions, respectively. Then, *in vivo* experiments were carried out to compare clock gene expression and plasma melatonin levels in the pineal of salmon and seabass reared under a 12L: 12D photoperiod. Seabass is a teleost species suggested to display endogenous clock controlled production of pineal melatonin (Migaud et al. 2007). Finally, post-hoc analyses of salmon and seabass 5' region of the Aanat 2 sequences were carried out in order to determine the role E-box elements may have in the clock gene regulation of *Aanat2* and endogenous melatonin synthesis.

3. MATERIALS AND METHODS

Animals, housing and tissue sampling

Atlantic salmon in vitro and in vivo studies

For both the i*n vitro* and *in vivo* studies fish used were a standard farmed stock origin (mixed sex) and were housed at the Niall Bromage Freshwater Research Facilities (Institute of Aquaculture, Stirling, UK, 56.04N, -4.00E) under ambient thermal conditions (2.2 ± 0.1 ^{o}C i*n vitro* study, 4.6 ± 0.2 ^{o}C i*n vivo* study) and were fed in excess throughout the day hours with the use of automated feeders.

In late January 2010, ~400 (46.1 ± 2.7 g) salmon parr were acclimated to 12L:12D photoperiod with the light phase extending from 07:00 to 19:00. After 4 weeks acclimation period, ~70 salmon parr were sacrificed by lethal anaesthesia (2-phenoxyethanol 1ml/L Sigma) followed by decapitation. Pineal organs were dissected out by exposing the dorsal surface of the brain by making a rostro-caudal incision in the horizontal plane extending from the eyes to the end of the cranium. During this incision the pineal stalk was severed and the pineal gland was found resting in the pineal window in the liberated inverted cranial cap. Where necessary the pineal gland was removed with the aid of a dissection microscope and light. Once isolated the pineals were placed into fresh culture media (see below) maintained at 8^{o}C for a maximum of three hours before being placed under experimental culture conditions (see below). This tissue harvesting was repeated on two subsequent occasions with 140 fish for the 12D:12L and 24hD experiments.

The *in vivo* experiment was performed during March 2011. Seventy (64.0 ± 2.3 g) parr were acclimated to a 12L:12D photoperiod with the light phase extending from 07:00 to 19:00. After 4 weeks acclimation starting at 09:00 and then every four hours thereafter until 09:00 the following day, ten fish were anaesthetised in a lethal dose of 2-phenxoyethanol and 1 ml

of blood was withdrawn from the caudal peduncle using a heparinised syringe. Fish were decapitated and then a section of cranial cap removed as described above which encompassed the pineal window and was then stored in a RNA stabilisation solution (RNAlater®, Applied Biosystems). Within one hour of removing the blood samples, plasma was separated by centrifugation (30 minutes at 1500 G). Plasma aliquots were frozen in liquid nitrogen vapour prior to storage at - 70 ^{o}C. The cranial caps were stored in the RNA stabilisation solution for 24h at 4 ^{o}C and then with the aid of a dissection microscope the pineals were delicately removed from the cranial cap and frozen individually over liquid nitrogen vapour before being stored at - 70 ^{o}C. Nocturnal sampling was carried out under minimal dim red light according to Davie et al. (2009).

European seabass *in vivo* study

Fish for the seabass *in vitro* experiment were housed at the University of Murcia, circadian biology aquarium facility at the Algameca naval station (37.6 N, -0.98333W) near Cartagena, Spain. Seventy seabass (169.9 ± 10.6 g) were acclimated for 2 weeks to 12L:12D (lights on 06:00, lights off 18:00) at an ambient temperature of 16 ^{o}C. After the acclimation period all fish were sacrificed via lethal anaesthesia using clove oil, eugenol, (Guinama, Valencia, Spain) dissolved in 10 ml of ethanol at a final concentration of 50 µl/l. Lethal anaesthesia was rapidly followed by decapitation. Pineals were removed dorsally by thinning the tissue and bone around the pineal window then carefully removing the whole pineal with the aid of a dissection microscope. The pineal organ itself was then removed by carefully cutting the pineal stalk close to its origin and placing it into fresh culture media (see below) and maintained in at 17 ^{o}C in groups (35 per 100 ml) until culture conditions were established (see below).

Pineal cultures

Atlantic salmon pineal culture

All salmon pineal cultures were carried out at 8 ^{o}C in a light and temperature controlled chamber using RPMI – 1640 without phenol red culture medium (Sigma-aldrich ref R8755-10X1L Gillingham, UK) according to Migaud et al. (2007). Media was supplemented with 4.8 g Hepes Sodium salt (Sigma-aldrich, Gillingham, UK) per litre (buffer), 10 mg/L Penicillin-streptomycin solution and 5 mg/L Fungizone (amphotericn # B from Streptomyces Sp) to prevent any fungal and bacterial growth (Sigma-aldrich, Gillingham, UK). Prior to use the media pH was adjusted to 7.4 by adding hydrochloric acid (HCL) and was then sterilised through 0.2 µm filtration before being stored at 4 ^{o}C for no more than 72 hours. Aliquots of media were pre-warmed to the culture temperature prior to being used in the culture experiment. In all salmon *in vitro* experiments pineals were maintained in 20 ml glass vials (10 pineals/20 ml culture media) with a fine nylon mesh to prevent the pineals from floating on the surface of the media. Every 4 hours, 15 ml of media was removed from the culture vial and replaced with fresh, temperature equilibrated, media.

Three different experimental photoperiods were tested 12L:12D, 12D:12L and 24D using 70, 140 and 140 pineals respectively (Figure 1). For the 12L:12D experiment pineals were harvested between 09:00 and 12:00 on Day 0 and placed in culture vials by 13:00 and subjected to a 12L:12D photoperiod in synchrony with that experienced prior to harvest with a photophase from 07:00 to 19:00. Pineals were left in culture overnight undergoing regular media changes and then from ZT 13:00 on Day 1, 10 pineals were removed every 4 hours until 13:00 on Day 2 and instantly frozen over liquid nitrogen vapour and then samples were stored at -70 ^{o}C for later RNA extraction. At the same time three aliquots of culture media per time point were frozen for melatonin analyses. For the 12D:12L trial, at the end of the first

night the photoperiod was reversed to 12D:12L (Figure 1b) pineals (n=10) were then harvested from ZT 13:00 on Day 1, every 4 hours until 13:00 on Day 2, a further 24h cycle was then sampled from ZT 13:00 on Day 3, every 4 hours until 13:00 on Day 4. For the 24D trial, at the end of the first night the photoperiod was transferred to continuous darkness (Figure 1c) and pineals (n=10) were then harvested at comparable times as outlined for the 12D:12L trial.

European seabass pineal culture

As with the salmon 12L:12D culture the seabass *in vitro* culture was carried out over a 48 hour period during which pineal were exposed to 12L:12D with lights on at 06:00 and off at 18:00 at a constant temperature of 18 °C. The Culture medium was exchanged every 6 hours for the first 24 hrs and every 4h over the 24 hr culture duration. 1ml culture medium (for melatonin analysis) and individual pineals were removed and snap frozen on dry ice every 4 hours for 7 sample points. All nocturnal samples were carried out with the aid of a dim red light and were stored at -70°C.

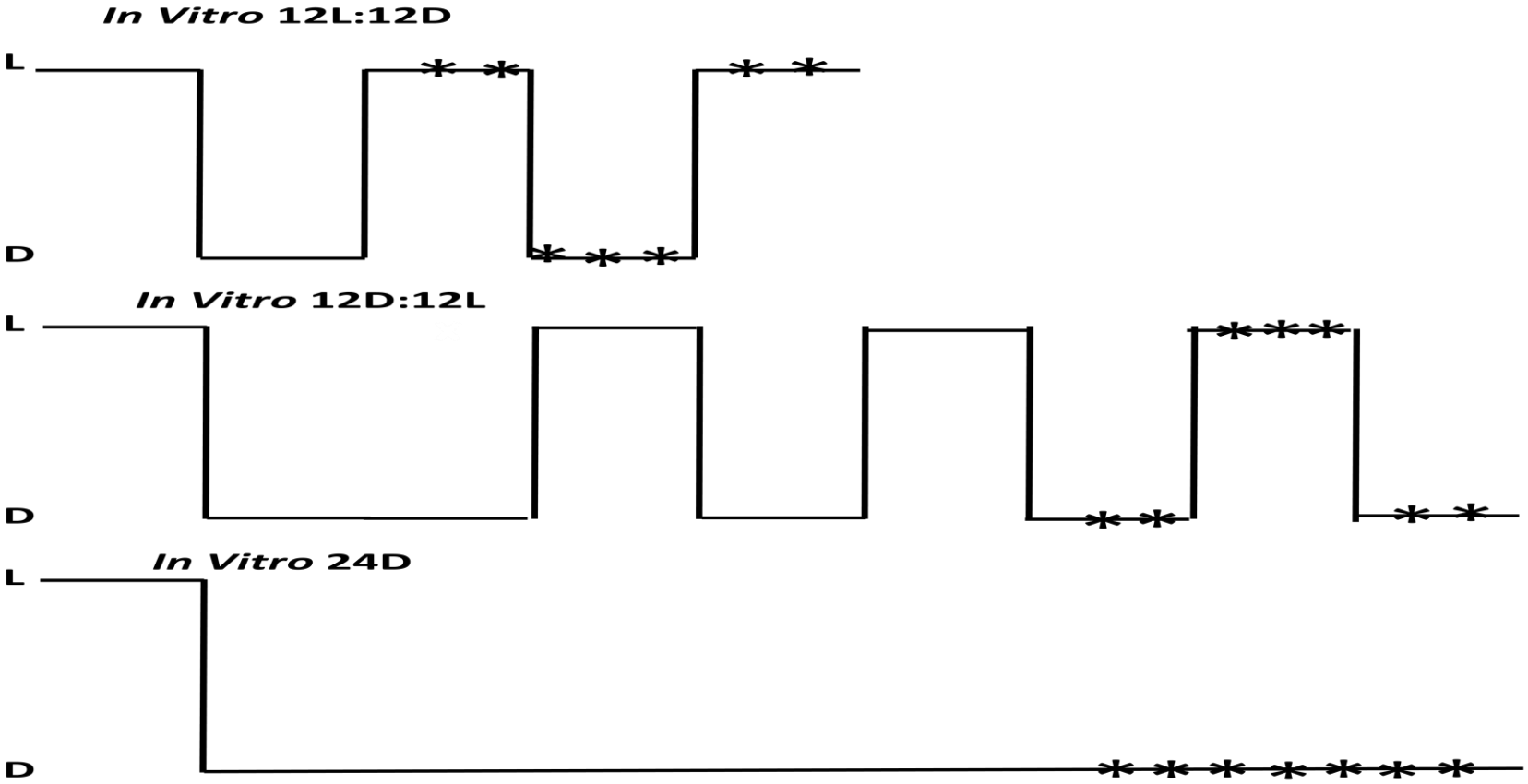


Figure 1: Schematic showing light dark cycles and sample points for the three *in vitro* culture conditions tested in salmon.

RNA extraction, DNase treatment and cDNA synthesis.

Individual pineal were homogenised in 500 µl of TRIzol® (Invitrogen UK) and total RNA extracted in accordance with the manufactures instructions. RNA pellets were rehydrated in 12 µl MilliQ water to achieve a final RNA concentration of approximately 100-500 ng/ul. Total RNA concentration was determined using ND-1000 Nanodrop spectrophotometer (Labtech Int., East Sussex, UK). In order to eliminate any genomic DNA contamination the remaining 10.5 µl totRNA was DNase treated following DNA-free™ kit guidelines for minimum volumes (Applied biosystems, Warrington, UK). cDNA was then reverse transcribed from 500 ng or 1 µg of DNase treated total RNA from the cultured and *in vivo* pineals respectively using random hexamer primers in a 20 µl total reaction volume according to manufactures protocol (High capacity reverse transcription kit without RNase inhibiter Applied biosystems). Final reactions were then diluted with DNA/RNA free H^2O to a final volume of 50 µl for both the seabass and salmon cultured pineal organs and 100 µl from the *in vivo* salmon pineal which equates to a 1:10 dilution in every case. Diluted cDNA samples were then stored at -20 °C prior to analysis.

Molecular cloning of AANAT 2 and qPCR standards.

Sequences primers were available for the majority of clock genes investigated (Davie et al. 2009). However it was necessary to identify the *Aanat2* sequence in the Atlantic salmon. *Aanat2* sequence information for a variety of teleosts was acquired from NCBI search (National Centre for Biotechnology Information Table 1). Sequences information was compiled in BioEdit with the addition of a number of predicted salmon sequences generated from salmon expressed sequence tag (EST) database ASalBase (http://www.asalbase.org/sal-

bin/index) (Table 2). A number of primer pairs were then designed on the rainbow trout *Aanat2* sequence (ascension no NM_001124257.1), and a salmon *Aanat2* theoretical contig from a salmon contig cluster, EST cluster and genomic sequence (Table 2). Primer locations were designed with regard to the additional teleost sequence information. Primers designed were then tested by PCR on pineal and Brain cDNA. cDNA reactions sequence was cloned and sequenced as described below. Sequence identity was established via the alignment of *Aanat2* sequences in clustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The salmon fragments displayed an identity score of 96 % with the rainbow trout. qPCR primers for *Aanat2* were designed on this fragment using Primer Select (Lasergene® DNASTAR).

For each gene to be investigated qPCR primer pairs (Table 3) were tested by PCR. PCR products were then cloned and sequenced in order to generate standards for each qPCR assay. Partial cDNA sequences were generated by PCR using 0.5 µM of primers (Eurofins MWG Operon, Edersberg, Germany) (Table 3) one fortieth of the original cDNA synthesis reaction, Klear Taq polymerase with supplied buffer (Kbiosciences, UK), and 1 mM MgCl2 in a final volume of 20 µl using a routine PCR strategy: 15 min 95 °C followed by 30 cycles of 95 °C 20 s, X °C 20 s, 72 °C 1 min. The annealing temperature is denoted as X °C in the description as it varied with the different primer pairs (Table 3). All primer pairs generated a single PCR product and those products used for qPCR standards were cloned into a pGEM-T Easy vector (Promega, UK) and sequenced (CEQ-8800 Beckman Coulter Inc., Fullerton, USA). The identities of the cloned PCR products were then verified (100 % overlapping) using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Sequencing was performed using a Beckman 8800 autosequencer. Lasergene SEQman software (DNASTAR, www.dnastar.com) was used to edit and assemble DNA sequences. ClustalW was used to generate multiple alignments of deduced protein sequences (Thompson et al. 2000). MEGA version 4 was used to deduce and

bootstrap phylogenetic trees using the neighbour joining method (Saitou & Nei 1987; Tamura et al. 2007) (Figure 2).

Table 1: Available teleost *Aanat2* sequences and Atlantic salmon ESTs utilised for the generation of a salmon *Aanat2* partial sequence.

Species (Latin)	Species (Common)	Ascension number	Source
Danio rerio	Zebrafish	NM_131411.1	NCBI
Carassius auratus	Gold fish	GU205782.1	NCBI
Oryzias latipes	Medaka	NM_001104846.1	NCBI
Solea senegalensis	Senegalese sole	GQ340973.1	NCBI
Paralichthys olivaceus	Olive flounder	HQ883478.1	NCBI
Oncorhynchus mykiss	Rainbow trout	NM_001124257.1	NCBI
Scophthalmus maximus	Turbot	EF033250.1	NCBI
Sparus aurata	Gilt head seabream	AY533403.2	NCBI
Esox lucius	Pike	AF034082.1	NCBI
Salmo salar	Atlantic Salmon	Cluster ID# 3912632	ASalBase
Salmo salar	Atlantic Salmon	Cluster ID# 3920741	ASalBase
Salmo salar	Atlantic Salmon	S0250N08SP6	ASalBase

Table 2: *Aanat2* primer pairs, sequences, and location on rainbow trout partial sequence.

Primer	**Region**	**Location on RT**	**Primer sequence 5' - 3'**
***Aanat2* A**	ORF	372bp - 1040bp	F: AGGTCAGCCGCTCTCCGTTCC
			R: CCAGTGCTAGGGTTGATGTGATTATGA
***Aanat2* B**	ORF + 3'	373bp - 1620bp	F: GGTCAGCCGCTCTCCGTTCCT
			R: TGGTGCTGCAGCTGAGATTGATGG
***Aanat2* C**	ORF + 3'	373bp- past end of RT sequence	F: GGTCAGCCGCTCTCCGTTCCTC
			R: CTGCAGCGCCTCAATGACAAAGTG
***Aanat2* D**	5'+ ORF (partial)	115bp- 796bp	F: AGACAGGCAGATAGAAAGCACAGAGCA
			R: CAGGTAGCGCCACAGCAGGATG
***Aanat2* E**	ORF(partial) + 3'	771bp - 1566bp	F: TCAGCCCAGTAAGTGACCATCATGACACAT
			R: GTTGCAACCTGGTCTGGACGGTCAAC

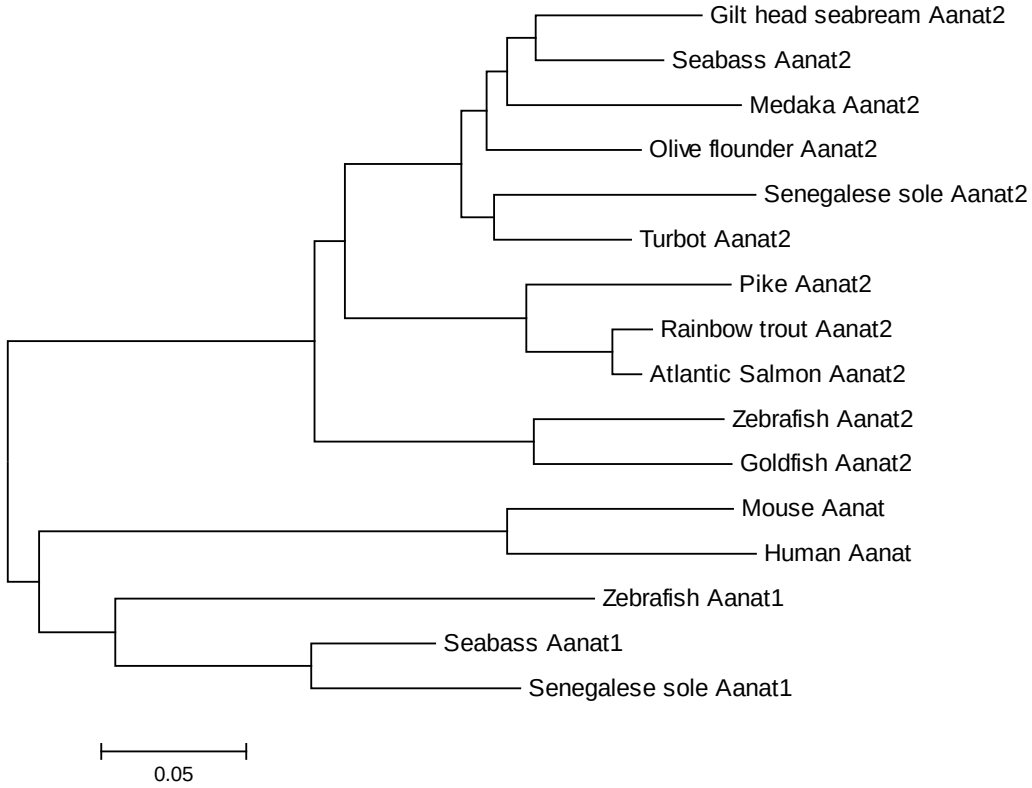


Figure 2: Phylogenetic tree of the Atlantic salmon *Aanat2* sequence in relation to other teleost *Aanat1* and 2 sequences and mammalian *Aanat*. The Atlantic salmon sequence displays highest similarity to the rainbow trout *Aanat2*. The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA4 (Tamura et al 2007). Assention numbers: Gilt head seabreem *Aanat2* AY533403, Seabass *Aanat2* DLA_VIII_005510|aanat2|arylalkylamine, N-acetyltransferase |LG8|11349220|11350343|-|ENSDARP00000002650 in linkage group 8 from the European seabass genome sequencing project draft 1 (unpublished), Medaka *Aanat2* NM_001104846, Olive flounder H *Aanat2* Q883478, Senegalese sole *Aanat2* GQ340973, Turbot *Aanat2* EF033250, Pike *Aanat2* AF034082, Rainbow trout *Aanat2* NM_001124257, Zebrafish A2 NM_131411, Goldfish *Aanat2* GU205782 Zebrafish *Aanat1* AY349158 Seabass *aanat 1* EU378922, Senegalese sole *Aanat 1* GQ340971, Human *Aanat* NG_015976, Mouse Aanat BC119139.

qPCR

Expression of the target genes was measured by absolute quantification with all samples being normalised with *β-actin* mRNA expression a proven stable reference gene in the Atlantic salmon and European seabass (Davie et al. 2009; Herrera-Perez et al. 2011). All cDNA for qPCR were synthesised as described previously and qPCR primers (Table 3) were used at a concentration 0.7 pM, with one tenth (*in vitro*) and one fifth (*in vivo*) of the total cDNA synthesis reaction and 10 µl ABsolute™QPCR SYBR-green qPCR master mix (Thermo scientific, Leon-Rot, Germany). The ABsolute™QPCR SYBR Green Mix was made up of Thermo-Start™ DNA polymerase, a proprietary reaction buffer, dNTP's and SYBR Green I with Mg++ at a concentration of 3 mM in the final 1× reaction. Additionally 3µl DNA/RNA free H_2O.was added to each reaction to a total reaction volume of 20 µl. All qPCR assays were carried out in a Techne Quantica thermocycler (Techne, Quantica, Cambridge, UK) in a thermo cycling programme consisting of a 15 minute initiation stage at 95°C this is followed by 45 cycles of 3 temperature steps; 95 ^{o}C for 15 s anneal x°C (See Table 3 for target specific annealing temperatures) for 15 s and 72 ^{o}C for 30s. This was followed by a temperature ramp from 70 – 90 °C for melt-curve analysis to verify that no primer–dimer artefacts were present and only one product was generated from each qPCR assay. Quantification was achieved by translating CT values of unknown samples from a parallel set of reactions containing a serial dilution of spectrophotometrically determined linearised plasmid containing partial cDNA sequences generated as described above. All samples were run in duplicate. Each qPCR plate included non-template controls. In the *in* vitro salmon 12L:12D experiment the expression of *Aanat2, Clock, Per1 and Per2* was assayed. After analysis of 12L:12D qPCR results *Aanat2* and *Per1* were analysed in *the in* vitro 12D:12L and 24 D experiments (due to absence of rhythmic expression in the 12L:12D experiment). For the salmon *in vivo* experiment, qPCR for *Aanat2, Cry2, Clock, Per1* and

Per2 was carried out (*Cry2* was analysed in order to compare with previous investigations) while for the seabass i*n vitro* experiment *Clock*, *Per1* and *Annat2* were analysed (due to limited RNA extracted from the tissue).

Table 3: Primer sequences and annealing temperatures used for qPCR.

Primer Name	Sequence (5'-3')	Anneal
***β Actin* - Forward**	ATC CTG ACA GAG CGC GGT TAC AGT	61°C
***β Actin* - Reverse**	TGC CCA TCT CCT GCT CAA AGT CCA	61°C
***Aanat2* - Forward**	GCT CTC CCT GGG CTG GTT TGA AG	62°C
***Aanat2* - Reverse**	CAT GGA TGT GCA CTG CCG AGG TT	62°C
***Cry2* - Forward**	GAG GGC ATG AAG GTG TTT GAG GAG	59 °C
***Cry2* - Reverse**	GTG GAA GAA CTG CTG GAA GAA GGA	59 °C
***Clock* - Forward**	AGA AAT GCC TGC ACA GTC GGA GTC	64°C
***Clock* - Reverse**	CCA CCA GGT CAG AAG GAA GAT GTT	64°C
***Per1* - Forward**	AGG GGG TCA TGC GGA AGG GGA AGT	66°C
***Per1* - Reverse**	TGG GCC ACC TGC ATG GG CTC TGT	66°C
***Per2* - Forward**	GCT CCC AGA ATT CCT AGT GAC AAG	60°C
***Per2* - Reverse**	GAA CAG CCC TCT CGT CCA CAT C	60°C
***β Actin* - Forward**	TGG CCG CGA CCT CAC AGA C	59 °C
***β Actin* - Reverse**	TCC AGG GCG ACA TAG CAC AGT TT	59 °C
***Aanat2* - Forward**	ACG CCG CAG GAT GCC ATC AGT GTA	62 °C
***Aanat2* - Reverse**	TCC TTG TCC CAG CCA GAG CCA ATG	62 °C
***Clock* - Forward**	CAG ACA AGT GCC AGG ATT CAG	55 °C
***Clock* - Reverse**	CAG CGG TGT GCG AGG ATT T	55°C
***Per1* - Forward**	CGG ACA GCA GGT TTT TAT CGA	54 °C
***Per1* - Reverse**	GAA AAA ACA CCA GCA CAG GC	54 °C

Melatonin radioimmunoassay

Melatonin in blood plasma and culture medium from the *in vitro* and *in vivo* pineal experiments was measured by radioimmunoassay (RIA) using a protocol adapted from Migaud et al (2007). The sensitivity of the assay, defined as the smallest quantity of melatonin statistically distinguishable from the zero standard was 1.95 pg ml^{-1}. The assay was performed on 250 μl of plasma while *in vitro* culture medium samples were diluted 1:50

with assay buffer (20 µl culture medium and 230 µl buffer). All samples were assayed in duplicate.

Data analysis

Results are presented according to zeitgeber time ZT where 0 = when lights are switched on (Table 4). The effect of time on mean expression levels was first analysed by one-way analysis of variance (ANOVA) followed by Turkey post-hoc test (where $P \leq 0.05$) (Minitab 16 Statistical Software, Minitab inc, United States). To analyse circadian rhythms of expression, the goodness of fit of each daily expression profile was checked against a cosine wave function using acro circadian analysis programs (Refennetti R., University of South Carolina, USA; http://www.circadian.org/softwar.html). (Refinetti 2006). Acro analysis determines both the significance, acrophase (peak in expression) mean and amplitude of raw data using the equation **Y= A + B * cos (C *X –D)** whereby Y is level of gene expression as a percentage of the mean A is the baseline, C is the frequency multiplier and D is the acrophase of the data set (Davie et al. 2009). In order to determine if there was a significant difference between the acrophase of melatonin acrophase as determined by acro ± SEM was converted to degrees (24h cycle = 360, 1 min = 0.25°). Data was log transformed and ANOVA with turkey's test performed using Instat statistical software (V. 3.01 GraphPad Software Inc., La Jolla, California, USA). A significant circadian rhythm was deemed present when p value was less than 0.05 in for all statistical analysis.

Table 4: Zeitgeber Time (ZT) and Real Time (RT) conversion for each experiment

		Sample	**Point**						
Atlantic salmon	**(Salmo salar)**	**Light on**	**1**	**2**	**3**	**Light off**	**4**	**5**	**6**
12L:12D Salmon *In vitro*	**Zeitgeber Time**	00:00	02:00	06:00	10:00	12:00	14:00	18:00	22:00
	Real Time	07:00	09:00	13:00	17:00	19:00	21:00	01:00	05:00
12D:12L Salmon *In Vitro*	**Zeitgeber Time**	00:00	02:00	06:00	10:00	12:00	14:00	18:00	22:00
	Real Time	19:00	21:00	01:00	05:00	07:00	09:00	13:00	17:00
24D:00L Salmon *In Vitro*	**Zeitgeber Time**	X	02:00	06:00	10:00	X	14:00	18:00	22:00
	Real Time	X	09:00	13:00	17:00	X	21:00	01:00	05:00
12L:12D Salmon *In Vivo*	**Zeitgeber Time**	00:00	03:00	07:00	11:00	12:00	15:00	19:00	23:00
	Real Time	07:00	10:00	14:00	18:00	19:00	22:00	02:00	06:00
12L:12D Seabass *In Vitro*	**Zeitgeber Time**	00:00	02:00	06:00	10:00	12:00	14:00	18:00	22:00
	Real Time	06:00	08:00	12:00	16:00	18:00	20:00	00:00	04:00

***Post hoc In silico* analysis of the 5' *Aanat2* promoter**

Since the completion of the qPCR assays, Atlantic salmon and European seabass genome information has been made available. Two *Aanat2* sequences containing the 5' region were identified using a NCBI whole genome shotgun (WGS) sequence blast in the *Salmo salar* genome (ascension numbers AGK01021084 and AGK001091293). The 5' region of each sequence compared to similar regions in non salmonid teleosts. Consequently CRX/OTX sites and E-box elements were identified and locations compared between species. In the European seabass the *Aanat2* coding sequence was identified

(DLA_VIII_005510|aanat2|arylalkylamine, N-acetyltransferase |LG8|11349220|11350343|-|ENSDARP00000002650) in linkage group 8 from the European seabass genome sequencing project draft 1 (unpublished). After initial investigation of E-box elements and CRX/OTX elements, the sequence was trimmed to 7124bp with approximately 3kb 5' and 3' either side of the coding sequence. Using the available sequence information it was not possible to determine extent of the seabass *Aanat2* mRNA sequence and the start and end of the *5'* and 3' UTR respectively.

4. RESULTS

Gene expression results are presented as a percentage of the mean totRNA expression for each photoperiod normalised to βActin. Melatonin results are presented as $pg.ml^{-1}$. All data is displayed as mean value per time point ± standard error of the mean (SEM).

Salmon *in vitro*

In the 12L:12D pineal culture mRNA for all genes investigated were expressed with mean expression levels ranging from 11,911,442 copies/ μg totRNA (*Aanat2*) to 100,207 copies/ μg (*Per2*) (Table 5) however no targets (*Aanat2*, *Clock*, *Per1* or *Per2*) displayed significant variation in expression over the 24 hour period (Figure 3). Melatonin measured within the culture media did display significantly rhythmic daily oscillations with peak levels occurring at the end of the nocturnal phase at ZT 01:00 ± 2.2h. Under the 12D:12L cycle and the 24h dark cycle no significant variation in expression could be measured in *Per1* or *Aanat2* mRNA levels (Figures 4 & 5). However, in both conditions, levels of melatonin in the medium did follow the experimental photoperiod pineal were exposed to. When exposed to the reversed photoperiod of 12D:12L peak melatonin levels were observed during the subjective dark phase at ZT 21:00 ± 2.6 (Table 5, Figure 3). Under constant darkness there was no significant

rhythm in melatonin levels over the duration of the 24h cycle however levels were comparable throughout to those measured at night in the 12L:12D trial (Figures 4 and 5).

Table 5: P value, Acro and ANOVA analysis (P < 0.05) with acrophase where appropriate. Time refers to Zeitgeber time (ZT) whereby 0 = lights on.

