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Polymorphism of the rod visual pigment between allopatric populations of the sand goby (\textit{Pomatoschistus minutus}): a microspectrophotometric study

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Summary

Absorbance spectra were measured by microspectrophotometry in retinal rods of sand gobies (\textit{Pomatoschistus minutus}) from four allopatric populations (Baltic Sea, Swedish west coast, English Channel and Adriatic Sea). Mean (± S.E.M.) wavelengths of maximum absorbance ($\lambda_{\text{max}}$) were 508.3±0.5 nm, 505.4±0.2 nm, 506.2±0.3 nm and 503.0±0.3 nm, respectively. Pairwise comparison between the populations (post-ANOVA Scheffe's test) shows that each of the $\lambda_{\text{max}}$ differences, except that between the Swedish west coast and the English Channel, is statistically significant ($P<0.05$). The shapes of the absorbance spectra indicated that the pigments were A1 rhodopsins with no measurable admixture of the A2 chromophore. Thus, the differences indicate polymorphism in the protein part (opsin) of the pigment. Convolution of A1 templates for $\lambda_{\text{max}}$ values 508.3 nm and 503.0 nm with quantum spectra of the downwelling light at two locations at the south-west coast of Finland indicated that a 13–19% improvement in quantum catch would accrue in the Baltic environment from the 5.3 nm red-shift of the rod pigment of Baltic compared with Adriatic sand gobies.

Key words: microspectrophotometry, rod photoreceptor, retina, sand goby, \textit{Pomatoschistus minutus}, polymorphism, vision.

Introduction

The rod photoreceptors of the retina chiefly subserve vision at low light levels. Effective photon catch is a necessary condition for reaching high signal-to-noise ratios in dim light and thus high visual sensitivity. This in turn requires, among other things, that the main absorption band of the visual pigment be in some sense tuned to match the illumination spectrum in the environment. Spectral tuning of the pigment can be achieved on an evolutionary time scale by amino acid substitutions in the protein part (the opsin) or on a physiological time scale by exchanging the chromophore; in vertebrates, either retinal (vitamin A1 aldehyde; denoted A1) or 3,4-dehydroretinal (A2). For a given chromophore, the absorbance spectra of all known visual pigments can be described by a common template having the wavelength of maximum absorbance ($\lambda_{\text{max}}$) as sole parameter (Dartnall, 1953; Govardovskii et al., 2000).

Aquatic environments offer a wide range of strongly profiled, yet reasonably stable or at least regularly recurring, spectral environments, making underwater vision a gratifying field for comparative studies. There is a vast literature on spectral adaptations in fishes (for reviews of older literature, see, for example, Lythgoe, 1972, 1988; Bridges, 1972; for more recent studies relating spectral absorbance to opsin structure, see, for example, Bowmaker et al., 1994; Hope et al., 1997; Hunt et al., 1996, 2001; Yokoyama and Tada, 2000).

However, little work has been done on incipient evolutionary adaptation between separated populations of a single species inhabiting spectrally different waters.

The sand goby (\textit{Pomatoschistus minutus}) is a small marine fish (adult length 5-10 cm) that occurs in considerable abundance along the coasts of Europe from northern Norway to the eastern Mediterranean, including the bracken-water of the Baltic Sea. This geographical span encompasses a wide range of different light environments. For example, the peak of the light spectrum at 30 m depth in the Baltic Sea lies around 550–560 nm, which is displaced by some 80 nm towards longer wavelengths compared with that of the Mediterranean (Jerlov, 1976; Lindström, 2000). As the gobies spend their off-breeding season at depths of several tens or even hundreds of meters (Koli, 1995), one would expect that the Baltic population would benefit from shifting $\lambda_{\text{max}}$ of the dim-light receptor towards longer wavelengths compared with its truly marine conspecifics.

