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Evolution of visual sensory system in the common barbel (*Barbus barbus*)

Evolve zrak u parmy obecné (*Barbus barbus*)

Diploma thesis

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Prohlášení

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Abstrakt

Obratlovci od společného předka zdědili pět tříd zrakových pigmentů (opsinů), které jsou exprimovány v tyčinkách a čípcích. Zatímco u mnoha skupin obratlovců došlo k redukci počtu opsinů, opsiny kostnatých ryb se rozrůznily a kostnaté ryby tak vynikají mezi obratlovci v počtu zrakových pigmentů i typů fotoreceptorů. Tomuto výjimečnému rozvoji napomohla též celogenomová duplikace specifická pro kostnaté ryby, k níž došlo přibližně před 350 miliony let. Zde se zabývám kostnatou rybou, která v nedávné době prošla další celogenomovou duplikací – parmou obecnou (*Barbus barbus*). Tato událost byla spojena s hybridizací (šlo tedy o allopolyploidizaci) a vedla ke vzniku 14 genů pro opsiny přítomných v genomu parmy, což je neobvykle vysoký počet i mezi kostnatými rybami. V této práci jsem zkoumala vliv takto rozvinutého repertoáru opsinů na zrakové schopnosti parmy obecné. Doplnila jsem referenční sekvence všech genů pro opsiny na základě celogenomových dat a odhalila jejich pozice na chromozomech. Analyzovala jsem transkripty sítnice a vytvořila vývojovou řadu expresních profilů opsinů. Geny všech opsinů byly v genomu zachovány funkční, ačkoliv jeden z nich (citlivý na zelené světlo, v této práci nazvaný RH2-4) nebyl nalezen v žádném transkriptu a vykazoval zvýšenou substituční rychlost na nesynonymních pozicích, což naznačuje uvolnění selekce. V rámci tří tříd opsinů (SWS1 citlivé na UV, SWS2 citlivé na modrou a RH2 citlivé na zelenou) jsem objevila pár opsinů s dělbou funkce v čase – larvální a adultní geny. V nejčasnější fázi vývoje parmy exprese larválních genů výrazně převyšuje expresi genů adultních. Následně fotoreceptory projdou synchronizovanou změnou exprese a všechny tři larvální geny (náležející do tří různých tříd opsinů) jsou nahrazeny adultními variantami. Podobný jev dosud nebyl u polyploidního druhu popsán a jeho účel a regulace na molekulární úrovni jsou stále nejasné. Dále jsem porovnávala sekvence aminokyselin napříč všemi opsiny v rámci každé třídy a vyhledala substituce, které ovlivňují jejich funkci (tj. substituce odlišující spektrální citlivost opsinů patřících do stejné třídy). Odhalila jsem zajímavé odlišnosti ve frekvenci aminokyselinových substitucí mezi jednotlivými třídami opsinů i uvnitř nich a pokusila jsem se tyto odlišnosti propojit se vzorci exprese opsinů. Objevila jsem a vizualizovala čtvercovou sítnicovou mozaiku (tj. pravidelný vzor v rozmístění čípků) na sítnici dospělé parmy pomocí fluorescenční in situ hybridizace (FISH). Stejnou metodou jsem určila i rozmístění exprese u dvojic opsinů, které vznikly ze stejného genu během nedávné allopolyploidizace a které byly zároveň exprimovány v dospělé sítnici. Zjistila jsem, že opsiny náležející do stejné třídy jsou vždy koexprimovány ve stejné buňce. Pro rozšíření kontextu této práce jsem navíc provedla fylogenetické analýzy genů pro opsiny pocházejících z parmy obecné, tří dalších druhů evropských parem a kapra obecného. Mé výsledky naznačují, že parma obecná disponuje mnohem rozvinutějším zrakem, než se myslelo, a že další výzkum tohoto druhu by mohl přinést nové poznatky o procesech souvisejících s polyploidizací u obratlovců.

Klíčová slova

smyslové soustavy, zrak, zrakové pigmenty, opsiny, evoluce, ryby, kaprovití, analýza transkriptomu, fluorescenční in situ hybridizace, fylogenetická analýza

Abstract

Vertebrates inherited five visual opsin classes from their common ancestor expressed in the rod and cone cells. While many vertebrate groups reduced the number of visual pigments they possess, opsins of teleost fishes have diversified. Today, teleosts stand out among all vertebrates in the number of visual pigments and photoreceptor types. This extraordinary development was also facilitated by the teleost-specific whole genome duplication that occurred approximately 350 Mya. Here I explored teleost species that recently underwent subsequent whole genome duplication – the common barbel (*Barbus barbus*). This event was accompanied by hybridization (i.e., it was allopolyploidization) and resulted in 14 opsin genes found in the genome, an unusually high number even among teleosts. In this thesis, I studied the effects of such an expanded opsin repertoire on the visual abilities of the common barbel. Based on the whole genome, I have completed the reference sequences of all opsin genes and revealed their positions on the chromosomes. I analysed retinal transcriptomes and constructed developmental series of opsin expression profiles. All opsin genes were preserved functional in the genome, even though one of them (green-sensitive, named RH2-4 in this thesis) was not found in the transcriptome and showed an increased substitution rate at non-synonymous sites, a possible sign of relaxed selection. I demonstrate that three opsin classes (UV-sensitive SWS1, blue-sensitive SWS2, and green-sensitive RH2) comprise the opsin pairs with a clear temporal division of function – larval and adult genes. At the earliest researched life stage, expression of all three larval genes greatly exceeds expression of adult ones. Subsequently, photoreceptors undergo a synchronized switch in opsin expression, and all three larval genes (within three distinct opsin classes) are gradually replaced by adult alternative copies. Such effect has never been reported before for the polyploid species, and its purpose and molecular regulation remain uncertain. I compared amino acid sequences of all opsins within each class looking for substitutions with functional consequences (i.e., substitutions that differentiate spectral sensitivity of opsins within the same opsin class). I revealed interesting variation in amino acid substitution rates both across and within opsin classes, and I attempt to link it to expression patterns. I have further discovered and visualized square cone mosaic (i.e., regular pattern in cone distribution) in the adult retina via fluorescent in situ hybridization (FISH). I used the same method to uncover spatial expression patterns of the opsin pairs that emerged in the recent allopolyploidization and were expressed simultaneously in the adult retina. I revealed that cone opsins from the same opsin class are always coexpressed in the same cell. Additionally, I constructed phylogenies of opsin genes from the common barbel, three other species from the European barbel clade, and the common carp to bring broader context to this study. My results suggest that the common barbel possesses a much more elaborated vision than thought before and that further research of this species could bring new insights into processes associated with polyploidization in vertebrates.

Key words

sensory systems, vision, retina, visual pigments, opsins, evolution, fish, cyprinids, transcriptome analysis, fluorescent in situ hybridization, phylogenetic analysis

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Introduction

Teleosts (Teleostei) are the most diverse vertebrate infraclass comprising more than half of all vertebrate species (over 35,000 to this day) (Fricke, Eschmeyer and Fong, 2022). They inhabited various water environments ranging from mountain streams to ocean depths. Their outstanding success was enabled by many unparalleled adaptations and life strategies. Yet, teleosts are the most understudied vertebrate group. Hundreds of species are still being described every year and some species inhabit such an inaccessible environment that they have never been observed alive (e.g., deep-sea fishes). Furthermore, most studies focused on a limited selection of model organisms (e.g., zebrafish, goldfish, Nile tilapia). For those reasons, there is still much to discover about teleosts and research of non-model species often brings surprising revelations of phenomena that we otherwise would not consider possible in vertebrates.

Since aquatic habitats represent a much more complex environment for vision than terrestrial ones, with many changeable optical parameters both in time and space, it is not surprising that we find fascinating visual adaptations in teleosts (Carleton *et al.*, 2020). Among the adaptations of visual apparatus, visual pigments (opsins) are exceptionally appealing to scientists due to their direct link between DNA sequence, RNA sequence, protein sequence, and function. Today, it is possible to infer the shifts in spectral sensitivity of the individual opsin from nucleotide substitutions in its DNA sequence (Yokoyama, 2008). Moreover, opsin gene repertoire tends to develop rapidly in teleosts and opsin genes often duplicate (Carleton, 2009; Cortesi *et al.*, 2015; Escobar-Camacho *et al.*, 2020). Visual pigments of teleosts are thus ideal model for the study of evolution.

Dynamic evolution of opsin genes in teleosts has been presumably aided by teleost-specific whole genome duplication that occurred approximately 350 Mya (Meyer and Van De Peer, 2005). Indeed, multiple opsins descending from this event are still preserved in some fishes (Escobar-Camacho *et al.*, 2020). While teleost-specific whole genome duplication is very ancient and opportunities to study associated processes are thus very scarce, many teleost species (sometimes even the whole lineages) underwent subsequent polyploidizations, and their research is now getting into the spotlight (Mable, Alexandrou and Taylor, 2011).

In this thesis, I study the visual sensory system of the evolutionary tetraploid teleost species – the common barbel (*Barbus barbus*). I focus on opsin genes originating in the recent polyploidization event, their evolution and utilization in this species.

The goals of this thesis are: (1) to identify all visual opsins in the common barbel; (2) to compare the sequences of “sister” opsins - opsin couples resulting from the recent polyploidization event; (3) to compare opsin expression profiles among different developmental stages; (4) to investigate and visualize retinal structure and potential cone mosaic; (5) to localize expression of individual opsins in the adult retina with special focus on sister opsins (if both expressed at the adult stage).

Literature overview

Vertebrate visual system anatomy

All vertebrates inherited the camera eye from their common ancestor (approx. 500 Mya) and thus share its main features (Lamb, Collin and Pugh, 2007). The eye has more or less spherical shape and is filled with liquid. The light passes through cornea, anterior chamber, pupil, posterior chamber, lens, and vitreous body and it is focused on retina, where it creates a two-dimensional image. The pupil is framed by iris that regulates its diameter. Fish eyes are adapted for underwater environment and differ from the eyes of terrestrial vertebrates in several characteristics (Fernald, 1988). The typical shape of the fish eye is hemispherical with very short axial dimension (see Figure 1). Since cornea and water have very similar refractive indices, cornea has no optical power and refraction must be achieved solely by lens.

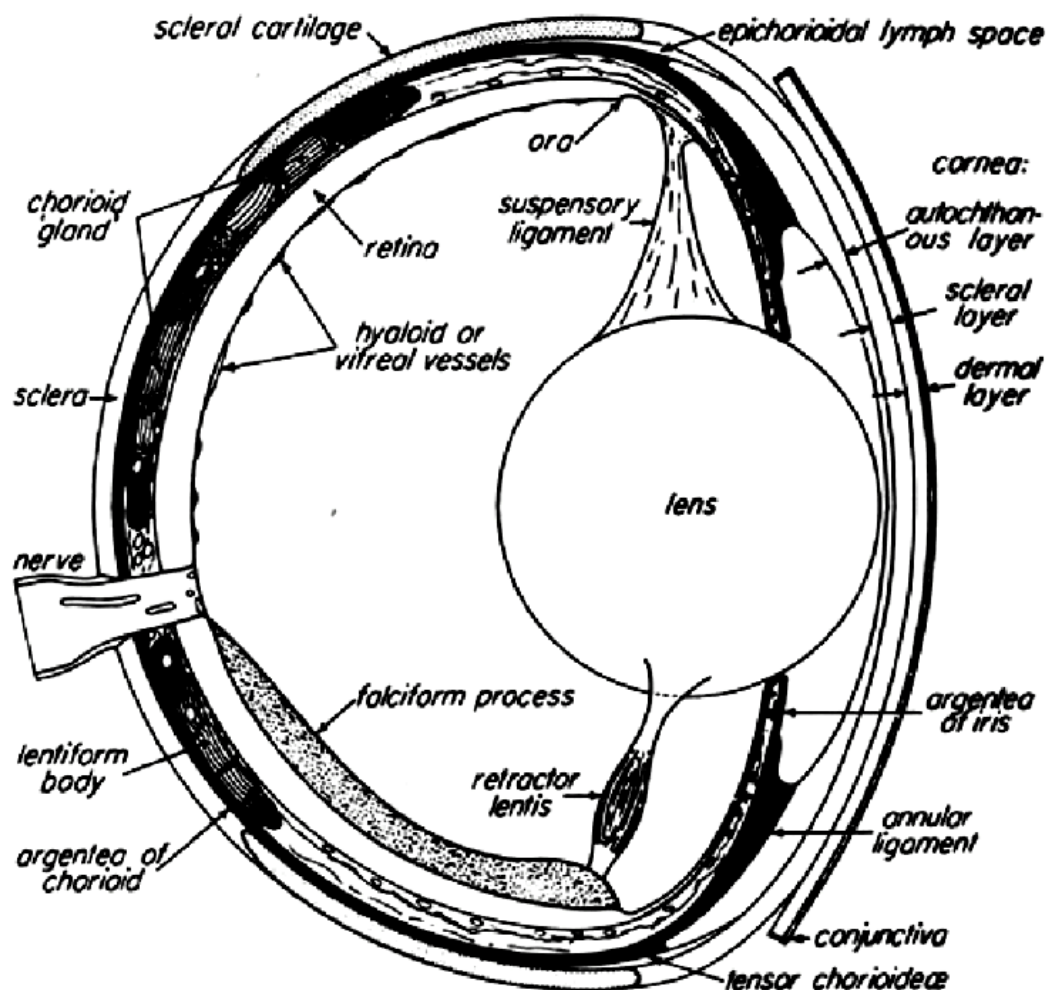


Figure 1: *Vertical section of generalized teleost eye.* Note the typical hemispherical shape of the eye and big spherical lens protruding through the pupil. All features might be further modified, and some structures may not be present in some species (from *Walls, 1942 in Fernald, 1988).

To provide sufficient refraction, fish lens must be spherical, even though this shape causes greater spherical and chromatic aberrations (Kröger, 2013). The fish lens usually protrudes through the pupil providing a broader visual field (Fernald, 1988). Unlike terrestrial vertebrate lens accommodating by the change in its curvature, the fish lens does not alter its shape. Instead, it is pushed towards and from the retina by specialized muscles while focusing the image. Interestingly, fish lenses often move also in nasal-temporal direction which enables different parts of the retina to focus on different distances: if the lens moves towards nasal pole of the eye, temporal part of the retina covering nasal visual field will be focused for near vision while nasal part of the retina covering temporal visual field focuses for far vision, which can be very beneficial (Fernald and Wright, 1985). Fish cornea, lens, and in some species even retina can contain yellow pigments that filter out the light of the shortest wavelengths (Muntz, 1973). Short-wavelength light scatters the most in the water and within the eye and thus its elimination might improve visual acuity.

Retina is the light-sensitive tissue that consists of ten cell layers (Figure 2A) (Stell, 1972). Most retinal cells are neurons (photoreceptors, amacrine cells, ganglion cells, bipolar cells, and horizontal cells) that are supported by Müller glia cells, the outer retinal layer is the pigment epithelium. This basic retinal structure is shared among all vertebrates. Photoreceptors are responsible for light detection and rest of the neurons for the first signal processing. The signal is conveyed to the brain via the optic nerve composed of ganglion cell axons (Brooks, Komáromy and Källberg, 1999).

Photoreceptor cells consist of five parts: outer segment, inner segment, soma, axon, and synaptic terminal (see Figure 2B) (Cohen, 1972; Baker and Kerov, 2013). The outer segment is a markedly enlarged and modified cilium with extensively folded membrane where visual pigments are located (Musser and Arendt, 2017). When the visual pigment captures a photon of suitable energy (i.e., light of suitable wavelength), it triggers a phototransduction cascade in the outer segment. This cascade subsequently leads to hyperpolarization of the cell that spreads through the plasma membrane to the inner segment, soma, and axon. Finally, the signal is passed on bipolar cell at the synaptic terminal (Baker and Kerov, 2013).

There are two types of photoreceptors in vertebrate retina that differ on morphological, physiological, and molecular level – rods and cones. The outer segment in rods is longer and thinner, whereas in cones it is shorter, bulkier, and tapered, as shown in Figure 2C&D (Cohen, 1972; Hunt and Collin, 2014). Rods are specialized for scotopic vision (in dim light conditions) and develop a strong response to very small stimuli (even a single photon). Their phototransduction cascade can amplify the signal greatly, but is also slower and easily saturated (Musser and Arendt, 2017). Cones are specialized for photopic vision across a wide range of light intensities. Their sensitivity is lower compared to rods, but their response and recovery is much faster and they are not easily saturated (Schnapf and Baylor, 1987; Musser and Arendt, 2017). In certain parts of the retina, cones of markedly smaller size accumulate in high densities

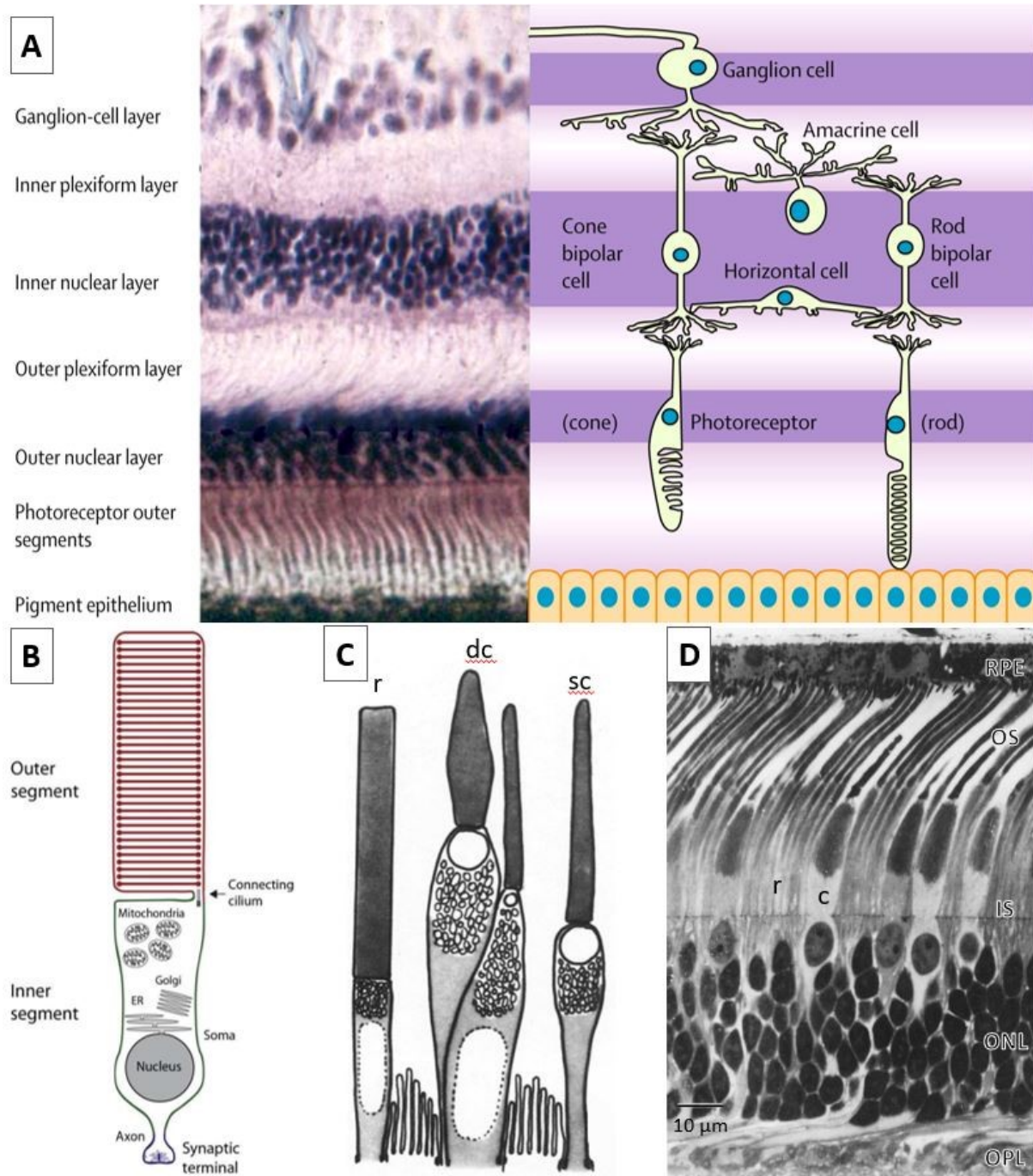


Figure 2: (A) **Photograph of retinal layers aligned with diagram of neuron types in vertebrate retina** (simplified after Hartong, Berson and Dryja, 2006); (B) **Generalized photoreceptor cell** (Baker and Kerov, 2013); (C) **Morphology of the most widespread photoreceptors in vertebrates** (after Szél et al., 1985); (D) **Photograph of rods and cones and adjacent retinal layers** (after Fisher et al., 1993); r – rod, c – cone, dc – double cone, sc – single cone, RPE – retinal pigment epithelium, OS – outer segments, IS – inner segments, ONL – outer nuclear layer (nuclei of photoreceptor cells), OPL – outer plexiform layer (synaptic terminals of photoreceptor cells). Note that the orientation of the rod/cone cells in A is flipped compared to B-D.

to mediate high acuity vision (Hunt and Collin, 2014). While rods are generally conserved within vertebrates, cones diversified into many subclasses (Bowmaker, 1995). They contain visual pigments of different spectral sensitivities and so enable colour discrimination (Musser and Arendt, 2017). Single cones with different visual pigments often differ also in size (they are usually referred to as long and short single cones) (Kunz, Ennis and Wise, 1983; Bowmaker, 1995). Some single cones merge to constitute double, triple, or even quadruple cones (see double cone in Figure 2C) (Lyall, 1957; Shand *et al.*, 2001). While triple and quadruple cones are very rare and appear only in some fishes (e.g., *Phoxinus laevis* or holocentrid fishes), double cones are present in retinas of all vertebrates except placental mammals and elasmobranchs (Lyall, 1956; Cameron and Pugh Jr, 1991; Hart, Lisney and Collin, 2006; De Busserolles *et al.*, 2021). In some teleosts, double cones can be even more frequent than single cones (Lyall, 1957; Musilova, Indermaur, *et al.*, 2019).

