Circadian rhythmicity and photic plasticity of myosin gene transcription in fast skeletal muscle of Atlantic cod (*Gadus morhua*)

Carlo C. Lazado, Kazue Nagasawa[§], Igor Babiak, Hiruni P.S. Kumaratunga[¤] and Jorge M.O. Fernandes*

Faculty of Biosciences and Aquaculture, University of Nordland, 8049 Bodø, Norway

Running title: Photic plasticity of cod myosins

*Corresponding author:
J.M.O. Fernandes, Ph.D.
E-mail: jorge.fernandes@uin.no
Tel.: +47 75517736
Fax: +47 75517457

Present Address:
[§]Laboratory of Aquacultural Biology, Graduate School of Agricultural Science, Tohoku University, 1-1 Tsutsumidori-amamiyamachi, Sendai, 981-8555 Japan
[¤]Department of Zoology, Faculty of Applied Sciences, University of Sri Jayewardenepura, Nugegoda, Sri Lanka

Abstract

The circadian rhythm is a fundamental adaptive mechanism to the daily environmental changes experienced by many organisms, including fish. Myosins constitute a large family of contractile proteins that are essential functional components of skeletal muscle. They are known to display thermal plasticity but the influence of light on myosin expression remains to be investigated in fish. In the present study, we have examined the circadian rhythmicity and photoperiodic plasticity of myosin gene transcription in Atlantic cod (*Gadus morhua*) fast skeletal muscle. *In silico* mining of the Atlantic cod genome resulted in the identification of 76 myosins representing different classes, many of which were hitherto uncharacterized. Among the 23 skeletal muscle-expressed myosin genes, *myh_tc*, *myh_n1*, *myh_n4*, *myo18a_2*, and *myo18b_2* displayed circadian rhythmicity and contained several circadian-related transcription factor binding sites (Creb, Mef2 and E-box motifs) within their putative promoter regions. Also, the circadian expression of these 5 *myosins* strongly correlated with the transcription pattern of clock genes in fast skeletal muscle. Under *ex vivo* conditions, *myosin* transcript levels lost their circadian rhythmicity. Nonetheless, different photoperiod regimes influenced the mRNA levels of *myh_n4*, *myo18a_2* and *myo18b_2* in fast skeletal muscle explants. Photoperiod manipulation in Atlantic cod juveniles revealed that continuous light significantly elevated mRNA levels of several myosins in fast skeletal muscle when compared to natural photoperiod. The circadian rhythmicity observed in some fast skeletal muscle *myosin* genes suggests that they may be under circadian clock regulation. In addition, the influence of photoperiod on their expression implies that *myosins* may be involved in the photic plasticity of muscle growth observed in Atlantic cod.

Keywords: Atlantic cod, circadian rhythm, environmental plasticity, epigenetics, myosin, skeletal muscle, photoperiod

1. Introduction

Most organisms adjust their behavior and physiology to the daily (circadian) cycle of day and night. This circadian rhythm is controlled by a complex molecular clock machinery that is highly conserved in the animal kingdom (Vatine et al., 2011, Katherine Tamai et al., 2003). The core system of the molecular clock is composed of interlocked auto-regulatory transcriptional-translational feedback loops that are regulated by clock genes and their proteins (Cahill, 2002, Dardente and Cermakian, 2007). In fish, the central clock is believed to be located in the pineal gland or retina (Falcón, 1999). Besides these organs, several tissues also express clock genes in a circadian rhythmic manner, thus indicating that there may be multiple peripheral oscillators (Whitmore et al., 1998, Whitmore et al., 2000, Tamai et al., 2005).

It is believed that the components of the clock system do not only regulate the core members of the transcriptional-translational loop but they also are regulators of other genes (McCarthy et al., 2007). The genes that are under the clock coordination are termed clock-controlled genes and they are responsible for integrating the clock mechanism and physiological pathways, eventually orchestrating biological processes in a circadian fashion (McCarthy et al., 2007, Amaral and Johnston, 2012). There is a paucity regarding the extent to what clock mechanisms regulate the transcriptional network in fast skeletal muscle in fish. Nevertheless, biological clocks are thought to play a key role in mammalian muscle physiology. For instance, *myoD*, a member of myogenic regulatory factors family, is believed to be under clock control. In mouse fast muscle, *MyoD* is expressed in a circadian manner and the absence of a functional clock mechanism disrupts the rhythmicity of gene expression, as well as both *Peroxisome proliferator activated receptor* γ *coactivator 1* α (*Pgc-1* α) and *Pgc-1* β, leading to structural and functional alterations at the cellular level in this tissue (Andrews et al., 2010). Further, the core enhancer (CE) in the promoter region of *MyoD* is necessary for its circadian expression, and the core clock genes, Circadian locomotor output cycles kaput (CLOCK) and

Brain and muscle Arnt-like protein-1 (BMAL1) bind to a conserved non-canonical E-box within the CE (Zhang et al., 2011). Moreover, in a transcriptome-wide study in mouse skeletal muscle, it was discovered that a total of 215 transcripts displayed a circadian expression pattern (McCarthy et al., 2007).

Myosin is a large group of structurally and functionally diverse superfamily of actin-based molecular motors that consists of more than 35 distinct classes (Odronitz and Kollmar, 2007). Myosin heavy chain genes are highly conserved throughout evolution (Ikeda et al., 2007) and they are expressed in a complex pattern during muscle fiber development (Ennion et al., 1999). In cultured smooth muscle cells, phosphorylation of myosin light chain displayed circadian rhythmicity, which could be abolished by pharmacological inhibition and knockdown of Rho-associated kinase 2 in mouse (Saito et al., 2013). Two other myosin genes, *Myh1* and *Myh10*, are expressed in a circadian pattern in adult mouse skeletal muscle (McCarthy et al., 2007).

The above studies on the importance of circadian rhythmicity for mammalian myosins imply that their counterparts in fish may also be under control of circadian clocks. Thus, the goal of the current study was to characterize the circadian rhythmicity of *myosin* gene expression in fast skeletal muscle of a teleost. Moreover, fish *myosins* are known to display thermal plasticity (Tao et al., 2004, Cole and Johnston, 2001, Watabe, 2002) but the influence of light in muscle growth plasticity remains to be determined. Atlantic cod is a particularly interesting species to study this phenomenon because somatic growth of juvenile fish is significantly affected by photoperiod manipulation, concomitantly with changes in expression of genes involved in epigenetic regulation, namely *mixed-lineage, leukemia* and *DNA (cytosine-5)-methyltransferases* (Nagasawa et al., 2012, Giannetto et al., 2013). To further explore the molecular mechanisms underlying the photic plasticity of muscle growth in Atlantic cod, the

105 present study also investigated the expression of multiple fast skeletal muscle *myosins* in
106 juvenile fish reared under different photoperiod regimes.
107
108
109 **2. Materials and Methods**
110 ***2.1.Ethics statement***
111 All experiments in this study concerning handling of live fish complied with the
112 guidelines set by the National Animal Research Authority (Forsøksdyrutvalget, Norway) and
113 were approved by the ethics committee of the Faculty of Biosciences and Aquaculture,
114 University of Nordland (UiN), Norway.
115
116 ***2.2. In silico mining of Atlantic cod myosins***
117 Ion Torrent™ PGM Sequencing of Atlantic cod fast skeletal muscle transcriptome
118 identified 11 *myosins* that were differentially expressed during a circadian cycle (Lazado,
119 Nagasawa, Kollias, Babiak, Johnston and Fernandes, unpublished). *In silico* mining was
120 performed to identify annotated and unannotated *myosins* in the Atlantic cod genome assembly
121 (www.ensembl.org/gadMor1; Accessed April 2013). Unannotated genes described as "novel"
122 were identified by BLAST similarity searches at the National Centre for Biotechnology
123 Information server (www.ncbi.nlm.nih.gov). Further, *myosins* from the tiger pufferfish
124 (*Takifugu rubripes*) genome (www.ensembl.org/Fugu4) were used to identify several other
125 unannotated *myosins* in Atlantic cod. To limit this study to skeletal muscle *myosins*, genes were
126 selected either by i) their presence in the Atlantic cod fast skeletal muscle transcriptome
127 (Lazado, Nagasawa, Kollias, Babiak, Johnston and Fernandes, unpublished) or ii) based on their
128 putative involvement in muscle physiology, as reported in the literature. Only these fast skeletal
129 muscle *myosin* genes were further characterized.

2.3.Circadian rhythm experiment

Juvenile Atlantic cod weighing 100.0 ± 6.0 g (mean ± standard deviation [SD]) were stocked in 250 m^3 painted fiber glass tanks at Mørkvedbukta Research Station of the University of Nordland. Sixty individuals were kept in each tank and a total of 9 tanks were used in the experiment; one for each sampling time point, in order to minimize stressing the fish throughout the experiment. Illumination was provided by fluorescent white light bulbs (Aura Light International AB, Karlskrona, Sweden) connected to an automated system that was programmed to provide a daily photoperiod regime of 12L:12D. A commercially available diet (Amber Neptun, Skretting AS, Stavanger, Norway) was delivered through automated belt feeders at a daily ration of 5 % (w/w) of the fish body weight. Water was supplied from 200 m depth of Saltenfjorden, and water temperature and dissolved oxygen were maintained at an average of 7 ^{o}C and 89 %, respectively. Fish were acclimated to these conditions for at least 3 weeks before sample collection during a circadian cycle.

Fish were sampled every 3 hours for a period of 24 h (Zeitgeber time: ZT0, 3, 6, 9, 12, 15, 18, 21 and 24). There was an approximate 30 min transition time (ZT12) between the presumptive day (ZT0-9) and presumptive night (ZT15-24). Ten fish were taken from each tank and immediately immersed in seawater containing 0.2 $g \cdot L^{-1}$ tricaine methanesulfonate (MS222; Sigma, Oslo, Norway). Sampling during the presumptive night was conducted in a room with minimal illumination (light intensity did not exceed 0.001 Klux) within 5 min. Fast skeletal muscle was excised from the area below the second dorsal fin. After removing the skin, fast muscle was washed with cold sterile 1 × phosphate buffered saline (PBS; Sigma, Steinheim, Germany) and immediately snap-frozen in liquid nitrogen. Samples were stored at – 80 ^{o}C until RNA extraction.

2.4.Photoperiod manipulation experiment

The photoperiod experiment is described in detail in two sister papers (Giannetto et al., 2013, Nagasawa et al., 2012). Briefly, two groups of 6-month-old juvenile Atlantic cod with an initial approximate mass of 2.7 ± 0.8 g were reared for six months under different photoperiod regimes at Mørkvedbukta Research Station. One experimental group was kept under continuous illumination (*LL*) while the other group was reared under the simulated natural photoperiod (*NL*) for Bodø, Norway (67°N, 14°E). The experiment was performed from January until July 2010. Day length continuously increased during this period and it was 6.3, 6.3, 7.1, 17 and 22 h at 0, 1, 7, 60 and 120 days, respectively (Giannetto et al., 2013). The initial stocking density was 130 fish per tank and 3 tanks per experimental group. During sampling, at least nine fish were taken from each experimental group and were humanely killed by immersion in seawater containing MS222, as above. Samples were collected at the start of the experiment (0) and after 1, 7, 60 and 120 days. Fast skeletal muscle samples were collected and stored as above.

