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EFFECT OF CONTINUOUS LIGHT ON DAILY LEVELS OF PLASMA MELATONIN AND CORTISOL AND EXPRESSION OF CLOCK GENES IN PINEAL GLAND, BRAIN, AND LIVER IN ATLANTIC SALMON POSTSMOLTS

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Continuous light is a common practice in salmon farming, where it is used to enhance growth, induce smoltification, and regulate puberty. However, knowledge about how different tissues receive information about daylength is limited. The aim of the present study was to evaluate the daily expression of clock (*Per1-like*, *Cry2*, and *Clock*), the nuclear transcription factor (peroxisome proliferator-activated receptor, PPAR; CCAAT/enhancer binding protein, C/EBP), and the endoplasmic reticulum (ER) stress (protein disulfide isomerase associated 3, PDIA3) genes in the pineal gland, brain, and liver of Atlantic salmon postsmolts reared under 12-h light:12-h dark (LD) regimes or under continuous light (LL) for 6 wks following transfer to seawater. All measured clock mRNAs displayed daily variations in one or more organs under LD, as well as plasma levels of melatonin. Similar variations were noted in the liver *c/ebpα*, pineal *c/ebpδ*, and *pdia3* mRNAs. Under LL, the clock and nuclear transcription factor mRNAs did not show any daily variation in the studied organs, with the exception of pineal *pdia3*. Furthermore, LL had the opposite effect on the levels of melatonin and cortisol, as observed by the increase in pineal *Clock*, *Per2, pparα*, and *c/ebpα* and *c/ebpδ* mRNAs and decrease in liver *Clock, Per2*, and *pparα* mRNAs compared to those under LD. The present findings show that the expression of clock genes is affected by the light across organs and that there is a relation between PPAR, C/EBP, and clock mRNAs; however, the functional role of the individual nuclear transcription factors related to this observation remains to be established in the pineal gland and liver. (Author correspondence: Tihu@nifes.no)

Keywords Atlantic salmon; Clock gene; Cortisol; Light; Melatonin; Nuclear transcription factor

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INTRODUCTION

Molecular clocks control the daily variations of wakefulness, stress hormones, lipid metabolism, immune function, and cell division, among other functions and processes (Duez & Staels, 2008). Genetic studies have identified components of the circadian pacemaker, including negative regulators, period (PER) and cryptochrome (CRY), as well as positive regulators, CLOCK and BMAL1, which are conserved in mammals where they constitute the core of the circadian clock described as transcriptional-translational feedback loops (Duguay & Cermakian, 2009; Gauger & Sancar, 2005). These feedback loops control the central and peripheral clocks that generate and maintain the functions and properties of various tissues and cell types during constant environmental conditions (Balsalobre, 2002; Ralph et al., 1990). Circadian clocks can be induced in some cultured cell lines and stem cells in vitro by different stimuli (Balsalobre et al., 1998; Huang et al., 2009a; Mendez-Ferrer et al., 2008). In contrast to the mammalian biological clock located in the suprachiasmatic nucleus (SCN) of the brain, to date the location of biological clock in fish is not known. Although the zebrafish is a useful model to study the effects of environmental stimuli, including light and temperature on circadian clock mechanisms (Hirayama et al., 2005; Lahiri et al., 2005), related studies are still lacking in other teleosts. Recently, *Clock* and *Per1* has been mapped to a quantitative trait locus to act as potential candidates for the timing of spawning in rainbow trout (Leder et al., 2006), and daily variations in clock gene expression have been shown in Atlantic salmon parr brains (Davie et al., 2009) and pineal glands (Huang et al., 2010b). These studies suggest the existence of a clock gene system that couples inputs from the environment to complex biological functions and controls the timing of lifecycle transitions in salmonids.

In seasonal mammals, robust annual cycles of adiposity, food intake, and energy metabolism are driven by daylength, which generates a diurnal melatonin profile and acts on neuroendocrine pathways (Rousseau et al., 2003). Salmonids also exhibit behavioral and physiological rhythms, of which the generation of the biological timing system is thought to control their daily and annual functions in relation to development, locomotor activity, sedation, skin pigmentation, oxygen consumption, and thermoregulation (Falcon et al., 2010). Seasonal changes in photoperiod are the most predictable environmental cues to modulate daily and annual rhythms. Daylength controls the release of pineal melatonin in the blood, with low and high levels during the light and dark phases, respectively (Falcon, 1999). In Atlantic salmon, photoperiod influences behavior (Oppedal et al., 2001); appetite (Nordgarden et al., 2003a); muscle lipid composition (Nordgarden et al., 2003b); development and growth of the axial muscle (Johnston et al., 2003), vertebral

column (Fjelldal et al., 2005), and gonads (Taranger et al., 1998); as well as plasma levels of melatonin (Porter et al., 1999), cortisol (Thorpe et al., 1987), growth hormone (GH), insulin-like growth, factor 1 (IGF-I) (Nordgarden et al., 2005), and sex steroids (Taranger et al., 1998). Cortisol and GH act together during smoltification and seawater adaption in regulating gill sodium-potassium adenosine triphosphatase (Na^+,K^+-ATPase) activity (McCormick, 2001). However, light-induced effects on the temporal relationship between physiological clocks and plasma levels of melatonin and cortisol are not clear in Atlantic salmon postsmolts.