Double and single cones in teleost retina are often arranged in a regular pattern – cone mosaic (Fernald, 1988). Cone mosaic is very typical for teleosts and missing in other vertebrate groups except one occurrence in geckos (Dunn, 1966; Allison *et al.*, 2010). The mosaic can be either of row type (with double cones in parallel orientation), square type (with double cones in orthogonal orientation forming edges of the squares), or triangular type (with double cones oriented in angles of 60° forming edges of the triangles) (Engström, 1963). All three mosaic types are shown in Figure 3. While the former two are widespread among fishes, the latter is restricted to the family Esociidae (Braekevelt, 1975; Ali and Anctil, 1976). Regardless of the cone mosaic type, once emerged pattern seems to be evolutionary conserved, because uniform distribution of cone types leads to better resolution (Tohya, Mochizukiz and Iwasaw, 2003). Rods fill the spaces between cones and do not exhibit any regular arrangement. Furthermore, photoreceptor types, size, and density often vary across the retina (Cheng and Flamarique, 2007b; Kondrashev, Gnyubkina and Zueva, 2012).

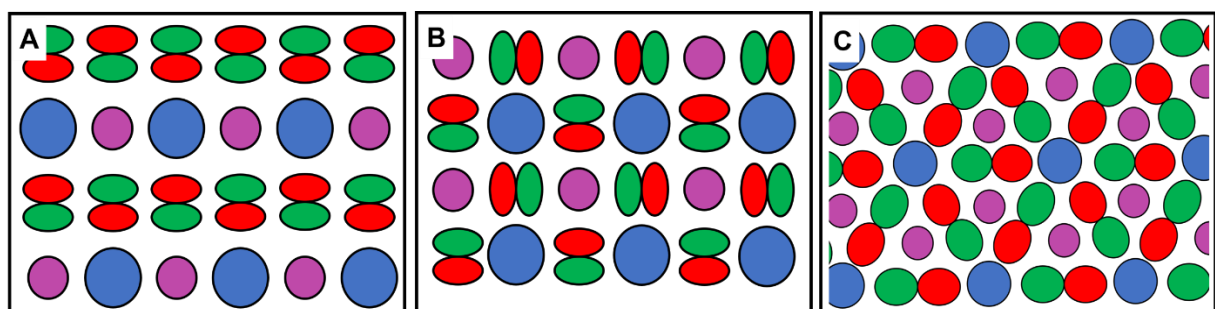


Figure 3: Diagrams of main cone mosaic types in fishes. (A) Row mosaic; (B) Square mosaic; (C) Triangular mosaic. Colours correspond to spectral sensitivity of cones. The arrangement can be further modified in some species – e.g., some cones might be missing or express different visual pigments.

Most teleost retinæ are duplex (i.e., consisting of both rods and cones), even though species with pure-rod retinæ were also described, especially among depth-dwelling species specialized for dim conditions (Locket, 1977; Musilova, Cortesi, *et al.*, 2019). Duplex teleost retinæ usually undergo retinomotor movements to move unemployed photoreceptors out of the way of incoming light and thus enhance the performance of active photoreceptors (Burnside and Nagle, 1983; Fernald, 1988). In the light-adapted state, cones are positioned closer to the inner (vitreous) side of the retina and exposed to the light while rods are situated at the outer side with outer segments embedded in pigment epithelium. The situation is reversed in dark-adapted state.

Vertebrate visual pigments

Vertebrate visual pigments are composed of two parts – transmembrane protein (opsin) with the special pocket, and a chromophore (aldehyde of vitamin A – retinal) that is embedded inside (Bowmaker, 1995). Retinal is the light-sensitive molecule that changes conformation after photon capture from 11-*cis* isomer to all-*trans* isomer. The suitable energy of the photon, and thus the wavelength of detected light, is determined by opsin type and its interaction with retinal (Yokoyama, 2008). Opsins are G-protein coupled receptors that consist of approximately 350 amino acids. Difference in amino acid sequence can result in the shift in spectral sensitivity of the visual pigment, especially in several positions known as tuning sites. So far, some 30 tuning sites have been discovered, some of them being specific for rod or cone opsins and others shared between the two (Yokoyama, 2008). This direct connection between the gene sequence, protein sequence, and function enables to track functional consequences of every substitution at DNA level, which makes opsins a very useful model system (Cortesi *et al.*, 2015).

Vertebrates possess five visual opsin classes inherited from their common ancestor with absorption maxima ($\lambda_{\max}$) between 360 and 560 nm. These opsins can detect light of wavelengths ranging from 320 to 700 nm. Four of these classes are expressed in cones: SWS1 (very short-wavelength sensitive; $\lambda_{\max} = 360\text{--}430$ nm), SWS2 (short-wavelength sensitive; $\lambda_{\max} = 440\text{--}460$ nm), RH2 (green-sensitive rhodopsin-like; $\lambda_{\max} = 470\text{--}510$ nm), LWS (long-wavelength sensitive; $\lambda_{\max} = 510\text{--}560$ nm). The cones expressing these opsin classes are frequently referred to as UV, blue, green, and red cones respectively (Musser and Arendt, 2017). While SWS1 and SWS2 are usually expressed in single cones, RH2 and LWS are expressed in double cones (Bowmaker *et al.*, 1994; Cheng and Flammarique, 2007b; Pignatelli *et al.*, 2010; Salbreux *et al.*, 2012; Remišová, 2019; Stieb *et al.*, 2019). The opposite halves of double cones might express either two distinct opsins or the same one. Additionally, more than one opsin gene might be expressed within a single cell. In such cases, the $\lambda_{\max}$ of the photoreceptor lies between the $\lambda_{\max}$ of coexpressed genes and the total absorbance spectra of the photoreceptor broadens (Carleton *et al.*, 2020). Coexpression thus might be used for spectral tuning of photoreceptors despite its negative effect on colour vision (two distinct opsins coexpressed in one cone provide only one colour channel) (Dalton *et al.*, 2014).

Cone opsin genes underwent turbulent evolution: some opsin classes were lost in some vertebrate lineages; others have diversified and shifted their spectral sensitivity. For example, therian mammals lost all vertebrate cone opsins except SWS1 and LWS during their nocturnal era (Musser and Arendt, 2017). The SWS1 and LWS opsins then mutated and shifted their $\lambda_{\max}$ – SWS1 to the blue part of the visual spectrum and LWS to the green part of the spectrum. Later, the LWS opsin gene diversified in some primates and gave rise to the new red-sensitive opsin. On the contrary, all cone opsin classes are still present in actinopterygians, birds, and some squamates. Furthermore, many teleosts possess more than four cone opsin genes resulting from various duplications (Carleton, 2009).

The fifth opsin class RH1 (rhodopsin; $\lambda_{\max} = 500$ nm) is expressed in rods. It is more conserved than cone opsins and present in all vertebrate groups. Losses of the RH1 gene from the genome of animals that use eyes are not known. However, in rare cases, such as in *Acipenser stellatus*, rhodopsin gene is present in the genome, yet not expressed (Košťátko, in prep.). Loss of RH1 expression is linked with the loss of rods, and *Acipenser stellatus* thus possess pure-cone retina (Khorshidi Sedhi and Shabanipour, 2018). On the other hand, there exist several fish species with multiple rhodopsin genes, e.g. cyprinids (*Cyprinus carpio*, *Danio rerio*), or some deep sea fishes (Diretmidae, Myctophidae, Stylephoridae) (Musilova, Cortesi, *et al.*, 2019). Uniquely in deep sea fishes, rhodopsins have diversified greatly and shifted their spectral sensitivities creating the potential for rod-mediated colour vision. Still, RH1 gene duplications and changes in its expression are far less common than similar processes in cone opsin genes.

Ontogenetic changes in fish retinæ

Fish reproduction typically takes place in the water environment, beginning with external fertilization of eggs and continuing with hatching, growth of the larvae, and their metamorphosis into juveniles. Even though metamorphosis in fishes is rather gradual process than rapid and strictly delimited transformation, we can still define the developmental period when the changes are the fastest. For example, *Danio rerio* undergoes metamorphosis between 14 and 28 days post-fertilization (dpf) (Flynn, Trent and Rawls, 2009).

It was demonstrated in many species, that structure and visual properties of fish retina can change conspicuously not only during metamorphosis itself, but also in the later life stages. These changes are often associated with the changes in lifestyle and transitions to the environments with different light conditions. Fish retinæ have the ability to grow and add new photoreceptors throughout the whole life, which makes them unique among vertebrates (Fernald, 1988). It also provides the potential to gradually adjust photoreceptor ratios even at adult stage. Retinæ grow both by stretching and by adding new photoreceptors at the retinal margin. Moreover, rods probably also emerge via cell division across the whole retina and occupy the spaces newly created by stretching (although some authors present contradictory results and offer other explanations of increasing rod-to-cone ratio (Johns, 1977)).

Still, the most striking differences were found while comparing the retinae before and after metamorphosis. Larval retinae differ from adult retinae in photoreceptor size, arrangement, and the recorded photoreceptor types. It was repeatedly demonstrated, that cones precede rods during retinal development (Shand, Archer and Collin, 1999; Shand *et al.*, 2001; Allison *et al.*, 2010). Usually, UV single cones expressing SWS1 opsin appear first. They are small in diameter and packed in a very tight pattern (Shand, Archer and Collin, 1999; Shand *et al.*, 2001). Even after appearance of other cone types, UV (and also blue) cones often remain in excess compared to the adult cone ratio (Spady *et al.*, 2006; Allison *et al.*, 2010) and the adult cone mosaic is not yet formed. Close packing of smaller photoreceptors in larval retina is indeed adaptive, because it enables the very small eye to achieve sufficient resolution (tight arrangement of small photoreceptors leads to increased photoreceptor density per visual angle and thus better resolution). The excess of UV and blue cones in larval retina might be an evolutionary constraint, but it was also argued that it could be adaptive, since fish larvae usually live near the water surface and feed on zooplankton that is well visible in UV light (Bowmaker and Kunz, 1987). Larval retina has also much lower proportion of rods which is the direct consequence of rod late development. Even some deep-sea fish species with pure-rod retinae as adults are known to have pure-cone retinae in the larval phase (Lupše *et al.*, 2021). The exact process how adult ratios of photoreceptors are achieved is not yet fully understood, but several mechanisms were proposed.

First, as mentioned earlier, the cone density could decrease as the retina grows and stretches, while rods emerge from precursor cells and fill the spaces (Fernald, 1988; Kunz *et al.*, 1994). Even if the precursor cells became depleted and new rods were added only at retinal margins after metamorphosis, as reported by some authors, retinal stretching could still increase rod to cone ratio – if rods resisted the retinal expansion and cone pattern thinned out, the overall rod-to-cone ratio would increase (Johns, 1977).

Second, larval retina is very small, so it would represent only minor part of the retina if preserved at the adult stage. New photoreceptors produced at retinal margin could thus outnumber the larval ones which could be an easy mechanism of establishing adult photoreceptor proportions. Indeed, such remnant of larval retina was identified in the proximity of optic nerve in zebrafish (Allison *et al.*, 2010). It differs from surrounding adult retina both in cone mosaic and photoreceptor ratios. Yet, the authors found some differences between larval remnant and larval retina photoreceptor composition.

Third, there is also evidence of the UV cones being partially or completely eliminated either during metamorphosis or later in life (Kunz *et al.*, 1994; Allison *et al.*, 2003, 2010). The apoptosis of UV cones can be linked to the cone mosaic rearrangement. Elimination of UV cones is often explained by fish transition to deeper water layers with no available UV light and thus no utilization for UV cones.

Finally, the photoreceptors can also undergo shift in spectral sensitivity – the change in opsin expression leading to the change of photoreceptor type. This shift might be accompanied by changes in photoreceptor morphology and molecular factors (e.g., components of phototransduction cascade). The

most common are shifts within a single opsin class that can only occur in species that experienced opsin gene duplication in the past. Strictly speaking, photoreceptor type remains the same in this scenario, even though its spectral sensitivity can be altered if the duplicated opsins have accumulated mutations that differentiated their absorption maxima. Since the presence of opsin gene duplicates is very common across fishes, so is the ontogenetic variation in their expression. For instance, percomorph fish *Pseudochromis fuscus* expresses SWS2A β at larval stage that is later replaced by SWS2A α at adult stage (Cortesi *et al.*, 2015). It is thought that transition occurs by the change in opsin expression in the same cell, not by replacing the cell with the new one. The switch in expression between the two copies of RH1 opsin gene was documented in the European eel (*Anguilla anguilla*), where it precedes transition from freshwater to the marine environment (Wood and Partridge, 1993). Transmutations between different cone classes are also reported – for example, UV cones switch their expression from SWS1 to SWS2 and thus transmute into blue cones in the fishes of the genus *Oncorhynchus* (Cheng and Flammarique, 2007a; Cheng and Flammarique, 2007b). The least presumable, yet proposed, is the transmutation between rods and cones. Despite extensive differences between the two photoreceptor types, fishes from the order Aulopiformes (*Coccothraustes atlantica* and *Scopelogadus* sp.) seem to switch expression during metamorphosis from RH2 to RH1 in significant percentage of their cones while keeping the original cone phototransduction cascade (Lupše *et al.*, 2021), so the rod vs. cone identity remains elusive in this case.

It is very difficult to track individual photoreceptors and follow their destinies during ontogeny. Therefore, a lot remains unknown in this field. Nevertheless, relative opsin expression profiles can be very informative on their own and they are much easier to reconstruct. (In the most studies, they extract RNA from the retina and use quantitative RT-PCR with specific primers to assess the relative percentages of all opsins (Spady *et al.*, 2006; Cortesi *et al.*, 2015; Chang *et al.*, 2020)). All studies comparing opsin expression profiles across several developmental series in various fish species revealed at least one significant change in expression level (Spady *et al.*, 2006; Shand *et al.*, 2008; Cortesi *et al.*, 2015, 2016; Chang *et al.*, 2020, 2021). The gradual decrease in expression of SWS1 and increase in LWS with age was reported repeatedly, which corresponds with the decline of UV cone density mentioned above (Spady *et al.*, 2006; Shand *et al.*, 2008; Matsumoto and Ishibashi, 2016; Härer, Torres-Dowdall and Meyer, 2017; Chang *et al.*, 2020). This shift of spectral sensitivity towards longer wavelengths is usually explained as adaptive and correlated with fish transition into deeper and darker water layers. Furthermore, the decrease in SWS1 expression is often accompanied by increase in SWS2 expression (Spady *et al.*, 2006; Matsumoto and Ishibashi, 2016; Härer, Torres-Dowdall and Meyer, 2017). These effects are thought to be related to each other and associated with habitat change too, because both opsins are sensitive to short-wavelength light but SWS2 matches better the available spectrum in deeper water. In addition, the switch in expression from SWS1 to SWS2 within the same

cone was also described, as mentioned above, giving even more evidence for the tight link between the two (Cheng and Flamarique, 2007a; Cheng and Flamarique, 2007b).

Where the gene duplicates are present, they are likely to evolve into the larval-specific and adult-specific opsin (as described in SWS2b/SWS2a and RH2A α /RH2A β in *Acanthopagrus butcheri*, in LWS-R/LWS-1 in *Lucania goodei*, or in above mentioned SWS2A β /SWS2A α in *Pseudochromis fuscus*), although the exact timing of their interchange and extend of the whole effect might vary (Shand *et al.*, 2008; Cortesi *et al.*, 2015; Chang *et al.*, 2021). The division of functions is not necessarily adaptive or beneficial at the beginning and might occur only to re-establish balance after the disruption of relative expression patterns caused by duplication. However, it provides the potential for both copies to accumulate mutations that advantage the life stage at which they are expressed. Differential expression across ontogeny thus leads to maintenance of more opsin genes in the genome (Spady *et al.*, 2006). In other species, such as *Oreochromis niloticus*, the ontogenetic shift in expression does not occur in one-for-one replacement mode, but rather in a complex scheme where some opsins increase and others decrease in their expression levels over development (SWS1 and RH2b are expressed only in larva and disappear during metamorphosis, RH2a β and SWS2b have the peak of expression in the juvenile, the expression of RH2a α gradually decreases from larva to adult, whereas the expression of LWS and SWS2a gradually increases (Spady *et al.*, 2006). If there are three and more opsin genes of the same class within the genome as a result of previous duplications, at least one of them is likely to be silenced through the whole development (e.g., LWS-2 and LWS-3 in *Lucania goodei*, RH2B in *Pseudochromis fuscus*) (Cortesi *et al.*, 2016; Chang *et al.*, 2021).

The common barbel biology and life history

The common barbel (*Barbus barbus*) is a European freshwater fish species from the family Cyprinidae. The name “barbel” refers to freshwater cyprinid fishes of various sizes that usually inhabit rivers. They are bottom-feeders and thus possess an inferior mouth with barbels that gave them the name. Such fishes can be found in Europe, Africa, and Asia, and used to be grouped in the subfamily Barbinae. However, taxonomical revisions revealed that barbels do not form a monophyletic group and the subfamily was later divided into several independent lineages (Tsigenopoulos *et al.*, 2002). The common barbel belongs to the European tetraploid barbel clade comprised of the genera *Barbus*, *Luciobarbus*, *Capoeta*, and *Aulopyge* (Tsigenopoulos *et al.*, 2003; Levin *et al.*, 2012; Wang *et al.*, 2013).

The common barbel is native to the temperate Europe, ranging from Pyrenees in the west to European border of Russia in the east. It is absent in Mediterranean region and northern Europe. Interestingly, there exists another population in eastern Balkan and Anatolia that has evolved separately from European lineage for at least several glacial cycles (Kotlík and Berrebi, 2001). The barbel inhabits upper parts of lowland rivers where the current is still fast and strong. If the conditions are favourable, it can

be very abundant and create shoals (see Figure 4). Therefore these parts of rivers are often referred to as barbel zones (Britton and Pegg, 2011).



Figure 4: The common barbels in the river Traun, Austria. Adults aggregate in the daytime to rest in the depressions of the river bottom. Photo by Zuzana Musilová.

