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RESEARCH ARTICLE

From crypsis to mimicry: changes in colour and the configuration of the visual system during ontogenetic habitat transitions in a coral reef fish

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ABSTRACT

Animals often change their habitat throughout ontogeny; yet, the triggers for habitat transitions and how these correlate with developmental changes – e.g. physiological, morphological and behavioural – remain largely unknown. Here, we investigated how ontogenetic changes in body coloration and of the visual system relate to habitat transitions in a coral reef fish. Adult dusky dottybacks, *Pseudochromis fuscus*, are aggressive mimics that change colour to imitate various fishes in their surroundings; however, little is known about the early life stages of this fish. Using a developmental time series in combination with the examination of wild-caught specimens, we revealed that dottybacks change colour twice during development: (i) nearly translucent cryptic pelagic larvae change to a grey camouflage coloration when settling on coral reefs; and (ii) juveniles change to mimic yellow- or brown-coloured fishes when reaching a size capable of consuming juvenile fish prey. Moreover, microspectrophotometric (MSP) and quantitative real-time PCR (qRT-PCR) experiments show developmental changes of the dottyback visual system, including the use of a novel adult-specific visual gene (*RH2* opsin). This gene is likely to be co-expressed with other visual pigments to form broad spectral sensitivities that cover the medium-wavelength part of the visible spectrum. Surprisingly, the visual modifications precede changes in habitat and colour, possibly because dottybacks need to first acquire the appropriate visual performance before transitioning into novel life stages.

KEY WORDS: Vision, Development, Gene duplication, Opsin, Colour change, Co-expression

INTRODUCTION

Throughout different life stages, animals may change their morphology, physiology and behaviour. Such ontogenetic variability often correlates with changes in diet, predation pressure or social status, which in turn are often associated with major habitat transitions (e.g. Booth, 1990; Childress and Herrnkind, 2001; Dahlgren and Eggleston, 2000; Evans and Fernald, 1990; Grant, 2007). However, despite a large body of literature on ontogenetic variability, studies looking at the development of multiple traits within individuals and how they relate to habitat transitions remain scarce. For example, it is well established that many animals alter some aspects of their visual system when shifting to novel habitats during ontogeny (Hunt et al., 2014), but how these changes interrelate with developmental changes in other traits such as body coloration remains poorly understood.

The complex and varied life histories of coral reef fishes make them particularly well suited for studies of the causes and consequences of ontogenetic habitat transitions. Most coral reef fishes experience a change in environment when moving from a pelagic larval phase in the open ocean to reef-associated juvenile and adult phases. In association with these migrations, the visual system as well as the pigmentation of the skin may be modified (Collin and Marshall, 2003; Evans and Browman, 2004; Evans and Fernald, 1990; Youson, 1988). Ontogenetic changes to the visual system are generally extensive and involve multiple morphological and/or physiological adaptations that cause a shift in peak spectral sensitivity (λ_{max}), which is used to adapt vision to varying light conditions or to solve novel visual tasks (Collin and Marshall, 2003; Evans and Browman, 2004; Evans and Fernald, 1990). This can be achieved through a gain or loss of different photoreceptor types in the retina (rod cells used for scotopic vision and/or various cone cell types used for photopic vision), qualitative and/or quantitative changes in the expression of visual pigments (opsins) within the photoreceptors themselves, or the use of different light-absorbing chromophores that bind to the opsin pigment: shorter wavelength sensitive vitamin A_1-based (retinal) or longer wavelength sensitive vitamin A_2-based (3,4-didehydroretinal) chromophores, respectively (Collin and Marshall, 2003).

Ontogenetic colour changes, in contrast, are less well documented in coral reef fishes, but generally include a change from transparent or silvery larval stages in the open ocean to often differently coloured juvenile and adult stages on the reef (Booth, 1990; Youson, 1988). While a transparent/silvery appearance may be used to camouflage fish larvae in open water light environments (McFall-Ngai, 1990), juvenile fish use their coloration for a number of strategies that facilitate access to food and reduce predation risks, including: aggressive mimicry, protective mimicry and several mechanisms of crypsis (Booth, 1990; Moland et al., 2005). When morphing into adults, however, many coral reef fishes become large enough or acquire appropriate defensive strategies to avoid predation. Coloration may from this point on also be used for sexual displays or during territorial behaviour (e.g. Booth, 1990; Kodric-Brown, 1998; Sale, 1993).

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List of abbreviations

dps	days post-settlement
JND	just noticeable difference
LWS	long-wavelength sensitive
LWS	long-wavelength sensitive opsin gene
MSP	microspectrophotometry
MWS	mid-wavelength sensitive
qRT-PCR	quantitative real-time PCR
RH1	rhodopsin 1 opsin gene
RH2Aα, *RH2Aβ*, *RH2B*	rhodopsin like 2 opsin genes
SL	standard length
SWS	short-wavelength sensitive
SWS1	short-wavelength sensitive 1 opsin gene
SWS2Aα, *SWS2Aβ*, *SWS2B*	short-wavelength sensitive 2 opsin genes
ΔL	luminance contrast
ΔS	chromatic colour contrast
λ_{max}	peak spectral sensitivity

The dusky dottyback, *Pseudochromis fuscus* Müller and Troschel 1849, is a small (maximum standard length ∼7 cm) predatory reef fish common to reefs throughout the Indo-Pacific ocean, including at our study site at Lizard Island, Australia, where both yellow and brown colour morphs can be found in sympatry (Munday et al., 2003). It has recently been shown that adult dottybacks flexibly adapt their colour from yellow to brown and vice versa to mimic the coloration of damselfishes (*Pomacentrus* spp.) in their surroundings (Cortesi et al., 2015a). By doing so, dottybacks gain multiple fitness benefits including an increase in predatory success on juvenile fish prey (aggressive mimicry) and habitat-associated crypsis (yellow morphs on live coral, brown morphs on coral rubble) that decreases predation risk (Cortesi et al., 2015a). It has also been shown that dottybacks, amongst other fish species, possess an additional gene that is part of a triplet of opsins responsible for visual discrimination in the short-wavelength 'violet – blue' region of the visible spectrum (*SWS2B*, *SWS2Aα* and *SWS2Aβ*; Cortesi et al., 2015b). Interestingly, in dottybacks, these opsins are spectrally distinct from one another and are differentially expressed between ontogenetic stages: larval dottybacks express *SWS2Aβ* (λ_{max}=457 nm), whereas adult dottybacks express *SWS2Aα* (λ_{max}=448 nm) and *SWS2Aβ* (Cortesi et al., 2015b). Finally, dottybacks are demersal spawners that guard their eggs until they hatch, after which larvae undergo a pelagic phase before returning to settle on coral reefs (Michael, 2004; Kuiter, 2004). Taken together, a pelagic larval phase, ontogenetic modifications of the visual system, adult-specific feeding and habitat associations provide a rich substrate for the study of multi-trait ontogeny and its relationship to habitat transitions.

In this context, we explored the relationship between habitat transitions and ontogeny in dottybacks using histological, neurophysiological and molecular approaches. We conducted a developmental time series in the laboratory and explored wild-caught dottyback specimens to examine when, and under what conditions, ontogenetic colour changes would take place. We then assessed how these changes related to modifications of the dottyback visual system by using a combination of microspectrophotometry (MSP) and quantitative real-time PCR (qRT-PCR) approaches. Finally, we used theoretical fish visual models from the perspectives of the dottyback and of a dottyback predator, the coral trout, *Plectropomus leopardus* (St John, 1999), to assess whether changes of the visual system and skin colour would benefit the various life history strategies dottybacks adopt throughout ontogeny.

MATERIALS AND METHODS

Study site and species

The field part of the study was conducted at Lizard Island (14°40′S, 145°27′E) and Heron Island (23°44′S, 151°91′E), Great Barrier Reef, Australia, between March 2007 and November 2013. Adult and juvenile dottybacks were collected on snorkel from shallow reefs (depth 2–5 m; yellow morphs from live coral, brown morphs from coral rubble, juveniles independent of habitat type) surrounding Lizard Island using an anaesthetic clove oil solution (10% clove oil, 40% ethanol, 50% seawater) and hand nets. Larval dottybacks and damselfishes (*Pomacentrus* spp.) were caught overnight at Lizard Island using light traps during the summer recruitment pulses in November 2007 and October–November 2013. Adult coral trout (N=1 Heron Island, no morphometrics; N=2 Lizard Island, total length 35.5 and 46 cm) were caught using de-barbed hooks and line in March 2007 (Heron Island) and November 2007 (Lizard Island). After capture, fish were placed in sealed bags of seawater, or in large plastic containers, and taken back to the laboratory for further examination. Coral trout and adult and larval dottybacks were used immediately for MSP, or eyes (adult and juvenile dottybacks) and in some cases the whole body (larval dottybacks) were stored on RNAlater (Life Technologies) for subsequent gene expression analysis. The skin of juvenile dottybacks was also used for cell histological assessments. Additional larval dottybacks and damselfishes were used for a developmental time series (see below). Fish sizes are reported in standard length (SL) throughout the study.

For the purpose of this study, we define larval dottybacks as those that are translucent (settlement stage larvae; 11–13 mm SL). After settlement has taken place (2–3 days), fish start to develop skin pigments and turn grey to light brown and are henceforth described as juveniles (SL≤48 mm). Adult stages are reached as soon as fishes adopt a mimic colour (either yellow or dark brown; SL≥43 mm; Figs 1 and 2). Juvenile and adult morphs were initially differentiated by eye based on their coloration, and the categorization was later reviewed based on the shape of their spectral reflectance curves (according to Marshall, 2000). Although our classification might not conform entirely to the traditional way ontogenetic stages in fishes are allocated (Balon, 1975), i.e. adult dottybacks in our study might not all have started to produce gametes, this classification coincides with two major life history transitions of dottybacks (also see Results and Discussion below).

Statistical analyses were conducted in R v.3.3.0 (R Core Team, 2013) using the package lme4 v.1.1-12 (Bates et al., 2015). Assumptions of normality and homogeneity of variance were assessed using histograms, residual plots and quantile–quantile plots.

Developmental time series

To investigate the course of ontogenetic colour change in dottybacks and eventual changes of the visual system associated with it, we placed single larval dottybacks (N=8) in holding tanks (40 cm×30 cm×25 cm) together with either yellow (*Pomacentrus amboinensis*) or brown juvenile damselfish (*Pomacentrus chrysurus*; four replicates per colour with five individuals each). Adult dottybacks are known to change their body coloration to imitate yellow and brown damselfishes (Cortesi et al., 2015a); therefore, we investigated whether juvenile dottybacks would adopt their mimic coloration immediately post-settlement.