Conditions	Gene/ Melatonin	P value–Acro analysis	Acrophase – Acro (ZT± SEM)	Significant Circadian Rhythm
Salmon *In vitro*	***Clock***	> 0.05	x	NS
12L:12D	***Per1***	> 0.05	x	NS
	Per2	> 0.05	x	NS
	Aanat2	> 0.05	x	NS
	Melatonin	<0.05	01:00±2.22	Sig
12D12L	***Per1***	> 0.05	x	NS
	Aanat2	> 0.05	x	NS
	Melatonin	<0.05	21:00±2.59	Sig
24D	***Per1***	> 0.05	x	NS
	Aanat2	> 0.05	x	NS
	Melatonin	> 0.05	x	NS
Salmon *In Vivo*	***Clock***	> 0.05	x	NS
12L:12D	***Cry2***	<0.05	20:00±2.30	Sig
	Per1	<0.05	00:00±2.03	Sig
	Per2	> 0.05	x	NS
	Aanat2	> 0.05	x	NS
	Melatonin	<0.05	20:00±2.50	Sig
Seabass *In Vitro*	***Clock***	> 0.05	x	NS
12L:12D	***Per1***	> 0.05	x	NS
	Aanat2	<0.05	13:00±2.19	Sig
	Melatonin	<0.05	21:00±1.32	Sig

Table 6: Mean mRNA expression as copy numbers per μg totRNA over 24 hrs sampling period for each gene/ condition.

Experiment	Photoperiod/Gene	Mean copy no/ mg totRNA	SEM
Salmon i*n vivo*			
12L:12D	Aanat2	18332385.60	1015936.77
	Clock	60528.70	4543.10
	Cry2	981172.06	49901.82
	Per1	971463.50	51522.86
	Per2	15811.32	1007.37
Salmon i*n vitro*			
12L:12D	Aanat2	11911442.85	1041731.67
	Clock	658747.62	71727.08
	Per1	1733338.98	94047.81
	Per2	100207.53	12076.89
12D:12L	Aanat2	7329494.88	626102.60
	Per1	2025735.69	109402.07
24D	Aanat2	7713770.44	489822.07
	Per1	2255335.64	144366.38
Seabass i*n vitro*			
12L:12D	Aanat2	1834792.64	167446.53
	Clock	544232.24	345805.87
	Per1	925596.87	163462.21

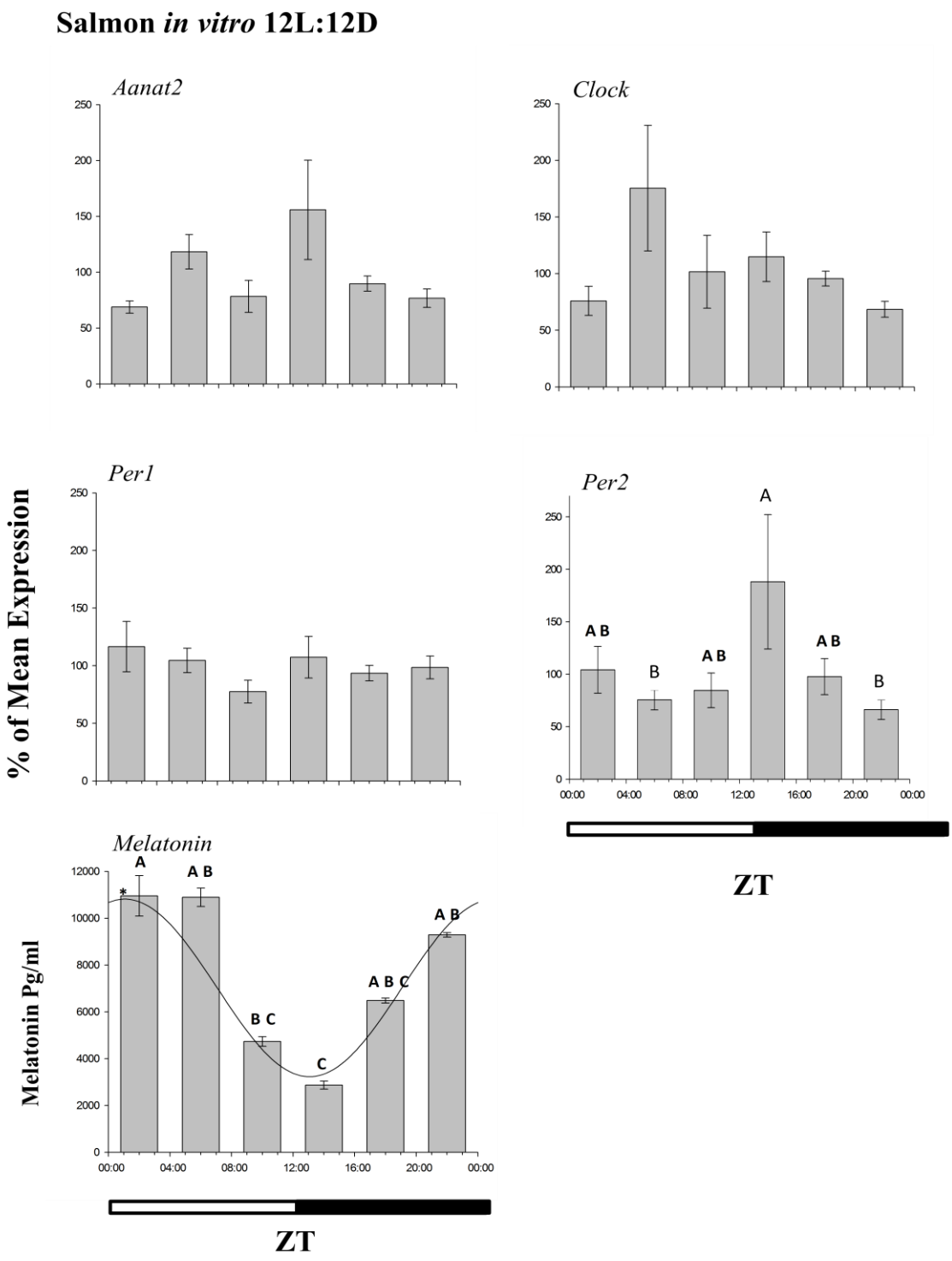


Figure 3: Diel profiles of *Clock*, *Per1*, *Per2*, *Aanat2* mRNA and melatonin from salmon pineal exposed *in vitro* to 12L:12D. Gene expression data is shown as a percentage of mean expression ±SEM. All results are presented in relation to zeitgeiber time (ZT) wherby ZT0 is the onset of light. The presence of a cosine wave denotes the presence of a significant circadian rhythm and different letters represents statistically significant difference between samples by way of ANOVA and Turkeys test where by $P<0.05$

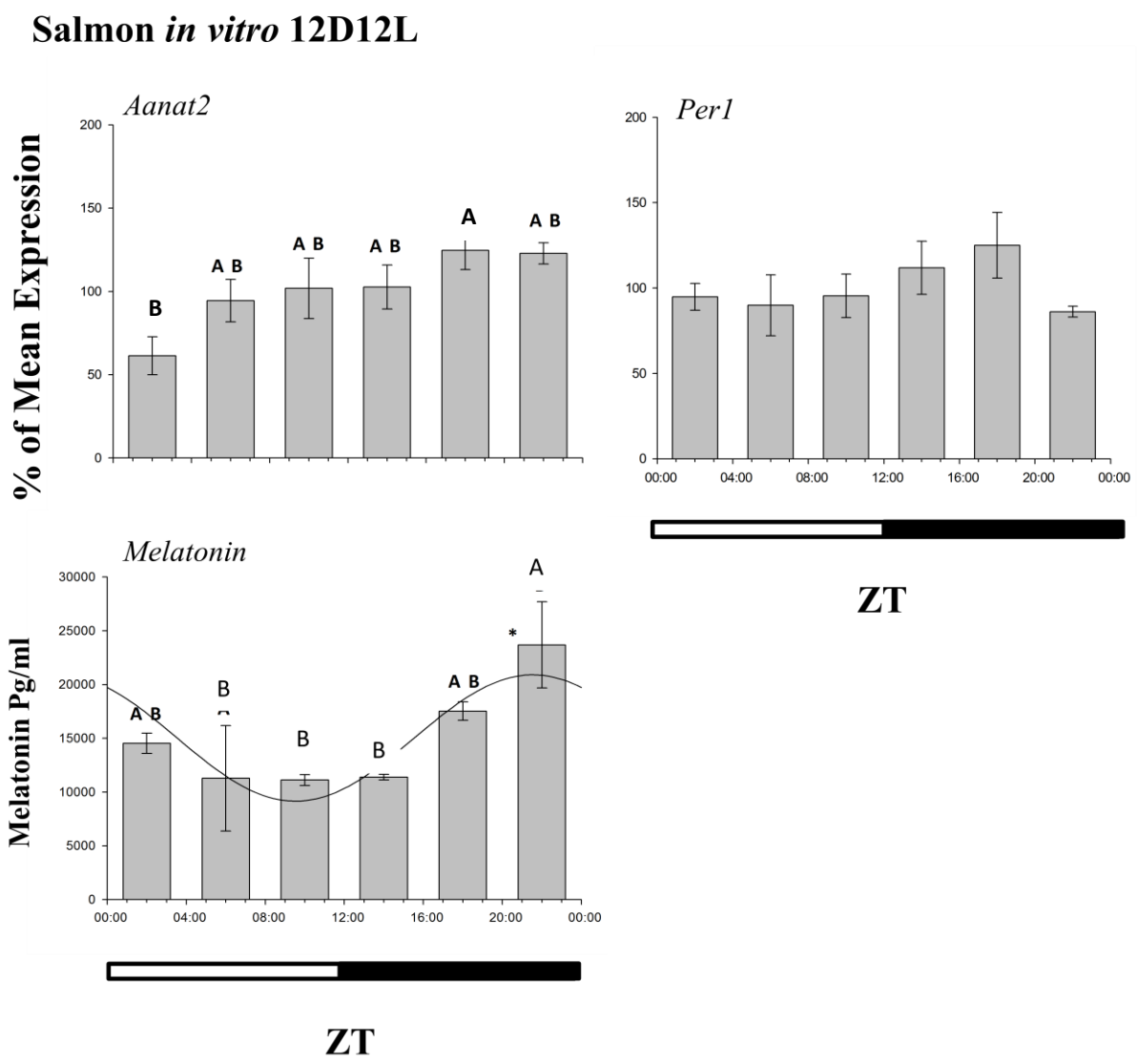


Figure 4: Diel profiles of *Per1 and Aanat2* mRNA and melatonin from salmon pineal exposed in vitro to 12D:12L Gene expression data is shown as a percentage of mean expression ±SEM. All results are presented in relation to zeitgeiber time (ZT) wherby ZT0 is the onset of light. The presence of a cosine wave denotes the presence of a significant circadian rhythm and different letters represents statistically significant difference between samples by way of ANOVA and Turkeys test where by $P<0.05$

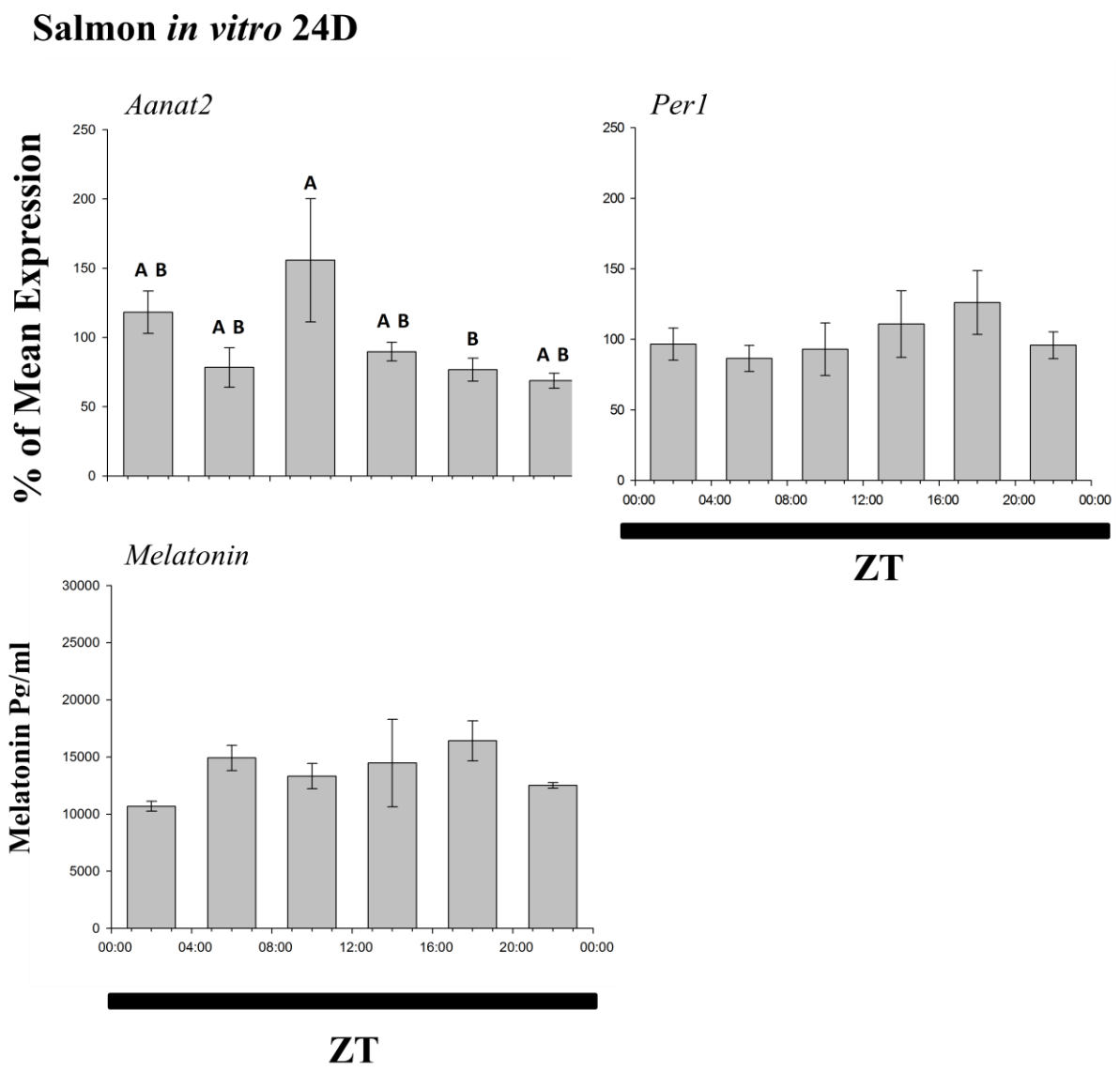


Figure 5: Diel profiles of *Per1 and Aanat2* mRNA and melatonin from salmon pineal exposed in vitro to 24D Gene expression data is shown as a percentage of mean expression ±SEM. All results are presented in relation to zeitgeiber time (ZT) wherby ZT0 is the onset of light (corisponding to 12L:12D *In Vitro*). The presence different letters represents statistically significant difference between samples by way of ANOVA and Turkeys test where by $P<0.05$

Salmon *in vivo*

Of the five target genes measured from pineal glands harvested from salmon both *Cry2* and *Per1* displayed a significant circadian cycle in expression with peaks in expression four hours

apart. The peak expression of *Cry2* occurred at ZT 20:00 ± 2.3h while *Per1* expression peaked at ZT 00:00 ± 2.0h. However *Aanat2, Clock,* and *Per2* displayed no significant variation in expression over the 24 hour period. Levels of circulating melatonin displayed a significant circadian rhythmicity with levels peaking in the middle of the dark phase at ZT 20:00 ± 2.5h (Table 5, Figure 6).

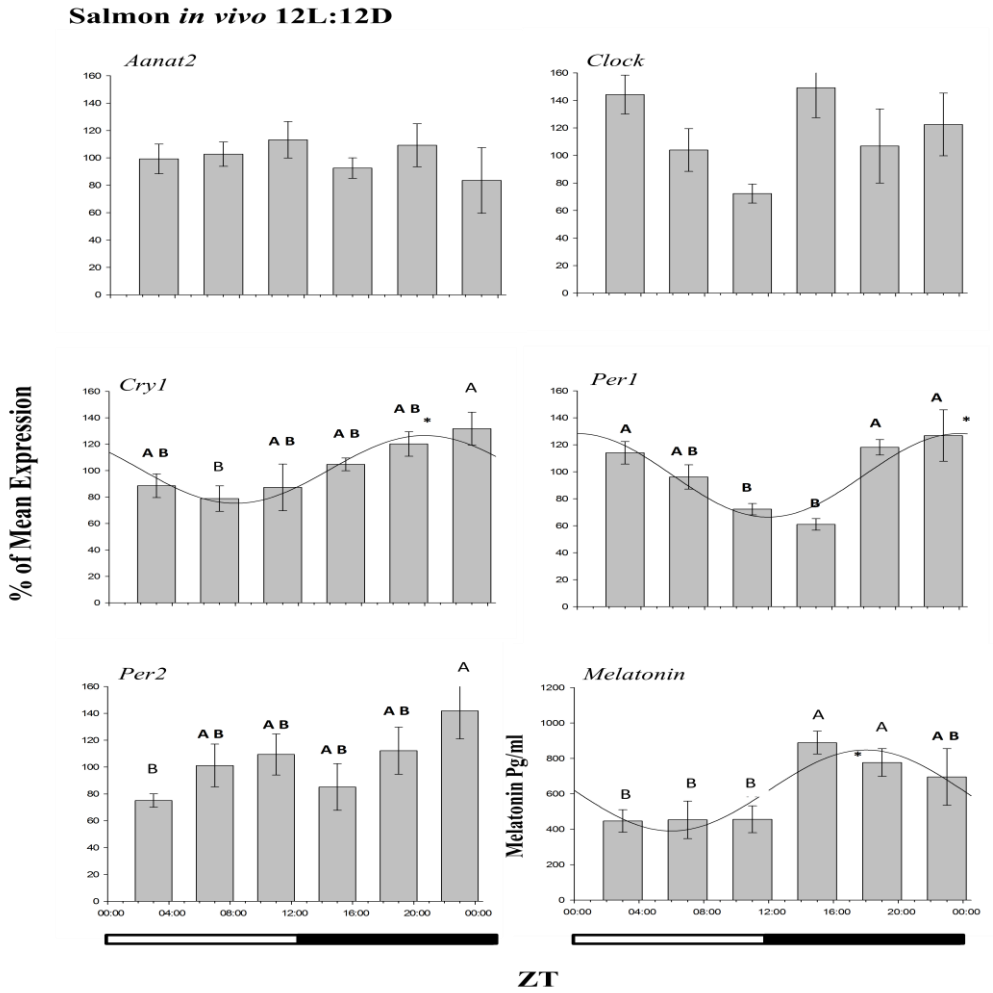


Figure 6: Diel profiles of *Clock, Cry 2, Per1, Per2 and Aanat2* mRNA and melatonin from pineal sampled in salmon exposed to 12L:12D *in vivo*. Results are displayed in relation to Zeitgeiber time (ZT), whereby ZT 0 is the onset of light. Gene expression data is displayed as the percentage of the mean ± the SEM and includes the spread of the data.The presence of a cosine wave denotes a significant circadian rhythm by acro analysis and The presence of different letters represents statistically significant difference between samples by way of ANOVA and Turkeys test where by $P<0.05$

Seabass *in vitro*

In the seabass i*n vitro* experiment neither *Clock* nor *Per1* displayed significant variation in expression over the 24 hour period however *Aanat2* did show rhythmic expression with the acrophase occurring at ZT 13:00 ± 2.2h just following lights off. The levels of melatonin released into the culture media also displayed a significantly circadian profile with the peak in release occurring at ZT 21:00 ± 1.3h towards the end of the dark phase (Tables 5 and 6, Figure 7).

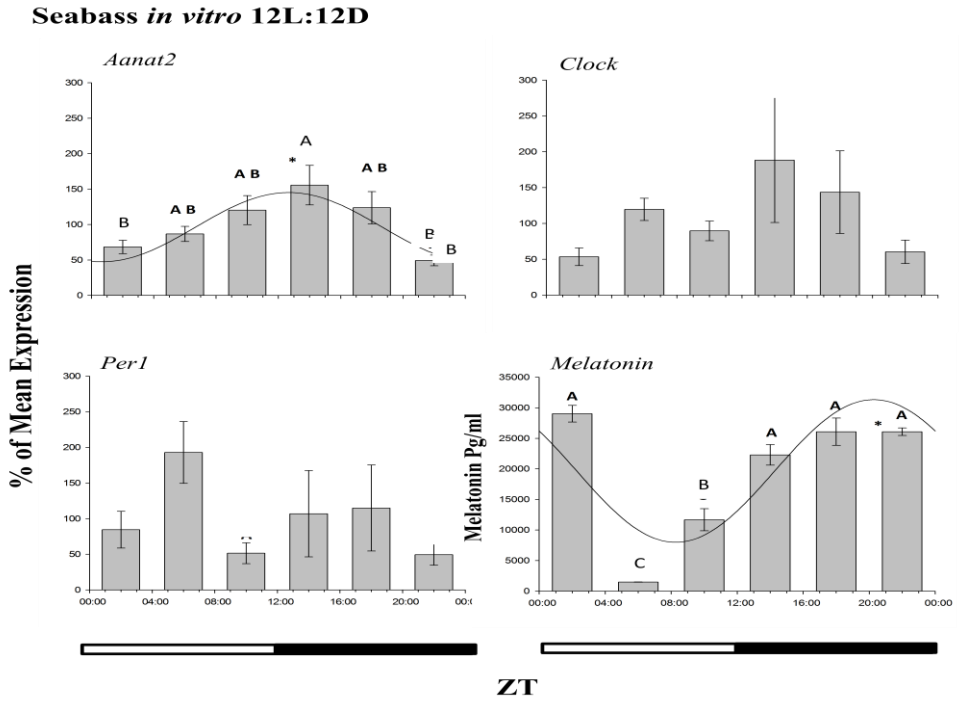


Figure 7: Diel profiles of *Clock, Per1* and *Aanat2* mRNA and melatonin from seabass pineal exposed in vitro to 12L:12D. Different letters for each time point denote statistical differences data while cosine wave represents the presence of a circadian rhythm. * represents the acrophase. Results are displayed in relation to Zeitgeiber time (ZT), whereby ZT 0 is the onset of light. Gene expression data is displayed as the percentage of the mean ± the SEM and includes the spread of the data.The presence of a cosine wave denotes a significant circadian rhythm by acro analysis and The presence of different letters represents statistically significant difference between samples by way of ANOVA and Turkeys test where by $P<0.05$

***In silico* analysis of the 5' *Aanat2* promoter**

Post hoc analysis of the Atlantic salmon genome revealed the presence of two Atlantic salmon *Aanat2* sequences (AGKD01021084 and AGKD010091293), potentially a consequence of the salmonid genome duplication. *In silico* analasyis of the *Aanat2* 5' region revealed the presence of an E-box element in the 5'region of the AGKD01021084 sequence (Apendix 1 and 2). E-box elements were located 1053 bp and 466 bp up-stream of the coding sequence and within the Atlantic salmon Aanat2 5' UTR. In the AGKD010091293 sequence 2 imperfect E-box elements (CATGTG and TACGTG) were additionally observed in the 5'UTR in locations corresponding to the E-box elements present in the AGKD01021084 sequence, approximatly 1kb and 466bp up-stream of the start codon. Photoreceptor conserved elements (PCEs) CRX/OTX were also identified up and down-stream of the coding sequence and within and outwith 5' and 3' UTR (Apendixes 1 and 2). For AGKD01021084, one CRX/OTX element (TAATC) was located in the 5'UTR 770 bp up-stream of the start codon. Down-stream of the stop codon 4 (T) and 3 (C) CRX/OTX elements were located in the 3'UTR. Nine CRX/OTX PCEs (6 TAATT and 3 TAATC) were present down-stream of the transcriptional start of thegene. For the AGKD010091293 sequence 5 PCEs (twoTAATT and three TAATC) were identified up-stream of the 5' UTR. One TAATT and one TAATC was located within the 5' UTR up-stream of the start codon. Four (T) and two (C) CRX/OTXs were present in the 3'UTR in this sequence.

Within the 3kb up-stream of the coding sequence of the seabass *Aanat2* gene 15 CRX/OTX elements and one E-box were identified (Apendixes 1 and 2). Two variants of CRX/OTX elements were found in this region, TAATT (10) and TAATC (5). The E-box element was 319 bp up-stream of the start codon. Down-stream of the coding sequence six CRX/OTX TAATC and two TAATT elements were present. In addition two E-box elements were located 2095 bp and 2143 bp down-stream of the stop codon (Figure 8, Apendixes 1 and 2).

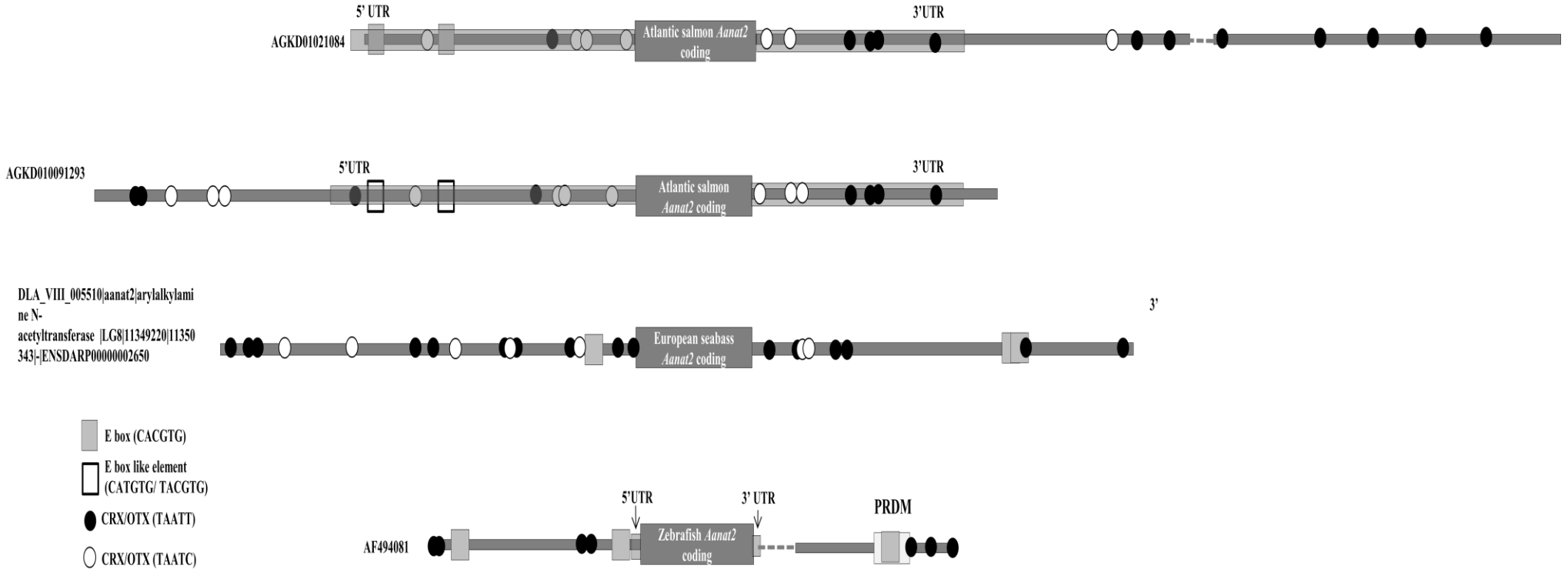


Figure 8: Schematic of the Atlantic salmon *Aanat2* contig with 5' region in comparison to zebrafish and European seabass (DLA_VIII_005510|aanat2|arylalkylamine N-acetyltransferase |LG8|11349220|11350343|-|ENSDARP00000002650).

5. DISCUSSION

Previous work on the teleost pineal has primarily focused on 24h profiles of melatonin in the pineal and plasma. In the majority of teleosts investigated the presence of endogenous melatonin rhythms have been described and have largely been attributed to regulation by a pineal clock. Salmonids appear to be the exception. In salmonid species pineal melatonin appears to not be endogenously regulated under constant conditions. It has been proposed that it may be a consequence of either clocks becoming decoupled from the melatonin synthesis pathway or the lack of a functional circadian clock in the salmonid pineal (Iigo et al. 2007). Results from the present experiment suggest that while clock mechanisms are present in Atlantic salmon pineal it is not capable of endogenous cycling and entrainment and thus the lack of endogenous rhythmic melatonin synthesis in pineal is most likely due to the decoupling of the melatonin synthesis pathway from the clock mechanisms.

The analysis of melatonin release acts as a verification of the pineal function. In all experiments performed, melatonin levels reflected the photic conditions as expected with significant circadian rhythms in melatonin being observed in all salmon and seabass experiments with the exception of the salmon 24D treatment. Under these conditions melatonin was continually elevated over the 24 hour period. The peak in melatonin release was in all cases during the dark phase. No significant difference was observed between the melatonin acrophase in all experiments with a significant circadian rhythm. Importantly current results are consistent with previous reports inferring a lack of endogenous melatonin production in the cultured Atlantic salmon pineal (Iigo et al. 2007). This consequently led to the hypothesis that pineal melatonin production in the salmonid pineal was somehow decoupled from the circadian clock.

In the current investigation no cycling of clock genes mRNA was observed in all salmon and seabass *in vitro* experiments. However rhythmic clocks were present in the salmon *in vivo*

experiment. Rhythmic expression of *Cry2* and *Per1* was only observed *in vivo* under 12L:12D photoperiod. Information on clock gene expression in the pineal, *in vivo* or *in vitro*, is sparse in teleosts. The studies by Huang et al. (2010 a & b) are the only *in vivo* reports of clock gene expression in pineal sampled from Atlantic salmon parr, smolts and post smolts. According to their results, *Per1* and *Cry2* were consistently expressed in parr and post smolts (under 12L:12D) but not in smolts (under constant light) (Huang et al. 2010a; Huang et al. 2010b). With regard to the current investigation the *in vivo* rhythmic expression of *Per1* and *Cry2* bares considerable resemblance to the Huang et al. (2010 a & b) experiments. The peak in expression of both clock genes was observed during the dark phase of the 24h cycle with the acrophase of *Cry2* observed 4h prior to that of *Per1* in current and previous results (Huang et al. 2010a; Huang et al. 2010b).

The current study additionally investigated 24h profiles of clock gene expression (*in vitro*) in the pineal of the European seabass, a species that was shown to display endogenous regulation of melatonin synthesis. As was the case in the cultured Atlantic salmon pineal organ neither of the *Per1* or *Clock* genes displayed significant rhythmic expression. This was surprising as the endogenous production of melatonin in teleost species such as the European seabass had previously been attributed to the cyclic expression of clocks in the pineal. However due to the small size of the seabass pineal, in comparison to the salmon pineal, only a limited amount of RNA (~ 5 μg totRNA) could be extracted from the individual pineal. As a result the expression of only two clock genes (*Clock* and *Per1)* could be investigated. The expression of other clock genes and homologs is unknown in the seabass pineal and further studies are clearly needed.

Knowledge on the cyclic clock gene expression in the pineal are much more advanced in mammals. Mammalian work provides a useful insight into the extra-pineal regulation as

mammalian pineals are not light sensitive. In rats, clock genes (*Bmal, Clock, Cry1, Cry2, Per1 – 3* and *Rev-erb* α) have all been shown to cycle in the pineal (Namihira et al. 1999; Fukuhara et al. 2000; Nakamura et al. 2001; Simonneaux et al. 2004; Wongchitrat et al. 2009; Wongchitrat et al. 2011;). However regulation of clock genes in the rodent pineal is gene dependent. *Per1* and *Cry2* mRNA expression is regulated by the suprachiasmatic nucleus (SCN) via the nocturnal release of the neurotransmitter norepinephrine (NE) while the other transcription factors appear to be endogenous in the rat (*Rattus norvegicus*) (Wongchitrat et al. 2011) and Syrian hamster *(Mesocricetus auratus)*(Wongchitrat et al. 2009). Wongchitrat et al. (2011) hypothesised that the endogenously expressed clock genes are synchronised by the SCN via NE control of *Per1* and its role in the circadian feedback loop. In aves, neurotransmitters have also been implicated in the control of clocks in the pineal i*n vivo* (Nagy & Csernus 2007). In the current investigation clock gene expression in the salmon pineal shares a number of characteristics with that of vertebrate species. In the rodent cyclic *Cry2* and *Per1*expression is lost in the isolated pineal, in the absence of NE input. Similarly in Atlantic salmon *in vivo* studies, both genes displayed circadian expression over a 24 hour Light Dark cycle. *In vitro*, under the same photic conditions, *Cry2* and *Per1* cycling ceases. This is a potential consequence of extra-pineal regulation as observed in rodents. However, in contrast to mammals and birds, no central circadian clock or SCN like structure has yet been identified in teleosts (Kulczykowska et al. 2010).

