Seasonal variations in clock gene-expression in Atlantic salmon (*Salmo salar*)

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Abstract

In homeothermic vertebrates inhabiting temperate latitudes, it is clear that the seasonal changes in daylength are decoded by the master circadian clock which through secondary messengers, like pineal melatonin secretion, entrains rhythmic physiology to local conditions. In contrast, the entrainment and neuroendocrine regulation of rhythmic physiology in temperate teleosts is not as clear, primarily due to the lack of understanding of the clock gene system in these species. In this study we analyzed the diel expression of the clock genes in brains of Atlantic salmon, a species that is both highly photoperiodic and displays robust clock-controlled behavior. Atlantic salmon parr were acclimated to either long (LD) or short day (SD) photoperiods for one month and thereafter sampled at 4 h intervals over a 24 h cycle. *Clock*, *Bmal1*, *per2,* and *cry2* were all actively expressed in salmon brain homogenates and, with the exception of *per2,* all displayed rhythmic expression under SD photoperiods that parallels that reported in zebrafish. Interestingly, daylength significantly altered the mRNA expression of all clock genes studied with *Clock*, *Bmal1,* and *per2* all becoming arrhythmic under the LD compared to SD photoperiod, while *cry2* expression was phase delayed under LD. It is thus proposed that the clock gene system is actively expressed in Atlantic salmon and, furthermore, as has been reported in homeothermic vertebrates, it appears that clock expression is daylength-dependent. (Email correspondance: andrew.davie@stir.ac.uk)

Keywords: Atlantic salmon, *Salmo salar*, circadian clock, photoperiodism, *Clock*, *Per2*

Introduction

Most temperate vertebrates demonstrate both daily and yearly rhythms in their biochemistry, physiology, and behavior which are entrained to local conditions by the daily and seasonal rhythms in photoperiod (Bromage et al., 2001; Foster, 2002). Recent years have seen significant progress in our understanding of the basis of photoperiodism in a wide range of organisms; however, this work has mainly concentrated on mammalian, invertebrate, or fungal models (Foster & Kreitzman, 2005). In the case of circadian rhythms, it is clear that entrainment is mediated via a self-sustaining endogenous clock which can maintain rhythms in the absence of environmental cues (Duffield, 2003; Dardente & Cermakian, 2007). The internal mechanism that sustains circannual rhythms is still an open debate; however, recent work in mammals and aves has identified that the circadian clock can express daylength-dependent variations in transcription profiles suggesting one route by which seasonal information can be integrated at the molecular level (e.g., Lincoln et al., 2002; Yasuo et al., 2003; Tournier et al., 2007). To date, information on clock function in poikilothermic vertebrates is very scarce, especially in teleosts, where the clock gene system has only been characterised in the zebrafish (*Danio rerio*) (Whitmore et al., 1998, 2000; Cermakian et al., 2000; Tamai et al., 2005). From these studies, it is evident that zebrafish possess homologues of both invertebrate and mammalian clock genes that exhibit rhythmic expression patterns comparable to those reported in both classes (Pando & Sassone-Corsi, 2002), leading to the suggestion that the teleost clock systems may represent an evolutionary link.

While the tropical teleost zebrafish has proven to be a particularly useful model for the study of basic circadian clock function, these animals do not have any apparent seasonal phenotype. In contrast, the salmonids, a family of temperate

teleosts, including Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), are highly seasonal and responsive to photoperiodic changes (Duston & Saunders, 1992). Furthermore, they display robust temporal organization (Stewart et al., 2002, 2006) as well as clear circadian (e.g., Sanchez-Vazquez & Tabata, 1998; Zaunreiter et al., 1998a, 1998b) and more importantly circannual rhythms (e.g., Eriksson & Lundqvist, 1982; Duston & Bromage, 1988; Randall et al., 1998) in their biochemistry, physiology, and behavior. However, despite these interesting features, studies of clock-gene cycling in these species are extremely limited. Previous studies have linked *Clock* along with *Period 1* (*Per1*) and *Brain and Muscle ARNT like protein -1* (*Bmal1*) to temporal variations in spawning time in rainbow trout (Leder et al., 2006), migration mode in Chinook salmon (*Oncorhynchus tshawytscha*) (O'Malley et al., 2007), and reproductive strategy in Atlantic salmon (Aubin-Horth et al., 2005). Furthermore, expression of the key transcription factor CLOCK which, with BMAL1 drives the rhythmic expression of the *per* and *cryptochrome (cry)* genes (Dunlap, 1999). has been localized in the visual centers of the brain of rainbow trout (Mazurais et al., 2000). However, despite clock systems being present in salmonids, diel profiles of clock-gene expression have not been characterized in these species so far. To better characterize the effects of photoperiod in teleosts and further explore and compare the seasonal clock cycling within vertebrates, this study investigated seasonal profiles of clock gene mRNA as expressed in the brain of Atlantic salmon.

