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Depth-dependent plasticity in opsin gene expression varies between damselfish (Pomacentridae) species

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Abstract

Phenotypic plasticity plays an important role in adapting the visual capability of many animal species to changing sensory requirements. Such variability may be driven by developmental change or may result from environmental changes in light habitat, thereby improving performance in different photic environments. In this study, we examined inter- and intraspecific plasticity of visual sensitivities in seven damselfish species, part of the species-rich and colourful fish fauna of the Great Barrier Reef in Australia. Our goal was to test whether the visual systems of damselfish were tuned to the prevailing light environment in different habitats and/or other aspects of their life-style. More specifically, we compared the opsin gene expression levels from individuals living in different photic habitats. We found that all species expressed rod opsin (RH1) used for dim-light vision, and primarily three cone opsins (SWS1, RH2B and RH2A) used for colour vision. While RH1 levels changed exclusively following a diurnal cycle, cone opsin expression varied with depth in four of the seven species. Estimates of visual pigment performance imply that changes in opsin expression adjust visual sensitivities to the dominant photic regime. However, we also discovered that some species show a more stable opsin expression profile. Further, we found indication that seasonal changes, possibly linked to changes in the photic environment, might also trigger opsin expression. These findings suggest that plasticity in opsin gene expression of damselfish is highly species-specific, possibly due to ecological differences in visual tasks or, alternatively, under phylogenetic constraints.

Keywords: gene expression, opsin, phenotypic plasticity, reef fish, vision

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Introduction

Animals rely on vision to navigate, to detect food and to recognize mates and foes (Endler 1992). As a result, visual systems are under strong selection to optimize signal detection dependent on the prevailing lighting conditions that can vary dramatically between species and populations (Lythgoe 1979; Endler 1992, 1993; Bowmaker 1995). Visual systems are ideal to study the processes involved in adaptation because changes at the molecular level are directly linked to visual phenotypes (Yokoyama & Yokoyama 1996; Bowmaker 2011; Hunt *et al.* 2014). More specifically, the spectral sensitivity of an organism is determined by differences in the wavelength of maximum absorbance (λ_{max}) of visual pigments. The visual pigments form the functional unit of the photoreceptor and are composed of an opsin protein that is covalently bound to a light-sensitive vitamin A-derived chromophore (Wald 1968). Differences in λ_{max} are generated by the type of chromophore and the structural variability of the opsin (Yokoyama 2008). Vertebrates possess five classes of opsins, a rod opsin (rhodopsin 1, RH1) used for scotopic vision, and four cone opsins used for photopic vision: the short-wavelength sensitive 1 (SWS1, UV-violet), the short-wavelength sensitive 2 (SWS2, violet-blue), the medium-wavelength

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sensitive (RH2, green; similar to RH1) and the long-wavelength sensitive (LWS, red) opsins (Yokoyama 2000).

Teleost fishes with their great diversity of natural habitats ranging from freshwater to marine, from coral reefs to open waters and from clear mountain streams to the light-deprived deep sea have become powerful models to study visual adaptations to varying ambient light conditions. To begin with, opsin genes have duplicated extensively and have undergone a variety of molecular changes in the evolutionary history of teleosts, creating visual systems with a great diversity in λ_{max}, which is crucial to adapt to the various aquatic light conditions (Hofmann & Carleton 2009; Cortesi *et al.* 2015). Further, mutations in the coding sequence of opsins can cause λ_{max} shifts presumably being tuned to the prevailing light environment (e.g. Yokoyama & Yokoyama 1996; Hunt *et al.* 2001; Carleton *et al.* 2005; Spady *et al.* 2005; Sugawara *et al.* 2005; Terai *et al.* 2006; Seehausen *et al.* 2008; Hofmann *et al.* 2009; Nakamura *et al.* 2013; Tezuka *et al.* 2014). Finally, qualitative and quantitative differences in opsin gene expression play an important role in long- or short-term adaptation to distinct light regimes in teleosts (Carleton & Kocher 2001). African cichlids from Lake Victoria, for example, express an interspecific complementary subset of opsin genes, which is shifted either towards violet or red sensitivities, depending on the prevailing light environment (Carleton *et al.* 2005; Hofmann *et al.* 2009). Differences in opsin expression profiles have also been observed between populations that are exposed to distinct light environments. For example, killifish (*Lucania goodei*) naturally occur in either clear or murky waters and show altered opsin expression profiles with λ_{max} matching the most abundant wavelengths, respectively (Fuller *et al.* 2004). Similarly, natural populations of black bream (*Acanthopagrus butcheri;* Shand *et al.* 2008), killifish (Fuller *et al.* 2004) and Lake Malawi cichlids (Hofmann *et al.* 2010) were found to differ in gene expression when compared to individuals raised under laboratory light conditions. Furthermore, ontogenetic changes in opsin gene expression, possibly linked to migrations between habitats or a change in diet, have been reported from a variety of fish species including the yellowfin tuna (*Thunnus albacares*; Loew *et al.* 2002), European eel (*Anguilla Anguilla*; Archer *et al.* 1995; Cottrill *et al.* 2009), rainbow trout (*Oncorhynchus mykiss*; Veldhoen *et al.* 2006), black bream (Shand *et al.* 2008), Nile tilapia (*Oreochromis niloticus*; Carleton *et al.* 2008), Pacific pink salmon (*Oncorhynchus gorbuscha*; Cheng & Flamarique 2004) and dusky dottyback (*Pseudochromis fuscus*; Cortesi *et al.* 2015). Also, plasticity in opsin expression in matured fish was observed in killifish that flexibly alter the expression levels of the same opsins within a few days when moved between light environments (Fuller & Claricoates 2011) and even within a few hours when exposed to diurnal changes in habitat lighting (Johnson *et al.* 2013).

Damselfishes (Pomacentridae), with currently 388 described species, occur on temperate to tropical coral reefs around the world (Allen 1991) and are a promising system to study the molecular basis for visual adaptations in fishes (Hofmann *et al.* 2012). Damselfishes are known for their diversity in coloration [ranging from drab hues of brown, grey and black to brilliant combinations of orange, yellow and neon blue (Randall *et al.* 1997)], but they also differ in diet (grazing herbivory, planktivory and corallivory) and lifestyle [solitary and school dwelling (Allen 1991)]. Colour patterns in damselfish are often highly contrasting and include UV and/or far-red components (Marshall 2000; Marshall *et al.* 2003a; Siebeck *et al.* 2010). Although the relationship between colour and vision remains elusive, there is behavioural evidence for colour discrimination in damselfishes (Siebeck *et al.* 2008). Moreover, vision has also been shown to play an important role in intra- and interspecific recognition (Katzir 1981; Siebeck *et al.* 2010). It is further known that damselfish possess five cone opsin (SWS1, SWS2B, RH2A, RH2B, LWS) and one rod opsin gene (RH1) (Hofmann *et al.* 2012), and microspectrophotometry (MSP) revealed that four to five (3–4 cone and one rod) of these opsin pigments are present within their retina at any one time (reviewed in Marshall *et al.* 2006; Siebeck *et al.* 2010; Marshall *et al.* 2015). During maturation, their light regime is changing drastically as damselfish, like the majority of coral reef fish, have an oceanic and pelagic larval phase and colonize the reef only at a later stage (Wellington & Victor 1989; Leis 1991; Victor 1991). Moreover, different species inhabit different light environments ranging from shallow and well-illuminated mid-shelf and outer crest reefs to more light-deprived muddy inshore or deeper reefs (Allen 1991; Randall *et al.* 1997). Finally, even individuals of the same species might, after settlement at the reef, populate different environments with respect to the light regime. Consequently, we would expect that their visual systems become rapidly adapted to the local light environment to maximize visual output.

To test this hypothesis and to, more generally, examine phenotypic plasticity in opsin gene expression, we investigated, in detail, the opsin gene expression in seven damselfish species native to the Great Barrier Reef (GBR), Australia, each with large population sizes and a wide intraspecific depth distribution: *Chrysiptera rollandi*, *Dascyllus aruanus*, *D. reticulatus*, *Pomacentrus amboinensis*, *P. coelestis*, *P. nagasakiensis* and *P. moluccensis*. All selected species have in common that they mainly feed on planktonic prey, are either found on

coral or rock rubble, or inhabit branching coral heads and are territorial – or at least become resident – once settled (Fricke 1977; Allen 1991; Sale 1991; Fishelson 1998, www.fishbase.org; own observations). Because of the similar ecology of the study species and their occurrence along a depth gradient, we were able to test whether the light regime induces plastic changes in their visual system. We specifically aimed to answer (i) whether differences in the light environment alter opsin gene expression (either through qualitative and/or quantitative changes in opsin gene expression) within damselfish species; and (ii) to what extent the plasticity in opsin gene expression varies between damselfish species. To this end, we used *quantitative real-time polymerase chain reaction (qRT-PCR)* experiments to compare opsin expression in seven damselfish species within which we sampled individuals at two different depth zones that varied in regard to their light regime and estimated how the quantum catch of visual pigments changes with depth. We furthermore tested how opsin expression changes on a daily basis (morning vs. afternoon).

