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RESEARCH ARTICLE

Variation in opsin transcript expression explains intraretinal differences in spectral sensitivity of the northern anchovy

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Abstract

Vertebrate retinal photoreceptors house visual pigments that absorb light to begin the process of vision. The light absorbed by a visual pigment depends on its two molecular components: protein (opsin) and chromophore (a vitamin A derivative). Although an increasing number of studies show intraretinal variability in visual pigment content, it is only for two mammals (human and mouse) and two birds (chicken and pigeon) that such variability has been demonstrated to underlie differences in spectral sensitivity of the animal. Here, we show that the spectral sensitivity of the northern anchovy varies with retinal quadrant and that this variability can be explained by differences in the expression of opsin transcripts. Retinal (vitamin A1) was the only chromophore detected in the retina, ruling out this molecular component as a source of variation in spectral sensitivity. Chromatic adaptation experiments further showed that the dorsal retina had the capacity to mediate color vision. Together with published results for the ventral retina, this study is the first to demonstrate that intraretinal opsin variability in a fish drives corresponding variation in the animal's spectral sensitivity.

Keywords: Anchovy, Cone photoreceptor, Chromophore, Optic nerve, Color vision

Introduction

It has long been known that the number and type of photoreceptors in vertebrates can vary with retinal location. A typical example is the absence of S cones (containing an SWS1 opsin) from within ~100 μm of the site of peak cone density in the human fovea in an otherwise trichromatic retina containing variable numbers of S, M, and L cones that give rise to variable spectral (color) sensitivity (Curcio et al., 1991; Roorda & Williams, 1999; McKeefy et al., 2007). Another species where intraretinal variation in cone photoreceptors leads to differential spectral sensitivity is the mouse. In this species, the majority of cones house two visual pigments, S (expressing an SWS1 opsin) and M (expressing an MWS/LWS opsin), and these are distributed along opposite dorso-ventral gradients wherein the S visual pigment dominates the ventral retina (Applebury et al., 2000; Baden et al., 2013). This variation in cone distributions underlies different spectral sensitivities, with higher sensitivity to ultraviolet (UV) and short wavelengths in the ventral retina (Calderone & Jacobs, 1995). In addition, by virtue of the stronger signal gain of the S cone and its preference for dark stimuli, the opsin gradient in this animal may primarily serve to improve achromatic contrasts in its surroundings (Baden et al., 2013). Other examples where intraretinal variation in cones underlies different spectral sensitivities are found in two species of birds: pigeon and chicken (Wortel et al., 1984, 1987; Remy & Emmerton, 1989). In these species, intraretinal variation in cone types was first assessed by quantifying the numbers of oil droplets, organelles that occur in the inner segments of cones and whose transparency varies with cone type (e.g., cones containing UV visual pigment are associated with colorless droplets). These studies showed enhanced UV sensitivity in retinal areas with greater numbers of colorless oil droplets.

Besides these four species (i.e., human, mouse, chicken, and pigeon), many other vertebrates also show morphological and/or spectral variation in photoreceptor types with retinal location (Calderone & Jacobs, 2003; Arrese et al., 2005; Takechi & Kawamura, 2005; Lukáts et al., 2005; Neitz et al., 2006; Cheng & Novales Flamarique, 2007; Chen et al., 2008; Temple et al., 2010; Hárosi & Novales Flamarique, 2012; Novales Flamarique et al., 2013; Isayama et al., 2014). This variation may be further amplified because photoreceptors can contain multiple visual pigments (Lukáts et al., 2005; Cheng & Novales Flamarique, 2007; Isayama et al., 2014; Savelli et al., 2018), and visual pigments may be modulated throughout life and, potentially, as a function of retinal location (Sakai et al., 2016; Dalton et al., 2017). The functional implications of such intraretinal variation are unknown. Most studies comprising measurements of an animal's spectral sensitivity have not investigated its retinal variation or associated topographical

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differences in cone distributions. Instead, these studies have analyzed intra- or interspecies differences in overall spectral sensitivity (McDonald & Hawryshyn, 1995; Leal & Fleishman, 2002; Sabbah et al., 2010; Fleishman et al., 2011), modeled cone contributions to general spectral sensitivity functions (DeMarco & Powers, 1991; Hughes et al., 1998; Risner et al., 2006; Sabbah et al., 2010), or investigated the implications of changes in cone phenotype or cone loss on spectral sensitivity (Novales Flamarique, 2013, 2016; Novales Flamarique & Wachowiak, 2015).

Besides the lack of information on intraretinal variation in spectral sensitivity and its underlying photoreceptor basis, the source of visual pigment variability, whether caused by differential opsin expression or chromophore type (retinal, vitamin A1, or 3,4-dehydroretinal, vitamin A2), is rarely demonstrated. At best, the preponderance of one chromophore type over another is inferred, indirectly, from semi-empirical models based on visual pigment absorbance parameters (Temple et al., 2010; Novales Flamarique, 2013; Novales Flamarique et al., 2013), that is, maximum wavelength of absorbance, λ_{max}, or bandwidth at half maximum absorbance, HBW (Hárosi, 1994). It is only in rare cases that direct measurements of chromophore type have been obtained (Kondrashev et al., 2013; Iwanicki et al., 2017; Savelli et al., 2018), despite the importance of this parameter in evaluating visual pigment absorbance.

In this study, we examined the spectral sensitivity in the dorsal retina of the northern anchovy to provide, along with previously published results from the ventral retina (Novales Flamarique, 2017), an assessment of intraretinal variability in the animal's spectral sensitivity. We then explored whether the variability in spectral sensitivity could be explained by differential expression of opsin or chromophore types. A previous study has shown that the ventral retina of this species can be divided into two functional regions: a ventro-temporal (VT) area that contains axially dichroic cones with two M visual pigments whose λ_{max}, at 514 and 516 nm are statistically the same, and a ventro-nasal (VN) region with cones that are not axially dichroic and house either M (λ_{max} ~ 518 nm) or L (λ_{max} ~ 556 nm) visual pigments (Novales Flamarique, 2017). Spectral sensitivity results suggested that the VN, but not the VT, retina mediates color vision. This was based on the presence of one peak (at 520 nm) in the spectral sensitivity function of the VT retina and two peaks (at 500 and 540 nm) in that of the VN retina. Behavioral experiments with free-swimming anchovies further showed that the animal can discriminate color and the polarization of light using the ventral retina (Novales Flamarique, 2017). Here, we hypothesized that any differences in spectral sensitivity between retinal sectors should reflect corresponding variations in opsin and/or chromophore types that make up the visual pigments.