While not the primary focus of this investigation comparison between *in vivo* and *in vitro* results provide some insight into the extra-pineal clock gene regulation and the Atlantic salmon circadian axis. In comparison to mammals the salmon circadian axis is hypothesised to be dependent on a photosensitive pineal and lack of a central circadian pacemaker (Migaud et al. 2010). The pineal has changed dramatically over the course of evolution, from a true photoreceptor in lower vertebrates to an endocrine gland (pinealocyte) in mammals (Migaud

et al. 2010). In mammals the circadian axis begins in the retina where photic information is interrogated in melanopsin containing retinal ganglion cells and transmitted via the retinohypothalamic tract (RHT) to the SCN (Foster & Hankins 2002; Foster & Hankins 2007). In the SCN the RHT interacts with the circadian clock and elevates NE release from post ganglionic sympathetic fibres to the pineal leading to melatonin synthesis (Maronde & Stehle 2007). In birds the situation appears to be more complex. Indeed, the retina, pineal and deep brain photoreceptors have all been implicated in the avian circadian axis. Moreover the relative contribution of each has been found to be highly species specific (Gwinner & Brandstatter 2001; Underwood et al. 2001; Karaganis et al. 2009). The generalised avian model represents an intermediate evolutionary state between the mammalian and teleost circadian axis (Migaud et al. 2010).

The teleost circadian axis was initially hypothesised to be simplified in comparison to mammals and birds. The phototransduction and melatonin synthesis pathways are considerably shortened. However more recently the teleosts this system has been shown to be highly species specific and considerably more diverse than initially described (Migaud et al. 2007). As previously described two circadian pathways for melatonin synthesis (salmonid and non salmonid) were initially hypothesised. However in 2007, Migaud et al. described three modes for the photic and circadian regulation of melatonin synthesis. In salmonids the pineal is predominantly responsible for both photo perception and the synthesis of melatonin. In species such as seabass photic input from the eye contributes significantly to the regulation of pineal melatonin synthesis. The relative contribution of the eye is even more important in tilapia and catfish whereby, removal of the eye results in the loss of elevated nocturnal levels of plasma melatonin (Migaud et al. 2007). The way in which photic information is perceived and the contribution of the retina and pineal in the control of melatonin synthesis had therefore been altered dramatically over the course of teleost evolution. From non clock

controlled photoreceptive organ to secretary gland containing a functional clock requiring photic input from retinal and the pineal to ultimately the loss of the photoreceptive pineal (Migaud et al. 2007; Migaud et al. 2010).

If the circadian axis has changed over the course of vertebrate evolution; however the melatonin biosynthesis pathway has remained much conserved. The nocturnal activity of AANAT regulates the highly conserved profile of melatonin synthesis and secretion and activity mirrors melatonin profile (Ganguly et al. 2001). In the absence of light the activity of the AANAT enzyme will increase (doubling time ~15 min.) and are rapidly inhibited with the onset of light (having time ~3.5 min.) (Klein 2007). However despite the conserved nature of the vertebrate melatonin profile and the action of AANAT in the melatonin biosynthesis pathway the regulation of AANAT is not conserved amongst vertebrates.

Teleosts are unique amongst vertebrates in that they have two forms of AANAT. AANAT1 and AANAT2 primarily located in the retina and the pineal. The tissue specific expression is regulated by photoreceptor conserved elements (PCEs) in the promoter region of the *Aanat* gene (Appelbaum & Gothilf 2006). PCEs control the expression of a number of genes involved in the synthesis of melatonin and photo transduction by binding to members of the orthodentical CRX/OTX transcription factor family expressed in both the pineal and the retina (Appelbaum et al. 2004). As a result of pineal specific expression in fish AANAT2's participation in the melatonin biosynthesis pathway regulates the nocturnal synthesis of the hormone (Falcon et al. 2011; Falcon et al. 2010).

In the majority of teleosts studied so far AANAT2 has been shown to be regulated at the level of protein activity and mRNA translation. At a protein level AANAT 2 activity is inhibited directly by the presence of light. In the absence of light the melatonin biosynthesis pathway is initiated. As part of this pathway elevated cAMP promotes the formation of the AANAT2/14-

3-3 protein complex via direct phosphorylation (Coon & Klein 2006). When light is present the pineal photoreceptors become hyperpolarized and cAMP and Ca^{2+} and levels are reduced. Under these conditions the AANAT2/14-3-3 complex becomes dissociated. Protein action then ceases and melatonin synthesis stops as AANAT2 is subjected to proteasomal degradation(Coon & Klein 2006) At a translational level the *Aanat2* gene is regulated by an independent circadian clock in the pineal via interactions with E-box elements present in the promoter region of the *Clock* and *Bmal* genes and the 5' end of the teleost *Aanat2* gene in species such as zebrafish(Coon & Klein 2006) However salmonids appear to be an exception to this.

In salmonids previous work has demonstrated that regulation of AANAT2 occurs at the level of protein activity only, and is independent of circadian clock work. As a result the AANAT driven rhythm of nocturnal melatonin would be directly regulated by light and not by the molecular mechanisms of the circadian clock (Falcon et al. 2010; Falcon et al. 2011). Absence of clock regulation of *Aanat2* has additionally been observed in the rainbow trout, another salmonid species (Coon et al. 1998; Falcon et al. 1998; Falcon et al. 2001; Falcon et al. 2003). The present investigation analysed the expression of *Aanat2* mRNA in the Atlantic salmon pineal *in vivo* and *in vitro* and in comparison to the European seabass, a species displaying endogenous regulation of melatonin and *Aanat2* (Migaud et al. 2007; Migaud et al. 2010Falcon et al. 2010) In salmon *in vivo* and *in vitro Aanat2* mRNA expression was continuously expressed under all treatments. Over the 24h sample period no significant difference in nocturnal / diurnal expression were observed. This is clearly confirming previously suggested lack of clock regulation of AANAT2 transcription.

In the seabass, *Aanat2* did display a circadian expression and elevated mRNA levels were observed at the beginning of the nocturnal phase (zt 12:38). This is consistent with the hypothesis that in teleosts displaying endogenous melatonin production AANAT2 activity is

regulated, in part, at a transcriptional level. In all probability this is achieved via E-box interaction between clock genes and *Aanat2* (Zilberman-Peled et al. 2007). Previous work in seabass has also described elevated nocturnal levels of *Aanat2* mRNA (Herrera-Perez et al. 2010). Similarly in the zebrafish pineal *Aanat2* mRNA is elevated at the end of the diurnal/ early nocturnal phase (Ziv et al. 2005; Falcon et al. 2010;). Clock gene regulation of the *Aanat2* gene has additionally been observed in the pike (Coon et al. 1998; Falcon et al. 1998; Falcon et al. 2001; Falcon et al. 2003)

Post-hoc *in silico* analysis of the Atlantic salmon 5' *Aanat2* sequence retrieved from a NCBI blast in the WGS revealed the presence of numerous photoreceptor conserved elements. CRX/OTX elements (TATT/C) were observed in the 5' sequence, the 5' UTR and down-stream of the *Aanat2* start codon within and down-stream of the 3'UTR. This is concurrent with results obtained in other teleosts such as gilthead seabream (*Sparus aurata*), pike (*Esox lucius*) and zebrafish (Zilberman-Peled et al. 2007). The importance of the single nucleotide change in the PCE in salmon and other teleosts is unknown. In the zebrafish the CRX/OTX elements are known to recruit orthodenticle homolog 5(OTX5) and mediate the pineal specific expression of *Aanat2* in the zebrafish pineal (Appelbaum & Gothilf 2006). In the Atlantic salmon it is unclear whether this function persists as the down-stream regulatory element, pineal – restrictive down-stream module (PRDM) has not been located in the 3'UTR. In zebrafish the PRDM, containing CRX/OTX elements, and E-box and 13bp repetitive motif, interacts with PCEs in the 5' region to mediate extra pineal expression of the *Aanat2* gene. In the current investigation Atlantic salmon *Aanat2* expression was not only observed in the pineal but in RNA extracted from whole brain homogenates. However levels were not quantified by qPCR and the significance of this on melatonin production is unclear. Two complete E-box elements in the *Aanat2* AGKD01021084 sequence and 2 imperfect E-box elements (GACCTG) AGKD010091293 were identified approximatly 1kb and 466bp up-

stream of the start codon. However in contrast to other teleosts the 5' UTR extends up-stream encompasing the E-box elements in both sequences. In teleosts displaying clock controled regulation of the *Aanat2* gene functional E-box elements are located up-stream of the 5'UTR as is the case in seabream, pike and zebrafish in which conical E-box are present in this region (Gothilf et al. 2002;Appelbaum & Gothilf 2006; Appelbaum et al. 2006; Zilberman-Peled et al. 2007). In the zebrafish this has been shown to be functional and the *Aanat2* circadian expression is driven by *Clock* and *Bmal* expression in the pineal (Appelbaum & Gothilf 2003).The absence of an E-box element up-stream of the 5'UTR in the Atlantic salmon *Aanat2* gene may account for the lack of circadian regulation and consequent melatonin production in the species.

In silico analysis of the seabass *Aanat2* 5'region revealed the presence of 15 PCEs within 3kb up-stream of the seabass *Aanat2* start codon and an E-box element 319 bp up-stream of the start codon. The current investigation could not determine the extent of the seabass *Aanat2* 5' and 3' UTR with available seabass sequence information. However this is similar to results in the zebrafish and the gilthead seabream where E-boxes are present within a 300bp of the coding sequence and up-stream of the end of the transcriptional gene and 5' UTR. It is the E-boxes up-stream of the 5'UTR that are considered to be functionally important in the clock gene regulation of *Aanat2* expression and subsequent endogenous control of pineal melatonin production in teleosts. As in the zebrafish two E-box elements were additionally observed approximately 2kb down-stream of the coding sequence (Figure 8). In the zebrafish, an E-box is present approximately 4kb down-stream of the transcriptional stop site and comprises part of the down-stream regulatory element, pineal – restrictive down-stream module (PRDM) (Appelbaum et al. 2004). This PRDM encompasses E-box elements, three CRX/OTX photoreceptor conserved elements and a 13bp repeated motif. The PRDM functions not only to enhance pineal specific expression but also interacts with up-stream elements to restrict

extra pineal *Aanat2* expression (Appelbaum et al. 2004). In silico analysis of the salmon *Aanat 2* gene has provided convincing evidence that support the lack of clock gene regulation of *Aanat 2* via E-box element and subsequent loss of endogenous pineal melatonin production.

6. CONCLUSIONS

Amongst teleosts a considerable number of reports have eluded to the presence of a functional clock in the pineal. However very little information is available on the presence and expression of clock genes in this tissue. The present study showed a lack of circadian expression in isolated pineal gland placed in culture of all clock genes investigated. *In vivo* results differed with *Per1* and *Cry2* genes in the pineal displaying circadian expression. These results confirm that clock genes are expressed in the salmon pineal but are not capable of independent cycling when isolated. Pineal clocks *in vivo* are likely driven by extra-pineal clocks located in the brain. *Aanat2* results were also contrasting between salmon and seabass with mRNA levels remaining high throughout the 24 h cycle in salmon while showing a significant circadian profile of expression in seabass. These results are consistent with the hypothesis that *Aanat2* mRNA is regulated by pineal clocks in non salmonid teleosts. This probably occurs via E-box interactions with clock genes, resulting in the endogenous production of melatonin. *In silico* analysis of the salmon and seabass 5' *Aanat2* promoter revealed the absence of an E-box element up-stream of the 5' UTR in salmon. The absence of this conserved element may account for the lack of clock gene regulation and endogenous cycling of the Atlantic salmon *Aanat2* gene and melatonin production in the pineal. Potential future work would likely focus on the localisation of clock gene expression in the brain and pineal organ of the Atlantic salmon. This may determine whether hierarchical structure is present in the Atlantic salmon brain and pineal clock work.

CHAPTER 6

CIRCADIAN EXPRESSION OF CLOCK GENES, *STEROL REGULATORY ELEMENT-BINDING PROTEINS* AND SREBP TARGETS IN THE LIVER OF THE ATLANTIC SALMON.

CIRCADIAN EXPRESSION OF CLOCK GENES, *STEROL REGULATORY ELEMENT-BINDING PROTEINS* AND SREBP TARGETS IN THE LIVER OF THE ATLANTIC SALMON.

1. ABSTRACT

In peripheral tissues such as the liver a number of clock genes and clock controlled gene mRNAs are expressed in a circadian manner. In mammals a number of genes involved in liver lipid and cholesterol homeostasis are rhythmically expressed and expression has been shown to be regulated by clock genes via *Rev-erb* α. In the Atlantic salmon liver lipid and cholesterol homeostasis is an area of considerable research interest both in terms of human health and improving the sustainability of commercial salmon feed. In order to better understand clock gene regulation of genes involved in the Atlantic salmon liver lipid metabolism the current investigation describes 24h expression of clock genes (*Bmal1, Clock, Per* 1 and *Per 2*), and cholesterol regulatory genes (*Srebp 1, Srebp 2, Fax, Lxr, Elovl5, Hmgcr* and *D6 Fad*) in the liver of salmon parr acclimated to a long day photoperiod, which have previously elicited rhythmic clock gene expression in the brain (see chapter 3). Results demonstrated the significant circadian expression of the clock gene *Bmal1* and cholesterol regulatory genes *Srebp1* and *Lxr*. The gene coding for the rate limiting enzyme in cholesterol synthesis, *Hmgcr*, was significantly elevated at ZT10 in comparison to ZT 22, this in contrast to mammals where mRNA expression of the gene and protein activity was elevated during the night. The rhythmic circadian expression of *Srebp 1* and *Bmal 1* is similar to previous results obtained in mice. However in contrast to mammals, *Per1, Per2, Fas,* and *Rev-erb* α did not display significant circadian rhythmicity in salmon. This investigation represents the first attempt to characterise 24h profiles of gene expression in the liver in the Atlantic salmon

which is an area of considerable interest for future research in this commercially important species.

2. INTRODUCTION

In most vertebrates clocks and clock genes are present in the majority of tissues and cell types. Their presence and cyclic expression drives circadian rhythms across a number of biological facets by initiating rhythmic transcription of a number of clock controlled genes in central and peripheral tissues (Ko and Takahashi. 2006). Amongst peripheral tissues, rhythmic expression in the liver transcriptome has been an area of considerable interest (Le Martelot et al. 2009) in the mammalian field while in teleosts research in this area is lacking. However to the aquaculture industry, factors which affect the regulation of fatty acid metabolism and cholesterol homeostasis in the tissue is an area of considerable importance (Minghetti et al. 2011). Cholesterol is fundamental to a number of biological processes including membrane fluidity and the synthesis of the bile acids necessary for the emulsification of dietary lipids. Excessive levels can lead to considerable health problems such as the formation of gallstones and hardening of the arteries (Schibler et al. 2010). In salmon lack of adequate cholesterol and long chain fatty acids and the regulation of processesinvolved in fat deposition have become an issue due to restriction of fish oil in the diet. Consequently the circadian regulation of cholesterol and fatty acid metabolism and the genes and enzymes involved is necessary for cholesterol homeostasis is of great interest (Le Martelot et al. 2009).

Understanding the mechanisms involved in regulation and absorption of fatty acids is of increasing importance, in particular with reference to the aquaculture industry (Minghetti et al. 2011). Salmon are an important source of omega 3 and polyunsaturated fatty acids (PUFA) in the human diet, an essential dietary component and vital for metabolism and healthy cardiovascular and neurological function (Eilander et al. 2007; Ruxton et al. 2007). Omega 3 have also been shown to be advantageous in the treatment of inflammatory diseases amongst others (Ruxton et al. 2007). This has resulted in increased demand and affordability

of oily fish species for human consumption. In order to meet this rising demand the culture of suitable species such as the Atlantic salmon is increasing, however, salmon are carnivorous and commercial diets have principally been based on fishmeal and oil from wild fisheries, exerting considerable pressure on the marine environment (Tacon & Metian 2008). Accordingly research has focused on the addition of novel lipid sources to dietary formulas. Lipid sources such as vegetable oils do not contain PUFA, or bioavailable cholesterol and, in the case of PUFA, vertebrates do not have the capacity to synthesise them from the nutrients available in vegetable oils (Burdge & Calder 2005; Tocher 2010). As a result further understanding of lipid regulation in commercially important species such as the Atlantic salmon is of fundamental importance (Minghetti et al. 2011). However reports of clock controlled genes and circadian regulation of fatty acid metabolism in the liver is limited in teleosts. In rodents research is considerably more advanced.

In rodents liver microarray investigations have shown between 8 to 10% of all mRNAs to be rhythmically expressed (Akhtar et al. 2002; Kornmann et al. 2007; Schibler et al. 2010). Most of these mRNAs encode for enzymes and regulators of specific importance to liver functions including fatty acid metabolism and cholesterol regulation (Schibler et al. 2010). In mice a number of genes intrinsically involved in cholesterol homeostasis and lipid metabolism have been shown to be mediated by REV-ERBα (nuclear receptor subfamily 1, group D, member 1) an essential component of the molecular clockwork (Akhtar et al. 2002; Le Martelot et al. 2009). These include SREBP 1c and targets of the *Sterol Regulatory Element-Binding Proteins* (SREBP) pathway such as hydroxymethylglutaryl-*CoA* reductase (HMG CoA-R). RE-VERBα is a key protein of the circadian feedback loop. *Rev-erbα* mRNA is rhythmically expressed and is subject to regulation by the negative components of the molecular clock, Cryptochrome (*Cry*) and Period (*Per*). Moreover the REV-ERBα protein has the capacity to influence the timing of the BMAL and the positive arm of the molecular

clockwork. *Rev-erbα* is not essential for the cycling of the molecular clock, however it is fundamental in the accuracy and fine tuning of the clock and has been implicated in adiposeness and fatty acid metabolism (Le Martelot et al. 2009) It is known to regulate a number of genes involved in cholesterol and lipid homeostasis in particular through sterol regulatory element binding protein pathways and SREBP target genes (Table 1 and Figure 1) (Le Martelot et al 2009).

In mammals the SREBP pathway is initiated in the liver with the circadian transcription of Insulin induced gene 2 (*insig2*) (Le Martelot et al 2009). The INSIG2 protein is resident in the endoplasmic reticulum (ER) and sequesters SREBP proteins to the ER where, it forms a complex with SREBP-cleavage activating proteins (SCAP). The formation of the SREBP:SCAP complex facilitates transfer from the ER to the golgi apparatus were it is cleaved by S1P and S2P. SREBP then moves to the nucleus were it acts on a number of target genes in the cholesterol synthesis pathway via presence of sterol regulatory elements (eg TCACNCCAC) which often contain an E-box element (Sato 2010). Under high concentrations of cellular cholesterol this processes is inhibited by the binding of SCAP to INSIG and SREBP is maintained in the ER (Sato 2010). In the nucleus the transcriptionally active SREBP triggers the synthesis of Hmg-coA reductase (*Hmgcr*) the rate limiting enzyme in cholesterol biosynthesis (Le Martelot et al. 2009). REV- ERB α also been demonstrated to be involved in the conversion of cholesterol to bile acid. It regulates the circadian expression of Cholesterol 7 alpha-hydroxylase CYP7A1, the rate limiting enzyme in the conversion of cholesterol to bile acid in hepatocyte cells (Le Martelot et al. 2009). This is hypothesised to be achieved via the circadian production of oxysterol, an oxidized derivative of cholesterol that mirrors the activity of HMG-CoA reductase and production of cholesterol. This likely

regulates LXR and the circadian transcription of *Cyp7a1* (Le Martelot et al. 2009). The temporal regulation of cholesterol and bile acid is necessary as constantly elevated levels can be toxic to cells (Schibler et al. 2010). The mechanisms involved in the synthesis of cholesterol conversion to bile acid appear to be under degree of circadian regulation via the orphan nuclear receptor REV-ERBα.

In the Atlantic salmon homologues of the genes involved in the circadian regulation cholesterol and bile acid homeostasis have been identified. Two *Srebp* sequences have been identified coding for proteins that align to mammalian SREBP1a and SREBP2 proteins with 54% and 49% identity respectively (Minghetti et al. 2011). In mice the presence of elevated cholesterol induces an increase in *Srebp 1* and *2* mRNA in addition to HMG-CoAR, *Elovl5a*, LXR and ACOX (Le Martelot et al. 2009). These sequences have additionally been identified in the Atlantic salmon (Morais et al. 2009; Minghetti et al. 2011). In mammals SREBPs and HMG-CoAR have been shown to be regulated by the circadian system via REV-ERBα. However in teleosts circadian regulation of the SREBP pathway is unknown. The aim of the present study was to determine daily patterns of mRNA expression for genes involved in cholesterol homeostasis and key components of the circadian clock in the liver of the Atlantic salmon.

Table 1: Abbreviation, full name, accession number and function of all genes investigated

Gene	Full Name	Ascension number	Gene Function
Srebp1	*Sterol Regulatory Element-Binding Protein 1*	TC148424	Indirectly involved in cholesterol synthesis. In mammals involved in sensing cholesterol availability in the ER
Srebp 2	*Sterol Regulatory Element-Binding Protein 2*	TC166313	Indirectly involved in cholesterol synthesis by regulating *Hmgcr*.
Lxr	*Liver x receptor*	FJ470290	Regulates cholesterol and fatty acids by activating Cyp7a , the rate limiting enzyme in the conversion of cholesterol to bile acid.
FadsD6	*D6 – Fatty acid desaturase*	AY458652	Required for the synthesis of highly unsaturated fatty acids
Elovl5a	*Elongation of very long chain fatty acids protein 5*	AY170327	Participates in the biosynthesis of long chain poly unsaturated fatty acids. Primarily the elongation of C18 and C20.
Hmgcr	3-hydroxy-3-methyl-glutaryl-CoA reductase	DW561983	Resulting HMGCoA reductase enzyme is the rate limiting enzyme in cholesterol synthesis.
Fas	*Fatty acid synthesis*	DW551395	Corresponding protein catalyzes fatty acid synthesis
Bmal1	*Brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like*	DY 735402	Forms part of the positive arm the circadian molecular clock. BMAL forms hetrodimer with CLOCK and initiates the transcription of *Per* and *Cry* genes.
Clock	*Circadian Locomotor Output Cycles Kaput*	CA 038738	In conjunction with BMAL forms the positive arm of the circadian clock and regulates negative elements *Cry* and *Per*.
Per 1	*Period1*		In conjunction with Cry genes forms negative components of the circadian clock.
Per 2	*Period2*	FM877775	In conjunction with Cry genes forms negative components of the circadian clock.
Rev-erb a	nuclear receptor subfamily 1, group D, member 1		REV-ERBa is regulated in a circadian manner by BMAL and via E-box elements regulates a number of clock controlled genes.

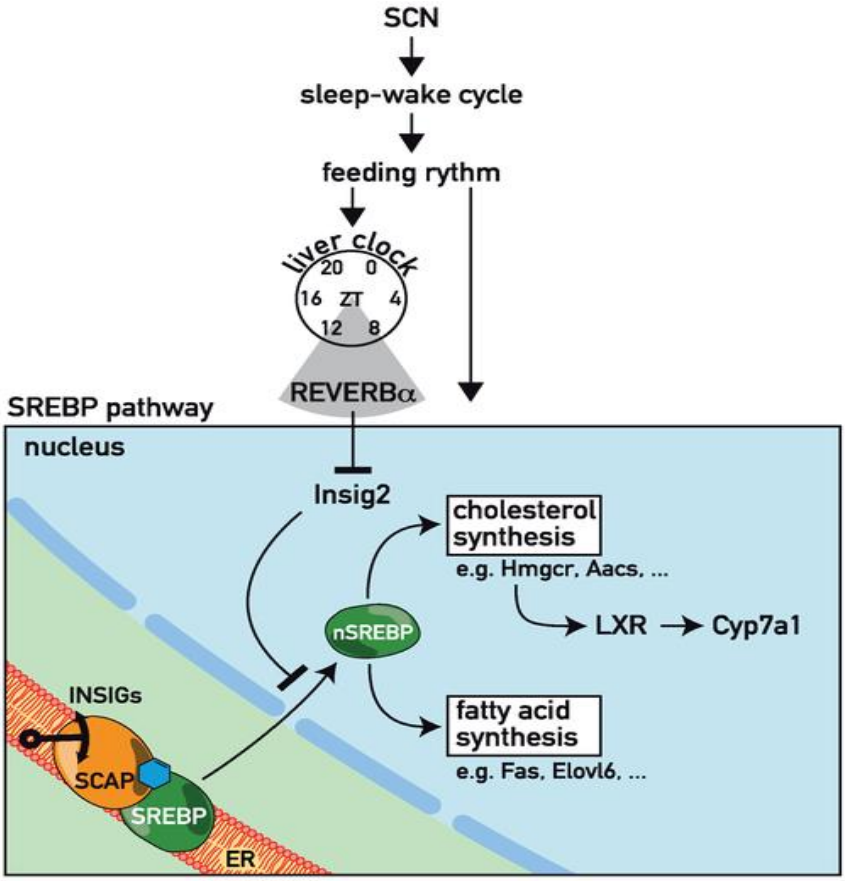


Figure 1. Circadian regulation of lipid and bile acid homeostasis, via *Rev-erb* α in mice. From Le Martelot et al 2009**.** The schematic above illiterates the circadian regulation of the liver lipid and bile acid homeostasis via REV-ERBα control of SREBP accumulation in the nucleus. In liver hepatocytes, REV-ERBα accumulates to maximal levels at ZT8–ZT12 and represses *Insig2* transcription, promoting the proteolytic activation and nuclear accumulation of SREBP proteins. In turn, the circadian activation of SREBP transcription factors drives the cyclic transcription of *Hmgcr*, encoding the rate-limiting enzyme of cholesterol biosynthesis. As a consequence the levels of oxysterols, which serve as ligands for LXR, also oscillate during the day, and cyclically activated LXR then controls rhythmic *Cyp7a1* transcription (Le Martelot et al. 2009)

3. MATERIALS AND METHODS

Experimental animals and sampling procedures

Liver samples previously reported in chapter 3 experiment 1 (LD photoperiod) were used for this investigation. In brief, these Atlantic salmon parr (Mean 24.9 ± 5.4g, 140.6 ± 7.8mm)

were acclimated to a LD (16h light: 08h dark) in early March 2009 when water temperature was on average 4.6 ± 0.7^{o}C at the Niall Bromage Freshwater Research Facilities (Institute of Aquaculture, Stirling, UK, 56: 02N). After 1 month liver tissue samples were collected (n = 6 individuals per sample point) every four hours over a 24h period. Experimental animals were sacrificed via a lethal anaesthesia and decapitation. Tissue samples were instantly frozen in liquid nitrogen stored at -70°C until use. A dim red light was used for nocturnal sampling. All experiments were carried out in accordance with accordance with the UK Animals (Scientific Procedures) Act 1986.

RNA extraction – cDNA synthesis

Approximately 100mg of liver tissue was homogenised in 1ml of TRIzol® (Invitrogen UK). RNA extraction was carried according to manufacture instructions. RNA pellets were rehydrated in MilliQ water in varying volumes to achieve a final RNA concentration of approximately 1000ng/ul. Total RNA concentration was determined by ND-1000 Nanodrop spectrophotometer (labtech Int., East Sussex, UK). In order to eliminate any DNA contamination 5μg of totRNA was treated with DNase enzyme following DNA-free™ kit guidelines (Applied biosystems, UK). cDNA was then synthesised using 1μg of DNase treated totRNA in 20μl reaction and random primers according to manufactures protocol, High capacity reverse transcription kit without RNase inhibiter. (Applied biosystems, UK) Final reactions were then diluted with DNA/RNA free H_2O to a final volume of 200μl. All cDNA reactions were stored at -20°C until use in qPCR.

Atlantic salmon *Rev-erb1α* Identification.

All qPCR assays used were previously established and verified in previous work (Chapter 3: *Per1*) by Davie *et al.* (2009) (*Clock, Bmal* & *Per2*) and Minghetti et al (2011) (*Srebp-1, Srebp2, Fas, Lxr, ElovL5, Hmgcr and D6 Fad*) with the exception of *Rev-erb1α. Salmo salar*

Rev-erb1α was identified as follows: two Atlantic salmon expressed sequence tag clones (Genbank ID: DY724083 and DY731913) were identified by BLAST analysis of published vertebrate *Rev-erb1α* sequences. 5' and 3' ends from the constructed contig were amplified using Rapid Amplification of cDNA Ends (RACE)-PCR with the RACE cDNAs generated from 1 µg of salmon whole brain total RNA as described in the manual using the SMART™ RACE kit (Clontech, USA). The 5' and 3' RACE amplicons were generated by two rounds of PCR using *Rev-erb* 5'R1 and *Rev-erb* 5'R2 primers or *Rev-erb* 3'F1 and *Rev-erb* 3'F2 respectively (Table 2). The final full-length sequence was confirmed by two rounds of PCR using nested primers designed to amplify end to end full length cDNAs (Rev-erb_full_F1: Rev-erb_full_R1 & Rev-erb_full_F2: Rev-erb_full_R2) (Table 2). All PCRs were run at an annealing temperature as listed in Table 2 and the extension time was 1 min/Kb of predicted PCR product, and 3 min were applied for unpredictable RACE PCR products. All primers were designed using Primer Select Ver. 6.1 program (DNASTAR, www.dnastar.com).

Table 2: Primer pairs and sequences for *Rev-erb α* identification including primer name, purpose, sequence and annealing temperature.