Studying four populations of sand gobies (Baltic Sea, Swedish west coast, English Channel and Adriatic Sea), we find small but consistent shifts in $\lambda_{\text{max}}$, from 503.0±0.3 nm (mean ± S.E.M.) in the Adriatic to 508.3±0.5 nm in the Baltic, with the two other populations falling in between. We show that even such a small shift may be significant for vision. Since the differences could not be explained by a chromophore change, they must indicate polymorphism of the opsin.
Fig. 1. Geographical locations and spectral light environments (A), individual rod absorbance spectra (B) and distributions of rod \( \lambda_{\text{max}} \) values (C) of sand gobies from the four populations studied. (A) The four locations from which gobies were collected. The wavelength range connected with each site gives the approximate band of maximum transmission of the respective water according to Jerlov (1976). The colour code used here and in all other figures is: red and violet, Tvärminne Zoological Station, Finland (sand gobies and common gobies, respectively, denoted B for ‘Baltic’); yellow, Kristineberg Marine Research Station, Sweden (sand gobies, denoted S for ‘Swedish’); orange, University of Plymouth, England (sand gobies, denoted E for ‘English’); blue and green, Chioggia Marine Biological Station, Italy (sand gobies and marbled gobies, respectively, denoted A for ‘Adriatic’). (B) Examples of rod absorbance spectra from individual sand gobies. Colour code as in A. The \( \lambda_{\text{max}} \) values of these individuals are 508.6 nm (B), 505.7 nm (S), 505.7 nm (E) and 501.9 nm (A). The spectra shown here and in subsequent figures have been Fourier-filtered, with 25 harmonics retained. (C) Distribution of the \( \lambda_{\text{max}} \) values in all 54 sand gobies on 1-nm bins. The different populations are distinguished by colour as in A and B: red (N=17), orange (N=10), yellow (N=9) and blue (N=18).

Materials and methods

Animals

The sand gobies [Pomatoschistus minutus (Pallas 1770)] were obtained from (1) the Baltic Sea at the south-west coast of Finland (Tvärminne Zoological Station, Hanko), (2) Kattegat at the west coast of Sweden (Kristineberg Marine Research Station, Fiskebäckskil), (3) the English Channel at Plymouth (University of Plymouth) and (4) the Adriatic Sea near Venice (Chioggia Marine Biological Station, Italy). These locations and the approximate bands of maximum spectral transmission of the respective waters according to Jerlov (1976) are indicated on the map in Fig.1A. The other species studied for comparison were the common goby [Pomatoschistus microps (Kröyer 1838)] from the Baltic Sea and the marbled goby [Pomatoschistus marmoratus (Risso 1810)] from the Gulf of Venice. The fish were kept in aquaria with the salinity of the respective habitat (from 0.6% in the Baltic via 2% in Sweden and the coast of England to 3.5% in the Adriatic). They were maintained at approximately 15°C and supplied with appropriate diet. The sand gobies from the Adriatic Sea were frozen at −18°C after netting, then stored in darkness at −70°C.

Recording

Before microspectrophotometrical (MSP) measurements, the living fish were dark-adapted overnight. The frozen Adriatic sand gobies were thawed individually for approximately 30 min in darkness at room temperature before dissection. All subsequent manipulations were performed under dim red light. The fish were decapitated and pithed. The eyes were dissected in physiological saline (teleost Ringer) containing: 110 mmol l\(^{-1}\) NaCl; 2.5 mmol l\(^{-1}\) KCl; 1 mmol l\(^{-1}\) CaCl\(_2\); 1 mmol l\(^{-1}\) MgSO\(_4\); 10 mmol l\(^{-1}\) NaHCO\(_3\); and 10 mmol l\(^{-1}\) glucose. The solution was buffered to pH 7.2–7.4 with 10 mmol l\(^{-1}\) Heps. The lens was removed, and pieces of retina separated from the pigment epithelium were transferred to a drop of Ringer on a cover slip and teased apart. Dextran (10–15%, \( M_r = 70 \text{kDa} \)) was added to the Ringer to prevent excess cell movements during recordings. The sample was covered with a second cover slip, sealed at the edges with vaseline and placed on the MSP stage.

