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Why UV vision and red vision are important for damselfish (Pomacentridae): structural and expression variation in opsin genes

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Abstract

Coral reefs belong to the most diverse ecosystems on our planet. The diversity in coloration and lifestyles of coral reef fishes makes them a particularly promising system to study the role of visual communication and adaptation. Here, we investigated the evolution of visual pigment genes (opsins) in damselfish (Pomacentridae) and examined whether structural and expression variation of opsins can be linked to ecology. Using DNA sequence data of a phylogenetically representative set of 31 damselfish species, we show that all but one visual opsin are evolving under positive selection. In addition, selection on opsin tuning sites, including cases of divergent, parallel, convergent and reversed evolution, has been strong throughout the radiation of damselfish, emphasizing the importance of visual tuning for this group. The highest functional variation in opsin protein sequences was observed in the short- followed by the long-wavelength end of the visual spectrum. Comparative gene expression analyses of a subset of the same species revealed that with SWS1, RH2B and RH2A always being expressed, damselfish use an overall short-wavelength shifted expression profile. Interestingly, not only did all species express SWS1 – a UV-sensitive opsin – and possess UV-transmitting lenses, most species also feature UV-reflective body parts. This suggests that damsels might benefit from a close-range UV-based 'private' communication channel, which is likely to be hidden from 'UV-blind' predators. Finally, we found that LWS expression is highly correlated to feeding strategy in damsels with herbivorous feeders having an increased LWS expression, possibly enhancing the detection of benthic algae.

Keywords: gene expression, herbivory, opsin, reef fish, sequence variation, UV reflectance

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Introduction

Coral reefs including the Great Barrier Reef off Australia's East Coast represent unique ecosystems, characterized by their richness in highly colourful and diverse organisms. Not surprisingly, visual communication plays a major role in the interactions between the inhabitants of coral reefs [reviewed in Marshall *et al.* (2015)]. Coral reef fishes, for example, use visual communication as a key mechanism for species recognition, warning signalling, mimicry, predation and sexual selection [reviewed in Marshall & Cheney (2011)]. Their variability in coloration and lifestyles, in addition to differences in the light environment they inhabit – all factors relevant for visual tasks – has resulted in a long-standing interest for visual ecologists for this particular group of animals (e.g. Longley 1917; Lorenz 1962; Loew & Lythgoe 1978; Losey *et al.* 2003). More recently, the focus has shifted towards the understanding of the molecular

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basis of visual adaptation in reef fishes, and substantial progress has been made through the study of visual pigment genes, the opsins and their evolutionary history (Hofmann *et al.* 2012; Cortesi *et al.* 2015b, 2016; Phillips *et al.* 2016; Stieb *et al.* 2016).

Visual opsin genes encode G-protein-coupled receptors that, together with a covalently bound, light-sensitive, vitamin A-derived chromophore, form the visual pigment, that is the functional unit of photoreceptors (Wald 1968). In vertebrates, visual opsins can be classified based on their photoreceptor specificity and shifts of their spectral sensitivities to different wavelengths of light. The common ancestor of all vertebrates most likely possessed one rod opsin (rhodopsin, RH1) used for dim-light vision, and four cone opsin genes used for colour vision: two short-wavelength sensitive (SWS1, ultraviolet (UV)-violet; and SWS2, violet-blue), a medium wavelength sensitive (RH2, green) and a long-wavelength sensitive (LWS, red) opsin (Yokoyama 2000). Structural variability of the opsin protein arising from differences in opsin gene sequence and the type of chromophore (A1 or A2), to which it is bound, define the wavelength of maximal absorbance (λ_{max}) of the visual pigment and in turn help determine the overall spectral sensitivity of an organism (Bowmaker 1990; Yokoyama & Yokoyama 1996). It is this coupling between an opsin genotype and its visual phenotype that allows us to draw a direct link between spectral tuning and functional adaptation, making the study of opsin gene evolution an especially worthwhile and exciting endeavour (Yokoyama 2000; Hunt *et al.* 2001).

Teleost fishes, by far the most species-rich clade of vertebrates, vary vastly in ecology and occur across a great range of different habitats, ranging from salt- to freshwater, the deep-sea to shallow creeks and from caves to brightly lit environments. Teleosts have, hence, become powerful model systems to study the evolution of opsin genes, especially in the context of adaptation and speciation (Seehausen *et al.* 2008; Ryan & Cummings 2013). In teleosts, extensive opsin gene duplications have created visual systems with a great diversity in spectral sensitivities (λ_{max}), often in relation to adaptations to novel photic environments (Hofmann & Carleton 2009; Cortesi *et al.* 2015b). Mutations in the coding sequence of opsins, on the other hand, led to shifts in λ_{max} in association with more subtle differences in lighting conditions (e.g. Yokoyama & Yokoyama 1996; Hunt *et al.* 2001; Terai *et al.* 2002, 2006; Carleton *et al.* 2005a; Sugawara *et al.* 2005; Hofmann *et al.* 2009; Larmuseau *et al.* 2009; Nakamura *et al.* 2013; Tezuka *et al.* 2014; Malinsky *et al.* 2015), predation density (Sandkam *et al.* 2015a) or species-specific habitat usage (Cummings & Partridge 2001). Besides the structural diversity, variation in opsin gene expression allows for a more flexible, possibly short-term, visual adaptation to changes in the prevailing light habitat (Fuller *et al.* 2004; Carleton *et al.* 2005a; Shand *et al.* 2008; Hofmann *et al.* 2009, 2010; Fuller & Claricoates 2011; Sandkam *et al.* 2015b; Stieb *et al.* 2016), predation density (Sandkam *et al.* 2015a) or feeding strategies (Hofmann *et al.* 2009; O'Quin *et al.* 2010).

In this study, we focus on the evolution of visual opsin genes in one of the most abundant and species-rich reef fish families, damselfishes (Pomacentridae). Most of the 388 described damselfish species inhabit tropical seas, mainly the Indo-Pacific, and are primarily associated with shallow, clear and light-rich waters. They exhibit a remarkable variety in ecology, behaviour and coloration, mirroring the high diversity in fishes found on coral reefs (Allen 1991). While some damsel species show vivid coloration, others appear drab. Damselfishes also vary in their social structure including shoaling and solitary living species and in their feeding strategy, which ranges from omnivores, territorial herbivores, water column feeding planktivores, to highly specialized corallivorous feeders. Interestingly, the damselfish diversification results from iterative convergent radiations with subclades presenting the same adaptive specifications to similar environments (Frédérich *et al.* 2013). The main trophic groups found in damselfish (herbivores, planktivores and omnivores) and phenotypic disparities being tightly linked to trophic ecology, like oral jaw morphology (Frédérich *et al.* 2008), have evolved repeatedly across the damselfish phylogeny [for trophic groups see Cooper & Westneat (2009); for trophic groups and linked morphologies see Frédérich *et al.* (2013)]. Previous research on the visual system of damselfish primarily focused on their physiology [for a review, see Marshall *et al.* (2006)] and behaviour (Thresher 1979; Katzir 1981; Siebeck *et al.* 2008, 2010), and only to some extent on the molecular basis of visual adaptation (Hofmann *et al.* 2012; Stieb *et al.* 2016). It has been established that damsels possess five cone opsin (SWS1, SWS2B, RH2A, RH2B and LWS) and one rod opsin gene (RH1) (Hofmann *et al.* 2012), and based on opsin gene expression in seven species, we also know that damselfishes mostly use RH1 together with SWS1, RH2B and RH2A for vision (Stieb *et al.* 2016). This fits in well with microspectrophotometric (MSP) data showing that the majority of damselfishes have visual pigments which are sensitive to UV and medium wavelengths (Loew & Lythgoe 1978; McFarland & Loew 1994; Hawryshyn *et al.* 2003; Losey *et al.* 2003; Marshall *et al.* 2006; Siebeck *et al.* 2010). Moreover, behavioural studies have shown that damsels indeed take advantage of this set-up to discriminate between colours (Siebeck *et al.* 2008). Finally, spectral reflectance measurements of damselfishes revealed that some species contain

UV colorations (Marshall 2000a). These UV markings could be perceived by con- and heterospecifics by means of the UV-sensitive visual pigment (Marshall 2000a; Marshall *et al.* 2006), which is supported by behavioural assays that show that the Ambon damsel (*Pomacentrus amboinensis*) uses facial UV patterns for species discrimination (Siebeck *et al.* 2010). Also, as most predatory reef fish lack the UV-sensitive visual pigment, these markings could serve as a 'secret predator-safe' communication channel (Marshall & Cheney 2011).