Materials and Methods

Animals and experimental procedures

All procedures were performed in accordance with the Animals (Scientific Procedures) Act, UK, 1986 under the approval of the local ethical review board and in accordance with the ethical standards of the journal (Portaluppi et al., 2008). Juvenile Atlantic salmon parr (26 ± 4 g) of a farmed stock origin (mixed sex), previously reared under a natural photo-thermal cycle at the Niall Bromage Freshwater Research Facilities (Institute of Aquaculture, Stirling, Scotland, 56 : 02 N), were transferred from the natural photo-cycle at that time (October 2nd 10.5L:13.5D) and acclimated for one month to either a long- (LD: 16L:8D) or short-day (SD: 8L:16D) photoperiod at ambient temperature (10.4 ± 0.3 °C). Food was offered in excess throughout the day and night.

Salmon were killed by lethal anaesthesia (2-phenoxyethanol, Sigma –Aldrich Co. Ltd, Poole, UK) followed by decapitation every 4 h over a 24 h period (n = 6 per time point per treatment). Brains including pituitary, though excluding the pineal gland, were dissected, homogenized in 1 ml TRIzol® Reagent (Invitrogen, UK) per 100 mg of tissue over ice, rapidly frozen, and stored at -70°C.

For the tissue expression studies, an additional six individuals maintained in the LD population were killed by lethal anaesthesia followed by decapitation, 2 h after sunrise and sunset only. Whole brain, eye, gonad, heart, and spleen, along with samples of gill, white muscle, kidney, liver, and intestine, were dissected, homogenized in 1 ml TRIzol® Reagent (Invitrogen, UK) per 100 mg of tissue over ice, rapidly frozen, and stored at -70°C.

RNA extraction and cDNA synthesis

Frozen homogenates were thawed and RNA extracted in accordance with guidelines (Invitrogen, UK). RNA pellets where reconstituted in 50 µl of MilliQ water. RNA quality checks were performed with a ND-1000 Nanodrop spectrophotometer (Labtech Int., East Sussex, UK). Furthermore 1 µg of total RNA was analyzed on a 1% agarose denaturing RNA gel electrophoresis showing ribosomal RNA of good quality. cDNA was synthesized using 1 µg of total RNA, 25 µM of anchored oligo dT_{20}, 500 µM dNTPs, 200 units of SuperScript^{TM} II RT reverse transcriptase with provided buffer (all from Invitrogen, Paisley, UK) in a final volume of 20 µl. Reactions were incubated for 60 min at 42°C followed by 70°C for 15 min. All samples were further diluted 1:10, and then stored at -70°C prior to qPCR analysis.

Synthesis of Clock, Bmal1, Per2, and Cry2 cDNA

Real-time quantitative PCR (qPCR) assays were used to quantify expression of *Clock*, *Bmal1*, *Per2,* and *Cry2* in the brain. Accession numbers of Norwegian Salmon Genome Project expressed sequence tag clones identified through BLAST analysis as *Clock*, *Bmal1*, *Cry2,* and published sequence (salmon *β-Actin*) used to design primers are shown in Table 1. For *Per2* primers (Per2f, Per2R) previously designed on Zebrafish (ENSDARG00000034503) (Whitmore D. *Pers. Com.*) generated a 543bp product designated *Salmo salar Per2* (FM877775). All primers were designed using PrimerSelect Ver. 6.1 program (DNASTAR, www.dnastar.com).

Partial cDNA sequences for each target were generated by PCR using 0.5 µM of primers (ClkF, ClkR; BmalF, BmalR; Per2F, Per2R; Cry2F, Cry2R), one eighth (2.5 µl) of the cDNA synthesis reaction, Taq polymerase with supplied buffer IV (ABgene, UK), and 1 mM $MgCl_2$ in a final volume of 25 µl using a touch-down PCR

strategy: 2 min 95°C, 5 cycles 95°C 20 s, 70°C 20 s, 72°C 1 min, 5 cycles 95°C 20 s, 68°C 20 s, 72°C 1 min, and 27 cycles 95°C 20 s, X°C 20 s, 72°C 1 min. Where annealing temperature X = 58, 60, 59, and 59°C for *Clock, Bmal1, Per2,* and *Cry2,* respectively. All primer pairs generated a single PCR product that was cloned into a 2.1 plasmid (Topo TA, Invitrogen, Paisley, 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/).

Sequence analysis

Sequencing was performed using a Beckman 8800 autosequencer. Lasergene SEQman software (DNASTAR, www.dnastar.com) was used to edit and assemble DNA sequences. ClustalW (Thompson et al., 2000) was used to generate multiple alignments of deduced protein sequences. MEGA version 4 was used (Tamura et al., 2007) to deduce and bootstrap phylogenetic trees using the neighbor joining method (Saitou & Nei, 1987).