Materials and methods

Animals

Adult northern anchovies (*Engraulis mordax*) were obtained from ocean net pens kept by Westport Seafood Ltd (Westport, WA) and transported to the University of Victoria Aquatic Facility (Victoria, BC, Canada) where they remained during the experimental period. Anchovies were kept in outdoor 5000-l recirculating saltwater tanks, fed frozen mysid, and experienced the natural daylight cycle throughout the study. The mean weight and total length (±S.D.) of the animals used were 22.8 ± 2.03 g and 15.7 ± 0.34 cm (n = 30). All experiments were approved by the Animal Care Committees of Simon Fraser University (protocol # 1126B-10) and the University of Victoria (protocol # 2013-005), which abide by regulations set by the Canadian Council for Animal Care.

Electrophysiology

The electrophysiological set-up to acquire optic nerve responses has been documented in detail before (Novales Flamarique & Wachowiak, 2015; Novales Flamarique, 2017) and will only be summarized here. The optical system for stimulus delivery consisted of a 150W xenon light source coupled to a monochromator whose output traversed a neutral density wheel and shutter before entering one end of a bifurcated liquid light guide. The other end of the light guide received input from a halogen source (Fiber-lite MI-150, Dolan-Jenner Industries, Boxborough, MA) whose output traversed a combination of barrier and neutral density filters; this was the background channel. The outputs of both channels overlapped, converging onto the common end of the liquid light guide. The latter fitted into a holder with ball-joint base that permitted its positioning at any angle with respect to the center of the fish's pupil. Experiments involved stimulation of the mid dorso-nasal (DN) and mid dorso-temporal (DT) retina by adjusting the angle of the fiber with respect to vertical and horizontal markers.

To generate a spectral sensitivity function, the fish was adapted to either a diffuse "white" (irradiance: 1.87×10^{14} photons cm^{-2}/s, λ range: 300–750 nm) or long wavelength (LW) (irradiance: 1.78×10^{14} photons cm^{-2}/s, λ range: 500–750 nm) light through the background channel of the bifurcated guide (Novales Flamarique, 2017). The fish adapted to this background for 1 h following which compound action potential recordings from the optic nerve were acquired to a 500-ms flash stimulus. For a given wavelength, the stimulus intensity was increased incrementally and the response at threshold plotted against log (stimulus intensity) and fitted with a third degree polynomial. The threshold value (20 μV) was chosen to lie in the lower linear part of the fit, characterized by a single major peak contribution to the optic nerve response. Sensitivity (i.e., the reciprocal of irradiance at threshold) was calculated for 14 wavelengths across the spectrum 360–650 nm to generate a spectral sensitivity function. All light measurements were obtained with a UV-visible liquid light guide (600 μm diameter input, 0.22 NA) connected to a USB 2000 spectroradiometer under the command of Spectrasuite software (Ocean Optics, Largo, FLA).

Analysis of spectral sensitivity data

We used the Simplex algorithm (Caceci & Cacheris, 1984) to fit the spectral sensitivity data with a combination of visual pigment absorbance curves using the equation (Sirovich & Abramov, 1977):

$$R = \left[k_i A_i^{\,p}(\lambda)\right]^{1/p},$$

where R is the response (sensitivity) function, $A_i{}^p$ is the absorbance of pigment i at light of wavelength λ, and p is an exponent resulting from the mathematical requirement that the spectral sensitivity function be differentiable at the origin (Sirovich & Abramov, 1977). The variables k_i are coupling constants resulting from the best fit of the model to the data. All response curves that are a function of one or multiple visual pigments must conform to the above equation. The coupling constants and the exponent p are determined by the inputs of each visual pigment to the spectral sensitivity function and the complex interactions between cone mechanisms (i.e., the cone photoreceptors which house the visual pigments

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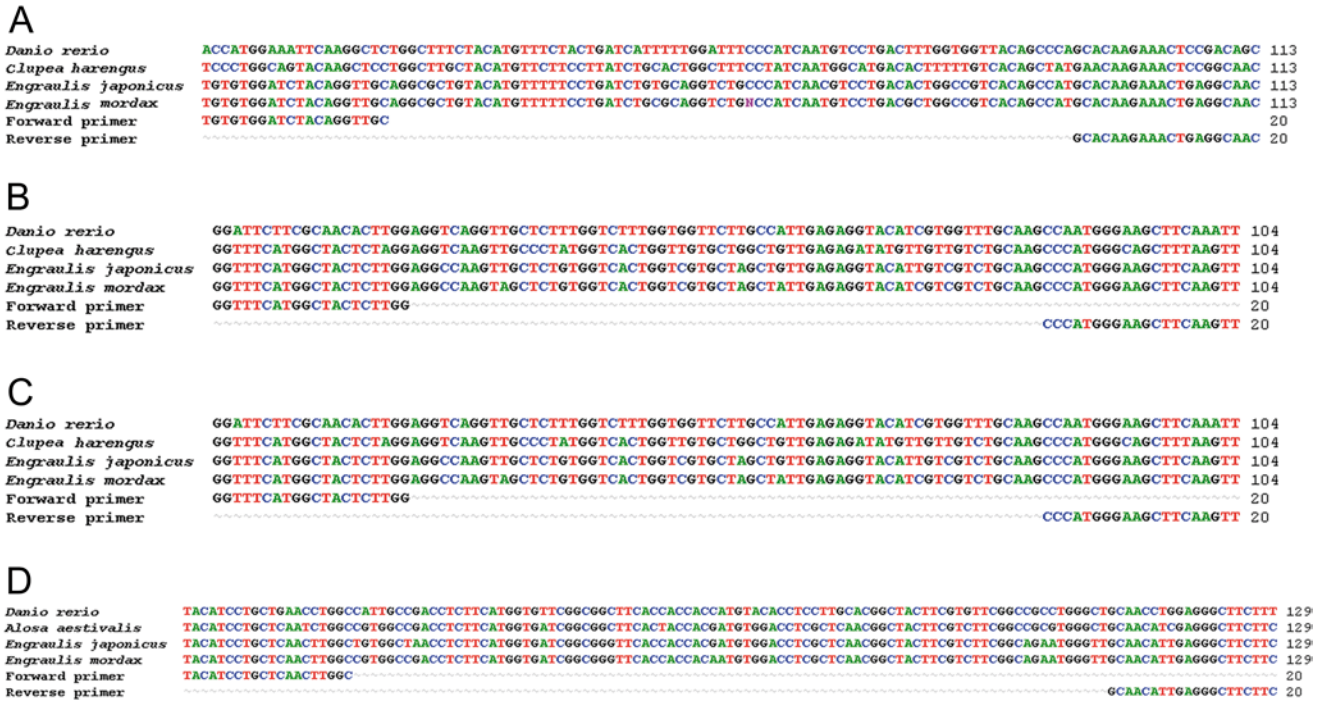


