



Molecular Evolution of Malacostracan Short Wavelength Sensitive Opsins

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Abstract

Investigations of the molecular mechanisms behind detection of short, and particularly ultraviolet, wavelengths in arthropods have relied heavily on studies from insects due to the relative ease of heterologous expression of modified opsin proteins in model organisms like *Drosophila*. However, species outside of the *Insecta* can provide information on mechanisms for spectral tuning as well as the evolutionary history of pancrustacean visual pigments. Here we investigate the basis of spectral tuning in malacostracan short wavelength sensitive (SWS) opsins using phylogenetic comparative methods. Tuning sites that may be responsible for the difference between ultraviolet (UV) and violet visual pigment absorbance in the *Malacostraca* are identified, and the idea that an amino acid polymorphism at a single site is responsible for this shift is shown to be unlikely. Instead, we suggest that this change in absorbance is accomplished through multiple amino acid substitutions. On the basis of our findings, we conducted further surveys to identify spectral tuning mechanisms in the order *Stomatopoda* where duplication of UV opsins has occurred. Ancestral state reconstructions of stomatopod opsins from two main clades provide insight into the amino acid changes that lead to differing absorption by the visual pigments they form, and likely contribute the basis for the wide array of UV spectral sensitivities found in this order.

Keywords Opsin · Ultraviolet · Vision · Arthropod · Stomatopod · Spectral tuning

Introduction

Ultraviolet (UV) visual sensitivity was once thought to be rare, but numerous studies have shown it to be quite prevalent, particularly amongst arthropods (Cronin and Frank 1996, Briscoe and Chittka 2001, Koyanagi et al. 2008, Kashiyaama et al. 2009, reviewed by Cronin and Bok 2016). The ability to see UV light confers potential advantages in contrast detection for foraging and predator avoidance (Losey et al. 1999; Johnsen and Widder 2001), navigation and orientation abilities (Seliger et al. 1994; Homberg et al. 2011), and a means of communication between conspecifics (Tovee 1995; Franklin et al. 2018). Early work on UV vision and photosensitivity was conducted mainly in insects (Lubbock 1881; Kühn 1924, 1927), which has resulted in robust

research output and relatively well understood molecular mechanisms of achieving UV sensitivity in this class of arthropods (Chase et al. 1997; Smith et al. 1997; Briscoe 1998, 2001; Townson et al. 1998; Salcedo et al. 2003). The same cannot be said for other members of the Pancrustacea, a clade containing the hexapods and the paraphyletic crustaceans (Bracken-Grissom and Wolfe 2020). While UV sensitivity has been documented physiologically in some non-insect pancrustaceans, there is less data available for the molecular components of UV vision, particularly the diversity and amino acid composition of opsin proteins.

Across pancrustacean lineages, the majority of documented variation in short wavelength spectral sensitivities outside of the *Insecta* comes from species in the class *Malacostraca* (Hariyama et al. 1986; Johnson et al. 2002; Marshall et al. 2003; Thoen et al. 2017). Short wavelength sensitive (SWS) is defined here as peak sensitivity to wavelengths below 440 nm (violet) while ultraviolet refers to wavelengths within the SWS portion of the spectrum which are below 400 nm. This distinction is based on characterizations of the short wavelength photoreceptor, or R8 cell, found in malacostracan visual systems

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(Hariyama et al. 1993; Cronin et al. 1994, 2014b; Kingston and Cronin 2015). R8 cell sensitivity in the violet range ($\lambda_{\max} = 440$ nm) has been recorded from the crayfish *Procambarus clarkii* (Cummins and Goldsmith 1981) and evidence for R8 cell UV light detection has been found in other species of decapods (λ_{\max} between 350 and 400 nm; Frank and Case 1988), stomatopods or mantis shrimp (λ_{\max} between 310 and 380 nm; Cronin et al. 2014a) and an isopod ($\lambda_{\max} < 340$ nm; Hariyama et al. 1993). These light detection abilities are enabled at the molecular level by opsin proteins, making them the target of evolutionary pressures impacting the sensitivity of homologous malacostracan photoreceptors.

Opsins, when bound to a vitamin-a derived chromophore, form a visual pigment capable of absorbing photons of light in a specific range of wavelengths and initiating cellular signaling known as the phototransduction cascade (Shichida and Imai 1998). Changes in amino acids at specific positions affect the folding pattern of the opsin protein and therefore its functionality (Pahlberg et al. 2006; Porter et al. 2007). These changes can be particularly important between closely related species where the chromophore is likely the same. Major changes in polarity (F–Y), hydrophobicity (S–A), size (I–V) or a combination of these elements (S–T) of amino acids close to the chromophore can shift the absorbance of the visual pigment formed (Lin et al. 1998; Janz and Farners 2001; Takahashi and Ebrey 2003; Salcedo et al. 2009; Nagata et al. 2019). These changes can be the result of random mutation or selective pressure at specific sites resulting in the adaptation of visual systems to specific environments (Lythgoe 1972; Warrant 2019).

Previous work by Salcedo et al. (2003) identified a single amino acid polymorphism at bovine rhodopsin position 90 as critical in tuning insect visual pigment absorbance. A substitution at this site from asparagine or glutamic acid to lysine in the visual pigments of *Drosophila* was found to change peak absorbance from visible ($\lambda_{\max} = 436$ nm) to UV ($\lambda_{\max} = 377$ nm) when modified visual pigments were expressed in transgenic flies. Position 90 had also been previously found to be important in UV light detection in bird visual pigments (Wilkie et al. 2000). This amino acid substitution has been often cited as the basis of UV vision in arthropods (Koyanagi et al. 2008; Kashiya et al. 2009; Hering and Mayer 2014; Wong et al. 2015; Cronin and Bok 2016; Pérez-Moreno et al. 2018), however its applicability outside of the insects has been called into question (Palcanda et al. 2022). Specifically, this amino acid position is lysine, the residue which should confer UV sensitivity in SWS opsins, from both the violet sensitive crayfish and the UV sensitive decapods, stomatopods, and isopod cited above. Thus, it appears that this single amino acid polymorphism is not solely responsible for UV absorbance in the Malacostraca.

