Anemonefish have finer color discrimination in the ultraviolet

Authors
Laurie J. Mitchell\textsuperscript{1,2,3}\textsuperscript{*}, Amelia Phelan\textsuperscript{1}, Fabio Cortesi\textsuperscript{2}, N. Justin Marshall\textsuperscript{2}, Wen-sung Chung\textsuperscript{2}, Daniel C. Osorio\textsuperscript{4}, Karen L. Cheney\textsuperscript{1}

Affiliations
\textsuperscript{1} School of Biological Sciences, The University of Queensland, Brisbane, QLD 4072, Australia.

\textsuperscript{2} Queensland Brain Institute, The University of Queensland, Brisbane, QLD 4072, Australia.

\textsuperscript{3} Marine Eco-Evo-Devo Unit, Okinawa Institute of Science and Technology, Onna son, Okinawa 904-0495 Japan.

\textsuperscript{4} School of Life Sciences, University of Sussex, Brighton, BN1 9QG, United Kingdom.

Correspondence
E-mail: laurie.mitchell@oist.jp
Abstract

In many animals, ultraviolet (UV) vision guides navigation, foraging, and communication, but few studies have addressed the contribution of UV vision to color discrimination, or behaviorally assessed UV discrimination thresholds. Here, we tested UV-color vision in an anemonefish (*Amphiprion ocellaris*) using a novel five-channel (RGB-V-UV) LED display designed to test UV perception. We first determined that the maximal sensitivity of the *A. ocellaris* UV cone was at ~386 nm using microspectrophotometry. Three additional cone spectral sensitivities had maxima at ~497, 515, and ~535 nm, which together informed the modelling of the fish’s color vision. Anemonefish behavioral discrimination thresholds for nine sets of colors were determined from their ability to distinguish a colored target pixel from grey distractor pixels of varying intensity. We found that *A. ocellaris* used all four cones to process color information and is therefore tetrachromatic, and fish were better at discriminating colors (i.e., color discrimination thresholds were lower, or more acute) when targets had UV chromatic contrast elicited by greater stimulation of the UV cone relative to other cone types. These findings imply that a UV component of color signals and cues improves their detectability, that likely increases the salience of anemonefish body patterns used in communication and the silhouette of zooplankton prey.
Many animals have ultraviolet (UV) sensitive (<400 nm) photoreceptors [reviewed by (1, 2)], and UV-vision contributes to behaviours including foraging (3, 4), celestial navigation (5, 6), mate selection (7–11), individual recognition (12), and aggressive displays (13, 14). But partly due to the technical challenge of producing UV-stimuli (15), few studies have tested its contribution to color discrimination, and it is unclear how UV sensitivity compares to that of visible range photoreceptors.

In vertebrates, cone photoreceptors in the retina mediate color vision. Most teleost fishes, reptiles, and birds have two morphological cone types: single cones and double cones. The latter is formed by the fusion of two cone cells (16–18). Photoreceptor spectral sensitivity is primarily determined by its photopigment(s) comprised of a G-coupled receptor opsin protein bound to a carotenoid-derived (Vitamin A1 or A2) chromophore, and can be modified by light-filtering in the eye (19, 20). Color vision can be defined as the ability to discriminate lights by their spectral composition regardless of their relative intensity. This requires a comparison of signals from different spectral types of photoreceptors, typically by chromatic opponent neurons (21, 22). To fully encode spectral information, an eye with n spectral receptor types require at least n-1 opponent mechanisms plus an achromatic (or luminance) mechanism (21). For example, birds are proposed to have UVS – SWS, SWS – MWS/MWS + LWS, and MWS – LWS (UVS, ultraviolet-sensitive; SWS, short-wavelength-sensitive; MWS, medium-wavelength-sensitive; and LWS, long-wavelength-sensitive) opponent systems (23, 24). The chromaticity (roughly hue and saturation) of a color can be defined by its location within an n-1 dimensional color space whose cardinal axes correspond to the activation of opponent signals (21), such as the 2-dimensional Maxwell’s triangle used for humans (25). Compared to human trichromatic color vision, adding a fourth cone allows tetrachromacy which expands the color gamut and can be represented by a tetrahedral color space (26, 27).
The presence of n-cone types does not mean that an animal has n-chromatic vision. For example, if the outputs of two spectral types of cones are summed, or a given cone type does not contribute to color discrimination. Thus, it has recently been found that the forward-looking retina of larval zebrafish (*Danio rerio*) forms a ‘strike zone’ (28). This zone contains a high density of enlarged UVS cones, which are suited to detecting zooplankton prey but make little contribution to chromatic opponency (28, 29). More generally, it is unknown whether UV chromatic contrast provides any major benefit to color discrimination in fishes, or in many animals for that matter, despite the presence of ample UV in many environments, including coral reefs, streams, grasslands, and forests (2, 30, 31).

Here we tested the UV and non-UV color vision capabilities of the false clown anemonefish, *Amphiprion ocellaris* (Figure 1A). Anemonefishes (genus, *Amphiprion*) are renowned for their symbiosis with sea anemones (Actiniaria spp.) (32) and their strict social hierarchy, which is determined by sex and body size (33, 34). Recently, anemonefishes were shown to have seven cone visual pigments, six of which are expressed in the adult *A. ocellaris* retina, where they produce four spectral types of cone (35). However, the exact cone spectral sensitivities in *A. ocellaris* were unknown, but estimated wavelengths of maximal sensitivity ($\lambda_{\text{max}}$) according to their assigned opsin(s) indicate one type of single cone containing a mix of UV-sensitive/SWS1 (est. 368 – 370 nm $\lambda_{\text{max}}$) and violet-sensitive/SWS2 (est. 406/407 nm $\lambda_{\text{max}}$) opsins, and three types of double cones with blue-green RH2 opsins (RH2B est. 497 nm $\lambda_{\text{max}}$ and RH2A est. 516-523 nm $\lambda_{\text{max}}$) in two separate members, and a third type with a mix of RH2A and LWS opsins (est. 560/561 nm $\lambda_{\text{max}}$) (33).