Materials and methods

Sample collection

Adult fish were sampled in water depths between 1 and 4 m (referred to as shallow) or between 10 and 15 m (referred to as deep) from coral reefs around Lizard Island (14°40′S, 145°27′E), Northern GBR, between 2012 and 2014. We sampled between six and 11 specimens per species and depth (see Table S1, Supporting information); if possible to determine, sex was recorded. Fish were caught using hand and barrier nets and kept in aquaria exposed to sunlight and a natural light cycle at the Lizard Island Research Station for no longer than 24 h before being anaesthetized using an overdose of clove oil (10% clove oil; 40% ethanol; 50% seawater) and killed by decapitation. Retinas were immediately dissected from the eyecup and stored in RNA-later (Ambion) for subsequent qRT-PCR experiments. Additionally, fin clips were preserved in 95% ethanol for subsequent genetic analysis. Tissues were sampled throughout the day between 8 am and 5 pm, and the date and time of dissection was noted (for an overview on sampling regime see Table S1, Supporting information). Although overall sampling spanned three field seasons and two years, specimens belonging to the same species (with the exception of *P. nagasakiensis*) were sampled in the same year and season. All experimental procedures were approved by The University of Queensland Animal Ethics Committee (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

Genomic DNA was extracted from fin tissue using a standard salt precipitation protocol (Laird *et al.* 1991). 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 subsequently reverse transcribed using the High Capacity RNA-to-cDNA kit (Applied Biosystems). RNA and DNA concentrations and purity were measured with a NanoDrop1000 Spectrophotometer (ThermoScientific).

Opsin sequencing

Publicly available opsin gene sequences for *P. amboinensis* were obtained from GenBank (accession numbers HQ286556, HQ286506, HQ286516, HQ286526, HQ286536, HQ286546). For the remaining six species, we Sanger-sequenced all five cone (SWS1, SWS2B, RH2B, RH2A and LWS) and the rod (RH1) opsin gene using damsel-specific primers reported in Hofmann *et al.* (2012) (see Table S2, Supporting information for details). Following the protocol of Hofmann *et al.* (2012), two overlapping fragments were PCR amplified for each opsin gene using cDNA as template, or, if not successful, genomic DNA. Red Taq DNA polymerase (Sigma) was used for PCR amplification, and products purified with ExoSapIT (USB, Cleveland, OH) were sequenced using the BIG DYE v.3.1 chemistry (Applied Biosystems) following the manufacturer's protocol on an ABI 3130*xl* genetic analyzer (Applied Biosystem). Sequences were aligned and edited using CODON CODE ALIGNER 3.5.6 (CodonCode Corporation, Dedham, MA); the sequences were also used as a template to design primers for the qRT-PCR experiments.

Phylogenetic reconstruction

In order to confirm the assignment of the newly obtained opsin sequences to the correct opsin gene, we compared their amino acid sequence with the opsin genes of zebrafish (*Danio rerio)*, Japanese rice fish (*Oryzias latipes)*, Bluefin killifish (*Lucania goodei*), a Lake Malawi cichlid (*Metriaclima zebra*) and Nile tilapia (*Oreochromis niloticus*) (GenBank accession numbers of these reference sequences are provided in Fig. 1). We then