Fig. 1. Alignment of northern anchovy (*Engraulis mordax*) partial opsin nucleotide sequences with those of the Japanese anchovy (*Engraulis japonicus*), zebrafish (*Danio rerio*), and herring (*Alosa eastivalis* or *Clupea harengus*). The alignment of primers is also indicated. (**A**) RH2-1, (**B**) RH2-2, (**C**) LWS, and (**D**) RH1.

and related circuitry) at the various levels of retinal processing. To compare spectral sensitivity between retinal quarters, we performed this analysis on data for the dorsal retina (from the present study) as well as previously published data for the ventral retina (Novales Flamarique, 2017).

The absorbance values used in the model were generated with an eighth-order polynomial template (Palacios et al., 1996) based on the λ_{max} of visual pigments measured by microspectrophotometry (Novales Flamarique, 2017). The VT quarter of the northern anchovy retina has a preponderance of two axially dichroic cone types with statistically similar visual pigments of λ_{max} at 514 nm (the short cone) and 516 nm (the long cone). The rest of the retina is primarily populated by nonaxially dichroic triple cones which have λ_{max} at 518 nm (lateral cones) and 556 nm (central cone) (Novales Flamarique, 2017). Thus, the λ_{max} of cone visual pigments used in the analysis of spectral sensitivity for the VT retina was 516 and 556 nm (the latter to account for any potential LW visual pigment presence), and for the rest of retinal quarters (i.e., VN, DN, and DT), the combination of 518 and 556 nm. To assess whether the spectral sensitivity results were best fitted by visual pigments based on retinal (vitamin A1) or 3-4-dehydroretinal (vitamin A2) chromophore, we performed the same analysis with the corresponding combinations of vitamin A2-based visual pigments. The latter were calculated using semi-empirical models (Hárosi, 1994) to be 549 nm (for the 516 visual pigment), 552 nm (for the 518 nm visual pigment), and 617 nm (for the 556 nm visual pigment).

