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 Aanat2 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.
ACKNOWLEDGEMENTS

For all their help and support I must first and foremost say thank you to my supervisors Dr Herve Migaud and Dr Andrew Davie for their help and guidance throughout my PhD project. They have provided excellent supervision from helping me to gain an initial understanding of my subject area, to help with 24h sampling and guidance in the lab. Most of all I would like to express my gratitude for their time and understanding during my write up period. It has been a privilege to work in such a stimulating and entertaining group

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1. Abstract
2. Introduction
## SPECIES LIST

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<th>Common Name</th>
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### LIST OF ABBREVIATIONS

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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>AAAD</td>
<td>Aromatic amino-acid decarboxylase</td>
</tr>
<tr>
<td>Aanat</td>
<td>Arylalkylamine N-acetyltransferas</td>
</tr>
<tr>
<td></td>
<td>Brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like</td>
</tr>
<tr>
<td>Bmal</td>
<td>Brain-pituitary-gonadal</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic AMP</td>
</tr>
<tr>
<td>CCAATβ</td>
<td>CCAAT enhancer binding protein beta</td>
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<tr>
<td>CDS</td>
<td>Coding sequence</td>
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<td>cDNA</td>
<td>Complementary DNA</td>
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<td>CK1</td>
<td>Casein kinase 1 epsilon</td>
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<td>Clock</td>
<td>Circadian Locomotor Output Cycles Kaput</td>
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<td>Cryptochrome</td>
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<td>D6 Fad</td>
<td>D6 fatty acid desaturase</td>
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<td>Dio1</td>
<td>Type I iodothyronine deiodinase</td>
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<tr>
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<td>Type II iodothyronine deiodinase</td>
</tr>
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<td>Type III iodothyronine deiodinase</td>
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<td>Deoxyribonucleic acid</td>
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<td>Elovl5</td>
<td>Elongation of very long chain fatty acids protein 5</td>
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<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
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<td>Eyes Absent 3</td>
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<td>FAO</td>
<td>Food and Agriculture Organization</td>
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<td>Fas</td>
<td>Fatty acid synthase</td>
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<td>FASPS</td>
<td>Familial advanced sleep phase syndrome</td>
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<td>FSH</td>
<td>Follicle-stimulating hormone</td>
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<td>GnRH</td>
<td>Gonadotropin-releasing hormone</td>
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<td>Hydroxindol - O-methyltransferase</td>
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<td>HMG-CoA reductase</td>
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<td>Kiss</td>
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<td>LD</td>
<td>Long Day</td>
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<td>LH</td>
<td>Luteinizing-hormone</td>
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<td>Lxr</td>
<td>Liver X receptor</td>
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<td>NCBI</td>
<td>National Centre for Biotechnology Information</td>
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<tr>
<td>PACAP</td>
<td>Pituitary adenylate cyclase-activating polypeptide</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>Pars distalis</td>
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<td>Per</td>
<td>Period</td>
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<td>Photoneuroendocrine system</td>
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<td>Definition</td>
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<td>PRDM</td>
<td>Pineal – restrictive down-stream module</td>
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<td>Quantitative PCR</td>
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<td>Nuclear receptor subfamily 1, group D, member 1</td>
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<td>Retino-hypothalamic tract</td>
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<td>Radioimmunoassay</td>
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<td>Related orphan nuclear receptors</td>
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<td>Tef</td>
<td>Thyrotroph embryonic factor</td>
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<td>Tryptophan hydroxylase</td>
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<td>Tshβ</td>
<td>Thyroid stimulating hormone Beta</td>
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<td>UTR</td>
<td>Untranslated region</td>
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<tr>
<td>VL-SCN</td>
<td>Ventrolateral SCN</td>
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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).
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**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
Chapter 1

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
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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.

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<th>Scales of rhythmic physiology and biological rhythms from ultradian to circannual rhythms</th>
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<td><strong>Ultradian (&lt; 24h)</strong></td>
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<td><strong>Tidal rhythms</strong></td>
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<td><strong>Circadian rhythms</strong></td>
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<td><strong>Lunar rhythms</strong></td>
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<td><strong>Infradian rhythms</strong></td>
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<td><strong>Circannual</strong></td>
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**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 feedback 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 (Ikegami 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
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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 heterodimers also activate the
transcription of *nuclear receptor subfamily 1, group D, member (Rev-erba)* and *Retinoic acid
related orphan nuclear receptors (Rora)*, 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).
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Figure 4: A network of transcriptional–translational feedback loops constitutes the mammalian circadian clock from Ko & Takahashi (2006). The feedback loop begins with CLOCK and BMAL proteins which form a heterodimer 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 complete 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 ubiquitylation (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 ubiquitinyluation. F-box domain containing protein Supernumerary limbs (SLMB). has been shown to be involved in the circadian clockwork in *D. melangaster*, 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.
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**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 CK1ε* in hamsters was a 22h circadian period in heterozygote animals (τ₀₀) and a 20h period in homozygote animals (τ₅₅). 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\textsuperscript{ldc} mutation (Bae et al. 2001). A mutation in the mouse clock gene (Clock\textsuperscript{Δ19/Δ19}) 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\textsuperscript{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 CK1\textsuperscript{δ} 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
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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), a tool 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
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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 *zCry* genes are rhythmically expressed in zebrafish tissues. Four of the *zCry* 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 *zCry1s* and *2s* exhibited different acrophase or peak in rhythmic mRNA expression. *zCry1a* and *b* peak during the day whilst *zCry2a* 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). *zCry3* and *zCry4* also appear to be homologues of mouse *Cry1* and are rhythmically expressed with highest levels of mRNA expression in the morning. However unlike *zCry1a/b* and *zCry2a/b* neither inhibited the transcriptional activation of the mammalian CLOCK: BMAL1 dimmers (Cahill 2002; Vatine et al. 2011). *zCry4*, 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 from mammals in the activities and expression patterns of these two zBmal 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 possess 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.
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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 patterns 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 $\beta_1$-adrenergic receptors, and increases intracellular $\text{Ca}^{2+}$ and protein kinase C activity via $\alpha_{1b}$-andrenergic receptors, consequently potentiating the $\beta_1$-adrenergic receptor activation of adenylyl cyclase. This process causes a large rapid increase in cAMP. In ungulates 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: $-\left[\text{Ca}^{2+}\right]_1$, unidentified rapid turnover protein repressor and endogenous clock control of cAMP (Coon & Klein 2006; Klein 2007). The melatonin pathway (Tryptophan $\rightarrow$ Hydroxytryptophan $\rightarrow$ N-acetylserotonin $\rightarrow$ 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.

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**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 upregulated 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 kisspeptin 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 underlying 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 T₄ displays very little biological activity (Dardente et al 2010). However when an iodine is removed, biologically active T₃ is formed via the activity of the deiodinase enzyme DIO2. Additionally, DIO3 catalyses the conversion of the active T₃ 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 T₄ to T₃ via the action of DIO2. Conversely under
Chapter 1

short day photoperiod release of TSH is low and DIO3 is stimulated to convert T3 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 T4 acceleration of ubiquitination and proteasome uptake, this in turn regulates the stability of T3 (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 synthesis 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 form. 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).

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
Chapter 1

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 organism's 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 undescribed. 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.
Chapter 1

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
Chapter 2

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-through tank systems ranging from 1-2m in diameter in fresh water and approximately 3m in diameter in marine systems (7 m³). 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).
Chapter 2

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°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°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 °C for later analysis. In order to remove the salmon pineal \textit{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°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 °C for later analysis.
### Table 1. Origin and housing for fish used for all experimental chapters

<table>
<thead>
<tr>
<th>Species</th>
<th>Experimental chapter</th>
<th>Origin</th>
<th>Year class</th>
<th>Housing</th>
</tr>
</thead>
</table>
| *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)                     |
|             | - 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)               |
|             | - 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)                     |
| *Salmo salar* | - 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 °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°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 °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.
Chapter 2

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 (amphotericin # 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 °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 °C. Seabass culture was conducted at 18 °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 acclimation 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°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 \(^{3}\text{H}\) (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\(_2\)O (DNA and RNA free sterile H\(_2\)O). Once all components were dissolved, buffer was stored at 4 °C until use.
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 H₂O. 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-³H] 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 ³H 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:

<table>
<thead>
<tr>
<th>Standard</th>
<th>Standard concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>100 μl (1 mg/ml) + 9.9 ml buffer</td>
</tr>
<tr>
<td>B</td>
<td>100 μl (10 μg/ml = A) + 9.9 ml buffer</td>
</tr>
<tr>
<td>C</td>
<td>100 μl (100 ng/ml = B) + 9.9 ml buffer</td>
</tr>
<tr>
<td>D</td>
<td>100 μl (100 ng/ml = B) + 4.9 ml buffer</td>
</tr>
</tbody>
</table>

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.
Chapter 2

**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 °C for 30 minutes.
- 100 μl of $^3$H radiolabel was added to all tubes, vortex all samples and incubate at 4 °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 °C for 15 minutes.
- Samples were then centrifuged at 2000 RPM at 4 °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.