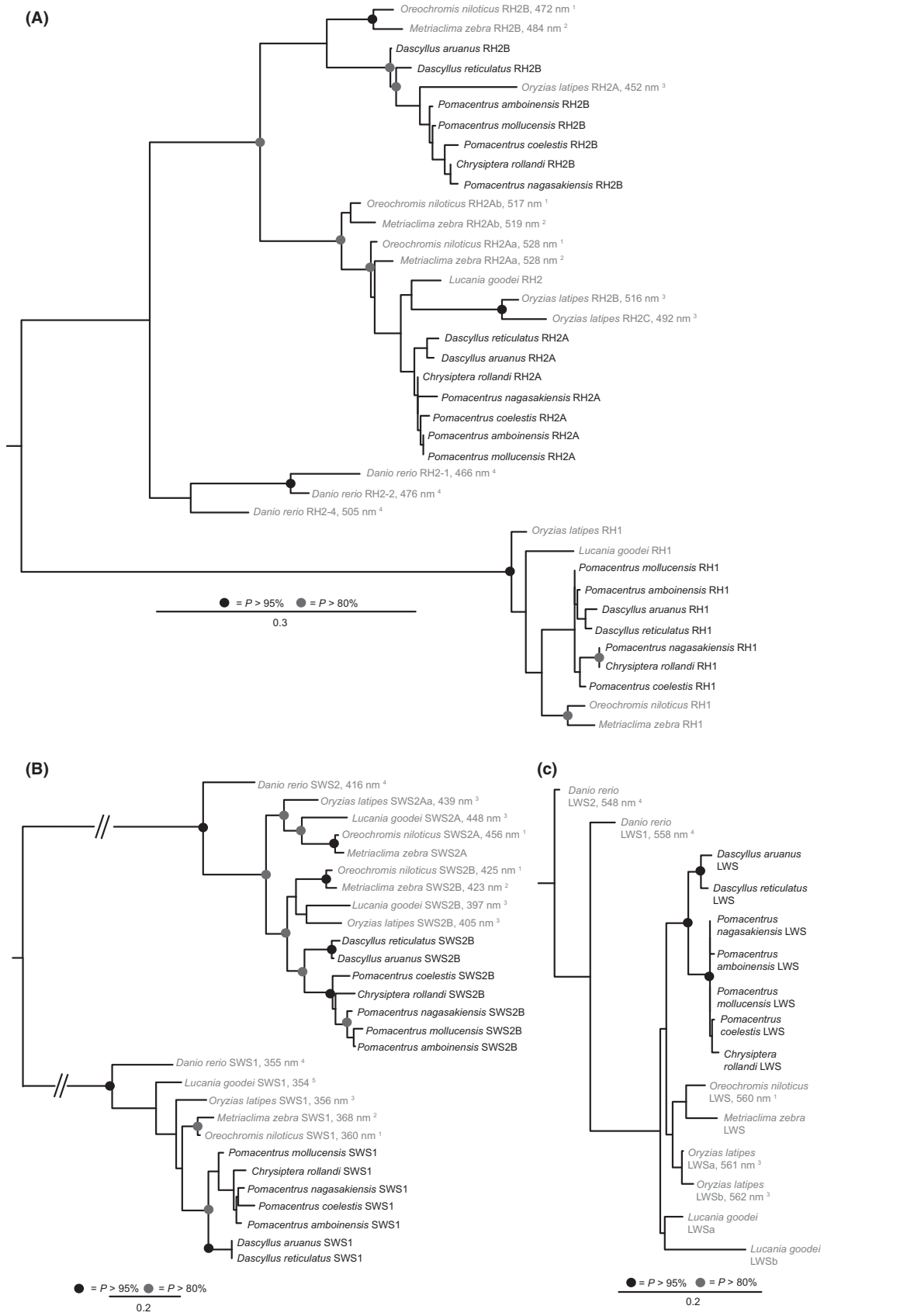

Fig. 1 Maximum likelihood (ML) trees based on the amino acid sequences of opsin genes. Shown are the phylogenetic relationships between RH1, RH2A and RH2B (A); SWS1 and SWS2B (B); and LWS (C) for the tested damselfish species (black font) compared to other fish species (grey font). Highly supported nodes are marked with black (>95%) or grey spheres (>80%), respectively. Known peaks of maximal absorbance (λ_{max}) from in vitro expression and reconstitution experiments are depicted: [1] Spady *et al.* (2006), [2] Parry *et al.* (2005), [3] Matsumoto *et al.* (2006), [4] Chinen *et al.* (2003), [5] Yokoyama *et al.* (2007). The following sequences were included: *C. rollandi* (SWS1, KU745452; SWS2B, KU745451; RH2B, KU745453; RH2A, KU745454; LWS, KU745456; RH1, KU745455), *D. rerio* (SWS1, AB087810; SWS2, BC062277; LWS1, AB087803; LWS2, AB087804; Rh2-1, AB087805; Rh2-2, AB087806; Rh2-4, AB087808), *D. aruanus* (SWS1, KU745446; SWS2B, KU745445; RH2B, KU745447; RH2A, KU745448; LWS, KU745450; RH1, KU745449), *D. reticulatus* (SWS1, KU745440; SWS2B, KU745439; RH2B, KU745441; RH2A, KU745442; LWS, KU745444; RH1, KU745443), *L. goodei* (SWS1, AY296735; SWS2A, AAP57197.2; SWS2B, AAP57196.1; RH2, AY296739; LWSa, AY296740; LWSb, AY296741; RH1, AY296738), *M. zebra* (SWS1, AF191219; SWS2A, AF247114; SWS2B, AF247118; RH2B, DQ088652; Rh2Aa, DQ088651; Rh2Ab, DQ088650; LWS, AF247126; RH1, AY775114), *O. niloticus* (SWS1, AF191221; SWS2A, AF247116; SWS2B, AF247120; Rh2Aa, DQ235683; Rh2Ab, DQ235682; Rh2B, DQ235681; LWS, AF247128; RH1, AY775108), *O. latipes* (SWS1, BAE78652; SWS2Aa, BAE78650; SWS2B, BAE78651; RH2A, AB223053; RH2B, AB223054; RH2C, AB223055; LWSa, BAE78645; LWSb, BAE78646; RH1, NP_001098165), *P. amboinensis* (SWS1, HQ286506; SWS2B, HQ286516; RH2B, HQ286526; RH2A, HQ286536; LWS, HQ286546; Rh1, HQ286556), *P. coelestis* (SWS1, KU745434; SWS2B, KU745433; RH2B, KU745435; RH2A, KU745436; LWS, KU745438; RH1, KU745437), *P. moluccensis* (SWS1, KU745428; SWS2B, KU745427; RH2B, KU745429; RH2A, KU745430; LWS, KU745432; RH1, KU745431) and *P. nagasakiensis* (SWS1, KU745422; SWS2B, KU745421; RH2B, KU745423; RH2A, KU745424; LWS, KU745426; RH1, KU745425).

calculated maximum likelihood phylogenetic hypotheses for each gene based on the amino acid sequences using PhyML (after Guindon & Gascuel 2003) on the web-based platform MOBYLE 1.5 (Neron *et al.* 2009), and applying a bootstrap analysis with 100 pseudo-replicates.

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

To examine intra- (between two depth zones) and interspecific differences in opsin gene expression, we subjected the RNA samples to *qRT-PCR* experiments, using a STEPONEPLUS Real-Time PCR System (Life Technologies). Each reaction contained ~1500 ng of total RNA, mixed in a 1/9 concentration with the SYBR Green master (Rox) dye (Roche) and a final primer concentration of 200 nM in a total volume of 20 μL. We constructed unique primers for each opsin gene and species, whereby either the forward or the reverse primer spanned 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 S3 and S4, Supporting information). To ensure that the correct products were amplified, we subjected one amplicon per gene and species to Sanger-sequencing, following the procedure described above. Following the strategy described in Cortesi *et al.* (2015) (for comparable methods see also normalization of reaction efficiencies in LT cichlids in O'Quin *et al.* (2010)), primer efficiencies (Table S4, Supporting information) were initially validated for each species using a fivefold dilution series (i.e. 1×, 0.2×, 0.04×, 0.008×, 0.0016×) of each species-specific opsin pool with a starting concentration of 0.1–0.5 nmol/μL so that cycle threshold (C_t) values of the dilution series encompassed the C_t values of the actual samples. The opsin pool contained equal ratios of fragments of each opsin gene that were amplified from cDNA from each tested species using the sequencing primers (see Table S2, Supporting information) to obtain a pool being specific for each species; products were cut out from the electrophoresis gel and purified using the QIAquick PCR Purification Kit (QiaGen). The molarity of opsin gene fragments was measured using an Agilent 2100 BioAnalyzer NanoChip (Agilent Technologies). qRT-PCR efficiency (E) was calculated for each reaction from the slope of the standard curve using the equation $E = 10^{(-1/\text{slope})}$ as implemented in the STEPONEPLUS software (LifeTechnologies), with an efficiency threshold of 2 – being equal to 100% ($E\%=[10^{(-1/\text{slope})}-1]\text{-}100$) – as indicator of a robust assay.

As is the current standard for comparative gene expression analyses in opsin genes (Carleton & Kocher 2001; Fuller *et al.* 2005; Spady *et al.* 2006; Hofmann *et al.* 2010; Cortesi *et al.* 2015), we did not include a housekeeping gene for the purpose of normalization for two main reasons: first, we were interested in the expression levels of opsin genes relative to each other; second, and more importantly, the usage of normalization genes (e.g. housekeeping genes) can be misleading, especially when comparing gene expression levels between different individuals (Bustin 2000, 2002). Instead, we measured the expression of each opsin gene as percentage of total opsin gene expression using an opsin gene pool as reference to normalize between qPCR plates (see Cortesi *et al.* 2015). Deep- and shallow-water individuals of different species were randomly assigned to each qPCR plate, and all experiments were carried out with three technical replicates. We used the following cycling conditions in our qRT-PCR experiments: 95 °C 10 min, 40 cycles 95 °C 15 s, and 61 °C 60 s. Each qRT-PCR amplification was validated by means of a melt curve analysis.

Following Carleton & Kocher (2001), relative abundance of each cone opsin gene was calculated based on the gene's critical threshold cycle (C_t) number being set above a background level (0.5). Relative gene expression was determined as a fraction of the total of cone opsin genes expressed for an individual according to:

$$T_i/T_{\text{all}} = (1+E_i)^{-C_{ti}} / \sum (1+E_i)^{-C_{ti}}$$

where T_i/T_{all} is the relative gene expression ratio for a given gene normalized by the total cone opsin genes expressed, E_i is the qRT-PCR efficiency for each gene, and C_{ti} is the critical cycle number for each gene. For RH1, the relative expression was calculated separately as a fraction of all opsin genes expressed.

Statistical tests

To test whether intraspecific opsin gene expression varies in relation to depth, we used the beta regression method based on the R package BETAREG (Cribari-Neto & Zeileis 2010), which allows handling of non-transformed data to model percentages and proportions. The beta distribution has a highly flexible shape and is, hence, suitable to fit the dependent variable (in our case the relative expression of each opsin gene) in the unit interval (0,1) with a mean related to a set of continuous and/or categorical regressors (in our case depth and time of day fish were dissected, respectively). Whereas time was measured continuously, we had to use a categorical factor for depth in our study due to sampling design. We caught fish either during dives in less than 4 m or during dives in more than 10 m. However, it was not possible to determine the exact depth at which an individual fish was caught within the two depth zones (shallow vs. deep) due to sampling logistics and the sizes of the nets used.

In our statistical analyses, we tested for an influence of time as on some days, deeper dives were conducted in the morning, whereas the dives to the shallow zone were performed in the afternoon (or *vice versa*), and fish were dissected, when possible, directly after the dives, but always within 24 h. To this end, we first determined the dependence of opsin gene expression on depth zone and on time independently. If both regressors had a significant effect on expression, they were tested together; in this case, only the results of the latter model are presented (see Result section and Table S5, Supporting information). In addition, because *P. nagasakiensis* individuals were collected during different seasons (summer and winter), we also tested for the influence of season on opsin expression in this species. If at least two regressors had a significant effect on expression, they were tested together and only the results of the latter model are presented (see Result section and Table S5, Supporting information).

Considering that damselfish may be able to adapt their visual system to changing light regimes makes it possible that the sampling regime in this study, namely that fish had been kept up to 24 h in aquaria until dissection, influences opsin expression. To account for this, we tested if the period kept in the tank had an influence on the expression pattern. As sex could only be determined reliably in very few individuals, we could not test for any potential sex bias on opsin gene expression. However, a random sampling regime makes it unlikely that sex had a major influence on opsin expression and thus would change our conclusions.

Statistical analyses were performed in R (R Core Team 2011) using the interface RSTUDIO (Version 0.98.1062).