Larval holding tanks (40 cm×30 cm×25 cm) were placed in daylight under shade cloth at the Lizard Island Research Station, with a constant supply of fresh seawater sourced directly from the

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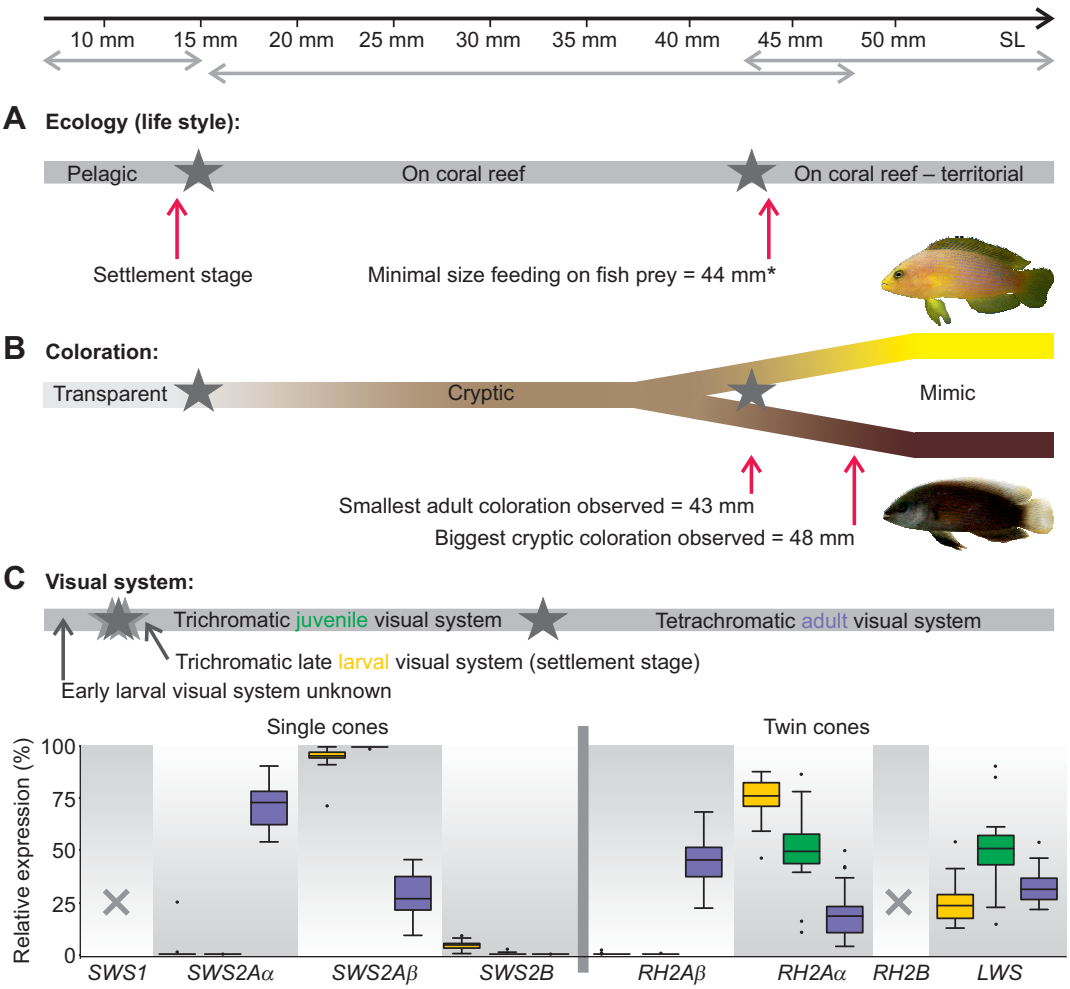

Fig. 1. Integrative approach to study multi-trait developmental adaptations during ontogenetic habitat shifts in the dusky dottyback, *Pseudochromis fuscus*. Developmental adaptations are marked with a star. (A) Dottybacks experience two major ontogenetic habitat transitions: settlement on coral reefs when returning from the pelagic environment as larvae, and reaching a size that enables them to feed on juvenile fish prey when turning into mimics as adults. (B) When returning to the reef, larval dottybacks are almost translucent (~13 mm in standard length, SL), after which they quickly become pigmented and cryptic against their habitat background, before changing to their mimic colorations when turning into adults (~43 mm). (C) Changes of the dottyback visual system precede ontogenetic colour change, probably because dottybacks need to alter their visual system to complete complex visual tasks before ontogenetic colour change can occur (see Discussion). The graph at the bottom shows the relative single (*SWS1* and *SWS2*s) and twin (*RH2*s and *LWS*) cone opsin gene expression measured by quantitative real-time PCR (qRT-PCR) for larval expression profiles (N=18), juvenile expression profiles (N=17) and adult expression profiles (N=18). The smallest dottyback to express an adult profile was 26 mm. Note that larvae/juveniles mainly express three cone opsin genes within their retina, while adults mostly express five (also see Fig. S2). Crosses indicate no expression of *SWS1* and *RH2B* genes. The box indicates Q2 and Q3, with the line indicating the median. The whiskers indicate Q1 and Q4 of the data, with dots marking outliers. *Holmes and McCormick, 2010.

ocean in front of the station. To make each tank into a reef mesocosm, we added 1 cm of sand substrate to the bottom of each tank, a live coral colony (cauliflower coral, *Pocillopora damicornis*, ~30 cm in circumference and ~10 cm in height) in the middle of the tank, and pieces of coral rubble (~20 cm in circumference and ~10 cm in height), placed in each corner. All larval fish were fed *ad libitum* with freshly hatched *Artemia nauplii*, twice daily.

To measure their size and take photographs of individual fish, dottybacks were temporarily removed from their tanks at different time points. Measurements were taken to the closest millimetre and photographs of various body parts were taken under a Zeiss Discovery v8 Stereoscope with an integrated AxioCam Erc5s microscope camera attached to a standard desktop computer running Zen2011 software (www.zeiss.com; Fig. 2). On day 34 post-settlement (dps), individuals from the developmental time series started to overlap in length (18–24 mm, mean±s.e.m. SL=22.3±0.8 mm) with the juveniles caught from the reef (N=16, 19–48 mm, 35.4±1.9 mm), and fish were then killed using an overdose of clove oil (40 mg l^{-1}). Sections of their skin were taken for histological assessments and the eyes were transferred to RNAlater for subsequent gene expression analysis. As a control, additional larval dottybacks were kept in four separate holding tanks without damselfishes. Control fish were killed with an overdose of clove oil (40 mg l^{-1}) either 1 dps (N=8) or 7–9 dps (N=8), before their bodies were transferred to RNAlater for subsequent gene expression analysis (see below).

Skin histological assessment

To assess the type of chromatophores (skin pigment cells) that were present at different ontogenetic time points, we took skin biopsies (0.5–1 cm^2) from fish at the end of the developmental time series (34 dps, N=8; see above) and of larger juveniles (N=3, 38.0±2.3 mm) located and caught from the reef in January–February 2012. Biopsies were taken from behind the

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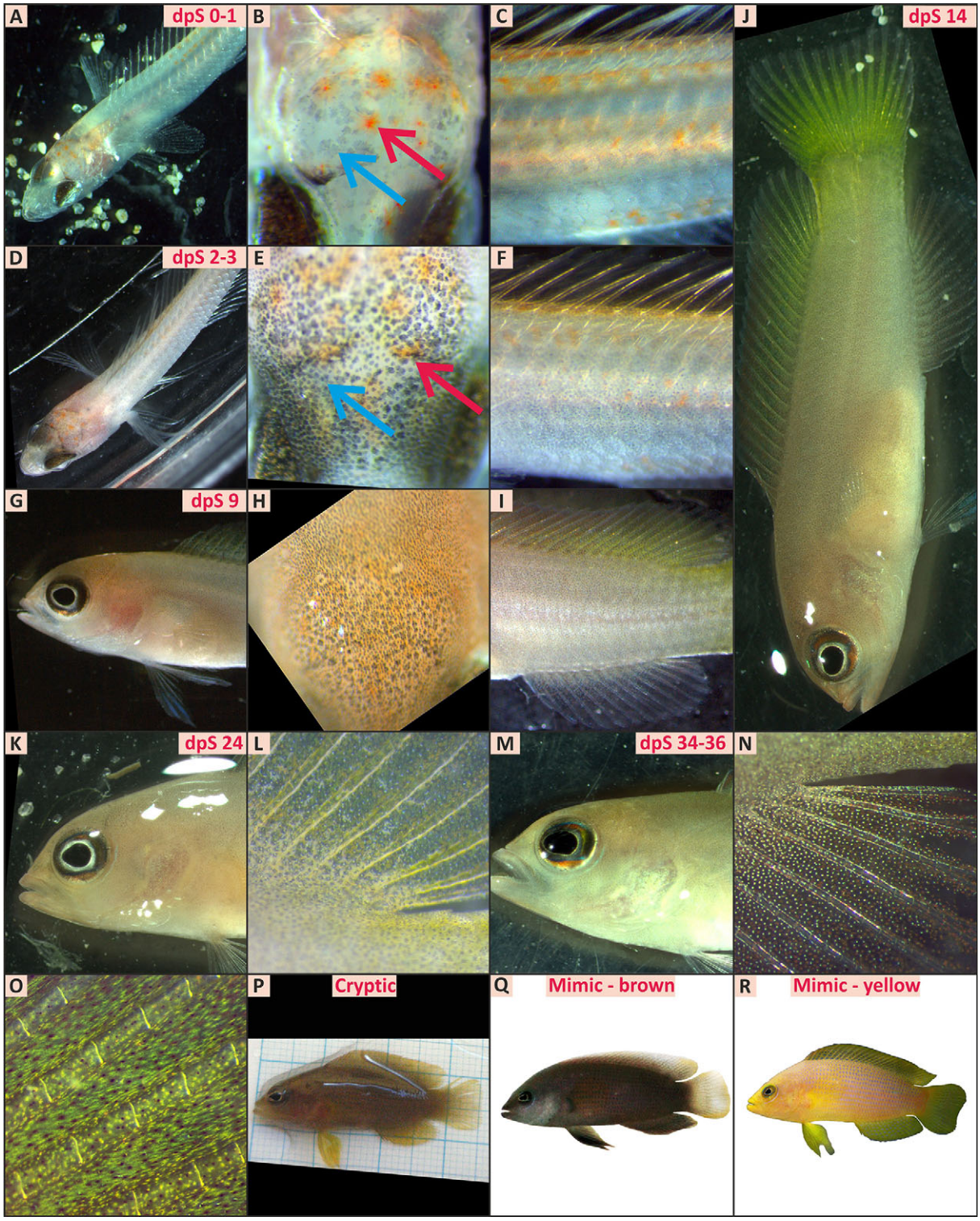

Fig. 2. Developmental time series tracing ontogenetic colour change in dottybacks. (A) When returning from the pelagic environment, larval dottybacks are almost translucent, showing only a little pigmentation on their cranial plate (B), and along the dorsal axis (C). (D–F) Within the first 2–3 days post-settlement (dps), black pigment rapidly starts to form inside melanophores and disperses over the whole body. (G–I) At 7–9 dps, dottybacks attain an overall grey to light-brown coloration, which is maintained (J,K,M,P) until juvenile dottybacks change into their mimic colorations as adults (Q,R). Note, yellow- and red-pigmented cells (xanthophores and erythrophores) first accumulate along the dorsal axis (C,F), spreading to the dorsal, caudal and anal fin (I,J,L,N,O), before migrating across the lateral axis to spread to the entire body (K,M,P). Red arrows point to developing xanthophores, blue arrows point to developing melanophores.

pectoral fin and were treated following the methods of Cortesi et al. (2015a). Results from juvenile fish were subsequently compared with skin histological assessments previously attained from adult dottyback morphs (data taken from Cortesi et al., 2015a; N=8 morphs each, yellow 55.4±3.1 mm, brown 61.6± 2.8 mm; Fig. 3).

