Stieb Sara (Orcid ID: 0000-0001-7158-3934)
Jardim de Queiroz Luiz (Orcid ID: 0000-0002-5524-6281)
Carleton Karen L (Orcid ID: 0000-0001-6306-5643)
Marshall N. Justin (Orcid ID: 0000-0001-9006-6713)

Long-wavelength-sensitive (*lws*) opsin gene expression, foraging and visual communication in coral reef fishes

Sara M. Stieb (SMS)[*1,2,3], Fabio Cortesi (FC)[3], Luiz Jardim de Queiroz (LJdQ)[1,2], Karen L. Carleton (KLC)[4], Ole Seehausen (OS)[1,2], N. Justin Marshall (NJM)[3]

[1]Centre for Ecology, Evolution and Biogeochemistry (CEEB), EAWAG Swiss Federal Institute of Aquatic Science and Technology, Kastanienbaum, Switzerland

[2] Institute of Ecology and Evolution, University of Bern, Switzerland

[3]Queensland Brain Institute, The University of Queensland, Brisbane, QLD 4072, Australia

[4]Department of Biology, University of Maryland, College Park, MD 20742, USA

[*]Corresponding author. E-mail: sara.stieb@eawag.ch

Abstract

Coral reef fishes are diverse in ecology and behaviour and show remarkable colour variability. Investigating the visual pigment gene (opsin) expression in these fishes makes it possible to associate their visual genotype and phenotype (spectral sensitivities) to visual tasks, such as feeding strategy or conspecific detection. By studying all major damselfish clades (Pomacentridae) and representatives from five other coral reef fish families, we show that the long-wavelength-sensitive (*lws*) opsin is highly expressed in algivorous and less or not expressed in zooplanktivorous species. *Lws* is also upregulated in species with orange/red colours (reflectance > 520 nm) and expression is highest in orange/red-coloured algivores. Visual models from the perspective of a typical damselfish indicate that sensitivity to longer

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wavelengths does enhance the ability to detect the red to far-red component of algae and orange/red-coloured conspecifics, possibly enabling social signalling.

Character state reconstructions indicate that in the early evolutionary history of damselfishes, there was no *lws* expression and no orange/red colouration. Omnivory was most often the dominant state. Although herbivory was sometimes dominant, zooplanktivory was never dominant. Sensitivity to long wavelength (increased *lws* expression) only emerged in association with algivory but never with zooplanktivory. Higher *lws* expression is also exploited by social signalling in orange/red, which emerged after the transition to algivory. Although the relative timing of traits may deviate by different reconstructions and alternative explanations are possible, our results are consistent with sensory bias whereby social signals evolve as a correlated response to natural selection on sensory system properties in other contexts.

Introduction

A central objective in biology is to understand changes in biological diversity through time and lineages, especially the processes of speciation and the emergence of species-rich clades. Ecological niches are multidimensional and ecological diversification may be positively correlated with niche dimensionality (Nosil & Sandoval, 2008). In this study we focus on the extremely species-rich group of tropical coral reef fishes that display extraordinarily high diversity in ecology, behaviour and (colour) phenotype (Randall et al., 1997). Reef fishes, especially those inhabiting shallow water coral reefs in the tropics, live in a light-flooded and spectrally diverse environment (Cox et al., 2021). Many are themselves, often conspicuously, colourful (Lorenz, 1962; Marshall, 2000a), and importantly, have evolved diverse visual sensitivities and tuning mechanisms (Carleton et al., 2020; Cortesi et al., 2020). The study of colourful coral reef fish living in a multidimensional adaptive landscape allows us to gain

valuable insights into the evolutionary interactions between vision, visual signalling traits, behaviour and ecology (Cortesi et al., 2020).

In teleosts, a multitude of tuning mechanisms, including opsin gene evolution via duplications and deletions, sequence variability and (co)expression, is used to presumably optimise vision (i.e., spectral sensitivities) for the prevailing light environment and/or visual tasks [reviewed in Carleton et al. (2020)]. In the photoreceptor cells of the retina, opsins, together with a vitamin A-derived chromophore, form the functional unit of visual pigments that absorb light and constitute the first step in visual processing (Wald, 1968; Yokoyama, 2008). Vertebrate opsins can be classified based on their genealogy, photoreceptor specificity, and the spectral sensitivity they confer. Rod photoreceptors express a single rod opsin type (rhodopsin, *rh1*) used for scotopic vision. Cone photoreceptors, on the other hand, express four basic types of cone opsins, which mediate photopic (colour) vision: two short-wavelength (UV-blue)-sensitive genes (*sws1* and *sws2*), a mid-wavelength (green)-sensitive gene (*rh2*), and a long-wavelength (red)-sensitive gene (*lws*) (Yokoyama, 2008).

Coral reef fishes of the superorder Acanthopterygii have evolved a number of different visual tuning mechanisms and a set of spectral sensitivities peaking anywhere between the ultraviolet (UV) and the red spectrum of light (350 – 600 nm) (Carleton et al., 2020; Cortesi et al., 2020; Losey et al., 2003; Luehrmann et al., 2019; Marshall et al., 2019; Phillips et al., 2016; Siebeck & Marshall, 2001; Stieb et al., 2017). While medium-wavelength-sensitivity (i.e., from blue to green) is well matched to the most prevalent light on coral reefs (Losey et al., 2003), spectral sensitivities at either end of the spectrum (i.e., UV and red) seem more likely to represent adaptations to one or more specific visual tasks. For example, damselfishes (Pomacentridae) use their UV vision to detect UV patterns of con- or heterospecifics (Siebeck et al., 2010). Herbivorous algae-feeding damselfishes also display enhanced red sensitivity (Stieb

et al., 2017) as it increases algal contrast (due to the red and far-red-reflecting part of chlorophyll) when seen against the reef background (Marshall et al., 2003). Long-wavelength-sensitivity may furthermore play a role in inter- or intraspecific communication in species that use red colour signals, such as many cichlids (Seehausen et al., 2008), guppies (Sandkam et al., 2018), medaka (Kamijo et al., 2018) or indeed other reef fishes (Marshall et al., 2003). Once evolved, sensitivity to a specific spectral range is likely to be exploited by other visual tasks, as has been shown for guppies (Endler, 1983; Grether et al., 2005; Kodric-Brown, 1989; Rodd et al., 2002) or Old World monkeys (Fernandez & Morris, 2017) that feed on reddish fruit and also use red for social signals.

In this paper, we focused on the emergence of long-wavelength-(red)-sensitivity and its function in coral reef fishes by extending our previous work on opsin gene evolution. We mainly focused on damselfishes. This speciose family of small to medium-sized reef fishes mirrors the high behavioural, ecological and colour diversity found among coral reef fishes more broadly (Allen, 1991). Importantly for this work, damselfishes are also one of the most studied reef fish families in terms of opsin evolution. Of the 21 species analysed thus far, only benthic herbivorous but not zooplanktivorous damselfishes expressed increased levels of *lws* producing red-sensitive visual pigments (Stieb et al., 2017). We now aimed to reveal whether a sensory bias towards red sensitivity in algal feeding damselfishes is also exploited in yellow-red colour signalling. Our predictions were that: 1) benthic herbivory and red colours would both correlate with enhanced red sensitivity as shown by higher *lws* expression, 2) red sensitivity has a functional benefit in detecting algae and red colour signals, 3) specialization for algae feeding is followed by the evolution of red signalling colours and, 4) following on from prediction 3, only algal feeding species have evolved red colouration. To test for a relationship of long-wavelength-sensitivity (*lws* expression) with feeding ecology (benthic herbivory), social

signalling (long-wavelength-reflecting colours; yellow, orange, and red), or both, we combined our previous work with newly sequenced retinal transcriptomes to generate a more extensive damselfish dataset. To go beyond the damselfish radiation, we also included pairs of benthic herbivorous and zooplanktivorous species from five other typical tropical reef fish families, including butterflyfishes (Chaetodontidae), angelfishes (Pomacanthidae), blennies (Blennidae), surgeonfishes (Acanthuridae), and labrids/wrasses (Labridae). To test for a functional benefit of seeing red, we next computed a damselfish visual system and modelled whether red sensitivity may improve the detection of benthic algae and the detection of yellow, orange, or red coloured conspecific signals. Lastly, by reconstructing ancestral character states, we assessed the sequence of emergence of *lws* expression, trophic groups and orange/red colour signals and their possible evolutionary interactions across the damselfish phylogeny.

