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RESEARCH ARTICLE

Environmental, population and life-stage plasticity in the visual system of Atlantic cod

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ABSTRACT

The visual system is for many fishes essential in guiding behaviors, such as foraging, predator avoidance and mate choice. The marine environment is characterized by large spatio-temporal fluctuations in light intensity and spectral composition. However, visual capabilities are restricted by both space limitations set by eye size and by the genomic content of light-absorbing opsin genes. The rich array of visual opsins in teleosts may be used differentially to tune vision towards specific needs during ontogeny and to changing light. Yet, to what extent visual plasticity is a pre-programmed developmental event, or is triggered by photic environment, is unclear. Our previous studies on Atlantic cod revealed an evolutionary genomic loss of UV-sensitive *sws1* and red-sensitive *lws* opsin families, while blue-sensitive *sws2* and green-sensitive *rh2* opsins had duplicated. The current study has taken an opsin expression approach to characterize visual plasticity in cod towards different spectral light during the larval stage, to maturation and extreme seasonal changes in the Barents Sea. Our data suggest that opsin plasticity in cod larvae is controlled by developmental programme rather than immediate light environment. The lack of expressional changes during maturation suggests a less important role for visual modulation related to mate choice. Although no seasonal effects on visual opsins were detected in migratory Northeast Arctic cod, the expressed opsin subset differed from the more stationary Norwegian coastal cod described in previous studies. Interestingly, these data provide the first indications of a population difference in actively used visual opsins associated with cod ecotypes.

KEY WORDS: Opsin, Retina, Larvae, Maturation, Population, Northeast Arctic cod

INTRODUCTION

The marine light environment rapidly changes with depth, mainly due to light being absorbed and scattered by the water and its components, which is a sharp contrast to life on land (Partridge and Cummings, 1999). The dynamic light environment has put pressure on a variety of visual adaptations that have both genetic and environmental influences (Hofmann and Carleton, 2009; Hofmann et al., 2010; Hunt et al., 2004; Partridge and Cummings, 1999). The eye size sets spatial limits to visual capabilities and demands strict prioritization in time and space (Evans and Browman, 2004; Moran et al., 2015). Consequently, many fishes have specialized vision for specific photic environments, and may also change visual capabilities during the course of development correlated to altered light ecology (Evans and Browman, 2004). Comparative studies have indicated that the light environment is important for the evolution of color vision, yet cannot alone account for the mechanisms underlying this correlation (Boughman, 2001; Fuller et al., 2004, 2010; Lythgoe et al., 1994; Seehausen et al., 2008; Travis and Reznick, 1998).

The signaling process of light clues used for vision is complex and involves light transmission, retinal reception and integration, then higher-order processing by the brain, ultimately leading to a response in animal behavior (Endler, 1992; Fuller et al., 2010; van der Sluijs et al., 2011). Environmental light may influence this process in three ways: (1) immediate effects on signal propagation and transmission; (2) induce variation of visual perception due to developmental plasticity; and (3) lead to genetic differences among species and populations due to history of selection in different habitats (summarized by Fuller et al., 2010). Interactions may involve developmental plasticity, genetics, rearing environment and immediate environment (Fuller et al., 2010).

Visual perception is largely dependent on the structure and function of the retina where rod and cone photoreceptors are the functional units (Reid and Usrey, 2008). Whereas the range of light spectra that is visible to a given species is determined by the variety of cone opsin genes expressed, the ability to sense low-intensity light requires rods expressing the rhodopsin pigment (Yokoyama, 2000a). Hence, the visual pigment component, opsin, has an essential role of directly translating light information (photons) from the outer environment to generate an image projected to the brain. The cone opsins used for color vision are distinguished into separate classes based on distinct spectral sensitivities within the UV [SWS1, maximum wavelength (λ_{max}) 350–440 nm], blue (SWS2, λ_{max} 430–470 nm), green (RH2, λ_{max} 460–530 nm) and red (LWS, λ_{max} 520–575 nm) range of the spectra (Yokoyama, 2000a). Based on the opsin sequence and expression patterns, one can make assumptions about visual color sensitivity and, in some cases, even visual-guided behaviors such as foraging, predator avoidance and mate choice (Fuller and Claricoates, 2011; Fuller and Johnson, 2009; Fuller et al., 2010; Hofmann and Carleton, 2009; Horth, 2007).

Although the genomic array of opsins restricts the potential of light discrimination and sensitivity, adaptive phenotypic plasticity may further locally adapt species or populations to different light (Kawecki and Ebert, 2004; Larmuseau et al., 2009, 2010; Spady et al., 2005; Yokoyama, 2000b). Comparative studies have shown that changes in opsin expression may be used to tune visual sensitivity (Carleton and Kocher, 2001; Hofmann and Carleton, 2009; Spady et al., 2006). As a consequence, visual systems are often under strong natural selection, and phenotypic plasticity in visual systems may help organisms adjust to changing conditions (Hofmann and Carleton, 2009). One example is Rainbow trout

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(*Oncorhynchus mykiss*) that experiences both loss and gain of UV vision through degeneration/regeneration of UV cones timed to sea–river migration (Allison et al., 2006). Phenotypic plasticity has also been shown to have evolutionary consequences as it facilitates colonization of novel habitats, and the synergistic change in environment and sensory systems can promote population differentiation and speciation (Price et al., 2003; Seehausen et al., 2008). A less dramatic change in sensitivity includes differential chromophore usage, where a switch of vitamin A1 and A2 has been reported in fish migrating between freshwater and marine habitats (Bowmaker et al., 2008; Carleton, 2009; Enright et al., 2015; Temple et al., 2006; Toyama et al., 2008).

The teleost visual system of fishes is particularly diverse and likely reflects environmental heterogeneity, including variety in light (Levine and MacNichol, 1982). In our previous work on Atlantic cod, we elucidated the genetic basis and developmental plasticity of opsin expression (Valen et al., 2014, 2016). Interestingly, we found that cod has lost SWS1 and LWS opsins, sensitive to UV and red light, respectively. In contrary, both SWS2 and RH2 have tandem-duplicated, resulting in two and three paralogs of each subfamily, respectively (Valen et al., 2014). Comparative studies have shown that having a wide array of opsin gene sets is a typical teleost feature, which is a result of numerous duplication events and retention of favorable gene paralogs (Lagman et al., 2013; Larhammar et al., 2009; Rennison et al., 2012). Studies in cichlids have shown that different light environments have led to the contemporary evolution of visual opsins and expression patterns (Hofmann et al., 2010). Modulation of vision plays a crucial role in tuning towards environmental light and may be achieved through triggering of differential opsin expression (Fuller and Claricoates, 2011). Cichlids show some of the largest known shifts in visual sensitivity that result from modulated expression of seven cone opsin genes (Parry et al., 2005). The mechanisms regulating opsin gene expression are largely unknown and have only recently become more clear (Carleton et al., 2010; O'Quin et al., 2011; Schulte et al., 2014; Takechi et al., 2008). Both genetic architecture and gene regulatory factors are involved in opsin gene regulation (O'Quin et al., 2011; Schulte et al., 2014).

Several fishes undergo natural ontogenetic changes in opsin expression, often suggested to correspond to changes in photic environment (Carleton et al., 2008; Cheng and Flamarique, 2007; Cottrill et al., 2009; Schweikert and Grace, 2017; Shand et al., 2002; Veldhoen et al., 2006). In Atlantic cod, we have shown that *rh1* and *sws2/rh2* opsin gene duplicates are used differentially during development from larval to juvenile transition (Valen et al., 2016). Hence, larval vision is purely driven by color vision, while the ability for low-sensitivity vision appears later on, which is typical for indirect developing species (Evans and Browman, 2004; Evans and Fernald, 1990). The difference in sensitivity among cichlid species has been attributed to heterochronic shifts in developmental opsin programmes (Carleton et al., 2008). Together, indicating that ontogenetic changes in visual opsins are determined by a multitude of factors, such as photic environment, ecology, life strategy and evolutionary history.

