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Validation of Endogenous Reference Genes for qPCR Quantification of Muscle Transcripts in Atlantic Cod Subjected to Different Photoperiod Regimes

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1. Introduction

Atlantic cod (*Gadus morhua*, L.) is a commercially important species worldwide and overfishing has contributed to a decline of wild stocks below sustainable levels. This has stimulated aquaculture production of this species, which has increased remarkably over the last decade to over 20,000 tonnes in 2008 (FAO). Nevertheless, cod farming still faces several production bottlenecks related to larval quality, nutrition, diseases and precocious sexual maturation. The early onset of sexual maturation at around two years in farmed conditions seriously restricts the profitability of the industry. Sexual maturation and the subsequent spawning result in loss of appetite, reduced feed conversion and increased mortality rate (Karlsen et al., 2006), which leads to an increase in the production time required to reach the desired harvest size.

Photoperiod manipulation, typified by continuous light illumination, has been used to delay sexual maturation to some extent in several aquaculture species, including Atlantic salmon (*Salmo salar*, L.) (Endal et al., 2000), European sea bass (*Dicentrachus labrax*, L.) (Begtashi et al., 2004) and Atlantic cod (Davie et al., 2003; Hansen et al., 2001; Norberg et al., 2004). The application of continuous light from the summer solstice prior to maturation is thought to mask the photoperiod signal that acts as a trigger for gonadal development and spawning (Davie et al., 2003). Taranger et al. (2006) have shown that gonadal maturation of cod kept in sea cages can be delayed by three to five months through application of continuous broad-spectrum light. In addition to inhibiting sexual maturation, photoperiod manipulation has a direct effect on somatic growth, particularly during juvenile stages (Davie et al., 2007; Taranger et al., 2006). In fact, short-term application of continuous light was found to induce a 5 to 9% increase in body weight when compared to cod reared under normal photoperiod conditions and significant differences can still be observed at harvesting size, nearly three years later (Imsland et al., 2007). In spite of its obvious relevance for the aquaculture industry, the molecular basis of this growth plasticity induced by light cues is not known. In order to better control the precocious sexual maturation of farmed cod, it is crucial to identify the transcriptional networks related to this phenomenon and to understand how they are influenced by photoperiod.

Muscle is the main tissue supporting fish growth. Teleost myogenesis is a complex phenomenon which involves a number of molecules regulating distinct phases of this process. The development and formation of muscle involves either hypertrophy (expansion of muscle fibre by absorption of myoblast nuclei) or hyperplasia (formation of fibres on the surface of an existing muscle fibre) (Johnston, 1999). The progression of muscle formation is associated with the sequential expression of key genes from the myogenic regulatory factors (MRFs) family, which include *myoblast differentiation 1 (myoD), myogenic factor 5 (myf-5), myogenin (myoG) and myogenic factor 6/myogenic regulatory factor 4 (myf-6/MRF4)* (Watabe, 1999). Another molecule of significant importance in muscle development is myosin heavy chain (*myhc*), which serves as marker of muscle development in several studies (Johnston, 1999). *Myhc* genes code for a family of ATP-dependent motor proteins that are involved in muscle contraction (Ikeda et al., 2007). *Myhc* activity can be used to monitor fish growth, since most fish have a continuous hyperplasic growth throughout their lifespan and *myhc* is actively involved in muscle protein synthesis (Dhillon et al., 2009).

Quantification of transcript levels by real-time PCR (qPCR) is currently the method of choice, since it is reliable and sensitive enough to quantify even lowly expressed mRNAs in small amount of target tissues (Bustin, 2002). For example, in tiger pufferfish (*Takifugu rubripes*, Temminck & Schlegel) this technique has been used to validate suppression subtractive hybridization results (Fernandes et al., 2005), to examine how temperature affects expression of the growth-related genes *myoG* (Fernandes et al., 2006) and *forkhead box protein K1 (foxk1)* (Fernandes et al., 2007a) during embryonic development, and to examine differential regulation of splice variants of the master transcription factor *myoD1* (Fernandes et al., 2007b). In spite of its enormous potential, relative qPCR quantification has several pitfalls that must be carefully considered (Bustin and Nolan, 2004). In particular, selection of suitable reference genes with even expression in all samples is critical to normalise qPCR data and the use of non-validated reference genes can lead to erroneous conclusions that are biologically meaningless (Fernandes et al., 2008). It is a general consensus that a versatile reference gene stable under various experimental conditions does not exist. Before proceeding to quantifying the expression of a target gene, it is necessary to select the most appropriate reference genes for each species and tissue for a particular experimental setup. A sensible practice involves testing multiple genes for each experiment and using statistical applications to identify the best combination of the two or three most stable genes that will be used to normalise qPCR data (Andersen et al., 2004; Vandesompele et al., 2002).