Methods

Specimen collection

Specimens were either collected from reefs surrounding Lizard Island (14° 40 S, 145° 27 E), Australia, using SCUBA and hand nets under the Great Barrier Reef Marine Park Permit (G12/35005.1) and the Queensland General Fisheries Permit (140763), or obtained from an aquarium supplier (Cairns Marine Pty Ltd, Cairns, Australia), collecting fishes from the Northern Great Barrier Reef. Fish used for molecular analysis were anaesthetized with an overdose of clove oil and killed by decapitation within 24 h after capture. Retinas were dissected out and preserved in RNAlater (Ambion) until further processing. Dissection took place during the daytime, at least 1 h after dawn and before dusk, respectively. The relative cone opsin expression has previously been shown not to be affected by time of day in several damselfish species (Stieb et al., 2016). Further, we used an opsin expression normalizing by proportion of

cone type that was assigned the best method for removing time-of-day variation (Yourick et al., 2019). 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).

Opsin gene studies

To investigate and quantitate opsin gene expression among damselfishes, we compiled a dataset for 39 species [based on our previous results, n = 21 (Luehrmann et al., 2018; Stieb et al., 2016, 2017, 2019), and newly sequenced transcriptomes, n = 18, (Bioproject PRJNA747115: SAMN21876388-SAMN21876434; Table S1)]. We were further interested in comparing the relative opsin gene expression between herbivorous and zooplanktivorous species-pairs from various other reef fish families (n = 5 coral reef fish families). For this, we compiled a dataset based on previous results (Phillips et al., 2016; Tettamanti et al., 2019) and generated new transcriptomes for the remaining species following our previously established protocols (Luehrmann et al., 2019; Musilova et al., 2019). To confirm the assignment of newly obtained opsin sequences to the correct opsin gene type/family, we used a fish-opsin reference dataset to reconstruct maximum-likelihood amino acid trees using PHYML (100 bootstrap iterations) (Dereeper et al., 2008).

(a) Transcriptomic sequencing and processing

Retinas were homogenized using a TissueLyser LT (Qiagen, Netherlands) and total RNA was extracted with the RNeasy Mini Kit (Qiagen, Netherlands) including an optional DNAse digestion step. RNA was quality checked with an Agilent 2100 BioAnalyzer 6000 NanoChip (Agilent Technologies, USA). RNAseq libraries were made using the TruSeq RNA Sample Preparation Kit v.2 (Illumina, San Diego, USA), and transcriptomes were sequenced as 125 bp paired reads on the Illumina platform (HiSeq2000 v4). Samples were multiplexed at 12 samples per lane, obtaining 4–51 million sequenced reads per sample.

Transcriptomes were processed following previously published methods (Cortesi et al., 2015; de Busserolles et al., 2017) using the online Bioinformatics platform Galaxy v.1.0.4 (Research Computing Centre, The University of Queensland, Australia) (Afgan et al., 2015). In short, data were converted using FASTQ Groomer, quality checked using FastQC, and trimmed using customized settings in Trimmomatic. Trinity was used for *de-novo* assembly of transcripts, with a group pair distance of 250 bp, and minimum inchworm kmer coverage of 2. Further bioinformatics analyses were performed using Geneious software (Version 9.0.4). Assembled transcripts were then mapped to known and publicly available opsin genes of reference species (see Figure S1). To manually check for gene duplications, we followed previously described methods (Cortesi et al., 2015; de Busserolles et al., 2017). Briefly, after identification of candidate gene coding sequences, unassembled reads were mapped to the opsin gene repertoire of the species using medium-sensitivity settings (70% identity threshold). Deviating reads were then extracted by working from single polynucleotide polymorphism (SNP) to SNP by exploiting paired-end matching to cover gaps, and their consensus sequence was used as species-specific reference for repeated high-specificity (100% identity) mapping of unassembled reads until maximum obtainable sequence length was reached. To analyse differences in relative cone opsin gene expression, we mapped the unassembled filtered PE reads against the CDSs of genes extracted from the transcriptomes [as per Cortesi et al. (2015) and de Busserolles et al. (2017)].

(b) Relative opsin gene expression given as proportional single and double cone expression

The coral reef fishes examined here possess single and double cones (two single cones fused together) which either express *sws* or *rh2* and/or *lws* genes (Stieb et al., 2019), respectively. Consequently, quantitative cone opsin expression is given as proportional expression of the total single (*sws1*s and *sws2s*) and total double cone expression (*rh2*s and *lws*), respectively. Proportional gene expression was then calculated according to

$$T_i/T_{all\ (single\ resp.\ double\ cones)} = N_i/\sum N_i \qquad (1)$$

where $T_i/T_{(single\ resp.\ double\ cones)}$ is the gene expression ratio for a given gene T_i normalized by the total genes expressed in all single cones or in all double cones $T_{(single\ resp.\ double\ cones)}$, and N_i is the number of mapped reads for a given gene divided by its length.

Because cone opsin expression is given as a fraction of the total single (*sws1*s and *sws2b*) and total double cone expression (*rh2*s and *lws*), respectively, we re-analysed expression data gained from Stieb et al. (2016; 2017) for damselfish and from Phillips et al. (2016) for labrids to calculate the proportional expression of single and double cone opsin genes, respectively.

Spectral reflectance

We compiled spectral reflectance data on live specimens [as per Marshall et al. (2003)] for 35 species [newly generated, n = 10; from the literature, n = 25 (Cheney & Marshall, 2009; Marshall, 2000b; Siebeck, 2002; Stieb et al., 2017)], following the colour categorization in Marshall (2000b).

The spectral reflectance of different areas of the fish was measured at a 45° angle using a 200 nm 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 UV (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.

Relationship of proportional opsin gene expression with diet and colouration

To identify possible evolutionary correlations between damselfish cone opsin expression and either trophic groups or long wavelength (yellow, orange and red) colouration, we computed phylogenetic generalised least squares regressions (PGLS) using the caper package (Orme et al., 2013) in R (Development Core Team, 2011). The PGLS regression estimates a maximum likelihood (ML) value of the phylogenetic scaling factor lambda (Pagel's λ), with λ = 1 indicating complete phylogenetic dependence and λ=0 indicating no phylogenetic effect. To compare opsin expression to feeding ecology, we placed species into three different trophic groups: herbivores, zooplanktivores, or omnivores (Table S2), with omnivores known to forage on both zooplankton and algae. To test for relationships of opsin expression to different patterns of fish colouration, we grouped species first based on having yellow colouration (reflectance starting beyond 500 nm) and second, based on having orange/red (reflectance starting beyond 520 nm) colouration (including colouration of fins or bodies, or patches thereof). We first determined the historical evolutionary dependence of opsin gene expression on trophic groups, yellow, and orange/red colouration independently. If more than one of these variables had a significant effect on expression, they were subsequently tested together. Finally, we were interested in how long-wavelength sensitivity is affected when orange/red colouration is attributed to a trophic group. For this, we tested proportional double cone *lws* expression in species showing no orange/red colouration versus species showing orange/red colouration within the different trophic groups. This test was only possible for omnivores and herbivores as none of the zooplanktivores reflected in orange/red.

To further explore the relationship between proportional opsin gene expression and feeding strategy, we compared herbivorous and zooplanktivorous species pairs from various other reef fish families (herbivore vs. zooplanktivore): butterflyfishes (*Chaetodon ulietensis* vs.

Hemitaurichthys polyepis), angelfishes (*Centropyge bicolor* vs. *Genicanthus watanabei*), blennies (*Escenius bicolor* vs. *Meiacanthus atrodorsalis*), surgeonfishes (*Acanthurus blochii* vs. *Naso brevirostris*), and labrids (*Chlorurus sordidus* vs. *Bodianus mesothorax*). Those species were chosen based both on their occurrence at the sampling location, reefs surrounding Lizard Island, and on their trophic group (herbivore or zooplanktivore) without respect to colouration.

Visual modelling

We performed visual modelling as we were interested in whether an additional long-wavelength-sensitive visual pigment (LWS) might enhance the fish's capability to detect benthic algae and/or conspecifics. For this, modelling was performed using a typical UV-transmitting damselfish lens [we used the lens of *Dacyllus aruanus* (Stieb et al., 2017)]. We constructed a trichromatic damselfish visual system with known peak spectral sensitivities (λ_{max}) [gained from microspectrophotometry measurements in *Pomacentrus amboinensis* (Siebeck et al., 2010)] of 370 nm (SWS1), 480 (RH2B), and 523 (RH2A) [for matching visual pigments and opsin genes, see (Stieb et al., 2016)], and then added the fourth visual pigment (LWS) with a range of 525–565 nm λ_{max} (5 nm increments) [a λ_{max} of 560 nm was measured in *Pomacentrus melanochir* (Loew & Lythgoe, 1978)]. While the different members of double cones can express two distinct visual pigments (as for example LWS and RH2B), one member of double cones can also co-express two different opsin pigments (as for example LWS and RH2A), resulting in intermediate sensitivities as shown for the African cichlid fish, *Metriaclima zebra* (Dalton et al., 2015). These different scenarios are represented in our models by adding the fourth visual pigment (LWS) with a range of 525–565 nm λ_{max}. Evidence that individual members of double cones are used in colour vision as independent spectral channels comes from behavioral studies in the reef fish *Rhinecanthus aculeatus* (Pignatelli et al., 2010).

