

Conspicuous visual signals do not coevolve with increased body size in marine sea slugs

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Abstract

Many taxa use conspicuous colouration to attract mates, signal chemical defences (aposematism) or for thermoregulation. Conspicuousness is a key feature of aposematic signals, and experimental evidence suggests that predators avoid conspicuous prey more readily when they exhibit larger body size and/or pattern elements. Aposematic prey species may therefore evolve a larger body size due to predatory selection pressures, or alternatively, larger prey species may be more likely to evolve aposematic colouration. Therefore, a positive correlation between conspicuousness and body size should exist. Here, we investigated whether there was a phylogenetic correlation between the conspicuousness of animal patterns and body size using an intriguing, understudied model system to examine questions on the evolution of animal signals, namely nudibranchs (opisthobranch molluscs). We also used new ways to compare animal patterns quantitatively with their background habitat in terms of intensity variance and spatial frequency power spectra. In studies of aposematism, conspicuousness is usually quantified using the spectral contrast of animal colour patches against its background; however, other components of visual signals, such as pattern, luminance and spectral sensitivities of potential observers, are largely ignored. Contrary to our prediction, we found that the conspicuousness of body patterns in over 70 nudibranch species decreased as body size increased, indicating that crypsis was not limited to a smaller body size. Therefore, alternative selective pressures on body size and development of colour patterns, other than those inflicted by visual hunting predators, may act more strongly on the evolution of aposematism in nudibranch molluscs.

Introduction

Animals that contain toxic or unpalatable chemicals often use conspicuous colouration and distinct body patterning to communicate unprofitability to visual hunting predators. Such warning colouration, or

aposematism, is found in a wide range of organisms including insects, snakes, molluscs, fish and amphibians (Poulton, 1890; Cott, 1940; Ruxton *et al.*, 2004). How such warning or aposematic colouration evolves has puzzled scientists for decades and has been limited by a lack of comparative studies investigating real prey species. Conspicuousness is a key feature of warning signals, as conspicuous signals are more likely to be detected and learned by predators, and memorized for longer (Rothschild, 1984; Roper, 1994; Lindstrom *et al.*, 2001; Aronsson & Gamberale-Stille, 2008). A conspicuous

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signal must differ from its background in colour, pattern and/or luminance from the perspective of the intended receiver (Endler, 1978, 1991). High contrast against the background increases signal efficiency and initial wariness by predators (Roper & Cook, 1989; Lindstrom *et al.*, 2001; Ruxton *et al.*, 2004), and the speed and strength of avoidance learning (Gittleman & Harvey, 1980). However, no simple method exists for quantifying the conspicuousness of an object against its background (but see Endler, 2012). Furthermore, the relative importance of each component and how they interact is often unclear (but see Osorio *et al.*, 1999; Aronsson & Gamberale-Stille, 2008).

Experimental evidence suggests that an increase in body size and/or in pattern element size within the visual display strengthens the avoidance response of warning colouration by predators (Gamberale & Tullberg, 1996b, 1998; Lindstrom *et al.*, 1999; Nilsson & Forsman, 2003). Domestic chicks have been shown to have an unlearned aversion to larger-sized insect prey (Gamberale & Tullberg, 1996a, 1998), and larger pattern elements in artificial prey caused blue tits to learn signals more rapidly and provided enhanced avoidance of unpalatable prey (Lindstrom *et al.*, 1999). Predator selective pressures may therefore cause species that have acquired aposematic colouration to increase overall body size and/or increase body pattern elements relative to body size (e.g. widening of stripes or size of dots). Alternatively, species with larger body size may be more likely to evolve conspicuous colouration. If predator selective pressures influence the evolution of aposematic displays, we would expect to find an evolutionary correlation between conspicuousness and body size of aposematic species. Indeed, a comparative analysis of poison frogs (Dendrobatidae) (Hagman & Forsman, 2003) indicated that body size was correlated with an increase in conspicuous colouration, in terms of brightness quantified by human assessment and computer analysis of digital colour photographs. However, Nilsson & Forsman (2003) failed to find such a correlation in moths, but lifestyle was thought to confound the results as a shift from lone behaviour to gregariousness was also partnered with a decrease in body size.