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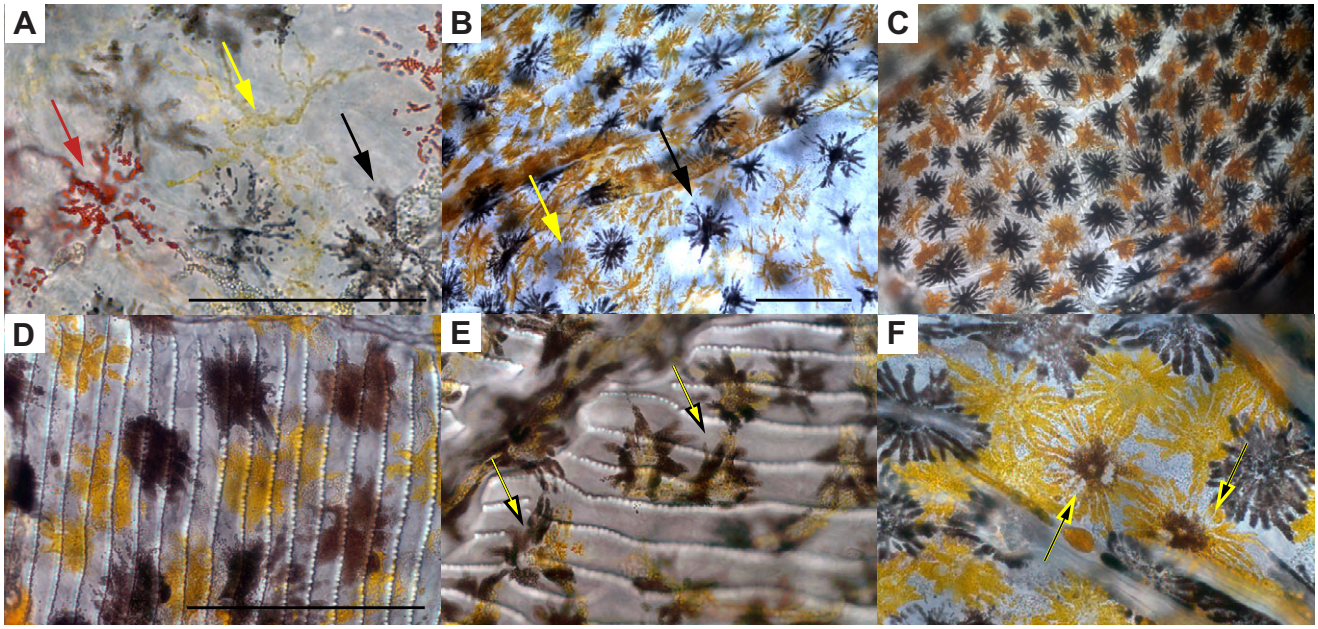

Fig. 3. Skin micrographs of dottybacks across ontogeny. (A) Typical skin biopsies of juvenile dottybacks at the end of the developmental time series (~24 mm SL). Arrows depict red-pigmented cells (presumable erythrophores), yellow-pigmented cells (presumable xanthophores) and black-pigmented cells (melanophores). The red cells are absent in the skin (B,C) and scales (D) of larger juvenile dottybacks (~38 mm) and adult dottybacks (yellow and brown morphs, ~58 mm). Instead, larger dottybacks show low numbers (<1%) of 'hybrid' cells containing yellow and black pigment within their scales (E) and skin (F) (*sensu* Bagnara and Hadley, 1973). Scale bars: 100 µm.

MSP

We used MSP to measure the spectral absorbance of different photoreceptor types in the retina of larval (*N*=1) and adult dottybacks (*N*=3), and of adult coral trout (*N*=3). MSP and raw absorbance spectra were analysed following the methods of Hart et al. (2011) and fitted with visual pigment absorbance spectrum templates of Stavenga et al. (1993) to be used for subsequent fish visual models (see below; Table 1, Fig. 4; Table S1, Fig. S1). Both the dottyback and coral trout contained single as well as twin cones within their retina. The individual members of the twin cones had a very similar overall morphology; however, one member generally contained a shorter shifted mid-wavelength sensitive visual pigment (MWS), while the other member contained a longer shifted long-wavelength sensitive visual pigment (LWS) (this was not always the case for the coral trout twin cones; see Results). Single cones contained a short-wavelength sensitive pigment (SWS).

Opsin genes, synteny and their phylogeny

Dottyback opsin genes were searched for in the genomic raw reads of the specimen that was sequenced as part of the whole-genome sequencing project at the Centre for Ecological and Evolutionary Synthesis (CEES) in Oslo, and the opsin gene sequences of the Nile tilapia, *Oreochromis niloticus* (Spady et al., 2006), were used as a reference against which to map the reads. Mapping and extraction of dottyback opsins followed the methods described in Cortesi et al. (2015b). Opsin sequences from 16 species were subsequently combined with the dottyback sequences to generate a dataset for the phylogenetic reconstruction of genes (Fig. 5). Genomes from three species were accessed from the Assembly or the SRA databases in GenBank (http://www.ncbi.nlm.nih.gov/genbank) and opsin genes were extracted following the methods of Cortesi et al. (2015b). Additional single gene coding sequences from 14 species were directly accessed from GenBank.

Table 1. Spectral characteristics of visual pigment found in the scotopic rod and the photopic single and twin cone photoreceptors of the dusky dottyback, *Pseudochromis fuscus*

	Single cones		Twin cones					
	SWS		MWS member		LWS member	Broad spectra		Rod
	SWS2Aα	*SWS2Aβ*	*RH2Aβ*	*RH2Aα*	*LWS*	*RH2Aα* & *RH2Aβ*	*LWS* & *RH2Aβ*	*RH1*
λ_{max}	Adult	Adult & larval	Adult	Adult & larval	Adult & larval	Adult	Adult	Adult & larval
Pre-bleach absorbance spectra (nm)	447.5±0.9	456.8±1.5	512.5±0.7	524.1±0.7	560.6±1.7	522.8	551.8±2.1	497.8±0.5
Difference spectra (nm)	446.7±1.0	456.1±1.4	513.4±0.1	524.3±1.0	561.5±1.8	524.2±0.9	554.3±2.2	502.4±0.5
No. cells pre-bleach/difference spectra	11/16	4/5	2/2	19/19	9/9	1/2	12/12	24/28

The pigment spectral range and corresponding opsin gene are given below the morphological distinction. λ_{max} data means±s.e.m. were obtained from adults/larvae as shown.

Note that most twin cones contained a mid-wavelength sensitive (MWS) and a long-wavelength sensitive (LWS) member with absorbance spectra that fitted an A_1 visual template (Stavenga et al., 1993). However, some twin cone members showed unusually broad absorbance spectra that are likely to be caused by pigment co-expression within outer segments (see Discussion).

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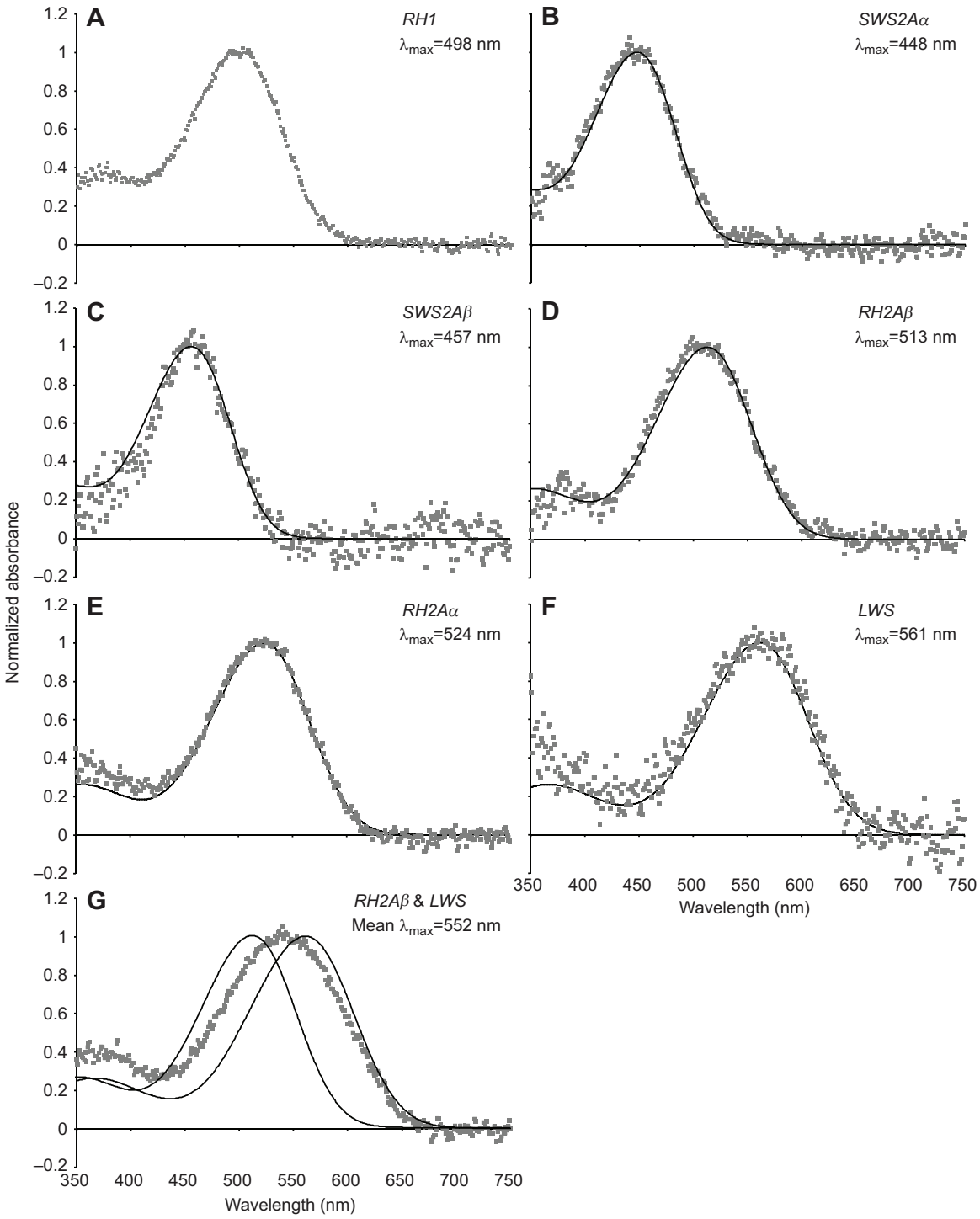

Fig. 4. Normalized pre-bleach absorbance spectra of the dottyback visual pigments measured with microspectrophotometry (MSP). (A) The visual pigment found in the rod photoreceptor used for scotopic vision (*N*=24). (B,C) The 'violet–blue' short-wavelength sensitive (SWS) single cones (B, short SWS, *N*=11; C, long SWS, *N*=4). (D) The 'short-green' mid-wavelength sensitive (MWS) member of the twin cones (*N*=2). (E) The 'long-green' MWS member of the twin cones (*N*=19). (F) The 'red' LWS member of the twin cones (*N*=9). (G) The mean of the broad absorbance spectra found mostly in the LWS member of twin cones and thought to be the result of co-expression of RH2Aβ and LWS visual pigments (*N*=13). The corresponding opsin genes are shown in the top right of each panel. Spectra were fitted with vitamin A_1-based rhodopsin templates of the appropriate λ_{max} calculated using the equations of Stavenga et al. (1993). Note that in G, no visual template was fitted, but instead the visual templates for the short MWS-twin and LWS-twin are shown.