We first calculated the quantum catch of different photoreceptors as they viewed the light reflected off different targets. For visual differentiation, we used the receptor noise limited model to quantify the relative differentiation of two colour targets [e.g. Vorobyev et al. (2001)] providing the results in terms of just noticeable differences (JNDs), i.e., the threshold at which two objects should be distinguishable from one another under bright illumination. Algae detection was calculated as the ability to distinguish benthic algae against different backgrounds (sand, rock, coral). Conspecific detection included comparing colours of selected damselfish species (*Chromis viridis* was chosen to represent a species with no yellow and no orange/red reflectance; *Pomacentrus amboinensis* and *Pomacentrus coelestis* were chosen to represent species with yellow reflectance but no orange/red; and *Pomacentrus moluccensis*, *Chrysiptera cyanea*, and *Amphiprion biaculeatus* represented species with orange/red reflectance) against each other or against the ambient illuminant, and also against the host anemone in the case of the anemonefish *Amphiprion biaculeatus*.

(a) Quantum catch equations

Reflectance spectra from different targets used for quantum catch calculations were gathered as follows: fishes or the anemone were illuminated by sidewelling irradiance and algae and substrates by downwelling irradiance, both obtained from previous light measurements around Lizard Island (Stieb et al., 2016); reflectance data from the host anemone (*Entacmaea quadricolor*) of the anemonefish *Amphiprion biaculeatus* was measured anew, reflectance data from algae, the average reef, and rubble background was taken from Marshall et al. (2003), average sand background was taken from Cortesi and Cheney (2010) and damselfish spectral reflectance was measured anew or taken from Marshall (2000a) and Stieb et al. (2017); for ambient illuminant, as a target, we used horizontal radiance obtained from previous measurements in reefs around Lizard Island (Stieb et al., 2016).

Quantum catch of horizontal radiance was assumed to be independent of viewing distance and for a given photoreceptor i can be calculated from

$$Q_{rad,i} = k_i \int_{300nm}^{750nm} I_{rad,\lambda} L_\lambda A_{\lambda,i} d\lambda \quad (2)$$

where $I_{rad,\lambda}$ is the horizontal radiance, L_λ is the lens transmission, $A_{\lambda,i}$ is the wavelength dependent photoreceptor absorptance using the Govardovskii (Govardovskii et al., 2000) opsin templates calculated for the respective λ_{max}, and k_i is the von Kries correction for colour constancy:

$$k_i = \frac{1}{\int_{300nm}^{750nm} I_{irrad,\lambda} L_\lambda A_{\lambda,i} d\lambda} \quad (3)$$

Here, we included this normalization for consistency with previous studies and for plotting the quantum catches in a trichromatic visual space. However, this correction did not impact the final differentiation calculations for either colour or luminance, as comparisons between targets involved ratios of quantum catches, where the von Kries factor k_i cancels out.

The quantum catch of a receptor i that views a target with reflectance spectra R_λ illuminated by the sidewelling or downwelling irradiance, at zero viewing distance was given by

$$Q_{refl,i} = k_i \int_{300nm}^{750nm} I_{irrad,\lambda}\ R_\lambda\ L_\lambda A_{\lambda,i} d\lambda \quad (4)$$

For simplicity, we did not include the effects of viewing distance that would act to decrease the contrast values with distance due to scattering.

(b) Visual differentiation equations

Because very little is known about damselfish photoreceptor opponency, modelling was performed using the receptor noise limited (RNL) model which does not require any explicit understanding of post-photoreceptor processing (Vorobyev et al., 2001; Vorobyev & Osorio,

1998). Price et al. (2019) indeed demonstrated that the RNL model approximately matches those based on knowledge of post-photoreceptor processes and on explicit consideration of opponent channels. To determine colour differentiation, we first calculated the colour contrast between two objects (either comparing two targets or one target compared to the ambient illuminant) for each photoreceptor i as the log of the quantum catch ratio:

$$\Delta f_i = ln \frac{Q_{i,target\ 1}}{Q_{i,target\ 2}} \quad (5)$$

These are then combined to include the input of all three (respectively four) receptors as

$$\Delta S = \left(\frac{\omega_1^2 (\Delta f_2 - \Delta f_3)^2 + \omega_2^2 (\Delta f_3 - \Delta f_1)^2 + \omega_3^2 (\Delta f_1 - \Delta f_2)^2}{(\omega_1\omega_2)^2 + (\omega_1\omega_3)^2 + (\omega_2\omega_3)^2} \right)^{1/2} \quad (6)$$

where the noise value, ωi for receptor i depends on the Weber fraction for a single receptor, ν_l taken to be the L cone, and the relative number density of photoreceptor i as compared to the L cone (Koshitaka et al., 2008):

$$\omega_i = \nu_i \sqrt{\frac{n_L}{n_i}} \quad (7)$$

As per Stieb et al. (2019), damselfishes have one single cone for each pair of double cones so that n_S: n_M: n_L = 1 : 2 : 2. We further set the Weber fraction ν_l, to 0.1, based on colour experiments in other fishes (Champ et al., 2016; Cheney et al., 2019; Escobar-Camacho et al., 2017).

Ancestral character state reconstruction

To reconstruct ancestral states for proportional double cone *lws* expression, trophic groups and orange/red colouration, we used a damselfish phylogeny modified from *The Fish Tree of Life* (Rabosky et al., 2018). For this, we concatenated sequence data and constructed maximum-likelihood trees (100 bootstrap iterations) using PHYML (Guindon & Gascuel, 2003) in Geneious v.9.0.5. Nile tilapia (*Oreochromis niloticus*) and the black surfperch (*Embiotoca jacksoni*) were used as outgroups to root the tree. As no genetic markers were available for *Parma unifasciata,* we used markers for *Parma oligolepis* as a surrogate to place *Parma unifasciata* in the phylogeny.

Ancestral reconstruction analyses of *lws* expression, trophic groups and orange/red colouration were performed using the package corHMM (Beaulieu et al., 2021) in R environment (R Core Team, 2021). corHMM can model ancestral reconstructions that assume a correlation between two or more traits in a way that the traits co-evolve with each other. This allowed us to model the transition of one trait being dependent on the transitions of the other traits. First, we reconstructed ancestral states for each trait independently. For trophic groups, we coded extant species in three possible states (herbivores, omnivores or zooplanktivores). For orange/red colouration, we used binary states (absence or presence). Expression level of *lws* was originally obtained as a continuous trait (proportional double cone expression in %). Since corHMM can only handle discrete data, *lws* expression was coded into four different categories: 0-0.9%, 1-9.9%, 10-19.9% and 20-31%. The last three categories were chosen in 10% interval; as 31% (highest observed value) was the sole value above 30%, it was included in the range 20-31%. The first category below 1% expression represents species that have either no or a minor *lws* expression. Then, we performed the ancestral state reconstructions of these traits assuming their evolution to be dependent on each other. We also allowed all possible combinations between traits. For each ancestral reconstruction, we ran three different analyses, each based on a

different transition rate configuration: i) allowing different transition rates among states (ARD; All Rates Differ), ii) constraining forward and reverse transitions to have the same rates (SYMmetrical rates, SYM), and iii) constraining all transitions to have the same rate (Equal transition Rates, ER). For orange/red colouration, SYM and ER become identical models because this trait is binary. To find which of these transition rate models is the best fit for our data, we used the corrected Akaike information criterion (AICc). Since some of the tips of our internal groups had missing information, we also set corHMM to reconstruct their most likely state.

We also used BayesTraits V3.0.1 (Pagel & Meade, 2006) to reconstruct *lws* expression in its original form, i.e., as a continuous trait. We followed the advice in the BayesTrait's manual, and we first ran a random walk maximum likelihood (ML) analysis of *lws* expression on the damselfish tree to obtain a general estimate of the most likely gene expression level at the root of the tree (parameter α), which resulted in a value of *c.* 1.5%. Then we ran a new analysis based on a random walk Markov chain Monte Carlo (MCMC) approach in order to estimate ancestral states of all nodes and tips with missing data. Given that our preliminary ML analysis pointed to a low *lws* expression at the root of the tree, we set our MCMC with a strong prior (exponential prior with a mean of 1%). The observation that long-wavelength sensitivity (assuming *lws* expression) is rarely found among coral reef fishes (Losey et al., 2003; Luehrmann et al., 2019; Phillips et al., 2016; Stieb et al., 2017), and seemingly is associated with specific behaviors, supports the usage of a strong prior on low *lws* expression at the root of the damselfish tree. For comparative purposes, we also set BayesTraits to reconstruct *lws* expression assuming an uninformative prior (uniform distribution, varying from 0 to 31%). For both settings, we ran three independent analyses with 100'000'000 generations each, with estimated parameters sampled every 10'000th generation. Mixing and convergence of the runs were confirmed.