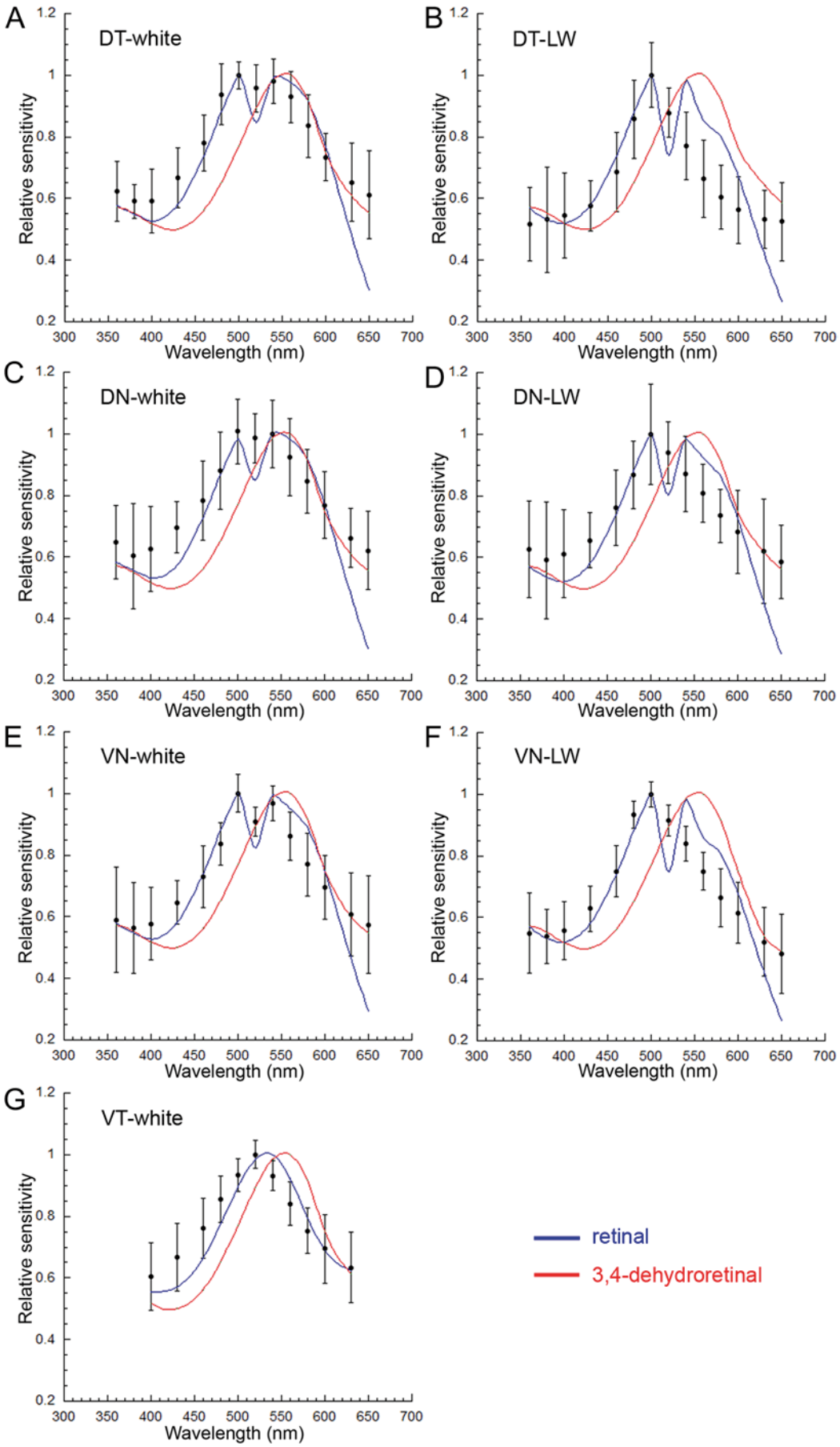
High performance liquid chromatography

Following dark-adaptation for 2 h, five anchovies were killed and the retinas were removed from the eyecups. Each retina was then placed in a 1.5-ml Eppendorf tube and frozen in liquid nitrogen until processing for the presence of chromophore types: retinal (vitamin A1) and 3,4-dehydroretinal (vitamin A2). Retinas were homogenized by sonication in a mixture containing 60 μl of 1.92 M hydroxylamine sulfate (neutralized with 1 N KOH) and 300 μl of methanol. The homogenate was cooled on ice, following which 300 μl of dichloromethane and 150 μl of ddH_2O were added and the mixture shaken vigorously. After the addition of 600 μl of *n*-hexane, the solution was centrifuged at 1000 g for 5 min at 4°C. The dichloromethane/hexane (top) layer was removed and placed in a 1.5-ml Eppendorf tube. This procedure was then repeated on the remaining, lower layer, after which the second extract was combined with the first and evaporated under vacuum for 1 h.

Table 1. *Percent similarity between northern anchovy* (Engraulis mordax) *partial opsin nucleotide sequences and those of Japanese anchovy* (Engraulis japonicus)*, zebrafish* (Danio rerio)*, and herring* (Alosa eastivalis *or* Clupea harengus). *Sequences are presented in Fig. 1*

Opsin	Percent similarity	Species
RH2-1	67.3	*Danio rerio*
RH2-1	70.8	*Clupea harengus*
RH2-1	97.3	*Engraulis japonicus*
RH2-2	80.8	*Danio rerio*
RH2-2	85.6	*Clupea harengus*
RH2-2	97.1	*Engraulis japonicus*
LWS	81.8	*Danio rerio*
LWS	81.0	*Clupea harengus*
LWS	96.7	*Engraulis japonicus*
RH1	83.7	*Danio rerio*
RH1	92.2	*Alosa aestevalis*
RH1	96.9	*Engraulis japonicus*

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The resulting, dried extract was dissolved in 100 μl of *n*-hexane and injected into a 5 μm YMC-Pack Silica 2.1 × 250 mm column (YMC America) attached to a high performance liquid chromatography (HPLC) system. The mobile phase running through the column flowed at a rate of 0.8 ml per minute and contained 7% ether and 0.075% ethanol in *n*-hexane. The computer-driven detector monitored absorbance at 360 and 400 nm and produced chromatograms which permitted identification of vitamin A1 and A2 oximes based on retention time in comparison with results from an all-trans-retinal (A1) standard (Sigma-Aldrich, St. Louis, MO).

Reverse transcriptase quantitative polymerase chain reaction

Ten dark adapted anchovies were euthanized and the left eyeballs were removed. The retinas were then extracted from the eyeballs and each cut into quarters (i.e., ventro-nasal, VN; ventro-temporal, VT; dorso-nasal, DN; and dorso-temporal, DT sectors). Each quarter was put into an RNAse-free 1.5-ml Eppendorf tube, capped, and immediately immersed in liquid nitrogen. The retinal quarters, each in its own tube, were stored at −80°C until processing for RNA extraction. This was carried out using the illustra RNAspin Mini RNA Isolation Kit (GE Healthcare, Mississauga, Ontario, CA) according to the manufacturer's instructions.

Primer pairs for amplification of RH1, RH2-1, RH2-2, and LWS opsin transcripts in the northern anchovy retina were designed based on sequences available in Genbank for the Japanese anchovy, *Engraulis japonicus* (Kondrashev et al., 2013), using the primer design software Primer3Plus. The accession numbers for the Japanese anchovy opsins are AB731902.1 (RH1), AB731899.1 (RH2-1), AB731900.1 (RH2-2), and AB731901.1 (LWS). The amplicons obtained were processed by agarose gel electrophoresis, sequenced (GENEWIZ Inc., South Plainfield, NJ), and aligned against published sequences for the Japanese anchovy (*Engraulis japonicus*), zebrafish (*Danio rerio*), and herring (*Alosa aestivalis* or *Clupea harengus*) using the multiple sequence alignment program ClustalW (Fig. 1). The RH1 partial sequence concurred with that reported previously for the northern anchovy (Van Nynatten et al., 2015) and was ~97% similar to the corresponding sequence from the Japanese anchovy (Fig. 1 and Table 1). Similarly, regardless of the opsin type, all partial sequences from the northern anchovy were >67% similar to corresponding ones from the other fishes analyzed, and ~97% to those from the Japanese anchovy (Fig. 1 and Table 1). The high similarity with herring (*Alosa aestivalis*) opsins appears to be paralleled by the absorbance properties of visual pigments in the double cones and rod of the latter species (λ_{max} of M: 517 nm, L: 556 nm, and rod: 510 nm) and the exclusive presence of A1 retinal chromophore, as assessed by microspectrophotometry (Novales Flamarique & Hárosi, 1999).

