Molecular Evolution of Ultraviolet Visual Opsins and Spectral Tuning of Photoreceptors in Anemonefishes (Amphiprioninae)

Laurie J. Mitchell 1,*, Karen L. Cheney 1, Martin Lührmann 2, Justin Marshall 2, Kyle Michie 2,3, and Fabio Cortesi 2,*

1School of Biological Sciences, The University of Queensland, Brisbane, Queensland, Australia
2Queensland Brain Institute, The University of Queensland, Brisbane, Queensland, Australia
3King’s College, Cambridge, United Kingdom

*Corresponding authors: E-mails: laurie.mitchell@uqconnect.edu.au; fabio.cortesi@uqconnect.edu.au.

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Abstract

Many animals including birds, reptiles, insects, and teleost fishes can see ultraviolet (UV) light (shorter than 400 nm), which has functional importance for foraging and communication. For coral reef fishes, shallow reef environments transmit a broad spectrum of light, rich in UV, driving the evolution of diverse spectral sensitivities. However, the identities and sites of the specific visual genes that underly vision in reef fishes remain elusive and are useful in determining how evolution has tuned vision to suit life on the reef. We investigated the visual systems of 11 anemonefish (Amphiprioninae) species, specifically probing for the molecular pathways that facilitate UV-sensitivity. Searching the genomes of anemonefishes, we identified a total of eight functional opsin genes from all five vertebrate visual opsin subfamilies. We found rare instances of teleost UV-sensitive SWS1 opsin gene duplications that produced two functionally coding paralogs (SWS1a and SWS1b) and a pseudogene. We also found separate green sensitive RH2A opsin gene duplicates not yet reported in the family Pomacentridae. Transcriptome analysis revealed false clown anemonefish (Amphiprion ocellaris) expressed one rod opsin (RH1) and six cone opsins (SWS1b, SWS2B, RH2B, RH2A-1, RH2A-2, LWS) in the retina. Fluorescent in situ hybridization highlighted the (co-)expression of SWS1b with SWS2B in single cones, and either RH2B, RH2A, or RH2A together with LWS in different members of double cone photoreceptors (two single cones fused together). Our study provides the first in-depth characterization of visual opsin genes found in anemonefishes and provides a useful basis for the further study of UV-vision in reef fishes.

Key words: visual opsins, spectral tuning, reef fish vision, anemonefishes, ultraviolet, gene duplication.

Significance

Many coral reef fishes possess ultraviolet (UV) vision that is facilitated by UV-sensitive (SWS1) visual opsin proteins; however, the identities, sites, and evolution of SWS1 genes are largely unknown and are important to understanding how reef fish vision has evolved to suit life on the reef. In the genomes of anemonefishes, we found eight functionally coding visual opsin genes, of which two were duplicate SWS1 genes flanking a third pseudogenized copy, and one was expressed in the retina of anemonefish, Amphiprion ocellaris. Our findings provide new insights into the evolution of opsin gene diversity and spectral tuning of photoreceptors in an iconic group of reef fishes, particularly how gene duplication has produced multiple copies of SWS1 potentially at and above the family level.
Ultraviolet (UV) vision is widespread across the animal kingdom (Jacobs 1992; Tovée 1995) and is relied upon for many essential behaviors, including foraging (Church et al. 1998; Sittari et al. 2002; Boulcott and Braithwaite 2005; Novales Flamarique 2013), mate selection (Andersson and Amundsen 1997; Bennett et al. 1997; Smith et al. 2002; Rick and Bakker 2008a) and detecting potential competitors (Rick and Bakker 2008b; Siebeck et al. 2010; Bohórquez-Alonso et al. 2018). Underlying vision are the opsins, which are G-protein-coupled receptors with a Lysine residue (Lys296) that forms a Schiff base linkage to a carotenoid-derived (Vitamin A1 or A2) chromophore to form visual pigments mediating light absorbance from around 300 to 700 nm (Collin et al. 2009). UV-sensitivity is mediated by visual pigments with sensitivities shorter than 400 nm and is found in many vertebrates (Bowmaker 2008), including multiple teleost lineages (Lin et al. 2017; Musilova et al. 2019). Yet, for the majority of the approximately 30,000 teleost species we still lack detailed information on the identities, sites, and molecular evolution of specific genes and regulatory pathways that facilitate vision, including UV-sensitivity.

Visual opsins from all five vertebrate opsin subfamilies can be found in teleosts, including the UV-sensitive or very-short-wavelength-sensitive 1 (SWS1), short-wavelength-sensitive 2 (SWS2), medium-wavelength-sensitive, rhodopsin 1 (rod opsin, RH1) and rhodopsin-like 2 (RH2), and long-wavelength-sensitive (LWS) opsins (Yokoyama S and Yokoyama R 1996; reviewed by Carleton et al. 2020; Musilova et al. 2021). All of these families arose from an ancient vertebrate opsin that underwent multiple whole-genome and individual gene duplication events (Van de Peer et al. 2009), the latter of which facilitated the acquisition of novel visual opsin copies in multiple teleost lineages (Chinen et al. 2003; Matsumoto et al. 2006; Hofmann and Carleton 2009; Rennison et al. 2012; Musilova et al. 2019; Escobar-Camacho et al. 2020). Further changes to the available opsin gene repertoire can include their preservation and/or resurrection via gene conversion, a homogenizer of paralogous genes (Katju and Bergthorsson 2010; Cortesi et al. 2015), whereas modifications to opsin gene function mostly occur via amino acid substitutions (i.e., mutation) at “key-tuning sites” that determine the wavelength of maximum absorption ($\lambda_{max}$) sensitivity (Yokoyama 2000). Currently most of what is known on teleost opsin gene evolution pertains to the SWS2, RH1, RH2, and LWS subfamilies for which functional paralogs have been described (Chinen et al. 2005; Rennison et al. 2012; Cortesi et al. 2015), whereas the duplication and retention of SWS1 paralogs are rare (Rennison et al. 2012; Lin et al. 2017; Musilova et al. 2019).