In all ancestral state reconstructions aforementioned, we assigned the outgroups (Nile tilapia and the black surfperch) with no data on *lws* expression, colouration or trophic group to not impact ancestral trait states (both outgroup taxa belong to clades with considerable variation in all of our traits).

Results

Opsin expression profiles of damselfish species are listed in Table S2, and opsin expression profiles of the other reef fish families (labrids, butterflyfishes, angelfishes, blennies, and surgeonfishes) are listed in Table 1 and Table S3. Protein-based maximum likelihood trees revealed that the newly extracted reef fish opsins (Genbank # OK350470–OK350614; Table S4) grouped with well-described opsin classes from other fish species (Figure S1a and b). Colour categories, including the presence or absence of yellow and orange/red, for damselfish and species from the other reef fish families are summarised in Table S5. The presence of orange/red is found in ten damselfishes (Table S2) and five of the other reef fish species (Table 1). Reflectance data from various damselfish species with orange/red colouration are shown in Figure 1b(ii) and Figure S2a(ii). Reflectance data from various damselfish species with yellow or other colours are shown in Figure S2a(iii and iv). Spectral reflectance of the remaining species measured in this study are presented in Figure S3. Trophic groups of damselfish species are listed in Table S2; trophic groups of the other reef fish families are listed in Table 1.

In summary, our results revealed that: Not only benthic herbivory, but also orange/red signalling colours correlate with enhanced red sensitivity, i.e., enhanced *lws* expression in damselfishes. This trend was also found when going beyond the damselfish radiation: in pairs of zooplanktivorous and benthic herbivorous species from five other reef fish families, short- versus long-wavelength shifted opsin combinations co-varied with zooplanktivory and

herbivory, respectively. Also, orange/red colours were only found in the herbivorous species. Red sensitivity, modelled from a damselfish's visual perspective, is likely to provide a functional benefit in detecting algae and orange/red colour signals. And, ancestral state reconstructions discovered that sensitivity to long wavelength (increased *lws* expression) emerged in association with algivory (omni- and herbivory) only, and that orange/red colour signals emerged after the transition to algal feeding had occurred.

1) In accordance with previous studies (Luehrmann et al., 2018; Stieb et al., 2016, 2017, 2019), our transcriptomic analysis revealed that all damselfish species expressed *sws1*, *rh2a*, and *rh2b*, with only a subset of species additionally expressing *sws2b* and/or *lws*. Here, gene expression of longer wavelength (as indicated by increased *lws* expression) opsins is associated both with feeding ecology and colouration (Table S6 summarizes PGLS results):

(a) Proportional double cone expression of *lws* (simplified as *lws* expression from now on) is associated with benthic herbivory. Among damselfishes, expression of *lws* was significantly associated with feeding ecology after correction for multiple testing, though it had a strong phylogenetic dependence ($\lambda = 0.795$, $F_{1,35} = 11.43$, $P = 0.001787$). Zooplanktivores showed little to none, omnivores some, and herbivores substantial levels of *lws* expression [Figure 1a(i), S4a].

(b) Proportional double cone expression of *rh2a* (simplified as *rh2a* expression from now on) and *lws* co-vary with orange/red colouration. We further found a significant positive correlation between the expression of *lws* and orange/red, but not yellow colouration (orange/red: $\lambda = 0$, $F_{1,23} = 7.456$, $P = 0.01192$; yellow: $\lambda = 0$, $F_{1,23} = 1.13$, $P = 0.2987$) and a very strong negative correlation between the *rh2a* expression and orange/red, but not yellow colouration (orange/red: $\lambda = 0$, $F_{1,23} = 15.12$, $P = 0.0007411$; yellow: $\lambda = 0$, $F_{1,23} = 0.5968$, $P = 0.4477$) [Figure 1a(ii), S4b].

(c) *Lws* opsin gene expression is highest in orange/red omni- and herbivores. When food and orange/red colouration were tested together, only a trend ($\lambda = 0$, $F_{2,22} = 3.836$, $P = 0.03297$) for a correlation between orange/red colouration and *lws* expression was observed [Table S6; Figure 1a(iii)]. When species with and without orange/red colouration were compared within their respective trophic groups, orange/red omnivores showed a significant *lws* upregulation relative to other omnivores ($\lambda = 0$, $F_{1,10} = 16.36$, $P = 0.002346$) [Figure 1a(iii)], while a trend towards higher expression was also noticeable for orange/red herbivores ($\lambda = 0$, $F_{1,4} = 8.083$, $P = 0.04672$) [Figure 1a(iii)].

While most species within pairs from the other reef fish families (labrids, butterflyfishes, angelfishes, blennies, and surgeonfishes) expressed a core set of the same three opsin genes, they did differ in the expression of additional genes resulting in one species having a short- and the other species having a long-shifted expression profile (Table 1 and S3). Here, short- versus long-wavelength shifted opsin combinations co-vary with feeding ecology and fish colouration as they do for damselfishes. Across families, benthic herbivores had long-wavelength-shifted visual systems with pronounced *lws* expression: in butterfly- and angelfishes, only herbivores expressed *lws*; in blennies, surgeonfishes, and labrids, the herbivores expressed higher levels of *lws* compared to the zooplanktivores. In contrast, for all reef fish families, zooplanktivores expressed a shorter shifted single cone opsin combination compared to herbivores. Importantly, for most within-family species contrasts, the herbivorous species showed an orange/red colouration, while the zooplanktivorous species had no orange/red colouration.

2) By computing a damselfish visual system, our visual models (results listed in Table S7) show that adding a LWS pigment enhances the differentiation of algae and orange/red colouration. Most algae colours against different backgrounds [for algae and background reflectance, see Figure 1b(i) and Figure S2a(i)] increased in contrast (in terms of JND) when

going from potentially tri- to tetrachromatic vision [Figure 1c(i) and Figure S2b(i)]. When comparing fish colours [for orange/red-coloured damselfish species, see Figure 1b(ii) and Figure S2a(ii); for non-orange/red species, see Figure S2a(iii and iv)], adding the fourth spectral channel mostly improved the contrast of red [(Figure 1c(ii) and Figure S2b(ii)], but not of yellow or other colours [Figure S2b(iii and iv)], against the ambient illuminant and against other fish colours. In the case of the red anemonefish, *Amphiprion biaculeatus*, it also increased its contrast against the host anemone [Figure S2b(ii); for anemone reflectance, see Figure S2a(i)].

3) Reconstructions of character states across the damselfish phylogeny using the package corHMM revealed ancestral states and possible evolutionary relationships among *lws* expression (given as proportional double cone expression of *lws*), trophic ecology and orange/red colour signalling (Figure 2, S5-S7). AICc results for competitive models based on distinct transition rate arrangements (ER – all rates are equal, ARD – all rates differ, and SYM – rates for forward and reverse transitions are same) are given in Table S8. ER was the best fit for our traits in all ancestral reconstructions, except the reconstruction with trait-dependence between trophic ecology and *lws* expression, which was better described by ARD (Figure 2 and S5). Reconstructions with less support are shown in Figures S6 and S7.

Ancestral reconstructions of each individual trait (*lws* expression, trophic groups, and orange/red colour) are shown in Figure S5 and display similar patterns as the trait-dependent analyses. Ancestral state reconstruction of proportional double cone *lws* expression as a continuous trait (using BaysesTrait) did differ depending on the prior used (Figure S8). When assuming an uninformative prior (uniform distribution with lower and upper bounds of 0 and 31%, respectively), the deepest nodes suggest very high upregulation of *lws* expression, which tends to decrease toward the tips (Figure S8a). A more constrained prior (exponential distribution with mean equals to 1), produced more similar results to the corHMM

reconstructions, with most ancestral nodes showing very low levels of *lws* expression (Figure S8b).

When looking at the evolutionary interactions between trophic groups and *lws* expression, basal nodes showed the lowest *lws* expression (0-0.9%), and this low *lws* expression was either combined with omnivory or herbivory (Figure 2a). Among basal nodes, omnivory was often, herbivory sometimes and zooplanktivory never the dominant state. As omni- and herbivory both include algal feeding, algal feeding with highest probability precedes the emergence or increase of *lws* expression. Importantly, when reconstructions of internal branches become less ambiguous, we note that zooplanktivory always retained zero to the lowest *lws* expression levels (e.g., zooplanktivorous species of the genus *Chromis* or the genus *Neopomacentrus*). Herbivory, on the other hand, tends to correlate with higher *lws* expression as seen, for example, in the herbivorous Stegastinae, and in several species within Pomacentrinae like *Dischistodus prosopotaenia* and *Dischistodus perpicillatus* as well as *Pomacentrus wardi*, *Pomacentrus australis*, *Pomacentrus adelus* and *Pomacentrus chrysurus, Chrysiptera brownrigii* and *Neoglyphididon nigroris*. However, internal branches being dominated by omnivory can either retain a low *lws* expression (e.g., within the genus *Dascyllus*) or be associated with an increased *lws* expression (e.g., within Amphiprionini).