Here, we investigate the coding regions and expression patterns of visual opsin genes in Pomacentridae. A comparison of a phylogenetically representative set of damselfish species has previously led to the conclusion that visual pigments may have been under strong selection during the radiation of damselfish (Hofmann *et al.* 2012). In this study, we address the question whether or not selection of known visual pigment tuning sites remains strong among groups of closely related damselfish species. Further, by including species from Hofmann *et al.* (2012) covering the damselfish phylogeny, we were able to screen whether the repeated diversification found in trophic groups (Cooper & Westneat 2009) and linked morphological traits (Frédérich *et al.* 2013) is also reflected in visual phenotypes. To this end, we investigated in total 31 damselfish species with 27 belonging to the clades of the *Pomacentrinae* and the *Chrominae* (Cooper *et al.* 2009). In addition, we examined whether opsin gene expression varies across 23 species belonging to our two clades of focus. Remarkable ecological and behavioural diversity of damselfish, as well as their highly variable colorations (Allen 1991; Randall *et al.* 1997), are strong indicators for the adaptive importance of spectral tuning. We were particularly interested to see whether there was a link between similar opsin expression phenotypes and dietary specialization (the studied damselfish species can essentially be divided into planktivores and herbivores), as well as an association between expressing SWS1 presumably conferring UV vision and having UV-reflective colours. We chose these two parameters – diet and UV reflectance – as they were, to begin with, accessible for almost all taxa included in this study. On the other hand, information about other potentially interesting ecological variables such as microhabitat preferences, degree of sociality (schooling vs. solitary species) or territoriality was often missing or not relevant for the species in question. For example, although territoriality is usually associated with herbivory in reef fishes (e.g. Ceccarelli *et al.* 2001), most Pomacentrids are territorial independent of feeding habits (e.g. Randall *et al.* 1997). More importantly, diet is one of the ecological variables, which is known to be related to visual abilities. UV perception, for example, enhances the efficiency to forage on zooplankton (Loew *et al.* 1993; Browman *et al.* 1994; Novales Flamarique 2016) as shown by increased expression of the UV-sensitive opsin (SWS1) in planktivorous cichlids (Hofmann *et al.* 2009; O'Quin *et al.* 2010). Herbivorous fishes, on the other hand, may benefit from long-wavelength-biased visual systems advancing their ability to detect benthic algae (Marshall *et al.* 2003a). This parallels the importance of long-wavelength sensitivity to feeding ecology for discrimination of green leaves (Lythgoe & Partridge 1989) and yellow and orange fruits (Osorio & Vorobyev 1996; Regan *et al.* 1998) as reported for the terrestrial environment. Finally, based on the hypothesis that small reef fishes such as damsels, may use a UV-based communication signal that is hidden from 'UV-blind' predators (Marshall & Cheney 2011), we expected to find that species, which reflect in the UV, would express the UV-sensitive opsin (SWS1) and have UV-transmitting lenses.

Material and methods

Species studied

In this study, we focused on 31 damselfish species (Table 1) native to the Great Barrier Reef (GBR), Australia. For this, we combined data collected in this study including gene sequences, gene expression, lens transmission and spectral reflectance with previously published data on opsin gene sequences (Hofmann *et al.* 2012; Stieb *et al.* 2016), gene expression (Stieb *et al.* 2016), lens transmission (Siebeck & Marshall 2007), spectral reflectance (Marshall 2000a; Siebeck 2002) and feeding categories (Allen 1991; Ceccarelli *et al.* 2001; Curtis-Quick *et al.* 2012; www.australianmuseum.net.au/fishes, www.fishbase.org); only data from mature specimen were considered.

Sample collection

Specimens collected over the course of this study were caught between 2012 and 2014 from coral reefs around Lizard Island (14°40′S, 145°27′E), northern GBR, using hand and barrier nets and kept in aquaria being exposed to sunlight and a natural light cycle at the Lizard Island Research Station for no longer than 24 h. Additionally, two species (*Chromis nitida* and *Neopomacentrus cyanomos*) were collected by a professional collector (Cairns Marine) off the coast of Cairns, northern GBR. Fish were anaesthetized with an overdose of clove oil (10% clove oil; 40% ethanol; 50% sea water), killed by decapitation, and retinas were dissected from the eyecup and immediately stored in RNAlater (Ambion) for subsequent molecular analysis.

All experimental procedures were approved by The University of Queensland Animal Ethics Committee

Table 1 Summary of damselfish relative opsin gene expression, UV body reflectance, lens transmission (T50 = wavelength of 50% transmission) and diet (P = planktivores, H = herbivores)

Species	*n*	Relative opsin expression						UV refl.	Lens (T50)	Diet
		SWS1	SWS2B	RH2B	RH2A	LWS	RH1			
Chrominae										
Chromis nitida	6*	5.7 ± 2.3	4.7 ± 1.4	45.3 ± 4.9	44.4 ± 6.0	0.5 ± 0.5	79.9 ± 5.9	N/A	361–364*,¶	P‡‡,¶¶
Chromis viridis	8*	15.8 ± 5.3	0	35.6 ± 3.8	47.9 ± 4.0	0.7 ± 0.8	81.7 ± 8.2	Yes‡	336*	P‡‡,¶¶
Dascyllus aruanus	19†	13.0 ± 4.4	0.5 ± 0.9	49.6 ± 4.8	36.7 ± 4.4	0.4 ± 0.6	77.6 ± 12.0	Yes‡	328*,¶	P‡‡,¶¶
Dascyllus reticulatus	13†	13.3 ± 1.8	0	49.8 ± 4.8	36.7 ± 4.4	0.2 ± 0.2	73.0 ± 9.4	Yes*	345¶/355*	P¶¶
Pomacentrinae										
Acanthochromis polyacanthus	7*	31.8 ± 9.3	0	35.3 ± 6.1	32.0 ± 3.8	0.8 ± 0.7	97.7 ± 1.8	No*	350*	P**
Amblyglyphidodon curacao	8*	16.3 ± 11.5	0	36.3 ± 16.7	45.3 ± 15.3	1.2 ± 1.7	18.6 ± 5.7	Yes*,‡	340–350*	P (H)**, ††,‡‡,§§,¶¶
Amblyglyphidodon leucogaster	6*	11.8 ± 4.0	0	47.3 ± 3.3	40.9 ± 1.8	0.5 ± 0.5	24.2 ± 3.7	Yes*,‡	350¶	P**,††,¶¶
Chrysiptera brownriggii	4*	23.4 ± 2.6	1.4 ± 1.4	13.6 ± 3.2	59.9 ± 5.5	1.7 ± 1.4	67.0 ± 14.0	N/A	N/A	H‡‡
Chrysiptera cyanea	8*	13.8 ± 3.4	0	52.8 ± 11.8	24.4 ± 7.7	9.0 ± 3.1	70.9 ± 13.6	Yes*,‡	326–339*,¶	H‡‡,§§
Chrysiptera rollandi	14†	12.8 ± 5.9	0	48.6 ± 3.4	38.0 ± 4.2	0.5 ± 0.3	57.6 ± 15.5	Yes*	348¶	P‡‡,§§
Dischistodus perspicillatus	4*	17.2 ± 3.6	0	36.9 ± 7.4	44.1 ± 5.0	1.7 ± 1.5	70.3 ± 10.7	N/A	355¶	H‡‡,§§,¶¶
Dischistodus prosopotaenia	5*	48.0 ± 9.9	1.0 ± 1.6	11.0 ± 4.1	38.3 ± 8.9	1.7 ± 0.7	62.6 ± 18.5	Yes*,‡	350¶	H§§,¶¶
Neopomacentrus azysron	2*	11.4 ± 5.1	0	43.1 ± 2.5	42.9 ± 0.5	2.6 ± 2.1	62.8 ± 0.3	Yes*,‡	324¶/335*	P§§
Neopomacentrus cyanomos	5*	8.7 ± 3.3	12.2 ± 0.6	29.3 ± 3.0	49.1 ± 2.1	0.7 ± 0.5	73.0 ± 2.3	N/A	362*	P§§
Neoglyphidodon nigroris	2*	7.5 ± 0.1	1.3 ± 0.1	48.5 ± 2.2	33.6 ± 1.3	9.2 ± 0.8	56.6 ± 1.3	No*	N/A	H¶¶
Pomacentrus adelus	2*	11.7 ± 3.4	0	48.7 ± 2.7	29.7 ± 2.6	10.0 ± 1.9	N/A	N/A	N/A	H¶¶
Pomacentrus amboinensis	21†	14.7 ± 5.9	0	44.0 ± 10.4	40.1 ± 6.8	1.2 ± 1.0	73.5 ± 8.7	Yes§	345*	P (H) ‡‡,§§
Pomacentrus chrysurus	5*	25.4 ± 9.5	0	30.6 ± 6.0	34.9 ± 6.0	9.0 ± 2.8	62.4 ± 12.2	Yes‡	339*	H‡‡,¶¶
Pomacentrus coelestis	15†	15.1 ± 5.7	0	45.4 ± 5.2	38.7 ± 2.8	0.8 ± 0.5	65.1 ± 16.0	Yes*	339¶/344*	P (H) ‡‡
Pomacentrus moluccensis	13†	20.6 ± 6.6	0	38.3 ± 9.1	37.3 ± 6.6	3.7 ± 2.5	77.6 ± 9.0	Yes‡,§	349–373*,¶	P (H) ‡‡,§§
Pomacentrus nagasakiensis	17†	11.8 ± 5.4	0	49.6 ± 3.0	38.0 ± 7.4	0.5 ± 0.5	60.3 ± 14.4	Yes*,‡	341*	P‡‡
Pomacentrus pavo	8*	16.1 ± 5.4	0	46.9 ± 2.7	36.5 ± 4.6	0.5 ± 0.5	62.9 ± 9.8	Yes*	350*	P (H) ‡‡
Pomacentrus wardi	2*	16.2 ± 2.1	0	41.8 ± 2.0	38.7 ± 0.4	3.4 ± 0.3	85.6 ± 2.5	Yes‡	340¶	H‡‡,¶¶
Diverse clades										
Abudefduf sexfasciatus		Only used for opsin sequence analyses***								
Amphiprion akindynos										
Chrysiptera rex										
Dascyllus trimaculatus										
Neopomacentrus bankieri										
Parma oligolepis										
Plectroglyphidodon dickii										
Stegastes gasconynei										

N/A not available.

*Measurements obtained from this study. †Stieb *et al.* (2016). ‡Marshall (2000a). §Siebeck (2002). ¶Siebeck & Marshall (2007). **www.australianmuseum.net.au/fishes. ††Allen (1991). ‡‡www.fishbase.org. §§Curtis-Quick *et al.* (2012). ¶¶Ceccarelli *et al.* (2001). ***Hofmann *et al.* (2012).

[QBI/223/10/ARC/US AIRFORCE (NF) and QBI/192/13/ARC], and fish were collected under the Great Barrier Reef Marine Parks Permit (G12/35005.1) and Queensland general fisheries permit (140763).

