CHARLES UNIVERSITY
FACULTY OF SCIENCE

Study programme: Zoology

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TRANSCRIPTOMICS AND DEVELOPMENTAL PLASTICITY OF SENSORY SYSTEMS IN FISHES

TRANSKRIPTOMIKA A VÝVOJOVÁ PLASTICITA SMYSLOVÝCH SYSTÉMŮ U RYB

Doctoral thesis

Supervisor: Zuzana Musilová, Ph.D

Prague, 2022

DECLARATION OF ORIGINALITY / PROHLÁŠENÍ O ORIGINALITĚ

I declare that this thesis has not been submitted for the purpose of obtaining the same or another academic degree earlier or at another institution. My involvement in the research presented in this thesis is expressed through the authorship order of the included publications and manuscripts and explained in detail in the "Outline of publications" section of the dissertation. All literature sources I used when writing this thesis have been properly cited.

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STATEMENTS OF CONTRIBUTION / PROHLÁŠENÍ O PŘÍSPĚVKU

I declare that my research for this dissertation was conducted in collaboration with colleagues and scientists from several countries. My personal contribution to the experimental design, data collection, analyses, and preparation of manuscripts for each chapter is explained in detail below.

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As a supervisor of the PhD thesis and to the best of my knowledge, I confirm that the contribution of Nik Lupše, MSc, to the chapters and appendices of this thesis corresponds to what is explained below in detail.

Jakožto vedoucí této disertační práce a podle mého nejlepšího vědomí potvrzuji, že uvedený podíl práce kandidáta odpovídá realitě tak, jak je uveden u jednotlivých kapitol níže.

Prague, December 2022 / Praha, Prosinec 2022,

Zuzana Musilová

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PUBLICATIONS

Chapter 1

Lupše, N., Cortesi, F., Freese, M., Marohn, L., Pohlman, J.-D., Wysujack, K., Hanel, R., Musilova, Z. (2021). Visual gene expression reveals a cone to rod developmental progression in deep-sea fishes. *Molecular Biology and Evolution* 38(12):5664-5677 (doi:10.1093/molbev/msab281)

Chapter 2

Lupše, N., Kłodawska, M., Truhlářová, V., Košátko, P., Kašpar, V., Bitja Nyom, AR., Musilova, Z. (2022). Developmental changes of opsin gene expression in ray-finned fishes (Actinopterygii). *Proceedings of the Royal Society B: Biological Sciences* 289:20221855; (doi:10.1098/rspb.2022.1855)

Chapter 3

Lupše, N., Indermaur, A., Bitja Nyom, AR., Musilova, Z. Plasticity of the visual system in the cichlids from Barombi Mbo and Bermin crater lakes. *Manuscript in preparation*.

PREFACE AND ACKNOWLEDGMENTS

First and foremost, I would like to thank my supervisor Dr Zuzana Musilová for her time, support, patience and guidance during my studies. This research would not have been possible without her.

I would also like to express my gratitude to Dr Fabio Cortesi from the Queensland Brain Institute (University of Queensland, Australia), whose smile and enthusiasm is contagious – surf's up! My thanks also go to other truly astonishing and global researchers I have met during this enchanting journey: Prof Karen Carleton (University of Maryland, USA), Dr Adrian Indermaur (University of Basel), Dr Ben Sandkam (Cornell University, USA) and so many more. Even the smallest chat we had meant the world.

A big thanks goes to all the fellow colleagues from the FishEvo group. Without you, this last half a decade would have certainly been less dynamic. I would especially like to thank Gina Sommer for all the conversations we had during the long hours of pipetting and all the desserts we ate while contemplating about our studies. My gratitude also goes to all the research staff and technical crew that is in more detail listed in each of the chapters. I would have certainly not come to this point without you!

A huge round of applause also goes to Richard Wood – not only did he understand what it means to live abroad, away from everything you know . . . at times, he and Greg made me laugh like there was no tomorrow.

Last but certainly not the least, I would like to express my warmest gratitude to my family. Žiga, Sabina and Ivan, without you, I would not have been the man I am today. You are bringing so much joy to my life. Although we often found ourselves looking at opposite sides of the Moon, being separated by vast oceans and landmasses, I never stopped thinking about you. Thanks for all the love, support and all the long phone calls that made me feel closer to home. A huuuuuge hug also goes to my beautiful fiancée. Natalija, in addition to being the most wonderful and accepting person I have ever met, you have also believed this moment would come. Since day one. Hvala!

ABSTRACT

Organisms depend on sensory input to survive and thrive. Vision is a key sensory system to many vertebrates, including ray-finned fishes (Actinopterygii). Sight is enabled by the retina composed of cone and rod photoreceptors, each characterised by its own set of opsin proteins that together with the chromophore form the photo-sensitive pigment. Vision is energetically very costly and so it is often adapted to specific photic conditions to best match available wavelengths of light. This Ph.D. thesis focuses on the evolution and development of opsin gene expression in ray-finned fishes. It mainly aims to explore how ontogenetic differences of visual capabilities across the fish phylogeny relate to ecological conditions. In some species, ecological shifts between developmental stages can affect their physiology, including vision. In this thesis I focused on the molecular differences of the visual system between developmental stages, mostly focusing on larvae and adults. The first chapter of the thesis focuses on developmental changes in deep-sea fishes, a unique group of organisms that has evolved unconventional adaptations to maximise photon capture in an otherwise photon-depleted environment. Most deep-sea fishes start their lives in the shallow, sun-lit, predator and food abundant epipelagic layer of the ocean. As they mature, they undergo a drastic vertical migration only to settle in the deeper layers of the meso- or bathypelagic. Through a comparative analysis of 20 species belonging to eight teleost orders, we studied the visual (opsin and phototransduction cascade) gene expression in different stages, and found how deep-sea fishes change from larval to adult vision. The second chapter of the thesis is focused on the developmental changes of the opsin gene expression across the actinopterygian phylogeny. To investigate the largest dataset to date, we studied vision in different developmental stages (embryos, larvae, juveniles and adults) of 63 species, belonging to 23 orders, and further specifically focused on species belonging to orders Polypteriformes, Acipenseriformes, Cypriniformes, Aulopiformes and Cichliformes. The third chapter of the thesis discusses an even faster mechanism by which organisms tend to adapt to rapid environmental changes – the phenotypic plasticity. We studied this phenomenon in two Cameroonian crater lake cichlids, *Coptodon flava* (Lake Bermin) and *Stomatepia pindu* (Lake Barombi Mbo), which we experimentally raised in different light regimes, discovering a strong plastic response namely in the long-wavelength (=red) part of the light spectrum. Phenotypic plasticity of the visual system might, therefore, potentially help adaptation to the changing environment.

ABSTRAKT

Organismy využívají smyslové soustavy k vnímání svého prostředí a tím ke svému přežití, kompetici i rozmnožování. Zrak klíčovým smyslem pro mnoho obratlovců včetně paprskoploutvých ryb (Actinopterygii). Světločivným orgánem zraku je sítnice, která se skládá z čípkových a tyčinkových fotoreceptorů, z nichž každý je charakterizován svou vlastní sadou opsinových proteinů, které spolu s chromoforem tvoří fotosenzitivní pigment. Vidění je energeticky velmi nákladné, a proto se zrak často rychle přizpůsobuje konkrétním světelným podmínkám, aby co nejlépe odpovídal dostupným vlnovým délkám světla. Tato dizertační práce se zaměřuje na evoluci a vývoj (=ontogenezi) exprese opsinových genů u paprskoploutvých ryb, tedy jejím hlavním cílem je prozkoumat, jak souvisí ontogenetické rozdíly zraku u různých fylogenetických skupin ryb s ekologickými podmínkami prostředí. U některých druhů je známo, že ekologické změny během ontogeneze mohou ovlivnit jejich fyziologii, včetně funkce zraku. V této práci se zaměřuji právě na molekulární podstatu zraku a jeho rozdíly mezi jednotlivými vývojovými stádii, zejména tedy mezi larvami a dospělci. První kapitola mojí práce je zaměřena na vývojové změny u hlubokomořských ryb, tedy jedinečné skupiny organismů, u které se v evoluci vyvinuly adaptace k maximalizaci zachycení fotonů v prostředí chudém na světlo. Hlubokomořské ryby začínají svůj život v mělké vodě, tedy s dostatkem světla, potravy i predátorů. Teprve jak dospívají, dochází u nich k vertikální migraci do větších hloubek, tj. do mezo- nebo bathypelagiálu. Pomocí srovnávací analýzy 20 druhů patřících do osmi řádů teleostních ryb jsme studovali genovou expresi zrakových genů (tj. opsinů a genů fototransdukční kaskády) u různých vývojových stádií a odhalili jsme, že zrak se u larev a dospělců hlubokomořoských ryb výrazně liší. Druhá kapitola mé práce se zaměřuje na ontogenetické změny exprese opsinových genů napříč paprskoploutvými rybami. K tomu jsme využili data set různých vývojových stádií (embrya, larvy, juvenilové a dospělci) 63 druhů patřících do 23 řádů ryb, a dále jsme se detailněji zaměřili na druhy patřící do řádů Polypteriformes, Acipenseriformes, Cypriniformes, Aulopiformes a Cichliformes. Třetí kapitola mé práce pojednává o fenotypové plasticitě, tedy efektivním mechanismu, kterým se organismy přizpůsobují změnám prostředí během života jedince. Tento fenomén jsme studovali u dvou kamerunských cichlid z kráterových jezer, druh *Coptodon flava* (jezero Bermin) a druh *Stomatepia pindu* (jezero Barombi Mbo), které jsme experimentálně odchovávali v různých světelných režimech. Odhalili jsme, že zraková výbava (exprese opsinů) je do určité míry plastická, zejména v dlouhovlnné (=červené) části světelného spektra, a může tak hrát roli v přizpůsobení se měnícím se podmínkám prostředí.

OUTLINE OF PUBLICATIONS

The thesis is composed of three chapters in which, overall, I investigate visual palettes of fishes, how visual gene expression develops, and which factors drive such a variation observed across different actinopterygian orders. Two of the mentioned publications have already been published in very distinguished journals, and the third is a manuscript in preparation. I am the first author in all three cases, and my contribution to each manuscript is detailed below.

Table 1: Overview of my contribution to each chapter

			Contribution					
Chapter	Original investigation	Journal abbreviation	Experimental design	Data collection	Formal analysis	Methodology	Visualisation	Manuscript writing
1	Lupše et al. 2021	*Mol Bio Evo*		x	x	x	x	x
2	Lupše et al. 2022	*Proc Roy Soc B*		x	x	x	x	x
3	Lupše et al. *(in prep)*	-	x	x	x	x	x	x

Chapter 1 investigates how a major ecological transition from epipelagic larvae to mesopelagic adults affects the development of the visual system of deep-sea fishes. Although morphological investigations into the adult deep-sea fish retina often found pure rod retinae (Ali & Anctil 1976), modern molecular studies (e.g., Musilova et al. 2019) revealed deep-sea fish genomes also contain cone opsins. When they are of use, if at all, remained unanswered. To answer this, we studied opsin gene expression in larvae and adults of 20 species of deep-sea fishes belonging to eight distant teleost orders (Argentiniformes, Aulopiformes, Beryciformes, Myctophiformes, Pempheriformes, Scombriformes, Stomiiformes, and Trachichthyiformes). A comparative transcriptomic analysis revealed that the larval retina predominantly expresses cone opsins (*RH2* in most cases), while adults predominantly or only express the rod opsin (*RH1*) which aids vision in dim-light conditions in which they live. This cone-to-rod progression seems to hold true for vertebrates in general (e.g., La Vail et al. 1991, Mears et al. 2001). A genome inspection revealed expanded opsin repertoires of some species (up to seven *RH2* copies) and convergent losses of other opsin classes (e.g., *LWS*). Furthermore, a transcriptomic analysis of phototransduction cascade genes, such as transducins and arrestins, revealed a similar ontogenetic trajectory to that of opsins; a molecular mismatch discovered in some aulopiform species, on the other hand, potentially indicates the presence of transmuted

photoreceptors previously thought to be found only in some squamates (Simoes et al. 2016; Schott et al. 2019), but also in a deep-sea fish (de Busserolles et al. 2017).

Chapter 2 expands on the idea that many fish species undergo age-related ecological transitions, hypothesising further that this in turn guides the development of sensory systems, including vision (Carleton et al. 2020). To elaborate on this, we analysed a comprehensive dataset of de-novo built and publicly available transcriptomes (N=215) of embryos, larvae, juveniles and adults belonging to 63 species and 23 actinopterygian orders. Analysing genes involved in the phototransduction cascade, and focusing on opsin genes specifically, we show in this chapter that general patterns of opsin development do exist: specifically, *LWS* becomes increasingly important for fishes as they mature, while *SWS1* seems to bear lesser importance for visual performance and remains expressed at functionally important levels only in rare occasions. We also present detailed analyses of expanded opsin repertoires in 14 selected species from the orders Polypteriformes, Acipenseriformes, Cypriniformes, Aulopiformes and Cichliformes, and present taxon-related stage-specific gene copies. Finally, our study provides further evidence for the cones-first rods-later hypothesis (e.g., La Vail et al. 1991, Mears et al. 2001, Lupše et al. 2021), as expression levels of the rod opsin increase with age in basically all actinopterygian species examined herein.

Chapter 3 focuses on yet another mechanism by which fish vision can adapt to novel photic conditions on a short time scale. Through the rapid emergence of novel phenotypes, phenotypic plasticity is believed to mediate evolution (reviewed in Pfennig et al. 2010), and an increasing amount of evidence suggests it provided a basis for the explosive adaptive radiation of one of the most famous model groups for evolutionary studies, the cichlids (Cichlidae) (Schneider & Meyer 2017). To explore how plastic opsin gene expression really is, we designed an experiment consisting of four tanks, each with its own light regime (full spectrum, short-wavelength, medium-wavelength, long-wavelength) in which two species of Cameroonian crater lake cichlids, *Coptodon flava* and *Stomatepia pindu*, were reared from larvae till adulthood. Individuals were sampled at week 1, week 2, week 4 and month 6 for opsin gene expression to be quantified. Our experimental design revealed all artificial light regimes instigated a plastic response, with long-wavelength (red) shifted ambient light triggering the strongest. Although responses differed between species that belong to two separate radiations, it seems that cichlids in general are fast to adapt to rapid spectral changes

(e.g., Dalton et al. 2015), suggesting vision might indeed play a role in this family's prolific diversification.

INTRODUCTION

An organism's survival depends on its perception of the world around it. Vision, which is essential for many organisms, underlies predator avoidance, prey detection, recognition of conspecifics and potential mates, adjustment of circadian rhythm, colour-based aposematism and much more (Land & Nilsson 2012, Cronin 2014). Animals have evolved structurally and molecularly diverse visual systems that range from simple eye spots (ocelli) of planarian flatworms to extremely complex eyes of insects and crustaceans (compound), cephalopods and vertebrates (both camera style) (Land & Nilsson 2012). Amongst the latter, teleosts (bony fishes), which are highly speciose and occupy a vast array of niches within the spectrally heterogeneous aquatic environment, have evolved some of the most prolific visual systems on Earth (Carleton et al. 2020, Fricke et al. 2022).

Ecology and the light environment

Teleosts are found in a variety of aquatic habitats, ranging from freshwater (e.g., streams, rivers, ponds, lakes) to marine ecosystems (e.g., coral reefs, open ocean or the deep-sea) (Nelson 1994). Accordingly, they differ in feeding preferences (e.g., planktivory, carnivory, herbivory), activity patterns (e.g., diurnal, nocturnal, crepuscular), as well as the depth at which they live in case of marine fishes (e.g., epipelagic, mesopelagic, bathypelagic). Spectral characteristics of these environments are governed by physical properties of light and water, with absorption and scattering effect of the water column constituents causing the intensity of light to decrease with depth, narrowing the spectrum to mostly blue-light (Jerlov 1976, Land 2003). In the case of oceans, the intensity of sunlight passing through them declines about 400 times within the first 100 m – epipelagic zone (Warrant & Locket 2004; Jerlov 1976), before reaching the intensity of starlight at around 700 m – mesopelagic zone - (Clarke & Denton 1962; O'Carroll & Warrant 2017) until finally, no photons reach depths greater than 1000 m (bathypelagic zone). Below this margin, the only sources of light are bioluminescent flashes produced by deep-sea organisms, which are often also found in the blue-green centre of the spectrum (Denton 1990, de Busserolles 2020). In clear shallow layers, the visual environment also changes throughout the day, with reduced light intensity and persistence of mostly shorter wavelengths during twilight hours. During night-time, low levels of light are sourced from the moon and the stars (McFarland 1986). Weather, season and latitude also influence the spectral properties of an environment (Lalli & Parsons 1995). Finally, constituents of the water, such as phytoplankton, dissolved inorganic and organic matter, and

silt in the water column (Jerlov 1976) also dictate the quantity and quality of photons that attenuate along the gradient (Jerlov 1976, Munz & McFarland 1977). As a result, shorter wavelengths attenuate more rapidly in turbid or nutrient-rich water bodies and so the longer (red) wavelengths prevail, a phenomenon opposite to that seen in clear water bodies such as the open ocean. All in all, photic conditions are spatially and temporally variable, resulting in visually heterogeneous environments. Due to energetic costs of vision and natural selection, fish visual systems therefore tend to adapt to local environments (Carleton et al. 2016).

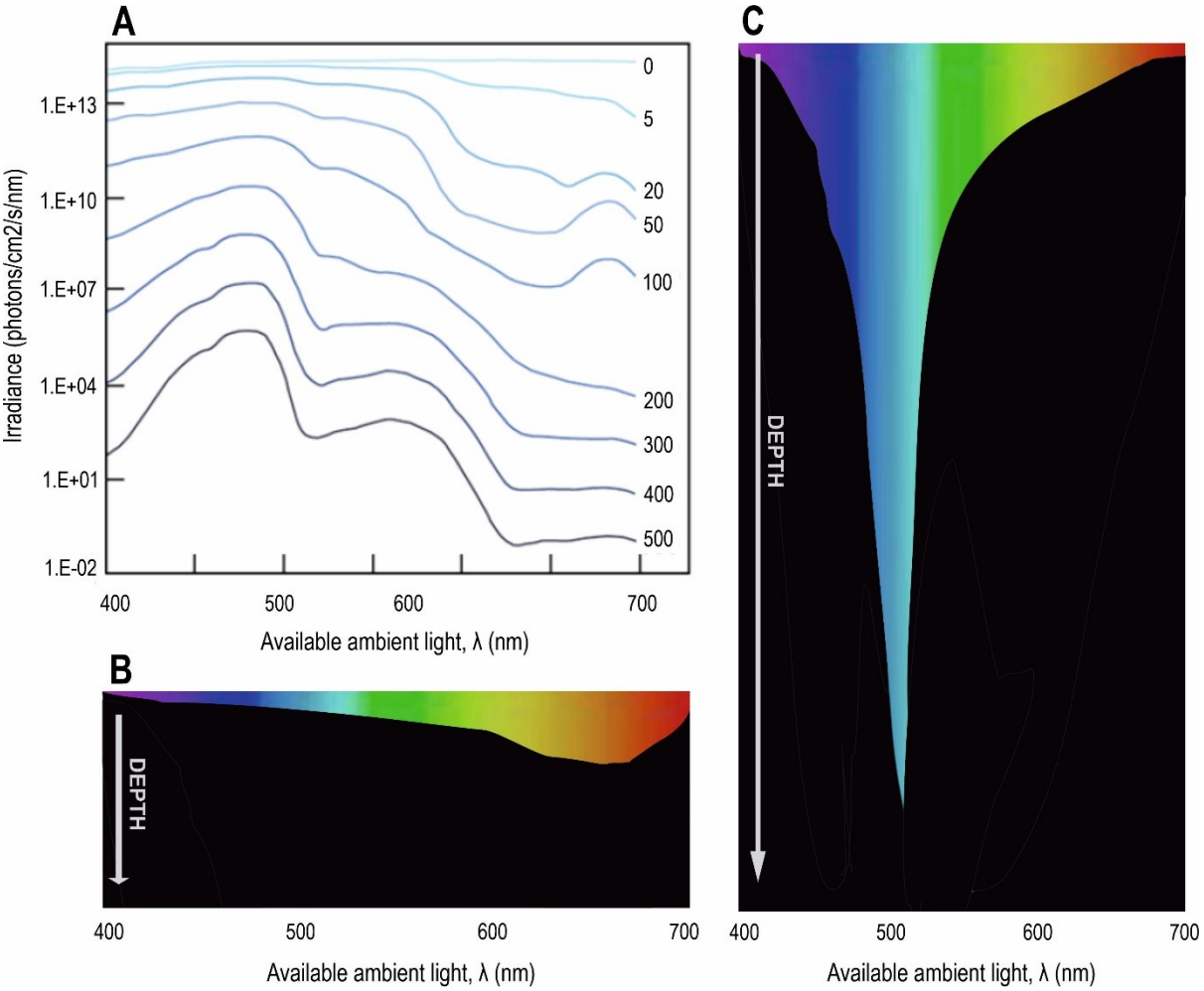

Figure 1. A. *Irradiance and spectral quality of light at different depth (marked in metres (m) on contour lines).* ***B.*** *Longer wavelengths dominate deeper layers in turbid or eutrophic water bodies (e.g., Lake Victoria)* ***C.*** *Clear oceanic waters enable greater penetration of photons, and greater depths of the mesopelagic tend to be dominated by blue part of the spectrum. Adapted and modified from Warrant and Johnsen (2013) and Musilova et al. (2021).*

Teleost vision

Anatomy of the eye

The main organ of the teleost visual system is the high-resolution image-forming camera eye, which bears strong similarity to that of other vertebrates (Lamb et al. 2007). The eye is surrounded by an opaque sclera, so light can only enter through the pupil which is framed by the iris. In case of water dwelling teleosts, the lens, which is the only part of the eye responsible for refraction and focus, is spherical and its shape can't be adjusted. Thus, focus is achieved by moving the lens horizontally back and forth, but also the naso-temporally (Fernald & Wright 1985, Ott 2006). Opposite the lens is the light-sensitive tissue, the retina. It normally consists of ganglion, amacrine, bipolar, horizontal, and photoreceptor cells (Masland 2012, Baden et al. 2020), which are located at its outside (inverted retina), as opposed to eyes of cephalopods (Land & Nilsson 2014). They relay the perceived inverted mirror image to other neurons leading to the optic nerve until finally, the signal is received and processed by the brain (Brooks et al. 1999).

Photoreceptors

The teleost retina is normally composed of two photoreceptor types, rods and cones, which are highly specialised sensory neurons in nature (for exceptions, please see section Transmuted photoreceptors). They differ morphologically, physiologically, and molecularly. The outer segment in rods, which provide scotopic vision in low light conditions and can respond to single-photon stimuli, is typically longer and thinner, whereas in cones, which are specialised for high-acuity photopic vision, it is shorter and cone-shaped (Cohen 1972, Hunt & Collin 2014). Cones diversified into many subclasses with differing spectral sensitivities (Land & Nilsson 2014). They can be subdivided into single and double cones (i.e., two joined single cones) (Pignatelli et al. 2010). In teleost fishes, single cones usually express short-wavelength-sensitive opsins, while double cones express medium- and long-wavelength-sensitive opsins (Carleton et al. 2020). When arranged in a regular pattern, they form so-called cone mosaics, typical of teleosts, but rare in other vertebrates (Dunn 1966, Fernald 1985).

Table 2: Morphological and molecular differences between photoreceptor types. Table adapted and modified from Fogg et al. 2022. Data from Lamb 2013, Hunt et al. 2014, Kawamura & Tachibanaki 2012.

	Rods	**Cones**
Type of vision	Scotopic (dim-light)	Photopic (brigh-light)
Sensitivity	Higher	Lesser
Acuity	Lesser	Higher
Shape of outer segment	Rod-shaped	Cone-shaped
Lenght of outer segment	Longer	Shorter
Width of outer segment	Thinner	Thicker
Opsin genes	*RH1*	*SWS1, SWS2, RH2, LWS*
Transduction and recovery	Slower	Faster

Phototransduction and visual pigments

The biochemical process of converting light (e.g., energy of the photons) into a electrochemical signal, which is interpreted by the brain, is called phototransduction (Hunt et al. 2014, Lamb 2020). The cascade begins with the absorption of photons through visual pigments located in the folded membrane of the outer photoreceptor segments (Land & Nilsson 2012). It triggers a conformational change of the chromophore (from cis to trans), giving way to further steps of the cascade leading to hyperpolarisation of the photoreceptor membrane (Purves et al. 2001). More specifically, visual pigments are composed of the G-protein couple receptors called opsins (ca. 350 amino acids) that are bound to a vitamin A-derived chromophore (Bowmaker 1995). The amino acid sequence of the opsin protein, the chromophore type and how the opsin is bound to the chromophore determines the wavelength of light that the visual pigment absorbs (i.e., peak spectral sensitivity or λ_{max}). Teleosts generally have four cone opsin (*SWS1*, *SWS2*, *RH2*, *LWS*) and one rod opsin class (*RH1*) (Carleton et al. 2020). Some species express only a subset of these subclasses, while some further expanded their repertoires and possess several duplicates, most commonly related to *RH2* (e.g., Musilova & Cortesi 2021). Rod opsin gene duplications are scarcer, most often restricted to deep-sea fishes (Musilova et al. 2019, Lupše et al. 2021). Colour vision per se is dependent on the excitation of at least two differently tuned opsins and photoreceptors (Krauskopf et al. 1982).

Table 3: Differences in spectral sensitivity (colour) and peak absorbance (nm) between major opsin classes: UV-sensitive *SWS1*, blue-sensitive *SWS2*, green-sensitive *RH2*, red-sensitive *LWS* and the rod opsin *RH1*. Table adapted from Fogg et al. 2022, data from Carleton et al. 2020.

