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 pressure or social status, which in turn are associated with 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 (\(\lambda_{\text{max}}\)), 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).
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, 2015). Taken together, a pelagic larval phase, ontogenetic transitions 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
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 cm²) 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
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).

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.
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*

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<th>Single cones</th>
<th>Twin cones</th>
<th>Broad spectra</th>
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<td>SWS</td>
<td>MWS member</td>
<td>LWS</td>
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<td>SWS2α</td>
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<td>SWS2β</td>
<td>RH2Aβ</td>
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<td>$\lambda_{\text{max}}$</td>
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<tr>
<td>Pre-bleach absorbance spectra (nm)</td>
<td>447.5±0.9</td>
<td>456.8±1.5</td>
<td>512.5±0.7</td>
</tr>
<tr>
<td>Difference spectra (nm)</td>
<td>446.7±1.0</td>
<td>456.1±1.4</td>
<td>513.4±0.1</td>
</tr>
<tr>
<td>No. cells pre-bleach/difference spectra</td>
<td>11/16</td>
<td>4/5</td>
<td>2/2</td>
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The pigment spectral range and corresponding opsin gene are given below the morphological distinction. $\lambda_{\text{max}}$ 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 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).
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+ model and an MCMC search with two independent runs and four chains each in...
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 duplicates in the Nile tilapia, Oreochromis niloticus (O’Quin et al., 2011).

**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).
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: Pillai1,16=0.3, P=0.2; twin cones: Pillai1,16=0.2, P=0.4), small juveniles 7–9 and 34 dps (MANOVA, single cones: Pillai1,15=0.3, P=0.2; twin cones: Pillai1,15=0.3, P=0.1), and larger juveniles and adult morphs (yellow and brown dottybacks; MANOVA, single cones: Pillai2,15=0.5, P=0.2; twin cones: Pillai2,15=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 λmax; see Results) to calculate ΔL between dottybacks and habitat types:

\[
ΔL = \ln(Q_{LWShabitat}) - \ln(Q_{LWSdottyback}).
\]

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 (SWS2AB, RH2A, LWS) were modelled as trichromatic and those with adult expression (SWS2Aa, SWS2AB, RH2Aa, LWS or LWSRH2Aa; see Results) as tetrachromatic using different visual sensitivities for the LWS member of the adult twin cones: first, a vitamin A1-based visual template (561 nm λmax) and second, a broader absorbance spectrum presumably derived from opsin co-expression (552 nm λmax; 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 A1-based templates for the MWS (507 nm λmax) and LWS (532 nm λmax) members of the twin cones or using a broad absorbance spectrum for both twin cone members (522 nm λmax), respectively (Fig. 6, Fig. S1).

Spectral sensitivity curves were multiplied by the lens transmission cut-off (dottyback T50=435 nm; coral trout T50=411 nm; Siebeck and Marshall, 2001) to generate species-specific visual templates (Fig. 6D). 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...
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, \( \Delta S \)) and luminance contrast (\( \Delta L \)) (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 (\( RH2A \)) and either an \( \alpha \)-based LWS (\( LWS \) 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, \( \Delta S \) was lower when visual models were computed using the broad absorbance spectra. Coral trout image credit: G. A. C. Phillips.
Do 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 $\lambda_{\text{max}}$ 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 $\lambda_{\text{max}}$ at 448 nm ($N=11$ cells; Fig. 4B) and cones that occurred throughout ontogeny with a visual pigment having a mean $\lambda_{\text{max}}$ 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 $\lambda_{\text{max}}$ at 524 nm (long MWS, $N=19$ cells; Fig. 4E) and a second member containing a LWS (‘red’) visual pigment with a mean $\lambda_{\text{max}}$ 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 $\lambda_{\text{max}}$ 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 $\lambda_{\text{max}}$ of 552 nm (Fig. 4G). Moreover, we also found one MWS member with a broad absorbance spectrum at 523 nm $\lambda_{\text{max}}$ (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-rodopsin, 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 pigments (SWS1, SWS2Aa, SWS2Ab 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: RH2Aa and RH2Ab, 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%; SWS2Aa, 1.6±1.4%; SWS2Ab, 94.1±1.4%; RH2Aa, 74.9±2.5%; and LWS, 23.9±2.5%. However, both SWS2B and SWS2Aa 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%; RH2Aa, 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: SWS2Aa, 71.7±2.5%; SWS2Ab, 28.2±3.2%; RH2Aa, 20.5±3.0%; RH2Ab, 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
change and habitat specialization take place. Moreover, these data together with the MSP measurements enabled us to assign visual pigments (and sensitivities) to opsins genes: SWS2A at 448 nm $\lambda_{\text{max}}$ (adult specific), SWS2B at 457 nm $\lambda_{\text{max}}$, RH2A at 524 nm $\lambda_{\text{max}}$, RH2A at 512 nm $\lambda_{\text{max}}$ (adult specific) and LWS at 561 nm $\lambda_{\text{max}}$ (Table 1 and Fig. 4).