Work by Henze and Oakley (2015), in which an arthropod opsin tree was reconciled with a species tree to provide information about the evolutionary history of this class of proteins, indicated that three main SWS opsin clades exist in the *Pan-crustacea*. The SWa and SWb clades were found to be lost in the decapods and all members of the *Hexapoda* while the SWc clade contained opsins from the *Hexapoda* and other pancrustacean orders including the decapods. The presence of SWS opsins from multiple clades in some malacostracan species corresponds with the observation of multiple types of SWS photoreceptors in those same taxa, particularly in the order *Stomatopoda*. Up to three SWS opsin transcripts have been found to be expressed in a single species of stomatopod (Bok et al. 2014; McDonald et al. 2022; Palcanda et al. 2023). Microspectrophotometry (MSP) measurements from two of the visual pigments found in the most well characterized species *Neogonodactylus oerstedii* revealed differences in peak absorbance. A visual pigment formed with an opsin from the stomatopod specific clade cUV1 had a peak absorbance at 334 nm while the clade cUV2 visual pigment had a slightly higher absorbance at 383 nm, both in the UV wavelengths (Bok et al. 2014). Stomatopod UV vision is amongst the most well studied in the *Malacostraca*, spanning species (Cronin et al. 1994; Marshall and Oberwinkler 1999; Bok et al. 2014, 2018) and life stages (McDonald et al. 2022, 2023). This order therefore provides an opportunity to look more closely at the amino acid changes that may result in different spectral absorbances within the UV and the evolutionary mechanisms that resulted in this unusual level of diversity.

Here we investigate how differences in opsin amino acid sequence may relate to spectral tuning of putative SWS visual pigments in the class Malacostraca. Using a combination of selection analyses and protein modeling, we present hypothesized spectral tuning sites and specific amino acid changes which may account for the shift from violet to UV absorption in malacostracan SWS visual pigments. In addition, we explore sequence level differences between major clades of stomatopod SWS opsins, taking steps to elucidate the ancestral sequence that gave rise to each and the evolutionary changes that resulted in differing UV spectral absorbances of visual pigments in the order. By identifying potentially important spectral tuning sites, we contribute to the growing breadth of knowledge of UV visual pigment diversity and function in arthropods and provide targets for future mutagenesis studies in this diverse group.

Methods

Malacostracan Opsin Data Collection

Malacostracan opsin sequences from the SWS spectral clade were identified following methods described in Palcanda

et al. (2022). In brief, publicly available opsin sequences and assembled and raw RNAseq data from malacostracans were downloaded from the National Center for Biotechnology Information (NCBI) databases. All data available at the time of analysis was included except in the case of the decapods where relatively plentiful RNAseq data meant that representative species from different infraorders could be selected and searched for opsins. In addition to the Decapoda, orders Amphipoda, Anaspidacea, Bathynellacea, Cumacea, Euphausiacea, Isopoda, Leptostraca, Lophogastrida, Mysida, Stomatopoda, and Tanaidacea were also searched for SWS opsins. Raw RNAseq data was trimmed using Trimmomatic v.0.36 (Bolger et al. 2014) and assembled using Trinity v.2.11.0 (Grabherr et al. 2013) on the National Center for Genome Analysis Support's Carbonate computing cluster at Indiana University. Opsin sequences were identified from assemblies using the Phylogenetically Informed Annotation (PIA) pipeline on the University of California Santa Barbara Galaxy platform (Speiser et al. 2014). Opsin sequences that did not contain residues for at least 70% of sites identified as being within 5 Å of the visual pigment chromophore were discarded, resulting in a minimum amino acid sequence length of 140 from the decapod *Cambarus rusticiformis* and maximum length of 394 from the stomatopod *Neogonodactylus oerstedii* (Supplementary Table S1).

PCR Amplification of Stomatopod SWS Opsins

Stomatopod SWS opsin fragments from the previously described cUV1 and cUV2 clades (Bok et al. 2014), were amplified using polymerase chain reaction (PCR). Primers were designed using published stomatopod opsin sequences (Supplementary Table S1) to target the region spanning transmembrane helices three to six, which contains the majority of sites within 5 Å of the opsin chromophore. Only one opsin type in cUV1, the cUV1.2 opsin type was successfully amplified. (cUV1.2 forward 5'-GCMTGGGCTGCCAAATCTWYGGMWTC-3' and reverse 5'-CRTTCACTCTAGCYGCCTGCTTCTTG-3'; cUV2 forward 5'-CATCTGGGGCAAAYTGGGSGTGAC-3' and reverse 5'-CTGGATCATCGACATCARRGGAGTC-3'). Unsuccessful attempts were made to amplify a larger portion of the opsin sequence, including the chromophore binding lysine in the seventh transmembrane helix. These efforts resulted in non-target amplification, possibly due to high levels of sequence similarity between stomatopod opsins.