Important for the association of orange/red colour signals with *lws* expression is the fact that basal nodes and most internal branches have no orange/red colour signals and no or very low *lws* expression (Figure 2b). Transitions to orange/red colouration are accompanied with an increase in *lws* expression at the tips of several species within Pomacentrinae (e.g., in *Pomacentrus moluccensis, Chrysiptera cyanea* and *Neoglyphidodon nigroris*) and in basal nodes of Stegastinae and Amphiprionini that include the species with the highest *lws* expression among all damselfishes. Prominently, orange/red colour signalling is always associated with a

rise in *lws* expression, but a rise in *lws* expression is also seen in algal feeding species that lack orange/red colour signalling (like the herbivorous *Dischistodus prosopotaenia* as well as *Pomacentrus wardi*, *Pomacentrus australis*, and *Pomacentrus chrysurus*). However, no clear pattern can be observed as to whether increased *lws* expression precedes or follows the emergence of orang/red colour signals.

Finally, for the interaction of trophic groups and orange/red colour signals, it is most notable that orange/red colour signals are missing at basal nodes and evolve exclusively on branches reconstructed as subtending algal feeding clades (like the herbivorous Stegastinae and omnivorous Amphiprionini) or first emerge on the tips of the tree, i.e., in present-day algal feeding species (Figure 2c). In contrast, omni- and herbivory evolved independently from and before orange/red colouration across the damselfish phylogeny. This is also true for the clades Amphiprionini and Stegastinae in which orange/red colour signals occur early in internal branches but an omnivorous or herbivorous state, respectively, lacking orange/red signals still precedes the origin of the colour signals.

Discussion

The mechanisms for shifting spectral sensitivities to long wavelengths in fishes are diverse. Long-wavelength shifts can be achieved by changes in LWS sequence structure (Carleton et al., 2005), a chromophore shift (A1 to A2), or may include yellow/orange carotenoid-based optical filters (de Busserolles et al., 2015; Douglas et al., 1998; Kondrashev, 2008; Saarinen et al., 2012; Siebeck et al., 2003; Terai et al., 2017). This study highlights that in major reef fish families, long-wavelength shifts in visual sensitivity have been achieved by turning on or increasing proportional double cone *lws* opsin gene expression (simplified as *lws* expression from now on). An increase in LWS opsin protein implies that more photoreceptors

across the retina increase their photon catch at long (red) wavelengths, making them overall more sensitive to red. While visual models (this study) provide theoretical support that adding LWS increases the fish's ability to detect red signals, only behavioural experiments can directly provide evidence for colour vision and response to red signals.

Here we show that in damselfishes and likely in several other reef fish families, *lws* gene expression and by virtue long-wavelength-(red)-sensitivity is related to benthic herbivory and orange/red colouration. Moreover, orange/red colour signals only evolved in association with algal feeding. For a summarizing figure with all tested coral reef fish species and traits, see Figure 3.

Opsin gene expression tuned to feeding ecology*: lws *associated with benthic herbivory

Studying 39 damselfish species with representatives from four of the five subfamilies, we found that *lws* expression was highest in herbivores feeding on benthic algae followed by omnivores. In contrast, almost no expression was found in zooplanktivores [Figure 1a(i)]. Corresponding patterns were also found across phylogenetically diverse reef fish families (Figure 3). Moreover, across these families, either parts or the entire visual palette/repertoire was shifted towards longer wavelengths in benthic herbivorous species. Examples are a shift from *sws1* (347–383 nm) to *sws2* (397–482 nm), *sws2b* (395–425 nm) *to sws2a (439–475 nm),* or *sws2aα* (448 nm) to *sws2aβ* (457 nm) in single cones, and/or a shift from *rh2b* (472-484 nm) to *rh2a* (518–528 nm) or *rh2* (452–537 nm) to *lws* (501–573 nm) in double cones (Table 1 and S3) [λ_{max} gained from (Bowmaker, 2008; Cortesi et al., 2015; Hofmann & Carleton, 2009a; Musilova et al., 2021; Spady et al., 2006; Yokoyama, 2008)].

Sensitivity to either end of the visible spectrum of the light has previously been associated with foraging in various vertebrates. For example, UV-sensitivity in fishes is generally thought to enhance the efficiency of predating on UV-absorbing or scattering zooplankton

(Browman et al., 1994; Loew et al., 1993; Rick et al., 2012). Although not all zooplanktivorous reef fishes expressed *sws1*, they always expressed shorter-shifted single cone opsins (i.e., *sws2α* instead of *sws2β* or *sws2βα* instead of *sws2αα*) compared to herbivorous species (Table 1 and S3).

In terrestrial forest species, long-wavelength-sensitivity improves distinguishing between items of brown forest litter (Lythgoe & Partridge, 1989). Similarly, long-wavelength-sensitivity in primates helps increasing the contrast of yellow and orange fruit against green foliage (Osorio & Vorobyev, 1996; Regan et al., 1998). Among insects, a shift to longer wavelength sensitivity is associated with shifts in diet and body colour (Martínez-Harms et al., 2012; van der Kooi et al., 2021). In the marine environment, green and brown algae broadly reflect in the green-red range (~500–650 nm) with a secondary, chlorophyll-generated peak in the far-red [> 700 nm, Figure 1b(i) and Figure S2a(i)]. Simulations show that to see algae against a typical coral reef background, a reef fish may get away with two photoreceptors sensitive at 510 and 580 nm λ_{max} (Marshall et al., 2003). Herbivorous damselfishes seem to approximate this optimum with a RH2A and LWS-based pigment pair that are sensitive to 520 and 560 nm λ_{max}, respectively (Marshall et al., 2006; Stieb et al., 2016). Indeed, damselfish visual models, including those constructed here, also indicate that the addition of an LWS-pigment increases the detection of algae against diverse backgrounds [Figure 1c(i) and Figure S2b(i)]. However, long-wavelength-sensitivity may not only facilitate algal detection but may be beneficial for benthic feeding more generally. For example, *lws* is highly expressed in several blennies that feed predominantly on detritus or benthic algae (Cortesi et al., 2019).

Opsin gene expression tuned to fish colouration*: *lws associated with orange/red

In addition to feeding ecology, we found that orange/red colouration in damselfish was associated with a change in double cone gene expression; *lws* was increased while *rh2a*

reduced in damselfish species [Figures 1a(ii) and S2, Table S6]. Similarly, for butterfly- and angelfishes, *lws* was only expressed in species with orange/red colouration and *lws* expression was enhanced in the orange-tailed bicolor blenny, *Ecsenius bicolor*, and the ringtail surgeonfish (it has a yellow/orange blotch behind the eye), *Acanthurus blochii* (Figure 3, Table 1).

The function of orange/red colouration for intraspecific communication and mate choice is known for some freshwater and marine fishes. In Lake Victoria cichlids, different light regimes are associated with divergent visual sensitivities of *lws*, ultimately contributing to speciation through sensory drive based on sexually selected (red) male breeding colouration (Maan & Sefc, 2013; Seehausen et al., 2008). Across guppy populations, *lws* coding and expression are associated with red colouration in males (Sandkam et al., 2015), and knocking out *lws* in medaka (*Oryzias latipes*) reduces grey-orange colour distinction (Kamijo et al., 2018). All labrids investigated so far express *lws* with some species expressing up to five copies of the gene [Table 1 and Cortesi et al. (2021); Musilova et al. (2019); Phillips et al., (2016)]. Since many labrids show complex patterns of colouration dominated by green, red, and far-red components (Marshall et al., 2003) and some species display sexual dimorphism in (red) colouration (Hodge et al., 2020), long-wavelength-sensitivity in this group is likely to facilitate intraspecific communication at close-range (Marshall, 2000a; Michiels et al., 2008).