CCAAT/enhancer binding protein (C/EBP) in the mollusc eye (Hattar et al., 2002), C/EBPδ in the hamster pineal gland (Maronde et al., 2007), and peroxisome proliferator-activated receptor (PPARα) in the rat liver (Lemberger et al., 1996) have been identified as clock-controlled genes with diurnal expression. In addition, PPARα is also involved in the circadian regulation of lipid metabolism (Oishi et al., 2005). In Atlantic salmon, PPAR and C/EBP display important roles in regulating lipid metabolism in hepatocytes, adipocytes, and myocytes (Huang et al., 2010a; Kleveland et al., 2006; Vegusdal et al., 2004), and stress-induced *pdia3* mRNA expression was found in the liver of smolts suffering from hypoxia (Huang et al., 2009b). Less is known about the association of clock and nuclear transcription factor mRNAs in teleost.

The clock gene system has previously been shown in Atlantic salmon parr and smolts (Davie et al., 2009; Huang et al., 2010b), whereas the expression of clock and clock-controlled genes and their response to light has not been reported in neural and peripheral tissues in postsmolts. In order to better understand the daily expression of clock and nuclear transcription factor mRNAs in Atlantic salmon in seawater, we described the plasma levels of melatonin and cortisol, in addition to the expression of clock (*Per1-like, Per2, Cry2*, and *Clock*), nuclear transcription factor (*ppar* and *c/ebp*), and endoplasmic reticulum (ER) stress (*pdia3*) mRNAs in the pineal gland, brain, and liver of postsmolts reared under 24 h of continuous light (LL) or a 12-h light:12-h dark (LD) cycle.

MATERIALS AND METHODS

Fish Stock

The underyearling Atlantic salmon (*Salmo salar* L.) smolts used in the present experiment were produced at the Institute of Marine Research (IMR), Matre Research Station, western Norway. The fish were from the Aquagen strain, and smoltification was induced by an artificial photoperiod commonly used in commercial salmon farming. The fish had been reared under LD for 6 wks, followed by LL for 6 wks in freshwater until December 14, 2007, when they were transferred to experimental

seawater tanks. On December 14, the smolts had a low condition factor, lacked parr marks, and were silvery, and the mean gill Na^+,K^+-ATPase activity was 9.2 mmol adenosine diphosphate (ADP)/mg protein/h (min value 6.9, max value 12.5), which confirmed complete smoltification.

Experimental Design

On December 14, 2007, 300 underyearling smolts (mean weight 196 g) were randomly allocated to six square, white, covered seawater tanks ($1 \times 1 \times 0.43$ m), with 50 fish/tank. For the following 6 wks (from December 14, 2007 to January 25, 2008), triplicate groups in separate tanks were employed with LD or LL. For illumination, two 18-W fluorescent daylight tubes (OSRAM L 18W/840 LUMILUX; OSRAM, Ausburg, Germany) were used to produce 960 lux underwater in the center of the tank. The light of the LD tanks was switched on at 08:05 h and off at 20:05 h. Both the LD and LL groups were fed by disc feeders that continuously release pellets into the water during the lighted period for the LD group. The feed used was a commercial salmon feed (Nutra Olympic; Skretting, Averøy, Norway), and the groups were fed by automatic feeders (ARVO-TEC T Drum 2000; Arvotec, Huutokoski, Finland). The fish were starved 24 h prior to sampling. The light and feeding were controlled automatically by a PC-operated system (Normatic, Norfjordeid, Norway). The temperature was stable at 8.9°C, and the oxygen saturation of the outlet was kept >80%.

Sampling of Atlantic Salmon Tissues for Expression Analysis

The samplings were performed on January 24–25, 2008, with sampling performed every 4 h; the first sampling started at 12:00 h on day 1 and the last started at 12:00 h on day 2 (day 1: 12:00, 16:00, 20:00, 24:00 h, and day 2: 04:00, 08:00, and 12:00 h). At each sampling, 3 fish/tank were anesthetized with benzokain (40 mg L^{-1}) (Benzoak® Vet; A.C.D., Braine-L'alleud, Belgium) and measured for fork length and body weight. Blood samples were then taken from the caudal vessel using a heparinized tuberculin syringe fitted with a 23-gauge needle. Plasma was separated by centrifugation and stored at −80°C until analysis. The night sampling of individual fish of the LD tanks was performed under a dim-red light. The fish were then decapitated with a scalpel, and the tissues were dissected from the carcass and immediately frozen in liquid nitrogen. During the sampling, all fish were anesthetized at the same time, and then 1 fish/tank was sequentially measured and dissected to omit systematic errors caused by time-related differences in gene expression between the tanks and light groups within the subsamplings. The experimental protocol and procedures were performed in

accordance with both international ethical standards (Portaluppi et al., 2008) and the National Animal Research Authority in Norway.

Levels of Plasma Melatonin and Cortisol

Melatonin levels were determined by radioimmunoassay (RIA) kit, in which the plasma sample was incubated with assay buffer, ^{125}I-melatonin, and rabbit anti-melatonin antiserum, as described in the manufacturer's instructions (IBL, Hamburg, Germany). The cortisol concentration was measured by RIA kit (DiaSorin, Minnesota, USA), in which 10 μL plasma samples were applied in 1 mL tracer-buffer before incubation with ^{125}I-cortisol and rabbit anti-corticosterone antibody followed by the standard protocol (Stefansson et al., 1989).