Reverse transcriptase quantitative polymerase chain reactions were prepared in a 20 μl volume containing 4 ng of cDNA using the iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA). Thermal cycling conditions were 2 min denaturation at 95°C, followed by 40 cycles of 5 s denaturation at 95°C and 20 s of annealing-elongation at 58°C for RH1 and RH2-2 or at 59°C for RH2-1 and LWS. After amplification, a melting curve analysis was performed (65° to 95°C, with increment of 0.5°C every 5 s) to confirm the specificity of the amplicon. Non-template controls were used as negative controls. The relative expression of a given opsin mRNA was calculated using the $\Delta\Delta C_t$ method (Livak & Schmittgen, 2001) with Cytochrome c oxidase I (*coxI*; accession number NC_003097.1) as the species-specific housekeeping gene, which showed stable expression throughout the retina. Each C_t value (i.e., the cycle number at which the signal reaches the threshold of detection) used in the calculations is the mean of two replicates from the same reaction. The relative expression of each opsin gene (n = 10 per retinal sector) with respect to the housekeeping gene was compared between sectors by ANOVA followed by S–N–K and Tukey grouping tests with $\alpha = 0.05$.

Results

Spectral sensitivities

The spectral sensitivities of the DN, DT, and VN sectors of the retina were similar (Fig. 2 and Table 2). Under the white adaptation background, spectral sensitivity was characterized by two peaks, at 500 and 540 nm (Fig. 2A, 2C, and 2E) and decreased progressively beyond these wavelengths. The Simplex-derived coupling constants indicated that the M visual pigment was the primary input to the results (Table 2) and that a negative interaction should exist between the M and L cone mechanisms, as suggested by the dip between the 500 and 540 nm peaks when the curve fit involved vitamin A1-based visual pigments (Fig. 2A, 2C, and 2E). When these retinal sectors were subjected to the LW background, the spectral sensitivity functions became narrower and sensitivity decreased substantially for wavelengths above the single peak, located at 500 nm (Fig. 2B, 2D, and 2F). In accordance with these results, the Simplex-derived coupling constants indicated an even greater difference in input between the M and L cone mechanisms (compared with equivalent recordings under the white adaptation background, Table 2), and this was as expected from the suspected greater bleaching of the L visual pigment. The best fits to the data implied a negative interaction between the M and L cone mechanisms when vitamin A1-based visual pigments were used in the modeling (Fig. 2B, 2D, and 2F). By contrast, regardless of the retinal sector or adaptation background, curve fits involving the vitamin A2-based visual pigments were worse (greater sums of squares, Table 2) and could not reproduce the negative interaction between the M and L cone mechanisms suggested by the data (Fig. 2A–2F). The spectral sensitivity of the VT retina was unlike that from the other quarters in that it consisted of a single peak at 520 nm (Fig. 2G; Novales Flamarique, 2017). In line with this result, the Simplex-derived curve fit comprised a single peak and showed the main contribution to arise from the M visual pigment (Table 2).

Fig. 2. Relative spectral sensitivities of the four retinal quarters in the northern anchovy. (**A** and **B**) Relative spectral sensitivity (±S.D., n = 4) of the DT retina under the white adapting background (**A**) and under the LW adapting background (**B**). (**C** and **D**) Relative spectral sensitivity (±S.D., n = 4) of the DN retina under the white adapting background (**C**) and under the LW adapting background (**D**). (**E** and **F**) Relative spectral sensitivity (±S.D.) of the VN retina under the white adapting background (**E**) (n = 6) and under the LW adapting background (**F**) (n = 5). (**G**) Relative spectral sensitivity (±S.D., n = 6) of the VT retina under the white adapting background. Panels **E**–**G** adapted from the data in Novales Flamarique (2017). The functions are Simplex-derived best-fits to the data given combinations of visual pigments based on retinal (vitamin A1, in blue) or 3-4-dehydroretinal (vitamin A2, in red) chromophore. The model parameters are given in Table 2.

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Table 2. *Best-fit Simplex-derived parameters and corresponding least sum of squares (SS) for the spectral functions depicted in Fig. 2. The k_M and k_L coupling constants are the inputs of the M and L visual pigments (or cone mechanisms) to the spectral sensitivity function. The λ_{max} of the M and L visual pigment combinations used in the fitting process are indicated in the table. The SS statistic is derived from the sum of differences between mean spectral sensitivity data and the corresponding values predicted by the Simplex algorithm. Retinal sectors: DT, dorso-temporal; DN, dorso-nasal; VN, ventro-nasal; and VT, ventro-temporal*

Adapting background	Retinal sector	Visual pigments	k_M	k_L	P	SS
White	DT	M (518), L (556)	7.83×10^5	8.42×10^4	31.388	0.314
White	DT	M (552), L (617)	3.71×10^5	0.164	29.825	0.376
LW	DT	M (518), L (556)	3.24×10^{12}	83.5	118.41	0.351
LW	DT	M (552), L (617)	4.36	0.0530	10.142	0.611
White	DN	M (518), L (556)	1.10×10^3	488	16.465	0.339
White	DN	M (552), L (617)	1.53×10^5	0.287	27.085	0.351
LW	DN	M (518), L (556)	1.25×10^5	2.88×10^3	30.023	0.397
LW	DN	M (552), L (617)	1.84×10^5	0.0210	33.710	0.518
White	VN	M (518), L (556)	1.62×10^3	210	20.603	0.263
White	VN	M (552), L (617)	5.78×10^4	0.00701	31.732	0.336
LW	VN	M (518), L (556)	2.23×10^{11}	800	99.010	0.234
LW	VN	M (552), L (617)	990	0	33.522	0.549
White	VT	M (518), L (556)	2.51	0.707	2.6550	0.086
White	VT	M (552), L (617)	3.12×10^5	0.057	32.553	0.421