Teleost retinas contain both single and double cone (i.e., two-fused single cones that may be optically coupled) photoreceptors, the former of which expresses SWS1 and/or SWS2 opsin to convey UV and violet/blue sensitivity, respectively (Dalton et al. 2017; Stieb et al. 2019), whereas sensitivity to longer wavelengths is conveyed by the expression of RH2 and/or LWS in double cones (Carleton et al. 2005, 2008; Parry et al. 2005; Spady et al. 2006; Dalton et al. 2015). It is changes in the opsin protein that drive most of the variation in visual pigment spectral tuning (Nickle and Robinson 2007; Carleton et al. 2020), particularly in the UV-region (~360–400 nm) (Yokoyama et al. 2016). Changes in the polarity and/or charge of amino acid residues at sites near the binding pocket can induce a short- or long-wavelength shift in spectral sensitivity (Carleton et al. 2005; Terai et al. 2006; Carleton 2009). The cumulative tuning effects of these sites may be used to estimate the peak spectral absorbance of visual pigments (Yokoyama et al. 2008), including SWS1-based pigments (Shi and Yokoyama 2003; Yokoyama et al. 2016). Further alterations to the spectral tuning of vision can be achieved by differential opsin expression (Fuller et al. 2004; Johnson et al. 2013; Cortesi et al. 2016; Shimmura et al. 2017) and/or coexpression of opsins in the retina (Dalton et al. 2014, 2017; Cortesi et al. 2015, 2016; Luehrmann et al. 2019; Stieb et al. 2019), which can adjust to match changes in light environment such as with depth, turbidity, and diet (Fuller and Claricoates 2011; Novales Flamarique 2013; Dalton et al. 2015; Stieb et al. 2016).

In shallow environments, such as coral reefs, which are rich in UV-wavelengths, small-bodied teleost fishes often possess SWS1 opsin genes expressed in single cones (Phillips et al. 2016; Stieb et al. 2016, 2017, 2019) and have UV-transmissive lenses (Siebeck and Marshall 2001, 2007; Losey et al. 2003). UV-vision in reef fishes is thought to aid the detection of UV-reflecting zooplankton prey (Stieb et al. 2017) and/or facilitate a short-distance communication channel hidden from predators, most of which lack UV-sensitive photoreceptors (Losey et al. 1999; Marshall et al. 2006; Siebeck et al. 2010). However, despite the widespread nature of UV-sensitivity and its importance in reef fishes, its genetic basis remains largely uncharacterized, which we aimed to address in this study.

To do this, we used anemonefishes [family, Pomacentridae (damselishes); subfamily, Amphiprioninae], which are an iconic group of reef fishes that obligately associate with one or more species of sea anemone. They are also sequential hermaphrodites living in strict social hierarchies governed by body size; the largest fish is the female, the second largest is the male and all smaller fish are sexually immature subordinates (Buston 2003). Amphiprioninae is split into two broad clades (Rolland et al. 2018), from which the visual system of one species (Amphiprion akindynos) belonging to the major clade (25 spp.) has been previously characterized in detail (Stieb et al. 2019). However, little is known on the visual systems of other anemonefishes within this clade, nor species from the minor clade (3 spp.). Amphiprion akindynos possesses short-wavelength-sensitive single cones (~400 nm
λ_{\text{max}}) mostly expressing SWS1 and a small area in the retina coexpressing SWS1 with SWS2B opsins, and mid- to long-wavelength-sensitive double cones (498, 520, 541 nm λ_{\text{max}}) expressing RH2B, RH2A, and LWS opsins (Stieb et al. 2019). The spectral tuning of their photoreceptors may help to enhance the detection of zooplankton prey and/or enhance the chromatic contrast of their own UV-reflective skin patterns, which may benefit species recognition (Stieb et al. 2019). Whether UV-vision is of general importance to anemonefishes is unknown but characterizing visual opsin gene diversity and expression patterns in a wider range of species could begin to reveal the extent of its importance across Amphilprioninae.

The public availability of short- and long-read sequenced genomes for 11 species of anemonefishes has made it possible to study in detail the evolution of SWS1 opsin genes in this group of reef fishes. We first identified the SWS1 opsin genes and other visual opsin genes found in the genomes of anemonefishes and provided information from their synteny analysis and phylogenetic reconstruction. Second, we identified the visual transduction and shut-off pathway genes that regulate opsin activity to show their synteny in all examined species. Finally, we quantified opsin gene expression levels and observed spatial patterns of cone opsin expression using fluorescence in situ hybridization (FISH) in the retina of the false-clove anemonefish (Amphiprion ocellaris), which belongs to the minor clade and is therefore suitable for an interclade comparison with previously published data on A. akindynos (Stieb et al. 2019).

**Results**

**Anemonefish Visual Opin Genes: Identification, Phylogeny, and Synteny**

Our *in silico* searches in the genomes of anemonefishes and Pomacanthus moluccensis yielded a total of eight fully coding opsin genes belonging to five opsin classes including one rod opsin RH1, two UV-sensitive SWS1 opsins, a single violet-sensitive SWS2 opsin, two to three blue-green-sensitive RH2 opsins, and a single yellow-red-sensitive LWS opsin (fig. 1A; supplementary table 1, Supplementary Material online). All visual transduction pathway genes and shut-off genes present in other vertebrates/fish species were also identified in A. ocellaris and Amphiprion percula, with no extra duplicates for these genes found (supplementary table 2, Supplementary Material online).