<table>
<thead>
<tr>
<th>Tube number</th>
<th>Standard conc</th>
<th>+ 250 µl</th>
<th>+ buffer µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 &amp; 2</td>
<td>500</td>
<td>Standard D</td>
<td>none</td>
</tr>
<tr>
<td>3 &amp; 4</td>
<td>250</td>
<td>Standard C</td>
<td>none</td>
</tr>
<tr>
<td>5 &amp; 6</td>
<td>125</td>
<td>Standard C</td>
<td>250</td>
</tr>
<tr>
<td>7 &amp; 8</td>
<td>62.5</td>
<td>5 &amp; 6</td>
<td>250</td>
</tr>
<tr>
<td>9 &amp; 10</td>
<td>31.3</td>
<td>7 &amp; 8</td>
<td>250</td>
</tr>
<tr>
<td>11 &amp; 12</td>
<td>15.6</td>
<td>9 &amp; 10</td>
<td>250</td>
</tr>
<tr>
<td>13 &amp; 14</td>
<td>7.8</td>
<td>11 &amp; 12</td>
<td>250</td>
</tr>
<tr>
<td>15 &amp; 16</td>
<td>3.9</td>
<td>13 &amp; 14</td>
<td>250</td>
</tr>
<tr>
<td>17 &amp; 18</td>
<td>1.95</td>
<td>15 &amp; 16</td>
<td>250(mix &amp; remove 250)</td>
</tr>
<tr>
<td>19 &amp; 20</td>
<td>0.0</td>
<td>None</td>
<td>250</td>
</tr>
<tr>
<td>21 &amp; 22</td>
<td>None specific binding</td>
<td>None</td>
<td>450</td>
</tr>
</tbody>
</table>

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 °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 °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 °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 H₂O 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 °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 H₂O.
Chapter 2

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 DNasefree enzyme in each reaction and gently mixed and samples were incubated at 37 °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.
Chapter 2

Final DNase treated RNA was quality and concentration was analysed by nanodrop spectrophotometer and stored at -70 °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 H₂O
- 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 °C for 10 minutes followed by 37 °C for 120 minutes and 85 °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 °C.
Table 3: Concentration of totRNA used for cDNA synthesis for qPCR experiments

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

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.

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

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 neighbor 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.

<table>
<thead>
<tr>
<th>Species (Latin)</th>
<th>Species (Common)</th>
<th>Ascension number</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Danio rerio</em></td>
<td>Zebrafish</td>
<td>NM_131411.1</td>
<td>NCBI</td>
</tr>
<tr>
<td><em>Carassius auratus</em></td>
<td>Gold fish</td>
<td>GU205782.1</td>
<td>NCBI</td>
</tr>
<tr>
<td><em>Oryzias latipes</em></td>
<td>Medaka</td>
<td>NM_001104846.1</td>
<td>NCBI</td>
</tr>
<tr>
<td><em>Solea senegalensis</em></td>
<td>Senegalese sole</td>
<td>GQ340973.1</td>
<td>NCBI</td>
</tr>
<tr>
<td><em>Paralichthys olivaceus</em></td>
<td>Olive flounder</td>
<td>HQ883478.1</td>
<td>NCBI</td>
</tr>
<tr>
<td><em>Oncorhynchus mykiss</em></td>
<td>Rainbow trout</td>
<td>NM_001124257.1</td>
<td>NCBI</td>
</tr>
<tr>
<td><em>Scophthalmus maximus</em></td>
<td>Turbot</td>
<td>EF033250.1</td>
<td>NCBI</td>
</tr>
<tr>
<td><em>Sparus aurata</em></td>
<td>Gilt head seabream</td>
<td>AY533403.2</td>
<td>NCBI</td>
</tr>
<tr>
<td><em>Esox lucius</em></td>
<td>Pike</td>
<td>AF034082.1</td>
<td>NCBI</td>
</tr>
<tr>
<td><em>Salmo salar</em></td>
<td>Atlantic Salmon</td>
<td>Cluster ID# 3912632</td>
<td>ASalBase</td>
</tr>
<tr>
<td><em>Salmo salar</em></td>
<td>Atlantic Salmon</td>
<td>Cluster ID# 3920741</td>
<td>ASalBase</td>
</tr>
<tr>
<td><em>Salmo salar</em></td>
<td>Atlantic Salmon</td>
<td>S0250N08SP6</td>
<td>ASalBase</td>
</tr>
</tbody>
</table>
Table 7: Aanat2 primer pairs, sequences, and location on rainbow trout partial sequence.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Region</th>
<th>Location on O. mykiss</th>
<th>Primer sequence 5' - 3'</th>
<th>Annealing temperature °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aanat2 A</td>
<td>ORF</td>
<td>372bp - 1040bp</td>
<td>F: AGGTCAGCCGCTCTCCGT TCC</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: CCAGTGCTAGGGTTGATG TGATTATGA</td>
<td></td>
</tr>
<tr>
<td>Aanat2 B</td>
<td>ORF + 3'</td>
<td>373bp - 1620bp</td>
<td>F: GGTCAGCGCTCTCCGT TT CCT</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: TGGTGCTGCAGCTGAGAT TGATGG</td>
<td></td>
</tr>
<tr>
<td>Aanat2 C</td>
<td>ORF + 3'</td>
<td>373bp - past end of RT sequence</td>
<td>F: GGTCAGCGCTCTCCGT TT CCT</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: CTGCAGCGCCTCAATGAC AAAGTG</td>
<td></td>
</tr>
<tr>
<td>Aanat2 D</td>
<td>5'+ ORF (partial)</td>
<td>115bp - 796bp</td>
<td>F: AGACAGGCAGATAGAAAA GCACAGAGCA</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: CAGGTAGCGCCACAGCAG GATG</td>
<td></td>
</tr>
<tr>
<td>Aanat2 E</td>
<td>ORF(partial)+ 3'</td>
<td>771bp - 1566bp</td>
<td>F: TCAGCCCAGTAAGTGACC ATCATGACACAT</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: GTTGCAACCTGGTCTGGA CGGTCAGC</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 2

**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](http://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 manufacturer's 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, (Dio2seqaF/R, Dio2seqbF/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.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer sequence Forward 5’-3’</th>
<th>Primer sequence reverse 5’-3’</th>
<th>Anneal °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dio2</td>
<td>GGCAGCGCATGCTGACCTCG</td>
<td>ACCAGCCCCGTCTCGACCCA</td>
<td>62</td>
</tr>
<tr>
<td>Dio2seqa</td>
<td>CCATGGGCCCGTGCTCCTT</td>
<td>CATGTGGCGTAAGTCTGGGTTGCT</td>
<td>65</td>
</tr>
<tr>
<td>Dio2seqb</td>
<td>AACGTGGGCCCTACGGCGTGT</td>
<td>TGCTGTGCTTGCTCTACGGCT</td>
<td>65</td>
</tr>
<tr>
<td>Dio2qPCR</td>
<td>GGACGAGTGCCCGCTGCTGGACTT</td>
<td>GAAGCGGGCCAGGGTGCTGATGA</td>
<td>68</td>
</tr>
</tbody>
</table>

**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
Chapter 2

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.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTper1 pub</td>
<td>AGAGCCCATCCCCACCCAGCAGTT</td>
<td>TCGGCCCGTCAGGAAGGAGA</td>
</tr>
<tr>
<td>1Per1</td>
<td>CTGCTGTCGACCAGCTCGGAGCACGAC</td>
<td>GGCGCCAAAAGCTCCGAAAACATGGTG</td>
</tr>
<tr>
<td>2Per1</td>
<td>CTGCGCTGCAAGCCAGAGCGTCTC</td>
<td>GCAGCTGGGAGTGTGACTGGTGAGTGGGAACATGGTG</td>
</tr>
<tr>
<td>3Per1</td>
<td>GCCCTGCGCGCTGCCCCAACTG</td>
<td>GCGCCCGACGATAAACGACCACCTTC</td>
</tr>
</tbody>
</table>

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-erb*α 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.

<table>
<thead>
<tr>
<th>Name</th>
<th>Purpose</th>
<th>Sequence 5’-3’</th>
<th>Anneal Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rev-erb 5’R1</em></td>
<td>RACE-PCR</td>
<td>GCCCCAGTTGTCCACCTCTCCGGTTATGT</td>
<td>60 °C</td>
</tr>
<tr>
<td><em>Rev-erb 5’R2</em></td>
<td>RACE-PCR</td>
<td>AATGGCGGGGCTTTTGGGTGGATG</td>
<td>60 °C</td>
</tr>
<tr>
<td><em>Rev-erb 3’F1</em></td>
<td>RACE-PCR</td>
<td>TACCCCCAAAGACGAACCCAACCACCA</td>
<td>60 °C</td>
</tr>
<tr>
<td><em>Rev-erb 3’F2</em></td>
<td>RACE-PCR</td>
<td>GGGAGGGCTTGCTAGACACCAC</td>
<td>60 °C</td>
</tr>
<tr>
<td><em>Rev-erb_full_F1</em></td>
<td>Full length outer PCR</td>
<td>AGGCCGACTTGGAAGACTGC</td>
<td>57 °C</td>
</tr>
<tr>
<td><em>Rev-erb_full_R1</em></td>
<td>Full length outer PCR</td>
<td>GTCTATTGGCCTTACCCCTATCA</td>
<td></td>
</tr>
<tr>
<td><em>Rev-erb_full_F2</em></td>
<td>Full length inner PCR</td>
<td>GTTCAGACCTGCACCGATAGAGC</td>
<td>62 °C</td>
</tr>
<tr>
<td><em>Rev-erb_full_R2</em></td>
<td>Full length inner PCR</td>
<td>TAGCCGCCCCAAACCACCACGTC</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 2

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 (K Biosciences 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 H20 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 15 minutes at 95°C followed by 35 cycles of 95 °C for 30 seconds, anneal for 30 seconds (anneal temperatures were generally first tested at mean TM of primer pair – 4 °C) followed by 72 °C extension (1 minute per Kb of expected product) this is followed by a final extension step of 72 °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.

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

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 H₂O to 5 µl

The ligation reaction is then mixed by pipetting gently and incubated overnight at 4 °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 °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 °C for 90 minutes. Cells were then gently centrifuged to collect cells at the bottom of the
Chapter 2

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 °C. Plates were then stored at 4 °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 H2O 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 °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 H2O

**LB Broth and Agar Plates**

LB Broth 500 ml
- 5 g tryptone
- 5 g NaCl
- 2.5 g yeast extract
Chapter 2

- 500 ml dH₂O
- 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 dH₂O
- 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

**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 H₂O
- <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 °C for 20 seconds, 50 °C for 20 seconds and 60 °C for 4 minutes.
Table 11: Quantity of DNA template for sequencing from Beckman 8800 auto sequencer protocol dependent on product size.