Samples for PCR consisted of adult stomatopod eyes or whole larvae preserved in RNAlater. Adult stomatopods were identified using morphology at the time of collection and DNA barcoding was used to identify larval specimens and confirm the identity of adults (Palcanda et al. 2020). RNA was extracted from preserved tissues using a RNeasy Mini Kit (Qiagen) following manufacturer protocols. An

on-column DNase digestion was performed to remove residual DNA and extracted RNA was quantified using a Qubit fluorometer. Reverse transcription of ~20 ng of RNA was performed using the SuperScript IV Reverse Transcriptase kit (Invitrogen) and the resulting cDNA was used to perform PCR. PCR mixes were conducted using Quick-Load Taq 2X Master Mix (NEB) following manufacturer protocols with 25 µl reactions containing 0.2 mM of each primer and 10–30 ng of cDNA. The cycling parameters for each PCR were a single 3 min incubation at 95 °C; 40 cycles of 15 s at 95 °C denaturing, 30 s at 58 °C annealing, and 1 min at 68 °C elongation; and a final elongation of 5 min at 68 °C. PCR amplicons were cleaned using EXO-SAP-IT (Thermo Fisher) and sequenced at the Advanced Studies in Genomics, Proteomics and Bioinformatics facility at the University of Hawai'i at Mānoa (Honolulu, Hawai'i). Thirteen sequences of SWS opsins from eight stomatopod species were generated, (Supplementary Table S1). These sequences included previously unpublished opsins from *Acanthosquilla derijardi*, *Chorisquilla hystrix*, *Chorisquilla tweediei*, *Gonodactylus chiragra*, *Gonodactylus smithii*, *Lysiosquillina maculata*, *Gonodactylus platysoma*, and *Haptosquilla trispinosa* with sequences from the latter two species isolated from larval samples. New data was combined with previously published sequences (Porter et al. 2013; Bok et al. 2014; Palcanda et al. 2023) for a final dataset consisting of 24 opsin sequences from 13 species of stomatopod. Newly generated PCR data was not added to the malacostracan dataset though previously published stomatopod opsin sequences were included.

Phylogenetic Analyses of Malacostracan Opsins

Translated malacostracan opsin sequences were compiled into a MAFFT alignment (Katoh et al. 2002) using automatic algorithm selection and a Blosum62 scoring matrix with an open gap penalty of 1.53 in Geneious R10 (Kearse et al. 2012). ModelFinder (Kalyaanamoorthy et al. 2017) was used to select a LG substitution matrix and a maximum likelihood phylogeny with 1000 bootstrap iterations was inferred using RAxML (Stamatakis 2014), accessed through the CIPRES platform (Miller et al. 2010). Malacostracan middle wavelength sensitive opsins (Accession nos. QIW86026, MN939404, BAA09132) were used as an outgroup and clades were delineated based on monophyly as well as conserved amino acid residues at potential spectral tuning sites. This process was repeated with stomatopod SWS opsins with a middle wavelength sensitive opsin from *Neogonodactylus oerstedii* used to root the tree (Accession no. QIW86026). Clades in the malacostracan SWS opsin tree were named in concordance with the opsin gene tree used in Henze and Oakley (2015) using the naming scheme from the reconciled tree in the same study.

Identification of Potential Spectral Tuning Sites and Ancestral State Reconstruction

Swiss-PdbViewer (Guex and Peitsch 1997) was used to fit a *Neogonodactylus oerstedii* UV opsin (NCBI accession no. AIF73507) to the structure of a previously published jumping spider rhodopsin protein (PDB ID 6I9K; Varma et al. 2019) and identify residues predicted to be within 5 Å of the chromophore which have been shown to affect spectral tuning (Neitz et al. 1991; Chan et al. 1992; Sharkey et al. 2023). This process was repeated for each SWS opsin sequence included in this study to verify that positions near the chromophore were correctly identified.

Tests of selection were used to identify additional potential tuning sites. A focus was placed on sites under positive diversifying selection which may account for differences in absorbance between clades of SWS opsins. One selection test, MEME, employs a maximum likelihood approach to detect both episodic and pervasive positive selection (Murrell et al. 2012). A likelihood ratio test resulting in a minimum *p*-value of 0.05 was used to infer positive selection. The other selection test used was FUBAR, which uses a Bayesian approach to detect positive or negative selection resulting in a posterior probability of positive and negative selection at each site (Murrell et al. 2013). Sites with a posterior probability of positive selection over 0.9 were reported. Both analyses were used to identify regions of the opsin sequence which may be undergoing adaptive evolution and were run on the Datamonkey webserver utilizing HyPhy software (Weaver et al. 2018; Kosakovsky Pond et al. 2020). Only sites under positive selection were considered in this analysis. Both tests of selection used here rely on sequence alignments as input and cannot compensate for missing data. Our malacostracan opsin phylogeny showed poor support at some basal branches but was congruent with previously published phylogenetic reconstructions from arthropods (Henze and Oakley 2015; Palecanda et al. 2022). Thus, we believe that using tests of selection on this dataset can still be useful in understanding SWS opsin evolution. Tests of selection were performed separately for the stomatopod dataset in order to reduce noise from less similar sequences (Murrell et al. 2012, 2013) and take advantage of the more well supported stomatopod UV opsins phylogeny and dataset. A maximum likelihood reconstruction of ancestral opsins from two main stomatopod SWS clades was carried out using the BaseML program in the PAML package (Yang 1997; Xu and Yang 2013) to further analyze how amino acid changes over time may contribute to diverging visual pigment absorbances.

To identify locations with a high probability of contributing to spectral tuning, sites which were within 5 Å of the chromophore or were found in either the MEME or FUBAR analysis to be under selection were compared within each

dataset. Using the opsin phylogenies generated in this study, differences in amino acid composition between sequences from species with known UV sensitivity (Frank and Case 1988; Hariyama et al. 1993; Bok et al. 2014) and species with known violet sensitivity (Cummins and Goldsmith 1981) as well as their close taxonomic relatives were noted for the Malacostraca dataset. Sites with amino acid differences corresponding to the two major opsin clades found in the order (Bok et al. 2014) were noted in the Stomatopod dataset. Sites at which variation existed within UV or violet sensitive clades or where amino acids residues were invariable between species with characterized SWS visual systems could not be interpreted. Sites are referred to by the opsin protein domain they are part of (e.g., transmembrane helix number) and position using bovine rhodopsin sequence numbering.