In this study, we examined this hypothesis using an intriguing, understudied model system, namely nudibranchs (opisthobranch molluscs, commonly known as sea slugs). To do this, we quantified the conspicuousness of animal body colouration using new ways of quantifying the spatial frequency of body patterns (intensity variance and power spectrum analysis) and compared this to spectral contrast measurements, which is frequently the sole measure of conspicuousness in studies of animal colour patterns. We then used Bayesian phylogenetic regression analysis to assess how these measures of conspicuousness related to animal body size.

Materials and methods

Study species

Nudibranchs exhibit tremendous species-level diversity with over 3000 species worldwide. Our sampling covered representative species from infraorder Doridacea (families: Aegiridae, Chromodoridae, Dendrodorididae, Discodoridae, Dorididae, Phyllidiidae and Polyceridae) and infraorder Aeolidida (families: Glaucidae, Facelinidae and Flabellinidae) (Table S1). Most nudibranchs contain secondary metabolites, including isocyanides, diterpenes and sesquiterpenes (Faulkner & Ghiselin, 1983; Cimino *et al.*, 1985; Avila, 1995), which protect the animals from predatory attacks (Avila, 1995; Mollo *et al.*, 2005). These chemicals are often localized in selected parts of the body (Avila & Paul, 1997; Somerville *et al.*, 2006; Wagele *et al.*, 2006) and can be diet-derived or produced *de novo* (Cimino *et al.*, 1983; Cimino & Sodano, 1993; Fontana *et al.*, 1994). Nudibranchs also range in their visual displays and include those that are highly cryptic against their background habitat to those that exhibit bold and distinct body colouration, which are used as aposematic signals. Although information on the identity of potential nudibranch predators is limited, predators are thought to include fish (e.g. pufferfish, triggerfish and wrasse) and other invertebrates such as crabs, sea spiders and other opisthobranchs. However, fish predators are considered to be the main selective pressure that drives the evolution of conspicuous colours and patterns due to their ability to detect colour (Siebeck *et al.*, 2008) and their di- or trichromatic visual system (Marshall *et al.*, 2006). We measured nudibranch body lengths from live individuals that were collected for spectral reflectance measurements and used nudibranch identification books with detailed body length information (Cobb & Willian, 2006; Debelius & Kuitert, 2007; Coleman, 2008). We used mean body length from a minimum of eight individual measurements for each species; measurements for juveniles were omitted. In this study, average body size ranged from 1.0 to 10.0 cm (Table S1).

Phylogenetic reconstruction

We used a Bayesian inference approach to estimate the phylogenetic relationships between 76 nudibranch species for which we were able to collect pattern and/or colour data. Phylogenetic relationships were reconstructed using published COI and 16S gene sequences from GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) for 49 species. We also sequenced both genes for an additional 16 species, COI for an additional eight species and 16S for an additional three species (Accession Numbers listed in Table S1). Three pleurobranch species: *Pleurobrancha meckeli*, *Bathyberthella antarctica* and *Thompsonia antarctica* were used as outgroups to root the trees.

DNA was extracted using a Qiagen DNeasy blood and tissue kit, and sequences were amplified using primers and protocols as in Wilson *et al.*, (2009). PCRs were carried out using illustra PuRe taq Ready-to-go beads (GE Healthcare, Piscataway, NJ, USA), and amplicons were directly purified using ExoSAP-IT (USB, Cleveland, OH, USA) prior to sequencing. Sequences were edited and reconciled in Sequencher (Genecodes, Ann Arbor, MI, USA). Sequences were aligned using the Q-INS-i strategy in Multiple Alignment using Fast Fourier Transform (MAFFT) (Kato & Toh, 2008), which takes into account secondary structure. The resulting alignment length of COI data comprised 658 bp and 16S data comprised 527 bp. We then used Gblock (Castresana, 2000) for the 16S alignment, implementing the least stringent options to remove areas of ambiguous alignment in an explicit, repeatable manner. This resulted in a 391 bp alignment, comprising 74% of the original 527 positions. Geneious v6.0.5 (Cimino & Sodano, 1993) was used to concatenate COI and 16S alignments, which resulted in a final dataset of 1049 bp.