Sample preparation for opsin gene studies

Retinas were homogenized using the high-speed benchtop homogenizer FASTPREP24 (MP Biomedicals Europe), and total RNA was extracted using TRIZOL according to the manufacturer's protocol (LifeTechnologies). To remove any possible DNA contamination, we subsequently treated the samples with DNase following the DNA-free protocol (Ambion). RNA was reverse transcribed using the High Capacity RNA-to-cDNA kit (Applied Biosystems). Genomic DNA was extracted from fin tissue using a standard salt precipitation protocol (Laird *et al.* 1991). RNA and DNA concentrations and quality were determined using a NanoDrop1000 Spectrophotometer (ThermoScientific).

Opsin sequencing

Opsin sequences for damselfish SWS1, SWS2B, RH2B, RH2A, LWS and RH1 genes were obtained from GENBANK for 16 species (Hofmann *et al.* 2012; Stieb *et al.* 2016). For an additional 15 species, we de novo sequenced, using Sanger sequencing (see Table S1, Supporting information for species list and GenBank Accession nos), all five cone (SWS1, SWS2B, RH2B, RH2A and LWS) and the rod (RH1) opsin gene using damsel specific primers [Table S4, Supporting information; primer names and sequences obtained from Hofmann *et al.* (2012)]. Two overlapping fragments were PCR amplified with Red Taq DNA polymerase (Sigma) for each opsin gene using cDNA as template, or, if not successful, genomic DNA. Products were subsequently visualized by staining with GelRed on a 1.5% agarose gel, purified with ExoSapIT (USB, Cleveland, OH) and sequenced using the BIG DYE version 3.1 chemistry (Applied Biosystems) following the manufacturers protocol on an ABI 3130*xl* genetic analyser (Applied Biosystem).

Opsin gene sequence analysis and ancestral state reconstruction

Sequences were aligned and edited using CODON CODE ALIGNER 3.5.6 (CodonCode Corporation, Dedham, MA). Alignments for each opsin gene were exported to MEGA7 (Kumar *et al.* 2016), which was used to calculate nucleotide diversity (π).

To identify potential functional amino acid substitutions of the opsin protein only involved in spectral tuning, we followed the methods previously used for damselfish by Hofmann *et al.* (2012). In brief, we concentrated on amino substitutions that differ in their physical property (polar, nonpolar, acidic, basic) and are located in the transmembrane and retinal binding pocket regions [based on the crystal structure of bovine rhodopsin (Palczewski *et al.* 2000) as shown in the alignments of Carleton *et al.* (2005b)]. Further, we focused on sites that have been identified as tuning sites [for RH1 site 299 see Fasick & Robinson (1998) and Hunt *et al.* (2001); for all other tuning sites, see Yokoyama (2008)]. We refer in the text to each site by its location relative to bovine rhodopsin.

In addition, to test for site-specific signs of positive selection for all opsins, we used the codeml program in PAML (Yang 2007) and performed likelihood ratio tests (LRT) of model comparisons M1a vs. M2 and M8 vs. M8a [for a detailed description see Hofmann *et al.* (2012)] based on gene trees for each opsin gene. The Bayes empirical Bayes (BEB; Yang 2005) inferences were used to identify sites under positive selection in case of significant LRTs.

Codeml was furthermore used to perform ancestral state reconstructions of known tuning sites of opsin genes to test whether amino acid changes occurred among groups of closely related damselfish species. Our sampling regime allowed us to test for this within the monophyletic clades *Pomacentrinae* and *Chrominae* and furthermore on the genus level for species belonging to the genera *Pomacentrus*, *Neopomacentrus*, *Chrysiptera*, *Dischistodus* as well as for *Chromis* and *Dascyllus*. All those genera form, respectively, monophyletic groups, except for the polyphyletic genus *Chrysiptera* [*Chry. brownriggii* belongs to *Chrysiptera 1* and *Chry. rex*, *Chry. rollandi* and *Chry. cyanea* to *Chrysiptera 2* (Cooper *et al.* 2009)]. Ancestral states were reconstructed using a damselfish phylogeny, which was based on the mitochondrial gene *12s* and the nuclear gene *rag1* (Tang *et al.* 2004; Quenouille *et al.* 2004; Cooper *et al.* 2009; Hofmann *et al.* 2012; for Accession nos see Table S1, Supporting information). For this, we concatenated and aligned the sequences using MAFFT (Katoh *et al.* 2009) and constructed maximum-likelihood trees (100 bootstrap iterations) using PHYML (Guindon & Gascuel 2003) using the web-based bioinformatics interface Mobyle (Neron *et al.* 2009). *Pom. wardi* and *Pom. nagasakiensis* were excluded from both LRTs and ancestral state reconstruction due to lack of *12s* and *rag1* sequences. Moreover, we excluded species from single gene analyses, which were lacking parts of the transmembrane region: SWS1 (*Amblyglyphidodon leucogaster*, *Das. reticulatus*, *Pom. adelus*), SWS2B (*Amb. curacao*, *Amb. leucogaster*, *Chro. nitida*, *Chry. cyanea*, *Chry. rollandi*, *Das. reticulatus*, *Dis. perspicillatus*, *Dis. prosopotaenia*, *Neoglyphidodon nigroris*, *Neop. azysron*, *Pom. coelestis*), RH2A *(Chro.*

nitida, *Chry. rollandi*, *Das. reticulatus*, *Dis. prosopotaenia*), LWS (*Amb. leucogaster*, *Chro. nitida*, *Das. reticulatus*, *Dis. prosopotaenia*), RH1 (*Chro. rollandi*, *Das. reticulatus*, *Dis. prosopotaenia*).

Quantitative real-time polymerase chain reaction (qRT–PCR)

We quantified relative opsin gene expression using quantitative Real-Time Polymerase Chain Reaction (qRT–PCR) [SYBR Green master (Rox) dye (Roche)] on a StepOnePlus Real-Time PCR System (LifeTechnologies) in 23 damselfish species (16 novel and 7 from our previous study; Stieb *et al.* 2016) with between 2 and 23 individuals tested for each species. Following Carleton & Kocher (2001) and Stieb *et al.* (2016), relative cone opsin expression as a fraction of the total of cone opsin genes expressed, and relative rod opsin expression as a fraction of the total of all opsin genes expressed was calculated from the reaction efficiency and critical cycle number (C_t).

Following previously described protocols (Stieb *et al.* 2016), we constructed unique primers for each opsin gene and species with either the forward or the reverse primer spanning an exon–exon boundary (except for the intronless RH1) so that only cDNA would be amplified with a product length of 60–100 bp (Tables S5, S6, Supporting information). Primer efficiencies (Table S6, Supporting information) were initially validated for each species using a five orders of magnitude dilution series of each species-specific opsin pool. The opsin pool contained equal ratios of fragments of each opsin gene [molarity of fragments was measured using an AGILENT 2100 BIOANALYZER NANOCHIP (Agilent Technologies)] that were amplified from cDNA from each tested species using the sequencing primers (see Table S4, Supporting information) to obtain a pool being specific for each species; products were cut out from the electrophoresis gel, purified using the QIAquick PCR Purification Kit (QiaGen) and Sanger sequenced for verification. The pool of opsin genes in each plate was used as an internal reference to control for plate and/or template biases. All experiments were carried out with three technical replicates.

Spectral measurements to determine UV reflectance

We gained spectral reflectance data for 18 of the 23 species [our own measurements, $n = 12$; from the literature, $n = 11$ (Marshall 2000a; Siebeck 2002)] and followed the colour categorization used in Marshall (2000a) to define species using UV components: UV, UV-Hump/Blue, UV/Blue, UV/Green, UV/Yellow or White, respectively.

Spectral reflectance was measured on live specimens following the methods described in Marshall *et al.* (2003b). In short, the reflectance of different areas of the fish was measured at a 45° angle using a 200 μm bifurcated UV/visible optic fibre connected to a PX-2 pulse xenon light source (Ocean Optics) and an Ocean Optics (Dunedin, FL, USA) USB2000 spectrophotometer attached to a laptop computer running OOIBASE32 (Ocean Optics). A Spectralon 99% white reflectance standard was used to calibrate the percentage of light reflected at each wavelength from 300 to 800 nm. Spectral reflectance was measured for two to three individuals per species by measuring distinct colour patches (from a human point of view) as well as common areas that may reflect in the ultraviolet [UV; see Marshall (2000a)] such as the surroundings of the eyes and mouth, the operculum, fins and the caudal peduncle. At least ten measurements per area and individual were taken and subsequently averaged.

Lens transmission

Lenses have been shown to be the primary physical light-filter of the damselfish eye (Siebeck & Marshall 2007). Hence, we combined data from the literature ($n = 12$; Siebeck & Marshall 2001, 2007) and our own measurements ($n = 15$) of damselfish lens transmittance [as defined by the wavelength of 50% of maximal transmittance (T50)] to test whether eyes of our study animals would be UV blocking ($T50 > 400$ nm) or transmitting ($T50 < 400$ nm).

We measured lens transmission curves (300–800 nm) following previously published protocols (Siebeck & Marshall 2001, 2007). Light from a pulsed xenon light source (Ocean Optics, PX2, USA) was directed through the lens mounted above a pinhole and into a quartz fibre-optic cable coupled to a spectrometer (USB2000; Ocean Optics, Dunedin, USA). Five to ten measurements were made per individual and one to three specimens were averaged from each species. All transmission curves were normalized at 700 nm (for an example see Fig. 3b), and the wavelength at which 50% of the maximal transmittance (T50) was reached was determined using a linear regression (Douglas & McGuigan 1989; Siebeck & Marshall 2001).