Isolation of RNA and qRT-PCR Analysis

RNA isolation and DNase treatment of the tissue samples were performed using a standard RNA extraction method (Invitrogen, Paisley, UK) and DNA-free™ DNase following the manufacturer's protocol. The total RNA, 500 ng/sample, was reverse transcribed using TaqMan RT reagents (ABI, Foster City, CA, USA) according to the manufacturer's instructions. A standard curve was obtained from a two-fold serial dilution of total RNA from the control fish, with six RNA concentrations covering between 31.25 ng and 1000 ng. The quantitative reverse transcriptase–-polymerase chain reaction (qRT-PCR) primer sequences (Table 1) were designed from expressed sequence tag (EST) sequences of *Per2*, *Cry2*, and *Clock*, obtained from the Salmon Genome Project (SGP) database, www.salmongenome.no, as described by Davie et al. (2009). In order to identify salmon *Per* ortholog from the EST sequence, PCR primers, 5′-CCCAGTGACCTACTGGACCTG-3′ and 5′-TGGTGCTCTCTGGTAAGG-3′, were designed to amplify a partial cDNA fragment (576 bp) from Atlantic salmon liver mRNA by PCR reaction. We refer to this gene as the *Per1-like* gene. The PCR primers for PPAR and C/EBP genes were designed from published sequences of Atlantic salmon (Huang et al., 2010a). The amplified PCR products of all three clock genes were sequenced to ensure correct mRNA sequences for quantification. The primers shown in Table 1 were used for SYBR Green assays (Roche Diagnostics, Germany) in a total volume of 20 μL containing 1 μL cDNA, 10 μL SYBR Green master, 0.2 μL (50 μM) of each primer, and 8.6 μL H_2O. Reactions were run on a Roche Lightcycler 480 (Roche Diagnostics) with 96 wells. The cycling conditions were composed of pre-incubation at 95°C for 5 min, followed by 45 cycles of amplification. Each cycle included 10 s of denaturation at 95°C, 20 s of primer annealing at 60°C, and 30 s of extension/synthesis at 72°C. The gene expression

TABLE 1 Primer sequences used in qRT-PCR of the reference and clock genes studied

Gene	Accession number	Forward primer	Reverse primer
pdia3	EJ457023	5′-CCAACGCCATGATCAAGAA-3′	5′-CAGCAGGTCCTTGTCCTTCA-3′
c/ebpα	EU668995	5′-AGACCTCGGCGAGATTTGT-3′	5′-TGTGGAATAGATCAGCCAGGAA-3′
c/ebpδ	EU668997	5′-TTGGGCGGTGGAGCCTAT-3′	5′-TTTCCTCGCCCGTGTCAT-3′
pparα	DQ294237	5′-TCCTGGTGGCCTACGGATC-3′	5′-CGTTGAATTTCATGGCGAACT-3′
Per1-like	DW576689	5′-CTGTCTCCTTGGGCACTGTGT-3′	5′-GAGTCGATGCTGCCAAAGTACTT-3′
Clock	CA038738	5′-GTTAGACGGCTTCTTCCTAGCAAT-3′	5′-CCACCAGGTCAGAAGGAAGATG-3′
Cry2	DY730105	5′-CCACAACATGGCAGGTGAATT-3′	5′-CAGACATCCAAAGCGGAGGTA-3′
Per2	FM877775	5′-GCTCCCAGAATTCCTAGTGACAAG-3′	5′-GAACAGCCCTCTCGTCCACATC-3′
β-Actin	BG933897	5′-CCAAAGCCAACAGGGAGAAG-3′	5′-AGGGACAACACTGCCTGGAT-3′
EF1AB	AF321836	5′-TGCCCCTCCAGGATGTCTAC-3′	5′-CACGGCCCACAGGTACTG-3′
ARP	AY255630	5′-GAAAATCATCCAATTGCTGGATG-3′	5′-CTTCCCACGCAAGGACAGA-3′

stability (M) was calculated with geNorm, and the three reference genes (*ARP*, *β-actin*, and *EF1AB*) revealed a low M value. The *geNorm* tool was used to determine a normalizing factor from the three reference genes (acidic ribosomal phosphoprotein [ARP], β-actin, and elongation factor 1 AB [EF1AB]) to postsmolts (LD and LL), and it was also used to calculate the normalized expression for the target genes (Vandesompele et al., 2002).

Statistical Analysis

Significant differences in both length and weight were tested by two-way nested analyses of variance (ANOVAs), with the tank as the random factor nested in the light group, and those in the plasma levels of melatonin and cortisol and in the mRNA expression of each clock, nuclear transcription factor, and *pdia3* genes were evaluated by one-way ANOVA (Statistica; Statsoft, Tulsa, USA). If the ANOVA test showed statistical significance ($p < .05$), a Newman-Keuls test was applied to determine all pairwise comparisons. Because ANOVA cannot assess rhythmicity, the cosinor method was used to test for circadian rhythmicity using normalizing data for each variable in three individual analyses (the average of the 3 fish/tank, n = 3 each timepoint) and in one cosinor test using the means for each timepoint (means of total fish from all the tanks). These analyses were performed by the cosinor periodogram (www.circadian.org/softwar .html), with significance of the periodicity determined by the zero-amplitude test of the cosinor procedure. The rhythm characteristics estimated by this method include the mesor (middle value of the fitted cosine representing a rhythm-adjusted mean), amplitude (half the difference between the minimum and maximum of the fitted cosine function), and acrophase (the time of the peak value expressed in h and min from local 00:00 h) (Table 2).