Quantitative PCR

All cDNA for qPCR was synthesized using Superscript II reverse transcriptase and supplied buffer components (Invitrogen, UK) and an oligo-dT containing primer (as described above). qPCR primers for *Clock*, *Bmal1*, *Per2*, *Cry2,* and the reference gene *β-actin*, (Table 1) were used at 0.5 µM, with one fortieth of the total cDNA synthesis reaction and SYBR-green qPCR mix (ABgene, UK) in a total volume of 20 µl. The thermal cycling protocol run in a Techne Quantica thermocycler (Techne, Quantica, Cambridge, UK) consisted of 15 min at 95°C followed by 45 cycles of 95°C for 15 s, X°C for 15 s,and 72°C for 30 s followed by a temperature ramp from

70 to 90°C for melt-curve analysis. The annealing temperature (X) were changed as follows; 64°C for *Clock,* 60°C for *Bmal1*, *Per2*, and *Cry2* and 61°C for *β-actin*. Mel-curve analysis verified the primer sets for each qPCR assay generated one single product and no primer-dimer artefacts. In addition, each qPCR product was than sequenced to confirm its identity and was found to be 100% identical to its relative sequence. Quantification was achieved by a parallel set of reactions containing standards consisting of serial dilution of spectrophotometrically determined, linearized plasmid-containing. Partial-salmon cDNA sequences generated as described above. All samples were run in triplicate together with non-template controls, standards, and internal controls to correct expression levels between plates. Only *Clock* and *Per2* were analyzed for the tissue expression study..

Normalization of qPCR results.

In a pilot study, the expression levels of five candidate reference genes: *α-tubulin* (DW570994), *β-actin* (AF012125), *GAPDH* (AM230811), *Elongation factor alpha* (AF321836), and *Elongation factor beta* (DY711785) were compared by qPCR in cDNA samples (n = 5) from three different external times under the two different photoperiod treatments (*i.e.,* six different conditions, data not shown). *β-actin* displayed the least variation over these conditions, as determined by geNorm-assisted analysis (Vandesompele et al., 2002) and was therefore used in the subsequent analysis of expression data.

Statistical analyses

Statistical analysis was performed using the InStat Statistical package (V 3.01; GraphPad Software Inc. USA). When considering the diel expression profiles from salmon brains, data was first assessed for normality with the Kolmogorov-Smirnov test and for homogeneity of variances by Bartlett's test. Means of the sample data were compared by two-way analysis of variance (ANOVA). Post hoc multiple comparisons were applied using Tukey's test. A significance of $p<0.05$ was applied to all statistical tests performed. All data are presented as mean ± SEM (standard error of the mean). Where a significant effect of time was observed by ANOVA, non-linear regression was used to fit the cosine function:

$$Y = A + B \times \cos(C \times X - D)$$

Where Y is the copy no. per µg of totRNA, A is the baseline copy no. per µg of totRNA, B is the wave amplitude (one half of the peak-to-trough variation determined by the cosine approximation), C is the frequency multiplier (set to fix period at 24 h), and D is the acrophase (peak time of the cosine approximation). This was performed in Acro version 3.5 (Refennetti R., University of South Carolina, USA http://www.circadian.org/softwar.html). Comparison of expression in tissues between external time (Ext) 06:00 and 22:00 h was performed by Student's *t* test.

Results

Seasonal clock gene expression patterns in whole brain extracts of Atlantic salmon

In accordance with (Daan et al., 2002), data are presented in relation to 'external time' (Ext), where Ext 00:00 h is defined as the middle of the dark phase. All four clock genes were expressed in the Atlantic salmon brain and their daily expression under both photoperiods is presented in Figure 1. In all cases, there was a significant interaction between photoperiod and external time ($p< 0.0001$). *Clock*, *Bmal1,* and *Per2* did not exhibit significant temporal variations in mRNA expression under the LD photoperiod, while there did show significant variations under the SD photoperiod ($p<0.05$). In the case of *Clock* and *Bmal1*, levels were elevated throughout the majority of the dark phase, while *Per2* peaked just prior to sunrise. *Cry2* was the only gene in which expression varied with time in both the LD and SD photoperiods. In both cases, expression was elevated through the dark phase.

Acrophase analysis was used to determine the peak expression in those cases displaying a significant variation over time (Table 2). Under a SD photoperiod, both *Clock* and *Bmal1* peaked close to the middle of the dark phase, at Ext 01:00 h, while *Per2* peaked just prior to sunrise, at Ext 05:00 h. The acrophase for *Cry2* was significantly advanced ($p<0.05$) to Ext 21:00 h in the SD treatment compared to Ext 01:00 h under the LD photoperiod.

Clock gene expression in tissues

A tissue-expression study of clock genes in Atlantic salmon was performed under only an LD photoperiod. As such, expression levels were generally low though there were significant differences in expression for *Clock* in the brain, muscle, and spleen and for *Per2* in the brain, intestine, and spleen between Ext 06:00 and 22:00 h

(Figure 2). These differences were generally < 1 fold change, which is in keeping with the variation observed in the brain study under the LD photoperiod.

Discussion

Studies of the clock-gene expression in teleosts are primarily limited to zebrafish (e.g., Whitmore et al., 2000; Pando et al., 2001), with only limited work being published in Rainbow trout (Mazurais et al. 2000) and the golden rabbitfish (*Siganus guttatus*) (Park et al., 2007; Sugama et al., 2008). However, the apparent parallels in the photic entraiment, robust temporal organization, and endogenous rhythmicity of physiological functions in salmonids, Atlantic salmon in particular (Randall et al., 1998; Bromage et al., 2001; Stewart et al., 2002, 2006), compared to seasonal mammals lead us to test the hypotheses that: firstly, the clock-gene system would be actively expressed in Atlantic salmon; and secondly, the expression profiles would be daylength-dependent.