Quantum catches

To test how the relative performance of each cone visual pigment changes with depth, we estimated its quantum catch (Q) using the equation:

$$Q = \int I(\lambda)R(\lambda)d(\lambda)$$

where I(λ) is the normalized irradiance spectrum, and R(λ) is the photoreceptor absorption calculated using the equations of Govardovskii *et al.* (2000). Because we were interested in the relative performance of the expressed cone visual pigments, we normalized the quantum catch of each single visual pigment by the sum of the quantum catches for single, and, in a second step, for double cone visual pigments. Irradiance was measured in February 2015 in the natural habitat of damselfishes at 2 and 15 m depths around midday and under blue sky with an Ocean Optics S2000 spectrometer (Dunedin, FL, USA). We modelled quantum catches using downwelling, upwelling and sidewelling irradiance, measured by pointing a 400 μm optical fibre (length 65 cm) with a cosine corrector attached (allowing light collection over a 180^0 sphere) from 1 m above the reef at the substrate, towards the surface, or away from the reef, respectively (see Marshall *et al.* (2003b)).

We used mean λ_{max} values from 14 different damselfish species (*Abudefduf abdominalis*, *Chromis ovalis*, *C. hanui*, *C. verater*, *C. viridis*, *C. vanderbilti*, *Dascyllus albisella*, *D. trimaculatus*, *D. melanurus*, *Plectroglyphidodon johnstonianus*, *Pomacentrus amboinensis*, *P. melanochir*, *P. coelestis* and *Stegastes fasciolatus*) with known sensitivities (Table 1; for a summary see Marshall *et al.* 2006, 2015) to generate photoreceptor absorption curves. In addition, we performed quantum catch estimates with

Table 1 Damselfish visual pigment sensitivities (λ_{max}) and relative cone opsin gene expression

		Single cone λ_{max} (nm)			Double cone λ_{max} (nm)		
Visual pigments		UVS	SWS	'blue' MWS	'blue' MWS	'green' MWS	LWS
Abudefduf abdominalis[1]		347	–	464	457	519	–
Chromis ovalis[1]		–	404	–	473	518	–
Chromis hanui[1]		355	–	482	470	514	–
Chromis verater[1]		–	410	–	471	514	–
Chromis viridis[2]		367	–	493	478	524	–
Chromis vanderbilti[1]		–	–	–	462	522	–
Dascyllus albisella[1]		376, 359	–	464	467	510	–
Dascyllus trimaculatus[2,3]		368, 360	–	485, 490	471, 490	512, 516	–
Dascyllus melanurus[2]		357	–	482	469	520	–
Pomacentrus amboinensis[4]		370	–	504	480	523	–
Pomacentrus melanochir[5]		–	–	502	502	–	560
Pomacentrus coelestis[3]		360	–	490	490	532	–
Stegastes fascialatus[1]		363	–	–	470	528	–
Mean damsel λ max		**362**	**407**	**486**	**475**	**519**	**560**
Relative opsin gene expression (this study)		**SWS1 (%)**	**SWS2B (%)**	**RH2B (%)**		**RH2A (%)**	**LWS (%)**
Chrysiptera rollandi	Shallow	14.9 ± 8.0	0	47.3 ± 4.5		37.3 ± 5.1	0.5 ± 0.2
	Deep	10.8 ± 2.8	0	49.9 ± 1.7		38.7 ± 3.8	0.6 ± 0.5
Dascyllus aruanus	Shallow	14.1 ± 5.1	0.1 ± 0.1	50.1 ± 5.7		35.3 ± 7.0	0.4 ± 0.6
	Deep	12.1 ± 4.1	0.7 ± 1.2	49.3 ± 5.6		37.5 ± 4.9	0.3 ± 0.6
Dascyllus reticulatus	Shallow	12.4 ± 2.3	0	49.8 ± 3.6		37.7 ± 2.2	0.2 ± 0.1
	Deep	14.0 ± 1.0	0	49.9 ± 6.3		35.8 ± 5.9	0.2 ± 0.3
Pomacentrus amboinensis	Shallow	15.7 ± 6.1	0	40.1 ± 10.6		42.4 ± 5.9	1.6 ± 1.0
	Deep	12.5 ± 5.1	0	50.3 ± 17.2		36.5 ± 7.0	0.6 ± 0.6
Pomacentrus coelestis	Shallow	20.8 ± 5.0	0	40.8 ± 5.2		37.6 ± 3.1	0.8 ± 0.5
	Deep	11.3 ± 1.9	0	48.4 ± 2.8		39.5 ± 2.6	0.8 ± 0.5
Pomacentrus moluccensis	Shallow	23.1 ± 7.0	0	31.5 ± 4.7		41.1 ± 5.0	4.3 ± 3.0
	Deep	17.3 ± 5.4	0	47.5 ± 4.8		32.3 ± 5.9	2.8 ± 1.9
Pomacentrus nagasakiensis	Shallow	8.4 ± 2.5	0	48.0 ± 1.9		43.1 ± 3.2	0.4 ± 0.3
	Deep	17.1 ± 3.9	0	51.0 ± 4.0		29.3 ± 4.4	0.8 ± 0.6
	Summer	7.7 ± 1.4	0	48.1 ± 1.9		43.8 ± 2.5	0.3 ± 0.2
	Winter	16.8 ± 3.7	0	50.6 ± 3.9		30.2 ± 4.9	0.8 ± 0.6

We suggest the following matching of visual pigments and opsin genes (in bold): ultraviolet-sensitive (UVS) pigment = SWS1, short-wavelength-sensitive (SWS) pigment = SWS2B, two medium-wavelength-sensitive (MWS) pigments with 'blue' MWS = RH2B (found in single and double cones; in single cones a possible coexpression of RH2B and RH2A), and 'green' MWS = RH2A, long-wave-length-sensitive (LWS) pigment = LWS. λ_{max} is obtained from previous studies with mean values across species shown in bold ([1]Losey *et al.* 2003; [2]Hawryshyn *et al.* 2003; [3]McFarland & Loew 1994; [4]Siebeck *et al.* 2010; [5]Loew & Lythgoe 1978).

known visual pigment absorbance for two of our test species independently, *P. amboinensis* (Siebeck *et al.* 2010) and *P. coelestis* (McFarland & Loew 1994).

Results

Opsin sequences and phylogeny

We sequenced SWS1, SWS2B, RH2B, RH2A, LWS and RH1 from *C. rollandi*, *D. aruanus*, *D. reticulatus*, *P. coelestis*, *P. nagasakiensis* and *P. moluccensis* (for GenBank accession numbers see Data accessibility, and Table S7, Supporting information) and obtained the coding sequence of the complete transmembrane regions for each species for RH1, RH2A and RH2B. For the remaining opsin genes, we obtained the complete transmembrane regions for some species, and only partial transmembrane regions for LWS in *P. nagasakiensis* and *D. reticulatus*, for SWS1 in *D. reticulatus*, and for SWS2B in *P. coelestis* and *D. reticulatus*.

The protein-based maximum likelihood trees confirmed the identity of the damselfish opsins, as they grouped together with well-described opsin classes

from other fish species (Fig. 1). This agrees with previous work, which reported six visual opsins in damselfishes: RH1, RH2A, RH2B, SWS1, SWS2B and LWS (Hofmann *et al.* 2012).

Opsin gene expression

None of the damselfish species examined here expressed SWS2B, whereas all species (and at both depth zones) expressed the UV-sensitive SWS1; both medium-wavelength-sensitive opsins, RH2B and RH2A; and the scotopic RH1. In addition, we found low levels of LWS expression in *P. moluccensis* and *P. amboinensis* (at both depth zones) (Fig. 2). Table 1 summarizes the relative expression percentages for each opsin gene in each species and in both depth zones.

When comparing individuals from the same species caught at different depths, only *P. amboinensis*, *P. moluccensis*, *P. coelestis* and *P. nagasakiensis* showed variation in cone opsin expression profiles (for relative opsin expression see Table 1 and Fig. 2; for beta regression statistics see Table S5, Supporting information). Here, the expression levels of cone opsin genes correlated (mostly) with depth, and not time of day. In *P. nagasakiensis*, cone opsin expression also correlated with season. In contrast, for all species (except *D. aruanus*), RH1 levels appeared to correlate with dissecting time of day (Fig. 3), but not with depth.