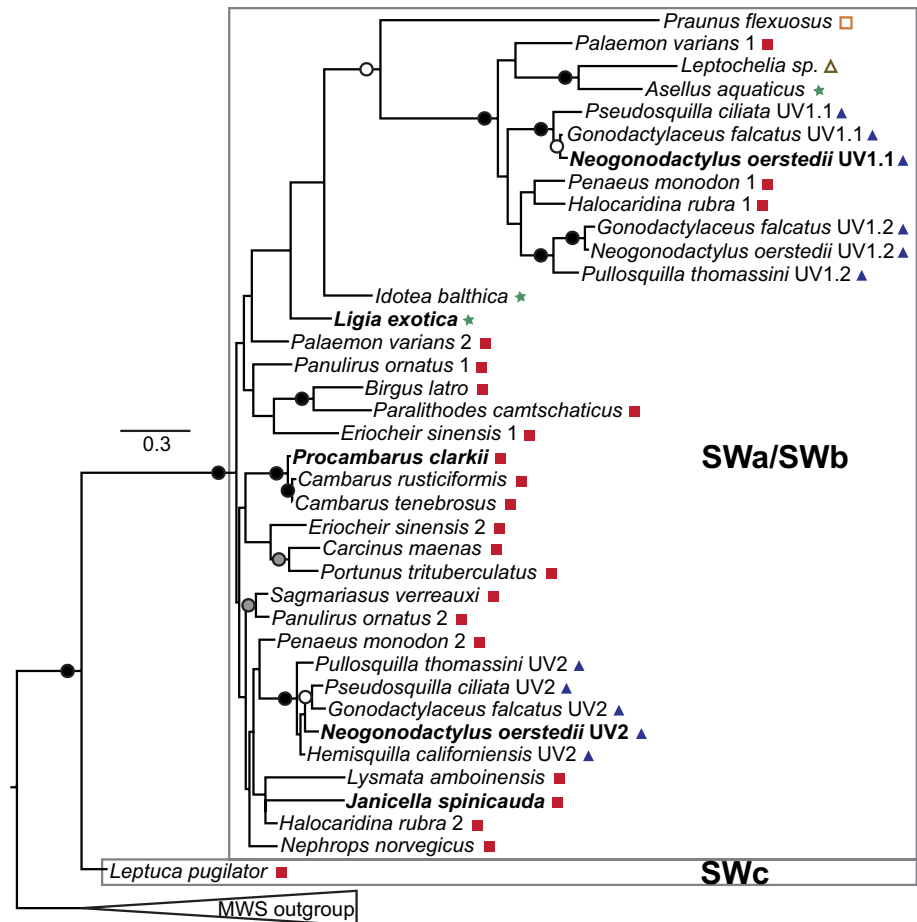
Results

A total of 38 SWS opsin sequences were identified from publicly available data, representing 27 species from five orders within the Malacostraca (Supplementary Table S1). All identified sequences possessed a lysine residue at bovine rhodopsin position 90. The majority of malacostracan SWS opsin transcripts (33 of 37) were identified from the orders Decapoda and Stomatopoda with the remaining individual transcripts identified from three species of isopod, one mysid, and one tanaid. A single SWS opsin was identified from the order Anaspidacea in the species *Anaspides tasminidae*, but this transcript did not have amino acids at the minimum 70% of sites identified as being close to the chromophore and was removed prior to analysis. A single SWS transcript was identified from 18 of the species surveyed, including members of all five orders where SWS opsins were found (Fig. 1). Seven species, five decapods and two stomatopods, were found to express two SWS opsin transcripts. A second SWS transcript was preliminarily identified from the isopod *Ligia exotica*, but it was removed due to missing amino acid residues. Two species of stomatopods were found to express three SWS opsin transcripts, previously confirmed in at least one species to form UV absorptive visual pigments (Bok et al. 2014). No SWS opsins were identified from species in the orders Amphipoda, Bathynellacea, Cumacea, Euphausiacea, Leptostraca, or Lophogastrida.

Malacostracan SWS Opsins and Potential Spectral Tuning Sites

In our malacostracan opsin phylogeny support for clades SWa and SWb, two of the main pancrustacean opsin groups described by Henze and Oakley (2015), was very low

Fig. 1 Maximum likelihood phylogeny of malacostracan short wavelength sensitive (SWS) opsin transcripts with middle wavelength sensitive (MWS) opsin transcripts used to root the tree. Clades are labeled as in Henze and Oakley (2015). Stomatopod opsins are labeled as in Bok et al. (2014) and all other transcripts are designated by number when multiple paralogs from the same species exist. Orders are designated by shapes with empty square = Mysida, empty triangle = Tanaidacea, solid square = Decapoda, solid triangle = Stomatopoda, and star = Isopoda. Opsins which form verified UV-sensitive photopigments are bolded. Bootstrap support for branches is indicated with white circles (70–79%), gray circles (80–89%), or black circles (90–100%) (Color figure online)



(Fig. 1). Therefore, for the purpose of our analyses we will refer to these previously defined clades jointly as SWa/SWb. Within the SWa/SWb clade, we found several well supported sub-clades of opsins that mapped to specific taxonomic lineages. A well supported (bootstrap support = 100%) clade of stomatopod opsins was recovered as well as decapod specific sub-clades including one composed of brachyuran crab sequences and one containing crayfish sequence with 68% and 100% bootstrap support respectively (Fig. 1). Not all clades were taxon specific with one well supported clade (bootstrap support = 100%) formed by sequences from stomatopods, decapods, an isopod and a tanaid. One transcript from the decapod *Leptuca pugilator* (accession no. ADQ01811) fell outside the rest of the malacostracan SWS opsins, with high support for this position (bootstrap support = 100%). This is keeping with this sequence's placement in the previously named pancrustacean SWc clade (Henze and Oakley 2015).