The aim of the present research paper was to identify suitable reference genes for relative quantification by qPCR of growth- and maturation-related genes that may be affected by photoperiod manipulation. Five commonly used reference genes were evaluated, namely: *β-actin* (*actb*), *acidic ribosomal protein* (*arp*), *eukaryotic elongation factor 1a* (*eef1a*), *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*) and *ubiquitin* (*ubi*). Their transcript levels in the above fast muscle samples were determined by qPCR using SYBR chemistry. GeNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004) were used to evaluate expression stability of above candidate genes. In addition, to demonstrate the importance of using validated reference genes in qPCR analysis, *myhc* expression was examined. Here, we showed the impact of normalisation strategies (i.e., different individual candidate genes versus the normalisation factor from the two best validated reference genes) on *myhc* expression levels and the necessity of validation to select the most stable reference gene in each experimental plot.

2. Materials and methods

2.1 Photoperiod experiment and sampling

2.1.1 Fish husbandry

Atlantic cod juveniles, *Gadus morhua* L. with an initial size of 2.7 ± 0.8 g (mean ± standard deviation [SD], n=123) were divided into six tanks (250 m^3) with an open flow system at a density of approximately 130 individuals per tank. Sea water was continuously supplied to each tank at 7.4 ± 0.4 °C. A commercial diet (Amber Neptun, Skretting AS, Stavanger, Norway) was provided daily by automatic belt feeders, at 5% (w/w) body weight of the fish and adjusted on a weekly basis.

2.1.2 Photoperiod experiment

Each group of fish in three tanks was either kept under continuous light (LL) or reared under a normal light regime (NL) that corresponded to natural environmental photoperiod conditions in Bodø (67°N), Norway during 6 months from January to July 2010. Day light time was recreated indoors using white light fluorescent tubes (Aura Light International AB, Karlskrona, Sweden) and controlled by a scheduled timer according to local sunrise and sunset times in Bodø. Light intensity on the central surface of each tank was 120 Lux.

2.1.3 Sample collection

The fish were killed by immersion in seawater containing 0.2 $g \cdot L^{-1}$ tricaine methanesulfonate (MS222; Sigma, Oslo, Norway). Fast muscle samples were carefully dissected from the trunk area below the second dorsal fin from six fish at the start of the experiment and 0.5, 1, 7, 30, 60, 120 and 180 days thereafter. Tissues were snap-frozen in liquid nitrogen and stored at –80 °C until RNA extraction.

2.1.4 Ethics statement

All procedures of fish rearing and tissue sampling were in accordance with the guidelines set by the National Animal Research Authority (Forsøksdyrutvalget, Norway).

2.2 Real-time PCR (qPCR)

2.2.1 Primer design

To validate expression stability of reference genes, five candidate genes (*β-actin* (*actb*), *acidic ribosomal protein* (*arp*), *elongation factor 1 alpha* (*eef1a*), *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*), *ubiquitin* (*ubi*) and *myosin heavy chain* (*myhc*)) were selected and analysed for qPCR validation.

2.2.2 RNA extraction and cDNA synthesis

Total RNA was extracted from the fast muscle samples above and used to synthesized cDNA as detailed elsewhere (Campos et al., 2010). Two micrograms of total RNA were used for cDNA synthesis by reverse transcription, following treatment with gDNA wipe out buffer (Qiagen, Nydalen, Sweden) to remove genomic DNA contamination.

