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Conspicuousness is correlated with toxicity in marine opisthobranchs

F. CORTESI & K. L. CHENEY

School of Biological Sciences, The University of Queensland, QLD, Australia

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Abstract

Aposematism is defined as the use of conspicuous colouration to warn predators that an individual is chemically or otherwise defended. Mechanisms that drive the evolution of aposematism are complex. Theoretical and empirical studies show that conspicuousness can be either positively or negatively correlated with toxicity as once aposematism is established, species can allocate resources into becoming more conspicuous and/or increase secondary defences. Here, we investigated the evolution of conspicuousness and toxicity in marine opisthobranchs. Conspicuousness of colour signals was assessed using spectral reflectance measurements and theoretical vision models from the perspective of two reef fish signal receivers. The relative toxicity of chemicals extracted from each opisthobranch species was then determined using toxicity assays. Using a phylogenetic comparative analysis, we found a significant correlation between conspicuousness and toxicity, indicating that conspicuousness acts as an honest signal when signifying level of defence and provides evidence for aposematism in opisthobranchs.

Introduction

Aposematism is defined as the use of conspicuous colouration to warn predators that a species is chemically or otherwise defended (Poulton, 1890; Cott, 1940). The mechanisms that drive the evolution of aposematic signals are complex and have received much attention in recent literature (Speed & Ruxton, 2005a, 2007; Darst *et al.*, 2006; Blount *et al.*, 2009). Aposematic species are thought to initially evolve from defended, inconspicuous (cryptic) species (e.g. Sillén-Tullberg & Bryant, 1983; Guilford, 1988). Once aposematism is established, resource allocation by a species becomes an important evolutionary factor: should a species invest in becoming more conspicuous, more defended or both? A positive correlation between signal strength and level of toxicity has been shown in the conspicuous and highly toxic Dendrobatid frog family and in the Asian lady bird beetle *Harmonia axyridis* (Summers & Clough, 2001; Bezzerides *et al.*, 2007). However, theory also predicts that highly defended prey should evolve less conspicuous colouration because their chances of surviving attacks are enhanced, and therefore, costs involved with conspicuous signalling can be reduced (Leimar *et al.*, 1986; Speed, 2001; Speed & Ruxton, 2005b). Support for this contradictory theory has been shown in three closely related Dendrobatid frog species: *Epipedobates parvulus*, *E. bilinguis* and *E. hahneli*. The most toxic species was only moderately conspicuous (*E. parvulus*), and the most conspicuous species was only moderately toxic (*E. bilinguis*) (Darst *et al.*, 2006).

Empirical evidence to corroborate these two hypotheses is limited, and studies have generally failed to consider the phylogenetic relatedness between large numbers of species (Darst *et al.*, 2006) or conspicuousness from a signal receiver's perspective (e.g. Summers & Clough, 2001; Bezzerides *et al.*, 2007; but see Darst *et al.*, 2006). Indeed, when investigating the function or evolution of coloured signals, it is important to consider the spectral sensitivity and visual abilities of the signal receiver, the light environment and the background against which the signal is viewed (Endler, 1990).

On coral reefs, marine opisthobranchs (which include Nudibranchia) provide an ideal model system to investigate the co-evolution of conspicuousness and toxicity. They display some of the most diverse and spectacular

Correspondence: Karen L. Cheney, School of Biological Sciences, The University of Queensland, St Lucia, QLD 4072, Australia.
Tel.: +61 7 3365 2855; fax: +61 7 3365 1655; e-mail: k.cheney@uq.edu.au

colours and patterns found in nature, which function as advertisement signals (aposematism and deimatism) and provide concealment (crypsis) to protect themselves from predation (Edmunds, 1987). Indeed, Gosliner & Behrens, (1990) estimated that 50% of opisthobranch species are aposematic. As a secondary defence, opisthobranchs contain chemicals that are produced *de novo* or deposited as secondary metabolites from their diet (Cimino & Ghiselin, 1998, 1999; Cimino *et al.*, 1999; Marin *et al.*, 1999). Defensive chemicals found within opisthobranchs are mainly alkaloids, many of which are terpenes and their derivates, and are typically stored in the outer body parts of the opisthobranch, either in the epidermal layer or in mantle glands (Carte & Faulkner, 1983; Cimino *et al.*, 1999; Fontana *et al.*, 1999, 2000; Ungur *et al.*, 1999).

