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„Gene Expression and Development in Cephalopod Eyes"

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Table of contents

List of abbreviations

b	base pairs
BCIP	5-bromo-4-chloro-3-indolyl phosphate
ddH_2O	double-distilled water
DIG	digoxigenine
EDTA	ethylenediaminetetraacetic acid
h	hours
kb	kilobase pairs
M	molar
mg	milligram
min	minute
ml	millilitre
mm	millimeter
NBT	nitro blue tetrazolium chloride
ng	nanogram
PBS	phosphate-buffered saline
rpm	revolutions per minute
UTP	uridine triphosphate
µg	microgram
µl	microlitre

Introduction

Cephalopod molluscs are enigmatic metazoans that consistently surprise us with their anatomical, molecular, physiological, and behavioural features (Albertin *et al*., 2022). Among their countless physiological and anatomical peculiarities, many cephalopods are quick learners with large brains, comparable in mass to those of vertebrates (Nixon & Young, 2003). Especially in consideration of the short lifespan and rapid ontogeny of cephalopods, their cognitive abilities are startling (Wang and Ragsdale, 2018).

Compared to other molluscs, coleoid cephalopods, e.g., all cephalopods but nautiluses, stand out since they have a highly centralized nervous system and are visual predators (Nixon & Young, 2003). They exhibit several organs that are often referred to as 'vertebrate-like` such as their closed cardiovascular system that was found to express an ortholog to vertebrate receptors during the formation of the vascular endothelium, which is a rare tissue type among invertebrates (Yoshida *et al*., 2010). Similar examples of convergent evolution of cephalopod structures to those of vertebrates can be found in cephalopod sensory and nervous systems. Camera lens type eyes are a well-known example, but more analogues to vertebrate structures are present such as the statocysts, which even show macula and crista type receptors akin to those of the vertebrate inner ears (Budelmann, 1995).

Input from sensory systems arrives in the peduncle lobe, which shows dense packing of nerve cells and was considered by some authorities as the cephalopod equivalent of the vertebrate cerebellum, coordinating the animal's movement based on sensory input (Nixon & Young, 2003).

Although on the first glance cephalopod camera lens type eyes exhibit remarkable similarities to those of vertebrates, important differences must be noted. While in vertebrates only the lens is formed by an epidermal placode and the rest of the eye via evagination from lateral derivatives of the neural tube, the cephalopod eye forms entirely from an epidermal eye placode via four consecutive invagination steps during ontogeny (Harris, 1997). In addition, the cephalopod eye has a single-layered, everted and rhabdomeric retina, while the vertebrate retina is multi-layered, inverted and has several types of ciliary receptor cells (Imarezene *et al*., 2017).

Although ontogeny clearly indicated the convergent nature of both eye designs, first studies on the genetic underpinnings of eye development shocked the scientific community since several evolutionarily highly conserved gene orthologs are involved in eye formation in both remotely related taxa. For instance, cephalopods and other molluscs employ gene pathways in

the formation of eyes or ocelli, that are orthologous to the *Pax6*-pathways of vertebrates (Tomarev *et al*., 1997; Navet *et al*., 2017). Such morphological and molecular parallels in the ontogeny of visual organs raise the question of how they evolved and if their evolutionary history is as similar as the result. Comparing different eye types across the Mollusca could deepen our understanding of the origin of cephalopod eyes. To achieve this, also non-model organisms that hold important phylogenetic positions such as *X. notoides* must be investigated.

Pygmy squids (Idiosepiidae) are excellent candidates for gene expression studies on cephalopods. Their small size allows to conduct *in situ* hybridisation experiments of the entire embryo (whole-mount *in situ* hybridisation experiments). Adults are usually about 10 – 20 mm in length, while eggs, embryos and even hatchlings measure only 0,6 – 1 mm (Reid & Strugnell, 2018).

Idiosepiid cephalopods are also a well-suited group for the study of the evolution of decabrachian cephalopods. Contrary to their internal phylogeny (Reid & Strugnell, 2018) the position of Idiosepiidae within decabrachian cephalopods remains poorly resolved (Uribe & Zardoya, 2017). Currently idiosepiid cephalopods are considered to belong to the sister clade of all teuthids (i.e., oegopsids & myopsids) and within that clade they form the sister clade to the Sepiidae together with the Sepiolidae (Tanner *et al.,* 2017.). Due to this unique phylogenetic position, new findings about idiosepiid ontogeny and their visual system may deliver valuable insights among others into the evolution of cephalopod eyes, photoreceptors, and opsins.

Whole mount *in situ* hybridization experiments to visualize the expression of candidate genes can be conducted in all embryonic stages up to hatching after stage 30 in the idiosepiid squid *X. notoides* due to its miniscule size. Other authors have already explored the expression of various transcription factors during pygmy squid embryogenesis (Yoshida *et al*., 2015b; Wollesen *et al*., 2014). While *Pax6* was already investigated in the pygmy squid, its possible downstream targets, such as opsins, have not yet been explored via whole mount *in situ* hybridization experiments. This study aims to fill this gap of knowledge by mapping the expression of possible *Pax6* downstream targets during *X. notoides* ontogeny.

Five genes from two groups were chosen according to their suspected function and due to extensive research conducted on them in other cephalopods, unravelling a myriad of unanswered questions, which allows a comparative approach. Thus, rhabdomeric *Rhodopsin* and the associated *Retinochrome* as well as the ciliary *Xenopsin* from the opsin gene family

were selected. Additionally, two *Reflectin* genes were added, as *Reflectins* are an apomorphy of cephalopods and poorly researched. Reflectin proteins are not photosensitive themselves but are hypothesized to be able to scatter and reflect light wavelength-specific (Cai *et al*., 2019) and could therefore be important in cephalopod vision, camouflage, and intraspecific communication.

Opsins are well-studied in model organisms and the rhodopsin-retinochrome system was first described in a squid (Hubbard & St. George, 1958). Retinochrome is the associated photoisomerase of rhodopsin and thus expression of those proteins' genes is likely to occur in the same tissues and cells, most importantly in the retina of the eye. Here, retinal and rhodopsin cause the visible darkening of the eye during ontogeny. Retinal appears orange in the retina as it is the vitamin-A-derived chromophore, whose isomerization to all-*trans*-retinal by rhodopsin induces phototransduction. All-*trans*-retinal is re-isomerized to 11-*cis*-retinal by retinochrome, also powered by photon absorption (Vöcking *et al*., 2021).

In *X. notoides*, *Rhodopsin*-expression is expected to first occur in the eye and later in the skin as in *Sepia officinalis (*Bonade *et al*., 2020). In line with this Master thesis, it is furthermore suspected that at least *Rhodopsin* and *Retinochrome* are spatially co-expressed.

During the last years, the enigmatic *Xenopsin* received considerable attention (Passamaneck *et al*., 2011; Vöcking *et al*, 2017), however its transcript has not been precisely localized (Bonade *et al*., 2020). For this reason, we localized *Xenopsin*-transcripts by whole mount *in situ* hybridization experiments.

While the development of the lens in cephalopods is steered by *Six3*, notably reduced in nautiluses (Ogura *et al.*, 2013), the expression of *Rhodopsin* and *Retinochrome* is regulated by *Pax6* (Tomarev *et al.*, 1997). The four splicing variants of *Pax6* were found to be expressed in four different tissues in embryos of *Idiosepius paradoxus* (Yoshida *et al*., 2015a). Those *Pax6* splicing variants missing the homeodomain are suspected to steer the development of the eye reflectors (Yoshida *et al*., 2015a). In cephalopod eye reflectors light scattering is achieved by unstructured proteins called reflectins. Since they are suspected downstream targets of *Pax6* and a cephalopod apomorphy of enigmatic origin, *Reflectin* genes were included in our *in situ* hybridization experiments as well. We anticipate *Reflectins* to be expressed in the eye reflectors and potentially in other tissues too, as seen in *Euprymna scolopes* (Crookes *et al.*, 2004). Furthermore, we expect *Rhodopsin* to be expressed in the skin of *X. notoides* as seen in *Octopus bimaculoides* (Ramirez & Oakley, 2015), *Sepia officinalis* (Mäthger *et al*., 2010; Kingston *et al*., 2015; Bonade *et al.*, 2020), *Sepia latimanus* and *Doryteuthis pealeii*

(Kingston *et al.*, 2015). As *Retinochrome* is associated with *Rhodopsin* it might also be detected in the skin of *X. notoides*.

Our study shows that *Xenopsin* as well as opsin-encoding genes are also expressed in extraocular regions of the developing embryo suggesting that these regions are either photosensitive, or that opsin-encoding genes may play a role in other processes than only photosensation.

Methods

Phylogenetic analysis

A *Xipholeptos notoides* transcriptome, which is derived from pooled embryonic stages 16-30 and adults was mined for candidate genes. Thus, localization of expression was determined solely via *in situ* hybridization experiments and not via tissue- or stage-specific transcriptomes. Amino acid sequences of candidate gene orthologues of other organisms were obtained from the NCBI database. To find *X. notoides* equivalents of the known sequences in the transcriptome, the 'blastp' command of ncbi_blast+ for Ubuntu in a Windows Subsystem for Linux (WSL, v20.04.3 LTS: GNU/Linux 5.10.16.3-microsoft-standard-WSL2 x86_64) was used. Prior to this, a protein database was constructed from the provided transcriptome in nucleotide FASTA format, using the 'makeblastdb' function of ncbi_blast+. Whenever a sequence of high similarity was identified in the transcriptome, it was reblasted against cephalopod orthologs by BLASTx searches in the NCBI database.

Five candidate gene sequences from two separate gene families were obtained. It was not possible to produce one large opsin amino acid sequence alignment, as the *Xno-xenopsin* and *Xno-retinochrome* nucleotide sequences did not overlap. This is due to the *Xno-xenopsin* sequence comprising only the starting part of the open reading frame (ORF) and the *Xno-retinochrome* sequence only comprising he terminal portion of the open reading frame. Many publicly available xenopsin amino acid sequences are confined to the anterior portion of the ORF, which seems to be a widespread phenomenon. Thus, amino acid alignments for rhodopsin, xenopsin and retinochrome and an alignment of all available reflectin amino acid sequences found on NCBI GenBank were performed including yet existing deduced amino acids of cephalopods, molluscs, other spiralians, and deuterostomes. Sequences for the alignments were obtained by the BLASTx and BLASTp tools of the NCBI GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and combined with protein sequences extracted from the supplementary material provided by Ramirez *et al.* (2016), which are all available on UniProt. By including protein sequences of this publication, it was possible to complement the sequences from the NCBI GenBank with quality checked protein sequences from UniProt. The final sequence alignments used in the phylogenetic analysis can be found in our supplementary data.

Alignments were performed using the MUSCLE function of MEGA-X (MEGA-X v.10.1.5. x64, https://www.megasoftware.net/), followed by a manual alignment. After completion of

the alignments, they were exported as amino acid sequences in FASTA format via MEGA-X as well. Subsequently, jModelTest-2.1.10 (Posada, 2008) and ModelTest-NG v0.1.7 (Darriba *et al.*, 2020; Flouri *et al.*, 2015; https://github.com/ddarriba/modeltest) were used to find the appropriate substitution models for phylogenetic analysis. JModelTest was run on Windows 10, while ModelTest-NG was run on WSL (v3.2.6 x64 for WSL, v20.04.3).

The amino acid substitution model used for phylogenetic analysis of the *Reflectin* sequence alignment was JTT-DCMUT+I+G4 and the amino acid substitution model for the opsin sequence alignments were variations of the basic amino acid substitution model LG. For the rhodopsin and retinochrome amino acid alignments LG+G4 was used and for the xenopsin amino acid alignment LG+G4+F was employed. Phylogenetic analyses were primarily conducted using RAxML Black Box (https://raxml-ng.vital-it.ch/#/). Additionally, Bayesian phylogenetic analysis results and posterior probability values were obtained via MrBayes (v3.2.6 x64 for WSL, v20.04.3) at a generation number of 2 000 000 and by utilizing the dynamic model finding option of MrBayes (prset aamodel=mixed).