Chromophore type in the anchovy retina

HPLC analyses of retinal extracts revealed the presence of a main absorbance peak at ~9.2 min retention time and a secondary peak at ~22.2 min retention time (Fig. 3A). These peaks were analogous to those exhibited by the all-trans A1 retinal standard (Fig. 3B) and corresponded to all-trans retinal oxime (peak 1) and to anti-all-trans retinal oxime (peak 2). As such, the retina of the northern anchovy only has all-trans A1 retinal as chromophore. Had there been any 3-4-dehydroretinal (vitamin A2) chromophore, the chromatogram from the retina would show additional peaks about 1 and 3 min following peaks 1 and 2, respectively, as has been demonstrated for the retinas of some freshwater fishes that contain vitamin A2 chromophore (Kondrashev et al., 2013; Iwanicki et al., 2017).

Opsin transcript expression in different sectors of the retina

The expression of opsin transcripts was heterogeneous (Fig. 4). RH2-1 and RH2-2 showed the highest means in the VT sector ($F = 21.29$, $P < 0.0001$ for RH2-1 and $F = 5.51$, $P = 0.0034$ for RH2-2; Fig. 4A and 4B). The RH2-1 mean, in particular, was significantly different from those in other retinal sectors. Conversely, LWS had the lowest mean in the VT sector and was most abundant in the DN sector ($F = 2.73$, $P = 0.0058$; Fig. 4C). The RH1 transcript was, statistically, evenly distributed throughout the retina ($F = 1.42$, $P = 0.254$; Fig. 4D). These results indicate a preponderance of RH2 opsins, and lowest abundance of LWS opsin, in the VT sector of the retina.

Discussion

Variable spectral sensitivities in a functionally compartmentalized retina

The retina of the northern anchovy, like that of other anchovies in the family Engraulididae (Fineran & Nicol, 1978; Heß et al., 2006; Kondrashev et al., 2012), is unusual among vertebrates in that it contains cones whose outer segment lamellae are oriented parallel to their lengths (Novales Flamarique, 2011). These cones are of two types, long (with cuneate outer segments) and short (with bilobed outer segments), are located in the VT retina, and alternate forming rows with lamellae that are orthogonal to each other. Such axially dichroic cones (Novales Flamarique & Hárosi, 2002) have been shown to possess the same visual pigment in multiple anchovy species (though the λ_{max} varies between species; Kondrashev et al., 2012, 2013; Novales Flamarique & Hárosi, 2002; Novales Flamarique, 2017) and, in the northern anchovy, mediate polarization sensitivity of the animal (Novales Flamarique & Hawryshyn, 1998; Novales Flamarique, 2017). The rest of the retina lacks these cone types and, instead, has single and triple cones with lamellae oriented perpendicular to their lengths, the latter as in other vertebrates studied (Hárosi & Novales Flamarique, 2012). In both the Japanese anchovy and the northern anchovy, the triple cones (Heß, 2009; Novales Flamarique, 2011) contain two different visual pigments (Kondrashev et al., 2013; Novales Flamarique, 2017) and, in the northern anchovy, such cones in the VN retina have been proven to mediate spectral (color) discrimination (Novales Flamarique, 2017). Thus, in the northern anchovy, the ventral retina is not only structurally but functionally compartmentalized for color and polarization vision.

The results from this study show that spectral sensitivity is similar between the VN retina and the dorsal retina, regardless of the sector analyzed (DN or DT). Both dorsal quarters showed broad spectral sensitivity curves with two peaks in the middle wavelengths (at 500 and 540 nm), and a dip in between, indicating a negative interaction between a minimum of two cone mechanisms, and the potential for color vision. The presence of at least two cone mechanisms was further demonstrated by adaptation to a LW background resulting in a narrower sensitivity function peaking at 500 nm with greatly diminished sensitivity beyond this wavelength. These results (Fig. 2 and Table 2) indicate the presence of a long wavelength cone mechanism, which may be founded on the LWS visual pigment present in the central cone of triple cone units, located throughout the dorsal and nasal retina (Novales Flamarique, 2011). The short wavelength sensitivity could be mediated by the lateral cones of triple cone units, which house