We used PartitionFinder v1.1.0 (Lanfear *et al.*, 2012) to search for the best partitioning scheme and model of sequence evolution for our dataset. Evolutionary models were chosen from the ones available in MrBayes, with linked branch lengths, a search for all possible schemes, and the Bayesian information criterion (BIC) as criterion for model selection. The resulting partitioning scheme comprised four subsets: 16S, first, second and third COI codon positions. GTR + G was chosen to be the best model of evolution for the second codon position of COI, and GTR + G + I was chosen to be the best model for 16S, the first and third codon position of COI. Subsequent Bayesian inference was conducted using MrBayes, v.3.2.1 (Ronquist *et al.*, 2012), using a MCMC search with two independent runs and four chains each. All partitions were set to be variable, and parameters (shape, pinvar, statefreq and revmat) were unlinked to allow each partition to evolve independently. Each run produced ten million generations, with trees sampled every 1000 generations (10 000 trees per run). A majority-rule consensus tree (Fig. 1) was constructed to illustrate the phylogenetic relationship between nudibranch species. We also show density plots of 1000 trees created in DensiTree v2.0.1 (Bouckaert, 2010) (Fig. S2).

Quantification of conspicuousness

We focused on quantifying two main aspects of conspicuousness: (i) analysis of first- and second-order image statistics to determine the brightness contrast and spatial frequency of the nudibranchs' pattern and how closely the pattern matches the background (as per Zylinski *et al.*, 2011); and (ii) spectral contrast both within the nudibranch body pattern and against background (as per Dalton *et al.*, 2010; Wang, 2011).

(i) First- and second-order image statistics

We obtained digital images of nudibranch species taken in the Indo-Pacific region by ourselves, a variety of other scientific researchers and recreational divers. Any photographs that were under or over exposed (i.e. containing large areas of white or black), or not focused, were excluded and only photographs that had >85% background habitat in the images were included. We ensured that we had a minimum of eight independent (e.g. different individuals and locations) images for each species (range 8–48 per species; Table S1). For this analysis, we had a total of 61 species.

The average length of each nudibranch species was used to calibrate image scale relative to known average body lengths (Table S1). Each photograph was scaled to approximate a scene viewed by a fish observer with an optical resolution of 10 cycles per degree (Collin & Pettigrew, 1989) and a viewing distance of 10 cm. This scaling method provided an estimate of the visual information available to a hypothetical fish predator, which is the most relevant perspective for an analysis of nudibranch conspicuousness.

The position of each nudibranch was manually identified by tracing the animal's outline with a computer mouse. Six background samples were then identified by shifting the nudibranch outline to random positions within the background area of the image. Each of these seven image samples (one nudibranch and six background samples) was split into its three RGB colour channels. Fish are thought to use their double cones for luminance vision (Kelber *et al.*, 2003), which generally lie in the green part of the visual spectrum at around 500 nm (Lythgoe, 1979; Marshall *et al.*, 2006); therefore, we used only the green channel for our analysis to approximate likely photon catch and therefore intensity viewed by such fish predators (for full discussion of this see Vorobyev *et al.*, 2001; Stevens *et al.*, 2007).

First- and second-order image statistics were then calculated for each nudibranch and background sample. The first-order statistic used was intensity variance, a measure of the distribution of brightness contrast within the sample. This was calculated as the standard deviation of pixel intensity values. Samples containing a range of intensity values, for example a pattern with mixture of light and dark patches, generate a high intensity variance value, whereas plain patterns produce a low value. The second-order statistic used was the two-dimensional power spectrum, an estimate of the spatial frequency of patterns within the sample area. This is calculated in a similar way to one-dimensional frequency analysis, in which a sequence of values (e.g. a line of pixel values extracted from an image transect; Fig. 2a,b) is converted into a measure of its frequency components using a Fourier transform (Fig. 2c; Fig. S1 for more examples). Each image