RESULTS

Survival, Somatic Growth, and Plasma Levels of Melatonin and Cortisol

There was no mortality in either of the groups during the 6-wk experimental period, and no significant differences in fork length and body weight between the fish under LD (27.9 ± 0.3 cm, 249 ± 7.3 g) and LL (27.5 ± 0.2 cm, 227 ± 6.1 g) at the end of the experiment. Under LD conditions, plasma melatonin levels were typically low during the light phase and gradually increased during the dark phase (cosinor, $p < .01$) (Figure 1) in contrast with no significant difference at any timepoint in melatonin levels under LL (ANOVA, $p = .55$). There was no significant

TABLE 2 Summary of clock gene expression and plasma melatonin from cosinor analysis in postsmolts

Gene	Tissue	Mesor ± SD	Amplitude ± SD	Acrophase	Cosinor *p*
		12-h light:12-h dark (LD)			
Per1-like	Liver	0.58 ± 0.02	0.19 ± 0.01	23:21 ± 0.46	<.01
	Brain	0.29 ± 0.01	0.16 ± 0.01	06:46 ± 0.02	<.05
	Pineal	0.36 ± 0.01	0.20 ± 0.03	03:54 ± 0.25	<.01
Cry2	Brain	0.44 ± 0.01	0.24 ± 0.01	05:44 ± 0.22	<.05
	Pineal	0.25 ± 0.01	0.15 ± 0.01	23:48 ± 0.23	<.01
Clock	Liver	0.76 ± 0.04	0.21 ± 0.04	04:00 ± 1.29	<.05
	Brain	0.38 ± 0.02	0.25 ± 0.03	08:52 ± 0.10	<.05
Per2	Brain	0.43 ± 0.02	0.35 ± 0.03	08:34 ± 0.22	<.05
	Pineal	0.38 ± 0.01	0.15 ± 0.02	23:53 ± 0.75	<.05
c/ebpα	Liver	0.51 ± 0.03	0.14 ± 0.03	23:58 ± 0.50	<.05
c/ebpδ	Pineal	0.51 ± 0.17	0.20 ± 0.04	04:14 ± 1.11	<.05
pdia3	Pineal	0.30 ± 0.01	0.10 ± 0.01	21:32 ± 0.90	<.01
Melatonin	Plasma	1033 ± 94	1199 ± 129	04:28 ± 0.17	<.01
		Continuous light (LL)			
pdia3	Pineal	0.58 ± 0.02	0.25 ± 0.02	20:21 ± 0.52	<.05

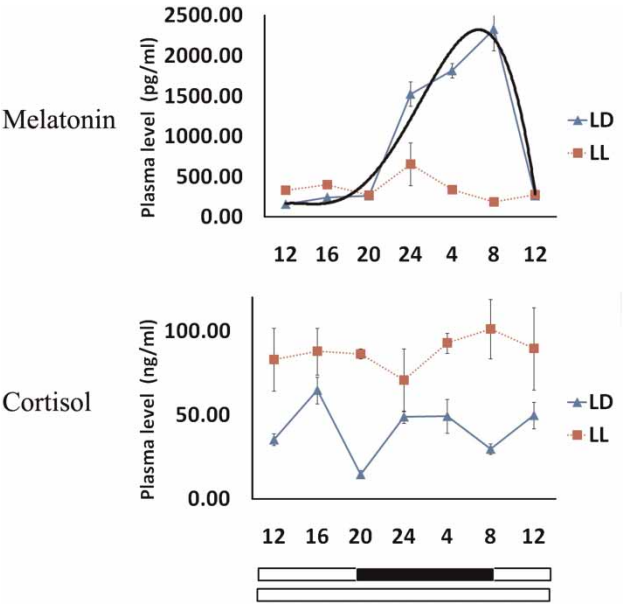

FIGURE 1 Daily plasma levels of melatonin and cortisol in Atlantic salmon postsmolts. Plasma levels of melatonin and cortisol were measured from postsmolts reared under 12 h of light:12 of h dark (LD) or 24 h of continuous light (LL) for 6 wks following transfer to seawater. Circadian rhythms were determined by three separate cosinor analyses and the statistical significance is reported in the text. Each value is the mean ± SEM (n = 3; 3 fish/tank). LD, 12-h light:12-h dark; LL, continuous light. N = 9; 3/tank.

daily variation in plasma cortisol in fish reared under LL (ANOVA, p = .86) or LD (ANOVA, $p < .01$; cosinor, $p = .97$), although the level was two-fold higher under LL compared to LD (ANOVA, $p < .001$) (Figure 1).

Variations of Daily Clock Gene Expression in Salmon Postsmolts

Under LD, there was significant daily variation of *Per1-like* in the pineal gland, brain, and liver; of *Cry2* and *Per2* in the pineal gland and brain, and of *Clock* in the brain and liver (Figure 2). The mesor and amplitude were similar in the pineal gland and brain for *Per2*, *Per1-like*, and *Cry2*, and their acrophases, respectively, occurred in the pineal gland 2 h before (23:53 h), 2 h after (03:54 h), and 2 h before (23:48 h) the middle of the dark period (02:00 h), respectively, whereas their