Twenty-five amino acid sites within the malacostracan opsins were identified exclusively from protein reconstructions as being within 5 Å of the chromophore. Tests of selection from MEME found five additional sites that were under positive selection, and one site was identified both as

being close to the chromophore and under positive selection (Supplementary Table S2). The FUBAR analysis did not uncover any sites under positive selection. The majority of sequences included in the malacostracan dataset come from species without spectrally determined SWS sensitivity. Because of this, spectral tuning sites were only reported if they may differentiate between the characterized visual systems of crayfish (violet sensitive), and decapod, stomatopod, and isopod species with documented UV sensitivity (Table 1). All stomatopod taxa were assumed to have UV sensitive visual systems based on spectral sensitivity, retinal absorbance, and behavioral evidence from multiple species (Cronin et al. 1994; Marshall and Oberwinkler 1999; Bok et al. 2014, 2018). Six sites were found that fit the reporting conditions. These were located in cytoplasmic loop one (CL1), transmembrane helix three (H3), extracellular loop two (EL2), and transmembrane helix five (H5), (Table 1). The site in CL1 (position 49) was identified through MEME selection analysis, while the five other sites were identified exclusively by proximity to the chromophore as potentially important in spectral tuning. The remaining 25 sites identified though protein reconstruction or selection analysis were either invariable (eight sites) or contained variation between

Table 1 Amino acid positions in malacostracan SWS opsins identified as potential spectral tuning sites by proximity to the chromophore (*), or MEME test for selection (‡) where variation corresponds with amino acid differences between violet sensitive crayfish (sequence in

bold; Cummins and Goldsmith 1981) and malacostracan species with verified UV spectral sensitivity or visual pigment absorbance (Hariyama et al. 1986; Frank and Case 1988; Bok et al. 2014)

	Species	λ_{\max}	Protein domain	Position	CL1	H2	H3		EL2		H5
					49‡	90	114*	122*	186*	188*	208*
	<i>Neogonodactylus oerstedii</i>	334 nm (MSP)	F	K	G	P	S	T	W		
	<i>Ligia exotica</i>	383 nm (ERG)	F	K	G	V	T	S	F		
	<i>Procambarus clarkii</i>	440 nm (MSP)	M	K	A	V	T	T	F		
	<i>Janicella spinicaudata</i>	390 nm (ERG)	F	K	A	I	?	?	?		
	<i>Neogonodactylus oerstedii</i>	383 nm (MSP)	F	K	A	I	T	S	F		

The method of characterization (ERG = electroretinogram, MSP = microspectrophotometry) is listed next to each spectral or absorbance peak in nm. Positions are listed using bovine rhodopsin sequence numbering. Position 90, at which a polymorphism between asparagine or glutamate and lysine forms the basis for ultraviolet sensitivity in insects (Salcedo et al. 2003) is also shown. Question marks indicate a residue that was missing at the end of a sequence

groups which were not spectrally characterized (17 sites, see Supplementary Table S2).

Stomatopod SWS Opsins and Potential Spectral Tuning Sites

Within the stomatopod dataset, which included both RNAseq and PCR sequence data, phylogenetic analysis uncovered higher opsin copy numbers per species compared to most other Malacostraca surveyed (Fig. 2). From RNAseq data, one species, *Hemisquilla californiensis*, was found to express a single SWS opsin. Two species, *Pullosquilla thomassini* and *Pseudosquilla ciliata*, expressed two SWS opsin transcripts and two species—*Neogonodactylus oerstedii* and *Gonodactylaceus falcatus*—expressed three SWS opsin transcripts each, the most of any species included in this study. These opsin transcripts clustered in two clades, named cUV1 and cUV2 with cUV1 further subdivided into cUV1.1 and cUV1.2 sub-clades (Bok et al. 2014). Opsin sequences from type cUV1.2 and/or cUV2 were obtained from each of the additional species sampled via PCR.

Twenty-four sites were identified in the stomatopod dataset exclusively from protein reconstruction as being within 5 Å of the chromophore. Two additional sites were identified as being under positive selection by MEME, and two sites were identified both by protein reconstruction and positive selection analysis (Supplementary Table S3). The FUBAR analysis found no evidence of positive selection at any site. Of the 28 total sites identified in this dataset, 11 were invariable or nearly so, with one sequence identified from *Chorisquilla tweediei* using PCR having a threonine at position 204 while all other sequences contained a valine (Supplementary Table S3). Sites where variation in amino acid residue corresponded to the division of stomatopod opsin clades cUV1 and cUV2 were identified and the ancestral state of those