To examine whether a positive correlation between conspicuousness and toxicity exists in marine opisthobranchs, we first measured the spectral reflectance of opisthobranch colour signals and their background habitat. Second, a colour opponent discrimination theoretical vision model was used (Vorobyev & Osorio, 1998) to quantify the conspicuousness of opisthobranch colour patches in terms of spectral contrast, both against their background and between colours within an opisthobranch pattern. This was carried out from the perspective of two distinct reef fish visual systems, with and without ultraviolet (UV) sensitivity. Third, toxicity assays were conducted on extracted opisthobranch chemicals using a brine shrimp assay following the protocol of Meyer *et al.* (1982). Finally, a phylogenetic comparative analysis was used to test for a correlation between conspicuousness and toxicity.

Materials and methods

Study site and species

Twenty opisthobranch species (n = 3–10 per species), comprising of members from the suborders Nudibranchia (n = 14 species), Cephalaspidea (n = 3) and Sacoglossa (n = 3), were located using SCUBA on coral reefs around Lizard Island (14°40′S; 145°28′E), Great Barrier Reef, Australia; North Stradbroke Island (27°35′S; 153°27′E), and Mooloolaba (26°40′S; 153°07′E), Southeast Queensland, Australia, at depths between 3 and 7 m. Opisthobranchs were placed in plastic vials, transported back to shore and then held in tanks with running sea water or air pumps for no longer than 48 h until their spectral reflectance could be measured. After measurements were taken, opisthobranchs were frozen and stored at −18 °C.

Spectral reflectance measurements of opisthobranchs and background habitat

Spectral reflectance measurements of opisthobranch colours were obtained using an Ocean Optics (Dunedin, FL, USA) USB2000 spectrometer and a laptop computer running Ocean Optics OOIBASE32 software. Opisthobranchs were measured in the laboratory in a tray containing enough sea water to cover each individual completely. The spectral reflectance of each distinct colour patch > 4 mm^2 (n = 1–6 depending on species) was measured through a 200-μm bifurcated optic UV/visible fibre connected to a PX-2 pulse xenon light (Ocean Optics). A Spectralon 99% white reflectance standard (LabSphere, NH, USA) was used to calibrate the percentage of light reflected at each wavelength from 300–800 nm. The bare end of the fibre was held at a 45° angle to prevent specular reflectance. At least ten measurements per colour patch were taken and then averaged. Colour measurements were taken from at least three individuals per species, with the exception of *Chelidonura inornata* and *Chelidonura varians*, of which we only acquired two individuals. Spectra were categorized by the wavelength at which light was reflected and the shape of reflectance curves, as per a previous categorization of reef fish colours (Marshall, 2000).

We also measured the spectral reflectance of background habitats that each individual was found upon using an underwater spectrometer. At the location where each opisthobranch was collected, measurements of the respective background habitat within a 5 cm radius of the opisthobranch were taken using the USB2000 spectrometer enclosed in an underwater housing (Wills Camera Housings, Vic., Australia). Data were stored using a Palm-Spec computer running Palm-Spec software (Ocean Optics). Measurements were taken with a modified (shortened, 60 cm) 1000-μm UV/visible fibre using underwater video lights (Sunray 200; Light and Motion, USA) that emit light in the 350–800 nm range. At least ten measurements were taken of the substrate and then averaged. A Spectralon 99% white reflectance standard was again used to calibrate the percentage of light reflected. For heterogeneous backgrounds, equally coloured areas were judged by eye, and then each substrate type was measured.

Visual modelling of colour signals

We used the Vorobyev–Osorio colour opponent discrimination model (Vorobyev & Osorio, 1998; Vorobyev *et al.*, 2001) to assess the conspicuousness of different opisthobranch species in terms of spectral contrast from the perspective of two potential trichromatic fish species: the UV-sensitive damselfish, *Stegastes fasciolatus* (λ_{max} = 363 nm, 470 and 528 nm) (Losey *et al.*, 2003), and the blue/green–sensitive triggerfish, *Rhinecanthus aculeatus* (λ_{max} = 420, 480 and 530 nm) (NJ Marshall, unpublished data). These fish were chosen because they have markedly different visual sensitivities, and both fish are likely to encounter opisthobranchs. Although they themselves may not be potential predators of opisthobranchs, they are used in this study to represent the visual systems of a

range of reef fish species (Losey *et al.*, 2003). Many opisthobranch species reflect colours in the ultraviolet (UV, < 400 nm) (Fig. 1, unpublished data); therefore, sensitivity in the UV may affect the way in which opisthobranch colours are perceived. These species will subsequently be referred to by their genus name only.