Two UV-sensitive opsins, SWS1α and SWS1β, were identified in all examined anemonefishes. Those found in *A. percula* and *A. ocellaris* matched the sequence predictions on Ensembl (Ensembl transcript ID: ENSAOCT0000002771.1, ENSAET00000018561.1, ENSAET00000018598.1, ENSAET0000002830.1, ENSAET00000018621.1). Like in other examined teleosts (Lin et al. 2017), the RH2 genes in *A. percula* (fig. 1B) and *A. ocellaris* were found in tandem, spanning a region of approximately 29 kb and flanked by identical genes immediately up- and down-stream of the syntenic region. Orthologous RH2A genes were found to be highly conserved within pairs of sister-species, including: 1) *A. percula* and *A. ocellaris* (sharing RH2A-1 = 99.6% and RH2A-2 = 99.3% similarity), and 2) *A. akallopisos* and *A. perideraion* (sharing RH2A-1 = 99.4% and RH2A-2 = 99.1% similarity).

One violet-sensitive SWS2B opsin and red-sensitive LWS opsin were identified in all anemonefishes, and those in *A. percula* and *A. ocellaris* matched sequence predictions on Ensembl (Ensembl transcript ID: ENSAOCT00000024298.1, ENSAET00000033644.1, ENSAOCT00000031935.1, ENSAET00000033670.1). The chromosomal resolution of the *A. percula* genome revealed the locations of the RH2 and SWS2B-LWS syntenic regions (fig. 1B) in-tandem on Chromosome 6, separated by approximately 8.6 Mb; a conserved syntenic region shared by many other Perciform species (Musilova et al. 2019).

Like most teleost fishes (Musilova et al. 2019), anemonefishes were found to possess a single RH1 (fig. 1A), with a conserved RH1 syntenic region (fig. 1B). Those found in A.
Fig. 1.—Summary of anemonefish visual opsin genes and their synteny. (A) Visual opsin genes mapped on the species tree (modified from Tang et al. [2021]) along with their range of estimated peak spectral absorbance ($\lambda_{max}$) values. A detailed opsin gene phylogeny is presented in supplementary figure 2, Supplementary Material online. (B) Synteny of anemonefish visual genes according to the chromosomal arrangement in Amphiprion percula, including opsin gene coding regions (boxes depict single exons) and their flanking genes (black). Areas highlighted in yellow indicate regions where recombination occurred between opsin gene paralogs. Opsin gene acronyms stand for: RH1, rhodopsin 1 (rod opsin); RH2, rhodopsin-like 2; SWS2, short-wavelength-sensitive 2; LWS, long-wavelength-sensitive; SWS1*, short-wavelength-sensitive 1/short-wavelength-sensitive pseudogene. *Opsin genes mapped from raw transcriptome reads of the A. akindynos retina (from Steeb et al. [2019]). Image credit: A. nigripes, Ewa Barska via Wikimedia Commons; A. polymnus, Jens Petersen via Wikimedia Commons; A. bicinctus, Patryk Krzyzak via Wikimedia Commons; A. frenatus, Vincent Chen via Wikimedia Commons; A. ocellaris and A. akindynos, Valerio Tettamanti via direct permission.
ocellaris and A. percula genomes matched predicted sequences on Ensembl (Ensembl transcript ID: ENSAOCG00000018646, ENSAPEG00000012211).

Note that the SW51 pseudogene and RH2A-2 paralog were first found in the highly resolved genomes of A. percula and A. ocellaris that were published by two independent lab groups that used different sequencing and assembly strategies (Tan et al. 2018; Lehmann et al. 2018), making it unlikely that they are the product of assembly artefacts. Because the draft genomes of other anemonefishes are comparatively poorly resolved, the SW51 pseudogene, the full coding region of SW51x, and RH2A paralogs where present, were extracted using the raw-read mapping approach in those cases. This incomplete or incorrect assembly of highly repetitive genomic regions is an inherent problem of short-read draft genomes (Richards 2018; Rice and Green 2019). To corroborate our results, we also used raw-read mapping on an independently sequenced short-read genome of A. ocellaris (bioproject: SRX5249785; Marcionetti et al. 2019).

Phylogenetic analyses placed all the identified opsin genes into distinct homologous clusters with their predicted opsin classes (supplementary fig. 2, Supplementary Material online). Furthermore, the translated and aligned protein sequences for the identified genes exhibited typical opsin traits including the conserved chromophore binding site residue (K296) and intact seven transmembrane domains. Phylogenetic analysis of RH1, SW52B, RH2B, and LWS in anemonefishes (supplementary fig. 2, Supplementary Material online) showed a typical pattern of species-relatedness that mostly resembled the inferred phylogenies reported elsewhere (Tang et al. 2021). RH2A and SW51 phylogenies showed a clear separation of opsin gene paralogs, where SW51x and SW51β formed two clusters, and RH2A-1 and RH2A-2 formed two clusters (supplementary fig. 2, Supplementary Material online). Lone RH2A genes found in seven species mostly grouped with RH2A-2 genes, with the exception of A. melanopus and A. biauculeatus RH2A-1 genes.

### Anemonefish Opin Gene Conversion Analysis

We found evidence of gene conversion in both the RH2A and SW51 duplicates (fig. 1B). GARD analysis revealed three major breakpoints in the coding sequences of RH2A-1 and RH2A-2. Alternative tree topologies based on the different RH2A breakpoint regions (supplementary fig. 3A and B, Supplementary Material online) supported recombination only at one located in Exons 4–5 (891–1,059 bp), as evident by a lack of orthologous clustering according to opsin gene that was recovered in trees based on a nonrecombined segment (1–890 bp), to reflect the phylogenetic relationships observed in the full opsin gene tree. Analysis of the aligned RH2A opsin protein sequences identified only one known key tuning site (aa site 292; site number given according to bovine rhodopsin) (Yokoyama and Jia 2020) located within the region of recombination.