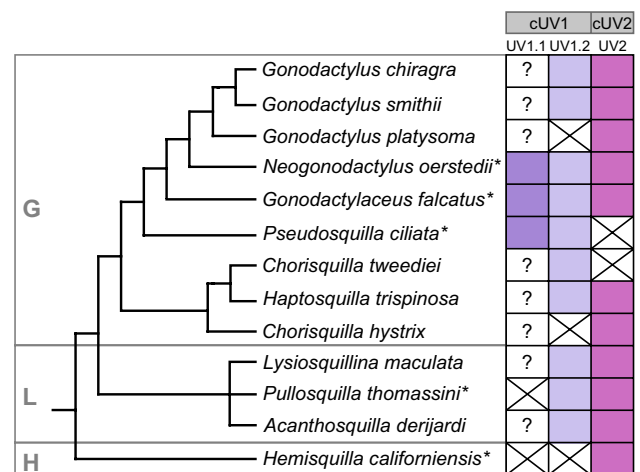


Fig. 2 Phylogenetic supertree of Stomatopod species with the presence or absence of opsins belonging to stomatopod UV opsin clades, cUV1.1, cUV1.2, and cUV2 (Bok et al. 2014) mapped across it. Relationships between species were reconstructed from multiple published phylogenies (Barber and Erdmann 2000; Porter et al. 2010; Van Der Wal et al. 2017). Stomatopod superfamilies are designated with letters; G=Gonodactyloidea, L=Lysiosquilloidea, H=Hemisquilloidea. Species for which RNAseq data is available are indicated with a star (*). Opsins that were identified are colored in for each species and opsins which were not identified in RNAseq data or were not successfully amplified using PCR are marked with an 'X'. Opsins for which PCR primers were not available are marked with question marks (Color figure online)

positions were compared. Ten sites, located in structures H3, EL2, H5, and transmembrane helix six (H6), were found which showed amino acid variation corresponding with clade structure and differing ancestral states between clades. (Table 2; Supplementary Fig. S1). The remaining seven sites showed no evidence of clade structure (Supplementary Table S3). Reconstructed ancestral cUV1 and cUV2 stomatopod opsins had high posterior probabilities

(above 0.9) at all potential spectral tuning sites except positions 118 (PP = 0.74) and 178 (PP = 0.86) in the reconstructed cUV2 sequence (Supplementary Table S3 for full list of positions and posterior probabilities).

Discussion

Our analysis of SWS opsin evolution within malacostracans provides new insight into the variability and complexity of short wavelength light detection in this comparatively less studied group. Candidate amino acid positions were identified for spectral tuning of SWS opsins in the Malacostraca as a whole, as well as within the order Stomatopoda which contains species with multiple characterized UV spectral sensitivities. Our results provide support for the hypothesis that more than one amino acid polymorphism could be responsible for the shift from violet to UV absorbance in malacostracan visual pigments and differences in UV absorbance between stomatopod visual pigments. Shifts in visual pigment absorbance caused by multiple simultaneous amino acid changes to an invertebrate opsin have recently been demonstrated in scallop visual systems by Smedley et al. (2022). This work suggests that while single or a small number of changes may be sufficient to tune vertebrate visual pigments, this may not be the case for all invertebrate systems. Heterologous expression of opsin proteins, the production of opsins outside of their native system, has been possible in insects for some time (Townson et al. 1998; Salcedo et al. 1999; Wakakuwa et al. 2010) but this technology has only recently been successfully applied to other pancrustaceans (Li enard et al. 2022). This study provides a basis for future heterologous expression of Malacostracan SWS opsins.

Decapods Possess Opsins from Clade SWa/SWb

Opsins from clades SWa and SWb were inferred to have been lost in decapods by Henze and Oakley (2015) based on their reconciled tree. While clades SWa and SWb were not recovered with high support in this study, possibly due to the exclusion of non-malacostracan opsin sequences, increased sampling of the decapods resulted in identification of SWS opsin transcripts that clustered with our combined SWa/SWb clade. This included transcripts from *Palaemon varians*, *Penaeus monodon*, and *Halocaridina rubra*, forming a well-supported clade with stomatopod, isopod, and tanaid opsin transcripts. These decapod species were not included in Henze and Oakley (2015)'s analysis. Limited sampling from some malacostracan orders, notably *Bathynellacea*, *Cumacea*, *Leptostraca*, and *Lophogastrida*, which were all represented by a single species, means that a lack of SWS opsins in these orders is not conclusive. In addition, because the data for this study was generated from publicly available sources, tissue type was not standardized (see Palecanda et al. 2022 for details). This means that SWS opsins expressed outside of the specific tissue sampled in each study would not be detected, and opsin copy numbers for some species may be underestimated. Genomic data from malacostracans is needed to make inferences about the gain and loss of opsins within lineages.

Amino Acid Changes that Affect Spectral Absorbance

This study focused on potential spectral tuning sites which were either close to the visual pigment chromophore or identified as being under positive selection, with the majority of sites discussed falling in the former category. We found these methods could be applied across orders and did not require detailed analysis of individual opsin protein structures, which are not yet available in high resolution.

Table 2 Amino acid positions in stomatopod UV opsin sequences identified as potential spectral tuning sites by proximity to the chromophore (*) and/or MEME test for selection (‡) where variation corresponds with clade structure

Protein domain	H3				EL2		H5			H6	
	113*	114*	118*	122*	178*	188*‡	207*	208*	212*	261*	
Opsin clade	cUV1 Anc.	Y	G	A	P	F	T	L	W	W	F
	cUV1.1 (3)	Y	G	A	P	F ₂ ? ₁	T ₂ ? ₁	L ₂ ? ₁	W ₂ ? ₁	W ₂ ? ₁	F ₂ ? ₁
	cUV1.2 (9)	F ₂ Y ₁ ? ₆	G ₃ ? ₆	A ₇ G ₁ ? ₁	P ₇ A ₁ ? ₁	F ₈ ? ₁	T ₈ ? ₁	L ₈ ? ₁	W ₈ ? ₁	W ₈ ? ₁	F ₂ ? ₇
	cUV2 Anc.	F	A	T	I	Y	S	I	F	Y	W
	cUV2 (12)	F ₁₀ L ₁ ? ₁	A ₁₁ ? ₁	S ₆ T ₃ N ₂ ? ₁	I ₁₁ ? ₁	F ₈ Y ₄	S	I	F	Y	W ₁₁ - ₁

Positions are listed using bovine rhodopsin sequence numbering and opsins are divided into cUV1.1, cUV1.2, and cUV2 spectral clades with the number of sequences in each group given in parenthesis. In cases where variation among sequences exists at an amino acid position, identity is shown with subscript denoting the number of sequences in which a particular residue was found. Amino acid identity at the stated position from ancestral state reconstructions for cUV1 and cUV2 opsins are shown in bold. Question marks indicate a residue which was not captured at one or the other end of a sequence; dashes represent a gap in the alignment used to compare positions between sequences

However, recent work by Sharkey et al. (2023) has suggested that structures outside of the chromophore binding pocket may also affect spectral tuning and should be considered in future analyses. Therefore, the sites identified here should not be viewed as an exhaustive list, and detailed protein structures may be necessary to fully account for spectral shifts within SWS visual pigments.