The model calculates the 'distance' (ΔS) between the colours in a dichromatic, trichromatic or tetrachromatic visual space, depending on the number of receptor types of the signal receiver. Colours that appear similar within each visual system result in low ΔS values, whereas those that are chromatically contrasting are high in value. This model assumes that the luminosity signal is disregarded, that colours are encoded by an opponent mechanism judged using the known cone sensitivity of the signal receiver and that colour discrimination in the perceptual space is limited by noise originating in the receptors and determined by the relative proportion of each photoreceptor (Vorobyev & Osorio, 1998; Vorobyev *et al.*, 2001). The receptor quantum catch, q_i, in photoreceptor of type i (i.e. cone cell) is calculated as (modified from eqn 1 of Vorobyev & Osorio, 1998):

$$q_i = \int_{\lambda} R_i(\lambda)S(\lambda)I(\lambda)d\lambda \qquad (1)$$

where λ denotes wavelength, R_i (λ) denotes the spectral sensitivity of a receptor i, $S(\lambda)$ is the reflectance spectrum of the colour patch, $I(\lambda)$ is the irradiance spectrum entering the eye and integration is over the range 300–700 nm. Colour distances were calculated with an illumination measured at 5 m depth.

Colour distances were calculated between each opisthobranch colour patch and the corresponding background habitat (colour/background) for each individual and then averaged for each species. Colour distances were also calculated between each adjacent colour patch on the opisthobranch (colour/colour). The maximum colour distance (ΔS) for each species was used as our measure of conspicuousness and varied considerably between opisthobranch species for both signal receivers (Table S1).

The Weber fraction *(ω)* was based on the relative proportion of receptor types, and here, we assumed a 1 : 2 : 2 ratio [short-wavelength-sensitive (SWS) cones to medium-wavelength-sensitive cones (MWS) to long-wavelength-sensitive cones (LWS); S : M : L] for our signal receivers, which was set according to morphological studies of fish retina (NJ Marshall, unpublished data). Because of the lack of behavioural data, the LWS noise threshold was set at 0.05, which represents a conservative visual performance, being half the sensitivity of the human LWS system (Wyszecki & Styles, 1982).

To compare the spectral contrast of colours while considering a coral reef environment, colour distances were calculated using illumination measurements at a

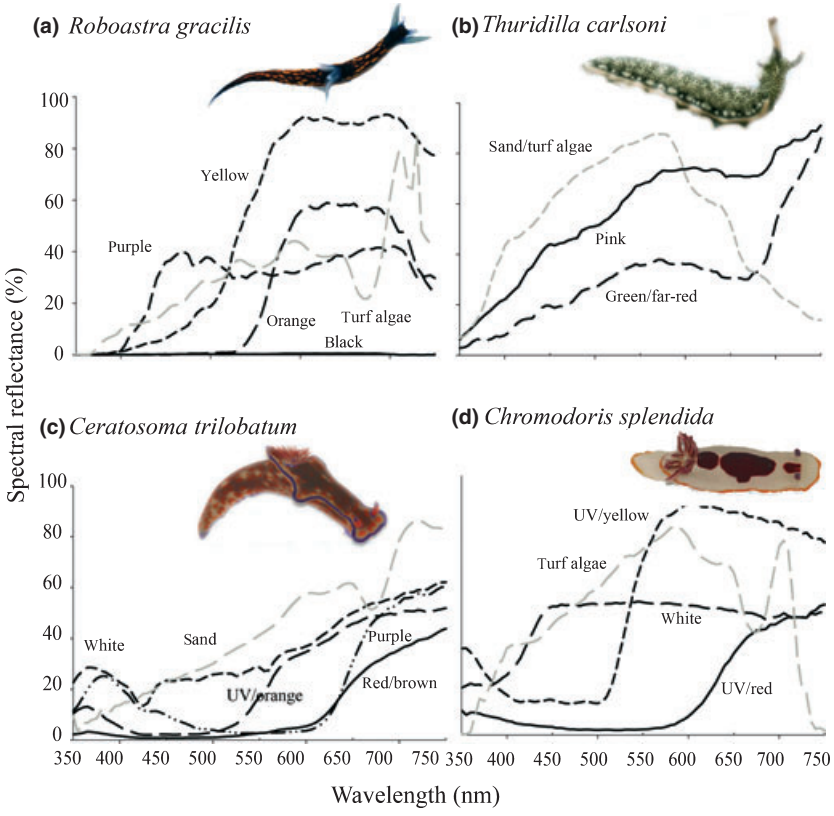

Fig. 1 Spectral reflectance (%) of four representative opisthobranch species. Grey lines indicate background colours.

water depth of 5 m. Illumination measurements were taken using the underwater spectrometer on reefs at each study site with a cosine corrector providing an 180° hemisphere. We measured both down-welling light (by holding the fibre 1 m away from the reef and pointing upwards) and side-welling light (holding the fibre at a distance of 1–2 m from and pointing horizontally at the reef); however, we found no significant differences in our overall conclusions, regardless of which measure we used.