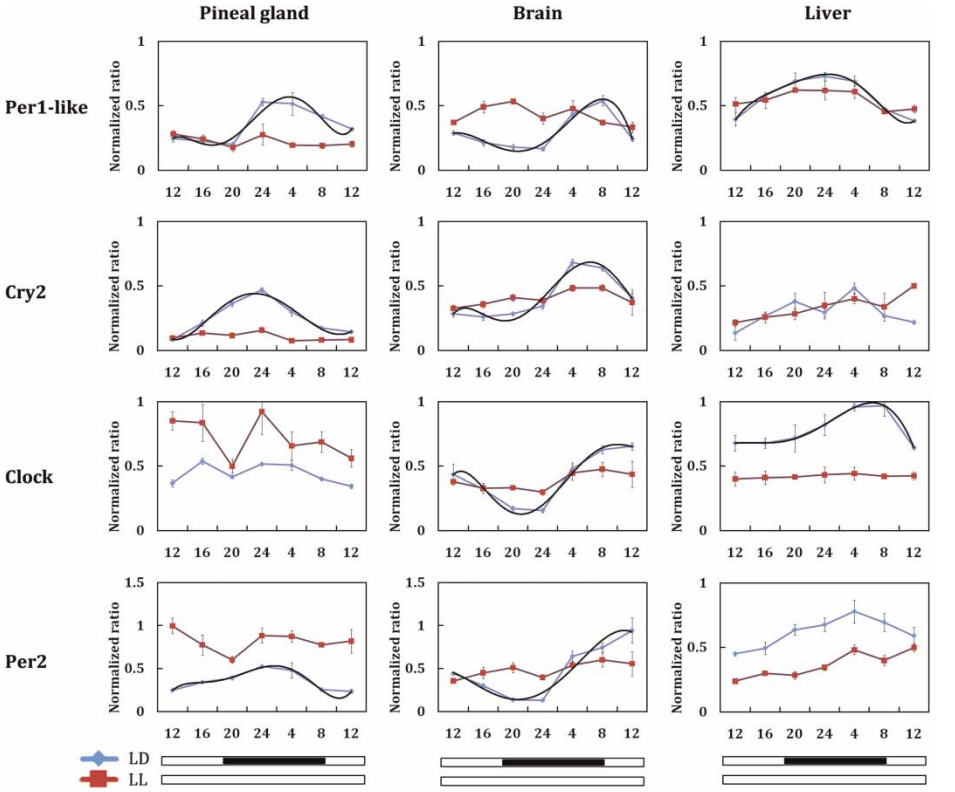

FIGURE 2 Daily expression of clock gene mRNAs in the pineal gland, liver, and brain of Atlantic salmon postsmolts. Expression of clock gene mRNAs in the pineal gland, brain, and liver harvested from postsmolts reared under 12 h of light:12 h of dark (LD) or 24 h of continuous light (LL) for 6 wks following transfer to seawater and normalized to three endogenous control genes, *ARP*, *β-actin*, and *EF1AB*. Circadian rhythms were determined in three separate cosinor analyses, and the statistical significance is shown in Table 2. Each value is the mean ± SEM (n = 3; 3 fish/tank). LD, 12-h light:12-h dark; LL, continuous light; n = 9, 3/tank.

acrophases in the brain for *Per1-like* and *Cry2* occurred late in the dark period at 06:46 and 05:44 h, respectively (Table 2). In contrast, the acrophase of brain *Per2* and *Clock* was early in the light phase, at 08:34 and 08:52 h, respectively (Table 2). The acrophases of liver *Per1-like* and *Clock* occurred 2.5 h before (23:21 h) and 2 h (04:00 h) after the middle of the dark period, respectively (Table 2). Of the studied genes and organs, the pineal and liver *Per1-like* and the pineal *Cry2* demonstrated the most pronounced oscillation ($p < .01$; Table 2), and only pineal *Per1-like* demonstrated a strong response after the shift between the light and dark spans (marked increase between 20:00 and 24:00 h; Figure 2).

Under LL, none of the clock RNAs showed significant daily variations in any of the analyzed tissues, but the overall levels of the *Clock* and *Per2* increased in the pineal gland and decreased in the liver of LL-reared fish (Figure 2).

Rhythmic Expression of Nuclear Transcription Factor and ER Stress-Related Genes

The mRNA expression of *c/ebpα* and *c/ebpδ* displayed significant daily variations in the liver and pineal gland, respectively, with acrophases 2 h before (23:58 h) and 2.2 h (04:14 h) after the middle of the dark period (Figure 3, Table 2). The *pparα* expression did not show significant rhythms in either the pineal gland or liver. There were no rhythms of these mRNAs in fish under LL, but mRNA levels of the liver *pparα* were repressed by LL and those of the pineal *pparα*, *c/ebpα*, and *c/ebpδ* were up-regulated by LL when compared to those under LD. The *pdia3* expression showed significant daily variations in the pineal gland of fish reared under both LD and LL, but their respective acrophases were different under the two light conditions (Figure 3, Table 2).

DISCUSSION

The present study is the first to demonstrate the effect of light on daily variations in the expression of genes encoding for clock proteins, nuclear transcription factors, and ER stress protein in seawater postsmolts of Atlantic salmon. We observed daily variations of plasma melatonin levels under LD, which were elevated in the dark phase and suppressed in the light phase. This nocturnal rise in plasma melatonin disappeared under LL. These results are in accordance with reports on the diurnal rhythms of plasma melatonin levels in salmonids (Iigo et al., 1997; Randall et al., 1995). Although significant daily variations of cortisol levels were not seen in postsmolts (LD), lower levels occurred during the transition between the day and night spans, which was in contrast with the nocturnal increase in plasma cortisol reported in smolts under