Absorbance spectra were recorded with a single-beam, computer-controlled, fast-wavelength scanning microspectrophotometer built at the University of Helsinki (Govardovskii et al., 2000; Ala-Laurila et al., 2002). The basic design is
described in Govardovskii and Zueva (2000). A halogen lamp served as a light source, and spectral scanning was achieved through a diffraction grating attached to the head-moving lever of a Seagate ST-225 computer hard disk drive. The grating was moved by a stepper motor grid, whose position was controlled by a computer-driven step motor. Recordings were made on isolated rod outer segments (OSs) or the outer segments of rods still attached to small pieces of retina. OS dimensions were approximately 3 μm x 30–40 μm. The size of the measuring beam was adjusted to match the sample, typically to approximately 75% of the OS width and nearly the full OS length. The beam was linearly polarized in the plane of the discs. A baseline measurement was obtained by scanning a clear area adjacent to the cell. The OS was then scanned, and the ratio between the two measurements gave the absorbance spectrum. The wavelength calibration was checked regularly, at least at the beginning and at the end of each experiment, against the spectrum of a ‘blue glass’ standard, the spectrum of which had been accurately determined in a Hitachi spectrophotometer. The recordings were carried out at room temperature. For further technical details, refer to Govardovskii et al. (2000) and Ala-Laurila et al. (2002).

**Analysis**

The data were stored on the computer hard disk for later analysis. The details of the analysis can be found in Govardovskii et al. (2000), and only a brief description is given here. Raw spectra from single cells were averaged and normalized within each individual, and the resulting individual spectra were corrected for zero offset. The position of the zero-line was computed as a straight line least-square fitted to the long-wave tail of the spectrum between 650 nm and 750 nm, where the absorbance of the visual pigment is close to zero. High-frequency noise components were removed by Fourier filtering, retaining 25–35 harmonics. Finally, the mean, zero-line-corrected and filtered spectrum from each individual was fitted with the A1 template of Govardovskii et al. (2000), giving the λ_{max}. Differences in λ_{max} between the populations were statistically evaluated using the SPSS 10.0.7 program. The pairwise comparisons were based on Scheffe’s test after an initial analysis of variance (ANOVA) had indicated the presence of significant between-population differences.

**Results**

**Sand gobies**

Absorbance spectra were recorded from rods of 18 individual sand gobies from the Adriatic Sea (A), nine from the west coast of Sweden (S), 17 from the Baltic Sea (B) and 10 from the English Channel (E). In each individual, 35 cells were recorded on average [the mean per individual ranged from 15 (S) to 65 (E)]. Fig. 1B shows typical examples of spectra for individual fish from the four populations after zero-line correction, smoothing and normalization to unity peak absorbance. For each individual spectrum, λ_{max} was determined by fitting of the Govardovskii et al. (2000) template for A1 pigments.

The histogram in Fig. 1C shows the distribution of λ_{max} values obtained by fitting all the individual sand goby spectra. The different populations are symbolized by the colours that mark the respective locations in Fig. 1A. The means (± S.E.M.) of λ_{max} calculated across individuals within each population were 503.0±0.3 nm (A), 505.4±0.2 nm (S), 506.2±0.3 nm (E) and 508.3±0.5 nm (B). An initial ANOVA indicated that there are significant differences between populations (P<0.001). Pairwise comparison based on Scheffe’s test indicated that the Adriatic population differs from each of the others (A vs B, P<0.01; A vs S or E, P<0.01), as does the Baltic population (B vs S or E: P<0.05). By contrast, the difference between S and E is not significant (P>0.8).