Three breakpoints were also reported in the SW51x and SW51β duplicates. Alternative tree topologies supported the notion of gene conversion in one of these areas in Exons 4 and 5 (839–1,017 bp), where SW51 orthologs had partial clusters that differed from the clear separation observed in trees based on a nonrecombined segment (341–509 bp) and in the full opsin gene phylogeny. Analysis of the aligned SW51 opsin protein sequences did not yield any known tuning site changes within the recombined region.

### Analysis of Visual Opin Tuning Sites and λ_max Value Estimation

Protein sequence analysis (table 1) revealed highly conserved SW51 opsin that shared 95.0–96.2% similarity. Closer inspection of the protein sequences showed some of the differences between paralogs occurred at known SW51 tuning sites (Shi and Yokoyama 2003; Yokoyama et al. 2008), and 90% were within the seven transmembrane domains. Two sites were found to have a nonpolar to polar shift including A118S and A114S (table 1), with the former known to induce a ~15 nm shift in opsin λ_max (Shi and Yokoyama 2003) (see supplementary fig. 4, Supplementary Material online, for a comprehensive list of all variable aa sites). All SW51 protein sequences had conserved F86 and S90 aa sites, an invariable feature of UV-sensitive opsins (Cowling et al. 2002). Estimated λ_max values for SW51 opsin sequences ranged from 356 to 362 nm for SW51x, and 368 to 370 nm for SW51β (fig. 1A). SW51x and SW51β opsin protein sequences were near-identical to those found in Onyrias latipes (λ_max value = 356 nm, Matsumoto et al. 2006) and P. amboinensis (λ_max value = 370 nm, Siebeck et al. 2010), respectively. Note, all λ_max values reported should be treated as rough estimates, as they do not consider the effect of potential unknown tuning sites and/or interactive tuning effects between sites. Therefore, physical absorption measurements will be necessary to verify our approach.

Multiple opsin protein tuning sites were identified in RH2B (table 1) and used to infer a single estimated λ_max value of 497 nm (fig. 1A). No separate estimates for the RH2A paralogs could be made due to a lack of differences in identifiable known tuning site effects between the copies. One variable amino acid site had a known tuning effect; F158I/L that causes a ~10 nm shift (table 1), which was used to infer RH2A λ_max estimates = 516–523 nm for most species, and a slightly broader range in A. nigripes, A. polymnus, and A. sebae (estimated λ_max value = 516–528 nm) (fig. 1A). Other notable but unaccountable variable aa sites included Y37F and T266V, both of which consistently alternated state between RH2A paralogs (supplementary fig. 4, Supplementary Material online).

Two variable aa sites were found with known tuning effects in LWS opsins (table 1) and were used to give
estimated $\lambda_{\text{max}}$ values = 560/561 nm in most species (fig. 1A), except for A. nigripes, A. polymnus, A. sebae, and A. bicinctus (estimated $\lambda_{\text{max}}$ values = 553–561 nm). Anemonefish SWS2B opsin yielded six variable aa sites with known tuning effects (table 1) and were used to infer estimated $\lambda_{\text{max}}$ values = 406/407 nm, which were consistent across anemonefishes.

RH1 opsin protein sequences were highly conserved across anemonefishes, with one variable aa site with a known tuning effect; D83N that causes a 6 nm shift. This was used to infer estimated $\lambda_{\text{max}}$ values = 491–499 nm, which were consistent across anemonefishes.

### Visual Gene Expression Analysis of A. ocellaris

Analyses of retinal transcriptomes revealed that under our aquarium lighting the A. ocellaris retina ($N = 4$) expressed one rod opsin, RH1 (mean ± SD = 70.6 ± 11.5%), and six cone opsins including four double cone opsins: RH2B (35.3 ± 3.2%), RH2A-1 (43.1 ± 6.7%), RH2A-2 (10.1 ± 11.7%) and LWS (11.5 ± 6.8%), and two single cone opsins: SWS1β (59.1 ± 9.4%) and SWS2B (40.8 ± 8.7%) (fig. 2). No trace of SWS1α expression was detected in three out of the four retinas, and only a small amount (1.5%) was detected in one retina. No apparent difference in opsin gene expression was found between males ($n = 2$) and females ($n = 2$) (fig. 2).

### FISH of A. ocellaris Retina

Brightfield viewing of the retina revealed a regular square mosaic arrangement with a central single cone surrounded by four double cones (fig. 3D, H, and I). FISH analyses showed that short-wavelength-sensitive opsins, SWS1β and SWS2B, were mostly coexpressed in single cone photoreceptors (fig. 3A–D).

Medium- and longer-wavelength-sensitive opsins, RH2B, RH2A, and LWS, were exclusively expressed in double cone photoreceptors (fig. 3E–I). Among these, RH2A and RH2B were expressed throughout the retina in separate double cone members (fig. 3E–H).

LWS was expressed across the retina and only observed to be coexpressed with RH2A (fig. 3I–L). However, although RH2A and RH2B fluorescent signal strength varied little between different cells, LWS signal strength varied noticeably, suggesting that quantitatively LWS expression may have been more variable. In some double cone members, RH2A appeared to be the only expressed opsin, with little or no expressed LWS apparent judging by a very dim or nondetectable fluorescent signal.