Primer design and synthesis

Forward and reverse primers for the candidate gene sequences were designed with ApE (A plasmid Editor, by M. Wayne Davis; ApE v.3.0.9, October 14, 2021, https://jorgensen.biology.utah.edu/wayned/ape/). It was assured that the GC content was around 50% and primer lengths are between 25 and 40 base pairs (Table 01). The melting temperature of all primers ranges between 60 °C and 63 °C, which was achieved by lowering the GC (guanine, cytosine) content of the primers. Upstream of the reverse primer the T7-RNA-Polymerase promoter sequence (TAATACGACTCACTATAGGGAGA) was added to ensure direct reverse transcription of this template into the riboprobe later. Primers were purchased from Microsynth Austria GmbH.

Table 01. The candidate genes, their transcriptomic contig ID and the respective nucleotide sequences. The prefix 'Ino' refers to *Idiosepius notoides,* which is the workflow name of *Xipholeptos notoides*. After the transcriptome was assembled X. *notoides* was elevated into its own genus in 2018 (Reid & Strugnell).

gene	contig name	forward primer	reverse primer without t7promoter
Ino_r-opsin	comp167212 c0 seq3	GTCCACTGGTTTTACTGTAGTGGGTTC	GTGGGTATCCTTGTGGTGGGTATC
Ino_retinochrome1	comp52955 c0 seq1	CTGGTCCGGTCTGAGTCTTATTCCAG	CAATTTCCAATAGACCAACAAATACATATTAGTGTGTCTC
Ino_xenopsin	comp473175 c0 seq1	CAATAGTACCGAAGTATATTATCTGATGTCGCAAAC	GACGTAGCGGTCAACCGAAATCG
Ino_reflectin1	comp168894 c0 seq2	GTTGAGAACAACAGCAACCGTACCATC	CGTATTGGTCTTGATAGCGTCCTTGC
Ino_reflectin2	comp180415 c0 seq1	CACCCAAAGTCTTCCAACGTGTATCAG	GAAGGGTCAGTATAACGACCCTGCATG

PCR and gel electrophoresis

Using a cDNA library generated from pooled developmental stages and adults, candidate gene sequences were amplified via touchdown PCR and size-selected via gel electrophoresis. Primers for five candidate genes (*Rhodopsin*, *Retinochrome*, *Xenopsin*, *Reflectin1* and *Reflectin2*) were used (Table 01). The tool of choice to amplify candidate gene transcripts from the cDNA library of *X. notoide*s was a touchdown PCR, which starts with a higher annealing temperature for primers which is subsequently lowered every five cycles to cover a broader annealing temperature range. In total, four thermoprofiles were employed, utilizing five annealing temperatures each. In this way, temperature ranges from 64 °C to 53 °C were covered. Most successful were the thermoprofiles employing the higher temperatures from 64-59 °C and 63-58 °C. Seven PCRs were necessary until all gene sequences were amplified successfully.

PCR products were then size-selected via several gel electrophoreses, using a gel composed of SybrGreen, 50 ml TAE (Tris base, acetic acid and EDTA) buffer, and 1.5 – 2 % agarose. The size markers of choice were Quantitas Fast (100 b – 2 kb) and Quantitas Pro (100 b – 3 kb) (Biozym Scientific GmbH, Hessisch Oldenburg, Germany).

Sequencing

Purified PCR products were Sanger sequenced by Microsynth Austria GmbH at a concentration of 17 ng/µl. To determine nucleic acid concentrations the PCR products were measured using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, USA). Sequencing results are provided via an online database the next day.

Sample preparation for *in situ* hybridisation experiments

Samples were reared and fixed by Dr. Tim Wollesen as described in earlier publications (Wollesen *et al.*, 2010). Individual embryos were classified according to the embryonic stage system established and commonly used in Idiosepiids (Yamamoto, 1988). The animals were assigned to a developmental stage mainly based on the anatomy of the eyes, mantle, and the inner yolk mass. Idiosepiids hatch as stage 30 embryos with less developed tentacles. In some cases, a portion of their external yolk sac remains and development into a free-swimming juvenile is completed outside of the egg membranes (Yamamoto, 1988).

Riboprobe synthesis and whole-mount *in situ* hybridisation experiments

Anti-sense riboprobes for all five candidate genes for the *in situ* hybridization experiments were synthesized using a RNA labelling mix, which incorporates digoxigenin-UTP in the transcripts at approximately every 20-25th nucleotide (Roche Applied Science, Penzberg, Germany). Concentrations of PCR products were measured with a NanoDrop 2000 and incubated with a T7 Polymerase (reverse transcriptase) and DIG RNA labelling mix (digoxigenin-labelled nucleotides) at 37 °C for 2 h. Transcription was stopped by adding 1 µl EDTA (ethylenediaminetetraacetic acid, pH 8.0), 1.25 µl 4 M LiCl (lithium chloride) and 37 µl of chilled 99 % ethanol and the reaction was left for anoth
er 2 h at -80 °C. Then, the precipitated riboprobes were centrifuged, the ethanol was removed and the riboprobes were dissolved again in ribonuclease-free ddH_2O and immediately stored at -80 °C for later use in the *in situ* hybridization.

Prior to riboprobe use in *in situ* hybridization experiments nucleic acid concentrations were measured using a NanoDrop 2000 to add the correct working concentration for *in situ* hybridization experiments.

Whole-mount *in situ* hybridization experiments were conducted as described in earlier publications (Wollesen *et al.,* 2010; Wollesen *et al.*, 2014). Embryos stored in 75 % ethanol and 100 % methanol were rehydrated in a PBT buffer solution (PBS + 0.1 % Tween-20). For de-calcification the samples were rotated in PPE (1 ml paraformaldehyde + 0.5 ml x10 PBS + 0.5 ml 0.5 M EDTA + 3 ml ddH_2O) for 60 minutes at room temperature. To increase permeability for probes and antibodies, the animals were digested in Proteinase-K for 8 min (40 mg/ml in PBT) at 37 °C. Prior hybridization embryos were prehybridized over night at 63 °C. On the next day riboprobes were added at a concentration of 1 µg/ml and the samples were incubated over night at 63 °C. After several washes the samples were stepped in block solution for 3 hours at room temperature before addition of the DIG – Antibody solution (1: 2500 = AB: block solution). The Antibody reaction was carried out overnight at 4 °C. Subsequently, the colour reaction buffer, based on alkaline phosphatase (AP) mediated BCIP/NBT colour reaction, was added and the samples were observed carefully until the stain was complete. To stop the colour reaction the successfully stained samples were stepped in PBT and post-fixed in 4 % paraformaldehyde. Finally, a DAPI (4′,6-diamidino-2-phenylindole) stain was applied as well (3 µl DAPI/1 ml PBT). The stained embryos were then mounted in a TDE clearing buffer (2,2′-thiodiethanol) and documented in the following days.

Documentation

Samples successfully stained via *in situ* hybridization experiments were documented primarily by bright field microscopy via an Olympus BX53 light microscope (Olympus Life Science, Shinjuku, Tokio, Japan) and subsequently scanned with a Leica TCS SP5 II scanning system (Leica Microsystems, Wetzlar, Germany). Image stacks obtained by CLSM were rendered in Fiji (Schindelin *et al*., 2012; Fiji v.2.5.0) and Amira (Thermo Fisher Scientific, Waltham, USA).

Results

Eye pigmentation

Dark red pigmentation was observed in the eyes, a condition due to increased expression of *Retinal*, the vitamin-A-derived chromophore required for rhodopsin, retinochrome and xenopsin proteins to allow phototransduction. Eye pigmentation may be used as an indicator for the presence of photosensitive proteins. In our animals, eye pigmentation was detected in stage 24 (Figure 05), and clearly visible in all hatchlings (Figures 08 & 09). Eye pigmentation seems to increase dramatically between stages 26 and 30 (hatchlings).

Phylogenetic analyses of candidate genes

The phylogenetic analyses confirmed the identity of all candidate genes of *X. notoides*. Gene orthologs are most similar to other cephalopod sequences. As reflectins are a cephalopod apomorphy, the phylogenetic analysis of reflectin1 and reflectin2 amino acid sequences includes an unidentified protein from *Mytilus coruscus* as an outgroup. The gene contains a repetitive region similar to the reflectin repeat domain and was found in the NCBI GenBank via the BLASTp function.

Rhodopsin

The xno-r-opsin amino acid sequence shows high sequence similarity with a rhabdomeric rhodopsin amino acid sequence found on UniProt that is also derived from *X. notoides*. Together these two sequences sit at the base of a cluster formed by decabrachian sequences (Figure 01). The octobrachian sequences form a sister taxon to the decabrachian group and the *Nautilus pompilus* sequence sits at the base of the cephalopod group. The remaining molluscan sequences cluster with the cephalopod sequences, followed by other invertebrates,

while vertebrate rhodopsins and melanopsins are the most dissimilar to the cephalopod rhodopsins (Figure 01. Two opsin sequences from *Trichoplax adherens* were chosen as outgroup to the rhodopsins and melanopsins in this analysis.