The retinas of anemonefish are unusual from that of more commonly studied teleosts, such as goldfish (*Carassius auratus*) and zebrafish (both in the family, Cyprinidae), which have two single cones maximally sensitive to UV (SWS1 pigment, 355 – 365 nm $\lambda_{\text{max}}$) and violet-blue (SWS2 pigment, 415 – 450 nm $\lambda_{\text{max}}$), and double cones with separate ‘red’ (LWS) and
‘green’ (RH2) members (36, 37). As in larval zebrafish, anemonefish UV/violet cones are most abundant in a region of highest acuity or area centralis, but here, rather than being for prey detection, UV-sensitivity is thought to enhance the chromatic contrast of their UV-orange and UV-white colors for intraspecies communication (38) (Fig 1 A-C). Underwater UV-photography revealed that the light ‘white’ bars of A. percula, a sister species of A. ocellaris, have strong UV-contrast against adjacent dark ‘orange’ skin and the background of sea anemone tentacles (Fig 1A). Spectrometry of A. ocellaris skin confirms that both colors reflect UV: UV-white and UV-orange (Fig 1B and C). The natural background of anemonefishes is the tentacles of a host sea anemone (e.g., Stichodactyla gigantea) whose pronounced UV absorption below ~350 nm (Fig 1D) coincides with the peak reflectance of anemonefish UV/white (Fig 1B), resulting in a dim appearance of the anemone compared to the fish’s bright UV/white bars (Fig 1A).

Fig. 1. Colors of anemonefish skin and host sea anemone tentacles. (A) Underwater photographs of anemonefish taken in the human visible (RGB) spectrum and UV-only.
Normalised spectral reflectance shown for *A. ocellaris* (B) UV-white bars, (C) UV-orange, and (D) sea anemone (*Stichodactyla gigantea*) tentacles. Lines represent mean spectral reflectance (*n* = 3), and shaded areas depict s.e.m. bounds. Note: images in (A) depict *A. percula*, the sister species of *A. ocellaris*, which shares an almost identical appearance and spectral reflectance.

To investigate the colour discrimination capabilities of anemonefish to UV and non-UV signals, we first confirmed the sensitivity of all four cones using microphotospectrometry, and then conducted a behavioral experiment to examine how the three types of double cones, and the UV cones, contribute to color discrimination. Specifically, we asked whether discriminability was better for colors with higher UV chromatic contrast than those without (i.e., non-UV colors). To do this, we used nine different sets of test colors produced by a five-channel (RGB-V-UV) LED display (15) customized to the *A. ocellaris* visual system. The contribution of photoreceptors to color vision were evaluated by the Receptor Noise Limited (RNL) model (19), which fits the psychophysical data of many species by assuming that color discrimination is set by chromatic opponent mechanisms whose performance is limited by noise arising in the photoreceptors (39, 40). The discrimination threshold (1ΔS) corresponds to a minimally distinguishable difference between two colors (19). Departures from RNL model predictions can provide evidence for post-receptoral mechanisms affecting color vision, including lateral inhibition (i.e., excited neurons suppressing neighboring neuron activity) (41), chromatic opponent and higher-level processes such as color categorization (42, 43).
Results

Spectral sensitivities of A. ocellaris

We first measured the lens transmittance and photopigment spectral sensitivities of A. ocellaris. The lens absorbed some UV wavelengths, with 50% transmission (T50) at 322, 340 and 341 nm in the three fish measured (mean T50 = 334 nm; Fig 2A). Microspectrophotometry (MSP) of the cone pigments found one type of single cone (U) with a maximal wavelength sensitivity (λmax) in the UV (mean λmax: 386 ± 5.0 nm; n = 4, N = 4 fish; Fig 2A). The three additional spectral cone types are double cones with λmax values of about 497 nm (M1), 515 nm (M2) and 531/538 nm (L) (Fig 2A). These photoreceptors could be assigned specific visual pigments according to their previously identified opsin protein component (Fig 2A) (35). One type of rod photoreceptor was present (mean λmax value = 502 ± 4.0 nm; n = 7 cells, N = 4 fish; Supplementary Figure 1). All double cone absorbance spectra fitted a retinal (Vitamin A1) derived chromophore visual pigment template, while the single cone absorbance was likely due to the coexpression of UV- and violet- sensitive visual pigments, as has previously been shown to be the case in A. ocellaris (35). In vivo photoreceptor spectral absorbance curves were given by the product of lens transmission and photoreceptor spectral absorbance measurements (Fig 2A). Two measurements hinted at a third MWS (M3) double cone type (508/509 nm λmax, N = 1 fish; Supplementary Figure 1), but the spectral overlap with the M2 cone meant it is unlikely that M3 makes a separate contribution to color vision. Discrimination threshold estimates for target sets that included its input or substituted it with M2 provided a worse overall fit (see Supplementary Figure 2).
Fig. 2. Cone spectral sensitivities of *A. ocellaris* and the LED display used for testing the discriminability of nine different sets of colors. (A) Normalised average lens transmission of *A.*
ocellaris (N=3), and absorbance curves (in arbitrary units) fitted to the averaged absorbance spectra of the cones (N=9). In parentheses are the identities of (co-)expressed visual opsins that principally determine the spectral sensitivities of A. ocellaris cones (35): ‘SWS1/SWS2’ = UV/short-wavelength-sensitive 1 and violet/short-wavelength-sensitive 2 (U) (λ max: 386 ± 5.0 nm, n = 4, N = 4 fish), ‘RH2B’ = medium-wavelength-sensitive rhodopsin-like 2B (M1) (λ max: 496.7 ± 3.0 nm, n = 3, N = 3 fish), ‘RH2A’ = medium-wavelength-sensitive rhodopsin-like 2A (M2) (λ max: 515 ± 2.1 nm, n = 6, N = 3 fish), and ‘RH2A/LWS’ = long-wavelength-sensitive (L) (λ max: 531/538 nm, n = 2, N = 1 fish). Thin broken lines depict the upper and lower bounds of the standard deviation for lens transmittance. Supplementary Figure 1 gives individual absorbance spectra for all photoreceptors. (B) RGB-V-UV LED display with an example stimulus. Fish were trained to discriminate a target dot from distractor dots and peck it to receive a food reward. (C) Tetrahedral receptor space of A. ocellaris showing the nine tested color sets (and achromatic point in black), and (D) the normalized spectral radiance of nominally saturated example targets and the average grey distractor. Bar plots depict the relative receptor stimulation (quantum catches) evoked by colors or grey for A. ocellaris against the PTFE screen of the LED display. ‘U’ = ultraviolet-sensitive, ‘M1’ = medium-wavelength-sensitive 1, ‘M2’ = medium-wavelength-sensitive 2, and ‘L’ = long-wavelength-sensitive cone types. Note, color names have no implication for their appearance to the fish. Image credit: A. ocellaris taken by Valerio Tettamanti.