### Discussion

**Visual Opsin Gene Diversity in Anemonefish Genomes**

Anemonefish genomes revealed eight visual opsin orthologs belonging to all five ancestral vertebrate classes of opsin (Davies et al. 2012) including SWS1, SWS2B, RH1, RH2A, RH2B, and LWS, along with additional SWS1 and RH2A duplicates. We found instances of UV-sensitive SWS1 opsin gene duplication events that produced two functional paralogs (SWS1α and SWS1β) and a pseudogene. The expressed
remains unclear whether the gene duplication event that
Pomacentridae possess
SWS1 Chrominae (Musilova et al. 2019), and hence some
ocellaris opsins. Here, we show that
gene loss in the damselfish ancestor (Cortesi et al. 2015).
Amphiprion ocellaris aquarium lighting (see supplementary fig. 1, Supplementary Material)
Wavelength-sensitive 1; Huntington, 2019). Conversely, two
opsin duplicates. Thus, it
remains unclear whether the gene duplication event that
produced SWS1α and SWS1β was lineage- or subfamily-specific. More genomic data are needed to resolve whether this SWS1 gene duplication occurred at the base of Pomacentridae or multiple times independently in its radiated lineages.

Interestingly, we found evidence of a second gene duplication event that produced a third, pseudogenized SWS1 paralog in anemonefishes. The homology of this pseudogene was split roughly 50/50 between the SWS1α and SWS1β genes across anemonefish species with no clear phylogenetic pattern. This suggests the pseudogene either originated multiple times independently or more likely, because there is no clear phylogenetic pattern that subsequent gene conversion occurred after its duplication. The presence of this SWS1 pseudogene across all examined members of both the minor and major anemonefish clades suggests that it emerged at the very least in the last common ancestor of both anemonefish clades.

**Tuning of Anemonefish Single Cone Pigments and Potential Functional Significance**

All SWS1α and SWS1β opsin protein sequences have conserved alanine at site 86 and serine at site 90, crucial for UV-sensitivity (Shi and Yokoyama 2003; Tada et al. 2009). Furthermore, both anemonefish SWS1 duplicates have intact open reading frames but only SWS1β is expressed at considerable levels in the adult retina. The (co)expression of SWS1β/SWS2B opsin genes in the single cones of A. ocellaris is revealed by FISH strongly implicates UV-vision mediated by SWS1 and SWS2B visual pigments in the single cones. Similarly, in A. akindynos SWS1 and SWS2B are coexpressed in some single cones (Stieb et al. 2019), although the amount of SWS2B was substantially lower (~10% of single cone opsin expression) than in A. ocellaris (~41%). This lower expression of SWS2B in A. akindynos is due to it being highly localized within small, dorso-temporal (i.e., forward-looking) area in the retina of high acuity that may aid specific tasks such as foraging and intraspecific communication (Stieb et al. 2019). Whether any such spatial pattern in opsin expression exists in the A. ocellaris retina needs further investigation using detailed retinal topographic mapping of expressed cone opsin genes. We found no sex-related differences in opsin gene expression levels in A. ocellaris, an aspect shared by A. akindynos (Stieb et al. 2019). Because these two species are representative of the two major anemonefish clades, it suggests that visual gene expression is independent of sex in these fishes.

The function of UV-vision in anemonefishes remains unclear but consideration of other UV-sensitive teleosts could provide important insights. Juvenile rainbow trout (Oncorhynchus mykiss) express SWS1 opsin in their single cones that conveys UV-sensitivity to improve zooplanktivory efficacy by enhancing prey contrast (Novales Flamarique and
Similar foraging benefits conveyed by UV-sensitivity have been shown in perch (Loew et al. 1993), cichlids (Jordan et al. 2004), sticklebacks (Rick et al. 2012), and zebrafish (Novales Flamarique 2016; Yoshimatsu et al. 2020). Indeed, in damselfishes higher SWS1 expression correlates strongly with zooplanktivory (Stieb et al. 2017), and anemonefishes are life-long zooplanktivores (Fautin and Allen 1997) that could similarly benefit from the enhanced UV contrast of prey in the water column.

Fish also use UV-signals for communicating with rivals and mates, as reported in the guppy (Smith et al. 2002), stickleback (Rick and Bakker 2008a, 2008b), and a damselfish (Siebeck et al. 2010). In A. akindynos, the coexpression of SWS1 and SWS2 is believed to increase the chromatic contrast of its skin color patterns that may improve the detection of conspecifics (Stieb et al. 2019). The use of UV-signaling in communication is worth further investigation in anemonefishes, as they possess UV-reflective skin patterns (Marshall et al. 2006; Stieb et al. 2019) that could similarly benefit from the enhanced UV contrast of prey in the water column.

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In principle, the advantages of UV-vision should be conveyable by a single UV-sensitive opsin, and the functional significance of possessing SWS1 duplicates remains unknown. Another teleost species found to have two functionally coding SWS1 duplicates is the smelt, Pecoglossus altivelis and like A. ocellaris it only expresses one paralog in adults (Minamoto and Shimizu 2005). As suggested in P. altivelis, it is possible that SWS1α is expressed in A. ocellaris during specific seasons such as winter months on the reef, when higher visibility from lower turbidity may promote the more efficient transmission of shorter wavelengths of light (Stieb et al. 2016). Similarly, increased SWS1 opsin expression in the Nagasaki damsel, P. nagasakiensis, during winter has been suggested to be a visual tuning response for taking advantage of the higher UV (Stieb et al. 2016). Seasonal changes in opsin gene expression levels have been reported to alter color perception in widespread taxa (see review by Shimmura et al. [2018]).