The combined opsin dataset was aligned using the l-ins-i algorithm in MAFFT 6.8 (Katoh and Toh, 2008) and the most appropriate model of sequence evolution was estimated in jModeltest v.2 (Darriba et al., 2012), using the Akaike information criterion (AIC) for model selection. A Bayesian inference phylogenetic hypothesis was calculated on the CIPRES platform (Miller et al., 2010), using the GTR+I+Γ model and an MCMC search with two independent runs and four chains each in

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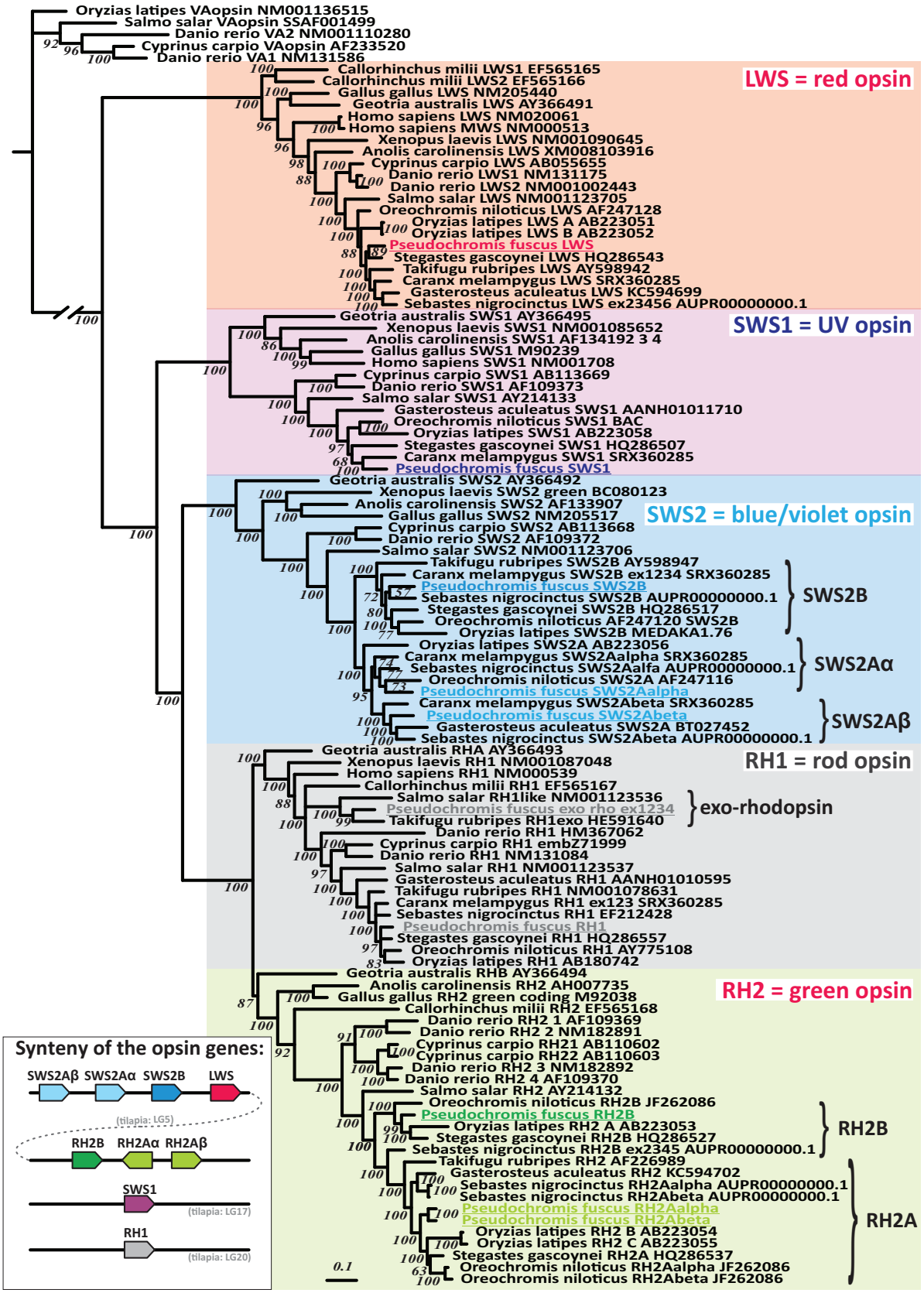

Fig. 5. Vertebrate opsin gene phylogeny and gene synteny of dottyback opsins. The dottyback genome contains nine visual opsin genes (eight cone genes used for photopic vision and one rhodopsin gene used for scotopic vision) and the pineal gland exo-rhodopsin. Note that in addition to having three *SWS2* genes (Cortesi et al., 2015b), dottybacks possess an additional *RH2A* gene, which is similar in synteny to the *RH2A* duplicates in the Nile tilapia, *Oreochromis niloticus* (O'Quin et al., 2011).

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MrBayes v.3.2.1 (Ronquist et al., 2012). Each run was set to 10 million generations, with trees sampled every 1000 generations (i.e. 10,000 trees/run) and a burn-in of 25%. Vertebrate-ancestral opsin gene sequences (VA-opsins) from four fish species were used as outgroups to reconstruct the phylogenetic relationship between opsins. The Dottyback genome data have been submitted to GenBank; other accession numbers are depicted after the species names in Fig. 5.

Opsin gene expression

To investigate whether the expression of cone opsins changed throughout ontogeny, we extracted RNA from the whole head of larvae prior to settlement (N=10, 11–13 mm, 12.2±0.4 mm) and at 1 dps (N=8, 12–13 mm, 12.8±0.3 mm), and small juveniles from the developmental time series 7–9 dps (N=8, 13–15 mm, 13.9±0.3 mm) and 34 dps (N=8, 18–24 mm, 22.3±0.8 mm). Additionally, RNA was extracted from retina tissue of larger juveniles (N=7, 19–41 mm, 31.8±3.1 mm) and adult morphs (N=6 each; yellow, 51–68 mm, 57.5±3.1 mm; brown, 49–65 mm, 58.5±2.7 mm) located and caught from the reef between April 2011 and February 2012. Importantly, juveniles from the reef overlapped in size with individuals from the developmental time series and reached all the way to the adult size class.

RNA extraction and qRT-PCR experiments were conducted following the methods of Stieb et al. (2016). In brief, unique primers were designed for each cone opsin gene, whereby either the forward or the reverse primer spanned an exon–exon boundary to warrant cDNA amplification (Table S2). Primer efficiency was validated using a fivefold dilution series of an opsin pool with a starting concentration of 0.1–0.5 nmol μl^{-1}, making sure that the critical threshold cycle (Ct) values of the dilution series encompassed the Ct values of the samples (Table S2). The opsin pool contained equal ratios of fragments of each opsin gene that were amplified from cDNA (measured on an Agilent 2100 BioAnalyzer, Agilent Technologies). Opsin expression was calculated for short-wavelength sensitive genes (*SWS1* and *SWS2* expressed in single cones) and long-wavelength sensitive genes (*RH2* and *LWS*, expressed in twin cones) separately as the fraction of total opsin gene expression within either single or twin cones, using the opsin pool as a reference to normalize between PCR plates. Individuals from different ontogenetic stages were randomly assigned to each RT reaction plate, and experiments were carried out with three technical replicates each (for further details on the approach, refer to Carleton and Kocher, 2001 and Stieb et al., 2016).

Expression data were transformed to the natural logarithm to compare opsin gene expression between different ontogenetic stages. Initially, a principal component analysis (PCA) followed by MANOVA revealed three distinct groups among ontogenetic stages: larvae prior to settlement and 1 dps (MANOVA, single cones: $Pillai_{1,16}$=0.3, P=0.2; twin cones: $Pillai_{1,16}$=0.2, P=0.4), small juveniles 7–9 and 34 dps (MANOVA, single cones: $Pillai_{1,15}$=0.3, P=0.2; twin cones: $Pillai_{1,15}$=0.3, P=0.1), and larger juveniles and adult morphs (yellow and brown dottybacks; MANOVA, single cones: $Pillai_{2,15}$=0.5, P=0.2; twin cones: $Pillai_{2,15}$=0.3, P=0.5). Importantly, PCA revealed that one large juvenile from the reef at 19 mm overlapped in expression with the small juvenile expression profile (Fig. S2A). Ontogenetic stages were subsequently joined into three different subgroups for expression analysis: larval expression (N=18), juvenile expression (N=17) and adult expression (N=18; Fig. 1; Fig. S2).

Measurement of body coloration and visual models of colour discrimination

Spectral reflectance measurements of juvenile dottybacks (N=6, 38.0±3.2 mm) located and caught between April and May 2011 were obtained following the methods of Cortesi et al. (2015a). Juvenile spectra were combined with measurements previously attained from adult dottybacks (yellow, N=31; brown, N=32), and from the yellow (*Pomacentrus amboinensis* and *P. moluccensis*) and brown (*P. chrysurus*) damselfishes they imitate as adults (N=8 each; data taken from Cortesi et al., 2015a; Fig. 6A). These spectra were then used in theoretical fish visual models (Vorobyev and Osorio, 1998; Vorobyev et al., 2001) to determine: (i) whether an adult expression profile would change the ability of dottybacks to discriminate between damselfishes compared with a juvenile expression profile (Fig. 6E), and (ii) how the predatory coral trout may perceive juvenile and adult dottybacks against a coral rubble or live coral background (Fig. 6F; for measurements of background spectra, see Cortesi et al., 2015a; Fig. 6B).

