1 # *In vitro* and *ex vivo* models indicate that the
2 # molecular clock in fast skeletal muscle of
3 # Atlantic cod is not autonomous

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Abstract

The hypothesis that the circadian rhythm is exclusively regulated by a central clock has been challenged by the discovery of peripheral oscillators. These peripheral clocks are known to have a direct influence on the biological processes in a tissue or cell. In fish, several peripheral clocks respond directly to light, thus raising the hypothesis of autonomous regulation. Several clock genes are expressed with daily rhythmicity in Atlantic cod (*Gadus morhua*) fast skeletal muscle. In the present study, myosatellite cell culture and short-term cultured fast skeletal muscle explant models were developed and characterized, in order to investigate the autonomy of the clock system in skeletal muscle of Atlantic cod. Myosatellite cells proliferated and differentiated *in vitro* as shown by the changes in cellular and myogenic gene markers. The high expression of *myogenic differentiation 1* (*myod1*) during the early days post-isolation implied the commitment to myogenic lineage and the increasing mRNA levels of *proliferating cell nuclear antigen* (*pcna*) indicated the proliferation of the cells *in vitro*. Transcript levels of myogenic marker genes such as *pcna* and *myogenin* (*myog*) increased during five days in culture of skeletal muscle explants, indicating that the muscle cells were proliferating and differentiating under *ex vivo* conditions. Transcript levels of the clock gene *aryl hydrocarbon receptor nuclear translocator-like 2* (*arntl2*) in myosatellite cells showed no daily oscillation regardless of photoperiod manipulation. On the other hand, mRNA levels of the clock gene *circadian locomotor output cycles kaput* (*clock*) showed circadian rhythmicity in 5-day-old skeletal muscle explant under different photoperiod regimes. Expression of *arntl2*, *cryptochrome2* (*cry2*), *period 2a* (*per2a*) and *nuclear receptor subfamily 1, group D, member 1* (*nr1d1*) was not rhythmic in muscle explants but photoperiod manipulation had a significant effect on mRNA levels of *cry2* and *per2a*. Taken together, the lack of rhythmicity of molecular clocks *in vitro* and *ex vivo* indicates that the putative peripheral clock in Atlantic cod fast skeletal muscle is not likely to be autonomous.

Keywords: Atlantic cod; circadian rhythm; peripheral clock; muscle; myosatellite cell; photoperiod

1. Introduction

Physiology and behavior of fish are markedly influenced by daily, lunar and annual light cycles, as a consequence of rotation and translation movements of the Earth and the Moon around the Sun [1]. The daily cycling pattern of biological processes (circadian rhythm) is the outcome of an interlocked auto-regulatory transcriptional and translational/post-translational feedback loop consisting of a complex network of genes and their proteins [2, 3]. The classical view is that a central "master clock" orchestrates the signals that are then transmitted to different cells, tissues and organs of an organism. The discovery of peripheral oscillators had posed the intriguing hypothesis that the regulation of circadian rhythmicity could be autonomous in cells or tissues. Clock genes in fish are not only expressed in the pineal gland, the probable central clock in teleosts, but they are also found in other peripheral tissues [4-8].

Self-sustained peripheral clocks became an integral area of chronobiological research, since they could provide an important model system for the elucidation of many aspects of clock function that are more difficult or impossible to study in the central oscillator itself [9]. Notably in zebrafish, it has been shown that some peripheral tissues and cells are photosensitive and the circadian clocks present within individual cells could be directly entrained by light both in cultured tissues and in dispersed cell cultures [10-12]. This distinctive feature is in contrast with the peripheral clock system in mammals but it resembles to some extent the photosensitivity of molecular clocks in peripheral tissues of *Drosophila melanogaster* [13]. The regulation of peripheral oscillators is complex and poorly understood, particularly on whether the rhythm, which is greatly influenced by the daily light/dark cycle, is autonomously regulated. The discovery of circadian organization in various species will likely lead to the identification of a broad spectrum of interactions between clocks and various organs and cells, depending on their physiological functions [14].

There is evidence that fishes have a functional clock system in fast skeletal muscle, based on the identification of several conserved molecular components of the core clock machinery and their rhythmicity during a daily/circadian cycle [5, 15, 16]. In some teleost species, muscle growth displays photic plasticity [15, 17], thus making them a particularly interesting model to study the muscle peripheral clock system and its entrainment by light. It is known that photoperiod is an important environmental factor in driving a number of physiological processes in Atlantic cod (*Gadus morhua*), namely somatic growth and sexual maturation [17-19]. In particular, Atlantic cod juveniles reared under continuous illumination were 13% heavier than those kept under natural photoperiod conditions for 120 days, and this effect was associated with changes in expression of muscle- and DNA methylation-related genes [15, 17, 20].

Previously, we have shown that a number of clock genes were expressed in the fast skeletal muscle of Atlantic cod, and 8 of them displayed daily rhythmic expression [16]. This suggests the presence of a peripheral clock system in Atlantic cod skeletal muscle but it is not clear whether it is autonomous or not. To address this question, we have developed an *in vitro* myosatellite cell culture and an *ex vivo* muscle explant model to examine changes in transcript levels of rhythmic genes during a daily cycle under different photoperiod conditions.

2. Materials and Methods

2.1 Animal ethics and husbandry

All procedures concerning animal handling and experimentation complied with the standards set by the National Animal Research Authority (Forsøksdyrutvalget, Norway). All fish used in the experiment were from the cultured stocks maintained at Mørkvedbukta Research Station of the University of Nordland (Bodø, Norway). These juvenile Atlantic cod were maintained at a constant illumination (*LL*; 24L:0D) provided by fluorescent white light

bulbs (Aura Light International AB, Karlskrona, Sweden). A commercial diet (Amber Neptun, Skretting AS, Stavanger, Norway) was provided daily at 5 % (w/w) of the fish body weight. All fish used in the experiments were fasted for 24 h prior to tissue sampling. The following physico-chemical parameters were maintained and monitored daily: temperature at 7.0 ± 0.5 ºC and dissolved oxygen at 90 ± 2 % saturation.

2.2 Isolation and culture of Atlantic cod myosatellite cells

The isolation of Atlantic cod myosatellite cells was carried out following previously published protocols [21, 22] with some modifications. Nine fish weighing approximately 100 g were euthanatized by immersion in seawater containing 0.5 $g \cdot L^{-1}$ tricaine methanesulfonate (MS222: Sigma, Oslo, Norway). The dorsolateral fast skeletal muscle was excised, washed with cold, sterile 1 × phosphate buffered saline (PBS, Sigma) and immediately placed in cold Dulbecco's Modified Eagle's medium (DMEM; pH = 7.4; 300 $mOsmoL \cdot L^{-1}$) supplemented with 9 mM $NaHCO_3$, 20 mM HEPES, 15 % (v/v) horse serum and antibiotics (100 $U \cdot ml^{-1}$ penicillin, 100 $\mu g \cdot ml^{-1}$ streptomycin, 0.25 $\mu g \cdot ml^{-1}$ gentamicin) (Sigma). The tissue samples used were free of any discernible traces of blood or skin contamination. They were mechanically dissociated and the fragments were centrifuged (300 *g*, 5 min) and washed twice with cold DMEM. Following a two-stage enzymatic digestion, the fragments were resuspended in DMEM with collagenase (0.2 % final concentration) and incubated at 18 ºC for 75 min with gentle orbital agitation (VWR, Oslo, Norway). The resulting suspension was centrifuged for 10 min at 300 *g*. The fragments were then washed twice with cold DMEM, aspirated 10 times to dissociate the cells and centrifuged again for 20 min at 300 *g*. They were then resuspended in a DMEM-trypsin solution (Sigma, 0.1% (v/v) final concentration in DMEM), incubated for 20 min at room temperature with gentle orbital agitation and then centrifuged for 1 min at 300 *g*. The supernatant was diluted in 4 volumes of cold complete medium (DMEM with 10 % [v/v]

fetal bovine serum and antibiotics) and the trypsin dissociation step was repeated once. After centrifuging the supernatant for 20 min at 300 *g*, the resulting cell pellet was resuspended in complete medium and triturated 5 times to dissociate the cells. The suspension was filtered sequentially through 100 µm and 40 µm nylon cell strainers and centrifuged for 20 min at 300 *g*. The collected cell pellets were suspended in complete medium thereafter. Isolated cells from three fish were pooled and considered as one biological replicate. Two mL of cell suspension, containing approximately 10^5 to 10^6 cells per mL, were seeded onto culture plates coated with laminin (Sigma; 50 µg/mL). The high affinity of satellite cells to laminin resulted in a satellite cell-enriched culture. The cells were incubated at 15 ºC, and washing and medium changes were done every day during the first 7 days of culture and every two days thereafter. Unless specified otherwise, the cells were cultured under a 12L:12D photoperiod regime with a maximum white light intensity of 0.115 Klux. During sampling, approximately three-fourths of the cultured media was aspirated, the cells were scraped off and the cell-enriched suspension was immediately transferred to a microcentrifuge tube. Samples were taken at 1, 3, 5, 7, 14, 22, and 30 days post-isolation (dpi) and stored at -80 ºC until RNA extraction.