Analyzing opsin expression levels in the larval and/or early-juvenile anemonefish retina may also reveal whether the shorter-wavelength-sensitive SWS1α is expressed during earlier life stages and whether an ontogenetic shift from SWS1α to SWS1β coincides with change(s) in light environment, particularly during the settlement stage when pelagic larvae return to the reef to seek a host anemone. Ontogenetic changes in cone opsin expression levels as a response to change in habitat have been reported in other reef fishes,
for example, spotted unicornfish (Naso brevirostris) (Tettamanti et al. 2019) and dusky dottyback (Pseudochromis fuscus) (Cortesi et al. 2016). Alternatively, the SW51x opsin may be expressed in other tissues and/or organs, where animal opsins have been demonstrated to serve other sensory modalities, for example, nonimage forming vision, thermosensation, hearing (see review by Leung and Montell [2017]), taste (Leung et al. 2020), or potentially a nonsensory related function such as early eye and cranial development (see Novales Flamarique et al. 2021).

Tuning of Anemonefish Double Cone Pigments and Potential Functional Significance

The anemonefish double cones expressed a variety of opsins including two RH2A paralogs (RH2A-1 and RH2A-2), RH2B and LWS, almost all of which orthologs can be found in other pomacentrids (Hofmann et al. 2012; Stieb et al. 2016, 2017). However, to the best of our knowledge, this is the first report of an RH2A duplication in a pomacentrid. Similar RH2A duplications have been reported in most percomorphs (as reviewed by Musilova et al. [2019, 2021]). It was previously difficult to separate RH2A duplicates in pomacentrids due to their high degree of similarity, coupled with the low-resolution genomes available. Indeed, completely assembled RH2A-2 genes could not be recovered in the draft genomes of A. akallopisos and A. perideraion, which were only detected by mapping against their raw paired-end genome reads. Neither approach recovered intact sets of duplicate RH2A opsin genes in 7 of the 11 anemonefishes likely due to gene loss, or alternatively due to incompleteness of genomic data.

The high level of similarity between detected RH2A orthologs was partly caused by gene conversion but could also be indicative of a fairly recent origin in the two clades of sister species (A. ocellaris/A. percula and A. akallopisos/A. perideraion). Regardless, it appears that RH2A genes are evolving quite dynamically in anemonefishes. Gene conversion at the fourth and fifth exons may act to preserve the opsin-chromophore binding site (Lys296), as is likely the case in SW51. Additionally, the region of gene conversion in RH2A encompasses a known tuning site, bovine rhodopsin site 292, where a large shift of ~11 nm occurs when Ala is substituted with Ser (Yokoyama et al. 2007). Thus, preservation of this site by gene conversion may also have importance in preventing large shifts in the spectral tuning of RH2A visual pigments. RH2A regions lacking any detected gene conversion held a few variable amino acid sites between duplicates with unknown tuning effects. Some of these sites exhibited changes in polarity which may impact spectral tuning, particularly at sites Y37F and T266V whose identities alternated between paralogs. Subject to their exact tuning, the differential expression of RH2A opsin in the double cones may improve blue-green sensitivity, as has been suggested in cichlids to aid the viewing of nuptial skin colors and/or colonizing different depths (Weädick and Chang 2012; Dalton et al. 2014).

In the A. ocellaris retina, FISH indicated that RH2A opsin and RH2B opsin were always expressed in separate double cone members, whereas LWS was almost exclusively coexpressed with RH2A. One potential benefit of coexpressing RH2A/LWS is to increase light absorption and thus, enhance luminance contrast (Dalton et al. 2014). In African cichlids, the coexpression of RH2A/LWS is mostly limited to the ventral (i.e., upwards looking) retina, which likely aids the detection of distant dark objects such as predators against downwelling space light (Dalton et al. 2014). Whether a similar distribution of LWS is present in the A. ocellaris retina requires further investigation. Behavioural experiments involving fish reared under different light regimes (Fuller and Claricoates 2011; Dalton et al. 2015), or by taking a reverse genetic approach to assess the effect of specific opsin gene knockouts (Homma et al. 2017) could elucidate the function of opsin gene coexpression in anemonefishes.

Conclusions and Future Directions

Here we have shown that anemonefishes possess seven to eight visual opsin genes including duplications of the SW51 gene, and RH2A gene in some species. The presence of two functional SW51 opsin genes in all examined anemonefishes suggests a pomacentrid or at least anemonefish-specific SW51 gene duplication event, and a possibly lineage-specific second duplication event that produced an SW51 pseudogene. Moreover, most of these opsin genes were found expressed in the adult retinae of A. ocellaris. Our reported visual opsin gene expression levels and cone opsin mRNA labeling provide an initial glance at the opsin expression profile in the retina of captive A. ocellaris, and therefore, comparisons with wild anemonefish are required to assess differences associated with lighting, seasonality and/or ontogeny. Finally, we hope this information on the anemonefish visual system will encourage their use as a focus organism for investigating the mechanistic basis and function of UV-vision in reef fishes.

Materials and Methods

Anemonefish Visual Opsin Genes: Identification, Phylogeny, and Synteny

All genetic sequence analyses including the visual inspection, mapping, and alignment of genes were performed using Geneious Prime (v. 2019.2.1). Our in silico searches of anemonefish visual opsin genes involved annotating the regions containing the SW51, SW52, RH2, LWS, and RH1 opsin genes, along with their immediate upstream and downstream (flanking) genes in the genomes of 11 species of anemonefish. The publicly available assembled genomes for anemonefishes were accessed from various sources including: A. ocellaris (Tan et al. 2018; accession number:
Visual opsins were detected by mapping individual reference exon sequences from *Oreochromis niloticus* (accession numbers: AY775108, AF247128, JF262086, JF262087) and *Pseudochromis fuscus* (accession number: KP004335) using low specificity (>70% similarity) against anemonefish genomes. The full coding regions from duplicate *SWS1* and *RH2A* opsins were detected in the high-quality long-read genome assemblies of *A. percula* (chromosome-resolution) and *A. ocellaris* (scaffold-resolution). However, only partially assembled opsin gene duplicates could initially be detected in the short-read genome assemblies of the other nine anemonefishes. Hence, to reconstruct the full coding sequences in those species we used a second approach that took advantage of the genomic raw-reads to back-map paired-end reads against the reference *A. percula*, *SWS1* and *RH2A* genes. This was followed by a “manual” approach that extracted highly similar gene duplicates by moving from one single-nucleotide polymorphism (SNP) to another and taking advantage of paired-end information to breach gaps between SNPs (as per Musilova et al. [2019]). To complement our data set and further support opsin gene identity, we also remapped the transcriptomic reads from *A. akindynos* (accession number: SRX5993365; Stieb et al. [2019]) against reference opsin gene sequences. Because that study did not specifically search for *SWS1* or *RH2* duplicates, we repeated the back-mapping approach using the transcriptomic raw reads in this case.