In the following, we report significant depth-dependent changes in cone opsin expression. SWS1 expression was lower in the deeper zone in *P. coelestis*. The expression of RH2B was higher in deep-water living *P. moluccensis* and *P. coelestis* compared to their shallow-water conspecifics. Relative RH2A expression levels were lower in the deep zone in *P. moluccensis*, *P. amboinensis* and *P. nagasakiensis*. Lastly, LWS expression was lower in deep-water living *P. amboinensis*. A correlation with dissecting time in cone opsin genes was found in one species only, namely in *P. nagasakiensis* for RH2B. *D. aruanus*, *D. reticulatus* and *C. rollandi* showed no changes in cone opsin expression with respect to depth or time. A significant correlation of cone opsin expression to season was observed in *P. nagasakiensis*. Here, SWS1 expression was higher in winter compared to summer (Fig. 2G). Further, relative expression levels of RH1 were affected by the time of day of dissecting in six of the seven species examined: *C. rollandi*, *D. reticulatus*, *P. amboinensis*, *P. coelestis*, *P. moluccensis* and *P. nagasakiensis*. In all these cases, RH1 showed higher expression levels in the morning compared to the afternoon (Fig. 3). *D. aruanus* was the only species to feature a significant overexpression of RH1 in the samples from the deeper zone (data not shown). This result should be taken cautiously, though, as we were only able to obtain four qPCR data points from the shallow zone for RH1. Finally, the period fish were kept in tanks (up to 24 h) had no influence on opsin gene expression (Table S5, Supporting information), with the exception of RH1 in *P. amboinensis*.

Quantum catches

The relative irradiance spectra at 2 and 15 m depths showed the typical attenuation across the light spectrum with depth, with the longer wavelengths of the spectrum being mostly affected, followed by differences in the UV part of the spectrum (Fig. 4A) (McFarland & Munz 1975; Jerlov 1976; Lythgoe 1979). Consequently, the estimated relative quantum catches of cone visual pigments differed with respect to water depth. In the following, we present detailed estimates made for cone visual pigments using mean λ_{max} values for 14 different damselfish species (Fig. 4B) with known sensitivities (Table 1; for a summary see Marshall *et al.* 2006, 2015). Based on the λ_{max} values of these species, three different types of single cone visual pigments have been categorized in damselfish: a UV-sensitive (UVS), a short-wavelength sensitive (SWS) and a medium-wavelength sensitive ('blue' MWS) single cone. Double cone members with sensitivities in the medium wavelengths ('blue' MWS and 'green' MWS) and long wavelengths (LWS) have been described. SWS single cones are only found in two damselfish species (*Chromis ovalis* and *C. verater*) and LWS double cones only in one (*Pomacentrus melanochir*). This is in agreement with our expression profiles showing that the tested species primarily expressed cone opsins matching the UVS, 'blue' and 'green' MWS visual pigments (for more detail on matching opsin genes to visual pigments see Table 1 and the discussion). Therefore, we only estimated quantum catch changes for those visual pigments and have performed them for single and double cones separately. For single cones, the quantum catch of the UVS visual pigment is much higher in the shallow zone (from shallow to deep, the quantum catch is declining 49.4% for downwelling light, 58.9% for sidewelling light and 51.9% for upwelling light), whereas the quantum catch for the 'blue' MWS visual pigment is higher in the deeper zone (from shallow to deep, the quantum catch is rising 14.3% for downwelling light; 15.8% for sidewelling light, 15.6% for upwelling light) (Table S6, Supporting information, Fig. 4C). For double cones, the quantum catch of the 'blue' MWS visual pigment is slightly higher in the deeper zone (from shallow to deep, the quantum catch is rising 4.8% for downwelling light; 4.2% for sidewelling light, 5.4% for upwelling light), but the quantum catch for the 'green' MWS visual pigment is decreasing (from shallow to deep, the quantum catch is declining 4.2% for downwelling light; 3.4% for sidewelling light,

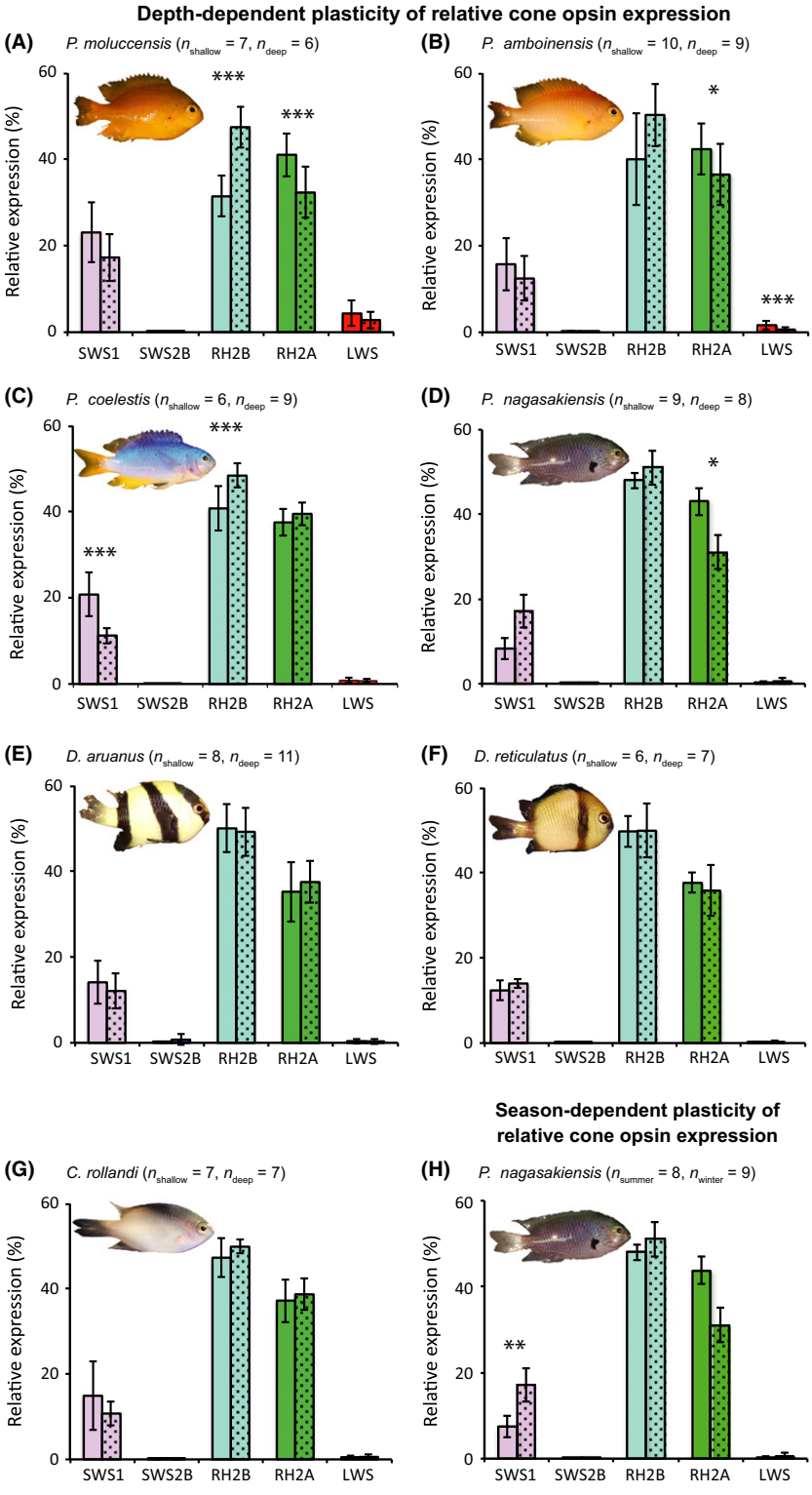

Fig. 2 Mean relative opsin expression measured using *qRT-PCR* for each of the seven damselfish species included in this study. (A–G) Filled bars are individuals caught in shallow waters (1–4 m), and dotted bars are individuals caught in deeper waters (10–15 m). (H) Here, filled bars are individuals caught in summer and dotted bars are individuals caught in winter. Opsin genes that revealed a significant regression in expression with depth (and not time) (A–G), respectively, with season (and not depth or time) (H) are marked with an asterisk (≤0.05 *, ≤0.01 **, ≤0.001 ***). For illustration purposes, we used columns and error bars (±SD) to visualize the dependence of opsin gene expression on depth or season. All species expressed SWS1, RH2B and RH2A; only, *P. moluccensis* (A) and *P. amboinensis* (B) showed additional minor expression of LWS. While expression varied with depth in at least one opsin gene in all four *Pomacentrus* species (A–D), it was stable in the *Dascyllus* (E, F) and *Chrysiptera* (G) species. In *D. aruanus*, *D. reticulatus* and *C. rollandi* (E–G), RH2B is distinctly higher expressed compared to RH2A in the shallow and deep group; the expression of RH2B is still noticeably higher in the deep group but becomes almost equal to RH2A expression in the shallow group of *P. coelestis* and *P. nagasakiensis* (C, D) and even switches to a lower expression compared to RH2A in the shallow group of *P. moluccensis* and *P. amboinensis* (A, B). (H) SWS1 was higher expressed in winter compared to summer in *P. nagasakiensis*. (D and H) Please note that significance levels are the result from testing all three regressors (season, depth and time) together (see Table S5, Supporting information).