Relationship of opsin expression with diet

To identify relationships between relative cone opsin expression (SWS1, SWS2B, RH2B, RH2A and LWS) and diet, we computed phylogenetic generalized least squares regressions (PGLS) using the caper package (Orme *et al.* 2013), which incorporates phylogenetic information of the tested species. The PGLS regression estimates a maximum-likelihood (ML) value of the phylogenetic scaling factor lambda (λ) with $\lambda = 1$ indicating

complete phylogenetic dependence and $\lambda = 0$ indicating no phylogenetic effect. To resolve the phylogenetic relationship between damselfishes of which we had expression data ($n = 23$), we reconstructed their phylogeny based on *rag-1* and *12s*, as described above. The only genetic information available for *Pom. nagasakiensis* comes from Quenouille *et al.* (2004) using *ATPase 6/8* and *Cytochrome b*. For our phylogeny, we manually added *Pom. nagasakiensis* next to *Pom. chrysurus* according to branch length estimates by comparing branch length proportions between the tree presented in Quenouille *et al.* (2004) and our tree for shared species. As no molecular data were available for *Pom. wardi*, we placed it next to other *Pomacentrus* species in multiple random positions. Consequently, we generated different phylogenies with varying polytomies within the genera *Pomacentrus*, which were then used to compute PGLSs. However, we did not find any qualitative difference in our findings, when using either phylogenetic hypothesis (two of the tested phylogenies are shown in Table 3). To compare relative opsin expression to diet, we placed species into two different foraging categories: herbivorous and planktivorous (for references on the diet of species, see Table 1). However, some species that are listed as being either herbivorous or planktivorous may, to a lesser extent, also feed on other resources. To account for the possibility of flexible feeding strategies, we reran the PGLS placing those five species with variable diets into their alternative feeding category (*Amb. curacao*, *Pom. amboinensis*, *Pom. coelestis*, *Pom. moluccensis* and *Pom. pavo*; alternative feeding strategies are denoted in brackets of Table 2).

All statistical analyses were performed in R (R Development Core Team 2011) using the interface RSTUDIO (Version 0.98.1062). Significance levels were adjusted for multiple testing ($n = 20$) using a Bonferroni correction.

Results

Opsin gene sequences, sequence diversity and test for positive selection

We obtained sequences for SWS1, SWS2B, RH2B, RH2A, LWS and RH1 genes for a total of 31 damselfish species, and the complete transmembrane region was successfully retrieved for RH2B from all species, for SWS1 and RH1 from 28 species, for RH2A and LWS from 27 species and for SWS2B for 19 species (Fig. S1, Supporting information). Amino acid alignments of all opsin genes are shown in Fig. S1 (Supporting information), with the transmembrane and retinal binding pocket regions, thought to be important for spectral tuning, circled and highlighted in grey, respectively.

There was considerable sequence variation both in the nucleotide and amino acid sequences for all visual opsins (Table 2). The nucleotide diversity (π) was highest in SWS1 (0.0802) followed by SWS2B (0.0535) and LWS (0.0543) and lowest for the RH genes (RH2B with 0.0307, RH2A with 0.0258 and RH1 with 0.0276).

The number of changes in potential spectral tuning sites (sites that have been identified as spectral tuning sites and sites with different physical properties located in the retinal binding pocket, labelled in Fig. S1, Supporting information as 3, 4 and 5, respectively) across all species varied between genes, with 13 sites for SWS1, 12 for SWS2B, 4 for RH2B, 1 for RH2A, 7 for LWS and 3 for RH1. Within *Pomacentrinae,* we identified six modified tuning sites in SWS1, eight in SWS2B, three in RH2B, none in RH2A, six in LWS and two in RH1; within *Chrominae*, we found six changes of tuning sites in SWS1, three in SWS2B, two in RH2B, one in RH2A, four in LWS and two in RH1. Out of these damselfish specific amino acid changes, the following have previously been identified as spectral tuning sites (Fasick & Robinson 1998; Hunt *et al.* 2001; Yokoyama 2008) in the corresponding opsin gene (site numbers refer to bovine rhodopsin): SWS1 (F46I or F46A; F49I, F49L or F49C; S97A; M109L; S114A; V116I; S118A), SWS2B (F46V or F46L; S118T), RH2B (M207L), RH2A (none), LWS (S164A) and RH1 (N83D; S299A). Table 2 shows the gene specific location of each tuning site, as well as their location relative to bovine rhodopsin.

Model comparisons using codeml in PAML revealed that, with the exception of SWS2B, damselfish opsin genes are under positive selection, with M1a vs. M2 and M8 vs. M8a for SWS1 ($P = 4.10E{-}05$; $P = 6.00E{-}06$), RH2B ($P = <1E{-}05$; $P = <5E{-}06$), RH2A ($P = <1E{-}05$; $P = <5E{-}06$), LWS ($P = 2.83E{-}04$; $P = 8.00E{-}06$) and RH1 ($P = <1E{-}05$; $P = <5E{-}06$) (for a summary of PAML results see Table S2, Supporting information). The PAML model 8/8a comparison resulted in three positively selected sites in damselfish SWS1, ten in RH2B, ten in RH2A, seven in LWS and three in RH1. Only one site in RH2B (M207L, P > 95%) and one site in LWS (S164A, P > 99%) are known tuning sites for the corresponding gene, and one of the RH2B (G109A or G109S, P > 95%) and two of the LWS (F46V or F46I, P > 99%; V116T or V116I, P > 99%) positively selected sites are known as tuning sites for a different opsin gene (Yokoyama 2008).

Ancestral state reconstruction

Irrespective of the selection test, we reconstructed the ancestral state for variable tuning sites for SWS1, SWS2B, RH2B, LWS and RH1 (Table 2; Fasick & Robinson 1998; Hunt *et al.* 2001; Yokoyama 2008) using PAML; no variation in known tuning sites was identified for RH2A. Based on the ancestral state reconstruction, we

Table 2 Overview of damselfish opsin sequence variation and amino acid sites used for ancestral state reconstruction

	SWS1	SWS2B	RH2B	RH2A	LWS	RH1
Nucleotide diversity (π)	0.0802	0.0535	0.0307	0.0258	0.0543	0.0276
Functionally variable aa sites [with positively selected sites (P > 95%) shown in brackets]						
In transmembrane region						
All damsels	35 (3)	31	9 (5)	7 (6)	13 (3)	11 (2)
Pomacentrinae	22	17	9	4	9	8
Chrominae	18	10	4	3	6	5
In retinal binding pocket						
All damsels	6	8	1 (1)	0	6 (2)	2
Pomacentrinae	2	4	1	0	4	2
Chrominae	4	1	1	0	3	2
Variable aa sites (positively selected)						
At known tuning site for any opsin						
All damsels	9	7	4 (2)	1	5 (3)	3
Pomacentrinae	6	5	3	0	4	2
Chrominae	4	3	2	1	4	2
At known tuning site for this opsin						
All damsels	7	2	1 (1)	0	1 (1)	2
Pomacentrinae	4	3	1	0	1	2
Chrominae	4	1	1	0	0	2

Sites used for ancestral state reconstruction: Position relative to bovine rhodopsin	Position in the corresponding damselfish opsin					
46*	39	52	—	—	—	—
49*	42	—	—	—	—	—
83*	—	—	—	—	—	83
97*	90	—	—	—	—	—
109*	102	—	—	—	—	—
114*	107	—	—	—	—	—
116*	109	—	—	—	—	—
118*	111	124	—	—	—	—
164*	—	—	—	—	177	—
207*	—	—	208	—	—	—
299†	—	—	—	—	—	299

*Yokoyama (2008). †Fasick & Robinson (1998), Hunt *et al.* (2001).

identified amino acid changes in closely related species belonging to the same genus in all opsin genes and most spectral tuning sites (Fig. 1, Fig. S2, Supporting information). Interestingly, we observed cases of parallel, divergent and reverse evolution in closely related species that are also present in more distantly related taxa (Fig. 1).

The change from serine to alanine in RH1 (S299A) is one example of parallel evolution (Fig. 1a) occurring in single species of the same genus (seen in *Neop. cyanomos* and absent in *Neop. azyscron* and *Neop. bankieri*; seen in *Chro. nitida* and absent in *Chro. viridis*) and spanning across the phylogeny (including the genera *Stegastinae*, *Chrominae* and diverse genera of *Pomacentrinae*). In this case, amino acid substitutions were changed to the same codon. However, in RH2B (M207L), independent changes from methionine to leucine using different codons (Fig. 1b) occurred within the same clade [a change from ATG to CTG occurred in *Pom. coelestis* and *Pom. pavo* whereas a change from ATG to TTG occurred in *Acantochromis polyacanthus* and A*mphiprion akindynos* (*Pomacentrinae*)] and across clades (a change from ATG to TTG was observed in *Das. trimaculatus* and *Das. reticulatus* (*Chrominae*); a change from ATG to CTG was observed in *Abudefduf sexfasciatus* (*Abudefdufinae*) and *Stegastus gascoynei* (*Stegastinae*)). Cases of different codon use in parallel evolution are also found in LWS (A164S; Fig. 1c).

Evidence for divergent evolution was observed, for example, in SWS1 (F49C/L/I; Fig. 1d). Here, divergent evolution occurred between closely related species belonging to the same genus (a change from phenylalanine to cysteine occurred in *Neop. azysron* and *Neop. cyanomus*, but *Neop. bankieri* changed to isoleucine;

Table 3 Summary of PGLS (phylogenetic generalized least squares regression) comparing cone opsin gene expression with foraging preferences. Results of two phylogenetic hypotheses (A and B) with different placements of *Pom. wardi* are presented. All tests were performed with *Amb. curacao*, *Pom. amboinensis*, *Pom. coelestis*, *Pom. moluccensis*, and *Pom. pavo* being categorized as planktivores (first value) and herbivores (second value)

Opsin	Foraging preferences	Phylogeny	Lambda λ (ML)	Degrees of freedom	*F*-statistic	*P*-value
SWS1	Planktivores vs. Herbivores	Phylogeny A	0.0	1, 21	0.5854	0.4527
			0.0	1, 21	1.718	0.2041
		Phylogeny B	0.0	1, 21	0.5889	0.4514
			0.0	1, 21	1.722	0.2036
SWS2B	Planktivores vs. Herbivores	Phylogeny A	0.799	1, 21	0.9234	0.3475
			0.831	1, 21	0.1441	0.7081
		Phylogeny B	0.788	1, 21	0.8122	0.3777
			0.820	1, 21	0.1294	0.7226
RH2B	Planktivores vs. Herbivores	Phylogeny A	0.0	1, 21	0.5984	0.4478
			0.941	1, 21	4.092	0.056
		Phylogeny B	0.0	1, 21	0.5807	0.4545
			0.0	1, 21	3.861	0.06278
RH2A	Planktivores vs. Herbivores	Phylogeny A	0.0	1, 21	3.75	0.06637
			0.0	1, 21	1.302	0.2666
		Phylogeny B	0.0	1, 21	3.653	0.06973
			0.0	1, 21	1.285	0.2698
LWS	Planktivores vs. Herbivores	Phylogeny A	0.123	1, 21	20.35	0.0001913**
			0.0	1, 21	14.81	0.0009335*
		Phylogeny B	0.154	1, 21	20.8	0.0001702**
			0.0	1, 21	14.91	0.0009044*

*<0.0025.
**<0.0005.
(Bonferroni corrected significance levels).