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

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 Na₂-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 °C freezer) 95 % ethanol/ nanopure H₂O and vortex to mix. Centrifuge at 14,000 rpm for 15 minutes at 4 °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 °C for
2 minutes) 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 H₂O 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 °C for 15 s anneal x°C (See Table 12 for target specific annealing temperatures) for 15 s and 72 °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 aβ-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

<table>
<thead>
<tr>
<th>Species</th>
<th>Primer Name</th>
<th>Sequence (5’-3’)</th>
<th>Anneal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmo salar</td>
<td>β Actin - Forward</td>
<td>ATCCTGACAGAGCGCGGTTACAGT</td>
<td>61°C</td>
</tr>
<tr>
<td></td>
<td>β Actin - Reverse</td>
<td>TGCCCATCTCTGCTCAAGTCCA</td>
<td>61°C</td>
</tr>
<tr>
<td>Reference genes</td>
<td>EF - α - Forward</td>
<td>TCTGGAGACGCTGCTATTGTTG</td>
<td>61°C</td>
</tr>
<tr>
<td></td>
<td>EF - α - Reverse</td>
<td>GACTTTGTGACCTTGCGGCTGGAG</td>
<td>61°C</td>
</tr>
<tr>
<td>Clock genes</td>
<td>Aanat2 - Forward</td>
<td>GCTCTCCCTGGGCTGGTTGAAG</td>
<td>62°C</td>
</tr>
<tr>
<td></td>
<td>Aanat2 - Reverse</td>
<td>CATGGATGTGCACGCGGAGTT</td>
<td>62°C</td>
</tr>
<tr>
<td></td>
<td>Bmal - Forward</td>
<td>GCTACTTGCAACGCTATGTC</td>
<td>64°C</td>
</tr>
<tr>
<td></td>
<td>Bmal - Reverse</td>
<td>GCTGCGCTCTGTAATGTCTTCA</td>
<td>64°C</td>
</tr>
<tr>
<td></td>
<td>Cry2 - Forward</td>
<td>GAGGGCATGAAAGGGTGGTGGAGGAG</td>
<td>59°C</td>
</tr>
<tr>
<td></td>
<td>Cry2 - Reverse</td>
<td>GTGGAAGAAAATCTGCGAAAGAGAA</td>
<td>59°C</td>
</tr>
<tr>
<td></td>
<td>Clock - Forward</td>
<td>AGAAATGCCTGCACAGTCGAGTTC</td>
<td>64°C</td>
</tr>
<tr>
<td></td>
<td>Clock - Reverse</td>
<td>CCACCAGTGCTAGGAAAGATGTT</td>
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</tr>
<tr>
<td></td>
<td>Per1 - Forward</td>
<td>AGGGGGTCTGAGCAAGGGGGAAGT</td>
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</tr>
<tr>
<td></td>
<td>Per1 - Reverse</td>
<td>TGGGCCACCTGACATGGGGCTGCTG</td>
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</tr>
<tr>
<td></td>
<td>Per2 - Forward</td>
<td>GCTCCCGAGATCTCCTAGGAGTC</td>
<td>60°C</td>
</tr>
<tr>
<td></td>
<td>Per2 - Reverse</td>
<td>GAACAGCCCTCTCGTCCACATC</td>
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</tr>
<tr>
<td></td>
<td>Rev-erb α – Forward</td>
<td>CCCCCAAGACGAACCCAACCAAGAC</td>
<td>61°C</td>
</tr>
<tr>
<td></td>
<td>Rev-erb α – Reverse</td>
<td>AGAGGAGGCAAAGCGCACCATA</td>
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</tr>
<tr>
<td>Seasonal genes</td>
<td>Dio1 - Forward</td>
<td>GACAACAGAGCACTTGTTGACT</td>
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</tr>
<tr>
<td></td>
<td>Dio1 - Reverse</td>
<td>GCCTGCGCAATGTAGACCACC</td>
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</tr>
<tr>
<td></td>
<td>Dio2 - Forward</td>
<td>GGACGAGTGCCGCTGCTGGACTT</td>
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<tr>
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<td>GAAGGCGGCCAGGGTGGCTGATGA</td>
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</tr>
<tr>
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<td>Dio3 - Forward</td>
<td>CCTGGCTGCTGTTCAAGCCGT</td>
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</tr>
<tr>
<td></td>
<td>Dio3 - Reverse</td>
<td>ATCTGGAAGGCGCGTGGAGGAG</td>
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</tr>
<tr>
<td></td>
<td>Eya3 - Forward</td>
<td>GGGCATCAACGACGACGCTTT</td>
<td>64°C</td>
</tr>
<tr>
<td>Gene</td>
<td>Primer 1</td>
<td>Primer 2</td>
<td>Temperature</td>
</tr>
<tr>
<td>-------------</td>
<td>--------------------------------</td>
<td>--------------------------------</td>
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<tr>
<td>Eya3</td>
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<td></td>
</tr>
<tr>
<td>Tshβ</td>
<td>GAGCTCGCCGGACCACGTTTCT</td>
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<td>Tshβ-Reverse</td>
<td>AGTGGCAGCTGAGGGCTACGGG</td>
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<tr>
<td>Liver lipid genes</td>
<td>D6 Fad- Forward</td>
<td>GTGAATGGGGATCCATAGCA</td>
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<tr>
<td>D6 Fad-Reverse</td>
<td>AAACGAACGGACAACCAGAC</td>
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<td></td>
</tr>
<tr>
<td>Elov5-Forward</td>
<td>ACAAGACAGGAATCTCTTCAGATTAA</td>
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<td>Fas-Forward</td>
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<tr>
<td>Fas-Reverse</td>
<td>CAGGGCCAAAAGGAGTAGG</td>
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<td>Hmgcr-Forward</td>
<td>CCTTCAGCCATGAACTGGAT</td>
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<td>Hmgcr-Reverse</td>
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<tr>
<td>Lxr-Forward</td>
<td>GCCGCCGCTATCTGAAATCTG</td>
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<td>Lxr-Reverse</td>
<td>CAATCCGGCAACCAATCTGTAGG</td>
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<td>Srebp1-Forward</td>
<td>GCCATGCAGCAGTTTCTTCAGA</td>
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<tr>
<td>Srebp1-Reverse</td>
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<td>Srebp2-Forward</td>
<td>TCGGCAGCTCCTGAGTATT</td>
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<td>Srebp2-Reverse</td>
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<td></td>
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<tr>
<td>Dicentrarchus β Actin</td>
<td>TGGCCGCAGCACACCAGAC</td>
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<tr>
<td>labrax β Actin</td>
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<td>Aanat2-Forward</td>
<td>ACGCCGCAGGATGCCATCAGTG</td>
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<td></td>
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<tr>
<td>Aanat2-Reverse</td>
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<td>Clock-Forward</td>
<td>CAGACAAGTGCCAGGATTCAG</td>
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<td>Clock-Reverse</td>
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<td>CGGACAGCAGTTTTTATCGA</td>
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<tr>
<td>Per1-Reverse</td>
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</tr>
<tr>
<td>C</td>
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<td>ITC2</td>
<td>2</td>
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<tr>
<td>H</td>
<td>10^x</td>
<td>ITC4</td>
<td>4</td>
</tr>
</tbody>
</table>

**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 ($10^5$).
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
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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 fluorescence 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 \( Y = A + B \times \cos (C \times X - D) \) 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.
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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
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 \textit{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 (\textit{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 (\textit{Clock, Cry2, Per1 and Per2}) in the brain over the course of a year at the natural equinox and solstices. The expression of \textit{Per1} was analysed in both experiments in addition to \textit{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 °C. Fish were sampled on the 6th and 7th 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 acclimation 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).

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

* Last sample point was unable to be repeated due to adverse weather conditions.
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°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).
Table 2: PCR primer names and sequences used for Per1 identification.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>RTper1 pub</td>
<td>AGAGCCCATCCCCACCCAGCAGTT</td>
<td>TCGGCCCGTCAGGAAGGAGA</td>
</tr>
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<td>1Per1</td>
<td>CTGCTGTCGACCAGCTCGGAGCACGAC</td>
<td>GGCGCCAAAAAGCTCCGAAAACATGGTG</td>
</tr>
<tr>
<td>2Per1</td>
<td>CTGCGCTGCAAGCCAGAGCGTCGCC</td>
<td>GCAGCTGGGAGTGTGACTGGTGGTGAAGAT</td>
</tr>
<tr>
<td>3Per1</td>
<td>GCCCTGCCGCTGCCCAGTCCGACACTG</td>
<td>GCGCCCGACGATAAACGCGACC TTC</td>
</tr>
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</table>

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 experiment 1 and 1/10 for experiment 2), 3 μl DNA/RNA free H₂O 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 °C for 15 s anneal x°C (see Table 3 for target specific annealing temperatures) for 15 s and 72 °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.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’-3’)</th>
<th>Anneal</th>
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<tr>
<td>β-Actin - Forward</td>
<td>ATCCTGACAGAGCGCGGTTACAGT</td>
<td>61°C</td>
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<tr>
<td>β-Actin - Reverse</td>
<td>TGCCCATCTCCTGCTCAAAGTCCA</td>
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</tr>
<tr>
<td>Clock - Forward</td>
<td>AGAAATGCCTGCACAGTGGAGTC</td>
<td>64°C</td>
</tr>
<tr>
<td>Clock - Reverse</td>
<td>CCACCAGGTCAAGGAAGATGTT</td>
<td>64°C</td>
</tr>
<tr>
<td>Cry2 - Forward</td>
<td>GAGGGCATAAGGTTGTTGAGGAG</td>
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<td>Cry2 - Reverse</td>
<td>GTGGAAGAACTGCTGGAAGAAGGA</td>
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<tr>
<td>EF - α - Forward</td>
<td>TCTGGAGACGCTGCTATTGTT</td>
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<td>EF - α - Reverse</td>
<td>GAC TTGTGACC TTGCCGCTTGGAG</td>
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</tr>
<tr>
<td>Per1 - Forward</td>
<td>AGGGGTACATGCGAAGGGAAGT</td>
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</tr>
<tr>
<td>Per1 - Reverse</td>
<td>TGGGCCACCTGCATGGGCTCTG</td>
<td>66°C</td>
</tr>
<tr>
<td>Per2 - Forward</td>
<td>GCTCCCAGATGCTGCTGACAGA</td>
<td>60°C</td>
</tr>
<tr>
<td>Per2 - Reverse</td>
<td>GAACAGCCTCCTCGTCCACATC</td>
<td>60°C</td>
</tr>
</tbody>
</table>

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 \( \beta\text{-Actin, ELF-}\alpha \) and \( \text{GAPDH} \). In the Atlantic salmon liver samples, \( \text{ELF-}\alpha \) 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 \( \mu \text{g} \) total RNA (normalised to the appropriate reference gene).

\[ \text{Potential House keeping Genes in the Liver} \]

\[ \text{ExT (Long Day Photoperiod)} \]

**Figure 2:** 24h expression of \( \beta\text{-Actin, EF}\alpha \) and \( \text{Gapdh} \) 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 (Refenetti R., University of South Carolina, USA; [http://www.circadian.org/softwar.html](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 \[ Y = A + B \cos(C \times X - D) \] 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).