Toxicity bioassay

We tested the relative toxic properties of extracted chemicals from each species of opisthobranch with a brine shrimp lethality assay, which is considered a useful tool for preliminary assessment of toxicity in marine organisms (Carballo *et al.*, 2002), and has been used previously when assessing opisthobranch toxicity (Gunthorpe & Cameron, 1987). Chemical extractions were conducted as per a previous study investigating the toxicity of opisthobranchs (Gunthorpe & Cameron, 1987). Briefly, frozen opisthobranchs were thawed at room temperature and homogenized using a mortar and pestle. The resulting mass was soaked for 72 h in 200 mL methanol at 4 °C. The methanol was decanted and evaporated using a rotary evaporator under reduced pressure in a water bath at 40 °C. The resulting aqueous–organic solution was then partitioned in a separating funnel in a 1 : 1 dichloromethane : distilled water mixture. The dichloromethane was evaporated using a rotary evaporator under reduced pressure in a water bath at 35 °C, and crude chemicals were transferred into 10-ml glass vials. Remaining chemical extracts that could not be transferred easily were diluted in 100% ethanol, transferred into 10-ml glass vials and left under a fume hood to ensure ethanol evaporation. All chemicals were then frozen at −4 °C until further use.

Brine shrimp bioassays were then conducted as per Meyer *et al.*, 1982. Frozen chemicals were thawed, and the weight of the compound was recorded. A solution of 10 mg compound per mL methanol was prepared. We then transferred 5, 50 and 500 μL of the solution to 1.5-cm discs of filter paper. The discs were placed in 5-mL plastic vials and left to dry for 24 h. Final concentrations of 10, 100 and 1000 μg mL^{-1} were obtained by adding 5 mL seawater to each vial during the process (see below). Control discs were prepared using methanol alone. Three replicates per dose level and control, per species of opisthobranch were prepared. Brine shrimp eggs (San Francisco Bay Brand, NY, USA) were hatched in a 1-litre glass bottle filled with double-distilled water. The hatch mix was suspended in the water and kept under aeration for 48 h, before the phototropic nauplii could be collected by pipette. Ten of the nauplii were counted against a lighted background and transferred into the test vials, which were then filled with artificial sea water to make 5 mL. A drop of dry yeast suspension was added for food supply. The vials were kept under constant illumination over a 24-h time period before survivors from each dose and control were counted out under a 4.5× dissecting microscope. The percentage of death per treatment was calculated as: % deaths = (1 − test population after treatment/control population after treatment) * 100 (Abbott, 1987) (Table 1; Fig. 3).

Phylogenetic comparative analysis

Sequence acquisition and alignment

Partial 16S rDNA gene sequences of fourteen species were taken directly from GenBank (http://www.ncbi.nlm.nih.gov/). However, sequence data were not available for six species. In these cases, we used species from the same genus: *Phyllidia elegans* for *Phyllidia picta*, *Glossodoris pallida* for *Glossodoris atromarginata*, *Flabellina verrucosa* for *Flabellina rubrolineata*, *Hypselodoris bennetti* for *Hypselodoris whitei*, *Aegires punctilucens* for *Notodoris citrina* and *Notodoris gardineri* (also known as *Aegires citrina* and *Aegires gardineri*; Fahey & Gosliner, 2004) to generate branch structure, and then species used in this study were added as polytomies. *Onchidella floridana* (Pulmonata) was included as an out-group species for rooting (Vonnemann *et al.*, 2005). Sequences were initially aligned using ClustalW (Thompson *et al.*, 1994) in BioEdit (Hall, 1999), and manual adjustments were made afterwards by eye. Alignment gaps, representing putative insertion–deletion (indel) sites, were coded as character

Table 1 Mortality (%) of brine shrimp (n = 30) after 24 h of exposure to opisthobranch chemical extracts.