2.2.3 qPCR amplification

qPCR reactions were conducted with the primer sets indicated on table 1. Quantification of transcripts were analysed by qPCR with SYBR Green chemistry (SYBR Green I Master, Roche) on a LightCycler® 480 (Roche) as previously described (Campos et al., 2010). Fifty-fold diluted muscle cDNA were run in duplicate, and minus reverse transcriptase and no template controls were included in the reactions. Thermocycling parameters were as follows: 95°C for 15 min, followed by 45 cycles of 15 s at 94°C, 20 s at 60°C and 20 s at 72°C. Five-point standard curves of a 2-fold dilution series (1:1, 1:2, 1:4, 1:8 and 1:16) were prepared from pooled RNA that was reverse transcribed as above. These dilution curves were used to calculate amplification efficiencies of the PCR reactions (Fernandes et al., 2006). Cycle threshold (C_t) values were determined by the LightCycler® 480 software with a fluorescence level arbitrarily set to 1.

Gene	GenBank	Sequence	Size (bp)	E (%)	Reference
actb	AJ555463	Fw: TGACCCTGAAGTACCCCATC Rv: TCTTCTCCCTGTTGGCTTTG	162	77	(Lilleeng et al., 2007)
arp	EX741373	Fw: TGATCCTCCACGACGATGAG Rv: CAGGGCCTTGGCGAAGA	113	86	(Olsvik et al., 2008)
eef1a	CO541820	Fw: CACTGAGGTGAAGTCCGTTG Rv: GGGGTCGTTCTTGCTGTCT	142	84	(Lilleeng et al., 2007)
gapdh	AY635584	Fw: GGTCGCAACCGCAAGGT Rv: TGACCGTTGAGCATTTCCTTCT	83	88	(Hall et al., 2006)
ubi	EX735613	Fw: GGCCGCAAAGATGCAGAT Rv: CTGGGCTCGACCTCAAGAGT	69	87	(Olsvik et al., 2008)
myhc	AY093703	Fw: CAGAAGCTATAAAAGGTGTCCG Rv: GCAGCCATTCTTCTTATCCTCCTC	86	81	(Koedijk et al., 2010)

Table 1. Primers used in this study. Primer sequences, Genbank accession numbers, amplicon sizes and PCR efficiencies are indicated.

2.3 Data analyses

2.3.1 Expression stability analyses

Raw qPCR data were converted to expression level using the above dilution curves. These were then analysed for expression stability using the statistical applications GeNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004).

2.3.2 Statistical analyses

Differences in expression levels of *gapdh*, *actb*, *eef1a*, *arp, ubi* and *myhc* during the photoperiod manipulation experiment were examined by one-way ANOVA with Holm-Sidak post-hoc tests. ANOVA assumptions were checked prior to carrying out the analyses and when the data did not follow the Gaussian distribution or did not meet the equal variance requirements, a Kruskal-Wallis one-way ANOVA on ranks with Dunn's test for post-hoc comparisons was used instead. Statistical analyses were performed with the SigmaStat statistical package (Systat software, London, UK). In all cases, significance was set at $P < 0.05$.

3. Results and discussion

3.1 Validation of reference genes

Specificity of qPCR reactions was confirmed by melting curve analysis, which revealed a single dissociation peak for each gene. Global variation on expression profiles of the candidate reference genes can be observed in Fig.1. Mean C_t values of the candidate reference genes were 19.99, 18.36, 18.03, 17.76 for *actb*, *ubi*, *eef1a* and *arp*, respectively, whereas the median C_t value for *gapdh* (25.96) was above the range of values for the other four genes. Expression of *ubi* showed the least variation across samples, in contrast to *gapdh*, *actb* and *eef1a*.

Detailed expression of the individual reference genes during the 6-month photoperiod manipulation experiment is presented in Fig. 2, showing a differential expression trend between sampling points and photoperiod regimes. There were no significant differences ($P < 0.05$) on the overall expression amongst any of the candidate reference genes (*arp*: $P = 0.997$; *eef1*: $P = 0.735$; *ubi*: $P = 0.124$; *gapdh*: $P = 0.386$; *actb*: $P = 0.554$). It is also important to note that the transcript levels of *actb*, *ubi*, *eef1a* and *arp* were almost the same based on the close range of their C_t values and all showed a perceptible increase on their transcript levels at the last 3 sampling points. In contrast, transcript levels of *gapdh* were relatively lower compared with the other reference genes as characterized by having higher C_t values. There was an apparent difference on the expression of *actb* and *gapdh* between treatments.