Maturation represents a major life event of fishes and, combined with spawning, may be linked to dramatic habitat shifts that affect opsin expression (Allison et al., 2006; Archer et al., 1995). Also, stickleback, cichlids and guppies change visual sensitivity upon mating through differential cone opsin expression (Carleton et al., 2010; Laver and Taylor, 2011; Shao et al., 2014). Still, whether this is developmentally programmed or triggered by environment, or a combination, is unknown.

Efforts to discern apart developmental plasticity of opsin regulation from plasticity towards light changes in fish have so far been focused on a few species (Fuller and Claricoates, 2011; Fuller et al., 2010; Hofmann et al., 2010; Shand et al., 2008). Hence, comparative knowledge including how these operate separately and how they may interact in species with different life strategies is lacking. This could be a key issue as eye development is fundamentally different in most marine species where color vision and scotopic vision is introduced stepwise (Evans and Browman, 2004). In contrast, direct developing fish typically have both visual capabilities functional from early on. A model has been put forth where the ecological versus developmental constraints on the visual system depend on developmental stage upon hatching (Evans and Browman, 2004). Hence, it may be likely that opsin expression plasticity towards immediate light is restricted by developmental programme but may also include 'developmental windows of opportunity' in which tuning towards environment may occur.

Atlantic cod is one of the most important fisheries species in the Northern Atlantic and has a key role as an ecosystem apex predator (Ottersen et al., 2014). Previous studies on Atlantic cod and their response to variation in light environment have mainly focused on foraging, growth, survival and maturation, mostly linked to optimization of aquaculture conditions (Puvanendran and Brown, 2002; Sierra-Flores et al., 2015; Taranger et al., 2006; Vollset et al., 2011). These studies demonstrated that cod responded differently in these traits to various light intensities, wavelength and photoperiod. However, the underlying molecular mechanism of light reception was only recently described by our group (Valen et al., 2014, 2016). The change of visual capabilities in Atlantic cod is likely linked to changes in ecology from planktonic foraging in the epipelagic to active predatory lifestyle in both deep and shallow waters. Previous light experiments suggested a cod population difference in growth and survival in response to varying light (van der Meeren et al., 1994; Van der Meeren and Jørstad, 2001).

In nature, Atlantic cod display divergent feeding behaviors depending on spawning ground, termed ecotypes (Karlsen et al., 2013). Whereas the Norwegian coastal cod (henceforth NC cod) remain more or less stationary, the Northeast Arctic cod (NEA cod) migrates north of the Arctic Circle, an area characterized by dramatic seasonal changes in photoperiod. Genome analyses have associated cod population differences with certain genomic regions, which includes variation within the rhodopsin *rh1* gene itself (Hemmer-Hansen et al., 2013; Pampoulie et al., 2015; Sarvas and Fevolden, 2005). Yet, so far, it is not known whether this may cause a population difference in visual sensitivity due to gene variation or by differential gene regulation.

Recent advances in genome sequencing have given access to the whole genome of several teleosts. This genomic backbone provides the framework for visual function. To understand how various genes are used functionally in the organism and in response to the environment, analysis of gene activity is central. We have taken a gene expression approach focusing on Atlantic cod to gain insight into how a marine teleost uses its opsin gene complement during ontogeny and in response to environmental changes. Previously, we have shown dramatic ontogenetic changes in visual opsin expression profile (Valen et al., 2016). In this study, we attempt to discern apart developmental- and life-history-driven opsin regulation from environmental-driven plasticity and unravel potential population effects. In summary, we will use our previously published methods on visual opsins to: (1) characterize the potential of phenotypic plasticity in NC cod larvae in response to different light regimes; (2) investigate opsin expression during

maturation in NC cod; and (3) characterize expression levels in NEA cod, and compare results with previous data on NC cod. We will also check for potential seasonal tuning in visual opsins in NEA cod.

MATERIALS AND METHODS

Biological material

Fertilized NC cod (*Gadus morhua* Linnaeus 1758) used in the current study for the characterization of different wavelength light on cone opsins were obtained from Parisvannet Research Station, Institute of Marine Research (IMR), Bergen, Norway. Embryos from one egg batch/group were transported to Bergen High Technology Centre at stage 9 days post-fertilization (dpf) and raised in black 15-liter tanks (see Fig. S1 for set-up). All tanks had oxygenated seawater running through, set to 6°C (for set-up, see below). Black tanks have been considered the best for marine larval rearing as they closely represent natural conditions in terms of background and light regime (Duray et al., 1996; Monk et al., 2008). Embryos were kept under similar white light conditions until 17 dpf [equal to 2 days post-hatching (dph)], then split into three replicate tanks with different light regimes (see Fig. S1). The developmental phase of the developing larvae exposed to different light treatments in the current study corresponds to our previous observations of this stage involving dramatic shifts in cone opsins (Valen et al., 2016). The light regime for all treatments was 14 h:10 h light:dark, simulating approximate day length in Bergen, Norway, in March–April. When approaching the time of natural feeding when yolk sac resources were exhausted (17 dpf), larvae were fed daily natural zooplankton enriched with microalgae (*Rhodomonas* and *Isocrysis*) of ∼3000 prey items liter^{-1}. The zooplankton were harvested from Marine Biological Station Espegrend, Department of Biology, Norway. Prior to sampling, cod larvae were transferred to Petri dishes with buffered seawater containing metacaine (MS-222) sedative (Sigma–Aldrich, St Louis, MO, USA) and then to RNAlater® (Ambion, Waltham, MA, USA). Samples were stored at 4°C for 24 h, then transferred to −80°C until further analysis. The necessary permit for the use of larval cod in the current study was obtained from the local IACUC (permit number 6388).

Maturing NC Atlantic cod (∼2.5 years) were donated from Austevoll Research Station, IMR. Prior to sampling, fish were sedated with buffered MS-222 (Sigma–Aldrich) until movement ceased, then euthanized with a blow to the head and bled out by cutting the main artery. Eyes from 11 fish, including six females (average length 57.5 cm) and five males (average length 54.8 cm), were sampled in November 2014. Sex was determined based on gonadal features, and all fish were characterized as maturing following the gonadal staging index proposed by ICES (Bucholtz et al., 2007). The dissected eyes were transferred to RNAlater® (Ambion) for real-time quantitative PCR (qPCR). To allow optimal penetration of RNAlater® (Ambion) through the tissue, incisions were made in the cornea and the lens was carefully removed. Samples were first kept at 4°C (24–48 h), then stored at −80°C.

The NEA cod used for the characterization of seasonal effects on visual opsin expression were obtained as part of research and ecosystem surveillance cruises organized by the IMR associated with Norwegian national fisheries management (for further information: http://toktsystem.imr.no/cruises/). Cod were sampled during the winter survey (N=10, length 9.1–25 cm) in the Barents Sea with bottom trawls (Campelen 1800, St John's, NL, Canada) with the Helmer Hanssen research vessel (12–18 February 2014, cruise id: 1395, nr: 2014202) and during early autumn (N=10, length 19–26 cm) from the ecosystem survey with G.O. Sars research vessel (August and beginning of September 2014, cruise id: 1414, nr: 2014116). Eyes were sampled and treated in a similar procedure as described for NC cod, except that, for NEA cod, the right eyes were transferred to 4% paraformaldehyde–phosphate-buffered saline (PFA–PBS) (Sigma–Aldrich, St Louis, MO, USA) fixative for *in situ* hybridization studies, in parallel with the left eyes being sampled in RNAlater® (Ambion) for qPCR.