**Color discrimination thresholds**

Anemonefish were trained to peck a rewarded target pixel that differed in chromaticity from grey distractor pixels (Fig 2B) (44, 45). The grey level of the distractor pixels was varied to prevent the use of brightness information. We then measured the fishes’ accuracy for nine sets of target
colors (Fig 2C, D). Each set lay on a line radiating from the central achromatic (grey) point in the
anemonefish color tetrahedron, so that they varied in saturation but not hue. Four of the nine
colors we collectively refer to by ‘UV colors’ (UV, UV-blue, UV-red, and Violet-green; Fig 2C)
had increasing UV/violet LED emission, while the five remaining (Blue, Green, Red, Orange, and
Purple; Fig 2C) were ‘non-UV colors’ without increasing UV saturation. Four of the test colors
also excited spectrally non-adjacent receptors more than intermediate receptors (Violet-green,
UV-Red, Orange, and Red), and so were non-spectral colors, i.e., the human equivalent of
‘purple’ (e.g., [27]), which could not be matched by a mixture of a monochromatic light with grey
(Fig 1D).

We conducted a total of 3921 test trials (N = 11 fish, n = 9 color sets, N = 84 colors, n = 8
– 17 trials per target, mean = 10 trials). Our (50%) probability of correct choices for determining
the threshold is more accurate than more commonly used (70% to 75%) in paired choice tests
(46), as the experiment involved a choice of 1 out of 38 pixels. Color differences were specified
by the RNL model for anemonefish (where 1 ΔS is defined as the discrimination threshold).

Because noise levels in A. ocellaris cones are unknown, we found the best fit of the RNL model
to the data (47, 48). This best fit predicted receptor noise of σ = 0.11 in the UV (single) cones,
and σ = 0.14 for the three types of double cone, which gave the following thresholds for the nine
test colors: blue (mean ± s.e.m = 1.5 ± 0.1 ΔS), purple (1.6 ± 0.07 ΔS), green (1.2 ± 0.1 ΔS), red
(1.0 ± 0.04 ΔS), orange (0.8 ± 0.07 ΔS), UV-blue (0.8 ± 0.07 ΔS), UV (0.8 ± 0.09 ΔS), Violet-
green (0.4 ± 0.02 ΔS), and UV-red (0.9 ± 0.05 ΔS). These values compare to a receptor noise
estimate of σ = 0.05 in another reef fish [43, 45, 49]. Because vectors are origin bound, the
vectors representing colors in anemonefish color space at 180° with each other can be considered
as complementary pairs, and when mixed in equal proportions, should be achromatic (50). Based
on the approximately polar azimuth angles among some color thresholds (φ ± 180; Fig 3B, C), we
were able to identify two pairs of what are likely complementary colors: 1) Green (φ = 297°) and UV (φ = 127), and 2) Purple (φ = 128) and Violet-green (φ = 298).

To examine the possibility that only a subset of cones contributes to color vision in anemonefish and one cone type might only serve achromatic tasks (i.e., trichromacy), we also compared the tetrachromatic model fit to that of four possible trichromatic models, where the input from one cone type was systematically dropped. None of the trichromatic models predicted the discrimination of all test colors from grey to an expected 1 ΔS (two-way ANOVA, range of estimated mean difference = 0.101 to 0.40, F = 18.1, all p < 0.05; Fig 4A). The tetrachromatic model (UM1M2L) had the closest fit (two-way ANOVA, range of estimated mean difference = -0.006, F = 18.1, p = 0.910; Fig 4A), which was followed by the UM1L model missing the M2 cone (two-way ANOVA, estimated mean difference = 0.101, F = 18.1, p = 0.046; Fig 4A).
**Fig. 3.** Anemonefish color discrimination thresholds and their hue angles in tetrahedral space. (A) Color discrimination thresholds shown as a function of the proportion of correct choices by anemonefish for targets with a range of chromatic contrasts (ΔS). Error bars denote 0.95 CIs. Discrimination thresholds are values calculated per fish (Fish ID) and are demarked by.
vertical lines. Each plotted point represents the mean proportion of correct choices from one fish
(n = 8 to 17 trials). Note, the x-axis has been truncated to ≤4.5 ΔS for presentation purposes. For
curves showing all the data see Supplementary Figure 3. (B) Relative positions of equal
discriminability marked by intersecting lines in the receptor space of *A. ocellaris* for target sets of
colors that varied in chromaticity (ΔS) in four principal directions representing the relative
stimulation of the ultraviolet- (U), medium 1- (M₁), medium 2-wavelength sensitive (M₂), and
long-wavelength sensitive (L) photoreceptors. Star symbols and circles adjacent to color sets
indicate whether UV receptor input was needed for discriminating threshold colors from grey as
determined by a positive elevation vector angle (Θ >0), and which colors potentially form
complementary pairs according to polar azimuth vector angles (approximately φ ± 180°)
respectively. Intersecting lines and adjacent numbers refer to the average discrimination threshold
of each target set. The black dot represents the average grey distractor spectra which the receptor
space is centered on (i.e., the origin). The inset is the reverse view of the receptor space to show
the Orange and Violet-green sets clearly. ΔS was calculated using a receptor noise standard
deviation (σ) of 0.11 for single cones and 0.14 for double cones. (C) Summary of hue angles
calculated in degrees using *XYZ* Cartesian coordinates according to noise-corrected threshold ΔS
values. Elevation ‘Θ’ describes the ‘vertical’ angle (-90° ≤ Θ ≤ 90°) of threshold vector position
above (positive) or below (negative) the plane of chromaticity facilitated by the double cones
(M₁, M₂, L), where a 90° direction corresponds to the U cone vertex. Azimuth is the ‘horizontal’
angle (0° ≤ φ ≤ 360°) from the L cone at 0°/360° with the U cone axis normal to the equatorial
plane. See Methods for hue angle equations and Supplementary Table 1 for relative quantum
catches of cones and *XYZ* Cartesian coordinates for vectors. UV colors (i.e., those produced by
higher UV/violet LED emission) are shown in bold.
**Fig. 4.** Discrimination threshold fit comparisons among different RNL model scenarios and color sets. (A) Comparison between tetrachromatic (UM\(_{1}\)M\(_{2}\)L) and trichromatic modelling in the accuracy of predicting color discrimination thresholds. For each model, the individual thresholds (ΔS) per color set were subtracted from the expected threshold value of 1 ΔS (demarked by the broken line), and the overall average difference was calculated. Visual models either included input from all cones (tetrachromatic) or dropped input from one of the four cone types (trichromatic): UV-sensitive cone ‘U’, medium-wavelength sensitive cone 1 ‘M\(_{1}\)’, medium-wavelength sensitive cone 2 ‘M\(_{2}\)’, or long-wavelength sensitive cone ‘L’. Error bars indicate upper and lower 0.95 confidence limits. ‘ns’ indicates \(p > 0.05\), ‘*’ indicates \(p = 0.05 > 0.001\), and ‘**’ indicates \(p <0.001\). All \(p\)-values were calculated by two-way ANOVA with Fisher’s Least Significant Difference test. See Supplementary Figure 4 for a multiple model comparison of individual color discrimination thresholds. (B) Pairwise comparisons of *A. ocellaris* discrimination thresholds (ΔS) between color sets with or without UV contrast. Letters denote statistical significance (\(p < 0.05\)) between groups. Grey bars indicate mean values while error bars represent ± 1 s.e.m. The broken, horizontal line demarks the RNL model assumed threshold (ΔS = 1). ΔS were calculated using a receptor noise standard deviation (σ) of 0.11 for single cones and 0.14 for double cones. Statistical significance was calculated using a Linear Mixed Effects Model (LMM) with multiple paired comparisons. See Supplementary Data 1 for full LMM results.
Lower discrimination thresholds induced by UV