The adult barbels are very rheophilous – they avoid the areas with low water velocity and can resist a very strong current. Generally, bigger specimens are found in areas with faster water flow (Britton and Pegg, 2011). They prefer gravel bottoms and feed on benthic invertebrates (especially molluscs and insect larvae) (Hanel and Lusk, 2005). During the summer period, they are most active at dusk and dawn, but in the spring and autumn, they exhibit diurnal activity too (Baras, 1995). The common barbel is a potamodromous species – during the mating period (from April to June), the barbels form large shoals and migrate upstream to their mating grounds. The length of their journey depends on availability of suitable habitat and also on individual strategy – while most fish are “sedentary” and travel shorter distances, some are “mobile” and travel as far as 20 km (Lucas and Batley, 1996; Britton and Pegg, 2011). Barbels spawn in groups and lay eggs directly on the gravel in shallow riverbays (Hanel and Lusk, 2005). The time of hatching and rate of early development depends mainly on water temperature – it was experimentally assessed that the development is fastest at 26 – 28 °C (Kamiński *et al.*, 2013). It usually takes 4 – 8 days for larvae to hatch. Newly-hatched barbel larvae are generally less developed than larvae of other cyprinids – their alimentary tract is obstructed, they do not have any eye pigment, their swim bladder is flat, and they only lay on the bottom feeding from the yolk sacs (Nowosad *et al.*, 2021). Later, the larvae might move among the gravel, but they cannot emerge from it until their swim bladder is filled (Vilizzi and Copp, 2013). This developmental phase is sometimes called “interstitial.” While studied in captivity, the eye retinal pigment epithelium appeared 2 days post-hatching (dph),

larvae performed negative phototaxis 4 dph, and their retina was fully developed at 7 dph when they also initiated exogenous feeding (Nowosad *et al.*, 2021). The swim bladder filled between 7 and 10 dph, so larvae could leave the substrate and swim actively. (In another study, the larvae emerged from the gravel already 6 dph which again shows how outer conditions influence the speed of development (Vilizzi and Copp, 2013)). Interestingly, barbel larvae do not stay in water column for long. After several days, when they exceed 2 cm in total length, they return to the bottom, and soon the first pair of barbels appear (20 dph). Since this return, their body shape and pigmentation resemble that of adult fish. They tend to aggregate and in the wild, they still prefer shallow river bays with low water velocity and avoid deep water. However, comparison with juvenile barbels from gravel banks and riffles revealed a significant overlap in diet – all fishes fed on chironomid and Ephemeroptera larvae (Bischoff and Freyhof, 1999). It is thus reasonable to consider 2 cm in total length as a turning point between larval and juvenile stage. Juveniles (and sometimes also eggs and larvae) eventually drift downstream, where they encounter new suitable habitats as they grow (Copp *et al.*, 2002; Britton and Pegg, 2011). The males mature at the age of 2–3 years, females at 4–5 years. Females grow faster, they are usually larger than males, and live longer (up to 18 years) (Hanel and Lusk, 2005).

Very little is known about the visual ability of the common barbel. Based on the lack of retinal pigment epithelium and absent response to the light, newly hatched larvae are supposed to be blind (Nowosad *et al.*, 2021). Despite retina being fully differentiated 7 dph when larvae initiate independent feeding, photoreceptor layer is still described as “poorly developed” in Nowosad *et al.* (2021). We shall thus presume not very elaborated vision, at least at this stage. Microscopic observations of adult retina revealed both single cones (long and short) and double cones, but they were not arranged in any regular pattern (Compere and Poncin, 1996). Since regular cone mosaic is tightly linked with vision quality in teleosts, the authors concluded that adult barbels have multichromatic, yet inferior vision. Such assumption is indeed reasonable as adult fish tend to crepuscular activity and superior colour vision would be of little use. On the other hand, cyprinid fishes are reported to have a cone mosaic, either row or square type, even though it can contain some irregularities (Marc and Sperling, 1976; Bowmaker and Kunz, 1987; Zaunreiter, Junger and Kotrschal, 1991; Allison *et al.*, 2010). The presence of the cone mosaic will thus be re-examined in this study. Moreover, Truhlářová (pers. commun.) revealed expression of uniquely high number of opsin genes in the retina of adult barbel. It seems unlikely that colour vision of species that uses so many opsins would be really inferior. Examination of opsin gene repertoire and their utilization in the common barbel is therefore another aim of this study.

Whole genome duplication in the common barbel

Polyploidization (or whole genome duplication) is an event when the complete chromosome set of an organism, and thus also the gene dose, duplicates. Such a massive change on genomic level (sometimes described as “genome shock”) imposes a great challenge for an organism to overcome. Polyploid

evolution has been intensively studied in plants that are known to survive on various ploidy levels more easily than animals. Lower percentage of polyploid animals (and especially vertebrates) was first explained by their sexual reproduction and sex chromosomes – it was thought that polyploidization disrupts meiosis and dosage compensation, so the polyploid individual becomes an evolutionary dead end (Orr, 1990). However, later studies demonstrated that sex chromosome systems alone cannot explain the incidence of polyploid species (Mable, 2004). The new and complex view is that polyploidization disrupts genome networks and subsequently also relative expression patterns (Wertheim, Beukeboom and Van De Zande, 2013). This was termed “transcriptomic shock” by Wertheim, Beukeboom and Van De Zande (2013). If the organism survives transcriptomic shock, it becomes a neopolyploid and enters the process of re-diploidization to achieve the balance on chromosomal (meiosis) and regulatory (genome networks) level (Mable, Alexandrou and Taylor, 2011; Wertheim, Beukeboom and Van De Zande, 2013). Such diploidization includes substantial gene loss because all genes are in excess at the beginning. In the most common scenario, the excess gene copies accumulate negative mutations, they are silenced, pseudogenized and eventually, they are eliminated from the genome. However, some duplicated genes also diversify and acquire new functions (neo-functionalization) or divide the tasks in original common function (sub-functionalization). The divergence between duplicate gene copies might manifest itself as the change in amino acid sequence or in the regulatory control of the genes. For example, differential expression patterns across various tissues were found in *Squalius alburnoides* hybrids with *S. pyrenaicus* – genes from progenitor species were silenced specifically in every organ to achieve the dosage compensation (but each tissue silenced different genes) (Pala *et al.*, 2010).

There are two modes how polyploidization can arise: autopolyploidization (whole genome duplication occurs within the species and all chromosomes thus have the same progenitor) and allopolyploidization (whole genome duplication occurs via hybridization between closely related species and chromosomes come from two different progenitors) (Wertheim, Beukeboom and Van De Zande, 2013). Allopolyploidy seems to be more frequent than autopolyploidy, at least in vertebrates (Mable, Alexandrou and Taylor, 2011). However, this observation could be biased because hybrid origin of allopolyploids makes them easier to distinguish from parental species (Spoelhof, Soltis and Soltis, 2017). Autopolyploids have lower genetic variation than allopolyploids because genetic variation increases with genetic distance between progenitors. So far, it seems that allopolyploids undergo more complex genetic remodeling than autopolyploids, and that redundant gene copies obtained from different progenitors are often distinct enough to acquire new functions and to be retained in the genome (Spoelhof, Soltis and Soltis, 2017). On the other hand, conservative genes with low mutation rates are more likely to sub-functionalize in allopolyploids than fast-evolving ones, suggesting that there might exist a “threshold of resemblance” beyond which sub-functionalization cannot develop (Sémon and Wolfe, 2008). Nonetheless, most studies focusing on effects of auto- and allopolyploidy were conducted on plants, often researched only one type of polyploid, and very few compared closely related auto- and

allopolyploids. For this reason, we still cannot fully understand the effects of genome duplication until more research is done to fill the gaps in our knowledge, especially in vertebrates.

More than 450 Mya, two rounds of whole genome duplication occurred in the ancestor of all living vertebrates (Dehal and Boore, 2005). Most of the genes were later lost, but some were retained, and the ancient duplications are still detectable in their positions on the chromosomes. It is hypothesized that the presence of excess gene copies allowed increase in mutation rate and that many genes evolved faster before reestablishment of one copy only. It is possible that these ancient duplications enabled subsequent diversification of vertebrates. The third round of ancient whole genome duplication occurred approximately 350 Mya in the lineage leading to teleost fishes (Meyer and Van De Peer, 2005). This event termed “teleost-specific duplication” is often perceived as the key to the remarkable success of this infraclass – today, teleosts comprise approximately 35,000 species and they account for half of the vertebrate diversity (Fricke, Eschmeyer and Fong, 2022).

In present days, polyploidization still has an important role in vertebrate evolution. Despite being lethal in birds and mammals due to general disruption of development, whole genome duplication can be withstood by reptiles and it is even quite frequent in amphibians and fishes (Mable, Alexandrou and Taylor, 2011; Luo *et al.*, 2014). Most of the polyploid species within these groups are temperate freshwater external breeders with large number of gametes (Mable, Alexandrou and Taylor, 2011). Since the major cause of polyploidization in amphibians and fishes are unreduced gametes, it was inferred that development in unstable environment might promote the unreduced gametes formation and that polyploidy might thus be associated with environmental stress (Mable, Alexandrou and Taylor, 2011; Peer, Mizrachi and Marchal, 2017). Notably, recent polyploidization events are not evenly distributed across amphibian and fish phylogeny. Especially in fishes, there are entire families derived from polyploid ancestor, and others with very high incidence of polyploid species. Polyploid fishes seem to be more common in lineages outside Neoteleostei. For example, Acipenseriformes comprise several lineages on various ploidy levels (Birstein and Vasiliev, 1987; Birstein, Hanner and DeSalle, 1997). The whole order Salmoniformes underwent autopolyploidization sometime between 25 and 100 Mya, which provides the opportunity for comparison with other polyploid fishes that are mostly allopolyploids (Allendorf and Thorgaard, 1984). Significant number of polyploid species belong to the order Cypriniformes (Luo *et al.*, 2014), including the common barbel. Interestingly, several independent cyprinid lineages previously grouped together as “barbels” differ in their established ploidy – some are diploid, some tetraploid, and one African lineage is even hexaploid (Tsigenopoulos *et al.*, 2002). These lineages underwent recent polyploidizations that are unique and independent to each monophyletic clade. The polyploidization event in European tetraploid barbel clade (comprising the common barbel) must have occurred after the clade diverged from the closely related cyprinid fishes and before it diversified into current genera/species. Wang *et al.* (2013) date the divergence of European barbels from other cyprinids to 16.6–15.5 Mya, and the separation of the genus *Aulopyge* from the rest of the barbel

lineage to 10.6 Mya. Based on this estimation, polyploidization in the common barbel occurred 11–16 Mya. Levin *et al.* (2012) estimate the time of separation of European barbel clade to 35 Mya and the time of split of *Aulopyge* from the rest of the lineages to 30.9 Mya, which puts the polyploidization event much deeper into the past, between 31 and 35 Mya. It was demonstrated that the lineage of European tetraploid barbels is of hybrid origin (Chenuil, Galtier and Berrebi, 1999). The putative progenitors are not yet known, but chromosomes of the common barbel can be assigned to the two sets according to their origin, i.e. the two subgenomes “a” and “b” have been identified (unpublished, genome available from: www.ncbi.nlm.nih.gov; CM042264:CM042313). Very similar situation with the two subgenomes was also found in the common carp (*Cyprinus carpio*), in this case published in (Xu *et al.*, 2019).

Materials and methods

Sampling

In my project, I analysed retinæ of larvae and adults of the common barbel (*Barbus barbus*) for their transcriptomes, and I have applied specific FISH staining on the adult retina.

Larval samples were collected from the captive breeding facility in FROV Vodňany, obtained in collaboration with Pavel Lepič after the artificial reproduction of the species. We sampled larval stages at 8, 14, 21, 28, and 42 days post-fertilization (dpf). The fish were reared under controlled conditions: the water temperature was between 20 and 21 °C and the water was replaced daily to keep constant chemical parameters. Ever since the hatching, 90% of the water tank area was shadowed by the black board. The larvae spent the first days after hatching at the bottom due to limited locomotor abilities (8 dpf stage in my study). They entered the water column 11 – 12 dpf and started an active search for food (14 and 21 dpf stages). After this short period of live in the water column, they started to spend more time near the bottom again (28 dpf stage) and eventually developed body shape, pigmentation, and lifestyle very similar to adults (42 dpf stage) (Lepič, pers. commun.).

All adult specimens for our analyses were caught in the wild by electrofishing. We sampled at two localities on the river Berounka – Liblín [GPS: 49°55'07.9"N 13°32'43.9"E] and Třímány [GPS: 49°56'16.7"N 13°35'23.6"E], at one locality on the river Sázava – Pikovice - 49°52'42.2"N 14°25'37.1"E, and at one locality on the river Vltava – Rájov [GPS: 48°51'04.2"N 14°21'58.9"E]. Electrofishing was conducted by authorized person (Miroslav Švátora, Zuzana Musilová) and upon agreement and permission from Český rybářský svaz. We used an anaesthetic MS-222 (tricaine methanesulfonate) to sacrifice the fish. We fixed the whole specimens for larval developmental series, in adults we extracted and fixed only eyes. The fixation protocol depends on the intended analysis and thus will be described later.

All samples used for this study are listed in Tables 1 and 2, Figure 5 shows representatives of all developmental stages.

Table 1: List of the common barbel specimens used for RNA isolation and subsequent analysis of transcriptome. The captive breeding facility is in FROV Vodňany. All samples were sequenced on Illumina platform in the Novogene company.

Sample	Stage	Locality	Sampled	Sequenced	Reads in total
roz1	8 dpf	Captive breeding	21.05.2021	April 2022	21 233 448
roz2	8 dpf	Captive breeding	21.05.2021	April 2022	32 719 284
roz3	8 dpf	Captive breeding	21.05.2021	April 2022	30 450 108
roz4	8 dpf	Captive breeding	21.05.2021	April 2022	23 492 512
roz5	8 dpf	Captive breeding	21.05.2021	April 2022	26 734 814
14d1	14 dpf	Captive breeding	27.05.2021	April 2022	22 234 360
14d2	14 dpf	Captive breeding	27.05.2021	April 2022	23 419 782
14d3	14 dpf	Captive breeding	27.05.2021	April 2022	24 964 094
14d4	14 dpf	Captive breeding	27.05.2021	April 2022	24 836 474
14d10	14 dpf	Captive breeding	27.05.2021	April 2022	17 085 892
21d1	21 dpf	Captive breeding	03.06.2021	April 2022	19 514 206
21d2	21 dpf	Captive breeding	03.06.2021	April 2022	26 933 090
21d3	21 dpf	Captive breeding	03.06.2021	April 2022	31 015 048
21d4	21 dpf	Captive breeding	03.06.2021	April 2022	26 849 882
21d10	21 dpf	Captive breeding	03.06.2021	April 2022	24 857 332
28d1	28 dpf	Captive breeding	10.06.2021	April 2022	24 200 338
28d2	28 dpf	Captive breeding	10.06.2021	April 2022	25 626 184
28d3	28 dpf	Captive breeding	10.06.2021	April 2022	30 793 720
28d4	28 dpf	Captive breeding	10.06.2021	April 2022	23 536 636
6wk1	42 dpf	Captive breeding	24.06.2021	April 2022	24 331 644
6wk2	42 dpf	Captive breeding	24.06.2021	April 2022	22 985 104
6wk3	42 dpf	Captive breeding	24.06.2021	April 2022	23 458 076
6wk4	42 dpf	Captive breeding	24.06.2021	April 2022	24 117 204
6wk5	42 dpf	Captive breeding	24.06.2021	April 2022	25 471 524
104B7	adult	Rájov	31.05.2019	May 2021	44 951 446
box1B2	adult	Pikovice	26.08.2016	2016	48 226 130
91B8	adult	Liblín	20.06.2018	March 2019	19 968 482
box5H9	adult	Pikovice	30.04.2018	March 2019	17 528 880

Table 2: List of samples successfully stained by FISH. The samples used for optimization of the method are not listed. All samples came from common barbels caught in the wild.

Sample	Stage	Locality	Sampled	Eye	Retinal part	Staining
119F3	adult	Liblín	20.07.2020	right	-	LWS1 & LWS2
ZK_DNA_1B7	adult	Liblín	04.08.2021	left	dorsal	SWS1 & SWS2-1
ZK_DNA_1B7	adult	Liblín	04.08.2021	left	dorsal	RH2-2 & RH2-1
119F1	adult	Liblín	20.07.2020	left	dorsal	RH2-2 & LWS2

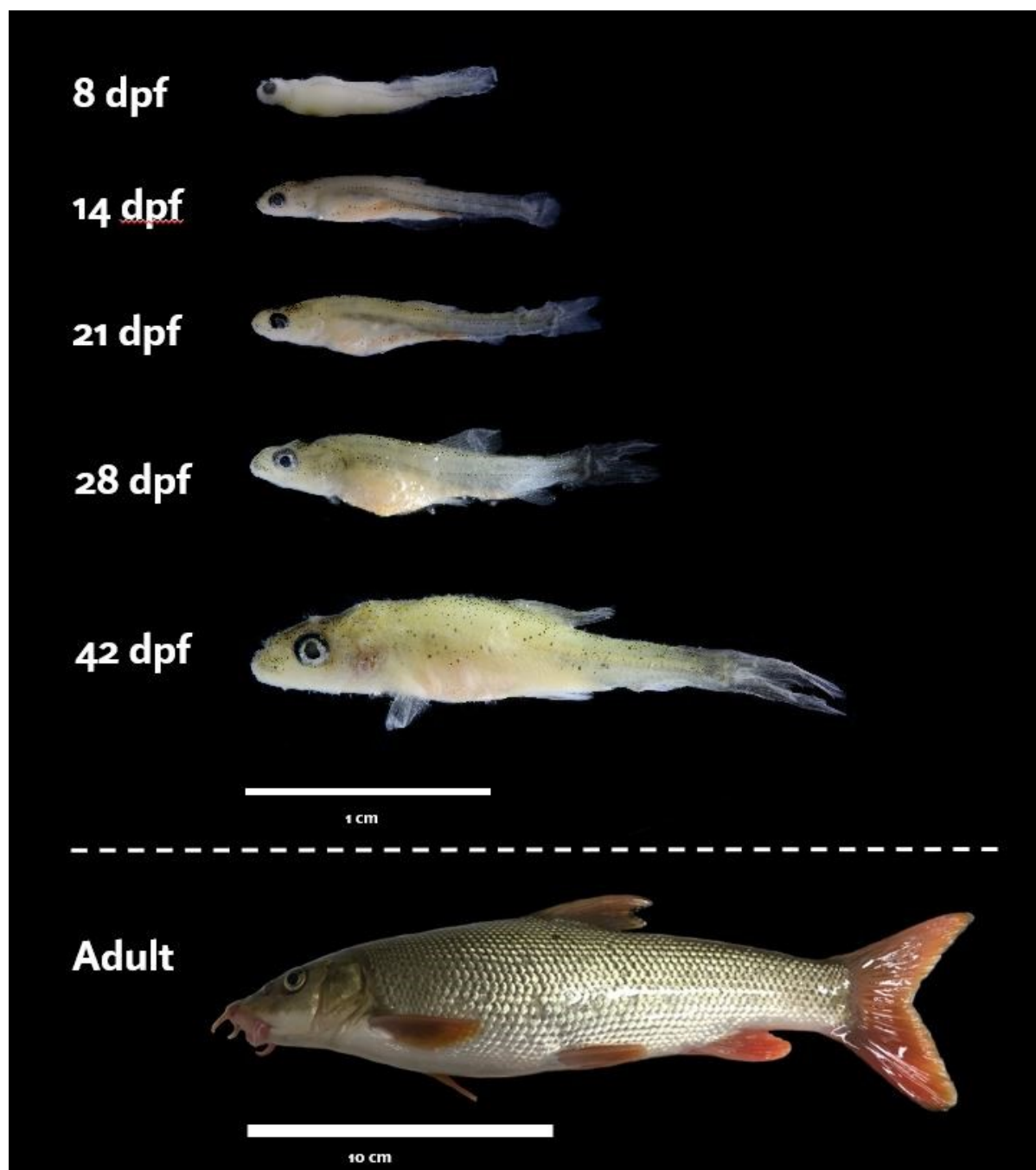


Figure 5: Photographs of representatives of all life stages used in this study. First five stages are depicted in the same measuring scale, the scale for adult specimen is different for spatial reasons.

RNA isolation and transcriptome analysis

Samples for RNA isolation were fixed in RNA-later solution and stored at -80 °C. RNA isolation was conducted in the sterile box in the designated genomic lab. Larval eyes were used without further dissection. Adult eyes were cut open, and retinæ carefully dissected out. We used Qiagen kits for RNA isolation: RNeasy Mini Extraction for adults and RNeasy Micro Extraction for larvae. Larval eyes/adult retinæ were homogenized on MagNA Lyser in the tubes containing β -Mercaptoethanol, RLT buffer, and glass beads. The solution was transferred to 1,5 ml tubes and centrifuged. The supernatant was transferred into the new tube, mixed with 70% EtOH, and transferred to the column. After the wash with RW1 buffer, DNase in RDD buffer was added for 15 minutes to digest unwanted nuclear acid. Then the column membrane was washed again with RW1 and RPE buffer. RNA was eluted into RNA-free water. The concentration and quality of RNA isolates were immediately measured on Nanodrop. Sufficient samples were aliquoted and sent for a further quality check on the Bioanalyzer Instrument (Agilent) to obtain the RNA profile, and on Quantus to measure the concentration. Both measurements were performed in the SeqLab of the Faculty of Science, Genomic laboratory OMICS BIOCEV, Vestec. RNA isolates were stored at -80 °C.