	Lethality (%)		
Opisthobranch species	10 μg mL^{-1}	100 μg mL^{-1}	1000 μg mL^{-1}
Chelidonura inornata	25	100	100
Roboastra gracilis	0	45	100
Sagaminopteron ornatum	50	89	100
Phyllidiella pustulosa	0	10	97
Phyllidia varicosa	0	17	67
Phyllidia picta	0	0	60
Glossodoris atromarginata	3	12	47
Ceratosoma trilobatum	0	14	35
Chromodoris splendida	0	21	32
Flabellina rubrolineata	0	11	30
Elysia ornata	0	0	27
Chelidonura varians	0	0	24
Phyllidia ocellata	3	11	24
Notodoris gardineri	0	0	17
Thuridilla gracilis	0	0	11
Hypselodoris whitei	0	10	10
Notodoris citrina	0	0	10
Risbecia tryoni	0	0	8
Hypselodoris obscura	0	0	7
Thuridilla carlsoni	0	0	0

states (1 = character present, 0 = character absent). The total analysed alignment length of 16S comprised 463 base pairs. The alignment and resulting trees are deposited in TREEBASE (http://www.treebase.org).

Phylogenetic reconstruction

We assessed the phylogenetic relationship using Maximum likelihood (ML) and Bayesian inference approaches. RAxML version 7.0.4 (Stamatakis *et al.*, 2005) was used for ML reconstruction with default settings (GTRGAMMA model) on the web-interface (http://www.phylo.org/) using rapid bootstrap analysis (1000 replicates) and the search option for best scoring ML tree (Stamatakis *et al.*, 2008). Bayesian inference was conducted using MRBAYES v3.1.2 (Huelsenbeck & Ronquist, 2001), using a Metropolis Chain Monte Carlo search. Each set produced five million generations by sampling every 1000 generations. The first 1000 trees (=1 000 000 generations) were removed as burn-in. Only clades with significant support values (> 70% bootstrap; > 0.80 posterior probabilities) were used for subsequent analysis; others were collapsed and treated as polytomies for the comparative analysis (Fig. 2).

Statistical analysis

Colour distances (ΔS) were first log-transformed to meet the assumptions of parametric testing. To assess whether colour distances were significantly different between signal receivers, we used a General Linear Mixed Model (GLMM) with log colour distance as the response variable, signal receiver and colour category as fixed factors and opisthobranch species as a random factor. Highly nonsignificant interactions ($P > 0.25$) were omitted from the model. There was a significant difference in colour distances between signal receivers for opisthobranch colours against their background ($F_{1,19.5} = 125.3$, $P < 0.001$) and between colours within an opisthobranch pattern ($F_{1,115} = 14.3$, $P = 0.001$); therefore, analyses for each signal receiver were conducted separately.

A linear regression model was first used to test for a relationship between conspicuousness and toxicity without considering the relatedness of species. Relative toxicity was defined as the percentage of dead brine shrimps at a concentration of 1000 μg/mL. To consider whether a correlation existed between conspicuousness and toxicity while considering phylogenetic relatedness between species, we used a Generalized Least Squares (GLS) regression model. To select the most parsimonious model, we ran the analysis using the Grafen (1989), Martins & Hansen (1997), Brownian (Felsenstein, 1985) models using corGrafen, corMartins, corBrownian packages in the ape package (http://ape.mpl.ird.fr) and selected the model with the lowest AIC value (corGrafen). To resolve polytomies, we used the multi2di function in the ape package for R and introduced zero-length branch lengths for the branch within the polytomy. We adjusted the degrees of freedom to account for soft polytomies in our phylogenetic tree (Garland & Diaz-Uriarte, 1999). All phylogenetic regression analysis was conducted in R v. 2.4.1. (http://www.r-project.org/).

Results

Conspicuousness of opisthobranchs

The most common colour categories found on opisthobranch molluscs were white ($n = 9$ species), black ($n = 8$), brown ($n = 8$) and purple ($n = 8$). Opisthobranchs were found on turf algae, sand, live coral, sponge and tunicates (Fig. 1, Table S1). Overall, colour distances were significantly higher for the UV-sensitive fish *Stegastes* compared to *Rhinecanthus* (mean ± SE: colour/background *Rhinecanthus* 8.8 ± 7.4, *Stegastes* 20.7 ± 1.6, paired *t*-test $t_{55} = 9.6$, $P < 0.001$; colour/colour: *Rhinecanthus* 13.5 ± 1.8, *Stegastes* 20.1 ± 2.3, paired *t*-test $t_{57} = 4.80$, $P < 0.001$). The highest colour distances were found on *Roboastra gracilis* between colour (orange) and background and between colours within the pattern (black and orange) (Fig. 1; Table S1). The lowest colour distances were found on *Thuridilla carlsoni* between colours and background (*Rhinecanthus:* green/far-red; *Stegastes:* pink) and between colours within the pattern (green/far-red and pink, for both signal receivers) (Fig. 1; Table S1).

