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Short communication

Influence of continuous light treatment on expression of stress biomarkers in Atlantic cod

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ABSTRACT

Continuous light treatment during early juvenile stages in *Gadus morhua* is a common farming management practice but the effects of these unnatural light conditions on fish stress have received scant attention. In the present study we investigated how continuous illumination affects transcription levels of key stress-related and antimicrobial peptide genes in juvenile Atlantic cod. Gene expression quantification by real-time PCR revealed higher levels of transcripts coding for antioxidant enzymes, namely *superoxide dismutase*, *catalase* and *glutathione reductase* in liver of fish reared under continuous illumination, concomitantly with a 43% decrease in glutathione content. Transcription of antimicrobial peptides such as *piscidins, hepcidin* and *cathelicidin* was also affected by constant illumination. Overall, the significant changes in liver transcript levels of these biomarkers in response to continuous light may be an adaptation to light stress.

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1. Introduction

Photoperiod control is one of the most common methods applied to delay sexual maturation in many economically important farmed fish, including Atlantic cod (*Gadus morhua* L.), with the aim of preventing weight loss and reduction in flesh quality (Karlsen et al., 2006). It is well documented that the use of continuous light during specific life stages, can stimulate somatic growth and postpone sexual maturation up to 12 months in cod (Hansen et al., 2001). To date, the effect of these unnatural light conditions on expression of key immune- and stress-related genes has not been investigated. Nevertheless, studies on other vertebrates have demonstrated light toxicity at several levels in cells. In rat harderian gland subjected to continuous light, the temporal differences in glutathione reductase (Gsr) and catalase (Cat) activities between circadian phases were markedly diminished suggesting an involvement of melatonin in the antioxidant enzyme activities (Tomas-Zapico et al., 2003). Wood et al. (2008) studied the effects of visible light on cultured rat RGC-5 cells and demonstrated DNA break down and a significant increase of reactive oxygen species (ROS) production. Further, alterations in photoperiod have profound effects on testosterone, estradiol, and cortisol levels, all hormones implicated in the immune cell function (Kaattari and Ottinger, 2000). In fish, alterations in the natural light can also cause stress and compromise their welfare and general well-being (Wendelaar Bonga, 1997). It is known that prolonged changes in the natural photoperiod adversely affect the immune response of rainbow trout, but the fish rapidly resume normal function after returning to a normal photoperiod (Leonardi and Klempau, 2003).

The aim of this study was to investigate how continuous light affects the stress status of cod by using a set of stress biomarkers on two groups of juvenile Atlantic cod reared under different photoperiod conditions. Several biomarkers have been frequently used to evaluate a range of toxic effects in fish due either to pollutants in natural aquatic environments (Brunelli et al., 2010; De Domenico et al., 2013; Fasulo et al., 2012) or to controlled pollutant exposure in laboratory (Brunelli et al., 2011). We have hypothesized that light can be an additional factor leading to enhanced ROS production in cells. Hence, transcript levels of genes coding for enzymes involved in detoxification of free radicals, such as *superoxide-dismutase-1* and *superoxidedismutase-2* (*sod1–2*), *gsr* and *cat*, were evaluated by real-time PCR. Moreover, the mRNA expression rate of a panel of antimicrobial polypeptides (*cathelicidin-1*, *hepcidin* and *piscidin-1* and *-2*) was also investigated.

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2. Materials and methods

2.1. Photoperiod manipulation

The experiment was performed as detailed in our previous sister papers (Giannetto et al., 2013; Nagasawa et al., 2012). Briefly, 6 month old juveniles Atlantic cod were reared under a simulated natural photoperiod (NL group) for Bodø, Norway (67°N, 14°E) or exposed to continuous light (LL group) in three replicate 250 m^3 tanks for each experimental group, from January until May 2010. A commercial diet (Amber Neptun, Skretting AS) corresponding to 5% (w/w) of the fish body weight was daily provided by automatic belt feeders. Sea water was pumped from 200 m depth at a nearly constant temperature of 7 °C and oxygen saturation was above 85%. Fluorescent white light tubes (Aura Light International AB) were used to illuminate the tanks evenly, with a light intensity of approximately 120 lx near the water surface in the centre of the tanks. Liver samples of nine fish from each photoperiod group (three individuals from each tank) were collected at the start of the experiment and after 8, 24 h, 7 days, 1, 2 and 4 months thereafter. At each sampling the fish were anesthetized with 0.5 g L^{-1} tricainemethanesulfonate (Sigma, Oslo, Norway) and liver tissue was carefully dissected, snap-frozen in liquid nitrogen and stored at −80 °C until RNA extraction. The mean weights of the fish throughout the experiment are reported in Supplementary Table S1. All procedures were conducted in accordance to the guidelines set by the National Animal Research Authority (Forsøksdyrutvalget, Norway).