Experimental set-up: effect of different wavelength light on cone opsins

In our light treatment experiment, we used five different light regimes on NC cod larvae: white light (LD), continuous light (LL), blue light (B), green light (G), and red light (R). The LD, B, G and R groups followed a 14 h:10 h light:dark cycle, whereas light was kept on (24 h day^{-1}) in the LL group. The light source used was connected LED strips (RGB LED Strip Starter Kit, North Light, Riga, Latvia); see Fig. S1. Both intensity of light (mW m^{-2} nm^{-1}) and wavelength distribution (nm) of each channel (LD, B, G, R) were measured using a RAMSES/SAM-ACC-UV-VIS (350–900 nm wavelength range) irradiance sensor (TriOS GmbH, Rastede, Germany) with associated software MSDA-XE (TriOS, version 8.8.13 2012-06-28). Light measurements obtained from the MSDA-XE software were plotted in Statistica (version 12, Dell Inc., Round Rock, TX, USA). For additional information including spectral distribution, see Fig. S1. Intensity of the LED light could be adjusted in 10 steps, and the step corresponding to approximately the same intensity (∼0.2 mW m^{-2} nm^{-1}) was used in the experiment. In each light treatment, larvae were distributed into three tanks consisting of buckets (15 liters) with plankton mesh in the bottom to allow water circulation (Fig. S1).

RNA extraction, cDNA synthesis and visual opsin expression studies

For the light experiment, total RNA was extracted from pools of 10 larvae each from three replicate tanks (N=10×3) at 7 dph and 12 dph, representing 5 and 10 days of light treatment, respectively. In addition, RNA from a pool of 20 larvae at 2 dph was isolated from the white light tank as an opsin expression reference prior to exposure of the various light regimes. RNA isolation was performed on whole larvae using column-based Total RNA Purification Kit (Norgene Biotek Corp., Thorold, ON, Canada), according to the manufacturer's protocol.

On retinal tissue from NEA cod and maturing NC cod, total RNA was isolated by phenol–chloroform extraction (removed from sclera) as previously described (Chomczynski, 1993; Valen et al., 2014). RNA from all samples was treated with Turbo DNase free kit (Ambion). Synthesis of cDNA single strand was performed on 700 ng of DNase-treated RNA as input, according to Valen et al. (2014). A minus reverse-transcription enzyme (minRT) control was included by pooling RNA from all larval samples and all adult retina samples. In addition to a minRT control, a non-template control was also included in the qPCR. No signals were detected in either control, indicating no genomic contamination. Primers used in qPCR for all visual opsins in cod have previously been published by our group, along with qPCR reaction and cycling conditions (Valen et al., 2016).

Threshold value for qPCR was set manually to a fixed value for all samples, well above baseline fluorescence. Cycle threshold values were efficiency corrected and normalized to an internal housekeeping gene, i.e. *ubuiqitin*, which is ranked as the best out of three tested (*rpl4* and *ef1a*) by the NormFinder algorithm [MDL, 2004, Aarhus, Denmark (Andersen et al., 2004)]. Relative expression of opsins (rather than proportional values) has been suggested to be the best choice for making conclusions concerning which opsins are differentially regulated (Fuller and Claricoates, 2011).

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In order to visualize the spatial retinal pattern of opsin-expressing photoreceptors in NEA cod, we also performed *in situ* hybridization studies in parallel to qPCR on a subset of eyes from the winter survey. The procedure in cod for analyzing visual opsin expression by sectional *in situ* hybridization, including synthesis of opsin-specific probes, has previously been described by our group (Valen et al., 2014). Sections were mounted in 70% glycerol (Sigma–Aldrich) in 1× PBS. Images were taken with a Leica 6000B microscope (Leica Microsystems, Wetzlar, Germany) and contrast/brightness adjusted with Adobe Photoshop CS5 (2010, Adobe Systems Inc., San Jose, CA, USA).

Statistical analysis

All statistical analyses were performed in Statistica 12.0. (Dell Inc.). For the light exposure experiment, the total number of individuals (N) is given for the following stages and treatments: 2 dph: N=20 (20×1 tank), 7 dph (LD/LL/B/G/R): N=30 (10×3 tanks) per treatment, 12 dph: LD; N=10 (10×1 tank), LL; N=30 (10×3 tanks), B; N=20 (10×2 tanks), G; N=10 (10×1 tank), R; N=20 (10×2 tanks). The normalized and efficiency corrected q-PCR CT values were first tested for normality distribution and homogeneity of variance, which are assumptions of analysis of variance (ANOVA) tests (see Valen et al., 2016). ANOVA statistical tests were then used to determine differentially expressed genes between light treatments and stages (one-way ANOVA: treatment, and main-effects ANOVA: treatment×stage). In case of significant ANOVA (P<0.05), Tukey's HSD *post hoc* test and Bonferroni test were used to identify significant differences. As not enough tank replicates were present for all data points (2 dph and 12 dph), the power of the ANOVA test was reduced and results were thus interpreted with some caution. It should be emphasized that ANOVA interprets N=1 for one tank, which represents a pool of at least 10 larvae. For analysis of the effect of NC cod maturation, including different retinal regions on differential expression of visual opsins, a main-effects ANOVA was performed (gene×part of retina×gender). A similar analysis was performed on NEA cod but including seasonal effects (gene×part of retina×season). All of the expressional data were tested for homogeneity of variances using Levene's test and for normality distribution using a Shapiro–Wilk test. In case of significant ANOVA (P<0.05), a Tukey's HSD *post hoc* test and Bonferroni test were performed. See Tables S1–S23 for more detailed information on the statistical analysis.

RESULTS

Visual opsin expression in cod larvae exposed to different spectral light

To investigate the plasticity of the cod retina to various spectral lights during a phase of rapid eye growth, the regulation of visual opsin genes was assessed quantitatively by qPCR. The different spectral light treatments of larvae did not have any significant effect on cone opsin expression after 5 days (7 dph) or after 10 days of treatment