Components of both the positive and negative arms of the canonical clock system were successfully identified and cloned, and qRT-PCR assays were established to profile mRNA expression in whole brain isolates of salmon parr acclimated to different photic seasonal states. If we first consider the positive arm, results showed that both *Clock* and *Bmal1* were up-regulated during the scotophase under SD conditions, with expression peaking at Ext 01:00 h (mid-dark). *Clock* and *Bmal* also displayed rhythmic expression in zebrafish; however, the expression peaks occurred earlier, being closer to sunset, except in *in vitro* studies when expression peaks during the middle of the night as we report in salmon (Whitmore *et al.,* 1998, 2000; Cermakian et al., 2000; Pando et al., 2001). In homeothermic vertebrate species, such as aves (Larkin et al., 1999; Yoshimura et al., 2000; Helfer et al., 2006) and mammals (Lincoln et al., 2002; Tournier et al., 2003; Tournier et al., 2007), *Clock*

and *Bmal* expression patterns can not be easily summarized, as data can be conflicting with the presence or not of rhythmic expression, of *Clock* in particular, being dependent on tissue/location and species studied. It also appears the peak expression can vary from late photophase through to mid-scotophase (Larkin et al., 1999; Yoshimura et al., 2000; Lincoln et al., 2002) the significance of this variation is not clear.

When we consider elements of the negative arm of the feedback loop, *Per2* expression peaked just prior to sunrise, at Ext 05:00 h. *Per2* has been described as being light-induced in teleosts (Ziv & Gothilf, 2006), with expression levels peaking at ZT3 (3 h post-sunrise) in zebrafish cell line Z3 (Pando et al., 2001) or towards the end of the photophase in Golden rabbitfish (Sugama et al., 2008). Importantly, Pando et al. (2001) observed that in the Zebrafish cell line Z3, both *Per1* and *Per3* genes anticipated the onset of dawn, while *Per2* did not exhibit an increase in expression prior to the initiation of the light phase. The current findings do not confirm such a pattern in salmon and more closely match those reported for *Per1* in teleosts (Pando et al., 2001; Park et al., 2007). Although we cannot conclusively confirm the identity of this period gene in salmon at this time, primers used to generate the partial sequence were designed for zebrafish *Per2* spanning the PAS domain and alignment and phylogenetic analyses of the 543bp fragment (Figure 3) revealed the salmon partial cds is most closely related to zebrafish *Per2* (>80% identity) ,which both lie within the *Per2a* node of teleost Period genes in accordance with the classification of Wang (2008). Clearly, however, the full-length sequence needs to be determined and phylogenetic analysis performed to truly state the identity of this period product in salmon.

The *Cry2* results, with expression peaking in the early night, draw similarities with zebrafish findings (Cahill, 2002). In zebrafish, *Cry1a, 1b, 2a,* and *2b* homologues are all rhythmically expressed and have all been shown to play a functional role in clock-gene cycling through the inhibition of CLOCK:BMAL induced transcription (Kobayashi et al., 2000; Cahill, 2002). However, *Cry1* homologues were shown to peak during the day, while *Cry2* peaks in the evening. Clearly a future priority is to identify the number of salmon homologues for all of these clock genes, a task possibly further complicated by the salmonid genome duplication (Allendorf & Thorgaard, 1984). Thereafter, cataloguing their expression profiles under classic experimental paradigms like LD, LL, DD, and DL will be crucial in describing this new clock system. Not withstanding this, the current dataset does confirm that key elements of the traditional vertebrate clock system are present and being rhythmically expressed in this species, and more importantly it also demonstrates that these elements display differential expression patterns in relation to daylength.

These results are the first to describe the effect of photoperiod on circadian clock-gene rhythmicity in a temperate teleost species. Seasonal changes in daylength have been shown to regulate expression patterns of the clock system in sheep (Lincoln et al., 2002), mouse (Steinlechner et al., 2002), rat (Sumova et al., 2003), as well as Syrian, Siberian, and European Hamsters (Messager et al., 2000; Tournier et al., 2003, 2007), and Japanese quail (Yasuo et al., 2003). In salmon, there is a striking difference in expression patterns between LD and SD photoperiods in all genes investigated, with *Clock*, *Bmal,1* and *Per2* all displaying rhythmic expression under a SD but not a LD photoperiod. Meanwhile, only *Cry2* was rhythmic under both LD and SD, with the photoperiod significantly altering the duration of elevated