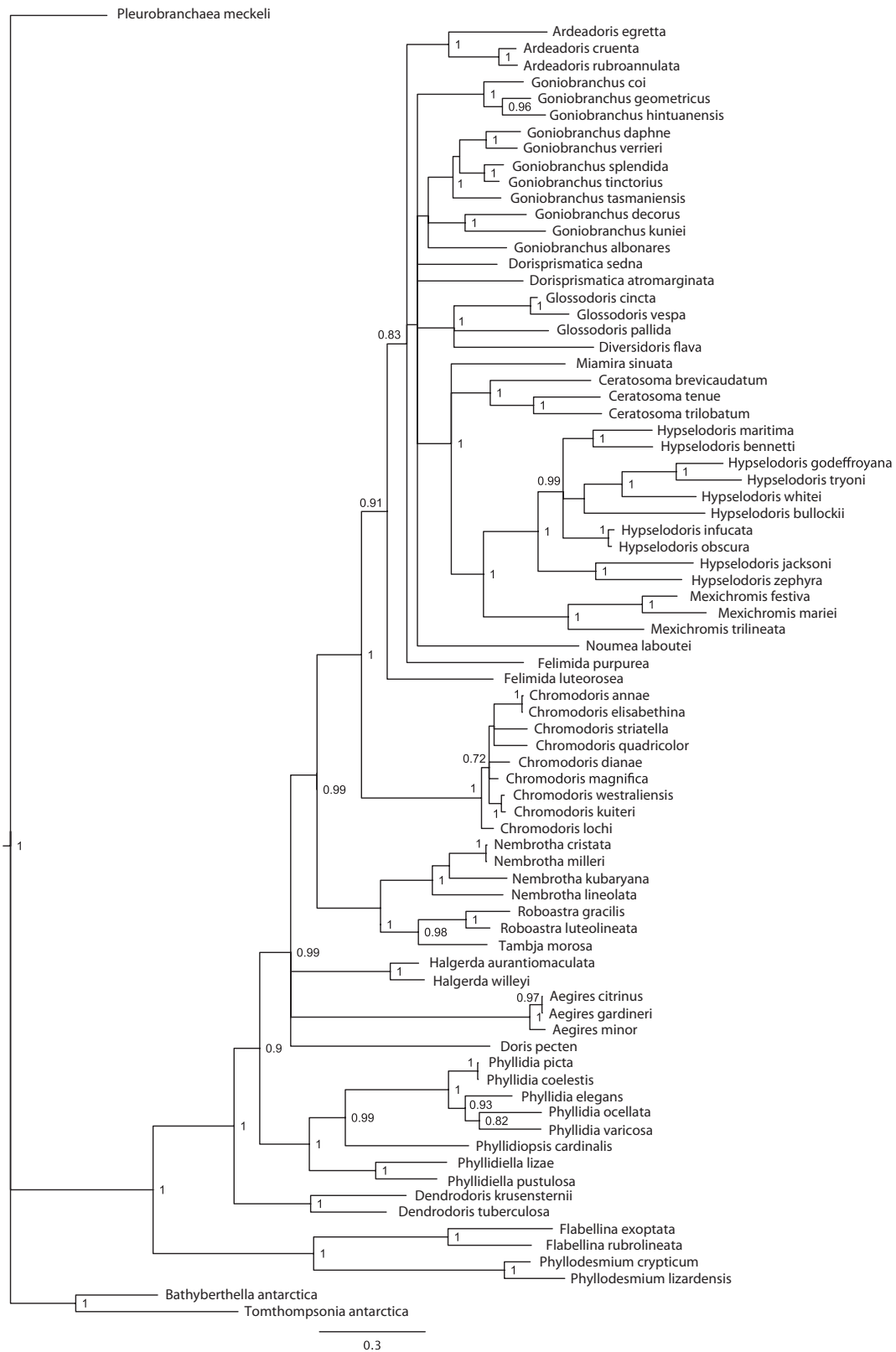


Fig. 1 Bayesian 50% majority rule consensus phylogram based on CO1 and 16S rDNA sequence data. Only support values for Bayesian posterior probabilities (≥ 0.7) are shown.

sample was extracted from the image and padded with black pixels to make a rectangular shape. A two-dimensional discrete Fourier transform (Matlab function *fft2*) was performed on the sample (Fig. 2d), and the resulting amplitudes were rotationally averaged to produce a log-scaled power spectrum curve (Fig. 2e; see Field, 1987 for details). These image statistics were then used to quantify the conspicuousness of each nudibranch against their background by calculating the difference in area beneath power spectrum curves (nudibranch – background curve) and absolute difference between power spectrum curves. Both of these measurements gave similar results, so we present the former. This method could not be used to compare patterns between images (within pattern analysis) due to edge effects from the border between the sample and the padded area. Image intensity variance and power spectrum are potentially vulnerable to changes in camera exposure settings; however, performing within-image comparisons of sample and background measures largely controls for such effects.