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

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<td>FSSHRTVEY</td>
<td>COQTESSVY</td>
<td>AGQAGGA</td>
<td>AEGGAV</td>
<td>ES-4-GEN-3</td>
<td>NEVQGA</td>
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<td>DMRG</td>
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<td>114</td>
<td>115</td>
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</tr>
</tbody>
</table>

Note: The table and diagram represent amino acid sequences or nucleotide sequences extracted from the document. The numbers indicate positions in the sequence.
Figure 4: Partial Atlantic salmon Per1 protein alignment with other available teleost sequence information.

Figure 5: 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
Chapter 3

*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 *Per2* 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).

<table>
<thead>
<tr>
<th>Gene / Photoperiod/ tissue</th>
<th>Acro analysis</th>
<th>ANOVA</th>
<th>Significant Circadian Rhythm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P Value</td>
<td>Acrophase (ExT±SEM)</td>
<td>P Value</td>
</tr>
<tr>
<td>Brain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clock SD</td>
<td>&gt;0.05</td>
<td>-</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Clock 12:12</td>
<td>&gt;0.05</td>
<td>-</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Clock LD</td>
<td>&lt;0.05</td>
<td>05:00 ± 2.68</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Per1 SD</td>
<td>&gt;0.05</td>
<td>-</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Per1 12:12</td>
<td>&gt;0.05</td>
<td>-</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Per1 LD</td>
<td>&gt;0.05</td>
<td>-</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Per2 SD</td>
<td>&gt;0.05</td>
<td>-</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Per2 12:12</td>
<td>&gt;0.05</td>
<td>-</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Per2 LD</td>
<td>&lt;0.05</td>
<td>05:00 ± 2.45</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Fin</td>
<td></td>
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</tr>
<tr>
<td>Clock SD</td>
<td>&lt;0.05</td>
<td>21±2.2</td>
<td>&gt;0.05</td>
</tr>
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<td>Clock 12:12</td>
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<td>-</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Clock LD</td>
<td>&gt;0.05</td>
<td>-</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Per1 SD</td>
<td>&gt;0.05</td>
<td>-</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Per1 12:12</td>
<td>&gt;0.05</td>
<td>-</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Per1 LD</td>
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<tr>
<td>Per2 SD</td>
<td>&gt;0.05</td>
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</tr>
<tr>
<td>Per2 12:12</td>
<td>&gt;0.05</td>
<td>-</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Per2 LD</td>
<td>&gt;0.05</td>
<td>-</td>
<td>&gt;0.05</td>
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<tr>
<td>Liver</td>
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</tr>
<tr>
<td><strong>Clock SD</strong></td>
<td>&gt;0.05</td>
<td>-</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td><strong>Clock 12:12</strong></td>
<td>&lt;0.05</td>
<td>15:00 ± 2.33</td>
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</tr>
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<td><strong>Clock LD</strong></td>
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<td>-</td>
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</tr>
<tr>
<td><strong>Per1 SD</strong></td>
<td>&lt;0.05</td>
<td>13:00 ± 2.64</td>
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</tr>
<tr>
<td><strong>Per1 12:12</strong></td>
<td>&gt;0.05</td>
<td>-</td>
<td>&lt;0.05</td>
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</tr>
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<td><strong>Per2 SD</strong></td>
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<td>&lt;0.05</td>
</tr>
<tr>
<td><strong>Per2 12:12</strong></td>
<td>&gt;0.05</td>
<td>-</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td><strong>Per2 LD</strong></td>
<td>&gt;0.05</td>
<td>-</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td><strong>Melatonin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SD</strong></td>
<td>&lt;0.05</td>
<td>01:00 ± 1.33</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td><strong>12:12</strong></td>
<td>&lt;0.05</td>
<td>23:00 ± 0.95</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td><strong>LD</strong></td>
<td>&lt;0.05</td>
<td>05:00 ± 1.56</td>
<td>&lt;0.05</td>
</tr>
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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
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(January 2010) samples compared to all others. In contrast \textit{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 \textit{Per1} expression was statistically higher in January and March 2010 sample sets. Finally, \textit{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).
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**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).

<table>
<thead>
<tr>
<th>Sample Set</th>
<th>Acro analysis</th>
<th>ANOVA</th>
<th>Significant Circadian Rhythm</th>
</tr>
</thead>
<tbody>
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<td><strong>Clock</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>October 09</td>
<td>&gt;0.05 NS</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>January 10</td>
<td>&gt;0.05 NS</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>March 10</td>
<td>&gt;0.05 NS</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>July 10</td>
<td>&lt;0.05 01±02:30</td>
<td>&lt;0.05</td>
<td>Sig</td>
</tr>
<tr>
<td>Oct 10</td>
<td>&gt;0.05 NS</td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Cry2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>October 09</td>
<td>&gt;0.05 NS</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>January 10</td>
<td>&lt;0.05 06±02.75</td>
<td>&lt;0.05</td>
<td>Sig</td>
</tr>
<tr>
<td>March 10</td>
<td>&gt;0.05 NS</td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>July 10</td>
<td>&lt;0.05 05±02.00</td>
<td>&lt;0.05</td>
<td>Sig</td>
</tr>
<tr>
<td>Oct 10</td>
<td>&gt;0.05 NS</td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Per1</strong></td>
<td></td>
<td></td>
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</tr>
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<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>January 10</td>
<td>&gt;0.05 NS</td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>March 10</td>
<td>&gt;0.05 NS</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>July 10</td>
<td>&gt;0.05 NS</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Oct 10</td>
<td>&gt;0.05 NS</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Per2</strong></td>
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<tr>
<td>October 09</td>
<td>&gt;0.05 NS</td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>January 10</td>
<td>&gt;0.05 NS</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>March 10</td>
<td>&gt;0.05 NS</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>July 10</td>
<td>&gt;0.05 NS</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Oct 10</td>
<td>&lt;0.05 23±2.44</td>
<td>&lt;0.05</td>
<td>Sig</td>
</tr>
<tr>
<td><strong>Melatonin</strong></td>
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<td></td>
<td></td>
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<td>October 09</td>
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</tr>
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<td>Sig</td>
</tr>
<tr>
<td>Oct 10</td>
<td>&lt; 0.05 23:00±1.61</td>
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<td>Sig</td>
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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 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
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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 \textit{Per1} gene. \textit{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 \textit{Clock} and \textit{Per2} was observed in brains sampled from fish exposed to LD. \textit{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 \textit{Clock}, \textit{Bmal} and \textit{Per2} in the brain of salmon parr under SD photoperiod. Yet the phase and shape of the \textit{Clock} and \textit{Per2} expression between the two contrasting results share important characteristics. Results from Davie et al. (2009) and the present the acrophase of \textit{Clock} and \textit{Per2} expression occurs at similar times during the circadian cycle. Peak expression of \textit{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 \textit{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 \textit{Clock} and \textit{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 locomotor 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
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 experiment 1 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 experiment 1 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 experiment 1 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
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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.
Chapter 4

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 photoperiodic 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
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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 Sixl 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.
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.
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 Tshβ not present in the microarray. Further investigation was subsequently carried utilising a qPCR expression study to determine 24h patterns of Dio2, Eya3 and Tshβ 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 1m³ 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 H₂O to a final volume of 200 µl (experiment 1) and 100 µl (experiment 2). Brain cDNA reactions were then stored at -20°C.

**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 fluorescence 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 µl 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 Dio1-3, 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 H₂O 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 °C for 15 s anneal x°C (See Table 1 for target specific annealing temperatures) and 72 °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
\[ Y = A + B \times \cos (C \times X - D) \]

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.