expression, resulting in the acrophase being advanced by 4 h in the SD compared to LD photoperiods. Lincoln et al. (2003) hypothesized that daylength could be differentially interpreted at the molecular level, either through modulation of the amplitude of expression or through phase control of expression of key genes. A more recent study (Wagner et al., 2008) has demonstrated, at least in Soay sheep, that the refined model is a synergy of both phenomena. It appears that in this seasonal mammal, the definition of the "critical daylength window" of responsiveness to long day photoperiod is based on the phase relationship of *Per1* and *Cry1* expression in combination with amplitude changes of *Per1* expression levels (Wagner et al., 2008). The current preliminary findings in Atlantic salmon show similarities to this work as clear amplitudinal variations of *Clock*, *Bmal1,* and *Per2* expression were observed in response to daylength, while *Cry2* appeared to show a phase relationship. This could suggest the basic molecular mechanisms involved in the interpretation of seasonal changes in daylength might be conserved among vertebrates. However, the study of the circadian organization and the clock-gene system in salmon is only in its infancy and does not, at this stage, allow direct comparisons to be made.

One key point that has to be addressed in future research is the localization of clock-gene expression in the salmon brain. The current expression data are from whole brain homogenates, which do not allow us to exclude the possibility the profiles could be an averaging of expression in more than one discrete location which could well be cycling out of phase (Carr et al., 2003; Johnston et al., 2005). While it has been proposed that there are no 'master' clock centers apparent in zebrafish (Whitmore et al., 1998, 2000), due to the diversity of circadian organization evident in teleosts (Migaud et al., 2007), one should draw no conclusions over the whole vertebrate class. It has been suggested that a 'master clock' is located within a SCN-

like structure within the hypothalamus in salmonids from indirect evidence obtained by retinal neural tract tracing, multi-unit activity recordings, 2-DG metabolism studies, and melatonin receptor studies (Holmqvist et al., 1992; Falcon et al., 2007). In fact, previous work in Rainbow trout localized *Clock* (Mazurais et al., 2000) and *Per 1* (Brierley et al., 1999) expression to regions associated with the processing of visual information (e.g., periventricular layer of the optic tectum) (*Clock and Per1*) as well as the hypothalamus and pituitary (*Per1*), which is comparable to what has been reported in zebrafish (Whitmore et al., 1998). However, in these studies only *Per1* showed significant temporal variation; in light of the current results, this is possibly due to the fact the authors only examined animals acclimated to a long-day photoperiod. Clearly, more accurate spatial definitions of clock expression are vital to determining whether 'master' clocks truly exist in salmonids. Considering the extensive research into peripheral clock systems in zebrafish and higher vertebrates, it is perhaps not surprising the tissue expression study confirmed that clock genes are expressed throughout a wide range of tissues in salmon. It is impossible to draw any strong conclusions concerning temporal variations in gene expression within this tissue screen, as only two samples were profiled. Variation is limited (<1-fold change) throughout, which could be due to a range of reasons including, daylength-specific expression profiles (e.g., Lincoln et al., 2002), tissue specific phase shifts in expression (e.g., Carr et al., 2003) or even non-cycling expression. This can only be clarified through more comprehensive investigations of the peripheral clocks to state if they are functional and by which route they may be entrained (i.e., centrally or independently).

While photoperiod is known to strongly influence the physiology and behavior of salmonids (Duston & Saunders, 1992; Bromage et al., 2001), the mechanism by

which photic information is perceived, interpreted, and then used to regulate many physiological events, like smoltification, migration, puberty, and spawning, is still unclear. In salmonids, the melatonin synthesis pathway has been well characterized; light perceived by the pineal, alone, is responsible for the daily rhythms in melatonin synthesis (Porter et al., 1996; Migaud et al., 2007). Furthermore, the synthesis of melatonin is directly related to daylength and temperature (Gern et al., 1992; Randall et al., 1995; Porter et al., 2001); but, uniquely among the teleosts studied so far, it is not regulated by any endogenous clock mechanism (Bolliet et al., 1996; Iigo et al., 2007). It remains to be determined whether the clock mechanism has been truly lost from the pineal gland or has rather become decoupled from the melatonin synthesis pathway. While early studies have demonstrated that pinealectomy and melatonin dosing can influence the timing of physiological events, like spawning and smoltification, no conclusive link has yet been found (Mayer et al., 1997; Porter et al., 1998). It is of course possible that photic entrainment of fish physiology could operate independently of the melatonin pathways, as has previously shown in aves (Brandstatter, 2003).

In a continuous effort to unravel the photic entrainment of physiology in salmonids, recent findings have brought attention to the clock system. Leder et al. (2006), when re-examining an existing QTL data set for spawning time in Rainbow trout, mapped *Clock* as well as *Per1* to quantitative trait loci that explained up to 50% of the variance in spawning time, while other candidate genes, including components of the melatonin synthesis pathway (*AANAT-1* and *AANAT-2*), were not significantly associated. Furthermore, while Stewart et al. (2002, 2006) identified a genetic component to temporal order of migration in Atlantic salmon, O'Malley et al. (2007) demonstrated that similar variations in Chinook salmon were associated with allelic

variations in *Clock*. From these published findings and current results, it is evident that the clock system is present, cycles in a daylength-dependent manner, and furthermore is functionally associated with the temporal organization of physiology. While this might not come as a surprise, seeing that the transcription/translation negative feedback loop at the core of the clock molecular mechanism appears to be highly conserved from *Drosophila* to humans (Dunlap, 1999), it still provides valuable basic information and a starting point for the elucidation of the salmonid circadian system. Future research into clock systems in salmonids may thus prove fruitful in providing the missing link in the neuroendocrine pathways linking the perception of light to the entrainment of physiology that has to date remained elusive. Such findings will ultimately be of great scientific interest as well as vast commercial importance in fish (Bhattacharya et al., 2007; Chemineau et al., 2007; Meseguer et al., 2008).