(ii) Spectral contrast

To assess conspicuousness in terms of spectral contrast, spectral reflectance measurements were collected for 61 nudibranch species ($n = 1\text{--}6$ individuals per species). Nudibranchs were located using SCUBA on coral reefs at depths from 1 to 15 m in Australia: Lizard Island (14°40'S; 145°28'E) and Heron Island (23°29'S; 151°11'E), Great Barrier Reef; North Stradbroke Island (27°35'S; 153°27'E), Mooloolaba (26°40'S; 153°07'E) and Gold Coast (27°25'S; 153°25'E), Southeast Queensland; and in Indonesia: Palau Hoga, (05°28'S; 123°45'E). Nudibranchs were placed in plastic vials or bags and held in containers with air pumps for no longer than 48 h. Spectral reflectance measurements of nudibranch colours were measured using an Ocean Optics (Dunedin, FL, USA) USB2000 spectrometer and a laptop computer running Ocean Optics OOIBASE32 software. Nudibranchs were placed in a tray containing enough seawater to cover each individual completely, and the spectral reflectance of each distinct colour patch $> 4 \text{ mm}^2$ 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, North Sutton, NH, USA) was used to calibrate the percentage of light reflected at each wavelength from 300 to 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 per individual were taken and then averaged.

To estimate the conspicuousness of nudibranch colour pattern based on spectral contrast from the perspective of a potential trichromatic reef fish predator, we used the Vorobyev–Osorio theoretical vision model (Vorobyev & Osorio, 1998). As per previous studies (Cheney & Marshall, 2009; Cortesi & Cheney,

2010), we assumed a 1 : 2 : 2 ratio for the weber fraction (ω), LWS noise threshold was set at 0.05. To account for the light environment in which the colours would be viewed, colours were modelled using illumination measurements at a water depth of 5 m (as per Cheney & Marshall, 2009). We considered the effects of signal transmission through water to be negligible, as most coral reef fish would view nudibranchs from a relatively close distance (approximately 1–2 m).

The model calculates the 'colour distance' (ΔS) between colours in a trichromatic visual space. Colours that appear similar within each visual system result in low ΔS values, whereas those that are chromatically contrasting are high in value. We modelled colours from the perspective of a trichromatic, benthic feeding fish species: the Picasso triggerfish, *Rhinecanthus aculeatus* ($\lambda_{\text{max}} = 413, 480, 530 \text{ nm}$; Cheney *et al.*, 2013). This fish was chosen because they are likely to encounter nudibranchs and are representative of a common visual system found in a range of reef fish species (Losey *et al.*, 2003; Marshall *et al.*, 2006). We also show results from a potential dichromatic fish predator in the supplementary information (Fig. S4).

To measure **against background spectral contrast**, we measured the spectral reflectance of coral reef background habitats (coral, turf algae, sponge, sand and specific habitats such as xeniid soft corals, which are used by the cryptic nudibranch *Phyllodesmium lizardensis*) using an underwater spectrophotometer (as per Cortesi & Cheney, 2010). Colour distances were calculated between each nudibranch colour patch and the background habitat on which it was found most frequently. To estimate **within pattern spectral contrast**, colour distances were calculated between each nudibranch colour patch with a diameter (circular) or width (stripe) $> 3 \text{ mm}$ for each individual and then averaged for each species. Species that only had one colour patch ($n = 4$) were removed from this analysis ($n = 59$ species).

Comparative analysis of traits

We used a Bayesian approach, employing the methods outlined in de Villemereuil *et al.* (2012). We took the first 10 000 trees (with branch lengths) from the output of two MrBayes chains. We discarded the first 2500 of each as burn-in, and combined the two resulting sets of trees (15 000 trees). Species that did not have trait data (pattern data or spectral contrast) for a particular analysis were pruned from trees. The trees were then converted to correlation matrices using the function *vcv.phylo* in package *ape* version 3.0-8 for R version 3.0.1 (Paradis *et al.*, 2004; R Core Team, 2013). The trait data and the correlation matrices were used as input to a Markov Chain Monte Carlo analysis using program JAGS (Plummer, 2012). We ran three separate chains for each analysis, with different starting values. We fitted simple linear regression models, regressing each col-