4.7% for upwelling light) (Table S6, Supporting information, Fig. 4D for sidewelling light).

In summary, this suggests that both the shortest (UVS with λ_{max} = 362 nm) and the longest ('green' MWS with λ_{max} = 519 nm) wavelength visual pigments decreased their quantum catch with depth, whereas the quantum catch of the two medium-wavelength sensitive visual pigments ('blue' MWS with λ_{max} = 486 nm in single cones, respectively, 475 nm in double cones) increased their quantum catch with depth (Fig. 4B,C).

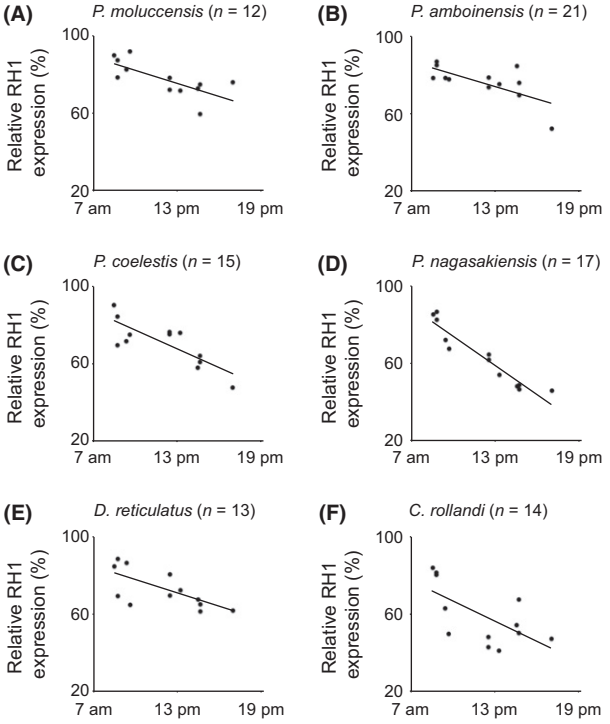

Fig. 3 The relative expression of RH1 is plotted against time and trend lines are added. In the course of the day, RH1 expression declines for (A) *P. moluccensis*, (B) *P. amboinensis*, (C) *P. coelestis*, (D) *P. nagasakiensis*, (E) *D. reticulatus* and (D) *C. rollandi*. *D. aruanus* is not illustrated as it was excluded from statistical analyses (see result section).

Note that the overall changes in quantum catch remained the same when we used λ_{max} values for *P. amboinensis* and *P. coelestis* separately (see Table S6, Supporting information).

Discussion

The objective of this study was to quantify intraspecific differences in opsin gene expression under different light conditions in natural populations of seven damselfish species between two depth zones. We further tested if and how the prevailing light environment inhabited by these populations shapes such variability. Our approach allowed us to address the question of what role phenotypic plasticity may play for sensory adaptation in one of the most species-rich lineages of marine fishes.

Damselfish have five cone opsin genes. In this study, we show that adult damselfish rely primarily on the expression of three of these cone opsin genes: SWS1, RH2B and RH2A. This occurs in seven species in three different genera; only, *P. moluccensis* and *P. amboinensis* showed additional minor expression levels of LWS (Fig. 2A,B). This is consistent with physiological studies based on MSP, which suggest that damselfish primarily use three to four cone visual pigments within their retina [see Table 1 shown for 13 different damselfish species; reviewed in Marshall *et al.* (2006, 2015); for *P. amboinensis* see Siebeck *et al.* (2010); and for *P. coelestis* see McFarland & Loew (1994)].

With respect to intraspecific cone opsin expression levels, we find that four of seven species show changes in gene expression between the two depth zones. In the following, we discuss the species-specific plasticity of opsin gene expression, and the dynamics in opsin gene expression profiles over depth and the light environment. To do so, we first describe how we can use damselfish visual pigment absorbance gained from MSP data to match with the likely opsin genes that are expressed in these photoreceptors.

Finally, we show that expression of the dim-light vision gene RH1 changes consistently over the course of the day across species.

Matching visual pigment sensitivities with cone opsin genes

A drawback in interpreting depth-related changes in opsin gene expression on the basis of quantum catch models is that correlating visual pigment absorbance from particular cone types with specific opsin genes is never completely safe unless the peak absorbances are determined in visual pigments that are synthesized in vitro. Unfortunately, in vitro reconstitution of visual pigments is not available for damselfish. However, such experiments have been performed in cichlid fishes where they found that the shorter-wavelength SWS opsins are expressed in single cones, whereas the longer-wavelength RH2 and LWS genes are expressed in double cones (Carleton *et al.* 2005, 2008; Parry *et al.* 2005; Spady *et al.* 2006). As cichlids are phylogenetically closely related to damselfish (see, e.g. Mabuchi *et al.* 2007), we assume that the association of opsin genes to cone types is comparable.

In addition, our own results allow reasonable assumptions on the sensitivities of visual pigments corresponding to particular opsin genes. As our experiments revealed high expression levels for SWS1, RH2A and RH2B, none for SWS2B, and only very low levels for LWS in two species (see Table 1), we suggest the following classification matching opsin genes to visual sensitivities (Table 1): UV-sensitive (UVS) single cone = SWS1, short-wavelength sensitive (SWS) single cone = SWS2B, medium-wavelength sensitive ('blue' MWS) single cone = one of the RH2 genes – most probably RH2B and double cones with either two medium-wavelength ('blue' MWS and 'green' MWS) sensitivities = RH2B and RH2A, or with one member having a long-wavelength sensitivity (LWS) = LWS. This is