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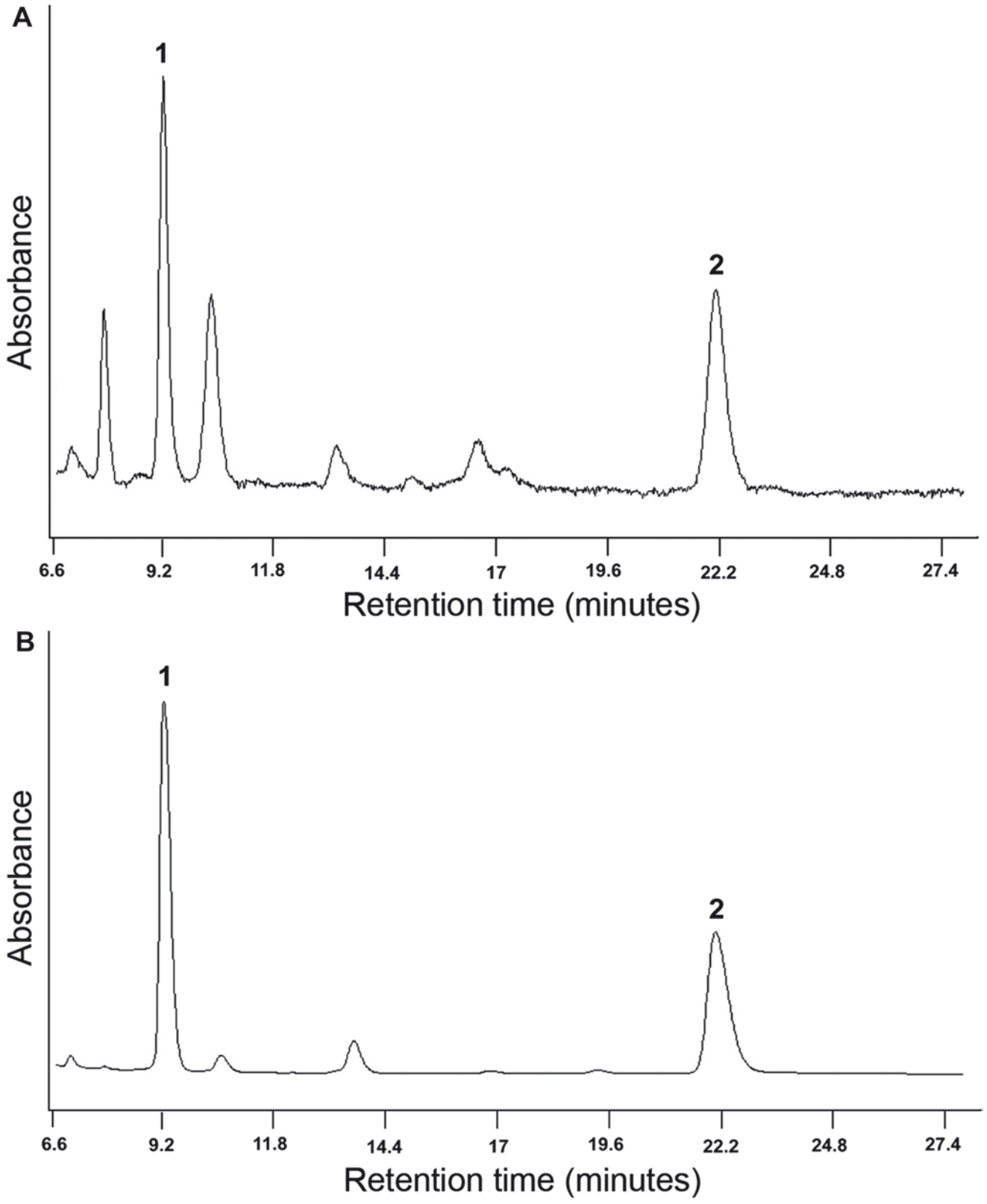


Fig. 3. Representative HPLC absorbance profiles of (**A**) northern anchovy retina extract and (**B**) the all-trans retinal (vitamin A1) standard. Peak 1 corresponds to all-trans retinal and peak 2 to anti-all-trans retinal.

an M visual pigment (Novales Flamarique, 2017). It is noteworthy that the outer segment of the central cone is much smaller than that of the lateral cone (Heß, 2009; Novales Flamarique, 2011; Kondrashev et al., 2016), and this may explain, through a negative interaction between cone types, the diminished sensitivity at longer wavelengths (e.g., 560 nm) compared to that at shorter

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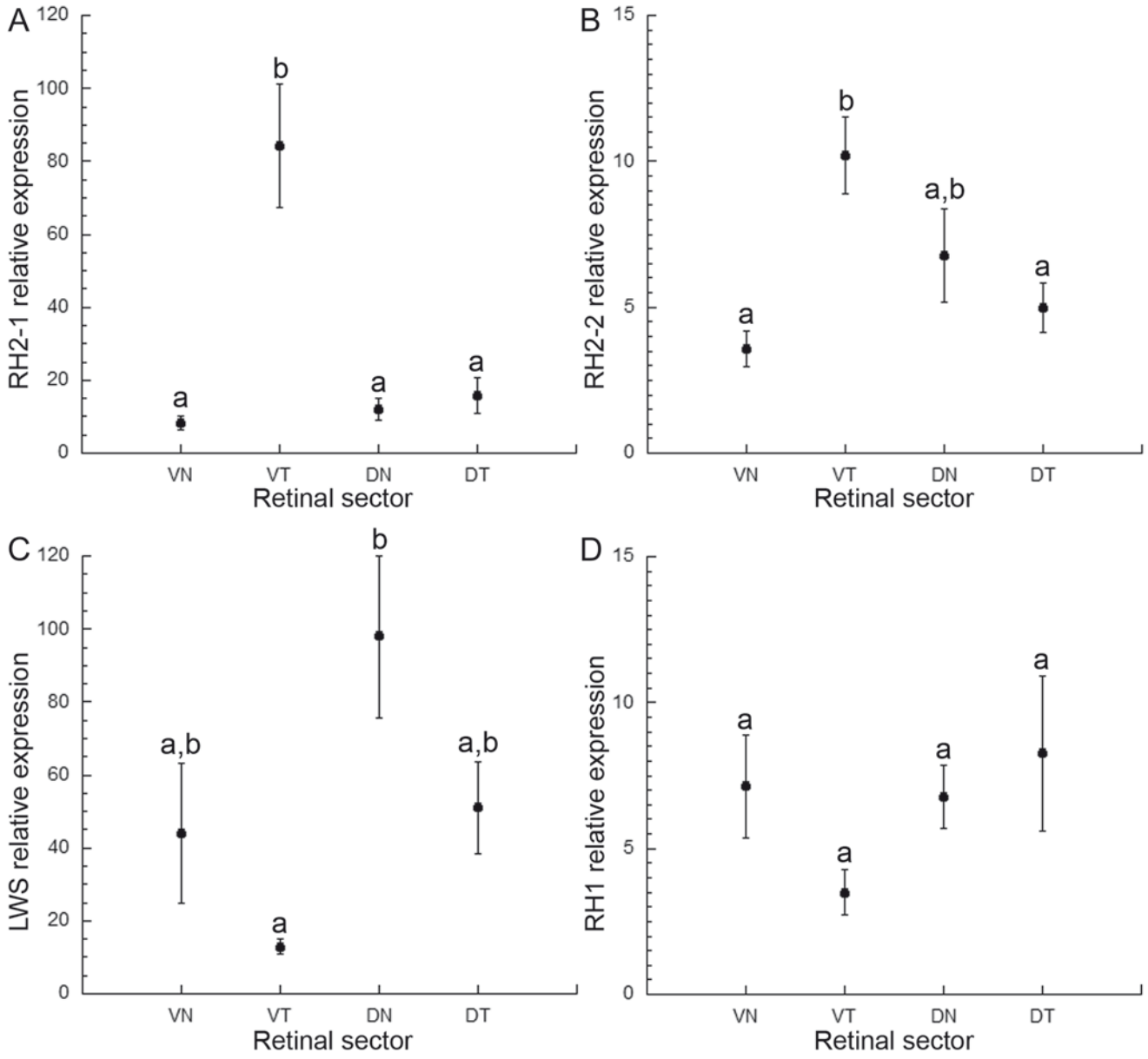