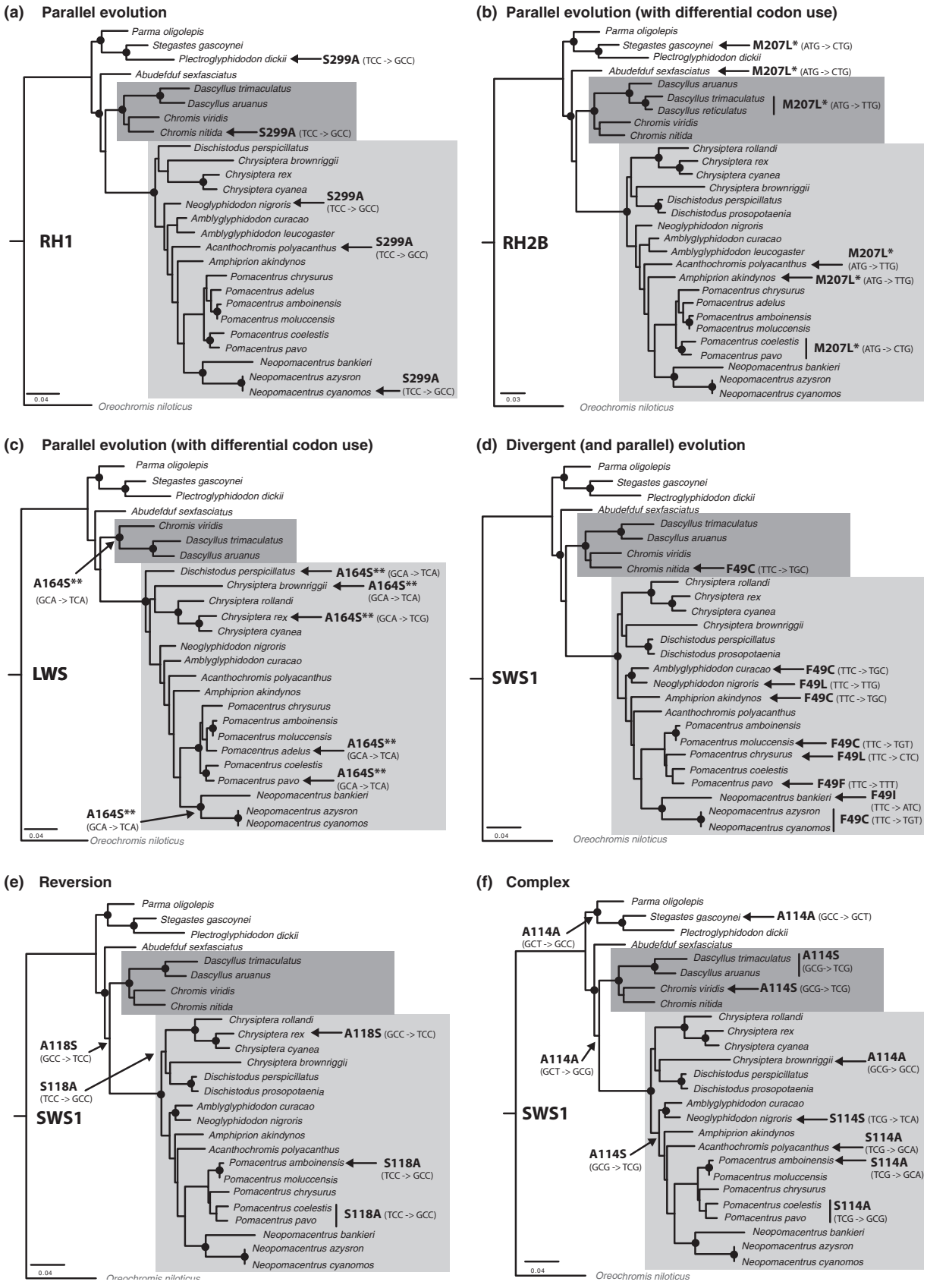
likewise, *Pom. moluccensis* changed to cysteine, but *Pom. chrysurus* to leucine). In addition, divergent evolution is also observed across different genera of *Pomacentrinae* (*Neopomacentrus*, *Pomacentrus*, *Amphiprion* and *Amblyglyphidodon*) and different damselfish clades (*Pomacentrinae* and *Chrominae*). Interestingly, in this case, the same amino acid phenotypes also occurred through differential codon use (the change to cysteine is either encoded by TGC or TGT; the change to leucine is either encoded by TTG or CTC).

An example for reverse evolution was found for site 118 in SWS1 (Fig. 1e). In this instance, a change from alanine to serine was found in two clades: *Chrominae* and *Pomanecentrinae*. Within the genus *Pomacentrus*, a change back to alanine only occurred in *Pom. amboinensis*, *Pom. pavo* and *Pom. coelestis*. A change back from the derived serine to the ancestral alanine also occurred in the genus *Chrysiptera*. Strikingly, a second reversion back to serine occurred in *Chry. rex*.

A complex scenario including parallel and convergent evolution, differential codon use and reversion at different levels of relatedness between species was observed in SWS1 site 114 (Fig. 1f). To begin with, the amino acid substitution S114A occurred repeatedly within the *Pomacentrinae* and differential codon use was observed, for instance, between closely related species of the genus *Pomacentrus* (alanine is encoded by GCG in *Pom. pavo* and *Pom. coelestis*, but by GCA in *Pom. amboinensis*). A reversion was, for instance, observed on the clade level in *Pomacentrinae*. The basal *Pomacentrina*e change from alanine to serine was reversed in *Aca. polyacanthus* and several species of closely related *Pomacentrus*. When comparing across damselfish, parallel evolution was suggested by the change from alanine to serine as seen in the basal *Pomacentrina*e and in several *Chrominae* species; the use of alanine across damselfish, on the other hand, indicates a case of convergent evolution.

Opsin expression variation

The relative opsin gene expression for 23 damselfish species (n = 2–21 individuals per species) is shown in Fig. 2 and Table 1. All species showed a high expression of SWS1 (5.7–48%), RH2B (11–52%) and RH2A (24.4–59.9%). In addition, although LWS was weakly expressed (<2%) in most species, eight species featured an LWS expression of around 2–10%. Five species also showed a low expression of SWS2B (<2%); however, only in two species was SWS2B expression more pronounced (~5%). Relative expression of RH1 was high



(>55%) in most species and only the genus *Amblyglyphidodon* exhibited a weaker expression (<25%).

Spectral reflectance

We found UV reflectance (see Table S3, Supporting information for specific UV components) on various body parts including species-specific patterns on the head or fins of most species [n = 16; Table 1; Fig. 2; see Fig. 3a for examples of UV-reflective colour patches in six different damselfish species (i–vi)]; the only exceptions were *Aca. polyacanthus* and *Neoglyphidodon nigroris* where no UV coloration could be identified. However, while scanning across the whole body and UV videography of these species minimized the risk of missing colour patches in the UV range – invisible to the human observer – it does not exclude the possibility that some UV patterns might have been overlooked.

Lens transmission

We found that lenses of all tested damselfish species (n = 20) were UV-transmitting (Table 1). The transmission curves of an exemplary damselfish (Humbug damsel, *Das. aruanus*) UV-transmitting lens and a UV-blocking lens [data obtained from Siebeck & Marshall (2001)] of a predatory reef fish (Coral trout, *Plectropomus leopardus*) are shown in Fig. 3b.

Relationship of opsin expression with diet

We found that only LWS expression is strongly correlated to diet (Table 3), whereby species that are herbivorous express higher levels of LWS [Fig. 3c; ML values of λ indicated that there was no or only minor (<0.2) association with phylogeny]. These results remained consistent, independent of whether the topology of the phylogeny or feeding categories were varied (see Material and Methods). A strong phylogenetic signal was only observed for the SWS2B opsin (Table 3).