2.5.Circadian and photoperiod regulation in fast skeletal muscle explants

Fast skeletal muscle explants were prepared essentially as described by Funkenstein et al. (2006), with some modifications. Juvenile cod were obtained from Mørkvedbukta Research Station. The fish were reared under constant illumination (*LL*) with the above *ad libitum* feeding regime and under the same water conditions detailed in section 2.3. Tissue samples from the dorsolateral region of anesthetized ~150 g Atlantic cod were divided into smaller fragments (approximately 4 mm × 4 mm area and 1-2 mm height), blotted on a sterile tissue paper to remove excess media and pressed firmly on a laminin-coated multiwell plate (BD Falcon™, New Jersey, USA). After 45 min, Dulbecco's Modified Eagle's Medium supplemented with 9 mM NaHCO3, 20 mM HEPES, 15 % 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) was carefully added. The explants

were cultured at 15 °C and the medium was replenished daily. For 5 days, explants were cultured under 3 different photoperiod regimes: i) constant illumination (*LL*; 24L:0D), ii) constant darkness (*DD*; 0L:24D) and iii) equal length of day and night (*LD*; 12L:12D). After 5 days, samples were collected every 3 h for a period of 24 h, immediately immersed in liquid nitrogen and stored at – 80 °C until RNA extraction.

2.6. RNA extraction, cDNA synthesis and primer design

Total RNA was extracted from the samples using the *mir*Vana™ miRNA Isolation kit (Ambion, Oslo, Norway). After quantification by spectrophotometry using a Nanodrop® ND-1000 (Thermoscientific, CO, USA), RNA quality was assessed by denaturing electrophoresis on a 1.2 % (w/v) agarose gel. The quality of RNA samples was further assessed with an Agilent 2100 Bioanalyzer™ using the Eukaryote Total RNA Pico Series II kit (Agilent Technology Inc., CA, USA). Only samples with an RNA Integrity (RIN) value above 9 were used. cDNA was synthesized from a 1 µg/mL total RNA by QuantiTect Reverse Transcription kit (Qiagen, Nydalen, Sweden).

Specific primers for skeletal muscle *myosins* were designed with PerlPrimer (www.perlprimer.sourceforge.net). To avoid amplification of contaminating genomic DNA, primers were designed to cross intron/exon borders. Three candidate reference genes were used to normalize the expression of *myosin* genes: *acidic ribosomal protein* (*arp*), *ubiquitin* (*ubi*) and *elongation factor 1-alpha 1 (eef1a)* (Nagasawa et al., 2012, Nagasawa et al., 2011). Primer sequences and thermocycling conditions are provided in Supplementary Table S1. Primers for some *myosin* genes were not successfully designed.

2.7.Quantitative real-time PCR (qPCR)

Transcript levels of fast skeletal muscle *myosins* in the muscle samples was quantified by real-time PCR (qPCR) on a LightCycler® (Roche, Basel, Switzerland) with SYBR Green I chemistry (Roche) The qPCR reaction using a diluted sample was performed following this thermocycling protocol: initial denaturation at 95 ºC for 15 min, followed by 45 cycles of 15 s at 94 ºC, 20 s defined annealing temperature per primer set (Supplementary Table S1) and 20 s at 72 ºC. Five-point standard curves of 2-fold dilution series were prepared from a pooled cDNA in order to calculate amplification efficiencies, as detailed elsewhere. All reactions were run in duplicate including minus reverse transcriptase and no template controls. The cycle threshold (C_T) values were generated from the built-in LightCycler® software and fluorescence arbitrary value was set to 0.8. The geometric averages of *arp* and *ubi* obtained from GeNorm (http://medgen.ugent.be/~jvdesomp/genorm/) were used to calculate the relative expression of each gene.

2.8. In silico identification of transcription factor binding sites

The 5 kb genomic regions upstream of *myosin* genes displaying circadian rhythmicity were analyzed *in silico* for the presence of circadian-related transcription factor binding sites (*circadian*TFBS). The upstream sequences of five circadian rhythmic *myosins* were retrieved from Ensembl and *circadian*TFBS were analyzed using rVista 2.0 (http://rvista.dcode.org/) with matrix similarity set at 0.90. The search for *circadian*TFBS was focused on MEF2, CREB and E-BOX motif, which were previously identified as key regulators of circadian-related transcription of several genes (Bozek et al., 2009, Zhang et al., 2012). The upstream region of *myh_n4* was not included in the characterization as the available sequence in Ensembl comprised only of N repeats.

2.9. Data analysis

Differences in the transcript levels of *myosins* during a circadian cycle were analyzed 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 parametric 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 followed by SNK post-hoc test was used instead. The same approach was also used to determine differences in the expression of *myosin* genes at a specific time point in fast skeletal muscle explants exposed to different illumination conditions. For the photoperiod experiment in juvenile Atlantic cod, differences in mRNA levels of *myosins* in relation to light treatment were determined by two-way ANOVA followed by SNK post-hoc test. The level of significance was set at $P < 0.05$.

To evaluate circadian rhythmicity of *myosin* transcripts, a COSINOR analysis was performed by fitting a periodic sinusoidal function to normalized transcript levels across the nine time points, using the formula: $f(t) = M + A\cos(t/pi/12 - \phi)$, 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. Transcript levels were considered to display a circadian rhythm if $P < 0.3$ (Velarde et al., 2009).

Correlation analyses (n = 6) were conducted to determine the relationship between mRNA levels of *myosins* with circadian rhythmicity and transcription patterns of clock genes that were earlier shown to be rhythmically expressed in fast skeletal muscle of Atlantic cod (Lazado, Kumaratunga, Nagasawa, Babiak, Giannetto and Fernandes, unpublished). Statistical

dependence was measured by Pearson's correlation (r) or Spearman rank order correlation (ρ) for parametric and non-parametric data sets, respectively.

3. Results and Discussion

3.1.Multiple skeletal muscle myosins in Atlantic cod

In silico mining of *myosins* led to the identification of 76 *myosin* genes from different families. A list of myosins found in the Atlantic cod genome is given in Supplementary Tables S2a and S2b. Myosins I and II are the most abundant members of the myosin family present in nearly all eukaryotic cells (Lodish et al., 2000) and constitute approximately 51 % of the Atlantic cod *myosin* genes identified in the present study. From this large repertoire of *myosin* genes, 51 were annotated and identified in the Atlantic cod genome assembly. Remarkably, there were 25 *myosins* that were unannotated and were categorized as "novel" *myosins*. The identity of these novel *myosins* is given in Supplementary Table S2b. Approximately 48 % of these "novel" *myosins* are from the Myosin I and II families. The presence of several myosin paralogs in Atlantic cod, such as *myo1*, *myo10*, *myo15 and myo18*, could be explained by tandem duplications, as well by the whole-genome duplication event that occurred in ray-finned fishes (Panopoulou and Poustka, 2005).

From this diverse group of myosins, there were a total of 23 putative fast skeletal muscle *myosin* genes identified (Table 1), 11 of which had been identified in a sister study of the Atlantic cod fast skeletal muscle transcriptome (Supplementary Tables S2a and S2b; Lazado, Nagasawa, Kollias, Babiak, Johnston and Fernandes, unpublished). Fourteen putative skeletal muscle *myosins* could be annotated in the cod genome assembly and were represented by several paralogs, namely *myosin 3* (*myo3*), *myo15*, *myo18*, *myosin heavy chain 11* (*myh11*) and *myosin heavy chain phosphorylatable* (*mylpf*). All novel *myosins* analyzed in this study were

277 from the *myh* type. Although large and complex, each isoform of vertebrate sarcomeric *myosins,*
278 particularly from the myosin heavy chain group, is encoded by a separate gene (Ennion et al.,
279 1999). Only 17 of the 23 skeletal muscle *myosins* were subjected to further characterization
280 because the design of primers for *myo3*, *myo15*, *myh11_2* and *myh_n8* was unsuccessful.
281

282 ***3.2.Circadian rhythmic expression of several fast skeletal muscle myosins***

283 The expression of five *myosin* genes in fast skeletal muscle was found to have circadian
284 rhythmicity under a 12L:12D photoperiod regime (Fig. 1). Circadian rhythmic expression was
285 demonstrated by *myh_tc* (P = 0.04), *myh_n1* (P = 0.01), *myh_n4* (P = 0.10), *myo18a_2*
286 (P = 0.12) and *myo18b_2* (P = 0.06). The circadian expression of *myh_tc*, *myh_n1* and *myh_n4*
287 had an acrophase during the presumptive night while transcript levels of *myo18* paralogs peaked
288 during the presumptive day. The circadian parameters defining the rhythmicity of expression
289 are given in Supplementary Table S3. The circadian expression profiles of all *myosins* examined
290 are shown in Supplementary Fig. 1, including *myosin* genes that did not display circadian
291 rhythmicity. Though a number of myosins did not display circadian rhythmicity, there were
292 significant temporal differences in their expression throughout a daily light/ dark cycle with the
293 exception of *myh11_1*, *myh_n5*, *myh_n6, myh_n7* and *myl1*. The circadian rhythmic expression
294 of *myosins* supports the hypothesis that the physiology of fast skeletal muscle in Atlantic cod
295 may be under circadian control. The exact function of *myo18* in teleost fast skeletal muscle is
296 not yet known but in human, MYO18A acts as an actin-crosslinker with multiple regulatory
297 modulators that targets interacting proteins or complexes to the actin-based cytoskeleton (Taft
298 et al., 2013). Our data indicate that Atlantic cod *myo18* may have an important role in the
299 circadian-related muscle functions, since its two paralogs displayed circadian rhythmic
300 expression.

Studies in mice have shown that circadian regulation has a potential role in the function of myofilaments, in which the myosins are important structural components. Clock protein was localized within the myofilament Z-disc of cardiomyocytes (Qi and Boateng, 2006) and has been demonstrated that contractile activity and energy usage within the myofilaments led to nuclear translocation of Clock protein. Mice deficient in Rev-erbA α exhibited alterations in contractile protein content, particularly showing a shift in myosin heavy chain composition (Pircher et al., 2005, Downes et al., 1995). The circadian rhythmicity of *myosin* transcripts observed in our study suggests that components of the contractile mechanism may be under circadian control, to some extent at the transcriptional level. Moreover, the varying peaks of expression observed between Atlantic cod *myosin* paralogs may be related to different physiological changes during a daily day/night cycle, since different *myosin* isoforms are likely to have different functional properties.