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Table 1: Primer name, Sequence, predicted amplicon size, and GenBank accession number for the different genes studied.

Name	Sequence	Product size	Accession Number
ClkF	5’ – AGGCAACACTCGGAAGATGGACAA – 3	628 bp	CA 038738
ClkR	5’ – AGGAGATGTGCACTGTGGAAGAAC – 3’		
qpcr ClkF	5’ – AGAAATCGCTGCACAGTCGGAGTC – 3’	196 bp	
qpcr ClkR	5’ – CCACCAGGTCAGAAGGAAGATGTT – 3’		
BmalF	5’ – GAATAGCAGCCAGGGCAACCAAC – 3	632 bp	DY 735402
BmalR	5’ – CGCATCCGACGACAAACAGAAAG – 3’		
BmalqpcrF	5’ – GCCTACTTGCAACGCTATGTCC– 3’	90 bp	
BmalqpcrR	5’ – GCTGCGCCTCGTAATGTCTTCA– 3’		
Per2F	5’ – CAGTGTGTTCTACAGCTTCACCAC– 3’	543 bp	FM877775
Per2R	5’ – AAGCTGGACCAGCTGGTGTC– 3’		
Per2qpcrF	5’ – GCTCCCAGAATTCCTAGTGACAAG – 3’	88 bp	
Per2qpcrR	5’ – GAACAGCCCTCTCGTCCACATC – 3’		
Cry2F	5’ – CAACTTCGACCTGACCTCTCCACA – 3’	590 bp	DY 730105
Cry2R	5’ –CCAAAGCCCACAGGACAGTAGCAG – 3’		
Cry2qpcrF	5’ – GAGGGCATGAAGGTGTTTGAGGAG – 3’	108 bp	
Cry2qpcrR	5’ – GTGGAAGAACTGCTGGAAGAAGGA – 3’		
ActinF	5’ – AGTGTTGTCCCTGTACGCCTCTG – 3’	681 bp	AF 012125
ActinR	5’ – CTCGTCGTACTCCTGCTTGCTGAT – 3’		
ActinqpcrF	5’ – ATCCTGACAGAGCGCGGTTACAGT – 3’	112 bp	
ActinqpcrR	5’ – TGCCCATCTCCTGCTCAAAGTCCA – 3’		

Table 2: Photoperiod regulates the acrophasic expression of multiple clock genes in the salmon brain. Acrophases (circadian peak times) were calculated by non-linear regression fit of a cosine function (See Methods). Data are expressed as acrophase ± 95% confidence intervals. n.s. = no significant variation in expression over the 24 h under the specified photoperiod condition.

	Acrophase ± S.E.M. (External time, h)	
	LD	SD
Clock	n.s.	01:00 ± 1:45
Bmal	n.s.	01:00 ± 2.38
Per2	n.s.	05:00 ± 2.67
Cry2	01:00 ± 2.28	21:00 ± 1.75

Figure Legends

Figure 1. Photoperiod affects diurnal expression of clock genes in the brain of Atlantic salmon. Diurnal expression profiles of *Clock, Bmal1, Cry2, and Per2* mRNA in Salmon brains. Fish were acclimated to LD (closed circle) or SD (open circle) for one month. Each value is mean ± SEM of six individuals. Horizontal solid and open bars represent dark and light phases, respectively, of the light-dark cycle.

Figure 2. Expression of *Clock* and *Per2* at Ext 06:00 and 22:00 h in an array of tissues of Atlantic salmon previously acclimated to LD for one month. Each value is mean ± SEM of six individuals. * indicates a significant difference between time points, Student's *t* test.

Figure 3. Phylogenetic tree analysis of teleost *Per2* genes with Mouse *Per2*, Zebrafish *Per3*, *1a,* and *1b* as outliers. The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Neil, 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 (Felenstein, 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). Accession numbers: Atlantic salmon *Per2* (FM877775), Zebrafish *Per2* (ENSDARG00000034503), Fugu *Per2a* (SINFRUG00000138842), Tetradon *Per2a* (GSTENG00028368001), Medaka *Per2a* (ENSORLG00000016612), Stickleback *Per2a* (ENSGACG00000013485), Medaka *Per2b* (ENSORLG00000015456), Stickleback *Per2b* (ENSGACG00000005662),

Golden Rabbitfish *Per2* (EF208027), Tetradon *Per2b* (GSTENG00026769001), Fugu *Per2b* (SINFRUG00000130362), Mouse *Per2* (NM_011066), Zebrafish *Per3* (ENSDARG00000010519), Zebrafish *Per1a* (ENSDAEG00000056885), Zebrafish *Per1b* (ENSDARG00000012499).