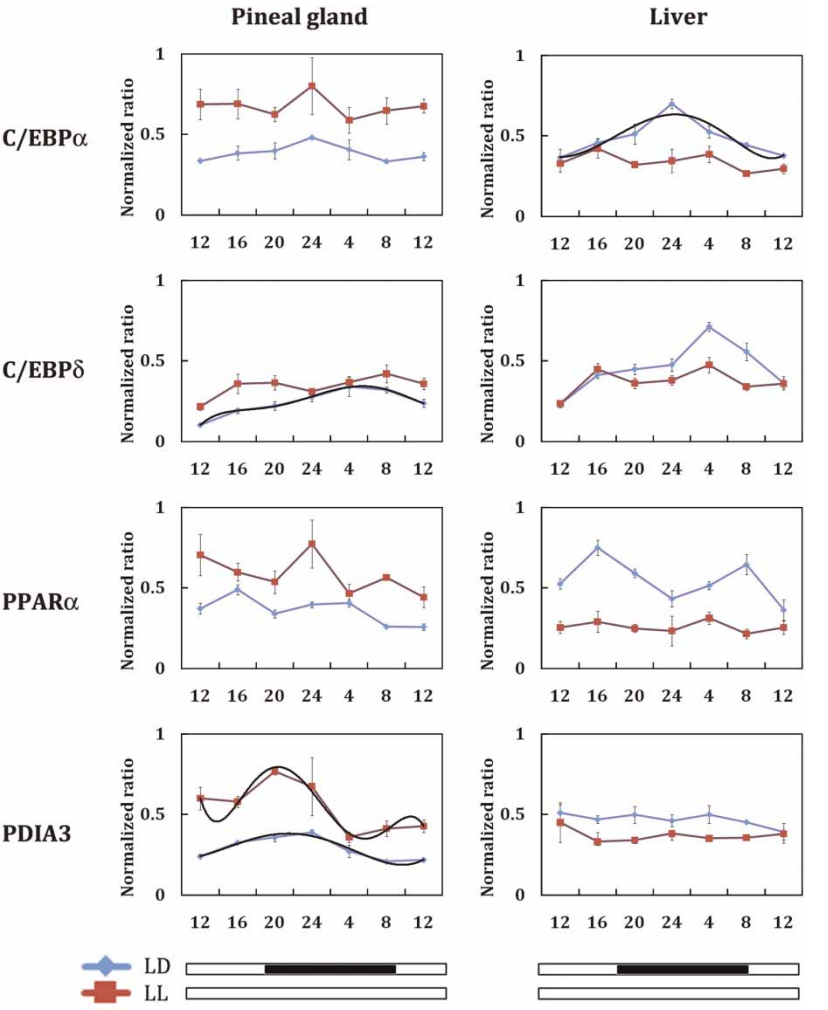

FIGURE 3 Daily expression of nuclear transcription factor and *pdia3* mRNAs in the pineal gland and liver of Atlantic salmon postsmolts. Expression of PPAR, C/EBP, and *pdia3* mRNAs in the pineal gland and liver harvested from postsmolts reared under 12 h of light:12 h of dark (LD) or 24 h of continuous light (LL) for 6 wks following transfer to seawater and normalized to three endogenous control genes, *ARP*, *β-actin*, and *EF1AB*. Circadian rhythms were determined in three separate cosinor analyses and the statistical significance is shown in Table 2. Each value is the mean ± SEM (n = 3; 3 fish/tank). LD, 12-h light:12-h dark; LL, continuous light; n = 9, 3/tank.

natural winter photoperiods (Ebbesson et al., 2008). Some studies have reported daily variations of cortisol with different acrophases in fish, such as rainbow trout (Polakof et al., 2007), goldfish (Singley & Chavin, 1975), carp (Kuhn et al., 1986), eel (Li & Takei, 2003), and sea bass (Cerda-Reverter et al., 1998), which may be due to different light regimes and species. LL induced overall higher plasma cortisol levels compared to LD. In rainbow trout, glucocorticoid receptors are present in the pineal

gland and melatonin production is inhibited by administration of the synthetic glucocorticoid dexamethasone (Benyassi et al., 2001). Similar results were found in mammals, in which adrenocorticotropin-stimulated cortisol was inhibited by the administration of melatonin (Torres-Farfan et al., 2003). Moreover, maternal melatonin suppression increases cortisol production in the newborn rat (Torres-Farfan et al., 2004). Therefore, there may be an inverse relationship between melatonin and cortisol in Atlantic salmon under LL, which needs to be studied in more detail. Cortisol is known to be involved in the endocrine response to stress (Boujard & Leatherland, 1992). In the present study, however, it is unlikely that the cortisol levels were a result of stress during the capture procedure, since they did not reveal high levels in both LD and LL. In teleost, it is known that cortisol increases salinity tolerance through its effect on mineral corticoids and ion transport proteins (Kiilerich et al., 2007; McCormick et al., 2008). Thus, cortisol levels appear to be influenced by light, although the biological function of the constant increase of levels induced by LL is as yet unknown in postsmolts.

Here, the rhythmic expression of the pineal and liver *Per1-like* peaked at midnight, which is in accordance with reports in goldfish retina *Per1* (Velarde et al., 2009). The peak of brain *Per1-like* that occurred at dawn was similar to that of *Per1b* (previously referred to as *Per4*) in the golden rabbitfish brain (Park et al., 2007). In zebrafish, rhythmic expression of *Per1a* (previously referred to *Per1*), *Per1b*, *Per2*, and *Per3*—with different timing of their maximal expression—was reported in an in vitro embryo system, in which those of *Per1a*, *Per1b*, and *Per3* were activated before light-onset (Pando et al., 2001; Vallone et al., 2004). In mice, three *Per* genes—*Per1*, *Per2*, and *Per3*—are rhythmically expressed with different light responses in the SCN, but only *Per1* and *Per2* have been identified as important elements of the feedback loops (Takumi et al., 1998a, 1998b; Zylka et al., 1998). We have described the expression of Atlantic salmon *Per1-like* gene in parr and smolts (Huang et al., 2010b), in which the putative amino acid sequence derived from a cDNA fragment of *Per1-like* showed greatest homology with golden rabbitfish PER1b (Park et al., 2007). Since the full sequence data were not obtained, it is necessary to obtain a large sequence in order to clarify the identity of *Per1* expression.