Name	**Purpose**	**Sequence 5'-3'**	**Anneal Temperature**
Rev-erb 5'R1	RACE-PCR	GCCCCAGTTGTCCACCTCTCCGTTATGT	60 °C
Rev-erb 5'R2	RACE-PCR	AATGGCGGGCTTTGGGTGGATG	60 °C
Rev-erb 3'F1	RACE-PCR	TACCCCCAAGACGAACCCAACA	60 °C
Rev-erb 3'F2	RACE-PCR	GGGAGGCTTGCTAGACACCAT	60 °C
Rev-erb_full_F1	Full length outer PCR	AGGCCGACTTGGAAACTGC	57 °C
Reverb_full_R1		GTCTATTGGCCTTACCCCTATCA	
Rev-erb_full_F2	Full length inner PCR	GTTCAGACCTGCACCGATAGAGC	62 °C
Rev-erb_full_R2		TAGCCGCCCAACCACCACTGTC	

qPCR

In order to determine diel patters of clock gene expression qPCR was carried out on clock genes *Bmal 1, Clock, , Per1, Per2, Rev-erb1α* and cholesterol regulating genes *Srebp-1, Srebp2, Fas, Lxr, ElovL5, Hmgcr and D6 Fad*. *Elongation factor alpha (EF-α)* was used as a reference gene in the liver as it displayed the highest degree of stability in comparison to other potential house keeping genes (See further details below). qPCR primer sequences and annealing temperatures are described in Table 3. All samples were run in duplicate and assays were preformed as follows 95 ^{o}C for 15 minutes and 45 cycles of 95 ^{o}C for 15s, anneal for 15s and 72 ^{o}C for 30s. This was followed by a temperature ramp from 70 – 90°C for melt-curve analysis to verify that no primer–dimer artefacts were present and only one product was generated from each qPCR assay. Quantification was achieved by a parallel set of reactions containing standards consisting of serial dilution of spectrophotometrically determined, linearised plasmid containing partial cDNA sequences.

qPCR normalisation and statistical analysis

In the liver, geNorm analysis was carried out on three potential house keeping genes over the long day liver diel profile to determine the most stable and appropriate reference gene for this tissue. Of the three genes studied (*β-Actin, EF-α* and *GAPDH)* analysis highlighted elongation factor α (*EF-α)* as the most appropriate housekeeping gene (Figure 2).

Table 3: Primer sequence information and annealing temperature for qPCR assays

Gene	**Primer Sequence F 5'-3'**	**Primer Sequence R 5'-3'**	**Anneal° C**
Srebp 1	GCCATGCGCAGGTTGTTTCTTCA	TCTGGCCAGGACGCATCTCAC ACT	**63**
Srebp 2	TCGCGGCCTCCTGATGATT	AGGGCTAGGTGACTGTTCTGG	**63**
Lxr	GCCGCCGCTATCTGAAATCTG	CAATCCGGCAACCAATCTGTA GG	**58**
D6Fa ds	GTGAATGGGGATCCATAGCA	AAACGAACGGACAACCAGAC	**60**
Elovl5 a	ACAAGACAGGAATCTCTTTCAGA TTAA	TCTGGGGTTACTGTGCTATAGT GTAC	**58**
Hmgc r	CCTTCAGCCATGAACTGGAT	TCCTGTCCACAGGCAATGTA	**60**
Fas	ACCGCCAAGCTCAGTGTGC	CAGGCCCCAAAGGAGTAGC	**60**
Bmal1	GCCTACTTGCAACGCTATGTCC	GCTGCGCCTCGTAATGTCTTCA	64
Clock	AGAAATGCCTGCACAGTCGGAGT C	CCACCAGGTCAGAAGGAAGAT GTT	64
Per 1	AGGGGGTCATGCGGAAGGGGAA GT	TGGGCCACCTGCATGGGCTCT GT	66
Per 2	GCTCCCAGAATTCCTAGTGACAA G	GAACAGCCCTCTCGTCCACATC	60
Rev-erba	CCCCCAAGACGAACCCAACAAG AC	AGAGGGAGGCAAAGCGCACCA TTA	61
Ef1a	CTGGAGACGCTGCTATTGTTG	GACTTTGTGACCTTGCCGCTTG AG	61

Analysis of Variance (ANOVA) was used to determine a significant effect of time and Turkey's test was used to determine the significance of differences between sample time points and mean of different sample sets. (InStat® 3.1, Graphpad software inc). Data from each tissue/ photoperiod was then fitted to a cosine wave in order to determine the presence of a significant circadian rhythm. Raw data was analysed using acro circadian analysis programs (Refennetti R., University of South Carolina, USA;

http://www.circadian.org/softwar.html) Acro analysis determines both the significance, acrophase (peak in expression) mean and amplitude of raw data using the equation **Y= A + B * cos (C *X –D)** whereby Y is level of gene expression as a percentage of the mean A is the baseline, C is the frequency multiplier and D is the acrophase of the data set.(Davie et al. 2009) A significant circadian rhythm was deemed present when *p* value was less than 0.05 for all statistical analysis. The expression of a number genes involved in the circadian clock and genes involved in salmon parr liver lipid metabolism were was analysed over a 24h period in fish acclimated to a long day photoperiod (16L:08D). All results are presented in relation to zeitgeber time (ZT) where by ZT 00:00 occurred at lights on and lights of was at ZT 16:00.

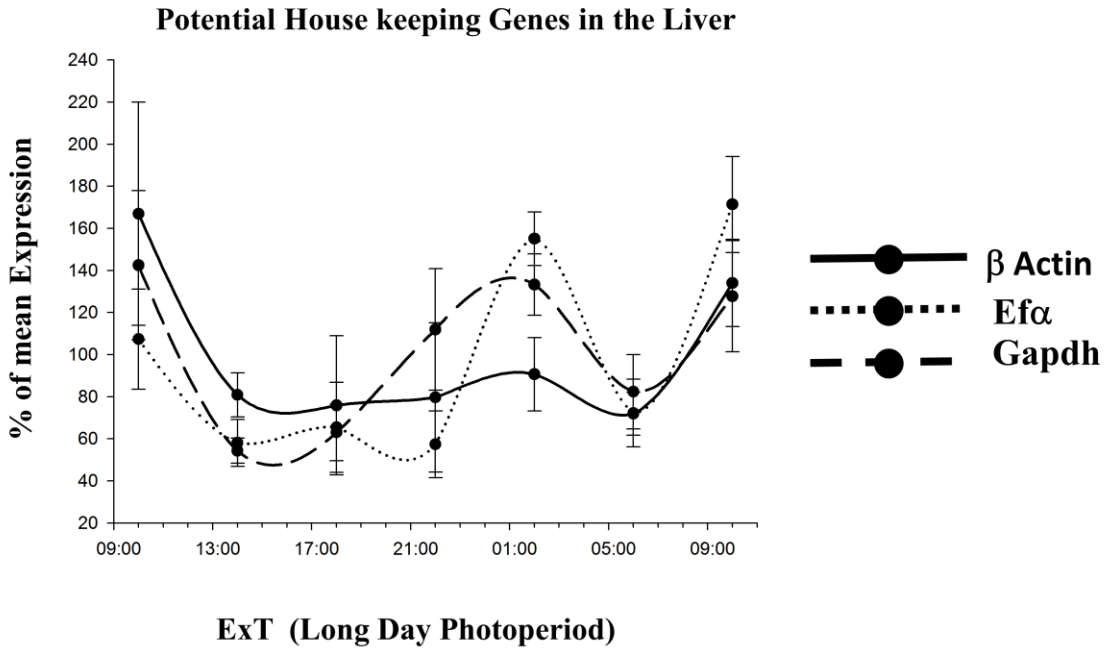


Figure 2: 24h expression of *β–Actin*, *EFα* and Gapdh in the liver of Atlantic salmon parr acclimated to experimental long day photoperiod for potential use as a housekeeping gene in the liver.