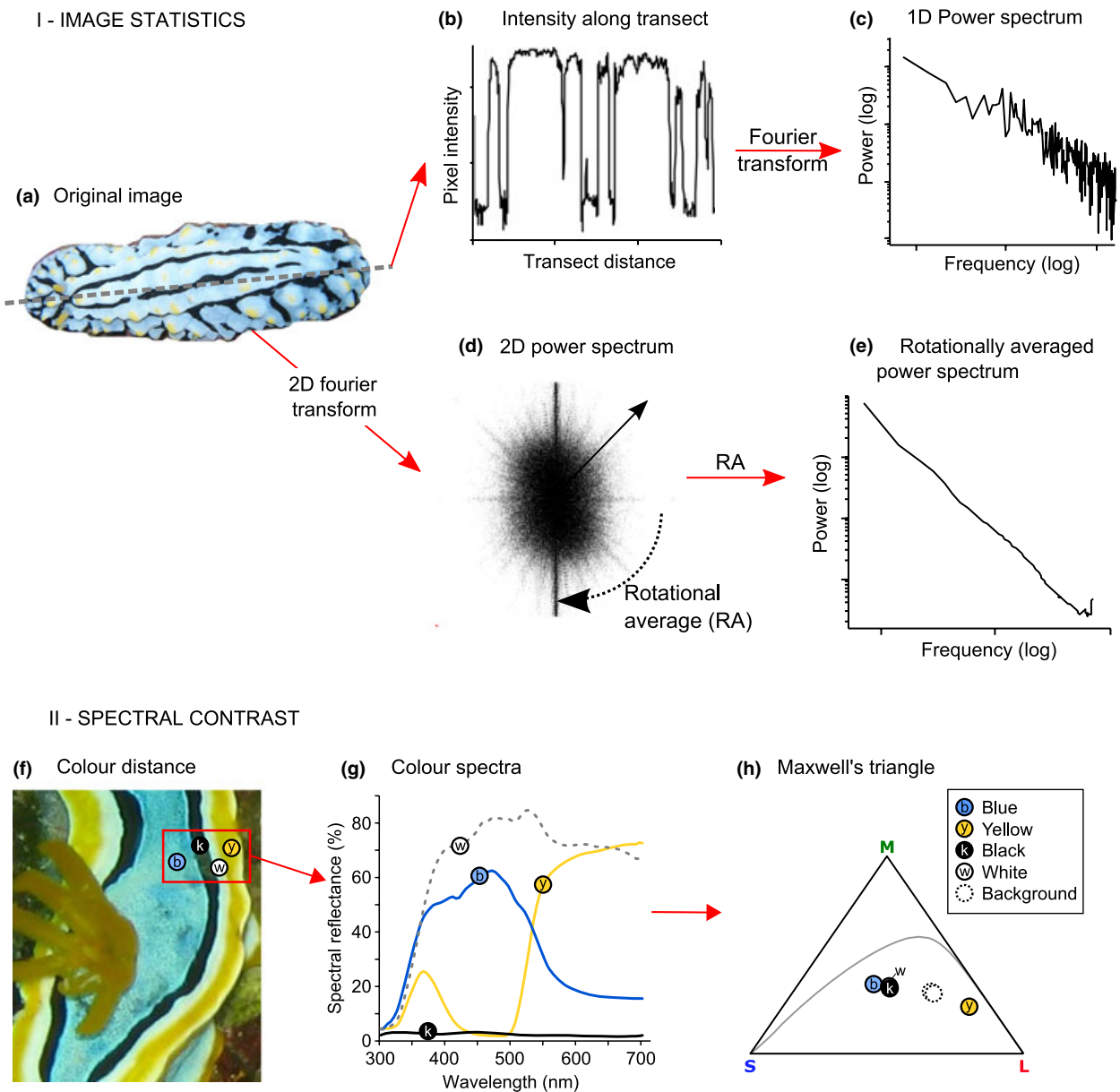


Fig. 2 Methods used for quantifying conspicuousness. (I) Spatial frequency was calculated using the two-dimensional Fourier transform. The principle is first illustrated in one dimension. A transect through an image (a – grey dashed line) is used to extract a slice of pixel intensity values (b), and the frequencies contained within are characterized with a Fourier transform (c). The slope of the frequency–power relationship is a rough measure of the distribution of frequencies within the image. Two-dimensional Fourier transforms have a similar approach, but transects are sampled through the image in all orientations, producing a two-dimensional map of frequency distributions (d) which is then rotationally averaged into a one-dimensional power spectrum (e). (II) Distance in visual space between different nudibranch colours: blue [b], black [k], white [w] and yellow [y] patches on a nudibranch (f). First the reflectance spectral frequency is measured for each colour (g). This is then modelled from the perspective of the colour vision system of a potential fish predator (e.g. *R. aculeatus*, this study) (h) and colour distances between spectra calculated using the Vorobyev–Osorio model (Vorobyev & Osorio, 1998).