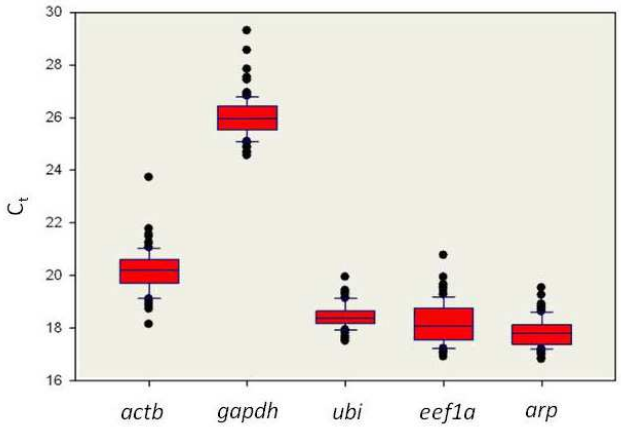

Fig. 1. Overall expression patterns of candidate reference genes in the muscle of Atlantic cod reared under different photoperiod regimes. Raw cycle threshold (C_t) qPCR data of individual reference genes in all samples (n=96) are represented as box-and-whisker plots. Median values are indicated by a solid line inside the boxes.

Expression stability indices of the candidate reference genes as assessed by geNorm varied with time and photoperiod regime (Table 2). *Arp* and *ubi* were identified as the most stable reference genes. Specifically, *arp* was the most stable in fish group under normal photoperiod (0 h, 6 h, 1 d, 1 w, 1 m and 6 m), while *ubi* was the most stable in the group

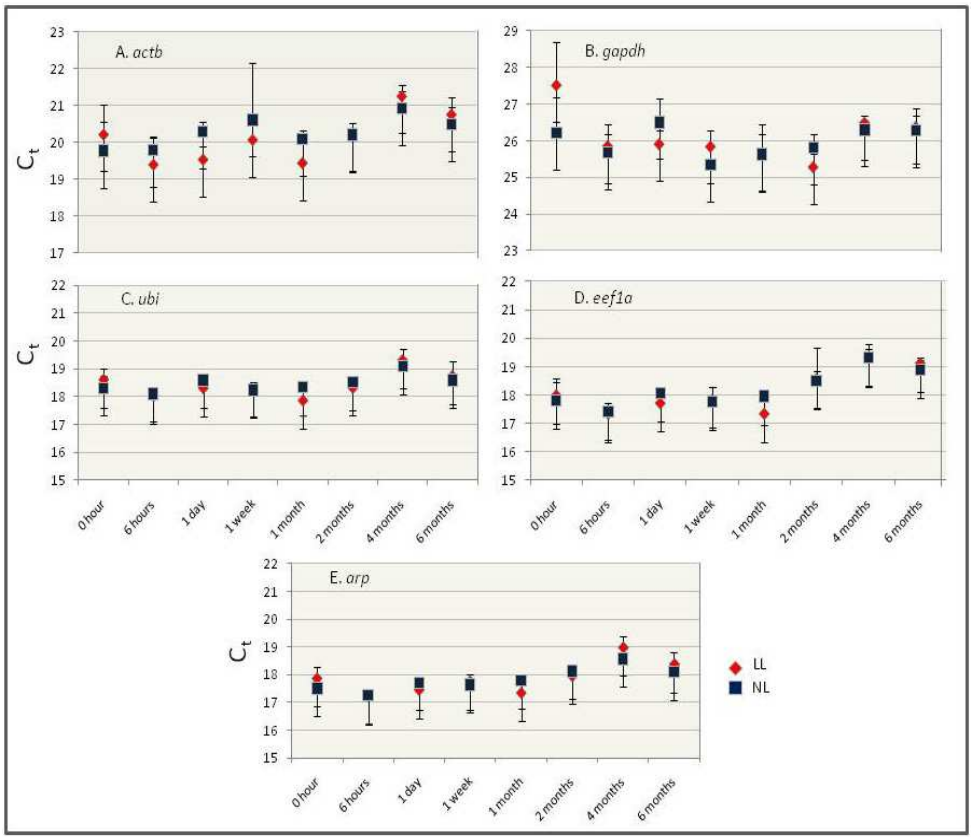

Fig. 2. Expression profiles of potential reference genes in fast muscle of cod kept under two photoperiod regimes: continuous illumination (LL) or normal photoperiod (NL). Data are presented as mean ± SD of the raw cycle threshold (C_t) values of A) *actb*, B) *gapdh*, C) *ubi*, D) *eef1a* and E) *arp* as determined by real-time qPCR (n=6).