3.3.Correlation of myosin circadian expression with clock genes in fast skeletal muscle

In a sister study, we have shown that the expression of several muscle-related genes was changing during a circadian cycle and the expression of some muscle-related genes in circadian rhythmicity, such as *myogenic factor 5 (myf5)* and *muscleblind-like 1 (mbn11),* correlated with the expression of clock genes in fast skeletal muscle (Lazado, Kumaratunga, Nagasawa, Babiak, Giannetto and Fernandes, unpublished). This observation raises the hypothesis that some muscle-related genes may be at least partly regulated by clock genes. In the current study, expression of circadian rhythmic *myosins* was compared with transcript levels of Atlantic cod clock genes that were previously shown to display circadian rhythmic expression in fast skeletal muscle (Supplementary Table S4). *Myh_tc (ρ = 0.550)* and *myh_n1 (ρ = 0.717)* transcript levels positively correlated with expression of *aryl hydrocarbon receptor nuclear translocator-like 2* (*arntl2*), a member of the positive arm of the core clock system. On the other hand, circadian

expression of *myo18a_2* and *myo18b_2* positively correlated with transcript levels of two *cryptochrome* genes (*cry2* and *cry3*), which belong to the negative arm of the transcriptional feedback loop. Expression of the two *myh* genes *myh_n1* and *myh_n4* negatively correlated with transcript levels of *clock*, *neuronal PAS (Per-Arnt-Single-minded) domain-containing protein 2* (*npas2*), *nuclear receptor subfamily 1, group D, member 1* (*nr1d1*) and *nr1d2a*. In mammals, there is evidence supporting the regulatory role of clock genes in the proper functioning of myosins in the fast skeletal muscle. It has been shown in Bmal1 (Arntl1)-deficient mice that altered expression of two myosin heavy chain isoforms leads to cardiomyopathy (Lefta et al., 2012). In another study, expression of myosins decreased in *Clock* $^{\Delta 19}$ and *Bmal1*$^{-/-}$ mutant mice and this resulted in the alteration of myofilament organization (Andrews et al., 2010). Hitherto, there is no clear evidence that teleost myosins are regulated by clock genes but our results corroborate this hypothesis, since there is a strong correlation between the mRNA level of *myosins* and clock gene transcript levels in the fast skeletal muscle of Atlantic cod during a circadian cycle. Given the diverse nature of myosins, it would be interesting to study how clock genes are interacting with the different myosin isoforms in fish.

3.4. Presence of circadian-related transcription factor binding sites in the putative promoter region of myosins

In silico characterization of TFBS involved in the circadian regulation of clock or clock-controlled genes identified *circadian*TFBS (Creb, Mef2, and E-box motifs) in the 5 kb putative promoter regions of *myh_tc*, *myh_n1*, *myo18a_2* and *myo18b_2* (Fig. 2). Creb TFBS were found in several locations within the first 3 kb upstream region of *myh_tc* and *myh_n1*. In particular, Creb TFBS were located at -1231, -2194, -2253 bp upstream in *myh_tc* and at -1181, -2065, -3030 bp upstream of *myh_n1* (Fig. 2A, B). A Mef2 consensus sequence was also identified at several locations in the upstream region of two *myh* genes (-810, -883, -1157, -

1402 bp in *myh_tc* and at -997, -1201, -1233, -1549 bp in *myh_n1*). In addition, one E-box motif was identified at -3062 bp of the putative promoter region of *myh_tc*. As for the two paralogs of *myo18*, Creb TFBS were found at -990 bp and -882 bp upstream of *myo18a_2* and *myo18_b2*, respectively. Besides the three Creb TFBS located between -1.5 and 2.5 kb of *myo18a_2*, Mef2 TFBS and an E-box motif were identified at positions -2441 and -3787 bp, respectively.

The MEF2, CREB and E-Box motifs are some of the main regulatory factors of various genes with circadian rhythmic expression (Bozek et al., 2009). For instance, the circadian expression of *MyoD* in mouse skeletal muscle is regulated by the non-canonical E-box in its promoter region (Zhang et al., 2012). Besides having a role in skeletal muscle commitment and synergizing with *MyoD* (Al Madhoun et al., 2011), MEF2 plays an essential regulatory role in the normal circadian behaviour in *Drosophila* (Blanchard et al., 2010). CREB, which plays key roles in differentiation of embryonic skeletal muscle progenitors and survival of adult skeletal muscle (Stewart et al., 2011), has also been shown to be a circadian transcriptional regulator of the suprachiasmatic nucleus clock (Lee et al., 2010). The presence at multiple locations of the above transcription factor binding sites in the putative promoter regions of *myh_tc*, *myh_n1*, *myo18a_2* and *myo18_2* implies their possible regulatory role in the circadian rhythmic expression of these *myosin* genes. Variations in acrophase and amplitude between paralogs could be attributed to differences in the number and location of these and other *circadian*TFBS in the putative promoter region of these genes.

3.5. Myosin expression in fast skeletal muscle explants

The presence of autonomous clocks is typified by their circadian rhythmic gene expression even when the tissue or cells have been excised from the organism, and this has been shown in model species such as the zebrafish (Carr and Whitmore, 2005). In Atlantic cod fast skeletal muscle explants, the transcript levels of *myh_tc*, *myh_n1*, *myh_n4, myo18a_2* and

376 *myo18b_2* did not display circadian rhythmicity, even under a 12L:12D cycle (Fig. 3A-E).
377 Assuming that the transcriptional control of circadian rhythmicity of *myosins* is an output of
378 the circadian clocks, the present results support the hypothesis that the clock present in Atlantic
379 cod fast skeletal muscle is likely to be dependent on regulatory neural signals from the central
380 clock.

381 The circadian response is markedly influenced by light history (Glickman et al., 2012).
382 Different photoperiod regimes did not significantly influence the circadian expression of
383 *myh_tc* and *myh_n1* (Fig. 3A, B). However, significant differences were observed between
384 photoperiod treatments in the circadian expression of *myh_n4*, *myo18a_2* and *myo18b_2* (Fig.
385 3C-E). In particular, the transcript levels of *myh_n4* (Time [*t*] 6 – 18h) in fast skeletal muscle
386 explants cultured under constant conditions (*LL* and *DD*) were significantly higher than in the
387 group cultured under *LD*. On the other hand, *myo18b_2* had generally lower transcript levels
388 under *LL* (significant differences noted at t = 6, 15 and 18 h) than under *LD* and *DD* conditions.
389 There was no clear trend for *myo18a_2* expression but significant differences between
390 photoperiod treatments were found at t = 6, 15 and 24 h. Taken together, these observations
391 imply that the transcription of *myosins* in fast skeletal muscle explants particularly of *myh_n4*,
392 *myo18a_2* and *myo18b_2* is significantly affected by photoperiod conditions. In addition, the
393 photoperiodic-associated changes suggest that the response mechanisms in different light
394 regimes may vary between *myosin* paralogs.
395

3.6. Influence of photoperiod on the transcription of fast skeletal muscle myosins with circadian rhythmicity

The influence of temperature has been the main focus of studies on the plasticity of myosins in fish, particularly in *myh* genes (Kobiyama et al., 2006, Tao et al., 2004). It is relevant to study the effect of photoperiod on myosin plasticity in Atlantic cod fast muscle, since light has a remarkable impact on somatic growth of Atlantic cod both at phenotypic and transcriptional levels (Nagasawa et al., 2012, Giannetto et al., 2013). Amongst the *myosins* with circadian rhythmicity, rearing under continuous light (*LL*) generally resulted in a significant elevation of their mRNA levels compared with natural photoperiod (*NL*), with the exception of *myh_n4* (Fig. 4). At the 60th day of light treatment, transcript levels of *myh_tc* and *myo18a_2* were significantly higher in *LL* than in *NL* conditions. The significant elevation of *myosin* transcripts in *LL* group was observed even earlier in *myh_tc*, as its expression was approximately 55 % higher than in *NL* group after 7 days. At the last day of sampling, a significant effect of continuous illumination on *myosin* mRNA levels was observed for *myh_n1* and *myo18b_2.* Interestingly, at 60 days before the last sampling, *myo18b_2* mRNA levels under *NL* regime were significantly higher than in *LL* group. In our sister study, transcripts of one *myh* paralog (identified as *myh3* in the Atlantic cod genome assembly) were significantly elevated under *NL* regime as well (Nagasawa et al., 2011). In addition to the observed differences in *myosin* expression between the different light treatments at a given time point, *myosin* transcript levels (particularly of *myh_tc* and *myo18a_2*) increased throughout the duration of the experiment and this pattern was evident regardless of photoperiod regimes. Given the remarkable effect of photoperiod on their mRNA levels, it is plausible that *myosins* are involved in the molecular network regulating the photic plasticity of muscle growth in Atlantic cod. Most differences in *myosin* transcript levels between photoperiod treatments were observed after 60 days (Fig. 4; Supplementary Fig. 2), concomitantly with significant changes

in growth parameters that were previously reported, namely a 13 % weight increase in *LL* fish compared to the *NL* group after 120 days (Nagasawa et al., 2012). It is noteworthy that *myosins* without circadian rhythmicity were differentially expressed with light treatment from 60 days and thereafter (*myh11_1, myh_n3, myh_n5, myh_n6, myo18a_1* and *myl1*), with significantly higher transcript levels under *LL* than *NL* conditions (Supplementary Fig. 2). We have also observed that in addition to weight gain, there were changes in muscle fiber size associated with photoperiod manipulation (Nagasawa, Giannetto, Lazado and Fernandes, unpublished). It would be compelling to investigate whether the elevated transcription of *myosins* observed in the present study is related to the phenotypic changes observed during photoperiod treatment in this species. Nevertheless, their differential expression of *myosin* genes with photoperiod suggests that they may be involved in the epigenetic regulation of skeletal muscle growth in Atlantic cod.

3.6. Conclusions

This is the first study to demonstrate that *myosin* transcript levels oscillate with a circadian pattern in fast skeletal muscle of a teleost. The *circadian*TFBS identified by *in silico* analysis in the putative promoter region of these *myosin* genes could be involved in the regulation of their circadian rhythmicity but this hypothesis needs to be experimentally confirmed. The correlation between *myosin* transcripts with circadian rhythmicity and molecular clocks implies a possible transcriptional control from the core circadian clock machinery. However, the loss of rhythmicity under *ex vivo* conditions supports the hypothesis that the clock system in Atlantic cod skeletal muscle may be under the regulatory control by the central clock. Exposure to continuous illumination *in vivo* was associated with an increase in transcript levels of *myh_tc*, *myh_n1*, *myo18a_2* and *myo18b_2.* Taken together, our results

indicate that some *myosin* genes may be clock-controlled and may also be involved in the photic plasticity of muscle growth observed in Atlantic cod.

Acknowledgements

This research was financed by a grant from the Research Council of Norway (ref. 190350/S40) to J.M.O. Fernandes. The technical assistance of Hilde Ribe and Katrine Klippenberg from the Mørkvedbukta Research Station (Univerity of Nordland, Norway) is gratefully acknowledged. The authors would also like to express their appreciation to Dr. Lech Kirtiklis (University of Warmia and Mazury in Olsztyn, Poland), Dr. Alessia Gianetto (University of Messina, Italy), Dr. Arvind Sundaram (University of Nordland, Norway) and Spyros Kollias (University of Nordland, Norway) for their help with the photoperiod and circadian rhythm experiments.