2.3 Characterization of Atlantic cod myosatellite cells in culture

Morphological observations were conducted daily using a phase contrast microscope equipped with a Leica camera (Leica Microsystem, Germany). The index of cellular proliferation was determined by the CyQuant® Direct Cell Proliferation Assay (Invitrogen, Oslo, Norway). Spectrophotometric determination of proliferation based on the intact DNA and cellular integrity was quantified by fluorescence at excitation/emission maxima of 508/527 nm. Creatine phosphokinase activity of the developing myofibers was used as an index of cellular differentiation, since it has been known to correlate with myoblast differentiation [23]. It was determined by colorimetry using the EnzyChrom™ Creatine Kinase assay kit (Bioassay

Systems, CA, USA). Cellular apoptosis was related to the activity of caspase-3. The corresponding colorimetric assay was performed with the Caspase-3/CPP32 assay kit (BioViosion, CA, USA), which is based on the detection of the chromophore ρ-nitroaniline after cleavage from labeled substrate DEVD- ρNA. The above indices were determined at 1, 3, 5, 7, 10, 14, 17, 22, 27 and 30 dpi.

2.4 Photoperiod manipulation of myosatellite cell cultures

Myosatellite cells were isolated as described above and the culture medium was changed daily, except on the sampling day. For the first 3 days, the cultures were incubated at 12L:12D (*LD*) photoperiod regime and morphological changes were monitored every day under a light phase microscope. The cells were then split in three groups that were kept under i) equal length of light and dark (*LD;* 12L:12D), ii) continuous illumination (*LL*; 24L:0D) and iii) total darkness (*DD*; 0L:24D). On the fourth day after the photoperiod change, samples were taken every 3 h during a 24 h period from each photoperiod treatment group as described above. The cells were immediately snap-frozen and stored at -80 ^{o}C for further analysis.

2.5 Short-term culture of fast skeletal muscle explants of Atlantic cod

Muscle explants were obtained following the method of Funkenstein et al. [24], with minor modifications. After the fish were euthanatized, a fast skeletal muscle fragment from the dorsolateral region was excised, washed with cold PBS and immediately placed in cold DMEM supplemented with antibiotics. The excised muscle fragment, devoid of any traces of blood and skin, was then subdivided into smaller pieces (approximately 4 mm × 4 mm area and 1–2 mm height) for explant culture. Tissue fragments were first blotted on a clean and sterile tissue paper to remove excess media and pressed down firmly but gently onto a laminin-coated surface of a multi-well plate. DMEM supplemented with 9 mM $NaHCO_3$, 20 mM HEPES, 15 % (v/v) fetal

bovine serum and antibiotics was carefully added after approximately 45 min. The explants were cultured at 15 ºC under a 12L:12D light regime with a maximum white light intensity of 0.115 Klux. Microscopic observations and change of medium were done every day for 5 days. On the sampling day, approximately three-fourths of the culture media was aspirated and the explant was removed. The resulting suspension mixture was then transferred to a microcentrifuge tube and stored at -80 ºC for further analysis. Samples were taken at 0, 3 and 5 days in culture for gene expression analysis of myogenic markers genes.

2.6 Photoperiod manipulation of 5-day old muscle explants

Fast skeletal muscle explants were prepared as described above and cultured for 5 days under *LD, LL* or *DD* photoperiod regimes. The culture medium was changed daily except on the sampling day. Samples were then collected every 3 h for a period of 24 h as described above, snap-frozen in liquid nitrogen and stored at – 80 ºC prior to RNA isolation.

2.7 RNA isolation, reverse transcription and qPCR analysis

Total RNA from myosatellite cells and muscle explants was isolated using the MasterPure™ RNA Purification kit (Epicentre®, USA) and TRIzol® (Invitrogen), respectively, following the manufacturers' protocols. Total RNA was quantified on a Nanodrop® ND-1000 Spectrophotometer (Thermoscientific, USA) and its quality was assessed by running a denaturing RNA electrophoresis on a 1.2 % (w/v) agarose gel. First strand cDNA synthesis was performed with the QuantiTect Reverse Transcription kit (Qiagen, Nydalen, Sweden).

Primers used in this study are provided in Supplementary Table S1, along with their annealing temperature. Expression of clock and myogenic genes was quantified by quantitative real-time PCR (qPCR) with the LightCycler® SYBR Green I master chemistry (Roche, Basel, Switzerland) on a LightCycler® 480 thermocycler (Roche). The qPCR reaction was performed

according to the following thermocycling protocol: initial denaturation at 95 ºC for 15 min, followed by 45 cycles of 15 s at 94 ºC, 20 s at defined temperature for each primer set and 20 s at 72 ºC. In order to calculate amplification efficiencies, five-point standard curves of 2-fold dilution series were prepared from pooled cDNA. Cycle threshold (C_T) values were calculated through the LightCycler® 480 software using a fluorescence arbitrary value set to 0.8.

Relative expression values were normalized using the geometric average of the *acidic ribosomal protein* (*arp*) and *ubiquitin* (*ubi*) using the geNorm algorithm (http://medgen.ugent.be/~jvdesomp/genorm/) for each experimental setup, as reported [25, 26].

2.8 Statistical analyses

Significant temporal differences for the various cellular indices (proliferation, differentiation and apoptosis) and transcript levels were determined with the SigmaStat Statistical Package (Systat software, London, UK). ANOVA assumptions were checked and if the data set did not follow a Gaussian distribution with equal variance, they were log-transformed before conducting a one-way ANOVA. Pairwise comparisons were done by Student–Newman–Keuls (SNK) post-hoc tests. For non-parametric data, a Kruskall-Wallis ANOVA on ranks with an SNK post-hoc test was used instead. The same statistical approach was used to determine differences between photoperiod treatments at a given time point. The level of significance was set at $P < 0.05$.

The COSINOR algorithm was used to determine if the transcript levels of clock genes displayed daily rhythmicity [7]. The analysis was performed by fitting a periodic sinusoidal function to normalized gene expression values across the nine time points, using the formula: $f(t) = M + A\cos(t/pi/12 - \varphi)$, where $f(t)$ is the gene expression level at given time, mesor (M) is the mean value, A is the sinusoidal amplification of oscillation, t is time in hours and φ is the acrophase (peak time of the approximating sinusoidal function). The statistical significance p

of the approximated 24 h waveform was defined by the noise/signal of the amplitude. $p < 0.3$ was the threshold to consider that gene expression followed a daily rhythmic pattern [16]. Phase shifts were calculated based on changes of acrophase relative to the LD group. A positive shift means that the acrophase was advanced, while a negative shift denotes a delay relative to the LD group.

3. Results

3.1 Morphological characterization of myosatellite cells in culture

The distinct spindle shape morphology of myosatellite cells could be observed at 1 dpi, even if some satellite cells had an irregular appearance (Fig. 1A). This shape irregularity diminished as the culture progressed and by 3 dpi most cells were spindle-shaped (Fig. 1B, insert). The cells started to elongate and form a network in the succeeding days. At 7 dpi, approximately 30 to 40 % of the satellite cells had already at least 2 nuclei (Fig. 1C) and numerous small myotubes were observed with their nuclei centrally placed (Fig. 1C, insert). At 14 dpi, a network of myotubes started to be more prominent (Fig.1D) and became more complex as the culture progressed. An intricate web-like network could be observed in the culture at 30 dpi and by this point, over 90 % of the myosatellite cells had fused into multinucleated myotubes (Fig. 1E).