Opsins	**RH1**	**SWS1**	**SWS2**	**RH2**	**LWS**
Colour	Blue-green	UV - violet	Blue – violet	Green	Red-green
Peak (nm)	447–525	347–383	397–482	452–537	501–573

Transmuted photoreceptor cells

Photoreceptor transmutation is a process by which photoreceptors undergo a shift in spectral sensitivity, which can happen molecularly and/or morphologically. Within-cell-type switch of different cone or rod opsin gene copies are presumed to be common (Wood & Partridge 1993, Cortesi et al. 2015), as well as shifts from one cone opsin class to another (e.g., from *SWS1* to *SWS2* as seen in *Oncorhynchus*; Cheng & Flamarique 2007). More dramatic and challenging to the classic rod vs. cone identity, however, is the transmutation between cones and rods. which results in an intermediate hybrid cell type that combines both rod and cone-like features. This phenomenon has been detected previously in reptiles (Simoes et al. 2016; Schott et al. 2019), some deep-dwelling teleosts (de Busserolles et al. 2017, Wagner et al. 2019, Lupše et al. 2021) and amphibians (Mariani 1986). Organisms might physiologically benefit from the use of one transmuted cell instead of two individual, less than optimal photoreceptors (Fogg 2022). It is common for the molecular machinery to not match the morphology, resulting in e.g., rod-like cells with a cone molecular machinery (de Busserolles et al. 2017, Simoes et al. 2016, Schott et al. 2019). On the other hand, some aulopiforms exhibit an opposite mismatch at the molecular level (Lupše et al. 2021): Adults of *Coccorella atlantica* and *Scopelarchus* spp. seem to combine a more stable rod opsin in a more sensitive rod-shaped cell, while keeping the cascade larval-like, i.e., cone-specific. The latter could allow for a faster physiological response of the retina (Kawamura & Tachibanaki 2012). A similar but opposite molecular mismatch (cone opsin, rod-specific cascade, rod-like cone photoreceptor) has been observed in salamanders (Mariani 1986). Transmutation between photoreceptor types might not be such a rare phenomenon, especially in the case of organisms living in extreme ecological conditions where optimisation and effectiveness of vision is that much important.

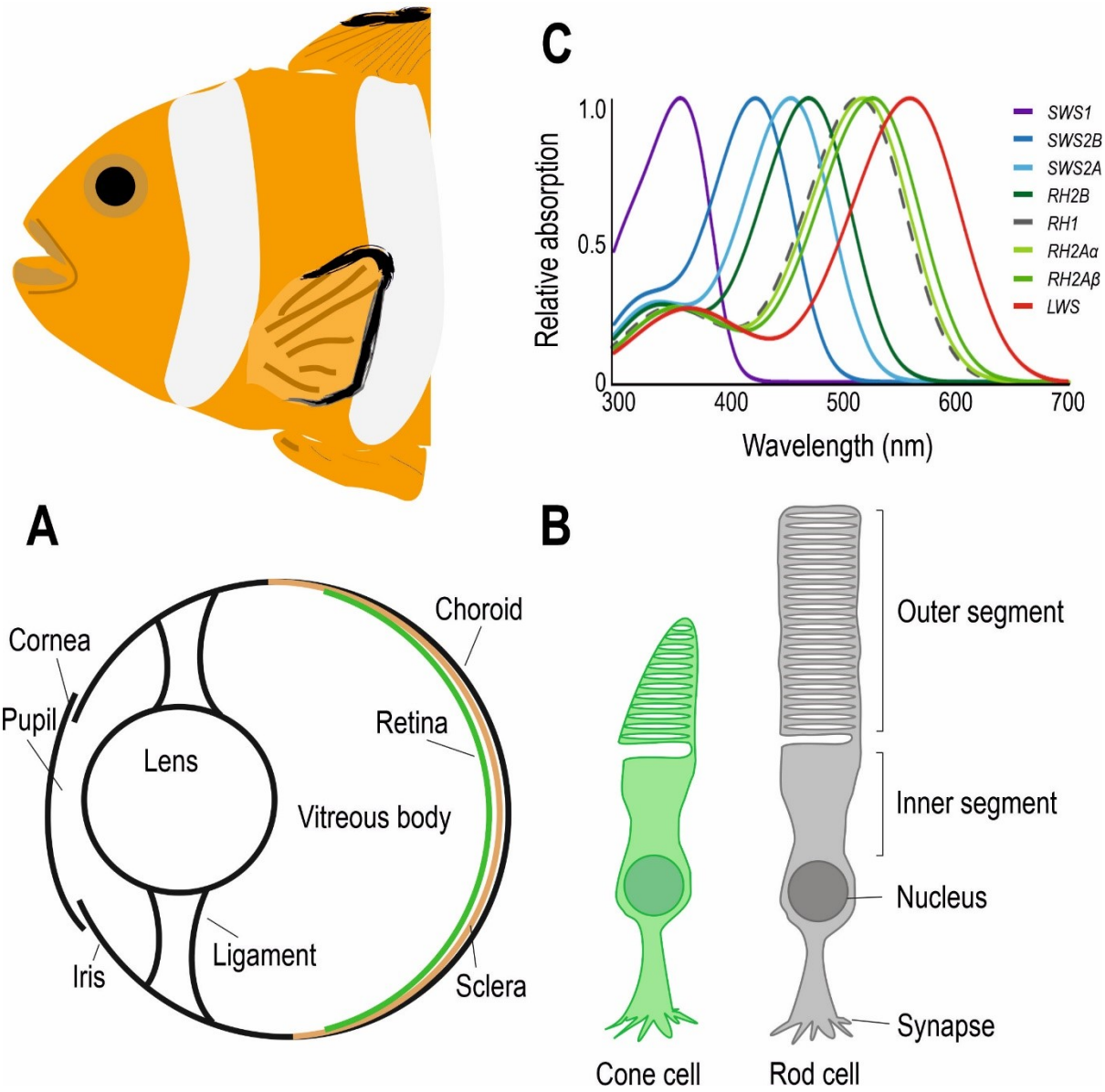

Figure 2. *The visual system of teleosts.* ***A.*** *Anatomy of the eye.* ***B.*** *Photoreceptor cells.* ***C.*** *Spectral sensitivities of eight tilapian opsins, of which seven are cone opsins SWS1(λ_{max}: 360 nm), SWS2B (425 nm), SWS2A (456 nm), RH2B (472 nm), RH2Aβ (517 nm), RH2Aα (528 nm), LWS (560 nm), and one is a rod opsin RH1 (516 nm). λ_{max} values from Spady et al. (2006). Adapted and modified from Musilova et al. 2021.*

Adaptation

Diversity in the visual system of vertebrates, including teleosts, is thought, but not always, to be the product of ecologically driven selection pressure to select for vision that best matches the lighting conditions of the local environment (Munz & McFarland 1977, Hunt et al. 2014, Carleton et al. 2016, Schweikert et al. 2018). Vision itself can vary in several traits, such as resolution, brightness sensitivity, but also spectral sensitivity. The presence of certain wavelengths of light will guide the evolution of visual pigments that are most sensitive to them,

and the brightness will dictate the number and type of cells (cone or rod) present. For example, fishes inhabiting deep-sea habitats dominated by shorter wavelengths have previously been shown to have visual systems molecularly adapted to the blue-green portion of the visual spectrum (sculpins: Hunt 1997; cichlids: Sugawara et al. 2005, Malinsky et al. 2015, Musilova et al. 2019, Ricci et al. 2022; salmonids: Eaton et al. 2020; damselfishes: Stieb et al. 2016; holocentrids: Munz & McFarland 1973, Yokoyama & Takenaka 2004; deep-sea fishes: Lupše et al. 2021) and that morphologically, retinae of fishes living in the deep-sea mostly or only exhibit (more tightly packed) rod photoreceptors with enlarged outer segments, arranged in multiple banks (de Busserolles et al. 2020). In contrast, red-shifted spectral sensitivities have been detected in teleosts living in turbid water (Weadick et al. 2012, Liu et al. 2016, Escobar-Camacho et al. 2017). Abovementioned adaptations to diverse ecological and visual demands can be achieved either through duplication or gene loss (Carleton et al. 2020), functional diversifications of opsin gene duplicates (Yokoyama 2008, Yokoyama & Yia 2020), or by regulation of the opsin gene expression, either through ontogeny (Lupše et al. 2022) or plastically within the same developmental stage (e.g., Fuller & Claricoates 2011, Dalton et al. 2015, Härer et al. 2017, Nandamuri et al. 2017, Luehrmann et al. 2018).

Gene duplication and loss

Throughout vertebrate evolution, some opsin gene classes (and copies within) were lost, while others re-emerged or got expanded via duplication and functional diversification of existing genes (Hunt et al. 1998, reviewed in Musilova et al. 2021). Extant teleost fishes expanded their visual palettes (Musilova et al. 2019), thus exceeding other vertebrates with their plethora of differentially tuned opsins that arose either via whole genome duplications (Meyer & Van de Peer 2005, Lamb 2020), tandem duplications most often observed in cone opsins (Lin et al. 2017, Musilova & Cortesi 2021) or retrotransposition, involving the *RH1* (Fujiyabu et al. 2019). Consequently, as reviewed by Musilova et al. (2021), up to three *SWS1* copies have been detected in anemonefish, Pomacentridae (Mitchell et al. 2020), four copies of *SWS2* in the humphead wrasse, Labridae (Dong et al. 2020), eight copies of *RH2* in soldierfish, Myripristinae (Musilova et al. 2019) and five copies of *LWS* in wrasses (Labridae), fighting fish (Osphronemidae), and brown trout (Salmonidae) (Dong et al. 2020, Cortesi et al. 2021). In general, double cone opsin duplicates (*RH2* and *LWS*) seem to be more common than single cone (*SWS1* and *SWS2*) duplicates (Musilova & Cortesi 2021), and finding numerous rod opsin duplicates is even rarer, most often found in lineages in need of an extremely sensitive

visual apparatus (Musilova et al. 2019, Lupše et al. 2021) – currently, the all-vertebrate record (38 copies of *RH1*) sits with the silver spinyfin, *Diretmus argenteus* (Musilova et al. 2019).

While duplications increase the total number of opsin genes, gene-loss and pseudogenization, on the other hand, deplete the basis for functional novelty. Loss of opsin genes is presumed to be ecologically driven; for example, deep-sea fishes inhabiting depth layers lacking longer-wavelengths simply lost the red-sensitive *LWS* from their genomes because they do not need it (Musilova et al. 2019, Lupše et al. 2021).

Functional diversification

Functional adaptation of visual pigments can also be achieved through point mutations located at sites deemed as key-spectral tuning sites (Yokoyama 2008, Yokoyama & Yia 2020). Mutations at these positions, often due to proximity to the retinal binding pocket, shift the peak spectral sensitivity (λ_{max}) of the photopigment, possibly aiding optimisation of vision to a specific light environment (Lupše et al. 2021, Musilova et al. 2021). Exact details on how a certain mutation affects opsin's spectral (and other, non-spectral) properties, how this effect differs between opsin classes, and how amino acid sites - whether tuning or not - interact, are often scarce (reviewed in Musilova et al. 2021)– thus far, most work has been done on *RH1*, also due to a well-described crystal structure of the bovine *RH1* (Palczewski et al. 2000, Yokoyama 2008). Although in vitro protein regenerations and phylogenetic comparisons aid our understanding of spectral shifts (Yokoyama 2008, Musilova et al. 2019, Yokoyama & Yia 2020, Lupše et al. 2021), in vivo spectral absorbance measurements using microspectrophotometry are needed, wherever possible, to determine the exact λ_{max} of a photopigments.

Developmental changes of gene expression

Organisms can actively change their ecological preferences, either plastically within the same developmental stage (for details, see section Plasticity), or during ontogeny. A much more rapid method of responding to different light conditions than selection on genetic variation is by regulating opsin gene expression (reviewed in Musilova et al. 2021). As a result, teleosts at any given time often express only a subset of opsin genes otherwise present in their genomes (Lupše et al. 2021, Musilova et al. 2021).

Reproduction in fish begins with external fertilisation of eggs. Once hatched, larvae continually grow until they develop (i.e., metamorphose) into juveniles and subsequently sexually mature adults. Development, which can be either direct or indirect, invokes significant

anatomical, physiological and behavioural changes (Evans & Browman 2004, Carleton et al. 2020). As a result, ecology also often varies between different developmental stages. For example, deep-sea fish larvae migrate from highly-illuminated upper layers of the epipelagic to photon-depleted depths of the mesopelagic (Moser and Smith 1993, Paraboles et al. 2019) or deeper zones; coral reef fish larvae migrate from open waters to more heterogeneous reef habitats where they settle as adults (Knowlton and Jackson 2001, Cortesi et al. 2016); larval eels living in the open ocean migrate into the rivers (Zhang et al. 2000, Cottrill et al. 2009); cichlid larvae shift from a planktivorous to a herbivorous diet (Ibrahim et al. 2015). As their ecological demands change, so do the challenges on the visual system. In general, teleost eyes function and adjust to local optima from the earliest stages onwards (Blaxter 1975, Fernald 1988, Wood & Partridge 1993, Cortesi et al. 2015). They adapt to specific ecological niches of each developmental stage, which results - either through regulation of opsin gene expression levels or a switch between opsin classes/copies - in ontogenetically differing vision of larvae and adults (e.g., Lupše et al. 2022). Although developmental progression of opsins expressed along a developmental axis varies from species to species (Lupše et al. 2022), generally, teleost retinal progression follows a seemingly general vertebrate cone-to-rod developmental trajectory (teleosts: Lupše et al. 2022, Sernagor et al. 2006; mice: Mears et al. 2001; rhesus monkey: La Vail et al. 1991).

Phenotypic plasticity

Not only do ecological preferences differ between developmental stages - environmental conditions, irrespective of individual's preferences, can also change during a very short period. More specifically, spectral properties of a certain environment can shift due to natural (e.g., weather and seasons) or anthropogenic causes (e.g., waste-based inflow of nutrients into an otherwise clear lake, followed by eutrophication; light pollution) (Anderson et al. 2002, Gaston et al. 2017). An ever-stronger line of evidence suggests that to cope with such fast and unpredictable changes of photic conditions, teleosts have evolved different levels of phenotypic plasticity. Plastic responses of opsin gene expression can be studied through aquarium-based experiments and human-induced changes in light conditions. Although there is some evidence from experiments that opsin gene expression can be developmentally plastic meaning that individuals are affected by differing light conditions to which they are exposed from early stages onwards (e.g., bluefin killifish: Fuller et al. 2010; cichlids: Smith et al. 2012, guppies: Ehlman et al. 2015, bream: Shand et al. 2008), this phenomenon isn't observed in all species and some, e.g., cave-dwelling mollies, remain unaffected by it (Tobler et al. 2010). Most

experiments, though, focused solely on determining the potential plasticity of adults. Results, however, are somewhat contradictory. While adults of some species respond plastically within weeks or even days (e.g., damselfishes and cardinalfishes: Luehrmann et al. 2018; cichlids: Dalton et al. 2015, Härer et al. 2017, Nandamuri et al. 2017; killifish: Fuller & Claricoates 2011), some respond minimally to changed light conditions (e.g., sticklebacks: Flamarique et al. 2013). A rather rapid plastic response can also occur at the chromophore level via regulation of the *Cyp27c1* expression, which results in a shift from the shorter-shifted A1 to longer-shifted A2-derived chromophore (Härer et al. 2018, Escobar-Camacho et al. 2019). Due to variable degree of plasticity observed throughout the teleost phylogeny, the latter might also constrain it, potentially resulting in taxa that lack plasticity of opsin gene expression altogether. In general, however, phenotypic plasticity aids one's fitness as it can promote faster exploitation of resources in a rapidly changing world without the need for a generational turn-over, and as such, is more likely to be selected for (reviewed in Pfennig et al. 2010). Thus, epigenetic control of gene expression is very likely one of the factors driving adaptive radiations (reviewed in Pfennig et al. 2010, Losos et al. 2000, Shimizu-Inatsugi et al. 2017, Stein & Bell 2019), including that of cichlids (Seehausen et al. 2006, Turner 2007, Muschick et al. 2011, Schneider & Meyer 2017, Ronco et al. 2021).

Cichlids – a celebrated example of an adaptive radiation

Cichlids (Cichlidae) are a group of mainly tropical freshwater teleosts with a Gondwanan distribution (Kocher 2004, Seehausen 2015), most famous for their species richness. As such, they are a textbook example of a phenomenon called adaptive radiation which presupposes ecological opportunities and rapid niche specialisation (Turner 2007). For example, last 5-7 million years produced more than 2000 species in East Africa alone (Salzburger 2018). Several factors are believed to have aided in cichlid phenotypic, behavioural and ecological diversification, including plasticity (e.g., opsin expression) and morphological novelties, such as pharyngeal jaws, which enabled evolution of different feeding strategies (Nandamuri et al. 2017, Conith & Albertson 2021). Indeed, cichlids trophically specialise on algae sponges, zooplankton, insects, other fishes, eggs, snails and much more - some are even filter feeders (Galvez et al. 2021). Selection on reproductive strategies, colouration, olfaction and acoustics, male courtship traits and parenting seems to also drive evolution of some species flocks either in allopatry or sympatry (reviewed in Rometsch et al. 2021, Barluenga et al. 2006, Svensson et al. 2017, Alter et al. 2017, Torres-Dowdall & Meyer et al. 2017). In general, the substrate for

the appearance of new cichlid species is provided by molecular mechanisms, i.e., gene duplication, accelerated coding sequence evolution, transposable element insertion and regulatory evolution (Brawand et al. 2014, Malinsky et al. 2018), and, namely, the ancestral hybridization creating enormous substrate for selection to act on, as recently shown in the Malawi and Victoria cichlids (Meier et al. 2017, Svardal et al. 2020). In addition, cichlid genomes are stable in terms of size and the number of chromosomes; these traits, as well as low nucleotide mutations rates, seem to promote cichlid hybridisation, which in turn aid their evolutionary boom (Stelkens et al. 2015, Svardal et al. 2021).

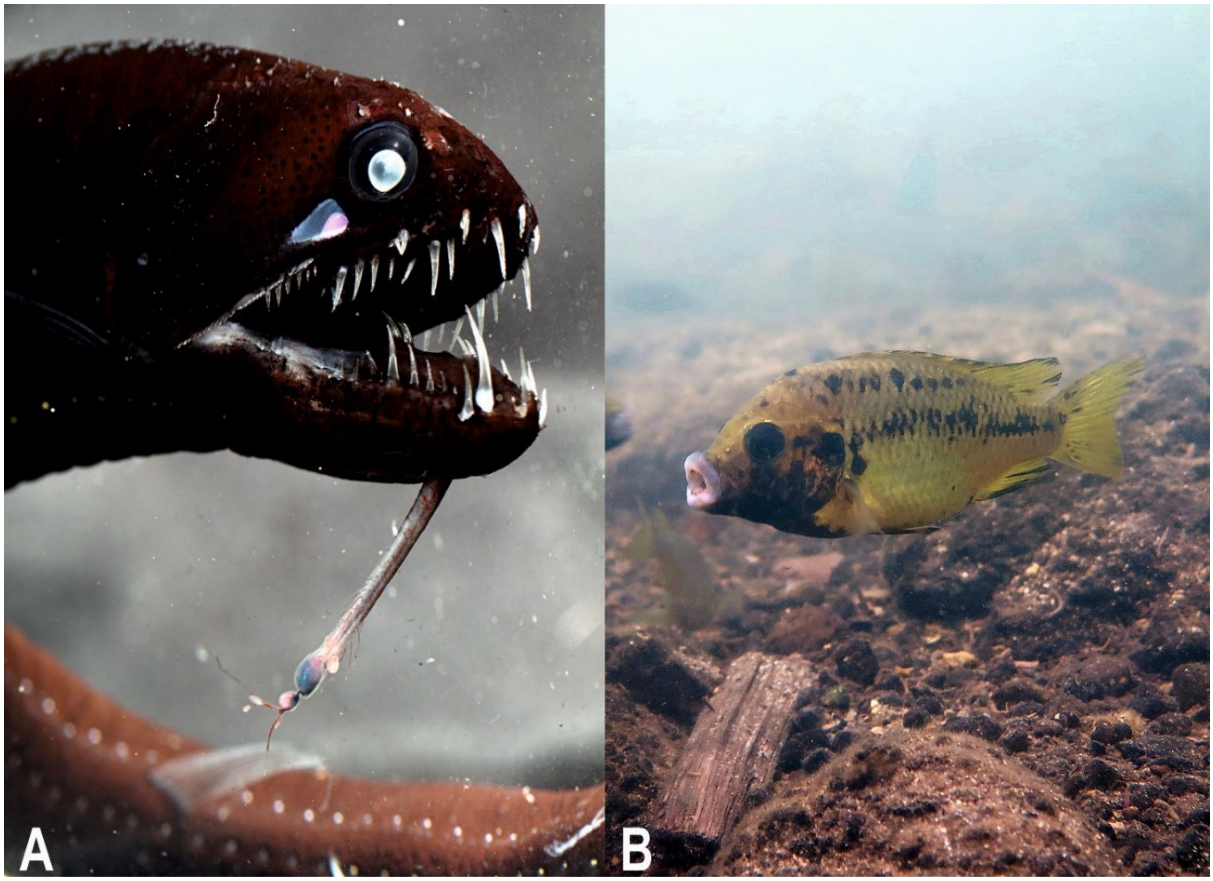

Figure 3. ***A*** *Echiostoma barbatum* (Stomiiformes), a deep-sea fish species ***B.*** *Pungu maclareni*, a cichlid from the Barombi Mbo crater lake. Photographs used with permission from Zuzana Musilová.

Deep sea fishes – living in the realm of darkness

The deep sea is the largest habitat on Earth. It is characterised by low temperature, high hydrostatic pressure, food scarcity, low oxygen availability and poor light conditions (Locket 1977). The shallowest zone, called the mesopelagic (200m – 1,000m), lies just below the highly lit epipelagic zone where photosynthesis is still present (Denton 1990). Here, residual daylight is present, but the quantity of photons decreases with increasing depth. Similarly, this layer of

water beholds mostly wavelengths of light corresponding to the blue-green part of the visible spectrum. Organisms tend to be active swimmers, also capable of vertical migrations, often possess large eyes and can exhibit counter illumination (Denton 1990, Hopkins & Gartner 1992, Land & Nilsson 2012, Afonso et al. 2014). Below 1000m is the bathypelagic zone, which is followed by the abyssopelagic (4,000 – 6,000m) and hadopelagic (6,000 – 11,000m). These areas are characterised by the lack of photons; here, the only source of light is bioluminescence and so, organisms tend to have smaller eyes, tend to be more passive, conserving energy and relying mostly on short bursts of speed or scavenging when feeding (Denton 1990, Land & Nilsson 2012).

Deep-sea fishes are not a monophyletic group – instead they are the result of convergent evolution and adaptation to the deep sea that occurred independently at least 22 times (Randall & Farrell 1997). In the depths of the deep-sea, successfully capturing a photon might represent a difference between life and death. Consequently, deep-sea fishes have evolved extremely sensitive visual systems with often enlarged eyes and increased pupil apertures (Locket & Crescitelli 1977, Warrant & Locket 2004) and the presence of tapeta lucidum (Locket 1977, Nicol 1989). Histologically, they have increased the raw number of (sometimes transmuted) photoreceptors, which possess enlarged outer segments; in addition, they are often pooled together to achieve higher neural summation, i.e., more photoreceptor cells converging on fewer retinal ganglion cells (Locket 1977; Wagner et al. 1998, Land & Nilson 2012, reviewed in de Busserolles et al. 2020, Lupše et al. 2021). An additional adaptation to the deep are pure rod retinae that are either single or multibank (Locket 1977; Wagner et al. 1998, Land & Nilson 2001). Pure rod retinae are commonly reported in adults in numerous deep-sea lineages (deep-sea eels: Hirt & Wagner 2005; e.g., in Atlantic argentine, deep-sea smelts, barreleyes and spookfishes, lightfishes, hatchetfishes, viperfishes, dragonfishes, slickheads, lanternfishes, spiderfishes, sabre-tooth fishes, pearleyes, tube-eyes, deep-sea anglers and ceratid seadevils: Ali & Anctil 1976). In some cases, though, cones persist morphologically as individuals mature, but the relative proportion of cones usually decreases throughout the development (Bozzano et al. 2007). Such a developmental trajectory is supported also by molecular evidence, indicating presence of all-cone retinae of some larval forms (Lupše et al. 2021). These results are in congruence with the ecology-driven developmental trajectory of deep-sea fishes - despite their name, many deep-sea fishes start their lives in the well-lit epipelagic zone, where photic conditions allow for a cone-based vision. As animals mature, they move to greater depths of the meso- or bathypelagic where a rod-dominated vision is selected for (Moser & Smith 1993, Paraboles et al. 2019, Lupše et al. 2021).

AIMS OF THE THESIS

This thesis aims to throw light on the evolution and development of vision in ray-finned fishes (Actinopterygii). As discussed in the introduction, many fishes undergo major ecological shifts within their lifetime – during these transitions, photic environments often change, forcing organisms to adapt. Vision is an essential, but also energetically very demanding sensory system. Consequently, opsin gene expression serves as a tell-tale sign of a light environment, as it is known to directly correspond to available wavelengths of light. Using a predominantly transcriptomic approach, I aimed to:

(i) Investigate developmental changes of opsin gene expression in one of the most enigmatic groups of organisms on Earth, the deep-sea fishes (Chapter 1)

(ii) Uncover general and taxon-specific developmental patterns of opsin gene expression across the actinopterygian phylogeny (Chapter 2)

(iii) Examine the phenotypic plasticity of opsin gene expression in Cameroonian crater lake cichlid species (Chapter 3)

(iv) Explore the expression of phototransduction cascade genes as to illuminate the photoreceptor cell identity (Chapter 1, 2)

(v) Discuss ecological pressures that shape the fish visual development (Chapter 1, 2, 3)

MATERIALS AND METHODS

In this section, I would like to give an overview of the methodology, which is detailed fully within respective chapters.

As this thesis mainly deals with gene expression (transcriptomic) analyses, samples needed to be fixed in such a way that RNA was preserved, and its integrity kept. In the case of smaller larval individuals, this was done by fixing them in RNAlaterTM (ThermoFisher) immediately upon capture. Adults, on the other hand, were due to their size either flash frozen upon collection, or euthanized by the MS222 overdose - eyes or retinae were then extracted and fixed in RNAlaterTM, and in all cases, tissue was stored at −80°C. Fin clips, which were stored in 96% ethanol, were also taken for the purpose of preserving DNA.