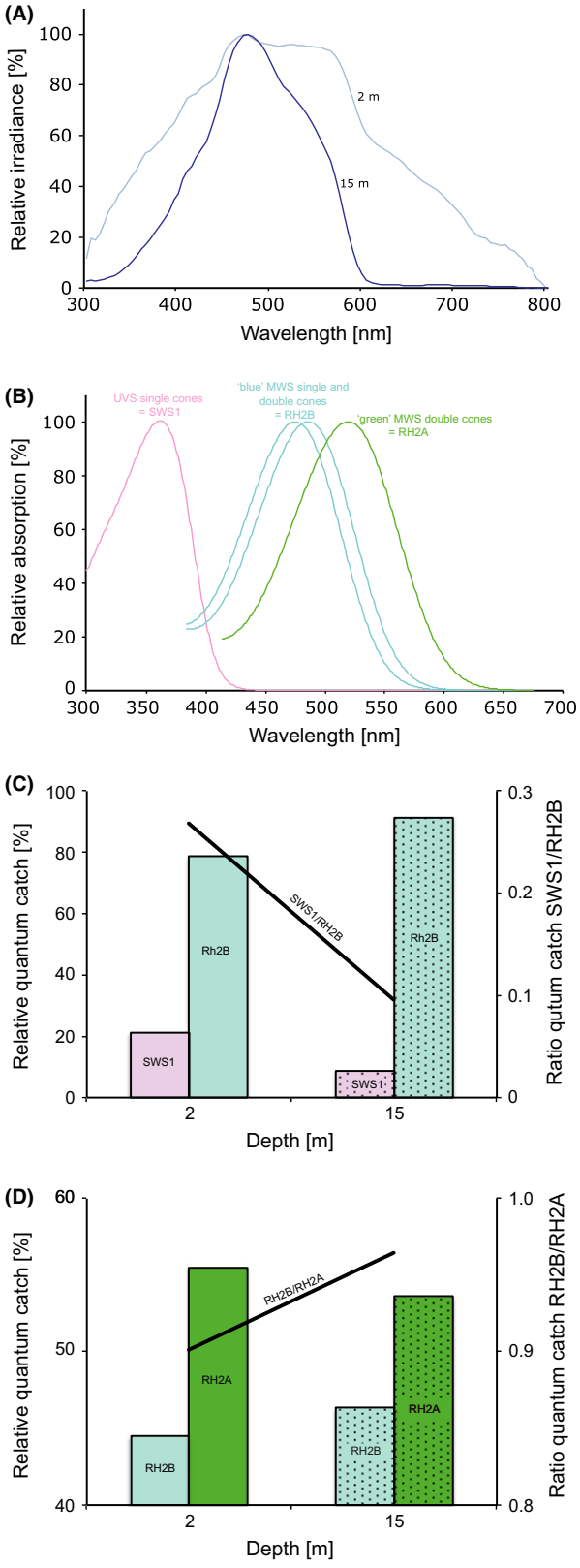

Fig. 4 (A) Relative spectral irradiance curves for 2 m (light blue) and 15 m (dark blue) depth at Lizard Island reefs. Only, irradiance for sidewelling light is shown. Note that with depth the short- and long-wavelength parts of the spectra are attenuated. (B) Idealized spectral absorbance curves for cone visual pigments (averaged for several damselfish species see Table 1). Note that the visual sensitivity of two medium-wavelength sensitive (MWS) visual pigment found in single and double cones ('blue' MWS, illustrated in blue) is nearly identical. We suggest the following matching of visual pigments identified by MSP to expressed opsin genes as follows: ultraviolet-sensitive (UVS) single cone = SWS1, 'blue' MWS single cone = RH2B (or possibly a co-expression of RH2A and RH2B), 'blue' MWS double cone = RH2B and 'green' MWS double cone = RH2A. (C) and (D) Estimated quantum catch for different visual pigments in the shallow (2 m, filled bars) vs. deeper (15 m, dotted bars) waters (using sidewelling irradiance spectra). The bar graphs refer to the left *y*-axis, whereas the line refers to the right *y*-axis. In single cones, the relative quantum catch of SWS1 is predicted to decrease and of RH2B to increase with depth (C); in double cones, the relative quantum catch of RH2B is predicted to increase and of RH2A to decrease with depth.

further supported by the following arguments: (i) The phylogenetic hypothesis of opsin genes demonstrate that the damselfish opsin classes group together with those of other fish species (Fig. 1) and our suggested matching of damselfish opsin genes to visual pigment sensitivities lies in the range of opsin-specific visual sensitivities known for other species. (ii) The SWS1 opsin gene produces a short-wavelength-ultraviolet- to violet-sensitive pigment in a diverse array of vertebrates, with λ_{max} of 360–440 nm (Yokoyama 2008). We can thus assume that the damselfish UV-sensitive (347–376 nm, see Table 1) pigment equals SWS1. (iii) SWS2B was not expressed in any of our tested species; however, its spectral range of 400–450 nm (Yokoyama 2008) would be consistent with SWS cones that have been found in some juvenile damselfish (MSP data from McFarland & Loew 1994). (*iv*) MSP data for the majority of damselfish species shows a second single cone being sensitive to medium wavelengths (464–504 nm, see Table 1), with a λ_{max} that is similar to one of the visual pigments found in double cones (Table 1, e.g. in *P. coelestis* single cone λ_{max} = 490 nm double cone λ_{max} = 490 nm) or lies in between the values of both double cone λ_{max} (Table 1, e.g. in *P. amboinensis* single cone λ_{max} = 504 nm lies between the double cone λ_{max} = 480 nm and 523 nm). This leads us to speculate that the second single cone expresses RH2 in spectral ranges of 450–530 nm (Yokoyama 2008). For example, *P. coelestis* is likely to express pure RH2B pigment in MWS single cones, while *P. amboinensis* appears to co-express RH2B with RH2A in MWS single cones. Opsin co-expression in adult fishes has so far only been reported for double cones (Dalton *et al.* 2014). Co-expression in single cones may

temporarily occur during development, when opsin switches take place as has been reported for salmon (Cheng & Flamarique 2004), salamanders (Isayama *et al.* 2014) and mice (Applebury *et al.* 2000).

Double cones in damselfish have two visual pigments, which are medium-wavelength sensitive (Table 1) and match spectral ranges of RH2 or partly LWS (RH2 467–516 nm, LWS 508–565 nm; Yokoyama 2008). Considering our findings that we either found no or only minor LWS expression, we suggest that damselfish double cone λ_{max} most likely matches RH2B (shorter-wavelength sensitivity = 'blue' MWS) and RH2A (longer-wavelength sensitivity = 'green' MWS). To conclusively gain insights into opsin coexpression (in single and double cones) and its possible role in spectral tuning to different depth, in situ hybridization experiments on whole retinas would be required (Dalton *et al.* 2014).

Plasticity of spectral sensitivity

Varying visual sensitivities among and within species may be tuned to habitat and behaviour by various mechanisms such as variation in photoreceptor size and distribution (shown for lanternfish (Myctophidea), de Busserolles *et al.* 2014), variation in pigment filters (shown for stomatopods, e.g. Cronin *et al.* 2014), structural alterations in opsin genes (RH1 in Lakes Malawi and Tanganyika cichlids, Sugawara *et al.* 2005; or LWS seen in Lake Victorian cichlids, Terai *et al.* 2002, 2006; Carleton *et al.* 2005) or changing opsin expression profile (shown for cichlids, e.g. O'Quin *et al.* 2010). The light environment that species, populations or individuals of the same species inhabit has a strong influence on visually based communication. As a consequence, it should be advantageous if the visual sensitivity is tuned to the prevailing light spectrum in order to optimize the functionality of the visual system (Munz & McFarland 1977; Lythgoe 1979). Optomotor responses in the damselfish *Dascyllus marginatus* suggest that individuals living in deeper waters have a higher light sensitivity and higher visual acuity than their shallow-water counterparts (Brokovich *et al.* 2010). Behavioural evidence that light environments favour specific male coloration and in turn female mating preferences has been suggested for killifish (*L. goodie*; Fuller & Noa 2010) and guppies (*Poecilia reticulata*; Cole & Endler 2015) and has also been shown to be linked to structural opsin gene alterations tuning visual sensitivities in cichlids (Seehausen *et al.* 2008). Varying opsin expression within species, other than developmentally driven changes, being linked to different light habitats have so far been only reported for cichlids (Hofmann *et al.* 2009, 2010; Smith *et al.* 2011), sticklebacks (Novales Flamarique *et al.* 2012) and killifish (Fuller & Claricoates 2011). In killifish, those intraspecific expression changes could be associated to some aspects of mating (Fuller & Noa 2010) and foraging behaviour (Fuller *et al.* 2010).

In this study, we found intraspecific variation in expression levels in at least one cone opsin in four Pomacentridae species. It is unclear, however, whether this variation is genetically based or triggered environmentally. Other fishes show a great diversity in genetic vs. environmental plasticity in opsin expression. Opsin expression in killifish can vary in quantitative expression levels in adults within several days due to changing light environment (Fuller & Claricoates 2011). In Lake Malawi cichlids, opsin expression has been shown to have a genetic component (O'Quin *et al.* 2012) that sets the framework of expressed opsin genes within which phenotypic plasticity can act (Hofmann *et al.* 2010). In sticklebacks, on the other hand, population differences in opsin expression related to different light habitats are heritable and not phenotypically plastic (Novales Flamarique *et al.* 2012).

The focal species of this study live in sympatry and most Pomacentridae undergo a pelagic larval phase (Sale *et al.* 1994) before returning to settle on reefs, making it likely that damselfish benefit from an ability to adapt their visual system to different light environments. Whether or not the observed plasticity is restricted to a critical phase or stays flexible throughout life still needs to be answered. Transplant experiments to different natural and artificial light environments are currently ongoing to test for environmentally driven opsin expression plasticity and to determine the flexibility through development in damselfish.

Cone opsin expression changes with different light habitats

Based on the assignments of peak visual pigment sensitivity, we can consider the quantum catch estimates for each visual pigment and how it varies with depth. The reduction of the relative irradiance spectra at the short- and long-wavelength ends of the spectrum with depth results in the relative quantum catch of the UVS visual pigment and the 'green' MWS visual pigment decreasing with depth. However, the 'blue' MWS visual pigment increases its quantum catches with depth (see Fig 3, Table S6, Supporting information). Visual sensitivities being adjusted to the prevailing light conditions gathered by changes in opsin expression is exactly what we observe in the following: *P. coelestis* shows a decrease in SWS1 (UVS-corresponding); RH2A ('green' MWS-corresponding) expression is decreased in *P. moluccensis*, *P. amboinensis* and *P. nagasakiensis;* and an increase in the RH2B ('blue' MWS-corresponding) expression is seen in *P. moluccensis* and *P. coelestis.* (Fig. 2, Table 1). These

results are in line with previous findings (for killifish see Fuller *et al.* 2004; for the black bream see Shand *et al.* 2008; for Lake Malawi cichlids see Hofmann *et al.* 2010), suggesting that opsin expression changes with λ_{max} matching the most abundant wavelengths; that is, sensitivity is increased in regions of the spectrum where light is abundant (Hofmann & Carleton 2009).