3.2 Cellular activity indices of myosatellite cells in culture

Proliferation and differentiation of the myosatellite cells occurred almost concurrently but apoptotic activity was observed throughout the culture period (Fig. 2). The myosatellite cells were at a non-proliferative stage from 1 to 3 dpi (Fig. 2A). Proliferation significantly increased at 5 dpi and increased further at 7 dpi. Thereafter, the rate of proliferation remained at the same level until 17 dpi. The second proliferative stage occurred at 22 dpi. Although significantly higher creatine kinase activity had been detected at 5 dpi, a pronounced increase was observed at 10 dpi, corresponding to a significant 72 % increase in the differentiation index. There was a constant rate of differentiation from 10 to 22 dpi, followed by a significant increase in creatine kinase activity at 27 (~53 %↑) and 30 (~42 %↑) dpi. There were three peaks of apoptotic activity in the myosatellite cell cultures. Apoptotic activity increased significantly from 1 to 5 dpi. Thereafter, there was a decrease in activity and the rate of apoptosis remained constant until 14 dpi. Activity increased again at 17 dpi and then at 27 to 30 dpi.

Changes in relative expression of *myod1* (marker of myogenic commitment), *myog* (terminal differentiation), *pcna* (cellular proliferation) and *des* (early stage of differentiation) during the culture period of myosatellite cells are shown in Figure 3. The highest level of *myod1* transcripts was observed at 1 dpi and decreased progressively until 22 dpi, when the lowest *myod1* expression was observed (Fig. 3A). Expression of *myog* and *des* showed no significant changes during the course of the culture period (Figs. 3B, D). Transcription levels of *pcna* increased during the first 7 days of culture with a significant 60 % increment at 7 dpi relative to 1 dpi (Fig. 3C). Its levels decreased significantly at 14 dpi to basal levels and then remained constant until the late culture stages.

3.3 Daily rhythm and photoperiod manipulation in myosatellite cells

From the 8 clock genes previously identified to be daily rhythmic [16], only *aryl hydrocarbon receptor nuclear translocator-like 2* (*arntl2;* also known as *brain and muscle Arnt-like 2, bmal2*) was significantly expressed in myosatellite cells (Fig. 4). *circadian locomotor output cycles kaput* (*clock*) transcripts were also present but at levels that could not be accurately quantified by qPCR. Albeit the significant temporal changes in the transcript levels of *arntl2,* it did not display daily rhythmicity under a 12L:12D light regime, as shown in Fig. 4 and Supplementary Table S2. A similar pattern was observed when myosatellite cells were exposed to continuous illumination (*LL*) or total darkness (*DD*). Nonetheless, changes in photoperiod significantly influenced the transcript levels of *arntl2* in the cell cultures. Myosatellite cells exposed to *LL* showed significantly less *arntl2* transcripts, particularly at 15, 18 and 24 h, than the group cultured under *LD* or *DD* conditions.

3.4 Daily rhythm and photoperiod manipulation in short-term cultured skeletal muscle explants

Myogenic marker genes were expressed during the 5-day culture of fast skeletal muscle explants (Fig. 5A-D). Sustained proliferation and differentiation in the explants were typified by the expression of myogenic marker genes, particularly *myog* (Fig. 5B) and *pcna* (Fig. 5C). The transcript levels of *myog* were similar during the first 3 days of culture but a significant 20 % increase was noted at 5 days in culture (Fig. 5B). An increasing trend on *pcna* transcript levels was observed (Fig. 5C). In particular, a significant ~32% increase in transcript levels was noted in the muscle explants at 5 days in culture.

Transcript levels of the clock genes *arntl2*, *clock*, *cryptochrome* 2 (*cry2*), *period 2a* (*per2a*) and *nuclear receptor subfamily 1, group D, member 1* (*nr1d1;* also known as *rev-erb alpha*) were determined in 5-day old fast skeletal muscle explants (Fig. 6, Supplementary Fig. S1). Under *LD*, only *clock* transcripts showed rhythmic expression, which was largely

unaffected by changes in photoperiod regime (Table 1). Photoperiod manipulation resulted to phase shift on the expression of *clock* particularly delaying the acrophase by an approximate -1.2 h under *DD* conditions. Generally, muscle explants cultured under *LL* conditions had lower *clock* mRNA levels compared to *LD* and *DD*; significant differences between photoperiod treatments were noted at 0 and 24 h (Fig. 6A). Changes in photoperiod regime influenced the transcript levels of *cry2* and *per2a* at certain times of the daily cycle (Fig. 6B, C). Under *LL* conditions, *cry2* mRNA levels were relatively lower compared to *LD* and *DD* conditions, and significant differences were noted at 15 and 24 h (Fig. 6B). On the other hand, culturing the muscle explants under *LD* conditions resulted in significantly lower *per2a* transcript levels, especially at 9 and 12 h (Fig. 6C).

4. Discussion

The rhythmic expression of several clock genes in fast skeletal muscle of Atlantic cod suggests the presence of a peripheral clock in this tissue [15, 16]. In the present study, we developed *in vitro* (myosatellite cell culture) and *ex vivo* (muscle explant) models of muscle growth to ascertain its autonomy.

Myosatellite cells are small spindle-shaped cells with a heterochromatic nucleus and are situated between the sarcolemma and the basal lamina of fully differentiated skeletal muscle fibers [21]. These stem cells play a pivotal role in muscle growth and regeneration [27]. Atlantic cod myosatellite cells were able to proliferate and differentiate *in vitro.* The morphological characteristics of Atlantic cod satellite cells were similar to the reported features found in other teleosts species, particularly their spindle-shape morphology [21, 22, 28, 29]. The observed irregular shape of some satellite cells at the early days of culture could have been due to the enzymatic digestion and mesh passages. Among the myogenic regulatory factors, MyoD is a key factor in determining muscle lineage [30] and the high levels of *myod1* transcripts observed

during the early days of myosatellite cell culture in Atlantic cod indicate that the isolated cells are committed to the myogenic program. Myogenic cell cultures are characterized by distinct phases where cells first proliferate, and then fuse to form multinucleated myotubes [31]. Atlantic cod myosatellite cells actively proliferated during the first week in culture, as indicated by high levels of *pcna* transcripts and a high proliferation index. The trend of apoptosis rate in the culture may be related to a continuous cellular turnover. Nearly 100 % confluency was observed after 20 dpi, which could account for the constant rate of proliferation and elevated apoptotic rate observed at the later time points. The morphological observations during the culture period showed that myosatellite cells were able to differentiate under *in vitro* conditions but this process was not always well correlated with the differentiation index (creatine kinase activity) and the expression of *myog*. This apparent discrepancy could be related to the different parameters these indices were measuring.

Myosatellite cell cultures are most useful to investigate the different phases of the myogenic program, from commitment of individual cells to differentiation into functional muscle fibers. On the other hand, muscle explants can demonstrate how the muscle continues to function as a tissue unit, rather than just a cell type. Therefore, muscle explants do not only provide an approximation of the *in vivo* conditions for cell proliferation and differentiation but also enable a closer comparison with muscle regeneration events *in vivo* [24]. The differential regulation of some myogenic markers within 5 days of culture of the muscle explants indicates that myogenic activity of the muscle was sustained under *ex vivo* conditions. In particular, proliferation and differentiation processes may have occurred in the muscle explants, as shown by the differential expression of *pcna* and *myog*. These observations are corroborated by a previous *ex vivo* study in sea bream (*Sparus aurata*) muscle explants [24]. Given the above features, both the myosatellite cell culture and muscle explants can be used as models to

investigate the autonomy of some centralized biological processes in skeletal muscle, such as the circadian rhythm.

The loss of daily rhythmicity of most clock genes in myosatellite cells and muscle explants supports the hypothesis that the molecular clocks earlier identified in Atlantic cod muscle lack autonomy. In contrast, several zebrafish peripheral oscillators (including fast skeletal muscle) display conserved rhythmicity under *in vitro* conditions [10, 11, 32]. Our data show that the autonomy of muscle clocks may vary among teleost taxa. It is plausible that the clock system of fast skeletal muscle is regulated under an endocrine signaling pathway, where output signals from the “master clock” are necessary to drive the rhythmicity of the clock components. Even if the control of peripheral oscillators is still largely unknown in fish, there is evidence in mammals that the suprachiasmatic nucleus (the central clock) output pathways serve as input for the synchronization of peripheral clocks. For example, several circulating metabolites such as corticosterone, glucose and cholesterol have been implicated as regulators of peripheral oscillators in mammals [33]. It is also plausible that the peripheral oscillator in Atlantic cod muscle is under neuronal control, similarly to what has been observed in mammalian models [34]. A recent study in blind cavefish (*Phreatichthys andruzzii*) revealed that the lack of light responsiveness of peripheral cells was due to a mutation in photopigments [35]. It would be pertinent to investigate if a similar mutation could explain, at least partly, the absence of synchronization by light in Atlantic cod skeletal muscle models. We have not investigated if the muscle cells express photopigments such as melanopsins in culture but we have previously reported that cryptochromes, a group of genes coding for photosensitive proteins, are expressed in Atlantic cod skeletal muscle [16]. Further, we cannot conclusively exclude light as a synchronization factor in Atlantic cod skeletal muscle, since the present study and previous reports suggest that this tissue is responsive to changes in lighting regimes [15, 16, 17, 19].