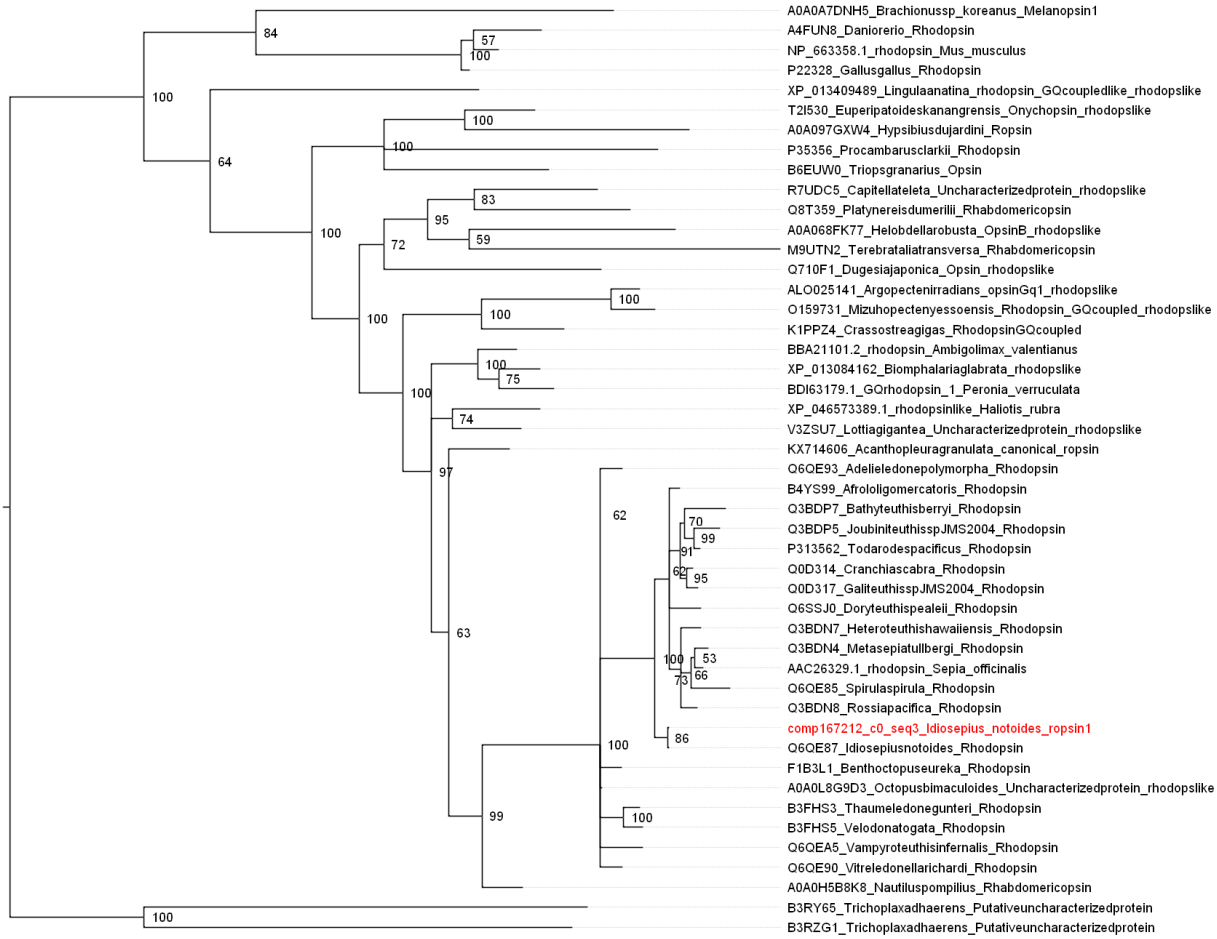

Figure 01. Rhodopsin Bayesian inference tree obtained using MrBayes (v3.2.6 x64 for WSL, v20.04.3) at 2 000 000 generations with two *Trichoplax adherens* opsins serving as outgroup. Node labels indicate posterior probabilities in per cent. The prefix 'Ino' refers to *Idiosepius notoides*, which is the workflow name of *Xipholeptos notoides*. After the transcriptome was assembled *X. notoides* was elevated into its own genus in 2018 (Reid & Strugnell). Black tip labels are the sequence name and the respective databank ID (see supplementary data), whereas a red tip label indicates a gene sequence obtained from our *X. notoides* transcriptome.

Retinochrome

In the cephalopods *Idiosepius paradoxus*, *Euprymna scolopes* and *Sepia spp*. two copies of *Retinochrome* are present, i.e., *Retinochrome1* and *Retinochrome2*. Within our retinochrome alignment, the xno-retinochrome amino acid sequence from our *X. notoides* transcriptome is

closest to an ortholog of *Idiosepius paradoxus* and other bilaterian retinochrome1 amino acid sequences (Figure 02). Thus, *Xno-retinochrome* is most likely a *Retinochrome1* gene ortholog. As outgroup two opsin sequences from the placozoan *Trichoplax adherens* were chosen (Figure 02).

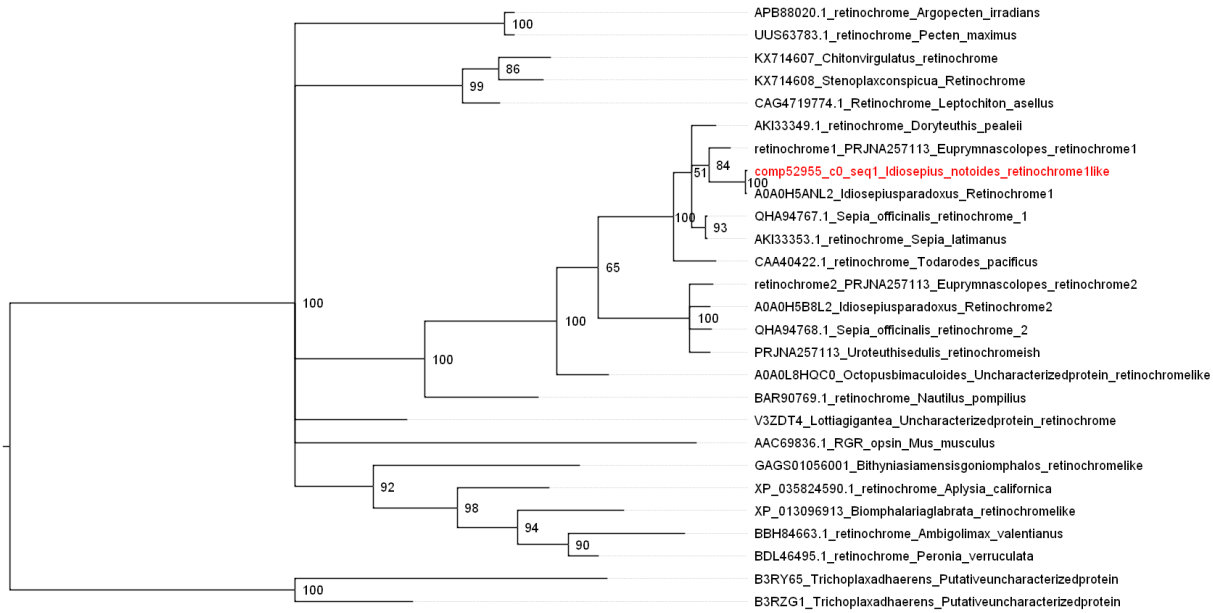

Figure 02. Retinochrome Bayesian inference tree obtained by phylogenetic analysis utilizing MrBayes (v3.2.6 x64 for WSL, v20.04.3) at 2 000 000 generations with two *Trichoplax adherens* opsins serving as outgroup. Node labels indicate posterior probabilities in per cent. The prefix 'Ino' refers to *Idiosepius notoides*, which is the workflow name of *Xipholeptos notoides*. After the transcriptome was assembled *X. notoides* was elevated into its own genus in 2018 (Reid & Strugnell). Black tip labels are the sequence name and the respective databank ID (see supplementary data), whereas a red tip label indicates a gene sequence obtained from our *X. notoides* transcriptome.

Xenopsin

In both the maximum likelihood and the Bayesian analysis xno-xenopsin amino acid sequences clusters with the other cephalopod xenopsin amino acid sequences. This cephalopod cluster appears as a sister taxon to the oysters (Figure 03). Scallops in turn form a sister taxon to the group consisting of oysters and cephalopods. The deeper bilaterian node did not resolve well and was collapsed (Figure 03). Cnidarian xenopsins, except for the sequence of *Nematostella vectensis*, form a sister taxon to the other groups which consists mainly of molluscan and flatworm sequences. Two placozoan opsins (*Trichoplax adherens*) were chosen as outgroup.

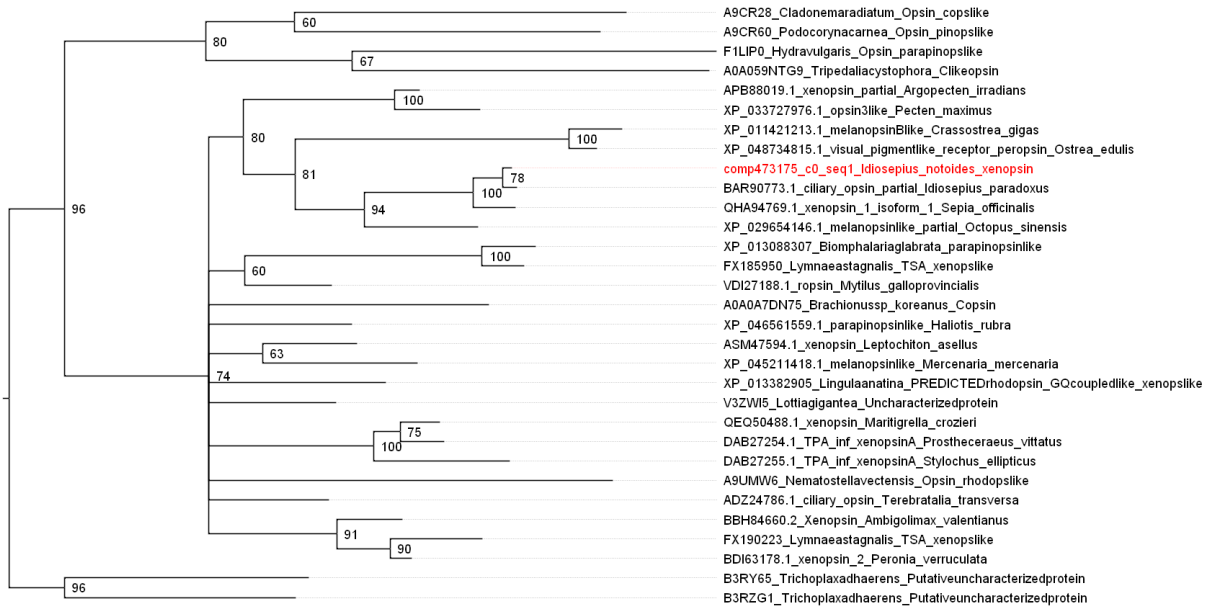

Figure 03. Xenopsin phylogenetic tree obtained by Bayesian phylogenetic analysis using MrBayes (v3.2.6 x64 for WSL, v20.04.3) at 2 000 000 generations with two *Trichoplax adherens* opsins serving as outgroup. Node labels indicate posterior probabilities in per cent. The prefix 'Ino' refers to *Idiosepius notoides*, which is the workflow name of *Xipholeptos notoides*. After the transcriptome was assembled *X. notoides* was elevated into its own genus in 2018 (Reid & Strugnell). Black tip labels are the sequence name and the respective databank ID (see supplementary data), whereas a red tip label indicates a gene sequence obtained from our *X. notoides* transcriptome.

Reflectin1 and Reflectin2

Both *X. notoides* reflectins include several methionine-rich reflectin domains (see supplementary data). The position and number of reflectin domains is most similar to the loliginid sequences in this alignment (Figure 04). The xno-reflectin2 amino acid sequence shows highest sequence similarity with the sequences of *Doryteuthis pealeii* and *Doryteuthis opalescens*, while the xno-reflectin1 amino acid sequence clusters with the reflectin of *Loligo forbesi*. *Sepia officinalis* and *Euprymna scolopes* have both numerous reflectin proteins, which cluster amongst themselves (Figure 04).

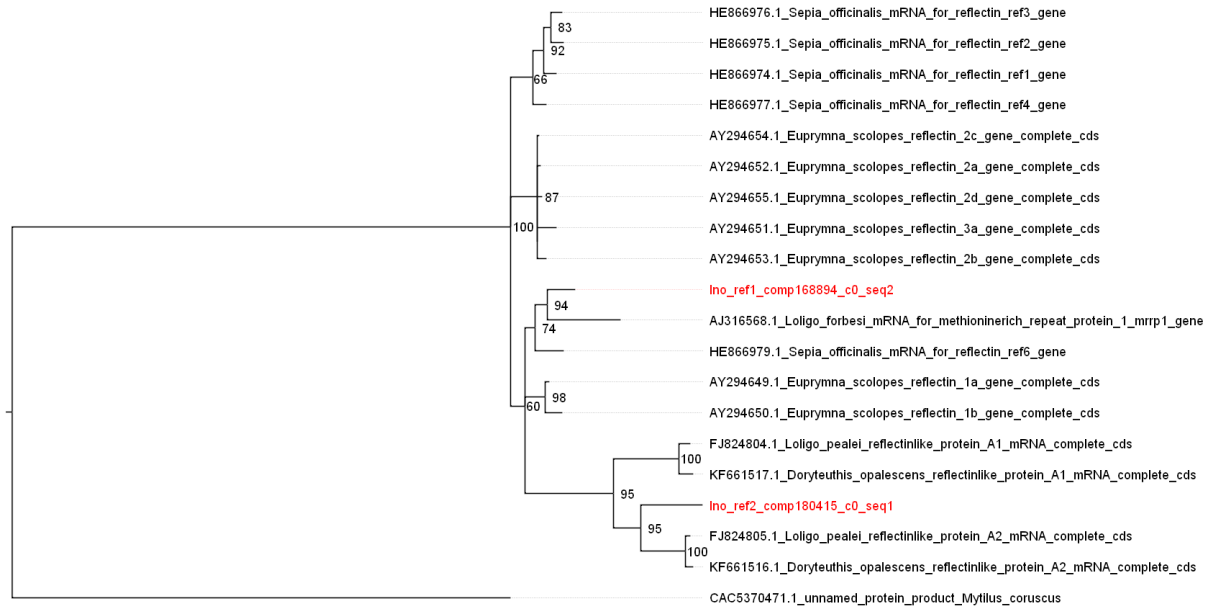

Figure 04. Reflectin phylogenetic tree obtained by Bayesian phylogenetic analysis using MrBayes (v3.2.6 x64 for WSL, v20.04.3) at 2 000 000 generations with an unidentified protein sequence of *Mytilus coruscus* serving as outgroup. Node labels indicate posterior probabilities in per cent. The prefix 'Ino' refers to *Idiosepius notoides*, which is the workflow name of *Xipholeptos notoides*. After the transcriptome was assembled *X. notoides* was elevated into its own genus in 2018 (Reid & Strugnell). Black tip labels are the sequence name and the respective databank ID (see supplementary data), whereas a red tip label indicates a gene sequence obtained from our *X. notoides* transcriptome.