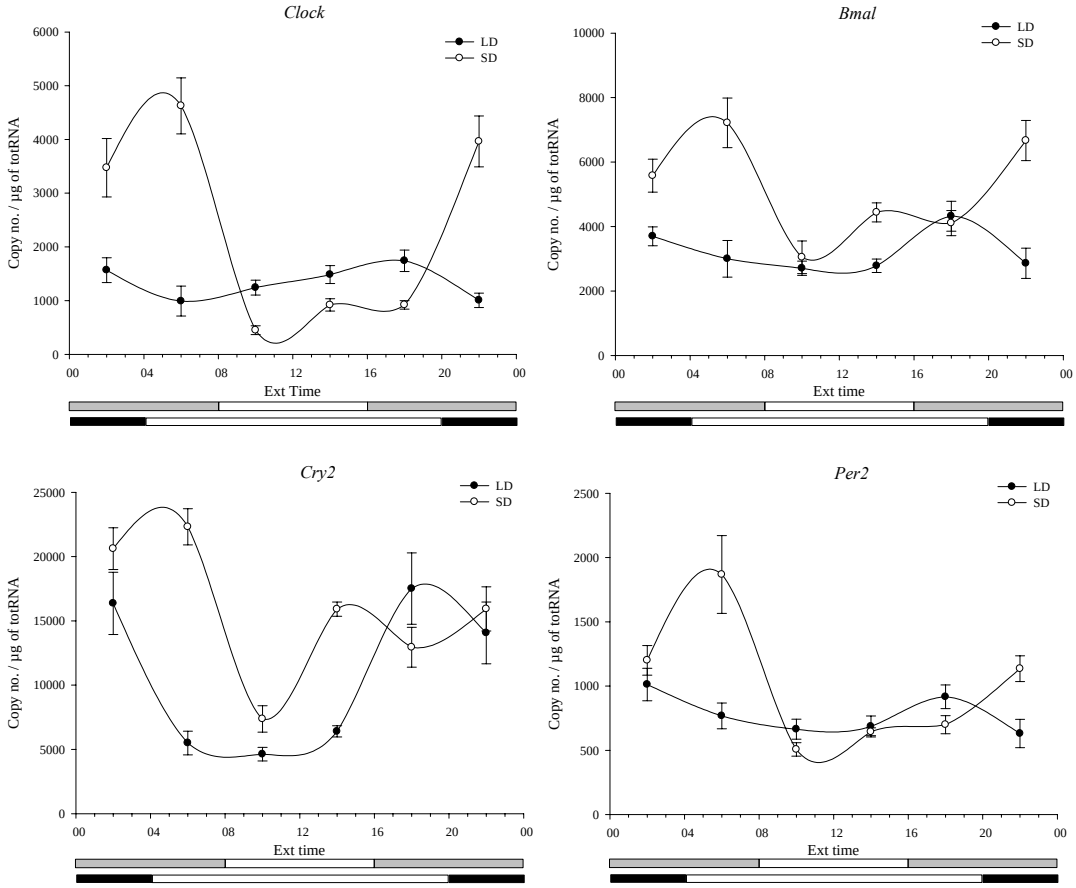
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Figure 1.