457 **Reference**s

458 Al Madhoun, A.S., Mehta, V., Li, G., Figeys, D., Wiper-Bergeron, N. Skerjanc, I.S. 2011.
459 Skeletal myosin light chain kinase regulates skeletal myogenesis by phosphorylation of
460 MEF2C. EMBO J. 30**,** 2477-2489.
461 Amaral, I.P.G. Johnston, I.A. 2012. Circadian expression of clock and putative clock-
462 controlled genes in skeletal muscle of the zebrafish. Am. J. of Physiol. Reg. Integr.
463 Comp. Physiol. 302**,** R193-R206.
464 Andrews, J.L., Zhang, X., Mccarthy, J.J., Mcdearmon, E.L., Hornberger, T.A., Russell, B.,
465 Campbell, K.S., Arbogast, S., Reid, M.B., Walker, J.R., Hogenesch, J.B., Takahashi,
466 J.S. Esser, K.A. 2010. CLOCK and BMAL1 regulate MyoD and are necessary for
467 maintenance of skeletal muscle phenotype and function. Proc. Natl. Acad. Sci. U.S.A.
468 107**,** 19090-19095.
469 Blanchard, F.J., Collins, B., Cyran, S.A., Hancock, D.H., Taylor, M.V. Blau, J. 2010. The
470 transcription factor Mef2 is required for normal circadian behavior in Drosophila. J.
471 Neurosci. 30**,** 5855-5865.
472 Bozek, K., Relógio, A., Kielbasa, S.M., Heine, M., Dame, C., Kramer, A. Herzel, H. 2009.
473 Regulation of clock-controlled genes in mammals. PLoS ONE. 4, e4882.
474 Cahill, G.M. 2002. Clock mechanisms in zebrafish. Cell Tissue Res. 309**,** 27-34.
475 Carr, A.J.F. Whitmore, D. 2005. Imaging of single light-responsive clock cells reveals
476 fluctuating free-running periods. Nat. Cell Biol. 7, 319-321.
477 Cole, N.J. Johnston, I.A. 2001. Plasticity of myosin heavy chain expression with temperature
478 acclimation is gradually acquired during ontogeny in the common carp *Cyprinus carpio*
479 L.. J. Comp. Physiol. B. 171**,** 321-326.
480 Dardente, H. Cermakian, N. 2007. Molecular circadian rhythms in central and peripheral clocks
481 in mammals. Chronobiol. Int. 24**,** 195-213.

482 Downes, M., Carozzi, A.J. Muscat, G.E.O. 1995. Constitutive expression of the orphan
483 receptor, Rev-erbA α, inhibits muscle differentiation and abrogates the expression of
484 the myoD gene family. Mol. Endocrinol. 9**,** 1666-1678.
485 Ennion, S., Wilkes, D., Gauvry, L., Alami-Durante, H. Goldspink, G. 1999. Identification and
486 expression analysis of two developmentally regulated myosin heavy chain gene
487 transcripts in carp *Cyprinus carpio*. J. Exp. Biol. 202**,** 1081-1090.
488 Falcón, J. 1999. Cellular circadian clocks in the pineal. Prog. Neurobiol., 58**,** 121-162.
489 Fernandes, J.M.O., Kinghorn, J.R. Johnston, I.A. 2007. Differential regulation of multiple
490 alternatively spliced transcripts of *MyoD*. Gene, 391**,** 178-185.
491 Fernandes, J.M.O., Mommens, M., Hagen, Ø., Babiak, I. Solberg, C. 2008. Selection of
492 suitable reference genes for real-time PCR studies of Atlantic halibut development.
493 Comp. Biochem. Physiol. B, Biochem. Mol. Biol. 150**,** 23-32.
494 Funkenstein, B., Balas, V., Skopal, T., Radaelli, G. Rowlerson, A. 2006. Long-term culture of
495 fast skeletal muscle explants from *Sparus aurata*. Tissue Cell. 38**,** 399-415.
496 Giannetto, A., Nagasawa, K., Fasulo, S. Fernandes, J.M.O. 2013. Influence of photoperiod on
497 expression of DNA cytosine-5 methyltransferases in Atlantic cod. Gene. 519**,** 222-230.
498 Glickman, G., Webb, I.C., Elliott, J.A., Baltazar, R.M., Reale, M.E., Lehman, M.N. Gorman,
499 M.R. 2012. Photic sensitivity for circadian response to light varies with photoperiod. J.
500 Biol. Rhythms. 27**,** 308-318.
501 Ikeda, D., Ono, Y., Snell, P., Edwards, Y.J.K., Elgar, G. Watabe, S. 2007. Divergent evolution
502 of the myosin heavy chain gene family in fish and tetrapods, Evidence from comparative
503 genomic analysis. Physiol. Genomics. 32**,** 1-15.
504 Katherine Tamai, T., Vardhanabhuti, V., Arthur, S., Foulkes, N.S. Whitmore, D. 2003. Flies
505 and Fish, Birds of a Feather. J. Neuroendocrinol. 15**,** 344-349.

506 Kobiyama, A., Hirayama, M., Muramatsu-Uno, M. Watabe, S. 2006. Functional analysis on
507 the 5′ -flanking region of carp fast skeletal myosin heavy chain genes for their
508 expression at different temperatures. Gene. 372**,** 82-91.
509 Lee, B., Aiqing, L., Hansen, K.F., Ruifeng, C., Jae Hwa, Y. Obrietan, K. 2010. CREB
510 influences timing and entrainment of the SCN circadian clock. J. Biol. Rhythms. 25**,**
511 410-420.
512 Lefta, M., Campbell, K.S., Feng, H.Z., Jin, J.P. Esser, K.A. 2012. Development of dilated
513 cardiomyopathy in Bmal1-deficient mice. Am. J. Physiol. Heart Circ. Physiol. 303**,**
514 H475-H485.
515 Lodish, H., Berk, A., Lawrence Zipursky, S., Matsudaira, P., Baltimore, D. Darnell, J. 2000.
516 Myosin, The Actin Motor Protein, New York, W.H. Freeman.
517 Mccarthy, J.J., Andrews, J.L., Mcdearmon, E.L., Campbell, K.S., Barber, B.K., Miller, B.H.,
518 Walker, J.R., Hogenesch, J.B., Takahashi, J.S. Esser, K.A. 2007. Identification of the
519 circadian transcriptome in adult mouse skeletal muscle. Physiol. Genomics. 31**,** 86-95.
520 Nagasawa, K., Giannetto, A. Fernandes, J.M.O. 2012. Photoperiod influences growth and MLL
521 mixed-lineage leukaemia expression in Atlantic cod. PLoS ONE. 7, e36908.
522 Nagasawa, K., Lazado, C.C. Fernandes, J.M.O. 2011. Validation of endogenous reference
523 genes for qPCR quantification of muscle transcripts in Atlantic cod subjected to
524 different photoperiod regimes. In: Muchlisin, Z. (Ed.), Aquaculture. InTech. Available
525 from: http://www.intechopen.com/books/aquaculture/validation-of-endogenous-
526 reference-genes-for-qpcr-quantification-of-muscle-transcripts-in-atlantic-c.
527 Odronitz, F. Kollmar, M. 2007. Drawing the tree of eukaryotic life based on the analysis of
528 2,269 manually annotated myosins from 328 species. Genome Biol. 8, R196.
529 Panopoulou, G. Poustka, A.J. 2005. Timing and mechanism of ancient vertebrate genome
530 duplications - The adventure of a hypothesis. Trends Genet. 21**,** 559-567.

Pircher, P., Chomez, P., Yu, F., Vennström, B. Larsson, L. 2005. Aberrant expression of myosin isoforms in skeletal muscles from mice lacking the rev-erbA α orphan receptor gene. Am. J. Physiol. Regul. Integr. Comp. Physiol. 288**,** R482-R490.

Qi, L. Boateng, S.Y. 2006. The circadian protein Clock localizes to the sarcomeric Z-disk and is a sensor of myofilament cross-bridge activity in cardiac myocytes. Biochem. Biophys. Res. Commun. 351**,** 1054-1059.

Saito, T., Hirano, M., Ide, T., Ichiki, T., Koibuchi, N., Sunagawa, K. Hirano, K. 2013. Pivotal role of rho-associated kinase 2 in generating the intrinsic circadian rhythm of vascular contractility. Circulation. 127**,** 104-114.

Stewart, R., Flechner, L., Montminy, M. Berdeaux, R. 2011. CREB is activated by muscle injury and promotes muscle regeneration. PLoS ONE. 6**,** e24714.

Taft, M.H., Behrmann, E., Munske-Weidemann, L.C., Thiel, C., Raunser, S. Manstein, D.J. 2013. Functional characterization of human myosin-18A and its interaction with F-actin and GOLPH3. J. Biol. Chem. 288**,** 30029-30041.

Tamai, T.K., Carr, A.J. Whitmore, D. 2005. Zebrafish circadian clocks, Cells that see light. Biochem. Soc. Trans. 33**,** 962-966.

Tao, Y., Kobayashi, M., Liang, C.S., Okamoto, T. Watabe, S. 2004. Temperature-dependent expression patterns of grass carp fast skeletal myosin heavy chain genes. Comp. Biochem. Physiol. B, Biochem. Mol. Biol. 139**,** 649-656.

Vatine, G., Vallone, D., Gothilf, Y. Foulkes, N.S. 2011. It's time to swim! Zebrafish and the circadian clock. FEBS Lett. 585**,** 1485-1494.

Velarde, E., Haque, R., Iuvone, P.M., Azpeleta, C., Alonso-Gomez, A.L. Delgado, M.J. 2009. Circadian clock genes of goldfish, *Carassius auratus*, CDNA cloning and rhythmic expression of period and cryptochrome transcripts in retina, liver, and gut. J. Biol. Rhythms. 24**,** 104-113.

556 Watabe, S. 2002. Temperature plasticity of contractile proteins in fish muscle. J. Exp. Biol.
557 205**,** 2231-2236.
558 Whitmore, D., Foulkes, N.S. Sassone-Corsi, P. 2000. Light acts directly on organs and cells in
559 culture to set the vertebrate circadian clock. Nature. 404**,** 87-91.
560 Whitmore, D., Foulkes, N.S., Strähle, U. Sassone-Corsi, P. 1998. Zebrafish Clock rhythmic
561 expression reveals independent peripheral circadian oscillators. Nat. Neurosci. 1**,** 701-
562 707.
563 Zhang, X., Patel, S.P., Mccarthy, J.J., Rabchevsky, A.G., Goldhamer, D.J. Esser, K.A. 2011.
564 A non-canonical E-box within the MyoD core enhancer is necessary for circadian
565 expression in skeletal muscle. Nucleic Acids Res. 40, 3419-3430.

Figure captions

Figure 1. Circadian rhythmicity of *myosin* transcript levels in Atlantic cod fast skeletal muscle. A) *myh_tc*, B) *myh_n1*, C) *myh_n4*, D) *myo18a_2* and E) *myo18b_2*. Relative expression data are presented as mean±SEM of six individual fish per sampling point. The dashed line is the best-fit sinusoidal curve of the qPCR expression data based on the circadian parameters calculated by COSINOR (Supplementary Table S3). Statistical differences ($P < 0.05$) between time points are indicated by different letter notations. The panel above the graph represents the photoperiod regimes: white bar = presumptive day; gray bar = light-dark transition; black bar = presumptive night.

Figure 2. Circadian-related transcription factor binding sites in the 5 kb upstream region of Atlantic cod *myosin* genes. A) *myh_tc*, B) *myh_n1*, C) *myo18a_2* and D) *myo18b_2*. *circadian*TFBS mapped to the putative promoter regions of *myosins* with circadian rhythmic expression are CREB (blue), E-Box, (red) and MEF2 (green). The black box indicates the putative coding region of each gene.

Figure 3. Expression of *myosin* genes in 5-day old Atlantic cod fast skeletal muscle explants cultured at different photoperiod regimes. A) *myh_tc*, B) *myh_n1*, C) *myh_n4*, D) *myo18a_2* and E) *myo18b_2*. Values presented are mean±SEM from two independent experiments. The dashed line is the best-fit sinusoidal curve of the qPCR expression data based on the circadian parameters calculated by COSINOR (Supplementary Table S5). Statistical differences ($P < 0.05$) between photoperiod regimes at a given time point are denoted by different letters.