The visual models calculate the chromatic distance between two colours (ΔS) within the visual 'space' of the fish based on an opponent mechanism, which is limited by the noise of the different photoreceptors (Vorobyev and Osorio, 1998; Vorobyev et al., 2001), whereby, ΔS=1 is an approximate threshold of discrimination, ΔS<1 indicates colours are chromatically indistinguishable, and ΔS>1 indicates colours are discriminable from one another (just noticeable difference, JND; e.g. Cheney et al., 2014; Boileau et al., 2015). In addition, the coral trout might also use differences in luminance contrast (ΔL) to detect dottybacks against their habitat background. In general, coral reef fishes are assumed to use the LWS receptor to perceive differences in ΔL, with some direct evidence in damselfishes (Siebeck et al., 2014). Hence, we used the differences in the natural logarithm quantum catch (Q) of the coral trout LWS receptor (522 or 532 nm λ_{max}; see Results) to calculate ΔL between dottybacks and habitat types:

$$\Delta L = \ln(Q_{\mathrm{LWSdottyback}}) - \ln(Q_{\mathrm{LWShabitat}}). \quad (1)$$

Members of twin cones have previously been shown to contribute individually to colour vision in some coral reef fishes (Pignatelli et al., 2010). Consequently, dottybacks with juvenile expression (*SWS2Aβ*, *RH2Aα*, *LWS*) were modelled as trichromatic and those with adult expression (*SWS2Aα*, *SWS2Aβ*, *RH2Aα*, *LWS* or *LWS/RH2Aβ*; see Results) as tetrachromatic using different visual sensitivities for the LWS member of the adult twin cones: first, a vitamin A_1-based visual template (561 nm λ_{max}) and second, a broader absorbance spectrum presumably derived from opsin co-expression (552 nm λ_{max}; Figs 4 and 6). Because broad absorbance spectra were also found in the coral trout twin cones, we modelled its visual system to be either trichromatic or dichromatic. These models were computed using two A_1-based templates for the MWS (507 nm λ_{max}) and LWS (532 nm λ_{max}) members of the twin cones or using a broad absorbance spectrum for both twin cone members (522 nm λ_{max}), respectively (Fig. 6, Fig. S1).

Spectral sensitivity curves were multiplied by the lens transmission cut-off (dottyback T_{50}=435 nm; coral trout T_{50}=411 nm; Siebeck and Marshall, 2001) to generate species-specific visual templates (Fig. 6C,D). Cone receptor ratios were based on previously conducted morphological assessments of coral reef fish retinas (N.J.M., unpublished) and set to 1:4 (SWS:LWS) for dichromatic, 1:2:2 for trichromatic (SWS:MWS:LWS) and

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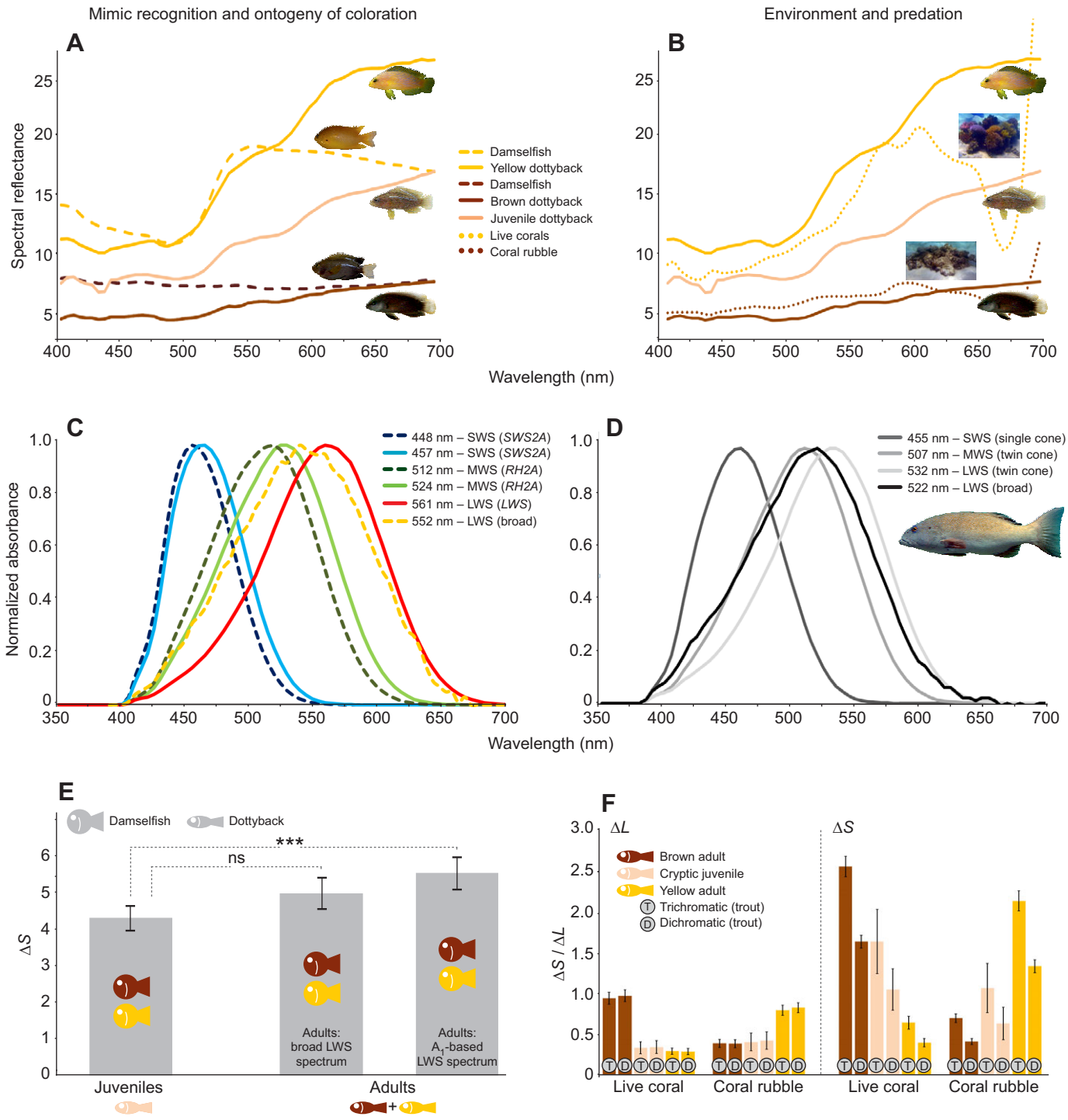

Fig. 6. Theoretical fish vision models used to investigate the possible benefits of ontogenetic changes in dottybacks. (A,B) Mean spectral reflectance measurements of juvenile (*N*=6) and adult (yellow, *N*=31; brown, *N*=32) dottybacks and (A) the damselfish they mimic as adults (yellow and brown, *N*=8 each) and (B) the habitat types that dottybacks are found on [yellow morphs on live coral, brown morphs on coral rubble (Munday et al., 2003) and juveniles across habitat types]. (C,D) Visual templates of juvenile and adult dottybacks (C) and the predatory coral trout, *Plectropomus leopardus* (D; see also Fig. 4, Fig. S2). In C, the dashed spectral curves are adult specific while the continuous curves belong to both larval/juvenile and adult dottyback visual systems. The genes corresponding to the visual pigments found in different photoreceptor types are shown (see also Fig. 4). These visual templates were used to calculate the chromatic (hue, ΔS) and luminance contrast (ΔL) (Vorobyev and Osorio, 1998; Vorobyev et al., 2001) between yellow and brown damselfish models (*Pomacentrus amboinensis* and *P. moluccensis*, yellow; *P. chrysurus*, brown) when perceived by different dottyback stages (*P. fuscus*; E), and between dottybacks and different habitat types when perceived by the coral trout (*P. leopardus*; F). In E, juvenile dottybacks were modelled to have three spectral sensitivities (trichromacy), while adult dottybacks were modelled to have four spectral sensitivities (tetrachromacy) using the long MWS (*RH2A*α) and either an A_1-based LWS (*LWS*) or a broad absorbance spectra-based LWS (putative *RH2A*β and *LWS* co-expression) for the twin cone members. In F, the coral trout was modelled as a trichromat using separate values for the MWS and LWS twin cone members, or as a dichromat using the broad absorbance spectra for both twin cone members. Details on statistical values in F are provided in Table 2. Note that, independent of the visual receiver, ΔS was lower when visual models were computed using the broad absorbance spectra. Coral trout image credit: G. A. C. Phillips.

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1:1:2:2 (SWS:SWS:MWS:LWS) for tetrachromatic visual systems. To account for the light environment under which fish and the background habitat are viewed, we modelled colour discrimination using illumination measurements taken from their natural environments at a water depth of 5 m (as per Cortesi et al., 2015a).

To examine whether dottybacks with a juvenile or an adult expression would differ in their ability to discriminate between damselfish colours (N=8 yellow, N=8 brown damselfishes; 64 pairwise comparisons), we used a linear mixed model (LMM) in lmerTest v.2.0-11 (R package lme4) with ΔS square-root transformed as the response variable. Signal receiver (juvenile, adult, adult co-expression) was set as fixed factor, and damselfish identities were set as random factors. We used likelihood ratio tests to compare a model with random intercepts-only to a model with random slopes and intercepts (models fitted by maximum likelihood). However, we found no significant difference between approaches and the final model was computed using random intercepts-only. Linear models (LMs) were used to investigate whether the coral trout would perceive juvenile and adult dottybacks differently when seen against various habitat backgrounds (trichromatic and dichromatic results were analysed separately). The nature of significant differences was further examined using Tukey–Kramer HSD means comparison tests.

RESULTS

Do dottybacks change colour during ontogenetic habitat shifts?

When larval dottybacks settle onto reefs after their pelagic larval stage, they are translucent and show only a few pigmented chromatophore cells, mostly along the dorsal axis and on the cranial plate (Fig. 2A–C). Within the first 2–3 dps, pigments rapidly start to form and to disperse over the whole body (Fig. 2D–F). At 7–9 dps, fish attain an overall grey to light-brown coloration (Fig. 2G–I). This coloration is maintained (Fig. 2K,M,P) until juvenile dottybacks change to either dark brown or yellow colour morphs as adults, when feeding and habitat specializations take place (Figs 1 and 2Q,R).

While melanophores (black pigment cells) immediately spread across the whole body, erythrophores and xanthophores (red and yellow pigment cells) first accumulate along the dorsal axis (Fig. 2C,F), spreading to the dorsal and caudal fin (Fig. 2I,J,L), before migrating across the lateral and ventral axis to spread across the entire body (Fig. 2K,M). At the end of the developmental time series, at 34 dps, juvenile dottybacks possessed a mixture of melanophores, erythrophores and xanthophores within their skin (Fig. 3A). However, erythrophores were absent in the skin of larger juvenile and adult dottybacks (Fig. 3B–D). Instead, in addition to melanophores and xanthophores, we sporadically found ‘mosaic’ cells (*sensu* Bagnara and Hadley, 1973) within the skin of these specimens (<1% of overall chromatophores), i.e. chromatophores that contained black and yellow pigments and thus appeared to be at a transitional stage between melanophores and xanthophores (Fig. 3E,F).