The circadian secretion of melatonin by the pineal gland and retina is a direct output of the central clock in many species of vertebrates [36]. The effect of melatonin treatment on circadian rhythmicity has been shown previously in a resynchronization of oscillatory circadian rhythm genes *Dbp* and *Per2* [37]. However, administration of exogenous melatonin *in vitro* and *ex vivo* did not significantly influence the rhythmicity of the molecular clocks in Atlantic cod muscle (data not shown), suggesting that melatonin has an indirect effect in this peripheral clock.

The CLOCK protein is one of the key components of the positive arm of the transcriptional-translational feedback loop that regulates the circadian rhythm both in teleosts and mammals [2, 5, 6]. Although most clock genes lost their rhythmicity under *in vitro* and *ex vivo* conditions, it is possible that the rhythmic transcription of *clock* in fast skeletal muscle may not be entirely dependent on the central clock and it has a self-sustaining oscillation. The rhythmicity of *clock* transcript levels under *ex vivo* conditions is corroborated by previous observations in zebrafish peripheral tissues [6] and *clock* is likely to be an indispensable component of the circadian clock in Atlantic cod fast skeletal muscle. The absence of CLOCK in *Drosophila* shut down the entire circadian program, thus demonstrating its crucial role in the circadian clock system [38]. It would be relevant to investigate the influence of the observed *clock* rhythmicity in the overall circadian clock system in fast skeletal muscle of Atlantic cod. Given the nature of the molecular network of circadian rhythm which is highly dependent on the outputs generated by the transcriptional-translational feedback loop [1], we can still regard the clock system in fast skeletal muscle of Atlantic cod to be non-autonomous, even if the rhythmicity of *clock* transcript levels was maintained *ex vivo*. The daily media changes could have affected the expression levels of clock genes, since serum shock is known to be a powerful entrainment factor for mammalian cells [39]. The absence of rhythmicity in transcript levels of

all genes examined except *clock*, further supports the hypothesis that fast muscle cells of Atlantic cod lack an autonomous circadian oscillator.

Photoperiod manipulation has a remarkable influence on skeletal muscle of Atlantic cod both at phenotypic and transcriptional levels [15, 16, 19]. Some fish (e.g., zebrafish) contain individual cells and tissues containing circadian pacemakers that are directly light responsive [3, 6, 32, 40]. In the present study, the clock genes analyzed in both models displayed arrhythmic expression both in LD and in constant photoperiod conditions (LL and DD), with the exception of *clock*. Nevertheless, their mRNA levels could be significantly influenced by changes in photoperiod, namely the 2-fold reduction in *cry2* transcript levels observed under LL conditions in muscle explants compared to LD or DD. Interestingly, the rhythmicity of *clock* transcript levels in muscle explants was unaffected by changes in photoperiod, supporting the above hypothesis that Atlantic cod *clock* may have a self-sustaining oscillation in muscle under *ex vivo* conditions. The parameters defining the rhythmicity of *clock* revealed that under *DD*, its expression was delayed by approximately 1.2 h. Photoperiodic sensitivity of circadian rhythm is phase-dependent [41] and these phase shifts could derive from either an enhancement of photic input or an altered entrainment state of the clock [42]. The observed phase shift in *clock* transcript levels could be a differential response mechanism to photoperiod changes in fast skeletal muscle of Atlantic cod.

In summary, the myosatellite cell culture and muscle explant developed in this study can be used as *in vitro* and *ex vivo* models, respectively, to investigate the influence of different environmental conditions on muscle physiology and to explore the autonomy of some biological processes in skeletal muscle. The expression profile of clock genes under different photoperiod regimes *in vitro* and *ex vivo* supported previous observations that light has a remarkable influence on muscle physiology in Atlantic cod. However, all the clock genes analyzed except *clock* lost their rhythmicity under *in vitro* and *ex vivo* conditions. Taken

together, our data indicate that the peripheral clock in fast skeletal muscle of Atlantic cod is not likely to be autonomous and probably depends on signals generated by the transcriptional-translational feedback loop of the central clock.

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Figure captions

Figure 1. Myosatellite cells of Atlantic cod in culture at A) 1 day post-isolation (dpi), B) 3 dpi, C) 7 dpi, D) 14 dpi and E) 30 dpi. A typical spindle-shape morphology was conspicuously observed at 3 dpi. Atlantic cod myosatellite cells were able to proliferate and differentiate into myotubes under *in vitro* conditions. Scale bar corresponds to 100 µm. Insets show magnified photos of cells stained with May-Grunwald and observed by phase contrast microscopy. The scale bar represents 50 µm.

Figure 2. Characterization of myosatellite cells in culture. Cellular indices of **A)** proliferation, **B)** differentiation and **C)** apoptotis during *in vitro* culture of myosatellite cells**.** Values are expressed as mean ± SEM from two independent experiments (n = 6). Significant temporal differences are indicated by different letters.

Figure 3. Expression profile of key myogenic marker genes in Atlantic cod myosatellite cells in culture. Transcript levels of **A)** *myod1*, **B)** *myog*, **C)** *pcna* and **D)** *des* were quantified by qPCR in myosatellite cell cultures. The values are presented as mean ± SEM from two independent experiments (n = 6). Significant differences are indicated by different letters.

Figure 4. Photoperiod manipulation of *arntl2* gene expression in myosatellite cell cultures. qPCR analysis of *arntl2* expression during a daily cycle under different photoperiod regimes (*LD*, *LL* and *DD*)**.** The broken line is the periodic sinusoidal function of transcript levels in one daily cycle, obtained from the COSINOR parameters shown in Supplementary Table S2. Values are expressed as mean ± SEM from two independent experiments (n = 6). Significant

differences between photoperiod treatments at a given time of a daily cycle are indicated by different letters.

Figure 5. Expression of some key myogenic markers in Atlantic cod fast skeletal muscle explant in culture. Transcript levels of **A)** *myod1*, **B)** *myog*, **C)** *pcna* and **D)** *des* were quantified by qPCR during the short-term culture of fast skeletal muscle explants. Values are expressed as mean ± SEM from two independent experiments (n = 6). Significant temporal differences are indicated by different letters.

Figure 6. Expression of clock genes in a 5-day old muscle explant of Atlantic cod under different photoperiod regimes. Transcript levels of **A)** *clock*, **B)** *cry2*, **C)** *per2a* are presented as mean ± SEM from two independent experiments (n = 6). The expression profile of *arntl2* and *nr1d1* is provided in Supplementary Figure S1. The broken line represents the periodic sinusoidal function of the transcript levels throughout a daily cycle, based on the COSINOR rhythmicity parameters shown in Table 1. Significant differences between photoperiod treatments at a given time are indicated by different letters.

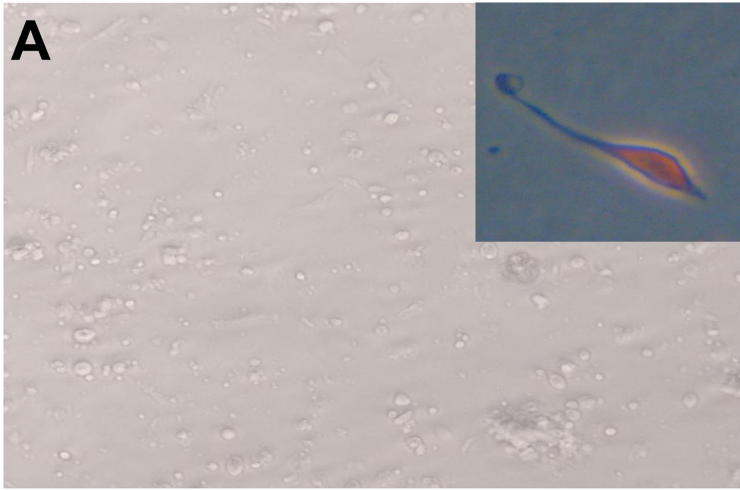
Table 1. Rhythmicity parameters of the expression of clock genes determined by COSINOR in fast skeletal muscle explants of Atlantic cod upon exposure to different photoperiod conditions [1].