**Table 1** Primers sequences used (5’-3’) and annealing temperatures for PCR and qPCR assays, including primer pairs for Dio2 identification.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer sequence Forward 5’-3’</th>
<th>Primer sequence reverse 5’-3’</th>
<th>Anneal °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dio2</td>
<td>GGCAGCGCATGCTGACCTCG</td>
<td>ACCAGCCCCGTCTCGACCCA</td>
<td>62</td>
</tr>
<tr>
<td>Dio2seqa</td>
<td>CCATGGGCCCGTGCTCCTT</td>
<td>CATGTGGCGTAAGTCTGGGTTGCT</td>
<td>65</td>
</tr>
<tr>
<td>Dio2seqb</td>
<td>AACGTGGGCTACGGCGTGT</td>
<td>TGCTGTGCCCTGCTCTACGGCT</td>
<td>65</td>
</tr>
<tr>
<td>Actin qPCR</td>
<td>ATCCTGACAGAGCGCGGTTACA GT</td>
<td>TGCCCATCTCTGCTCAAGTCCCA</td>
<td>61</td>
</tr>
<tr>
<td>Dio1qPCR</td>
<td>GACAACAGACCCTGGTTGCTGA CT</td>
<td>GCCTGCAGCAATGTAGACCACC</td>
<td>62</td>
</tr>
<tr>
<td>Dio2qPCR</td>
<td>GGACGAGTGCGCCCTGCTGGAC TT</td>
<td>GAAGGCGGCGAGGCTTGATGA</td>
<td>68</td>
</tr>
</tbody>
</table>
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. *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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Full name</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Eya3</em></td>
<td>Eyes absent homolog 3</td>
<td>(Dardente et al. 2010)</td>
</tr>
<tr>
<td><em>Six1</em></td>
<td>SIX-family protein 1</td>
<td>(Dardente et al. 2010)</td>
</tr>
<tr>
<td><em>Tef</em></td>
<td>Thyrotroph embryonic factor</td>
<td>(Dardente et al. 2010)</td>
</tr>
<tr>
<td><em>Tshb</em></td>
<td>Thyroid stimulating hormone beta</td>
<td>(Hanon et al. 2008)</td>
</tr>
<tr>
<td><em>Tshr</em></td>
<td>Thyroid stimulating hormone receptor</td>
<td>(Dardente et al. 2010)</td>
</tr>
<tr>
<td><em>Dio1</em></td>
<td>Type I iodothyronine deiodinase</td>
<td>(Lechan &amp; Fekete 2005)</td>
</tr>
<tr>
<td><em>Dio2</em></td>
<td>Type II iodothyronine deiodinase</td>
<td>(Lechan &amp; Fekete 2005)</td>
</tr>
<tr>
<td><em>Dio3</em></td>
<td>Type III iodothyronine deiodinase</td>
<td>(Lechan &amp; Fekete 2005)</td>
</tr>
</tbody>
</table>
**Table 3**: Numbers of microarray probes displaying significant differences between experimental conditions.

<table>
<thead>
<tr>
<th>P value</th>
<th>Photoperiod SD vs. LD</th>
<th>Day vs. Night</th>
<th>Photoperiod/Day night interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.05</td>
<td>2989</td>
<td>2832</td>
<td>957</td>
</tr>
<tr>
<td>&lt;0.02</td>
<td>1449</td>
<td>1259</td>
<td>338</td>
</tr>
<tr>
<td>&lt;0.01</td>
<td>802</td>
<td>707</td>
<td>174</td>
</tr>
<tr>
<td>&lt;0.005</td>
<td>453</td>
<td>308</td>
<td>71</td>
</tr>
<tr>
<td>&lt;0.001</td>
<td>120</td>
<td>128</td>
<td>10</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Fold Change</th>
<th>Photoperiod SD vs. LD</th>
<th>Day vs. Night</th>
<th>Photoperiod/Day night interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;1</td>
<td>5892</td>
<td>5892</td>
<td>5892</td>
</tr>
<tr>
<td>&gt;1.5</td>
<td>532</td>
<td>494</td>
<td>1418</td>
</tr>
<tr>
<td>&gt;2</td>
<td>192</td>
<td>175</td>
<td>598</td>
</tr>
<tr>
<td>&gt;3</td>
<td>79</td>
<td>54</td>
<td>250</td>
</tr>
<tr>
<td>&gt;5</td>
<td>30</td>
<td>13</td>
<td>111</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Description</th>
<th>Probe</th>
<th>Blast type</th>
<th>Blast result</th>
<th>Short name</th>
<th>P value</th>
<th>Day / Night</th>
<th>Photo/ Day night</th>
<th>Fold change</th>
<th>Day /Night</th>
<th>Photo/ Day night</th>
</tr>
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<tbody>
<tr>
<td>Ssa#STIR18614</td>
<td>RefSeq_Hit Def</td>
<td>Salmo salar selenocysteine lyase (scly), mRNA &gt;gi</td>
<td>223648997</td>
<td>gb</td>
<td>BT059544.1</td>
<td>Salmo salar clone ssal-rgf-511-328 Selenocysteine lyase putative mRNA, complete cds</td>
<td>Cry1</td>
<td>0.9946</td>
<td>0.0001</td>
<td>0.0038</td>
</tr>
<tr>
<td>Ssa#DW579347</td>
<td>RefSeq_Hit Def</td>
<td>PREDICTED: Oreochromis niloticus cryptochrome-1-like (LOC100694774), mRNA</td>
<td>Cry1</td>
<td>0.1112</td>
<td>0.0005</td>
<td>0.0050</td>
<td>-1.1818</td>
<td>1.6146</td>
<td>2.1461</td>
<td></td>
</tr>
<tr>
<td>Omy#S27583073</td>
<td>RefSeq_Hit Def</td>
<td>Oncorhynchus mykiss selenoprotein Ja (selja), mRNA</td>
<td>Cry2</td>
<td>0.0034</td>
<td>0.0613</td>
<td>0.2778</td>
<td>1.5029</td>
<td>1.2602</td>
<td>-1.8940</td>
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</tr>
<tr>
<td>Ssa#S30289725</td>
<td>RefSeq_Hit Def</td>
<td>PREDICTED: Cavia porcellus period circadian protein homolog 2-like (LOC100713579), mRNA</td>
<td>Per1</td>
<td>0.0735</td>
<td>0.0322</td>
<td>0.9397</td>
<td>-1.1302</td>
<td>1.1632</td>
<td>1.3147</td>
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</tr>
<tr>
<td>Ssa#CL285Ctg1</td>
<td>RefSeq_Hit Def</td>
<td>Salmo salar CCAAT/enhancer binding protein (C/EBP), beta (cebpb), mRNA &gt;gi</td>
<td>209152840</td>
<td>gb</td>
<td>BT044870.1</td>
<td>Salmo salar clone ssal-rgf-506-052 CCAAT/enhancer-binding protein beta putative mRNA, complete cds</td>
<td>CCAAT β</td>
<td>0.0586</td>
<td>0.0012</td>
<td>0.4554</td>
</tr>
<tr>
<td>Ssa#STIR07904</td>
<td>RefSeq_Hit Def</td>
<td>Salmo salar CCAAT/enhancer binding protein (C/EBP), beta (cebpb), mRNA &gt;gi</td>
<td>209152840</td>
<td>gb</td>
<td>BT044870.1</td>
<td>Salmo salar clone ssal-rgf-506-052 CCAAT/enhancer-binding protein beta putative mRNA, complete cds</td>
<td>CCAAT β</td>
<td>0.0013</td>
<td>0.0028</td>
<td>0.8080</td>
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<tr>
<td>Ssa#CL344Ctg1</td>
<td>RefSeq_Hit Def</td>
<td>Oncorhynchus mykiss CCAAT/enhancer binding protein beta2 (LOC100379112), mRNA &gt;gi</td>
<td>90019517</td>
<td>gb</td>
<td>DQ423470.1</td>
<td>Oncorhynchus mykiss CCAAT/enhancer binding protein beta2 mRNA, complete cds</td>
<td>CCAAT β2</td>
<td>0.0017</td>
<td>0.0131</td>
<td>0.3282</td>
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<tr>
<td>RefSeq_Hit</td>
<td>Def</td>
<td>CCAAT/enhancer-binding protein beta (LOC100136165), mRNA</td>
<td>DIO 1</td>
<td>DIO 2</td>
<td>DIO 3</td>
<td>DIO 3</td>
<td>DIO 3</td>
<td>DIO 3</td>
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<tr>
<td>Ssa#S35697434</td>
<td>RefSeq_Hit</td>
<td>Oncorhynchus mykiss CCAAT/enhancer-binding protein beta (LOC100136165), mRNA</td>
<td>0.0404</td>
<td>0.9566</td>
<td>1.1808</td>
<td>1.3049</td>
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<tr>
<td>Ssa#S35582016</td>
<td>RefSeq_Hit</td>
<td>PREDICTED: Oreochromis niloticus Type I iodothyronine deiodinase (dio1), mRNA</td>
<td>0.0322</td>
<td>0.9269</td>
<td>0.0183</td>
<td>-1.2021</td>
<td>-1.0231</td>
<td>1.5336</td>
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<td></td>
</tr>
<tr>
<td>Ssa#STIR15458</td>
<td>RefSeq_Hit</td>
<td>Oncorhynchus mykiss deiodinase, iodothyronine, type II (dio2), mRNA</td>
<td>0.0076</td>
<td>0.5060</td>
<td>0.8359</td>
<td>1.6111</td>
<td>-1.074</td>
<td>1.5612</td>
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<td></td>
</tr>
<tr>
<td>Omy#TC151869</td>
<td>B2GO_Blas txHit</td>
<td>iodothyronine deiodinase type III</td>
<td>0.0068</td>
<td>0.5118</td>
<td>0.1950</td>
<td>1.2026</td>
<td>-1.0389</td>
<td>-1.0389</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ssa#DW562425</td>
<td>B2GO_Blas txHit</td>
<td>IOD3_SPAAURecName: Full=Type III iodothyronine deiodinase; AltName: Full=Type-III 5'-deiodinase; AltName: Full=Type 3 DI; AltName: Full=DIOIII; AltName: Full=5DIII</td>
<td>0.0443</td>
<td>0.2534</td>
<td>0.8150</td>
<td>1.2068</td>
<td>-1.0202</td>
<td>1.3343</td>
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<tr>
<td>Omy#S34424874</td>
<td>RefSeq_Hit</td>
<td>PREDICTED: Oreochromis niloticus eyes absent homolog 3-like (LOC100705151), mRNA</td>
<td>0.0041</td>
<td>0.0131</td>
<td>0.9030</td>
<td>-1.3561</td>
<td>1.2844</td>
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</table>
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 (IX135096). 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 selenocysteine 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 selenocysteine 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 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
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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 *Eya3* 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 patterns 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
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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-Gso-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
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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 $Tsh\beta$ 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 $Tsh\beta$ 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 $Tsh\beta$ 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 $Tsh\beta$ 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
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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.
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3. MATERIALS AND METHODS

Animals, housing and tissue sampling

*Atlantic salmon in vitro and in vivo studies*

For both the *in 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 °C in vitro* study, *4.6 ± 0.2 °C in 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°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-phenoxyethanol 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 °C. The cranial caps were stored in the RNA stabilisation solution for 24h at 4 °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 °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 °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 °C in groups (35 per 100 ml) until culture conditions were established (see below).
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Pineal cultures

Atlantic salmon pineal culture

All salmon pineal cultures were carried out at 8 °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 (amphotericin # 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 °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 °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
Chapter 5

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 pineals 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/μl. 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 inhibitor Applied biosystems). Final reactions were then diluted with DNA/RNA free H₂O 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.