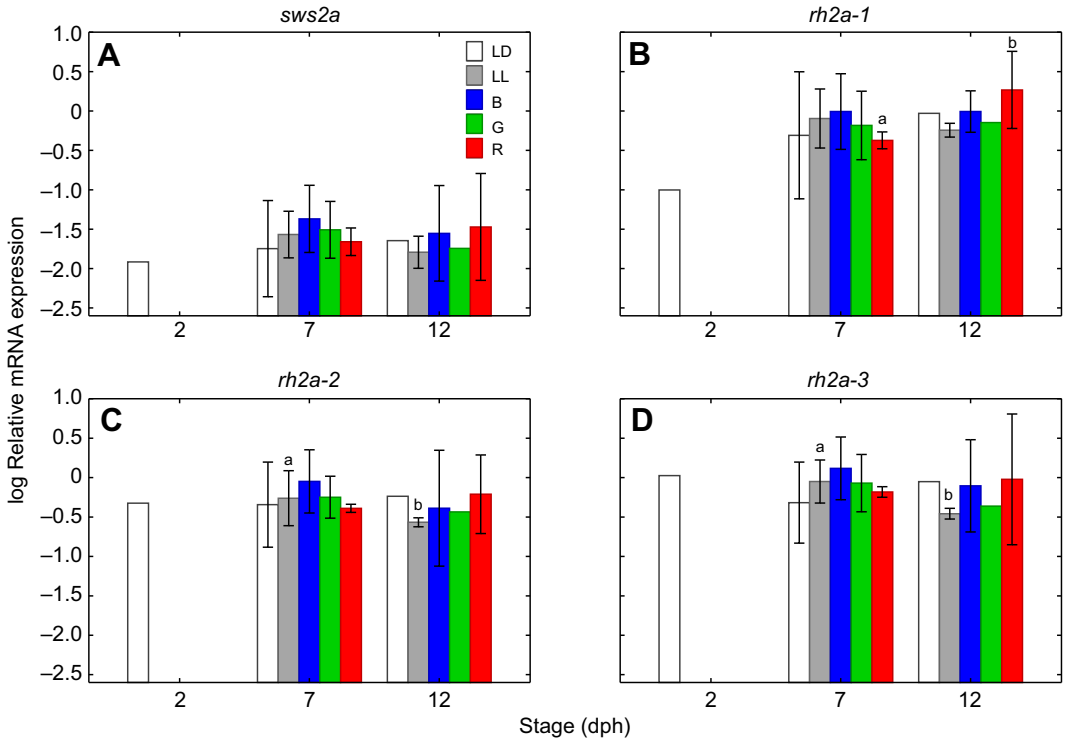

Fig. 1. Different spectral light treatment of cod larvae and effect on cone opsin expression. Cone opsin mRNA expression levels were measured by quantitative real-time PCR (qPCR) using SYBR green assay and opsin-specific primers. The qPCR cycle threshold (Ct) values were efficiency corrected and normalized to an internal housekeeping gene, *ubiquitin*, then plotted as log-transformed values (*y*-axis). To gain a complete picture of potential spectral effects on opsin gene regulation, opsin expression is presented for all cone opsins except *sws2b*, which showed too low expression to be exactly quantified. The 2 days post-hatching (dph) stage represents opsin expression in a pool of 20 larvae from a common tank at the start of feeding and just prior to light treatment. The 7 dph and 12 dph stages represent 5 and 10 days of light treatments, respectively (*x*-axis). The LD group represents white light day/night rhythm (see the Materials and methods section), LL represents constant white light day/night, B represents blue light, G represents green light and R represents red light. Data are presented as average opsin expression±s.d. of a pool of ∼10 larvae in three tanks for 5 days of light treatment. Due to larval mortality, 10 days of light treatment included N larvae: LD, N=10 (10×1 tank); LL, N=30 (10×3 tanks); B, N=20 (10×2 tanks); G, N=10 (10×1 tank); and R, N=20 (10×2 tanks). Different letters indicate statistically different expression (P<0.05) between stages within a light treatment group, using a main-effects ANOVA (treatment×stage), followed by a Tukey's HSD *post hoc* test.

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(12 dph) (Fig. 1). Furthermore, most light regimes included variation in cone opsin expression, resulting from differences in average gene expression between tanks (Fig. 1A–D). For blue-sensitive *sws2a* expression, no significant temporal changes were detected from 2 dph to 12 dph (Fig. 1A). The *rh2a-1* expression increased from 2 dph (LD group) prior to light treatment to 7 dph and 12 dph. This trend of increasing *rh2a-1* expression was seen in all light groups; however, a significant increase (P<0.05) from 7 dph to 12 dph was seen in the red light-treated larvae. *rh2a-2* expression showed less change from the 2 dph to 12 dph stage; however, a significant decrease in expression was found from 7 dph to 12 dph in the constant light (LL) group. The overall *rh2a-3* expression showed a slight decrease from 2 dph to 12 dph and, similar to *rh2a-2*, a significant decrease was detected from 7 dph to 12 dph in the LL group. For a clearer visualization of temporal changes of visual opsins within each light treatment, see Fig. S2. The *sws2b* expression was set to 0 in the current study, as mRNA levels were below the detectable range of qPCR.

Expression of visual opsins during maturation in NC cod

In order to unravel potential effects of maturation, including sex-related differences affecting opsin regulation, expression levels of all visual opsins were investigated by qPCR. By analyzing mRNA expression of visual opsins in maturing 2 year old cod, the highest expressed gene was found to be *rh1*, followed by *rh2a-1* and the least expressed gene was *sws2a* (Fig. 2A). Expression levels of *rh2a-2*, *rh2a-3* and *sws2b* were all below detectable levels. Our comparisons of visual opsin expression between female and male maturing cod did not detect any significant differentially expressed opsins (Fig. 2B). However, the expression levels varied slightly more among male cod compared with females. By comparing opsin expression in dorsal and ventral retina, no topographic differences in opsin expression levels were found (Fig. 2C,D). Yet, this analysis revealed that the opsin expression variance observed in the male group could mainly be attributed to ventral retina (Fig. 2D). No such regional difference in visual opsin expression variance was observed for female fish (Fig. 2C).

Effect of population and season on visual opsin expression in NEA cod from the Barents Sea

As the overall expression pattern of visual opsins in NEA cod has remained unknown, and also to what extent extreme seasonal changes in available light may influence vision through opsin regulation, both quantitative and qualitative analyses were performed. The quantitative assessment of visual opsin mRNA expression by qPCR revealed that the highest expressed gene in NEA cod was *rh1*, followed by *rh2a-1*, *rh2a-2* and *sws2a*, while the lowest expressed gene was *rh2a-3* opsin *(rh1>rh2a-1>rh2a2/sws2a>rh2a3*) (Fig. 3A). The *sws2b* opsin was not expressed in high enough levels to be detected by qPCR. By comparing visual opsin expression in NEA cod sampled in February (winter) and September (early autumn), we could not detect any seasonal effects on opsin gene-expression level (Fig. 3B). However, we did detect a regional difference in *rh2a-2* expression where the ventral retina showed significantly higher expression (P<0.05) compared with the dorsal retina (Fig. 3C,D). This regional difference was detected in NEA cod sampled both during winter and early autumn. The spatial tissue expression patterns of opsins were investigated by *in situ* hybridization studies (Fig. 4A–R), which together supported the

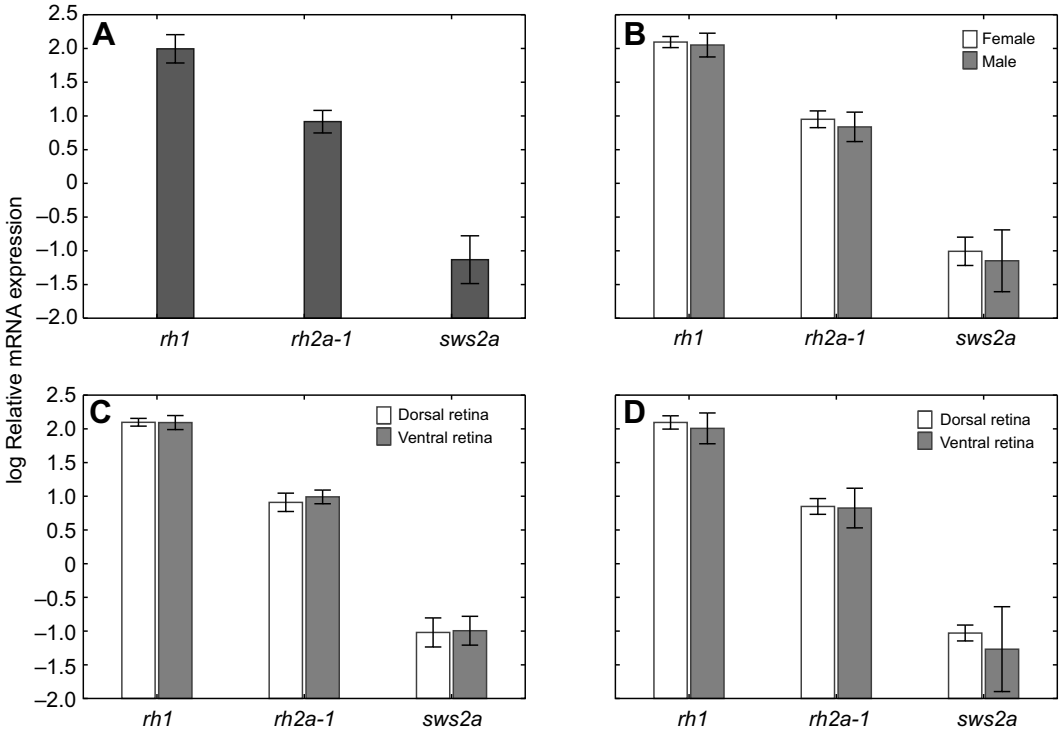

Fig. 2. Visual opsin expression during maturation of male and female Atlantic cod. Visual opsin mRNA expression was measured by qPCR in maturing 2 year old cod to unravel potential opsin regulation during maturation. Expression levels were plotted as efficiency corrected, relative (housekeeping gene *ubiquitin*) and log-transformed values (*y*-axis) for *rh1*, *rh2a-1* and *sws2a* (*x*-axis). (A) Average visual opsin expression for all 2 year maturing fish analyzed. (B) Expression values separated for different sexes. (C,D) Regional visual opsin expression is presented for dorsal and ventral retina in female (C) and male (D) cod. Data are presented for N=6 females and N=5 males (total N=11) as mean expression±s.d. Statistical analysis using main-effects ANOVA did not reveal any significant effects of sexes or retinal regions.