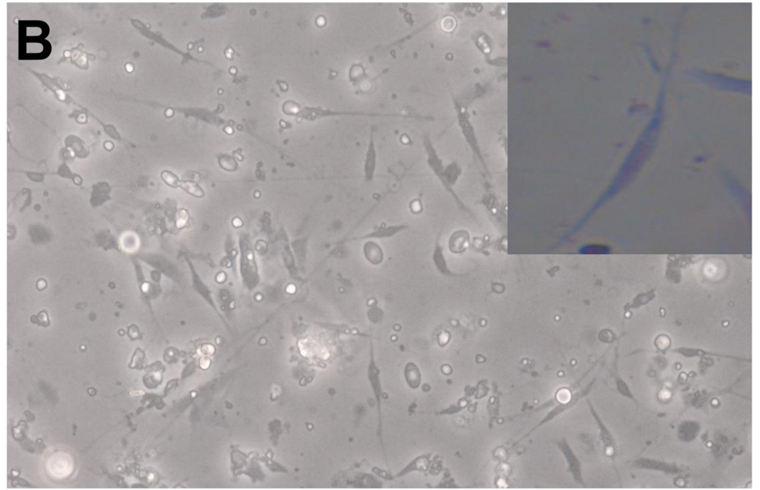
Gene [2] and treatment	Mesor	Amplitude	Peak of expression/ Acrophase (h)	*p* [3]
arntl2-LD	0.48	0.15	12.4	0.31
arntl2-LL	0.41	0.07	14.2	0.63
arntl2-DD	0.40	0.07	14.1	0.63
***clock*-LD**	0.46	0.14	10.5	0.12
***clock*-LL**	0.40	0.13	10.5	0.13
***clock*-DD**	0.49	0.10	9.25	0.10
cry2-LD	0.53	0.07	22.0	0.41
cry2-LL	0.37	0.04	23.1	0.89
cry2-DD	0.54	0.03	23.4	0.85
per2a-LD	0.45	0.16	18.2	0.33
per2a-LL	0.52	0.10	15.4	0.50
per2a-DD	0.55	0.10	15.6	0.50
nr1d1-LD	0.46	0.03	19.3	0.86
nr1d1-LL	0.48	0.05	23.3	0.44
nr1d1-DD	0.39	0.01	19.3	0.93

[1] *Photoperiod conditions: equal length of light and dark (LD; 12L:12D; gray shading), continuous light (LL; 24L:0D) and constant darkness (DD; 0L:24D; black shading).*

[2] *Genes highlighted in bold displayed significant daily rhythmicity.*

[3] The *p* value is defined as the noise/signal ratio of the oscillation amplitude obtained from the COSINOR analysis. $p < 0.3$ indicates daily rhythmicity.