<table>
<thead>
<tr>
<th>Species (Latin)</th>
<th>Species (Common)</th>
<th>Ascension number</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Danio rerio</em></td>
<td>Zebrafish</td>
<td>NM_131411.1</td>
<td>NCBI</td>
</tr>
<tr>
<td><em>Carassius auratus</em></td>
<td>Gold fish</td>
<td>GU205782.1</td>
<td>NCBI</td>
</tr>
<tr>
<td><em>Oryzias latipes</em></td>
<td>Medaka</td>
<td>NM_001104846.1</td>
<td>NCBI</td>
</tr>
<tr>
<td><em>Solea senegalensis</em></td>
<td>Senegalese sole</td>
<td>GQ340973.1</td>
<td>NCBI</td>
</tr>
<tr>
<td><em>Paralichthys olivaceus</em></td>
<td>Olive flounder</td>
<td>HQ883478.1</td>
<td>NCBI</td>
</tr>
<tr>
<td><em>Oncorhynchus mykiss</em></td>
<td>Rainbow trout</td>
<td>NM_001124257.1</td>
<td>NCBI</td>
</tr>
<tr>
<td><em>Scophthalmus maximus</em></td>
<td>Turbot</td>
<td>EF033250.1</td>
<td>NCBI</td>
</tr>
<tr>
<td><em>Sparus aurata</em></td>
<td>Gilt head seabream</td>
<td>AY533403.2</td>
<td>NCBI</td>
</tr>
<tr>
<td><em>Esox lucius</em></td>
<td>Pike</td>
<td>AF034082.1</td>
<td>NCBI</td>
</tr>
<tr>
<td><em>Salmo salar</em></td>
<td>Atlantic Salmon</td>
<td>Cluster ID# 3912632</td>
<td>ASalBase</td>
</tr>
<tr>
<td><em>Salmo salar</em></td>
<td>Atlantic Salmon</td>
<td>Cluster ID# 3920741</td>
<td>ASalBase</td>
</tr>
<tr>
<td><em>Salmo salar</em></td>
<td>Atlantic Salmon</td>
<td>S0250N08SP6</td>
<td>ASalBase</td>
</tr>
</tbody>
</table>
### Table 2: Aanat2 primer pairs, sequences, and location on rainbow trout partial sequence.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Region</th>
<th>Location on RT</th>
<th>Primer sequence 5’ - 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aanat2 A</td>
<td>ORF</td>
<td>372bp - 1040bp</td>
<td>F: AGGTCAGCCGCTCTCCGTTCC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: CCAGTGCTAGGGTTGATGTGATTATGA</td>
</tr>
<tr>
<td>Aanat2 B</td>
<td>ORF + 3’</td>
<td>373bp - 1620bp</td>
<td>F: GGTCAGCCGCTCTCCGTTCT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: TGGTGCTGAGCTGAGATTGTGG</td>
</tr>
<tr>
<td>Aanat2 C</td>
<td>ORF + 3’</td>
<td>373bp - past end of RT sequence</td>
<td>F: GGTCAGCCGCTCTCCGTTCTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: CTGCAGCGCCTCAATGACAAAGTG</td>
</tr>
<tr>
<td>Aanat2 D</td>
<td>5’+ ORF (partial)</td>
<td>115bp - 796bp</td>
<td>F: AGACAGGCAGATAGAAAAGCACAGAGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: CAGGTAGCGCCACAGCAGGATG</td>
</tr>
<tr>
<td>Aanat2 E</td>
<td>ORF(partial) + 3’</td>
<td>771bp - 1566bp</td>
<td>F: TCAGCCCAGTAAATGACCATCATGACA CAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: GTTGCAACCTGGTGGACGGTGCAAC</td>
</tr>
</tbody>
</table>
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 seabream *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.
Chapter 5

qPCR

Expression of the target genes was measured by absolute quantification with all samples being normalised with \( \beta\)-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 \( \mu \)l ABsolute\textsuperscript{TM}QPCR SYBR-green qPCR master mix (Thermo scientific, Leon-Rot, Germany). The ABsolute\textsuperscript{TM}QPCR SYBR Green Mix was made up of Thermo-Start\textsuperscript{TM} DNA polymerase, a proprietary reaction buffer, dNTP\'s and SYBR Green I with Mg++ at a concentration of 3 mM in the final 1x reaction. Additionally 3\( \mu \)l DNA/RNA free H\textsubscript{2}O was added to each reaction to a total reaction volume of 20 \( \mu \)l. 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\(^\circ\)C this is followed by 45 cycles of 3 temperature steps; 95 \(^\circ\)C for 15 s anneal x\(^\circ\)C (See Table 3 for target specific annealing temperatures) for 15 s and 72 \(^\circ\)C for 30s. This was followed by a temperature ramp from 70 – 90 \(^\circ\)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 \textit{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 \textit{in vitro} 12D:12L and 24 D experiments (due to absence of rhythmic expression in the 12L:12D experiment). For the salmon \textit{in vivo} experiment, qPCR for Aanat2, Cry2, Clock, Per1 and
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*Per2* was carried out (*Cry2* was analysed in order to compare with previous investigations) while for the seabass *in 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.

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

**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 ≤ 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 (Refenetti R., University of South Carolina, USA; [http://www.circadian.org/softwar.html](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 \( Y = A + B \times \cos (C \times X - D) \) 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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Point</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Light off</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atlantic salmon</td>
<td>Light on</td>
<td>00:00</td>
<td>02:00</td>
<td>06:00</td>
<td>10:00</td>
<td>12:00</td>
<td>14:00</td>
<td>18:00</td>
</tr>
<tr>
<td>12L:12D Salmon In vitro</td>
<td>Zeitgeber Time</td>
<td>00:00</td>
<td>02:00</td>
<td>06:00</td>
<td>10:00</td>
<td>12:00</td>
<td>14:00</td>
<td>18:00</td>
</tr>
<tr>
<td></td>
<td>Real Time</td>
<td>07:00</td>
<td>09:00</td>
<td>13:00</td>
<td>17:00</td>
<td>19:00</td>
<td>21:00</td>
<td>01:00</td>
</tr>
<tr>
<td>12D:12L Salmon In Vitro</td>
<td>Zeitgeber Time</td>
<td>00:00</td>
<td>02:00</td>
<td>06:00</td>
<td>10:00</td>
<td>12:00</td>
<td>14:00</td>
<td>18:00</td>
</tr>
<tr>
<td></td>
<td>Real Time</td>
<td>19:00</td>
<td>21:00</td>
<td>01:00</td>
<td>05:00</td>
<td>07:00</td>
<td>09:00</td>
<td>13:00</td>
</tr>
<tr>
<td>24D:00L Salmon In Vitro</td>
<td>Zeitgeber Time</td>
<td>X</td>
<td>02:00</td>
<td>06:00</td>
<td>10:00</td>
<td>X</td>
<td>14:00</td>
<td>18:00</td>
</tr>
<tr>
<td></td>
<td>Real Time</td>
<td>X</td>
<td>09:00</td>
<td>13:00</td>
<td>17:00</td>
<td>X</td>
<td>21:00</td>
<td>01:00</td>
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<tr>
<td>12L:12D Salmon In Vivo</td>
<td>Zeitgeber Time</td>
<td>00:00</td>
<td>03:00</td>
<td>07:00</td>
<td>11:00</td>
<td>12:00</td>
<td>15:00</td>
<td>19:00</td>
</tr>
<tr>
<td></td>
<td>Real Time</td>
<td>07:00</td>
<td>10:00</td>
<td>14:00</td>
<td>18:00</td>
<td>19:00</td>
<td>22:00</td>
<td>02:00</td>
</tr>
<tr>
<td>12L:12D Seabass In Vitro</td>
<td>Zeitgeber Time</td>
<td>00:00</td>
<td>02:00</td>
<td>06:00</td>
<td>10:00</td>
<td>12:00</td>
<td>14:00</td>
<td>18:00</td>
</tr>
<tr>
<td></td>
<td>Real Time</td>
<td>06:00</td>
<td>08:00</td>
<td>12:00</td>
<td>16:00</td>
<td>18:00</td>
<td>20:00</td>
<td>00:00</td>
</tr>
</tbody>
</table>