However, we observe the opposite in the relative SWS1 expression changes in *P. nagasakiensis* – namely a higher SWS1 expression in the deeper samples (albeit not statistically significant when season was also incorporated in the test). Interestingly, such a decline of sensitivity in regions of the spectrum where light is abundant was reported for the blue acara (*Aequidens pulcher*). Here, rearing fish under blue light conditions resulted in a reduction in the number of photoreceptors being sensitive to the corresponding wavelength (Kröger *et al.* 1999; Wagner & Kröger 2000) and is interpreted as a compensatory mechanism that helps maintain colour constancy (Wagner & Kröger 2005).

Based on our results showing that season alone had a significant effect on SWS1 expression in *P. nagasakiensis*, we propose that in this case, seasonal differences may explain the inverted changes in SWS1 expression with depth (Fig. 2G, Table S5, Supporting information). All individuals of the six other tested damselfish species plus all *P. nagasakiensis* deep-caught individuals ($n = 8$) were sampled in June/July (Australian winter), most *P. nagasakiensis* shallow individuals (8 out of 9) were sampled in February (Australian summer); only one of the 'shallow' individuals was also sampled in winter. This individual has a relative SWS1 expression of 14% that is considerably higher than average SWS1 expression of 8% in shallow individuals from the summer. Seasonality is expected to have an effect on water visibility around Lizard Island with highest visibility in winter and a decrease in visibility in summer. This would suggest that the greater visibility in winter might result in more short-wavelength light, which in turn might produce a higher quantum catch at shorter wavelengths, and a higher expression of SWS1. Thus, expression differences in *P. nagasakiensis* may not exclusively be the result of a different light environment produced by depth but also by seasonality and consequently provides a hint that opsin expression in damselfish may indeed stay plastic throughout an individual's lifetime.

Why do some species have plastic opsin expression?

Our expectation that damselfish, which are initially pelagic and then settle into different possibly final light environments, might benefit of some degree of plasticity in their visual system was only partly confirmed: while cone opsin expression varied according to sampling depth in all four *Pomacentrus* species, it was stable in the *Dascyllus* and *Chrysiptera* species. We can only speculate that plasticity of opsin expression might be favoured in some species, while in others it may be advantageous to have constant expression. It is not clear whether the discrepancies in the degree of plasticity are due to ecological differences in visual tasks or, alternatively, limited by phylogenetic constraints between species. The fact that all species from the genus *Pomacentrus* show plasticity, while the *Dascyllus* and *Chrysiptera* species do not, suggests some sort of genetic control. Referring to the latest phylogeny of Pomacentridae (Cooper *et al.* 2009), it is possible that the molecular basis for plasticity has only evolved in the genus *Pomacentrus*. Although *Pomacentrus* belongs to the same clade (Pomacentridae) as the genus *Chrysiptera*, it is clearly separated from the latter as well as from the genus *Dascyllus*, which belongs to a different clade (Chrominae). However, a more widespread sampling regime spanning more members of different clades is needed to validate a genetic component for plasticity in Pomacentridae.

Most Pomacentridae are territorial and only move within a few metres once settled (Fricke 1977; Allen 1991; Sale 1991; Fishelson 1998) – our own observations confirm this for the tested species. Nevertheless, more detailed species-specific observations need to be carried out in order to examine whether some species might be more stenotopic than others, which might in turn influence their degree of plasticity. Also, we need to better understand the species-specific ecological constraints derived from visual demands like species recognition, predator avoidance, sexual selection or food detection. Moreover, whether or not the observed quantitative changes in opsin expression levels are relevant to visual tasks and colour vision is unknown at this stage and can only be verified by behavioural assays in controlled light environments. However, studies in Lake Victorian cichlids (Carleton *et al.* 2005; Maan *et al.* 2006; Terai *et al.* 2006; Seehausen *et al.* 2008), in the killifish (Fuller 2002; Fuller & Travis 2004) and in sticklebacks (Boughman 2001) provide evidence that male coloration and female perceptual sensitivity can be directly linked to the photic environment resulting in a match of male signals to female visual preferences favouring speciation through sensory drive. In damselfish, it is unknown if and how species use their coloration for species recognition or mate choice. *P. amboinensis* was the first damselfish in which colour discrimination has been shown (Siebeck *et al.* 2008) and uses fine species-specific UV-reflective facial patterns – the only difference in its appearance from *P. moluccensis* – for species discrimination (Siebeck *et al.* 2010).

Colour spectra in reef fish are often very conspicuous at close range but well camouflaged at a distance (Marshall 2000; Marshall & Cheney 2011). These colour

patterns might alter the ability and need for colour vision vs. contrast detection between our tested species with changing light environments. Interestingly, when comparing the species showing opsin gene expression changes with depth to those that do not alter their expression profiles, one notices that *P. amboinensis*, *P. moluccensis*, *P. nagasakiensis* and *P. coelestis* are more continuously yellow or bluish coloured when compared to the more contrasting patterns of *D. aruanus* and *D. reticulatus*, although *C. rollandi* is also more uniformly coloured. The striped outline of *D. aruanus* and *D. reticulatus* is maximally conspicuous when they are above the coral head which may help for intraspecific communication but when they hide in the coral branches they appear cryptic against the background from the perspective of predators (Marshall & Cheney 2011; Phillips *et al.* 2013).

Whether or not contrasting body patterns enhance the need for better contrast detection rather than colour discrimination compared to less patterned damselfish species and the possible impact on depth-related change of opsin expression remains unknown. However, a recent study in guppies suggested that colour pattern or chromatic cues change their appearance with changing light habitats, whereas achromatic components change very little and provide some sort of contingency against environmental change (Cole & Endler 2015; see also Seehausen 2015).

The trade-off between colour discrimination and contrast detection and its potential effect on population differences in opsin expression exist. A study in Lake Malawi cichlids revealed that co-expression of two opsin genes in double cones [partially fused cone cells that are known to have an absorbance often matching the background spectra of their environment (Temple *et al.* 2010)] results in increased contrast detection and at the same time can lower colour discrimination (Dalton *et al.* 2014). This may partially explain different expression profiles found in populations living in dissimilar light habitats (Hofmann *et al.* 2010).

Plasticity of the rod opsin (rhodopsin 1, RH1) shows diurnal variation

Another finding of our study is that even though changes in cone opsin expression occurred according to depth, variation in RH1 expression was predominantly affected by time of day with a steady decrease over the course of the day and lowest expression in the late afternoon to dawn. This outcome is in accordance with previous studies in the cichlid *Haplochromis (Astatotilapia) burtoni* showing that rhodopsin transcript level fluctuates in a daily rhythm with a peak in the late morning followed by a steady decrease over the course of the day (Korenbrot & Fernald 1989; Halstenberg *et al.* 2005). It also demonstrates the potential flexibility of opsin expression relative to light in all of these species.

In conclusion, our data suggest that damselfish rely primarily on expression of the SWS1, RH2B and RH2A opsin genes for photopic vision. Within these expressed cone opsin genes, four damselfish species showed intraspecific variation in gene expression according to water depth. Estimates of visual pigment quantum catch suggest that changes in opsin expression adjust visual sensitivities to coincide with the prevalent light environment. We also show that plasticity in opsin expression in damselfish is highly species-specific with some species showing a stable expression profile along the depth gradient. Finally, seasonal differences, which may go hand-in-hand with changes of the photic environment, might also influence opsin expression. Thus, further studies need to be carried out that take into account the diverse visual needs and varying ecologically relevant factors between species to unravel the complexity of the damselfish visual system.

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S.M.S, K.L.C., N.J.M. and W.S. designed the study. S.M.S., F.C. and N.J.M. performed the experiments. S.M.S. and K.L.C. analyzed the data. S.M.S., K.L.C. 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 numbers (KU745421–KU745456) are listed in the Supporting information, Table S7 (Supporting information). Primer sequences used for qPCR are made available in the Supporting information, Table S3 (Supporting information).

Supporting information

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

Table S1 Summary of sampling regime

Table S2 Primer combinations used for PCR and sequencing in each species

Table S3 Primer names and sequences used for qPCR

Table S4 Summary of qPCR primer combinations and efficiencies for each species

Table S5 Summary of beta regression models

Table S6 Relative quantum catch estimates for visual pigments in damselfish with changing depth

Table S7 Genbank accession numbers of damselfish opsins sequenced in this study