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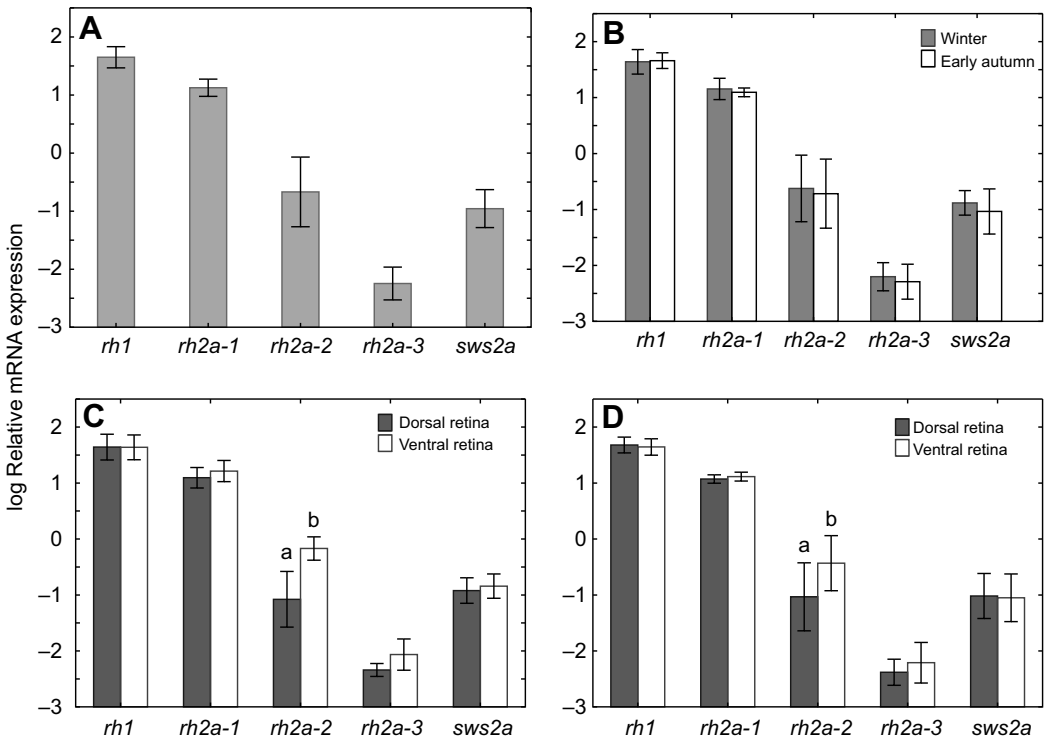

Fig. 3. Visual opsin expression in Northeast Arctic (NEA) cod. (A) To obtain an overview of opsin expression and potential seasonal regulation in NEA cod, opsin mRNA expression levels were quantified by qPCR from eye samples collected during winter and early autumn in the Barents Sea. The bars show efficiency-corrected mRNA expression levels and log-transformed values (*y*-axis) for all visual opsins, except *sws2b*, which was not detected by qPCR. (B) Seasonal expression profiles of visual opsins. (C,D) Regional dorsal/ventral expression during winter (C) and early autumn (D). Different letters in C and D show significant ($P<0.05$) differences in gene expression between retinal regions using main-effects ANOVA. Expression values are represented as means±s.d. for $N=10$ fish sampled during winter and during early autumn (total $N=20$).

quantitative estimations performed by qPCR. While *rh1*, *rh2-a1* and *sws2a* are expressed in all retinal regions (Fig. 4P–R/A–C/J–L, respectively), the *rha-2*- and *rh2a-3*-expressing cones are mostly localized to ventral retina, to a lesser degree in dorsal retina and almost absent in between (Fig. 4D–I). Cones expressing *sws2b* could not be detected in any retinal regions (Fig. 4M–O).

DISCUSSION

To gain insight into the plasticity of the visual photoreceptive system in cod, the current study investigated the activity of visual opsin genes in response to: (1) larval rearing under different spectral light (NC cod); (2) maturation in NC cod; and (3) season in NEA cod. The resulting data suggest limited phenotypic plasticity of visual opsins to the analyzed conditions. These findings may suggest degree-limited capacity of visual tuning to photic environmental changes or during maturation. Surprisingly, our current study on NEA cod revealed a population difference in visual opsin usage compared with our previous studies in NC cod. To our knowledge, this is the first study to demonstrate plasticity in visual opsins linked to different cod population ecotypes. Each of the three conditions will be discussed separately.

Developmental plasticity and effect of different spectral light on opsin expression

Analysis of visual opsin expression in cod larvae reared under different spectral light did not show any immediate response to light environment. Our data show that the overall temporal changes in opsin expression from 2 dph to 12 dph correlate well with the pattern observed between 4 dph and 22 dph in a previous study (Valen et al., 2016), where the larvae were developed under broad-spectrum light/dark (LD) conditions (Fig. 5) (Karlsen et al., 2015). In both experiments, we found an upregulation of *rh2a-1* expression, concomitant with a decrease in *rh2a-2* and *rh2a-3* expression. The *sws2a* expression is less regulated, although a slight upregulation from 2 dph to 12 dph seemed to be present. In the current study, *sws2b* opsin levels proved too low for exact quantification, despite previous successful detection in cod larvae using similar experimental conditions (Valen et al., 2016). Whether this was caused by variation in larval rearing conditions or by intra-population differences is unknown.

The developmental stages of NC cod larvae used in the current study have previously been shown to include large ontogenetic changes in green-sensitive *rh2a* cone opsin expression (Valen et al., 2016) (see Fig. 5). This, combined with our current lack of response to different spectral light, along with temporal expression patterns indicate that opsin usage is ontogenetically pre-programmed during this phase of development. Consequently, the ability of adaptive plasticity in cone opsins towards spectral environment is likely to be limited in larval cod.

Although different spectral light did not alter visual opsin expression under the given conditions, some significant temporal changes were seen in the continuous light and red-light groups. The more significant increase in *rh2a-1* in red light, and decrease in *rh2a-2* and *rh2a-3* in continuous light, suggest a possible difference in the timing of developmental changes in these light regimes. However, as red light had no effect on *rh2a-2* and *rh2-a3*, or continuous light did not affect *rh2a-1*, inconsistency in light effect may indicate the involvement of mechanisms other than light alone. Nevertheless, the overall temporal *rh2a-1* expression from 2 dph to 12 dph indicates an expressional increase similar to that previously