Preparation of transcriptomic libraries for Illumina

I prepared cDNA libraries from RNA isolates using NEBNext Ultra II Directional RNA Library Prep Kit for Illumina. I followed the protocol, but I worked with half volume of all solutions. First, I diluted the total RNA with nuclease-free water to a final volume 25 μ l so the final amount of RNA would always be either 600 ng, 400 ng or 200 ng. (This amount depended on RNA isolate concentration, and it later determined number of cycles in the last PCR amplification.) Oligo dT Beads were washed in Binding Buffer and then added to the RNA sample. The tube was heated at 65°C for 5 minutes in a thermal cycler to denature the RNA and facilitate binding of the mRNA to the beads. Then I cooled the solution on ice, placed the tube on the magnetic rack and washed the beads twice with Wash Buffer to get rid of the unwanted RNA fragments. After washing, I added 25 μ l of Tris Buffer to the beads and heated the sample at 80°C for 2 minutes to do the first elution of the mRNA from the beads. When the sample cooled to 25°C, I added Binding Buffer so the mRNA would re-bind to the beads. After 5-minute incubation at room temperature, I placed the sample to the magnetic rack and washed the beads again with Wash Buffer. Following the careful removal of all of the supernatant, I suspended the beads in the First Strand Synthesis Reaction Buffer pre-mixed with Random Primer Mix. I incubated the solution for 4 minutes at 94°C to fragment the mRNA. Then I transferred the supernatant containing fragmented mRNA to the new tube and placed it on ice. Keeping the tube on ice, I assembled the first strand cDNA synthesis reaction by adding NEBNext Strand Specificity Reagent and NEBNext First Strand Synthesis Enzyme Mix to the tube. I incubated the sample for 10 minutes at 25°C, then for 50 minutes at 42°C, and then for 15 minutes at 70°C to synthesize the first strand cDNA. I let the sample cool to 4°C on ice

and immediately assembled the second strand cDNA synthesis reaction – I added NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix, NEBNext Second Strand Synthesis Enzyme Mix, and Nuclease-free Water to the sample. I incubated the sample for 1 hour at 16°C. After second strand cDNA synthesis, I added SPRIselect Beads to the sample and let sample stand for 5 minutes at room temperature so the cDNA would bind to the beads. Then I placed the tube on the magnetic rack and washed the beads twice with freshly prepared 80% ethanol. I quickly air dried the beads after the second wash and then added 0.1X TE Buffer to elute the cDNA from the beads. I transferred 25 µl of the supernatant to a clean tube and placed it on ice. In this tube, I assembled the end preparation reaction. I added NEBNext Ultra II End Prep Reaction Buffer and NEB Next Ultra II End Prep Enzyme Mix and mixed the reaction thoroughly. The sample was incubated for 30 minutes at 20°C and then for 30 minutes at 25°C. In the meantime, I prepared solution for the following reaction: I diluted NEBNext Adaptor in ice-cold Dilution Buffer. If the starting total amount of RNA was 600 ng or 400 ng, I used 20-fold dilution, for 200 ng I used 25-fold dilution. When the end prep incubation ended, I placed the sample on ice and assembled the ligation reaction by adding Diluted Adaptor (prepared in advance), NEBNext Ligation Enhancer and NEBNext Ultra II Ligation Master Mix. I mixed the reaction well and incubated the sample for 15 minutes at 20°C. Then I added USER Enzyme to the tube and incubated the sample for another 15 minutes at 37°C. Afterwards, I carried out size selection of adaptor ligated DNA according to chapter 6.2 in the protocol. (For my libraries, I replaced chapter 1.8 by chapter 6.2.) Before the size selection procedure, I added 48.25 µl of nuclease free water to the sample to reach the starting volume 96.5 µl as stated in the protocol. Then I added 15 µl of SPRIselect Beads and incubated the solution for 5 minutes at room temperature so the beads would bind the unwanted large DNA fragments. Afterwards, I placed the tube on the magnetic rack, carefully transferred the supernatant to the new tube and discarded the beads. I added 47 µl of SPRIselect Beads to the supernatant and incubated the solution at room temperature for another 5 minutes. This time the beads binded DNA fragments that I wanted to keep. I transferred the tube to the magnetic rack, discarded the supernatant and washed the beads twice with freshly prepared 80% ethanol. Then I quickly air dried the beads and eluted DNA by adding 8.5 µl of 0.1X TE Buffer. After 2-minute incubation at room temperature, I placed the tube on the magnetic rack and transferred 7.5 µl of supernatant to the new tube. Then I proceeded with the final PCR amplification according to chapter 1.9.1A in the protocol. I added NEBNext Ultra II Q5 Master Mix, Universal PCR Primer, and Index (X) Primer to the tube with the sample. (Index (X) Primer is the barcode – (X) stands for the specific barcode number. I used different barcodes for all my samples so they could be later pooled for one Illumina lane.) I performed PCR amplification with cycling conditions from the protocol. The number of cycles depended on starting amount total of RNA – I used 12 cycles for samples with 600 ng and 400 ng and 13 cycles for samples with 200 ng. After the cycling finished, I purified the PCR reaction product using SPRIselect Beads. I added 22.5 µl of the beads to the sample and incubated the solution for 5 minutes at room temperature. Then I placed the tube on the magnetic rack and discarded the supernatant. I washed the beads containing target DNA twice with 80% ethanol

and quickly air dried the beads after the second wash. I added 17 µl of 0.1X TE Buffer to elute the DNA from the beads. I placed the tube on the magnetic rack again, transferred the supernatant to the new tube and stored the sample at -20°C. I sent the aliquot to BIOCEV for the quality check on Bioanalyzer. If the analysis revealed the presence of unwanted fragments in the sample, I later performed one more size selection with SPRIselect beads which was designed specifically for every sample according to the SPRI Based Size Selection protocol. When all the samples were prepared and cleaned, I pooled them together according to their DNA concentration so that the amount of DNA from all the samples in the pool was equal. The pool was sequenced on the Illumina platform in the Novogene company on the commercial basis.

Transcriptomic data processing

For the transcriptomic analysis I received in total 26 samples with 17 – 49 million reads per sample. All samples passed the basic quality check criteria, and none had to be removed because of the bacterial contamination. I supplemented the dataset with two samples of adult specimens processed by Veronika Truhlářová. (See Table 1 for the complete list of samples.)

Opsin expression profiles construction

For the work with sequences, I used Geneious Prime 2020.2.4 (<https://www.geneious.com>, Biomatters). First, I had to get the reference set of the barbel opsin genes. I acquired the partial set of barbel opsin gene references based on the adult transcriptome previously sequenced in our working group. I mapped several transcriptomes against this reference, I searched for possibly missing new genes (= not present in adults) and I generated their sequences. I have also confirmed my set of genes by the analysis of the high-quality chromosome-level barbel genome, which became available in the meantime (www.ncbi.nlm.nih.gov; CM042264:CM042313; accessed in June 2022, scheduled to be fully released on 17th October, 2022). I verified the correct sequence of all opsin gene references. Then I mapped all 26 transcriptomes against the complete set of opsin references and counted all the reads that assembled to each opsin in every sample. I normalized the results for gene length and total number of paired reads in each sample and I expressed them as fragments per kilobase million (FPKM). (I divided the read counts for each opsin by total number of reads divided by 1,000,000 to get the fragments per million (FPM) value. Then I divided FPM by length of corresponding sequence in kilobases.) Since the FPKM values were incomparable among different samples, I expressed them as percentages. I counted the percentages separately for rod and cone opsins. I processed 4-5 retinal samples for every developmental stage (8 dpf, 14 dpf, 21 dpf, 28 dpf, 42 dpf, and adult) and I executed the computation in Excel. I present the data as boxplots that were created in RStudio 1.1.463 (RStudio Team, 2016).

Analysis of amino acid substitutions

I compared genes belonging to the same opsin class looking for the differences in their amino acid sequence after translation. I aligned the genes together with *Bos taurus* rhodopsin (RHO) mRNA for

positional reference, I translated the alignment and aligned acquired amino acid sequences again. Then I noted all the sites with amino acid substitution between the opsin genes from the same class. I later inspected whether any of the mutated sites had been proved to alter spectral sensitivity of the opsin (i.e., whether any of the amino acid substitutions occurred in key tuning sites) (Yokoyama, 2008). I categorized the substitutions in key tuning sites into four groups with increasing probability of influence on opsin spectral sensitivity: (1) Key tuning site for another opsin class (not proved for the opsin class where substitution was detected); (2) Key tuning site for this opsin class (the site is proved to influence spectral sensitivity in this opsin class, but amino acids detected at the site are not); (3) Amino acid substitution proved to shift $\lambda_{\max}$ in another opsin class (the site is proved to influence spectral sensitivity in this opsin class and amino acids involved in substitution are proved to shift $\lambda_{\max}$ at this site in another opsin class); (4) Amino acid substitution proved to shift $\lambda_{\max}$ in this opsin class (this particular amino acid substitution at this particular site is proved to shift $\lambda_{\max}$ in this opsin class).

Chromosome mapping of opsin genes

I obtained the *Barbus barbus* chromosome assembly from GenBank (www.ncbi.nlm.nih.gov/CM042264:CM042313). To find the chromosomes carrying opsin genes, I created the list of exons from all barbel opsin genes and used it as a reference. First, I mapped all chromosomes against this reference and kept the chromosomes that mapped for further inspection. In the second step, I reversed the approach – I used the selected chromosomes as a reference (one by one), and I mapped opsin exons against them. This way I revealed the position of all opsin genes on the chromosomes, and I was also able to identify one more gene originally not found in the transcriptomic references (see Results). I schematically depicted chromosomes with opsin genes in PowerPoint (Figure 6).

Phylogenetic analysis

I used the set of opsin gene references for opsin gene phylogeny in the common barbel to identify the gene pairs that emerged in the most recent whole genome duplication – sister genes. Then I supplemented the dataset with complete set of opsin gene references from the common carp (*Cyprinus carpio*), that was completed by Zuzana Musilová and confirmed with the carp genome. I ran the second phylogenetic analysis for barbel and carp opsin genes to confirm different origins of allopolyploidy in these species and to find homologies among the opsin genes. Finally, I supplemented the dataset with opsin gene references from *Barbus caucasicus*, *Luciobarbus mursa*, and *Aulopyge huegelii*. These genes were found by me in the retinal transcriptome of adult specimens using the same method as described above for the common barbel references, and they are preliminary results of another project ongoing in our working group. (Specimens of *Aulopyge huegelii* were collected in Croatia and kindly provided by Jasna Vukič, and the samples of *Barbus caucasicus* and *Luciobarbus mursa* originate from Azerbaijan.) However, the sets of opsin references could not be completed from genomes, because they are not yet available for these species. I performed the third phylogenetic analysis to demonstrate that recent

allopolyploidization event was shared among the barbel species and separate for the common carp, and to identify the relationships between the genes. I used the vertebrate ancient opsin (VA) from the common carp as outgroup in all three analyses.

First, I aligned the sequences using the MAFFT alignment plugin (Katoh, Rozewicki and Yamada, 2018) in Geneious Prime 2020.2.4 (<https://www.geneious.com>, Biomatters) with default settings. All sequences were complete (beginning with start codon and ending with stop codon) and no further editing was done. I performed the Bayesian analysis in MrBayes on XSEDE (Huelsenbeck and Ronquist, 2001) that ran at CIPRES (phylo.org, Miller, Pfeiffer and Schwartz, 2010) with following parameters: 5 000 000 MCMC generations (every 1000th tree was saved), four MCMC chains, and two runs. I inspected the results of Bayesian analyses in Tracer, none of the three required further modifications. I summarized trees in MrBayes using default settings – majority-rule consensus and burnin 25%. The final appearance of phylogenetic trees was created in FigTree software.

Fluorescent in-situ hybridization (FISH)

I used FISH for visualization of the cone photoreceptors in the retina according to their opsin expression. In total I performed four combinations visualizing all six single and double cone opsins expressed in the adults. The protocol for the FISH has been optimized from several sources (the original protocol from the research group of Fabio Cortesi, University of Queensland, Brisbane has been updated and customized by Anna Pospíšilová following her personal expertise with the technique) and I describe it below.

All chemicals that were prepared specifically for this protocol are listed in Table 5.

Preparation of the probes for fluorescent in situ hybridization

First-strand cDNA synthesis

RNA isolate from the retina of adult specimen was used for cDNA synthesis, which was done with Evrogen Mint-2 cDNA synthesis kit (SK005). For first-strand cDNA synthesis, I pipetted 3 µl of the isolate to the PCR tube and added 1 µl of CDS-1 Adapter and 1 µl of Plug-Oligo-1 Adapter to the reaction. I incubated the sample at 70°C for 2 minutes and held it at 42°C. In the meantime, I prepared RT Master Mix for the following reaction: I mixed 2 µl of 5X First-Strand Buffer, 1 µl of DTT, 1 µl of dNTP and 1 µl of Mint Reverse Transcriptase. Then I added 5 µl of RT Master Mix to the sample and incubated it for 20 minutes at 42°C. After incubation, I pipetted 5 µl of IP Solution and incubated for 30 minutes at 42°C. Then I cooled the sample on ice and deposited first-strand cDNA to the freezer (-20 °C).

Second-strand cDNA synthesis

I assembled the reaction by adding following ingredients in the given order: 40 µl PCR Grade Water, 5 µl 10X Encyclo Buffer, 1 µl dNTP Mix, 2 µl PCR Primer – M1, 1 µl first-strand cDNA (prepared

previously from RNA isolate), and 1 µl of Encyclo Polymerase Mix. I performed PCR amplifications under the conditions stated in the protocol. (I had carried out evaluative PCR in advance to reveal the ideal number of cycles – I had aliquoted the reaction into several PCR tubes and set different number of cycles for each. Then I performed electrophoresis and compared all products on the gel. That way I had established the number of cycles that led to the clean product of highest possible concentration, and I could later use it for full-scale preparation of dsDNA.) After the amplification, I purified the sample by Min Elute PCR purification Kit according to its protocol and eluted the dsDNA to 50 µl of PCR Grade Water. I stored DNA at -20°C.

Template production

To produce template (i.e., probes), I used specifically designed primers available in our group (the primers aim to amplify the 450 – 500 bp product between the exons 1 and 3 for RH2, exon 1 and 4 for SWS1, exon 3 and 3'UTR in SWS2 and exon 5 and 3'UTR in LWS gene(s)). I diluted the primers in advance to the working concentration. I assembled the PCR reaction by adding following components: 10 µl PPP Master Mix (Top-Bio), 7 µl PCR Water, 0.5 µl Primer F, 0.5 µl Primer R, 2 µl dsDNA. I set cycling conditions as stated in the protocol with 35 cycles and annealing temperature set at 56°C. Afterwards, I performed electrophoresis and cut the product from the gel. I extracted the product using Gel/PCR DNA Fragments Extraction Kit (GENEAID) according to Gel Extraction Protocol from the kit. I eluted the template to 20 µl of PCR Grade Water and measured its concentration and purity on Nanodrop. I stored the product at -20°C to be later labelled with fluorescein or digoxigenin and used for FISH.

Probe labelling

Probes had to be prepared and labelled at least one day before the start of the hybridization process and stored at -20°C overnight. I chose the probe templates of the two genes that I wanted to visualize in the retina. The gene with presumably lower expression was labelled with fluorescein (FL) and the gene with presumably higher expression with digoxigenin (Dig). I assembled the labelling reactions in the separate PCR tubes according to Table 3. 100 – 200 ng of template was required for the reaction while maximum template volume in reaction was 13 µl. I used template concentration values acquired from Nanodrop to count the volume of the template and DEPC water that should be pipetted into each reaction. I incubated the sample for 2 hours at 37°C. In the meantime, I prepared DNase in RDD Buffer (1:7). Afterwards, I added 2 µl of the mixture to each sample and incubated for 15 more minutes at 37°C. Then I pipetted 0.5 µl of EDTA to each probe and let the samples stand for 5 minutes at room temperature. After that, I moved the probes to the freezer.

Table 3: *Probe labelling reaction.*

PROBE	FL probe	Dig probe
100–200 ng template	X µl	X µl
DEPC water	(13–X) µl	(13–X) µl
10x Dig labelling mixture	-	2 µl
10x FL labelling mixture	2 µl	-
10x transcription buffer (from T3 polymerase box)	2 µl	2 µl
RNase inhibitor	1 µl	1 µl
RNA polymerase T3	2 µl	2 µl
Total volume	20 µl	20 µl

Preparation of retinae

For FISH, we used only retinae of adult specimens. The eye was extracted from the head and the cornea pierced and cut in dorsal direction to mark the orientation of the eye. The eye was immersed in 4% paraformaldehyde (PFA) in PBS (phosphate-buffered saline) for 30 minutes at room temperature and then moved to the fridge for at least 16 hours. Then I removed the fixative and rinsed the sample three times for 5 minutes in PBS. After that, the eye was immersed into 70% methanol that was replaced by 100 methanol after 5 minutes. The sample was stored at -20°C until dissection.

Before dissection, I transferred the samples from methanol to PBS by way of decreasing methanol concentrations (75%, 50%, 25%). I removed the fat and extra tissue attached to the eye and then I cut away the cornea. I took out the lens and made 4 cuts from the sites of the eye cup so that the previously made dorsal cut was positioned in the middle of the dorsal wedge. Then I carefully removed sclera and choroid from all four wedges and cut away its remains around the optic nerve. I transferred the retina to the clear PBS solution, cleaned away the vitreous body attached to the inner side, and peeled away the pigment layer attached to the outer side of the retina. When the retina was clean, I used increasing methanol concentrations to transfer it back to 100% methanol and I stored it at -20°C until hybridization.

Hybridization day 1

I rinsed the retina in 50% xylene in methanol for 5 minutes and then in 100% xylene for 30 minutes. Afterwards, I replaced xylene with 100% methanol and rinsed the retina for another 30 minutes. I rehydrated the tissue using decreasing methanol concentrations in PBST (0.1% Tween in PBS) and then I rinsed the retina in PBST twice for 15 minutes. I diluted proteinase K in PBST and transferred the retina into this solution. During digestion, I removed the remnants of pigment layer and cleaned the retina. After 15-30 minutes, I removed the proteinase K solution and quickly washed the retina in PBST. Then I re-fixed the retina in 16% paraformaldehyde in phosphate buffer with 5% sucrose. I removed the fixative after 15 minutes and washed the retina in PBST three times. During those 20-minute washes, I

prepared the prehybridization solution according to Table 4. I replaced PBST with pre-heated prehybridization solution (~300 µl per retina) and incubated the sample on the thermo-shaker at the lowest possible RPM for 2 hours at 56°C. Before the end of prehybridization, I prepared the hybridization solution with the labelled probes. I took the probes out of the freezer and aliquoted 2 µl of each into PCR tubes. I boiled the aliquotes for 7 minutes at 99°C and chilled them on ice before adding to hybridization solution. The hybridization solution was prepared according to Table 4. I replaced the prehybridization solution with hybridization solution and hybridized the retina overnight at 56°C with RPM set at the lowest possible value.

Table 4: Prehybridization and hybridization reaction components.

	Prehybridization solution	Hybridization solution
Prehyb/Hyb solution	261.6 µl	130.8 µl
Probe 1 (FL)	-	1.5 µl
Probe 2 (Dig)	-	1.5 µl
H ₂ O	38.4 µl	16.2 µl
Total	300 µl	150 µl

Hybridization day 2

I discarded the hybridization solution, quickly washed the retina with 50% formamide in 2x SSCT and set the temperature of the thermos-shaker at 65°C. Then I washed the retina twice for 30 minutes in 2x SSCT, once for 15 minutes in 2x SSCT and twice for 30 minutes in 0.2x SSCT, keeping the sample on the heated thermo-shaker. Afterwards, I let the retina stand for 5 minutes at room temperature and rinsed it in MAB-Tween (0.1% Tween in maleate buffer). Then I replaced MAB-Tween with 1x Roche molecular blocking solution (1x RMB) and left the sample for 2 hours at RT. In the separate tube, I mixed 1.5 µl of anti-fluorescein in 150 µl of 1x RMB. Then I replaced 1xRMB with this solution and incubated the retina overnight at 4°C.

Hybridization day 3

I quickly washed the retina in MAB-Triton (0.1% Triton in maleate buffer) and then I performed three 30-minute washes with MAB-Triton and one 15-minute wash with PBST at room temperature. In the meantime, I prepared the Tyramide Dye (TSA 488) solution using Tyramide SuperBoost™ Kit with Alexa Fluor™ Tyramide. First, I prepared H₂O₂ and Reaction Buffer solutions in 1,5 ml tubes: I pipetted 1 ml ddH₂O into both tubes and added 1 drop of H₂O₂/Reaction Buffer from the kit. Then I mixed 150 µl of the Reaction Buffer solution, 1.5 µl of the H₂O₂ solution, and 1.5 µl of the TSA 488 in the separate tube. From now on, all steps had to be protected from light. I incubated the retina in the TSA solution for two hours. Then I washed it three times for 10 minutes in PBST. I incubated the retina for 45 minutes at RT in 3% H₂O₂ to deactivate the peroxidase. After that, I washed the retina three times in PBST again.