Visual transduction and shut-off pathway genes were also identified in the *A. percula* and *A. ocellaris* genomes by mapping against predicted gene sequences obtained from Ensembl v. 97 (ensembl.org, accession date: August 15, 2019) [Zerbino et al. [2018]]. The coding sequences were confirmed by mapping assembled transcripts from the *A. ocellaris* retina against the genes from Ensembl.

Phylogenetic trees based on the nucleotide alignment (MAFFT v. 7.388; Katoh and Standley [2013]) of 135 visual opsin-coding sequences were generated using Bayesian inference in MrBayes v. 3.2.7a (Huelsenbeck and Ronquist [2001]) in a workflow run through CIPRES (Miller et al. [2012]) and viewed using Figtree v. 1.4.4 (Rambaut 2018). We included *Anolis carolinensis* vertebrate ancestral opsin (accession number: NM_001293118) as an outgroup, and additional opsin sequences from distantly related species to show the grouping of anemonefish opsin genes relative to those found in other teleost fishes obtained from GenBank (www.ncbi.nlm.nih.gov/genbank) last accessed August 18, 2021) including *Oreochromis niloticus* (AY775108, JF262088, JF262086, JF262087), *Pseudochromis fuscus* (accession numbers: KP004335, KP017247), *Danio rerio* (AB087811, HM367062, AB087803, NM_001002443, AF109369, KT008394, NM_182892, NM_131254, KT008398, KT008399), *O. latipes* (AB180742, XM_004069094, NM_001104694, AB223057, AB223058), and *Gasterosteus aculeatus* (KC774627, KC774623, KC594701, KC774625, KC774626). The opsin tree was reconstructed under a GTR+I+G model selected based on the best-fit model Akaike Information Criterion (AIC) from Jmodeltest2 (Darriba et al. [2012]) with default parameters. The Bayesian reconstructions included MCMC searches for 10 million generations with two independent runs and four chains each, a sampling frequency of 1,000 generations and a burn-in of 25%.

### Anemonefish Opsin Gene Conversion Analysis

Visual opsin gene duplicates were tested for gene conversion: a phenomenon commonly found in teleosts (Owens et al. 2009; Watson et al. 2011; Nakamura et al. 2013; Cortesi et al. 2015; Sandkam et al. 2017; Escobar-Camacho et al. 2017, 2020; Matsumoto et al. 2020). This was analyzed using the program GARD (Genetic Algorithm for Recombination Detection) (Kosakovsky Pond et al. 2006) on the aligned whole coding sequences of the *RH2A* and *SWS1* duplicates, respectively. Segments between reported breakpoint sites were identified as possible regions of recombination and corroborated by comparing different phylogenetic tree topologies based on those limited to suspected regions of recombination, and regions not suspected of recombination. Note that species with only a single *RH2A* gene were excluded from this analysis.

### Analysis of Visual Opsin Tuning Sites and \( \lambda_{\text{max}} \) Value Estimation

Comparisons between anemonefish opsin protein sequences and those of other fishes with known \( \lambda_{\text{max}} \) values were made to infer the spectral tuning effects of individual amino acid (aa) sites. Estimates of anemonefish opsin \( \lambda_{\text{max}} \) values were calculated from the known spectral absorbances of teleost opsins that have been thoroughly studied using either microspectrophotometry or direct measurement via in vitro reconstitution of opsin proteins including those found in *Oreochromis niloticus* and *Maylandia zebra* (Parry et al. 2005), *O. latipes* (Matsumoto et al. 2006), *Lucania goodei* (Fuller et al. 2003), *Pomacentrus amboinensis* (Siebeck et al. 2010), and *Dascyllus trimaculatus* (McFarland and Loew 1994). Our analysis involved identifying variable amino acid residues located at sites within the retinal binding pocket attributed to a shift in polarity and/or substitutions at previously reported tuning sites in those of other species. Opsin protein sequences were aligned using MAFFT alignment (MAFFT v.
7.388; Katoh and Standley 2013) with bovine (Bos taurus) rhodopsin as a template (PDB accession number: 1U19).

Animals and Ethics Statement
Anemonefish (A. ocellaris) (N = 7) (supplier Gallery Aquatica, Wynnum, QLD, Australia) used for opsin gene expression level analysis and FISH were housed in recirculating aquaria at the Queensland Brain Institute at the University of Queensland, Australia. Experiments were conducted in accordance with the University of Queensland’s Animal Ethics Committee guidelines under the approval number: QBI/304/16.

Visual Gene Expression Analysis of A. ocellaris
Adult retinas from two female (mean standard-length = 45 mm) and two male (mean standard-length = 30 mm) A. ocellaris were sampled for opsin gene expression analysis. Teleost opsin gene expression levels can be highly plastic with detectable change occurring within as little as 1-month exposure to different lighting (Fuller and Claricoates 2011), and therefore, we kept fish under broad-spectrum lighting (Supplementary Figure 1, Supplementary Material Online) for a minimum of 3 weeks.