In the reef environment, orange/red can become highly conspicuous against water or coral backgrounds near the surface, at least for short-distance viewing, but may help for camouflage against the background or within a group of similarly coloured fishes (Cortesi et al., 2015; Marshall, 2000b; Marshall et al., 2003a; Marshall et al., 2019). Our damselfish visual models show that long-wavelength-sensitivity (given by the expression of LWS) increases the colour contrast of orange/red colours when perceived against other fish colours, the ambient illuminant, or specific backgrounds [Figure 1c(ii) and Figure S2b(ii)], which may be

advantageous for species recognition and/or mate choice. Colour patterning has been shown to often be a trustworthy signal for intraspecific communication (Marshall et al., 2006; Sibeaux et al., 2019). *Amphiprion biaculatus* and *Chrysiptera cyanea* both exhibit colour patterns visualized with increased contrast when adding long-wavelength sensitivity [Figure 1c(ii) and Figure S2b(ii)]. The orange tail of male *Chrysiptera cyanea* has been related to sexual selection and mating success in this species (Wacker et al., 2016). Anemonefish have a striking appearance with white stripes and orange/red body colourations and show the highest *lws* expression among damselfishes (Table S2, Figure 3). Having a cone type containing a long-wavelength-sensitive pigment combined with a relatively short single cone photoreceptor (Stieb et al., 2019) seems to increase the colour contrast of the striped pattern and thus may be important for conspecific detection and recognition. But seen from a distance through the eyes of (perhaps relatively red-blind) predators, orange-to-red anemonefishes may blend in with their anemone, which is also often red or orange [Figure S2a(i)].

The evolution of red sensitivity, body colouration, and herbivory in reef fish

Supporting a scenario in which fish vision, colouration, and trophic ecology are co-evolving, we discovered repeated evolutionary shifts in spectral sensitivity to longer wavelengths (given by increased *lws* expression) that appeared to be adaptations to algal feeding. Further, we found that orange/red colouration in damselfish is only present in benthic algal feeding species [Figure 1a(iii), Figure 2c]. Where ancestral nodes are reconstructed as lacking orange/red colouration, such colouration may then have evolved independently in several extant omnivorous or algivorous species, as well as in the ancestors of the herbivorous Stegastinae and omnivorous Amphiprioninae (Figures 2c). However, there are ancestral state reconstructions that are less clear than others (e.g., basal nodes in Figure 2a and 2c) or result in different scenarios of transition rates (Figures 2a and 2c compared to Figures S7a and S7c).

The sensory drive hypothesis (Endler, 1992; Ryan & Cummings, 2013) suggests that long-wavelength-sensitivity evolved initially in adaptation to feeding strategy, but is now also co-evolving with red social signals. It has been suggested, for example, that in primates and guppies, a visual system tuned to find red coloured food is likely to have pre-dated female preference for males displaying red colouration (Endler, 1983; Fernandez & Morris, 2017; Grether et al., 2005; Rodd et al., 2002). In damselfishes, long-wavelength-sensitivity is evolutionarily associated with orange/red colouration and algal feeding, but never with zooplanktivory. Also, long-wavelength-sensitivity can evolve in the absence of orange/red colouration but never in the absence of algal feeding, and benthic algal feeding species have the highest *lws* expression. Finally, orange/red colouration is exclusively emerging with and most likely after algal feeding. Hence, ancestral state reconstructions suggest that a sensory bias is at play with the following scenario: (1) feeding on algae (but not zooplankton) favours (2) the evolution of long-wavelength-sensitivity (higher *lws* expression) that (3) is now leading to the evolution of orange/red social signals, which in turn causes selection for (4) increasing *lws* expression even further.

Conclusions

Our results indicate that variation among coral reef fishes in sensitivity to light of longer wavelengths may have evolved as an adaptation to feeding ecology and that, once evolved, it subsequently facilitated the evolution of orange/red fish colour elements, possibly facilitating social signalling. Our study reveals how the evolutionary feedbacks between variation in ecology, here foraging strategy, and social signalling, mediated by variation in visual sensitivity, might help explain the astonishing colour diversity of reef fishes. We call for more behavioural

studies to test the predictions that our hypothesis makes with regard to feeding performance and behavioural interactions.

Data accessibility

Newly generated opsin gene sequences ([dataset Stieb 2021] OK350470- OK350614; see Table S6) and retinal transcriptomes ([dataset Stieb & Cortesi, 2021] Bioproject PRJNA747115: SAMN21876388-SAMN21876434) have been deposited in GenBank. The R-code and all spectral measurements used for visual models, alignments and R-code used for ancestral state reconstructions and corresponding figures as well as alignments of newly sequenced opsin genes have been deposited to Dryad (doi:10.5061/dryad.qv9s4mwgf).

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Author contributions

SMS, KLC, FC, OS and JM designed the study. SMS, FC and JM caught the specimens, and took body reflectance measurements. SMS and FC prepared retinal tissue for RNA sequencing and analysed the data. OS, LJdQ and SMS performed ancestral state reconstructions. KLC and SMS performed visual models. All authors contributed to writing the manuscript and approved the final version.

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Table 1: Proportional single and double cone opsin expression (mean and standard deviation per species) in 10 reef fish species newly sequenced in this study or taken from [1](Tettamanti et al., 2019), [2](Phillips et al., 2016) (for individual expression data, see Table S3). Also indicated are trophic groups (H: herbivore, P: zooplanktivore, D: detrivore, Bi: benthic invertebrates, G: gastropods) as per [3](www.fishbase.org), [4](www.australianmuseum.net.au/fishes), [5](Randall, 1985), [6](Steene, 1978), [7](Cvitanovic et al., 2007), [8](Wilson, 2000), [9](Choat et al., 2004), [10](Sandin & Williams, 2010), [11](Allen et al., 2003), [12](Cowman et al., 2009), and yellow (>500nm) and/or orange/red reflectance (>520nm) gained from this study or adapted from [13](Marshall, 2000b), and [14](Cheney & Marshall, 2009).

		proportional single cone expression				proportional double cone expression			overall expression		trophic	yellow	orange/red
	n	*sws1*	*sws2b*	*sws2aα*	*sws2aβ*	*rh2b*	*rh2a*	*lws*	cone	rod	group	(>500nm)	(>520nm)
Chaetodontidae – Butterflyfishes													
Chaetodon ulietensis	3	-	-	24.4 ± 4.1	75.6 ± 4.1	4.8 ± 2.7	79.8 ± 11.0	15.5 ± 11.9	15.9 ± 7.3	84.1 ± 7.3	H, (BI)[3, 4]	yes[13]	yes[13]
Hemitaurichthys polylepis	3	-	86.9 ± 1.5	13.1 ± 1.5	-	56.9 ± 5.6	43.1 ± 5.6	-	14.5 ± 4.6	85.6 ± 4.6	P[3, 4, 5]	yes	no
Pomacanthidae – Angelfishes													
Centropyge bicolor	3	-	-	-	100	1.4 ±1.4	94.4 ± 3.5	4.1 ± 2.0	15.3 ± 7.4	84.7 ± 7.4	H[3, 6], BI [4]	yes[13]	yes[13]
Genicanthus watanabei	2	-	-	34.3 ± 20.5	65.7 ± 20.5	52.8 ± 4.1	45.9 ± 4.6	-	30.8 ± 4.8	69.2 ± 4.8	P[3, 6]	yes	no
Blennidae – Blennies													
Ecsenius bicolor	3	(<0.01)	-	16.4 ± 7.0	83.5 ± 7.0	0.6 ± 0.1	57.5 ± 10.2	41.9 ± 10.2	37.1 ± 22.4	62.9 ± 22.4	H: D[7,8]	no[14]	yes[14]
Meiacanthus atrodorsalis	3	0.9 ± 1.4	-	98.3 ± 1.2	0.8 ± 1.1	60.2 ± 10.5	22.4 ± 3.7	17.4 ± 7.0	53.3 ± 15.6	46.7 ± 15.6	P, (BI)[3, 4]	yes[14]	no[14]
Acanthuridae – Surgeonfishes													
Acanthurus blochii	4	-	-	100	-	2 ± 0.5	56.9 ± 1.3	41.1 ± 1.3	16.1 ± 4.5	84 ± 4.5	H[3, 5, 7]	yes	yes
Naso brevirostris	3[1]	-	100	-	-	56.1 ± 1.9	38.1 ± 1.6	5.8 ± 0.4	17.3 ± 1.8	82.7 ± 1.8	P[3, 9]	yes	no
Labridae – Labrids/Wrasses													
Chlorurus sordidus	1[2]	-	100	-	-	-	53.4	46.6	22.4	77.6	H[3, 10] H: D[10]	yes	yes
Bodianus mesothorax	1[2]	7.6	92.4	-	-	41.3	43.4	15.3	42.7	57.3	P[11], G[12]	N/A	N/A

Figure 1 Relationship of damselfish *lws* expression to trophic groups and colouration. a) *Lws* expression (n = 39; given as proportional double cone expression) is significantly upregulated in omnivores and herbivores (algae feeders) compared to zooplanktivores (i), and in species with orange/red colouration compared to species not reflecting in red (ii). Within trophic groups, proportional double cone *lws* expression is significantly increased in red compared to non-red omnivores and shows a trend to be increased in red compared to non-red herbivores (iii). Boxplots: box indicates Q2 and Q3, with the line indicating the median; whiskers indicate Q1 and Q4 of the data, with dots marking outliers. Statistics: phylogenetic generalized least squares regression (PGLS); Bonferroni corrected (n = 4), p * < 0.0125, ** < 0.0025, *** < 0.00125. (b) Normalised spectral reflectance measurements of algae [more algae examples shown in Figure S2a(i)], and various backgrounds [coral, rubble, sand, more backgrounds shown in Figure S2a(i)] [data modified from Cortesi & Cheney (2010) and Marshall (2000b)] (i), and body parts of one exemplary damselfish species reflecting in orange/red (> 520nm) [more species reflecting in orange/red shown in Figure S2a(ii)] (ii). (c) Visual models show that adding LWS (going from a trichromat with SWS1, RH2B and RH2A to a tetrachromat with SWS1, RH2B, RH2A and LWS expressed) increases the colour contrast (higher colour JND values) of algae viewed against diverse backgrounds (i) [see also Figure S2b(i) for more visual models], and of red body colouration viewed against other body colours and against ambient illuminant (ii) [see also Figure S2b(ii) for more visual models]. Note that adding LWS with a range of 525 - 565 nm λ_{max} is a result from different (co)-expression scenarios: LWS may either be coexpressed with another double cone opsin (RH2s) or solely expressed.