Colors with positive UV chromatic contrast were identifiable by a positive elevation angle ($\Theta > 0$) and had significantly lower thresholds ($\text{UV} = 0.8 \Delta S, \Theta = 81^\circ$; Violet-green $0.4 \Delta S, \Theta = 53^\circ$; UV-red $0.9 \Delta S, \Theta = 71^\circ$ and UV-blue $0.8 \Delta S, \Theta = 10^\circ$; Fig 3B and 4B) than three of the five with negative UV chromatic contrast ($\Theta < 0$) (Blue $1.5 \Delta S, \Theta = -45^\circ$; Green $1.2 \Delta S, \Theta = -78^\circ$, and Purple $1.6 \Delta S, \Theta = -58^\circ$; Fig 3B and 4B) (LMM, all paired comparisons $p < 0.05$; Fig 4B). Red ($1.0 \Delta S, \Theta = -4^\circ$), Orange ($0.8 \Delta S, \Theta = 6^\circ$), and UV-blue thresholds had low UV chromatic contrast and were located near the equatorial plane ($\Theta = 180^\circ \pm 10.0^\circ$) (Fig 3B). Both identified pairs of complementary colors had positive and negative UV chromatic contrasts, where the former had significantly lower discrimination thresholds for UV/Green (LMM, estimate $\Delta S_{\text{UV-Green}} = -0.40, \text{se} = 0.11, z = -3.22, p = 0.012$; Fig 4B) and Violet-green/Purple (LMM, estimate $\Delta S_{\text{Violet-green-Purple}} = -1.21, \text{se} = 0.11, z = -11.0, p < 0.0001$; Fig 4B).

Psychometric functions of the nine color sets differed significantly, with blue, purple, green, UV, and UV-blue sets having more gradual functions (Fig 3A) than orange, red, violet-green, and UV-red sets (binomial generalized linear mixed-effects model/GLMM, all $p \leq 0.01$; for GLMM results and pairwise comparisons see Supplementary Data 1). A more gradual incline was indicative of a higher error rate for relatively high $\Delta S$ targets, and a higher $\Delta S$ asymptote for discrimination performance. The differences in these functions were not attributable to the order in which colors were presented and followed no obvious pattern. Because some color sets were introduced later than others in the experiment (refer to Methods for the introduced order of color sets), this may have contributed to discrepancies in psychometric functions and thresholds. To determine whether the presentation-order of color sets affected discrimination performance, we reassessed two fish per color set at the end of the experiment. We found that all color sets had either no change or only minor differences in discrimination thresholds ($\text{range} = 0 - 0.4 \Delta S$ shift, ...
mean ± s.e.m. = 0.1 ± 0.02 ΔS shift; see Supplementary Figure 5 for psychometric curve comparisons).

**Discussion**

We have shown that the anemonefish UV cone which has a peak sensitivity (\(\lambda_{\text{max}}\)) at 386nm contributes to tetrachromatic color vision with the three spectral types of double cones (\(\lambda_{\text{max}}\) 495nm, 515nm and c. 535nm). This suggests that the cones containing all four combinations of the main pigment types (SWS1/SWS2, RH2B, RH2A, RH2A/LWS) (35) contribute separately to color vision, and that the UV cone has a comparatively high sensitivity. These conclusions are striking given the evidence for co-expression of the RH2A and LWS pigments in the same double cone member, and of the SWS1 and SWS2 pigments in the single cones (35). There is experimental evidence for tetrachromacy in a few other species, including goldfish (51, 52) and chicken (53), but to our knowledge this is the first demonstration by testing the minimally saturated hues that can be distinguished from grey, which clearly suggests that anemonefish have a 3-D color space. This advance was made possible by our five channel LED display customized to anemonefish vision (Fig 2B). The display also allowed us to show that anemonefish can discriminate a wide variety of non-spectral colors from grey, which would be highly difficult with monochromatic test lights (15, 27).

A major finding was the importance of the UV receptor in anemonefish color vision. By providing a rare comparison of discrimination thresholds among relatively unexplored UV-
regions of animal color space, we show that positive UV chromatic contrast improved the
discrimination of four of the nine color sets (UV, UV-blue, Violet-green, and UV-red). The
estimated noise ($\sigma = 0.11$) in the UV cone was lower than in the three double cones ($\sigma = 0.14$).
Furthermore, fitting the RNL model revealed that the discrimination thresholds for these colors
were substantially lower than three of the nine color sets (Blue, Green, and Purple) which had
negative UV chromatic contrast, i.e., the relative stimulation of the single cone was lower than all
three double cones. This difference in discriminability was most convincingly shown by the
complementary color pairs UV/Green and Violet-green/Purple, which are theoretically equidistant
in $\Delta S$ from grey ($50$) but had large disparities in psychophysical threshold distances. This
asymmetry between color discrimination thresholds cannot be directly attributed to noise in the
early stages of the visual pathway such as photoreceptor noise or chromatic opponent neurons in
the retina. One possibility is that activation of the UV receptor suppresses noise in the visual
pathway or enhances the saliency of colors for anemonefish. The high sensitivity to violet-green,
which was found in all six of the fish tested is consistent with the heightened saliency of this
color.

**Positive UV chromatic contrast benefits anemonefish color discrimination**

UV-contrast sensitivity has been reported in multiple animals, such as common goldfish (41, 52),
zebrafish (54), budgerigars (*Melopsittacus undulatus*) (24), and hummingbirds (*Selasphorus
platycercus*) (27). Previously, *A. ocellaris* demonstrated an ability to detect UV targets (15),
which did not strictly require color vision, and like the larval zebrafish (28), one anemonefish
(*Amphiprion akindynos*) has been found to have UV cones most concentrated in the frontal visual
field (i.e., the centrotemporal retina) (38). It is unknown whether *A. ocellaris* also has a peak UV
cone abundance in its centrotemporal retina; however, this seems quite likely given that they
share multiple key features with their larger cousin *A. akindynos* (38), including similar cone
spectral sensitivities and photopigment diversity (35), and common ecological aspects (life
history, sea anemone habitat, social hierarchy, diet). In the zebrafish, UV cones seem to make
little input to color vision (28), while in anemonefish UV cones make a disproportionately strong
input. This strong UV contribution to color discrimination suggests that anemonefish color
patterns are highly salient to conspecifics and could benefit UV-signaling used in social
communication (55).