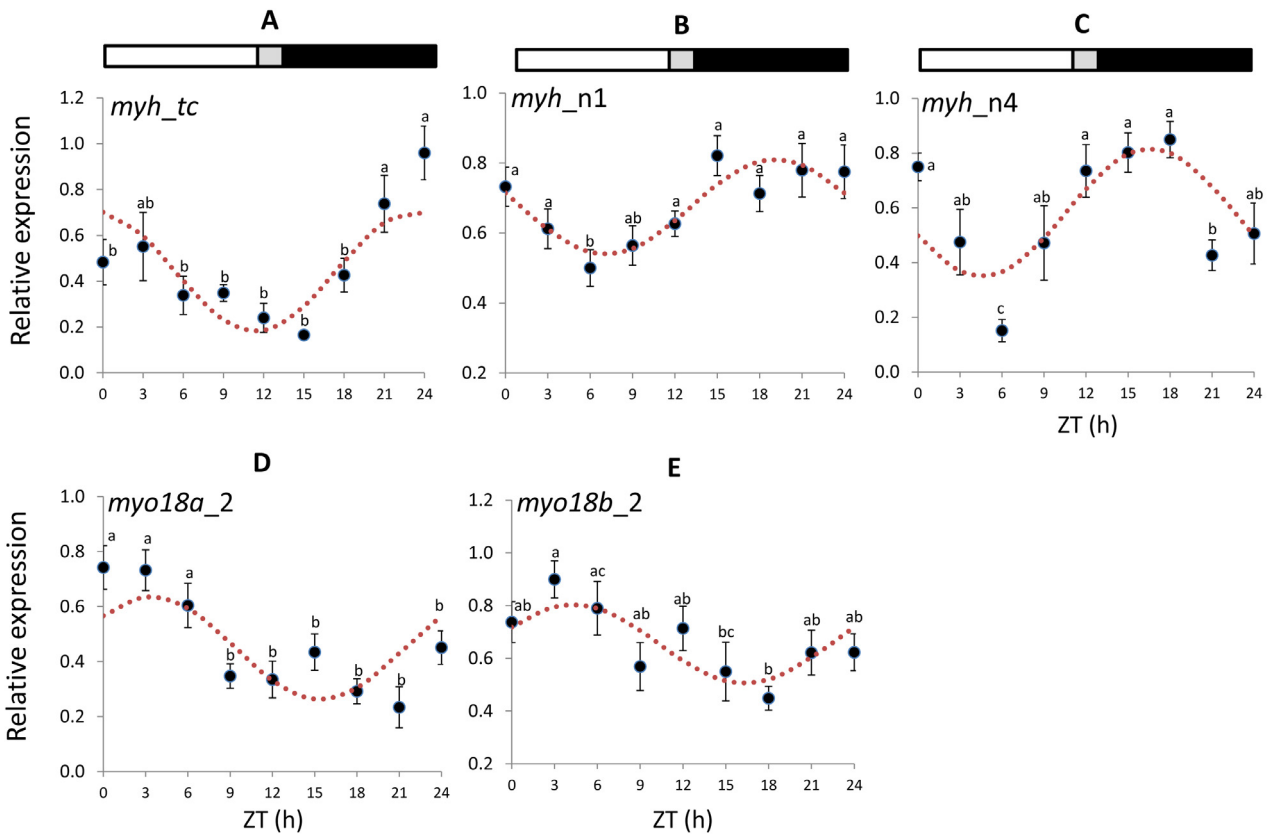
Figure 4. Photoperiod-associated changes in *myosin* expression. A) *myh_tc*, B) *myh_n1*, C) *myh_n4*, D) *myo18a_2* and E) *myo18b_2*. Relative transcript levels of *myosin* genes in fast skeletal

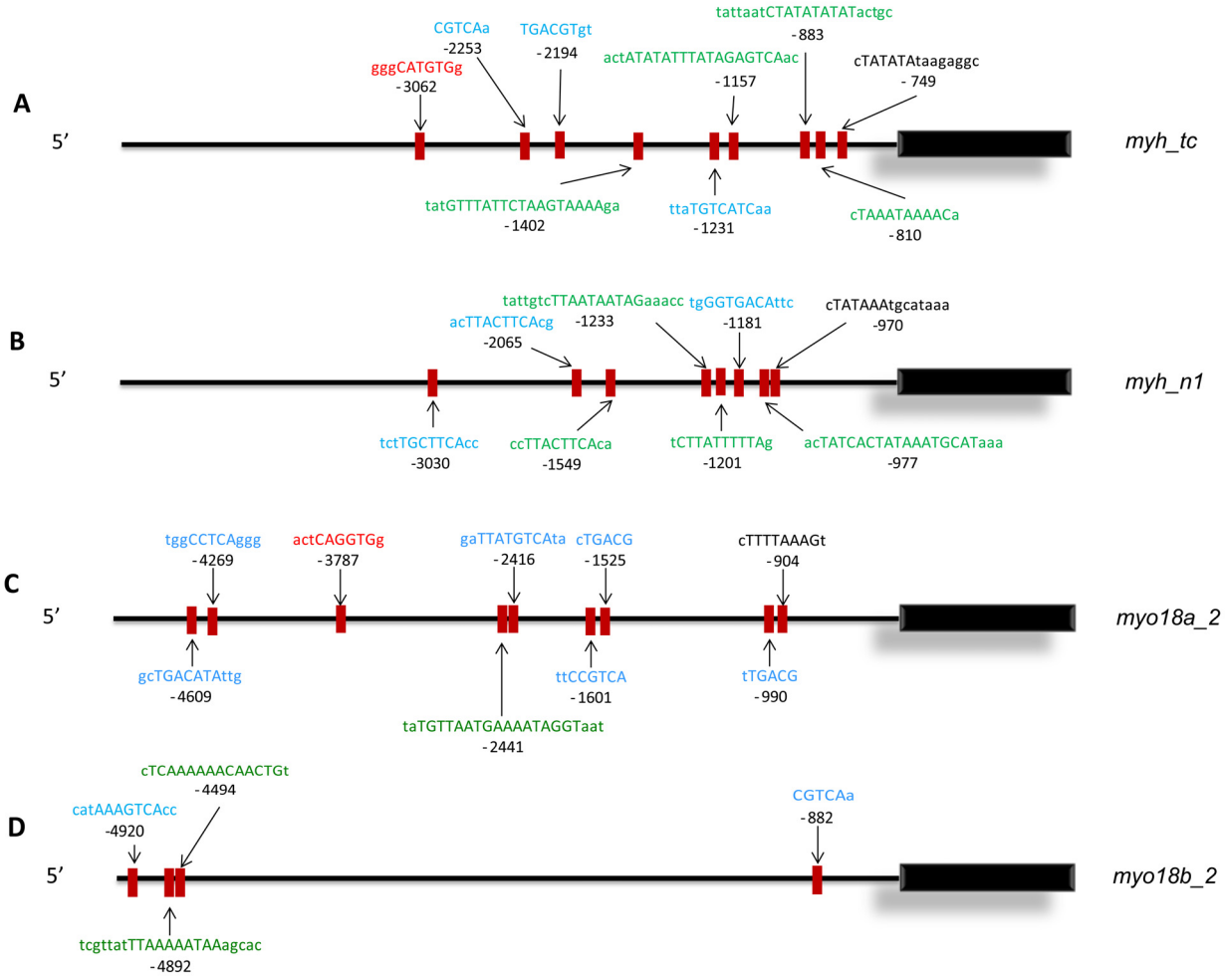
592 muscle of juvenile Atlantic cod is presented as mean±SEM from six individual samples. Different
593 numbers indicate significant differences ($P < 0.05$) between time points within the *LL* group,
594 whereas different letters refer to significant differences ($P < 0.05$) between time points in the *NL*
595 group. Asterisks (*) represent significant differences ($P < 0.05$) between the *LL* and *NL* groups at
596 the same sampling point. Notation, *LL* = continuous light (24L,0D); *NL* = simulated natural
597 photoperiod in Bodø, Norway (see Methods section).
598

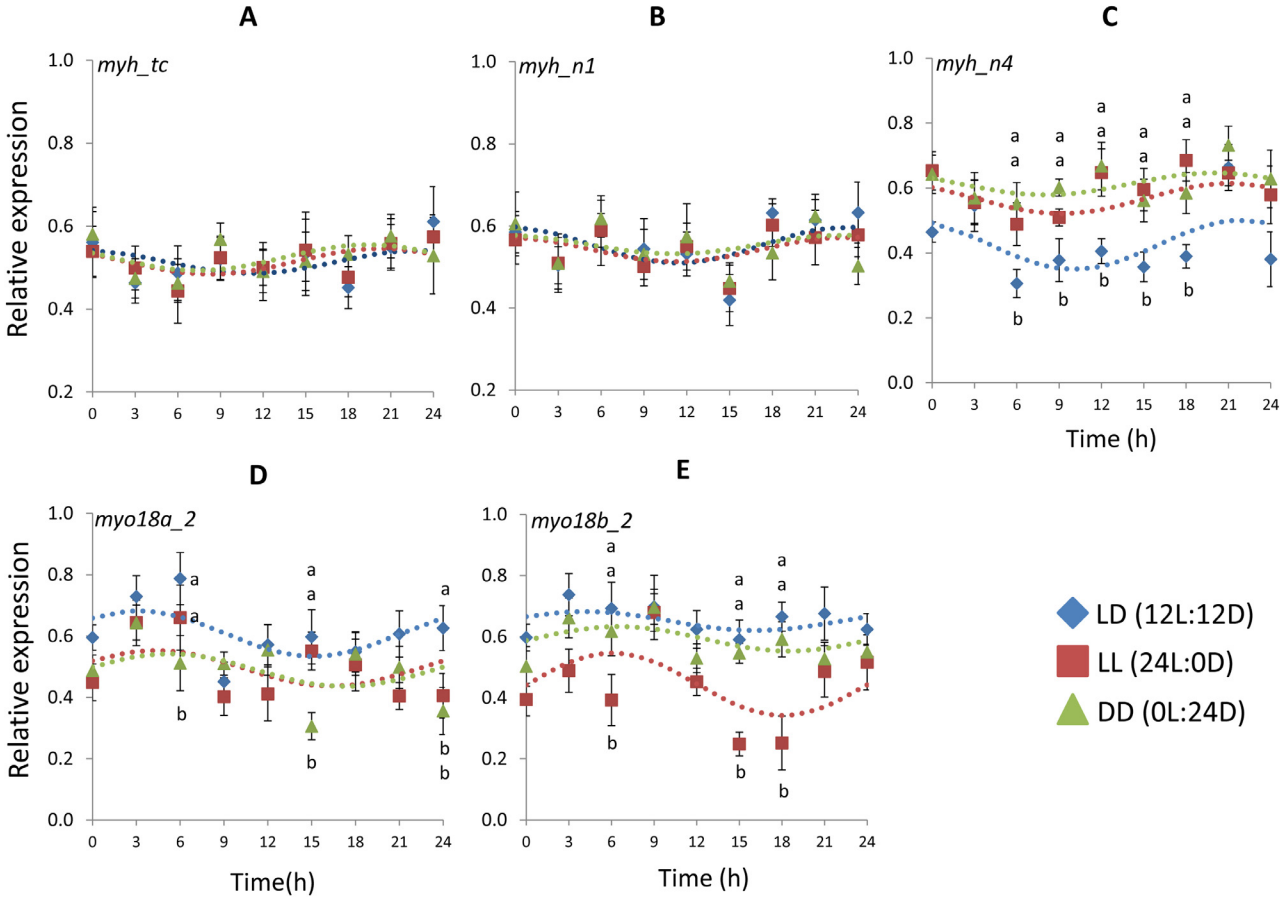
Table 1. Fast skeletal muscle myosins of Atlantic cod analyzed in this study.