reared under continuous light (6 h, 1 d, 1 w, 1 m and 2 m). *Actb* and *gapdh* were two of the least stable reference genes, with *gapdh* as the least stable in 3 sampling points (0 h, 6 h and 1 m) regardless of photoperiod regime. Collating all the stability indices of each reference gene, the order of stability from the most to the least was as follows: *arp* > *ubi* > *eef1a* > *actb* > *gapdh*. Pairwise comparisons revealed that *arp* and *ubi* were the best pair for two-gene normalisation with a joint stability value of 0.138 (Fig. 3A).

The validation software, NormFinder identified *arp* (0.084) and *gapdh* (0.259) as the most and least stable reference genes, respectively. The overall ranking reference genes from the most to the least stable was as follows: *arp* > *ubi* > *actb* > *eef1a* > *gapdh* (Fig. 3B). It was also determined that the best pair of candidate reference genes was *arp* and *ubi* with a joint stability value of 0.084. This pairwise result is similar to the result in geNorm that the best pair for two-gene normalisation was *arp* and *ubi*. It was also found that regardless of photoperiod regimes, *gapdh* was the least stable gene at 0 h, 6 h, 1 m and 4 m, corresponding broadly to the results obtained in geNorm.

		geNorm					NormFinder				
Time	Photoperiod	*arp*	*eef1a*	*ubi*	*actb*	*gapdh*	*arp*	*eef1a*	*ubi*	*actb*	*gapdh*
0 h	LL	0.389	0.425	0.401	0.445	0.862	0.023	0.031	0.025	0.001	0.342
	NL	0.372	0.389	0.381	0.464	0.602	0.027	0.016	0.034	0.049	0.145
6 h	LL	0.363	0.349	0.329	0.640	0.665	0.001	0.002	0.001	0.174	0.190
	NL	0.159	0.171	0.206	0.166	0.326	0	0.003	0.013	0.001	0.047
1 d	LL	0.182	0.189	0.153	0.258	0.195	0.007	0.007	0.001	0.026	0.010
	NL	0.298	0.357	0.313	0.356	0.602	0.010	0.025	0.013	0.013	0.160
1 w	LL	0.264	0.347	0.250	0.430	0.388	0.004	0.032	0.001	0.069	0.051
	NL	0.540	0.623	0.544	1.390	0.676	0.001	0.072	0.001	0.904	0.094
1 m	LL	0.287	0.277	0.275	0.367	0.605	0.014	0.009	0.009	0.020	0.164
	NL	0.230	0.298	0.231	0.295	0.542	0	0.022	0	0.013	0.134
2 m	LL	0.389	0.946	0.367	0.382	0.429	0.021	0.419	0.009	0.007	0.010
	NL	0.173	0.193	0.179	0.166	0.184	0.009	0.012	0.011	0.004	0.008
4 m	LL	0.210	0.225	0.239	0.224	0.321	0.008	0.010	0.016	0.006	0.044
	NL	0.188	0.239	0.186	0.316	0.313	0	0.011	0	0.039	0.039
6 m	LL	0.329	0.332	0.387	0.469	0.390	0.020	0.006	0.053	0.085	0.033
	NL	0.180	0.208	0.192	0.252	0.350	0	0.004	0.004	0.016	0.052

Table 2. Expression stability indices of the five reference genes, as determined by geNorm and NormFinder applications. Relative mRNA level were determined in fast muscle of cod kept under two photoperiod regimes: continuous illumination (LL) and normal photoperiod (NL). The most and the least stable reference genes are shaded in blue and red, respectively.