When returning from the pelagic environment, larval dottybacks measured 11–13 mm, after which fish continuously grew until reaching 18–24 mm at the end of the developmental time series at 34 dps. Juvenile dottybacks caught from the reef (independent of habitat type) ranged from 19 to 48 mm and did not differ in coloration from dottybacks that were raised with either yellow or brown damselfish in our developmental time series. The smallest dottyback to adopt a mimic coloration was 43 mm for yellow morphs and 44 mm for brown morphs.

Does the dottyback visual system change during ontogenetic habitat shifts?

Using MSP, we found seven different types of visual pigments within dottyback retinas, of which two were adult specific (summarized in Table 1). Rods contained a MWS pigment with a mean λ_{max} at 498 nm (N=24 cells; Fig. 4A). There were two spectrally distinct types of single cones containing SWS (‘blue’) pigments: adult-specific cones containing a visual pigment with a mean λ_{max} at 448 nm (N=11 cells; Fig. 4B) and cones that occurred throughout ontogeny with a visual pigment having a mean λ_{max} at 457 nm (N=4 cells; Fig. 4C; see also Cortesi et al., 2015b). Most dottyback twin cones were made up of a member containing a MWS (‘green’)-sensitive visual pigment with a mean λ_{max} at 524 nm (long MWS, N=19 cells; Fig. 4E) and a second member containing a LWS (‘red’) visual pigment with a mean λ_{max} at 561 nm (N=9 cells; Fig. 4F). However, we also found one twin cone in adult dottybacks that contained two shorter shifted MWS pigments with a mean λ_{max} at 512 nm (short MWS, N=2 cells; Fig. 4D). In addition, the LWS members of twin cones in adult fish were found to sporadically depict unusually broad absorbance spectra (N=12 cells), with a mean λ_{max} of 552 nm (Fig. 4G). Moreover, we also found one MWS member with a broad absorbance spectrum at 523 nm λ_{max} (see Discussion on the possible origin of these broad spectra; Fig. 4G, Table 1).

Using whole-genome sequencing, we recovered 10 different opsin genes from the dottyback genome, nine of which are orthologous to visual opsin genes from other vertebrates and similar in synteny to the visual opsin genes of the Nile tilapia (O’Quin et al., 2011), and one of which is orthologous to exo-rhodopsin, the opsin gene expressed in the pineal gland of fishes (Mano et al., 1999; Fig. 5). Phylogenetic analyses revealed that dottyback visual opsins belong to the known visual opsin gene families in percomorph fishes (Rennison et al., 2012), including one rod opsin gene used for scotopic vision (*RH1*) and six cone opsin genes used for photopic vision: four ‘UV–blue’-sensitive genes (*SWS1*, *SWS2Aα*, *SWS2Aβ* and *SWS2B*; see also Cortesi et al., 2015b), one ‘blue–green’-sensitive gene (*RH2B*) and one ‘red’-sensitive gene (*LWS*). In addition, we discovered a novel, possibly dottyback-specific duplication of the ‘green’-sensitive *RH2A* gene: *RH2Aα* and *RH2Aβ*, which cluster together in the phylogeny (Fig. 5).

Independent of ontogeny, dottybacks did not express the UV-sensitive *SWS1* or the green-sensitive *RH2B* genes (Fig. 1C; Fig. S2). Larval dottybacks were found to express three single (*SWS2*) and two twin cone (*RH2* and *LWS*) opsins within their retina (percentage of overall single or twin cone opsin expression): *SWS2B*, 4.2±0.5%; *SWS2Aα*, 1.6±1.4%; *SWS2Aβ*, 94.1±1.4%; *RH2Aα*, 74.9±2.5%; and *LWS*, 23.9±2.5%. However, both *SWS2B* and *SWS2Aα* were expressed at very low levels and are therefore unlikely to be used for vision. Juvenile dottybacks, in contrast, were found to express one single and two twin cone opsins: *SWS2Aβ*, 99.4±0.1%; *RH2Aα*, 50.2±4.7%; and *LWS*, 49.6±4.7%. Finally, dottybacks with an adult expression profile were found to express two single and three twin cone opsins: *SWS2Aα*, 71.71±2.5%; *SWS2Aβ*, 28.23±2.5%; *RH2Aα*, 20.5±3.0%; *RH2Aβ*, 45.8±2.7%; and *LWS*, 33.2±2.0% (Fig. 1C; Fig. S2).

The largest juveniles with juvenile expression profiles were found to be between 19 mm (wild caught) and 24 mm (developmental time series), and the smallest juvenile with an adult expression profile was found to be 26 mm (wild caught). Hence, the transition between the juvenile and the adult expression profile occurs when dottybacks reach ∼25 mm, well before the juvenile to adult colour

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change and habitat specialization take place. Moreover, these data together with the MSP measurements enabled us to assign visual pigments (and sensitivities) to opsin genes: *SWS2Aα* at 448 nm λ_{max} (adult specific), *SWS2Aβ* at 457 nm λ_{max}, *RH2Aα* at 524 nm λ_{max}, *RH2Aβ* at 512 nm λ_{max} (adult specific) and *LWS* at 561 nm λ_{max} (Table 1 and Fig. 4).

Coral trout visual system

The coral trout rod cells contained a MWS pigment with a mean λ_{max} at 497 nm (N=22 cells; Fig. S1A), while single cones contained a SWS pigment with a mean λ_{max} at 455 nm (N=10 cells; Fig. S1B). Similar to the dottybacks, the coral trout twin cone members were found to have absorbance spectra that were broader than would be expected based on the presence of only a single pigment binding either an A_1 or an A_2 chromophore. However, in this case, broad absorbance spectra were found for almost every cell and often both twin cone members had a similar spectral absorbance ranging from 507 to 532 nm λ_{max} (mean λ_{max}=522 nm, N=48 cells; Fig. S1C).

Colour discrimination by juvenile and adult dottybacks and by the predatory coral trout

Using theoretical vision models, we found that the chromatic contrast (ΔS) between differently coloured damselfish models increased for adult dottybacks compared with juvenile dottybacks (ΔS brown versus yellow damselfish: adult dottybacks with A_1-based LWS=5.5±0.5; adult dottybacks with broad LWS spectrum=5.0±0.4; juvenile dottybacks=4.3±0.3; LMM: χ^2_2=16.9, P<0.001). However, while adult dottybacks with an A_1-fitted LWS had a significantly higher ΔS compared with juvenile dottybacks (pairwise *post hoc* Tukey contrast: z=−4.2, P<0.001), this difference was not apparent when using the broad LWS spectrum (pairwise *post hoc* Tukey contrast: z=−0.1, P=0.1; Fig. 6E).

From the perspective of the predatory coral trout, we found that when perceived against different habitat backgrounds, there was a significant difference between juvenile and adult dottybacks for colour (ΔS: LM, dottyback colour×habitat type, trichromat: $F_{2,134}$=134.9, P<0.001; dichromat: $F_{2,134}$=124.2, P<0.001) and luminance contrast (ΔL: LM, dottyback colour×habitat type, trichromat: $F_{2,134}$=55.0, P<0.001; dichromat: $F_{2,134}$=48.4, P<0.001; Fig. 6F). While adult yellow and brown morphs have previously been shown to match their habitat (yellow on live coral and brown on coral rubble; Cortesi et al., 2015a), we found no difference in colour and luminance contrast for juveniles against either habitat type (ΔS and ΔL values as well as pairwise *post hoc* Tukey contrast tests are summarized in Table 2; Fig. 6F). However, although using different chromaticity models did not change our conclusions and ΔL remained similar between models, ΔS was consistently lower for the dichromatic models than for the trichromatic models (Table 2, Fig. 6F).

DISCUSSION

Using a multidisciplinary approach, we show that dottybacks experience two major ontogenetic habitat shifts, which are associated with multi-trait developmental modifications. Starting their life as translucent larvae, dottybacks are likely to be well camouflaged within the open water of the pelagic environment. Upon returning to the reef to settle, larvae quickly become pigmented and adopt a coloration that, independent of the habitat background, appears cryptic from the perspective of their predators. The smallest adult dottybacks from our study were ∼43 mm, which coincides with the predicted minimum size at which dottybacks are capable of feeding on juvenile fish prey (Holmes and McCormick, 2010; Fig. 1A). Hence, adopting their characteristic mimic coloration at this ontogenetic stage is likely to deliver substantial fitness benefits in terms of deceiving and capturing prey, and – at the same time – maintaining cryptic benefits due to model-associated habitat specialization (see also Cortesi et al., 2015a, for further details on multiple fitness benefits of this mimicry system).

Table 2. Summary of the chromatic and luminance (achromatic) contrast between dottyback ontogenetic stages when perceived against different habitat backgrounds by the coral trout, *Plectropomus leopardus*

Background	Visual system	Developmental stage	ΔS	t	P	ΔL	t	P
Live coral	Trichromatic	Adult (yellow)	0.6±0.1	−13.1	<0.001	0.3±0.04	−8.9	<0.001
		Adult (brown)	2.5±0.1			1.0±0.1		
	Dichromatic	Adult (yellow)	0.4±0.1	−12.6	<0.001	0.3±0.04	−8.2	<0.001
		Adult (brown)	1.6±0.1			1.0±0.1		
Coral rubble	Trichromatic	Adult (yellow)	2.1±0.1	10.0	<0.001	0.8±0.1	5.9	<0.001
		Adult (brown)	0.7±0.1			0.4±0.05		
	Dichromatic	Adult (yellow)	1.3±0.1	9.7	<0.001	0.8±0.1	5.4	<0.001
		Adult (brown)	0.4±0.04			0.4±0.05		
Live coral	Trichromatic	Adult (yellow)	0.6±0.1	−4.1	0.001	0.3±0.04	−0.8	1.0
		Juvenile (grey)	1.6±0.4			0.3±0.1		
	Dichromatic	Adult (yellow)	0.4±0.1	−4.0	0.001	0.3±0.04	−0.7	1.0
		Juvenile (grey)	1.0±0.2			0.3±0.1		
Coral rubble	Trichromatic	Adult (brown)	0.7±0.1	1.6	0.6	0.4±0.05	0.7	1.0
		Juvenile (grey)	1.1±0.3			0.4±0.1		
	Dichromatic	Adult (brown)	0.4±0.04	1.4	0.7	0.4±0.05	0.5	1.0
		Juvenile (grey)	0.6±0.2			0.4±0.1		
Live coral	Trichromatic	Juvenile (grey)	1.6±0.4	−1.6	0.6	0.3±0.1	0.6	1.0
Coral rubble			1.1±0.3			0.4±0.1		
Live coral	Dichromatic		1.0±0.2	−1.8	0.5	0.3±0.1	0.5	1.0
Coral rubble			0.6±0.2			0.4±0.1		

Plectropomus leopardus visual system was modelled as trichromatic or dichromatic.
Note that modelling the coral trout as either a dichromat or a trichromat did not change the overall results. However, while luminance contrast values (ΔL, means±s.e.m.) stayed consistent, chromatic contrast values (ΔS, means±s.e.m.) were always lower for the dichromatic compared with the trichromatic models. Tukey *post hoc* tests.