	Gene name	Abbreviation	Ensembl ID	Blast Hit	% Identity
Annotated	Myosin IIIA*	*myo3a*	ENSGMOG00000015600		
	Myosin IIIB*	*myo3b*	ENSGMOG00000002507		
	Myosin XVA*	*myo15a (1 of 2)*	ENSGMOG00000010621		
	Myosin XVA*	*myo15a (2 of 2)*	ENSGMOG00000007696		
	Myosin XVIIIA	*myo18a (1 of 2)*	ENSGMOG00000018558		
	Myosin XVIIIA	*myo18a (2 of 2)*	ENSGMOG00000010045		
	Myosin XVIIIB	*myo18b (1 of 2)*	ENSGMOG00000005484		
	Myosin XVIIIB	*myo18b (2 of 2)*	ENSGMOG00000005499		
	Myosin, heavy chain 11, smooth muscle*	*myh11 (1 of 2)*	ENSGMOG00000005651		
	Myosin, heavy chain 11, smooth muscle	*myh11 (2 of 2)*	ENSGMOG00000009607		
	Myosin light chain, phosphorylatable, fast skeletal muscle	*mylpf (1 of 2)*	ENSGMOG00000005541		
	Myosin light chain, phosphorylatable, fast skeletal muscle	*mylpf (2 of 2)*	ENSGMOG00000013719		
	Myosin, light chain 1, alkali; skeletal, fast	*myl1*	ENSGMOG00000000267		
	Myosin, light chain 9, regulatory	*myl9*	ENSGMOG00000017056		
Unannotated	Novel	*myh_tc*	ENSGMOG00000011194	Myosin heavy chain	96%; *Coryphaenoides yaquinae*
	Novel	*myh _n1*	ENSGMOG00000016381	Myosin heavy chain	85%; *C. yaquinae*
	Novel	*myh _n2*	ENSGMOG00000009472	Myosin heavy chain	88%; *Oryzias latipes*
	Novel	*myh _n3*	ENSGMOG00000016313	Myosin heavy chain	93%; *Coryphaenoides cinereus*
	Novel	*myh _n4*	ENSGMOG00000011161	Myosin heavy chain	91%; *Saurida wanieso*
	Novel	*myh _n5*	ENSGMOG00000015700	Myosin heavy chain	99%; *Gadus chalcogrammus*
	Novel	*myh _n6*	ENSGMOG00000016449	Myosin heavy chain	97%; *G. chalcogrammus*
	Novel	*myh _n7*	ENSGMOG00000006802	Myosin heavy chain	96%; *Coryphaenoides acrolopis*
	Novel*	*myh _n8*	ENSGMOG00000009501	Myosin heavy chain	86%; *Danio rerio*

NOTE: * not included in the characterization as primers were not successfully designed.


A
B
C
D
E
myh_tc
myh_n1
myh_n4
myo18a_2
myo18b_2
Relative expression
ZT (h)


A
5′
CGTCAa
-2253
TGACGTgt
-2194
gggCATGTGg
-3062
actATATATTTATAGAGTCAac
-1157
tattaatCTATATATATactgc
-883
cTATATAtaagaggc
- 749
tatGTTTATTCTAAGTAAAAga
-1402
ttaTGTCATCaa
-1231
cTAAATAAAACa
-810
myh_tc
B
5′
tattgtcTTAATAATAGaaacc
-1233
tgGGTGACAttc
-1181
acTTACTTCAcg
-2065
cTATAAAtgcataaa
-970
tctTGCTTCAcc
-3030
ccTTACTTCAca
-1549
tCTTATTTTTAg
-1201
acTATCACTATAAATGCATaaa
-977
myh_n1
C
5′
tggCCTCAggg
-4269
actCAGGTGg
-3787
gaTTATGTCAta
-2416
cTGACG
-1525
cTTTTAAAGt
-904
gcTGACATAttg
-4609
taTGTTAATGAAAATAGGTaat
-2441
ttCCGTCA
-1601
tTGACG
-990
myo18a_2
D
5′
cTCAAAAAACAACTGt
-4494
catAAAGTCAcc
-4920
CGTCAa
-882
tcgttatTTAAAAATAAagcac
-4892
myo18b_2


A
B
C
D
E
myh_tc
myh_n1
myh_n4
myo18a_2
myo18b_2
Relative expression
Time (h)
Time(h)
LD (12L:12D)
LL (24L:0D)
DD (0L:24D)

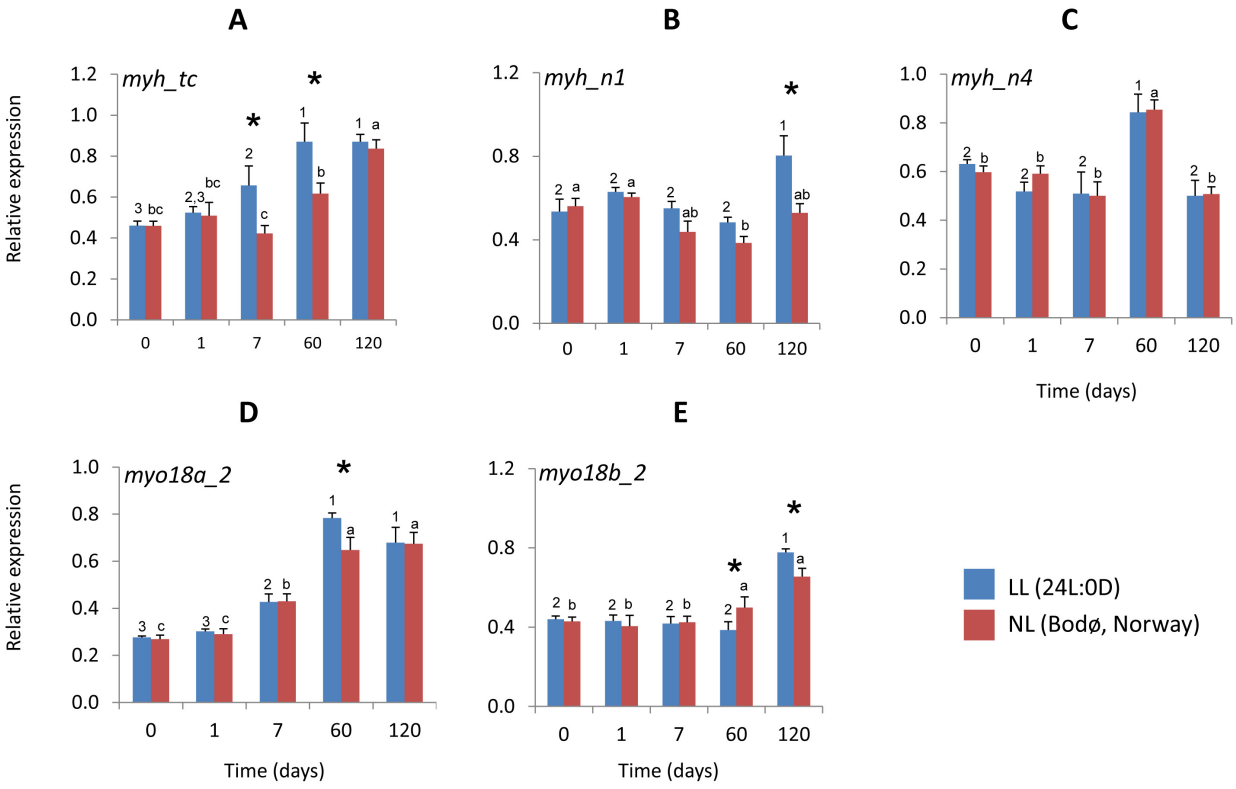

A
B
C
D
E
myh_tc
myh_n1
myh_n4
myo18a_2
myo18b_2
Relative expression
Time (days)
0
1
7
60
120
LL (24L:0D)
NL (Bodø, Norway)

Table S1. Primer sequences (5' to 3'), amplicon size (bp), annealing temperature (^{o}C) and PCR efficiency (E) of *myosin* and reference genes used in this study.

Gene name	Amplicon size (bp)	Annealing Temperature ^{o}C	E	Primer sequences (5' to 3')	Reference
myh_tc	142	60	98.4	F: TTTAAAGCTGGTCTTCTGGGT R:AAGATGGCTTCCCTCCTCTC	This study
myh_n1	127	60	83.8	F: GAAATCCTCAAACAAACTGCTG R: CCAAATTCTCCCTGAACTGTG	This study
myh_n2	246	60	85.0	F: CACTGCTGATGAGAAGATTGG R: TGAGTTATGGACCTGTGGAC	This study
myh_n3	195	60	86.7	F: AACAAGGTGAAGAACCTGACTG R: GTGAACCCTCAAGATCATCCA	This study
myh_n4	169	60	91.4	F: GTGTCATCCAGTACTTTGCCA R: CCTGATGAATTTACCAAAGCGA	This study
myh_n5	136	60	91.9	F: TCACATACCAGACTGAGGAG R: CTTGGACAGGTAGGAGTTGG	This study
myh_n6	205	60	81.8	F: TCTTCTTCATCCTCTCCAGGT R: GCACTCTCAGAACACAAGCC	This study
myh_n7	139	60	83.5	F: CCAGCAGACTCTTGATGACC R: CCAGTGAACCTTCAAGATCATCC	This study
myo18a_1	224	60	99.9	F: GAGAGGACCCAGATCAAGAG R: GATGTCCATTTCCAGTTCGT	This study
myo18a_2	141	60	84.1	F: AACACGAGCTGGAAATGGAC R: AGGTCTTCATTGTCATCACTCTC	This study
myo18b_1	248	60	80.2	F: AAGAGGTTTGAGGTGCTGGT R: GTCGGCCTGTAATGTCTGTC	This study
myo18b_2	135	60	93.9	F: CAAGCAGAGGAGGTTTGACAG R: CTGTAGGTTGGCCCTTAGAG	This study
myh11_1	194	60	109	F: CGTCAAATTCTCCAAGCCCA R: ACTCTGTCAGCATCTTTCCA	This study
mylpf_1	175	60	98.4	F: CAAAGGTTGGTCATCTCCTCAG R: GTCTTCCTCACCATGTTCGG	This study
mylpf_2	300	60	89.7	F: CAGAGACGGTATCATCAGCA R: CCACATGTTCTTGATCTCCTCAG	This study
myl1	107	60	88.9	F: GTATGCTACAACCAGATCGCC R: GGAGTTCATGTCTTCGTCGG	This study
myl9	249	62	92.9	F: GCCTTCAACATGATTGACCA R: ATGGATCACACCAGATCCCT	This study
arp	113	60	90.3	F:TGATCCTCCACGACGATGAG R:CAGGGCCTTGGCGAAGA	Olsvik et al.2008
eef1a	79	60	89.0	F: CACTGCGGTGAAGTCCGTTG R: GGGGTCGTTCTTGCTGTCT	Lilleeng et al. 2007
ubi	69	60	91.5	F: GGCCGCAAAGATGCAGAT R: CTGGGCTCGACCTCAAGAGT	Olsvik et al.. 2008

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, 47.