Molecular lab work was done in the laboratories of the Faculty of Science, or at the BIOCEV in Vestec, Czech Republic. In the case of DNA, when needed for barcoding purposes, we followed DNA extraction, polymerase chain reaction, gel electrophoresis and purification protocols. Specifically, we resorted to standard PCR conditions throughout, using the thermocycler Mastercycler (Eppendorf) and visually checking the gel electrophoresis to determine successful amplification. As a pre-mix for the PCR protocol, we used the Multiplex PCR Master Mix (QIAGEN). PCR products were purified using the Exo-CIP PCR clean up protocol (New England Biolabs). Sanger sequencing for the *COXI* gene was done at BIOCEV.

The library preparation and genomic sequencing of some deep-sea samples was outsourced (details in Chapter 1). RNA was extracted in-house using standard kits listed in respective chapters. Its integrity was checked either at the faculty or at BIOCEV facilities, before proceeding with the library preparation protocol using library prep kits (see Chapter 1, 2 for details), which prepares the sample for the Next Generation Sequencing on the Illumina platform. The protocol itself was at times, together with the NGS sequencing, outsourced (for details, see Chapters 1-3).

Once transcriptomes were obtained, they were quality checked with FastQC (Andrews 2017) and analysed using the Geneious software (Kearse et al. 2012). To obtain the most precise expression levels, reads were first mapped to a general reference dataset using the medium-sensitivity settings, enabling us to capture most opsin class-specific reads. A new, species-specific reference was then mapped against with medium-low sensitivity settings as to obtain final expression values. In case of multiple opsin gene copies, the latter were disentangled following Musilova et al. (2019). Other genes studied, e.g., arrestins, transducins, *cyp27c1* underwent the same expression quantification methodology, and so did publicly

available transcriptomes that were obtained from the Sequence Read Archive (NCBI) using a very specific set of search terms, described in detail in Chapter 2.

The identity of each gene, including the *COXI* barcoding fragment, was confirmed using the Basic Local Alignment Search Tool (BLAST, NCBI). Phylogenetic reconstruction of *COXI*, *RH2* and *RH1*, used mostly for Chapter 1, was done with Bayesian estimation using MrBayes (Ronquist et al. 2012).

To investigate phenotypic plasticity (Chapter 3), we reared laboratory born generation F2 under artificial lighting conditions, instigated by us. To achieve this, we covered all the sides of all four tanks with filter sheets, which spectral properties were known beforehand, thus modifying photic conditions accordingly. The resulting experimental setup consisted of four light regimes: full-spectrum, short-wavelength (=blue), medium-wavelength (=green) and long-wavelength (=red) shifted, and fishes were reared under such conditions until adulthood, with a subset of individuals being sampled after week 1, week 2, week 4 and month 6.

CONCLUSIONS

This thesis provides novel insights into the development and plasticity of fish vision and has several major outcomes. In more detail, through transcriptomic analyses of visual gene expression, with a focus on opsin genes, we uncovered general actinopterygian ontogenetic patterns that are mostly depicted in the age-related increase of the red-sensitive *LWS,* and in a decrease of the UV-sensitive *SWS1* cone opsin; adults also express significantly higher levels of the rod opsin (*RH1*) gene as compared to larvae, suggesting actinopterygians aren't exempt from the general cone-to-rod retinal development that is characteristic of vertebrates. Additionally, we scrutinised specific taxa to uncover the expansion of opsin repertoires in several lineages and describe age-specific switches between and within cone opsin classes. Furthermore, we put an extra emphasis on the visual development of deep-sea fishes, as they represented a unique conundrum of their own. Our study explored major differences in retinal expression between larvae and adults and presented special cases of gene duplications and functional changes of amino-acid sequences. Furthermore, we revealed that the development of other phototransduction cascade genes, such as *GNAT*, matches that of opsins in some cases and not in others, which might be indicative of otherwise rarely described transmuted photoreceptors. Finally, to provide additional evidence for epigenetic control of vision in fishes, we investigated plastic visual responses of Cameroonian crater lake cichlids. Rearing them under different artificial light regimes showed that not only are Barombi Mbo and Bermin lake cichlids able to regulate the expression of different sets of opsin genes depending on the developmental stage – they are also capable of a response on a much shorter time scale, suggesting phenotypic plasticity could aid their persistence in an ever-changing natural environment.

Although this thesis presents (i) general and detailed accounts of ecology-driven developmental changes of visual gene expression in ray-finned fishes, (ii) illuminates individual interesting and unique evolutionary adaptations and (iii) showcases the potential value of phenotypic plasticity, future research is needed to better understand the evolutionary importance of fish visual development and plasticity. Open questions remain, and further studies focusing on non-model organisms and a whole array of phototransduction genes, as set out in this thesis, are needed. In addition, investigations into epigenetic control, extraocular opsins, neuronal circuits and transmuted photoreceptors through a combination of molecular,

physiological and histological methods would benefit our understanding of molecular mechanisms underlying fish, and vertebrate vision in general.

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- CHAPTER 1 –

Lupše, N., Cortesi, F., Freese, M., Marohn, L., Pohlman, J.-D., Wysujack, K., Hanel, R., Musilova, Z. (2021). Visual gene expression reveals a cone to rod developmental progression in deep-sea fishes. *Molecular Biology and Evolution* 38(12):5664-5677 (doi:10.1093/molbev/msab281)

*Presented on the cover of the January 2022 issue

Volume 39 • Number 1 • January 2022

MOLECULAR BIOLOGY AND EVOLUTION

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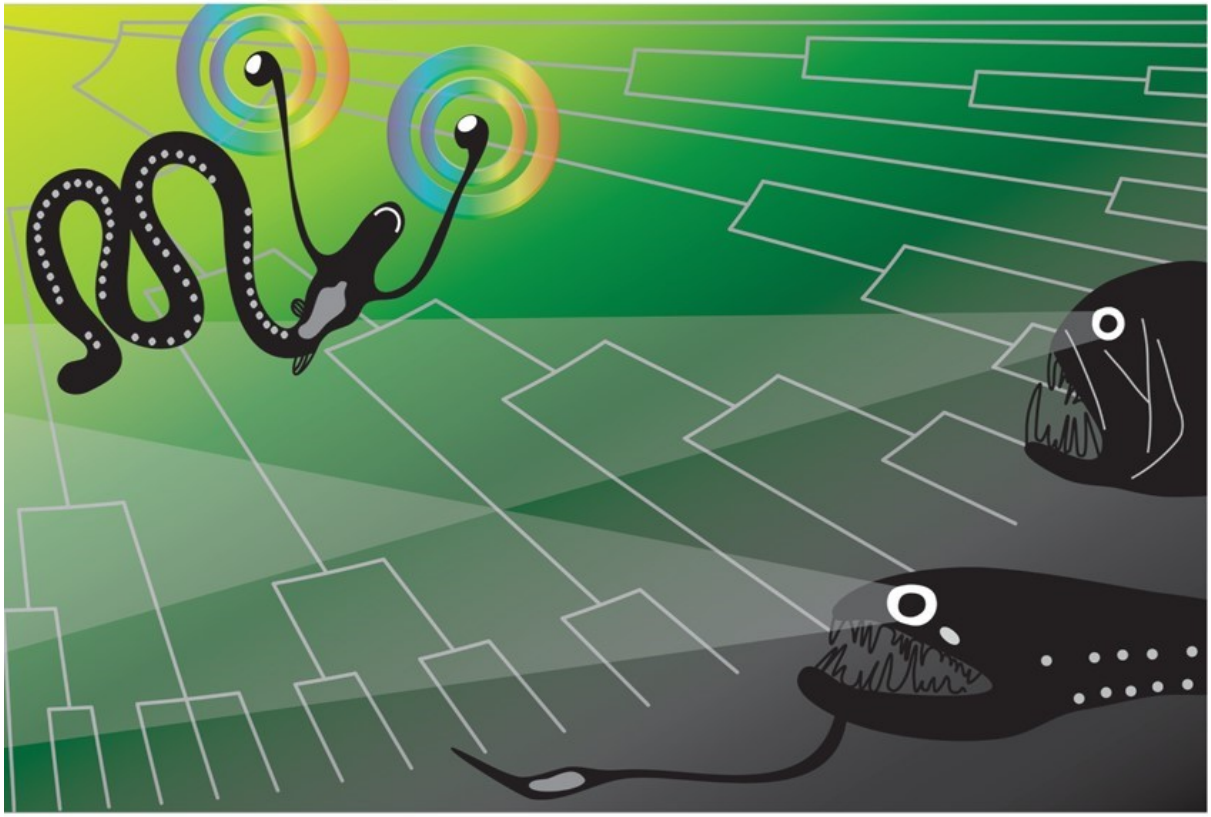

Society for Molecular Biology and Evolution

Online ISSN 1537-1719

Visual Gene Expression Reveals a cone-to-rod Developmental Progression in Deep-Sea Fishes

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Associate editor: Belinda Chang

Abstract

Vertebrates use cone cells in the retina for color vision and rod cells to see in dim light. Many deep-sea fishes have adapted to their environment to have only rod cells in the retina, while both rod and cone genes are still preserved in their genomes. As deep-sea fish larvae start their lives in the shallow, and only later submerge to the depth, they have to cope with diverse environmental conditions during ontogeny. Using a comparative transcriptomic approach in 20 deep-sea fish species from eight teleost orders, we report on a developmental cone-to-rod switch. While adults mostly rely on rod opsin (*RH1*) for vision in dim light, larvae almost exclusively express middle-wavelength-sensitive ("green") cone opsins (*RH2*) in their retinas. The phototransduction cascade genes follow a similar ontogenetic pattern of cone—followed by rod-specific gene expression in most species, except for the pearleye and sabretooth (Aulopiformes), in which the cone cascade remains dominant throughout development, casting doubts on the photoreceptor cell identity. By inspecting the whole genomes of five deep-sea species (four of them sequenced within this study: *Idiacanthus fasciola*, *Chauliodus sloani*; Stomiiformes; *Coccorella atlantica*, and *Scopelarchus michaelsarsi*; Aulopiformes), we found that they possess one or two copies of the rod *RH1* opsin gene, and up to seven copies of the cone *RH2* opsin genes in their genomes, while other cone opsin classes have been mostly lost. Our findings hence provide molecular evidence for a limited opsin gene repertoire in deep-sea fishes and a conserved vertebrate pattern whereby cone photoreceptors develop first and rod photoreceptors are added only at later developmental stages.

Key words: **opsin, evolution, mesopelagic, adaptation, convergence, phototransduction, vision, gene expression, rhodopsin.**

Article

Introduction

Vision is a primary sense used by most vertebrates for navigation, predator avoidance, communication and to find food and shelter. At its initiation, vertebrate vision is enabled by cone (photopic, color vision) and rod (scotopic) photoreceptors in the retina containing a light absorbing pigment that consists of an opsin protein covalently bound to a vitamin-A-derived chromophore (Lamb 2013). The absorbance of photons by the chromophore leads to a conformational change of the opsin protein, which initiates a photoreceptor-specific G-protein-coupled phototransduction cascade, propagating the signal to the brain (Downes and Gautam 1999; Larhammar et al. 2009; Lamb 2019). It is thought that the development of the visual system follows a conserved molecular pattern whereby cone specific genes are activated first before the rod molecular pathway is initiated later during ontogeny (Mears et al. 2001; Shen and Raymond 2004; Sernagor et al. 2006). However, whether this is the case for all vertebrates and especially for those that have retinas that contain only rods as adults, remains unclear.

Changes in the light environment, ecology, and phylogenetic inertia are thought to be primary drivers for visual system diversity in vertebrates (Hunt et al. 2014). For example, most mesopelagic deep-sea fishes (200–1,000 m depth), either living strictly at depth or migrating to the shallows at night, have evolved visual systems that are sensitive to the dominant blue light (~470–490 nm) of their environment (Turner et al. 2009). Furthermore, as the daylight and the bioluminescent light emitted by deep-sea critters are quickly dimmed with depth and distance, deep-sea fish visual systems have evolved peculiar morphologies to maximize photon capture including barrel-eyes, reflective tapeta and the use of rod-dominated and in many cases rod-only retinas that might be stacked into multiple banks (reviewed in de Busserolles et al. (2020)). However, most mesopelagic fishes start their lives in the shallow well-lit epipelagic zone (0–200 m depth) (Moser and Smith 1993; Sassa and Hirota 2013). Consequently, their visual systems must cope with a variety of light intensities and spectra throughout development.

Studies investigating the gene expression in the retina of deep-sea fishes are scarce and usually focus on a selected few species (Zhang et al. 2000; Douglas et al. 2016; de Busserolles et al. 2017; Musilova et al. 2019a; Byun et al. 2020). In adults, species with pure rod retinas tend to only express rod opsin(s) (Douglas et al. 2016; Musilova et al. 2019a), albeit two species of pearlsides (*Maurolicus* spp.) have been found to express cone-specific genes (i.e., cone transduction pathway and opsin genes) inside rod-looking cells (de Busserolles et al. 2017). It remains unknown whether deep-sea fishes that have a low proportion of cone photoreceptors as adults (Munk 1990; Collin et al. 1998; Bozzano et al. 2007; Pointer et al. 2007; Biagioni et al. 2016) also express cone-specific genes at any stages of their lives or whether these fishes rely on the rod machinery alone. To investigate whether the retinal development in deep-sea fishes follows a similar cone-to-rod molecular pathway as found in other vertebrates or whether some species start their lives with the rod pathway activated, we set out to sequence the retinal transcriptomes of 20 deep-sea fish species, including the larval stages in ten species, belonging to eight different teleost orders (Argentiniformes, Aulopiformes, Beryciformes, Myctophiformes, Pempheriformes, Scombriformes, Stomiiformes, and Trachichthyiformes). We further investigated the genomic repertoire in five selected species.

Results and Discussion

Opsin Gene Repertoire in the Genome

In teleost fishes, gene duplications and deletions followed by functional diversification have resulted in extant species having between 1 and 40 visual opsin genes within their genomes (Musilova et al. 2019a, 2021). These genes are defined by their photoreceptor specificity, their phylogeny, and their spectrum of maximal sensitivity (λ_{max}) and fall within five major classes, four cone opsins ("ultraviolet or UV sensitive" *SWS1*: 347–383 nm, "blue" *SWS2*: 397–482 nm, "green" *RH2*: 452–537 nm, and "red" *LWS*: 501–573 nm) and one rod opsin ("blue-green" rhodopsin, *RH1* or Rho: 447–525 nm) (Carleton et al. 2020). We analyzed the whole genomes of five deep-sea species (sawtail fish *Idiacanthus fasciola*, viperfish *Chauliodus sloani*; both Stomiiformes; sabretooth *Coccorella atlantica*, pearleye *Scopelarchus michaelsarsi*; both Aulopiformes; and fangtooth *Anoplogaster cornuta*; Trachichthyiformes), four of them sequenced for the purpose of this study. All species possess one or two copies of the rod opsin *RH1* gene, and one to seven copies of the *RH2* cone opsin (fig. 1). All other cone opsin classes, that is, the *SWS1*, *SWS2* (except for the fangtooth) and *LWS* are missing and have been putatively lost during evolution in these five species. This is in accordance with the observation that the *LWS* gene abundance decreases with the habitat depth (Musilova et al. 2019a). Such a limited genomic repertoire most likely represents an evolutionary response to the deep-sea scotopic environment where the shortest (UV-violet) and longest (red) wavelengths of light are filtered out first in the water column, as opposed to middle-range wavelengths that can penetrate to greater depths (reviewed in Musilova et al. 2021; de Busserolles et al. 2020; Carleton et al. 2020). The increased *RH2* diversity observed in the two aulopiform species, on the other hand, illustrates the versatility of this cone opsin class and confirms its dominance in various dimmer-light habitats (Musilova and Cortesi 2021). Here, we confirm that *RH2* is undoubtedly the most important (and often the only) cone opsin gene present in deep-sea fish genomes.

Visual Opsin Gene Expression

Transcriptomic sequencing of 20 deep-sea teleost species revealed that deep-sea fishes mainly express rod opsins and/or green-sensitive cone opsins (*RH2*s) in their retinas (fig. 1 and table 1). While larvae mostly expressed *RH2*, adults and juveniles mostly expressed *RH1* and in a few cases a combination of both. We found none or very low expression of any of the other cone opsin genes: the red sensitive *LWS* was not expressed at all, the UV sensitive *SWS1* was only found in the larva of the whalefish, *Gyrinomimus* sp. (Beryciformes), and the blue/violet sensitive *SWS2* only in the larvae of the whalefish, and the fangtooth, *A. cornuta* (Trachichthyiformes) (fig. 1 and table 1). Differences in gene expression patterns are likely to be driven by ontogenetic transitions in light habitat from bright to dim environments and by changes in ecological demands, as discussed in more detail below.

Similar to the opsin genes, we also detected ontogenetic differences in the expression of phototransduction cascade genes (fig. 2). Here, we focused on the comparison of five species from three teleost orders for which we had both larval and adult specimens available and found that the cone-specific genes were mostly expressed in the larval stages (e.g., cone transducin, *GNAT2*), while adults from three species mostly expressed rod-specific genes (e.g., rod transducin, *GNAT1*; fig. 2*B*). Hence, at the molecular level, the visual systems of deep-sea fishes start out with a cone-based expression pattern. Furthermore, in the fangtooth, where samples from various sized specimens were available, we found that the cone-specific expression was gradually replaced with the rod profile as the fish grew (fig. 2*C* and table S1). This sequence is similar to the visual development in shallower living fishes (e.g., Atlantic cod (Valen et al. 2016), zebrafish (Sernagor et al. 2006)) and terrestrial vertebrates (e.g., mice (Mears et al. 2001), rhesus monkey (La Vail et al. 1991)), where cone photoreceptors are first to develop, followed by temporally and spatially distinct rods (Raymond 1995; Shen and Raymond 2004). The cone-to-rod developmental sequence is therefore likely to be shared across vertebrates, even in species that have pure rod retinas as adults.

Ontogenetic Shift in Expression Profiles and the Transition Phase

The observed developmental changes in the visual system are best explained by the different habitats larval and adult deep-sea fishes inhabit. In general, deep-sea fish larvae live in the shallow epipelagic zone (Moser and Smith 1993) where ambient light levels are sufficiently high to warrant a cone-based visual system. After metamorphosis, deep-sea fishes start to

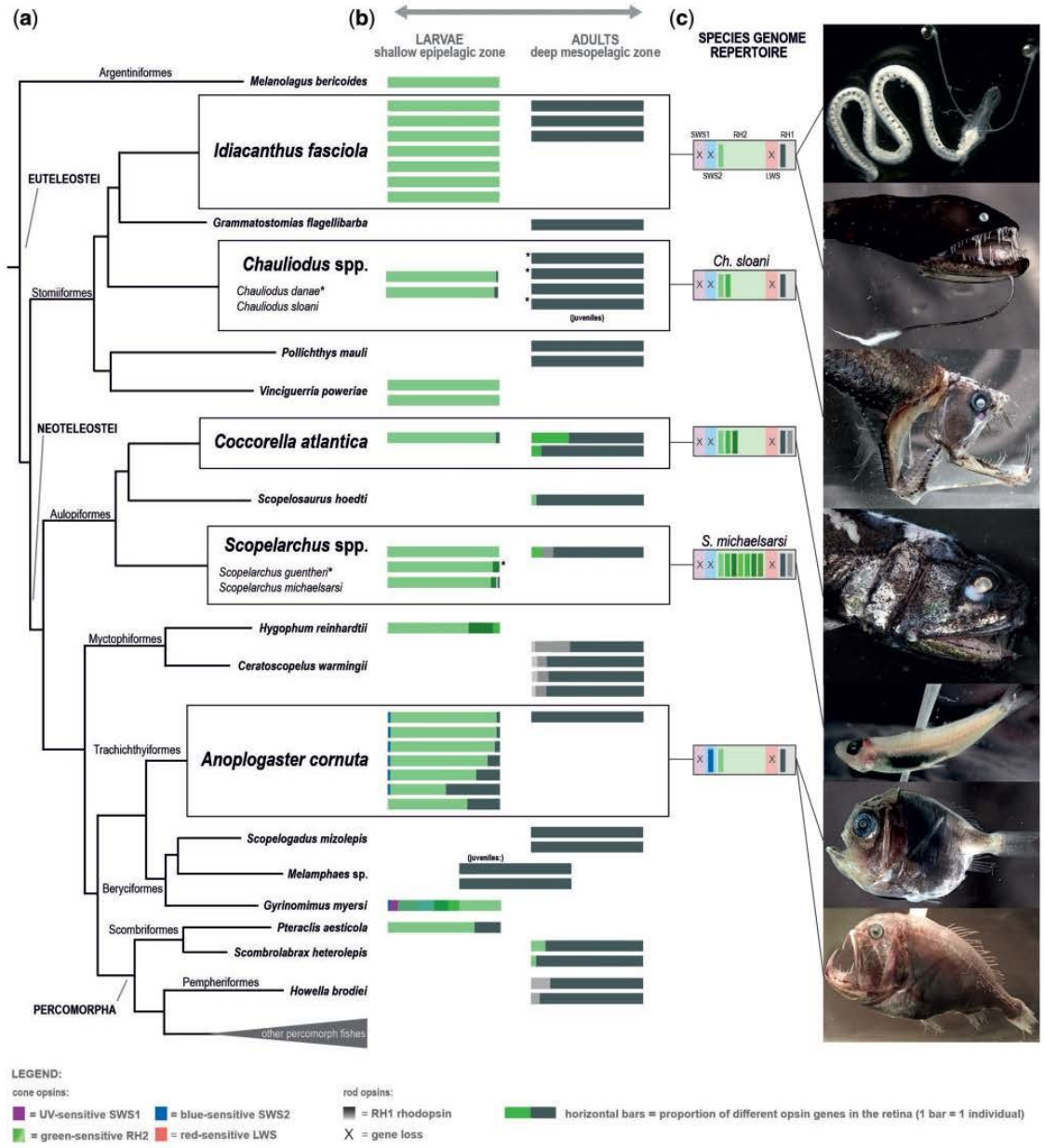

Fig. 1. Cone and rod opsin gene expression in larval and adult deep-sea fishes. (*A*) Simplified phylogeny of the 20 deep-sea fish species for which the retinal transcriptomes were sequenced (topology after Betancur et al. 2017). Boxes highlight the five species for which both larval and adult samples were available. (*B*) Proportional opsin gene expression for each individual (horizontal bars) at different developmental stages. Different colors correspond to cone (colors) or rod (shades of grey) opsin genes, depicted as the proportional expression over the total sum of visual opsins expressed. Different shades of the same color represent multiple copies of the same gene class. Based on the opsin gene expression, the larvae (left column) show a pure-cone or cone-dominated retina, while the adults (right column) have a pure-rod or rod-dominated visual system. Juvenile specimens in two species had an adult expression profile. Note that some species expressed multiple *RH1* copies (*Scopelarchus*, *Howella brodiei*, and *Ceratoscopelus warmingii* adults) or multiple *RH2* copies (*Gyrinomimus* sp. larva, *Hygophum reinhardtii* larva). Notably, adults and larvae of *Scopelarchus* sp. and *Coccorella atlantica* expressed different copies of *RH2* (more details in fig. 2). Details about the samples and expression levels are listed in table 1. (*C*) The genomic repertoire of the visual opsins is shown for five species: *Idiacanthus fasciola*, *Chauliodus sloani*, *Coccorella atlantica*, *Scopelarchus michaelsarsi* (all this study), and *Anoplogaster cornuta* (Musilova et al. 2019a). The rod *RH1* opsin and the cone *RH2* opsin genes are present in all studied species in one or multiple (up to seven) copies. The *SWS2* opsin gene was found only in the fangtooth, and the *SWS1* and *LWS* are missing from all five studied genomes.

Table 1. Samples Used in the Study and Results of the Opsin Gene Expression in the Eyes or Retina.