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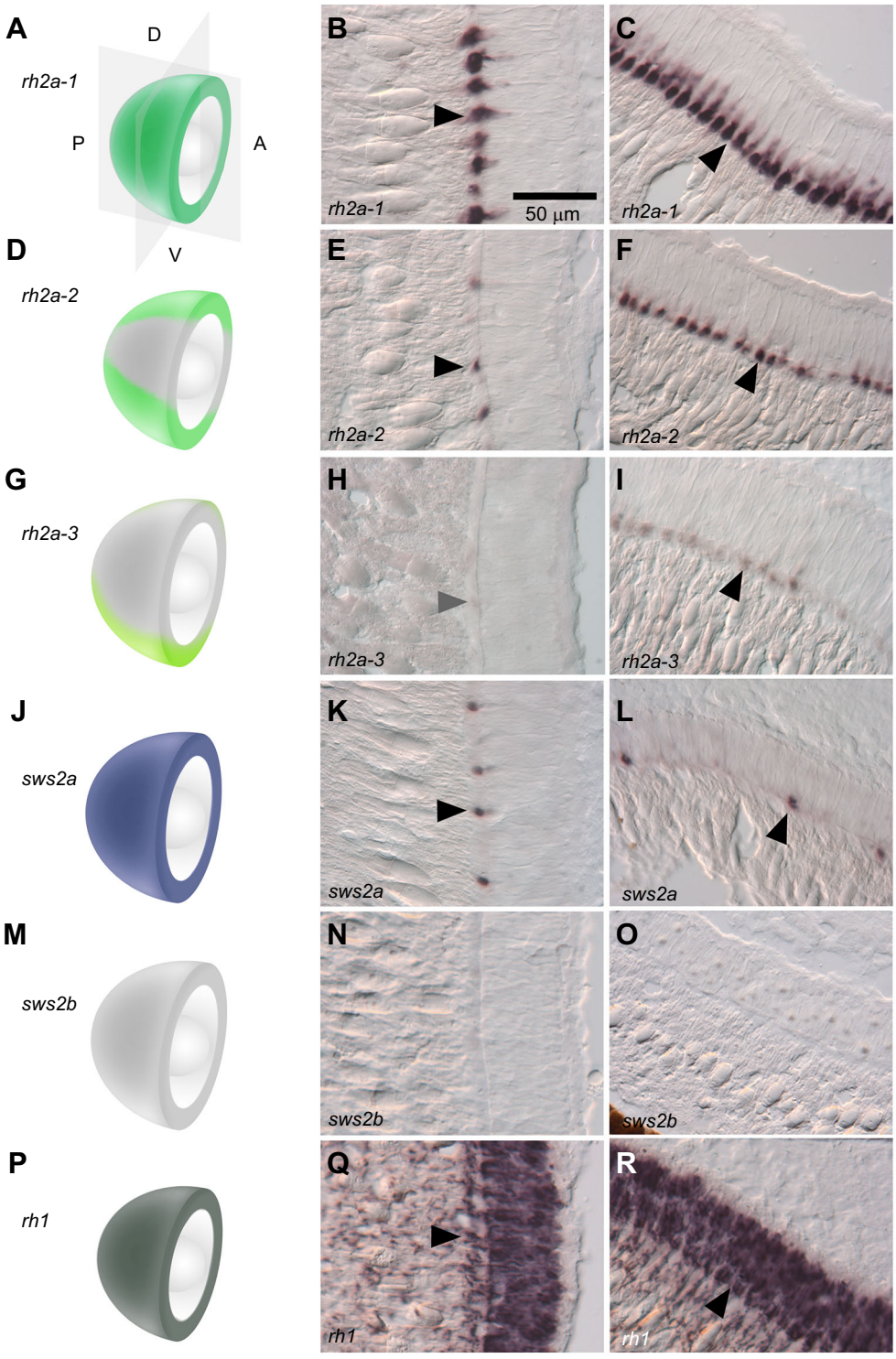

Fig. 4. Retinal spatial expression patterns of visual opsins in Northeast Arctic (NEA) cod. Left-hand side: a schematic summary of visual opsin spatial mRNA expression in the NEA cod retina based on the *in situ* hybridization technique using opsin-specific DIG-labelled probes. (A) Axis of orientation, where D is dorsal, V is ventral, A is anterior, P is posterior, C is central retina, and CMZ is circumferential marginal zone. (A,D,G) Green-sensitive *rh2a* expression; (J,M) blue-sensitive *sws2* expression; (P) *rh1* expression. Retinal tissue expression of *rh2* opsins is shown in the central retina (B,E,H) and in the ventral retina in proximity to CMZ (C,F,I). Whereas *rh2a-1* is expressed in cones throughout the retina (A–C), *rh2a-2* and *rh2a-3* are predominantly expressed in ventral retina, and detected to varying degrees in the dorsal retina (D–I). Cones expressing *sws2a* were found in all retinal regions (J), and tissue expression is shown in central (K) and ventral retina (L). No cones expressing *sw2b* could be detected in NEA cod retina (M–O). Rods expressing rhodopsin (*rh1*) were present in all retinal regions (P–R). Black arrowheads indicate cones and rods expressing the respective opsin whereas the gray arrowhead in H indicates possible weak *rh2-3* expression in central retina. Scale bar (50 µm) shown in B is the same for all images.

reported (Valen et al., 2016). The overall less-apparent change of *rh2a-2* and *rh2a-3* corresponds to previous findings, including a slight decrease towards the 12 dph stage (Fig. 5). The overall similarities further suggest that opsins were unaffected by spectral and potential intensity differences in the currently used LED light and previously used tungsten–halogen light sources (Karlsen et al., 2015; Sierra-Flores et al., 2015; Valen et al., 2016).

The large variation within most treatments is most likely to be a result of differential larval growth and survival success related to the period after start of feeding (Puvanendran and Brown, 1999). Hence, the slight variation in temporal opsin profiles among light groups may thus represent more and less developed larva. It is likely that a constant light environment allows more hours for visual feeding and, as a consequence, may increase growth as previously suggested in cod (Puvanendran and Brown, 2002). A recent study showed improved growth and survival of cod larvae reared in blue/green light compared with red light (Sierra-Flores et al., 2015). However, effects of various light on growth were most obvious at 60 dph, indicating more prominent long-term effects (Sierra-Flores et al., 2015). The improved performance in these light conditions

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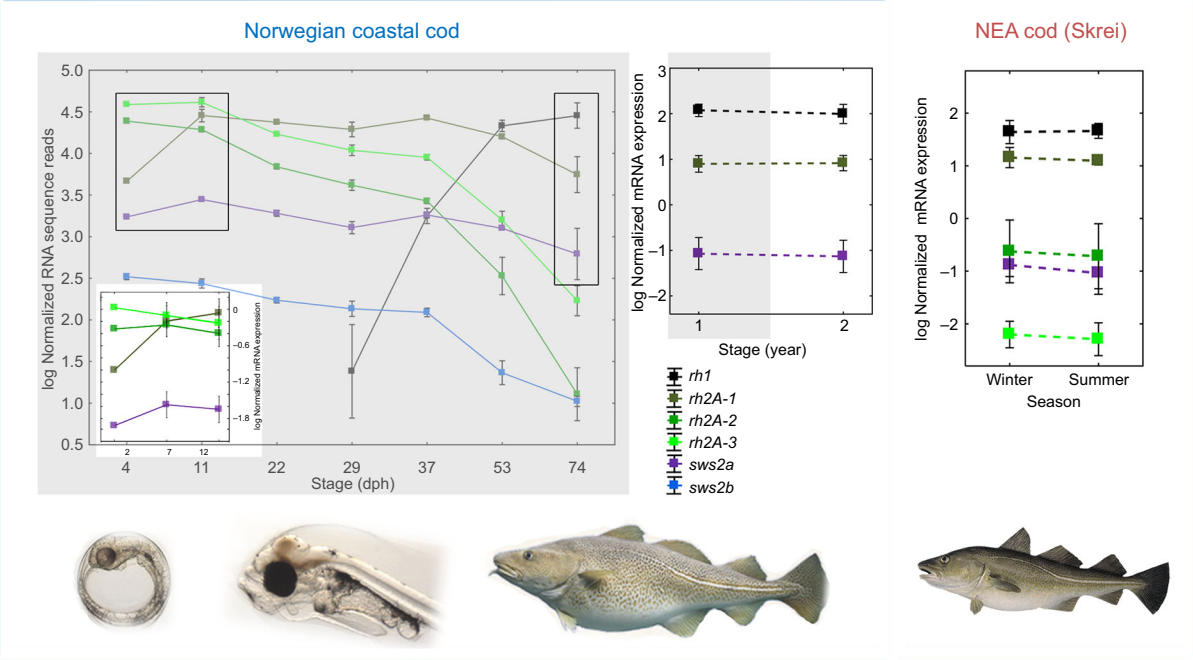

Fig. 5. Visual opsins in Norwegian coastal cod (blue frame) and plasticity related to different light and maturation. Gray areas represent previously published results on visual opsins using RNA-Seq and qPCR during development (Valen et al., 2016). Areas in gray marked with black boxes highlight developmental periods where relative opsin profile correlates with current detected profiles marked by white in graphs. A summary of all data representing the overall temporal expression is shown in white covering 2–12 dph (left corner) and of males and females (right-hand side). A comparison between current findings and previous studies, suggests similar patterns that indicate less degree of plasticity towards environmental light or to maturation. See Fig. S2 for detailed information on temporal patterns of visual opsins in response to separate light treatments. The red box shows visual opsin expression in immature Northeast Arctic cod (NEA cod) during winter and early autumn. Photo credit: Adult cod, Institute of Marine Research, Bergen, Norway.

correlates well with cod larvae being naturally adapted to blue/green-dominated light in the marine environment. It is intriguing to speculate whether the poor performance in red light is related to the genomic loss of LWS cones (Valen et al., 2014).