4. RESULTS

Rev-erb1α

A 2984bp sequence was isolated which contained an 1818bp coding sequence and a 352bp 5'untranslated region (UTR) and a 814bp3' UTR (Figure 3). Within the coding sequence the predicted DNA binding domain and ligand binding can be seen (Figure 4). Phylogenetic analysis of the deduced amino acid sequence for *Rev-erb1α* in relation to other vertebrate *Rev-erb1α & β* sequences shows the transcript grouped within the teleost *Rev-erb1α* cluster with 65-77% identity with other vertebrate *Rev-erb*1α sequences (Figure 5).

```
3     ATG GGG GAG CAA AAC CAG GCC GAC TTG GAA ACT GCG GTT CAG ACC   47
48    TGC ACC GAT AGA GCC TGT GAC AGC ATA GAC GCT TTT CGT AAT TTT   92
93    TTT CAC TGG CAG TGC TCA CAG AGA AGT GAT TCA CAT TAC GCG GGA   137
138   ACT TGC TCG ATA GCA GAT GCA TCC TTT GGT ATA GTT TAT TAA CCT   182
183   TGG TTC TAA GTA TTT TAA TGA GTA TTC GAA CTC AAA CAG AGC TTT   227
228   TAT TAT AGT TAC ATA ATG ACA TCG TTA TAR CAG AGA ATA GAG GAC   272
273   CTG CTT TTT TGC CAA GAA CCT CGC CAG AAG GGA AAT TAT CCT TTC   317
318   CTA AAC TGT TTT CTA TTT TCA CCG ATA TCT ATA TGA ATG TTT TGG   362
                                                  M   F   W     2
363   AGC TCA GGA TGG CAA CAA CAG CAA TGG ACA AAT AAC AAC AAT ACA   407
3      S   S   G   W   Q   Q   Q   Q   W   T   N   N   N   N   T   17
408   GGG GGC GTA ATC TCC TAC ATT GGC TCC AGT GGC TGC TCA CCG AAT   452
18     G   G   V   I   S   Y   I   G   S   S   G   C   S   P   N   32
453   CAC ACC AGC CCG GTG TCT ATG TAC AGT GAG AAC TCC TTC GGA GCC   497
33     H   T   S   P   V   S   M   Y   S   E   N   S   F   G   A   47
498   TCC TTC CCT CCC TCC CCC AAT GGT TCC CAG AGC TTC TCC AAT GCC   542
48     S   F   P   P   S   P   N   G   S   Q   S   F   S   N   A   62
543   TAC TCT GGC AGC GGC AGC TCC AGC TCC TCC AAT GGT GAT GAT GGC   587
63     Y   S   G   S   G   S   S   S   S   S   N   G   D   D   G   77
588   AAC TCC TCT TCC GGT TCC GGA GGG TCC CCA AGG CCT AGG GGT CGT   632
78     N   S   S   S   G   S   G   G   S   P   R   P   R   G   R   92
633   AAT GAC AGC AGC ATC TCT CGC TGC TCC CCC AGC AAG TCC GTG GCA   677
93     N   D   S   S   I   S   R   C   S   P   S   K   S   V   A   107
678   AGC CTT ACC AAA CTG AAT GGG ATG GTG CTG CTG TGT AAA GTG TGT   722
108    S   L   T   K   L   N   G   M   V   L   L   C   K   V   C   122
723   GGA GAC GTC GCC TCA GGC TTC CAC TAT GGG GTC CAT GCC TGT GAG   767
123    G   D   V   A   S   G   F   H   Y   G   V   H   A   C   E   137
768   GGC TGC AAG GGA TTC TTC CGA CGC AGT ATC CAG CAG AAC ATC CAG   812
138    G   C   K   G   F   F   R   R   S   I   Q   Q   N   I   Q   152
813   TAC AAA AAG TGC CTG AAG AAC GAG ACC TGC ACC ATC ATG AGG ATT   857
153    Y   K   K   C   L   K   N   E   T   C   T   I   M   R   I   167
858   AAC CGC AAC CGC TGC CAG CAG TGC CGC TTC AAA AAG TGT CTG TCC   902
168    N   R   N   R   C   Q   Q   C   R   F   K   K   C   L   S   182
903   GTG GGC ATG TCC CGC GAT GCT GTT CGC TTT GGC AGA ATA CCT AAA   947
183    V   G   M   S   R   D   A   V   R   F   G   R   I   P   K   197
948   CGT GAG AAG CAG CGC ATG CTG GCA GAG ATG CAG AGC GCC ATG AAC   992
198    R   E   K   Q   R   M   L   A   E   M   Q   S   A   M   N   212
993   AAC ATG AAC AAC ATG CAA AAC GAG TTC CAG CTG GCC AGC CTG ACT   1037
213    N   M   N   N   M   Q   N   E   F   Q   L   A   S   L   T   227
1038  CAC AAC TCT CCC CCT TCT CCC ACT TCC TCT TCT CCC TGC CCG GGT   1082
228    H   N   S   P   P   S   P   T   S   S   S   P   C   P   G   242
1083  CTG ACT GTG GCA CCC CAG CCT CAG GCC CTG CCT TTT GCC CCT TCC   1127
243    L   T   V   A   P   Q   P   Q   A   L   P   F   A   P   S   257
1128  CCT TCT CCT CCA GCA CAA GCT CCT GCT TCC CTT CTG CCC CCA CCA   1172
258    P   S   P   P   A   Q   A   P   A   S   L   L   P   P   P   272
1173  TCC ACC CAA AGC CCG CCA TTG TTG GCC AGC TCT CCA CCC CTG TGC   1217
273    S   T   Q   S   P   P   L   L   A   S   S   P   P   L   C   287
1218  CCC AGC CCT GGG GTG GAC TGC ACC ATA ACG GCC ATT GCC CGG GCG   1262
288    P   S   P   G   V   D   C   T   I   T   A   I   A   R   A   302
1263  CAC CGT GAG ACC TTC GTC TAC GCC CAC GAC AAG CTG GGC GAG TCC   1307
303    H   R   E   T   F   V   Y   A   H   D   K   L   G   E   S   317
1308  ACG AGA CAA CAT AAC GGA GAG GTG GAC AAC TGG GGC TCC AAC TAC   1352
318    T   R   Q   H   N   G   E   V   D   N   W   G   S   N   Y   332
1353  TGC CCT GCT GGC TAC CAT CAG AAC GGC CTC AAC ACA ATC TAC CAC   1397
333    C   P   A   G   Y   H   Q   N   G   L   N   T   I   Y   H   347
1398  CAC AAC AAC AAC GTG GCC CTC CAG CAC CAT GGC TTC CAC GCC ATG   1442
348    H   N   N   N   V   A   L   Q   H   H   G   F   H   A   M   362
1443  TCT GAC AGA CAT CAC CAG AAC AAC GGC AAG CAG TTC CAC AAC ACC   1487
363    S   D   R   H   H   Q   N   N   G   K   Q   F   H   N   T   377
1488  AAC CTG TTC GGG AGC CAC CAA AGC ACT GAG ACC AGC AGC GTC CCC   1532
378    N   L   F   G   S   H   Q   S   T   E   T   S   S   V   P   392
1533  CAG GGG CAG AAC TTC CCA TGG AAA AAC CAC AAG GAC ATT GTG CTG   1577
393    Q   G   Q   N   F   P   W   K   N   H   K   D   I   V   L   407
1578  GCA TGT CCA ATG AAC ATG TAC CCC CAA GAC GAA CCC AAC AAG ACC   1622
408    A   C   P   M   N   M   Y   P   Q   D   E   P   N   K   T   422
1623  CCC CAG GAG ATC TGG GAA GAC TTC TCA CTC AGC TTC ACG CCG GCT   1667
423    P   Q   E   I   W   E   D   F   S   L   S   F   T   P   A   437
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1668  GTG CGT GAG GTG GTG GAG TTC GCC AAG CAC ATT CCA GGG TTC AGT   1712
438    V   R   E   V   V   E   F   A   K   H   I   P   G   F   S    452
1713  GCA CTC TCY GAG AAC GAC CAA GTC ACC CTG CTC AAG GCC GGC ACC   1757
453    A   L   X   E   N   D   Q   V   T   L   L   K   A   G   T    467
1758  TTT GAG GTC TTA ATG GTG CGC TTT GCC TCC CTC TTC AAC ATG AAG   1802
468    F   E   V   L   M   V   R   F   A   S   L   F   N   M   K    482
1803  GAG CAG ACT GTC ACC TTC ATC TCG GGT ACC ACC TAC AGC CTG GAG   1847
483    E   Q   T   V   T   F   I   S   G   T   T   Y   S   L   E    497
1848  GCC CTG AAG GGC ATG GGC ATG GGA GGC TTG CTA GAC ACC ATG TTT   1892
498    A   L   K   G   M   G   M   G   G   L   L   D   T   M   F    512
1893  GAA TTC AGC GAG AAG CTC AAC TCC CTG GAG CTC ACG GCC GAG GAG   1937
513    E   F   S   E   K   L   N   S   L   E   L   T   A   E   E    527
1938  CTG GGT CTC TTC ACC GCT GTA GTG CTA GTG TCT GCA GAT CGC TCA   1982
528    L   G   L   F   T   A   V   V   L   V   S   A   D   R   S    542
1983  GGC ATC GAG AAC CTA AAC TCT GTG GAG CTG CTT CAG GAG TCT CTG   2027
543    G   I   E   N   L   N   S   V   E   L   L   Q   E   S   L    557
2028  ATC AAA GCG CTG CGT GCC CTG GTC AGC AAG AGC AAC CCC TGC GAC   2072
558    I   K   A   L   R   A   L   V   S   K   S   N   P   C   D    572
2073  GCC TCT CGC TTC ACC AAG CTG CTG CTC AAG ATG CCC GAC CTG CGC   2117
573    A   S   R   F   T   K   L   L   L   K   M   P   D   L   R    587
2118  ACA CTC AAC AAT GTG CAC TCA GAG AAG CTG CTG TCC TTC CGC ATC   2162
588    T   L   N   N   V   H   S   E   K   L   L   S   F   R   I    602
2163  GAC GTA TAA GAC TGT CAC CAC TGA TGA CAA GAA AAC CCA TCT GGA   2207
603    D   V   *
2208  CCC GTC GCC CAT CTC CAC CTC ACA GAC AGT GGT GGT TGG GCG GCT   2252
2253  AAG GTG CGG GGC AAG TTT GGG GAG GTG ACA GGG GAT CTG ATT CCG   2297
2298  CCT GCT CGC TTG CTC CCT TCA GGG ACA CCC GCC TCC GAG TTG ACT   2342
2343  GAT GTG CGC ATC AGC CAA TGA CAT GGC CGA AAG ACA TTG ACT GAC   2387
2388  AAC TGA TAG GGG TAA GGC CAA TAG ACC CAT GGA GAT TAG GGA GAC   2432
2433  AAA GCC CTC TAC ATA AAT TAT TTT TGG AGG TTG GGG TGG TCG CAG   2477
2478  CTC AAG ACT GCC CAG AAC AAG TGC CCC CTC TAC ACC AGC TGG TCT   2522
2523  GAG GGG GCC AGA GAC CAT TTG TTG GTT GAG CCG AAC CAA AAG AAG   2567
2568  CAA TTG GGC TTT ACT ACC CCA CAT GTC CAG CTA GGC CTG GTT CCT   2612
2613  TCC AAG ATT CTG CTA GAT GCA TTT TAA AAC CAT CTG CTC TGT TTT   2657
2658  ATC ATG ACT GTA AAT AAT TAG TAA AAT ATG TGC AGT GTT GTA AAC   2702
2703  TTT ATT CAT ACT CCA AAC TGA GTT TGT ACA AAT TTG TAA ATA TCT   2747
2748  AGC TAC AAA CAT CAA TAA GCT TAA CTT GAA ATG TTT GAA AAA TGT   2792
2793  GTA TAA TAT ATA TAT TTT TTT GTT AAG ATG AAA TTC TCT AGT GCT   2837
2838  CAT AGT TAT AAA TGT GTG GGT ACA AAT GTA TAA AAT ACA AAT TGA   2882
2883  GTG TAT GCG TAC AAA AAA TAA TAT CCA GAA CTG TAT TTC AAA AAT   2927
2928  GTT TTA TGT AAT GAA GAC AAT TAT AAA AAA AAA AAA AAA AAA AAA   2972
2973  AAA AAA AAA AAA   2984
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Figure 3: Atlantic salmon *Rev-erb* α sequence with deduced protein sequence.

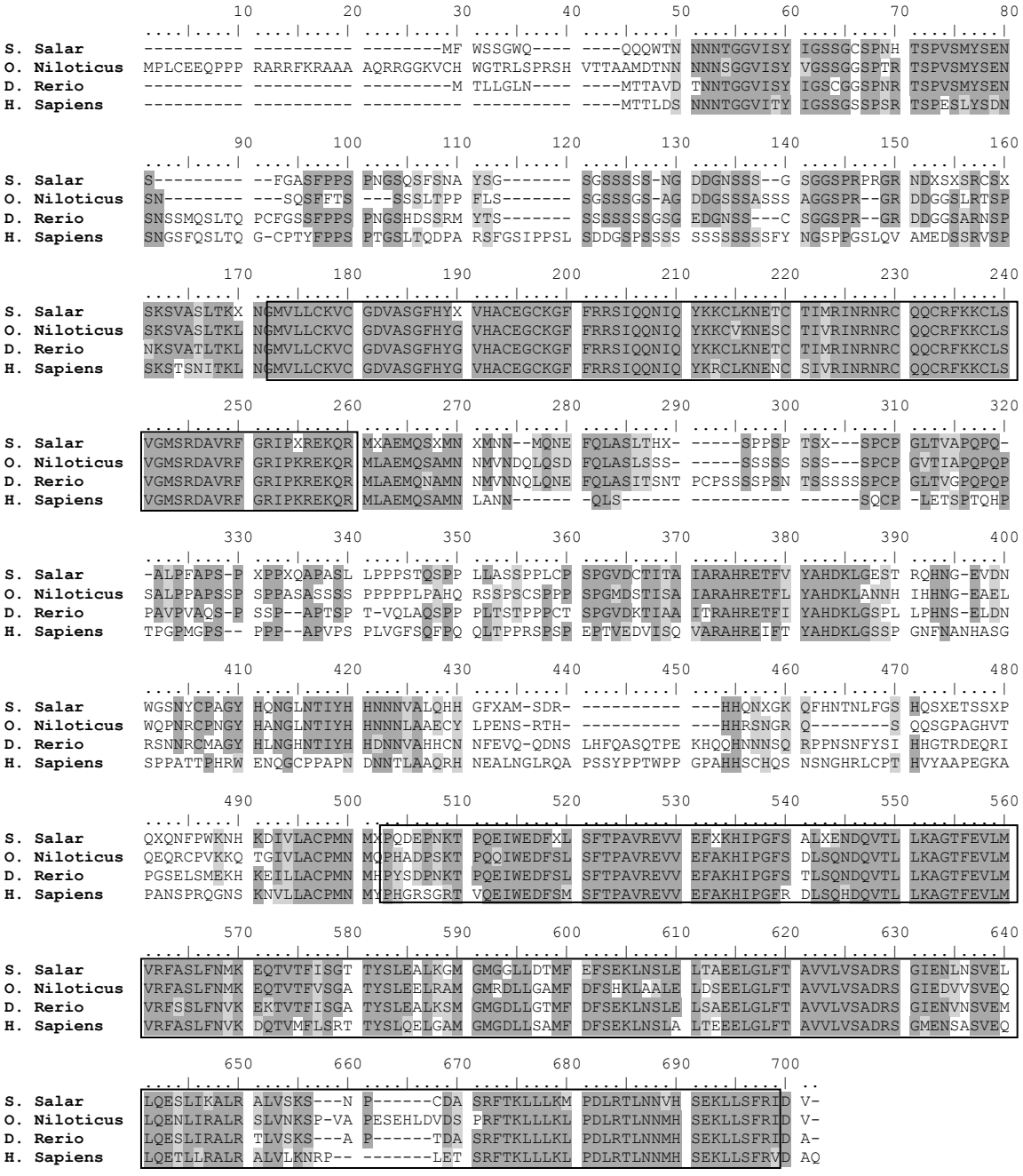

Figure 4: Alignment of the deduced amino acid sequence for salmon, tilapia, zebrafish and human *Rev-erbα*. The conserved amino acids are shaded. Predicted DNA binding domain (top) and ligand binding domain (bottom) identified using a CDD search (Marchler-Bauer et al. 2011) are boxed.

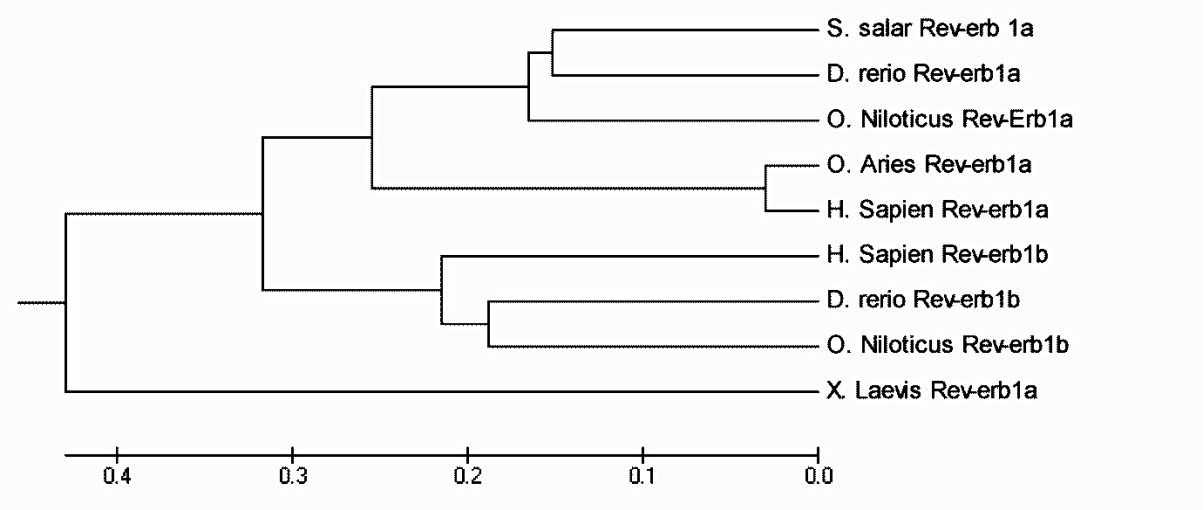

Figure 5: Phylogenetic analysis of the deduced amino acid sequence for *Rev-erb*1α in relation to other vertebrate *Rev-erb*1α & β sequences The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007).

Liver lipid and cholesterol homeostasis gene expression

Of the five clock genes investigated all were expressed in the liver. However, only *Bmal 1* displayed a significant circadian pattern of expression when results were fitted to a cosine wave using Acro analysis (Refinetti 2006). Amongst the genes involved in the liver lipid metabolism *Lxr* and *Srebp1* displayed a significant circadian profile of expression. However for *LxR* no significant effect of time was observed when expression per time point was analysed by ANOVA and turkey's test for difference. The acrophase of both *Bmal1* and *Shrebp1* occurred at between 3 and 4 hours prior to lights off at ZT13:00±3.9 and 13:00±2.4 respectively. Peak *Lxr* expression occurred at ZT 13:00±2.7, (see Table 4 and Figure 6).

Table 3: P value of 24h profiles of gene expression Acro and ANOVA analysis and acrophase where significant rhythm is present.

	Acro		**ANOVA**
Gene	**P value**	**Acrophase ZT**	**P value**
Bmal1	<0.05	13:00±3.9	<0.05
Clock	>0.05	-	<0.05
Per 1	>0.05	-	>0.05
Per 2	>0.05	-	<0.05
Rev-erb a	>0.05	-	>0.05
D6Fads	>0.05	-	>0.05
Elovl5a	>0.05	-	>0.05
Fas	>0.05	-	>0.05
Hmgcr	>0.05	-	<0.05
Lxr	<0.05	13:00±2.73	>0.05
Srebp1	<0.05	13:00±2.41	<0.05
Srebp 2	>0.05	-	>0.05

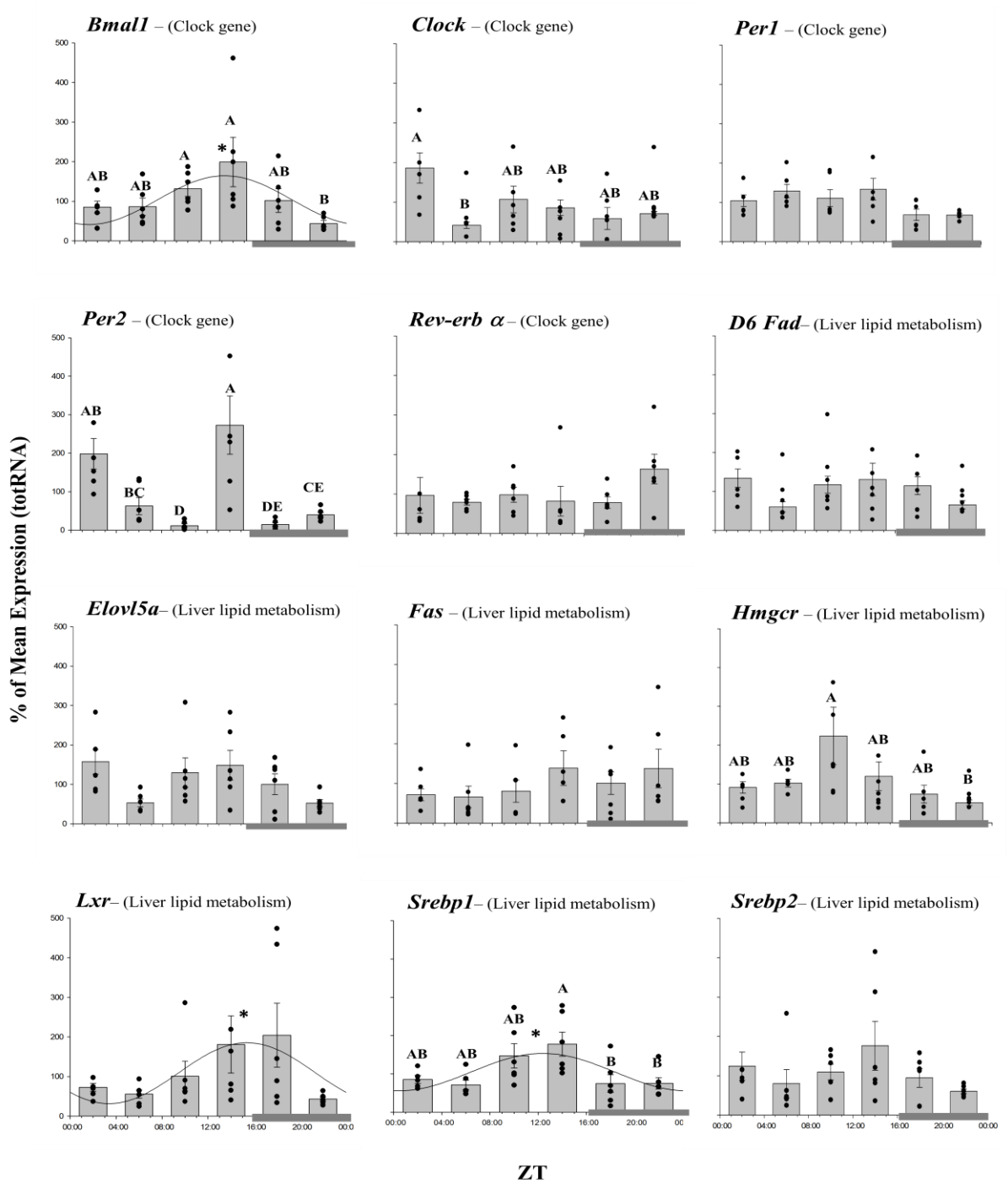

Figure 6: 24h expression profiles of clock genes and genes involved in the liver lipid metabolism in the liver of salmon parr acclimated to LD photoperiod. Results are displayed in relation to Zeitgeiber time (ZT), whereby ZT 0 is the onset of light. Gene expression data is displayed as the percentage of the mean ± the SEM and includes the spread of the data.The presence of a cosine wave denotes a significant circadian rhythm by acro analysis. The presence of different letters represents statistically significant difference between samples by way of ANOVA and Turkeys test where by $P<0.05$

5. DISCUSSION

In vertebrates it is now recognised that the expression of a number of genes involved in cholesterol, bile acid and liver fatty acid homeostasis are under circadian regulation (Le Martelot et al. 2009). In the Atlantic salmon lipid and cholesterol regulation is an area of considerable interest, in particular, the regulation of dietary lipid uptake and the optimisation of polyunsaturated fatty acids synthesis with regard to human consumption. Understanding the molecular mechanisms underpinning fatty acid homeostasis and potential endogenous regulation is an essential component of this. In order to provide a preliminary evidence for the role of the circadian clock in cholesterol and fatty acid homeostasis the mRNA expression of five clock genes, *Bmal1, Clock, Per1, Per2 and Rev-erb 1α,* and seven genes involved in the regulation of lipid metabolism, *D6 Fad, Elovl5a, Fas, Hmgcr, Lxr Srebp1* and *Srebp2,* were measured over a 24h period in the liver. Long day liver cDNA from chapter 3 was used for the current investigation as rhythmic clock gene expression in the brain was previously observed (Chapter 3).

Results showed that the clock gene *Bmal1* in addition to *Srebp1* and *Lxr* were rhythmically expressed in the salmon liver. Mammalian homologues of these genes have also been shown to follow a circadian pattern of expression over a 24h period. In rodents *Srebp1a* is known to be regulated by the circadian clock via the actions of *Rev-erb α* and in mice rhythmic expression of *Srebp1c* has been observed in the liver (Le Martelot et al. 2009) and adipose tissue (Yang et al. 2006). In salmon *Srebp1* mRNA displayed a circadian profile with a peak of expression at ZT 12:24 during the photophase of the LD cycle. This is not consistent with previous reports in mice where the acrophase of *Srebp1*expression occurred during the dark phase. In addition *Fas* mRNA profile was not significantly rhythmic in salmon as opposed to previous findings in rodents. Le Martelot et al (2009) reported the circadian expression of *Fas* mRNA in mice to be regulated via the action of the SREBP1c protein in *Rev-erb α*

knockout mice. In the Atlantic salmon only one *Srebp1* isoform has been identified that displays considerable similarities in sequence identity with the mammalian *Shrebp1a* and *Shrebp1c* (Morais et al. 2011). However despite the rhythmic expression of *Srebp1* in the Atlantic salmon liver *Fas* did not display a circadian profile of mRNA expression. Moreover previous reports have described *Fas* mRNA expression as being strongly associated with dietary composition (Morais et al. 2011). In salmon the expression of *Hmgcr* was not circadian, although mRNA expression was significantly different with elevated expression at ZT 10:00 in comparison to ZT22:00. This contrasts with rodents where *Hmgcr* displays a circadian profile of expression and the acrophase occurred during the night (Sahar & Sassone-Corsi 2012) or at the onset of the scotophase at ZT 12:00. Similarly in humans HMGCR enzyme activity is elevated during the night. Consequently cholesterol lowering drugs that target HMGCR as the rate limiting enzyme in cholesterol biosynthesis work more effectively at night (Sahar & Sassone-Corsi 2012). In mammals circadian regulation of cholesterol and lipid homeostasis additionally occurs via *Lxr* and the corresponding protein LXR.

In salmon *Lxr* displayed a statistically significant circadian regulation. In mammals LXR is thought to be regulated by clocks via *Rev-erb* α. However regulation appears to be at protein level as opposed to transcriptional level of mRNA expression. Consequently *Lxr* mRNA is not rhythmically expressed however circadian rhythms are observed in protein levels and activity in mammals. In mice mRNA levels of *lxr*α and *lxr*β remain unchanged in *Rev-erb* α knockout compared to wild type mice (Akhtar et al. 2002). In salmon *Lxr* mRNA did display a statistically significant circadian regulation. In salmon initial differences in the circadian expression of a number of genes in comparison to mammals were observed and further evidence is required to determine if this may be attributed to differential expression of clock genes in the liver. In rodents REV-ERB is considered to be fundamental in the in clock

mediated regulation of the SREBP pathway (Figure 1)(Le Martelot et al. 2009). Accordingly Rev-*erb* α mRNA displays robust circadian expression in the mammalian liver in addition to other metabolically important tissues such as adipose tissue and muscle (Yang et al. 2006). In the liver of the Atlantic salmon this was not the case. Rev-*erb* α mRNA was not rhythmically expressed and there was no statistical difference between diurnal and nocturnal levels of mRNA as has previously been reported in rodents. In mammals *Rev-erb* α is the key connection between the liver molecular clock and the liver lipid metabolism. In order to investigate such processes in the Atlantic salmon it was necessary to identify and characterise the *Rev-erb* α gene in the Atlantic salmon. A 2984bp sequence was isolated containing 1818bp of coding sequence. The sequence identified bared considerable similarity to other vertebrate *Rev-erb* sequences. Within the coding sequence a DNA binding domain and ligand binding regions were identified. A 2984bp sequence was isolated which contained an 1818bp coding sequence and a 352bp 5'untranslated region (UTR) and a 814bp3' UTR.

In mammals a number of other clock genes have been shown to cycle over 24h in the liver including *Bmal1* and *Per* genes. In mice the acrophase of *Bmal*, *Per1* and *Per2* occurred between ZT 10:00-14:00 and 14:00 – 18:00 respectively (Akhtar et al. 2002). This is in accordance with current results where peak *Bmal1* expression occurred at approximately ZT13:00h. In contrast to results obtained in mice, *Per1* and *Per2* were not rhythmically expressed in the salmon liver. In rodents the cyclic expression of genes involved in lipid metabolism and cholesterol homeostasis is largely attributed to top down-regulation from the suprachiasmatic nucleus (SCN), known as the master circadian oscillator, via *Rev-erb* α and the positive and negative components of the molecular feedback loop. The SCN has been shown to mediate cyclic expression of rhythmically expressed genes in the mouse liver

(Akhtar et al. 2002). When the intact SCN is lesioned or destroyed the hepatic expression of clock genes *Per2* and *Bmal1* amplitude of circadian rhythm was considerably suppressed (Akhtar et al. 2002). Significant rhythmic expression of genes not part of the core circadian clock was also abolished in liver in the absence of an intact SCN (Akhtar et al. 2002). In mice it is clear that the expression of clock genes and a number of clock controlled genes in the liver is highly regulated by the SCN. It is thought that this extends to the regulation of rhythmically expressed genes involved in cholesterol and lipid homeostasis in the liver. In mammals the SCN is fundamental in the synchronisation of molecular clocks and biological rhythms in central and peripheral tissues. However in teleosts the hierarchical structure of clocks is not so clear and the presence of an SCN or SCN-like structure has yet to be identified. Amongst teleosts the majority of work on clocks has been conducted in the zebrafish. In contrast to mammals peripheral clock gene oscillations are not under the control of a SCN-like structure but clocks are self sustaining and individually entrainable by light and have been documented persisting in *in vitro* tissue culture experiments and cultured cell lines (Carr et al. 2006; Kaneko et al. 2006; Vatine et al. 2011; Whitmore et al. 1998). It has been hypothesised that each cell is photoreceptive with possible candidates including extra retinal opsins, flavin containing oxidase and photoreceptive cryptochrome genes (Vatine et al. 2011). Previous work in the Atlantic salmon, as described in chapter 3, has shown clock gene expression in the liver in comparison to central tissues. Moreover 24h profiles of gene expression differed with regard to differing seasonal photoperiod. Consequently results from this chapter and chapter 3 provide evidence of functional clocks in the liver with the potential to regulate a number of physiological processes.

This study has shown the significant circadian expression of important genes in the regulation of cholesterol and lipid homeostasis in *Srebp1* and *Lxr*. However in this preliminary investigation we are unable to determine whether this is a result of regulation by the circadian

clock or food availability. In higher vertebrates the genes involved in the cholesterol and lipid metabolism are additionally regulated by the feeding/ fasting cycle and diet. In mammals the SREBP pathway and target genes have been shown to be regulated by food (Horton et al. 1998; Le Martelot et al. 2009; Schibler et al. 2010). In fish, as in rodents, food has been shown to entrain clocks in the liver and result in altered rhythms in clock gene expression between the central tissue and the liver (Damiola et al. 2000; Stokkan et al. 2001). In both goldfish and zebrafish the expression of clock genes in the liver has been attributed to feeding (Feliciano et al. 2011; Lopez-Olmeda et al. 2010). This is most likely a result of the presence of food availability as a zeitgeber signal in the liver. Under constant conditions (24 hours light) feeding time was able to entrain *Per1* rhythms in the liver (Lopez-Olmeda et al. 2010). However central clock regulation in the liver and potential control of SREBP pathway remains to be characterised in fish.

Peripheral clocks such as those in the liver are considered to be highly flexible and have the capacity to be synchronised by a number of entrainment cues and are modulated by a variety of different pathways. In particular, in fish, clockwork mechanisms in the liver have been demonstrated to be entrained by photoperiod, food availability and temperature (Feliciano et al. 2011; Lopez-Olmeda et al. 2010). In mammals a variety of regulatory mechanisms have been hypothesised including the role of clock gene Per1, feeding (via PPARa and heat shock proteins), glucocorticoid signals from the SCN and cytoskeleton signalling (Escobar et al. 2009; Schibler et al. 2010). However the mechanisms linking feeding as a zeitgeber signal, clocks in the liver and rhythmically expressed lipid metabolism genes in the liver are not understood, particularly in teleosts.

6. CONCLUSIONS

In summary the current investigation provides preliminary evidence for the circadian expression of a number of genes involved in cholesterol and lipid homeostasis in the liver. In the Atlantic salmon *Bmal1, Srebp1* and *Lxr* were all rhythmically expressed in the liver. Furthermore peak expression for *Bmal1* in the present study match previous results obtained in mammals. However in contrast to previous results in mice the rhythmic mRNA expression of SREBP target genes was not observed in the Atlantic salmon. Moreover the diurnal elevation of *Hmgcr* contrasts with the nocturnal peak in the expression of the same gene in rodents. In mammals the molecular components of the liver lipid metabolism, as described above, are regulated by clock genes via *Rev-erb* α. The mRNA expression of which is circadian in nature (Le Martelot et al. 2009). However in the Atlantic Salmon *Rev- erb* α was constitutively expressed over a 24h period in the liver. The significance of this is unknown in the Atlantic salmon requires and further investigation. Any future investigations will first need to determine whether the genes involved in cholesterol and lipid homeostasis display significant circadian expression in the liver independently from feeding. Furthermore the identification of other genes involved such as *CYP7A1*or *Insig2* and identification of Regulation by other nuclear receptors such as Ppar α and γ may also be beneficial to future research in this area.

CHAPTER 7

SUMMARY OF CONCLUSIONS

SUMMARY OF CONCLUSIONS

The section below details a summary of the main findings and conclusions from each chapter.

Chapter 3: Seasonal melatonin and clock gene expression in the brain, fin and liver of the Atlantic salmon *(Salmo salar).*

- Large differences in the presence and phase of significant circadian rhythms in clock gene mRNA expression was shown between the brain, fin and liver in experiment 1.
- Daylength dependent clock gene expression was observed in the brain and liver for *Clock* and *Period* genes. In the brain both *Clock* and *Per2* were rhythmically expressed under long day photoperiod in contrast to previous reports of SD dependent expression. However no apparent consensus between genes and 24h circadian expression between experement1 and 2 was found.
- Amplitudinal changes were present, in mean clock gene expression between seasonal sample points. Mean expression individual clock genes responded differently to seasonal sample point and amplitudinal changes differed between experiments 1 and 2 inferring that changes can not be accounted for by photoperiod alone and may be dependent on other factors such as salinity, temperature or life history.
- Seasonal amplitudinal differences in mean nocturnal levels of melatonin were observed and could not be accounted for by photoperiod alone as results differed between artificial and SNP photo treatments.

Chapter 4: Photoperiod regulation of *Deiodinases, Eyes absent 3* and *Thyroid stimulating hormone beta* in the Atlantic salmon *(Salmo salar).*

- Genes involved in the mammalian molecular switch for photoperiod responsiveness in mammals (*Dio1, Dio2, Dio3, Eya3* and CCAATs) displayed photoperiod differences in expression when analysed by microarray.
- qPCR studies revealed daylength dependent differences in the circadian and mean expression of *Dio1 – 3, Eya3* and *Tshβ. Dio2* and *Dio3* were responsive to LD and SD photoperiods, respectively. However *Eya3* and *Tshβ* were responsive to SD photoperiod in microarray validation and LD photoperiod in qPCR study.

Chapter 5: Comparative study of clock gene expression and melatonin in the Atlantic salmon *(Salmo salar)* and European seabass *(Dicentrarchus labrax)* pineal.

- Significant circadian clock gene expression was present *in vivo* but was absent *in vitro* in the Atlantic salmon pineal.
- Constitutively elevated *Aanat2* mRNA expression in the Atlantic salmon was present under all conditions. In the seabass significant circadian, nocturnally elevated, *Aanat2* mRNA expression was observed.
- Comparative post hoc *in silico* analysis of the Atlantic salmon and European seabass 5' promoter region of the *Aanat2* gene reviled the presence of conserved E-box elements in a location similar to that previously describe in teleosts with clock controlled *Aanat2*. In the Atlantic salmon no functional E-box element was present in the promoter of the gene.

Chapter 6: Circadian expression of clock genes, *Sterol Regulatory Element-Binding Proteins* and SREBP targets in the liver of the Atlantic salmon.

- Rhythmic expression of *Bmal* and liver lipid genes *Srebp1* and *Lxr* was observed in liver of LD acclimated Atlantic salmon parr. However in contrast to mammals *Per1, Per2*, *Fas*, and *Rev-erb* α did not display significant circadian rhythmicity in salmon.

CHAPTER 8

GENERAL DISCUSSION

Chapter 8

GENERAL DISCUSSION

This investigation was undertaken in order to gain a better understanding of photoperiod regulation of molecular clocks and the genes involved in the seasonal control of physiology in the Atlantic salmon *(Salmo salar).* The molecular components of the circadian clock are relatively well characterised in mammals and involvement of clock genes and circadian melatonin production from the pineal has been demonstrated in the regulation of seasonal physiology, in particular via deiodinase regulation of thyroid hormone bioactivity (Dardente et al. 2010). In teleosts the majority of work has been carried out in the zebrafish where the expression of clock genes has now been relatively well described in a wide variety of tissues and cell types (Vatine et al. 2009). However the zebrafish is a tropical species lacking distinct seasonal organisation of physiology. The Atlantic salmon, on the other hand, is a highly seasonal teleost species displaying a high level of temporal organisation of its physiology (Davie et al. 2009). Furthermore photoperiod, melatonin and clock genes have all been associated with seasonal migration; maturation and reproduction in salmonid species (Aubin-Horth et al. 2005; Leder et al. 2006; Randall et al. 1995).

Davie et al (2009) demonstrated photoperiod dependent circadian expression of clock genes, i.e. *Clock*, *Bmal* and *Per2* in the Atlantic salmon brain, inferring the presence of a functional circadian clock under a short day as opposed to long day seasonal photoperiod. This thesis set out to further investigate the photoperiod control of clock and seasonal gene expression in the species. In order to achieve this, four primary lines of investigation were established. Chapter 3 focuses was to determine whether circulating melatonin and clock gene expression in the brain, fin and liver are subjected to differential regulation by experimental and simulated natural seasonal photoperiod treatments. This led to chapter 4 which looked at the expression of genes involved in the molecular switch for photoperiod response in mammals (*Dio1-3*, *Eya3* and *Tshβ).* Chapter 5 then progressed to the pineal and the expression of clock genes *in*

vivo and *in vitro* and expression in relation to melatonin production in salmon in comparison to European seabass. The final experimental chapter (Chapter 6) provides one of the first examinations of a functional output of clocks in the Atlantic salmon targeting hepatic clocks and regulation of the liver lipid metabolism, an area of considerable commercial interest within the aquaculture industry. The following discussion outlines the key conclusions drawn from the four experimental chapters and the potential for future research in these areas.