In order to obtain more robust conclusions it is advisable to do a parallel validation of reference gene stability using different alternative software applications, since there is no each method uses different mathematical models that can lead to different outputs. For example, NormFinder takes all candidate reference genes into account and ranks them with the intragroup and intergroup variation, whereas geNorm sequentially excludes the worst gene ending with two and ranks genes with the degree of similarity of expression. The overall results from NormFinder and geNorm applied to our data revealed that the most stable genes were *arp* and *ubi* and these two is the most suitable pair for two-gene normalisation. In previous qPCR studies in cod, it was also shown that *arp* and *ubi* were the most stable genes and could be used in studying wild populations of cod living in contaminated areas (Olsvik et al., 2008) and ontogeny in cod larvae (Sæle et al., 2009). To optimise the results in selecting the most suitable reference gene, candidate genes should belong to different biological pathways, so as to minimise errors associated with co-regulation. Co-regulation is still possible between *ubi* and *arp*, since they fall on the same biological pathway as important molecules in protein degradation and elongation step of protein synthesis, respectively. However, since these genes were validated by two applications generating similar results, it is fair to consider them as stable in this experimental setup. Both applications identified *gapdh* as the most unstable reference gene. It has been observed that *gapdh* is regulated under varying physiological conditions, which could render this gene inappropriate as reference gene (Olsvik et al., 2005). In a study in cod where candidate reference genes were evaluated during ontogeny with emphasis on the development of gastrointestinal tract, *gapdh* was also rated as one of the least suitable normalisation genes regardless of categorisation and analysis (Sæle et al., 2009). It is also mentioned that in cod, there are two isoforms of *gapdh*, one that is muscle-specific and the other as a brain-specific. Validation of reference genes in cod exposed to thermal stress revealed that *gapdh* was also the least favourable gene for normalisation (Aursnes et al., 2011).

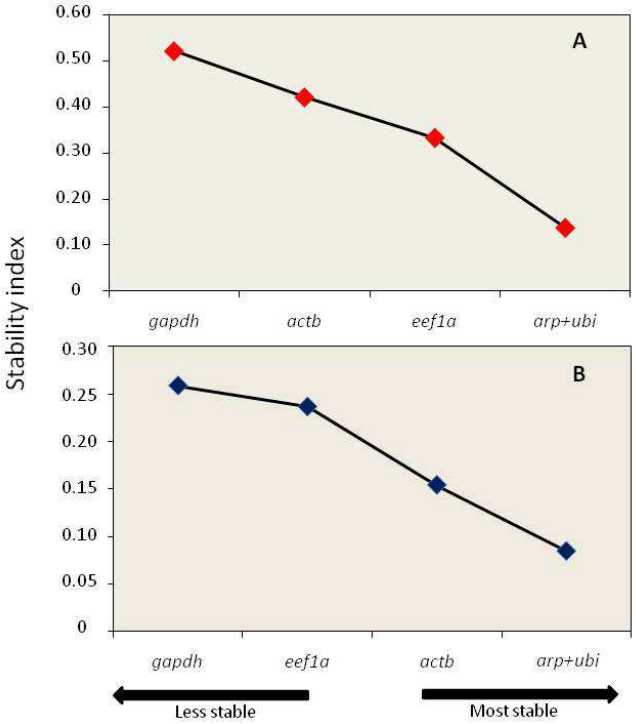

Fig. 3. Ranking of reference genes according to their expression stability in fast muscle of Atlantic cod reared under different photoperiod regimes. The average expression stability values were calculated with (A) geNorm and (B) NormFinder.

3.2 Influence of normalisation on photic-induced expression of *myhc* in cod muscle

Myosin is a ubiquitous eukaryotic motor that interacts with actin to generate the force for cellular movements as diverse as cytokinesis and muscle contraction (Cheney et al., 1993). This motor protein accounts for the majority of myofibrils, which themselves make up to two-thirds of muscle protein synthesis (Mommsen, 2001). For this reason, *myhc* has been used to study muscle growth and development in teleosts (Johnston, 2001). Environmental stimuli such as light influence most of the physiological processes in fish and muscle development is not an exception. The influence of photoperiod manipulation on the muscle physiology of Atlantic cod was assessed in this study by profiling the expression of this gene during a photoperiod manipulation experiment.