Species	Order	Median Species Depth (m)[a]	Stage	Code	Size (mm)	Date of Collection	Raw Reads	Reads After Bacteria Filtering	Gene Expression				Accession No.	Source
									RH1	*RH2*	*SWS1*	*SWS2*		
Anoplogaster cornuta	Trachichthyiformes	1000–2000	Larva	39016	4	April 2015	36,373,372	36,125,630	<0.01	0.99		<0.01	SAMN10473242	GenBank
			Larva	39017	4	April 2015	34,712,538	34,604,364	<0.01	0.99		<0.01	SAMN10473243	GenBank
			Larva	4_23	9	April 2015	41,592,840	41,519,070	0.02	0.98		<0.01	SAMN10473246	GenBank
			Larva	4_22	11	April 2015	75,109,270	74,990,214	0.11	0.89		<0.01	SAMN10473245	GenBank
			Larva	4_21	11-12	April 2015	28,945,804	28,869,552	0.22	0.78		<0.01	SAMN10473244	GenBank
			Larva	261s03	12	30.3.2017	48,458,044	48,457,472	0.47	0.53		<0.01	SAMN20741930	This study
			Larva	126A1	20	1.3.2020	79,371,594	79,347,702	0.28	0.72			SAMN20746021	This study
			Adult	56H6	102	6.-7.4.2017	11,658,040	11,635,610	1				SAMN20748547	This study
Ceratoscopelus warmingii	Myctophiformes	400–500	Adult	300s03	63	7.-8.4.2017	49,078,974	44,114,639	0.66 RH1-1 0.01 RH1-2 0.33 RH1-3				SAMN20752508	This study
			Adult	S1	n/a	June 2014	8,796,786	8,789,961	0.94 RH1-1 0.02 RH1-2 0.04 RH1-3				SAMN10473255	GenBank
			Adult	S2	n/a	June 2014	8,655,248	8,649,342	0.93 RH1-1 0.02 RH1-2 0.05 RH1-3				SAMN10473256	GenBank
			Adult	S3	n/a	June 2014	7,904,714	7,899,617	0.94 RH1-1 0.01 RH1-2 0.05 RH1-3				SAMN10473257	GenBank
Chauliodus sloani	Stomiiformes	800–900	Larva	67I2_2	11	3.4.2017	34,445,252	34,371,394	0.01	0.99			SAMN20769443	This study
			Larva	67I1	17	3.4.2017	20,701,686	20,683,764	<0.01	0.99			SAMN20769824	This study
			Juvenile	109B6	20	5.4.2017	24,854,866	24,835,646	1				SAMN20771653	This study
Chauliodus danae	Stomiiformes	800–900	Juvenile	109C7	24	30.3.2017	32,865,334	32,834,300	1				SAMN20771814	This study
			Juvenile	109A2	20	5.4.2017	25,389,318	25,344,182	1				SAMN20777471	This study
			Juvenile	109D7	96	30.3.2017	18,540,248	18,533,484	1				SAMN20777490	This study
Coccorella atlantica	Aulopiformes	300–400	Larva	109G8	21	30.3.2017	26,376,620	26,359,060	0.01	0.99 RH2-1			SAMN20793157	This study
			Adult	56C7	77	25.3.2017	36,794,070	36,790,854	0.69	0.31 RH2-2			SAMN20793158	This study
			Adult	297_7	68	6.-7.4.2017	43,948,534	40,511,279	0.96	0.04 RH2-2			SAMN20793260	This study
Grammatostomias flagellibarba	Stomiiformes	500–600	Adult	56H8	138	6.-7.4.2017	9,775,520	9,742,864	1				SAMN20794729	This study
Gyrinomimus myersi	Beryciformes	1000–2000	Larva	S25	n/a	April 2015	19,270,096	19,122,986	<0.01	0.45 RH2-1 0.13 RH2-2 0.08 RH2-3 0.11 RH2-4 0.15 RH2-5	0.07	<0.01	SAMN10473271	GenBank
Howella brodiei	Pempheriformes	500–600	Adult	56D8	76	25.3.2017	56,905,264	43,936,178	0.17 RH1-1 0.83 RH1-2				SAMN20797093	This study
			Adult	56D9	68	25.3.2017	61,909,674	61,590,270	0.07 RH1-1 0.93 RH1-2				SAMN20799523	This study
Hygophum reinhardtii	Myctophiformes	100–200	Larva	67I2_1	5	3.4.2017	17,222,626	17,122,880		0.26 RH2-1 0.68 RH2-2 0.06 RH2-3			SAMN20799641	This study
Idiacanthus fasciola	Stomiiformes	600–700	Larva	71C2	22	22.3.2017	23,382,468	23,379,360		1			SAMN20800764	This study
			Larva	67D2	22	31.3.2017	6,647,384	6,623,496		1			SAMN20800770	This study
			Larva	71C1	18	22.3.2017	23,632,774	23,497,715		1			SAMN20800774	This study
			Larva	67I7	25	5.4.2017	25,713,960	25,701,768		1			SAMN20800775	This study
			Larva	71B9	14	22.3.2017	43,695,620	43,633,424		1			SAMN20801851	This study
			Larva	67B8	17	29.3.2017	28,333,642	28,313,024		1			SAMN20804813	This study
			Larva	109A1	41	5.4.2017	27,834,274	27,818,750		1			SAMN20804946	This study
			Adult	228s01	210	22.-23.3.2017	21,704,528	21,622,274	1				SAMN20805116	This study
			Adult	67F8	178	1.4.2017	15,744,604	15,739,004	1				SAMN20805515	This study
			Adult	67B6	75	29.3.2017	15,439,954	11,368,044	1				SAMN20834669	This study
Melamphaes sp.	Beryciformes	600–700	Juvenile	4_26	n/a	April 2015	38,583,370	38,534,507	1				SAMN10473273	GenBank
			Juvenile	4_28	n/a	April 2015	35,489,970	35,431,421	1				SAMN10473274	GenBank

(continued)

Table 1. Continued

Species	Order	Median Species Depth (m)[a]	Stage	Code	Size (mm)	Date of Collection	Raw Reads	Reads After Bacteria Filtering	Gene Expression			Accession No.	Source
									RH1	*RH2*	*SWS1*	*SWS2*	
Melanolagus bericoides	Argentiniformes	800–900	Larva	71H3	7	26.3.2017	26,219,646	26,210,402		1			SAMN20834672 This study
Pteraclis aesticola	Scombriformes	0–300	Larva	109H4	8	30.3.2017	19,918,922	19,870,484	0.22	0.78			SAMN20844147 This study
Pollichthys mauli	Stomiiformes	300–400	Adult	109I2	34	30.3.2017	55,767,498	54,875,747	1				SAMN20845044 This study
			Adult	109I3	36	30.3.2017	68,655,686	67,082,709	1				SAMN20848441 This study
Scombrolabrax heterolepis	Scombriformes	800–900	Adult	,56E3	91	25.3.2017	65,770,014	65,527,135	0.98	0.02			SAMN20857047 This study
			Adult	,56E4	77	25.3.2017	60,643,652	60,472,479	0.93	0.07			SAMN20857932 This study
Scopelarchus guentheri	Aulopiformes	200–300	Larva	109H1	21	30.3.2017	37,542,802	37,416,486		0.96 RH2_larval			SAMN20859427 This study
										0.04 RH2-2_larval			
Scopelarchus michaelsarsi	Aulopiformes	300–400	Larva	109B9	21	5.4.2017	9,971,250	9,919,148		1 RH2_larval			SAMN20860823 This study
			Larva	71B7	20	22.3.2017	43,695,620	43,682,534	<0.01 RH1a	0.96 RH2_larval			SAMN20865446 This study
									<0.01 RH1b	0.03 RH2-2_larval			
			Adult	272_10	66	2.4.2017	45,565,202	43,729,338	0.80 RH1a	0.10 RH2_adult			SAMN20867027 This study
									0.10 RH1b				
Scopelogadus mizolepis	Berycifomes	800–900	Adult	,57E2	43	5.4.2017	5,160,876	5,160,672	1				SAMN20867114 This study
			Adult	300s01	62	7.-8.4.2017	15,448,240	15,420,650	1				SAMN20867573 This study
Scopelosaurus hoedti	Aulopiformes	500–600	Adult	297_9	69	6.-7.4.2017	43,948,534	42,248,053	0.98	0.02			SAMN20872996 This study
Vinciguerria poweriae	Stomiiformes	100–300	Larva	109H2	18	30.3.2017	25,863,628	25,755,100		1			SAMN20878005 This study
			Larva	109C8	16	30.3.2017	22,914,004	22,858,398		1			SAMN20882114 This study

[a]https://obis.org/.

submerge deeper and take up a life at different depths in the mesopelagic or even bathypelagic (below 1,000 m depth) zone, where the sun- and moonlight is gradually replaced by bioluminescence as the main source of light (Denton 1990). In this extremely dim environment, rods work at their best and cone photoreceptors would be obsolete for the most part at least. Rod-based vision is also favored in those deep-sea species that exhibit diel vertical migrations to feed in the plankton rich surface layers at night (de Busserolles et al. 2020). In addition, we discovered that in some species there was a switch in the expressed type of cone *RH2* opsin (fig. 1). For example, in Aulopiformes, the larvae expressed an alternative *RH2* copy that is presumably sensitive to longer wavelengths of light compared to the *RH2* that was found in adults (table 2). This clearly shows that larval and adult deep-sea fishes rely on different opsin expression profiles, which is similar to ontogenetic changes in opsin gene expression in diurnal shallow-water fishes such as freshwater cichlids (Carleton et al. 2016) and coral reef dottybacks (Cortesi et al. 2015, 2016) or between the freshwater and deep-sea maturation stages in eels (Zhang et al. 2000).

Our data furthermore suggest that the ontogenetic change in visual gene expression precedes morphological changes such as metamorphosis from larva to juvenile and also habitat transitions. For example, in the fangtooth, the larvae which were collected from the shallows (0–300 m) showed increasing amounts of *RH1* expression with growth, despite displaying larval phenotypes throughout (horns and small teeth; fig. 1). A similar pattern of changing opsin gene expression ahead of metamorphosis has also been reported from shallow-water fishes such as European eels (Bowmaker et al. 2008), dottybacks (Cortesi et al. 2016), and surgeonfishes (Tettamanti et al. 2019). Interestingly, all our fangtooth larvae (including the smallest individual with a total length of 4 mm) already expressed a small amount of *RH1* (fig. 2C). Whether fangtooth start their lives with a pure cone retina or low-levels of rod opsin expression are normal even in preflexation larvae remains therefore unclear. In addition to the green-sensitive cone opsin *RH2*, the smallest fangtooth larvae also expressed low levels of the blue-sensitive *SWS2*, potentially conferring dichromatic color vision to the early-life stages of this species (fig. 1).

Photoreceptor Cell Identities

Interestingly, two aulopiform species, the Atlantic sabretooth, *C. atlantica*, and the Bigfin pearleye, *S. michaelsarsi*, despite expressing mostly *RH1* as adults, retained a cone-dominated phototransduction cascade expression profile akin to the one found in the larval stages (fig. 2 and table S1). This begs the question whether the photoreceptors they are using are cones or rods in nature. Initially described in snakes and geckos (Simoes et al. 2016; Schott et al. 2019) and recently also in a deep-sea fish (de Busserolles et al. 2017), it appears that the dichotomy of rods and cones is not always as clear cut as one might think. For example, adult deep-sea pearlsides, *Maurolicus* spp. have a retina that expresses ~99% *RH2* and ~1% *RH1* with corresponding cone and rod phototransduction gene expressions. Their photoreceptors, however, are all rod-shaped and careful histological examination has

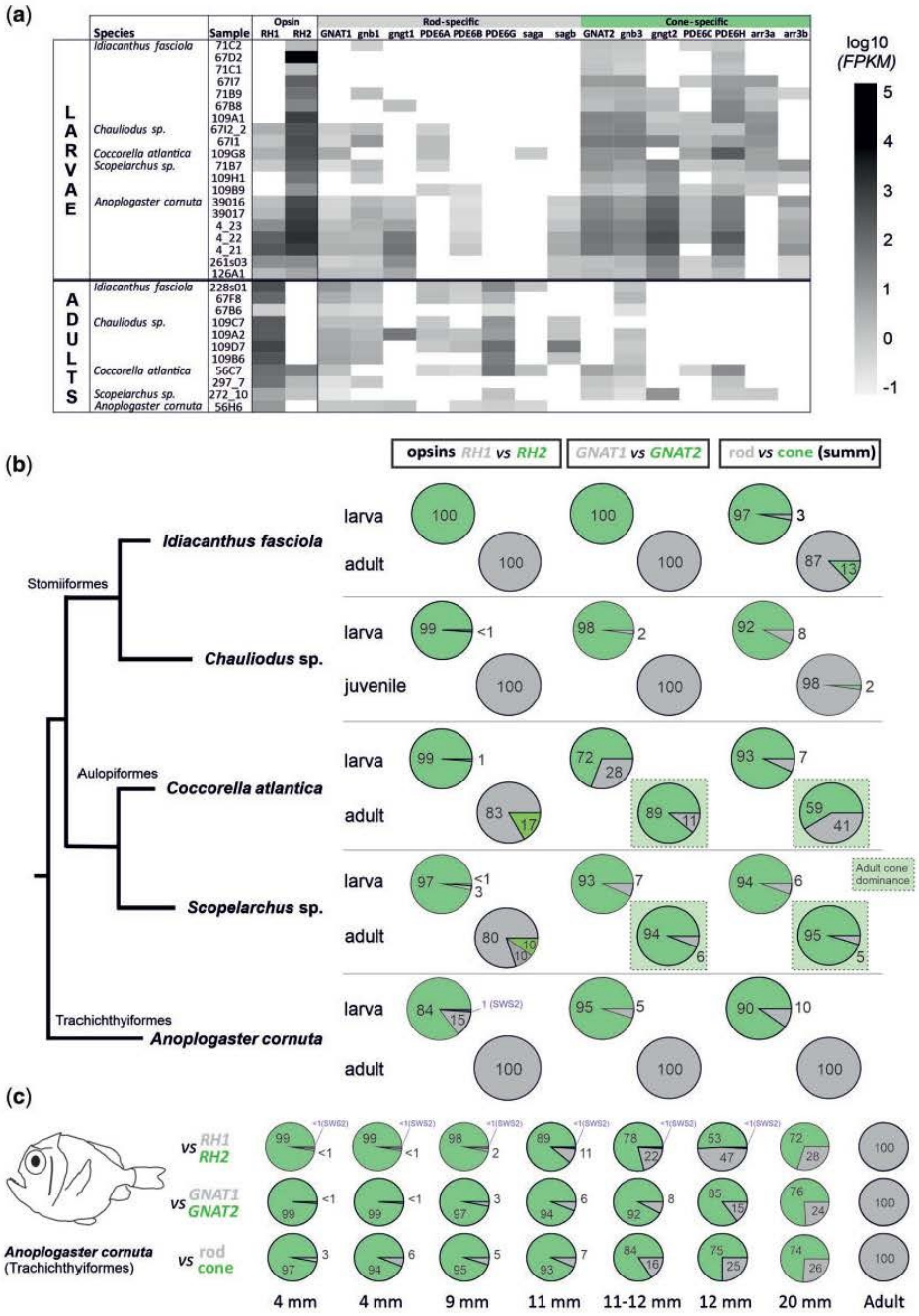

FIG. 2. Phototransduction cascade gene expression in the retina of five deep-sea fish species. (A) Heat map of the expression of individual phototransduction cascade genes for each sample, based on normalized numbers of reads (FPKM). (B) Pie charts comparing mean values of relative expression of the opsin genes (rod *RH1* and cone *RH2*), photoreceptor-specific cascade transducin genes (rod-type *GNAT1* and cone-type *GNAT2*), and all cascade genes excluding opsins (photoreceptor-specific transducins, arrestins and phosphodiesterases) summarized. The green rectangles highlight the two aulopiform species with the discordance between the opsin type (rod-specific) and phototransduction cascade genes (cone-specific) in adults. (C) Focus on the common fangtooth (*Anoplogaster cornuta*) transitional phase shown as a sequence for seven larval and one adult sample. Size given as standard length (SL). Note that all fangtooth larvae expressed both *RH1 and RH2*, with an increasing proportion of *RH1* to *RH2* as the larvae grew in size (with the exception of the largest larva where *RH1*:*RH2* was 28:72). Smaller larvae also expressed the *SWS2* gene. Except for the adult, all other individuals had traits of larval phenotypes (dorsal and ventral horns and small teeth; fig. 1) and were collected relatively shallow between 0 and 300 m using plankton trawls.

Table 2. Key-Tuning Amino Acid Sites in the Cone Opsin *RH2* Gene.

Species	Order	83	122	207	255	292	λ_{max} (nm)	Reference
Bovine RH1		D	E	M	I	A	500	Yokoyama (2008)
Ancestral teleost		D	Q	M	I	A	488	Yokoyama and Jia (2020)
Melanolagus bericoides	Argentiniformes	G	Q	.	V	.		
Coccorella atlantica adult	Aulopiformes	G	Q	.	.	.		
Coccorella atlantica larval	Aulopiformes	G	Q	.	V	.		
*Scopelarchus michaelsarsi*_ RH2_adult	Aulopiformes	?	Q	I	C	T		
Scopelarchus guentheri _RH2_larval	Aulopiformes	G	Q	.	V	.		
*Scopelarchus michaelsarsi*_ RH2_ larval	Aulopiformes	G	Q	.	V	.	505	Pointer et al. (2007) (for *S. analis*)
*Scopelarchus guentheri*_ RH2-2_ larval	Aulopiformes	G	Q	.	C	.		
*Scopelarchus michaelsarsi*_ RH2-2_ larval	Aulopiformes	G	Q	.	C	.		
Scopelosaurus hoedti	Aulopiformes	G	Q	.	.	.		
Gyrinomimus myersi RH2-1	Beryciformes	G	Q	.	F	.		
Gyrinomimus myersi RH2-2	Beryciformes	G	Q	L	F	.		
Gyrinomimus myersi RH2-3	Beryciformes	G	Q	L	F	.		
Gyrinomimus myersi RH2-4	Beryciformes	G	Q	L	F	.		
Gyrinomimus myersi RH2-5	Beryciformes	G	Q	L	F	.		
Lepisosteus platyrhincus (shallow outgroup)	Lepisosteiformes	G	.	.	.	.		
Hygophum reinhardtii RH2-1	Myctophiformes	G	Q	.	V	.		
Hygophum reinhardtii RH2-2	Myctophiformes	G	Q	.	V	.		
Hygophum reinhardtii RH2-3	Myctophiformes	G	Q	.	V	.		
Lepidopus fitchi RH2-1	Scombriformes	G	.	.	V	.	496	Yokoyama and Jia (2020)
Lepidopus fitchi RH2-2	Scombriformes	G	Q	.	V	.		
Lepidopus fitchi RH2-3	Scombriformes	G	Q	.	V	.	506	Yokoyama and Jia (2020)
Lepidopus fitchi RH2-4	Scombriformes	G	Q	.	V	.		
Pteraclis aesticola	Scombriformes	G	Q	L	.	.		
Scombrolabrax heterolepis	Scombriformes	G	Q	.	.	.		
Aristostomias scintillans	Stomiiformes	G	Q	L	V	.	468	Yokoyama and Jia (2020)
Chauliodus sp.	Stomiiformes	G	Q	.	F	.		
Grammatostomias flagellibarba	Stomiiformes	G	Q	.	.	.		
Idiacanthus fasciola	Stomiiformes	G	Q	.	V	.		
Vinciguerria poweriae	Stomiiformes	G	Q	.	F	.		
Anoplogaster cornuta	Trachichthyiformes	G	Q	.	.	.		

shown that these consist of a tiny proportion of true rods and a majority of transmuted rod-like cones (de Busserolles et al. 2017). In the case of pearlsides, and also in geckos and snakes, the opsin and phototransduction genes correspond to each other making it possible to distinguish photoreceptor types at the molecular level. However, in the aulopiforms, high expression of rod opsin is seemingly mismatched with high levels of cone phototransduction gene expression (fig. 2). In salamanders, the opposite pattern can be found whereby a cone opsin is combined with the rod phototransduction cascade inside a rod looking cone photoreceptor (Mariani 1986). Anatomically, the retina of *S. michaelsarsi* is composed of mostly rods with low numbers of cone cells (Collin et al. 1998), while the adult retina of *Evermanella balbo*, an evermannellid species related to *C. atlantica*, appears to consist of two differently looking rod populations (Wagner et al. 2019). It is therefore likely, as found in pearlsides (de Busserolles et al. 2017), that these fishes have a high proportion of transmuted rod-like cone photoreceptors, but that they use *RH1* instead of a cone opsin as the visual pigment. Alternatively, a proportion of true rods might make use of the cone phototransduction cascade. Either way, combining more stable rod opsin in a rod-shaped cell with the cone-specific cascade is likely to increase sensitivity while also maintaining high transduction and recovery speeds of cells (Baylor 1987, Kawamura and Tachibanaki 2012, Luo et al. 2020). Histology, fluorescent in situ hybridization and ideally physiological recordings are needed to ultimately disentangle the identity of photoreceptor cells in aulopiforms.

Evolutionary History of Deep-Sea Fish Opsins

While the majority of adult fishes relied on a single *RH1* copy, we found three species that expressed multiple *RH1* copies: The Warming's lanternfish, *Ceratoscopelus warmingii* (Myctophiformes), expressed three different *RH1* genes, and *S. michaelsarsi* and the basslet, *Howella brodiei* (Pempheriformes), expressed two copies each. Larvae and a few adult deep-sea fishes mostly expressed a single *RH2* copy, except for the pearleyes, *Scopelarchus* spp., and the Reinhardt's lanternfish, *Hygophum reinhardtii*, which expressed up to three larval copies each, and the whalefish (*Gyrinomimus* sp.) which expressed five larval copies (fig. 1).

The *RH1 and RH2* phylogenies revealed that most deep-sea fish visual opsins cluster together by species or order (fig. 3). For example, in the whalefish all *RH2s* are clustered together suggesting that these genes are lineage or species-specific duplicates (fig. 3*B*). However, there were a few exceptions, suggesting more ancient duplication events. In *Scopelarchus* the two *RH1* copies are not in a sister relationship and in fact result in different clusters, suggesting that these copies originated in the common ancestor of aulopiforms or perhaps even earlier (fig. 3*A*). Similarly, the *RH2s* in aulopiforms (*Scopelarchus*, *Coccorella*) cluster by ontogenetic stage, making it likely that the

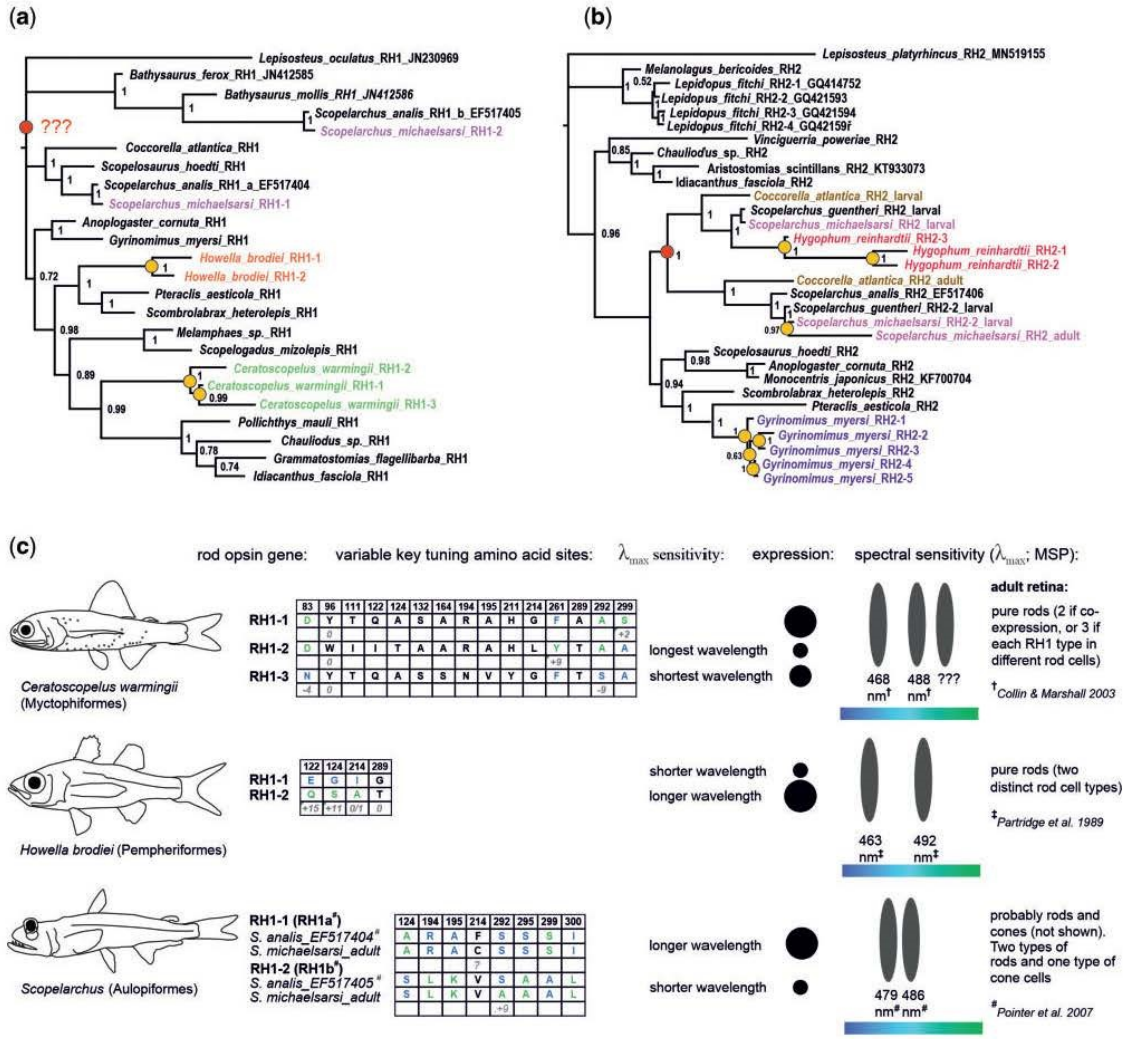

FIG. 3. Gene trees of the (A) *RH1* and (B) *RH2* opsin genes found in the retinal transcriptomes of deep-sea fishes. Species with multiple copies are highlighted in color. Additional gene sequences from public databases are listed with their GenBank accession numbers. Note the topology within Aulopiformes; the adult *RH2s* of *Coccorella atlantica and Scopelarchus* cluster together as do the major larval *RH2s*. Yellow circles mark lineage-specific gene duplication events, while red circles pinpoint the ancestral duplication of *RH1* impacting the *Scopelarchus* genus, and the duplication of *RH2* in the aulopiform ancestor (or at least the common ancestor of *Coccorella and Scopelarchus*). (*C*) Key tuning spectral site mutations in species with multiple rod opsins and indicative wavelength shifts based on previous in vitro experiments. Known shifts are listed in nanometers if available. Blue and green letters in the tables stand for the shorter- and longer-shifting amino acid variants, respectively. Multiple different rod opsins have been found in three species, *Ceratoscopelus warmingii* (Myctophiformes), *Howella brodiei* (Pempheriformes), and *Scopelarchus michaelsarsi* (Aulopiformes). Note that the *RH1* copies in *Scopelarchus* seem to show a mixed pattern—the longer-wavelength sensitive copy (*RH1a*; confirmed by in vitro measurements by Pointer et al. (2007) carries also several shorter-shifting amino-acid sites as compared to *RH1b*). Assignment of the longer and shorter wavelength sensitive photoreceptor to a rhodopsin sequence is marked next to the tables. Rhodopsin gene expression marks dominant (large circles) and less abundant (small circles) copies. For functional interpretation of the rod cells in the visual system we considered microspectrophotometry measurements from # = Pointer et al. (2007), † = Collin and Marshall (2003), and ‡ = Partridge et al. (1989).

developmental switch in gene expression was already present in the aulopiform ancestor (fig. 3*B*).