*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.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Gene/ Melatonin</th>
<th>P value–Acro analysis</th>
<th>Acrophase – Acro (ZT±SEM)</th>
<th>Significant Circadian Rhythm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmon <em>In vitro</em></td>
<td><em>Clock</em></td>
<td>&gt; 0.05</td>
<td>x</td>
<td>NS</td>
</tr>
<tr>
<td>12L:12D</td>
<td><em>Per1</em></td>
<td>&gt; 0.05</td>
<td>x</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td><em>Per2</em></td>
<td>&gt; 0.05</td>
<td>x</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td><em>Aanat2</em></td>
<td>&gt; 0.05</td>
<td>x</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Melatonin</td>
<td>&lt;0.05</td>
<td>01:00±2.22</td>
<td>Sig</td>
</tr>
<tr>
<td>12D12L</td>
<td><em>Per1</em></td>
<td>&gt; 0.05</td>
<td>x</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td><em>Aanat2</em></td>
<td>&gt; 0.05</td>
<td>x</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Melatonin</td>
<td>&lt;0.05</td>
<td>21:00±2.59</td>
<td>Sig</td>
</tr>
<tr>
<td>24D</td>
<td><em>Per1</em></td>
<td>&gt; 0.05</td>
<td>x</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td><em>Aanat2</em></td>
<td>&gt; 0.05</td>
<td>x</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Melatonin</td>
<td>&gt; 0.05</td>
<td>x</td>
<td>NS</td>
</tr>
<tr>
<td>Salmon <em>In Vivo</em></td>
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<td>&gt; 0.05</td>
<td>x</td>
<td>NS</td>
</tr>
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<td>12L:12D</td>
<td><em>Cry2</em></td>
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<td>20:00±2.30</td>
<td>Sig</td>
</tr>
<tr>
<td></td>
<td><em>Per1</em></td>
<td>&lt;0.05</td>
<td>00:00±2.03</td>
<td>Sig</td>
</tr>
<tr>
<td></td>
<td><em>Per2</em></td>
<td>&gt; 0.05</td>
<td>x</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td><em>Aanat2</em></td>
<td>&gt; 0.05</td>
<td>x</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Melatonin</td>
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<td>20:00±2.50</td>
<td>Sig</td>
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<tr>
<td>Seabass <em>In Vitro</em></td>
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<td>x</td>
<td>NS</td>
</tr>
<tr>
<td>12L:12D</td>
<td><em>Per1</em></td>
<td>&gt; 0.05</td>
<td>x</td>
<td>NS</td>
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</tr>
<tr>
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<td>Melatonin</td>
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<td>21:00±1.32</td>
<td>Sig</td>
</tr>
</tbody>
</table>
Table 6: Mean mRNA expression as copy numbers per μg totRNA over 24 hrs sampling period for each gene/condition.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Photoperiod/Gene</th>
<th>Mean copy no/ mg totRNA</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Salmon in vivo</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>12L:12D</td>
<td>Aanat2</td>
<td>18332385.60</td>
<td>1015936.77</td>
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<tr>
<td></td>
<td>Clock</td>
<td>60528.70</td>
<td>4543.10</td>
</tr>
<tr>
<td></td>
<td>Cry2</td>
<td>981172.06</td>
<td>49901.82</td>
</tr>
<tr>
<td></td>
<td>Per1</td>
<td>971463.50</td>
<td>51522.86</td>
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<td></td>
<td>Per2</td>
<td>15811.32</td>
<td>1007.37</td>
</tr>
<tr>
<td><strong>Salmon in vitro</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>12L:12D</td>
<td>Aanat2</td>
<td>11911442.85</td>
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<tr>
<td></td>
<td>Clock</td>
<td>658747.62</td>
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<td></td>
<td>Per2</td>
<td>100207.53</td>
<td>12076.89</td>
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<tr>
<td>12D:12L</td>
<td>Aanat2</td>
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<td>626102.60</td>
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<td></td>
<td>Per1</td>
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<td>Aanat2</td>
<td>7713770.44</td>
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</tr>
<tr>
<td></td>
<td>Per1</td>
<td>2255335.64</td>
<td>144366.38</td>
</tr>
<tr>
<td><strong>Seabass in vitro</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>12L:12D</td>
<td>Aanat2</td>
<td>1834792.64</td>
<td>167446.53</td>
</tr>
<tr>
<td></td>
<td>Clock</td>
<td>544232.24</td>
<td>345805.87</td>
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<tr>
<td></td>
<td>Per1</td>
<td>925596.87</td>
<td>163462.21</td>
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</table>
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) whereby 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 zeitgeber time (ZT) whereby 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 zeitgeber time (ZT) whereby ZT0 is the onset of light (corresponding to 12L:12D *In Vitro*). The presence of 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 in 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
Chapter 5

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 analysis of the Aanat2 5’ region revealed the presence of an E-box element in the 5’region of the AGKD01021084 sequence (Appendix 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, approximately 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 (Appendixes 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 the gene. 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 (Appendixes 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, Appendixes 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|ENSDARP0000002650).
Chapter 5

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 in 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\(^{2+}\) 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. 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 downstream of the *Aanat2* start codon within and downstream 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 downstream regulatory element, pineal – restrictive downstream 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 approximately 1kb and 466bp up-
stream of the start codon. However in contrast to other teleosts the 5’ UTR extends up-stream encompassing 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 processes involved 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-erba 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-erba* 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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Full Name</th>
<th>Accession number</th>
<th>Gene Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Srebp1</em></td>
<td>Sterol Regulatory Element-Binding Protein 1</td>
<td>TC148424</td>
<td>Indirectly involved in cholesterol synthesis. In mammals involved in sensing cholesterol availability in the ER.</td>
</tr>
<tr>
<td><em>Srebp 2</em></td>
<td>Sterol Regulatory Element-Binding Protein 2</td>
<td>TC166313</td>
<td>Indirectly involved in cholesterol synthesis by regulating <em>Hmgcr</em>.</td>
</tr>
<tr>
<td><em>Lxr</em></td>
<td>Liver x receptor</td>
<td>FJ470290</td>
<td>Regulates cholesterol and fatty acids by activating <em>Cyp7a</em>, the rate limiting enzyme in the conversion of cholesterol to bile acid.</td>
</tr>
<tr>
<td><em>FadsD6</em></td>
<td>D6 – Fatty acid desaturase</td>
<td>AY458652</td>
<td>Required for the synthesis of highly unsaturated fatty acids.</td>
</tr>
<tr>
<td><em>Elov15a</em></td>
<td>Elongation of very long chain fatty acids protein 5</td>
<td>AY170327</td>
<td>Participates in the biosynthesis of long chain poly unsaturated fatty acids. Primarily the elongation of C18 and C20.</td>
</tr>
<tr>
<td><em>Hmgcr</em></td>
<td>3-hydroxy-3-methylglutaryl-CoA reductase</td>
<td>DW561983</td>
<td>Resulting HMGCoA reductase enzyme is the rate limiting enzyme in cholesterol synthesis.</td>
</tr>
<tr>
<td><em>Fas</em></td>
<td>Fatty acid synthesis</td>
<td>DW551395</td>
<td>Corresponding protein catalyzes fatty acid synthesis.</td>
</tr>
<tr>
<td><em>Bmal1</em></td>
<td>Brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like</td>
<td>DY 735402</td>
<td>Forms part of the positive arm of the circadian molecular clock. BMAL forms heterodimer with CLOCK and initiates the transcription of <em>Per</em> and <em>Cry</em> genes.</td>
</tr>
<tr>
<td><em>Clock</em></td>
<td>Circadian Locomotor Output Cycles Kaput</td>
<td>CA 038738</td>
<td>In conjunction with BMAL forms the positive arm of the circadian clock and regulates negative elements <em>Cry</em> and <em>Per</em>.</td>
</tr>
<tr>
<td><em>Per 1</em></td>
<td>Period1</td>
<td></td>
<td>In conjunction with <em>Cry</em> genes forms negative components of the circadian clock.</td>
</tr>
<tr>
<td><em>Per 2</em></td>
<td>Period2</td>
<td>FM877775</td>
<td>In conjunction with <em>Cry</em> genes forms negative components of the circadian clock.</td>
</tr>
<tr>
<td><em>Rev-erb a</em></td>
<td>nuclear receptor subfamily 1, group D, member 1</td>
<td></td>
<td>REV-ERBa is regulated in a circadian manner by BMAL and via E-box elements regulates a number of clock controlled genes.</td>
</tr>
</tbody>
</table>
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°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 H₂O 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*
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Rev-erb\(\alpha\) 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-erb\(\alpha\) 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 \(\mu\)g 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](http://www.dnastar.com)).

Table 2: Primer pairs and sequences for Rev-erb \(\alpha\) identification including primer name, purpose, sequence and annealing temperature.

<table>
<thead>
<tr>
<th>Name</th>
<th>Purpose</th>
<th>Sequence 5’-3’</th>
<th>Anneal Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rev-erb 5’R1</td>
<td>RACE-PCR</td>
<td>GCCCCCAGTTGTCCACCTCTCCGTATGT</td>
<td>60 °C</td>
</tr>
<tr>
<td>Rev-erb 5’R2</td>
<td>RACE-PCR</td>
<td>AATGGCGGGCTTTGGGATG</td>
<td>60 °C</td>
</tr>
<tr>
<td>Rev-erb 3’F1</td>
<td>RACE-PCR</td>
<td>TACCCCCAAGACGAACCAACA</td>
<td>60 °C</td>
</tr>
<tr>
<td>Rev-erb 3’F2</td>
<td>RACE-PCR</td>
<td>GGGAGGGCTTGCTAGACACCATT</td>
<td>60 °C</td>
</tr>
<tr>
<td>Rev-erb_full_F1</td>
<td>Full length outer PCR</td>
<td>AGGCCGACTTGGAACACTGC</td>
<td>57 °C</td>
</tr>
<tr>
<td>Reverb_full_R1</td>
<td></td>
<td>GTCTATGGGCTTACCCCTATCA</td>
<td></td>
</tr>
<tr>
<td>Rev-erb_full_F2</td>
<td>Full length inner PCR</td>
<td>GTTCAGACCTGCACGGATAGAGC</td>
<td>62 °C</td>
</tr>
<tr>
<td>Rev-erb_full_R2</td>
<td></td>
<td>TAGCCGCCAACCACCACTGTC</td>
<td></td>
</tr>
</tbody>
</table>
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qPCR