Color preferences and ecological significance of UV

An innate (or learnt) UV-preference in A. ocellaris could explain their acute discrimination,
where a higher attention to UV may be influenced by the color of their food or conspecifics. The
white bars of A. ocellaris appear to have strong UV contrast against adjacent dark orange skin and
sea anemone tentacles, as was shown to be the case in A. akindynos (38). Indeed, juvenile
anemonefish (A. akindynos) have a distinct UV coloration shown to signal subordinance (55).
Other suggested functions of anemonefish color patterns include warning coloration (56),
camouflage (56), species recognition (57), and mate recognition (58). Future studies on the
function of anemonefish coloration should include the UV and not restrict their spectral analysis
to longer wavelengths in the human visible spectrum. Moreover, attention should be paid to how
the appearance of these colors change with increasingly common sea anemone bleaching events
and the implications this may have for signaling efficacy. Another potential basis for a UV
preference in anemonefish could be to detect the UV-contrast of their common prey
(zooplankton) which can either scatter or absorb UV (28, 59, 60). Larval anemonefish (A.
biaxuleatus) can solely rely on UV illumination (peaking at 365 nm) for detecting prey (61),
which might be attributed to an achromatic UV channel as in larval zebrafish (28). More
generally, highly sensitive UV vision could help maintain the detectability of UV signals in
habitats with reduced UV photon availability (e.g., deep water, dense foliage cover, heavy
overcast), as suggested in goldfish (62). Anemonefishes typically inhabit shallow coral reefs about 1 – 15 meters) where UV is abundant; however, a similar mechanism for facilitating acute UV discrimination might exist in other marine fishes and benefit UV vision in deeper habitats even beyond 100 meters and at a maximum of 200 meters, where a conservative estimate indicates enough UV photons could sustain the visual sensitivity of surface-dwelling fishes (63).

An aversion towards blue and purple might explain their high discrimination thresholds (~1.5 ΔS) and variable psychometric functions. This variation may indicate individual differences in learning (e.g., categorical perception), or differences in attentiveness and motivation. Guppy (Poecilia reticulata) also poorly discriminate purple due to possible neophobia (64), and triggerfish have an aversion towards blue (65), which was explained based on its common use as an aposematic color, signaling unpalatability in reef invertebrates such as nudibranchs (66) and blue-ringed octopus (67).

Conclusion

We found the discriminability of colors by anemonefish varied depending on UV contrast, where positive UV chromatic contrast had lower discrimination thresholds. Thus, it appears that UV vision can aid the detectability of fine-scale differences in chromaticity and might benefit the viewing of natural UV-reflective objects (e.g., colors of prey and conspecifics). This raises new questions regarding the identity and function of neural mechanism(s) in the retina and/or brain that facilitate highly sensitive UV color discrimination. Although we did not explicitly test the extent of cone opponency in anemonefish, their psychophysical thresholds were best explained when all four cone types contributed to color vision suggesting tetrachromacy. The enhancement of UV color saliency in anemonefish likely has an ecological benefit for communicative signaling using their UV colors.
Materials and methods

Animals and ethics statement

Anemonefish (A. ocellaris) (N = 20) were acquired from a local aquarium store (supplier Gallery Aquatica, Wynnum, 4178 QLD, Australia). We used N = 9 (female = 3, mean total length = 4.5 ± 0.5 cm; male = 6, mean total length = 3.5 ± 0.5 cm) for taking measurements of cone spectral sensitivities, and N = 11 (female = 11, mean total length = 4.9 ± 0.3 cm) for behavioral experiments. Fish were housed individually in recirculating aquaria (60x30x30 cm) at the Queensland Brain Institute at The University of Queensland, Australia, and all experiments were conducted in accordance with The University of Queensland’s Animal Ethics Committee guidelines under approval numbers QBI/304/16 and SBS/077/17. For anatomical measurements anemonefish were euthanized by immersion in MS222 (500 mg/L) for 10 minutes and subsequent decapitation.

Underwater photography and measuring skin spectral reflectance

Underwater photographs of anemonefish (Amphiprion percula) were taken at Horseshoe Reef near Lizard Island on the Great Barrier Reef (Figure 1A). Photographs in the visible (RGB) were captured using an Olympus (TG-5) camera (with Nikon 60 mm Micro), while a UV-converted Nikon (D810) fitted with both a short-pass filter (UG 11) and far-red filter took UV images.

Reflectance spectra of A. ocellaris white and adjacent orange skin patches surrounding the head/operculum region were measured for three wild-caught, captive males (n = 2) and a female (n = 1), for which no major differences were found. Three replicate measurements per skin area (within roughly 0.5 cm² – 1 cm²) were taken across a 300 – 700 nm range using a spectrometer (USB4000 Ocean Optics) with a pulsed Xenon lamp (PX-2 Ocean Optics), and a bifurcated 200µm fibre optic cable (Ocean Optics). Reflectance measurements were captured out-of-water at a 45° angle directly against skin and relative to a Spectralon 99% diffuse reflectance standard.
(Lab-sphere, North Sutton, NH, USA). During the measuring process fish were restrained in a seawater-soaked towel to protect their eyes from the bright light and skin from desiccation. Exposure out of water was limited to a maximum of 1-minute, and post-measurement all fish recovered and were returned to their aquarium. Sea anemone reflectance was based on averaged (n = 10) measurements recorded in-situ using a submersible spectrometer (USB2000 Ocean Optics) with a 100µm fibre and relative to a 99% Spectralon reflectance standard positioned next to the anemone. Reflectance measurements of tentacles used natural daylight as a light source at midday during non-overcast conditions at ~3m depth.

**Lens transmission of A. ocellaris**

For the measurement of lens transmission in *A. ocellaris*, the lenses (n = 3 fish) were isolated from the hemisected eyecup and rinsed in PBS to remove any blood and vitreous. Spectral transmission (300-800 nm) was measured by mounting the lens on a drilled (1.0 mm diameter hole) metal plate between two fibers (50, 100 µm diameters) connected to an Ocean Optics USB4000 spectrometer and a pulsed PX2 xenon light source (Ocean Optics, USA). Light spectra were normalized to the peak transmission value at 700 nm (68), and lens transmission values were taken at the wavelength at which 50% of the maximal transmittance (T$_{50}$) was attained (68, 69). No pigmented ocular media was observed.