Similar to light-dependent *Per2* expression in zebrafish (Delaunay et al., 2000; Ziv et al., 2005), golden rabbitfish (Sugama et al., 2008), and goldfish (Velarde et al., 2009), brain *Per2* mRNAs oscillated under LD with a peak of expression at 08:34 h, which corresponds to the time of light-onset. However, the peak expression of pineal *Per2* occurred in the dark phase, as described in parr brain *Per2* under a short-day photoperiod (SD) by Davie et al. (2009). It is likely that *Per2* expression in the whole brain does not occur in parallel with that in the pineal gland, which does not contain a functional circadian clock as in other teleosts

(Falcon et al., 2010). We have unpublished data on pituitary clock gene expression in smolts showing that light-dependent *Per2* expression is antiphasic to *Per1-like*; this suggests that other brain areas contribute to internal circadian oscillators and that the regulation of *Per2* is different in salmonids compared to other teleosts (Sugama et al., 2008; Velarde et al., 2009; Ziv et al., 2005). Although we cannot explain why *Per2* is light-induced in the pineal gland but not in the liver and brain under LL in the present study, this finding can provide important information about the clock gene system in salmonids.

Pineal *Cry2* peaked at midnight and brain *Cry2* peaked at dawn, which is similar to the nocturnal expression of *Cry2* in goldfish retina (Velarde et al., 2009), Atlantic salmon parr brain (Davie et al., 2009), and zebrafish larva (Lahiri et al., 2005). The collection of these results suggests that Atlantic salmon *Per1-like* and *Cry2* mRNAs are expressed with daily variations in the brain and peripheral tissues of postsmolts. The peak expression of brain *Clock* was in the beginning of the light phase (08:52 h), and in liver *Clock* it was in the late night phase; in contrast, Atlantic salmon parr *Clock* peaked at midnight under SD (Davie et al., 2009), whereas it peaked during the light phase in mouse SCN (Abe et al., 1999). These variations of the peak time may imply that there is a different response of *Clock* expression in various organs, species, and under different photoperiods. Given that *Clock* expression is not rhythmic in the pineal gland under LD, we suggest that *Per1-like* and *Cry2* expression is not dependent on the rhythmic expression of *Clock* in the pineal gland, although it is unknown whether this organ acquires specific regulation of clock gene expression in postsmolts. The mechanisms regulating gene expression in the rat pineal gland are complex, because gene transcription in this organ is controlled by both the central clock in the SCN and the circadian clocks present in the pineal gland (Abe et al., 2002; Fukuhara et al., 2005). In contrast to light up-regulated *Clock* antiphasic to dark up-regulated *Per1-like* and *Cry2* in the pineal gland of parr under LD (Huang et al., 2010b), the brain transcriptional repressors and activators peaked before and after onset of daylight in postsomlts, respectively. Although it is uncertain if clock gene RNA levels can be compared between parr and postsmolts, the differences of expression under LD may be due to developmental changes. The acrophases of brain *Per1-like*, *Per2*, and *Cry2* occurred a few hours later than those of the pineal genes and brain *Per2*, and *Clock* peaked in the beginning of the light phase, suggesting that a set of genes that peaked during the day or later at night may be regulated by pathways that are indirectly activated by melatonin or perhaps by a "master clock" located within a SCN-like structure in the brain. Yet, in salmonids, pineal melatonin is directly regulated by light but not by a circadian clock (Iigo et al., 2007), and salmonids may have evolve a decentralized system in the pineal gland, independently of the

eyes (Migaud et al., 2007). There is to date no evidence for the presence of a functional circadian clock either in the pineal gland or retina, although they express clock genes in salmonids. Thus, further studies should clarify the role of the clock genes in the growth and stress axis and their expression in the hypothalamus.