**Coral trout visual system**

The coral trout rod cells contained a MWS pigment with a mean $\lambda_{\text{max}}$ at 497 nm ($N=22$ cells; Fig. S1A), while single cones contained a SWS pigment with a mean $\lambda_{\text{max}}$ 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 $\lambda_{\text{max}}$ (mean $\lambda_{\text{max}}=522$ 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 ($\Delta S$) between differently coloured damselfish models increased for adult dottybacks compared with juvenile dottybacks ($\Delta S$ brown versus yellow; 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: $\chi^2=16.9$, $P<0.001$). However, while adult dottybacks with an $A_1$-fitted LWS had a significantly higher $\Delta S$ 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 ($\Delta S$: 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 ($\Delta L$: 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 ($\Delta S$ and $\Delta L$ 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 $\Delta L$ remained similar between models, $\Delta S$ 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).

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<th>Background</th>
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<th>$P$</th>
<th>$\Delta L$</th>
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*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 ($\Delta L$, mean±s.e.m.) stayed consistent, chromatic contrast values ($\Delta S$, mean±s.e.m.) were always lower for the dichromatic compared with the trichromatic models. Tukey post hoc tests.
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 Brownman, 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 Brownman, 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 (SW51 ‘UV’ and SW52 ‘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 (SW52Aβ, 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 (SW52Aα; 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 reverse orientated 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 $\lambda_{\text{max}}$ 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 A1-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 A1 and A2 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 A2 chromophores within their photoreceptors (e.g. Toyama et al., 2008). Moreover, given that for both species the rod and SWS cones in and the dottyback also the ‘normal’ MWS and LWS cones are fitted by A1 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 A1-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-tissue 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 dotties start their lives well camouflaged within their respective habitats and while their visual systems quickly adapt to a lifestyle on coral reefs, changes in their mimic adult coloration and associated habitat specialization only occur once dotties 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.

Acknowledgements
We would like to thank Eva C. McClure, Peter A. Waldie and William E. Feeney for assistance in the field, Mark McCormick and Mark Meekan for the use of light traps, and the staff at the Lizard Island Research Station for logistical help. We would furthermore like to thank the members of the Teleost Genome Project at CEES, University of Oslo, for providing unpublished raw sequence reads, and two anonymous referees for valuable suggestions.

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.

Funding
F.C. was supported by an Australian Endeavour Research Fellowship (2012), Schweizerischer Nationalfonds zur Förderung der Wissenschaftlichen Forschung Doctoral Mobility and Early Postdoctoral Mobility Fellowships (148460; 165364), and a Doctoral Fellowship (2013) from the Lizard Island Research Station, a facility of the Australian Museum; Z.M. was supported by Novartis and a Doctoral Fellowship (2013) from the Lizard Island Research Station, a facility of the Australian Museum; S.M.S. was supported by an Australian Research Council QEII Research Fellowship (2014); N.S.H. was supported by an Australian Research Council QEII Research Fellowship (2012).

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

References


**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 \(\lambda_{\text{max}}\) *(n = 48).* Spectra are fitted with Vitamin A1 rhodopsin templates of the appropriate \(\lambda_{\text{max}}\) 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 \(\lambda_{\text{max}}\) 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 $\lambda_{\text{max}}$ (also see discussion in the main article; Fig. S1).

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<th>twin cone</th>
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<td>broad spectra (coexpression?)</td>
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Table S2. qRT-PCR and pool primers used in this study

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