Molecular Complexity of Deep-Sea Fish Visual Systems

The complexity of the deep-sea fish visual systems at the molecular level varied quite substantially. For example, the three Stomiiformes species: The Ribbon sawtail fish, *I. fasciola*, and two species of viperfish, *C. sloani* and *Ch. danae*, appeared to have a very basic visual set up; these fishes were found to express a single *RH2* cone opsin as larvae and a single *RH1* rod opsin as adults (fig. 1). On the contrary, several deep-sea fish orders examined here expressed more than one opsin gene. Adult lanternfishes and basslets have rod-only retinas but

expressed multiple *RH1* copies that have functionally diversified (fig. 3). Other species expressed both cone and rod opsins as adults (the aulopiform species and *Scombrolabrax*), which is somewhat similar to the opsin gene expression profiles found in shallow-living nocturnal reef fishes (Cortesi et al. 2020).

The most complex visual system in this study was found in *S. michaelsarsi*. In general, this species is known for its numerous morphological and anatomical adaptations to vision in the deep, including having barrel eyes with a main and an accessory retina, rods that are organized in bundles, large ganglion cells and corneal lens pads (Collin et al., 1998). The two copies of *RH1* (*RH1a* and *RH1b*) it expressed showed high sequence diversity differing in 79 out of 354 amino acids, eight of which are known key tuning sites likely to change the spectral sensitivity of the pigments via a shift in λ_{max} (fig. 3 and table 3) (Yokoyama 2008; Musilova et al. 2019a; Yokoyama and Jia 2020). This supports the findings by Pointer et al. (2007) who, using in vitro visual pigment expression in another pearleye species, *S. analis*, found two rod photoreceptors with different absorption maxima at 479 and 486 nm. Interestingly, Pointer et al. (2007) also speculate that another short-shifted opsin (previously measured in *S. analis* to have λ_{max} at 444 nm by Partridge et al., 1992) possibly belongs to the *SWS2* class. Our data however does not support this prediction as no SWS2 gene is found in the genome of *S. michaelsarsi*. The existence and identity of such short-sensitive opsins in pearleyes remains therefore elusive. The situation is less clear for the green-sensitive *RH2* opsin. While in *S. analis* cones have been found in the accessory and main retinas, in *S. michaelsarsi* cone photoreceptors appear restricted to the accessory retina alone (Collin et al. 1998). This is intriguing as it suggests substantial differences in visual systems even between closely related species from the same genus.

The Visual Ecology of Deep-Sea Fishes

We found molecular support for deep-sea visual adaptations on multiple levels:

(1) *Opsin gene diversity in the genome.* Data from the genomes of five deep-sea species revealed the diversity of the opsin genes (fig. 1C). Retinal transcriptomes in the Stomiiformes pointed towards a simple visual system that is based on a single expressed opsin gene at different developmental stages (*RH2* in larvae, *RH1* in adults) (fig. 1). A search for visual opsins in the stomiiform genomes sequenced for the purpose of this study (*I. fasciola and Ch. sloani*), as well as in several published genomes (Musilova et al. 2019a) revealed that Stomiiformes are likely to have lost all cone opsin gene classes except for *RH2* (fig. 1). In the case of *RH2*, they seem to only have a single or at most two gene copies, which is substantially less than other teleosts (Musilova et al. 2019a; Musilova and Cortesi, 2021). The stomiiform example, therefore, shows that a decrease in light intensity and the spectrum of light in the deep-sea may not only restrict gene expression at adult stages, but also lead to the loss of opsin genes altogether. Similarly, a loss in opsin and other vision-related genes (e.g., otx5b, crx) has previously been reported from shallow living fishes that are either nocturnal (Luehrmann et al. 2019), live in murky waters (Liu et al. 2019), or inhabit caves or similarly dim environments (Huang et al. 2019; Musilova et al. 2019a). Contrarily, the genomes of two aulopiform species (*C. atlantica and S. michaelsarsi*) revealed expanded cone opsin gene repertoires achieved mostly through *RH2* duplications (*C. atlantica* with three *RH2*s, and *S. michaelsarsi* with seven *RH2* genes; fig 1). Both species also possess two copies of the rod opsin gene. These species inhabit relatively shallower depths (300–400 m) compared to other deep-sea fishes such as the Stomiiformes (table 1). It is likely that having extra copies of *RH2* cone opsins may benefit their vision at these photon-richer depths. It is also possible that an evolutionary stochasticity and the gene content in the ancestor have contributed to the observed pattern. To be able to clearly state this, future research should be done on multiple aulopiform species.

(2) *Visual gene expression.* Previous work has found that the expression of the longest- (*LWS*—red) and shortest- (*SWS1*—UV) sensitive opsins is reduced or absent in deeper living coral reef fishes (Cortesi et al. 2020) and in fishes inhabiting deep freshwater lakes (Hunt et al. 1997; Sugawara et al. 2005; Musilova et al. 2019b), which is correlated with a loss of short- and long-wavelengths with depth. Also, many deep-sea fish lineages have lost *LWS* from their genomes (Musilova et al. 2019a; Cortesi et al. 2021). Supporting these findings, we show here that deep-sea fishes lack any *LWS* expression even in the shallow-living larval stages (fig. 1). Similarly, *SWS1* is not expressed in any of the species studied, except for in the larval whalefish, and is also absent from many deep-sea fish genomes (fig. 1) (Musilova et al. 2019a). However, shallow larval stages are likely to explain why all deep-sea fishes studied to date maintain at least some cone opsins in their genomes (Musilova et al. 2019a). Most deep-sea fish larvae expressed a single *RH2* gene, but the larvae of some species (fangtooth, whalefish, and lanternfish) expressed multiple cone opsin genes (fig. 1). This is likely to provide them with similar visual systems to the larvae of shallow-living marine (Britt et al. 2001) and freshwater species (Carleton et al. 2016), possibly aiding in detecting residual light and discriminating brightness and/or colors. Juvenile deep-sea fishes, on the other hand, showed rod-based expression profiles also found in the adult stages (fig. 1). This shift in opsin gene expression correlates with developmental changes in ecology. As opposed to the adults which are exposed to a narrow and dim light environment where food is scarce, larvae typically live in well-lit, broad spectrum shallow waters where food and predators are abundant (Moser and Smith 1993).

(3) *Functional adaptation in key spectral tuning sites.* When multiple *RH1* copies were expressed, they often showed

Table 3. Key-Tuning Amino Acid Sites in the Rhodopsin *RH1* Gene.

Species	Order	83	90	96	102	111	113	118	122	124	132	164	183	188	194	195	207	208	211	214	253	261	265	269	289	292	295	299	300	317	λ_{max} (nm)	References
Bovine RH1		D	G	Y	Y	N	E	T	E	A	A	A	M	G	P	H	M	F	H	I	M	F	W	A	T	A	A	A	V	M	500	Yokoyama (2008)
Ancestral teleost RH1		D	G	Y	Y		E	T	E	A	A	A	M		L	N	M	F	H	I	M	F	W	A	T	A	A	A	L	M		Musilova et al. (2019a)
Bathysaurus ferox	Aulopiformes	N	.	.	.	.	.	.	.	S	.	.	.	.	R	A	.	.	.	.	.	.	.	.	.	S	.	T	L	.	481	Collin and Marshall (2003)
Bathysaurus mollis	Aulopiformes	N	.	.	.	.	.	.	.	S	.	.	.	.	R	A	.	.	.	.	.	.	.	T	.	S	.	T	L	.	479	Collin and Marshall (2003)
Coccorella atlantica	Aulopiformes	N	.	.	.	S	.	.	M	.	.	.	.	.	R	A	.	.	.	C	.	.	.	.	.	.	S	.	I	.	480[c]	Douglas et al. (1998)
Scopelarchus michaelsarsi RH1a	Aulopiformes	N	.	.	.	.	.	.	.	.	.	.	.	.	R	A	.	.	.	C	.	.	.	.	.	.	S	S	I	.	486[a]	Pointer et al. (2007)
Scopelarchus michaelsarsi RH1b	Aulopiformes	N	.	.	.	.	.	.	.	S	.	.	.	.	L	K	.	.	.	V	.	.	.	.	.	.	S	.	L	.	479[a]	Pointer et al. (2007)
Scopelosaurus hoedti	Aulopiformes	.	.	.	.	.	.	.	.	.	.	.	.	.	R	A	.	.	.	.	.	.	.	.	.	.	S	S	I	.	.	
Gyrinomimus myersi	Beryciformes	N	.	.	.	.	.	.	.	.	.	.	.	.	R	A	.	.	.	.	.	.	.	.	.	.	S	.	I	.	.	
Melamphaes sp.	Beryciformes	.	.	.	.	.	.	.	Q	.	S	.	.	.	R	V	.	.	.	G	.	.	.	S	.	.	.	.	S	.	.	undetected
Scopelogadus mizolepis	Beryciformes	.	.	.	.	.	.	.	Q	G	S	.	.	.	R	V	.	.	.	G	.	.	.	T	.	.	.	.	S	.	.	488[d]	Douglas et al. (1998)
Lepisosteus oculatus (outgroup)	Lepisosteiformes	.	.	.	.	.	.	.	Q	S	.	.	.	.	L	K	.	.	.	L	.	Y	.	G	.	.	.	.	.	L	.		
Ceratoscopelus warmingii RH1-1	Myctophiformes	.	.	.	.	T	.	.	Q	.	S	.	.	.	R	A	.	.	.	G	.	.	.	.	A	.	.	.	S	.	.	488[b]	Collin and Marshall (2003)
Ceratoscopelus warmingii RH1-2	Myctophiformes	.	.	W	.	I	.	.	I	T	.	.	.	.	R	A	.	.	.	L	.	Y	.	.	.	.	.	.	I	.	.	undetected	Collin and Marshall (2003)
Ceratoscopelus warmingii RH1-3	Myctophiformes	N	.	.	.	T	.	.	Q	.	S	S	.	.	N	V	.	.	Y	G	.	.	.	.	.	.	S	.	I	.	.	468[b]	Collin and Marshall (2003)
Hygophum reinhardtii	Myctophiformes	.	.	.	.	A	.	.	Q	.	.	.	.	.	R	A	.	.	.	G	.	.	.	.	.	.	.	.	I	.	.		
Howella brodiei RH1-1	Pempheriformes	N	.	.	.	.	.	.	.	G	.	.	.	.	R	A	.	.	.	.	.	.	.	.	.	G	S	S	I	.	.		
Howella brodiei RH1-2	Pempheriformes	N	.	.	.	.	.	.	Q	S	.	.	.	.	R	A	.	.	.	A	.	.	.	.	.	.	S	S	I	.	.		
Pseudanthias aesticola	Scombriformes	.	.	.	.	Q	.	.	Q	.	.	.	.	.	R	A	.	.	.	.	.	.	.	.	.	.	.	S	I	.	.		
Scombrolabrax heterolepis	Scombriformes	.	.	.	.	Q	.	.	Q	.	.	.	.	.	R	A	.	.	.	.	.	.	.	S	.	.	S	S	I	.	.		
Chauliodus spp.	Stomiiformes	N	.	.	.	.	.	.	.	G	.	.	.	.	R	A	.	.	.	V	.	.	.	.	A	S	.	.	L	V	484	Collin and Marshall (2003)
Grammatostomias flagelibarba	Stomiiformes	N	.	.	.	.	.	.	.	.	.	.	.	.	R	A	.	.	.	V	.	.	.	.	.	S	.	.	L	.	480 - 487	Kenaley et al. (2014)
Idiacanthus fasciola	Stomiiformes	N	.	.	.	.	.	.	.	.	.	.	.	.	R	A	.	.	.	.	.	.	.	.	.	S	.	.	L	.	485	Collin and Marshall (2003)
Pollichthys mauli	Stomiiformes	N	.	.	.	.	.	.	.	.	.	.	.	.	R	A	.	.	.	.	.	.	.	.	.	S	.	.	L	.		
Anoplogaster cornuta	Trachichthyiformes	N	.	.	.	.	.	.	.	.	.	.	.	.	R	A	.	.	.	.	.	.	.	.	.	T	S	.	S	I	485	Collin and Marshall (2003)

[a]Value for *Scopelarchus analis*.
[b]Two pigments reported without assignment to the gene; see also fig. 3.
[c]Value for closely related species, *Evermannella balbo*.
[d]Value for *Scopelogadus beani*.

distinct differences in key amino acid sites that are likely to shift the spectral sensitivities of the pigments (fig. 3 and table 3) (Yokoyama 2008; Musilova et al. 2019a). Estimating spectral absorbance from these sites resulted in very similar λ_{max} values to those that were measured in vivo from the same or closely related species using microspectrophotometry (MSP) or similar techniques (Partridge et al. 1989; Collin and Marshall 2003; Pointer et al. 2007) (fig. 3 and table 3). We therefore integrated the available functional evidence (MSP values) with our molecular data (protein sequence and gene expression levels) to better understand the function of the visual system in the three species that expressed multiple rod opsins (fig. 3 and table 3).

The three different *RH1*s in *C. warmingii* differed in 15 key-tuning sites. Our data revealed a dominant rod opsin copy (*RH1-1*), and a shorter- (*RH1-2*) and a longer-shifted (*RH1-3*) copies with lower transcript levels (figs. 1 and 3). Previously, MSP in *C. warmingii* has revealed two distinct rod types with λ_{max} values of 488 and 468 nm (Collin and Marshall 2003), most likely corresponding to the *RH1a/RH1-1 and RH1b/RH1-3* genes, respectively. It is possible that multiple *RH1* copies are coexpressed within the same photoreceptor, something that has previously been reported for cone opsins in shallow-water marine (Savelli et al. 2018; Stieb et al. 2019) and freshwater fishes (Dalton et al. 2014; Torres-Dowdall et al. 2017). Coexpression could produce visual pigment mixtures that shift photoreceptor sensitivity and enhance visual contrast, aiding in predator–prey interactions or mate detection (Dalton et al. 2014). Alternatively, we predict that a third rod photoreceptor type with longer spectral sensitivity (*RH1-2*; fig 3C) exists, possibly overlooked during MSP, which can happen especially if the cell type is rare. While the function of having multiple rod opsins in *C. warmingii* remains to be investigated, several possible benefits for a multi-rod visual system have recently been proposed including that it might enable conventional or unusual color vision in dim light, it might be used to increase visual sensitivity, or enhance an object's contrast against a certain background (Musilova et al. 2019a).

In *H. brodiei*, the second *RH1* copy (*RH1-2*) differed in two key-tuning sites, E122Q (−15 nm) and G124S (−11 nm), known to cause major short-wavelength shifts in other fishes (Yokoyama 2008). This is in accordance with the MSP measurements in its sister species, *Howella sherborni*, which found two different rod types with spectral sensitivities of 463 and 492 nm (fig. 3) (Partridge et al. 1989). Having multiple differently tuned rod photoreceptors, one centered on the prevailing light (bioluminescence and/or ambient light ~480–490 nm) and a second one that is offset from it (i.e., the offset pigment hypothesis; Lythgoe 1966), may be beneficial to break counter illumination of prey—a way of active camouflage in mesopelagic organisms where ventral photophores emit bioluminescent light that matches the residual downwelling light (Denton et al. 1985). Hence, revealing an individual's silhouette could help to distinguish prey and predators from the background lighting, or visually finding mates. However, apart from lanternfishes with three (or more) and basslets with two rod opsins, or exceptional cases of tube-eye (6) and spinyfin (38), the majority of the deep-sea fishes seem to have only one rod opsin (Musilova et al., 2019a).

Differences in spectral sensitivity between photoreceptors can also be due to the chromophore type that binds to the opsin protein; visual pigments with a vitamin A1-based chromophore (typical in marine fishes) confer shorter-shifted λ_{max} values compared to those with a vitamin A2-based chromophore (typical in freshwater fishes) (Carleton et al. 2016). *Cyp27c1* is the enzyme responsible for converting A1- to A2-based chromophores (Enright et al. 2015), with high expression levels suggesting the presence of longer-shifted visual pigments. However, *Cyp27c1* was not expressed in our data set suggesting that the visual pigments of these deep-sea fishes are based on A1-retinal alone (table S2).

Conclusions

So far, the development of deep-sea fish vision at the molecular level had not been studied in detail and only limited morphological information is available. In this study, we compared opsin and visual gene expression between 20 deep-sea fish species revealing a major change in expression between larval and adult stages. While deep-sea fish genomes contain both cone and rod opsin genes, larvae rely on the cone pathway and adults switch to a rod-dominated or rod-only visual system. The cone- versus rod-specific phototransduction cascade genes follow the opsins in some lineages, however, not in aulopiforms, suggesting the presence of transmuted photoreceptor cells. We detected reduced opsin gene repertoires in the genomes of five deep-sea fish species composed only of one rod (*RH1*) and one or two cone (*RH2*, or *RH2 and SWS2*) opsin gene classes. Interestingly, we have identified lineage-specific opsin gene duplications, possibly allowing for increased visual sensitivity and/or color vision in the deep in some species. Overall, our molecular results support a conserved developmental progression in vertebrates whereby cones appear first in the retina and rod photoreceptors are added later during development.

Materials and Methods

Specimens used in this study were collected in the Sargasso Sea during three multipurpose fishery surveys conducted by the German Thünen Institute of Fisheries Ecology onboard the research vessels *Maria S. Merian* in March to April 2015, and *Walther Herwig III* in 2017 and in 2020. The sampling of adults occurred during both day and night at depths of 600–1,000 m using a mid-water pelagic trawl net (Engel Netze, Bremerhaven, Germany) with an opening of 30 × 20 m, a length of 145 m, and mesh sizes (knot to knot) from 90 cm decreasing stepwise to 40, 20, 10, 5, 4, 3, 2 cm, with a 1.5-cm mesh in the 27-m-long codend. The larvae were mostly collected using an Isaacs-Kidd Midwater Trawl net (IKMT; 6.2 m^2 mouth-opening, 0.5 mm mesh size; Hydro-Bios Apparatebau

GmbH) at depths of 0–300 m by double-oblique transect tows. Adult fish were flash-frozen at −80°C upon arrival on board and a fin clip was stored in 96% ethanol. Larval samples were fixed in RNAlater™ (ThermoFisher) and stored at −80°C until further use.

To sequence the whole genome of *I. fasciola*, *C. sloani*, *C. atlantica*, and *S. michaelsarsi*, the genomic DNA was extracted from the fin clip using the DNeasy Blood and Tissue kit (Qiagen) following the enclosed protocol. The library preparation and genome sequencing on Illumina NovaSeq platform (150 bp PE and the yield over 20 Gb per genome) has been outsourced to the sequencing center Novogene, Singapore (https://en.novogene.com/). To analyze the opsin gene repertoire, the raw genomic reads were mapped in Geneious software version 11.0.3 (Kearse et al. 2012) against the opsin references (single exons of all five opsin classes from the reference species: Nile tilapia, Round goby, Blind cavefish, and Spotted gar), as well as against the genes found in the transcriptomes of each species. The parameters were set to the medium sensitivity to capture all reads that matched any visual opsin gene. The captured reads mapping to all exons were then remapped against one reference per exon and the species-specific consensus sequence was generated. If present, multiple paralogous genes were disentangled manually, and the consensus sequence was exported for each variant (see below more details for the transcriptomic analysis). The obtained consensus sequences served as references for the second round of mapping, whereby all genomic reads were again mapped with the Low Sensitivity settings, and each reference was then elongated by the overlapping sequence. This step was repeated until the full gene region was covered. In case of *S. michaelsarsi*, we were not able to cover the full length of five out of seven *RH2* genes due to the repetitions and these genes were reported in two parts, always one covering the exons 1 and 2, and one covering exons 3, 4 and 5. The genomic raw reads are available from GenBank (BioProject PRJNA754116) and the opsin gene sequences are provided in Supplementary file 1.

Total RNA was extracted from the whole eyes using either the RNeasy micro or mini kit (Qiagen) and the extracted RNA concentration and integrity were subsequently verified on a 2100 Bioanalyzer (Agilent). RNAseq libraries for 31 samples were constructed in-house from unfragmented total RNA using Illumina's NEBNext Ultra II Directional RNA library preparation kit, NEBNext Multiplex Oligos and the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs). Multiplexed libraries were sequenced on the Illumina HiSeq 2500 platform as 150 bp paired-end (PE) reads. Library construction and sequencing (150 bp PE) for an additional 10 samples was outsourced to Novogene, Singapore (https://en.novogene.com/). We additionally reanalyzed 11 retinal transcriptomes previously published in Musilova et al. (2019a). Together, then, our data set comprised 53 samples of which, based on morphology, 26 were classified as larvae, 6 as juveniles and 21 as adults. Sample IDs, number of raw reads, individual accession numbers for BioProject PRJNA754116 and further parameters are listed in table 1. Single genes extracted from the transcriptomic data are available in Supplemetary file 1.

The sequence data was quality-checked using FastQC (Andrews 2017). Opsin gene expression was then quantified using Geneious software version 11.0.3 (Kearse et al. 2012). For each sample we first mapped the reads against a general fish reference data set comprising all visual opsin genes from the Nile tilapia, *Oreochromis niloticus*, and the zebrafish, *Danio rerio*, with the Medium-sensitivity settings in Geneious. This enabled us to identify cone and rod opsin specific reads. If present, paralogous genes were subsequently disentangled following the methods in Musilova et al. (2019a) and de Busserolles et al. (2017). Briefly, we created species-specific references of the expressed opsin genes and their several copies (Musilova et al. 2019a) and remapped the transcriptome reads with medium–low sensitivity to obtain copy-specific expression levels. If multiple opsin genes were found to be expressed, we report their proportional expression in relation to the total opsin gene expression (fig. 1). We used the same pipeline to quantify expression of phototransduction cascade genes in five focal deep-sea species (fig. 2 and table S1), and to search for the expression of the *cyp27c1* gene (table S2).

To analyze key amino-acid substitutions in *RH1 and RH2* and potential shifts in their absorbance, we first translated the opsin coding sequences into amino acid sequences, and then aligned them with the bovine *RH1* (GenBank Acc.No: M12689). We have specifically focused on the positions identified as key-tuning sites in Yokoyama (2008) and Musilova et al. (2019a). For details, see tables 2 and 3. Unfortunately, we were not able to estimate the sensitivity shift of rod opsin copies in *C. warmingii* as only four of the amino acids that were substituted at the 15 key-tuning amino acid sites corresponded with previously tested cases (Yokoyama 2008; Musilova et al. 2019a). Out of the three copies, *RH1-2* has three out of four longer-shifting amino acid variants in these four sites and we assume it is therefore red-shifted. *RH1-1* is most likely sensitive to 488 nm, and *RH1-3*, being the shortest, to 468 nm (fig. 3C).

A data set containing *RH1* opsin gene sequences mined from our newly sequenced transcriptomes and genomes and additional *RH1*s obtained from GenBank (GenBank accession numbers listed in fig. 3), were aligned using the MAFFT (Katoh et al. 2009) plugin as implemented in Geneious, and a phylogenetic tree was subsequently reconstructed using MrBayes version 3.2.1 (Ronquist et al. 2012) (fig. 3A). Trees were produced using the Markov chain Monte Carlo analysis which ran for 1 million generations. Trees were sampled every 100 generations, and the printing frequency was 1,000, discarding the first 25% of trees as burn-in. The evolutionary model chosen was GTR model with gamma-distributed rate variation across sites and a proportion of invariable sites. Posterior probabilities (PP) were calculated to evaluate statistical confidence at each node. We used the same approach with an *RH2*-specific reference data set to reconstruct the phylogenetic relationship between the transcriptome-derived deep-sea *RH2* genes (fig. 3B).

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

Acknowledgments

We would like to express our thanks to both scientific and technical crew of the Maria S. Merian and Walther Herwig III research cruises in 2015, 2017, and 2020. In addition, we thank Tina Blancke for help with the sample management, and Veronika Truhlářová for technical support and lab management. We would also like to thank three anonymous reviewers for their comments improving the final version of the manuscript. NL and ZM were supported by the Swiss National Science Foundation (PROMYS-166550), ZM by the PRIMUS Research Programme (Charles University), the Czech Science Foundation (21-31712S) and the Basler Stiftung fuer Experimentelle Zoologie, and FC by an Australian Research Council (ARC) Discovery Early Career Award (DECRA) Fellowship (DE200100620).

Data Availability

Newly sequenced transcriptomes and genomes have been submitted to the SRA database in NCBI under BioProject accession number PRJNA754116. Accession numbers for individual samples are listed in Table 1, and single genes extracted from the transcriptomic and genomic data are available in the Supplementary file 1.

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- CHAPTER 2 –

Lupše, N., Kłodawska, M., Truhlářová, V., Košátko, P., Kašpar, V., Bitja Nyom, AR., Musilova, Z. (2022). Developmental changes of opsin gene expression in ray-finned fishes (Actinopterygii). *Proceedings of the Royal Society B: Biological Sciences* 289:20221855; (doi:10.1098/rspb.2022.1855)

PROCEEDINGS B

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Research

Cite this article: Lupše N, Kłodawska M, Truhlářová V, Košátko P, Kašpar V, Bitja Nyom AR, Musilova Z. 2022 Developmental changes of opsin gene expression in ray-finned fishes (Actinopterygii). *Proc. R. Soc. B* **289**: 20221855. https://doi.org/10.1098/rspb.2022.1855

Received: 16 September 2022
Accepted: 7 October 2022

Subject Category:
Evolution

Subject Areas:
evolution

Keywords:
opsin, evolution, Actinopterygii, development, vision, gene expression

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Electronic supplementary material is available online at https://doi.org/10.6084/m9.figshare.c.6251658.

THE ROYAL SOCIETY PUBLISHING

Developmental changes of opsin gene expression in ray-finned fishes (Actinopterygii)

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Fish often change their habitat and trophic preferences during development. Dramatic functional differences between embryos, larvae, juveniles and adults also concern sensory systems, including vision. Here, we focus on the photoreceptors (rod and cone cells) in the retina and their gene expression profiles during development. Using comparative transcriptomics on 63 species, belonging to 23 actinopterygian orders, we report general developmental patterns of opsin expression, mostly suggesting an increased importance of the rod opsin (*RH1*) gene and the long-wavelength-sensitive cone opsin, and a decreasing importance of the shorter wavelength-sensitive cone opsin throughout development. Furthermore, we investigate in detail ontogenetic changes in 14 selected species (from Polypteriformes, Acipenseriformes, Cypriniformes, Aulopiformes and Cichliformes), and we report examples of expanded cone opsin repertoires, cone opsin switches (mostly within *RH2*) and increasing rod : cone ratio as evidenced by the opsin and phototransduction cascade genes. Our findings provide molecular support for developmental stage-specific visual palettes of ray-finned fishes and shifts between, which most likely arose in response to ecological, behavioural and physiological factors.