The expression of *c/ebpα* and *c/ebpδ* exhibited daily variations in the liver and pineal gland, respectively, consistent with observations of rhythmic mRNA expression of *c/ebp* in the eye of the mollusc (Hattar et al., 2002), *c/ebpβ* in murine epididymal adipose tissue (Bray & Young, 2007), and *c/ebpδ* in the hamster pineal gland (Maronde et al., 2007). It has been shown that C/EBP is involved in the regulation of arylalkylamine *N*-acetyltransferase (AANAT) mRNA in response to cyclic adenosine monophosphate (cAMP) activation via the cAMP-response element-CCAAT complex in the promoter region (Baler et al., 1997) and that a role of adrenergic/cAMP signal transduction in regulating rhythmic gene expression has been studied in rat pineal function (Bailey et al., 2009). These reports will provide a foundation for studies of the function of the salmon *c/ebpδ* in the pineal gland. In a report by Bozek et al. (2009), C/EBP is a suggestive candidate for circadian transcriptional regulators in a large-scale analysis of the CCG promoters, and circadian clock genes have been identified as targets of C/EBP in mouse NIH 3T3 cells (Gery et al., 2005). Thus, the rhythmic expression of salmon *c/ebpα, c/ebpδ*, and *Per1-like* supports the idea of a link between C/EBP proteins and the clock gene system. In addition, we found that expression of liver *pparα* mRNA showed a dual peak, one in the evening and the other at dawn, whose values were statistically significant different from other timepoint values by ANOVA, but not by 24-h waveform cosinor analysis. This may be because the variable we are measuring varies over time, but not in a circadian manner, as not all biological rhythms are circadian. Targets of liver PPARα are involved in cellular lipid catabolism (Yamazaki et al., 2002), and the daily variation in the expression of lipogenic and cholesterogenic genes were found to be attenuated or abolished in PPARα-null mice (Patel et al., 2001). As described in mammals (Jump, 2004), Atlantic salmon PPAR and C/EBP have been suggested to be fatty acid–regulated transcriptional factors that play important roles in lipid metabolism in hepatocytes, adipocytes, and myotubes (Huang et al., 2010a; Kleveland et al., 2006; Vegusdal et al., 2004). PPARα and C/EBPα may act as possible mediators for the daily rhythms of lipid metabolism in the liver, although this requires further study. Different sets of clock-controlled genes across tissues were observed in the study of cycling transcripts in mice (Panda et al., 2002; Storch et al., 2002), suggesting liver *c/ebpα*, *pparα,* and pineal *c/ebpδ*are putative clock-controlled genes in Atlantic salmon. PPARs are expressed in the rat brain, of which the specific location, degree of expression, and physiological roles vary among the

receptors (Ramanan et al., 2010). Consequently, it is important to further elucidate the role of individual nuclear transcription factors when interacting with tissue-specific ligands to perform to their own function coupled with clock gene expression in the pineal gland and liver.

The expression of C/EBP, PPAR, and clock genes did not show significant daily variations in the studied tissues under LL, with the exception of pineal *pdia3*. The mRNA expression of pineal *Clock*, *Per2*, *pparα*, *c/ebpα*, and *c/ebpδ* increased under LL, whereas the liver *Clock*, *Per2*, and *pparα* expression decreased compared to under LD. Nonetheless, the increasing or decreasing clock mRNAs were not observed in the brain, indicating a different role of clock genes across tissues. Although pineal *Per2*, *c/ebpα*, and *c/ebpδ* did not express at high levels in the studied organs during the day under LD, the increase of expression was observed under LL in the pineal gland, but not in the liver. It is unknown why there were opposite effects of LL on the expression of *Per2*, *Clock*, and *pparα* in the pineal gland and liver, and future studies should address this. These results will provide insight into clock gene system in salmonids and provide considerable clues as to what is happening under LL conditions. Contrary to a lack of daily variations in the plasma levels of melatonin and cortisol and the expression clock genes, continuous light can enhance growth and muscle fiber recruitment (Johnston et al., 2003), affect mineralization, and delay osteoid incorporation in Atlantic salmon postsmolts (Wargelius et al., 2009). Similar results have reported that continuous light exposure affects the growth rate, timing of smoltification, and reproduction (Krakenes et al., 1991; Saunders & Henderson, 1988; Stefansson et al., 1991). Light and feeding effects on the daily rhythms have been reported in clock genes of zebrafish (*Per1* and *Cry*) by Sanchez and Sanchez-Vazquez (2009) and humoral daily rhythms of sea bream by Lopez-Olmeda et al. (2009). In our experiments, LL-reared fish were exposed to continuous light but 12 h cycles of feeding and starvation each day and showed no variation in daily clock gene expression, indicating that light has a stronger effect on clock gene expression than feeding in salmon. However, the connection between the earlier observed effects of LL on growth and development and a disrupted rhythm in the expression of clock genes imposed by LL as observed here remains elusive.

The most interesting effect of light was observed on *pdia3* expression. The *pdia3* mRNA appears to be rhythmically expressed in the pineal gland under both LD and LL, but the acrophases differed. Multiple functions of PDIA3 are known, i.e., to catalyze the oxidation, reduction, and isomerization of intra- and intermolecular disulfide bond formation in ER (Khanal & Nemere, 2007). We previously demonstrated that liver-expressed *pdia3* and *c/ebpδ* may be important biomarkers for oxidant- and toxicant-induced stress in smolts (Huang et al., 2009b). Although

arrhythmic in the liver, the daily expression of *pdia3* and *c/ebpδ* was only present in the pineal gland under LD. Although it is unknown whether the significance of pineal *pdia3* and *c/ebpδ* transcripts is related to physiological function, their expression may be coupled with clock gene expression under LD in Atlantic salmon. Whether the observation that *pdia3* expression is not correlated with the arrhythmic expression of clock genes under LL is related to the idea that an endogenous growth rhythm exists in Atlantic salmon under LL (Oppedal et al., 2006) remains unknown. This connection and the importance of the rhythmic expression of pineal *pdia3* under both LD and LL need to be studied in more detail.

The present study indicates that light exerts an influence on the daily variation and overall expression of clock and nuclear transcription factor genes in the pineal gland, brain, and liver of Atlantic salmon postsmolts, as well as levels of melatonin and cortisol. It is possible that light-dependent variations of expression reported here reflect interplay between daily and annual regulation. These changes can promote new approaches for achieving a better understanding of how the clock gene system is associated with the environmental, physiological, and genetic interactions that occur in lifecycle transitions and their signaling pathway between the brain and periphery. On the other hand, the integration of continuous light for the timing of events, such as smoltification and sexual maturation, and its effect on the seasonal growth pattern, need to be studied in more detail.

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