Toxicity of opisthobranchs

Toxic extractions from each opisthobranch species showed partial or total lethality to brine shrimps at a concentration of 1000 μg/mL, with the exception of *Thuridilla carlsoni*. *Roboastra gracilis*, *Chelidonura inornata* and *Sagaminopteron ornatum* were found to cause 100% lethality at this concentration (Table 1).

Phylogenetic analysis

The results of the phylogenetic analysis were in general agreement with previously published trees on opisthobranch phylogenies (Wollscheid-Lengeling *et al.*, 2001; Vonnemann *et al.*, 2005) (Fig. 2). There was a significant association between conspicuousness and toxicity from the perspective of both signal receivers for colour/background measurements (*Rhinecanthus*: $r^2 = 0.27$, $n = 20$, $P = 0.02$; *Stegastes*; $r^2 = 0.35$, $n = 20$, $P = 0.01$; Fig. 3) and for colour/colour measurements (*Rhinecanthus*: $r^2 = 0.25$, $n = 19$, $P = 0.03$; *Stegastes*: $r^2 = 0.27$, $n = 19$, $P = 0.03$; Fig. 3).

Using the phylogenetic generalized least squares (GLS) regression model, there was also a significant association between conspicuousness and toxicity for colour/background measurements (*Rhinecanthus*: $t_{17} = 2.62$, $P = 0.019$; *Stegastes*: $t_{17} = 3.11$, $P = 0.007$) and colour/colour measurements (*Rhinecanthus*: $t_{17} = 2.37$, $P = 0.03$; *Stegastes*: $t_{17} = 2.40$, $P = 0.03$). However, we