Gene expression analyses

Summary table

Table 02. Character table showing the tissues where candidate gene expression occurs in developmental stages of *Xipholeptos notoides* as revealed via *in situ* hybridization experiments. Color code: blue, optic epithelia; green, olfactory organ; red, brain lobes; abbreviations: dll, dorsolateral lobe; lt, lentigenic tissue; mcl, magnocellular lobe; ol, optic lobe; oll, olfactory lobe; oo, olfactory organ; pov, parolfactory vesicle.

stage/tissue	*Rhodopsin*	*Retinochrome1*	*Xenopsin*	*Reflectin1*	*Reflectin2*
22	not studied	not studied	lt	not studied	not studied
23	lt	lt	lt	lt	lt
24	oo, pov, retina	mcl, oo	dll, oll, ol, oo	iris, oo	lt, oo
25	retina	not studied	not studied	not studied	not studied
26	retina	not studied	not studied	not studied	not studied
30	retina	retina	absent	iris	cornea, eyelids
adult	retina	retina	absent	absent	absent

Rhodopsin

Xno-r-opsin is expressed most strongly in the retina of stages 24 and 30 (Figure 05). In stage 23 individuals, no *Xno-r-opsin*-expression is detected in the retina, but in the lentigenic tissue of the eye cavity. Additionally, *Xno-r-opsin*-expression is found in individual cells of the olfactory organs and in the parolfactory vesicles of stage 24 individuals (Figure 05; Table 03). Stage 26 embryos and adults (not shown) exhibit *Xno-r-opsin*-expression exclusively in the retina of the eyes.

Table 03. Summary of *Xno-r-opsin*-expression in *Xipholeptos notoides* as revealed by *in situ* hybridization experiments.

Rhodopsin				
stage/tissue	eye	olfactory organ	parolfactory vesicle	documentation
23	lentigenic tissue	absent	absent	brightfield
24	retina	cell somata	photoreceptor cells	brightfield, confocal
25	retina	absent	absent	brightfield
26	retina	absent	absent	brightfield
30	retina	absent	absent	brightfield
adult	retina	absent	absent	brightfield

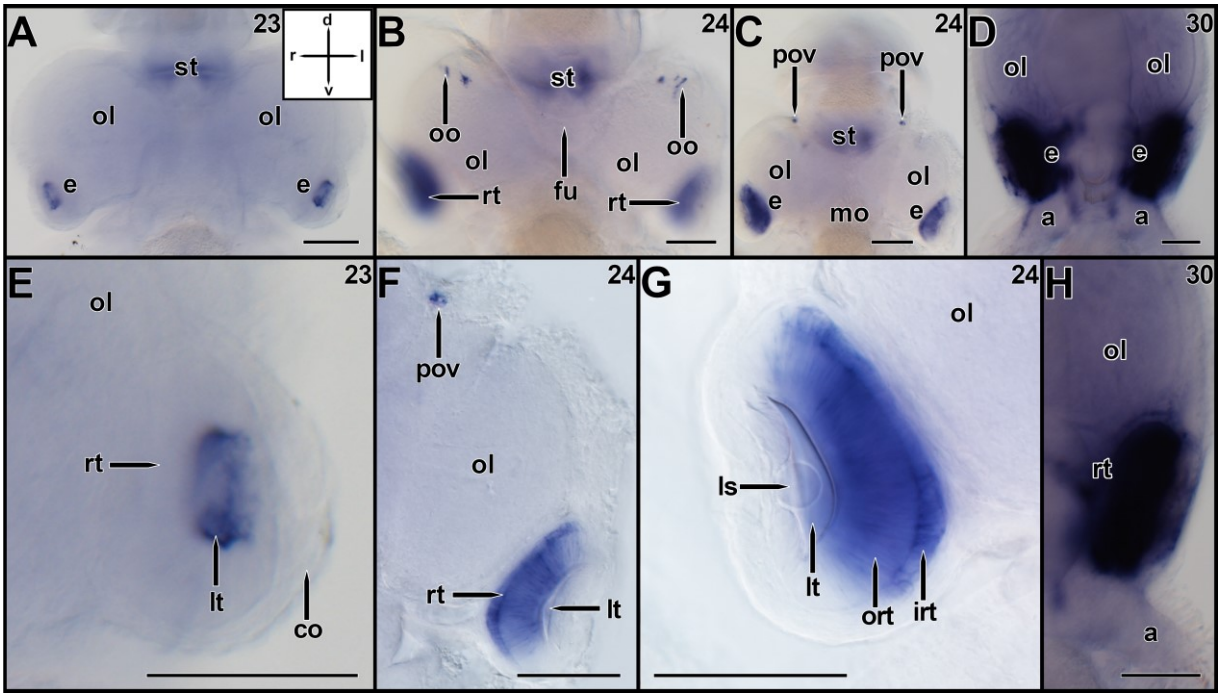

Figure 05. *Xno-r-opsin*-expression during development of *Xipholeptos notoides* as revealed via *in situ* hybridization experiments. (A) In stage 23 individuals *Xno-r-opsin*-expression is found in the lentigenic tissue (lt) of the posterior lens of the eye (e). **(B)** Stage 24 individuals show strong *Xno-r-opsin*-expression in the retina (rt) and in cells of the olfactory organ (oo). Ventrally to the statocysts (st) the funnel (fu) can be seen. **(C)** Strong *Xno-r-opsin*-expression in the eyes and parolfactory vesicles (pov) of a stage 24 individual. **(D)** In hatchlings, *Xno-r-opsin* is strongly expressed in the eyes, while no other structures showed significant staining. **(E)** Detail of the *Xno-r-opsin*-expression in the left eye of the stage 23 individual shown in (A). The primary cornea (co) covers the primary optic vesicle. **(F)** Posteriorly, *Xno-r-opsin*-expression is present in the parolfactory vesicle and throughout the retina of the same stage 24 animal as seen in (B). **(G)** The right eye of the stage 24 individual as depicted in (C) shows *Xno-r-opsin*-expression in the proximal cell bodies of the retina cells and in the distal rhabdomeres as well. The lentigenic tissue of the forming posterior lens (ls) is clearly visible. Here proximal cell bodies of the inner retina (irt) are distinguishable from the distal rhabdomeres of the outer retina (ort) **(H)** The left eye of the hatchling seen in (D) shows *Xno-r-opsin*-expression, leading to a very strong staining throughout the entire eye and surrounding tissues. Unspecific and diffuse staining can be seen in the cartilaginous and mineralizing tissue of the statocysts (st) and the mouth (mo). Abbreviations: a, arm; ol, optic lobe; anterior views with the dorsal side facing up, axes in the insert in (A) indicate the orientation of all panels, orientation is equal in all images: dorsal (d), ventral (v) and left (l), right (r); scale bars equal 0.1 mm in all panels.

Retinochrome1

Transcripts of this gene are first visible in the lentigenic tissue in the primary optic vesicle of stages 22 and more pronounced in stage 23 (Figure 06). In stage 24 individuals, *Xno-retinochrome1* is faintly expressed in the olfactory organ and probably co-expressed with

Xno-r-opsin. Staining also occurred in the developing magnocellular lobes of the periesophageal mass of stages 23 and 24 (Figure 06; Table 04).

In hatchlings, *Xno-retinochrome1* is co-expressed with *Xno-r-opsin* in the retina, allowing for phototransduction. Additionally, the retina is dark red in colour indicating the presence of retinal and adding to the possibility of light sensing ability in the eyes of late *X. notoides* embryos.

Table 04. Summary of *Xno-retinochrome1*-expression in *Xipholeptos notoides* as revealed via *in situ* hybridization experiments.

Retinochrome1

stage/tissue	eye	olfactory organ	periesophagal mass	documentation
23	lentigenic tissue	absent	magnocellular lobe	brightfield, confocal
24	absent	cell somata	magnocellular lobe	brightfield, confocal
30	retina	absent	absent	brightfield
adult	retina	absent	absent	brightfield

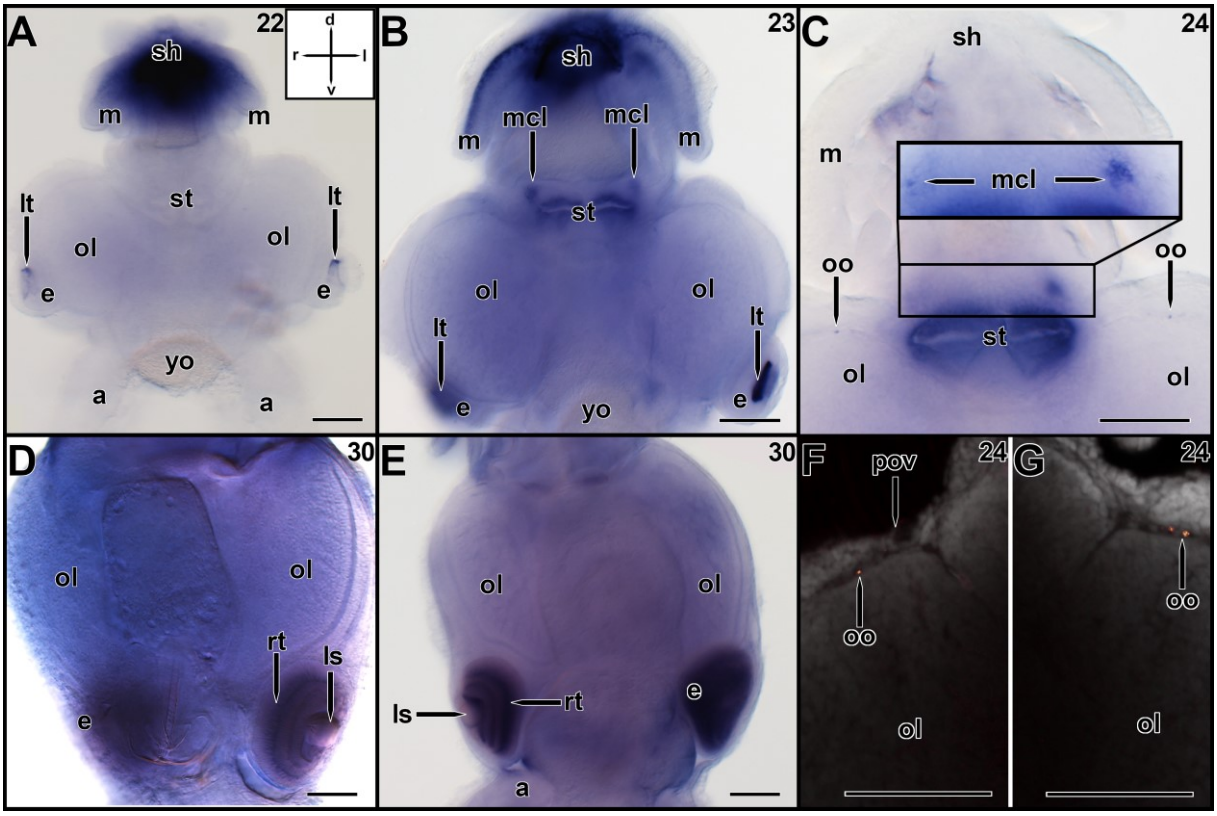

Figure 06. *Xno-retinochrome1*-expression during development of *Xipholeptos notoides* as revealed via *in situ* hybridization experiments. (A) In stage 22 beginning *Xno-retinochrome1*-expression is exhibited in the lentigenic tissue (lt) of the posterior lens (ls) in the developing eye (e). **(B)** Strong *Xno-retinochrome1*-expression in the lentigenic tissue of the posterior lens and dorsally of the statocysts (st) developing magnocellular lobes of the periesophageal mass (mcl) of a stage 23 embryo. **(C)** *Xno-retinochrome1*-expression