**Photoreceptor spectral sensitivities of A. ocellaris**

The spectral absorbance of *A. ocellaris* photoreceptors were measured using single-beam wavelength scanning microspectrophotometry (MSP). This procedure followed that outlined in detail elsewhere [see (70, 71)]. In summary, small pieces (~1 mm$^2$) of tissue were excised from the eyes of two-hour dark-adapted fish, then immersed in a drop of 6% sucrose (1X) PBS solution and viewed on a cover slide (sealed with a coverslip) under a dissection microscope fitted with an
infra-red (IR) image converter. A dark scan was first taken to control for inherent dark noise of
the machine and a baseline scan measured light transmission in a vacant space free of retinal
tissue. Pre-bleach absorbance measurements were then taken by aligning the outer segment of a
photoreceptor with the path of an IR measuring beam that scanned light transmittance over a
wavelength range of 300-800 nm. Post-bleach scans were then taken after exposing the
photoreceptor to bright white light for 60 seconds, and then compared to pre-bleach scans to
confirm the presence of a labile visual pigment. Confirmed visual pigment spectral absorbance
data was then analyzed using least squares regression that fitted absorbance data between 30%
and 70% of the normalized maximum absorbance at wavelengths which fell on the long-
wavelength limb. The wavelength at 50% absorbance was then used to estimate the maximum
absorbance ($\lambda_{max}$) value of the visual pigment by fitting bovine rhodopsin as a visual pigment
template (72, 73). This absorbance curve fitting was performed in a custom (Microsoft Excel)
spreadsheet, where the quality of fit of absorbance spectra between A1- and A2-based visual
pigment templates was also visually compared. Individual scans were binned on their grouping of
similar ($\leq$10 nm difference) $\lambda_{max}$ values, and then averaged and reanalyzed across fish to create
mean absorbance spectra (Supplementary Data 3).

**LED display and stimuli calibration**

To display the visual stimuli in our behavioral experiments we used a five-channel RGB-V-UV
LED display [for full design details, see (15)]. Note, that the violet channel had an emission that
emitted into the UV and violet, where it had higher overlap with the absorption curve of the UV
cone but is referred to as ‘violet’ to distinguish it by name from the shorter wavelength ‘UV’
LED. The display itself was held within a waterproof, 3D-printed case, with a PTFE screen that
acted as a light diffuser. A wide gamut of colors could be produced by modulating the relative
outputs of each LED to color mix the different channels.
Target and distractor colors were chosen to test anemonefish color discrimination along nine different sets of chromatic contrast including: UV, UV-blue, blue, purple, green, violet-green, UV-red, orange, and red. We first measured the spectral radiance ($\mu$M cm$^{-2}$ s$^{-1}$ nm$^{-1}$) of pixel colors using a spectrometer (Ocean Optics USB4000) with a 200 $\mu$m diameter UV-VIS fiber calibrated against a deuterium-halogen lamp (Mikropak DH2000-DUV, calibrated by Ocean Optics). An RPA-SMA holder (Thorlabs) maintained the fiber 1 mm directly above a pixel at a 90° angle.

The stimulus used for measuring discrimination thresholds was inspired by the Ishihara test of color vision deficiency (44), as per (45). Anemonefish were trained to discriminate a target pixel which differed in chromaticity from distractors (43). We ran the LED display via a Python script that pseudo-randomly assigned a target color to one out of 38-pixel coordinates, while the 37 remaining pixels were assigned as grey distractors. Note, we did not utilize the full-sized display due to fish being afraid of the LED display and not trainable when using a larger stimulus.

### Color selection and stimuli design

To estimate anemonefish photoreceptor excitation for target and distractor colors, receptor quantum catches ‘$q$’, were first calculated for each stimulus, ‘$S$’ (i.e., target and distractor radiance spectra in $\mu$M/cm$^2$/s$^{-1}$/nm) viewed under well-lit conditions given by:

$$q_i = k_i \int_{300}^{700} R_i(\lambda) S(\lambda) \, d\lambda,$$

where $k$ is a scaling coefficient for receptor adaptation to the background ambient light, $S_b$:

$$k_i = \frac{1}{\int_{300}^{700} R_i(\lambda) S_b(\lambda) \, d\lambda}. \tag{2}$$

$R_i(\lambda)$ was the normalized spectral absorbance of a given receptor type ‘$i$’ ($i = U, M_1, M_2, L$) multiplied by lens transmittance, and ‘$\lambda$’ denoted wavelength (nm). $S_b(\lambda)$ was the spectral radiance of the PTFE display screen (between the pixels) with all LEDs turned off and measured from 5.0...
cm in the experimental tank (for background radiance spectra see Supplementary Figure 6). This approach allowed for modelling spectral emission (from LEDs) rather than more commonly calculated for reflectance, as per (74). Integration was performed across the visible spectrum (i.e., 300 – 700 nm for *A. ocellaris*). Relative cone quantum catches (see Supplementary Data 2 for absolute quantum catches) were used to plot color loci in a tetrahedral color space (26, 75).

Next, we calculated the chromatic contrast or color distances (\(\Delta S\)) of target colors relative to the average distracter spectra using the log receptor noise limited (RNL) model (19, 46). This provided a visual representation of how target and distractor colors appeared to *A. ocellaris*. The RNL model assumes: 1) that \(\Delta S\) equates to a psychophysical threshold of one just noticeable difference between two stimuli of a given contrast, 2) color vision is conveyed by chromatic mechanisms independent of achromatic visual processes, and 3) that \(\Delta S\) is determined by the differences in receptor stimulation (\(\Delta q_i\)) elicited by two viewed stimuli, that is only constrained by receptor noise levels (\(e_i\)) for each of the four cone classes \(i = 1, 2, 3, 4\) for the U, M1, M2, L cone classes), or alternatively, for three cone classes in trichromat models \(i = 1, 2, 3\) for the U/M1/M2/L cone classes).

The contrast (\(\Delta q_i\)) for each receptor channel was calculated by,

\[
\Delta q_i = \ln \frac{q_{\text{target}}}{q_{\text{average distractor}}} 
\]

(3).

In the absence of direct noise measurements for *A. ocellaris* cones, we estimated cone noise levels (\(e_i\)) by,

\[
e_i = \sqrt{\frac{\sigma}{\eta_i}} 
\]

(4),

where ‘\(\sigma\)’, the numerator of the Weber fraction in (48), or ‘\(\nu_i\)’ in (19) is the standard deviation of noise within a photoreceptor, and ‘\(\eta\)’ is the ratio of the given cone type. We initially assumed that cone noise levels in *A. ocellaris* were like those reported in triggerfish and used a \(\sigma\)-value = 0.05 (49, 65, 76). Based on the regular mosaic of one single cone surrounded by four double cones in
the *A. ocellaris* retina (35), we used a relative cone abundance ratio of 1 : 2 : 1 : 1 (U : M1 : M2 : L) for a tetrachromatic visual system and 1 : 2 : 2 for a trichromatic visual system. The double cone ratio for the tetrachromatic scenario was based on in-situ hybridization experiments showing that *A. ocellaris* in our aquarium system express RH2B (M1) in one double cone member and either RH2A (M2) or RH2A with LWS (L) in the second double cone member (38).