Role of ontogeny versus environment on opsin plasticity

The lack of rapid light-induced effects on cone opsins, combined with dynamic changes during development (Valen et al., 2016), suggest that opsin changes in cod are pre-programmed developmental events. The lower degree of opsin plasticity towards environment may be linked to the continued post-embryonic retinal development, characteristic of indirect developing species [reviewed by Evans and Browman (2004)]. Typical for indirect developing fish is a prolonged larval stage with an undeveloped pure-cone retina (Evans and Fernald, 1990), which is also the case in cod (Valen et al., 2016). There are examples of changes in cone sensitivity during earlier life stages of other indirect developing fish (Archer et al., 1995; Cheng and Flamarique, 2007; Helvik et al., 2001; Shand et al., 2002, 1988). Yet, these changes have in most cases been attributed to ontogeny, and fewer studies have elucidated the role of the light environment independent of ontogeny.

An exception to this are studies in black bream, which, similar to cod, also have a pelagic pure-cone larva that later acquires rods (indirect eye development) (Blaxter and Staines, 1970; Shand et al., 2002). In black bream, cone opsin expression changes both during development and in response to rearing light environment (Evans and Fernald, 1990; Shand et al., 2008). These observations thus suggest that visual opsin gene activity can be regulated during periods of rapid transformation and eye growth and according to light environment. In contrast to cod, the more direct developing Bluefin killifish showed rapid light-induced responses in all cone opsins (SWS1, SWS2, RH2 and LWS) (Fuller and Claricoates, 2011; Fuller et al., 2010). Interestingly, it was also found that light condition experienced during development had larger effects on visual behavior (opsins) than immediate light treatments, indicating long-lasting developmental plasticity (Fuller et al., 2010). Hence, the studies mentioned above suggest that environmental long-term effects on opsins may occur. Thus, we cannot exclude that this may also be the case in cod; however, this requires studies of longer duration.

Furthermore, in contrast to the aforementioned species, cod have lost opsins sensitive to UV and red light, which may genetically restrict the potential of plasticity to various light input. In both killifish and black bream, more natural light situations were mimicked by light treatment, and demonstrated that these changes in light are sufficient to change opsin expression (Fuller et al., 2010; Shand et al., 2008). It is also likely that these species naturally experience larger variation in spectral light than cod and, in combination with more available visual opsins, have a greater in-built potential to change. Although we have used narrower bandwidth light that represents a more extreme situation and perhaps less natural, we hypothesize that visual opsins would be able to change if the ability for adaptive plasticity was present. In addition, we cannot exclude a missed developmental 'window of opportunity' prior either to sampling or after, along with undetected opsin changes. Together, both current and previous data suggest that variation in plasticity towards environment varies among fish, which may or may not be influenced by life strategy.

In nature, cod embryos and larvae are found in the upper epipelagic with multi-spectral light (Tupper and Boutilier, 1995).

Yet, variation in plankton, particulate matter and sediments changes spectral properties and consequently differs in the selective pressure put on visual adaptation (Partridge and Cummings, 1999). Furthermore, due to life strategy, prey detection and larval growth are crucial for increasing survival chances (Meekan and Fortier, 1996). Thus, having a pre-programmed larval vision may be speculated as a successful adaption towards a variable photic environment. Furthermore, cod seasonal spawning is closely tied to yearly algal and plankton blooms, which improves larval survival success (Kristiansen et al., 2011). Consequently, having a more constant predictable visual programme using all cone opsins present (Valen et al., 2014, 2016) may have proved a successful adaptation reflecting life strategy and ecology.

Visual opsin expression during maturation

Our data on visual opsin expression in maturing 2 year old NC cod show that the most expressed visual opsin is *rh1*, followed by *rh2a-1* and *sws2a* opsin. This profile is similar to our previous observations for late juvenile NC cod (Valen et al., 2016). These data thus suggest that the adult visual programme is established in the juvenile cod and maintained through maturation. Analysis of potential sex differences in visual opsins showed no significant differences between males and females, indicating that opsin expression is not used to tune differential sensitivity during maturation in cod. The male cod did however show higher variation in all opsins compared with females, yet whether this is sex dependent or caused by natural variation among samples is not known. However, by comparing male opsin expression in dorsal retina with ventral retina, there is clearly most expressional variation in the ventral region. These data could suggest topographic differences in opsin expression in some males; however, this remains speculative at this time. No regional differences in opsin expression were detected in the female group. Together, our data suggest that the visual system of males and females is similar and does not change during maturation. Yet, we cannot exclude a potential tuning of vision during spawning in the spring.

Common for many fishes displaying ontogenetic plasticity of vision is plasticity of SWS1 and LWS cones sensitive to the most extreme parts of the visible spectrum, i.e. UV and red, respectively (Allison et al., 2006; Shao et al., 2014). In fish, examples of both UV- and red-sensitivity changes during maturation have been reported (Allison et al., 2006; Shao et al., 2014). The *lws* opsin is actively used by Lake Victoria cichlids in response to water depth, coloration and preferences, and is suggested to even mediate speciation through sensory drive (Seehausen et al., 2008; Terai et al., 2002). In guppies, *lws* opsin is upregulated in the transition from juvenile to adult (Laver and Taylor, 2011). The female mating preference of male coloration has been hypothesized to favor males that contrast with their visual background (Boughman, 2001; Gray et al., 2008). It is tempting to speculate whether LWS opsin has a special function towards mate selection and, if present, makes it more likely that visual clues are central in courtship behaviors. If this may be the case, the loss of UV and LWS opsins in cod, combined with lack of differential opsin regulation during maturation, may be linked to the lack of sex-differential coloration of cod in general.

Localization and attraction of partners may also be mediated via other sensory systems, such as the olfactory and the auditory systems (Andersson, 1994). The natural mating behavior of cod is less known; however, studies have shown the involvement of male–male competition, including both acoustic and visual signal displays (Bekkevold et al., 2002; Brawn, 1961; Engen and Folstad, 1999; Hutchings et al., 1999; Rowe et al., 2007; Skjaeraasen et al., 2010). Apart from males displaying fin size movement during courtships, no visual color clues are known present (Brawn, 1961). Conclusively, our data combined with previous studies suggest that visual system tuning by opsins may not be a key process involved in mate choice. Likely, the well-documented studies of cod mating calls may represent a more central mating signal (Engen and Folstad, 1999; Nordeide and Kjellsby, 1999; Rowe and Hutchings, 2006).

A comparison between NC cod and NEA cod reveals population variation in visual opsin usage

The current expression profile of visual opsins in NEA cod reveals that all opsins except *sws2b* are expressed within a quantifiable range, which is supported by qualitative analysis of tissue-expression patterns. In contrast to late juvenile and maturing NC cod where retinal *rh2a-2* and *rh2a-3* expression is switched off, late juvenile NEA cod choose to express all three green-sensitive *rh2* opsins. These data thus indicate population differences in the complement of visual opsins used and thus suggest population-specific visual programmes in cod. To the authors' knowledge, this is the first report that documents a difference within the visual system between cod ecotypes resulting from differential opsin usage.