in stage 24 is detected in cells of the oolfactory organ (oo) and again in the magnocellular lobes (insert). **(D) & (E)** Hatchlings display strong *Xno-retinochrome1*-expression only in the retina (rt) of the eye. **(F) & (G)** CLSM images highlight the *Xno-retinochrome1*-expression as described in (C). These CLSM images only show one focal plane and more cells along the anterio-posterior axis in the olfactory organ showed *Xno-retinochrome1*-expression as well. The parolfactory vesicle (pov) can be seen in (F). Unspecific and diffuse staining can be seen in the cartilaginous and mineralizing tissue of the statocysts and shell field (sh). Abbreviations: a, arm; ol, optic lobe; y, yolk; anterior views with the dorsal side facing up, axes in the insert in (A) indicate the orientation of all panels, orientation is equal in all images: dorsal (d), ventral (v) and left (l), right (r); scale bars equal 0.1 mm in all panels.

Xenopsin

Xno-xenopsin-expression was documented via *in situ* hybridization experiments. In stage 22 weak expression and in stage 23 strong expression was observed in the lentigenic tissue in the developing eye of *X. notoides* embryos (Figure 07). *Xno-xenopsin* is expressed in the optic lobes, olfactory lobes, and the dorsolateral lobes in stage 24 individuals (Figure 07). Like other genes in this study, *Xno-xenopsin* is expressed in individual cells of the olfactory organ. Additionally, it is expressed in individual medulla cells of the optic lobes and strongly, although more diffusely, in the portion of the olfactory lobes most proximate to the olfactory organs. *Xno-xenopsin* is the only candidate gene to be expressed in the optic lobes, olfactory lobes, and the dorsolateral lobes.

Xno-xenopsin-expression was not found in a later stage, although *in situ* hybridization experiments were conducted in hatchlings and adults as well (Table 05).

Table 05. Summary of *Xno-xenopsin*-expression in *Xipholeptos notoides* as revealed via *in situ* hybridization experiments.

Xenopsin					
stage/tissue	eye	olfactory organ	olfactory lobe	dorso-lateral lobe	documentation
22	lentigenic tissue	absent	absent	absent	brightfield
23	lentigenic tissue	absent	absent	absent	brightfield
24	absent	cell somata	neuropil, cell somata	cell somata	brightfield, confocal
30	absent	absent	absent	absent	not found
adult	absent	absent	absent	absent	not found

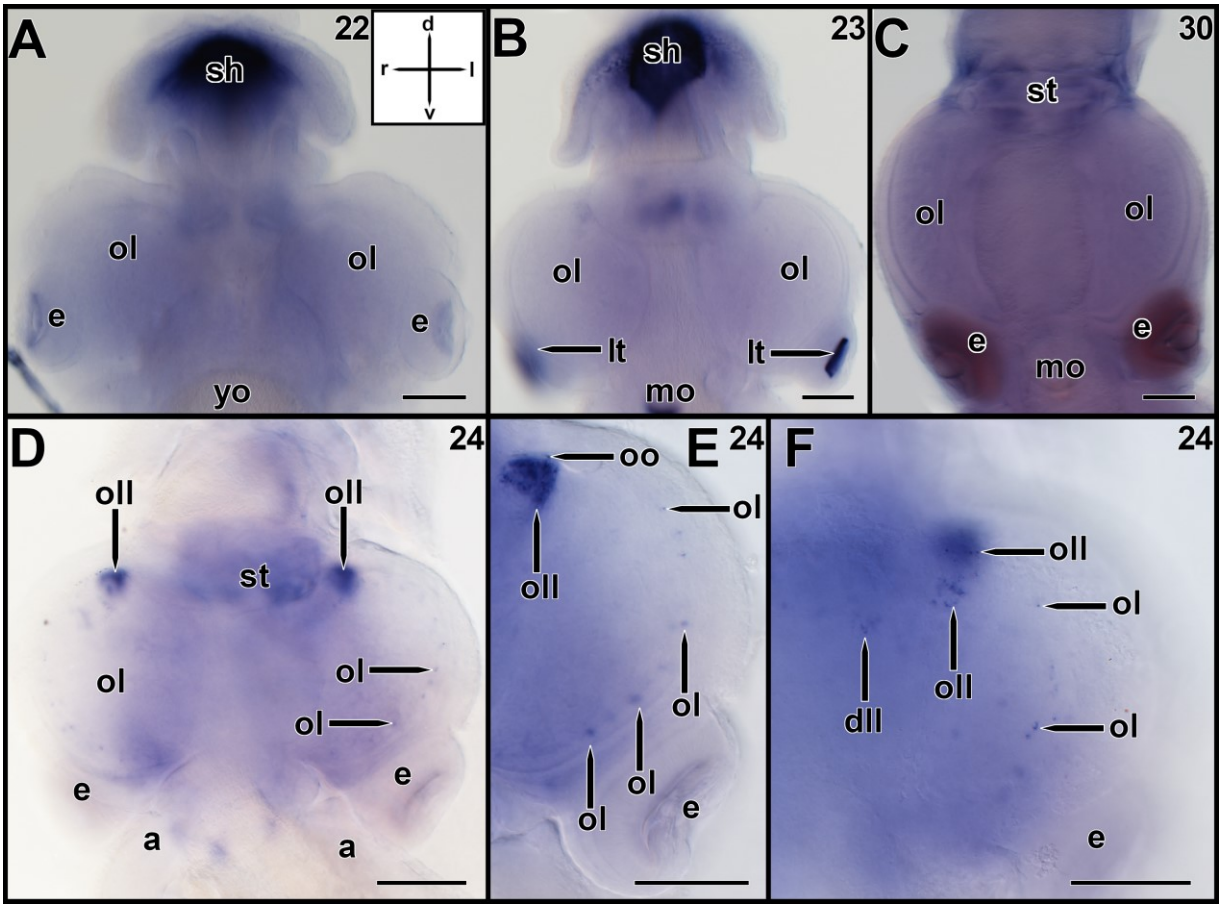

Figure 07. *Xno-xenopsin*-expression during development of *Xipholeptos notoides* as revealed via *in situ* hybridization experiments. (A) Stage 22 individuals show light *Xno-xenopsin*-expression in the lentigenic tissue (lt) of the eyes (e). **(B)** Strong *Xno-xenopsin*-expression is found in the lentigenic tissue forming the posterior part of the lens (ls) of stage 23 individuals. **(C)** No specific *Xno-xenopsin*-expression is detected in hatchlings of *X. notoides*. Note the distinct dark red colouration of the retina of the eyes due to retinal. **(D)** Significant and highly specific expression of *Xno-xenopsin* occurs in stage 24 individuals. *Xno-xenopsin*-expression appears in individual cells of the optic lobes and in the olfactory lobes (oll). **(E*)*** *Xno-xenopsin*-expression appears in the distal medulla of the optic lobe, bordering the cortex of the optic lobe. Note the strongly stained olfactory lobe (ol) besides the olfactory organ (oo) also exhibiting *Xno-xenopsin*-expression. **(F)** Anteriorly more cells show *Xno-xenopsin*-expression throughout the distal optic lobes, the olfactory lobes, and the dorsolateral lobes (dll). Unspecific and diffuse staining can be seen in the cartilaginous and mineralizing tissue of the statocysts, the mouth (mo) and shell field (sh). Abbreviations: a, arm; yo, yolk; anterior views with the dorsal side facing up, axes in the insert in (A) indicate the orientation of all panels: dorsal (d), ventral (v) and left (l), right (r); scale bars equal 0.1 mm in all panels.

Reflectin1

In stage 23 *Xno-reflectin1*-expression is strongest in the developing eye cavity, in the lentigenic tissue of the posterior lens (Figure 08). *Xno-reflectin1*-expression appears in the forming iris and in the olfactory organs of stage 24 embryos. Hatchlings show strong

expression in the iris. In hatchlings, *Xno-reflectin1* appears also in the tissue surrounding the eyes, even reaching the base of the arms anterio-laterally and the ventral head anteriorly (Figure 08, Table 06).

Table 06. Summary of *Xno-reflectin1*-expression in *Xipholeptos notoides* as revealed via *in situ* hybridization experiments.

Reflectin1				
stage/tissue	eye	olfactory organ	other tissues	documentation
23	lentigenic tissue	absent	absent	brightfield
24	iris	cell somata	absent	brightfield, confocal
30	iris	absent	around eyes	brightfield