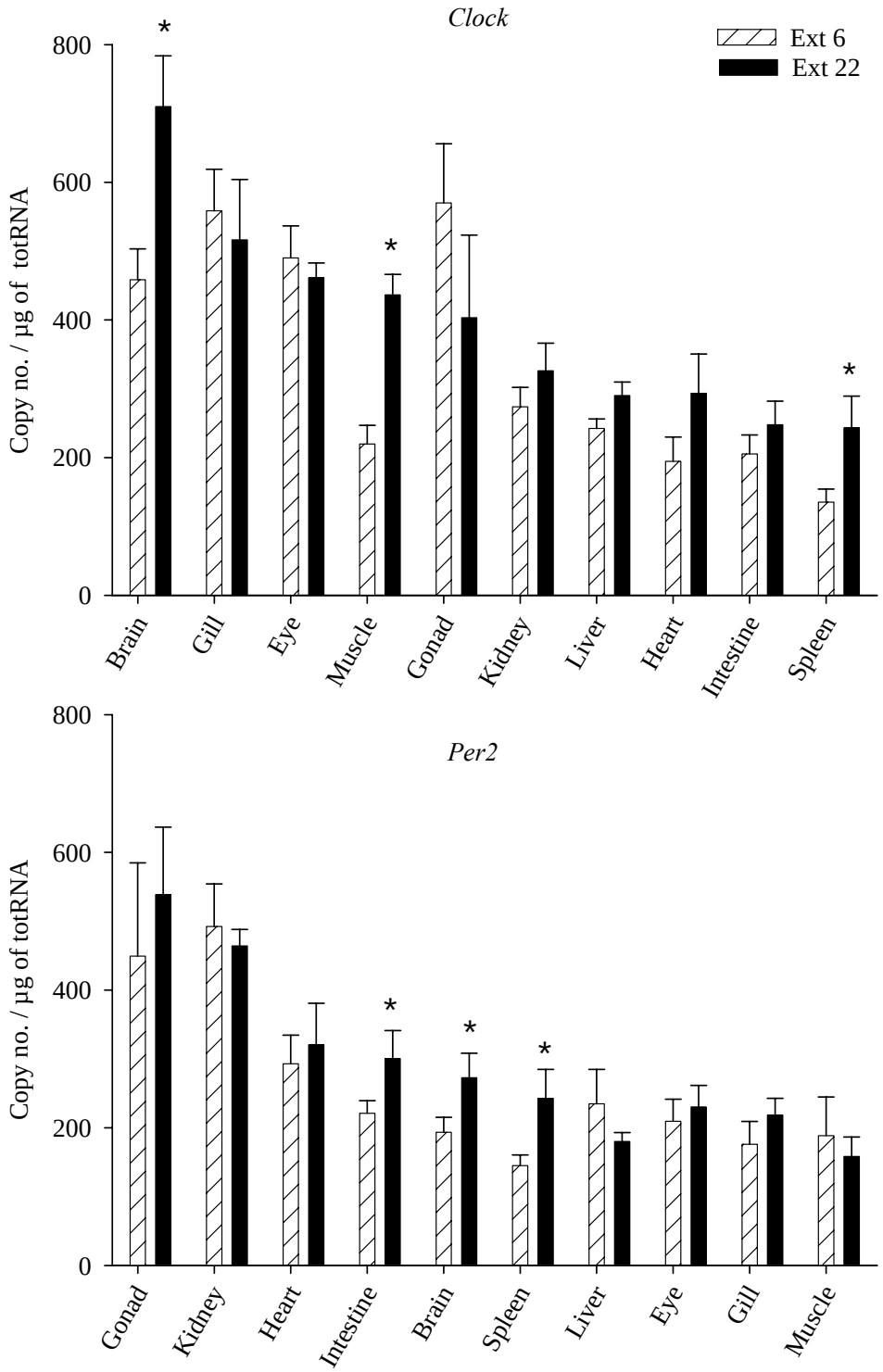
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Figure 2.

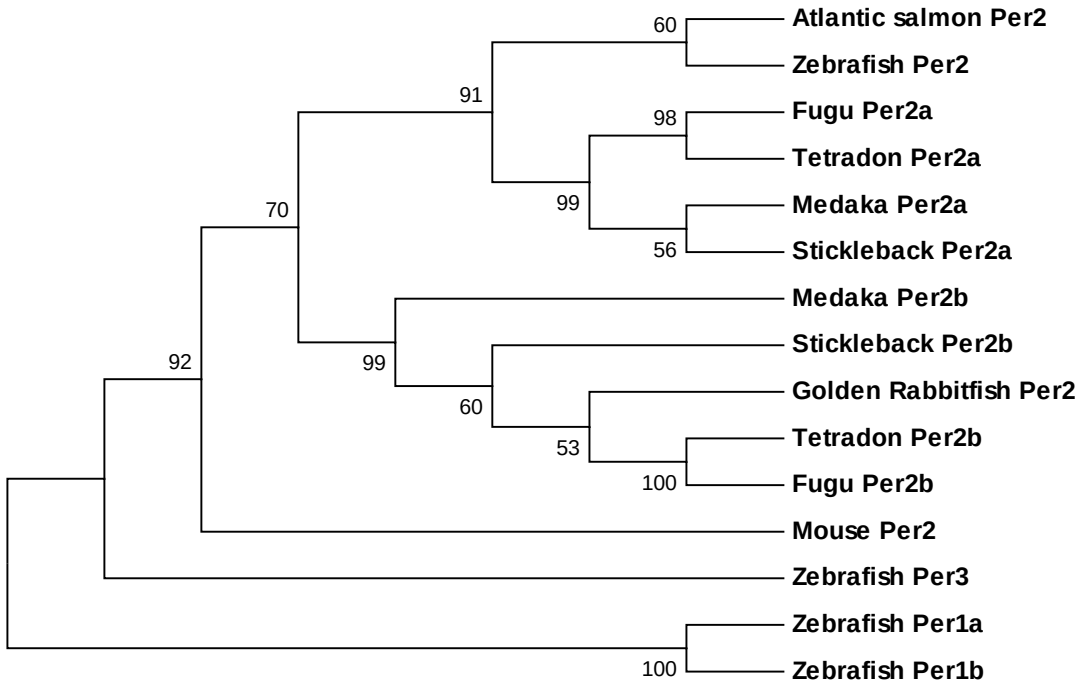
Atlantic salmon Per2
Zebrafish Per2
Fugu Per2a
Tetradon Per2a
Medaka Per2a
Stickleback Per2a
Medaka Per2b
Stickleback Per2b
Golden Rabbitfish Per2
Tetradon Per2b
Fugu Per2b
Mouse Per2
Zebrafish Per3
Zebrafish Per1a
Zebrafish Per1b
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Figure 3.