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Interestingly, we found that the type of chromatophore within the skin of dottybacks changes throughout ontogeny. Smaller juveniles have a combination of erythrophores, xanthophores and melanophores, while larger juveniles and adults lose erythrophores, and instead possess low numbers of mosaic cells containing both yellow and black pigments. Note, however, that the occurrence of adult orange dottyback morphs in Papua New Guinea indicates that, in some populations, erythrophores may be maintained throughout ontogeny (Messmer et al., 2005). Furthermore, as erythrophores and xanthophores are characterized by their carotenoid (red/orange)- and/or pteridine (yellow)-derived coloration (Fujii, 1993; Sköld et al., 2016), dottybacks may only possess one 'red–yellow' chromatophore type. Changes in hue of this chromatophore could then be achieved by varying the amount and/or type of pigment within the cell. Such trans-differentiation of chromatophore cells is a rarely described phenomenon in fish (Leclercq et al., 2009), but could also explain the mosaic cells we found in larger dottybacks. If cells were able to change their pigment content, then the non-developmental colour changes in adult mimics (Cortesi et al., 2015a) could occur without having to invest in the production of novel cellular structures. However, chromatographic approaches are needed to unambiguously distinguish between chromatophore types and pigment contents thereof in dottybacks.

The visual systems of coral reef fish larvae often undergo major morphological changes when the fish return to the reef and metamorphose into their juvenile phenotypes (Evans and Browman, 2004; Evans and Fernald, 1990). Generally, early-stage larvae possess a pure cone retina and are sensitive to shorter wavelengths of light, which is ideal for a life in a well-lit epipelagic environment (Britt et al., 2001; Evans and Browman, 2004; Evans and Fernald, 1990; Hunt et al., 2014). Our study did not include larval dottybacks from their early planktonic stages, which could explain why we only found very low levels or no expression of the shorter SWS (*SWS1* 'UV' and *SWS2B* 'violet') and MWS (*RH2B* 'blue–green') pigments. What we found instead is that at the time when dottyback larvae return from the pelagic environment, they possess a fully developed retina containing all photoreceptor types (single cones, twin cones and rods) that are also present in adults. These photoreceptors mainly express three longer-wavelength shifted cone opsins (*SWS2Aβ*, *RH2Aα*, *LWS*), theoretically providing settlement-stage fish with the ability to see colours likely to be necessary for survival on the reef (Evans and Fernald, 1990).

Using qRT-PCR, we showed that juvenile dottybacks change to an adult visual system when reaching ∼25 mm, thereby predating the ontogenetic colour change and juvenile to adult habitat transition, which only occurs when dottybacks are substantially larger (∼43 mm). While it has previously been shown that dottybacks express an additional blue opsin gene as adults (*SWS2Aα*; Cortesi et al., 2015b), we found that, just like in cichlids (Spady et al., 2006) and black bream, *Acanthopagrus butcheri* (Shand et al., 2008), larger dottybacks in addition start to express a second green opsin within their retina (*RH2Aβ*; Fig. 1; Fig. S2). Strikingly, the synteny of green genes, while unknown for black bream, is alike in dottybacks and cichlids, with the *RH2Aα* gene occurring in a prominent reversed orientation between the upstream *RH2B* and the downstream *RH2Aβ* genes (O'Quin et al., 2011; Fig. 5). However, it remains to be investigated whether these findings are instances of convergence or whether it is a more commonly occurring pattern in fishes that possess multiple *RH2A* genes, such as the Japanese rice fish, *Oryzias latipes* (Matsumoto et al., 2006), or the tiger rockfish, *Sebastes nigrocinctus* (Fig. 5).

Interestingly, in adult dottybacks, *RH2Aβ* was found to be the highest expressed twin cone gene, but pure RH2Aβ pigment was only found in two out of 43 cells. This suggests that the large absorbance spectra in the adult LWS twin cones may derive from the co-expression of *RH2Aβ* with *LWS* (*RH2Aβ* with *RH2Aα* in the case of the MWS twin cone). The proposed dottyback scenario of co-expression involving two orthologous green genes (*RH2A*) with a difference in λ_{max} of ∼10 nm and a longer shifted red gene (*LWS*), has recently been reported for the freshwater cichlid *Metriaclima zebra* (Dalton et al., 2014). In *M. zebra*, co-expressing multiple visual pigments within a single photoreceptor significantly enhances luminance discrimination, but the drawback seems to be a decrease in chromatic colour discrimination (Dalton et al., 2014). In support of these findings, we found a very similar pattern when modelling dottyback and coral trout visual tasks using the broad absorbance spectra instead of A_1-based visual templates. This suggests that pigment co-expression may serve a common function even across very distantly related species, which raises the question whether opsin co-expression has a long-lasting evolutionary history in fishes?

An alternative to opsin co-expression would be that both the dottyback and coral trout twin cone outer segments contained a mixture of A_1 and A_2 chromophores, something that has previously been found to cause broad absorbance spectra in frogs (Reuter et al., 1971). However, so far there are very few (if any) coral reef fishes that have been reported to contain A_2 chromophores within their photoreceptors (e.g. Toyama et al., 2008). Moreover, given that for both species the rod and SWS cones and in the dottyback also the 'normal' MWS and LWS cones are fitted by A_1 templates, it is unlikely that the broad spectra are due to chromophore mixtures. Nevertheless, methods such as *in situ* hybridization, gene knock-out approaches or chromophore extractions are necessary to unambiguously assess whether the broad spectra are caused by pigment co-expression or by chromophore mixtures.

Finally, the visual models showed that adult dottybacks might have an increased ability to distinguish between the colorations of the damselfishes they mimic compared with juvenile dottybacks, at least when relying on pure A_1-based LWS photoreceptors. Having excellent colour discrimination could be essential for dottybacks to determine the differences between the fishes they are going to mimic, which might partly explain why juvenile dottybacks switch their visual system well before ontogenetic colour changes take place. Interestingly, it has recently been observed that opsins are also expressed in a variety of non-eye tissues of fishes including the skin, where they are thought to mediate colour change via chromatophore light sensing (e.g. Chen et al., 2013; Davies et al., 2015). Whether the dottybacks also express opsins in their skin and how light sensing may contribute to colour change in this species warrants further investigation.

Using theoretical visual models as well as modelling only a few visual tasks, however, has its limitations. The assumption that juvenile dottybacks are trichromatic while adults are tetrachromatic, or, for that matter, that the coral trout is either dichromatic or trichromatic, needs to be verified by behavioural experimentation. Moreover, the models show that both juvenile and adult dottybacks should have colour vision and behavioural experiments are therefore needed to establish the significance (if any) of the changes in colour discrimination between different developmental stages. This is important because it is currently not understood what a change in JND beyond the discrimination threshold of 1 signifies for the animal, and whether the discrimination threshold varies depending on direction and position in colour space. Finally, behavioural

experiments are also needed to test the role putative opsin co-expression may play for vision in these species.

In conclusion, despite the evolutionary importance of ontogenetic habitat shifts, detailed studies investigating the triggers for the transitions and how these interrelate with multi-trait developmental adaptations remain scarce. Here, we examined ontogenetic habitat transitions in the dusky dottyback, an enigmatic mimic with the ability to imitate differently coloured model species in its surroundings. We show that dottybacks start their lives well camouflaged within their respective habitats and while their visual systems quickly adapt to a lifestyle on coral reefs, changes to their mimic adult coloration and associated habitat specialization only occur once dottybacks are big enough to feed on juvenile fish prey. Therefore, our study highlights the importance of comparative approaches to understand how species adapt and evolve to an ever-changing environment.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
F.C. conceived the study and designed the experiments together with N.J.M., K.L.C. and W.S. F.C., Z.M., S.M.S., N.S.H. and U.E.S. performed the experiments and analysed the data. F.C., Z.M., K.L.C., W.S. and N.J.M. wrote the initial manuscript. All authors reviewed and approved the final version of the manuscript.

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Data availability

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.139501.supplemental

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SUPPLEMENTARY FIGURES

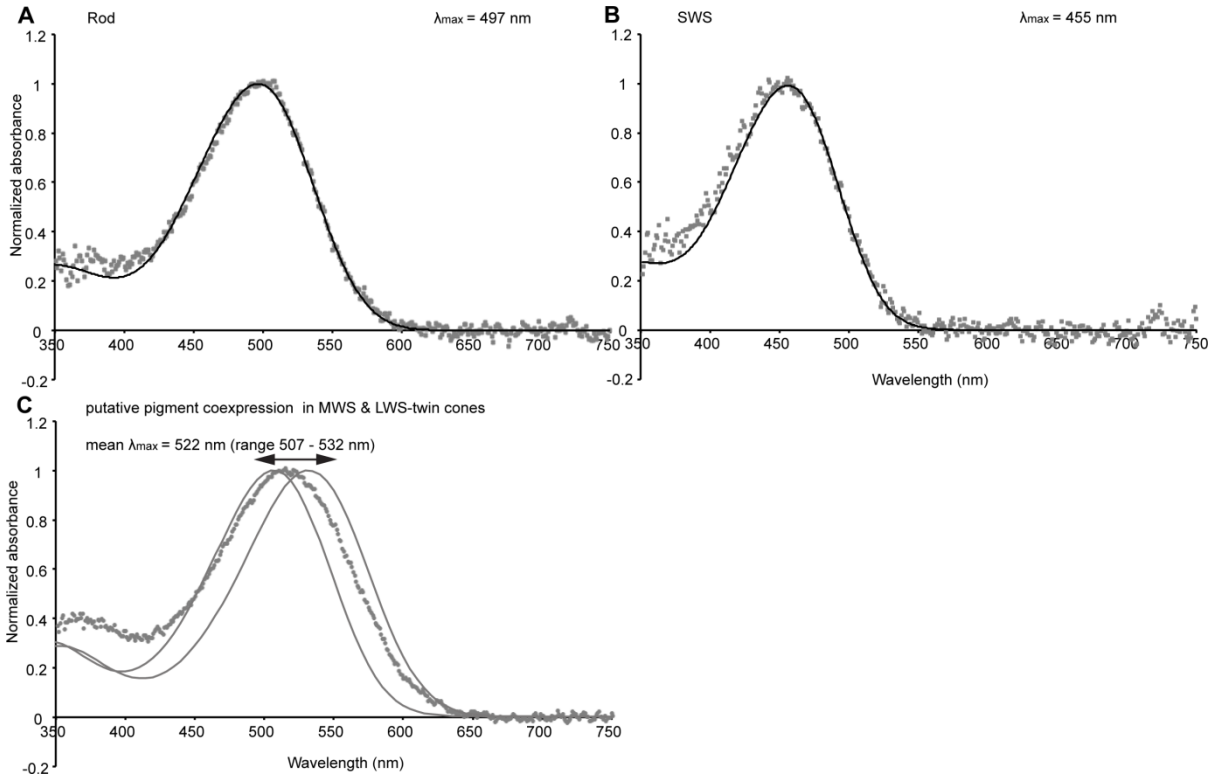

Fig. S1. Normalized pre-bleach absorbance spectra of the coral trout visual pigments (measured with MSP). (A) The visual pigment found in the rod photoreceptor used for scotopic vision (n = 22), (B) the 'blue' SWS single cone (n = 10), (C) the mean of the broad absorbance spectra found in the twin cones (MWS and LWS) and thought to be the result of a coexpression of two visual pigments with a range of 507 – 532 nm λ_{max} (n = 48). Spectra are fitted with Vitamin A1 rhodopsin templates of the appropriate λ_{max} calculated using the equations of Stavenga et al., 1993. Note that in (C) no visual templates were fitted, instead the A1 based visual templates for 507 nm and 532 nm λ_{max} are shown in grey.