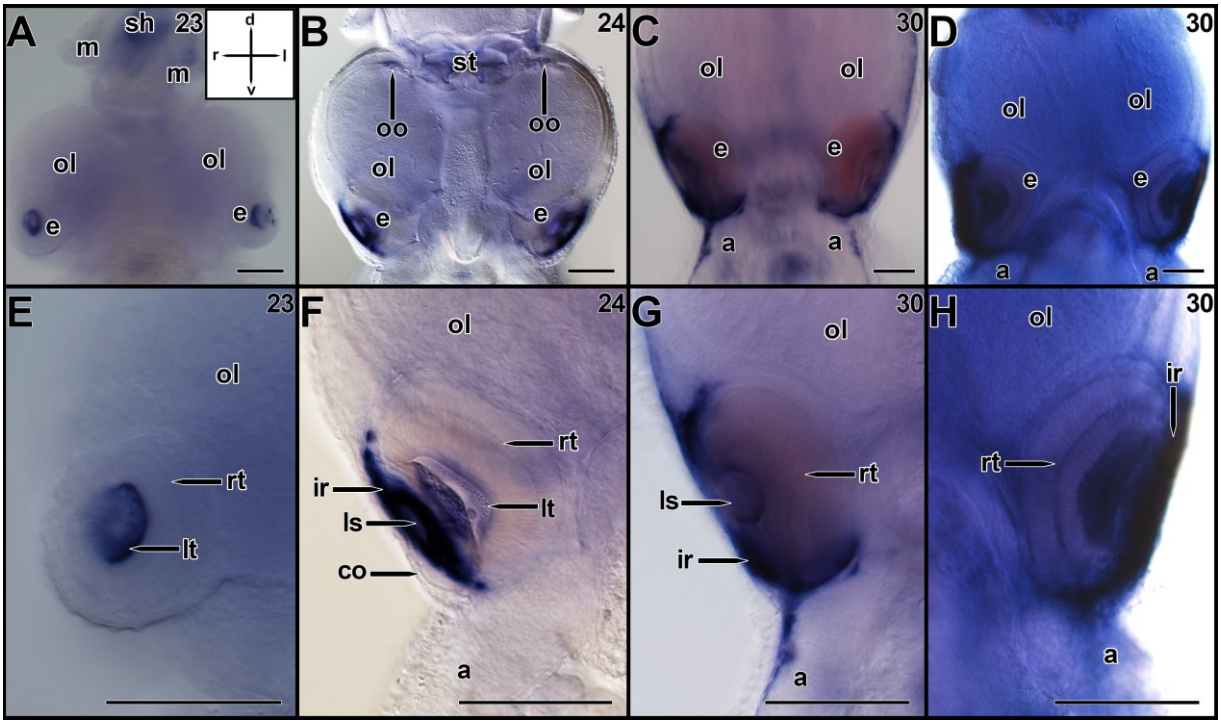

Figure 08. *Xno-reflectin1*-expression during development of *Xipholeptos notoides* as revealed via *in situ* hybridization experiments. (A) In stage 23 individuals, *Xno-reflectin1*-expression is found in the lentigenic tissue of the posterior lens of the developing eye (e). **(B)** Stage 24 individuals exhibit *Xno-reflectin1*-expression in the iris (ir) beneath the forming secondary cornea (co), and in individual cells of the olfactory organ (oo). **(C)** *Xno-reflectin1*-expression is displayed strongly in in the eyes (e) of hatchlings. The retina shows a dark red colouration due to retinal. **(D)** *Xno-reflectin1*-expression appears in the eye of this overstained hatchling as well. **(E)** Detail of the *Xno-reflectin1*-expression in the lentigenic tissue (lt) of the posterior lens of the right eye of a stage 23 individual. **(F)** *Xno-reflectin1*-expression is exhibited in the iris of the right eye of the stage 24 animal shown in (B). **(G)** Hatchlings display strong *Xno-reflectin1*-expression in the iris and adjacent tissues, like the base of the arms (a) and the tissue surrounding the eye laterally and anteriorly. **(H)** The enlarged left eye of an overstained individual exhibits *Xno-reflectin1*-expression in the same pattern as not overstained individuals. Unspecific and diffuse staining can be seen in the cartilaginous and mineralizing tissue of the statocysts (st) and

the shell field (sh). Abbreviations: m, mantle; ol, optic lobe; oo, olfactory organ; rt, retina; anterior views with the dorsal side facing up, axes in the insert in (A) indicate the orientation of all panels, orientation is equal in all images: dorsal (d), ventral (v) and left (l), right (r); scale bars equal 0.1 mm in all panels.

Reflectin2

In stage 23 *Xno-reflectin2* is strongly expressed in the developing eye cavity, namely in the lentigenic tissue of the posterior lens (Figure 09). *Xno-reflectin2* is expressed inside the eyes and lateromedially in the olfactory organs of stage 24 embryos. Stage 30 individuals show strong expression in and around the eyes (Figure 09). *Xno-reflectin2* is expressed in and around the eyelid of stage 30 individuals, laterally at the base of the arms and anteriorly in the epidermis of the head (Figure 09, Table 07).

Table 07. Summary of *Xno-reflectin2*-expression in *Xipholeptos notoides* as revealed via *in situ* hybridization experiments.

Reflectin2				
stage/tissue	eye	olfactory organ	other tissues	documentation
23	lentigenic tissue	absent	absent	brightfield
24	lentigenic tissue	cell somata	absent	brightfield, confocal
30	cornea	absent	eyelids	brightfield

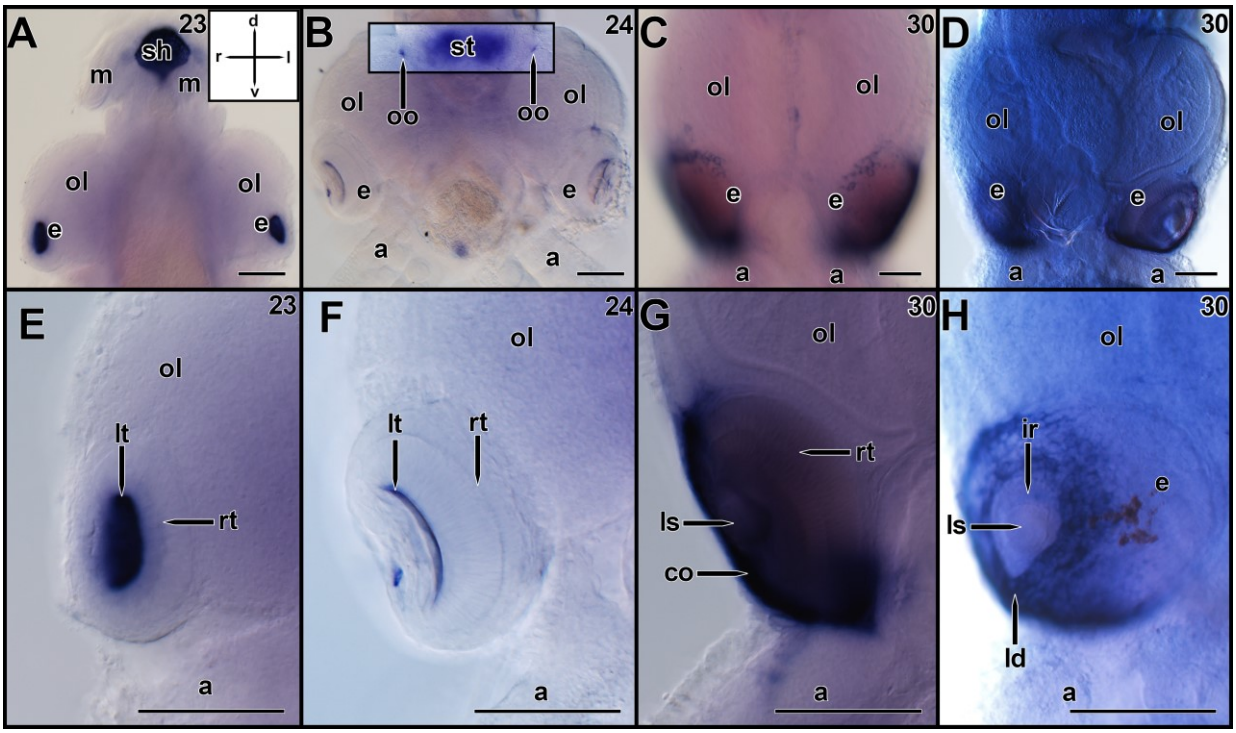

Figure 09. *Xno-reflectin2*-expression during development of *Xipholeptos notoides* as revealed via *in situ* hybridization experiments. (A) In stage 23 individuals, *Xno-reflectin2* is expressed in the developing eye (e). **(B)** Stage 24 individuals show *Xno-reflectin2*-expression in the lentigenic tissue of the posterior lens, and

lateromedially in the olfactory organ (oo), which here is shown as an insert from a more anterior focal plane of the same animal. **(C)** In hatchlings, *Xno-reflectin2*-expression appeared in the developed eye. **(D)** *Xno-reflectin2* is expressed strongly in the eye of another hatchling as well). **(E)** Xno-reflectin2-expression in the lentigenic tissue (lt) of the posterior lens (ls) in the developing eye of the same stage 23 individual seen in (A). **(F)** *Xno-reflectin2* is expressed in the lentigenic tissue of the posterior lens of a stage 24 eye. A more distal spot displays *Xno-reflectin2*-expression as well. The retina (rt) is not stained, but clearly visible. **(G)** *Xno-reflectin2*-expression in the secondary cornea of the eye (co) eye and the surrounding tissues. **(H)** *Xno-reflectin2*-expression in the eye lid (ld) of a hatchling. Overstained individuals show *Xno-reflectin2*-expression in the same pattern as not overstained individuals. Unspecific and diffuse staining can be seen in the cartilaginous and mineralizing tissue of the statocysts (st) and the shell field (sh). Abbreviations: a, arm; m, mantle; ol, optic lobe; oo, olfactory organ; anterior views with the dorsal side facing up, axes in the insert in (A) indicate the orientation of all panels, orientation is equal in all images: dorsal (d), ventral (v) and left (l), right (r); scale bars equal 0.1 mm in all panels.

Discussion

Phylogenetic analysis confirms the identity of all five candidate genes researched in this study. The xno-r-opsin and xno-retinochrome1 amino acid sequences contained the characteristic retinal binding amino acid lysine 296 (K 296) found in all retinal isomerizing opsins (Gühmann *et al.,* 2022). Lysine 296 binds retinal in tandem with glutamic acid 181 (E 181; Vöcking *et al*., 2021), which was also present in our xno-r-opsin and xno-retinochrome1 amino acid sequences. Our xno-xenopsin amino acid sequence was rather short, thus lacking its lysine 296. However, it was highly similar to the xenopsins of other cephalopods and also exhibits the E 181 amino acid.

Amino acid sequences of the *Xno-reflectin2* and *Xno-reflectin1* genes contained at least four reflectin repeat domains as seen in reflectins of other coleoid cephalopods, *i.e*., *Doryteuthis pealeii* and *Doryteuthis_opalescens* (Izumi *et al.*, 2010; DeMartini *et al.*, 2013a; DeMartini *et al.*, 2013b; DeMartini *et al*., 2015).

Rhodopsin **is also expressed in extraocular domains**

Our results corroborate the findings of Yoshida *et al.* (2015b) who suspected that *Rhodopsin* and the associated *Retinochrome* are largely found in the eye of at least *Idiosepius paradoxus.* In *X. notoides,* we find *Xno-r-opsin*-expression by *in situ* hybridization experiments mainly and strongly in the retina. Additionally, we report *Xno-r-opsin*-expression in the dorsal parolfactory vesicles, which are known to be photoreceptive in adult *Todarodes pacificus* (Hara & Hara, 1980) and *Liochranchia* sp. (Messenger, 1967). A novel finding is that *Xno-r-opsin* is also expressed in cells of the olfactory organs of stage 24 embryos of *X. notoides*.

Extraocular occurrence of *Rhodopsin* in cephalopods is known in *Octopus bimaculoides* (Ramirez & Oakley, 2015), *Sepia officinalis* (Mäthger *et al*., 2010; Kingston *et al*., 2015; Bonade *et al.*, 2020), *Sepia latimanus* and *Doryteuthis pealeii* (Kingston *et al.*, 2015). All these studies found *Rhodopsin* transcripts in tissue-specific transcriptomes. *Rhodopsin* and other molecules involved in phototransduction were also localized in cilia-bearing mechanoreceptor cells in the skin of adult *Octopus bimaculoides* via immunohistochemistry (Ramirez & Oakley, 2015). This hints at a dispersed light sensing ability of *O. bimaculoides*, which is supported by the documentation of light activated chromatophore expansion (LACE). LACE has been reported to be present in *Octopus vulgaris* previously (Florey, 1966; Packard & Brancato, 1993).

It was suggested that the extraocular light receptors in the light emitting organs of the bobtail squid (*Euprymna scolopes*) work in a feedback loop with the light organs (Tong *et al.*, 2009). Extraocular *Rhodopsin* has often been found in body parts that are excluded from the animal's field of view, e.g., in the mantle of species like *Doryteuthis pealeii* or *Sepia* spp. (Kingston *et al.*, 2015) and hence could play a role in countershading.

We could not detect any dermal expression of *Xno-r-opsin* or *Xno-retinochrome1* in embryos or adults of *X. notoides*. However, the absence of the rhodopsin protein cannot be assumed with certainty, yet it is probable. If dispersed dermal light sensing is indeed absent in Idiosepiidae, it was either lost in this cephalopod lineage or was never present in idiosepiids. If the latter scenario were the case, the Idiosepiidae could represent a basal decabrachian group that has not yet co-opted ocular *Rhodopsin* into its chromatophores, as known from Sepiida and Teuthida. If it were lost in the Idiosepiidae, this lineage would represent a derived group that has reduced its extraocular light sensing ability.