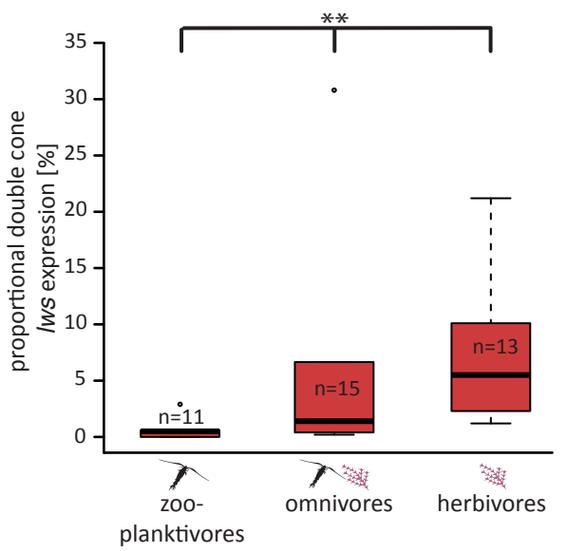
Figure 2 Ancestral reconstructions under a trait-dependent scenario between trophic groups and *lws* expression (measured as proportional double cone expression) (a), orange/red colouration and *lws* expression (b), and trophic groups and orange/red colouration (c). Reconstructions were performed using the R-package corHMM and highest support was given by ARD – all rates differ (a), and ER – all rates are equal (b and c). Phylogenetic tree was reconstructed using a maximum-likelihood damselfish phylogeny (n = d39) sourced and modified from *The Fish Tree of Life* (Rabosky et al., 2018); support values are given in (a). Note that no genetic markers were available for *Parma unifasciata*. To still be able to place it in the damselfish phylogeny, markers from *Parma oligolepis* were used. *Tips with missing data had their state reconstructed.

Figure 3: Coral reef fish phylogeny [maximum-likelihood damselfish tree (n = 39) sourced and modified from *The Fish Tree of Life* (Rabosky et al., 2018) in addition to rag 1 nuclear marker for *Pomacentrus wardi* (MW631536.1) *,* and maximum-likelihood tree for remaining reef fish families based on the rag1 nuclear and the 12s mitochondrial markers (AF108534.1 AF108541.1, EU167838.1, DQ533195.1, KY020162.1, LC049707.1, KC623832.1, LC049704.1, EF530093.1, FJ616393.1, LC069562.1, AY279584.1, JX189878.1, LC499313.1)] showing trophic groups [zooplanktivores, herbivores and omnivores (feeding on zooplankton and algae); note that species might also feed on other items as presented in detail in Table S2 for damselfish and Table 1 for remaining coral reef fish families], the presence of yellow (>500nm) and/or orange/red reflectance (>520nm) (note that no value means no yellow or orange/red colouration), and the expression of *lws* (presented as proportional double cone expression, note that no value means no expression). For references on opsin expression,

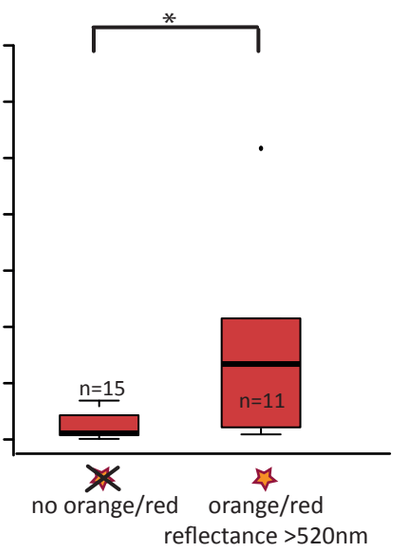
reflectance data and trophic groups for damselfishes, see Table S2 and S3, for other reef fish species, see Table 1.