A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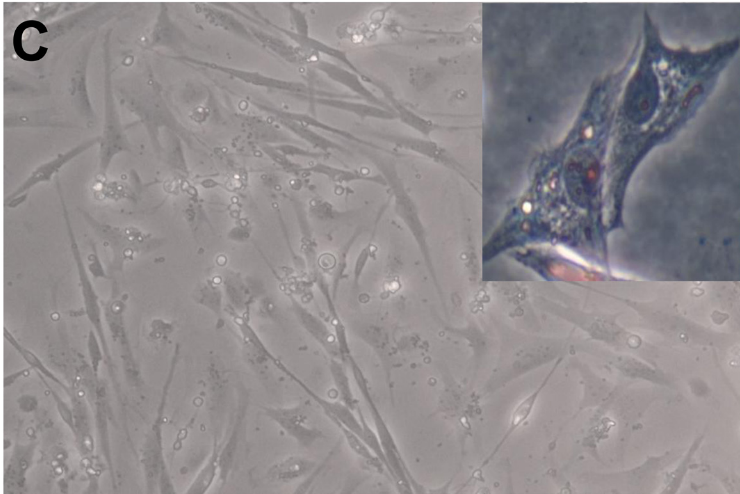
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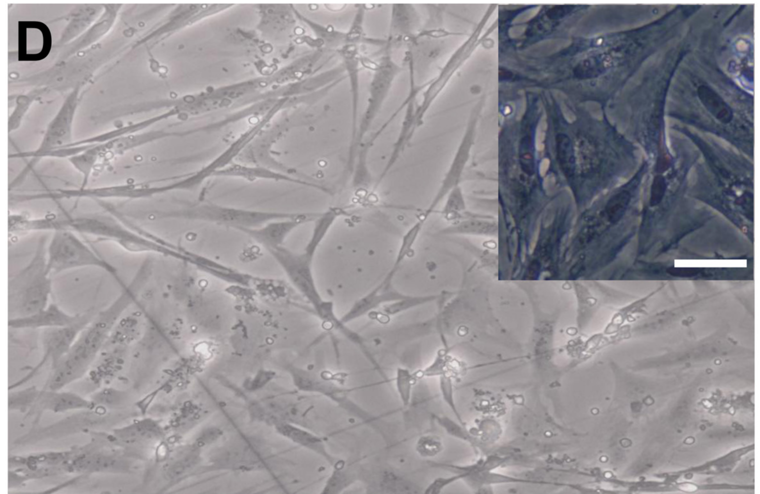
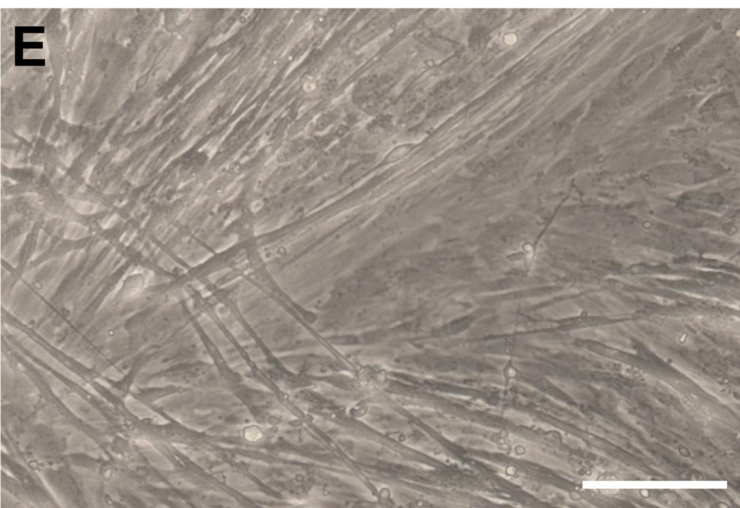
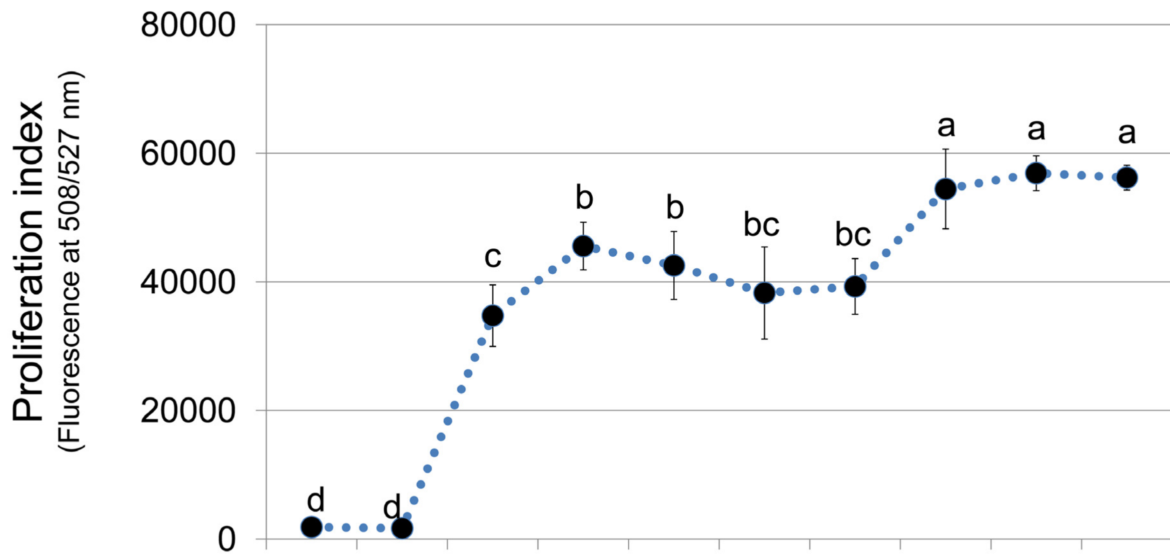
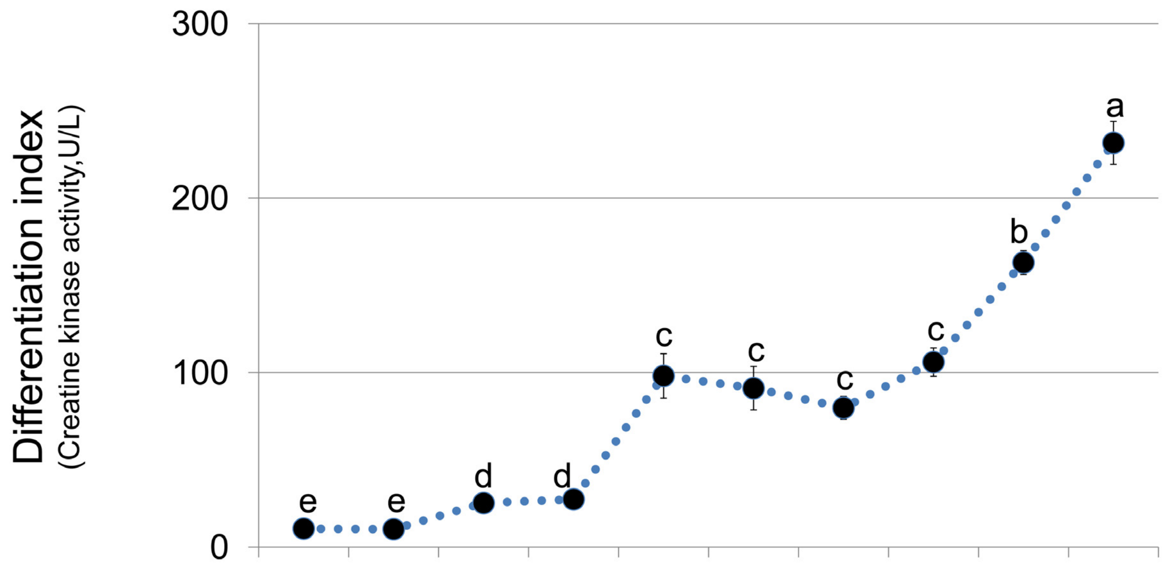
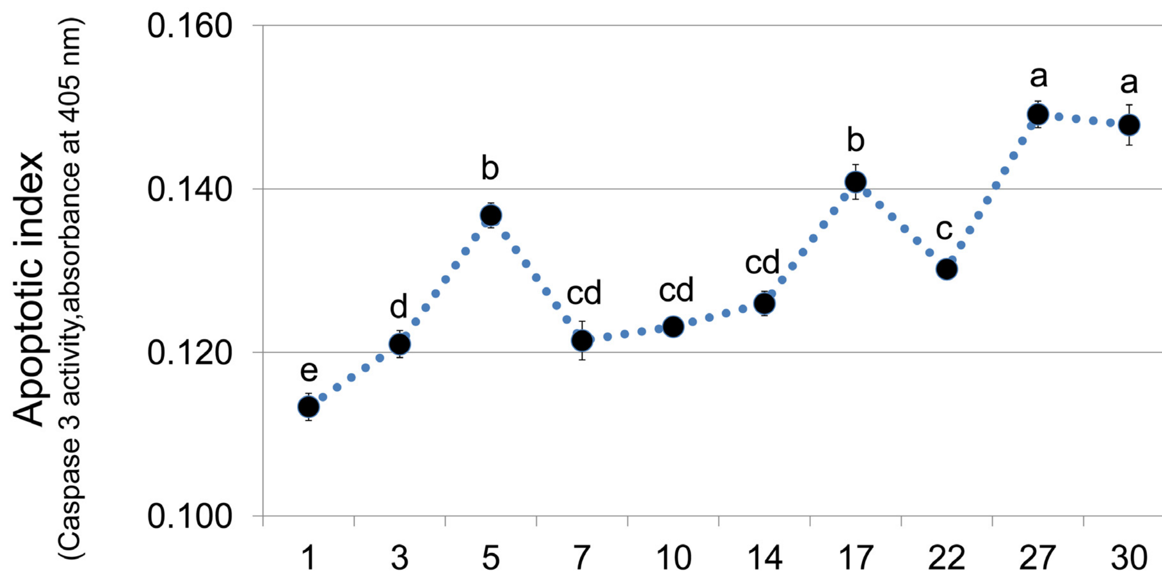
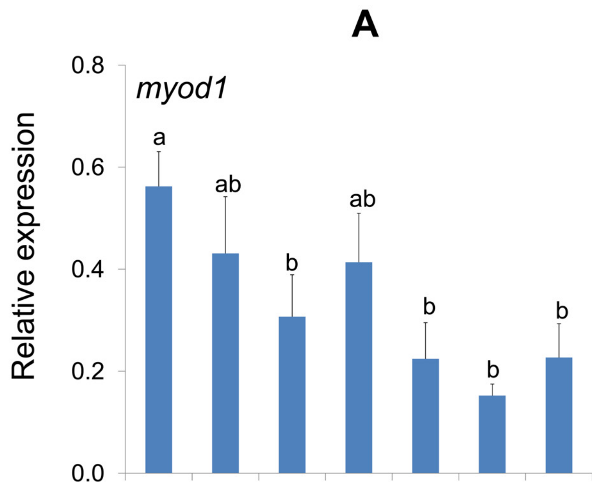
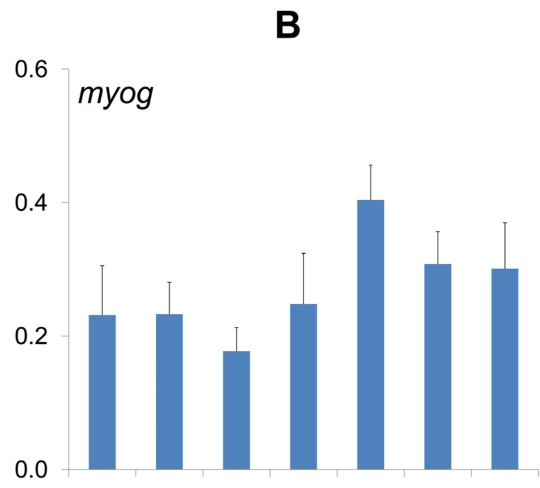
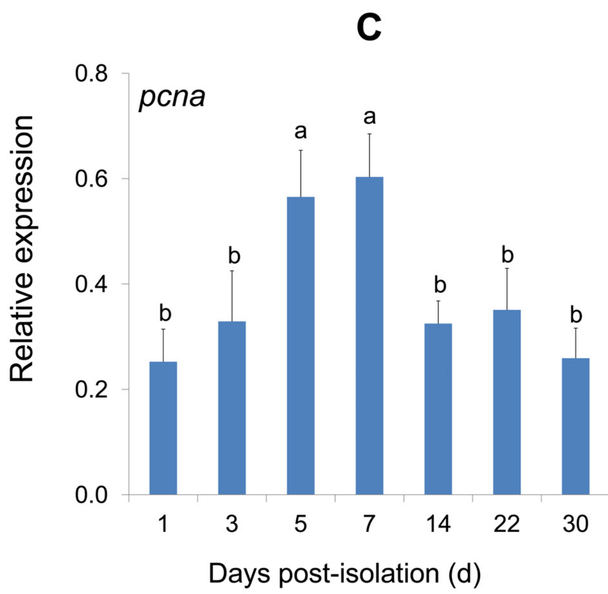
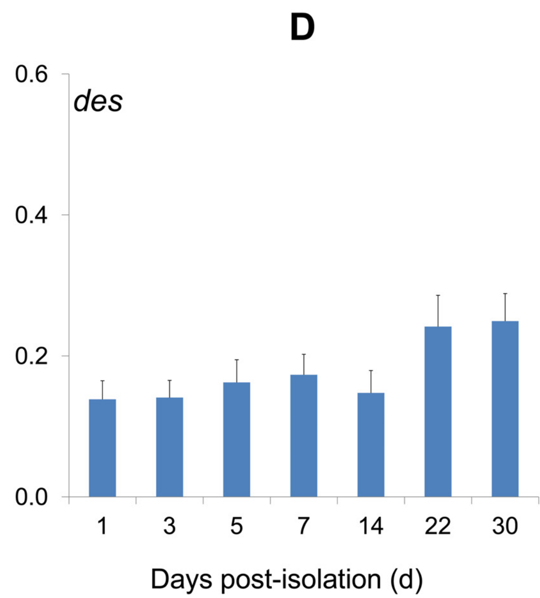