our or pattern variable on body size (cm), accounting for phylogenetic uncertainty by randomly sampling from the set of phylogenetic correlation matrices. In addition, we estimated Pagel’s λ for each regression,

which is a measure of phylogenetic signal in the data and flexibly accommodates some branch-length uncertainty (Pagel, 1999). We computed the posterior distributions (conditional on the observed data) for the slope

Fig. 3 Scatterplot of image statistics (intensity variance, difference in power spectrum slope) and spectral contrast (colour distance) against average body size of each nudibranch species, (a) against the background habitat and (b) within animal colour pattern. Highest-density predicted interval (HDPI) and regression lines show results of phylogenetic regression analysis. Bold HDPI values indicate those that did not exceed 0.

and intercept of the regression, the residual standard deviation (σ), and λ . We used Normal priors for the intercept and slope, both with zero mean and precision 10^{-6} . For σ , we used a Uniform prior on [0, 100] (Gelman, 2006). For λ , we used a Uniform prior on [0, 1]. Post-processing and convergence diagnostics were performed in R using the coda package (Plummer *et al.*, 2006). In addition, we performed checks of the models by comparing the discrepancy (we used square-root mean squared error) for the real data and simulated data from the models, and calculated posterior predictive *P*-values (Gelman *et al.*, 2004).

Results

Bayesian searches produced a tree similar to recently published work on nudibranchs (Johnson & Gosliner, 2012), with high support for many nodes (Fig. 1).

For all of our measures of conspicuousness, values close to 0 represent nudibranchs that closely match their substrate [e.g. *Phyllodesimum lizardensis*: intensity variance (against background) = 1.61; power spectra = 0.01; spectral contrast (against background) = 0.26; Fig. 3], whereas values further away from 0 indicate an increase in conspicuousness (e.g. *Chromodoris magnifica*: intensity variance (against background) = 28.7; power spectra = 0.07; spectral contrast (against background) = 31.2; Fig. 3).

There was a significant positive relationship between first-order (intensity variance against background) and second-order (power spectra) image statistics ($r^2_{59} = 0.06$, $P = 0.04$). However, we did not find a significant relationship between intensity variance (against background) or power spectra and spectral contrast (against background) (intensity variance: $r^2_{45} = -0.015$, $P = 0.57$; power spectra: $r^2_{45} = -0.02$, $P = 0.93$). There was also no relationship between intensity variance (within pattern) and spectral contrast (within pattern) ($r^2_{45} = 0.003$, $P = 0.30$).

Using phylogenetic regression models, there was a negative relationship between intensity variance (against background) and power spectra against body size, with the highest-density predicted interval (HDPI) not exceeding 0 for both variables (intensity variance: HDPI -2.72 to -0.18; power spectra: HDPI -0.007 to -0.003; Fig. 3i, ii). However, there was no relationship between intensity variance (within pattern) and body size (HDPI -1.78 to 1.10; Fig. 3iv).