Discussion

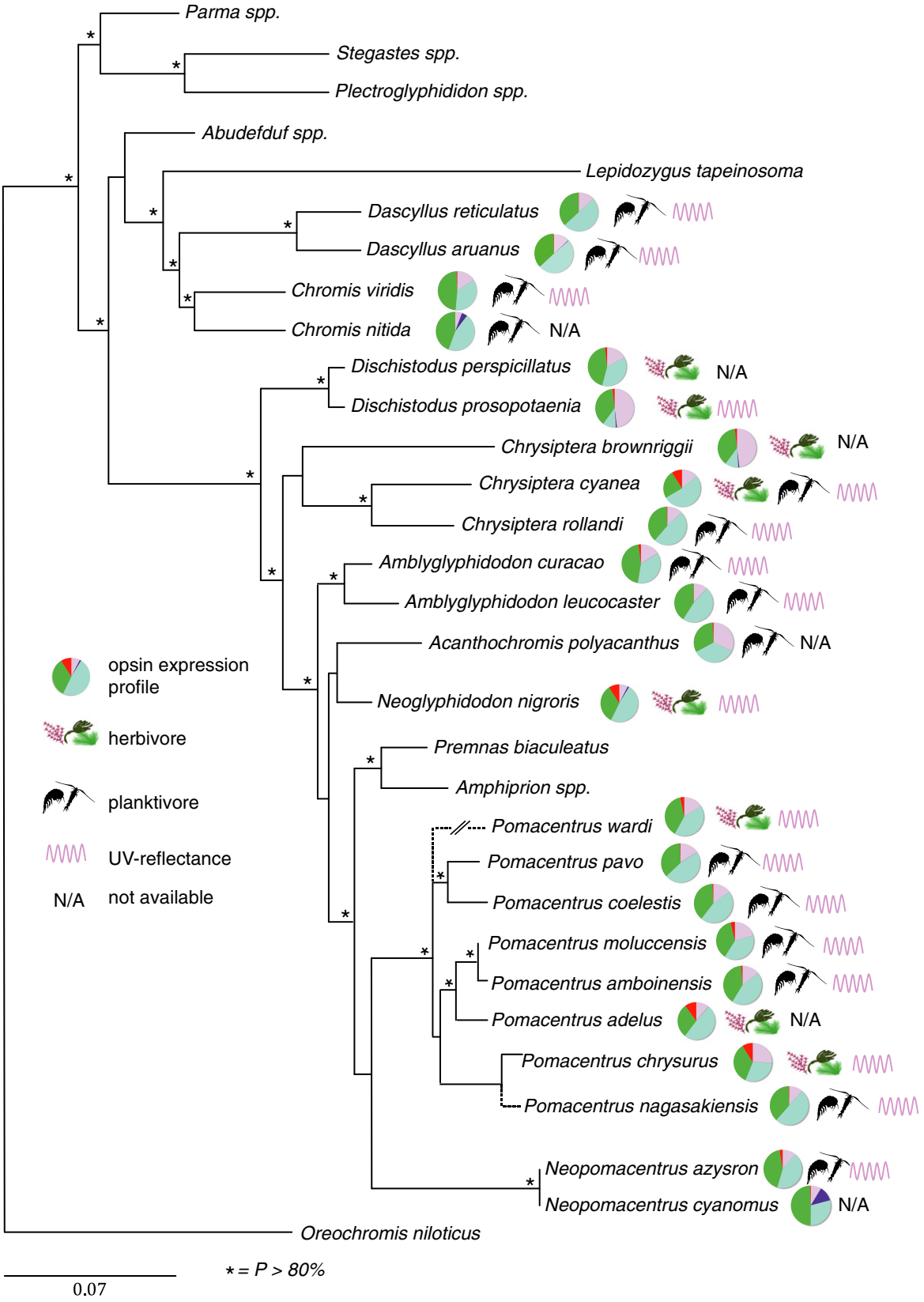
Damselfishes possess five cone opsin genes, which encode visual pigments sensitive to light ranging from the short to the long wavelengths of the visible light spectrum: a UV-sensitive SWS1, a violet-sensitive SWS2B, a blue-sensitive RH2B, a green-sensitive RH2A and a red-sensitive LWS [Fig. 3e; also see Stieb *et al.* (2016)]. Our intention was to compare opsin gene variation at the structural (coding sequence variation) and regulatory (expression variation) level and to then translate the molecular variation – from geno- to phenotype – to the visual diversity of damselfish ecology and behaviour. In the following, we discuss our findings focusing on opsin sequence variation, opsin expression, the importance of UV vision in damselfishes and the use of LWS for herbivores.

Opsin variation in damselfish: diversity in gene structure

Hofmann *et al.* (2012) had previously compared opsin gene variation in 10 species spanning the damselfish phylogeny and revealed that the opsins with the greatest potential functional diversity are those sensitive to either end of the spectral range. Furthermore, using ancestral state reconstructions, they demonstrated that some opsin genes were positively selected for over the course of the damselfish radiation. To test whether the variation found across the damselfish phylogeny remains strong among extant taxa, we compared opsin sequences and performed ancestral state reconstructions on a smaller phylogenetic scale focusing on closely related species of two of the most species-rich clades, *Chrominae* and *Pomacentrinae*.

We found that, independent of whether we compared sequence variation across damselfish species or within species belonging to either the *Pomacentrinae* or *Chrominae*, our results were in line with previous findings (Table 2; Hofmann *et al.* 2012): most functionally variable sites from the transmembrane region were found

Fig. 1 Reconstructions of amino acid changes at focal spectral tuning sites of damselfish opsins [following Yokoyama (2008)]. Changes at spectral tuning sites can be observed in both closely related species and across different clades of the damselfish phylogeny. Dark grey boxes encircle the clade *Chrominae* and light grey boxes the clade *Pomacentrinae*. Parallel evolution was observed in RH1 (a) at site 299, RH2B (b) at site 207 and LWS (c) at site 164. Amino acid changes in RH2B site 207 and LWS site 164 have arisen through differential codon use and have been identified to be under positive selection (*P > 0.95, **P > 0.99). Site 49 in SWS1 (d) shows evidence for divergent (and also parallel) evolution. An example of reversion is illustrated for site 118 in SWS1 (e). Finally, site 114 in SWS1 (f) shows a complex scenario comprising different types of evolution. Amino acids sites are numbered relative to their position in bovine rhodopsin; the corresponding positions in the damselfish opsin genes are shown in Table 2. Reconstructions were performed using the codeml package in PAML (Yang 2007). For an extensive list of sites under selection and ancestral state reconstructions of tuning sites, see Table S2 and Figure S2 (Supporting information). The maximum-likelihood (ML) consensus trees are based on the mitochondrial gene *12s* and the nuclear gene *rag1* (Cooper *et al.* 2009; Hofmann *et al.* 2012; Quenouille *et al.* 2004; Tang *et al.* 2004). Highly supported nodes (>80%) are marked with black spheres.



in the short-wavelength sensitive opsins, SWS1 followed by SWS2B; sites with polarity changes in the retinal binding pocket were most prevalent in SWS1, SWS2B and LWS; variation in known tuning sites for a specific opsin were highest in SWS1. Moreover, we also found low variation in focal tuning sites of the rod specific opsin, RH1. While changes in polarity of key amino acid sites can cause shifts of a few to up to tens of nm (e.g. Yokoyama 2000; Hunt *et al.* 2004; Yokoyama *et al.* 2016), interactions between various amino acid sites can also affect peak light absorbance (Yokoyama 2008). Moreover, amino acid substitutions do not always result in additive shifts of λ_{max} (Asenjo *et al.* 1994; Hauser *et al.* 2014). Therefore, it is important to confirm estimated λ_{max} values using MSP and/or behavioural studies.

We then used PAML to test whether potential functional sites are under positive selection, and only identified five focal tuning sites in RH2B and LWS (Table 2 and Table S2, Supporting information). Most of the other positively selected sites are indeed located in the transmembrane region but not in the retinal binding pocket and are consequently unlikely to tune peak visual pigment absorption. However, these variable sites may impact other aspects of opsin function [such as dimerization, visual pigment regeneration or pigment inactivation (Schott *et al.* 2014)] and require future studies to test their effect.

Further, based on ancestral state reconstructions of opsin-specific spectral tuning sites (Table 2), we clearly demonstrate that diversifying selection not only occurred in the damselfish radiation as evidenced by changes across the damselfish phylogeny [this study and Hofmann *et al.* (2012)], but in addition remained strong among members of closely related extant species belonging to the same genus (Fig. 1). Noteworthy is, for example, the reversion occurring in SWS1 at site 118 (Fig. 1e) that has previously been identified as a spectral tuning site (Yokoyama 2008) and therefore suggesting that changes could alter peak sensitivity. Moreover, we found evidence for parallel (and even convergent) evolution in visual phenotypes highlighting the importance of repeated adaptive radiations across the damselfish phylogeny as it is reported for trophic strategies and morphologies (Cooper & Westneat 2009; Frédérich *et al.* 2013). This underpins the importance of visual ecology in damselfish (Thresher 1979; Katzir 1981; Siebeck *et al.* 2008, 2010) and suggests that vision may be an important driver for damselfish diversification. However, to test whether cladogenesis is structured by visual molecular evolution in the damselfish radiation will require more extensive sampling of various species across the damselfish phylogeny followed by a similar approach of a suite of phylogenetic comparative methods as used by Frédérich *et al.* (2013).

Opsin variation in damselfish: similarity in gene expression

Despite the high diversity in their ecology and coloration, all damselfish tested in this study had a short-shifted cone opsin expression profile with SWS1, RH2B and RH2A always being expressed. Only a few species also expressed LWS.

The overall relative expression of the rod opsin (RH1) across damsel species was high (>55%), and only the two *Amblyglyphidodon* species, *Amb. curacao* and *Amb. leucogaster*, showed lower expression levels. We previously found that relative expression of RH1 shows a decrease in expression level over the course of the day (Stieb *et al.* 2016). As individuals of *Amb. curacao* and *Amb. leucogaster* were sampled throughout the day and only a slight variation in expression was observed (Table 1), this lower expression seems not to be related to sampling time, but may rather be related to phylogeny. However, more species of the genus *Amblyglyphidodon* need to be studied to thoroughly test this hypothesis.