D

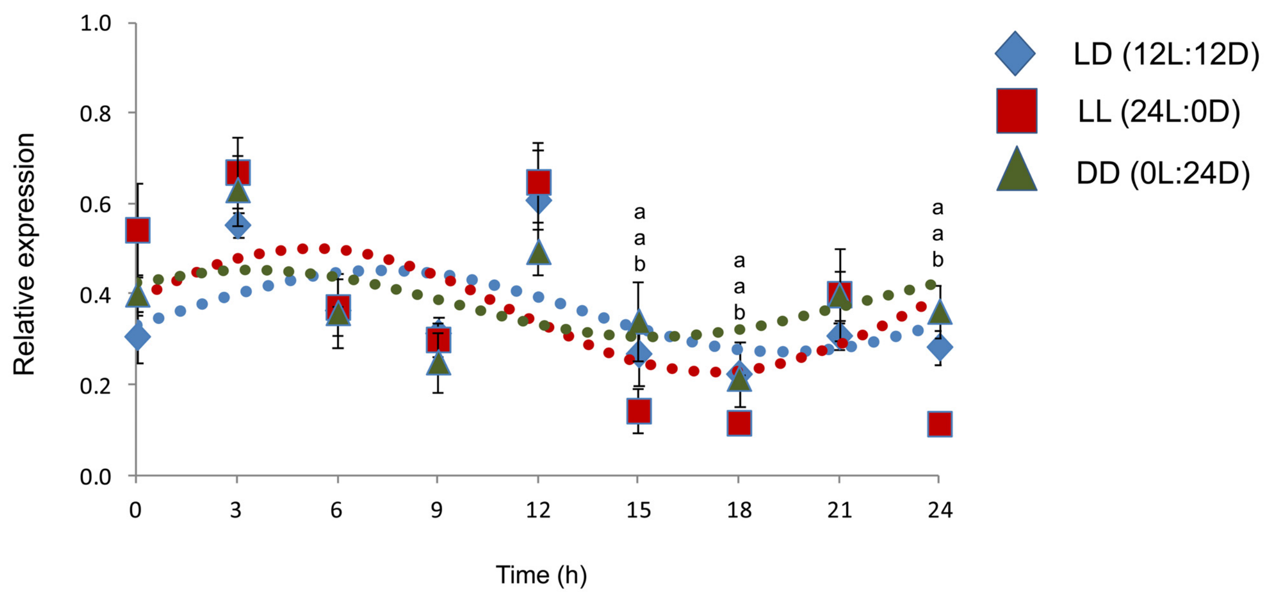

E


A
Proliferation index
(Fluorescence at 508/527 nm)
80000
60000
40000
20000
0
d
d
c
b
b
bc
bc
a
a
a

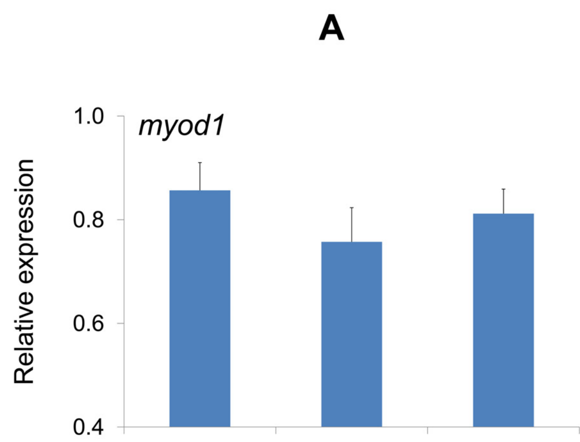

B
Differentiation index
(Creatine kinase activity,U/L)
300
200
100
0
e
e
d
d
c
c
c
c
b
a

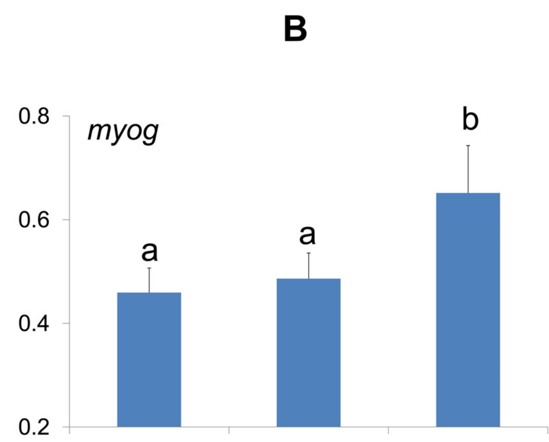

C
Apoptotic index
(Caspase 3 activity,absorbance at 405 nm)
0.160
0.140
0.120
0.100
e
d
b
cd
cd
cd
b
c
a
a
1
3
5
7
10
14
17
22
27
30
Days post-isolation (d)

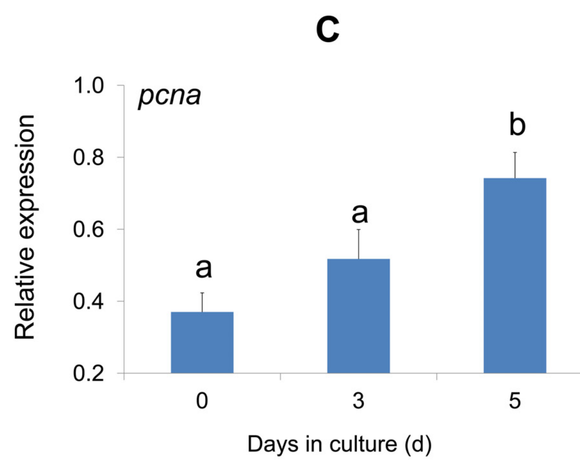

A
myod1
Relative expression
0.8
0.6
0.4
0.2
0.0
a
ab
b
ab
b
b
b

B
myog
0.6
0.4
0.2
0.0

C
pcna
Relative expression
0.8
0.6
0.4
0.2
0.0
b
b
a
a
b
b
b
1
3
5
7
14
22
30
Days post-isolation (d)

D
des
0.6
0.4
0.2
0.0
1
3
5
7
14
22
30
Days post-isolation (d)

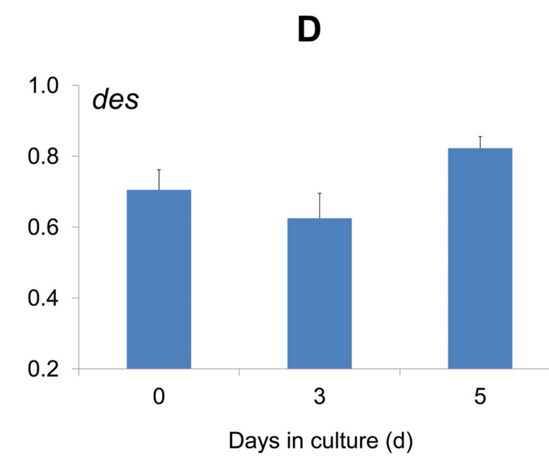


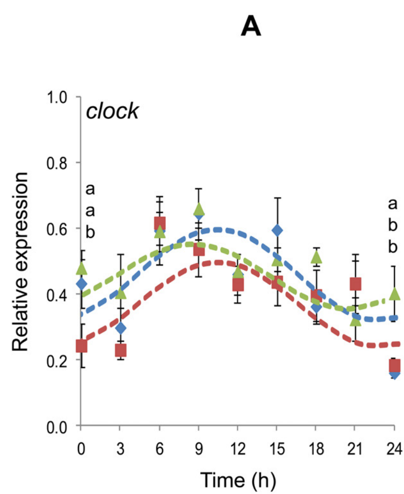
1.0
0.8
0.6
0.4
0.2
0.0
Relative expression
0
3
6
9
12
15
18
21
24
Time (h)
a
a
b
a
a
b
a
a
b
LD (12L:12D)
LL (24L:0D)
DD (0L:24D)