Isolated retinas were homogenized using a high-speed bench-top homogenizer and total RNA was extracted using the QiaGen RNeasy Mini Kit. RNA was purified from any possible DNA contamination by treating samples with DNase following the protocol outlined by the manufacturer (QiaGen). The integrity of the extract was subsequently determined using a Agilent Total RNA Nanochip on an Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA was sent to Novogene (https://en.novogene.com/, last accessed August 18, 2021) for library preparation and strand-specific transcriptome sequencing on a HiSeq2500 (PE150, 250–300 bp insert). Retinal transcriptomes were then filtered and transcripts de novo assembled on a customised Galaxy (v2.4.0.2; usegalax-y.org) (Afgan et al. 2016) workflow following the protocol described in de Busserolles et al. (2017). Teleost single cone and double cone photoreceptors are morphologically and physiologically distinct entities, and proportioning expression should be calculated separately between the two for a meaningful comparison (Yourick et al. 2019). Therefore, we calculated separate proportional cone opsin expression levels for A. ocellaris single and double cone genes. In brief, following de Busserolles et al. (2017), the number of mapped reads for each opsin gene was divided by its length (bp) and then normalized by the total number of reads mapped according to its cone type (i.e., against the combined single cone or double cone opsin gene expression).

A further step calculated separate proportional gene expression levels for SWS1 and RH2A gene paralogs, by first extracting all the reads that mapped against both paralogs (e.g., SWS1α and SWS1β) and remapping them against a highly heterozygous region (i.e., a region with a high number of SNPs) of the paralogous pairs, including 341–509 bp (on the second exon) of SWS1α and SWS1β, and 633–892 bp (on the third and fourth exons) of RH2A-1 and RH2A-2. Individual expression levels for paralogs were then recalculated by multiplying the normalized number of remapped reads for each paralog by the initial proportion of the combined paralog expression (i.e., that was originally calculated using whole coding regions).

Rod versus cone opsin expression was calculated as the total proportion out of all mapped opsin reads.

FISH of A. ocellaris Cone Opsins
Dual-labeling FISH was performed on whole mount retinas from three adult A. ocellaris, following standard protocols (Raymond and Barthel 2004; Allison 2010; Dalton et al. 2014, 2015) using eyes that were enucleated and prepared following methods outlined by Barthel and Raymond (2000) after 1-h dark adaptation. Retinal mRNA was reverse transcribed using a High Capacity RNA-to-cDNA Reverse Transcription Kit (Applied Biosystems). Riboprobe templates were synthesized from cDNA via standard PCR using Q5 High Fidelity DNA polymerase (New England Biolabs) and opsin specific primers (Supplementary Table 1, Supplementary Material Online). Amplicons were isolated via gel electrophoresis and gel extraction (Qiagen Gel Extraction Kit), followed by enrichment PCR using gel-extracted amplicons as cDNA template. Primers were designed to bind to the coding sequence of target opsins (LWS, RH2A, RH2B, SWS1β, SWS2B) and to contain T3 or T7 RNA polymerase promoter sequences at their 5’-ends (T3, reverse primer; T7, forward primer) to allow subsequent strand-specific RNA transcription from cDNA templates for riboprobe synthesis. Because of the high similarity between RH2A paralogs, it was not possible to design riboprobes accurate enough to bind exclusively to either RH2A-1 or RH2A-2. Antisense riboprobes were synthesized and labeled with digoxigenin-UTP (DIG) or fluorescein-UTP (FL) using DIG/FL RNA labeling mix (Roche). Hybridized, labeled riboprobes were detected using anti-digoxigenin or anti-fluorescein antibodies conjugated to horseradish peroxidase (Roche). Fluorescent tagging was performed using Alexa Fluor 594 or 488 dyes with streptavidin tyramide signal amplification (Invitrogen). Finally, retinas were mounted in 70% glycerol in PBS, photoreceptor side up, on microscopy slides with a coverslip.

Dual (RH2A/RH2B, RH2A/LWS, SWS2B/SWS1β) labeled photoreceptor cells were visualized and imaged at the Queensland Brain Institute’s Advanced Microscopy Facility using a CFI Apo Lambda S LW4D 40X/1.15 NA water immersion (SWS1β/SWS2B, RH2A/RH2B) and a CFI Apo Lambda 60X/1.4 NA oil immersion (RH2A/LWS) objective on a spinning disk confocal microscope (Diskovery, Andor Technologies, United Kingdom) built around a Nikon Ti-E body (Nikon Corporation, Japan) equipped with two Zyla 4.2 sCMOS cameras (Andor Technologies, United Kingdom).
Supplementary Material

Supplementary data are available at Genome Biology and Evolution online.

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Author Contributions

L.J.M., K.L.C., J.M., and F.C. conceived the study. L.J.M., K.M., and F.C. carried out gene identification and phylogenetic tree analyses. L.J.M. and F.C. conducted transcriptome analyses and calculated opsin gene expression levels. L.J.M. calculated estimates of pigment lambda max values. M.L. carried out FISH analyses. L.J.M. wrote the initial draft manuscript, and all authors contributed to the final version.

Data Availability

Assembled transcriptomes underlying this article are available in Genbank, and can be accessed with (bioproject accession number: PRJNA547682). Opsin gene CDSs extracted from transcriptomes are also publicly available in GenBank (TPA accession numbers: BK059177—BK059184). Output files from phylogeny construction, GARD analyses, all genome and raw-read mapped opsin gene sequences, and DNA/protein alignments are available in the University of Queensland’s Resource Data Manager platform, at (https://doi.org/10.14264/673b710, last accessed August 18, 2021).

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