During early studies it became apparent that clock genes are not homogeneously expressed in the Atlantic salmon across different tissue types. Results showed significant differences in clock gene expression with regard to the presence and phase of a significant circadian rhythm, between the brain, and peripheral tissues. The presence of differential clock gene expression have previously been described between the brain and the liver in Atlantic salmon post-smolts acclimated to a 12L: 12D photoperiod (Huang et al. 2010b). However it is difficult to make comparisons with the present investigation due to the different environmental conditions and physiological stages (freshwater vs. marine stages). Amongst other species of teleost fish there have been contrasting reports concerning the expression of clocks in central and peripheral tissues. In the zebrafish comparative circadian clock gene expression has been described in a wide variety of tissue and cell types (Whitmore et al. 1998). Moreover rhythms in mRNA expression have been shown to persist and have the capacity to be photo entrainable even when isolated in organ and cell culture (Whitmore et al. 2000). In other fish species such as the European seabass (*Dicentrarchus labrax*) (Sanchez et al. 2010) and Golden rabbitfish (*Siganus corallinus*) (Park et al. 2007) comparable *Per1* expression has been reported in brain, heart and liver and in the brain, retina and the liver respectively. However in the goldfish (*Carassius auratus*) differential expression of *Per1-3* and *Cry1-3* was reported between retina, liver and the gut tissues (Velarde et al. 2009). Amongst teleosts clock gene expression appears to be species and tissue specific. The

differences in central and peripheral expression may be a consequence of the potential presence a hierarchal structure present in the Atlantic salmon clock work.

A comparatively well described hierarchal structure for the mammalian circadian axis has now been described. Fundamental to this is the existence of the SCN as a central circadian pacemaker acting to synchronise clock gene oscillations throughout the body. However amongst teleosts no such structure has been identified. As previously described initial work in the zebrafish suggested each cell has the capacity to be entrained by light and sustain endogenous clock gene oscillations under constant conditions (Whitmore et al. 1998). The absence, as yet, of master circadian clock or SCN-like structure in teleosts lead to the hypothesis that the clear hierarchical structure observed in mammalian clocks may not be present in fish.

Despite more recent research suggesting the lack of a master circadian pacemaker in teleosts the evidence for a central oscillator in fish was reviewed by Holmqvist, Ostholm and Ekstrom in a 1992 book chapter of the same name. The authors summarised that the brain and neural organisation of vertebrates was varied and had the potential to be species specific. The review then suggested that a central oscillator in fish may not be present in the same location as in mammals and birds. The review then focused on areas of retinohypothalamic innervations in the Atlantic salmon as sites of a potential central oscillator. The authors identified hypothalamic areas located close to the floor of the third ventricle and the ventral hypothalamus in addition to the anterior periventricular nucleus as areas of particular interest. In addition to receiving an input from the retina there are a number of similarities between these structures and central oscillators in other vertebrates. For example the anterior periventricular nucleus is composed of densely packed neurons as in the SCN. However the presence of endogenous rhythmic activity in these regions is yet to be described. Possible future work should focus on the localisation of clock gene expression in the brain and pineal

organ of the Atlantic salmon using laser microdissection techniques. In situ hybridisation expression studies for a number of clock genes would be of assistance in determining concentrated regions of clock gene expression in the brain and whether or not differential circadian expression occurs throughout different central brain structures and the pituitary or the pineal organ. This could potentially lead to the identification of a master circadian oscillator that may be present within the Atlantic salmon brain.

Within the mammalian brain the SCN, the master circadian clock, synchronises clock gene expression and regulates physiology via hormonal and neural outputs (Maywood et al. 2007). The SCN relays information and establishes connectivity between clocks, thus constituting the hierarchical structure of the clock (Maywood et al. 2007). This then enables the synchronisation of physiology to the external environment. In terms of hormonal signalling the best described example of this is SCN regulation of nocturnally produced pineal melatonin. The melatonin signal binds to melatonin receptors in central and peripheral tissues and synchronises most physiological functions to environmental photoperiod often via the expression of clock genes, as described in the mammalian PT (Foster & Kreitzman 2009). With regard to neural connectivity a multitude of pathways promote clock connectivity, circadian information is relayed from the SCN via a region of MBH extending from the sub-paraven- tricular zone adjacent to the SCN, dorsally and caudally into the dorsomedial hypothalamus (Saper et al. 2005; Maywood et al. 2007). The neural signal is transduced into arousal and sleep-regulatory centres mediating sleep/ wake hormones (Maywood et al. 2007). In addition, extensive efferent pathways run to a diversity of autonomic centres (Kalsbeek et al. 2006; Kalsbeek et al. 2007; Maywood et al. 2007). Therefore providing evidence of connectivity and communication between clocks and linking clocks to physiological processes. However in spite of clear differential clock gene expression described in chapter 3 the extent of the connectivity between clocks is unknown in the Atlantic salmon. In addition

chapter 5 provides further suggestion for the connectivity of clocks in the Atlantic salmon brain and pineal as clocks were only present in the pineal in vivo.

The salmonid pineal has for a long time been described as the only non clock containing pineal amongst teleosts. However results from chapter 5 demonstrate rhythmic clock gene expression in the tissue *in vivo* but not *in vitro*. Thus suggesting some degree of extra-pineal input may be necessary to elicit circadian clock gene expression in the pineal. There are known to be a multitude of neural connections linking the brain with the pineal (Holmqvist et al. 1992) however the connectivity of clocks between the two tissues is unclear. In teleosts localisation of clock gene expression and identification of circadian and seasonal centres would be the first step in determining the connectivity and communication of clocks between the brain and the pineal. Secondly connectivity between clocks could be investigated by identifying important neural pathways in other vertebrates and with the use of lesion experiments.

However amongst teleosts the extent of connectivity between clocks is likely to be highly species specific. This is a consequence of the diversity of the teleost circadian light axis. In the majority of teleosts studied the pineal highly photosensitive providing extra retinal, source of photic input to the brain. Photic information is transmitted via extensive neural connections to the brain and melatonin binding to central and peripheral tissues. Research has demonstrated that within the teleost group the source and degree of circadian and seasonal photic input varies considerably between species (Migaud et al. 2007). For example in salmonids melatonin synthesis is reliant on photic input directly perceived by pineal, while in the seabass it is dependent on both the retina and the pineal and in the tilapia photoperiod information is primarily perceived in the retina (Migaud et al. 2007). Thus the variation in the teleost circadian light axis may have an impact clock gene expression in the pineal and the connectivity of clocks between tissue and cell types.

One interesting finding in chapter 5 was the absence of rhythmic *Aanat2* mRNA expression in the Atlantic salmon pineal in contrast to rhythmic, high at night; *Aanat2* expression observed in seabass, a species that displays clock controlled endogenous melatonin production. This was hypothesised to be a consequence of the absence of clock regulation of salmon *Aanat 2* at the transcriptome level. Unfortunately the Atlantic salmon genome was not publicly available during most of my doctoral work becoming available in 2012. The availability of the salmon genome has facilitated *in silico* analysis of non coding regions of the *Aanat2* gene Analysis of the Atlantic salmon and European seabass *Aanat2 5*' promoter region revealed the presence of functional E-box elements in the promoter of the seabass but not the salmon sequence, consequently providing a link between clocks and *Aanat2* in the seabass. This demonstrates one of many applications of the Atlantic salmon genome publication in this field.

As described above the availability of the salmon genome has facilitated in silico analysis of non coding regions of the *Aanat2* gene. This methodology could be expanded to analysis of the 5 and 3 prime regions of a wide variety of clock, clock controlled and seasonal genes in the Atlantic salmon. E-box, D-elements and RORs, rhythmicity and circadian output, could more easily be identified in the promoter regions of a gene. This could be expanded to other regulatory elements in the non-coding and coding sequence.

In addition the publication Atlantic salmon genome has enabled rapid identification of clock and seasonally important genes and their homologues. BLAST analysis using sequence information of desired target genes in other teleosts or vertebrate species would identify similar sequences in the Atlantic salmon. PCR primer pairs could subsequently be designed on the resulting sequence, as opposed to ESTs or other teleost sequences, and the product could be cloned or sequenced more rapidly than previously described in the general materials and methods (Chapter 2). In comparison to other vertebrates the salmonids have experienced

2 separate genome duplication events over the course of evolution from mammals(Davidson et al. 2010). Consequently there is the potential for the presence of multiple homologues of clock and seasonal genes. The publication of the Atlantic salmon genome is most likely the greatest advance in the study of the species biology and will continue to be of considerable value in the study of circadian and seasonal biology in the species.

Prior to undertaking this thesis the presence of daylength dependent clock gene expression under SD photoperiod had previously been reported in the brain of Atlantic salmon (Davie et al. 2009). However Chapter 3 describes contrasting results daylength dependent clock gene expression in response to LD conditions. It was hypothesised that this was a consequence of photoperiod history as rhythmic clock gene expression was present in the photoperiod that best represented the natural seasonal progression after acclimation from SNP. Photoperiod history can have a profound effect on salmonid physiology Randall and Bromage (1998) demonstrated that it is not a specific daylength that triggers seasonal process but daylength in relation to previous photoperiod experienced. Photoperiods usually considered to represent long days (18L: 06D) are recognised as short if fish have been previously exposed to extreme long days (22L: 02D). Similarly spawning can be advanced under conventional short day photoperiods (e.g. 06L:18D) if fish have previously been acclimated to extreme short days (02L:22D) (Randall & Bromage 1998; Randall et al. 1998). However when the expression of clock genes was investigated under SNP (Chapter 3) the effects of photoperiod history on the expression of clock genes was not observed. It is hypothesised that expression of clock genes is more apparent under a ridged experimental photoperiod than under constantly adjusting natural photoperiod conditions. In order to better understand the impact of photoperiod history on gene expression a series of trials could be set up as described above by Randall et al (1998) and Randall and Bromage (1998). However at the end of the acclimation period the

expression of both the amplitude and circadian expression of clocks and seasonal genes could be analysed as amplitude of clock gene expression is also likely to be of importance.

In mammals it is not only the phase shifting or presence of a significant circadian rhythm that is of importance. Amplitude appears to be associated with photoperiod changes in some cases. For example in the mammalian PT *Per1* amplitudinal changes in mRNA expression is suppressed in response to SD, in addition to seasonal changes in the phase of gene expression (Messager et al. 2000; Hazlerigg & Wagner 2006). In chapter 3 significant difference in the seasonal amplitude of clock gene expression were observed between SNP (experiment 2) sample points. Up to 7.9 fold change in amplitude was observed over the course of the year that can not be explained by photoperiod variations alone. Moreover seasonal amplitudinal differences described in the chapter were not consistent across all clock genes and the clock mechanisms as a whole did not change consistently with varying seasonality. However over the duration of this trial Atlantic salmon parr went through the smoltification process and were transferred from freshwater to salt water. It is unknown what effect such physiological events may have on the expression of clock genes. Moreover genomic regions containing a number of clock genes have been identified as quantitative trait loci (QTLs) for a number of life history events in salmonids (Leder et al. 2006; O'Malley et al. 2010). However it is unclear how amplitude changes in gene expression may impact on physiology.

Chapter 3 highlights differences in both the phase and amplitude of clock gene expression in the brain of salmon acclimated to experimental and SNP treatments. The contrasting results obtained may be a consequence of the differences between short term acclimation to constant photoperiod vs. a natural cycle of entrainment. Currently the existence of photorefractory states and the seasonal photoperiod mechanisms present in mammals have not yet been established in teleosts (Hazlerigg and Wagner. 2006). However research into these responsive and non-responsive conditions could be under taken in future by reporting clock and seasonal

gene expression under a wider variety of photoperiod treatments and differing acclamation periods ranging from changes in gene expression after 24h to several months. Such results would offer considerable insight into seasonal processes in the Atlantic salmon.

In order obtain a better understanding of the photoperiod regulation of seasonal processes and physiological events key genes involved the molecular photoperiod switch in mammals and birds was investigated in the Atlantic salmon. Elements of the "molecular switch" (*Dio1-3, Eya3* and CCAAT α and β were identified in the transcriptome study and displayed differing expression patterns between SD and LD photoperiod. Daily expression of seasonal genes was determined in samples from Davie et al (2009) (microarray validation) and from chapter 3 (qPCR study). This is one of the first studies looking at the mechanisms of the highly conserved molecular photoperiod switch in teleosts.

In agreement with the mammalian seasonal mechanism microarray and both qPCR reported LD up-regulation of *Dio2.* However contrasting results were obtained for *Eya3* and *Tshβ* expression. *Eya3* and *Tshβ* expression appeared to be dependent on photoperiod history, as discussed for clock gene expression. In mammals *Eya3* has been shown to be regulated by clocks via three conserved E-box elements in the promoter region of the gene which in turn regulates the expression of *Tshβ* (Dardente et al. 2010). Moreover *Tshβ* is directly regulated by clocks via conserved D-elements in mammals (Dardente et al. 2010). In order to investigate this *in silico* investigation identifying the presence of E-boxes and D-elements, in addition to other regulatory elements, could be undertaken for seasonal genes promoter regions in the Atlantic salmon. Despite the need for further investigation into the precise mechanisms and regulatory elements this highly conserved pathway is present in the Atlantic salmon. In order to elaborate on the evolutionary history of this mechanism it would be interesting to investigate the presence and expression of seasonal genes in more primitive

vertebrate species such as the hagfish or lamprey, thus confirming the ancestral nature of this highly conserved seasonal mechanism throughout vertebrate evolution.

A multitude of cues, other than photoeriod, have been shown to regulate rhythmic gene expression including temperature, feeding For example temperature has been shown to differentially regulate peripheral oscillations of *Per3* in zebrafish (Kaneko et al. 2006). In particular the presence of a food entrainable oscillator (FEO) with the potential to synchronise clock gene oscillations in metabolically important tissues such as the liver is an area of considerable interest. Previous reports in a number of teleosts have reported the presence of FEOs in teleosts. In the absence of photic cues *Per1* expression in the liver is hypothesised to be dependent on feeding time in the zebrafish (Sanchez & Sanchez-Vazquez 2009; Lopez-Olmeda et al. 2010). In the goldfish FEOs have been implicated in the regulation of clock gene oscillations and locomoter activity (Feliciano et al. 2011; Vera et al. 2007). In mammals restricted feeding in both mice and rats has resulted in altered clock gene expression between the brain and the liver (Damiola et al. 2000; Stokkan et al. 2001). Whether differential expression between the brain and the liver in the Atlantic salmon can be explained by feeding time is unknown. The presence of a food entrainable oscillator in the liver could not explain the differential expression of *Clock* and *Per1* as a result of exposure to different photic conditions. Results from the Atlantic salmon seasonal and peripheral expression trials clearly demonstrate the necessity for further research into the presence and entrainment of peripheral clocks in teleosts as no clear generalisations can be made between species or tissue type. Clearly, the study of clock mechanisms in fish is in its early days.

Future investigations within this field are necessary to investigate the role of non photic zeitgeber signals and unravel the molecular mechanisms driving daily and seasonal entrainment. Potential further investigation would benefit from more exhaustive tissue sampling over a 24h period, including functionally important tissues such as the heart,

kidney, gut, spleen or eye. More specifically with regard to the liver the existence of FEO and entrainment of clock gene expression in the liver could be established under controlled photic and temperature conditions and the application of restricted feeding as has previously been done in rodents (Damiola et al. 2000; Stokkan et al. 2001). Unravelling the mechanisms driving feeding entrainment is also of considerable interest. As yet the mechanisms that synchronise feeding entrainment and food availability to the molecular clockwork of the peripheral tissues are not described. In mammals a number of pathways have been hypothesised, including temperature (Schmutz et al. 2012), glucocorticoid signalling (Le Minh et al. 2001) and peroxisome proliferator-activated receptors (PPARs) (Asher et al. 2010). Moreover food metabolites and the hormones secreted by the feeding/ fasting cycle have been suggested as entrainment mechanisms (Damiola et al. 2000; Schmutz et al. 2012). In mammals tight coupling of the cell cycle in peripheral tissues and the molecular mechanisms of the clock are apparent. In particular the intracellular redox state of the cell is thought to mediate circadian rhythms in the liver. Nicotinamide adenine dinucleotide (NAD^+) dependent protein deacetylase SIRT1 and NAD itself, via regulation of *Cry1*, appear to be involved in the resetting of the clock in mammals (Asher et al. 2008). In teleosts potential mechanisms that mediate food and feeding entrainment of clock gene expression in the liver are unknown. However the suggested pathways of entrainment in mammals could be investigated in the Atlantic salmon.

To this end Chapter 6 set out as one of the first investigations linking clocks to functional physiological processes in teleosts. This chapter investigated the endogenous clock mediated regulation of cholesterol homeostasis and lipid metabolism in the liver of the Atlantic salmon. This is of particular importance to the aquaculture industry as the need for sustainable alternative feed sources for cultured species increases. Ultimately if the pathways involved in the entrainment of appetite and lipid metabolism and deposition can be determined then the

use of feed in culture can be optimised to align with the endogenous regulation of fat deposition with the economically optimal utilisation of nutrients. As reported in rodents results demonstrated significant circadian expression of some liver lipid and clock genes (*Srebp 1* Lxr and *Bmal 1)*. However in contrast to mammals, *Per1, Per2, Fas,* and *Reverb* α did not display significant circadian rhythmicity in salmon. Future work in this field should take the form of a more exhaustive investigation where nutritional factors and levels of fatty acid and cholesterol are controlled and effects in relation to clock gene and liver lipid gene expression are analysed. Moreover microarray or next generation sequencing technology could be utilised in order to identify circadian regulation of the liver transcriptome under different conditions. Future research in this area would be of considerable interest not only to circadian biologists but also the aquaculture industry.

Overall these doctoral studies have advanced knowledge on clock genes regulation and seasonality in the Atlantic salmon. Results from the current investigation provide new insight supporting a number of theories on the circadian and seasonal control of physiology at a molecular level in salmon. Further work is now required to confirm or reject hypotheses raised during this PhD

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APPENDIX

APPENDIX

Appendix 1 List of microarray probes with a P value of less than 0.001 with regard to photoperiod (LD Vs SD), Day vs. night and photoperiod/day night interaction.

Appendix 1a: Microarray targets (P<0.001) photoperiod.

Probe	Blast type	Blast results	Photoperiod P value	Photoperiod Fold change
Ssa#CX357917	RefSeq_Hit Def	Salmo salar DnaJ homolog subfamily C member 3 (dnjc3), mRNA >gi|209156215|gb|BT046078.1| Salmo salar clone ssal-rgf-541-017 DnaJ homolog subfamily C member 3 precursor putative mRNA, complete cds	4.34271E-07	1.3597882
Ssa#S35505093	RefSeq_Hit Def	Oryzias latipes geminin (LOC100049517), mRNA	1.69469E-06	1.3579277
Ssa#STIR26041	RefSeq_Hit Def	Salmo salar Sulfate transporter (s26a2), mRNA >gi|223648201|gb|BT059146.1| Salmo salar clone ssal-rgf-528-110 Sulfate transporter putative mRNA, complete cds	3.24503E-06	1.3364023
Ssa#STIR00009_3	RefSeq_Hit Def	Salmo salar interferon alpha 2 (ifna2), mRNA >gi|37499750|gb|AY216595.1| Salmo salar interferon alpha 2 (IFNA2) mRNA, complete cds	4.81148E-06	1.9038987
Ssa#TC90520	RefSeq_Hit Def	Danio rerio sestrin 1 (sesn1), mRNA >gi|49900305|gb|BC076550.1| Danio rerio sestrin 1, mRNA (cDNA clone MGC:91970 IMAGE:7041961), complete cds	8.64328E-06	-1.2405695
Ssa#S18889873	RefSeq_Hit Def	Salmo salar Stromal cell-derived factor 2-like protein 1 (sdf2l), mRNA >gi|209735729|gb|BT048933.1| Salmo salar clone ssal-rgb2-576-085 Stromal cell-derived factor 2-like protein 1 precursor putative mRNA, complete cds	8.93747E-06	1.2741431
Ssa#S18888857	B2GO_BlastnHit	taeniopygia guttata misc_rna miscrna	1.10187E-05	1.5867685
Ssa#TC112586	RefSeq_Hit Def	Salmo salar StAR-related lipid transfer protein 7 (star7), mRNA >gi|223649091|gb|BT059591.1| Salmo salar clone ssal-rgf-527-298 StAR-related lipid transfer protein 7 putative mRNA, complete cds	2.61508E-05	-1.2438473
Omy#S153303	RefSeq_Hit	Salmo salar p21-activated protein kinase-interacting protein 1-like	3.07212E-05	1.1935716

52	Def	(pk1ip), mRNA >gi|221220677|gb|BT057128.1| Salmo salar clone ssal-eve-528-068 p21-activated protein kinase-interacting protein 1-like putative mRNA, complete cds		
Ssa#TC100565	B2GO_BlastxHit	novel protein similar to galactosidase, beta 1 (GLB1, zgc:110823)	3.11393E-05	-1.4600939
Ssa#STIR24402	RefSeq_Hit Def	PREDICTED: Meleagris gallopavo jmjC domain-containing protein 8-like (LOC100547031), mRNA	3.8124E-05	1.3847582
Ssa#STIR14012	RefSeq_Hit Def	PREDICTED: Oreochromis niloticus ADP-ribosylation factor 6-like (LOC100704590), mRNA	3.92337E-05	1.180091
Ssa#S30241991	RefSeq_Hit Def	Salmo salar Mitotic spindle assembly checkpoint protein MAD2A (md2l1), mRNA >gi|209731291|gb|BT046714.1| Salmo salar clone ssal-rgb2-630-192 Mitotic spindle assembly checkpoint protein MAD2A putative mRNA, complete cds	4.69544E-05	1.5237253
Ssa#S30259657	RefSeq_Hit Def	Salmo salar Glyoxalase domain-containing protein 5 (glod5), mRNA >gi|209737605|gb|BT049871.1| Salmo salar clone ssal-rgb2-546-081 Glyoxalase domain-containing protein 5 putative mRNA, complete cds	5.73194E-05	1.5225122
Ssa#STIR09633	RefSeq_Hit Def	PREDICTED: Oreochromis niloticus heterogeneous nuclear ribonucleoprotein U-like (LOC100696161), mRNA	5.76409E-05	1.1871353
Ssa#STIR22167	B2GO_BlastxHit	ornithine decarboxylase 1	7.28839E-05	1.6053214
Ssa#STIR22851	RefSeq_Hit Def	PREDICTED: Oreochromis niloticus leucine-rich repeat-containing protein 58-like (LOC100702566), mRNA	7.29958E-05	1.3747792
Ssa#STIR17188	B2GO_BlastnHit	Gasterosteus aculeatus clone CNB77-A04 mRNA sequence	7.62252E-05	1.5995821
Ssa#S18890581	B2GO_BlastxHit	Hypothetical LOC559844	8.0997E-05	1.3982209
Ssa#S35663967	RefSeq_Hit Def	Drosophila simulans GD18867 (Dsim\GD18867), mRNA	8.88836E-05	-1.2218877
Ssa#STIR05038	RefSeq_Hit Def	Salmo salar DnaJ homolog subfamily C member 8 (dnjc8), mRNA >gi|209735127|gb|BT048632.1| Salmo salar clone ssal-evf-515-147 DnaJ homolog subfamily C member 8 putative mRNA, complete cds	9.68578E-05	1.1240298
Ssa#S35701995	B2GO_BlastxHit	Replication protein A 32 kDa subunit	9.83721E-05	1.3331934
Ssa#S18892255	RefSeq_Hit Def	Salmo salar interferon alpha 2 (ifna2), mRNA >gi|37499750|gb|AY216595.1| Salmo salar interferon alpha 2 (IFNA2)	9.90275E-05	2.1395192

		mRNA, complete cds		
Omy#CX144324	RefSeq_HitDef	PREDICTED: Cricetulus griseus dnaJ homolog subfamily B member 5-like (LOC100750493), miscRNA	0.000101577	1.4883635
Ssa#S31968671	B2GO_BlastnHit	bos taurus cysteine-rich with egf-like domains 1 complete cds	0.00010715	1.1170424
Ssa#S30293065	B2GO_BlastxHit	canopy homolog 2 precursor	0.000122221	1.3484056
Omy#S34423517	RefSeq_HitDef	Danio rerio kinesin family member 1B (kif1b), mRNA >gi|224830809|gb|FJ756939.1| Danio rerio strain TL Kif1b beta (kif1b) mRNA, complete cds	0.000124395	-1.4643961
Ssa#S31964109	RefSeq_HitDef	Salmo salar XIAP-associated factor 1 (xaf1), mRNA >gi|209737269|gb|BT049703.1| Salmo salar clone ssal-eve-575-311 XIAP-associated factor 1 putative mRNA, complete cds	0.000141673	2.5339084
Ssa#STIR18513	RefSeq_HitDef	Salmo salar Hydroxyacylglutathione hydrolase (glo2), mRNA >gi|209156247|gb|BT046094.1| Salmo salar clone ssal-rgf-541-193 Hydroxyacylglutathione hydrolase putative mRNA, complete cds	0.000144015	-1.3308626
Ssa#STIR00030_2	B2GO_BlastxHit	tumor necrosisalpha-induced protein 8-like protein 2	0.000155837	1.3888503
Omy#TC156750	B2GO_BlastnHit	mus musculus protein kinase c and casein kinase substrate in neurons 2 transcript variant mrna	0.000165898	-1.4772913
Ssa#S30278680	B2GO_BlastxHit	novel protein similar to vertebrate cell division cycle associated 7 (CDCA7, zgc:110113)	0.000167443	1.7135794
Ssa#STIR11202	RefSeq_HitDef	PREDICTED: Taeniopygia guttata misc_RNA (LOC100224413), miscRNA	0.000172111	1.3165977
Omy#S18162995	B2GO_BlastxHit	plectin 1	0.000199266	-1.3959866
Ssa#KSS2070	RefSeq_HitDef	PREDICTED: Oreochromis niloticus aminoacyl tRNA synthase complex-interacting multifunctional protein 1-like (LOC100705518), mRNA	0.000204141	1.1539145
Ssa#S18892256	RefSeq_HitDef	Salmo salar interferon alpha 1 (ifna1), mRNA >gi|37499748|gb|AY216594.1| Salmo salar interferon alpha 1 (IFNA1) mRNA, complete cds	0.00020507	2.2536354
Ssa#S35698416	RefSeq_HitDef	Sus scrofa MAK16 homolog (S. cerevisiae) (MAK16), mRNA	0.000220661	1.1865376
Ssa#S35481736	B2GO_Blas	plectin 1	0.000250279	-1.1682231

	tnHit			
Ssa#STIR22352	B2GO_BlastxHit	f-box protein 5	0.000252149	1.6575229
Ssa#S31990935	B2GO_BlastnHit	homo sapiens subfamily member 5 transcript variant mrna	0.000254525	1.4775491
Omy#CX034437	B2GO_BlastnHit	danio rerio zgc:175139 (zgc:175139) mrna	0.000261279	7.3411126
Ssa#S35606171	RefSeq_HitDef	Salmo salar HGV2 protein (hgv2), mRNA >gi|223672950|gb|BT060297.1| Salmo salar clone ssal-rgh-513-188 HGV2 putative mRNA, complete cds	0.000266111	1.246705
Ssa#STIR18839	RefSeq_HitDef	Salmo salar neutrophil cytosolic factor 1 (ncf1), mRNA >gi|209156151|gb|BT046046.1| Salmo salar clone ssal-rgf-540-075 SH3 and PX domain-containing protein 2A putative mRNA, complete cds	0.000279317	-1.237874
Omy#BX299135	B2GO_BlastnHit	rattus norvegicus transcript variant 1 mrna	0.000285092	-1.7144196
Ssa#S35663388	RefSeq_HitDef	Salmo salar thymocyte nuclear protein 1 (thyn1), mRNA >gi|209734429|gb|BT048283.1| Salmo salar clone ssal-evd-561-107 Thymocyte nuclear protein 1 putative mRNA, complete cds	0.000294519	1.316333
Ssa#KSS1929	RefSeq_HitDef	Salmo salar immediate early response 2 (ier2), mRNA >gi|209154611|gb|BT045276.1| Salmo salar clone ssal-rgf-517-071 Immediate early response gene 2 protein putative mRNA, complete cds	0.000301916	1.8813386