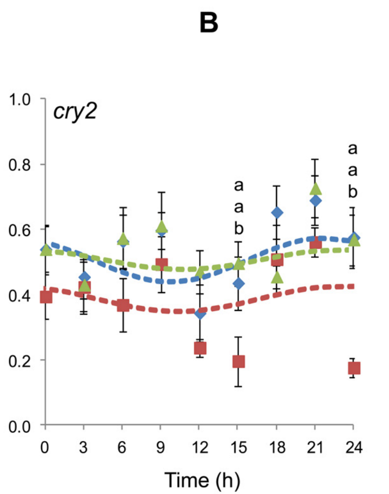

A
myod1
Relative expression
1.0
0.8
0.6
0.4

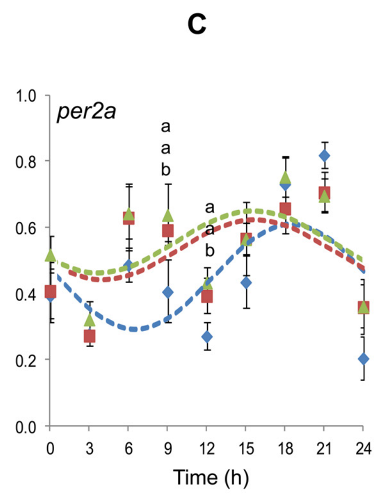

B
myog
0.8
0.6
0.4
0.2
a
a
b


C
pcna
Relative expression
1.0
0.8
0.6
0.4
0.2
a
a
b
0
3
5
Days in culture (d)


D
des
1.0
0.8
0.6
0.4
0.2
0
3
5
Days in culture (d)







LD (12L:12D) LL (24L:0D) DD (0L:24D)

Supplementary Table S1. Primers used in this study.

Gene name	Gene ID	Primer sequence (5’ – 3’)	Annealing temperature (^{o}C)	Amplification efficiency (E)	Amplicon size (bp)	References
arntl2	JX03864	F: TCTCAAAGGTTCCTCTTCTTCGT R: CACAAACAGAAAGCCATCGG	64	94.5	103	Lazado et al. (2014)
clock	JN643707	F: CTGGAGCACCTACCGGCGGA R: GCGGCGTGCGTCGACAGAGC	60	86.9	101	Lazado et al. (2014)
cry2	JN643709	F: GGCTGGATCTGCCCCGACG R: CCCAGCTCTTCCAGTGAGGG	60	86.1	121	Lazado et al. (2014)
per2a	JN643711	F: GCTCCCATGATGTTCAACGCT R: CCTCGATGATCTCAGATTCGG	64	81.9	186	Lazado et al. (2014)
nr1d1	KC204822	F: GACTTCTCCCTCTCCTTCAG R: CACCATTAGCACCTCAAAGG	60	107.6	135	Lazado et al. (2014)
myoD1	AF329903	F: GGCATGATGGATTTCAACGG R: CTCGAGAGGCACTCCAGGCT	60	78.7	108	Lazado et al. (2014)
myoG	JQ582407	F: AACTTCGACCGCATGCTGG R: GGTGGTGGATCCAGCCCTC	60	90.6	121	Nagasawa et al. (2012)
pcna	KC204826	F: CCTCAGCAGTATGTCAAAGATCC R: GATGGTTTCAAACACAAGAACGAG	60	112	105	This study
des	ENSGMOG00000001871	F: AGGACATCGCCAAGATGAAG R: GCATGTTGGTGGTGATTCTG	60	82.1	148	This study
arp	EX741373	F:TGATCCTCCACGACGATGAG R:CAGGGCCTTGGCGAAGA	60	93.4	113	Olsvik et al. (2008)
eef1a	EX721840	F: CACTGCGGTGAAGTCCGTTG R: GGGGTCGTTCTTGCTGTCT	60	84.4	79	Lilleeng et al. (2007)
ubi	EX735613	F: GGCCGCAAAGATGCAGAT R: CTGGGCTCGACCTCAAGAGT	60	86.9	69	Olsvik et al. (2008)

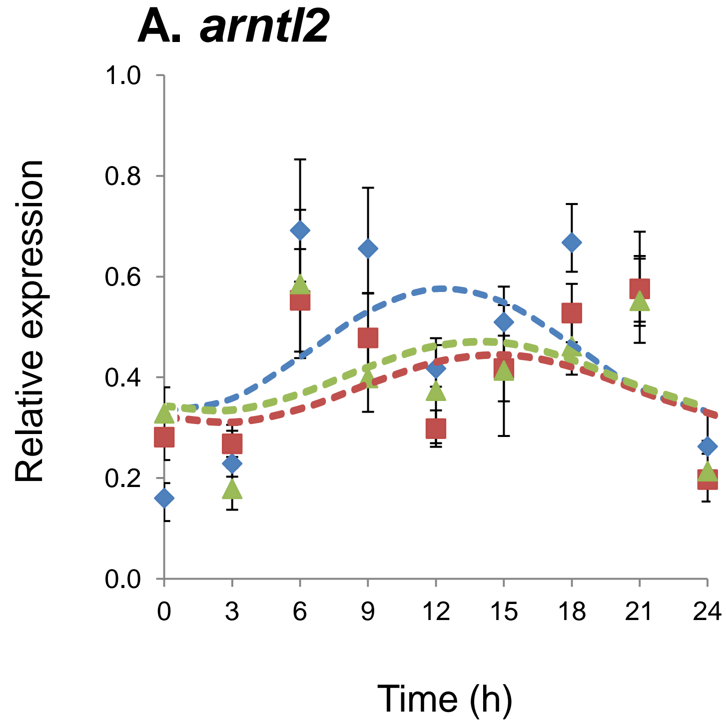
Lazado CC, Kumaratunga HPS, Nagasawa K, Babiak I, Giannetto A, Fernandes JMO (2014) Daily rhythmicity of clock gene transcripts in Atlantic cod fast skeletal muscle. PLoS ONE. http://dx.doi.org/10.1371/journal.pone.0099172
Olsvik PA, Søfteland L, Lie KK (2008) Selection of reference genes for qRT-PCR examination of wild populations of Atlantic cod *Gadus morhua*. BMC Research Notes 1.
Lilleeng E, Frøystad MK, Vekterud K, Valen EC, Krogdahl Å (2007) Comparison of intestinal gene expression in Atlantic cod (*Gadus morhua*) fed standard fish meal or soybean meal by means of suppression subtractive hybridization and real-time PCR. Aquaculture 267: 269-283.
Nagasawa K, Giannetto A, Fernandes JMO (2012) Photoperiod influences growth and MLL (mixed-lineage leukaemia) expression in Atlantic cod. PLoS ONE 7: e36908.

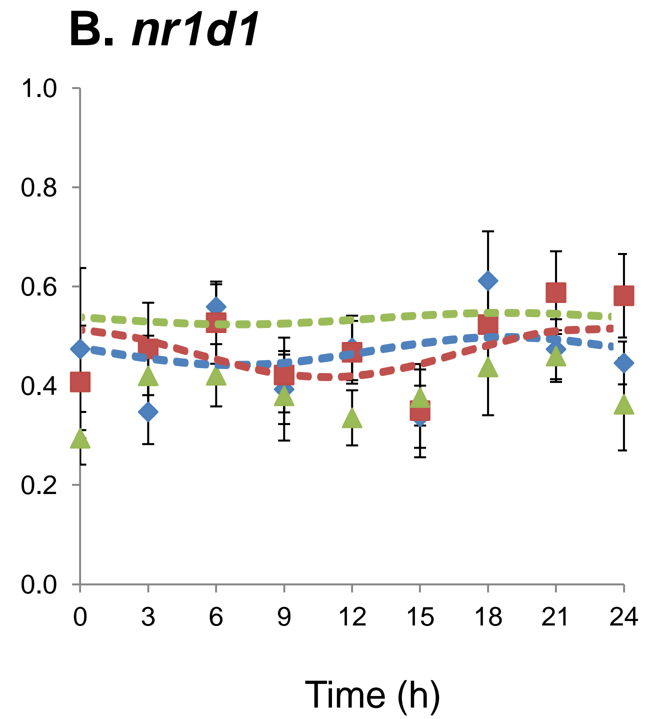
Supplementary Table S2. Rhythmicity parameters of *arntl2* expression in myosatellite cells of Atlantic cod upon exposure to different photoperiod regimes determined by COSINOR.

Treatment	Mesor	Amplitude	Peak of expression (h)	*P*
arntl2-LD	0.36	0.09	7.34	0.42
arntl2-LL	0.38	0.15	5.32	0.51
arntl2-DD	0.41	0.08	3.43	0.54

Note: Photoperiod regimes: equal length of light and dark (LD; 12L:12D), continuous light (LL; 24L:0D) and continuous darkness (DD; 0L:24D).

Supplementary Figure S1





LD (12L:12D) LL (24L:0D) DD (0L:24D)