In the separate tube, I mixed 1.5 µl of anti-digoxigenin in 150 µl of 1x RMB. I incubated the retina in this solution overnight at 4°C.

Hybridization day 4

I quickly washed the retina in MAB-Triton (0.1% Triton in maleate buffer) and then I performed three 30-minute washes with MAB-Triton and one 15-minute wash with PBST at room temperature. In the meantime, I prepared the Tyramide Dye (TSA 594) solution using Tyramide SuperBoost™ Kit with Alexa Fluor™ Tyramide. The procedure was the same as on the day 3, it differed only in the Tyramide Dye used. I incubated the retina in the TSA solution for two hours. Then I washed it six times for 5 minutes in PBST. (Repeated washes reduce the background fluorescence.) I incubated the retina for 45 minutes at RT in 3% H₂O₂, then I washed the retina six times in PBST again. I replaced PBST with 70% glycerol in PBST, let the retina for 10 minutes at RT and then replaced the solution for a fresh one. I mounted the retina in 70% glycerol in PBST, cone side down, and stored the mount at -20°C.

Table 5: List of solutions for FISH.

PBS (phosphate-buffered saline)	10x PBS bought ready to use, diluted to 1x PBS with H ₂ O
PBST	0.1% Tween in 1xPBS
10% Tween	100% Tween bought ready to use, diluted to 10% with H ₂ O
1.67x phosphate buffer with sucrose	0.20 g NaH ₂ PO ₄ (monohydrate); 1.63 g Na ₂ HPO ₄ (heptahydrate); 3.75 g sucrose; bring total volume to 45 ml with RNase free H ₂ O
16% paraformaldehyde (PFA) in phosphate buffer with sucrose	500 µl formaldehyde solution (Sigma, 36.5-38%); 1.5 ml 1.67x phosphate buffer with sucrose
Proteinkinase K in PBST	5 ml PBST + 1.5 µl pK frozen stock (20 mg/ml)
MAB = 5x maleic acid	0.5M maleic acid + 0.75M NaCl
MAB-Tween	10 ml 5x maleic acid; 40 ml DEPC water; 0.5 ml 10% Tween
MAB-Triton	10 ml 5x maleic acid; 40 ml DEPC water; 50 µl Triton X-100
10x Roche Molecular Blocking Solution (10x RMB)	10 g Roche Blocking Reagent powder; 20 ml MAB; 80 ml H ₂ O
1x Roche Molecular Blocking Solution (1x RMB)	4.5 ml 10x RMB; 8.1 ml MAB; 32.4 ml H ₂ O
20x SSC (saline-sodium citrate buffer)	Bought ready to use
50% formamide in 2x SSCT	20 ml DEPC water; 25 ml 100% formamide; 5 ml 20x SSC; 50 µl Tween
2x SSCT	45 ml DEPC water; 4.5 ml 20x SSC; 45 µl Tween
0.2x SSCT	50 ml DEPC water; 500 µl 20x SSC; 50 µl Tween
Prehyb/Hyb solution	3.6 ml TEN solution; 25 ml 100% Formamide; 10 ml 50% Dextran Sulfate; 5 ml 10x RMB

Microscopy imaging

All mounts were quickly examined under fluorescent microscope Olympus BX51. Only samples with clearly visible signal from both probes were selected for microscopy imaging. The subsequent imaging was conducted on laser scanning confocal microscope Zeiss LSM 800. I used objective Plan-Apochromat 63×/1.4 Oil and two lasers for excitation - Argon 488 nm and DPSS 561 nm. The emission spectrum for fluorescein-labelled probe was 499–553 nm (green) and for digoxigenin-labelled probe 596– 695 nm (red). I used Z-stacking mode for imaging, and I took a series of images covering the whole range of focal distances where signal of the probe was visible in red and green channel (~ 80 images per sample) with ZEN software. I conducted Z-stack imaging in corresponding range of focal distances also in transmission light.

Image processing

I used Fiji (Schindelin *et al.*, 2012) to process the images from Z-stacking. In each fluorescent channel, I selected only the images with desired signal (i.e., I omitted the images with prevailing background fluorescence) and I used them to create one composite image under the mode “Average Intensity.” Then I merged images acquired in both channels (red and green). I let the colour channels correspond the fluorescence of the probe, not the spectral sensitivity of the opsin. I added the scalebar and I adjusted the brightness and contrast in Affinity Photo.

The images in transmission light were processed separately in Fiji. I tried to choose only few images with the best visible photoreceptor structure and combine them in various modes. However, images from confocal microscope in transmission light were below sufficient quality to produce the image of the barbel retina in bright field, and these results are thus not presented

Results

Visual opsins in the common barbel

I found 14 opsin genes in the common barbel (*Barbus barbus*) – 13 genes were detected by mapping the transcriptome against the basic set of reference sequences of all examined life stages and one gene was later discovered in the genome. Out of 14 genes, ten genes coded cone opsins (red-sensitive: LWS1, LWS2; green-sensitive: RH2-1, RH2-2, RH2-3, RH2-4; blue-sensitive: SWS2-1, SWS2-2; UV-sensitive: SWS1-1, SWS1-2), and four rod opsins (RH1b-1, RH1b-2, RH1d1, RH1d2). Sequences of all genes were preserved functional (i.e., without stop codons), even though RH2-4 was not present in the transcriptome.

I identified the positions of opsin genes on the chromosomes of the common barbel. The genes are located on chromosomes 10a&b, 17a&b, 19a&b, and 21a&b (A and B corresponding to parental subgenomes), and their arrangement shows a pattern expected after the whole genome duplication – sister genes that originated in the recent whole genome duplication are always found at the corresponding positions on A and B chromosomes (see Figure 6). Each chromosome 10 carries two RH2 genes in tandem. Chromosomes 17a&b and 19a&b carry only one opsin gene, RH1d, and SWS1, respectively. The remaining opsin genes are located on chromosomes 21a&b. While RH1b is quite remote from the others, SWS2 and LWS are next to each other, with a clear linkage between the two.

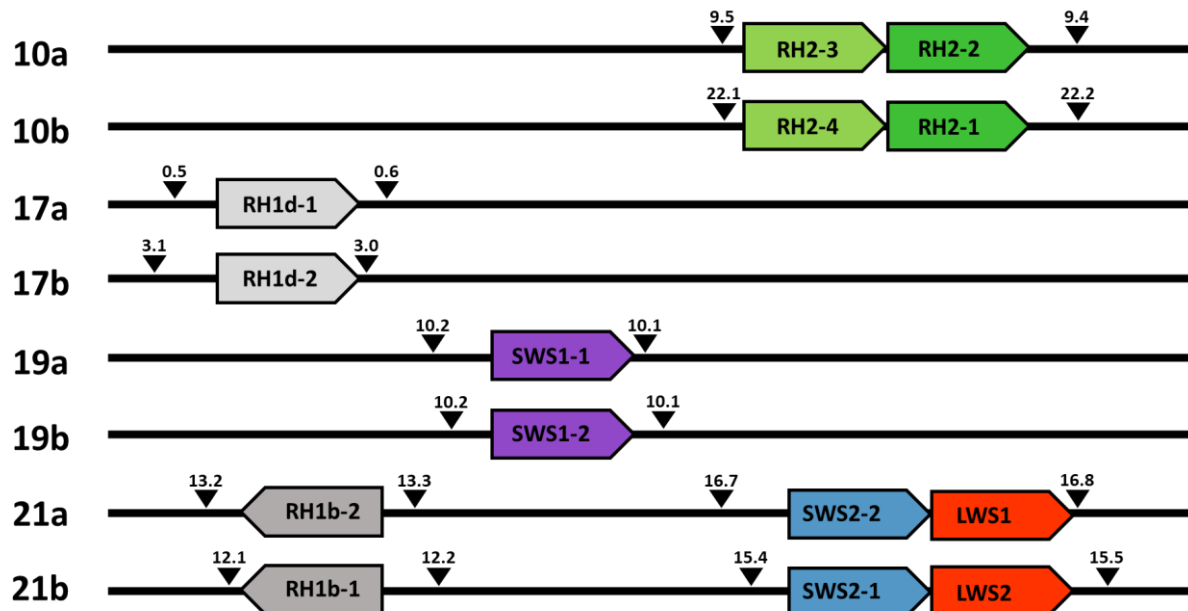


Figure 6: *Location of opsin genes on the chromosomes in the common barbel (B. barbus).* Black arrows mark positions along the chromosome in megabases. Chromosomes 10a, 17b, 19a, and 19b are reversed in this diagram to present the opsin genes accordingly between the sister chromosomes.

I decided to run the three following phylogenetic analyses and show their results separately to demonstrate different phenomena: (1) phylogenetic analysis of opsin genes from the common barbel to uncover their origin and mutual homologies; (2) phylogenetic analysis of opsin genes from the common barbel and another cyprinid evolutionary tetraploid species – the common carp (*Cyprinus carpio*) – to confirm that the recent polyploidization event was not shared by the two species; (3) phylogenetic analysis of opsin genes from the common carp, common barbel, and three other species from European tetraploid barbel clade to detect the homologies between the genes from the common barbel and the genes from the adult retinal transcriptome of other barbel species, and to confirm their common origin in the barbel-specific whole genome duplication shared with the common barbel.

I constructed the phylogenetic tree of opsin genes in the common barbel (see Figure 7) to confirm the pairs of sister genes determined by their positions on chromosomes in Figure 6. The phylogenetic tree shows a very clear pattern that is congruent with the pattern in gene positions – pairs of genes at the end of branches are sister genes from the recent whole genome duplication event (see splits with black asterisks in Figure 7). I also detected older duplication event in RH2 opsin class that was shared by both progenitor species later involved in allopolyploidization (yellow asterisk in Figure 7). Red asterisk in Figure 7 marks ancient RH1 duplication event shared among all cyprinid fishes (Morrow *et al.*, 2017).

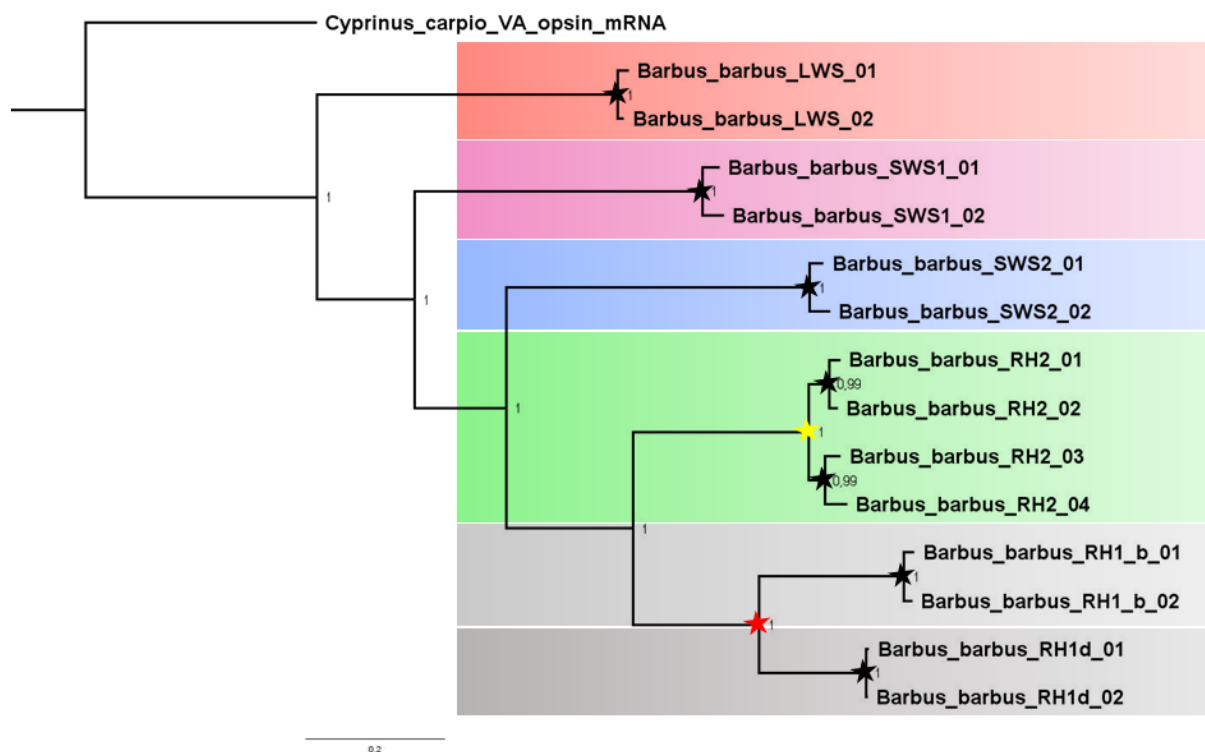


Figure 7: Opsin gene phylogeny in the common barbel (*B. barbus*). Bayesian majority-rule consensus tree, node labels represent Bayesian posterior probabilities, vertebrate ancestral opsin from the common carp used as outgroup. Colours in the background symbolize spectral sensitivities of cone opsins (violet stands for UV) and shades of grey highlight opsins expressed in rods. Black asterisks mark the recent allopolyploidization event, yellow asterisk marks preceding duplication of RH2 opsin gene, and red asterisk marks duplication of RH1 opsin gene in the ancestor of all cyprinids.

The phylogeny of opsin genes in the common barbel and the common carp demonstrates that both species underwent separate polyploidization events (Figure 8). While older duplication of RH1 is clearly shared by both species, phylogenetic pattern in RH2 opsin class is quite obscured. That is most likely due to presence of the pseudogene – when run without the pseudogene, the analysis suggests that older duplication of RH2 occurred before the split of the two species (the phylogeny is not presented in this thesis).

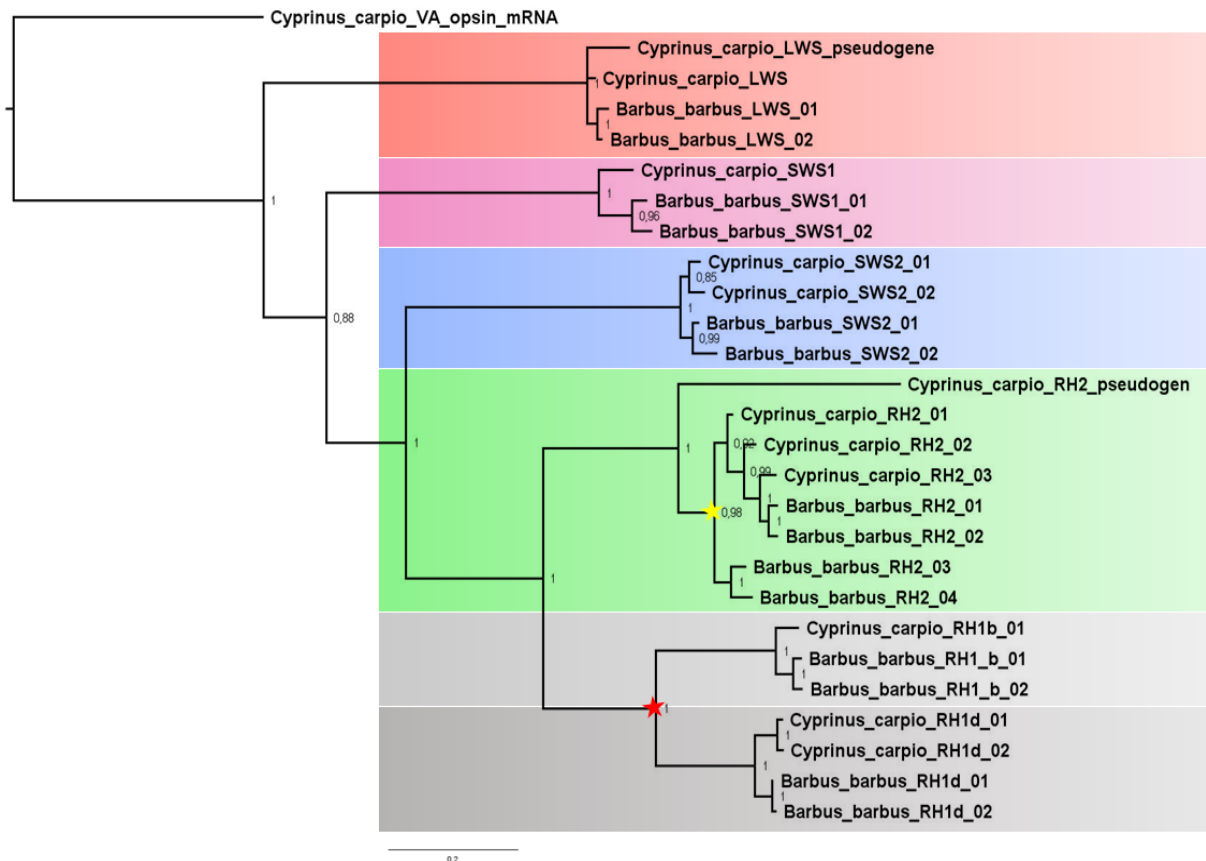


Figure 8: Phylogeny of opsin genes in the common barbel (*B. barbus*) and the common carp (*C. carpio*). Bayesian majority-rule consensus tree, node labels represent Bayesian posterior probabilities, vertebrate ancestral opsin from the common carp used as outgroup. Colours in the background symbolize spectral sensitivities of cone opsins (violet stands for UV) and shades of grey highlight opsins expressed in rods. Phylogenetic positions of opsin genes indicate two recent polyploidization events that occurred separately in the common barbel and common carp. Yellow asterisk marks barbel-specific duplication of RH2 opsin gene, red asterisk marks duplication of RH1 opsin gene in the ancestor of all cyprinids.

The phylogeny of opsin genes of four species from European tetraploid barbel clade (*Barbus barbus*, *Barbus caucasicus*, *Luciobarbus mursa*, and *Aulopyge huegelii*) together with *Cyprinus carpio* opsin genes confirms that tetraploidy in barbels originates from one event that occurred in their common ancestor, and that the common carp underwent separate recent polyploidization (Figure 9). It also shows that homologs of several common barbel opsin genes were not found in other barbel species.



Figure 9: Phylogeny of opsin genes in four species from European tetraploid barbel clade (*B. barbus*, *B. caucasicus*, *L. mursa*, *A. huegelii*) and the common carp (*C. carpio*). This analysis included all opsin genes from the genome of the common carp and common barbel, but only genes from adult transcriptomes of other barbel species. Bayesian majority-rule consensus tree, node labels represent Bayesian posterior probabilities, vertebrate ancestral opsin from the common carp used as outgroup. Colours in the background symbolize spectral sensitivities of cone opsins (violet stands for UV) and shades of grey highlight opsins expressed in rods. Phylogenetic positions of opsin genes in all classes except RH2 demonstrate that the three genera of European barbels (*Barbus*, *Luciobarbus*, and *Aulopyge*) shared the same polyploidization event while the common carp underwent separate whole genome duplication. Red asterisk marks duplication of RH1 opsin gene in the ancestor of all cyprinids. Arrows indicate expression patterns of cone opsins in the common barbel – yellow arrows show opsins that were found exclusively in the larval transcriptome, orange arrows opsins from the adult transcriptome, blue arrows opsins detected in the transcriptomes of all life stages, and grey arrow opsin that was not expressed at any stage. Note that homologs of larval and unexpressed genes are missing in adult transcriptomes of other European tetraploid barbels, suggesting the possibility of an expression pattern similar to the common barbel.

Those are particularly the genes expressed only in larval stages (SWS1-2, SWS2-2, RH2-3) or not expressed at all (RH2-4). (For expression patterns, see chapter Results – Opsin expression throughout development.) Since opsin references for other barbel species could be obtained only from adult retinal transcriptome, it is very likely that these sets of references are incomplete and homologs of the common barbel's larval genes might be expressed in larval stages of other barbels too, or at least present in the genome.

Opsin protein sequence differentiation and its functional consequences

I compared amino acid sequences of all opsins from each class and recorded all positions where amino acids differ (see Table 6). I found at least minimal variation in amino acid sequences within all opsin classes. Then I identified which of these amino acid substitutions occurred in key tuning sites (KTS) and thus potentially cause the differentiation of $\lambda_{\max}$ within the opsin classes (see colour highlights in Table 6).