A quick way for fish to red-shift their spectral sensitivity is to switch the visual-pigment chromophore from A1 to A2 (e.g. Dartnall and Lythgoe, 1965: Bridges, 1972). We therefore paid special attention to the question of whether the relative bathochromic shift of the Baltic rod pigment could be explained by some degree of A2 admixture. Since A1 and A2 spectra are of different shape, this can be studied by comparing the quality of fit of a pure A1 template with that achieved by some linear combination of A1 and A2 templates. The λ_{max} of the A1 pigment was always chosen so that the mixture should give the best possible fit to the main part of the recorded spectrum [the λ_{max} of the A2 component is tied to its A1 pair through the Harsy (1994) relationship]. In no individual could a perceptibly improved fit be achieved by adding A2, and when the assumed proportion of A2 was increased above approximately 2%, the fits clearly started to deteriorate. This is exemplified in Fig. 2A for the Baltic spectrum shown in Fig. 1B. The best-fitting template for a 95%:5% A1:A2 mixture is seen to run systematically above the recorded spectrum at long wavelengths. Fig. 2B illustrates the unacceptability of a conceivable ‘null’ hypothesis that Adriatic and Baltic fish actually have the same opsin and that the λ_{max} shift from 503.0 nm to 508.3 nm is achieved just by mixing in a certain percentage of A2 chromophore. Moving the peak to 508.3 nm would require a 39% admixture of A2, producing a curve shape that is quite incompatible with the recorded spectrum.

**Other gobies**

When collecting sand gobies, we happened to catch a few specimens of two other species of Pomatoschistus. It is of some interest to compare the λ_{max} of these close relatives with the range covered by the sand goby populations. Thus, rod absorbance spectra were also recorded from 11 common gobies (P. microps) from the Baltic Sea and two marbled gobies (P. marmoratus) from the Gulf of Venice (on average, 30 cells from each individual). The λ_{max} values obtained were 515.7±0.4 nm (P. microps) and 506.7 nm (the two P. marmoratus individuals studied both yielded the same λ_{max}). Fig. 3A shows individual spectra from these species together with the Adriatic sand goby spectrum from Fig. 1B. Fig. 3B summarizes the results as a histogram similar to Fig. 1C. For comparison, the λ_{max} ranges found in the
different sand goby populations have been plotted as bars above the histogram.

*Light environment*

The gobies go into deep waters after the breeding season, to some 40 m in the Adriatic Sea, starting in March, and at least tens of meters in the Baltic Sea, starting in August–September. It is in these conditions that there is least light overall, and spectral filtering by the water is most pronounced. Thus, these are the conditions where spectral adaptation of the rod pigment (as opposed to cone pigments) would be most important for visual sensitivity, and considerations of the ecological significance of rod $\lambda_{\text{max}}$ are likely to be most relevant. As an example, we shall compare the calculated quantum catches of an Adriatic and Baltic sand goby and a Baltic common goby, all assumed to live in a Baltic light environment.

The grey symbols in Fig. 4 show the spectral distribution of light at 10 m depth in a narrow bay of the Baltic Sea, Pojoviken, close to where the Baltic gobies were caught. The spectrum is reproduced from Lindström (2000; curve B in his Fig. 1). The three other curves are quantum catch spectra, obtained by convolving the 503 nm, 508.3 nm and 515.7 nm A1 templates with the light spectrum. It is immediately evident that the highest $\lambda_{\text{max}}$ value gives the best quantum catch (violet curve for the common goby), while the lowest value gives the worst (blue curve for the Adriatic sand goby). The total quantum catch in each case is proportional to the integral of the spectrum across wavelengths, i.e. the area under each curve. Thus, we find that the Baltic sand goby would catch 19% more quanta than the Adriatic sand goby in this environment. The Baltic common goby, however, is still 23% better than the Baltic sand goby and 47% better than the Adriatic sand goby.
Fig. 3. Comparison with the rod absorbance spectra of common goby and marbled goby. (A) Individual spectra of common goby from the Baltic Sea (violet) and marbled goby from the Gulf of Venice (green). The Adriatic sand goby spectrum from Fig. 2 is reproduced as a reference (blue). (B) Distribution of the \( \lambda_{\text{max}} \) values measured in 10 common gobies and two marbled gobies on 1-nm bins. For comparison, the \( \lambda_{\text{max}} \) ranges spanned by the four populations of sand gobies are shown as bars above the histogram. Colour code as in Fig. 1.