In order to determine diel patterns 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 °C for 15 minutes and 45 cycles of 95 °C for 15s, anneal for 15s and 72 °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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence F 5’-3’</th>
<th>Primer Sequence R 5’-3’</th>
<th>Anneal° C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Srebp 1</td>
<td>GCCATGCAGCAGTTTCTTTTCTCA</td>
<td>TCTGGCCAGGACGCATCTCA</td>
<td>63</td>
</tr>
<tr>
<td>Srebp 2</td>
<td>TCGCCGCCTCTGTATGATT</td>
<td>AGGGCTAGGTGACTGTTCTGG</td>
<td>63</td>
</tr>
<tr>
<td>Lxr</td>
<td>GCCGCCGCTATCTGAAAATCTG</td>
<td>CAATCCGCAACCATCTGTA</td>
<td>58</td>
</tr>
<tr>
<td>D6Fa ds</td>
<td>GTGAATGGGGATCCATGCA</td>
<td>AAACGAACGGCAACCAAGAC</td>
<td>60</td>
</tr>
<tr>
<td>Elovl5 a</td>
<td>ACAAGACAGGAATCTTTCAGAATTTAA</td>
<td>TCTGGGGTACTGCTGATGTCGTAC</td>
<td>58</td>
</tr>
<tr>
<td>Hmger</td>
<td>CCTTCAGCCATGAACTGGAT</td>
<td>TCCTGTCCACAGGCAATGTA</td>
<td>60</td>
</tr>
<tr>
<td>Fas</td>
<td>ACCGCCAAGCTCAGTGTGC</td>
<td>CAGGCCCCAAGGAGTAGC</td>
<td>60</td>
</tr>
<tr>
<td>Bmal1</td>
<td>GCCTACTTGCAACGCTATGCC</td>
<td>GCTGCGGCTCGTAAATGTCTTCA</td>
<td>64</td>
</tr>
<tr>
<td>Clock</td>
<td>AGAAATGCTGCACAGTCTGGAGT GC</td>
<td>CCACCAGGTCAGAAGGAAGAT GT</td>
<td>64</td>
</tr>
<tr>
<td>Per 1</td>
<td>AGGGGTCATGCGGAAGGGGAA GT</td>
<td>TGGGACCTGATGGGCTGT</td>
<td>66</td>
</tr>
<tr>
<td>Per 2</td>
<td>GCTCCCAGAATTTCCTAGTGAACAG</td>
<td>GAACAGCCCCTCTGTCACATC</td>
<td>60</td>
</tr>
<tr>
<td>Reverba</td>
<td>CCCCCAGACGAAACCCACAAG AC</td>
<td>AGAGGAGGCAAGCGCACCACAC G</td>
<td>61</td>
</tr>
<tr>
<td>Ef1a</td>
<td>CTGGAGACGCTGCTATTGTTG</td>
<td>GACTTTGTGACCTTGCGCTTG</td>
<td>61</td>
</tr>
</tbody>
</table>

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 $Y = A + B \cos(C \times X - D)$ 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 $\beta$–Actin, $EF\alpha$ 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.
Chapter 6

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-erb1α sequences (Figure 5).
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-erba*. 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-erb1α in relation to other vertebrate Rev-erb1α & 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 Bmal1 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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Acro P value</th>
<th>Acrophase ZT</th>
<th>ANOVA P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bmal1</td>
<td>&lt;0.05</td>
<td>13:00±3.9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Clock</td>
<td>&gt;0.05</td>
<td>-</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Per 1</td>
<td>&gt;0.05</td>
<td>-</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Per 2</td>
<td>&gt;0.05</td>
<td>-</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Rev-erb a</td>
<td>&gt;0.05</td>
<td>-</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>D6Fads</td>
<td>&gt;0.05</td>
<td>-</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Elovl5a</td>
<td>&gt;0.05</td>
<td>-</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Fas</td>
<td>&gt;0.05</td>
<td>-</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Hmgcr</td>
<td>&gt;0.05</td>
<td>-</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Lxr</td>
<td>&lt;0.05</td>
<td>13:00±2.73</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Srebp1</td>
<td>&lt;0.05</td>
<td>13:00±2.41</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Srebp 2</td>
<td>&gt;0.05</td>
<td>-</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>
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 Zeitgeber 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 Srebp1cexpression 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 lxra and lxrb 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 \(\alpha\) 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 \(\alpha\) 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 \(\alpha\) 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 \(\alpha\) 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 \(\alpha\) 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.
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 PPARα 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

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 experiment 1 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-paraventricular 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
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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
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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 acclimation 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 photoperiod, have been shown to regulate rhythmic gene expression including temperature, feeding. For example, temperature has been shown to differentially regulate peripheral oscillations of \textit{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, \textit{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 \textit{Clock} and \textit{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, *Perl, 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**
REFERENCES


Arrojo E Drigo, R. & Bianco, A.C. 2011. Type 2 deiodinase at the crossroads of thyroid hormone action. The international journal of biochemistry & cell biology, 43, 1432-1441.


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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.

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

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<td>S3568567</td>
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<td>S3024207</td>
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<td>S3200191</td>
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<td>S3567330</td>
<td>B2GO_BlastnHit</td>
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<td>S3558277</td>
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<td>Oncorhynchus mykiss Growth arrest and DNA-damage-inducible</td>
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<td>Ssa#S3024051</td>
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<td>Salmo salar limb and neural patterns a (lnpa), mRNA</td>
<td>0.000987987</td>
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### Appendix 1c: Microarray targets (P< 0.001) Photoperiod/ Day night interaction.

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<td>taeniopygia guttata misc_rna miscrna</td>
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<td>Ssa#STIR 25834</td>
<td>RefSeq_Hit Def</td>
<td>PREDICTED: Oreochromis niloticus heterogeneous nuclear ribonucleoprotein L-like, transcript variant 2 (LOC100698149), mRNA</td>
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<tr>
<td>Ssa#STIR 19299</td>
<td>RefSeq_Hit Def</td>
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<td>Ssa#S355 97250</td>
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<td>Ssa#DW5 77228</td>
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<td>Ssa#S355 96271_S</td>
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<td>Ssa#CB51 8090</td>
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<td>pol-like protein</td>
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Appendix

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

<table>
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<tr>
<th>Probe Name</th>
<th>Blast type</th>
<th>RefSeq_HitDef</th>
<th>Fold change photoperiod</th>
<th>p-value Photoperiod</th>
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<tr>
<td>Ssa#STIR1 5680</td>
<td>RefSeq_HitDef</td>
<td>Salmo salar adenosine monophosphate deaminase 1 (isoform M) (ampd1), mRNA &gt;gi</td>
<td>197631794</td>
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<tr>
<td>Ssa#STIR1 7814</td>
<td>RefSeq_HitDef</td>
<td>Oncorhynchus mykiss arylalkylamine N-acetyltransferase (aanat-2), mRNA &gt;gi</td>
<td>4585222</td>
<td>gb</td>
</tr>
<tr>
<td>Ssa#STIR0 0072_4</td>
<td>B2GO_Blastx Hit</td>
<td>interferon-inducible protein gig2</td>
<td>9.332867</td>
<td>0.000309485</td>
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<tr>
<td>Ssa#S23871 809</td>
<td>RefSeq_HitDef</td>
<td>Salmo salar fast myotomal muscle troponin-T-2 (LOC100196675), mRNA &gt;gi</td>
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<td>gb</td>
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<tr>
<td>Ssa#STIR1 8455</td>
<td>RefSeq_RNA</td>
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<tr>
<td>Ssa#CX357 274</td>
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<tr>
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<td>RefSeq_RNA</td>
<td>Salmo salar v-yes-1 Yamaguchi sarcoma viral related oncogene homolog (lyn), mRNA &gt;gb</td>
<td>BT045857.1</td>
<td>Salmo salar clone ssal-rgf-534-333 Tyrosine-protein kinase Lyn putative mRNA, complete cds</td>
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<td>Omy#CX03 4437</td>
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<td>S35528745</td>
<td>B2GO_Blastx</td>
<td>Hit</td>
<td>PREDICTED: similar to interferon-inducible protein Gig2</td>
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<td>TC167443</td>
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<tr>
<td>STIR00072_2</td>
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<td>KSS3721</td>
<td>RefSeq_HitDef</td>
<td>Salmo salar RAS guanyl-releasing protein 2 (grp2), mRNA &gt;gi</td>
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<tr>
<td>STIR0061_S</td>
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<td>STIR19294</td>
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<td>Salmo salar Low density lipoprotein receptor adapter protein 1 (arh), mRNA &gt;gi</td>
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<tr>
<td>STIR00118_4</td>
<td>RefSeq_HitDef</td>
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<tr>
<td>STIR15776</td>
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<td>Salmo salar myxovirus resistance 2 (mx2), mRNA</td>
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<td>PREDICTED: wu:fb81h03</td>
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Appendix 2b: Microarray targets (> 5 x Fold change) Day vs night.

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<tr>
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<td>RefSeq_HitDef</td>
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<td>RefSeq_HitDef</td>
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<tr>
<td>Omy#CA380549</td>
<td>B2GO_Blastx Hit</td>
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<tr>
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<td>RefSeq_HitDef</td>
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<td>RefSeq_RNA</td>
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<td>RefSeq_HitDef</td>
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### Appendix 2c: Microarray targets (> 5 x Fold change) Photoperiod/ Day night interaction (maximum)

<table>
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<th>P value Photoperiod / Day night</th>
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<td>Ssa#S23871809</td>
<td>RefSeq_HitDef</td>
<td>Salmo salar fast myotomal muscle troponin-T-2 (LOC100196675), mRNA &gt;gi</td>
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<tr>
<td>Ssa#S18892465</td>
<td>RefSeq_HitDef</td>
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<tr>
<td>Ssa#S35496061_S</td>
<td>B2GO_Blastx Hit</td>
<td>imap family member 8</td>
<td>50.791286</td>
<td>0.04563181</td>
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<tr>
<td>Ssa#STIR00118_4</td>
<td>RefSeq_HitDef</td>
<td>Salmo salar creatine kinase-1 (ckm1), mRNA &gt;gi</td>
<td>197632378</td>
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<td>B2GO_Blastx Hit</td>
<td>imap family member 8///&quot;PREDICTED: similar to GTPase, IMAP family member 7&quot;</td>
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<td>Ssa#TC106938</td>
<td>RefSeq_HitDef</td>
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<td>B2GO_Blastx Hit</td>
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<td>RefSeq_HitDef</td>
<td>PREDICTED: Monodelphis domestica THO complex subunit 1-like (LOC100013574), mRNA</td>
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<td>0.04348469</td>
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<tr>
<td>Omy#CA380549</td>
<td>B2GO_Blastx Hit</td>
<td>PREDICTED: wu:fb81h03</td>
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<td>RefSeq_RNA</td>
<td>Salmo salar v-yes-1 Yamaguchi sarcoma viral related oncogene homolog (lyn), mRNA &gt;gb</td>
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<td>Salmo salar clone ssal-rgf-534-333 Tyrosine-protein kinase Lyn putative mRNA, complete cds</td>
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<td>Omy#S15318281</td>
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