For comparison, raw expression data of *myhc* were normalised in two different ways: i) with the use of a two-gene normalisation factor from the most stable genes (*arp* and *ubi*) and, ii) with the least stable reference genes (Fig. 4). Using the best two-gene normalisation factor from geNorm, it was observed that from 0 h to 1 week the expression of *myhc* did not change significantly in either photoperiod regime. However, after a month of photoperiod manipulation, a significant difference was noted between treatments and the group exposed to normal photoperiod showed a significantly higher *myhc* expression than the group

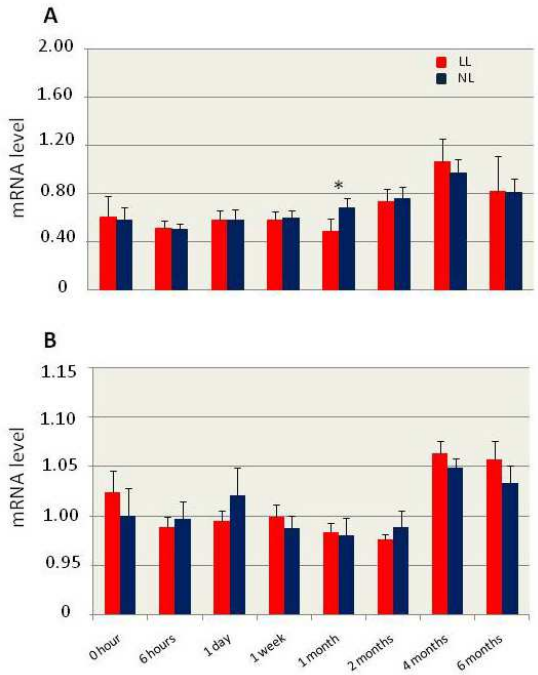

Fig. 4. Expression of *myhc* in the fast muscle of Atlantic cod subjected to different photoperiod regimes. A) Transcript levels of *myhc* gene normalised using *arp* and *ubi*, the best combination for a two-gene normalisation. B) Transcript levels of *myhc* gene normalised by the least stable reference gene, *gapdh* using $\Delta\Delta C_t$ method, Data are shown as mean ± SD of the normalised values (n=6). Asterisk (*) indicates that a significant difference was detected between photoperiod treatments ($P < 0.001$).

exposed to continuous light. Expression of *myhc* in the natural photoperiod group was approximately 40% higher than the expression in the continuous light group. From 2 to 6 months, *myhc* expression increased equally in both photoperiod regimes.

No significant difference was noted between light regimes throughout whole photoperiod manipulation experiment when *myhc* expression data were normalised using *gapdh*, the least stable reference gene. This stresses the importance of identifying suitable reference genes for a particular biological system, not only to draw robust conclusions but also to identify subtle and important differences in mRNA levels.

4. Conclusions

Though morphometric analysis is still an acceptable strategy in studying muscle growth in fish, molecular approaches have opened a new set of possibilities to study this phenomenon and to understand the role of key regulatory molecules in myogenesis. qPCR analysis is the most reliable method to quantify gene expression, provided that suitable reference genes are used for data normalisation. To the best of our knowledge, this report represents the first

validation of reference genes for qPCR quantification of muscle transcripts in Atlantic cod reared under different photoperiod regimes.

NormFinder and geNorm identified *ubi* and *arp* as the most suitable gene pair to normalise our expression data. Using this two-gene normalisation factor, a 40% difference in *myhc* transcript levels was observed between photoperiod conditions, which was not detected when data were normalised with *gapdh*. Therefore, it is clear that using inadequate reference genes for normalisation of qPCR data can lead to biologically meaningless conclusions.

This study represents a valuable resource for future gene expression studies aimed at investigating the molecular mechanisms of the photic-plasticity of muscle development in Atlantic cod. Moreover, it is applicable to more general related topics in aquaculture research, including growth and nutrition.

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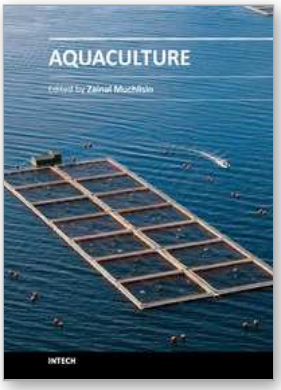

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This book provides an understanding on a large variety of aquaculture related topics. The book is organized in four sections. The first section discusses fish nutrition second section is considers the application of genetic in aquaculture; section three takes a look at current techniques for controlling lipid oxidation and melanosis in Aquaculture products. The last section is focused on culture techniques and management, ,which is the larger part of the book. The book chapters are written by leading experts in their respective areas. Therefore, I am quite confident that this book will be equally useful for students and professionals in aquaculture and biotechnology.

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