Ssa#S32000646	RefSeq_HitDef	Salmo salar Amyotrophic lateral sclerosis 2 chromosomal region candidate gene 4 protein (al2s4), mRNA >gi|304376642|gb|BT048119.2| Salmo salar clone ssal-rgb2-639-071 Amyotrophic lateral sclerosis 2 chromosomal region candidate gene 4 protein putative mRNA, complete cds	0.000307145	1.3308146
Ssa#STIR00072_4	B2GO_BlastxHit	CNS0GHZ1Tetraodon nigroviridis full-length cDNA	0.000309485	9.332867
Ssa#STIR15209	B2GO_BlastnHit	Salmo salar clone ssal-rgf-514-134, novel cds	0.000309692	1.3521045
Ssa#STIR11473	B2GO_BlastxHit	Salmo salar clone ssal-rgf-535-298 Nuclear factor interleukin-3-regulated protein putative mRNA, complete cds	0.000313191	1.1695461
Ssa#S18867054	RefSeq_HitDef	Salmo salar hexosaminidase A (alpha polypeptide) (hexa), mRNA >gi|209155853|gb|BT045897.1| Salmo salar clone ssal-rgf-536-069	0.00031378	1.335907

		Beta-hexosaminidase alpha chain precursor putative mRNA, complete cds		
Ssa#S35479035	B2GO_BlastxHit	Transcription factor jun-D	0.00032105	1.4772568
Ssa#STIR24828	RefSeq_Hit Def	Salmo salar Influenza virus NS1A-binding protein homolog A (ns1ba), mRNA >gi|223647697|gb|BT058894.1| Salmo salar clone ssal-rgf-511-317 Influenza virus NS1A-binding protein homolog A putative mRNA, complete cds	0.000324203	1.1333947
Ssa#STIR23746	RefSeq_Hit Def	Salmo salar Abhydrolase domain-containing protein FAM108B1 (f108b), mRNA >gi|223648771|gb|BT059431.1| Salmo salar clone ssal-rgf-535-234 Abhydrolase domain-containing protein FAM108B1 precursor putative mRNA, complete cds	0.000324644	1.1515381
Ssa#STIR09223	RefSeq_Hit Def	Oncorhynchus mykiss low molecular mass protein 2 (lmp2/d), mRNA >gi|5823091|gb|AF115540.1|AF115540 Oncorhynchus mykiss low molecular mass protein 2 (LMP2/d) mRNA, complete cds	0.000333325	1.7300459
Ssa#STIR26221	RefSeq_Hit Def	Salmo salar xeroderma pigmentosum, complementation group A (xpa), mRNA >gi|209734029|gb|BT048083.1| Salmo salar clone ssal-evd-532-367 DNA-repair protein complementing XP-A cells homolog putative mRNA, complete cds	0.000343195	1.2263672
Ssa#S48396675	B2GO_BlastnHit	gasterosteus aculeatus clone cnb156-d12 mrna sequence	0.000352369	1.3147539
Ssa#S18892114	B2GO_BlastxHit	novel protein similar to H.sapiens LGTN, ligatin (LGTN, zgc:63669)	0.00035723	1.179697
Ssa#S35538913	RefSeq_Hit Def	Salmo salar B-cell CLL/lymphoma 7A (bcl7a), mRNA >gi|209736733|gb|BT049435.1| Salmo salar clone ssal-eve-551-310 B-cell CLL/lymphoma 7 protein family member A putative mRNA, complete cds	0.000375406	1.1157467
Ssa#S35523441	B2GO_BlastxHit	slowmo homolog 2	0.000423879	1.2136298
Ssa#STIR11316	RefSeq_Hit Def	Salmo salar Retrovirus-related Pol polyprotein from transposon 17.6 (pol3), mRNA >gi|209153405|gb|BT044897.1| Salmo salar clone ssal-rgf-506-338 Retrovirus-related Pol polyprotein from transposon 17.6 putative mRNA, complete cds	0.000438722	1.2014903
Ssa#S48420820	B2GO_BlastnHit	PREDICTED: hypothetical LOC571373	0.000460623	-1.4504169

Ssa#S35688826	B2GO_BlastnHit	tetraodon nigroviridis full-length cdna	0.000475488	1.1498079
Ssa#STIR19614	RefSeq_HitDef	PREDICTED: Bos taurus mitochondrial import inner membrane translocase subunit Tim8 A-like (LOC781462), miscRNA >gi|297477814|ref|XR_083735.1| PREDICTED: Bos taurus mitochondrial import inner membrane translocase subunit Tim8 A-like (LOC781462), miscRNA	0.0004764	1.1715822
Ssa#S31964618	RefSeq_HitDef	Salmo salar Coiled-coil-helix-coiled-coil-helix domain-containing protein 1 (chch1), mRNA >gi|209731993|gb|BT047065.1| Salmo salar clone ssal-plnb-016-050 Coiled-coil-helix-coiled-coil-helix domain-containing protein 1 putative mRNA, complete cds	0.000492144	1.2203999
Ssa#STIR09872	RefSeq_HitDef	Danio rerio zgc:92744 (zgc:92744), mRNA >gi|60551986|gb|BC090909.1| Danio rerio zgc:92744, mRNA (cDNA clone MGC:103695 IMAGE:7263700), complete cds	0.000495066	1.2589003
Ssa#S35483299	RefSeq_HitDef	Salmo salar Ribonuclease T2 (rnt2), mRNA >gi|209733367|gb|BT047752.1| Salmo salar clone ssal-eve-558-088 Ribonuclease T2 precursor putative mRNA, complete cds	0.000495789	1.4445971
Ssa#S35667484	B2GO_BlastxHit	FK506-binding protein 11 precursor	0.000496809	1.3845326
Ssa#STIR07766	RefSeq_HitDef	Danio rerio glycogen synthase kinase binding protein (gbp), mRNA >gi|4240382|gb|AF060499.1|AF060499 Danio rerio glycogen synthase kinase binding protein (gbp) mRNA, complete cds	0.000513417	1.1126817
Ssa#S30242995	RefSeq_HitDef	Salmo salar CDC45-related protein (cc45l), mRNA >gi|209154837|gb|BT045389.1| Salmo salar clone ssal-rgf-520-024 CDC45-related protein putative mRNA, complete cds	0.000513671	1.920305
Omy#S19710910	B2GO_BlastxHit	si:dkey-189g17.2	0.000525142	-2.0448813
Ssa#S31984993	B2GO_BlastxHit	Cdc42 guanine exchange factor 9	0.000529996	-1.2590157
Ssa#KSS2969	RefSeq_HitDef	Salmo salar proteasome (prosome, macropain) inhibitor subunit 1 (psmf1), mRNA >gi|197632108|gb|BT043663.1| Salmo salar clone HM5_1345 proteasome (prosome, macropain) inhibitor subunit 1 (psmf1) mRNA, complete cds	0.000543516	1.1183414
Ssa#S35657423	B2GO_BlastnHit	taeniopygia guttata solute carrier family 23 (nucleobase transporters) member 2 mrna	0.000544248	-1.3034041

Ssa#TC80656	B2GO_BlastnHit	danio rerio deah (asp-glu-ala-his) box polypeptide 30 mrna	0.000559073	1.215863
Ssa#S35516272	B2GO_BlastxHit	danio rerio deah (asp-glu-ala-his) box polypeptide 30 mrna	0.000573261	1.1896373
Ssa#S30257510	RefSeq_Hit Def	Salmo salar 26S proteasome non-ATPase regulatory subunit 14 (psde), mRNA >gi|209736701|gb|BT049419.1| Salmo salar clone ssal-eve-510-036 26S proteasome non-ATPase regulatory subunit 14 putative mRNA, complete cds	0.000577046	1.1119843
Ssa#S37438815	RefSeq_Hit Def	Salmo salar CD3zeta-2 (LOC100136514), mRNA >gi|126362105|gb|EF421413.1| Salmo salar CD3zeta-2 mRNA, complete cds	0.000579029	1.4446131
Omy#S18143547	B2GO_BlastxHit	Cell growth-regulating nucleolar protein	0.000606254	1.2334342
Ssa#STIR03400	RefSeq_Hit Def	Salmo salar RING finger protein 181 (rn181), mRNA >gi|209735201|gb|BT048669.1| Salmo salar clone ssal-evd-554-047 RING finger protein 181 putative mRNA, complete cds	0.00066624	1.2444069
Ssa#STIR24672	RefSeq_Hit Def	Plasmodium yoelii yoelii str. 17XNL hypothetical protein (PY01084) partial mRNA 0	0.000683814	1.1371344
Ssa#STIR18396	B2GO_BlastnHit	Salmo salar neurogranin, TIP41-like protein (TIP41), MHC class II antigen beta chain (Sasa-DBB), MHC class II antigen alpha chain (Sasa-DBA), leucine rich repeat containing 35-like protein, and alpha-tectorin-like protein genes, complete cds	0.000693882	2.1171832
Ssa#KSS4925	B2GO_BlastxHit	FAM133	0.000708143	-1.1002774
Ssa#CL300Ctg1	RefSeq_Hit Def	Oncorhynchus mykiss low molecular mass protein 2 (lmp2/d), mRNA >gi|5823091|gb|AF115540.1|AF115540 Oncorhynchus mykiss low molecular mass protein 2 (LMP2/d) mRNA, complete cds	0.000715702	1.6027347
Ssa#STIR15964	B2GO_BlastnHit	Salmo salar clone 251P16 TCR-alpha/delta locus, genomic sequence	0.000719516	1.4593086
Ssa#S48415453_S	RefSeq_Hit Def	PREDICTED: Oreochromis niloticus phosphofurin acidic cluster sorting protein 1-like (LOC100712551), mRNA	0.000747729	-1.3209413
Ssa#STIR16805	RefSeq_Hit Def	Plasmodium berghei strain ANKA hypothetical protein (PB405526.00.0) partial mRNA 0	0.000751301	1.3397809

Ssa#S35605422	B2GO_BlastxHit	Kinetochore protein Spc25	0.00075551	1.5677475
Omy#S34314958	B2GO_BlastxHit	F-box protein 43	0.000757728	-2.2802875
Ssa#TC89601	RefSeq_Hit Def	PREDICTED: Canis lupus familiaris dual specificity phosphatase 4 (DUSP4), mRNA	0.000768498	1.4027525
Ssa#S35582968	RefSeq_Hit Def	PREDICTED: Oreochromis niloticus 4-hydroxybenzoate polyprenyltransferase, mitochondrial-like (LOC100708557), mRNA	0.000774685	1.217724
Ssa#CB513260	RefSeq_Hit Def	Mus musculus zinc finger protein 395 (Zfp395), mRNA	0.000785561	-1.6962222
Ssa#S18889609	B2GO_BlastxHit	Mitochondrial 39S ribosomal protein L3	0.000786205	1.1745192
Ssa#STIR07847	B2GO_BlastxHit	proliferating cell nuclear antigen	0.000802636	1.1788188
Ssa#STIR11638	RefSeq_Hit Def	PREDICTED: Taeniopygia guttata similar to solute carrier family 9 (sodium/hydrogen exchanger), isoform 3 regulator 1 (LOC100221704), mRNA	0.000807623	-1.2586586
Ssa#S30290706	B2GO_BlastxHit	Elongation factor Ts, mitochondrial precursor	0.000814043	1.1833819
Ssa#TC77494	RefSeq_Hit Def	Salmo salar 26S protease regulatory subunit S10B (prs10), mRNA >gi|209732643|gb|BT047390.1| Salmo salar clone ssal-rgb2-649-197 26S protease regulatory subunit S10B putative mRNA, complete cds	0.000818377	1.1397179
Ssa#STIR20713	RefSeq_Hit Def	Salmo salar Transmembrane protein 163 (tm163), mRNA >gi|209154219|gb|BT045080.1| Salmo salar clone ssal-rgf-511-188 Transmembrane protein 163 putative mRNA, complete cds	0.000827199	1.3631165
Ssa#STIR13580	RefSeq_Hit Def	Salmo salar acid phosphatase-like 2 (acpl2), mRNA >gi|209154093|gb|BT045017.1| Salmo salar clone ssal-rgf-509-370 Acid phosphatase-like protein 2 precursor putative mRNA, complete cds	0.00083703	1.155967
Ssa#DW543929	B2GO_BlastxHit	ubiquitin specific protease 16	0.000841251	1.2353141
Ssa#STIR22870	B2GO_BlastxHit	g-protein coupled receptor 173	0.000847638	-1.1705192
Ssa#S35505196	B2GO_BlastnHit	strongylocentrotus purpuratus kiaa1404 protein mrna	0.000872327	1.7846395

Ssa#S30240713	B2GO_BlastxHit	TRAF interacting protein TANK	0.000875307	1.2602183
Ssa#STIR42027	B2GO_BlastxHit	PREDICTED: hypothetical protein LOC497376	0.000886621	2.0059104
Ssa#DW006200	B2GO_BlastxHit	JAK1_MOUSERecName: Full=Tyrosine-protein kinase JAK1; AltName: Full=Janus kinase 1; Short=JAK-1	0.000890764	1.4455134
Ssa#S31979342	RefSeq_HitDef	Oncorhynchus mykiss LBP (LPS binding protein)/BPI (bactericidal/permeability-increasing protein)-1 (lbp/bpi-1), mRNA >gi|20387084|dbj|AB042025.1| Oncorhynchus mykiss LBP/BPI-1 mRNA for LBP (LPS binding protein)/BPI (bactericidal/permeability-increasing protein)-1, complete cds	0.000910454	1.4020058
Ssa#S30277130	RefSeq_HitDef	PREDICTED: Oreochromis niloticus cell division cycle-associated protein 7-like (LOC100695219), mRNA	0.000911391	1.556224
Ssa#STIR06082	RefSeq_HitDef	Salmo salar Butyrate response factor 1 (tisb), mRNA >gi|209155825|gb|BT045883.1| Salmo salar clone ssal-rgf-535-262 Butyrate response factor 1 putative mRNA, complete cds	0.000918498	1.3535284
Ssa#STIR22544	RefSeq_HitDef	Salmo salar uncharacterized LOC100194706 (LOC100194706), mRNA >gi|198285548|gb|BT043996.1| Salmo salar clone HM4_1183 hypothetical protein mRNA, complete cds	0.00092809	-1.3084229
Ssa#STIR25249	RefSeq_HitDef	Oncorhynchus mykiss succinate-CoA ligase GDP-forming alpha subunit (LOC100301669), mRNA >gi|223049432|gb|FJ607869.1| Oncorhynchus mykiss succinate-CoA ligase GDP-forming alpha subunit mRNA, complete cds	0.00093096	1.3721848
Ssa#S30275617	B2GO_BlastxHit	Hypothetical LOC559844	0.000931994	1.35186
Ssa#STIR15943	RefSeq_RNA	Sorghum bicolor hypothetical protein, mRNA 0	0.000938259	1.2566497
Ssa#KSS1926	RefSeq_HitDef	Salmo salar CL012 protein (cl012), mRNA >gi|221220325|gb|BT056952.1| Salmo salar clone ssal-evd-522-337 C12orf12 homolog putative mRNA, complete cds	0.000939976	-1.2184137
Ssa#S18888540	RefSeq_HitDef	Salmo salar MCM2 minichromosome maintenance deficient 2, mitotin (S. cerevisiae) (mcm2), mRNA >gi|223648101|gb|BT059096.1| Salmo salar clone ssal-rgf-525-100 DNA replication licensing factor mcm2 putative mRNA, complete cds	0.000943906	1.4045998

Ssa#S35555013	RefSeq_HitDef	Salmo salar eukaryotic translation initiation factor 4e 1a (eif4e1a), mRNA >gi|197631908|gb|BT043563.1| Salmo salar clone HM4_2316 eukaryotic translation initiation factor 4e 1a (eif4e1a) mRNA, complete cds	0.000951903	1.5291076
Ssa#STIR05647	B2GO_BlastxHit	cyclin-dependent kinase inhibitor 1c	0.000952302	-1.1979393
Ssa#STIR09453	RefSeq_HitDef	Salmo salar UDP-xylose and UDP-N-acetylglucosamine transporter (s35b4), mRNA >gi|209155471|gb|BT045706.1| Salmo salar clone ssal-rgf-529-070 UDP-xylose and UDP-N-acetylglucosamine transporter putative mRNA, complete cds	0.000986975	1.2851709
Ssa#STIR08978	RefSeq_HitDef	Salmo salar eukaryotic translation initiation factor 4e 1a (eif4e1a), mRNA >gi|197631908|gb|BT043563.1| Salmo salar clone HM4_2316 eukaryotic translation initiation factor 4e 1a (eif4e1a) mRNA, complete cds	0.000987007	1.5538536
Ssa#STIR25602	RefSeq_RNA	PREDICTED: Cricetulus griseus hypothetical protein LOC100763491 (LOC100763491), mRNA 0	0.000989892	-1.1491168
Ssa#STIR05514	RefSeq_HitDef	PREDICTED: Taeniopygia guttata hypothetical protein LOC100226116 (LOC100226116), mRNA	0.000993063	1.1572293

Appendix 1b: Microarray features (P< 0.001) Day vs. Night

Probe Name	Blast Type	Blast result	p-value Day/Night	Fold change DAY vs Night
Ssa#S18888857	B2GO_BlastnHit	taeniopygia guttata misc_rna miscrna	2.19E-09	-2.7527103
Omy#S18159750	RefSeq_HitDef	PREDICTED: Oreochromis niloticus hairy and enhancer of split-related protein helt-like (LOC100699318), mRNA	5.29E-09	-1.6576612
Ssa#STIR17188	B2GO_BlastnHit	Gasterosteus aculeatus clone CNB77-A04 mRNA sequence	7.80363E-08	-2.5095983
Omy#CX144324	RefSeq_HitDef	PREDICTED: Cricetulus griseus dnaJ homolog subfamily B member 5-like (LOC100750493), miscRNA	9.54753E-08	-2.635506
Ssa#S31990935	B2GO_BlastnHit	homo sapiens subfamily member 5 transcript variant mrna	1.36701E-07	-2.3011987
Ssa#S35479035	B2GO_BlastxHit	Transcription factor jun-D	1.6395E-07	-2.7889938
Ssa#S35547571	RefSeq_HitDef	Salmo salar Krueppel-like factor 2 (klf2), mRNA >gi|209150946|gb|BT044789.1| Salmo salar clone ssal-rgf-503-311 Krueppel-like factor 2 putative mRNA, complete cds	3.41669E-07	-1.755981
Ssa#STIR21014	RefSeq_ RNA	PREDICTED: Sus scrofa ras and Rab interactor 2-like (LOC100625300), mRNA 0	4.09511E-07	-1.3780068
Ssa#STIR19561	B2GO_BlastnHit	Gasterosteus aculeatus clone CH213-128O17, complete sequence	4.58192E-07	-2.1490364
Ssa#S35559072	B2GO_BlastxHit	Transcription factor jun-D	5.16809E-07	-2.1257377
Ssa#STIR22687	B2GO_BlastxHit	fos-like antigen 2	1.29385E-06	-2.5248806
Ssa#TC89601	RefSeq_HitDef	PREDICTED: Canis lupus familiaris dual specificity phosphatase 4 (DUSP4), mRNA	1.50468E-06	-1.9386196
Ssa#STIR077	RefSeq_HitDef	Danio rerio glycogen synthase kinase binding protein (gbp), mRNA	1.60343E-06	-1.2603525

66		>gi|4240382|gb|AF060499.1|AF060499 Danio rerio glycogen synthase kinase binding protein (gbp) mRNA, complete cds		
Ssa#STIR11473	B2GO_BlastxHit	nuclear factor interleukin-3-regulated protein	1.88686E-06	-1.307705
Ssa#STIR21137	B2GO_BlastxHit	axin1 up-regulated 1	2.24043E-06	-2.1064465
Omy#S34313882	RefSeq_HitDef	PREDICTED: Oreochromis niloticus fos-related antigen 2-like (LOC100693783), mRNA	5.59755E-06	-2.0284927
Ssa#STIR03176	RefSeq_HitDef	Oncorhynchus mykiss regulator of G-protein signalling 1 (rgs1), mRNA >gi|225705377|gb|BT074111.1| Oncorhynchus mykiss clone omyk-evo-508-055 Regulator of G-protein signalling 1 putative mRNA, complete cds	5.9233E-06	-3.093505
Ssa#STIR26242	RefSeq_HitDef	Danio rerio glycogen synthase kinase binding protein (gbp), mRNA >gi|4240382|gb|AF060499.1|AF060499 Danio rerio glycogen synthase kinase binding protein (gbp) mRNA, complete cds	6.90721E-06	-1.2014744
Ssa#STIR07425	RefSeq_HitDef	Oncorhynchus mykiss regulator of G-protein signalling 1 (rgs1), mRNA >gi|225705377|gb|BT074111.1| Oncorhynchus mykiss clone omyk-evo-508-055 Regulator of G-protein signalling 1 putative mRNA, complete cds	9.92858E-06	-2.238353
Ssa#S30295557	RefSeq_HitDef	Salmo salar myc target 1 (myct1), mRNA >gi|221220719|gb|BT057149.1| Salmo salar clone ssal-rgb2-575-330 Myc target protein 1 homolog putative mRNA, complete cds	1.96193E-05	-1.5723212
Ssa#STIR23362	RefSeq_HitDef	Salmo salar processing of precursor 5, ribonuclease P/MRP subunit (S. cerevisiae) (pop5), mRNA >gi|209735235|gb|BT048686.1| Salmo salar clone ssal-evf-514-117 Ribonuclease P/MRP protein subunit POP5 putative mRNA, complete cds	2.19095E-05	1.4105287
Ssa#S30291861	B2GO_BlastxHit	cofactor required for Sp1 transcriptional activation, subunit 8	2.20765E-05	1.2072647
Ssa#S35550804	B2GO_BlastxHit	Transcription factor jun-D	2.21619E-05	-1.5463029
Ssa#S48402625	B2GO_BlastxHit	predicted protein	2.84244E-05	1.9438599

Ssa#STIR10180	B2GO_BlastxHit	jun d proto-oncogene	2.95494E-05	-2.1189778
Ssa#STIR12647	RefSeq_HitDef	Oncorhynchus mykiss regulator of G-protein signaling 1 (rgs1), mRNA >gi|225705377|gb|BT074111.1| Oncorhynchus mykiss clone omyk-evo-508-055 Regulator of G-protein signaling 1 putative mRNA, complete cds	3.28515E-05	-2.7119741
Omy#S15286653	RefSeq_HitDef	Salmo salar SRY-box containing gene 2 (sox2), mRNA >gi|209151034|gb|BT044795.1| Salmo salar clone ssal-rgf-504-016 Transcription factor Sox-2 putative mRNA, complete cds	3.33409E-05	1.3452208
Ssa#CL382Ctg1	B2GO_BlastxHit	NF-kappa-B inhibitor alpha	3.37603E-05	-1.5018016
Ssa#STIR02511	RefSeq_HitDef	Oncorhynchus mykiss regulator of G-protein signaling 1 (rgs1), mRNA >gi|225705377|gb|BT074111.1| Oncorhynchus mykiss clone omyk-evo-508-055 Regulator of G-protein signaling 1 putative mRNA, complete cds	3.96779E-05	-2.5229366
Ssa#S30239956	B2GO_BlastxHit	NF-kappa-B inhibitor alpha	4.13226E-05	-1.7863648
Ssa#STIR12277	RefSeq_HitDef	Salmo salar Butyrate response factor 1 (tisb), mRNA >gi|209155825|gb|BT045883.1| Salmo salar clone ssal-rgf-535-262 Butyrate response factor 1 putative mRNA, complete cds	4.15788E-05	-1.4583147
Ssa#STIR08607	RefSeq_HitDef	Salmo salar Butyrate response factor 1 (tisb), mRNA >gi|209155825|gb|BT045883.1| Salmo salar clone ssal-rgf-535-262 Butyrate response factor 1 putative mRNA, complete cds	4.27796E-05	-1.3105443
Ssa#S32008293	RefSeq_HitDef	Salmo salar T-cell activation GTPase activating protein (tagap), mRNA >gi|209152907|gb|BT044873.1| Salmo salar clone ssal-rgf-506-073 T-cell activation Rho GTPase-activating protein putative mRNA, complete cds	4.29211E-05	-1.6876218
Ssa#S35685287	B2GO_BlastnHit	danio rerio hypothetical loc568734 mrna	4.55842E-05	-1.7405447
Ssa#STIR18843	RefSeq_HitDef	Salmo salar Dual specificity protein phosphatase 6 (dus6), mRNA >gi|223647661|gb|BT058876.1| Salmo salar clone ssal-rgf-509-353 Dual specificity protein phosphatase 6 putative mRNA, complete	4.67651E-05	-1.4212582

		cds		
Ssa#STIR21064	RefSeq_ RNA	Mus musculus neuropeptide FF-amide peptide precursor (Npff), transcript variant 2, non-coding RNA 0	4.72506E-05	-1.2690678
Ssa#S18890026	RefSeq_HitDef	PREDICTED: Oreochromis niloticus coenzyme Q-binding protein COQ10 homolog, mitochondrial-like (LOC100704234), mRNA	4.94725E-05	-1.894962
Ssa#S35566535	RefSeq_HitDef	Salmo salar CD4-like protein (LOC100136502), transcript variant 1, mRNA >gi|167538885|gb|EU409794.1| Salmo salar CD4-1-like protein mRNA, complete cds	4.98195E-05	-1.7715738
Ssa#S30239919	RefSeq_HitDef	PREDICTED: Oreochromis niloticus dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit-like (LOC100697727), mRNA	5.06962E-05	1.2469674
Ssa#KSS351	RefSeq_HitDef	Salmo salar Krueppel-like factor 2 (klf2), mRNA >gi|209150946|gb|BT044789.1| Salmo salar clone ssal-rgf-503-311 Krueppel-like factor 2 putative mRNA, complete cds	5.5467E-05	-1.463886
Ssa#STIR18614	RefSeq_HitDef	Salmo salar selenocysteine lyase (scly), mRNA >gi|223648997|gb|BT059544.1| Salmo salar clone ssal-rgf-511-328 Selenocysteine lyase putative mRNA, complete cds	5.63276E-05	-1.9870508
Ssa#STIR08375	RefSeq_HitDef	Salmo salar Ubiquitin-conjugating enzyme E2 variant 2 (ub2v2), mRNA >gi|209730345|gb|BT046241.1| Salmo salar clone ssal-evf-551-149 Ubiquitin-conjugating enzyme E2 variant 2 putative mRNA, complete cds	5.64781E-05	1.2151277
Ssa#S35703778	B2GO_BlastxHit	Immediate early response gene 5 protein	7.08272E-05	-1.4284151
Ssa#S18892092	RefSeq_HitDef	Salmo salar GLIPR1-like protein 1 (gprl1), mRNA >gi|209737963|gb|BT050050.1| Salmo salar clone ssal-rgb2-539-210 GLIPR1-like protein 1 precursor putative mRNA, complete cds	7.2077E-05	-1.4782301
Ssa#STIR14258	RefSeq_ RNA	PREDICTED: Hydra magnipapillata hypothetical protein LOC100204443 (LOC100204443), mRNA	7.73055E-05	1.4320724
Ssa#S35501046	RefSeq_HitDef	Oncorhynchus mykiss Group XIIA secretory phospholipase A2 (pg12a), mRNA >gi|225704885|gb|BT073865.1| Oncorhynchus mykiss clone omyk-evo-507-207 Group XIIA secretory phospholipase A2 precursor putative mRNA, complete cds	9.97931E-05	1.1515629

Ssa#S30275361	RefSeq_HitDef	Salmo salar EH-domain containing 3 (ehd3), mRNA >gi|209154681|gb|BT045311.1| Salmo salar clone ssal-rgf-518-062 EH domain-containing protein 3 putative mRNA, complete cds	0.000105953	1.3082215
Ssa#CL65Contig1	RefSeq_HitDef	Salmo salar C-X-C chemokine receptor type 4 (cxcr4), mRNA >gi|223647193|gb|BT058642.1| Salmo salar clone Contig2817 C-X-C chemokine receptor type 4 putative mRNA, complete cds	0.000131253	-1.8059281
Ssa#STIR15662	RefSeq_HitDef	Salmo salar ERBB receptor feedback inhibitor 1 (errfi), mRNA >gi|223648511|gb|BT059301.1| Salmo salar clone ssal-rgf-540-055 ERBB receptor feedback inhibitor 1 putative mRNA, complete cds	0.000139025	-1.238496
Ssa#STIR06082	RefSeq_HitDef	Salmo salar Butyrate response factor 1 (tisb), mRNA >gi|209155825|gb|BT045883.1| Salmo salar clone ssal-rgf-535-262 Butyrate response factor 1 putative mRNA, complete cds	0.000140482	-1.4623064
Ssa#STIR02585	RefSeq_HitDef	Danio rerio glycogen synthase kinase binding protein (gbp), mRNA >gi|4240382|gb|AF060499.1|AF060499 Danio rerio glycogen synthase kinase binding protein (gbp) mRNA, complete cds	0.000141513	-1.3029525
Ssa#STIR21002	RefSeq RNA	Drosophila melanogaster CG13840 (CG13840), mRNA	0.000148287	-1.2362767
Ssa#STIR13179	B2GO_BlastxHit	kruppel-like factor 9	0.000151313	-1.4936682
Ssa#STIR22553	B2GO_BlastnHit	Osmerus mordax clone omor-eva-507-053 ADP-ribosylation factor-like protein 5B putative mRNA, complete cds	0.000160632	-1.3542436
Ssa#S31981569	RefSeq_HitDef	Danio rerio heme oxygenase (decycling) 1 (hmox1), mRNA	0.00017486	-1.6563822
Ssa#STIR04448	RefSeq_HitDef	Danio rerio glycogen synthase kinase binding protein (gbp), mRNA >gi|4240382|gb|AF060499.1|AF060499 Danio rerio glycogen synthase kinase binding protein (gbp) mRNA, complete cds	0.000185447	-1.2435138
Ssa#S18886680	RefSeq_HitDef	Salmo salar Eukaryotic translation initiation factor 2C 3 (i2c3), mRNA >gi|223648891|gb|BT059491.1| Salmo salar clone ssal-rgf-513-072 Eukaryotic translation initiation factor 2C 3 putative mRNA, complete cds	0.000191131	1.1420635
Ssa#S1888870	B2GO_BlastxHit	novel protein similar to vertebrate proline rich region 18 (PRR18)	0.000200713	-1.3217409

2				
Omy#BX084067	B2GO_BlastnHit	dendrocolaptes certhia early growth response 1 exon 2 and partial cds	0.000206791	-2.9119499
Ssa#STIR02934	RefSeq_HitDef	Salmo salar ripply1 (ripp1), mRNA >gi|304376784|gb|BT049136.2| Salmo salar clone ssal-sjb-011-184 ripply1 putative mRNA, complete cds	0.000217255	1.3785561
Ssa#TC112586	RefSeq_HitDef	Salmo salar StAR-related lipid transfer protein 7 (star7), mRNA >gi|223649091|gb|BT059591.1| Salmo salar clone ssal-rgf-527-298 StAR-related lipid transfer protein 7 putative mRNA, complete cds	0.000217535	1.1887021
Ssa#S35590181	B2GO_BlastxHit	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	0.000230599	-1.7937212
Omy#BX309467	B2GO_BlastxHit	AXIN1 up-regulated 1	0.000234315	-2.5670185
Ssa#STIR05535	RefSeq_HitDef	Salmo salar potassium voltage-gated channel, Isk-related family, member 4 (kcne4), mRNA >gi|209730907|gb|BT046522.1| Salmo salar clone ssal-evd-562-054 Potassium voltage-gated channel subfamily E member 4 putative mRNA, complete cds	0.000236424	-1.4670478
Omy#S34424830	RefSeq_HitDef	PREDICTED: Anolis carolinensis midnolin-like (LOC100552822), partial mRNA	0.000242859	-2.007433
Ssa#STIR00009_3	RefSeq_HitDef	Salmo salar interferon alpha 2 (ifna2), mRNA >gi|37499750|gb|AY216595.1| Salmo salar interferon alpha 2 (IFNA2) mRNA, complete cds	0.000247907	-1.4697614
Ssa#STIR18303	RefSeq_HitDef	Salmo salar vaccinia related kinase 3 (vrk3), mRNA >gi|223647489|gb|BT058790.1| Salmo salar clone ssal-rgf-503-077 Serine/threonine-protein kinase VRK3 putative mRNA, complete cds	0.000254618	-1.151597
Ssa#STIR02171	RefSeq_HitDef	Salmo salar CCAAT/enhancer binding protein (C/EBP), delta (cebpd), mRNA >gi|209737603|gb|BT049870.1| Salmo salar clone ssal-evf-540-010 CCAAT/enhancer-binding protein delta putative mRNA, complete cds	0.000257118	-1.5041416
Ssa#EG91722	RefSeq_HitDef	Salmo salar Pleckstrin homology Sec7 and coiled-coil domains-	0.000267553	-1.7717628

0		binding protein (pscbp), mRNA >gi|209154393|gb|BT045167.1| Salmo salar clone ssal-rgf-513-381 Pleckstrin homology Sec7 and coiled-coil domains-binding protein putative mRNA, complete cds		
Ssa#STIR12498	RefSeq_HitDef	Salmo salar C-X-C motif chemokine 10 (cxl10), mRNA >gi|209733803|gb|BT047970.1| Salmo salar clone ssal-evd-534-187 C-X-C motif chemokine 10 precursor putative mRNA, complete cds	0.000268533	-1.6071593
Ssa#STIR26068	RefSeq_HitDef	Salmo salar TRAF2-binding protein (tifa), mRNA >gi|223648901|gb|BT059496.1| Salmo salar clone ssal-rgf-529-067 TRAF2-binding protein putative mRNA, complete cds	0.00027246	-1.225249
Ssa#S31979721	B2GO_BlastxHit	Cytochrome P450 20A1	0.000283607	1.2420185
Ssa#S30277290	B2GO_BlastxHit	Survival motor neuron protein 1	0.000306794	-1.1763536
Ssa#STIR29077	RefSeq_HitDef	Salmo salar DnaJ homolog subfamily C member 17 (djc17), mRNA >gi|221221903|gb|BT057741.1| Salmo salar clone ssal-rgb2-598-029 DnaJ homolog subfamily C member 17 putative mRNA, complete cds	0.000324114	-1.1516469
Ssa#S35563023	RefSeq_HitDef	Salmo salar partner of NOB1 homolog (S. cerevisiae) (pno1), mRNA >gi|209732321|gb|BT047229.1| Salmo salar clone ssal-sjb-017-162 RNA-binding protein PNO1 putative mRNA, complete cds	0.000334464	1.1158783
Ssa#STIR18230	RefSeq_HitDef	Salmo salar C-C chemokine receptor type 9 (ccr9), mRNA >gi|209156077|gb|BT046009.1| Salmo salar clone ssal-rgf-539-033 C-C chemokine receptor type 9 putative mRNA, complete cds	0.000348061	-2.1405168
Ssa#S31985678	RefSeq_HitDef	PREDICTED: Oreochromis niloticus transcription factor jun-D-like (LOC100693920), mRNA	0.000362643	-1.3625591
Ssa#STIR04546	RefSeq_HitDef	Salmo salar FAM32A-like (fa32a), mRNA >gi|209732905|gb|BT047521.1| Salmo salar clone ssal-eve-537-227 FAM32A-like putative mRNA, complete cds	0.000364193	-1.1020848
Ssa#DW543822	B2GO_BlastxHit	PREDICTED: G protein-coupled receptor 3	0.00037258	-1.8818725
Ssa#STIR136	B2GO_BlastnHit	Salmo salar clone ssal-rgf-527-229 BTG2 putative mRNA, complete	0.000377842	-1.2661139

81		cds		
Ssa#KSS4885	RefSeq_HitDef	Oncorhynchus mykiss Rhag (LOC100136711), mRNA >gi|160961518|gb|EF667352.1| Oncorhynchus mykiss Rhag mRNA, complete cds	0.000387367	1.4141538
Ssa#STIR07161	RefSeq_HitDef	Salmo salar SRY-box containing gene 2 (sox2), mRNA >gi|209151034|gb|BT044795.1| Salmo salar clone ssal-rgf-504-016 Transcription factor Sox-2 putative mRNA, complete cds	0.000388382	1.199601
Ssa#STIR12117	RefSeq_HitDef	Salmo salar jun B proto-oncogene (junb), mRNA >gi|209152284|gb|BT044843.1| Salmo salar clone ssal-rgf-505-157 Transcription factor jun-B putative mRNA, complete cds	0.000389325	-1.5340674
Ssa#S30284933	RefSeq_HitDef	Oncorhynchus mykiss Vesicle transport protein SEC20 (sec20), mRNA >gi|225705781|gb|BT074313.1| Oncorhynchus mykiss clone omyk-evo-507-334 Vesicle transport protein SEC20 putative mRNA, complete cds	0.000400854	1.1429853
Ssa#STIR16597	RefSeq_HitDef	Oncorhynchus mykiss insulin-like growth factor binding protein 3 (igfbp3), mRNA >gi|83571698|gb|DQ146966.2| Oncorhynchus mykiss insulin-like growth factor binding protein 3 (IGFBP3) mRNA, complete cds	0.000408961	-1.2581165
Omy#S34424583	RefSeq_HitDef	Salmo salar Butyrate response factor 2 (tisd), mRNA >gi|209155171|gb|BT045556.1| Salmo salar clone ssal-rgf-524-337 Butyrate response factor 2 putative mRNA, complete cds	0.000411628	1.2703125
Ssa#STIR16630	RefSeq_ RNA	Mus musculus neuropeptide FF-amide peptide precursor (Npff), transcript variant 2, non-coding RNA	0.000421923	-1.195154
Ssa#CK874004	B2GO_BlastnHit	danio rerio slit and trk like 4 protein mrna	0.000441593	1.4596949
Ssa#TC73119	RefSeq_HitDef	Oncorhynchus mykiss uncoupling protein 2B (LOC100136329), mRNA >gi|83270939|gb|DQ295328.1| Oncorhynchus mykiss uncoupling protein 2B mRNA, complete cds	0.000453838	-1.3596672
Ssa#S35555814	B2GO_BlastxHit	S100-A11	0.000458639	-1.7224529
Ssa#STIR11423	B2GO_BlastxHit	pim-1 oncogene	0.000463844	-1.3777499

Ssa#DW579347	RefSeq_HitDef	PREDICTED: Oreochromis niloticus cryptochrome-1-like (LOC100694774), mRNA	0.000488018	-1.6145815
Omy#BX870813	B2GO_BlastxHit	ZNF16	0.000502921	-1.7055439
Ssa#STIR15755	RefSeq_HitDef	Salmo salar charged multivesicular body protein 3 (chmp3), mRNA >gi|209155515|gb|BT045728.1| Salmo salar clone ssal-rgf-529-351 Charged multivesicular body protein 3 putative mRNA, complete cds	0.000503355	-1.9297497
Ssa#S35669880	RefSeq_HitDef	PREDICTED: Oreochromis niloticus probable histone deacetylase 1-B-like (LOC100711924), mRNA	0.000516899	-1.5937812
Ssa#STIR16411	RefSeq_HitDef	Salmo salar Cbp/p300-interacting transactivator 2 (cite2), mRNA >gi|209148285|gb|BT044666.1| Salmo salar clone ssal-rgf-002-160 Cbp/p300-interacting transactivator 2 putative mRNA, complete cds	0.000535478	-1.2958548
Ssa#STIR00109_4	RefSeq_HitDef	Salmo salar Catalase (cata), mRNA >gi|209155289|gb|BT045615.1| Salmo salar clone ssal-rgf-526-219 Catalase putative mRNA, complete cds	0.000539205	1.2198262
Ssa#S30240154	RefSeq_HitDef	Salmo salar small nuclear ribonucleoprotein polypeptide A (snrpa), mRNA >gi|209735589|gb|BT048863.1| Salmo salar clone ssal-evd-575-331 U1 small nuclear ribonucleoprotein A putative mRNA, complete cds	0.000541204	1.1201419
Ssa#S35694019	B2GO_BlastnHit	gallus gallus protein phosphatase regulatory subunit 3c mrna	0.000546163	1.4049615
Omy#S15262412	RefSeq_HitDef	Danio rerio immediate early response 5-like (ier5l), mRNA >gi|37590897|gb|BC059641.1| Danio rerio immediate early response 5-like, mRNA (cDNA clone MGC:73321 IMAGE:4789521), complete cds	0.000554379	1.3630253
Ssa#STIR12048	RefSeq_HitDef	Oncorhynchus mykiss SOX9 (LOC100135781), mRNA >gi|2780749|dbj|AB006448.1| Oncorhynchus mykiss SOX9 mRNA, complete cds	0.000568897	1.26207