Interestingly, amino acid sequences of LWS opsins differed only in five positions and none of them was KTS. Out of 22 substitutions identified between SWS1-1 and SWS1-2, four occurred in KTS determined for this opsin (with one of them being the exact couple of amino acids that were reported to shift $\lambda_{\max}$ in SWS1). The sequences of SWS2-1 and SWS2-2 differed at 36 positions including three positions reported as KTS for SWS2 and one position reported as KTS in another opsin class. One of these positions constituted the amino acid couple that was proved to differentiate $\lambda_{\max}$ in SWS2 and one other position constituted the amino acid couple that was proved to differentiate $\lambda_{\max}$ in SWS1. 49 variable positions were found within the RH2 opsin class. Two of these positions were KTS for RH2 and one for SWS2. RH2 tuning sites constituted amino acids that are known to influence spectral sensitivity in this opsin class. Remarkably, RH2-1 and RH2-2 differed only in six positions, while RH2-3 and RH2-4 differed in 25 positions with RH2-4 being the most differentiated opsin from the whole class. I found 86 variable positions within the RH1 opsin class, but only two of them were KTS for RH1 and another two were KTS for RH2 and SWS1. Moreover, none of these positions contained amino acids that would be reported to shift spectral sensitivity. While RH1b-1 and RH1b-2 differed in eleven positions, there was no amino acid substitution between RH1d-1 and RH1d-2.

Table 6: **Amino acid substitutions among all opsin classes in the common barbel.** Substitutions at key tuning sites are highlighted. Colours of highlight with increasing probability of influence on opsin spectral sensitivity: (1) Key tuning site for another opsin class (not proved for the opsin class where substitution was detected); (2) Key tuning site for this opsin class (the site is proved to influence spectral sensitivity in this opsin class, but amino acids detected at the site are not); (3) Amino acid substitution proved to shift λ_{max} in another opsin class (the site is proved to influence spectral sensitivity in this opsin class and amino acids involved in substitution are proved to shift λ_{max} at this site in another opsin class); (4) Amino acid substitution proved to shift λ_{max} in this opsin class (this particular amino acid substitution at this particular site is proved to shift λ_{max} in this opsin class). Where more than two genes are present, asterisks in the bottom rows mark the substitutions between sister genes that arose in the recent whole genome duplication.

Key tuning site for another opsin class				Key tuning site for this opsin class				AA substitution proved to shift λ_{max} in another opsin class				AA substitution proved to shift λ_{max} in this opsin class			
aa position	304	162	175	304	162	175	304	162	175	304	162	175	304	162	175
bovine RHO	V	K	I	A	I	A	I	A	I	A	I	A	I	A	I
LWS1	V	K	I	A	I	A	I	A	I	A	I	A	I	A	I
LWS2	M	R	L	V	V	V	M	R	L	V	V	V	M	R	L
aa position	8	14	45	50	57	19	22	33	37	43	46	48	49	53	57
bovine RHO	Y	S	I	V	T	H	M	S	S	S	S	S	S	S	S
SWS1-1	Y	S	I	V	T	H	M	S	S	S	S	S	S	S	S
SWS1-2	F	V	I	V	T	H	M	S	S	S	S	S	S	S	S
aa position	8	16	22	23	37	43	46	48	49	53	57	104	108	113	122
bovine RHO	D	E	T	S	N	H	A	T	F	I	L	K	V	V	V
SWS2-1	D	E	T	S	N	H	A	T	F	I	L	K	V	V	V
SWS2-2	D	E	T	S	N	H	A	T	F	I	L	K	V	V	V
aa position	2	7	11	13	17	24	36	39	42	44	45	56	60	61	100
bovine RHO	N	G	V	L	T	Y	Q	L	I	I	F	L	V	V	V
RH2-1	N	G	V	L	T	Y	Q	L	I	I	F	L	V	V	V
RH2-2	N	G	V	L	T	Y	Q	L	I	I	F	L	V	V	V
RH2-3	N	G	V	L	T	Y	Q	L	I	I	F	L	V	V	V
RH2-4	N	G	V	L	T	Y	Q	L	I	I	F	L	V	V	V
1 vs. 2 dif.	I	S	H	M	R	Y	Q	V	C	F	I	F	G	V	A
3 vs. 4 dif.	I	S	H	M	R	Y	Q	V	C	F	I	F	G	V	A
aa position	8	16	19	32	33	35	37	38	40	42	49	51	52	53	54
bovine RHO	D	V	V	A	S	L	F	Y	V	A	C	I	L	I	G
RH1b-1	D	V	V	A	S	L	F	Y	V	A	C	I	L	I	G
RH1b-2	D	V	V	A	S	L	F	Y	V	A	C	I	L	I	G
RH1d-1	A	I	V	A	S	L	F	Y	V	A	C	I	L	I	G
RH1d-2	A	I	V	A	S	L	F	Y	V	A	C	I	L	I	G
b1 vs. b2	A	I	V	A	S	L	F	Y	V	A	C	I	L	I	G
d1 vs. d2	A	I	V	A	S	L	F	Y	V	A	C	I	L	I	G
aa position	198	199	209	213	218	220	227	232	256	260	266	278	281	282	284
bovine RHO	T	E	V	S	I	S	L	V	L	A	L	H	S	Q	S
RH1b-1	T	E	V	S	I	S	L	V	L	A	L	H	S	Q	S
RH1b-2	T	E	V	S	I	S	L	V	L	A	L	H	S	Q	S
RH1d-1	V	N	I	T	V	F	E	I	G	T	N	T	E	G	L
RH1d-2	V	N	I	T	V	F	E	I	G	T	N	T	E	G	L
b1 vs. b2	V	N	I	T	V	F	E	I	G	T	N	T	E	G	L
d1 vs. d2	V	N	I	T	V	F	E	I	G	T	N	T	E	G	L
aa position	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211
bovine RHO	E	N	I	V	V	V	V	V	V	V	V	V	V	V	V
RH1b-1	E	N	I	V	V	V	V	V	V	V	V	V	V	V	V
RH1b-2	E	N	I	V	V	V	V	V	V	V	V	V	V	V	V
RH1d-1	E	N	I	V	V	V	V	V	V	V	V	V	V	V	V
RH1d-2	E	N	I	V	V	V	V	V	V	V	V	V	V	V	V
b1 vs. b2	E	N	I	V	V	V	V	V	V	V	V	V	V	V	V
d1 vs. d2	E	N	I	V	V	V	V	V	V	V	V	V	V	V	V
aa position	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293
bovine RHO	K	I	V	T	I	K	I	V	T	I	K	I	V	T	I
RH1b-1	K	I	V	T	I	K	I	V	T	I	K	I	V	T	I
RH1b-2	K	I	V	T	I	K	I	V	T	I	K	I	V	T	I
RH1d-1	K	I	V	T	I	K	I	V	T	I	K	I	V	T	I
RH1d-2	K	I	V	T	I	K	I	V	T	I	K	I	V	T	I
b1 vs. b2	K	I	V	T	I	K	I	V	T	I	K	I	V	T	I
d1 vs. d2	K	I	V	T	I	K	I	V	T	I	K	I	V	T	I
aa position	294	295	296	297	298	299	300	301	302	303	304	305	306	307	308
bovine RHO	A	L	V	V	V	V	V	V	V	V	V	V	V	V	V
RH1b-1	A	L	V	V	V	V	V	V	V	V	V	V	V	V	V
RH1b-2	A	L	V	V	V	V	V	V	V	V	V	V	V	V	V
RH1d-1	A	L	V	V	V	V	V	V	V	V	V	V	V	V	V
RH1d-2	A	L	V	V	V	V	V	V	V	V	V	V	V	V	V
b1 vs. b2	A	L	V	V	V	V	V	V	V	V	V	V	V	V	V
d1 vs. d2	A	L	V	V	V	V	V	V	V	V	V	V	V	V	V

Opsin expression throughout development

I constructed opsin expression profiles to assess variation in expression across six developmental stages: 8 dpf, 14 dpf, 21 dpf, 28 dpf, 42 dpf (juvenile), and adult, as shown in Figure 10 (dpf stands for days post-fertilization). I found remarkable variation in cone opsin expression. Opsins SWS1-2, SWS2-2, and RH2-3 were only expressed in larvae, and they were gradually replaced by another gene from the same class during development. This gene was a sister gene in case of SWS1 and SWS2 (SWS1-1 and SWS2-1 respectively), in case of RH2 it was a copy located on the same chromosome (RH2-3) (see Figure 6). The expression of these “adult” genes was very low in the youngest larval stages and the switch in expression was synchronized in all three classes – the level of expression of “larval” and “adult” gene was almost equal 14 dpf (see also Figure 11). Both LWS genes and RH2-1 gene were expressed in all developmental stages, whereas RH2-4 gene was not expressed in any. Expression of rod opsins was similar across all stages with RH1d-1 constituting ~55%, RH1d-2 ~ 45%, and RH1b-1&2 together 1% or less of rod opsin expression (see Figure 11).

When I summed up all cone opsins in each class and compared the levels of their expression, I did not find any conspicuous changes over development (Figure 12, left). Expression of SWS1 is slightly higher than expression of SWS2 in the 8 dpf stage. After the transition to adult expression pattern, expression of SWS2 becomes slightly higher than expression of SWS1. The level of expression of RH2 is slowly increasing throughout development. LWS is the most expressed cone opsin class at all stages except juvenile (42 dpf). LWS expression at the first stage (8 dpf) is remarkably high compared to other cone opsins.

Rod opsins constitute majority of opsin expression at all developmental stages. However, their proportion changes during development (Figure 12, right). At the first stage, rod opsins account for 82% of opsin expression. This percentage later decreases until it reaches its minimum at 21 dpf stage (63%). Since then, proportion of rod opsins increases, and it accounts for 89% of opsin expression in adults.

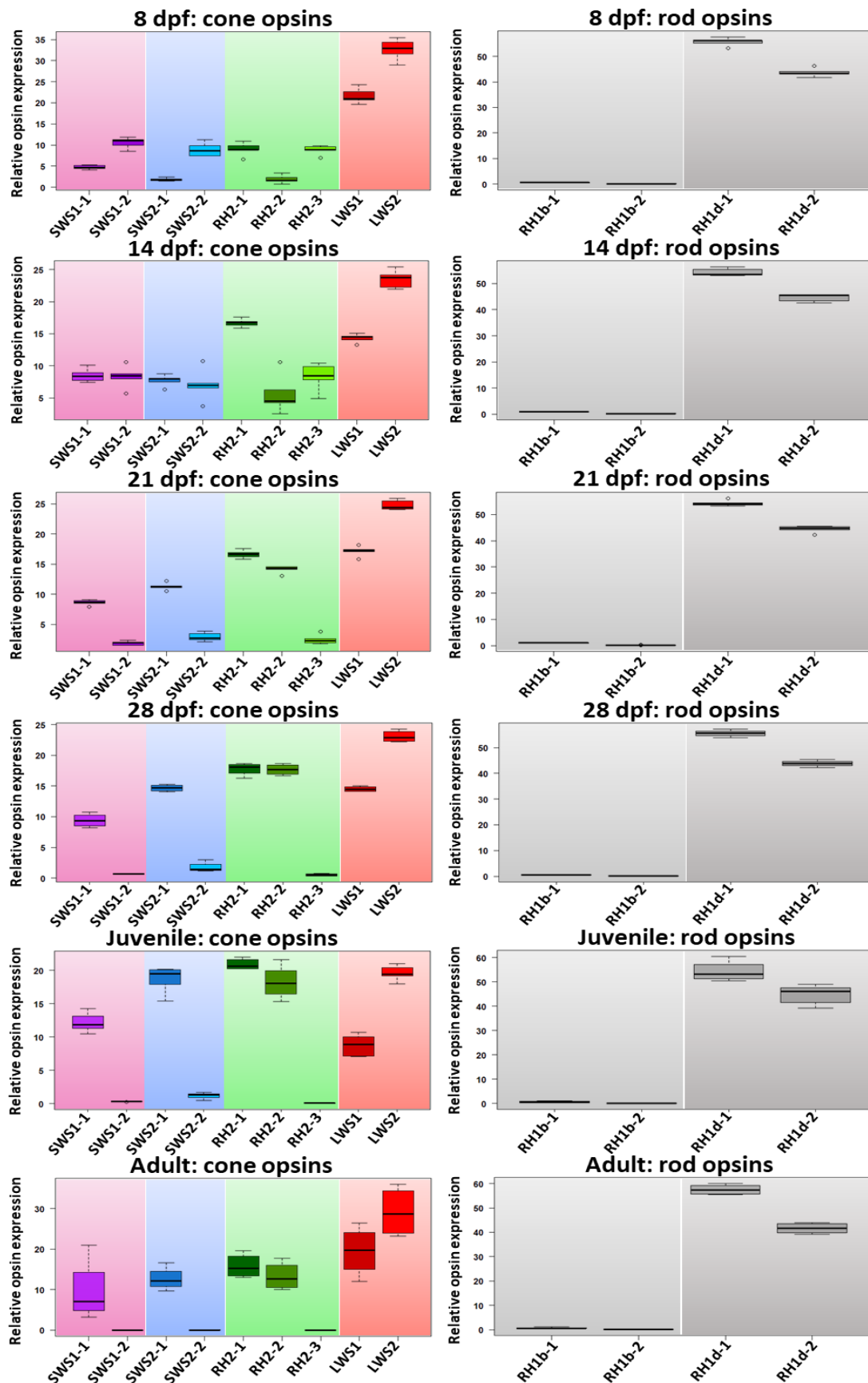


Figure 10: *Developmental series of opsin expression profiles in the common barbel. RH2-4 opsin was omitted because it is not expressed at any life stage. Colours represent spectral sensitivities of cone opsins (violet stands for UV), shades of grey were used for opsins expressed in rods.*

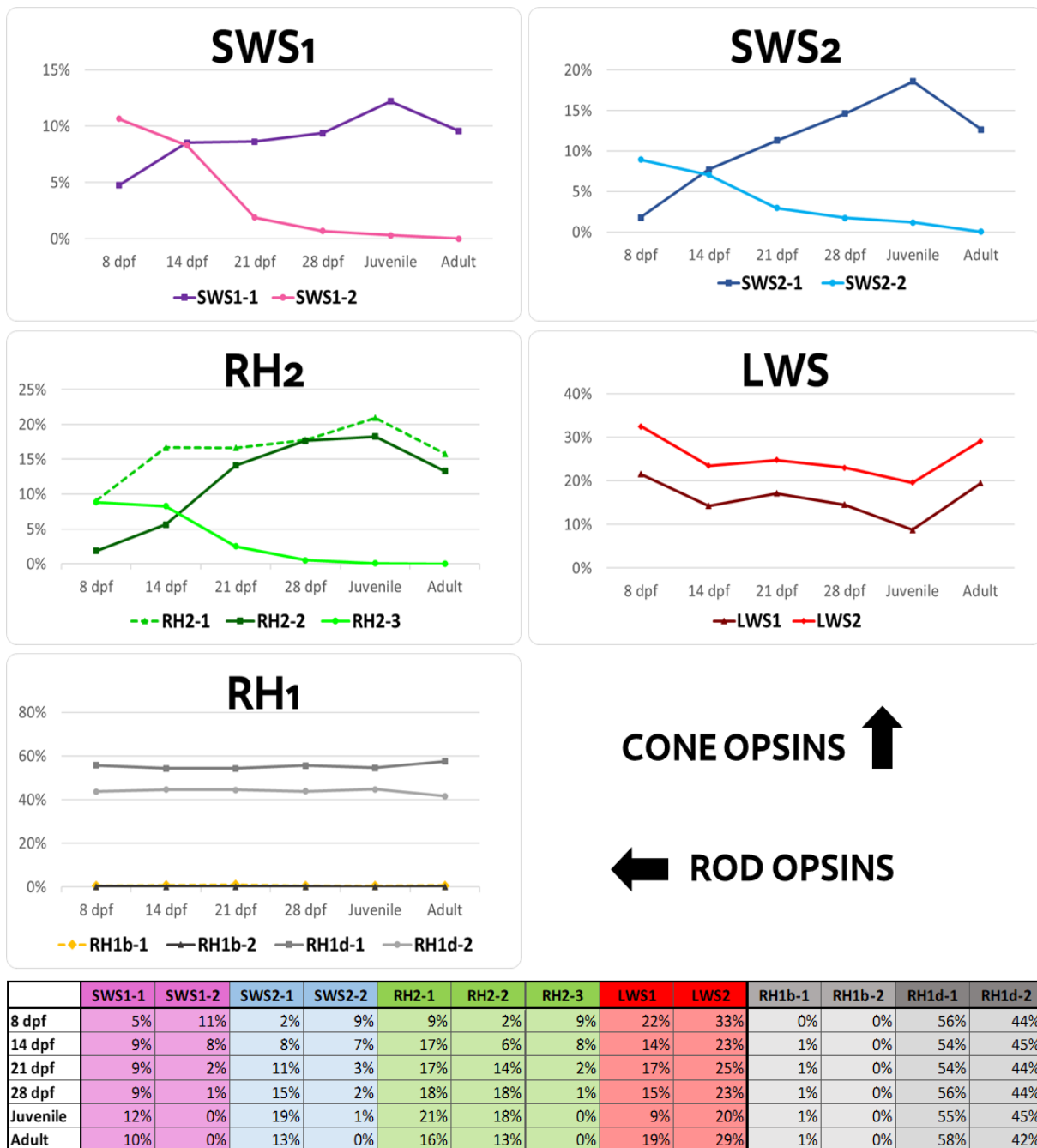


Figure 11: *Developmental changes in opsin expression within individual opsin classes. Table shows average percentages of all opsins in the retinal transcriptome at all developmental stages. Percentages of cone and rod opsins were counted separately. RH2-4 opsin was omitted because it is not expressed at any life stage.*

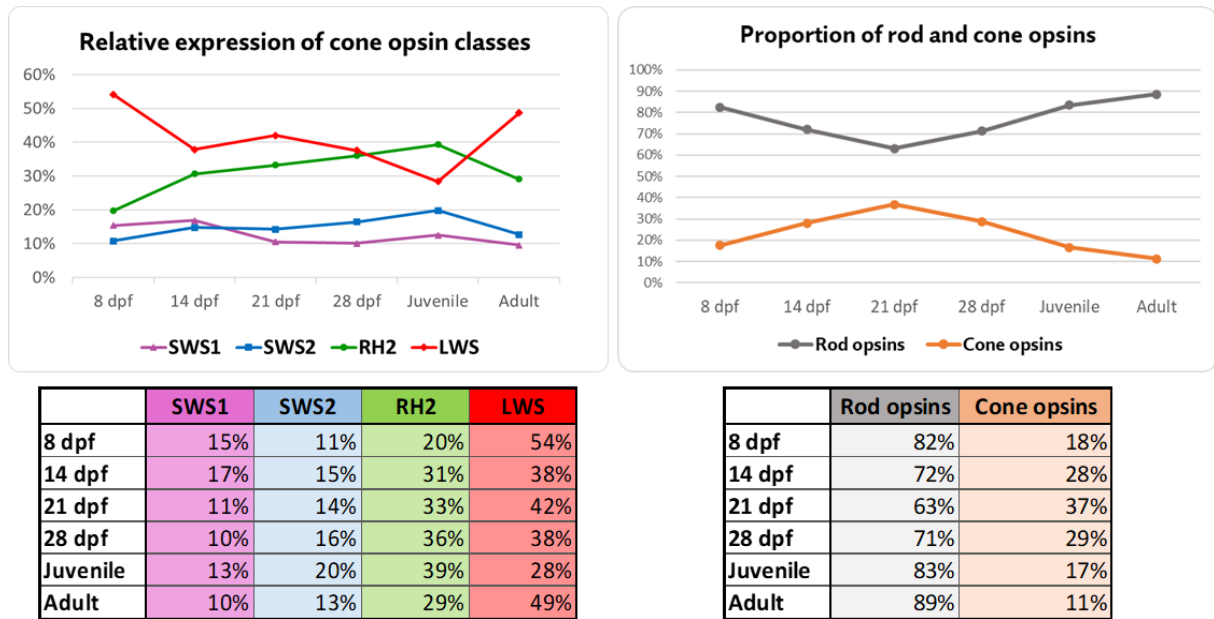


Figure 12: Developmental changes in expression among cone opsin classes (left) and developmental changes in proportion of rod and cone opsins in the retinal transcriptome (right). Left table shows average percentages of all opsin classes in the retinal transcriptome at all developmental stages. Right table shows average percentages of rod and cone opsins in the retinal transcriptome at all developmental stages.

Retinal structure and cone mosaic

I hybridized two combinations of cone opsin genes from different classes in the adult retina – SWS1 with SWS2 and RH2 with LWS. SWS1 and SWS2 are expressed in separate single cones (Figure 13, upper row). These cones are arranged in rows creating a very regular pattern. RH2 and LWS are expressed in opposite halves of the double cones (Figure 13, lower row). The double cones in my sample did not exhibit such undisputable mosaic pattern as single cones, but they were distributed evenly. Moreover, there are clues that they were originally regularly distributed in the gaps between single cones, but their orientation was disrupted during hybridization process. I have not identified any second class of double cones. I assume that all cones constitute square mosaic with possible irregularities in the common barbel.