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.

Table S2. List of *myosins* in Atlantic cod genome (gadMor1; accessed April 2013)

Table S2a. Annotated cod *myosins*

Gene name	Abbreviation	Ensembl ID
Cardiac muscle Myosin heavy chain 6 alpha	*b3svj6_gadmo*	ENSGMOG00000020224
Myosin, heavy chain 10, non-muscle	*myh10 (1 of 2)*	ENSGMOG00000013798
Myosin, heavy chain 10, non-muscle	*myh10 (2 of 2)*	ENSGMOG00000003775
*Myosin, heavy chain 11, smooth muscle**	*myh11 (1 of 2)*	ENSGMOG00000005651
Myosin, heavy chain 11, smooth muscle	*myh11 (2 of 2)*	ENSGMOG00000009607
Myosin, heavy chain 14, non-muscle	*myh14*	ENSGMOG00000005568
Myosin, heavy chain 7B, cardiac muscle, beta	*myh7b*	ENSGMOG00000012704
Myosin, heavy chain 9, non-muscle	*myh9 (1 of 2)*	ENSGMOG00000011235
Myosin, heavy chain 9, non-muscle	*myh9 (2 of 2)*	ENSGMOG00000013979
Myosin, light chain 1, alkali; skeletal, fast	*myl1*	ENSGMOG00000000267
Myosin, light chain 2, regulatory, cardiac, slow	*myl2*	ENSGMOG00000017657
Myosin, light chain 4, alkali; atrial, embryonic	*myl4*	ENSGMOG00000011523
Myosin, light chain 7, regulatory	*myl7*	ENSGMOG00000007319
*Myosin, light chain 9, regulatory**	*myl9*	ENSGMOG00000017056
Myosin light chain kinase	*mylk*	ENSGMOG00000013753
Myosin light chain kinase 3	*mylk3*	ENSGMOG00000014610
*Myosin light chain, phosphorylatable, fast skeletal muscle**	*mylpf (1 of 2)*	ENSGMOG00000005541
Myosin light chain, phosphorylatable, fast skeletal muscle	*mylpf (2 of 2)*	ENSGMOG00000013719
Myosin IB	*myo1b*	ENSGMOG00000001491
Myosin IC	*myo1c (1 of 2)*	ENSGMOG00000005827
Myosin IC	*myo1c (2 of 2)*	ENSGMOG00000000051
Myosin ID	*myo1d*	ENSGMOG00000012703
Myosin IE	*myo1e*	ENSGMOG00000015360
Myosin IF	*myo1f*	ENSGMOG00000011050
Myosin IG	*myo1g*	ENSGMOG00000011448
Myosin IH	*myo1h (1 of 2)*	ENSGMOG00000001197
Myosin IH	*myo1h (2 of 2)*	ENSGMOG00000012006

Myosin IIIA	*myo3a*	ENSGMOG00000015600
Myosin IIIB	*myo3b*	ENSGMOG00000002507
Myosin VA (heavy chain 12, myoxin)	*myo5a*	ENSGMOG00000015730
Myosin VB	*myo5b*	ENSGMOG00000019264
Myosin VC	*myo5c*	ENSGMOG00000003867
Myosin VI	*myo6a*	ENSGMOG00000001048
Myosin VI	*myo6b*	ENSGMOG00000010314
Myosin VIIA	*myo7a (1 of 2)*	ENSGMOG00000014332
Myosin VIIA	*myo7a (2 of 2)*	ENSGMOG00000011272
Myosin VIIB	*myo7b (1 of 2)*	ENSGMOG00000019141
Myosin VIIB	*myo7b (2 of 2)*	ENSGMOG00000013078
Myosin IXA	*myo9a (1 of 2)*	ENSGMOG00000019567
Myosin IXA	*myo9a (2 of 2)*	ENSGMOG00000008460
Myosin IXB	*myo9b (1 of 2)*	ENSGMOG00000013427
Myosin X	*myo10 (1 of 2)*	ENSGMOG00000017524
Myosin X	*myo10 (2 of 2)*	ENSGMOG00000015562
Myosin XVA	*myo15a (1 of 2)*	ENSGMOG00000010621
Myosin XVA	*myo15a (2 of 2)*	ENSGMOG00000007696
Myosin XVI	*myo16*	ENSGMOG00000018320
*Myosin XVIIIA**	*myo18a (1 of 2)*	ENSGMOG00000018558
*Myosin XVIIIA**	*myo18a (2 of 2)*	ENSGMOG00000010045
Myosin XVIIIB	*myo18b (1 of 2)*	ENSGMOG00000005484
*Myosin XVIIIB**	*myo18b (2 of 2)*	ENSGMOG00000005499
Myosin XIX	*myo19*	ENSGMOG00000014015

NOTE: * found in the sequenced muscle transcriptome

Table S2b. Unannotated cod *myosins* (Accessed April 2013).

Name	Ensembl ID	BLAST Hit	% Identity
novel*	ENSGMOG00000011194	Myosin heavy chain	96; *Coryphaenoides yaquinae*
novel	ENSGMOG00000009387	Myosin 4-like	85; *Oreochromis niloticus*
novel	ENSGMOG00000001779	Myosin 4	74; *Takifugu rubripes*
novel*	ENSGMOG00000016381	Myosin heavy chain	85; *C. yaquinae*
novel	ENSGMOG00000002171	Myosin 7	89; *T. rubripes*
novel	ENSGMOG00000003030	Slow myosin heavy chain 2	89; *Danio rerio*
novel	ENSGMOG00000004895	Myosin IE	81; *O.niloticus*
novel	ENSGMOG00000008969	Myosin VA-like	84; *O.niloticus*
novel	ENSGMOG00000009348	Myosin 4	79; *O.niloticus*
novel	ENSGMOG00000009352	Myosin XV	74; *T. rubripes*
novel*	ENSGMOG00000009472	Myosin heavy chain	88; *O. latipes*
novel	ENSGMOG00000010893	Myosin X-like	89*; O. niloticus*
novel	ENSGMOG00000011161	Myosin heavy chain	91; *Saurida wanieso*
novel*	ENSGMOG00000015700	Myosin heavy chain	92; *Gadus chalcogrammus*
novel	ENSGMOG00000016449	Myosin heavy chain	97; *G. chalcogrammus*
novel	ENSGMOG00000017570	Myosin X-like	71; *O.latipes*
novel*	ENSGMOG00000006802	Myosin heavy chain	96; *Coryphaenoides acrolopis*
novel	ENSGMOG00000016068	Myosin-4	74; *Ceratotherium simum*
novel	ENSGMOG00000002258	Myosin-7	91; *O. latipes*
novel	ENSGMOG00000016313	Myosin heavy chain	93*; Coryphaenoides cinereus*
novel	ENSGMOG00000003963	Myosin VA	66; *O. niloticus*
novel	ENSGMOG00000009501	Myosin heavy chain, fast skeletal muscle	86; *D. rerio*
novel[1]	ENSGMOG00000000857	Myosin polypeptide 6	91; *Salmo salar*
novel[1]	ENSGMOG00000019287	Myosin X-like	84*; O. niloticus*
novel[1]	ENSGMOG00000008991	Myosin light chain kinase smooth muscle	86*; Dicentrachus labrax*

NOTE: * found in the sequenced fast skeletal muscle transcriptome; [1]uncharacterized myosins in Fugu Genome that are found in Cod Genome but unannotated

Table S3. Rhythmicity parameters of *myosin* gene expression in Atlantic cod fast skeletal muscle.

Gene	**Rhythmicity parameters**				
	Period (h)	***Amplitude***	***Peak of expression/acrophase (h)***	***Mesor***	***P value***
myh_tc	24	0.261	23.4	0.444	0.04
myh_n1	24	0.135	19.1	0.675	0.01
myh_n2	24	0.072	9.58	0.668	0.41
myh_n3	24	0.071	4.39	0.434	0.72
myh_n4	24	0.223	16.6	0.583	0.10
myh_n5	24	0.009	17.4	0.675	0.94
myh_n6	24	0.042	2.42	0.641	0.77
myh_n7	24	0.042	1.52	0.535	0.64
myo18a_1	24	0.043	22.6	0.327	0.41
myo18a_2	24	0.122	3.28	0.299	0.12
myo18b_1	24	0.087	23.1	0.530	0.31
myo18b_2	24	0.149	4.32	0.655	0.06
myh11_1	24	0.038	4.08	0.618	0.47
mylpf_1	24	0.106	19.2	0.611	0.57
mylpf_2	24	0.098	19.3	0.561	0.54
myl1	24	0.071	21.4	0.637	0.39
myl9	24	0.114	1.47	0.349	0.31

NOTE: Genes that are in bold font and are underlined displayed rhythmic expression.

Table S4. Correlation of *myosin* expression with clock transcript levels during a daily cycle.

Myosin	Clock genes								
	arntl2	*clock*	*npas2*	*cry2*	*cry3*	*per2a*	*nr1d1*	*nr1d2a*	Test
myh_tc	0.550	-0.183	-0.067	0.883	-0.100	0.067	0.150	-0.450	SRO
myh_n1	0.717	-0.583	-0.767	0.050	-0.400	0.500	-0.750	-0.683	SRO
myh_n4	0.220	-0.093	-0.422	-0.180	-0.002	-0.016	-0.613	-0.323	PPM
myo18a_2	-0.144	-0.053	0.783	0.622	0.895	-0.688	0.070	-0.119	PPM
myo18b_2	-0.383	-0.138	0.777	0.546	0.707	-0.313	0.252	0.301	PPM

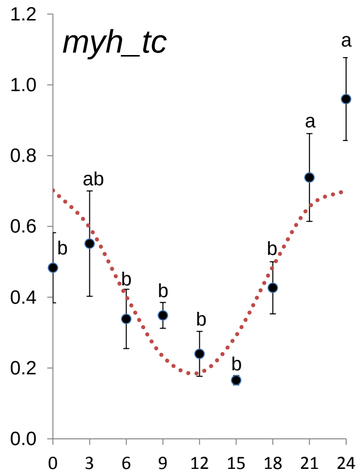
Note: SRO = Spearman Rank Order Correlation; PPM = Pearson Product Moment Correlation

Table S5. Rhythmicity parameters of *myosin* gene expression in 5-day old Atlantic cod fast skeletal muscle explants cultured under different photoperiod regimes.