1. Introduction

Fish visual systems are very diverse, and they vary in morphology, physiology and spectral sensitivity [1–3]. Vertebrate vision is enabled by cone and rod photoreceptors in the retina, which carry light-sensitive molecules composed of an opsin protein bound to a light absorbing, vitamin A-derived chromophore [4]. In fishes, there are usually four types of cone opsins (*SWS1* and *SWS2*; commonly found in single cones, whereas *RH2* and *LWS* in double cones; with the respective peak sensitivity ranges of 347–383 nm, 397–482 nm, 452–537 nm and 501–573 nm; [2]) used for photopic and colour vision, and one rod opsin (rhodopsin, *RH1* or Rho) for scotopic vision in dim-light conditions [2]. Through gene duplications followed by functional diversifications, extant teleost fishes reached a median of seven cone opsin genes within their genomes [5]. Throughout the phylogeny, teleost genomes contain more copies of double-cone genes (middle and longer wavelength sensitive; *RH2* and *LWS*) than that of single cones (shorter wavelength *SWS1* and *SWS2*). While the *SWS1* is often missing from the genome or seen in one, at best two copies [3] and *SWS2* seen in up to three copies [6], teleost genomes can contain up to eight copies of *RH2* [7] and up to five copies of *LWS* [8]. Unlike cone opsins, rod opsin duplicates are rarely

found, most often in mesopelagic lineages [5,9,10]. Higher copy number is considered beneficial by providing more 'substrate' for selection, as well as for alternative gene expression of the variants within the opsin type.

The formation of the eye, and expression of opsin genes, starts at the embryonic stage [11,12]. Still, eyes continue to grow, and new photoreceptors are being added throughout life [13]. Within the retina, cone photoreceptors are first to develop, followed by temporally and spatially distinct rods [14–16]. For example, in zebrafish, photoreceptor progenitor cells start out by first differentiating into cones before rods are added later during development [17], suggesting that vision changes with age. This cone-to-rod developmental sequence is likely shared across vertebrates (Atlantic cod: [18]; zebrafish: [17]; mice: [19]; rhesus monkey: [20] and appears to hold even for teleost species with an all-rod retina in the adult stage [10]).

Photic conditions can change spatially and temporally, resulting in a visually heterogeneous environment in which visual systems of fishes are expected to be under natural selection that favours those that match the local environment [21]. For example, longer and shorter wavelengths are scattered and filtered out with increasing water depth and consequently, fishes living in deep-water habitats such as sculpins of Lake Baikal [22], cichlids of lakes Malawi and Tanganyika [23,24], and African crater lakes [25,26], as well as deep-sea fishes [10,27] have visual systems sensitive to the blue-green part of the visible spectrum. Adaptation can be achieved either through functional diversification of opsin genes when mutations at key-spectral tuning sites shift the peak spectral sensitivity (λ_{max}) of the photopigment [28,29], or by regulation of the opsin gene expression. This can be achieved when a subset of opsin genes is expressed and altered among or within species and even within the same individuals during ontogeny [10,21,30,31].

Before reaching the juvenile or sexually mature adult stage, fish larvae undergo major anatomical, physiological, behavioural and quite often, ecological changes [2,32]. The developmental shift in habitat preference is often suggested to drive ontogenetic changes in opsin expression (e.g. cichlids: [21,33]; black bream: [34]; eel: [35]; squirrelfishes and soldierfishes: [36]; clown anemonefish: [37]; damselfishes: [38]; bluefin killifish: [39]; gambusia: [40]; rainbow trout: [41]; dottybacks: [42]; starry flounder: [43]; deep-sea fishes: [10,44]). However, habitat-related changes of photic conditions solely do not always result in different and stage-specific visual system modifications, as seen in the Atlantic cod [18] or the spotted unicornfish [45]. Shifts in diet (planktivory, carnivory and herbivory) and activity patterns (diurnal, nocturnal and crepuscular) [36,46,47], in addition to developmental or phylogenetic constraints seem to also play a role in shaping the visual diversity of fishes and potential age-related shifts of it.

Here, we aim to investigate ontogenetic changes of opsin and phototransduction cascade gene expression across ray-finned fishes, to estimate the presence and relative abundance of opsin gene classes, and to elucidate general and/or taxon-specific patterns. For the purpose of this study, we have sequenced and analysed (i) retinal transcriptomes of different developmental stages of 14 species, belonging to five major actinopterygian orders: *Polypterus senegalensis* (Polypteriformes), *Acipenser ruthenus* (Acipenseriformes), *Abramis brama* and *Vimba vimba* (both Cypriniformes), *Scopelarchus* spp. and *Coccorella atlantica* (both Aulopiformes), *Coptodon bemini*, *C. imbriferna*, *C. flava*, *C. snyderae*, *C. thysi*, *Sarotherodon linnellii*, *S. lohbergeri* and *Stomatepia pindu* (all Cichliformes from the Bermin and Barombi Mbo lakes). (ii) We have complemented this dataset by publicly available embryonic/larval/juvenile/adult transcriptomes belonging to 49 species and 21 orders, some of which have never been analysed for visual gene expression before. In total, the comprehensive dataset of 63 species from 23 ray-finned fish orders allows us to focus on the development of the opsin gene expression, and rod and cone cell identity throughout actinopterygian evolution.

2. Methods and materials

(a) Data and sample collection

Transcriptomes belonging to taxa deemed as focal groups, which were inspected for age-specific copies and presented in detail in figure 3, were obtained from specimens ($N = 73$) caught solely for the purpose of this study. In detail, 16 specimens were classified as larvae, 4 as juveniles, 3 as subadults and 50 as adults (figure 3; electronic supplementary material, table). *Polypterus senegalensis* larvae were collected in the rearing facility of the Department of Zoology, Charles University, and the adults were purchased from the aquarium trade. *Acipenser ruthenus* and cyprinids were collected at the rearing facility in Vodňany, and in local water bodies (adults: Velky Tisy pond, Klicava dam, Lipno dam; larvae: Vltava and Elbe rivers), Czech Republic, respectively. Both mesopelagic taxa, *Scopelarchus* spp. and *Coccorella atlantica*, were collected in the Sargasso Sea and originate from Lupše *et al.* [10]. Crater lake cichlids were collected in lakes Barombi Mbo and Bermin (Cameroon, West Africa) between 2013 and 2018 (research permit numbers: 0000047,49/MINRESI/B00/C00/C10/nye, 0000116,117/MINRESI/ B00/C00/C10/C14, 000002-3/MINRESI/B00/C00/C10/C11, 0000032,48-50/MINRESI/B00/C00/C10/C12). Larvae were caught by fine-meshed nets and fixed in RNAlaterTM immediately. Adults were collected using gill nets and selective capturing by snorkelling in the shallow-water zone. For all species, fin clips were taken from specimens and stored in 96% EtOH for subsequent molecular analyses. Larval samples were fixed in RNAlaterTM (ThermoFisher) and stored at −80°C until further use. Adults of all species were euthanized on site with eyes or retinae extracted, fixed in RNAlaterTM and stored at −80°C upon arrival at the laboratory.

To obtain publicly available transcriptomes used in this study (figure 1; electronic supplementary material, table), we searched the largest publicly available repository of high-throughput sequencing data, the Sequence Read Archive (SRA), using the following topic search term: *'(embryo* OR larva* OR juvenile* OR adult*) AND (retina* OR eye* OR head* OR whole*) AND (taxon name * OR fish*)'*. Whenever possible, we have analysed up to three specimens per stage per species (figure 1; electronic supplementary material, table). In the case of embryos, specimens closest to hatching were analysed. The entire dataset analysed, including de novo transcriptomes described below, includes 215 samples of which, based on morphology, 56 were classified as embryos, 40 as larvae, 25 as juveniles, 3 as subadults and 91 as adults (figures 1 and 3; electronic supplementary material, table). Sample IDs, number of raw reads, individual accession numbers for BioProject PRJNA841439 and further parameters are listed in the electronic supplementary material, table.

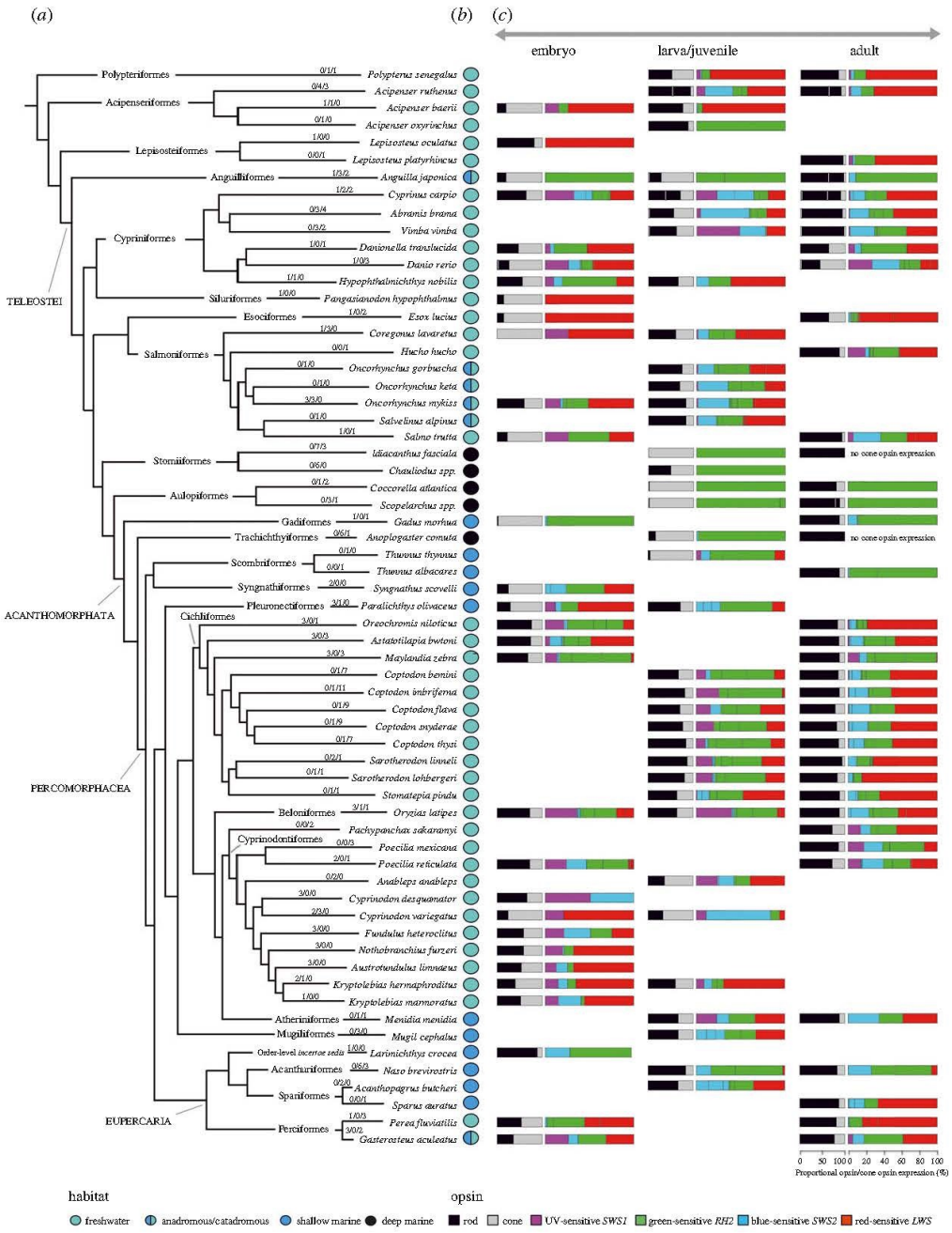

Figure 1. Opsin gene expression in different developmental stages of ray-finned fishes (Actinopterygii). (*a*) Simplified phylogeny of the 63 species, belonging to 23 orders, for which the transcriptomes were analysed (topology after [48]). Numbers above branches represent number of individuals per developmental stage analysed (embryo/larva + juvenile/adult). (*b*) Information on habitat preference, obtained from https://www.fishbase.de. Separation between the shallow and deep marine species is 200 m. Information on depth obtained from https://obis.org/. (*c*) Proportional opsin gene expression (horizontal bars) at different developmental stages. First (shorter) bar represents mean proportional expression of rod and cone opsins. Cone opsin expression (grey) is depicted as the sum of the expression of all four classes of cone opsin genes (*SWS1*, *SWS2*, *RH2* and *LWS*). If several rod opsin genes (black) were expressed, the different proportions of their expression are distinguished with white vertical bars. Second (longer) bar represents mean proportional expression of different cone opsins. Black vertical bars within gene classes separate different copies, if co-expressed. For details, see electronic supplementary material, table. (Online version in colour.)

(b) Transcriptome sequencing and analyses

Total RNA was extracted from the whole eyes or retinal tissue using either the RNeasy micro- or mini-kit (Qiagen). The extracted RNA concentration and integrity were verified on a 2100 Bioanalyzer (Agilent) and Qubit Fluorometer (Thermofisher Scientific). RNA-seq libraries were constructed

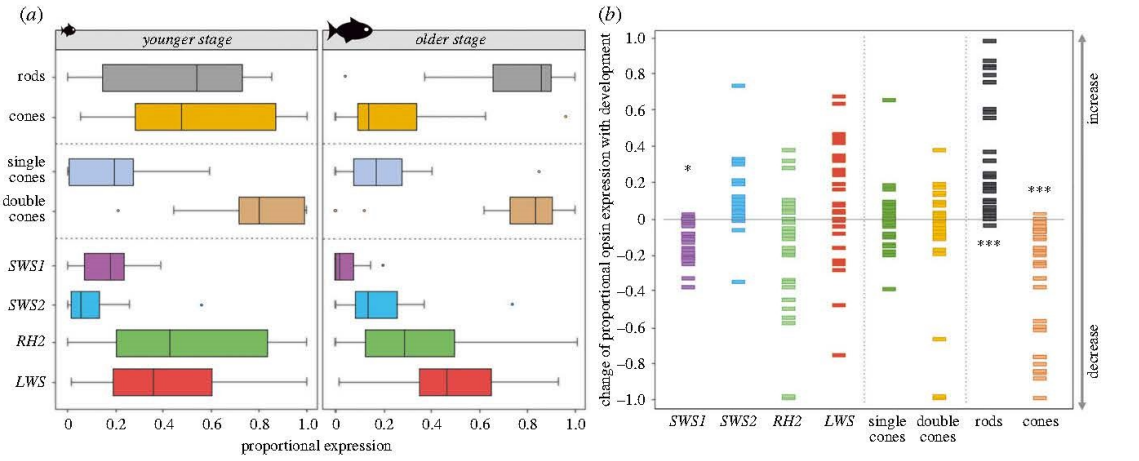

Figure 2. General patterns of age-related opsin expression changes. (*a*) Interquartile ranges (25th and 75th percentiles) and whiskers show data dispersion (proportional expression) across different opsins for the youngest and oldest analysed stage. Data medians are presented as solid vertical lines. To avoid over-representation of certain taxa (e.g. five Coptodon species), data points ($N = 32$) represent mean genus values and are comprised only of species that had at least two developmental stages analysed. (*b*) Change of opsin expression (positive/negative) with development, calculated as a difference between the mean opsin expression in the oldest and the youngest stage of a certain genus. Resulting values are represented by rectangles ($N = 32$), centred at the mean. The lower half of the plot (values below 0.0) shows a decrease, and the upper half (values above 0.0) an increase in proportional expression with age. Significant differences found by beta regression models are marked by asterisks (table 1 for details). (Online version in colour.)

in-house from unfragmented total RNA using Illumina's NEBNext Ultra II Directional RNA library preparation kit, NEBNext Multiplex Oligos and the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs). Multiplexed libraries were sequenced on the Illumina HiSeq 2500 platform as 150 bp paired-end reads. The sequence data was quality checked using FastQC [49]. Opsin gene expression was then quantified using Geneious software version 11.0.3 [50]. For each sample, we mapped the reads against a general genomic reference dataset for all visual opsin genes composed of Nile tilapia, zebrafish and the long-nose gar, using the Medium-sensitivity settings in Geneious. This enabled us to capture most of the cone and rod opsin-specific reads and create species-specific opsin references. If needed, paralogous genes were subsequently disentangled following the methods in Musilova *et al.* [5] and de Busserolles *et al.* [51]. Transcriptome reads were then re-mapped to the newly created (species-specific) references with medium-low sensitivity to obtain copy-specific expression levels. We report opsin gene proportional expression in relation to the total opsin gene expression which was calculated using FPKM (Fragments Per Kilobase of transcript Per Million reads), taking into account the library size, the length of each gene and number of mapped reads (electronic supplementary material, table). The above-mentioned quantification of opsin gene expression was also used on transcriptomes obtained from SRA. Identical pipeline was used for the quantification of *GNAT1*/2 genes in selected taxa (figure 3).

(c) Statistical analyses

To formally test whether opsin gene expression differs between developmental stages, we applied the beta regression models specifically designed to analyse the proportional datasets and percentages. We used the R package betareg [52], which allows handling of non-transformed data. The beta distribution has a highly flexible shape and is, hence, suitable to fit the dependent variable (in our case the proportional expression of each opsin gene) in the standard unit interval (0,1) with a mean related to a set of categorical regressors (in our case developmental stage). We tested the difference for each cone opsin gene class separately (i.e. *SWS1*, *SWS2*, *RH2* and *LWS*), then for the sum of single-cone (*SWS1* + *SWS2*), and double-cone opsins (*RH2* + *LWS*), and additionally also for rods (*RH1*) and cones (*SWS1* + *SWS2* + *RH2* + *LWS*).

3. Results and discussion

(a) General developmental patterns of opsin gene expression across the actinopterygian phylogeny—cone-to-rod developmental constraint

The analysis of the opsin gene expression in 63 ray-finned fishes revealed that, generally, the ratio of the rod opsin (*RH1* or Rho, λ_{max}: 447–525 nm) to cone opsin expression increases with age in analysed species (figures 1 and 2, table 1; electronic supplementary material, table; $p \leq 0.001$). This is in accord with the cone-to-rod development of the retina which starts with cone cells, and rods appearing only later [10,17,18]. The increasing rod : cone cell ratio is further confirmed by the expression of the phototransduction cascade gene *GNAT1* (rod specific) versus *GNAT2* (cone specific), figure 3*b*. Rod opsin and *GNAT1* usage increases significantly already during the larval and juvenile stage, before finally transforming into sexually mature adults with rod-dominant retina (figures 1 and 2; electronic supplementary material, table). It thus seems that larval vision is mostly driven by cone vision, while the ability to perform well in low-light conditions appears consequently, at later

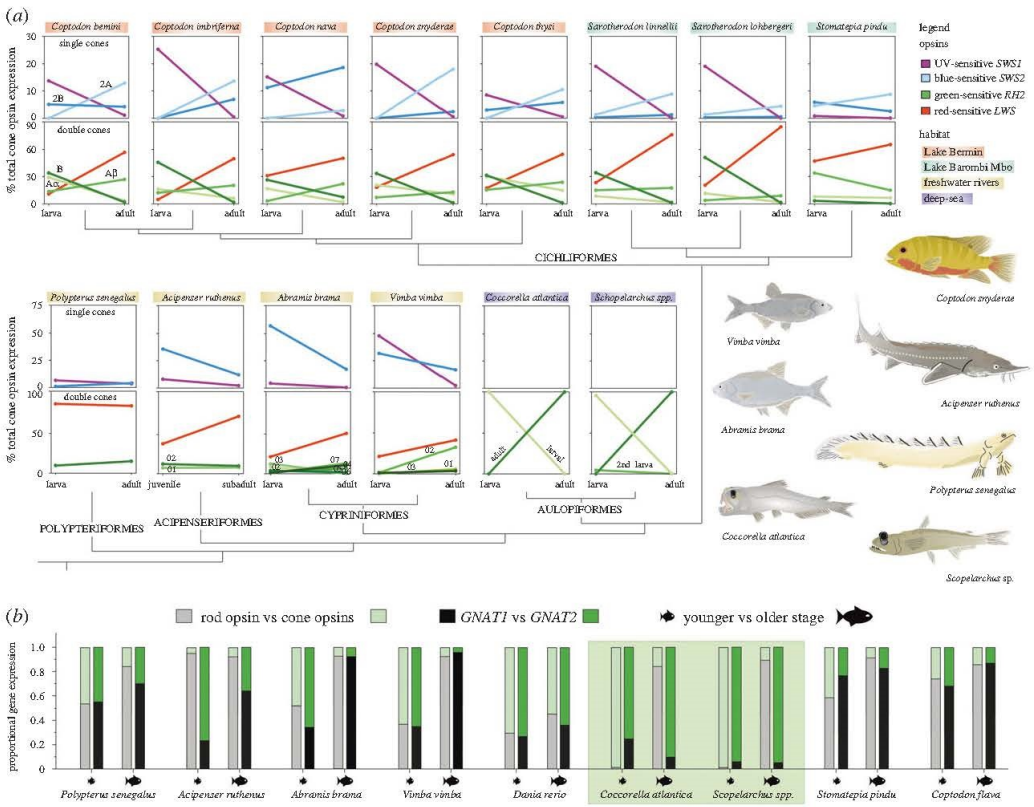

Figure 3. Cone opsin gene switches, age-specific copies and phototransduction cascade gene expression of representative taxa specifically sequenced for this study. (*a*) Detailed presentation of ontogenetic changes of opsin expression in selected polypteriform, acipenseriform, cypriniform, aulopiform and cichliform species. Interconnected dots are coloured according to specific single- and double-cone opsins and present mean expression values for specific developmental stages. In cases of gene duplications, copies are named and coloured with different shades. *Y*-axis scale, which is identical for all species depicted in the same row but differs between Cichliformes and all other fish orders, is labelled only on the left most axis. For details on number of individuals and exact values, see electronic supplementary table. (*b*) Ontogenetic changes of rod/cone opsin gene expression, and to it related shifts in expression of phototransduction cascade genes GNAT1 (rod specific) and GNAT2 (cone specific) for selected teleost taxa. Highlighted in green are special cases of the two aulopiform species that exhibit a discordance between the dominating opsin type (rod specific) and phototransduction cascade genes (cone specific) in adults [10]. (Online version in colour.)

Table 1. Statistical comparison between the younger and older developmental stages for 32 ray-finned fish genera. Summary of beta regression models specifically aimed at proportional datasets (opsin expression as a dependent variable from developmental stage) with the obtained *p*-values. Alpha levels of significance after the Bonferroni correction additionally marked as equivalent to: <0.001*** and <0.05*.

opsin gene(s)	*p*-value
SWS1	0.005*
SWS2	0.040
RH2	0.469
LWS	0.675
rods	1.6×10^{-9}***
cones	7.6×10^{-10}***
single cones	0.950
double cones	0.302

developmental stages [32,53]. Functionally, rods generally allow for an improvement in visual acuity and startle responses in fishes [54–56] and are also associated with motion sensitivity and the appearance of novel behaviours, such as schooling [57]. More specifically, higher rod expression increases individual performance of fishes living in the deep-sea [10,51]. Additionally, laboratory experiments have shown that the ability to follow a rotating stripe pattern (the optomotor drum) might be dependent on rod formation and retinal development, as it is not seen in stages or specimens lacking rods [58–60].

In the selected taxa (figure 3), we have specifically focused on the rod versus cone identity by quantifying the expression of the phototransduction cascade gene *GNAT1* or *GNAT2*, respectively. We found correspondence between the expression of phototransduction cascade gene type and the opsin type (i.e. cone *SWS1*, *SWS2*, *RH2*, *LWS* and rod *RH1*), and detected a clear increase of *GNAT1* : *GNAT2* ratio with ageing, with the exception of the Aulopiformes deep-sea fishes. In this group, a discordance between the dominating opsin type (rod specific) and phototransduction cascade genes (cone specific) in adults challenges the rod versus cone identity and suggests a presence of possibly partially transmuted photoreceptors, potentially similar but not identical to other vertebrates (snakes and geckoes: [61,62]; deep-sea fishes: [10,51,63]; salamanders: [64]). The overall intriguing

visual system of aulopiforms, hence, definitely needs to be investigated further and in more detail (figure 3, [10]).

(b) Developmental switch of the short-wavelength-sensitive opsin genes

A trend of age-related shifts in expression also appears within cone opsins (table 1). Our dataset shows a clear decrease in proportional expression of the UV-sensitive *SWS1* (λ_{max}: 347–383 nm) with age ($p = 0.005$; table 1). Although *SWS1* expression is usually low, it seems to be expressed more in early stages throughout the phylogeny (figure 1, table 1). On one hand, UV radiation can result in larval mortality; to mitigate negative effects of exposure, UV avoidance through detection of UV light and adjustments of vertical position is expected [65,66]. On the other hand, distinguishing wavelengths belonging to the UV part of the visual spectrum aids younger individuals that feed on zooplankton [67–69]. With ageing and a shift in diet, UV opsin expression might become irrelevant for some species [70], thus potentially explaining why some adults do not express *SWS1* (e.g. *Naso brevirostris* and *Oryzias latipes*), while others still do (e.g. *Danio rerio*, *Poecilia reticulata* and cichlids) (figure 1; electronic supplementary material, table). Adult expression of *SWS1*, when seen, seems to play a role in species and/or colour discrimination and mate selection (guppies: [71]; damselfishes: [72]; cichlids: [21]), male aggression (sticklebacks: [73]) or is associated with migration events (salmonids: [41]). The blue-sensitive *SWS2* cone opsin (λ_{max}: 397–482 nm) expression generally increases with age and generally replaces the *SWS1* gene in the single cones (figures 1 and 2; electronic supplementary material, table, table 1). Interestingly, while some fish (e.g. sturgeons and cyprinids) seem to ontogenetically decrease the proportion of both *SWS1* and *SWS2* opsins, other fish groups (e.g. cichlids) replace one type by another (figure 3). This switch in single-cone opsin expression between *SWS1* and *SWS2* has been shown before, e.g. by Spady *et al.* [74] in Nile tilapia or by Cheng and Flamarique [75] in rainbow trout, and it mostly keeps the total single-cone opsin expression similar between different developmental stages (figure 2).