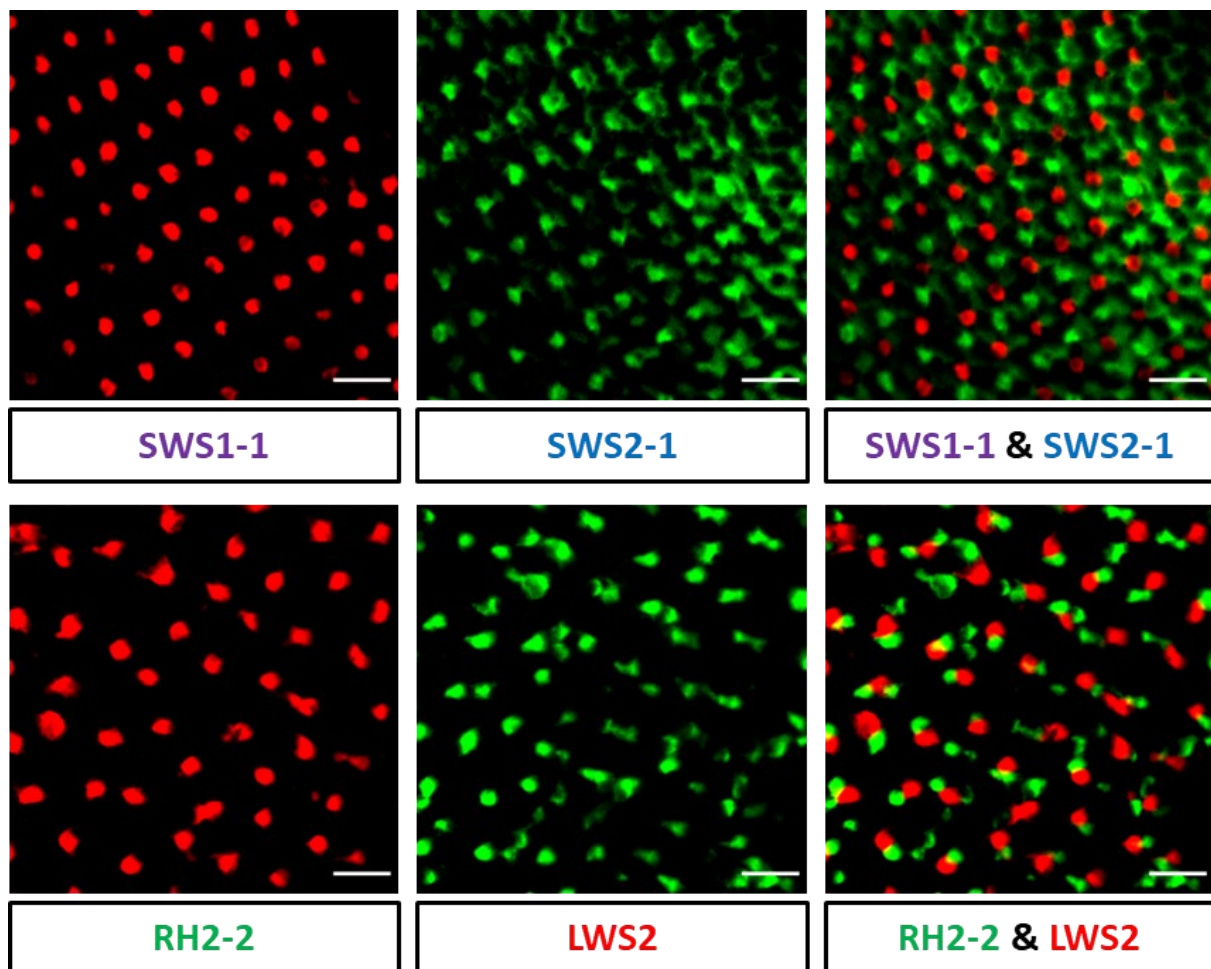


Figure 13: Fluorescent in situ hybridization labelling of photoreceptors in the retina of adult common barbel. Photoreceptors are labelled according to their opsin expression by specific probes. Colours in the images correspond the colour of the probe, not the spectral sensitivity of the opsin. First two columns show the images with separate probe signals, third column shows the composite images. Labelled opsins are stated bellow each image. **Note the separate expression of SWS1-1 and SWS2-1 in the distinct single cones in the first sample and separate expression of RH2-2 and LWS2 in the opposite halves of the double cones in the second sample.** Scale bar represents 20 μm .

Localization of expression of sister opsins in adult retina

There are two cone opsin classes represented by more than one gene in adult expression profile: RH2 and LWS. I hybridized both genes expressed at adult stage together within each class (i.e., RH2-1 with RH2-2 and LWS1 with LWS2). In both RH2 and LWS, I found their coexpression in corresponding halves of the double cones (see Figure 14).

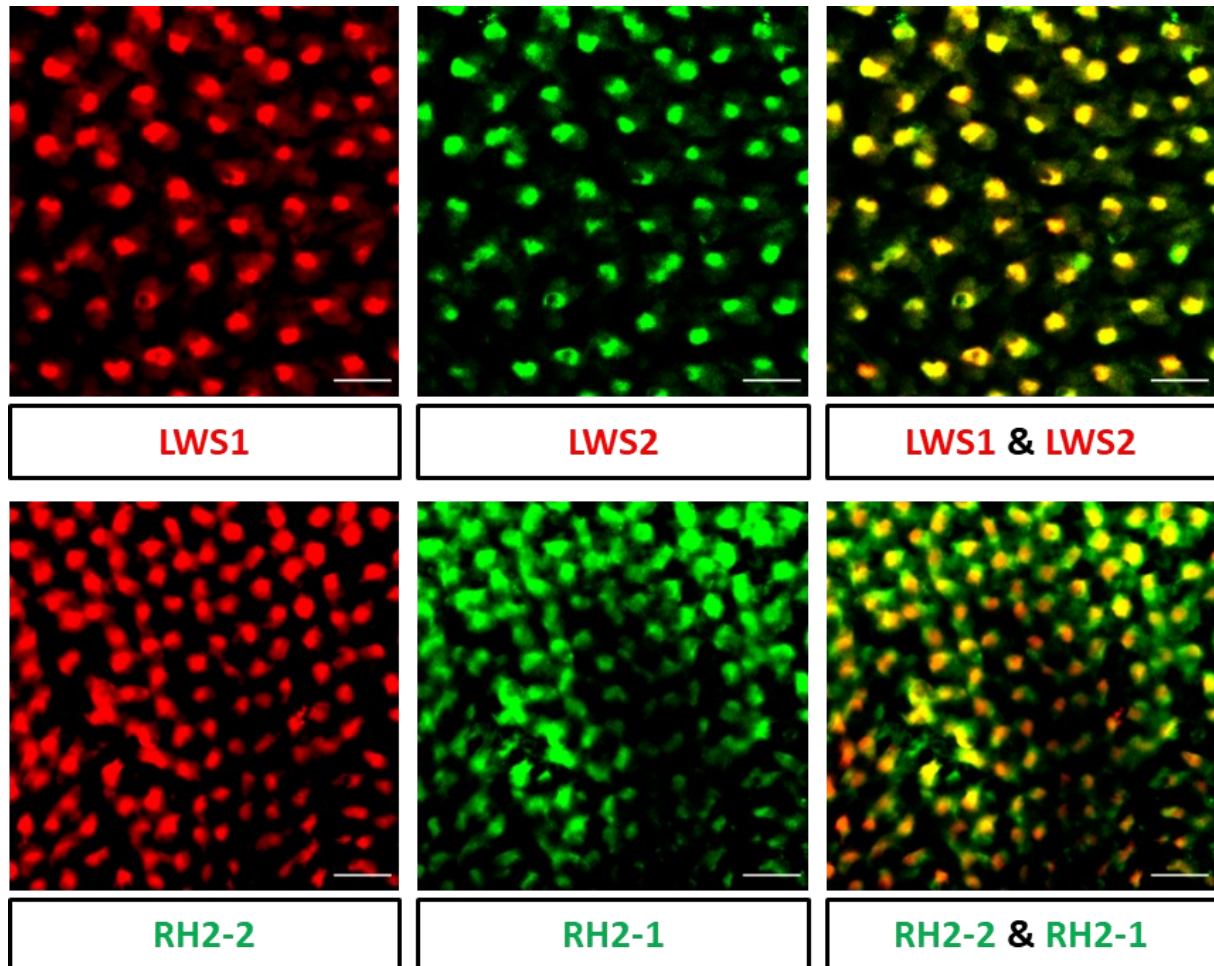


*Figure 14: Fluorescent in situ hybridization labelling of photoreceptors in the retina of adult common barbel. Photoreceptors are labelled according to their opsin expression by specific probes. Colours in the images correspond the colour of the probe, not the spectral sensitivity of the opsin. First two columns show the images with separate probe signals, third column shows the composite images. Labelled opsins are stated bellow each image. **Note the coexpression of sister genes in the same halves of the double cones in both samples.** Scale bar represents 20 μm .*

Discussion

Visual opsins in the common barbel

I detected 14 opsin genes in the genome of the common barbel (*Barbus barbus*), which is an unusually high number. The fundamental cause of such opsin gene richness is the recent whole genome duplication, even though two genes (RH1 and RH2) have already duplicated before (and hence the common barbel has four copies of each of these genes). The first duplication of RH1 is very old, it most likely occurred in the common ancestor of Ostarioclupeomorpha between 153 and 248 MyA (Morrow *et al.*, 2017). The second RH1 gene (named RH1b in this study) is therefore originally present in all cyprinid fishes. My results confirm that the RH1 gene duplication event is shared not only among European tetraploid barbels, but also with the common carp, independent polyploid cyprinid lineage, and are thus congruent with previous findings (see Figures 8 and 9). Furthermore, both progenitor species for European barbel clade involved in the recent allopolyploidization shared the same duplication of RH2 gene, as demonstrated both in the barbel opsin phylogeny and in the position of RH2 genes on the chromosomes of the common barbel (Figures 7 and 6). RH2 opsin is also the most variable opsin gene within cyprinids (and other teleosts too), with nearly every cyprinid fish having several copies of RH2, including some pseudogenes (Musilova and Cortesi, 2021; Truhlářová, in prep.). It is therefore noteworthy that both notional parental species to European barbels had the same two RH2 genes, and that none of these genes have later duplicated or pseudogenized in the common barbel. Unfortunately, dating this RH2 duplication event that preceded allopolyploidization is difficult, at least until the identity of progenitor species is revealed. Notably, my results also suggest that RH2 duplication event might be also shared with the common carp which considerably broadens the set of candidate progenitor species. Such analysis would require expanded taxon sampling including RH2 genes of some distantly related groups. Finding the parental/progenitor species has been out of the scope of my thesis. However, authors of the high-quality whole genome released recently (while the publication is still not present) will probably run this analysis in the future.

Most visual opsin gene classes in European barbels show the expected phylogenetic pattern: Each gene forms a separate clade with *Aulopyge huegelii* diverging first, followed by *Luciobarbus mursa*, leaving *Barbus barbus* and *Barbus caucasicus* grouped together (see Figure 9). *Aulopyge huegelii* sometimes deviates from this scheme (especially in RH2 and RH1 gene classes) which can be explained by the long time of separate evolution (at least 10.6 million years) and unique way of life (*Aulopyge* inhabits Balkan Dinaric karst and lives partly underground, so its visual pigments might have adapted to these specific light conditions) (Ludoški *et al.*, 2021). I have not found equivalents for the common barbel's SWS1-2, SWS2-1, RH2-3, RH2-4, and RH1b-2 in any other barbel species. Absence of these genes in other barbel species is best explained by the availability of material - I only had the opportunity to work with transcriptomes from adult retinae where these genes would not be found in the common

barbel either. (SWS1-2, SWS2-2, and RH2-3 were expressed exclusively in larvae while RH2-4 was not expressed at all, and I discovered it in the genome). It would be certainly very interesting to sequence the genomes and larval transcriptomes of other barbel species and see whether these genes are preserved and utilized in the same way as in the common barbel. The expression of RH1b-2 was particularly low in all researched species and thus it is not surprising that RH1b-2 was not expressed at all in some species. However, it is also possible that its expression was low enough to be overlooked.

Opsin protein sequence differentiation and its functional consequences

Comparison of amino acid sequences of opsins in the common barbel revealed that mutation rate varied substantially among the opsin classes (Table 6). Unsurprisingly, RH1 class comprised the highest number of variable positions (86 sites) mostly due to very deep division of RH1b and RH1d (at least 153 million years). On the other hand, very few of these positions were in the key tuning sites and none of them contained the amino acid couple that would be proven to change spectral sensitivity of the opsin. Furthermore, amino acid sequences of RH1d-1 and RH1d-2 were entirely identical – none of the substitutions on DNA level resulted in the change of amino acid which speaks for strong purifying selection to keep both copies functional. However, their nucleotide sequences differed only at nine sites which also brings the possibility of gene conversion in the past. RH1b-1 and RH1b-2 differed in 11 positions, yet none of them was a tuning site. These results indicate that RH1 opsins are extremely conserved, especially RH1d1&2 that account for ~99% of rod opsin expression.

Another remarkably conserved opsin class was LWS. Out of five amino acid changes, none had the potential to differentiate the spectral sensitivity of LWS1 and LWS2. LWS1&2 are both expressed at relatively high levels throughout the whole development, not unlike RH1d. Hence proposed explanations for the low incidence of substitutions at nonsynonymous sites might apply to both opsin classes. It was demonstrated that mutation rates in highly expressed genes should be about the same or higher than in the ones with lower expression levels (Kumar and Subramanian, 2002; Park, Qian and Zhang, 2012). However, the intensity of selection is not necessarily positively correlated with the level of expression. Duret, Mouchiroud and Bernard (2000) indicated that certain expression patterns can lead to stronger selection and elevated substitution rates at nonsynonymous sites – proteins that were expressed specifically in particular tissues had three times higher substitution rates at nonsynonymous sites than ubiquitous proteins. Any mutation in ubiquitous protein can negatively influence its function and imposes the risk of potential detrimental effect on various tissues that would indeed reduce the fitness of an organism. It is hence more advantageous to reduce the mutation rate in such proteins. On the contrary, mutations in tissue-specific proteins have generally lower potential to cause a substantial harm to the whole organism and they might bring some useful adaptations. I hypothesize that the same basic principle could apply not only to proteins that differ in localization of their expression, but also to proteins that are expressed differentially in time. If certain opsin (e.g., LWS1&2, RH1) is present at all

life stages, it can be considered ubiquitous in the terms of time. On the other hand, opsins specific for particular life stage (e.g., larva or adult) can be perceived as equivalents of tissue-specific proteins. While amino acid substitutions in life stage-specific opsins might make them match the visual requirements of the life stage at which they are expressed, similar processes in ever-present opsins do not bring such advantages, only the risk of functional deterioration that would affect all life stages. From this point of view, the low incidence of amino acid substitutions at the key tuning sites in LWS and RH1 genes that I observed does not seem unexpected at all. Moreover, RH1 is the only opsin class expressed in rods and so the only opsin class used in dim light. Since the spectrum of available light shifts towards red in mildly turbid water, LWS opsins are the most important cone opsins during daytime in the freshwater environment. The indispensability of both opsin classes should lead to stronger negative selection against substitutions in non-synonymous sites.

In the SWS1 and SWS2 opsin classes, I found several amino acid substitutions at the key tuning sites, including those that were earlier proven to shift spectral sensitivity of these opsins. It is quite convincing evidence that spectral sensitivity of sister opsin genes within these classes (i.e., SWS1-1&2 and SWS2-1&2) differentiated since the allopolyploidization event (11–35 Mya). Even though it is hard to estimate how much the spectral sensitivities of these opsins diverged, it is interesting that substitutions with direct impact on function accumulated in such a short evolutionary time. For comparison, difference between λ_{max} of the two SWS2 genes that arose in the percomorph-specific SWS2 duplication that occurred 110–130 Mya (SWS2A α and SWS2A β) is 9 nm in *Pseudochromis fuscus* (Cortesi *et al.*, 2015), while the amount of amino acid substitutions is comparable to what I found between SWS2-1 and SWS2-2 in the common barbel. Considering that barbel's opsins experienced several times shorter evolution, it seems that substitution rate in the common barbel is remarkably high. On the other hand, RH2 opsins that duplicated in Nile tilapia approximately 10 Mya have already differentiated their λ_{max} by 11 nm, and thus the rate of their evolution is comparable to common barbel's SWS1 and SWS2 (Spady *et al.*, 2006).

RH2 opsin class consists of four genes with distinct expression patterns. RH2-1 is expressed at all stages, RH2-2 is expressed in adult, RH2-3 is expressed in larva, and RH2-4 is not expressed at any stage. Since RH2-1/2 and RH2-3/4 originated in duplication event that preceded allopolyploidization, it is not surprising that most amino acid substitutions were found between these two couples, including one substitution at the key tuning site that is known to shift λ_{max} in RH2. I thus presume that λ_{max} of RH2-1/2 and RH2-3/4 diverged, even though I cannot estimate the extend of this divergence. Moreover, comparison of substitution rates within the RH2-1/2 and RH2-3/4 couples brought another intriguing revelation – I found 31 nucleotide substitutions between RH2-1 and RH2-2 that resulted only in six amino acid substitutions with none of them being proved to change spectral sensitivity. In contrary, RH2-3 and RH2-4 differed in 54 nucleotide sites which led to 25 amino acid substitutions, including the substitution at the key tuning site that was proved to shift λ_{max} in RH2. In other words, RH2-1/2 is comparable to LWS in substitution rate, whereas RH2-3/4 to SWS1 and SWS2. It is possible that the

explanation of the low substitution rate in LWS could apply to RH2-1 too because it is also expressed throughout the whole life, and hence it should be considered ubiquitous. Notably enough, the high number of amino acid substitutions between RH2-3 and RH2-4 seems to be caused by increased substitution rate in RH2-4 alone, making it the most distinct of all four RH2 genes. Since this gene was not utilized at any life stage, we may be witnessing the onset of the pseudogenization process in RH2-4 – the gene accumulates mutations at nonsynonymous sites that will eventually disable its potential function or introduce the presence of a stop codon.

Opsin expression throughout development

The detailed study of opsin expression during development uncovered the unique utilization of duplicated genes – in three opsin classes (SWS1, SWS2, and RH2), I found the larval-specific and adult-specific gene (see Figures 10 and 11). The existence of larval-specific and adult-specific genes itself is nothing unforeseen – such division of function after opsin duplication is in fact very prevalent in fishes and it was reported for various gene classes (e.g., SWS2 and RH2 in *Acanthopagrus butcheri*, SWS2 in *Pseudochromis fuscus*, or LWS in *Lucania goodei*) (Shand *et al.*, 2008; Cortesi *et al.*, 2015; Chang *et al.*, 2021). However, previous studies focused on genes descending from local duplications and none explored the expression patterns of duplicated opsins that emerged in the same polyploidization event. Furthermore, because the whole genome duplication in the common barbel was accompanied by hybridization, opsin genes were presumably slightly distinct since the very beginning, unlike the genes originating in local duplications. This initial variation could make it easier for opsin genes to sub-functionalize and divide functions. Indeed, I found the effect that has never been reported before. Not only that larval-specific and adult-specific genes were present in three classes (which is more than in any other fish studied before), but also the gradual exchange of the two copies was noticeably coordinated. All larval-specific opsins (SWS1-2, SWS2-2, and RH2-3) dominated in expression over adult-specific opsins (SWS1-1, SWS2-1, and RH2-2) in the first studied life stage, 8 dpf (days post-fertilization). At 14 dpf, the expression of larval-specific and adult-specific opsins nearly equalled. Then the adult-specific genes took over the expression and the larval genes became practically silenced already 21 dpf. Since this stage, the opsin expression pattern resembled the adult one and did not undergo further substantial changes. I aligned the expression profiles with the most important events in the development of the common barbel to find an explanation for the timing of the switch between larval and adult opsins (see Table 7). Any comparisons with literature were quite challenging because the rate of growth and development is greatly influenced by rearing conditions and their relationship is not linear (the specimens of the same size might be at different developmental stages and vice versa). To solve these problems, I took account of fish age, size, and morphology while applying findings of other researchers on my developmental series.