Comparisons of opsin sequence and gene expression between several fish families

By comparing opsin sequence and expression variation found in damsels to that reported for other coral reef

Fig. 2 Damselfish phylogeny showing the variability in cone opsin expression, feeding category and UV-body reflectance for the 23 tested species (according to Table 1). Pie charts illustrate the relative expression of the short-wavelength sensitive (SWS1 in light-violet and SWS2 in violet), the medium wavelength sensitive (RH2B in blue and RH2A in green) and the long-wavelength-sensitive (LWS in red) opsin genes. Note that all tested species express the UV-sensitive SWS1 and most of them also reflect in the UV, highlighting the role of UV communication in damselfish. Also note that the expression of LWS is increased in herbivorous species (for statistics, see Fig. 3C and Table 3). The maximum-likelihood (ML) consensus tree is based on the mitochondrial gene *12s* and the nuclear gene *rag1* (Cooper *et al.* 2009; Hofmann *et al.* 2012; Quenouille *et al.* 2004; Tang *et al.* 2004). Highly supported nodes (>80%) are marked with an asterisk. *Pom. nagasakiensis* was manually added next to *Pom. chrysurus* according to estimates of branch length proportions of shared species between this tree and a tree based on genetic information of *ATPase 6/8* and *Cytochrome b* (Quenouille *et al.* 2004). For *Pom. Wardi*, no such data is available.

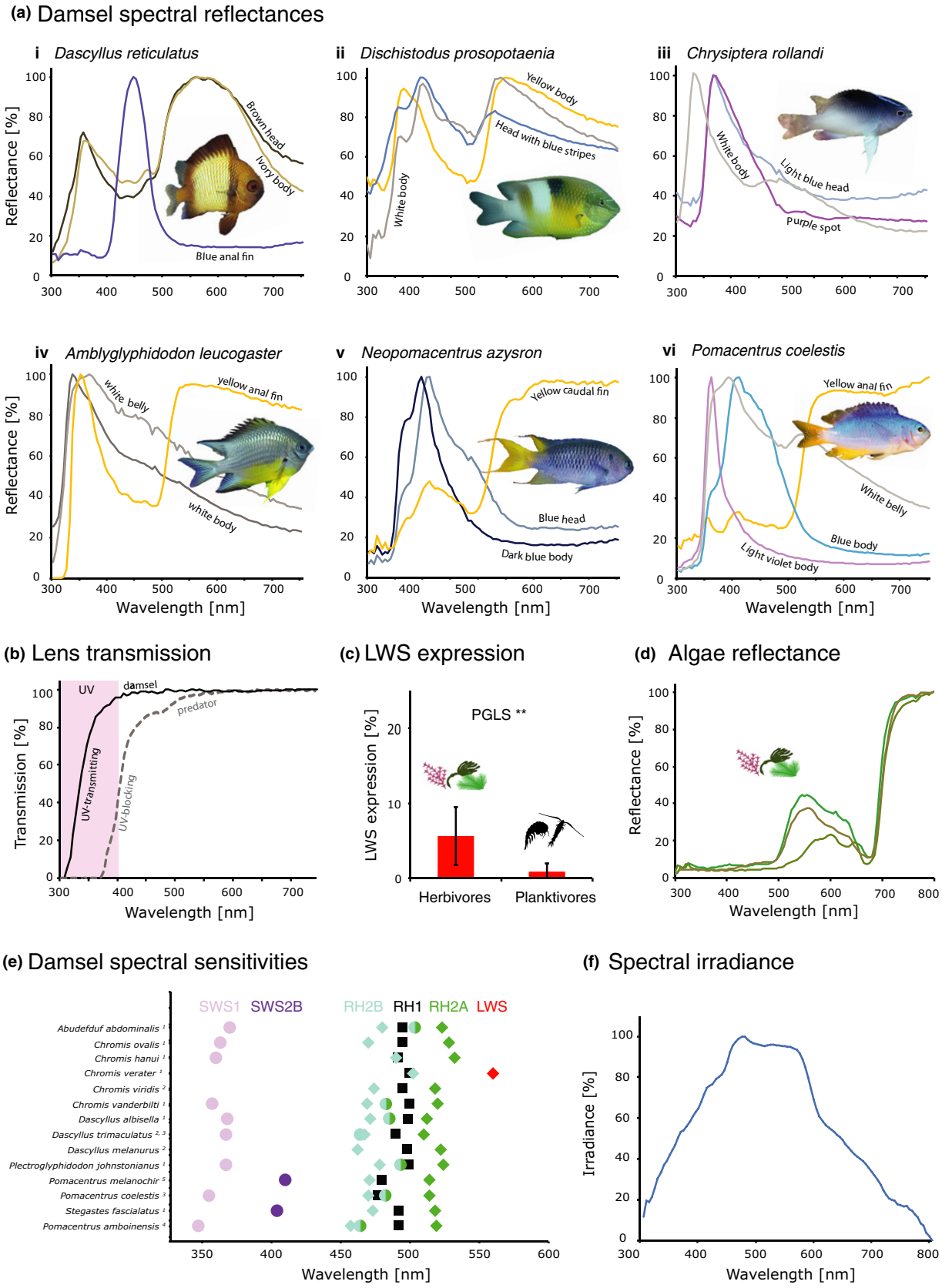


Fig. 3 (a) Normalized spectral reflectance measurements from six representative damselfish species with UV-reflective body parts spanning diverse genera from the clades *Chrominae* (i) and *Pomacentrinae* (ii–iv). The UV reflectance together with the overall expression of the UV-sensitive SWS1 (Table 1, Fig. 2) in species tested in this study highlights the role of UV communication in damselfish. For each species, only a subset of colours is illustrated. Fish pictures are kindly provided by Steve Parrish (i, v) and Gerald Allen (ii–iv). (b) Lens transmission curves showing an example of a damselfish (Humbug damsel, *Dascyllus aruanus*) UV-transmitting lens vs. an UV-blocking lens from a predatory reef fish [Coral trout, *Plectropomus leopardus*; data adapted from Siebeck & Marshall (2001)]. (c) Bar plots showing higher expression of LWS in herbivorous vs. planktivorous damselfishes. The phylogenetic generalized least squares regression (PGLS) confirmed that LWS expression is correlated to feeding strategies in damselfishes (**$P < 0.0005$, see Table 3). (d) Normalized spectral reflectance of different algae species [data modified from Marshall (2000b)] showing a broad peak in the green to red spectrum (~500–650 nm) and a second peak in the far-red (beyond 700 nm). (e) Spectral sensitivities of known damselfish species measured by microspectrophotometry (MSP; [1]Losey *et al.* 2003; [2]Hawryshyn *et al.* 2003; [3]McFarland & Loew 1994; [4]Siebeck *et al.* 2010; [5]Loew & Lythgoe 1978). Single cones are represented by dots, double cones by diamonds and rods by squares. Visual pigments are matched [as per Stieb *et al.* (2016)] to the supposd corresponding rod opsin gene (RH1, black) and cone opsin genes: ultraviolet-sensitive pigment (mauve) = SWS1, violet-sensitive pigment (violet) = SWS2B; blue-sensitive pigment (blue) = RH2B, green-sensitive pigment (green) = RH2A and red-sensitive pigment (red) = LWS. (f) Relative spectral irradiance curve [data modified from Stieb *et al.* (2016); measured at 2 m] for a typical shallow water light environment measured on reefs around Lizard Island where specimen tested in this study were sampled. Note that the full spectrum is covered with a broad peak around 470–570 nm.

fish like labrids (Labridae), or to that of the closely related cichlids (Cichlidae) from the East African Lakes, we can hypothesize how opsin variation might be linked to light environment and ecology. All these cases represent good examples of adaptive radiations [for cichlids and labrids, see Matschiner *et al.* (2010) and references therein; for damsels, see Frédérich *et al.* (2013)] and show a rich diversity in regard to ecology and coloration [for cichlids, see Salzburger (2009); for labrids and damsels, see Marshall (2000a)].

Similar to damselfish, labrids and cichlids were also found to show very high sequence variability in opsins encoding for the shortest and longest wavelength sensitive visual pigments (Spady *et al.* 2005; Hofmann *et al.* 2009; Phillips *et al.* 2016). However, while in labrids, damsels and Lake Malawi cichlids, the highest variability is found at the short end of the spectrum, in Lake Victoria cichlids, the highest variability is seen at the long end of the spectrum. This is most likely due to differences in light habitats between systems. The turbid waters of Lake Victoria are dominated by long-wavelength light, which may have resulted in stronger divergent selection on the LWS opsins (Hofmann *et al.* 2009). In contrast, the clear light habitat of shallow coral reefs, home to labrid and damsel species [at least to those species of this study and that of Phillips *et al.* (2016)], and the clear waters of Lake Malawi have a broad spectral range providing plenty of light at the short end of the visible spectrum.

Interestingly, although damsel and labrid species were both sampled from the northern Great Barrier Reef and share largely the same light environment (Fig. 3f), their opsin expression profiles are quite contrasting (this study; Stieb *et al.* 2016; Phillips *et al.* 2016). In labrids, opsin sensitivities ranging from the UV to the red have been observed; however, most species have visual systems that are lacking UV expression and are shifted to longer wavelength spectra expressing two different copies of the LWS gene (Phillips *et al.* 2016). The potential specialization to longer wavelength vision in fishes (Losey *et al.* 2003; Marshall *et al.* 2003a) has been suggested to be associated with intraspecific communication via fluorescent body parts that reflect beyond 600 nm (Michiels *et al.* 2008). In damsels, on the other hand, visual systems are shifted to the short wavelength with species ubiquitously expressing SWS1, therefore highlighting the importance of UV vision in damsels (Siebeck *et al.* 2010).

UV vision in damsels: a private communication channel based on SWS1 expression, UV-transmitting lenses and UV-reflective coloration

Species belonging to *Pomacentrinae* and *Chrominae*, at least the ones tested in this study, satisfy all prerequisites for UV vision: they inhabit coral reefs providing enough UV light to be detectable by visual systems (Losey *et al.* 1999; Fig. 3f), they possess UV-transmitting lenses (Table 1), and they express SWS1 (Table 1, Fig. 2). The SWS1 opsin gene produces a short-wavelength ultraviolet- to violet-sensitive pigment in a diverse array of vertebrates (Yokoyama 2008), and in damselfish most likely matches the UV-sensitive pigment with a λ_{max} of 347–376 nm (Fig. 3e; MSP data gained from nine damselfish species: Losey *et al.* 2003; Hawryshyn *et al.* 2003; McFarland & Loew 1994; Siebeck *et al.* 2010).