ΔS in tetrachromatic visual space was calculated by:

\[
\Delta S = \frac{(e_1 e_2)^2 (\Delta q_4 - \Delta q_3)^2 + (e_1 e_3)^2 (\Delta q_4 - \Delta q_2)^2 + (e_2 e_4)^2 (\Delta q_2 - \Delta q_1)^2}{(e_1 e_2 e_3)^2 + (e_1 e_2 e_4)^2 + (e_1 e_3 e_4)^2 + (e_2 e_3 e_4)^2}
\]  

(4),

and in trichromatic visual space was calculated by:

\[
(\Delta S)^2 = \frac{e_1^2 (\Delta q_3 - \Delta q_2)^2 + e_2^2 (\Delta q_3 - \Delta q_1)^2 + e_3^2 (\Delta q_1 - \Delta q_2)^2}{(e_1 e_2)^2 + (e_1 e_3)^2 + (e_2 e_3)^2}
\]  

(5).

Grey distractor spectra (N=13) were chosen to be <1 ΔS of the achromatic point of *A. ocellaris* and ranged between 0.3 ΔS to 0.8 ΔS of each other. To control for the potential use of achromatic (intensity) cues when discriminating targets, we selected 6 to 10 distractor greys (from the 13) per stimulus based on all four-cone quantum catches to encompass the highest and lowest target intensities (see Supplementary Data 2 for distractor quantum catches and LED input values).

We chose target colors that increased in ΔS away from grey distractors in nine different directions within *A. ocellaris* color space. Each color set comprised of between 6 to 11 colors that varied from a high-saturated target color which was deemed highly contrasting against the grey distractors, to a low-saturated target color which had low contrast (<1 ΔS) against the grey distractors. The number of contrasts per color set included: ten for UV; six for UV-blue and purple; nine for blue, green, red, and violet-green; 11 for UV-red; and seven for orange. Note, we refer to colors as either non-UV colors (e.g., blue, green, red, purple, orange) or UV-colors (e.g., UV-blue, violet-green, UV-red) based on whether contrast was induced predominantly by UV/violet LED input. Color sets were plotted as lines in the receptor noise corrected space of *A.
ocellaris using $\sigma = 0.11$ for single cones and $\sigma = 0.14$ for double cones. To visualize the
directionality of any asymmetrical contours in receptor space, an ellipsoid was manually created
using Adobe Illustrator (2020) to be approximately centered on the average grey distractor point
and intersect with the color discrimination thresholds.

Alternative models calculated $\Delta S$ values using more-conservative receptor $\sigma$-values
ranging from 0.05 to 0.15, to assess their fit with A. ocellaris behavioral thresholds. Lower single
cone noise ($\sigma = 0.04 - 0.11$) than double cones ($\sigma = 0.14$) was also modelled in case of different
inherent noise levels. Threshold predictions were also compared between models of trichromat
and tetrachromat vision in A. ocellaris, in case this could reveal any information on the
contribution of double cones to color vision. The closest model fit was determined based on
which had the smallest mean difference summed across all color lines from 1 $\Delta S$.

Note that it is likely that color thresholds are not set by noise in individual photoreceptors but
depend upon pooling of receptor signals across a fixed area of the retina (39). Consequently,
thresholds should depend upon the density of a given receptor type in the retinal cone array (39,
40). If relative receptor densities vary across the retina [e.g., (47, 77)] this might lead to variations
in color thresholds across the visual field.

Calculating hue angles

To gain additional information on the perceptual properties of colors including the type of UV
chromatic contrast and the presence of any complementary pairs, we calculated the elevation and
azimuth angles of vectors plotted in anemonefish color space which corresponded to the
psychophysical thresholds of color sets.

First, we converted $\Delta S$ to noise-corrected XYZ Cartesian coordinates using the ‘jnd2xyz’
function in the R package PAVO2 (85), which performs calculations based on the algorithm from
(Pike, 2012; Maia & White, 2018). This returned XYZ coordinates for color threshold vectors
representing the difference in receptor signal for the x axis \([L - (M_1 + M_2)]\), y axis \([M_1 - (M_2 + L)]\), and z axis \([U - (M_1 + M_2 + L)]\).

We then calculated elevation angle ‘\(\Theta\)’ by

\[
\Theta = \cos^{-1}\left(\frac{\alpha \cdot b}{|\alpha||b|}\right),
\]

where ‘\(\alpha \cdot b\)’ was the dot product of vectors ‘\(\alpha\)’ threshold XYZ position, and ‘\(b\)’ threshold XYZ position with the Z axis normal to the XY plane (i.e., zero), and |\(\alpha\)||\(b\)| were the magnitudes of each vector e.g., |\(\alpha\)| = \(\sqrt{a_x^2 + a_y^2 + a_z^2}\).

The product in units of radians was then converted to degrees and given appropriate signage to indicate relative position above (positive) or below (negative) the XY plane (\(-90^\circ \leq \Theta \leq 90^\circ\)).

Thus, giving an elevation angle where the vertex was at the grey point (origin) and position was relative to an (XY) equator to indicate UV receptor stimulation based on movement along the x axis.

The azimuth angle ‘\(\varphi\)’ was calculated by

\[
\varphi = \begin{cases} 
\arctan\left(\frac{x}{y}\right) & \text{if } x > 0 \\
\arctan\left(\frac{x}{y}\right) + \pi & \text{if } x < 0 \text{ and } y \geq 0,
\end{cases}
\]

negative azimuth angles were corrected by adding 360\(^\circ\), so that all values ranged between 0\(^\circ\) – 360\(^\circ\), where a bearing of 0\(^\circ\)/360\(^\circ\) marked the x axis describing L cone stimulation.

**Training and experiment**

During both training and the experiment, the LED display was presented in a section of the aquarium separated by a sliding, opaque door. This door was closed to kept fish from viewing the display while the stimulus was updated between trials, and only upon trial commencement was the door raised to allow fish to view and interact with the display. For both training and testing, a
morning (09:00 – 11:00) and afternoon (14:00 – 16:00) session were run, in which fish completed between 10 to 12 trials per day.