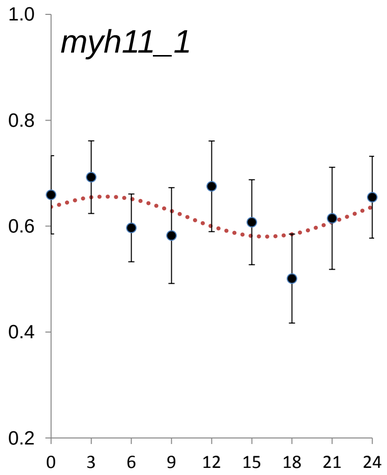
Gene	Photoperiod regime	Rhythmicity parameters				*P* value
		Period	Amplitude	Peak of expression (*h*)	Mesor	
myh_tc	*LD*	24	0.028	23.2	0.514	0.57
	LL	24	0.031	20.3	0.515	0.34
	DD	24	0.031	19.4	0.524	0.43
myh_n1	*LD*	24	0.044	23.4	0.554	0.42
	LL	24	0.030	23.3	0.543	0.48
	DD	24	0.023	23.1	0.555	0.67
myh_n4	*LD*	24	0.076	22.1	0.426	0.36
	LL	24	0.047	21.1	0.568	0.43
	DD	24	0.034	20.0	0.613	0.57
myo18a_2	*LD*	24	0.074	3.11	0.608	0.34
	LL	24	0.058	4.35	0.495	0.47
	DD	24	0.053	5.28	0.489	0.63
myo18b_2	*LD*	24	0.031	4.14	0.651	0.48
	LL	24	0.102	6.01	0.444	0.30
	DD	24	0.040	6.52	0.593	0.53

LD = 12L:12D; **LL** = 24L:0D; **DD** = 0L:24D

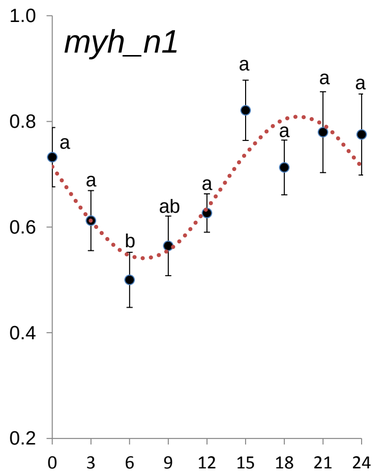
A



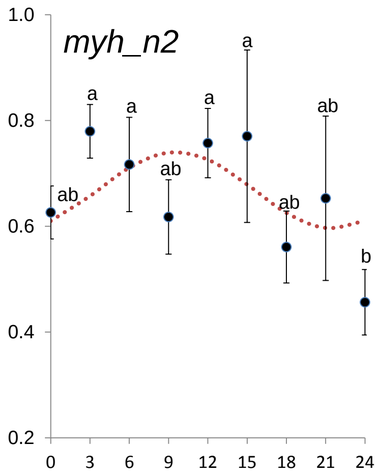
B



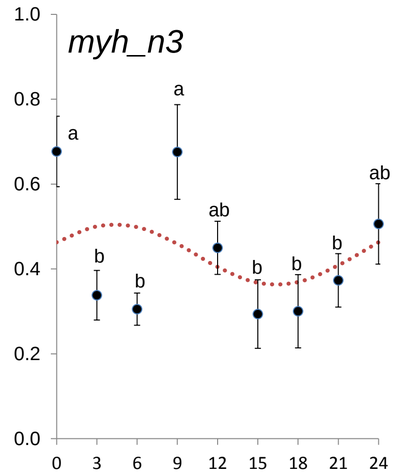
C



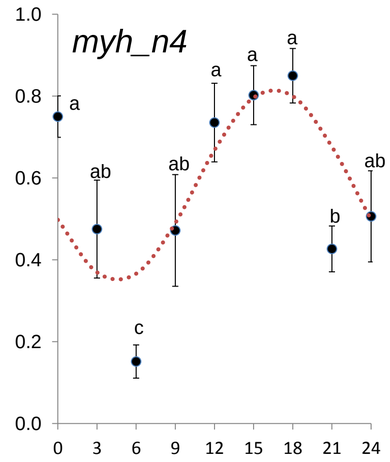
D



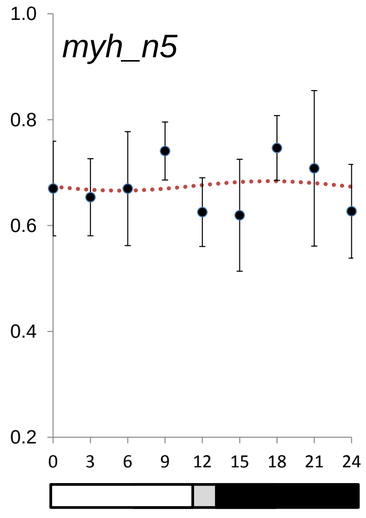
E



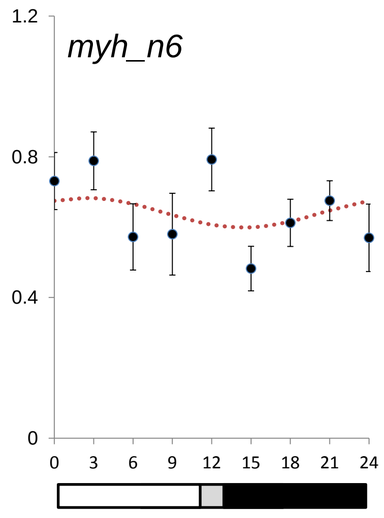
F



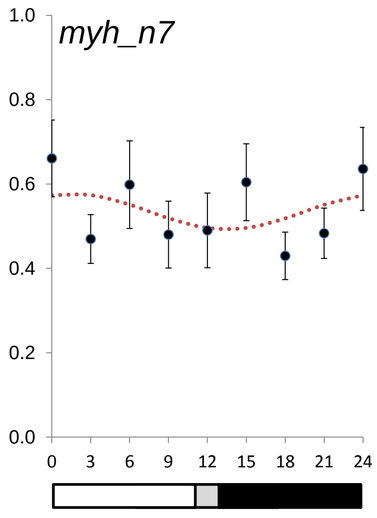
G



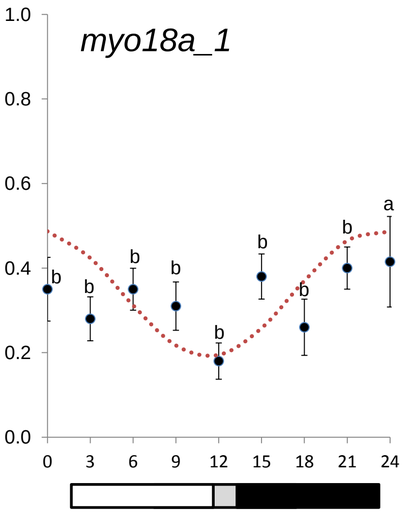
H



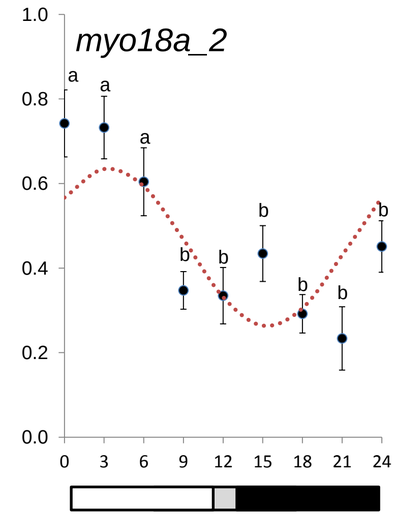
I



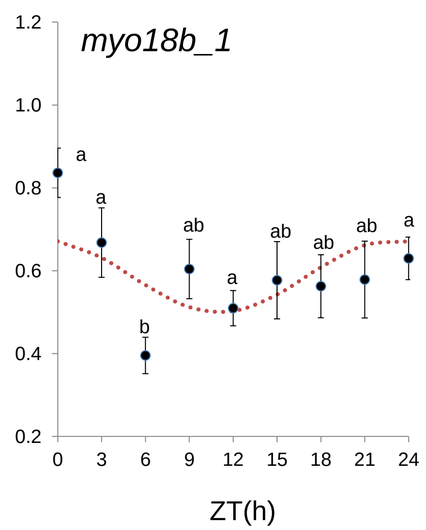
J



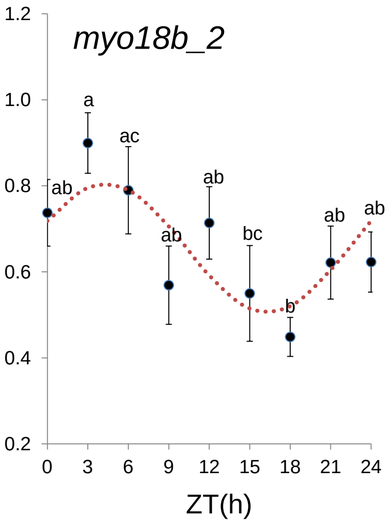
K



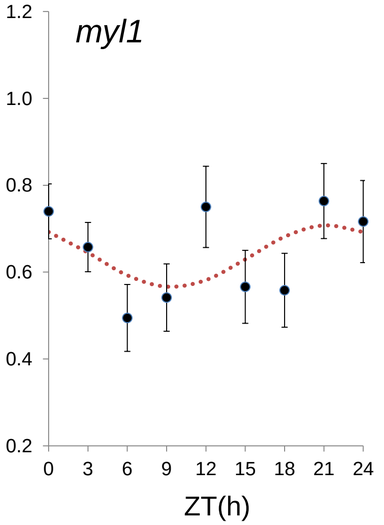
L



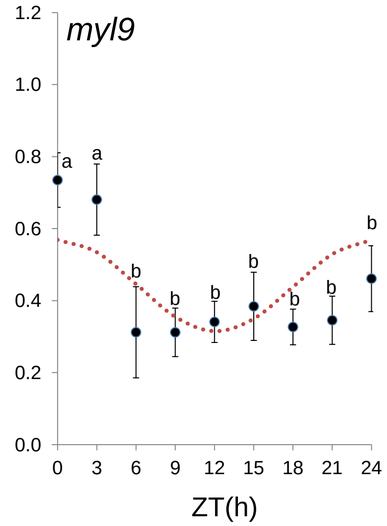
M



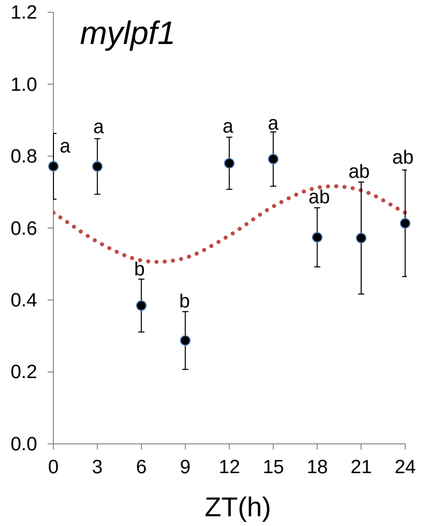
N



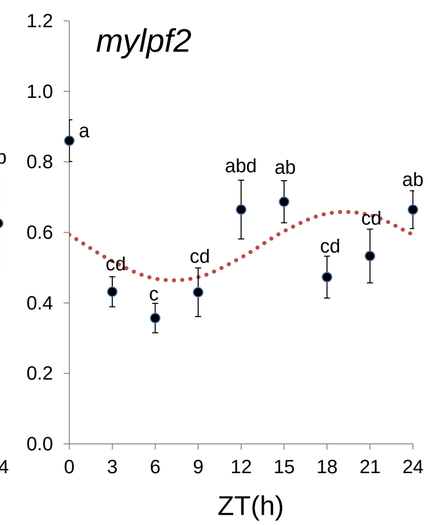
O



P



Q



A
myh_tc
B
myh11_1
C
myh_n1
D
myh_n2
E
myh_n3
F
myh_n4
G
myh_n5
H
myh_n6
I
myh_n7
J
myo18a_1
K
myo18a_2
L
myo18b_1
M
myo18b_2
N
myl1
O
myl9
P
mylpf1
Q
mylpf2
Relative expression
Time (days)
LL (24L:0D)
NL (Bodø, Norway)