Ssa#S31990659	RefSeq_HitDef	PREDICTED: Oreochromis niloticus partner of Y14 and mago A-like (LOC100711261), mRNA	0.00061372	1.2778387

Ssa#KSS2112	RefSeq_HitDef	Oncorhynchus mykiss Set1 complex component swd2 (swd2), mRNA >gi|225705949|gb|BT074397.1| Oncorhynchus mykiss clone omyk-evo-511-066 Set1 complex component swd2 putative mRNA, complete cds	0.000632403	1.187811
Ssa#S35581551	RefSeq_HitDef	Caenorhabditis briggsae Hypothetical protein CBG21705 (CBG21705) mRNA, complete cds	0.000660146	-1.1655862
Ssa#TC82366	RefSeq_HitDef	Oncorhynchus mykiss Growth arrest and DNA-damage-inducible protein GADD45 beta (ga45b), mRNA >gi|225703347|gb|BT073096.1| Oncorhynchus mykiss clone omyk-evn-507-243 Growth arrest and DNA-damage-inducible protein GADD45 beta putative mRNA, complete cds	0.000661091	-1.2672371
Ssa#S30290522	RefSeq_HitDef	Oncorhynchus mykiss T-cell activation Rho GTPase-activating protein (LOC100136248), mRNA >gi|51949898|gb|AY606035.1| Oncorhynchus mykiss T-cell activation Rho GTPase-activating protein mRNA, complete cds	0.000661392	-1.5076252
Ssa#STIR06600	RefSeq_HitDef	Salmo salar Interleukin-13 receptor alpha-2 chain (i13r2), mRNA >gi|209154781|gb|BT045361.1| Salmo salar clone ssal-rgf-519-098 Interleukin-13 receptor alpha-2 chain precursor putative mRNA, complete cds	0.000672303	-1.4360772
Ssa#STIR23565	RefSeq_HitDef	Salmo salar Ras association domain-containing protein 2 (rasf2), mRNA >gi|223648387|gb|BT059239.1| Salmo salar clone ssal-rgf-535-320 Ras association domain-containing protein 2 putative mRNA, complete cds	0.000676985	-1.4516588
Ssa#STIR13627	B2GO_BlastxHit	cox18 cytochrome c oxidase assembly homolog	0.000679845	1.1546925
Ssa#STIR24800	B2GO_BlastxHit	protein-interferon-inducible double stranded rna dependentrepressor of (p58 repressor)	0.000681999	2.3024435
Ssa#S35664360	RefSeq_HitDef	PREDICTED: Oreochromis niloticus regulator of G-protein signaling 1-like (LOC100707040), mRNA	0.00068337	-1.560424
Ssa#S18892255	RefSeq_HitDef	Salmo salar interferon alpha 2 (ifna2), mRNA >gi|37499750|gb|AY216595.1| Salmo salar interferon alpha 2 (IFNA2) mRNA, complete cds	0.000688507	-1.7782513

Ssa#STIR12210	RefSeq_HitDef	Salmo salar thioredoxin interacting protein (txnip), mRNA >gi|209155761|gb|BT045851.1| Salmo salar clone ssal-rgf-534-292 Thioredoxin-interacting protein putative mRNA, complete cds	0.000719704	1.3847609
Ssa#CL72Contig1	RefSeq_HitDef	Salmo salar Transaldolase (taldo), mRNA >gi|221219805|gb|BT056692.1| Salmo salar clone ssal-evf-517-302 Transaldolase putative mRNA, complete cds	0.000740438	1.2340382
Ssa#TC112786	RefSeq_HitDef	PREDICTED: Danio rerio brain-specific angiogenesis inhibitor 3-like (LOC100331635), mRNA	0.000744069	-1.320783
Ssa#STIR14663	RefSeq_HitDef	Salmo salar beta globin (LOC100136576), mRNA >gi|452794|emb|X69958.1| S.salar mRNA for beta-globin	0.000766158	-1.2322885
Ssa#STIR24329	RefSeq_RNA	PREDICTED: Oreochromis niloticus zinc finger protein 729-like (LOC100710208), mRNA	0.000766335	-1.2702931
Ssa#S35685673	B2GO_BlastxHit	NCK-associated protein 1-like	0.000838522	-1.3538387
Ssa#STIR15964	B2GO_BlastnHit	Salmo salar clone 251P16 TCR-alpha/delta locus, genomic sequence	0.000857651	1.4470521
Ssa#S30242075	B2GO_BlastnHit	unnamed protein product	0.000857935	1.284243
Ssa#S32001918	RefSeq_HitDef	Salmo salar CCL4-like chemokine (LOC100136511), mRNA >gi|126507270|gb|EF079664.1| Salmo salar CCL4-like chemokine mRNA, complete cds	0.00086156	-2.4121222
Ssa#S35673304	RefSeq_HitDef	PREDICTED: Oryctolagus cuniculus glutathione S-transferase mu 3 (LOC100356052), mRNA	0.000886549	1.1327995
Ssa#S31976370	RefSeq_HitDef	PREDICTED: Oreochromis niloticus insulinoma-associated protein 1-like (LOC100696889), mRNA	0.000903389	1.3944714
Ssa#STIR07483	B2GO_BlastnHit	M.truncatula DNA sequence from clone MTH2-59B18 on chromosome 3, complete sequence	0.000911307	-1.657095
Ssa#STIR11390	B2GO_BlastxHit	zinc finger protein 572	0.000953537	1.25753
Ssa#S35516179	B2GO_BlastnHit	gasterosteus aculeatus clone ch213- complete sequence	0.000971374	-1.4470485
Ssa#S3558277	RefSeq_HitDef	Oncorhynchus mykiss Growth arrest and DNA-damage-inducible	0.000983005	-1.3245772

7		protein GADD45 beta (ga45b), mRNA >gi|225703347|gb|BT073096.1| Oncorhynchus mykiss clone omyk-evn-507-243 Growth arrest and DNA-damage-inducible protein GADD45 beta putative mRNA, complete cds		
Ssa#S30240514	RefSeq_HitDef	Salmo salar limb and neural patterns a (lnpa), mRNA >gi|223649177|gb|BT059634.1| Salmo salar clone ssal-rgf-521-188 lunapark-A putative mRNA, complete cds	0.000987987	1.2357255

Apendix1c: Microarray targets (P< 0.001) Photoperiod/ Day night interaction.

ProbeName	Blast type	RefSeq_HitDef	p-value Photoperiod / Day Night	Fold change Photoperiod/ Day night
Ssa#S18888857	**B2GO_BlastxHit**	taeniopygia guttata misc_rna miscrna	3.76495E-05	-2.382842
Ssa#STIR25834	**RefSeq_HitDef**	PREDICTED: Oreochromis niloticus heterogeneous nuclear ribonucleoprotein L-like, transcript variant 2 (LOC100698149), mRNA	0.000111932	-1.5901239
Ssa#STIR19299	**RefSeq_RNA**	PREDICTED: Oreochromis niloticus leucine-rich repeat-containing protein 59-like (LOC100690486), mRNA	0.00023968	1.138391
Ssa#S35597250	**RefSeq_HitDef**	Oncorhynchus mykiss caspase-9 (LOC100136676), mRNA >gi|68270851|gb|DQ025755.1| Oncorhynchus mykiss caspase-9 mRNA, complete cds	0.000351221	-1.5712776
Ssa#DW577228	**RefSeq_HitDef**	PREDICTED: Danio rerio kelch-like protein 20-like (LOC556381), mRNA	0.000534406	1.5650208
Ssa#S30239693	**B2GO_BlastxHit**	novel protein	0.000665537	-1.265366
Omy#CX144324	**RefSeq_HitDef**	PREDICTED: Cricetulus griseus dnaJ homolog subfamily B member 5-like (LOC100750493), miscRNA	0.000883872	-2.4088717
Ssa#S35596271_S	**RefSeq_HitDef**	Salmo salar multiple coagulation factor deficiency 2 (mcfd2), mRNA >gi|304376408|gb|BT046423.2| Salmo salar clone ssal-evf-526-180 Multiple coagulation factor deficiency protein 2 homolog precursor putative mRNA, complete cds	0.000930625	-1.9259074
Ssa#CB518090	**B2GO_BlastxHit**	PREDICTED: leprecan-like 2	0.000949213	1.1958597
Ssa#S35693263	**B2GO_BlastxHit**	pol-like protein	0.000962005	-4.457587

Appendix 2 List of microarray probes displaying a fold change greater than 10 with regard to photoperiod (LD Vs SD), Day vs. night and photoperiod/ day night interaction.

Appendix 2a: Microarray targets (>5 x fold change) photoperiod long day vs. short day

Probe Name	Blast type	RefSeq_HitDef	Fold change photoperiod	p-value Photoperiod
Ssa#STIR25944	RefSeq_HitDef	Danio rerio ankyrin repeat domain 13C (ankrd13c), mRNA >gi|28839569|gb|BC047821.1| Danio rerio zgc:56077, mRNA (cDNA clone MGC:56077 IMAGE:5410010), complete cds	-46.455067	0.010115239
Ssa#STIR15680	RefSeq_HitDef	Salmo salar adenosine monophosphate deaminase 1 (isoform M) (ampd1), mRNA >gi|197631794|gb|BT043506.1| Salmo salar clone HM4_0643 adenosine monophosphate deaminase 1 (ampd1) mRNA, complete cds	15.261917	0.004162263
Ssa#STIR17814	RefSeq_HitDef	Oncorhynchus mykiss arylalkylamine N-acetyltransferase (aanat-2), mRNA >gi|4585222|gb|AF106006.1|AF106006 Oncorhynchus mykiss arylalkylamine N-acetyltransferase (AANAT-2) mRNA, complete cds	-10.924063	0.040280964
Ssa#STIR00072_4	B2GO_Blastx Hit	interferon-inducible protein gig2	9.332867	0.000309485
Ssa#S23871809	RefSeq_HitDef	Salmo salar fast myotomal muscle troponin-T-2 (LOC100196675), mRNA >gi|197632598|gb|BT043908.1| Salmo salar clone HM6_0331 fast myotomal muscle troponin-T-2 mRNA, complete cds	7.943922	0.012831342
Ssa#STIR18455	RefSeq_ RNA	Danio rerio zgc:56235 (zgc:56235), mRNA	7.8793855	0.003795717
Ssa#CX357274	B2GO_Blastx Hit	PREDICTED: similar to LYST-interacting protein 8, partial	-7.874486	0.008941274
Ssa#STIR17552	RefSeq_ RNA	Salmo salar v-yes-1 Yamaguchi sarcoma viral related oncogene homolog (lyn), mRNA >gb|BT045857.1| Salmo salar clone ssal-rgf-534-333 Tyrosine-protein kinase Lyn putative mRNA, complete cds	7.643616	0.035028446
Omy#CX034437	B2GO_Blastx Hit	unnamed protein product	7.3411126	0.000261279

Ssa#S35528745	B2GO_Blastx Hit	PREDICTED: similar to interferon-inducible protein Gig2	7.2423887	0.002074441
Omy#TC167443	RefSeq_HitDef	PREDICTED: Oreochromis niloticus glyceraldehyde-3-phosphate dehydrogenase-like (LOC100704894), mRNA	7.077523	0.01720346
Ssa#STIR00072_2	B2GO_Blastx Hit	interferon-inducible protein gig2	6.637287	0.002558014
Ssa#STIR00072_3	B2GO_Blastx Hit	interferon-inducible protein gig2	6.3724675	0.001621204
Ssa#KSS3721	RefSeq_HitDef	Salmo salar RAS guanyl-releasing protein 2 (grp2), mRNA >gi|209151713|gb|BT044818.1| Salmo salar clone ssal-rgf-504-236 RAS guanyl-releasing protein 2 putative mRNA, complete cds	6.264085	0.008033107
Ssa#S35496061_S	B2GO_Blastx Hit	imap family member 8	6.234745	0.20998588
Ssa#STIR19294	RefSeq_HitDef	Salmo salar Low density lipoprotein receptor adapter protein 1 (arh), mRNA >gi|221219625|gb|BT056602.1| Salmo salar clone ssal-rgb2-642-047 Low density lipoprotein receptor adapter protein 1 putative mRNA, complete cds	6.0253615	0.00652373
Ssa#STIR24047	RefSeq_HitDef	Oncorhynchus mykiss zinc exporter 1 (znt1), mRNA >gi|56406616|gb|AY742790.1| Oncorhynchus mykiss zinc exporter 1 (ZnT1) mRNA, complete cds	5.983663	0.023619961
Ssa#STIR00118_4	RefSeq_HitDef	Salmo salar creatine kinase-1 (ckm1), mRNA >gi|197632378|gb|BT043798.1| Salmo salar clone HM4_0185 creatine kinase-1 (ckm1) mRNA, complete cds	5.7848706	0.030929605
Ssa#STIR21664	RefSeq_HitDef	Salmo salar Transmembrane protein 149 (tm149), mRNA >gi|223648349|gb|BT059220.1| Salmo salar clone ssal-rgf-534-112 Transmembrane protein 149 precursor putative mRNA, complete cds	-5.710232	0.019659145
Ssa#STIR14473	B2GO_Blastn Hit	Salmo salar clone ssal-rgf-528-249, novel cds	5.634237	0.014026556
Ssa#STIR15776	RefSeq_HitDef	Salmo salar Hsp70-binding protein 1 (hpbp1), mRNA >gi|209153943|gb|BT044942.1| Salmo salar clone ssal-rgf-508-063 Hsp70-binding protein 1 putative mRNA, complete cds	5.5901675	0.01475773

Ssa#EG820908	B2GO_Blastn Hit	homo sapiens myoferlin transcript variant mrna	5.484561	0.023758586
Ssa#STIR25540	RefSeq_HitDef	Salmo salar Hsp70-binding protein 1 (hpbp1), mRNA >gi|209153943|gb|BT044942.1| Salmo salar clone ssal-rgf-508-063 Hsp70-binding protein 1 putative mRNA, complete cds	5.451452	0.015280915
Ssa#STIR23530	RefSeq_HitDef	Salmo salar Hsp70-binding protein 1 (hpbp1), mRNA >gi|209153943|gb|BT044942.1| Salmo salar clone ssal-rgf-508-063 Hsp70-binding protein 1 putative mRNA, complete cds	5.408449	0.025305893
Ssa#STIR15526	B2GO_Blastx Hit	family with sequence similaritymember a	5.381762	0.014945637
Ssa#STIR10575	B2GO_Blastx Hit	apoptosis-associated speck-like protein containing a card	5.2639	0.020625941
Con_CANDS_07	RefSeq_HitDef	Salmo salar myxovirus resistance 2 (mx2), mRNA >gi|1519385|gb|U66476.1|SSU66476 Salmo salar Mx2 protein mRNA, complete cds	5.2614775	0.016696358
Omy#CA380549	B2GO_Blastx Hit	PREDICTED: wu:fb81h03	5.238579	0.023726037
Ssa#S35496061	B2GO_Blastx Hit	PREDICTED: similar to CG5765-PA	5.1479087	0.31128702
Omy#BX877417	B2GO_Blastx Hit	PREDICTED: similar to CG5765-PA	-5.086931	0.027065517

Appendix 2b: Microarray targets (> 5 x Fold change) Day vs night.

ProbeName	Blast Type	RefSeq_HitDef	Fold Change Day/ Night	p-value Day/ Night
Ssa#STIR03571	RefSeq_HitDef	Salmo salar CD209 antigen-like protein D (c209d), mRNA >gi|304376625|gb|BT048043.2| Salmo salar clone ssal-evf-579-332 CD209 antigen-like protein D putative mRNA, complete cds	10.30289	0.03647512
Ssa#S35542336	RefSeq_HitDef	Salmo salar fast myotomal muscle actin (actc1), mRNA >gi|119721175|gb|AF304406.2| Salmo salar fast myotomal muscle actin mRNA, complete cds	-9.357814	0.0424916
Ssa#TC112890	RefSeq_HitDef	PREDICTED: Bos taurus protein phosphatase 4, regulatory subunit 1 (PPP4R1), mRNA	9.128924	0.02218637
Omy#S15262039	RefSeq_HitDef	PREDICTED: Oreochromis niloticus O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase), transcript variant 2 (OGT), mRNA	8.643788	0.00678337
Ssa#STIR22740	RefSeq_HitDef	Salmo salar Retinoid-binding protein 7 (ret7), mRNA >gi|209737323|gb|BT049730.1| Salmo salar clone ssal-eve-572-076 Retinoid-binding protein 7 putative mRNA, complete cds	-7.7628856	0.04234355
Omy#CA380549	B2GO_Blastx Hit	PREDICTED: wu:fb81h03	6.7699795	0.01150544
Ssa#STIR17814	RefSeq_HitDef	Oncorhynchus mykiss arylalkylamine N-acetyltransferase (aanat-2), mRNA >gi|4585222|gb|AF106006.1|AF106006 Oncorhynchus mykiss arylalkylamine N-acetyltransferase (AANAT-2) mRNA, complete cds	6.4541936	0.09821406
Ssa#TC102889	B2GO_Blastx Hit	ubinuclein 2	-6.3009925	0.03601006
Ssa#STIR00118_4	RefSeq_HitDef	Salmo salar creatine kinase-1 (ckm1), mRNA >gi|197632378|gb|BT043798.1| Salmo salar clone HM4_0185 creatine kinase-1 (ckm1) mRNA, complete cds	-6.099753	0.02778198
Ssa#STIR17552	RefSeq_RNA	Salmo salar v-yes-1 Yamaguchi sarcoma viral related oncogene homolog (lyn), mRNA >gb|BT045857.1| Salmo salar clone ssal-rgf-534-333 Tyrosine-protein kinase Lyn putative mRNA, complete cds	-6.0767064	0.05244112

Ssa#S35599996	RefSeq_HitDef	Salmo salar Vacuolar proton pump subunit H (vath), mRNA >gi|209154159|gb|BT045050.1| Salmo salar clone ssal-rgf-510-264 Vacuolar proton pump subunit H putative mRNA, complete cds	-5.444198	0.04255521
Ssa#S35553712	B2GO_Blastx Hit	PREDICTED: similar to Protein-glutamine gamma-glutamyltransferase 5 (Transglutaminase-5) (TGase 5) (Transglutaminase X) (TGase X) (TGX) (TG(X))	-5.3636265	0.01958431
Omy#CX034437	B2GO_Blastn Hit	danio rerio zgc:175139 (zgc:175139) mrna	-5.1907873	0.00120107

Appendix 2c: Microarray targets (> 5 x Fold change) Photoperiod/ Day night interaction (maximum)

Probe Name	Blast Type	RefSeq_HitDef	Fold change Photoperiod / Day night	P value Photoperiod/ Day night
Ssa#STIR15680	RefSeq_HitDef	Salmo salar adenosine monophosphate deaminase 1 (isoform M) (ampd1), mRNA >gi|197631794|gb|BT043506.1| Salmo salar clone HM4_0643 adenosine monophosphate deaminase 1 (ampd1) mRNA, complete cds	-188.05891	0.02087278
Ssa#S23871809	RefSeq_HitDef	Salmo salar fast myotomal muscle troponin-T-2 (LOC100196675), mRNA >gi|197632598|gb|BT043908.1| Salmo salar clone HM6_0331 fast myotomal muscle troponin-T-2 mRNA, complete cds	-82.176384	0.02412807
Ssa#S18892465	RefSeq_HitDef	Salmo salar rod opsin (LOC100136370), mRNA >gi|7271780|gb|AF201470.1|AF201470 Salmo salar retinal rod opsin mRNA, complete cds	57.997765	0.0321345
Ssa#S35496061_S	B2GO_Blastx Hit	imap family member 8	50.791286	0.04563181
Ssa#STIR00118_4	RefSeq_HitDef	Salmo salar creatine kinase-1 (ckm1), mRNA >gi|197632378|gb|BT043798.1| Salmo salar clone HM4_0185 creatine kinase-1 (ckm1) mRNA, complete cds	-42.523563	0.05925008
Ssa#S35496061	B2GO_Blastx Hit	imap family member 8///"PREDICTED: similar to GTPase, IMAP family member 7"	39.10866	0.01067536
Ssa#TC106938	RefSeq_HitDef	PREDICTED: Danio rerio GTPase IMAP family member 8-like (LOC100332582), partial mRNA	34.448006	0.01069007
Ssa#S30263425	B2GO_Blastx Hit	PREDICTED: glutamate receptor, ionotropic, kainate 1	33.054	0.01853157
Omy#S34312805	RefSeq_HitDef	PREDICTED: Monodelphis domestica THO complex subunit 1-like (LOC100013574), mRNA	-32.516174	0.04348469
Omy#CA380549	B2GO_Blastx Hit	PREDICTED: wu:fb81h03	-27.956371	0.68715185
Omy#TC167443	RefSeq_HitDef	PREDICTED: Oreochromis niloticus glyceraldehyde-3-phosphate dehydrogenase-like (LOC100704894), mRNA	-23.803677	0.16933796

Ssa#STIR25944	RefSeq_HitDef	Danio rerio ankyrin repeat domain 13C (ankrd13c), mRNA >gi|28839569|gb|BC047821.1| Danio rerio zgc:56077, mRNA (cDNA clone MGC:56077 IMAGE:5410010), complete cds	22.775366	0.4780948
Ssa#STIR17552	RefSeq_ RNA	Salmo salar v-yes-1 Yamaguchi sarcoma viral related oncogene homolog (lyn), mRNA >gb|BT045857.1| Salmo salar clone ssal-rgf-534-333 Tyrosine-protein kinase Lyn putative mRNA, complete cds	-22.511501	0.3692513
Omy#TC169899	RefSeq_HitDef	Salmo salar creatine kinase-1 (ckm1), mRNA >gi|197632378|gb|BT043798.1| Salmo salar clone HM4_0185 creatine kinase-1 (ckm1) mRNA, complete cds	-22.344875	0.05456895
Omy#S15262039	RefSeq_HitDef	PREDICTED: Oreochromis niloticus O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase), transcript variant 2 (OGT), mRNA	-19.644037	0.25646386
Ssa#STIR15526	B2GO_Blastx Hit	family with sequence similaritymember a	-19.496592	0.40282133
Ssa#DY718631	B2GO_Blastx Hit	Rho GTPase-activating protein 15	-19.19899	0.06144268
Ssa#CO470179	B2GO_Blastx Hit	sortilin 1	-19.01868	0.02209195
Ssa#STIR26062	RefSeq_RNA	Salmo salar sarcoglycan, epsilon (sgce), mRNA >gb|BT059367.1| Salmo salar clone ssal-rgf-510-082 Epsilon-sarcoglycan precursor putative mRNA, complete cds	17.366827	0.02664242
Omy#BX877417	B2GO_Blastx Hit	PREDICTED: similar to CG5765-PA	16.48222	0.09394795
Ssa#STIR03571	RefSeq_HitDef	Salmo salar CD209 antigen-like protein D (c209d), mRNA >gi|304376625|gb|BT048043.2| Salmo salar clone ssal-evf-579-332 CD209 antigen-like protein D putative mRNA, complete cds	-16.274656	0.6528121
Ssa#STIR00118_3	RefSeq_HitDef	Salmo salar creatine kinase-1 (ckm1), mRNA >gi|197632378|gb|BT043798.1| Salmo salar clone HM4_0185 creatine kinase-1 (ckm1) mRNA, complete cds	-15.622583	0.07504167
Ssa#TC110597	RefSeq_HitDef	PREDICTED: Oreochromis niloticus hypothetical protein LOC100708024 (LOC100708024), mRNA	-15.355853	0.00840261
Ssa#DY72949	RefSeq_HitDef	Salmo salar P2Y purinoceptor 8 (p2ry8), mRNA	-14.605243	0.0299134

2		>gi|209731413|gb|BT046775.1| Salmo salar clone ssal-rgb2-576-249 P2Y purinoceptor 8 putative mRNA, complete cds		
Ssa#STIR01237	RefSeq_HitDef	Salmo salar parvalbumin beta (prvb), mRNA	-14.603089	0.06488016
Ssa#STIR22740	RefSeq_HitDef	Salmo salar Retinoid-binding protein 7 (ret7), mRNA >gi|209737323|gb|BT049730.1| Salmo salar clone ssal-eve-572-076 Retinoid-binding protein 7 putative mRNA, complete cds	-14.582099	0.8457009
Ssa#S48371757	B2GO_Blastn Hit	mus musculus titin transcript variant n2- mrna	13.981991	0.04433387
Ssa#STIR00072_4	B2GO_Blastx Hit	interferon-inducible protein gig2	-13.783437	0.45199442
Ssa#S35514991	RefSeq_HitDef	PREDICTED: Anolis carolinensis 1,25-dihydroxyvitamin D(3) 24-hydroxylase, mitochondrial-like (LOC100555767), partial mRNA	-13.5553	0.12977287
Ssa#DY706808	RefSeq_HitDef	Schizosaccharomyces japonicus yFS275 SNF2 family ATP-dependent chromatin-remodeling factor snf21, mRNA	13.500755	0.04280261
Omy#TC172353	B2GO_Blastx Hit	hypothetical protein LOC100137110	-13.275645	0.04117857
Ssa#KSS3721	RefSeq_HitDef	Salmo salar RAS guanyl-releasing protein 2 (grp2), mRNA >gi|209151713|gb|BT044818.1| Salmo salar clone ssal-rgf-504-236 RAS guanyl-releasing protein 2 putative mRNA, complete cds	-13.146598	0.313945
Ssa#DW470032	B2GO_Blastx Hit	PREDICTED: similar to Serine/threonine-protein kinase ULK4 (Unc-51-like kinase 4)	-13.085985	0.11287892
Ssa#STIR19294	RefSeq_HitDef	Salmo salar Low density lipoprotein receptor adapter protein 1 (arh), mRNA >gi|221219625|gb|BT056602.1| Salmo salar clone ssal-rgb2-642-047 Low density lipoprotein receptor adapter protein 1 putative mRNA, complete cds	-12.469442	0.25085774
Omy#S19710960	B2GO_Blastx Hit	MOXD2_DANRERecName: Full=DBH-like monooxygenase protein 2 homolog; Flags: Precursor	-12.218872	0.03206809
Ssa#STIR04514	RefSeq_HitDef	Danio rerio splicing factor 3a, subunit 3 (sf3a3), mRNA >gi|49618980|gb|AY648757.1| Danio rerio splicesome-associated factor 61 mRNA, complete cds	-12.097144	0.02794942
Ssa#TC10288	B2GO_Blastx	ubinuclein 2	11.098908	0.68151367

9	Hit			
Ssa#CX354598	RefSeq_HitDef	PREDICTED: Oreochromis niloticus mitogen-activated protein kinase kinase kinase 5-like (LOC100703888), mRNA	11.048678	0.05112877
Ssa#STIR17814	RefSeq_HitDef	Oncorhynchus mykiss arylalkylamine N-acetyltransferase (aanat-2), mRNA >gi|4585222|gb|AF106006.1|AF106006 Oncorhynchus mykiss arylalkylamine N-acetyltransferase (AANAT-2) mRNA, complete cds	-10.928259	0.6217961
Ssa#TC108629	RefSeq_HitDef	Oncorhynchus mykiss complement factor H1 protein (LOC100136131), mRNA >gi|67904936|emb|AM039935.1| Oncorhynchus mykiss mRNA for complement factor H1 protein	10.873915	0.23662262
Ssa#CX357274	B2GO_Blastx Hit	PREDICTED: similar to LYST-interacting protein 8, partial	10.862316	0.6360513
Ssa#TC112890	RefSeq_HitDef	PREDICTED: Bos taurus protein phosphatase 4, regulatory subunit 1 (PPP4R1), mRNA	-10.806593	0.84464276
Ssa#STIR18085	RefSeq_HitDef	Salmo salar Zinc finger protein Xfin (xfin), mRNA >gi|223648569|gb|BT059330.1| Salmo salar clone ssal-rgf-541-357 Zinc finger protein Xfin putative mRNA, complete cds	10.689163	0.02011773
Ssa#STIR10575	B2GO_Blastx Hit	apoptosis-associated speck-like protein containing a card	-10.060478	0.39479604
Ssa#CB512841	RefSeq_HitDef	Salmo salar Serine/threonine-protein kinase PCTAIRE-2 (pctk2), mRNA >gi|223648663|gb|BT059377.1| Salmo salar clone ssal-rgf-523-300 Serine/threonine-protein kinase PCTAIRE-2 putative mRNA, complete cds	9.612992	0.03735065
Ssa#STIR18481	RefSeq_HitDef	Salmo salar inositol 1,3,4-triphosphate 5/6 kinase (itpk1), mRNA >gi|304376530|gb|BT047282.2| Salmo salar clone ssal-evd-502-275 Inositol-tetrakisphosphate 1-kinase putative mRNA, complete cds	-9.605071	0.01240713
Omy#CX034437	B2GO_Blastn Hit	danio rerio zgc:175139 (zgc:175139) mrna	9.415298	0.15348983
Ssa#S18835342	RefSeq_HitDef	Oncorhynchus mykiss differentially regulated trout protein 1 (LOC100136615), mRNA >gi|11095802|gb|AF281355.1| Oncorhynchus mykiss differentially regulated trout protein 1 mRNA, complete cds	9.407242	0.04288711

Ssa#STIR18455	RefSeq_RNA	Danio rerio zgc:56235 (zgc:56235), mRNA	-9.308748	0.7266945
Ssa#STIR24047	RefSeq_HitDef	Oncorhynchus mykiss zinc exporter 1 (znt1), mRNA >gi|56406616|gb|AY742790.1| Oncorhynchus mykiss zinc exporter 1 (ZnT1) mRNA, complete cds	-9.300929	0.5430023
Omy#CU073361	B2GO_Blastx Hit	0	-9.117006	0.94429696
Con_CANDS_07	RefSeq_HitDef	Salmo salar myxovirus resistance 2 (mx2), mRNA >gi|1519385|gb|U66476.1|SSU66476 Salmo salar Mx2 protein mRNA, complete cds	-9.113772	0.5720938
Ssa#TC109996	B2GO_Blastx Hit	PREDICTED: similar to transmembrane protein 74	9.051912	0.10629478
Omy#S32715052	RefSeq_HitDef	Danio rerio glucose phosphate isomerase b (gpib), mRNA	8.825852	0.1771449
Omy#CA377250	B2GO_Blastn Hit	danio rerio skin mucus antibacterial l-amino acid oxidase mrna	-8.788457	0.03553839
Ssa#TC64988	RefSeq_HitDef	Ixodes scapularis sodium-neurotransmitter symporter, putative, mRNA	-8.787946	0.06542971
Omy#S34421934	B2GO_Blastx Hit	Fstl4 protein	8.758008	0.3411463
Ssa#STIR20548	RefSeq_RNA	PREDICTED: Strongylocentrotus purpuratus uncharacterized LOC100892022 (LOC100892022), mRNA 0	-8.568612	0.04541114
Omy#S23945470	B2GO_Blastn Hit	salmo salar clone ssal-rgf-525-204 type-2 angiotensin ii receptor complete cds	-8.563399	0.03644463
Ssa#DY693369	RefSeq_HitDef	PREDICTED: Danio rerio mucolipin-3-like (LOC100001113), partial mRNA	-8.499387	0.03358924
Omy#CA362044	B2GO_Blastn Hit	danio rerio smith-magenis syndrome chromosome candidate 7-like mrna	8.331676	0.12315166
Ssa#STIR19014	RefSeq_HitDef	Arabidopsis thaliana uncharacterized protein (AT5G47020) mRNA, complete cds	8.1190195	0.23395196
Ssa#STIR25965	RefSeq_HitDef	Salmo salar histidine triad nucleotide binding protein 3 (hint3), mRNA >gi|221220801|gb|BT057190.1| Salmo salar clone ssal-sjb-013-304	-8.089872	0.04815245

		Histidine triad nucleotide-binding protein 3 putative mRNA, complete cds		
Omy#S26989086	B2GO_Blastx Hit	natriuretic peptide receptor type C/D	-7.9511538	0.04854657
Ssa#STIR21664	RefSeq_HitDef	Salmo salar Transmembrane protein 149 (tm149), mRNA >gi|223648349|gb|BT059220.1| Salmo salar clone ssal-rgf-534-112 Transmembrane protein 149 precursor putative mRNA, complete cds	7.905333	0.95997465
Omy#BX858059	RefSeq_HitDef	PREDICTED: Gallus gallus regulatory associated protein of MTOR, complex 1, transcript variant 1 (RPTOR), mRNA	7.7954855	0.5624641
Ssa#STIR11009	RefSeq_RNA	PREDICTED: Pan paniscus ATPase, Na+/K+ transporting, alpha 2 polypeptid	-7.6973977	0.00858717
Ssa#S35581889	B2GO_Blastx Hit	hypothetical protein LOC541512	-7.60477	0.03920256
Ssa#STIR00118_2	RefSeq_HitDef	Salmo salar creatine kinase-1 (ckm1), mRNA >gi|197632378|gb|BT043798.1| Salmo salar clone HM4_0185 creatine kinase-1 (ckm1) mRNA, complete cds	-7.5966835	0.20887837
Ssa#STIR15730	B2GO_Blastx Hit	loh11cr2a protein	-7.5199966	0.10930253
Ssa#CB513842	B2GO_Blastx Hit	homo sapiens synaptotagmin-like 4 transcript variant mrna	7.402076	0.00562732
Ssa#S35528745	B2GO_Blastx Hit	danio rerio interferon-inducible protein gig2 mrna	-7.377134	0.9898905
Omy#AB024294	RefSeq_HitDef	PREDICTED: Oreochromis niloticus structural maintenance of chromosomes protein 1A-like (LOC100708270), mRNA	7.345468	0.19001392
Ssa#S35553712	B2GO_Blastx Hit	gasterosteus aculeatus clone cfw240-a04 mrna sequence	7.234363	0.64013284
Ssa#S18885712	RefSeq_HitDef	Salmo salar ribosomal protein L6 (rpl6), mRNA >gi|197632288|gb|BT043753.1| Salmo salar clone HM6_0879 ribosomal protein L6 (rpl6) mRNA, complete cds	7.2024817	0.0319637
Ssa#STIR00072_3	B2GO_Blastx Hit	interferon-inducible protein gig2	-7.1893277	0.8294556
Ssa#S4841044	B2GO_Blastx	p90 autoantigen	7.0981174	0.01359439

1	Hit			
Ssa#STIR00072_2	B2GO_Blastx Hit	interferon-inducible protein gig2	-6.681376	0.97552335
Ssa#STIR18645	RefSeq_ RNA	Xenopus laevis MGC84433 protein (MGC84433), mRNA >gb|BC074417.1| Xenopus laevis MGC84433 protein, mRNA (cDNA clone MGC:84433 IMAGE:7019563), complete cds	-6.64759	0.14692286
Ssa#S43134841_S	RefSeq_HitDef	Salmo salar CD4-like protein (LOC100136502), transcript variant 1, mRNA >gi|167538885|gb|EU409794.1| Salmo salar CD4-1-like protein mRNA, complete cds	6.6394615	0.03276559
Ssa#S18892317	RefSeq_HitDef	Salmo salar myxovirus resistance 1 (mx1), mRNA >gi|1519383|gb|U66475.1|SSU66475 Salmo salar Mx1 protein mRNA, complete cds	-6.432686	0.1786227
Ssa#S35543300	RefSeq_HitDef	Oncorhynchus mykiss interleukin-12 beta chain (il12b), mRNA >gi|60416861|emb|AJ548829.1| Oncorhynchus mykiss mRNA for interleukin-12 beta chain (il12b gene), large transcript	6.413683	0.63579655
Ssa#STIR00154_2	RefSeq_HitDef	Salmo salar myxovirus resistance 1 (mx1), mRNA >gi|1519383|gb|U66475.1|SSU66475 Salmo salar Mx1 protein mRNA, complete cds	-6.401455	0.21282125
Ssa#EG820908	B2GO_Blastn Hit	homo sapiens myoferlin transcript variant mrna	-6.3793197	0.8796511
Omy#CA355482	B2GO_Blastx Hit	PREDICTED: similar to G protein-coupled receptor 82	-6.3504176	0.4450548
Omy#TC169598	RefSeq_HitDef	PREDICTED: Oreochromis niloticus tyrosine-protein kinase receptor UFO-like (LOC100708491), mRNA	6.3148384	0.17585228
Omy#S18100560	RefSeq_HitDef	Salmo salar E3 ubiquitin-protein ligase RNF8 (rnf8), mRNA >gi|223648921|gb|BT059506.1| Salmo salar clone ssal-rgf-518-310 E3 ubiquitin-protein ligase RNF8 putative mRNA, complete cds	-6.2592278	0.20490001
Ssa#S48371191	B2GO_Blastx Hit	glyceraldehyde-3-phosphate dehydrogenase-2	-6.2233033	0.17687623
Omy#S15318750	RefSeq_HitDef	PREDICTED: Oreochromis niloticus dihydropyrimidinase-related protein 2-like (LOC100711297), mRNA	6.21713	0.03414092

Ssa#TC110735	B2GO_Blastn Hit	unnamed protein product	-6.0302386	0.9408544
Ssa#DY724373	RefSeq_HitDef	PREDICTED: Oreochromis niloticus ubiquitin specific peptidase 15 (USP15), mRNA	-5.8833904	0.6674592
Ssa#S35549782	RefSeq_HitDef	Salmo salar ISG15-like protein (LOC100136541), mRNA >gi|62737687|gb|AY926456.1| Salmo salar ISG15-like protein mRNA, complete cds	-5.797771	0.5944774
Omy#BX909219	RefSeq_HitDef	PREDICTED: Canis lupus familiaris myosin XVIIIA, transcript variant 3 (MYO18A), mRNA	5.680153	0.02184267
Omy#S15318281	B2GO_Blastx Hit	PREDICTED: similar to Intersectin 2 (SH3 domain-containing protein 1B) (SH3P18) (SH3P18-like WASP associated protein) isoform 2	5.6185975	0.0052072
Ssa#S35542336	RefSeq_HitDef	Salmo salar fast myotomal muscle actin (actc1), mRNA >gi|119721175|gb|AF304406.2| Salmo salar fast myotomal muscle actin mRNA, complete cds	5.605844	0.56255096
Omy#S18154452	B2GO_Blastn Hit	bos taurus chromosome 15 open reading frame 44 ortholog mrna	-5.6055	0.02416926
Omy#CA363099	RefSeq_HitDef	PREDICTED: Oreochromis niloticus vesicle-trafficking protein SEC22a-like (LOC100698971), mRNA	5.596344	0.46691826
Omy#S34425192	RefSeq_HitDef	PREDICTED: Oreochromis niloticus dual specificity protein phosphatase 2-like (LOC100694883), mRNA	-5.5417557	0.2868468
Ssa#NP9934200	RefSeq_HitDef	T cell receptor alpha	-5.5125303	0.88004225
Ssa#STIR21048	RefSeq_RNA	PREDICTED: Hydra magnipapillata hypothetical protein LOC100203825 (LOC100203825), mRNA	5.479197	0.962878
Ssa#EG855563	RefSeq_HitDef	retinal pigment epithelium-specific protein 65kDa	-5.4648466	0.21535324
Ssa#KSS3245	RefSeq_HitDef	Salmo salar Ubiquitin-like protein (ubil), mRNA >gi|209736095|gb|BT049116.1| Salmo salar clone ssal-eve-520-325 Ubiquitin-like protein precursor putative mRNA, complete cds	-5.424694	0.02966759
Ssa#KSSb2554	RefSeq_HitDef	ZC3HE_DANRERecName: Full=Zinc finger CCCH domain-containing protein 14	5.408544	0.19645256
Ssa#TC96609	RefSeq_HitDef	Nectria haematococca mpVI 77-13-4 hypothetical protein, mRNA	5.395546	0.16349296

Omy#CX257435	RefSeq_HitDef	hypothetical protein LOC100036701	-5.3674746	0.04956973
Omy#CA342136	RefSeq_HitDef	danio rerio bat2-like protein mrna	-5.222133	0.5546996
Ssa#DW540683	RefSeq_HitDef	PREDICTED: Sus scrofa megakaryoblastic leukemia (translocation) 1 (MKL1), mRNA	5.141159	0.04583039
Ssa#S35549114_S	RefSeq_HitDef	Salmo salar SH3 and PX domain-containing protein 2B (spd2b), mRNA >gi|223647567|gb|BT058829.1| Salmo salar clone ssal-rgf-506-005 SH3 and PX domain-containing protein 2B putative mRNA, complete cds	-5.125886	0.03425171
Ssa#S30241271	B2GO_Blastx Hit	salmo salar clone bac complete sequence	5.095956	0.49406898
Ssa#DW566581	RefSeq_HitDef	PREDICTED: Ornithorhynchus anatinus uncharacterized protein KIAA0195-like (LOC100075929), mRNA	5.0861125	0.8602685
Ssa#KSSb2768	RefSeq_HitDef	PREDICTED: Oreochromis niloticus 3-hydroxyacyl-CoA dehydratase 1-like (LOC100709129), mRNA	-5.0698476	0.0294946