Identifying changes that control a second important functional property of visual pigments, thermal stability (Fyhrquist et al., 1998). Sequencing the opsins of individual fish (characterized by MSP) from the different populations may allow us to establish very specific correlations between amino acid substitutions and the spectral properties, the published amino acid sequence of the English Channel population (Archer et al., 1992) serving as a reference. Moreover, the results can be related to within-species phylogeny based on other markers, whereby the common goby and the marbled goby serve as outgroups.

**Functional significance of the differences in \( \lambda_{\text{max}} \)**

The qualitative consistency of the results may seem surprising, as the \( \lambda_{\text{max}} \) differences are so small as to leave doubts about their functional significance. This is true especially considering seasonal, tidal and other variations in the spectral transmission of the water as well as very different conditions encountered in the shallow and the deep waters inhabited at different times of the year.

With respect to the conditions relevant for rod pigment adaptation, the situation is less complex. Only in deep waters will the rod system consistently have to face the limits to visual sensitivity and quantum catch (together with thermal stability) become all important. Vision in shallow waters in brighter light has to solve other problems, which mainly concern the cone pigments (cf. Lythgoe, 1979, 1988).

In Fig. 4, we considered adaptation to the Baltic Sea in order to estimate how much quantum catch may vary with the observed differences in \( \lambda_{\text{max}} \). This is a reasonable example, as the Baltic red-shift of \( \lambda_{\text{max}} \) may probably be regarded as a recent adaptation from an original marine state. The gobies probably colonized the Baltic Sea in its last truly marine (Litorina) phase, starting some 7000 years ago and gradually developing into the present-day bracken-water condition. As shown in Fig. 4, moving \( \lambda_{\text{max}} \) from 503 nm to 508.3 nm would confer a 19% quantum catch advantage on a fish living at 10 m depth in Pojoviken Bay. Similar convolution with an open-sea Baltic spectrum measured at 20 m depth (Lindström, 2000; Fig. 1A) gives a 13% advantage for the Baltic sand gobies compared with the Adriatic ones. Although the ecological value of this is not negligible, it is worth noting that the closely related common goby (\( P. \) microps, \( \lambda_{\text{max}} = 515.7 \) nm) fares much better in the same environments, having a 31% and 47% quantum catch advantage over the Adriatic sand gobies in the

**Discussion**

**Polymorphism of the rod pigment between populations**

We find small but clear (statistically significant) differences in rod pigment \( \lambda_{\text{max}} \) in five of the six possible comparisons between pairs among the four sand goby populations. As there was no hint of A2 chromophore in any of the fish, the differences indicate polymorphism in the opsin. Since they are qualitatively well correlated with the differences in the respective spectral light environments, it seems justified to regard them as evolutionary adaptations. The greatest \( \lambda_{\text{max}} \) shift was found between the Baltic and Adriatic populations, which inhabit the two spectral ‘extremes’ (cf. Fig. 1A). By contrast, the two populations living in the two intermediate light milieus of the English Channel and the Swedish west coast did not differ significantly.

The spectral absorbance of visual pigments (determining the spectral sensitivity of the organism) is an interesting characteristic from an evolutionary point of view. It provides a case where a phenotypic trait subject to strong natural selection depends directly on changes in a single gene. A considerable amount of knowledge has been accumulated about amino acid changes that modify the absorption spectra of visual pigments (see, for example, Hunt et al., 1996, 2001; Yokoyama, 2000, 2002). A start has also been made towards
Baltic open sea and Pojoviken environments, respectively. The rather modest adaptation of the Baltic sand goby compared with the common goby could be due to different earlier histories but might also indicate functional constraints in the molecule that oppose further red-shifts along the routes open to this particular opsin. Opposing pressures could arise, for example, from a connection between increased long-wavelength sensitivity and increased thermal noise (Barlow, 1957; Donner et al., 1990; Milder, 1991). A general empirical correlation has been found between the thermal stability and $\lambda_{\text{max}}$ of visual pigments, although no strict physical relation exists (Firsov and Govardovskii, 1990; Fyhquist, 1999; Koskelainen et al., 2000). The loose but still significant correlation observed could be interpreted to mean that the cost in thermal noise incurred by shifting $\lambda_{\text{max}}$ varies depending on the exact amino acid sequence of the opsin, so that a red-shift that gives a net signal-to-noise profit when caused by a certain mutation or set of mutations in one opsin (e.g. the common goby) might give a net loss when implemented in the partly different molecular setting of another opsin (e.g. the sand goby).