Nowosad *et al.* (2021) report that retinal pigment layer in the eye appears 2 days post-hatching and that larvae (corresponding the 8 dpf stage in my study) performed negative phototaxis. Curiously enough, I discovered significant expression of all opsin classes at this stage suggesting that there is a potential for much more elaborated vision. We might not see the direct impact of the visual abilities on the behaviour due to limited mobility of the youngest larvae. The interchange of larval and adult opsins occurs 14 dpf when fish is already able to feed from external sources and swim freely in the water column. Nowosad *et al.* (2021) describe the retina as “fully differentiated” at this stage, but they also note that photoreceptor layer is still “poorly developed.” I find this claim very peculiar – first, I found regular opsin expression at this stage and since opsins are expressed in photoreceptors, I infer those photoreceptors must be already well developed in the retina. Second, as authors also reported themselves, fish at this stage are actively swimming in the water column and feeding exogenously, so it is hard to imagine that their vision should be anyhow disabled. Third, the photo of the retina presented by authors themselves shows nicely developed photoreceptor layer that should not have any major deficit in function. Still, I could not find any cause for synchronized switch between larval and adult opsins at this particular life stage – the larva has just left the bottom and initiated the short life period in the water column. It is going to live and feed in the water column for two more weeks before it returns to the bottom and initiates the “adult way of life,” and yet its opsin expression pattern already turns into the adult one. Opsin expression patterns of the two following stages (21 dpf and 28 dpf) closely resemble expression pattern of adult specimens, even though those larvae still inhabit the water column. As much as these results seem irrational, they are in compliance with several studies conducted on different fish species. For example, changes in opsin expression precede habitat transitions and metamorphosis in the

Table 7: Most important developmental events, changes in morphology, behaviour, and habitat across all life stages of the common barbel included in this study. The seventh column (Opsin expression) shows which copies of the genes that interchange (larval and adult) prevails at each life stage. Photographs were added to demonstrate the overall morphology of the specimens, they are not in the same measuring scale (see Figure 5 for photographs with scalebars). The information in the table are based on Bischoff and Freyhof (1999) Vilizzi and Copp (2013), Nowosad *et al.* (2021), and observation in breeding facility in FROV Vodňany where larvae for this study were sampled.

DPF	DPH	TL [mm]	Life stage	Developmental events	Habitat	Opsin expression	Photograph
8	4	10	larva	retinal pigment layer (since 2 DPH), negative phototaxis	interstitial, starts to emerge, aggregations	larval >> adult	
14	10	13	larva	fully developed retina, exogenous feeding, swimbladder filled	water column	larval = adult	
21	17	15	larva	yolk sack already absorbed	water column	adult >> larval	
28	24	18	larva	photoreceptor layer fully developed	slowly returning to the bottom	adult >> larval	
42	38	25	juvenile	barbs, adult body shape and pigmentation	bottom	adult >> larval	
years	years	260	adult	reproduction since 2–3 years in males, 4–5 years in females	bottom	adult	

fangtooth (*Anoplogaster cornuta*), the dusky dottyback (*Pseudochromis fuscus*), or in the European eel (*Anguilla anguilla*), albeit in the eel they occur much later in life, before their final spawning migration to the sea (Bowmaker *et al.*, 2008; Cortesi *et al.*, 2016; Lupše *et al.*, 2021). It is very hard to explain this phenomenon - Cortesi *et al.* (2016) hypothesize that fish might need to adjust their visual apparatus in advance so it could enable them invade new environments. However, the fact that the common barbel continues in the larval way of life with opsin expression perfectly adjusted to adult environment for almost two weeks makes this explanation seem insufficient, at least for this species.

Seeing the tight coordination of larval and adult opsin interchange across the three distinct opsin classes, I examined some possible straightforward explanations of this phenomenon. First, I inspected whether there could be genetic linkage between the three genes. I realised that they are located on three pairs of separate chromosomes: SWS1 genes on chromosomes 19a&b, SWS2 genes on chromosomes 21a&b, and RH2 genes on chromosomes 10a&b (see Figure 6). The possibility of genetic linkage was thus excluded. Second, I tested whether expression pattern of the genes could be explained by their origin (i.e., whether all larval genes come from one progenitor species and all adult genes from the other progenitor species). Surprisingly, I found three different arrangements while comparing SWS1, SWS2, and RH2 opsin classes: in SWS1, larval gene (SWS1-2) was located on chromosome A and adult gene (SWS1-1) on chromosome B. SWS2 had the inversed arrangement with larval gene (SWS2-2) on chromosome A and adult gene (SWS2-1) on chromosome B. Larval and adult gene in RH2 class did not originate in the recent whole genome duplication, but in the individual RH2 duplication that had preceded this event, and were therefore located on the same chromosome, chromosome A. The origin of the genes from one or the other parental species hence did not provide any explanation for strikingly similar expression patterns. I conclude that regulatory mechanisms of the whole process are more complex and cannot be explained from my data. However, it would be certainly interesting to search for them in the future. Even if the opsin gene copies were at first designated by chance to be expressed either in larva or in adult only to re-establish balance in opsin expression after polyploidization, comprehending the regulation of the whole phenomenon would help us to understand events after polyploidization in general.

Despite many studies reporting changes in the ratio of expression while comparing not individual opsins, but the whole classes, I have not found any pronounced change in the expression of opsin classes in the common barbel (see Figure 12). LWS was the most expressed class since the first larval stage (8 dpf). In fact, the first larval stage had the highest ratio of LWS to other cone opsin classes, which is quite uncommon – most studies conducted on various fish species described larval retina as “more short wavelength-sensitive” than adult retina. The expression of SWS1 and SWS2 opsin classes usually gradually decreases while expression of LWS gradually increases throughout development, which was interpreted as adaptive change linked to the transition to deeper water habitats (Spady *et al.*, 2006; Shand *et al.*, 2008; Matsumoto and Ishibashi, 2016; Härer, Torres-Dowdall and Meyer, 2017; Chang *et al.*,

2020). While the common barbel indeed undergoes the transition to deeper habitat – specifically, it leaves the water column and becomes the bottom feeder – its retina does not experience any substantial shift towards longer wavelengths. This revelation slightly challenges the usual explanation for increase in LWS expression that is regularly applied to species with much less pronounced habitat changes. I found moderate decrease in expression of SWS1 opsin class accompanied by moderate increase in SWS2 expression. Similar, but more conspicuous effect has been repeatedly described in fishes and has been once again interpreted as adaptive change associated with transition to deeper water (Spady *et al.*, 2006; Matsumoto and Ishibashi, 2016; Härer, Torres-Dowdall and Meyer, 2017). This theory could indeed apply to the common barbel, however, the change in SWS1 and SWS2 expression might be too small to have a significant effect on the colour vision.

Another frequently reported pattern is that cones precede rods in differentiation and that rod-to-cone ratio should be gradually increasing throughout development (Johns, 1977; Fernald, 1988; Kunz *et al.*, 1994). For example, larvae of some perciform species have pure-cone retinas and rods do not emerge until metamorphosis (i.e., more than 15 days post-hatching) (Shand, Archer and Collin, 1999; Shand *et al.*, 2001). The pattern I discovered in the common barbel was different. At all stages, rod opsins (RH1) constituted at least 60% of opsin expression. Interestingly, more than 80% of opsins in 8 dpf larvae were rod opsins and rod-to-cone ratio was initially decreasing, not increasing as expected (Figure 12, right). The lowest rod opsin expression (~ 63%) was in 21 dpf larvae. Then the proportion of rod opsins started to increase again, reaching almost 90% in adult fish. Overall, the rod-mediated vision seems to be essential for the barbel at all life stages that I have studied. Even though I cannot exclude the existence of initial pure-cone retina at some earlier stage, I observed predominant rod opsin expression at 8 dpf indicating that rods must have appeared very soon after cones in the common barbel, if not at the same time or earlier. In my future work, I plan to analyse retinal transcriptomes of even younger larvae to determine the onset of rod and cone opsin expression. The changes in rod-to-cone ratio are again being explained by transition to deeper water and change in the diet (Shand, Archer and Collin, 1999). Curiously, this explanation can be very well applied to the pattern that I discovered in the common barbel, although the pattern itself differs from what was observed in other fishes. At the first stage, 8 dpf, larvae live interstitially at the bottom, feed from the yolk sack and their RH1 expression is considerably high. As they emerge to the water column and feed exogenously at 14 dpf, rod opsin proportion decreases. The peak of cone opsin expression is at 21 dpf, in the middle of their water column period. At 28 dpf, rod-to-cone ratio starts to increase again as the fish slowly leave the water column and return to the bottom.

For future research, it would be very interesting to analyse developmental series of opsin expression in other species from European tetraploid barbel clade and in the common carp that underwent separate whole-genome duplication at the similar time. Comparisons among those species together with the

common barbel could indeed bring fascinating insights into turbulent events succeeding allopolyploidization.

Retinal structure and cone mosaic

I found SWS1 and SWS2 expression in distinct types of single cones, while RH2 and LWS were expressed in separate halves of the double cones, as shown in Figure 13. The common barbel thus fits in the expression scheme that was previously described for many other teleost species, with short-wavelength sensitive opsins expressed in single cones and long-wavelength sensitive opsins expressed in double cones (Bowmaker *et al.*, 1994; Cheng and Flamarique, 2007a; Pignatelli *et al.*, 2010; Salbreux *et al.*, 2012; Stieb *et al.*, 2019).

I observed a regular pattern in cone distribution in the common barbel – a square mosaic. My findings are in contradiction with the study of Compere and Poncin (1996) who reported an irregular distribution of photoreceptors. I assume that the mosaic was overlooked before because barbel's retina is very delicate and photoreceptors are hardly visible in the bright field – I was only able to reveal the regular pattern by fluorescence in situ hybridization which has not been performed on this species before. Still, the double cones were considerably misplaced in my sample, which I believe happened during manipulation with the retina. The existence of cone mosaic in the common barbel is not utterly surprising – the square mosaic with some irregularities should be typical for cyprinids (Marc and Sperling, 1976; Bowmaker and Kunz, 1987). It is thought worth mentioning that some cyprinid species, including the zebrafish, have the paradigmatic row mosaic (Zaunreiter, Junger and Kotrschal, 1991; Allison *et al.*, 2010). Nevertheless, since existence of any mosaic enhances resolution, my finding indicates that the common barbel has most likely better vision than has been thought before.

Localization of expression of sister opsins in adult retina

In the adult retina, I found parallel expression of both genes that arose in the recent whole genome duplication in two cone opsin classes – RH2 and LWS. The fact that these four genes – RH2-1, RH2-2, LWS1, and LWS2 – were the most expressed cone opsins in the adult retina indicates the vital importance of red, yellow, and green light for colour vision in the barbel. Even though I have found only few amino acid substitutions between these sister genes and none in the known key tuning sites, there still is a chance that their function, i.e., their absorption spectra, might have diverged. To further explore the potential functions of these sister genes, I localized their expression by FISH on the retinal whole mount (see Figure 14). RH2 and LWS are expressed in the opposite halves of double cones and all double cones that I observed coexpressed both sister genes (i.e., RH2-1 and RH2-2 were always expressed together in one half of double cone while LWS1 and LWS2 were coexpressed in other half). Unfortunately, I cannot fully exclude the eventuality of non-specific probe binding that would lead to coexpression-like results even in the case of separate expression of opsin genes. However, our laboratory experience proved that probes with a similar or even lower number of substitutions than the probes used

in this study bind specifically in other species, which makes the coexpression pattern demonstrated here much more convincing. The possibility of the sister opsins developing to provide new separate colour channels was thus excluded (only if the sister genes were expressed in separate cells and their $\lambda_{\max}$ diverged significantly, they could enhance colour discrimination). On the other hand, coexpression of different opsins within the same cell may also have its benefits. The coexpression of opsins with markedly distinct $\lambda_{\max}$ broadens the spectral sensitivity of the photoreceptor while coexpression of similar opsins might be used for spectral tuning of the photoreceptor (Dalton *et al.*, 2014; Carleton *et al.*, 2020). Since spectral sensitivity of the examined sister opsins is very similar, if not the same, the spectral tuning remains the only hypothetical benefit. The spectral tuning of photoreceptors via opsin coexpression in fishes was reported before – the cichlid species *Metriacroma zebra* coexpresses RH2B with RH2A β , RH2A α with LWS, and SWS1 with SWS2B in peripheral parts of the retina to adjust the photoreceptor spectral sensitivity to the background. That way it can easier recognize dark silhouettes, for instance approaching predators (Dalton *et al.*, 2014, 2017). The anemonefish *Amphiprion akindynos* coexpresses SWS1 and SWS2B in the retinal region with the highest cell density to make the spectral sensitivity of photoreceptors match the colour of conspecifics (Stieb *et al.*, 2019). However, the spectral divergence of these opsin couples is still bigger than what we could expect in the genes coexpressed in the common barbel. Furthermore, the expression pattern differs too: while only certain fraction of photoreceptors coexpressed both genes in *Metriacroma zebra* and *Amphiprion akindynos*, I did not observe any double cone expressing only one gene in the common barbel. The spectral tuning achieved by coexpression of the sister genes in the common barbel is thus very implausible and I consider the observed expression pattern not yet adaptive.

Conclusions

In this thesis, I studied vision in the evolutionary tetraploid species – the common barbel (*Barbus barbus*), with a special focus on visual pigments – opsins. I revealed that the recent allopolyploidization gave rise to 14 opsin genes in the common barbel genome and confirmed that this event occurred in the ancestor of the whole European barbel clade. Sequences of all 14 opsin genes were preserved functional in the genome, and 13 genes were also found in the transcriptome. I examined their sequences and studied their expression patterns in time and space to infer some hypotheses about the impact of such a rich opsin gene repertoire on visual abilities.

I demonstrated that differentiation of protein sequences has already led to differentiation of spectral sensitivities of opsins within some opsin classes. I also showed that the substitution rate varies significantly among opsin classes. The examination of variation in opsin expression throughout development uncovered temporal division of function of individual opsin genes within three cone opsin classes. These opsin classes comprised the larval gene gradually replaced by the adult gene as the fish developed. Furthermore, the switch between larval and adult opsin genes was synchronized across all three opsin classes – such coordinated effect has never been described in any other species. Curiously, this study did not confirm some trends frequently reported in other teleosts for the common barbel – e.g., constant increase in rod-to-cone ratio or shift in expression towards long-wavelength sensitive opsins throughout development.

Opsin-specific fluorescent in situ hybridization labelling revealed a square cone mosaic pattern in photoreceptor distribution in the adult retina of the common barbel. Shorter-wavelength sensitive opsins (SWS1 and SWS2) were expressed in distinct single cones and longer-wavelength sensitive opsins (RH2 and LWS) in separate halves of double cones, as usual among teleosts. The existence of a regular pattern indicates that the visual abilities of the common barbel might be better than thought before. Adult specimens expressed both RH2 (green-sensitive) and LWS (red-sensitive) genes that emerged in the recent allopolyploidization simultaneously at very high levels. However, all observed double cones coexpressed both sister genes (RH2 genes in one half and LWS genes in the other) and protein sequences of the genes differed only in positions that should not influence their spectral sensitivities. The localization of opsin expression that I observed thus does not enhance vision in the common barbel.

The study shows that some opsins from the recent whole genome duplication have already acquired differential expression patterns and separated their absorption maxima. Such results suggest that we are witnessing the ongoing diploidization process in the common barbel and its status of “evolutionary tetraploid” is thus questionable.

This study not only brought new insights into the function of the visual sensory system in the common barbel but also revealed a phenomenon that has not been described before in any species. My findings

thus demonstrate the importance of the study of non-model organisms. Furthermore, this study leaves some unresolved questions and proposes some directions for future research that would help us to comprehend evolutionary forces and regulatory mechanisms shaping the opsin repertoire in this beautiful yet enigmatic species.

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Attachments

Poster Presented at the International Congress of Neuroethology (ICN) 2022, Lisbon, Portugal



How does the common barbel (*Barbus barbus*) see?

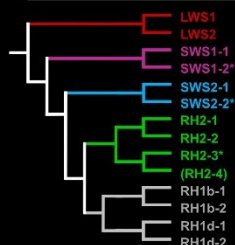
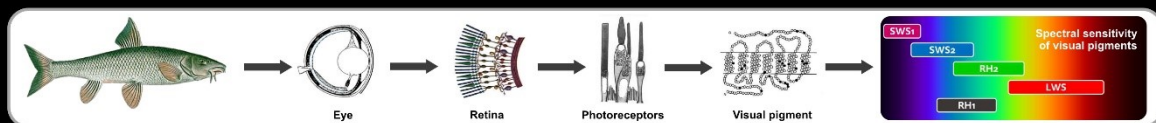
The effect of the whole-genome duplication on vision

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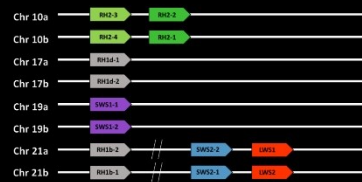
Teleosts outstand among vertebrates in the number of visual pigments and photoreceptor types. Such extraordinary repertoire was aided by the whole-genome duplication that occurred in their ancestor, approximately 350 Mya. Here we explore teleost fish species that has recently experienced subsequent whole-genome duplication – the common barbel (*Barbus barbus*). Does tetraploidy influence vision?



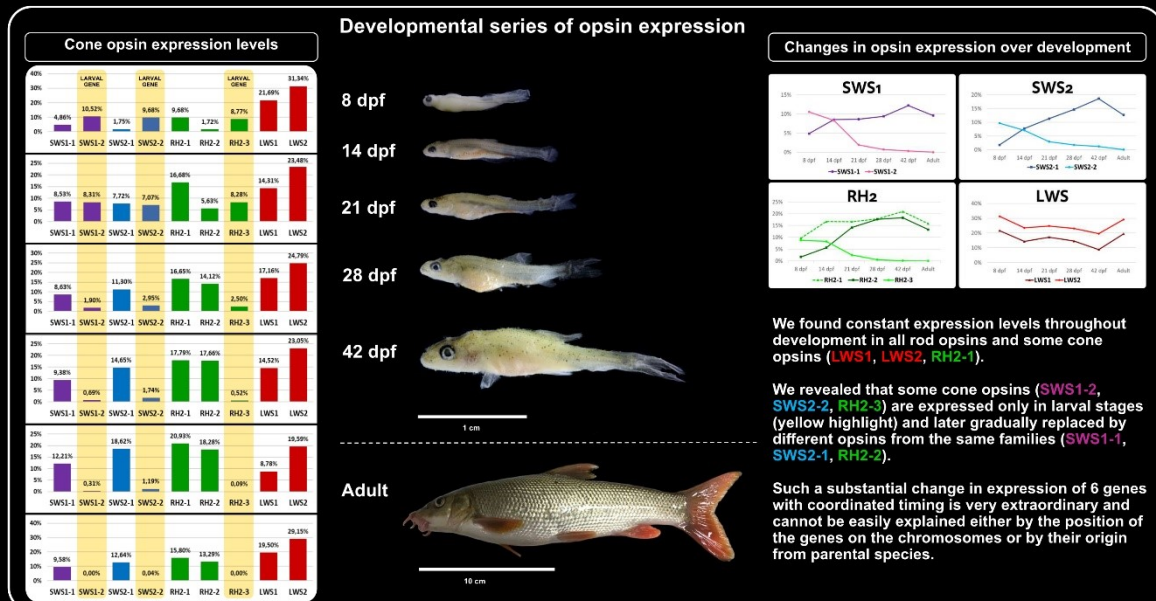
The whole-genome duplication resulted in 14 opsin genes.

Opsin phylogeny in the common barbel.

Colours correspond to the spectral sensitivity of opsins present in cones, RH1 rhodopsins in grey are expressed in rods. Note the opsin pairs that originated in the most recent whole-genome duplication (11–16 Mya) – **sister genes**. RH2 and RH1 opsin genes underwent separate duplications before this event. Asterisk * marks genes that are only expressed in larvae (see also the plots below). We did not find expression of RH2-4 gene at any developmental stage.



Position of opsin genes on the chromosomes. The common barbel is allopolyploid and chromosomes from parental species can still be distinguished.

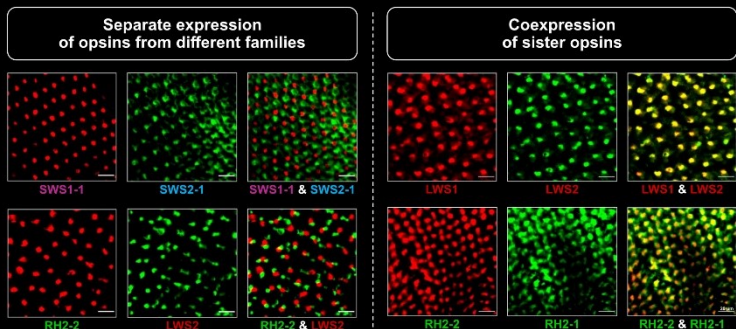
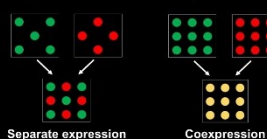


Visualization of opsin expression via Fluorescent In Situ Hybridization

We hybridized adult retinas with specific probes to localize opsin expression in photoreceptors.

SWS1 and **SWS2** are expressed in distinct single cones, while **RH2** and **LWS** in opposite halves of double cones.

We found coexpression of both **LWS** and **RH2** sister genes expressed simultaneously in adult retina.



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