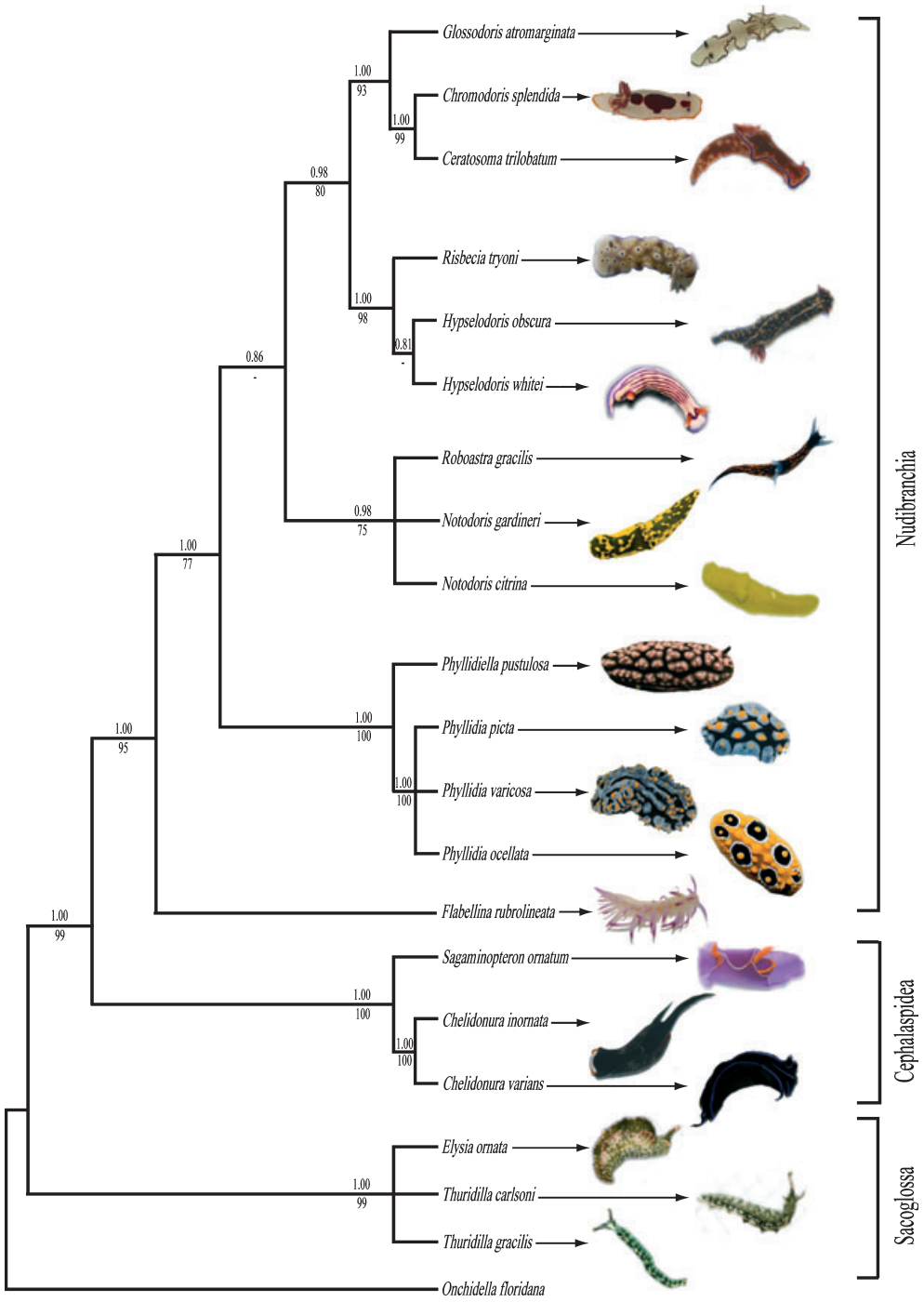

Fig. 2 Bayesian majority rule consensus phylogram of the 16S rDNA gene. Numbers above branches represent posterior probabilities from Bayesian analysis, and numbers below branches represent bootstrap values for nodes obtained by RAxML. Only values greater or equal to 0.8 and 70 are shown.

found little phylogenetic signal in our data (Grafen's rho < 0.26), and there was little difference between the GLS regression model and ordinary linear model for each signal receiver (ANOVA, d.f. = 3, $P > 0.93$).

Discussion

Our study reveals that the conspicuousness of marine opisthobranchs, measured in terms of maximum spectral

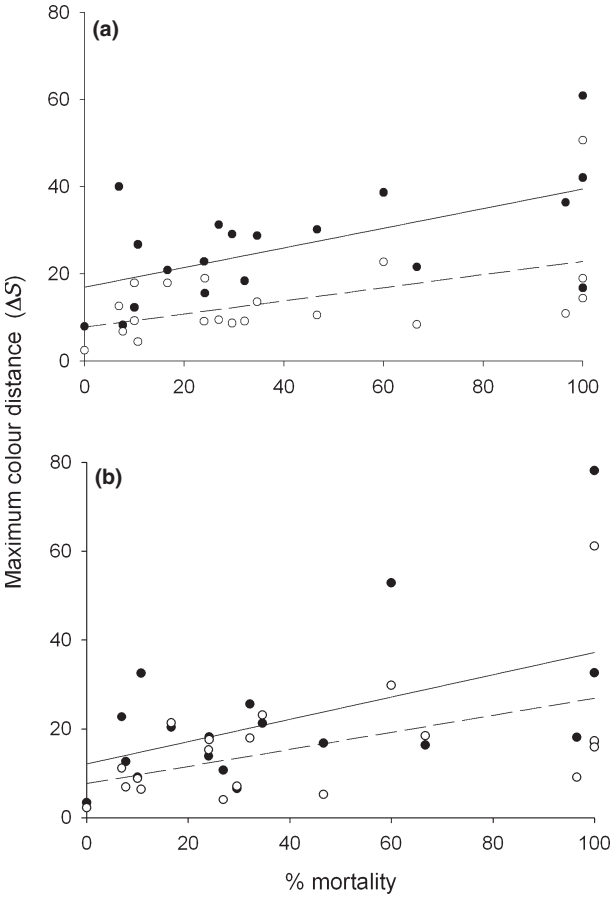

Fig. 3 Mortality of brine shrimp (%) in relation to maximum colour distance for (a) colour/background contrast and (b) colour/colour contrast. Dark circles and solid lines represent *Stegastes*, open circles and dashed lines *Rhinecanthus*.

contrast of an opisthobranch colour patch against its background, and between two colours within a pattern, was correlated with toxicity. This was found to be the case when we considered colour signals from the perspective of two reef fish visual systems, with and without UV sensitivity. Therefore, conspicuousness appears to be an honest signal of the strength of secondary defences within opisthobranchs and provides evidence that aposematism has evolved as a defensive strategy in this family (Yachi & Higashi, 1998; Servedio, 2000; Summers & Clough, 2001).

Contrasting theories predict that conspicuousness should either evolve with increased toxicity (Summers & Clough, 2001) or the evolution of aposematic displays favours conspicuousness or toxicity, but not both (Darst *et al.*, 2006). Speed & Ruxton (2007) developed an optimization model to account for differences between these two scenarios. The model predicts that when the costs (in terms of fecundity) involved in producing a warning display vary, but the costs of producing secondary defences are fixed, then a negative correlation will exist between conspicuousness and toxicity. In this scenario, prey species benefit from diminishing resource allocation into a warning display and increasing investment into secondary defences. However, if the costs of producing a warning display and secondary defence increase in relation to each other, then a positive correlation between warning display and toxicity can exist. In support of this, it has been suggested that antioxidant molecules may be used for both pigmentation and protection against accumulated toxins; therefore, their presence may explain a positive correlation between conspicuousness and toxicity (Blount *et al.*, 2009).