A similar argument can be advanced to explain why the sand gobies have not taken recourse to the obvious possibility of changing the chromophore to A2, as used by many fish and amphibian species. Switching from A1 to A2 red-shifts $\lambda_{\text{max}}$ by more than 20 nm (Dartnall and Lythgoe, 1965; Hárosi, 1994) but is also known to lower the minimum energy for photoactivation and increase the rate of thermal pigment activations and thus thermal noise (Donner et al., 1990; Milder, 1991; Koskelainen et al., 2000; Ala-Laurila et al., 2002). The signal-to-noise change could easily be negative. With respect to chromophore changes, however, it should further be noted that all photoreceptors in one retinal area apparently receive similar proportions of A1/A2 from the pigment epithelium (Loew and Dartnall, 1976; Makino-Tasaka and Suzuki, 1984), and optimisation of the A1/A2 ratio may equally well be driven by cone vision as by rod vision. Obviously, these arguments concern ‘ultimate’ causes of lack of A2. The ‘proximate’ cause might simply be that the fish lack the necessary enzyme for A1/A2 conversion.

**Polymorphism within the same region?**

Our mean $\lambda_{\text{max}}$ value for the English Channel sand gobies (506.2 nm) is significantly higher than the value of 500.8 nm reported by Archer et al. (1992) for specimens caught in the same area near Plymouth. In the 10 Plymouth individuals studied here, $\lambda_{\text{max}}$ ranged from 504.6 nm to 507.8 nm. The differences we are concerned with in the present work are small overall, so we have to ask whether the difference between our value and that of Archer et al. must indicate that different populations (in a statistical sense) have been sampled even in this local region. At this point there seem to be no strong reasons to think so. Firstly, trivial differences in $\lambda_{\text{max}}$ reported by different investigators arise just from using different visual-pigment templates. To evaluate this possibility, we fitted the spectral data of Archer et al. (1992, their fig. 1) with the Govardovskii et al. (2000) template. The best fit was obtained with $\lambda_{\text{max}}$=502 nm, but, admittedly, their data were certainly not consistent with $\lambda_{\text{max}}$=506.2 nm. Secondly, however, their data had been collected from a very small sample of rods in questionable condition, eight rods from five fish, while the typical dimensions of the rod OSs (or the measurement beam fitting within the OS?) are reported as 2 $\mu$m$\times$5 $\mu$m. In our material, the dimensions of the OSs were typically approximately 3 $\mu$m$\times$30–40 $\mu$m. In view of the small sample size and apparent fragmentation of the OSs, we would not regret the discrepancy between their and our $\lambda_{\text{max}}$ values as significant.

On the other hand, there is of course no reason to exclude the possibility of polymorphism within populations. This is the material on which natural selection operates. Bowmaker et al. (1975) have previously suggested that the wide variation they found in rod $\lambda_{\text{max}}$ between individuals of the frog *Rana temporaria* (from a single supplier but of unidentified provenance) could indicate genetic heterogeneity. It may be significant that the strongest suggestion of within-population polymorphism in our material is found among the Baltic sand gobies, which display a particularly broad $\lambda_{\text{max}}$ distribution spanning 5.7 nm (Fig. 1C).

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**Fig. 4.** Calculated spectral quantum catch for rods of Baltic common gobies (violet curve), Baltic sand gobies (red curve) and Adriatic sand gobies (blue curve) in Pojoviken Bay of the Baltic Sea (spectral distribution of light quanta measured at 10 m depth by Lindström, 2000; grey curve). The quantum catch spectra were obtained by convolution of the light spectrum with the respective rod absorbance spectra, all initially normalized to unity. The integrals of the quantum catch spectra give total quantum catches, which are related as 1 (blue), 1.19 (red) and 1.46 (violet).
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