Many studies of UV sensitivity in arthropods use the work of Salcedo et al. (2003) as their basis. In that publication, sequences in the “insect blue” opsin clade were compared with those in the “insect UV” clade and heterologous expression studies were used to determine that position 90 was responsible for shifting sensitivity in *Drosophila* from the short visible wavelengths to the UV. This utility of this position to identify UV sensitive opsins may hold true in insects which have published opsin sequences and spectrally characterized visual systems, for example bumblebees, butterflies, and crickets (Peitsch et al. 1992; Kitamoto et al. 2000; Spaethe and Briscoe 2005; Henze et al. 2012; Hu et al. 2014; Perry et al. 2016). In members of the order *Coleoptera*, where blue sensitive opsins have been lost and then recovered in some species through duplication of the UV sensitive opsin, a substitution at position 90 from a positively charged lysine residue to a negatively charged amino acid seems to correspond with blue sensitivity (Sharkey et al. 2017). However, this study shows that the existence of a lysine at position 90 does not necessarily indicate UV sensitivity in malacostracan opsins.

It is difficult to untangle whether the N/E-K polymorphism at position 90 is responsible for a shift towards lower wavelengths, though not necessarily always to UV sensitivity in malacostracans. Another insect example, this time from the locust *Schistocera gregaria* shows that a photoreceptor with violet sensitivity ($\lambda_{\max} = 430$ nm) can express opsins with a glutamic acid at position 90, indicating absorbance in the visible range (Bennett et al. 1967; Towner et al. 1997). In contrast, the crayfish SWS opsin has a lysine at this position, indicating UV sensitivity, despite being expressed in a photoreceptor with peak sensitivity at 440 nm (Cummins and Goldsmith 1981). Additional spectral and opsin sequence data are needed from malacostracan visual systems to determine whether violet sensitivity exists across this class or is exclusive to crayfish.

Potential Mechanisms for Spectral Tuning in Malacostracan SWS Visual Pigments

We did not find evidence of a single amino acid polymorphism like the N/E-K polymorphism described above that could explain the shift from UV to violet absorbance. However, several potential tuning sites were identified by comparing the amino acid sequences of species with demonstrated violet and UV sensitivity at positions which

could affect visual pigment absorbance. As in other cases the combined effect of several amino acid changes may be responsible for shifting absorbance of SWS opsins rather than individual amino acid substitutions (Lin et al. 1998; Takahashi and Ebrey 2003; Yokoyama and Tada 2003; Yokoyama et al. 2007; Smedley et al. 2022). In particular, work by Liénard et al. (2021, 2022) has suggested that multiple mutations within the same opsin protein domain may contribute to shifts in visual pigment absorbance.

The only site within the CL1 domain identified in this study, and the sole site identified exclusively by selection analysis in the malacostracan dataset was position 49 (Table 1). Though amino acid changes at this site have been shown to affect UV spectral tuning in vertebrates (Shi and Yokoyama 2003), the specific polymorphisms seen here were not described in previous studies.

The H3 opsin protein domain represents a strong target location for future mutagenesis studies as it contains two sites, 114 and 122, which are both close to the chromophore and have previous evidence of affecting spectral tuning. The substitution of alanine for glycine at position 114 in particular has been identified as contributing to the shift from UV to blue visual pigment absorbance in mammals (Table 1; Deeb et al. 2003). Substitutions at site 122, though not of the specific amino acids seen here, have been shown to contribute to a short wavelength shift in fish and newt visual pigments (Hunt et al. 2001; Takahashi and Ebrey 2003).

The EL2 domain similarly has high potential to affect spectral tuning due to its function as a “plug”, which folds back into the transmembrane domain of the opsin and maintains thermal stability (Janz et al. 2003; Porter et al. 2007). Two positions were identified in this domain, sites 186 and 188. Position 186, has been shown to be a mediator for the invertebrate Schiff base counterion in spider rhodopsin (Nagata et al. 2019). A serine at this position stabilizes the protonation of the Schiff base, and mutations resulting in amino acids with different properties, such as alanine, were found by Nagata et al. (2019) to affect the stability of the resulting visual pigment and in some cases the spectral sensitivity of the photoreceptor in which they were found. Here we found a S-T polymorphism at site 186, which represents a significant change in hydrophobicity. While the position of the aforementioned counterion in malacostracans is unknown, position 186 remains one of several possibilities making a change in amino acid properties at that site potentially interesting. The same polymorphism was found at site 188 though to our knowledge no previous studies have identified this site as contributing to spectral tuning.

Finally, the only site identified in domain H5—position 208—was one of the targets for spectral tuning in previous studies of lyconid butterflies (Liénard et al. 2021). Here a W-F polymorphism was observed while previous studies in

fish uncovered a F to Y mutation which contributed to long wavelength shifts in absorbance (Hunt et al. 2001).