(c) Middle- and long-wavelength-sensitive opsins in double cones

The ontogenetic switch in expression also occurs between the green-sensitive *RH2* (λ_{max}: 452–537 nm) and the red-sensitive *LWS* (λ_{max}: 501–573 nm) cone opsin types; plus switching between different *RH2* copies also commonly occurs (figure 3). Values for these typically double-cone opsins vary considerably across the fish phylogeny, albeit a possible weak general trend of a decrease in relative expression of *RH2*, and an increase of *LWS* with age is noticable (figures 1 and 2; electronic supplementary material, table; not significant—table 1), except for groups that completely lost the *LWS* opsin gene. In general, medium-wavelength opsins appear to be of use to all stages (figures 1 and 2; electronic supplementary material, table), perhaps due to the general presence of corresponding wavelengths in most habitats. Our overview data further seem to show that freshwater species exhibit dominance of red-sensitive *LWS* opsin gene expression, whereas in marine species, green-sensitive *RH2* gets to be more dominant (with exceptions) (figure 1). Namely, for species inhabiting the spectrally narrower deep sea at least during certain parts of their lives (Stomiiformes, Aulopiformes, Trachichthyiformes, Anguilliformes and Gadiformes), *RH2* seems to be the most important, if not the only cone opsin expressed (figure 1, [10]). On the other hand, the expression of *LWS* in adults might be a response to inhabiting freshwater habitats, such as turbid rivers and murky, eutrophic lakes (e.g. Lake Victoria) where usually, longer wavelengths penetrate to greater depths and are the most prevalent colour of the ambient light [21,76]. The expression of *LWS* is also beneficial for foraging in herbivorous reef fishes, providing them with the visual ability to discriminate benthic algae from coral reef backgrounds [77,78]. In some cases, increased *LWS* expression and expanded *LWS* repertoires might also be explained by sexual selection (e.g. in Poeciliidae), where females evolved mate preferences for red and orange male colouration [79].

(d) Age-specific cone opsin gene copies in the selected taxa

We have specifically focused and de novo sequenced retina transcriptomes of larvae/juveniles and adults of 14 actinopterygian species belonging to five orders spanning the ray-finned fish phylogeny. Apart from the aforementioned rod versus cone identity assessed by *GNAT* genes, we have additionally focused on switches between copies of the same opsin type in the selected taxa (figure 3; electronic supplementary material, table). Namely, we studied the visual opsin gene repertoire in two basal non-teleost fish groups, bichirs (Polypteriformes) and sturgeons (Acipenseriformes), and in teleost riverine cyprinids (Cypriniformes; Ostariophysi), crater lake cichlids (Cichliformes; Euteleostei) and deep-sea pearleyes and sabretooths (Aulopiformes; Euteleostei). The overall expression patterns are in most cases in accord with the general patterns discussed above (figure 3; electronic supplementary material, table), with exceptions seen in the deep-sea fishes (based on our earlier data from [10]).

In all species but the bichir, we found multiple copies within at least one opsin gene type, namely within the rod *RH1* opsin, and cone *SWS2* and *RH2* opsins. In some species (cyprinids, sturgeon, *Scopelarchus* spp.) we found simultaneous expression of two rod *RH1* copies (figure 1; electronic supplementary material, table). All three groups possess the two *RH1* genes in their genome resulting from three independent ancestral gene duplication events [3,10]. The *RH1* gene duplicates were lost in the later evolution of the euteleost crown group, and hence most teleost species carry only one *RH1* copy, a phenomenon similar to that seen in 'non-fish' vertebrates. These *RH1* copies do not show any sign of ontogenetic switch in studied species, as known, e.g. for eels [80]. On the other hand, we detected several cases of stage-specific copies within cone opsin genes. While *Acipenser ruthenus* and *Abramis brama* + *Vimba vimba* express only one *SWS2* copy, cichlids express two different *SWS2* genes (figure 3; electronic supplementary material, table); this corresponds to multiple copies found in their genome due to the neoteleost- and percomorph-specific *SWS2* gene duplications [6]. Most examined species show an expanded *RH2* repertoire (figure 3; electronic supplementary material, table) and the existence of clearly larval and adult-specific copies has been observed in cyprinids, cichlids and in the deep-sea aulopiforms (figure 3). The expression of multiple copies might

enhance colour vision by increased spectral resolution useful in a particular environment; however, reasons for these opsin switches are not yet completely understood. The presence of such stage-specific copies means that species adjust their vision to differing light environments not only through a change in opsin class expression, but also through preferential expression of opsin copies within a single class. In cichlids, a group for which the development of the visual system is probably best understood, a shift to longer wavelength copies is generally observed within a single opsin type (*RH2A* copies replacing *RH2B* with age) or among single-cone opsins (*SWS2* replacing *SWS1*) and has been reported before for different groups of cichlids (e.g. Malawi, [12]; Nile tilapia, [74]).

Mesopelagic deep-sea aulopiform species have a limited repertoire of cone opsin classes that reflects living in photon-depleted depths [10,26]. *Scopelarchus* spp. and *Coccorella atlantica* express only one cone opsin class, namely *RH2* (figure 3; electronic supplementary material, table). However, both expanded their *RH2* repertoires and express larval- and adult-specific copies that are thought to be functionally different and most likely best respond to different wavelengths shallow-water epipelagic larvae and mesopelagic deep-water adults encounter (figure 3; electronic supplementary material, table) [10]. Genomic analyses by Lupše *et al.* [10] reveal a total of three, and seven *RH2* cone opsin copies within the genomes of *Coccorella atlantica* and *Scopelarchus michaelsarsi*, respectively. Mesopelagic fish lineages in some cases expand rod opsin repertoires, which are better suited for dim-light conditions [10,26]. *Coccorella* and *Scopelarchus*, however, seem to inhabit relatively shallower and photon-richer depths than some other deep-sea fishes, such as Stomiiformes, and might thus benefit also from having extra copies of cone opsins [10].

We have collected a robust dataset combining not only our own, but also publicly available genetic data, deposited in databases. This allowed us to detect shared versus specific expression patterns among different fish groups. We are aware that the collected dataset has certain limitations and that many factors could not be controlled in this study. For example, this dataset is highly dependent on publicly available material, so there is no control over several potentially relevant factors, such as the sampling conditions, intraspecific variability, other tissues sequenced together with the eyes (as in the entire embryos), etc. Since not all stages are available for all species, we do not present any typical 'developmental time series' but rather snapshots of embryos, larvae, juveniles and adults; consequently, more subtle or time-restricted expression patterns could not be detected here. For the purpose of statistical analyses, we have restricted the public dataset only to species with two (or more) stages found (figure 2). To complement the public data we also include our own, controlled data in more detail (figure 3). Despite certain limitations, our combined dataset provides robust evidence for expression patterns shared across distantly related fish groups, as it highlights general trends, and more detailed conclusions achieved through in-detail analyses of species specifically sequenced within this study.

4. Conclusion

To conclude, this study aimed to identify general patterns of the visual opsin gene expression shared among ray-finned fishes, and to detect similarities in the ontogenetic changes between opsin gene types. We found that the rod : cone opsins ratio increased with age in fish species, supporting the conserved cone-to-rod developmental pathway. We also report the increasing importance of the *LWS*, and the decreasing importance of the *SWS1* opsin genes with age, observed across ray-finned fish phylogeny (e.g. in sturgeons, cyprinids and cichlids). We have further detected the existence of different stage-specific *RH2* copies, which are switched during development. To conclude, fish visual systems are evolutionary and developmentally very dynamic and future studies focused on particular fish groups promise to throw further light on exact mechanisms, patterns and reasons for this extreme sensory system diversity.

Data accessibility. The raw Illumina reads from RNA-seq of all studied individuals are deposited into the NCBI BioProject database with ID PRJNA841439. Individual accession numbers are listed in the electronic supplementary material, table.

The data are provided in the electronic supplementary material [81].

Authors' contributions. N.L.: conceptualization, data curation, formal analysis, methodology, visualization, writing—original draft and writing—review and editing; M.K.: methodology and writing—review and editing; V.T.: methodology and writing—review and editing; P.K.: methodology and writing—review and editing; V.K.: methodology and writing—review and editing; A.R.B.N.: methodology and writing—review and editing; Z.M.: conceptualization, formal analysis, methodology, visualization, funding acquisition, project administration, supervision and writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

Funding. N.L. and Z.M. were supported by the Swiss National Science Foundation (PROMYS – 166550), Z.M. by the PRIMUS Research Programme (Charles University), the Czech Science Foundation (21-31712S) and the Basler Stiftung fuer Experimentelle Zoologie. V.T. and P.K. were supported by GAUK (grant no. 1767618). V.K. was funded by Ministry of Education, Youth and Sports of the Czech Republic—the project Reproductive and Genetic Procedures for Preserving Fish Biodiversity and Aquaculture (CZ.02.1.01/0.0/0.0/16_025/0007370).

Acknowledgements. We would like to express our deep gratitude to Adrian Indermaur, Dmytro Omelchenko, the FishEcu team and Petra Horká for their help with the field sampling, and the Robert Černý group for providing the bichir larvae. We thank local people of the Barombi and Bemé village for allowing us to fish in the Barombi Mbo and Bermin lakes, and the Ministry of Scientific Research and Innovation in Cameroon for granting us research permits.

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- CHAPTER 3 –

Lupše, N., Indermaur, A., Bitja Nyom, AR., Musilova, Z. Plasticity of the visual system in the cichlids from Barombi Mbo and Bermin crater lakes. *Manuscript in preparation*.

Plasticity of the visual system in the cichlids from Barombi Mbo and Bermin crater lakes

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Keywords: opsin, evolution, Cichlidae, development, vision, gene expression

Abstract

Visual system in fish is known to adapt to the environmental conditions, either in evolution by nucleotide mutations and selection, or in development by phenotypic plasticity. Amongst vertebrates, cichlid fishes (f. Cichlidae) are one of the most noted examples of adaptive radiation, where plasticity is commonly reported. As an extremely speciose and ecologically diverse, yet evolutionary young group, cichlids have well-developed sensory systems, amongst which, vision seems to be primary. It is based on one rod, and seven cone opsin proteins – which arose through gene duplications - covering the entire spectrum from UV to red. Here, we investigate the development and phenotypic plasticity of opsin gene expression in two Cameroonian crater lake cichlids, *Coptodon flava* (Lake Bermin) and *Stomatepia pindu* (Lake Barombi Mbo), which we experimentally exposed to different ambient light treatments. We found the strongest plastic response of the visual system when fish developed in the long-wavelength (red) shifted ambient light, while the short- (blue) and middle- (green) wavelength triggered milder, yet noticeable plastic response. We found stronger response in the gene expression in the Bermin crater lake radiation compared to the Barombi Mbo cichlid radiation.

INTRODUCTION

The environment is an ever-changing heterogeneous entity to which organisms need to adapt to survive. Although accommodation to specific niches is achieved by genetic differentiation through generations (Schluter et al. 2000, Coyne & Orr 2004, Grant & Grant 2008), phenotypic plasticity – the emergence of multiple phenotypes from a single genotype in response to variation in the environment - represents a potentially much faster way of adaptation to a certain change in the environment (reviewed in Pfennig et al. 2010). Although previously documented across the animal kingdom (e.g., hymenopterans: Cahan et al. 2004, fish: Shaw et al. 2007, nematodes: Gutteling et al. 2007, arachnids: Brewer et al. 2015, squamates: Losos et al. 2000), it remains debatable how plasticity mediates evolution. Some argue that adaptive phenotypic plasticity allows populations to persist in novel environments by promoting survival and reproduction of phenotypically plastic individuals, thus providing a "genetic substrate" for a slower-paced selection and evolutionary change in the direction of the initial plastic response. Contrastingly, it is believed that non-adaptive phenotypic plasticity moves these novel phenotypes away from a local optimum, which in turn promotes evolutionary change through an increase in the strength of selection (Losos et al. 2000, Grether 2005, Crispo 2007, Ghalambor 2007, Ghalambor et al. 2015).

Light conditions of aquatic habitats change widely with depth or turbidity due to absorption and scattering (Jerlov 1976, Land 2003). They can also change less predictably, either because of natural or anthropogenic causes, and can consequently affect the detection of visual cues that are key to vast array of behavioural tasks (Anderson et al. 2002, Stuart-Fox et al. 2003, Sandkam et al. 2015, Gaston et al. 2017). Fishes occupy a variety of different aquatic habitats, and their visual systems are very diverse. The retina of their high-resolution image-forming camera eye consists of two photoreceptor types, i.e., cones (photopic vision) and rods (scotopic vision) (Lamb 2013). The membrane of the photoreceptor's outer segment contains photopigments, namely opsin proteins and chromophores (Lamb 2013). Rods possess rod opsins; cones, on the other hand, diversified into many different subclasses: ultraviolet-sensitive *SWS1*, blue-sensitive *SWS2*, green-sensitive *RH2* and red-sensitive *LWS*, and some of these classes exhibit functionally different duplicates (e.g., Carleton et al. 2020).

Fish visual systems differ immensely and are the product of selection for optimal vision, i.e. spectral tuning to a certain photic condition (Carleton et al. 2016). Adaptations to diverse ecological and visual demands, however, can be achieved either through duplication or gene loss (Carleton et al. 2020), functional (spectral) sensitivity-shifting diversifications of opsin

genes (Yokoyama 2008), or by the epigenetic regulation of the opsin gene expression (e.g., Luehrmann et al. 2018, Lupše et al. 2022).

The expression of opsin genes starts already at the embryonic stage when eyes and the retina, which continue to grow throughout life, form (Fernald 1995, Carleton et al. 2008). Fishes keep on adding photoreceptors with age, and just like other vertebrates, they seem to first develop cone photoreceptors (La Vail et al. 1991, Raymond 1995, Mears et al. 2001, Sernagor et al. 2006, Lupše et al. 2021). This general cone-to-rod progression supported by recent evidence of more specific developmental patterns observed across teleost fishes suggests that vision changes with age (Lupše et al. 2022). Moreover, aquarium-based experiments showed that teleost visual systems not only respond to habitat shifts through development – opsin gene expression also seems to adjust on a much shorter time scale, suggesting a certain degree of plasticity (damselfishes and cardinalfishes: Luehrmann et al. 2018; cichlids: Smith et al. 2012, Dalton et al. 2015, Härer et al. 2017, Nandamuri et al. 2017; killifish: Fuller et al. 2010, Fuller & Claricoates 2011; guppies: Ehlman et al. 2015; bream: Shand et al. 2008; cave mollies: Tobler et al. 2010).

Table 1: Literature review on phenotypic plasticity in fishes. * Colour treatments as stated by authors.

Species	Exposure begins as/sampled as	Color treatment*	Sampling at	Response	Study
Pomacentrus moluccensis, Pomacentridae	Adults/adults	- red - blue - green	- 1 month - 4 months - 6 months	Plastic (especially single cones)	Luehrmann et al. 2018
Pomacentrus amboinensis, Pomacentridae	Adults/adults	- red - blue - green	- 1 month - 4 months - 6 months	Plastic (especially single cones)	Luehrmann et al. 2018
Ostorhinchus cyanosoma, Apogonidae	Adults/adults	- red - blue - green	- 1 month - 4 months	Plastic (especially single cones)	Luehrmann et al. 2018
Metriaclima lombardoi, Cichlidae	Larvae/adults	- broad - narrow	- 6 months	Plastic (*LWS*, minimally also *SWS2B*)	Smith et al. 2012
Melanochromis auratus, Cichlidae	Larvae/adults	- broad - narrow	- 6 months	Plastic (*LWS*, minimally also *SWS2B*)	Smith et al. 2012

Metriaclima zebra, Cichlidae	Larvae/adults	- Full spectrum - red (and inverted)	- 6 months	Plastic (*LWS*, *RH2Aβ*, R*H2B, SWS1*	Dalton et al. 2015
Amphilophus cf. citrinellus, Cichlidae	Larvae/larvae+adults	- blue - cold white - warm white - red	- 1 week - 2 weeks - 6 months	Plastic throughout	Härer et al. 2017
Metriaclima mbenji, Cichlidae	Adults/adults	- narrow	- 3 days - 3 weeks - 5 weeks	Plastic *(*except *LWS, SWS2A* and *RH2A)*	Nandamuri et al. 2017
Metriaclima benetos, Cichlidae	Adults/adults	- broadband	- 3 or 7 days	Plastic (except *RH2A*)	Nandamuri et al. 2017
Lucania goodei, Fundulidae	Larve/adults	- clear water - "tea-stained"	- N/A ("sampled at maturation")	Plastic (*LWS*, *SWS2B* and *SWS1*)	Fuller et al. 2010
Lucania goodei, Fundulidae	Adults/adults	- clear water - "tea-stained"	- early (1 and 3 days) - middle (7 and 14 days) - late (21 and 28 days)	Plastic throughout	Fuller & Claricoates 2011
Poecilia reticulata, Poeciliidae	Larvae/adults	- clear water - turbid water	- N/A ("sampled at maturation")	Plastic (*RH2* and *LWS* copies)	Ehlman et al. 2015
Acanthopagrus butcheri, Sparidae	Larvae/adults	- broad spectrum - short wl-reduced	- larval - post-settlement - juvenile - adult	Plastic (mostly lws cone opsins)	Shand et al. 2008
Poecilia mexicana, Poeciliidae	Larvae/adults	- broad spectrum	N/A	Not plastic	Tobler et al. 2010

Cichlids (Cichlidae) are a group of tropical fishes noted for their morphological, behavioural and ecological diversity (Meyer 1993). They have had enormous success in occupying a vast array of ecological niches over a short period and are, as such, considered

classic models for adaptive radiation (Verheyen et al. 2003, Malinsky et al. 2018). Sensory systems, including vision, were key in shaping their evolution - their genomes contain one rod and seven cone opsin genes: *RH1*, *SWS1*, *SWS2A*, *SWS2B*, *RH2Aα*, *RH2Aβ*, *RH2B* and *LWS* (Carleton et al. 2016). However, it has been shown before that due to developmentally triggered ecological shifts, not all opsins are expressed at the same time (Fattah Ibrahim et al. 2015, Carleton et al. 2016, Lupše et al. 2022). Although East African lakes Victoria, Tanganyika and Malawi harbour the greatest and certainly the most studied cichlid assemblages (Carleton et al. 2016), smaller crater lakes of West Africa provide an excellent opportunity to study sympatric evolution and visual adaptation within smaller, but no less important assemblages (Turner 2007, Musilova et al. 2019b). Such are Cameroonian crater lakes Barombi Mbo and Bermin, with respective diameters of 2.5 km and 700m, which formed within the last million years ago (Trewavas 1972, Cornen et al. 1992, Stiassny et al. 1992). Barombi Mbo hosts 11 endemic and often morphologically and ecologically quite distinct cichlid species (Schliewen et al. 1994), and Bermin hosts a younger radiation of nine valid species (Stiassny et al. 1992). Both lakes undergo a seasonal variation in water clarity between the dry (November – April) and rainy season (June – August) (Trewavas et al. 1972), thus possibly providing a platform for visual plasticity.

To address the question of visual plasticity, we experimentally investigated opsin gene expression under different light regimes in two cichlid species, *Coptodon flava* (Barombi Mbo) and *Stomatepia pindu* (Barombi Mbo). With the aim of describing the effect ambient light has on ontogenetic patterns of opsin gene expression, and how these deviate from a species' developmental trajectory under full spectrum, we reared lab reared newly hatched individuals under different artificially induced light regimes (short wavelength, medium wavelength, long wavelength) and quantified their opsin gene expression at different ages from hatching until adulthood.

METHODS AND MATERIALS

Rearing conditions and sampling Wild fish were collected in lakes Barombi Mbo and Bermin (Cameroon, West Africa) between 2016 and 2018 (research permit numbers: 0000047,49/MINRESI/B00/C00/C10/nye, 0000116,117/MINRESI/ B00/C00/C10/C14, 000002-3/MINRESI/B00/C00/C10/C11, 0000032,48-50/MINRESI/B00/C00/C10/C12) and brought back to the aquarium breeding facilities of the Department of Zoology, Charles University (Czech Republic), where they were kept under standard fluorescence lamp

illumination. Laboratory born descendants (generation F2) were reared under different light regimes to test for changes in opsin gene expression. By using lab-reared second-generation fish, our aim was to eliminate unmeasured environmental effects. In more detail, after eggs have hatched and larvae started swimming freely (absence of egg-sack), we randomly sampled a subset of individuals for each species, i.e. stage zero, time point = 0. The remainder of individuals were equally split into four batches and put into four experimental tanks: the control or "full spectrum" (standard fluorescent illumination), "blue" (short-wavelength), "green" (medium wavelength) and "red" (long-wavelength) (Fig 1). Filtering was achieved using spectral filter sheets, which were put onto each of the tank's sides to allow for full effect (172 Lagoon Blue, 124 Dark Green, 182 Light Red; Lee Filters). Individuals were reared under these light regimes and randomly sampled at 7 days, 14 days, 1 month and 6 months – we chose 6 months as the final time point because many cichlids become sexually mature at that age and opsin expression reaches the adult phenotype (Carleton et al., 2008; O'Quin et al., 2011) (Fig 1). For exact number of individuals sampled at each stage (and for each light regime), and for details on their sizes, see Supplementary Table. Note that due to high mortality of *S. pindu* and low number of offspring this species has per clutch, we combined results of several experiments as to best cover all of the desired time points. For the duration of the experiment, fish were fed either live artemia or tubifex, depending on the age. All specimens were euthanised by the MS222 overdose. For the 7 days, 14 days and 1-month groups, whole fish were stored in RNAlater (ThermoFisher) at -80 °C; for 6-month-old fish, only eyes were kept stored. Regardless of the age, whole eyes were used for expression analyses.

Transcriptome sequencing and analyses Whole eye or retinal RNA was extracted using the RNeasy micro kit (Qiagen). After testing for RNA concentration and integrity on the 2100 Bioanalyzer (Agilent) and Qubit Fluorometer (Thermofisher Scientific), RNAseq libraries (as 150 bp paired-end reads) were outsourced at Biozentrum, Basel University (Switzerland) and sequenced on the Illumina HiSeq 2500 platform at the Genomics Facility Basel, Switzerland, or Novogene, Singapore (https://en.novogene.com/). Following the collection of data, FastQC was used for the quality-check of sequences (Andrews 2017) and opsin gene expression was quantified with Geneious software version 11.0.3 (Kearse et al. 2012). Our pipeline consisted of initially mapping reads against the genomic reference of a model cichlid species, the Nile tilapia, which enabled us to create species-specific references. For this, we used the medium-sensitivity settings. To obtain precise expression levels and capture all reads possible, we then re-mapped transcriptome reads with medium-low sensitivity to these newly created references.

Specimen information, number of raw reads and relative opsin gene expression that was calculated using FPKM (Fragments Per Kilobase of transcript Per Million reads) are presented in the Supplementary Table.

RESULTS AND DISCUSSION

Cichlid visual palettes under full spectrum light

Under full spectrum light, both cichlid species examined show a somewhat typical tilapian opsin gene expression development where younger individuals exhibit a visual palette more adapted to shorter wavelengths of light, as opposed to a longer shifted repertoire of adults (Carleton et al. 2008, Lupše et al. 2022) (Fig 2). In more detail and as seen in *Coptodon flava*, UV-sensitive cone (*SWS1*) opsin is mostly expressed in younger individuals and its expression becomes minimal as individuals sexually mature. On the other hand, the long-wavelength sensitive cone (*LWS*) opsin expression increases with age. Within the short-wavelength sensitive cone (*SWS2*) opsin class, only adults seem to express the *SWS2A*, while all individuals express the *SWS2B*, more so adults. As also seen in the Nile tilapia, expression of the middle-wavelength sensitive cone opsin copy *RH2B* also decreases with age, deeming the entire visual repertoire more long-shifted. *Stomatepia pindu* also shows a change in opsin gene expression with development, however less pronounced and slightly different to that of *Coptodon flava*. Interspecific changes have been detected, and a comparison between the youngest sampled fry (zero stage) shows that *S. pindu* expresses less *RH2B* and *SWS1* than *C. flava*, and more *LWS*, thus exhibiting a longer-shifted visual palette from the get-go. Intraspecifically, sexually immature individuals (age 2 and 4 weeks) show a shift in visual palettes related to aging: *SWS1*, *SWS2B* and *RH2B* expression decreases, and *LWS* expression increases with age, resulting in an even longer-shifted palettes of older fish.

Cichlid visual palettes under filtered light

In *Coptodon flava*, most drastic changes in opsin gene expression were observed under long-wavelength rearing conditions (Fig 2). More specifically, individuals responded to the "red" light regime already at the larval stage and a plastic response to a longer-shifted environment was visible throughout the development already after one week. In general, individuals exhibited lesser expression of shorter copies (e.g., *SWS1*, *RH2B*) and larger expression of longer-shifted opsins (*SWS2A*, *RH2Aα, RH2Aβ* and *LWS*) than their conspecifics which were exposed to full spectrum light (Fig 2). Response to the other two ambient light regimes, namely the "blue" and "green", was also plastic and different from the full spectrum, however less than

in the red light. *C. flava* responded to the "blue" (short wavelength) regime with an increase in *SWS1* expression, which seems to replace the *SWS2B* which expression dropped, as also observed in the adult stage. *LWS* expression of adults also decreased slightly, however the expression of *RH2Aβ* increased as compared to the control, and so did that of the "shortest" copy *RH2B* (Spady et al. 2006) (Fig 2). Larval individuals reared under the "green" light (middle-wavelength) seem to express more of the *SWS2B* copy, however as adults, they retain lower levels of *SWS2B* expression, but also *LWS* as compared to the control. On the other hand, expression of all three middle-wavelength sensitive *RH2* copies increases.