Other factors may also influence the coevolution of conspicuousness and toxicity within a species (Guilford, 1988). Sexual selection may favour brightly coloured males or females (Andersson & Iwasa, 1996), which may increase detection rates by predators and thus increase rates of mortality. An increase in secondary defence mechanisms may then evolve in response to this. However, opisthobranchs have very primitive eyes; therefore, colouration is very unlikely to be used in intraspecific communication (Edmunds, 1987).

We found little phylogenetic signal in our data, indicating that the correlation between conspicuousness and toxicity was independent of the relationship between organisms. If colour signals of a particular species are a function of diet and chemicals sequestered, then an apparent multiple evolution of conspicuousness may be caused by the diet specialization of individual species independent of their phylogenetic relationship. For example, *Phyllidia ocellata*, which was found to be mildly toxic and relatively inconspicuous, is known to sequester the toxins 10α-Isocyano-4-amorphene and Cavernothiocyanate from the sponge *Acanthella cf. Cavernosa*. *Phyllidia varicosa* on the other hand, a sister species of *P. ocellata* (Fig. 2), sequesters 2-Isocyanopupukeanane and 9-Isocyanopupukeanane from the sponge *Hymeniacidon* sp. and was found to be more toxic and more conspicuous than *P. ocellata* (Garson & Simpson, 2004). The ancestral Sacoglossans appeared more cryptic than the more recently derived Nudibranchia; therefore, conspicuousness may have evolved from cryptic ancestral states. However, this needs to be investigated further using more comprehensive analyses. Colour variation may also exist within an opisthobranch species. Juveniles are sometimes more lightly pigmented than adults, and pigments from food are often used to create colouration; therefore, individuals within the same species can differ in colour depending on the availability of particular foods (e.g. *Pteraeolidia ianthina*). However, for this study, we used species that showed little intraspecific variability in colour patterns.

Phylogenetic signals may also be clouded by the accuracy of trait data. We measured the maximum spectral contrast between two colours as our measure of conspicuousness. However, aposematic signals are

generally multimodal (Ruxton *et al.*, 2004); therefore, other visual components of colour patterns may be important in effective signalling to predators, such as luminance contrast and spatial distribution of colours within a pattern. For example, *Phyllidia ocellata* appeared to be relatively inconspicuousness in our measure of spectral contrast, but its distinct bold patterning (Fig. 2) suggests that luminance contrast may be important in interspecific signalling. However, colour contrast is considered more critical to the effectiveness of aposematic signalling than luminance contrast when predators have colour vision (Osorio *et al.*, 1999; Gamberale-Stille & Guilford, 2003). We also only investigated how potential reef fish predators with trichromatic visual systems viewed opisthobranch colour signals. Unfortunately, little is known about the identity of opisthobranch predators, so this was our best estimate of the selective pressures that could drive the evolution of colour signals in marine opisthobranchs derived from predation attempts observed in the field. However, other predators may include dichromatic reef fish, sea spiders and crabs, whose visual systems may also influence the evolution of warning signals in opisthobranchs.

Our measures of toxicity were somewhat crude; however, brine shrimp assays have been shown to provide a suitable initial screening for marine natural chemicals (Carballo *et al.*, 2002) and gave us a suitable relative measure of toxicity between species. Furthermore, brine shrimp assays have shown similar results to ichthyologic assays on *Cyprinodon variegates* when assaying toxins from a marine dinoflagellate (Moeller *et al.*, 2001). When consumed, terpenes and their derivatives affect the central nervous system; therefore, terpenes would likely affect vertebrates in a similar manner to invertebrates. Defensive chemicals may also be nontoxic but unpalatable, and responses may vary between taxa. Although many opisthobranchs contain chemicals that are unpalatable to a variety of species (e.g. Long & Hay, 2006), the link between toxicity and unpalatability has yet to be determined.

Why the appearance of prey varies from the highly cryptic to the very bright and conspicuous remains an intriguing phenomenon. Conspicuousness is costly in terms of attracting the attention of predators, and only well-protected species can generally afford this type of advertisement (Speed, 2000). Indeed, our results suggest that the most conspicuous marine opisthobranchs are the most toxic. However, further information is needed on the costs involved in producing warning displays and storing defence chemicals to fully understand the evolution of such signals.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Maximum colour distances (ΔS) found between colour patch on a species and its background.

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