While many animals including humans possess ocular filters that absorb UV light, several freshwater (Thorpe *et al.* 1993) and marine fish species (Siebeck & Marshall 2001) possess ocular media that transmit UV light, thus enabling the perception of UV light. And

indeed, several fish species are sensitive to UV as demonstrated via different approaches including behavioural assays (Siebeck *et al.* 2010), the identification of UV-sensitive photoreceptors using MSP or electrophysiological techniques [reviewed in Losey *et al.* (1999); Siebeck *et al.* (2006)], and more recently by opsin expression studies [for reef fish species see e.g. Phillips *et al.* (2016); Stieb *et al.* (2016)]. UV perception has been proposed to contribute to various functions of the visual system including colour vision, navigation and image analysis of polarized light and/or UV patterns, camouflage and crypsis, mate choice, species and individual recognition, and feeding strategy [reviewed in Losey *et al.* (1999); Siebeck *et al.* (2006)].

Although many reef fishes have UV-reflective body parts or patterns (Marshall *et al.* 2003b), many of them, often larger predators, have UV-blocking ocular filters (Siebeck & Marshall 2001), making it virtually impossible to perceive UV colours of con- or heterospecifics. This, together with the poor UV transmission in water, suggests that small reef fishes like damsels may benefit from a private close-range communication channel that is invisible to 'UV-blind' predators and concealed from other spectators at a distance (Losey 2003; Siebeck *et al.* 2006; Marshall & Cheney 2011). In fact, damselfish have become one of the best studied reef fish species with respect to UV communication. Damselfish are able to distinguish conspecifics from heterospecifics according to the UV component of their colour patterns (Siebeck *et al.* 2010). The Ambon damsel (*Pom. amboinensis*), for example, uses UV-reflective facial patterns, which are the only difference in its appearance from the Lemon damsel (*Pom. moluccensis*), for species and potentially individual based discrimination (Siebeck *et al.* 2010). Interestingly, UV markings in the Ambon damsel only develop when juveniles experience the socio-behavioural conditions of their natural environment, with the presence of a predator being a likely trigger (Gagliano *et al.* 2015). Our results clearly demonstrate the importance of UV vision in damselfish with all of the 23 investigated species being sensitive to UV light on the basis of SWS1 expression and UV-transmitting lenses ($n = 20$ tested; Table 1). Moreover, most damselfish have UV-reflective body parts (Table 1, Table S3, Supporting information; Fig. 3a) further supporting the existence of a 'secret' UV communication channel in these fishes.

In addition to the role of UV for communication, the expression of SWS1 across species and consequently UV sensitivity may also present an adaptation to feeding strategy, albeit we did not find any association between SWS1 expression and diet. Some teleost fishes that are able to perceive UV have been shown to increase their efficiency to forage on UV-absorbing zooplankton or other small organisms that appear as dark objects against a bright UV background (Loew *et al.* 1993; Browman *et al.* 1994; Novales Flamarique 2016). In addition, in African cichlids, SWS1-expression is increased in species that forage on zoo- and phytoplankton but also on algae when compared to species foraging on benthic invertebrates and fish (Hofmann *et al.* 2009; O'Quin *et al.* 2010). Notably, both cichlids and damsels have been shown to be opportunistic feeders that may switch between foraging on plankton and foraging on algae [for cichlids, see McKaye & Marsh (1983), for damsels, see Curtis-Quick *et al.* (2012)]. Thus, the overall relatively high SWS1 expression we found in damselfish, both in algae and planktonic feeders, may enhance their foraging efficiency.

Relationship of opsin expression with diet: LWS expression is associated with herbivory

Visual modelling in a variety of reef fish species has suggested that herbivorous fishes may benefit from long-wavelength-biased visual systems (Marshall *et al.* 2003a). These models predict that a visual pigment pair with sensitivities of 510 and 580 nm, or at least the presence of one long-wavelength-sensitive visual pigment with sensitivity above 500 nm, provides the best visual ability to discriminate average algae from average reef or coral backgrounds [for details of the visual model and similar approaches, see Marshall *et al.* (2003a) and Cortesi *et al.* (2015a)]. The reflectance spectra of the target signals, green or brown algae, are mostly generated by chlorophyll with a broad peak at green to red wavelengths (~500–650 nm) and a second peak in the far-red [beyond 700 nm; Fig. 3d, modified after data from Marshall (2000b)]. Thus, distinguishing algae from a reef background is maximized with a relatively long-wavelength visual pigment pair.

The damselfish RH2A opsin most likely produces a visual pigment with spectral ranges of 510–532 nm λ_{max}, and the damselfish LWS opsin a visual pigment with a spectral sensitivity of around 560 nm λ_{max} [Fig. 3e; for λ_{max} values of damselfish visual pigments quantified from MSP, see Loew & Lythgoe (1978), McFarland & Loew (1994), Hawryshyn *et al.* (2003), Losey *et al.* (2003) and Siebeck *et al.* (2010); for details of matching opsins to visual pigments in damselfish, see Stieb *et al.* (2016)]. Hence, combining damselfish RH2A with LWS would produce an opsin pair, which is similar to the predicted optimal pair for discriminating algae from average reef or coral backgrounds (Marshall *et al.* 2003a). This is supported by our results demonstrating that LWS expression is significantly correlated to diet in damselfish (Table 3), with herbivorous species having an increased expression of LWS (Fig. 3c). Whether or not

this association of LWS expression with herbivory can also be seen in other damselfish clades like, for example, the algae-feeding species of *Stegastinae* (Allen 1991), or whether it can even be transferred to other major herbivorous reef fish families such as the Scaridae (parrotfishes) and Acanthuridae (surgeonfishes; Randall *et al.* 1997), presents an exciting opportunity for future studies.

In the terrestrial environment, a comparable link of long-wavelength sensitivity to feeding ecology can be found. Lythgoe & Partridge (1989) used visual modelling to show that long-wavelength-biased visual pigment pairs are best to detect green leaves against forest litter (with one member being sensitive to 510–520 nm λ_{max} and the other member being sensitive to 570 nm λ_{max}), with the visual sensitivities found in tree shrews, squirrel monkeys and frogs matching those predictions. Further, spectral tuning in the long (around 560 nm λ_{max}) and medium (around 530 nm λ_{max}) wavelength sensitive pigments in primates have been shown to be advantageous to discriminate yellow or orange fruit from a background of green leaves (Osorio & Vorobyev 1996; Regan *et al*. 1998).

Conclusion

In summary, using an integrative approach, our results demonstrate that coral reef fish are particularly interesting models to study visual communication in natural environments, as they have astonishingly vivid colours and inhabit one of the most colourful and visually stimulating environments on earth. Visual communication plays an important role in damsel behaviour, which is reflected – on the molecular level – by the fact that selection on opsin genes was not only acting over the course of the damselfish radiation but still remains strong in extant members of closely related species. We found that opsin sequences differed considerably between species and identified amino acid substitutions that are likely to shift spectral sensitivities in SWS1, SWS2B, RH2B, LWS and RH1. Interestingly, most sequence variation affecting known spectral tuning sites was found in the short- and long-wavelength-sensitive genes (SWS1 and LWS) with the highest variation occurring in the UV-sensitive SWS1 opsin. Ancestral state reconstructions of the tuning sites highlight a complex evolutionary history with cases of parallel, divergent and convergent evolution, differential codon use, and reversion occurring across sites. Further, our data support the hypothesis that small reef fish might benefit from a 'predator-safe' UV-based communication system: not only have most damsels UV-reflective body parts and UV-transmitting lenses, they all also express the UV-sensitive SWS1 opsin gene, which in comparison shows the highest sequence variation at known spectral tuning sites. The expression of LWS, on the other hand, strongly correlated with herbivory, showing that feeding ecology may be driving spectral tuning in coral reef fishes. It will be interesting for future studies to further investigate how vision across the damselfish phylogeny might be related to other aspects of their highly diverse ecologies and behaviours.

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S.M.S., K.L.C., N.J.M. and W.S. designed the study. S.M.S., F.C., L.S. and N.J.M. performed the experiments. S.M.S., F.C. and L.S. analysed the data. S.M.S. and F.C. wrote the initial manuscript. All authors contributed to writing the manuscript and approved the final version.

Data accessibility

New opsin gene sequences have been deposited in the GenBank database, and Accession nos (KX766053–KX766142) are listed in Table S1 (Supporting information). Primer sequences used for qPCR are made available in Table S5 (Supporting information); and qRT-PCR critical cycle numbers (Ct) are provided in Table S7 (Supporting information).

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Genbank accession numbers (*accession numbers are requested and will be updated asap*) of damselfish opsins sequenced in this study (bold) or gained from [1]Hofmann *et al.* (2012) respectively [2](Stieb *et al.* 2016), and *12s* and *rag1* sequences gained from [1]Hofmann *et al.* (2012), [3]Cooper *et al.* (2009), [4]Quenouille *et al.* (2004), Tang *et al.* (2004).

Table S2 PAML analyses of damselfish opsin genes using codeml models M1a vs. M2 and M8 vs. M8a.

Table S3 Color categories for reef fish colors obtained from spectral reflectance measurements.

Table S4 Primer combinations used for PCR and sequencing for each species.

Table S5 Primer names and sequences used for qPCR.

Table S6 Summary of qPCR primer combinations and efficiencies for each species.

Table S7 Summary of critical cycle numbers (C_t) obtained by qRT-PCR reactions for each specimen and each species.

Fig. S1 Alignments for each of the six damselfish opsin genes.

Fig. S2 Reconstruction of amino acid changes at spectral tuning sites (following Yokoyama 2008) of damselfish opsins: (A) SWS1, (B) SWS2B, (C) RH2B, (D) LWS, and (E) RH1; RH2A did not show any changes at spectral tuning sites.