2.2. cDNA synthesis and quantitative real-time PCR (qPCR)

Total RNA was isolated from Atlantic cod liver collected at each sampling point using Qiazol (Qiagen), according to the manufacturer's instructions. cDNA synthesis was performed with the QuantiTect reverse transcription kit (Qiagen) after the evaluation of RNA integrity, concentration and purity as detailed elsewhere (Hagen et al., 2009). Gene expression quantification by real-time PCR was performed using the Rotor-Gene Q 2plex Hrm thermocycler (Qiagen) with SYBR Green chemistry (Qiagen) and gene-specific primers for selected oxidative stress (*catalase*, *glutathione reductase*, *superoxidedismutase-1* and *superoxidedismutase-2*) and health (*cathelicidin-1, hepcidin, piscidin-1* and *piscidin-2*) biomarkers (Supplementary Table S2). The *elongation factor* (*eef1a*), *acid ribosomal protein* (*arp*) and *ubiquitin* (*ubi*) were used as reference genes, since they had been previously validated by Nagasawa et al. (2012). Twenty-fold diluted liver cDNA samples were run in duplicate. No template and minus reverse transcriptase controls were included in each plate. The PCR efficiency was determined as described by Fernandes et al. (2006). Specificity of the reactions was confirmed from single-peak melting curves and amplicon sequencing.

2.3. Glutathione determination

The liver tissue was homogenized with 10 mM Tris–HCl buffer (pH 7.4) at a ratio of 0.9 ml of buffer per 0.1 mg of sample. After centrifugation at 9000*g* for 15 min at 4 °C, the supernatants were immediately analyzed for glutathione (GSH) content by spectrophotometric determination of the reduction of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) at 412 nm, as reported by Ellman (1959). The content of GSH in liver, expressed as μg of GSH per mg of protein, was determined from a standard acetyl cysteine (SH) calibration curve. Soluble proteins were quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific) and bovine serum albumin (PAA Laboratories, pH 7.0) as a standard.

2.4. Statistical analysis

Two-way analysis of variance followed by Student–Newman–Keuls post-hoc tests was performed in SigmaPlot (Systat software) to assess the statistical significance of any differences in GSH content, as well as in transcript levels of antioxidant enzymes and antimicrobial peptides between the two photoperiod groups. *P* values less than 0.05 were considered significant.

3. Results and discussion

3.1. Oxidative stress biomarkers

Continuous illumination was associated with changes in liver transcript levels of several genes that have traditionally been used as to quantify oxidative stress in fishes, including *cat*, *gsr*, *sod1* and *sod2* (van der Oost et al., 2003; Winston and Digiulio, 1991).

Cat mRNA levels were higher values in both the experimental groups early after just 8 h of exposure suggesting that this gene could be influenced by the cod circadian rhythm, as reported in zebrafish (Hirayama et al., 2007). Nevertheless, it is also plausible that stress contributed to the observed increase in *cat* transcription. *Cat* mRNA levels were 1.6- and 3.2-fold higher in the continuous light group (LL) than in the NL control at 2 and 4 months of treatment, respectively (Fig. 1A). The cytoplasmic *sod1* transcript levels did not vary significantly with time or light treatment throughout the experiment (Fig. 1B). In contrast, the *sod2* paralogue was generally up-regulated in the LL group, except for the 24 h time point. *Sod2* transcript levels were 1.9-, 2.4- and 2.5-fold higher in fish exposed to continuous illumination compared to the NL group at 1, 2 and 4 months, respectively (Fig. 1C). The *gsr* gene was expressed at higher levels in the LL group at all sampling points and the differences were particularly marked at 24 h and 2 months (nearly 4-fold higher than NL controls) (Fig. 1D). Statistically significant differences in *cat*, *sod2* and *gsr* transcript levels within the first 24 h of the experiment may be related to circadian rhythmicity.

Overall, transcript levels of genes involved in detoxification of free radicals are higher in fish reared under continuous illumination. This genes up-regulation may indicate an elevated antioxidant status in an attempt to neutralise the impact of ROS generation following the light exposure. The different trend between the cytoplasmic *sod1* and mitochondrial *sod2* gene expression is in accordance with previous studies (Fukui and Zhu, 2010). Since melatonin has a strong antioxidant action (Herrera et al., 2007), it is plausible that the oxidative stress is also related to decreased melatonin synthesis due to the lack of darkness in fish subjected to continuous light.

GSH content was not significantly affected by light treatment up to 24 h but after 1 week of exposure to continuous light, the GSH content was 43% lower in the LL group than in control fish. This difference decreased gradually to 29.4% and 24% at 1 week and at 1 month, respectively, reaching a minimum of 17.5% at the end of the experiment (Fig. 1E). It has been proposed that at moderate concentrations, ROS play an important role as regulatory mediators in signaling processes. Many of the ROS-mediated responses, such as those linked to the glutathione system, protect cells against oxidative stress and reestablish "redox homeostasis". The significant decrease of GSH in fish from the LL group compared to controls is consistent with the role of GSH in neutralizing excessive ROS and suggests an increased ROS production in Atlantic cod subjected to continuous illumination.

3.2. Antimicrobial peptides

It is known that several fish antimicrobial peptides are down-regulated by chronic stress (Noga et al., 2011). The present study

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