Expression of opsins in the developing olfactory organ due to olfactory cues?

Most unexpected was the expression of all five candidate genes in the olfactory organ in stage 24. It is especially remarkable, that each gene is expressed in different regions of the olfactory organ. Interestingly, in earlier (stages 22 & 23) as well as in later stages (stages 26 and 30) of *X. notoides*, expression of these genes could not be detected in this organ.

The olfactory organs of cephalopods are two epithelial pits lined with ciliated cells at both sides of the head (Polese *et al.,* 2016). In decabrachian cephalopods, such as *X. notoides*, the olfactory organs are situated dorsally of the optic lobes (Yamamoto *et al.*, 2003) and innervated by the adjacent olfactory lobe (Messenger, 1979).

In stage 24 co-expression of *Xno-retinochrome1* and *Xno-r-opsin* would theoretically allow for phototransduction to take place in the olfactory organ in this developmental stage if the mRNA would have been translated. However, pigmentation of the olfactory organs by retinal was not observed and *Xno-retinochrome1*-expression was weak compared to *Xno-r-opsin*-expression. Moreover, via *in situ* hybridization experiments only mRNA can be detected, and we do not know if these are in fact translated into proteins. Thus, phototransduction in the olfactory organs is improbable, especially considering the other sensory functions opsins can fulfill (Gühman *et al*., 2022). However, embryos of *Sepia officinalis* are known to react to olfactory cues of predators in the egg as early as stage 25 (Romagny *et al*., 2012). Thus, the expression of candidate gene transcripts in the olfactory organ could be triggered by olfactory cues of either conspecifics or other organisms.

We found significant expression of two unrelated gene groups, namely opsins and reflectins, in the olfactory organ in embryonic stage 24 just prior to increase in *Xno-r-opsin*-expression in the eyes of *X. notoides* (Figure 10). In *Sepia officinalis, R-opsin*-expression increases considerably in the embryonic stages 25 – 30 (Bonade *et al.,* 2020).

It was hypothesized that in *Idiosepius paradoxus* the *Pax6* splicing variants lacking the homeodomain regulate the expression of *Reflectin* genes. Incidentally, those homeodomain corrupted splicing variants, namely 1 and 3, are expressed around the eyes (Yoshida *et al*., 2015a). In other words, those *Pax6* splicing variants appear in the tissues giving rise to the eye reflectors, where we found expression of both *Xno-reflectin1* and *Xno-reflectin2* in stage 24 embryos and hatchlings of *X. notoides*. Although those homeodomain corrupted splicing variants do not occur in the olfactory organ, other *Pax6* splicing variants are indeed expressed in the optic lobe and the vicinity of the olfactory organ in *I. paradoxus* embryos (Yoshida *et al*., 2015a). Hence, it is tempting to assume a regulatory function of the olfactory organs during earlier ontogeny. Although our results are clear and well-documented, the number of samples was limited, and embryonic olfactory organ activity should be investigated exhaustively with a sufficient sample size. Our results are especially interesting as it is unclear if the mRNA of the five candidate genes is in fact translated. We deem migration of the mRNA unlikely due to its unstable nature and as this is only known in mammals (Haimovich *et al.*, 2017). It remains unknown if *Reflectin* activity is relevant in embryogenesis of *X. notoides*.

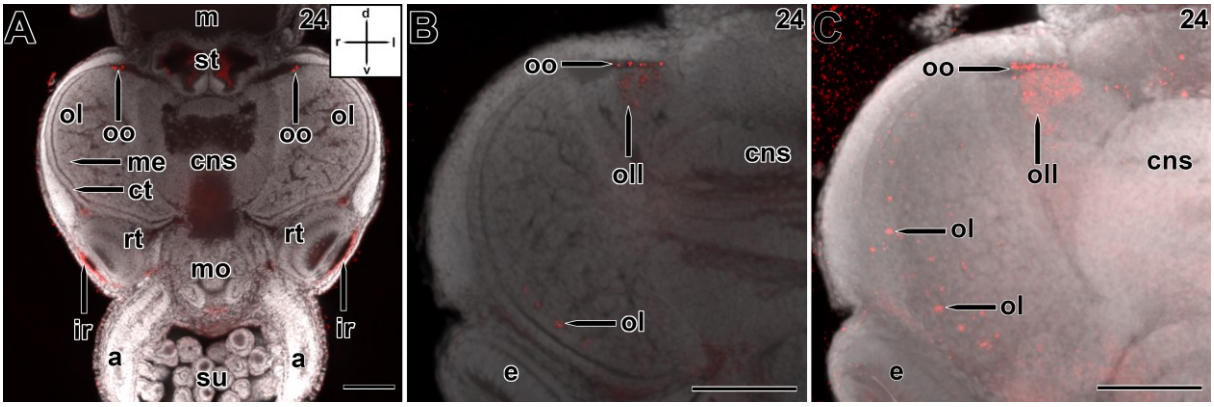

Figure 10. *Xno-reflectin1*-expression (A) and *Xno-xenopsin*-expression (B & C) in stage 24 individuals of *Xipholeptos notoides* as revealed via *in situ* hybridization experiments. Red colouration in the images indicates the stain of the DIG-specific antibodies binding to the riboprobes, while grey corresponds to the DAPI stain. Unspecific staining (red) can be seen in the cartilaginous and mineralizing tissues of the statocysts (st), mouth (mo) and in dust particles outside of the tissue. **(A)** This panel shows one representative layer of a confocal laser scanning microscopy (CLSM) image stack of a *X. notoides* individual showing *Xno-reflectin1*-

expression in cells throughout the olfactory organ (oo) and in the forming iris (ir) of the developing eye (e). **(B) and (C)** show a representative layer (B) and a z-project of all relevant layers (C) of a confocal laser scanning microscopy (CLSM) image stack of a stage 24 *X. notoides* individual. *Xno-xenopsin*-expression is found in individual cells of the olfactory organ (oo) and the distal medulla of the optic lobes (ol) of a stage 24 individual. In the olfactory lobe (oll) of the same individual *Xno-xenopsin*-expression occurs more diffusely, but considerably, as can be seen in the z-project image in (C). Abbreviations: a, arm; cns, central nervous system; m, mantle; rt, retina; su, suckers; anterior views with the dorsal side facing up, axes in the insert in (A) indicate the orientation of all panels, orientation is equal in all images: dorsal (d), ventral (v) and left (l), right (r); scale bars equal 0.1 mm in all panels.

The parolfactory vesicle as an extraocular photoreceptor during ontogeny?

In stage 24, *Xno-r-opsin*-expression occurred also in the dorsal parolfactory vesicles which are known to be extraocular photoreceptors in decabrachian cephalopods (Messenger, 1967; Hara & Hara, 1980; Kingston *et al*., 2015). The parolfactory vesicles of the deep-sea squid *Todarodes pacificus* have been shown to contain retinochrome and rhodopsin proteins in roughly equal amounts and are capable of retinal isomerization (Hara & Hara, 1980). These opsins also co-occur in epithelial cells of the parolfactory vesicles and are distributed within these cells similarly to rhabdomeric cells of the cerebral eyes (Hara & Hara, 1980). Although the cells of the inner lining of the parolfactory vesicles are akin to rhabdomeres they are not arranged as rigidly as those in the retina of the eyes (Hara & Hara, 1980). However, phototransduction very likely occurs in the parolfactory vesicles of adult squid (Messenger, 1979). Our results show the expression of *Xno-r-opsin* in the parolfactory vesicles of stage 24 embryos, a finding new for decabrachian cephalopods (Figure 05 & Figure 11). In contrast, *Xno-retinochrome1* could not be detected in the parolfactory vesicles, however, phototransduction may take place in subsequent embryonic stages that have not been investigated in this study (Figure 06).

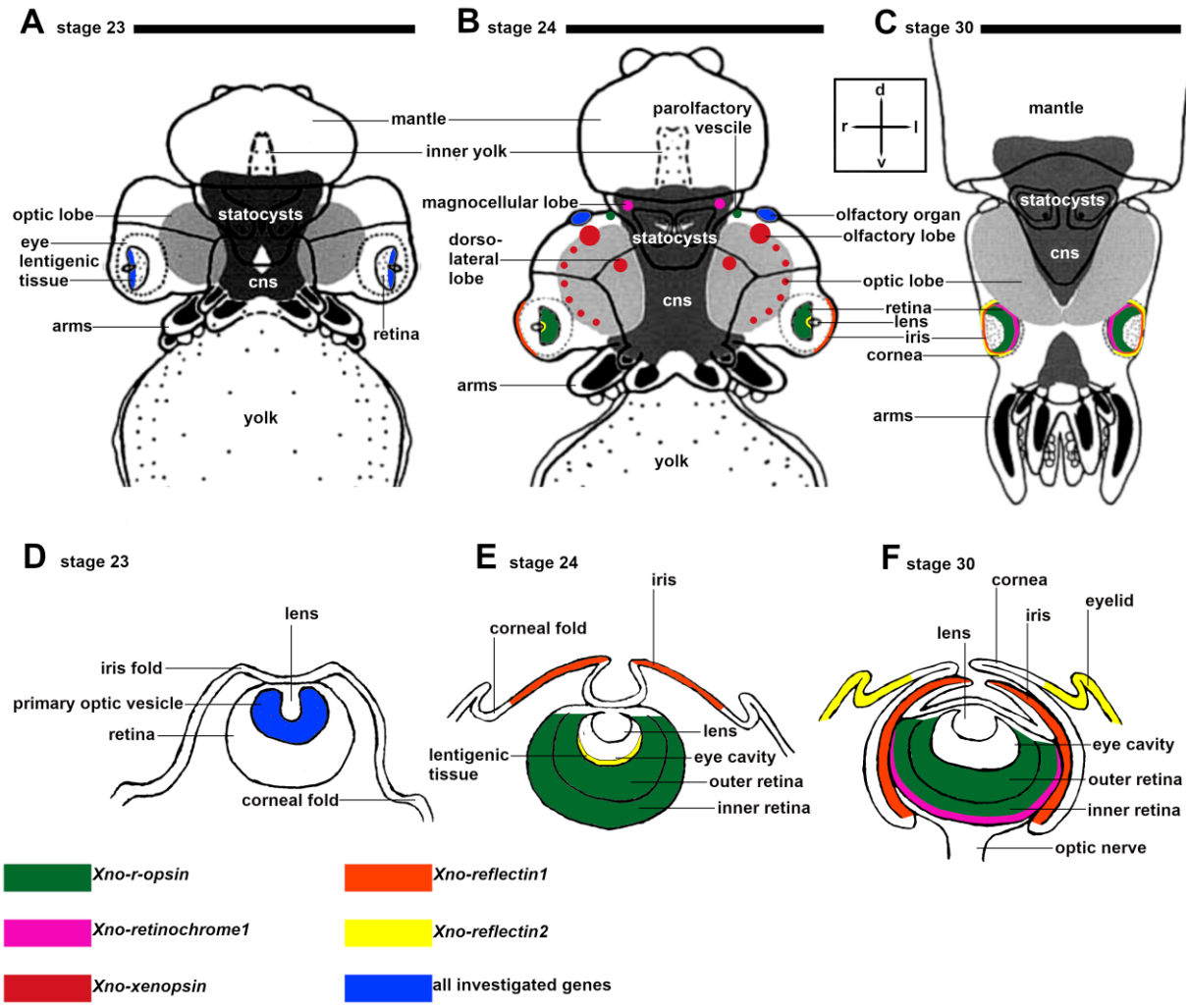

Figure 11. Schematic depiction of gene expression in the body (A - C) and in the eyes (D – F) during various developmental stages of *Xipholeptos notoides* as revealed via *in situ* hybridization experiments. (A) and (D) show gene expression of all investigated genes in the lentigenic tissue of the primary optic vesicle. Here (A) shows the whole body and some of the yolk of the embryo, while (D) is a sketch of the early developing eye in stage 23 embryos of *X. notoides*. **(B) and (E)** show expressional activity in stage 24 *X. notoides* embryos. (B) gives an overview of gene expression throughout the whole embryo, whereas (E) illustrates the expression of *Xno-r-opsin* and *Reflectin* genes in a stage 24 eye. *Xno-xenopsin*-expression is found in individual cells of the olfactory organ, the olfactory lobe, the dorso-lateral lobe, and the distal medulla of the optic lobes as seen in (B). *Reflectin* genes are mainly found in the eyes, but also in the olfactory organ. Note further the expression of *Xno-retinochrome1* in the magnocellular lobe and expression of *Xno-r-opsin* in the parolfactory vesicles. **(C) and (F)** depict the expression in stage 30 embryos, also known as hatchlings. Expression of the investigated genes is mainly found in and around the eyes. While (C) shows mainly the head portion of a hatchling, (F) provides a detailed scheme of gene expression in the consecutive tissue folds in the *X. notoides* eye. Abbreviations: cns, central nervous system; anterior views with the dorsal side facing up, axes in the insert in (C) indicate the orientation of panels (A) to (C): dorsal (d), ventral (v) and left (l), right (r); Drawings in panel (A) to (C) were modified after Yamamoto *et al*. (2003). Detailed drawings of the eyes were hand drawn and digitized by the author. Scale bars equal 0.6 mm in all panels.