Looking outside of protein structure, changes at positions 122, 186, and 208 may be important in extremely short wavelength tuning as polymorphisms were found at these positions between the characterized *N. oerstedii* cUV1.1 visual pigment (334 nm peak absorbance) and other longer wavelength UV visual pigments (383–390 nm peak absorbance; Table 1). The ability to express and manipulate malacostracan opsins in either in vitro or transgenic systems is necessary to test whether mutations at these sites can account for the shift between violet and UV sensitivity in this class.

Stomatopod Opsin Evolution and Spectral Tuning of SWS Visual Pigments

We are not able to predict with certainty what the ancestral copy number of SWS opsins is for stomatopods due to missing data, most notably from the superfamily *Squilloidea* and the cUV1.1 sub-clade. One species from the genus *Hemisquilla*, which has been placed as sister to all other extant taxa, had a single expressed SWS opsin (Fig. 2; Ahyong and Jarman 2009; Porter et al. 2010; Van Der Wal et al. 2017). Based only on the phylogenetic position of this species it's possible that a single UV sensitive opsin could represent the ancestral condition, however more data is needed to adequately support this claim. If such support were found the sets of SWS opsins expressed in *Lysiosquilloidea* and *Gonodactyloidea* could represent the evolution of expanded SWS, and specifically UV, opsin diversity. The existence of three SWS opsins in the family *Gonodactylidae* in particular corresponds with a complex UV filtering system capable of producing up to six distinct UV spectral peaks (Kleinlogel and Marshall 2009; Bok et al. 2015).

By far the highest number of potentially important spectral tuning sites for stomatopod SWS visual pigments were found in opsin protein domain H3 (Table 2, Supplementary Table S3). Sites 113, 114, 118, and 122 are all found in this helix and were identified based on proximity to the chromophore. Site 113 has been identified as a potential tuning site for arthropod opsins (Salcedo et al. 2003; Porter et al. 2007) and contains a F–Y polymorphism (Tables 1 and 2). Substitution of a tyrosine for phenylalanine has been shown to shift visual pigment absorption from UV to blue in amphibians (Takahashi and Ebrey 2003) and has also been mentioned as important for spectral tuning in previous studies (Chang et al. 1995; Chase et al. 1997). Experimental manipulation by Salcedo et al. (2003) did not find that substitutions between tyrosine and phenylalanine at this site sufficiently changed spectral sensitivity in *Drosophila* to account for the difference between UV and blue sensitivity, but even a small change may be enough to

tune visual pigment absorbance and shift sensitivity within the UV spectrum. Similarly, a serine to alanine substitution at site 118 has been implicated in causing a shift to shorter wavelengths in butterflies (Wakakuwa et al. 2010). The ancestral state at position 118 for cUV2 opsins is a threonine residue but half of the opsins in this clade have a serine at this position. Sites 114 and 122 were previously discussed in the context of Malacostracan spectral tuning. Both sites have strong evidence of differing ancestral states between the two clades.

As in the malacostracan dataset opsin domain EL2 also has the potential to be important in spectral tuning within the stomatopods. A phenylalanine to tyrosine substitution at one site in this domain, position 178, was found in butterflies to cause a short wavelength shift in visual pigment absorbance (Wakakuwa et al. 2010). Site 188 was the only potential tuning site in the stomatopod dataset identified both by proximity to the chromophore and selection analysis. Although there is strong evidence for differing ancestral states at this position the specific amino acids seen here have not previously been shown to affect spectral tuning at this site.

Domain H5 contains three sites which have the potential to affect spectral tuning. Site 207 is a strong candidate as it has been found to be important in determining the peak absorbance of opsins (Table 2; Madabushi et al. 2004) and a leucine to isoleucine substitution at this position, which is also seen here, contributed to a blue shift in bullfrogs (Yokoyama and Tada 2003). Site 208 was also identified in the malacostracan dataset with the same W–F polymorphism, which has not previously been linked to spectral tuning at this site. A tyrosine to tryptophan substitution at site 212 was previously shown by Smedley et al. (2022) to result in a blue shifted absorbance of visual pigments in scallops.

The stomatopod dataset was unique from the larger Malacostraca in that a single site in domain H6 was identified. Site 261 has been shown to be important in spectral tuning though the specific amino acid polymorphisms seen here were not observed in previous studies (Hunt et al. 2001; Takahashi and Ebrey 2003). As in the larger Malacostraca dataset, a combination of all or some of these amino acid changes may be responsible for the different absorption of stomatopod opsins from the two major clades.

The possibility remains that sites outside of the region searched here due to PCR limitations could be important in spectral tuning. While the section of the opsin between H3 and H6 does contain the majority of sites near the chromophore, there are exceptions and increased data would help to determine whether these sites, particularly in helix seven which contains the chromophore binding lysine, have the potential to affect visual pigment absorbance. Additional RNAseq data is needed to produce full length stomatopod opsin sequences.

Conclusions

From this study we can conclude that not all pancrustacean visual systems conform to expectations based largely on insect vision. The existence in the Malacostraca of SWS visual pigments that would be expected to absorb in the UV based on opsin sequence alone but have been determined experimentally to be maximally sensitive to longer wavelengths, is proof that conclusions based on the evolutionary history of one group of arthropods should be applied to others with caution. This also highlights the importance of developing heterologous expression systems for non-insect pancrustaceans so that the utility of potential tuning sites, like those put forward here, can be tested experimentally. It is clear that looking at opsin sequences alone is not sufficient to make definitive claims about visual pigment absorbance. The diversity of visual pigment absorbance within Malacostraca SWS visual systems, and the mechanisms in place to tune that absorbance, are potentially wide ranging, particularly in the order Stomatopoda. This study provides a starting point to further understand the evolutionary history of pancrustacean SWS visual pigments, and the changes that have taken place at the molecular level to produce such varied and unique visual systems.

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Declarations

Competing Interests The authors declare no competing interests.

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