Fish were initially enticed to peck the LED display by presenting a pseudo-randomly chosen high contrast pixel (blue, green, red, or UV) with a small piece of prawn meat smeared on it. Over a week, we gradually reduced the size of the smeared food and transitioned towards a food-reward (Formula One Ocean Nutrition pellets) delivered by forceps when fish pecked the single target pixel. Once anemonefish readily approached and pecked at the display without enticement, we introduced the grey distractor pixels alongside the target pixel. Fish were only rewarded when they correctly chose/pecked the target color within 60 seconds. They were deemed to have reached the training criteria for the discrimination task after maintaining a correct choice probability of 0.75 over five consecutive sessions. 11 anemonefish met this criteria (mean number of training trials ± sd = 8.0 ± 4) and underwent experimental testing.

For testing, like training, fish were only rewarded for pecking the target pixel. Trials were terminated if fish made more than one incorrect choice or exceeded 60 seconds, upon which fish were returned to behind the divider (starting position) without reward. Note, because of the numerosity of pixels (n=38) per stimulus and the potential for distractions, each fish was permitted to make up to one incorrect choice per trial. For each trial, we recorded whether fish made a correct or incorrect choice, time (seconds) after fish entered through the door till target detection (i.e., latency), tested color set, and target ΔS.

Each color set was tested using five or six individual anemonefish that completed a minimum of eight trials per target color per assigned set (mean ± sd = 10 ± 1.0). Fish were divided into two groups assigned different color sets, including: 1) Fish IDs 19, 20, 33, 34, and 36 which were assessed in order of testing with green, UV, purple, and UV-red, and 2) Fish IDs 21, 22, 24, 31, 32, and 35 which were assessed in order of testing with blue, UV-blue, violet-green, red, and orange.
Between each trial the target pixel contrast was pseudo-randomly assigned from a list of LED intensity values for each color set. Throughout the experiment, we included control trials (n=10) to ensure that no other cues were created by the controller or code when choosing the target pixel, this determined the random chance of fish making a correct choice by displaying a target pixel of zero contrast (i.e., grey). In none of the control trials did fish correctly peck the control target.

To verify that differences in discrimination thresholds were not influenced by the order in which each of the color sets were tested, we reassessed each of the nine sets at the end of the experiment using two anemonefish from each group. Behavioral thresholds and psychometric functions from this secondary assessment were then compared with the primary assessment. Although we found evidence that experience effects had influenced the shape or incline of the psychometric function for some color sets (e.g., UV, blue, green, and UV-blue), there was none indicating that experience had contributed to differences in color discrimination thresholds that remained unchanged in the reassessment. The direction and size of differences among color discrimination thresholds did not vary systematically over the course of the study.

Software and statistical analyses
All statistical analyses and color modelling were conducted using the statistical program R (v. 4.0.2) (78). Color distances were calculated using the RNL model and plotted in a tetrahedral space using the package ‘PAVO 2’ (79). Discrimination thresholds were determined by the point at which fish had a 0.5 probability of making a correct choice approximately at the inflection or steepest point of a sigmoid curve fitted to the behavioral data (Supplementary Data 4) using the package ‘quickpsy’ (80). A binomial test to determine the minimum number of correct choices required to be significantly above random choice (48, 81) gave a minimum threshold of
discrimination at 20% of correct choices for our experiment (probability of one trial success
=0.03, n = 10 trials per target, \( p = 0.04 \)), indicating that our threshold criterion was conservative.

The effect of color sets on anemonefish discrimination thresholds was assessed using a
linear mixed-effects model (LMM) run using function ‘lmer’ in the package ‘lme4’ (82).

Individual threshold \( \Delta S \) value was treated as the response variable, color sets as the fixed factor,
and fish ID was the random effect. A post-hoc, pair-wise analysis controlled for multiple
comparisons of threshold \( \Delta S \) values across all possible combinations of color sets using
Bonferroni adjustment (p.adjust, R base package ‘stats’). Another set of LMMs tested the effect
of color on the duration (latency) of trials, where trial latency (in seconds) was the response
variable, color set, target \( \Delta S \) and the first-order interaction between color set and target \( \Delta S \) were
fixed effects, and fish ID was a random effect. Nine models were run (one per color set), where
the intercept was assigned to a specific color to enable pairwise comparisons with the others.

To test whether the color set influenced the relationship between \( \Delta S \) and the proportion of
correct choices (i.e., did color influence how fish responded to the test and resulting shape of the
psychometric curve), a generalised linear mixed effects model (GLMM) was run (82). Variables
included anemonefish choice (0 = incorrect, 1 = correct) used as a binomial response variable, \( \Delta S \),
color set and the first-order interaction between the two variables treated as fixed factors, and Fish
ID entered as a random effect. Model p-values were corrected for multiple comparisons via
Bonferroni adjustment (‘p.adjust’, base R package ‘Stats’).

Residual diagnostics were performed for LMMs and GLMM using the package
‘DHARMa’ (83), that checked the distribution of residuals and verified there were no dispersion
issues.

Analysis of anemonefish skin reflectance and target color emission were performed using
the ‘vismodel’ and ‘spec2rgb’ functions in the package ‘PAVO 2’ (79).
References


    Ancestral circuits for vertebrate color vision emerge at the first retinal synapse. Sci Adv.

    coexpression in retinal regions that view different parts of the visual field. Proceedings of
    the Royal Society B: Biological Sciences. 281, 20141980 (2014).

31. Y. Qiu, Z. Zhao, D. Klindt, M. Kautzky, K. P. Szatko, F. Schaeffel, K. Rifai, K. Franke, L.
    Busse, T. Euler, Natural environment statistics in the upper and lower visual field are

    anemones: a guide for aquarists and divers (Western Australian Museum, 1997).

33. H. W. Fricke, Mating System, Resource Defence and Sex Change in the Anemonefish


    Evolution of Ultraviolet Visual Opsins and Spectral Tuning of Photoreceptors in
    Anemonefishes (Amphirioninae). Genome Biol Evol. 13 (2021),

36. J. K. Bowmaker, "Evolution of visual pigments and photoreceptors" in Vision and visual
    pp. 63–81.

37. D. Endeman, L. J. Klaassen, M. Kamermans, Action Spectra of Zebrafish Cone


44. S. Ishihara, “Tests for color-blindness” (Tokyo, Japan, Hongo Harukicho, 1917).


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**Author contributions:**

- **Conceptualization:** KLC, LJM, FC, NJM, AP
- **Methodology:** KLC, LJM, FC, NJM, AP, WC
- **Investigation:** LJM, AP, WC, NJM
- **Visualization:** LJM, KLC, DCO
- **Supervision:** KLC
- **Writing—original draft:** LJM
- **Writing—review & editing:** LJM, KLC, DCO, FC, NJM, WC, AP

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