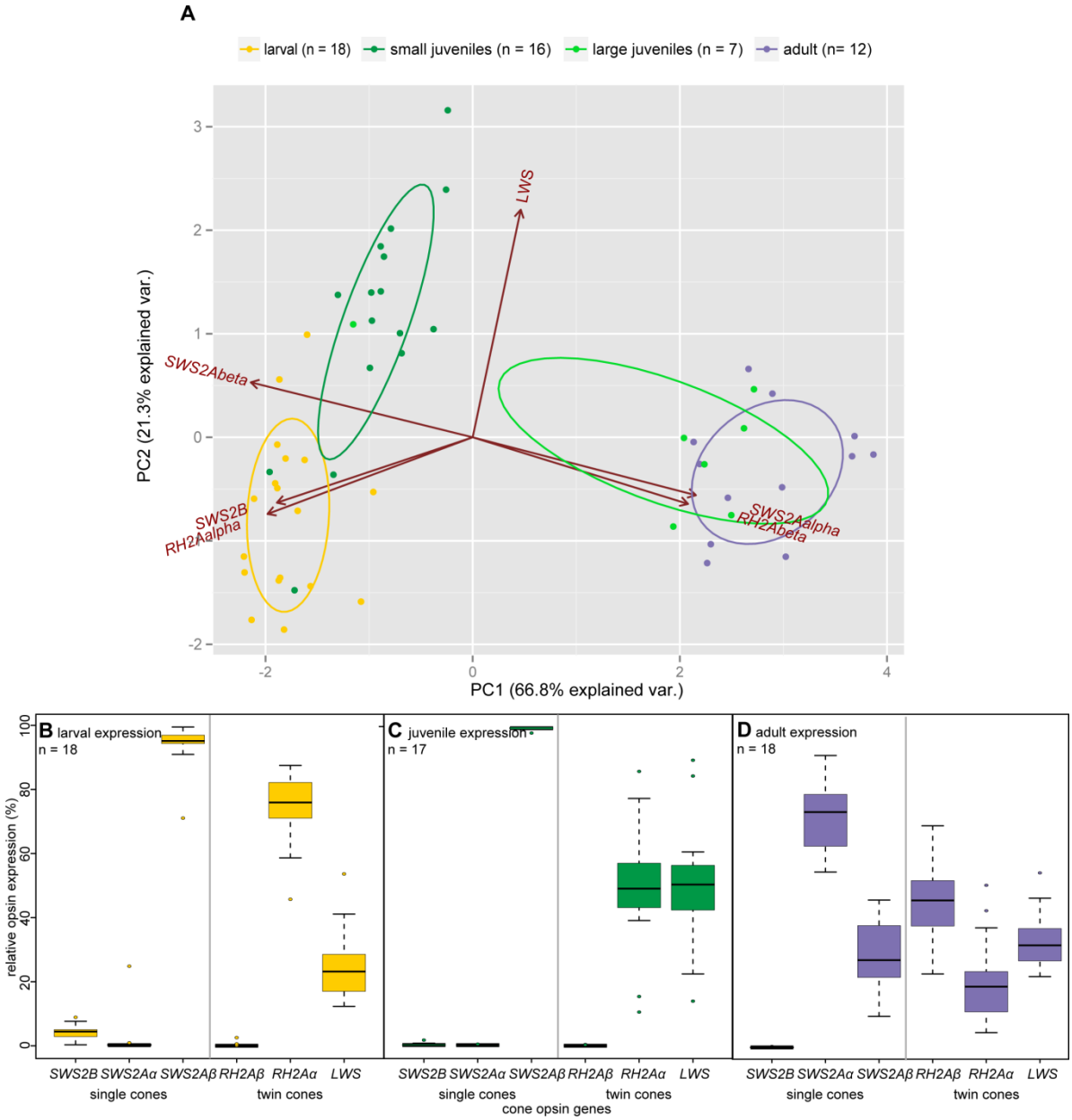

Fig. S2. Difference in opsin gene expression throughout dottyback ontogeny.

(A) A principle component analysis (PCA) shows dottyback cone opsin expression of larvae prior to settlement and one day post settlement (dps) in yellow (n = 18), small juveniles (7 – 9 dps and 34 dps) in dark green (n = 16), large juveniles in bright green (n = 7), and adults in violet (n = 12). The lines indicate differences in gene expression between individuals, separating ontogenetic stages into three distinct expression profiles: (B) larval-expression (n = 18), (C) juvenile-expression (n = 17), and (D) adult expression (n = 18). Note that the smallest of the large juveniles at 19 mm standard length (SL) clusters together with individuals of the juvenile-expression profile, while the remaining large juveniles (> 26 mm SL) already show an adult-expression profile. Gene expression was calculated for single and twin cone genes separately.

Table S1. Spectral characteristics of visual pigment found in the scotopic rod, and the photopic single cone and twin cone photoreceptors of the coral trout, *Plectropomus leopardus*. Both twin cone members showed broad absorbance spectra that are likely to be caused by pigment coexpression within outer segments with a range of 507 – 532 nm λ_{max} (also see discussion in the main article; Fig. S1).

	single cone	**twin cone**	**rod**
Morphological distinction	SWS	broad spectra (coexpression?) MWS & LWS	
λ_{max} mean ± s.e.			
pre-bleach absorbance spectra (nm)	455.4 ± 0.7	522.1 ± 0.9	496.5 ± 0.6
difference spectra (nm)	457.3 ± 1.6	522.8 ± 1.1	501.9 ± 1.2
no. cells pre-bleach/difference spectra	10 / 11	48 / 39	22 / 23

Table S2. qRT-PCR and pool primers used in this study

method	**gene (efficiency)**	**primer name**	**orientation**	**primer sequence**
qRT_PCR	*SWS1* (90%) qPCR primers	*Pfus_SWS1_2F*	forward	TTTTGGAGCCTTCAAGTTCACCAG
		Pfus_SWS1_23R	reverse	GATGTACCTGCTCCAGCCAAAG
qRT_PCR	*SWS2B* (94%) qPCR primers	*Pfus_SWS2B_1F1*	forward	CCGTGGGCTCCTTCACCTG
		Pfus_SWS2B_12R1	reverse	GGCTCACCATGCCTCCAATC
qRT_PCR	*SWS2Aα* (96%) qPCR primers	*Pfus_SWS2Aalfa_12F1*	forward	CATGGCAACACTCGGGGGTATG
		Pfus_SWS2Aalfa_2R1	reverse	CGCAAACACCCAGGTGAACC
qRT_PCR	*SWS2Aβ* (96%) qPCR primers	*Pfus_SWS2Abeta_1F2*	forward	GGTGAACTTGGCTGCCGCG
		Pfus_SWS2Abeta_12R1	reverse	CCATACCTCCAAGTGTTGCTAC
qRT_PCR	*RH2B* (91%) qPCR primers	*Pfus_RH2B_23R_new*	forward	TGTACCTCGACCAGCCCACC
		Pfus_RH2B_2F_new	reverse	TGTGGTCTGTAAACCTATGGGC
qRT_PCR	*RH2Aα* (tba) qPCR primers	*qPCR_RH2Aa_ex4_F1*	forward	GCTGCCTTCACCGCCCTC
		qPCR_RH2Aa_ex45_R1	reverse	GTCAGCATGCAGTTACGGAAC
qRT_PCR	*RH2Aβ* (tba) qPCR primers	*qRH2Abeta_ex2_F1*	forward	GGAGCTTCAAGTTCGGTGGAT
		qRH2Abeta_ex23_R1	reverse	ATGTACCTGGACCAGCCAGC
qRT_PCR	*LWS* (91%) qPCR pool	*PFus_LWS_34_F1*	forward	TGTCTCAACCTGTGGTATTACTGC
		PFus_LWS_4_R1	reverse	GGATCCCACCTGTGGCCCAT
Sanger sequencing	*SWS1* qPCR pool	*POOL_Pfus_SWS1_F*	forward	CTGTGTGCCATGGAGTCTGCC
		SWS1_R2d_dam	reverse	TCGTTGTGGGTGTACCAGTC
Sanger sequencing	*SWS2B* qPCR pool	*POOL_Pfus_SWS2B_F*	forward	GTGACTGGTACTGCCATCAATATC
		POOL_Pfus_SWS2B_R	reverse	AACGATGGTGAAGAAGGGGATGGAA
Sanger sequencing	*SWS2Aα* qPCR pool	*POOL_Pfus_SWS2Aalfa_F*	forward	CTCACTATTGCATGCACCGCC
		POOL_Pfus_SWS2Aalfa_R	reverse	GCCCATGCCCAGCATCGCT
Sanger sequencing	*SWS2Aβ* qPCR pool	*POOL_Pfus_SWS2Abeta_F*	forward	CTTACCGTTGCATGCACCGTG
		POOL_Pfus_SWS2Abeta_R	reverse	TCCACTCATCCCCAGCATCTTC
Sanger sequencing	*RH2B* qPCR pool	*RH2B_F2_Fuscus*	forward	TTA TCCTGGTTAACTTGGC
		Rh2B_R2c_dam	reverse	ATCACATAGGATTCGTTGTTG
Sanger sequencing	*RH2Aα* qPCR pool	*poolRH2Aalpha_ex1_F1*	forward	TCCAACAGGACTGGGATAAC
		poolRH2Aalpha_ex5_R1	reverse	CCATCCCAATAGTCGTCAG
Sanger sequencing	*RH2Aβ* qPCR pool	*poolRH2Abeta_ex1_F1*	forward	CCAACAGGACGGGGATTGT
		poolRH2Abeta_ex5_R1	reverse	GCCACCCATTCCAATAGTG
Sanger sequencing	*LWS* qPCR pool	*LWS_R4dFin_dam*	forward	CCCAAAACGAAGAACATGGA
		LWS_F6d_dam	reverse	AAGTTCAAGAAACTCCGTCA