Due to history of selection in different habitats, different lighting environments can lead to genetic variations in sensory system properties among fish populations (Endler et al., 2001; Fuller et al., 2005, 2010). Although a number of studies have examined opsin sequence variations related to spectral sensitivity, the extent to which these arise due to variable light environment is less clear (Fuller et al., 2010; Osorio and Vorobyev, 2008; Seehausen et al., 2008). The persistence of Atlantic cod populations through a history of extreme environmental variation, including sea ice, has been suggested to be a result of considerable inherent resilience (Bigg et al., 2008). Furthermore, evolutionary selection of genetic differences in opsins requires a long period of time, and may only cause subtle changes in sensitivity (Hofmann and Carleton, 2009). In contrast, a much more dramatic and rapid mode of changing sensitivity is by differential opsin regulation (Hofmann and Carleton, 2009). Hence, differential *rh2* opsin plasticity may represent local adaptation of vision to different environments among cod. No population genetic differences within any of the cone opsins represented in the current study have previously been associated with cod ecotypes.

In the initial survey of cod population differences, we used a qPCR assay and *in situ* probes designed for NC cod. Although primers were placed within less-conserved opsin regions, the assay works well on NEA cod, indicating highly similar opsin genes. Genetic differences between migratory and stationary cod ecotypes can be differentiated based on variation at the polymorphic pantophysin locus (Pan I), and multiple other genomic regions mostly in linkage group 1 (LG1), through single nucleotide polymorphism (SNP) analysis (Berg et al., 2016; Godø and Michalsen, 2000; Hemmer-Hansen et al., 2013; Karlsen et al., 2013; Kirubakaran et al., 2016; Nordeide and Båmstedt, 1998; Sarvas and Fevolden, 2005). The recent identification of polymorphic differences associated with rhodopsin suggests genetic differences in visual opsins between stationary and migratory Icelandic cod populations (Pampoulie et al., 2015). However, as these SNPs are not associated with previously reported functional phenotypes (Nakamura et al., 2013), and spectral analysis has not been performed, the functional significance remains uncertain. In our current study, we did not find any significant differences in rhodopsin (*rh1*) expression when comparing juvenile NC cod with juvenile NEA cod. Still, we cannot exclude that such differences may exist, either spectrally or undetected expressional variation.

Interestingly, these data also indicate different levels and possibly combinations of opsin-regulatory networks in cod populations. Variations within the *rx1* gene among closely related cichlid species have recently been shown to differentially regulate *sws2a* expression (Schulte et al., 2014). The same study showed that an ancestral polymorphism influenced *rx1* expression levels. Future studies targeting opsin-regulatory factors and associated population polymorphisms will be highly relevant to test visual adaption in cod ecotypes. Whether expressing three *rh2* opsins in later life stages of NEA cod is a result of dynamic environmental adaption, and/or functionally improves resolution towards green light, is currently unknown.

Sampled cod material and developmental stage: effect of size and age at maturation

Our opsin expression data on NEA cod were obtained from cod sampled wild in the Barents Sea. However, both mature NC cod and the late juveniles of our previous study were raised in captivity (Karlsen et al., 2015; Valen et al., 2016). It has been shown for both populations that age at maturation decreases in captivity compared with wild conditions (Godø and Moksness, 1987). Furthermore, NEA cod may take around 6–9 years to reach maturation whereas NC cod take around 2–4 years (Ajiad et al., 1999; Godø and Moksness, 1987; Svåsand et al., 1996). As we do not have information concerning gonadal features on NEA cod, we do not know for certain whether the NEA cod have reached the first maturation. However, previous studies report first-time spawning NEA cod from 60 cm and larger, more than triple the length of our averaged length of ∼20 cm (Ajiad et al., 1999; Bergstad et al., 1987). Thus, the cod used in the current study are most likely late juveniles. Previously, we showed that NC cod expresses all visual opsins during the larval stage whereas *rh2a-2* and *rh2-3* expression is almost completely lost in the 3 month juvenile cod (5 cm standard length) (Valen et al., 2016). Hence, we hypothesize that the observed differences are a consequence of population differences, and find it unlikely that the NEA cod opsin pattern is caused by an earlier developmental stage.

Limited visual opsin plasticity to seasonal change in NEA cod

Our overall comparisons on opsin expression between February and early September in the Barents Sea did not show any significant differences despite extreme seasonal variation in available light. These data thus suggest that visual tuning by opsin plasticity is minimal in cod north of the Arctic Circle despite experiencing a dark period. Yet, we cannot exclude alternative tuning by chromophore switch that has been shown to vary depending on season in fish (Temple et al., 2006; Ueno et al., 2005). However, most marine fishes display only vitamin A1, and the A1–A2 switch is typically associated with fish migrating between freshwater and seawater (Toyama et al., 2008). In general, very little is known concerning seasonal adaptions in vision of fishes inhabiting areas at high latitudes with large fluctuations in photoperiod. Studies of Antarctic notothenoid fishes initially suggested that LWS opsins were lost in these species, yet subsequent studies detected LWS in some fish (Miyazaki and Iwami, 2012; Pointer et al., 2005). This suggests that *lws* loss is not a common feature at high latitudes.

By comparing regional expression of visual opsins in NEA cod, we found that *rh2a-2* opsin was more highly expressed in ventral retina than in dorsal retina, indicating topographic differences in opsin expression. These differences were observed both in material sampled during February and September, indicating regionalization independent of season. In our previous developmental studies on NC cod opsins, we detected more cones expressing *rh2a-2* and *rh2a-3* in ventral retina during metamorphosis (Valen et al., 2016). Thus, the detection of higher levels of *rh2a-2* opsin in ventral retina of NEA cod has similarities to our previous findings in transforming juvenile NC cod. Future studies on older and mature NEA cod will be needed to elucidate whether *rh2a-2* and *rh2a-3* expression is sustained. Still, immature NEA cod do not express *sws2b*, which is similar to late juvenile NC cod (Valen et al., 2016), indicating a population difference in regulation of the green opsin locus whereas the blue opsins seem to be under similar regulation. It is also possible that differences between coastal and oceanic photic environments within the green part of the light spectra influence the expression of *rh2* genes differently as a visual adaptive mechanism.

Conclusions

Our initial investigations on cod visual opsin expression towards environmental light and to maturation indicate limited plasticity of tuning in cod. The developmental programme of visual opsins in larval cod appears to be robust towards immediate photic changes, yet does not exclude long-term effects. The lack of changes in visual opsins during maturation suggest that differential tuning of blue- and green-sensitive opsins plays a less important role during cod mating behavior. Interestingly, *rh2* opsins are differently expressed in NEA cod compared with NC cod, indicating phenotypic plasticity in visual systems related to cod ecotypes. Whether this relates to adaptations to different habitats, or to differences in gene regulation, is still unclear. Furthermore, the spatial similarities in cone opsin expression in NEA cod and earlier stages of NC cod may suggest shared aspects of opsin regulation. However, which factors are involved and how these operate in concert are currently unknown. Our initial survey on visual opsins in NEA cod does not indicate any major visual adaptation to season, despite the extreme variation in available light. The lack of *sws2* and *rh2* opsin plasticity towards different spectral light, maturation and season suggests that developmental programmes of vision prevail in cod. It is intriguing to speculative whether the lack of plasticity may be a consequence of evolutionary genomic loss of UV- and red-sensitive cone opsin genes. Cod may thus have less inherent genomic potential for tuning vision to different spectral light. Although this study has focused on a subset of factors, the method and approach have provided novel knowledge of visual system dynamics with implications extending beyond Atlantic cod.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: R.V., J.V.H.; Methodology: R.V., R.K., J.V.H.; Software: R.V.; Validation: R.V., R.K.; Formal analysis: R.V., R.K., J.V.H.; Investigation: R.V.; Resources: J.V.H.; Data curation: J.V.H.; Writing - original draft: R.V.; Writing - review & editing: J.V.H.; Visualization: R.V.; Supervision: J.V.H.; Project administration: J.V.H.; Funding acquisition: J.V.H.

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Supplementary information

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