Xenopsin is expressed in brain lobes and the olfactory organ

Ciliary opsins in cerebral eyes were long considered to be only found in vertebrates, until a ciliary photoreceptor expressing *C-opsin* was found in the larval eyes of a brachiopod (Passamaneck *et al.*, 2011). Later, invertebrate *C-opsins* were identified as their own clade and termed *Xenopsins*, belonging to one of the nine opsins thought to be present in the bilaterian common ancestor (Ramirez *et al*., 2016). Since then, *Xenopsin* was found to be co-expressed with *Rhodopsin* in the larval eyes of the polyplacophoran *Leptochiton asellus* (*Vöcking et al.*, 2017), in the eyes of the terrestrial slug *Ambigolimax valentianus* (formerly known as *Limax valentianus*; Matsuo *et al*., 2019) and in the larval eyes and adult extraocular photoreceptors of the flatworm *Maritigrella crozieri* (Rawlinson *et al*., 2019). Proteins involved in the vertebrate retinal re-isomerizing dark cycle were also found in the polyplacophoran *Leptochiton asellus,* hinting at the co-occurrence of ciliary and rhabdomeric retinal re-isomerization pathways (Vöcking *et al*., 2021).

In cephalopods, *Xenopsin* was detected in the transcriptome of the brain of *Sepia officinalis*, but not in the eyes (Bonade *et al.*, 2020). A *Xenopsin* (by Yoshida *et al.*, 2015b, termed as *C-opsin*) was found to be co-expressed with *r-opsins* in the eyes and gonads of *Idiosepius paradoxus* hinting towards an additional role apart from vision.

We provide the first gene expression pattern of a *Xenopsin* in cephalopods. Our results show *Xno-xenopsin*-expression in the lentigenic tissues in early eye formation and in the optic lobe, the olfactory lobe, the dorsolateral lobe and in the olfactory organ (Figures 07 & 10). The *Xno-xenopsin*-expression in the olfactory organ is consistent with the expression documented for other individuals from the same clutch of eggs, which exhibited expression of *Xno-r-opsin, Xno-retinochrome1* and two reflectins in cell somata of the olfactory organ. These findings suggest that the possibly monostable *Xenopsin* (Vöcking *et al*., 2021) might play a role in signal transduction in the nervous system, especially as its expression is strongest in the olfactory lobe and appears in other brain lobes that are interconnected with the olfactory organ (Messenger, 1979). Our results corroborate the enigmatic reputation of *Xenopsin*, raise further questions about its function both in cephalopods and in molluscs in general, and could hint at non-visual functions of opsins as suggested in earlier studies (Gühmann *et al.*, 2022).

Reflectins are expressed in the eyes and the olfactory organ

Reflectins are a group of genes with no known orthologs outside of Cephalopoda, except possibly a transposon from *Vibrio fischeri* (Crookes *et al.*, 2004; Guan *et al.*, 2017). They are unstructured proteins containing repetitive sequences and occur in iridiophores, also known as

iridiocytes, in platelets as well as in dynamically controlled leucophores as globulary aggregates (Cloney & Brocco, 1983). In leucophores they scatter all wavelengths, resulting in a bright white appearance of this skin layer when visible (DeMartini *et al*., 2013b). In the iridiophores the reflectins are embedded in platelets, called iridiosomes, forming bragg reflectors. Bragg reflectors are multi-layered mirrors, achieving reflectance or scattering of light by constructive phase interference (DeMartini *et al*., 2013a). In *Euprymna scolopes,* for which reflectins were first described, these platelets cannot dynamically change their density and thickness and thus have a fixed refractive index (Crookes *et al*., 2004). Dynamic colour change was first documented in *Doryteuthis pealeii* (Izumi *et al*., 2010) and later discovered in *Dorytheuthis opalescens* as well (DeMartini *et al.*, 2013a).

Our results show that *Reflectin* genes first appear in the lentigenic tissue of the posterior lens in stages 22 – 23. In stage 24 individuals, *Xno-reflectin1*-expression appears in the forming iris and in the olfactory organ, while *Xno-reflectin2* transcripts are located mainly in the lentigenic tissue of the posterior lens and in the olfactory organs (Figure 11). In hatchlings, each *Reflectin* gene shows a distinct expression pattern in and around the distal eyes. *Xno-reflectin1* appears throughout the extensive iris, while *Xno-reflectin2*-expression is exhibited in the cornea and eyelid. This spatially different expression patterns in the iris and cornea have been found previously in *Sepia officinalis* hatchlings (Andouche *et al.*, 2013) and might indicate different functions or division of labour between those two *Reflectins*. As *Reflectin* mRNA expression in *X. notoides* is limited to the eyes in adults it is compelling to suspect a role in vision for the corresponding proteins. *Xno-reflectin1*- and *Xno-reflectin2*-expression occurring in the olfactory organs is quite surprising, but expression of *Reflectins* can apparently be associated with the nervous system during cephalopod ontogeny, as previously shown (Duruz *et al.*, 2022). What functions reflectins may fulfil in the nervous system remains unknown.

Conclusion

In this Master thesis, gene expression of *Xenopsin* is for the first time reported in the brain of a cephalopod by *in situ* hybridization. Previously, *Xenopsin* was only revealed to be present in a brain transcriptome of the cuttlefish *Sepia officinalis* (Bonade *et al.*, 2020).

In our work, we focus on developmental stages of *X. notoides* in which the eyes form and we found the genes *Xno-r-opsin*, *Xno-retinochrome1* as well as Xno-*reflectin1* and *Xno-reflectin2* to be present and occur in distinct tissues and patterns (Figure 11). We support assumptions of

previously suspected regulation of *Reflectin*-expression by the homeodomain-deficient *Pax6* splicing variants 1 and 3 (Yoshida *et al.*, 2015a). We could not detect expression of *Reflectin* copies in the skin of *X. notoides* unlike shown in cephalopods relatively closely related to Idiosepiids (Tanner *et al*., 2017), like *Euprymna scolopes* and *Sepia officinalis* (Andouche *et al.*, 2013; Crookes *et al*., 2004). However, we found unexpected expression of genes encoding opsins and reflectins in the olfactory organ and various brain lobes.

Abstract

The eyes of squids, octopuses, and cuttlefish are a textbook example for evolutionary convergence, due to their striking similarity to those of vertebrates. For this reason, studies on cephalopod vision and photoreception are of importance for a broader audience. Previous studies showed that genes such as *Pax6,* or certain opsin-encoding genes, are evolutionarily highly conserved and play similar roles during ontogenesis in remotely related bilaterians. In this Master thesis, genes are identified and characterized which encode photosensitive proteins and reflectins. Via *in situ* hybridization experiments the expression patterns of five candidate genes including *Rhodopsin*, *Xenopsin*, *Retinochrome* and two *Reflectin* genes have been visualized in developing embryos of the pygmy squid *Xipholeptos notoides*.

In situ hybridization experiments revealed that *Rhodopsin* is not only expressed in the retina of *X. notoides*, but also in the olfactory organ and the dorsal parolfactory vesicles, a cephalopod apomorphy. The two *Reflectin* genes are expressed in the eyes and in the olfactory organ. These findings corroborate previous studies that found opsins in the transcriptomes of the eyes and several extraocular tissues of various cephalopods. Expression of *Rhodopsin*, *Xenopsin*, *Retinochrome* and the two *Reflectin* genes in the olfactory organ is a finding that has not been described yet. In other organisms, it has been shown that retinochrome and rhodopsin proteins are obligatorily associated with each other as both molecules rely on each other for retinal isomerisation. In addition, *Retinochrome* was detected in the retina of *X. notoides* and in the olfactory organ.

This study shows numerous new expression domains for opsin encoding genes in organs that have not been associated with photoreception before that suggest that either opsins may not only be involved in photoreception or organs such as the olfactory organ are involved in photoreception.

Zusammenfassung

Die Augen von Kalmaren, Oktopoden und Sepien gelten als Lehrbuchbeispiele für evolutionäre Konvergenz, da sie auffallend denen der Wirbeltiere ähneln. Aus diesem Grund sind Studien zum Sehvermögen und zur Photorezeption der Kopffüßer für ein breites Publikum relevant. Vorherige Studien zeigten, dass Gene wie *Pax6* und einige opsin-codierende Gene evolutionär hochkonserviert sind und selbst in weitschichtig verwandten Bilateria ähnliche Rollen spielen. In dieser Masterarbeit werden Gene identifiziert und charakterisiert die für photosensitive Proteine sowie Reflectine codieren. Via *In-situ*-Hybridisierungs-Experimente wurden die Expressionsmuster von fünf Genen (*Rhodopsin*, *Xenopsin*, *Retinochrom* und zwei für *Reflectine* codierende Gene) in sich entwickelnden Embryos des Zwergkalmaren *Xipholeptos notoides* visualisiert.

Die *In-situ*-Hybridisierungs-Experimente haben gezeigt, dass *Rhodopsin* nicht nur in der Retina von *X. notoides* exprimiert wird, sondern auch in den Riechorganen und den dorsalen parolfaktorischen Vesikeln, einer Apomorphie der Kopffüßer. Die beiden *Reflectin*-Gene werden in den Augen und ebenfalls in den Riechorganen exprimiert. Diese Ergebnisse untermauern vorherige Studien, welche Opsine in Transkriptomen von Augen und extraokulären Geweben diverser Cephalopoden nachweisen konnten. Die Expression von *Rhodopsin*, *Xenopsin*, *Retinochrom* und der beiden *Reflectin*-Gene in den Riechorganen ist eine Erkenntnis, die bisher nicht beschrieben wurde. In anderen Organismen wurde gezeigt, dass Retinochrom- und Rhodopsin-Proteine obligatorisch miteinander assoziiert sind, da beide Moleküle für die Isomerisierung von Retinal aufeinander angewiesen sind. Zusätzlich konnte in dieser Masterarbeit *Retinochrom* in der Retina und in den Riechorganen von *X. notoides* detektiert werden.

Diese Studie zeigt zahlreiche neue Expressionsdomänen für opsin-codierende Gene in Organen, die bisher nicht mit Photorezeption assoziiert wurden. Dies suggeriert, dass entweder Opsine nicht nur in der Photorezeption involviert sind oder Organe wie etwa die Riechorgane zur Photorezeption fähig sind.

List of figures

List of tables

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