Unfortunately, due to a smaller number of individuals of *Stomatepia pindu* available for the experiment and higher mortality of individuals of this species throughout the experiment, we unfortunately lack data for the adult stage (Fig 2). Still, *Stomatepia pindu*, as compared to *Coptodon flava*, shows a plastic response to changed light conditions, though not to such an extent. Changes were mostly observed in the expression of *RH2Aβ* and *SWS2A*, and in the case of the "green" regime, also in *SWS2B*. To describe potential plasticity and the degree of it more fully, especially that of the sexually mature stage, further long-term experiments are needed. It is clear, however, that the mouth brooder *Stomatepia pindu* develops an "adult" or longer-wavelength shifted visual phenotype sooner, and that its visual palette seems to be less plastic than that of a lineage-wise younger *Coptodon flava*. Besides possible behavioural or phylogenetic constraints, plasticity might also be somewhat limited by the already longer-shifted visual palette of this species.

It has been shown before that the development of teleost photoreceptors follows a what seems to be a conserved vertebrate cone-to-rod developmental sequence (Valen et al. 2016, Sernagor et al. 2006, Mears et al. 2001, La Vail et al. 1991, Lupše et al. 2021, 2022). In the case of teleosts, a rod-dominated adult retina brings many benefits (Evans & Fernald 1990, Fuiman 1993, Pankhurst et al. 1993, Hunter & Coyne 1982, Magnuson et al. 2020). This study gives further support to this claim, but also shows that the relative proportion of cone and rod opsin expression does not change plastically in response to any of the different light conditions to which fish were exposed, suggesting that plastic responses occur within cone opsins by replacing one gene class or copy with another (e.g., *SWS1* and *SWS2*), while maintaining the total cone opsin expression (Lupše et al. 2022) (Fig 2). Most interesting to note, however, is the fact that relative rod opsin expression isn't increasing constantly - at age of 4 weeks, relative rod opsin expression slightly decreases, before increasing again. As this holds true for all individuals studied within this study (Supp Table), and as cichlids are known to alter photoreceptor type and number (Carleton et al. 2020), this could suggest increased cone

importance and development at around this specific age, perhaps due to an ecological shift related to a larval-juvenile transition. To truly understand this phenomenon, however, anatomical and histological work is needed.

The effect of available light on individual opsin gene expression developmental trajectories

Our experimental design shows that the presence of certain wavelengths of light (full spectrum, short, medium or long-shifted) affects the production of opsin proteins that best correspond to sense available light. As such, plastic response seems to be maintained or even enhanced throughout the development as both larvae (week 1, 2 and 4) and adults (month 6) show deviation from a full-spectrum developmental trajectory. In fact, it seems that in the case of specific opsin genes, this deviation from "normal" is larger either in younger or older individuals, suggesting the presence of opsin gene plasticity and adaptability, especially in *Coptodon flava*, irrespective of age (Figs 3 & 4). Whether adults retain the ability to respond plastically to differing light conditions, when subjected to them as adults and not already as larvae, remains unknown. To investigate adult plasticity - independently of development, future experiments will need to subject them to changed light conditions without rearing them first, thus disabling a certain degree of retained adult plasticity.

Under full spectrum, UV-sensitive cone *SWS1* opsin expression in *C. flava* increases within the first two weeks, perhaps since it aids in planktivory or parent-offspring interactions in other species (Novales Flamarique 2013, 2016; Torres-Dowdall et al. 2017) (Fig 2). From week 2 onwards, its expression gradually decreases, and adults barely express it. Compared to the control, the "green" light had the least effect on *SWS1* expression, followed by the "blue" which increased its overall expression (Fig 3). The most plastic response was triggered by the "red" regime, which resulted in a large decrease of the expression of this gene. *S. pindu* shows minimal expression of *SWS1* throughout (below 0.5 per cent) (Fig 4).

In *C. flava*, Levels of short-wavelength sensitive *SWS2B* cone opsin gene seem to be rather constant within the first month under full spectrum conditions, before reaching much higher levels of expression as adults (Fig 3). "Red" light regime had the most effect on its expression, as larval individuals responded to it with higher levels of expression, followed then by a decrease and an adult phenotype with the lowest of expression of this gene. In *S. pindu*, levels vary depending on the regime individuals were reared under, but a decrease was detected in all, except the "red" (Fig 4).

Expression of *SWS2A* in *C. flava*, on the other hand, was none to minimal regardless of the rearing regime, and under full spectrum and "blue" light, it increased slightly (Fig 3). Yet again, "red" light had by far the most effect on it, as individuals living under these conditions expressed this gene on a level several times higher than conspecifics. In *S. pindu*, *SWS2A* was expressed most under full spectrum conditions at week 2; under "blue", "green" and "red", levels of expression were lower, but they did increase with age, reaching higher expression rates at week 4 and at month 6 in the case of the "green" adult (Fig 4).

Middle-wavelength sensitive cone opsin copy *RH2Aα* expression decreased with age under control conditions in *C. flava* (Fig 3). Plastic response was most evident in individuals reared under "blue" light, as they expressed more of this copy. Expression of *RH2Aα* in *S. pindu* was the highest under full spectrum conditions at week 2, and due to the biggest decrease with age compared to all other regimes, then also the lowest at week 4. Expression changed minimally between week 4 and month 6 in the "green" adult (Fig 4).

The "red" regime affected the *C. flava RH2Aβ* the most, as its expression was 2 to 3 times as high as that observed in any other light regime during the larval period, including the control (Fig 3). *RH2Aβ* expression decreased with maturation, and the levels of expression dropped close to that of the control, resulting in higher expression observed in adult fishes living in the "blue" and "green" tanks. The developmental trajectory under full spectrum light conditions revealed a rather stagnant expression of this *RH2* copy throughout the development. In *S. pindu*, all three artificial regimes produced higher levels of expression as compared to the control, but after an all-round age-related decrease, only fishes from the "green" tank kept levels higher than that of the control. Furthermore, levels increased slightly in the case of the adult from the "green" tank (Fig 4).

Under normal light conditions, approximately third of a *C. flava* larval visual palette is dedicated to the *RH2B* expression (Fig 3). As animals mature, *RH2B* decreases, and adults barely express it. Individuals that lived under red filtered light showed the most plastic response with barely expressing it throughout the development. Although *S. pindu* larvae that were sampled as "zero stage" did show some expression of *RH2B*, it seems that it essentially got lost within the first week as levels of expression in all individuals seem to be minimal and potentially non-functional (Fig 4).

Under control and "blue" light conditions, the *LWS* development in *C. flava* first shows an increase within week 1 and 2, followed by an increase till month 6 (Fig 3). Both middle and long-wavelength dominated environments guided a plastic response via an increase in *LWS* expression, however, only individuals living under the "red" regime expressed more *LWS* than

the control group. Plastic response of *S. pindu* was minimal, and in all cases, an increase in expression was detected within the first month. Levels of *LWS* expression in *S. pindu* are higher compared to *C. flava* from the earliest stage onwards (Figs 3 & 4).

In general, wavelengths of light that are present in a given habitat affect visual palettes of fish, and opsin gene expression seems to be a direct indicator of a light environment in which individuals live (Malinsky et al. 2015, Carleton et al. 2016, Musilova et al. 2019, Ricci et al. 2022). It has been hypothesised that cichlid vision in general, and opsin gene expression and its potential plasticity more specifically might have played a part in the explosive adaptive radiation of this prolific teleost family (Schneider & Meyer 2017). Our study on two non-model cichlid species gives further support to this claim but also shows that plasticity can be somewhat limited in certain taxa, either due to behavioural, phylogenetic, physiological, developmental and/or ecological constraints (reviewed in Introduction). The two focal species in our study, each originating from the two species flocks may also reflect different evolutionary history. The two lake radiations differ a lot in the age of the radiation, Bermin cichlids being evolutionary much younger than Barombi Mbo cichlids. Bermin cichlids also seem to demonstrate stronger response to the light condition, and hence, higher plasticity than the Barombi species. Whether higher plasticity may be linked to the age of radiation remains, however, unclear (as we simply test it in two systems only). Differing degrees of plasticity have also been seen in other ecologically relevant features, such as the pharyngeal jaw (Meyer 1987, Schneider et al. 2014). It seems that at least in theory, plasticity of opsin gene expression could mediate evolution by promoting survival and reproduction of individuals that can optically adjust to altered conditions (e.g., seasonal changes, eutrophication, algal blooms) – these individuals would be, in case of spectral changes, more able to persist as their optimal visual perception would allow them to outcompete their less plastic conspecifics (reviewed in Pfennig et al. 2010). Changes artificially induced by this study can in fact occur in nature where these cichlid species live (Trewavas et al. 1972), and so, plasticity observed in this study could potentially be found in nature. For example, increased rainfall and higher input of organic matter during the wet season can lead to higher levels of planktonic matter that in turn shifts available light to longer-wavelengths (represented herein by the “red” regime).

Conclusions

We show that vision of two Cameroonian crater lake cichlid species, *Coptodon flava* (lake Bermin species complex) and *Stomatepia pindu* (Barombi Mbo species group), can be altered in relation to available light via changes in opsin gene expression. The degree of their responses

varies, though, suggesting phenotypic plasticity needs to be assessed at the species level, taking into consideration a species' phylogenetic history, ecology, and behaviour. Out of all the regimes under which individuals were reared, the long-wavelength shifted light induced the most plastic response, suggesting a potential predisposition of these animals to seasonality (dry vs wet season) observed also in the wild. Our results support previous findings on visual plasticity in teleosts and suggest a key role of vision and phenotypic plasticity during the rapid adaptive radiation of cichlids.

Acknowledgements

We would like to express our gratitude to Dmytro Omelchenko, Gina Sommer and Monika Kłodawska for their help with field sampling, Zuzana Konvičková and Veronika Truhlářová for their help in the lab, and Karel Kodejš, Slavko Dobrotka, Vít Kaufman and Vojtech Miller for their help with aquarium fish facility. We thank local people of the Barombi and Bemé village to allow us to fish in the Barombi Mbo and Bermin lakes, and the Ministry of Scientific Research and Innovation in Cameroon to grant us research permits. NL and ZM were supported by the Swiss National Science Foundation (PROMYS – 166550), ZM by the PRIMUS Research Programme (Charles University), the Czech Science Foundation (21-31712S).

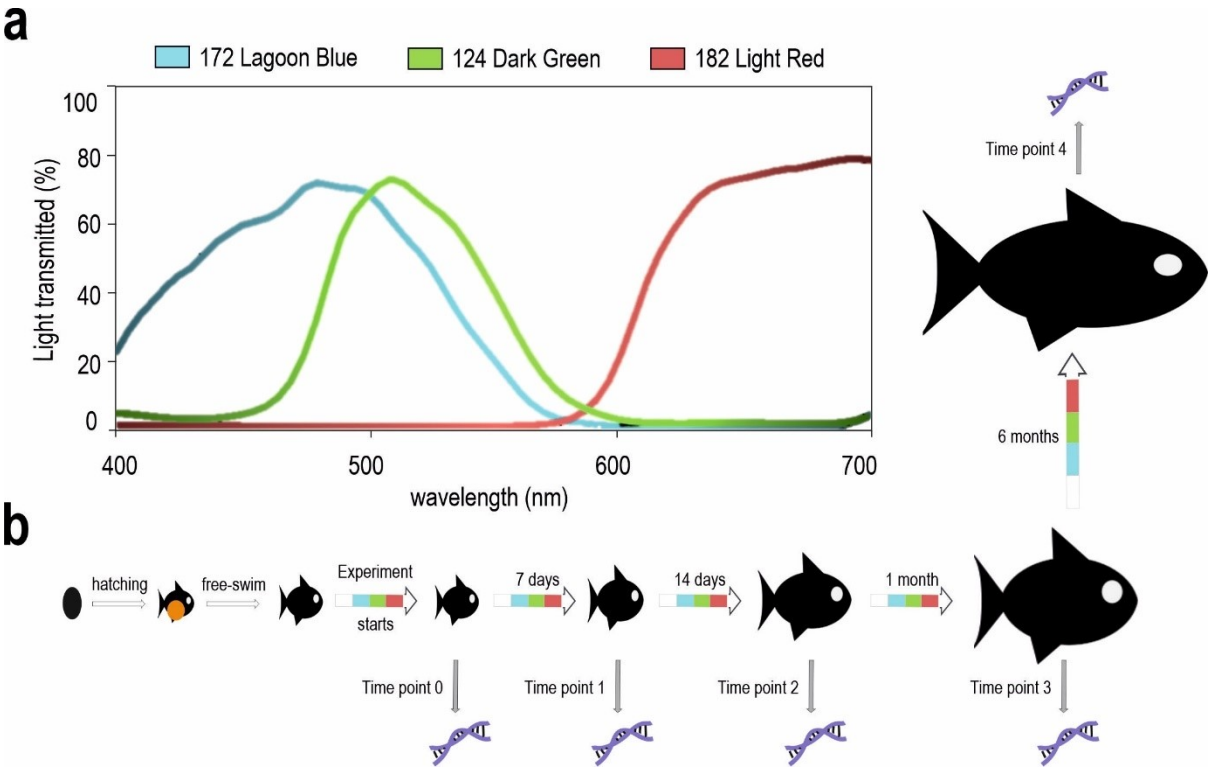

Fig. 1: Experimental design of the visual system plasticity. *(A) Spectral differences between the three light regimes under which fish were reared. Y-axis depicts the percentage of light of certain wavelengths that was transmitted by each filter. (B) A simplified scheme presenting key time points at which eye RNA was sampled. The coloured arrow presents the onset of the experiment.*

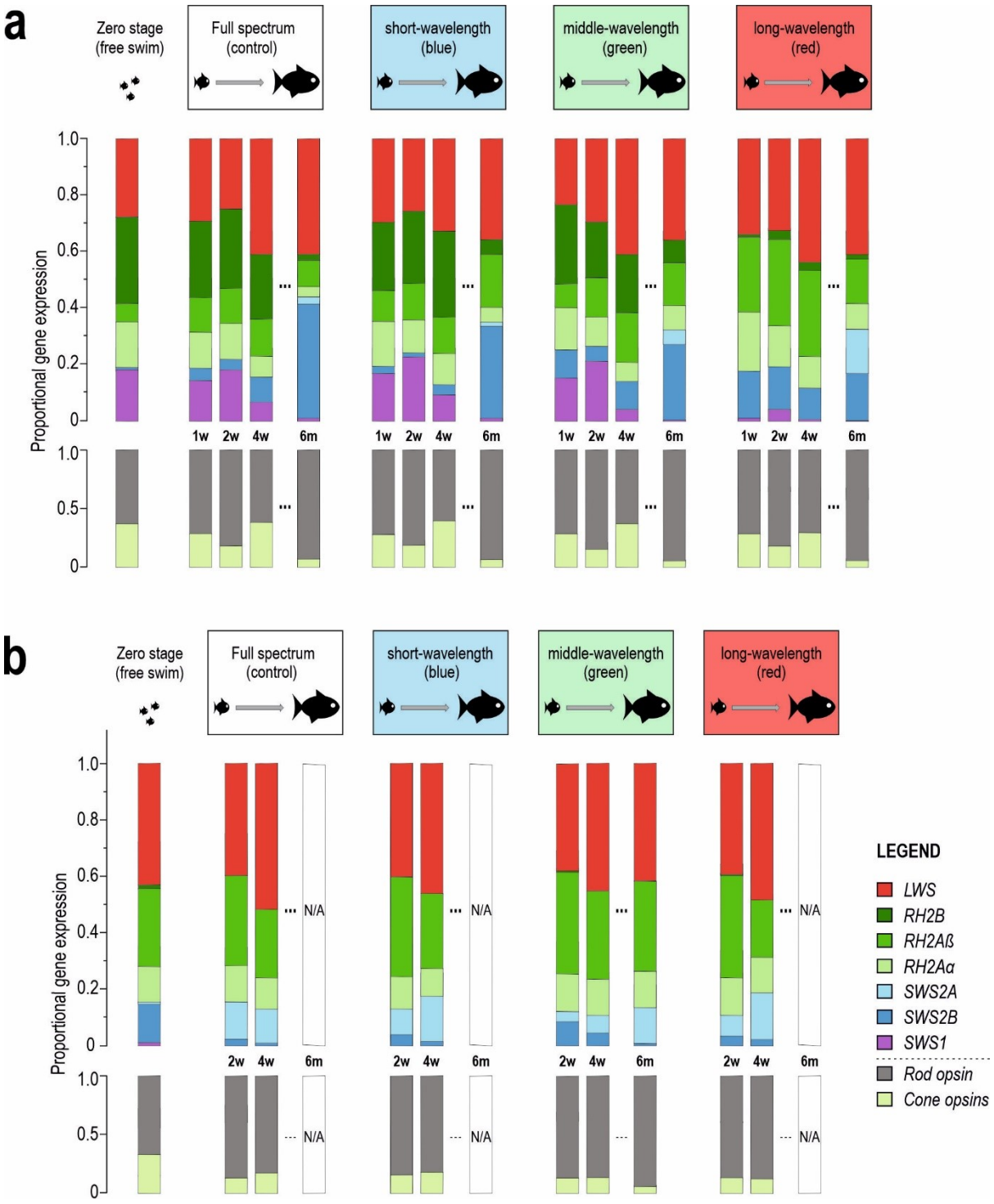

Fig. 2: Visual palettes of fish reared under the full light spectrum, short – ("blue"), middle- ("green") and long- ("red") wavelength shifted ambient light. *Developmental series of the visual opsin gene expression in Coptodon flava from the Bermin lake (A), and Stomatepia pindu from the Barombi Mbo lake (B). Above - cone opsin gene expression profile with the proportional gene expression, and below - relative cone vs rod opsin expression comparison. 1w = 1 week, 2w = 2 weeks, 4w = 4 weeks, 6m = 6 months. Note that due to difficulties in rearing S. pindu, and consequent high mortality, most adult samples (6 months) are missing.*

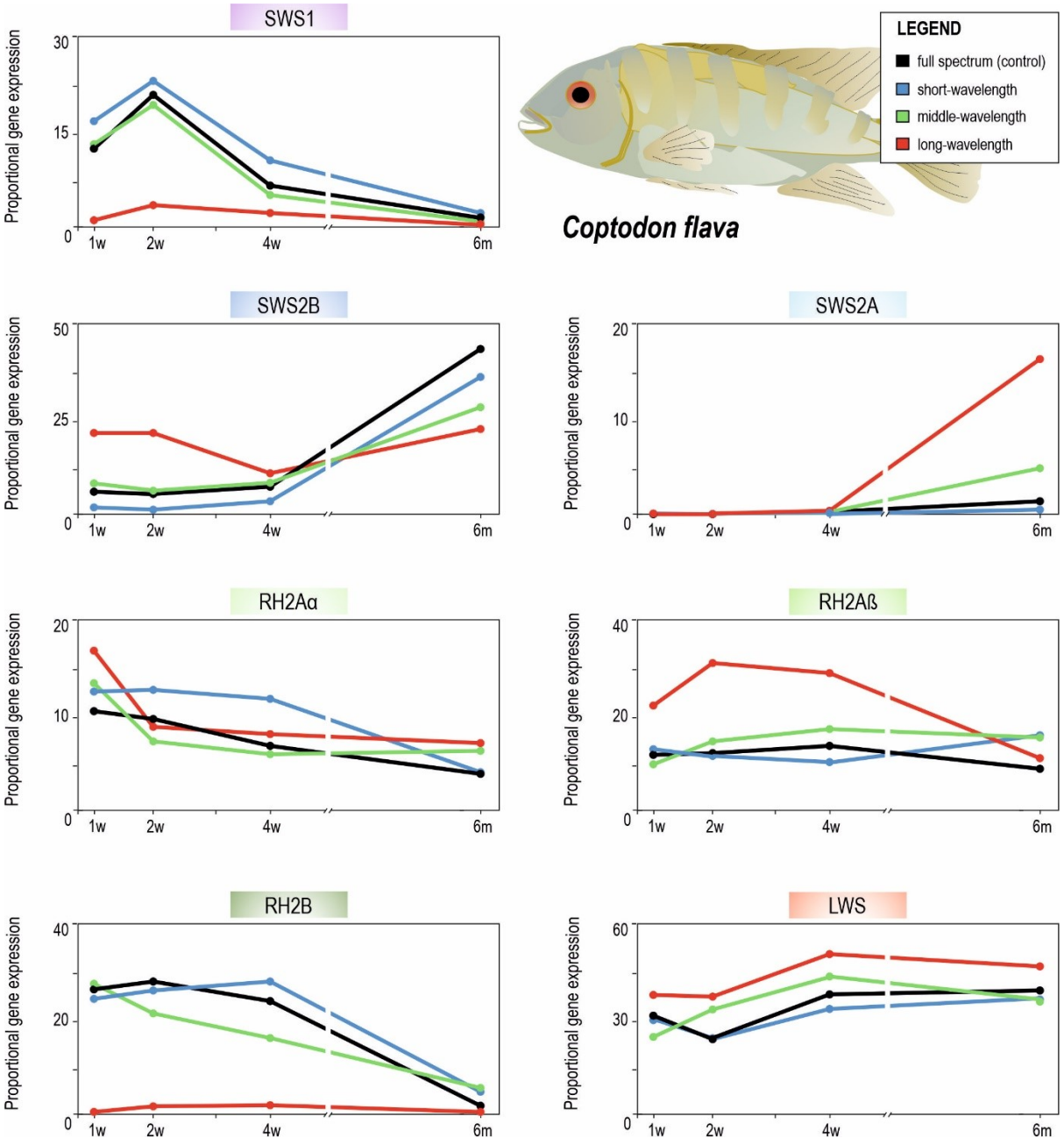

Fig. 3: Light regime-dependent variation in cone opsin gene expression in Coptodon flava. *Each plot presents shifts from the full-spectrum developmental trajectory of specific opsin gene expression that arises due to shifted spectral properties of rearing conditions. 1w = 1 week, 2w = 2 weeks, 4w = 4 weeks, 6m = 6 months.*

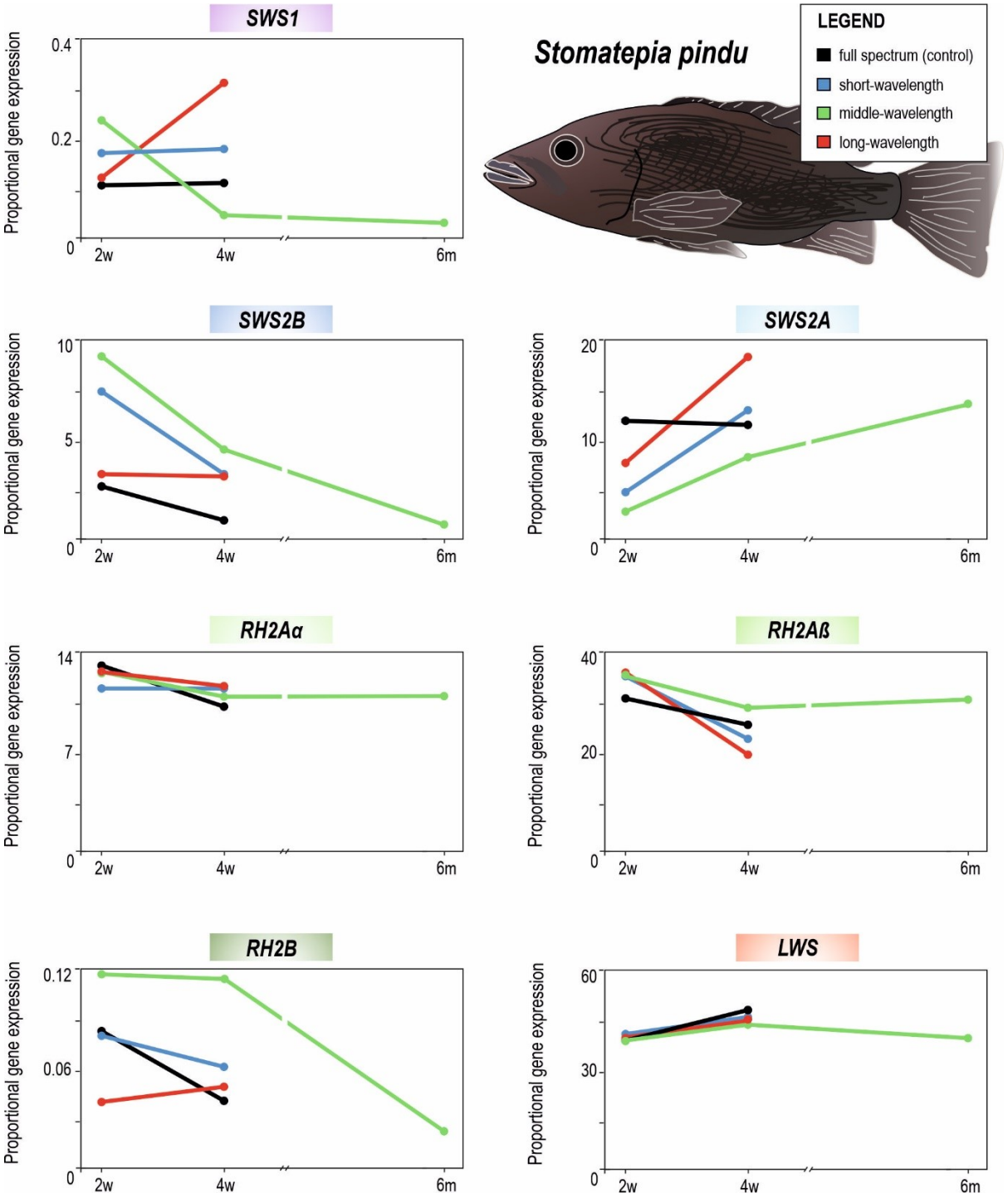

Fig. 4: Light regime-dependent variation in cone opsin gene expression in Stomatepia pindu*. Each plot presents shifts from the full-spectrum developmental trajectory of specific opsin gene expression that arises due to shifted spectral properties of rearing conditions. 1w = 1 week, 2w = 2 weeks, 4w = 4 weeks, 6m = 6 months. Note that due to difficulties in rearing S. pindu, and consequent high mortality, most adult samples (6 months) are missing.*

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