Fig. 4. Relative opsin transcript expression (mean ± S.E.) with respect to *coxI* as a function of retinal sector for (**A**) RH2-1, (**B**) RH2-2, (**C**) LWS, and (**D**) RH1 (n = 10 per sector). Means with the same letter (a or b) are statistically similar.

wavelengths (e.g., 500 nm). It is also possible that a short wavelength visual pigment may be present in anchovies (contributing, for instance, to the increased sensitivity at 480 nm in the DN retina, Fig. 2C) and may be housed in the single cones, whose absorbance is unknown. However, no SWS1 or SWS2 opsins, which would give rise to UV and short wavelength absorbing pigments, have been found in the retina of the Japanese anchovy (Kondrashev et al., 2013), even though an SWS1 opsin is present in the Japanese sardine (Miyazaki et al., 2017) and an S (447 nm) visual pigment is found in the blueback herring (Novales Flamarique & Hárosi, 1999), two close relatives. Together with previous results for the ventral retina, the present findings demonstrate a retina with variable spectral sensitivity and with the potential for color vision in all sectors except the VT region (Fig. 2), which is the only quarter known to mediate polarization vision (Novales Flamarique, 2017).

Differential opsin expression as the source of intraretinal variation in spectral sensitivity

As in the Japanese anchovy (Kondrashev et al., 2013), the retina of the northern anchovy contained only retinal (vitamin A1-based) chromophore. Thus, variability in the absorbance of visual pigments could only be due to differential expression of opsin types. RH2-1 expression was significantly greater in the VT retina, and the RH2-2 mean was 1.4 to 2.9 greater than corresponding means from other sectors. Conversely, the LWS mean associated with the VT sector was the lowest of all means; the ratio of means of LWS to the sum of RH2s was 0.13, 3.7, 2.5, and 5.2 for the VT, VN, DT, and DN sectors of the retina. These results show opposite trends in relative magnitude of RH2 and LWS expression between the VT sector and the rest of the retina.

The relative expression of the various opsin transcripts is in line with the results from visual pigment measurements and the variability

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in spectral sensitivity functions. Two, statistically similar, M visual pigments (with λ_{max} at 514 and 516 nm) were found in the VT retina among the axially dichroic cone population (Novales Flamarique, 2017). Accordingly, the spectral sensitivity function showed a single peak at 520 nm with reduced sensitivity in the shorter and longer wavelengths (Fig. 2G; Novales Flamarique, 2017). The preponderance of RH2 opsin transcript expression and lowest LWS expression in the VT retina thus agrees with what would be expected from the physiological findings. In fact, it is possible that expression of LWS within this area of the retina is not due to the axially dichroic cone population. This is because the area occupied by these cones does not cover the entire VT quarter and the results were therefore likely influenced by adjacent cone populations, which contain triple cones whose central element houses an L (λ_{max} ~ 556 nm) visual pigment.

The remaining quarters of the retina had greater expression of LWS over RH2 opsin transcripts, and this is also as expected from the physiological results. These areas of the retina are populated by triple cones with comparatively lower, but variable, numbers of single cones, and with at least two distinct visual pigments (Novales Flamarique, 2011, 2017). Accordingly, the optic nerve-derived spectral sensitivity functions showed two peaks in the middle wavelengths and the capacity for color discrimination (Fig. 2A–2F). Together, our results describe the first fish retina with intraretinal variability in spectral sensitivity that can be explained by differential expression of opsin transcripts.

RH1 transcript expression was evenly distributed across the retina and this may be indicative of similar rows of rods (3–5 elements in width) that alternate with those of cones throughout the retina (Novales Flamarique, 2011). The expression of the rod opsin transcript was lower overall than that of the sum of cone opsins, which is unusual for a duplex retina. A potential explanation may reside in the minute outer segment volume of individual rods compared with those of cones (Novales Flamarique, 2011; Hárosi & Novales Flamarique, 2012).

Potential ecological correlates of the northern anchovy's visual capabilities

The ecological advantages of a functionally segregated retina for color and polarization vision in the northern anchovy are unknown. It is likely that the polarization sensitive area, with its highly packed axially dichroic cones (Novales Flamarique, 2011), serves to detect small zooplankton prey that would disrupt the background polarization in the upper frontal field. Alternatively, it may also be used to detect avian predators against the downwelling polarized light field during crepuscular periods, when anchovies are commonly found near the water surface (Hayasi, 1963; Weimerskirch et al., 2012). Such predators would be perceived as shadows diving downwards and survival of the anchovy may depend on their early detection, when they are but small objects in the sky. Other areas of the retina, with the capability for color vision, may be more apt to detect predators based on reflections whose wavelength content stand out against the longer wavelength background (Novales Flamarique & Hawryshyn, 1997; Novales Flamarique et al., 2007), or to maintain cohesion with other fishes in the school whose quick turns would produce reflection flashes with high contrast against the background. However, having matching and offset visual pigments (and associated sensitivity) to the prevalent wavelengths in the background may not necessarily translate into visually mediated behaviors based on chromatic contrast. As is the mouse, whose dual-opsin expressing S cones in the ventral retina may be primarily devoted to achromatic detection of avian predators (Baden et al., 2013), triple cones in the VN retina of the anchovy may have additional physiological properties that endow them with a functional preference for detection of achromatic contrasts.

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