a. Relation of proportional double cone *lws* expression to

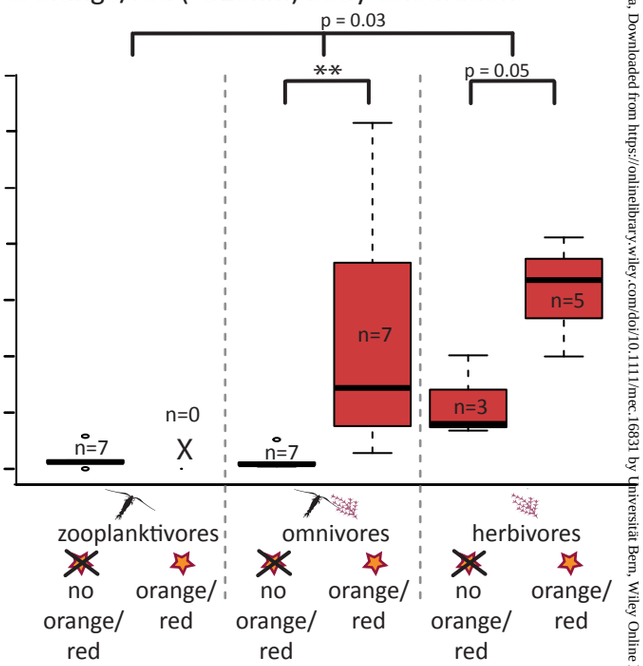
(i) trophic groups



(ii) orange/red (>520nm) body colouration

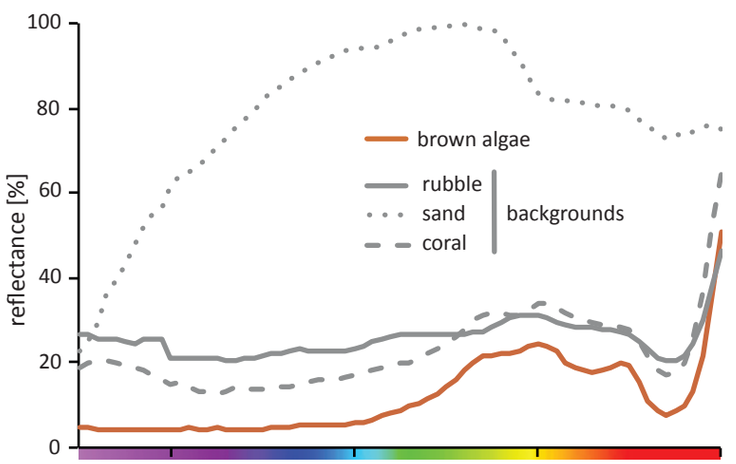


(iii) trophic groups & orange/red (>520nm) body colouration

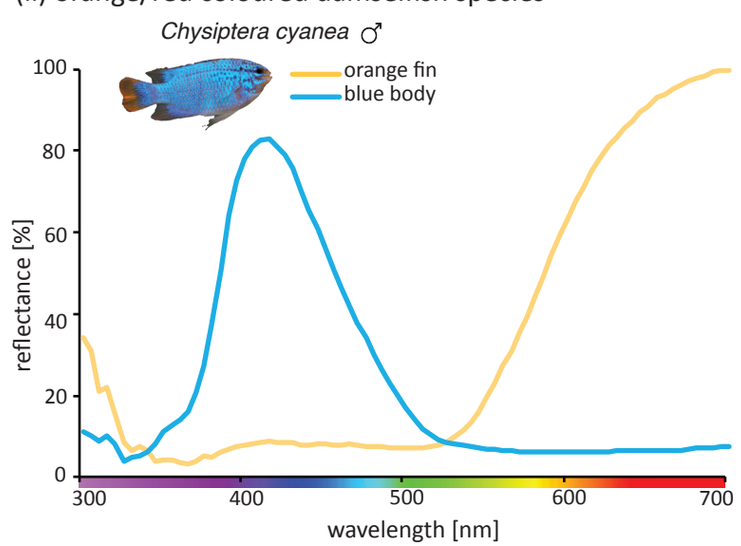


b. Spectral reflectance of

(i) algae and backgrounds

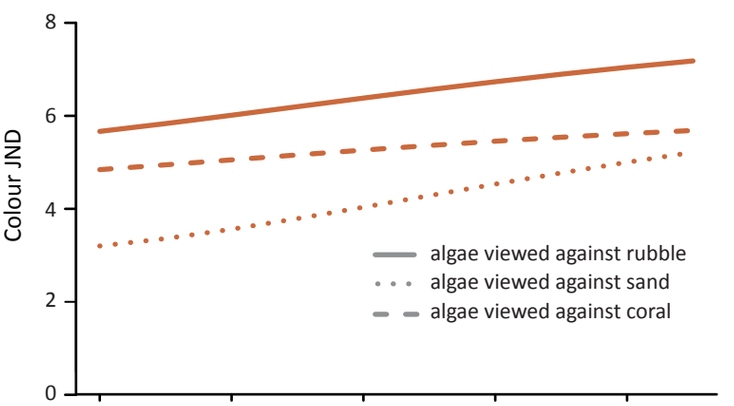


(ii) orange/red coloured damselfish species

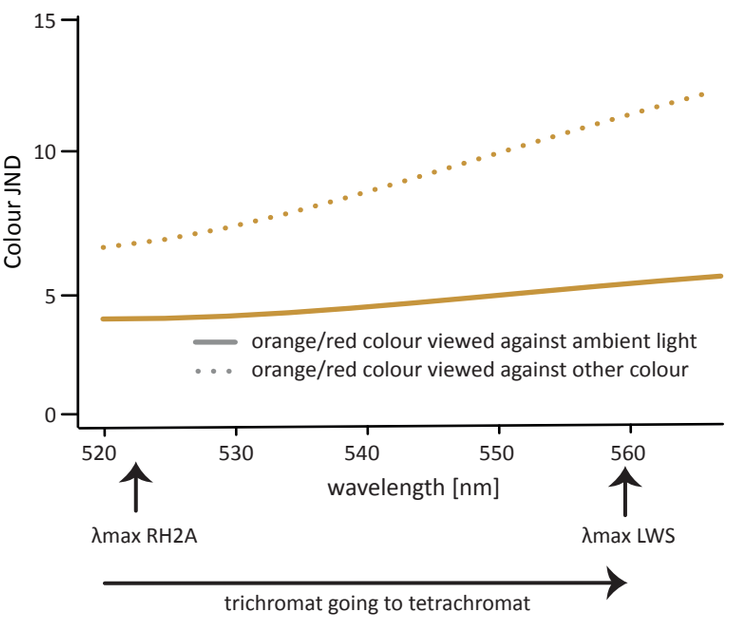


c. Visual modeling: does LWS help detect

(i) algae? yes

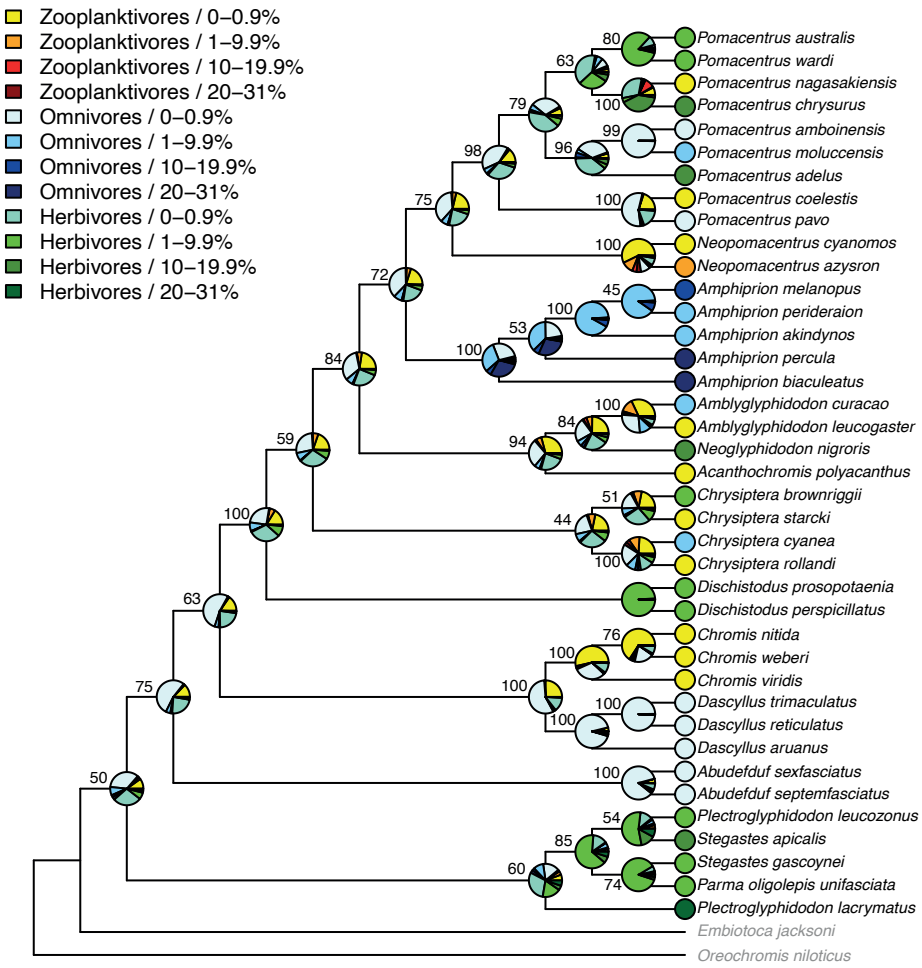


(ii) orange/red colouration (>520nm)? yes

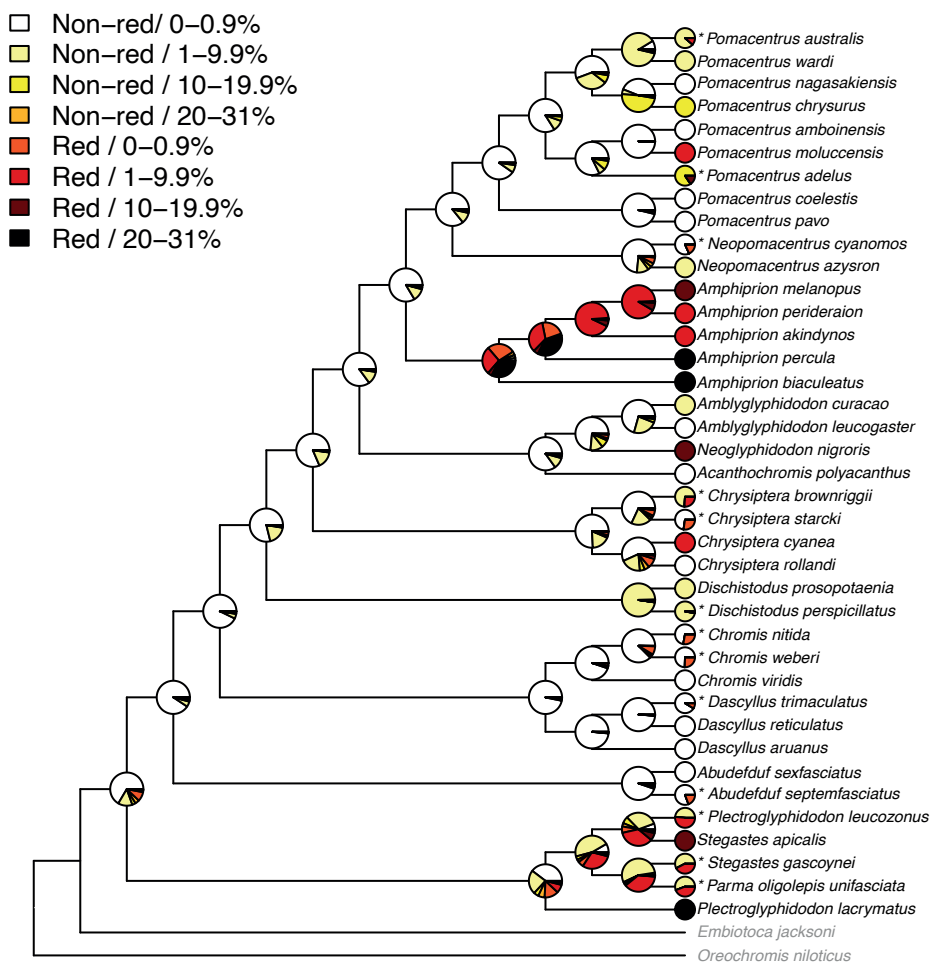


Reconstruction of transition rates for interaction between character states

a. Trophic groups & Proportional double cone *lws* expression



b. Orange/red colouration & Proportional double cone *lws* expression



c. Orange/red colouration & Trophic groups