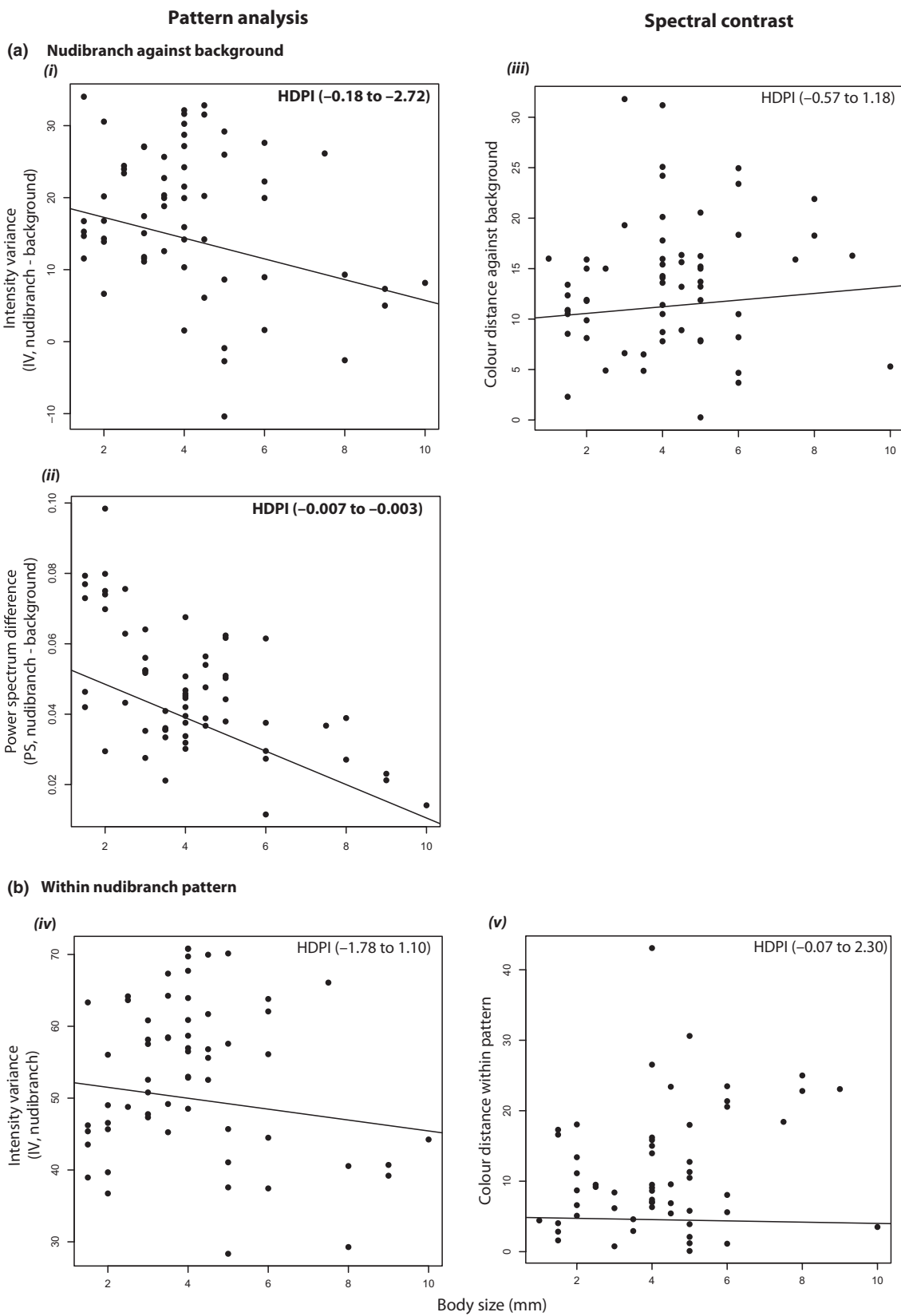
There was also no relationship between spectral contrast (against background) and body size (HDPI -0.57 to 1.18; Fig. 3iii), or spectral contrast (within pattern) and body size (HDPI -0.07 to 2.30; Fig. 3v).

Discussion

Here, we have used an understudied model system to investigate the hypothesis that the conspicuousness of aposematic signals coevolves with an increase in body size, using a new way of assessing the conspicuousness of animal signals by considering both pattern analysis and spectral contrast measurements. We did not find any evidence to support this hypothesis in our subsample of nudibranch species, on the contrary, we found that as body size increased, patterns in nudibranchs became less conspicuous, indicating that relatively large nudibranchs are capable of being cryptic. We found no relationship between spectral contrast and body size; nudibranchs that displayed the highest spectral contrast against the background habitat were only moderately sized, such as *Chromodoris elisabethina* (average 41 mm) and *Chromodoris magnifica* (45 mm).

Animals with large body size are predicted to evolve conspicuous colouration as cryptic colouration is often difficult to achieve in larger individuals (Cott, 1940). Indeed, cryptic colouration functions to decrease prey detection risk but may be costly in heterogeneous habitats, as it is difficult to match more than one visual background (Merilaita *et al.*, 2001). Many cryptic nudibranch species are indeed reliant on one habitat type, such as in this study, *Phyllodesmium lizardensis*, which closely resembles the xeniid soft corals it is found upon. This highly cryptic but moderately sized species (average 36 mm) is able to remain stationary as they house zooxanthellae in their digestive gland branches enabling individuals to meet their energy requirements through photosynthesis (Burghardt *et al.*, 2008). However, other large nonphotosynthetic cryptic nudibranchs including *Dendrodoris krusensternii* and *D. tuberculosa* were also located and included in our study. These species have an outer surface covered in tubercles, which helps skin texture to match their habitat. Other species such as octopi commonly use changes in skin texture to camouflage themselves against their background (Hanlon *et al.*, 2011). Conspicuous species should be easier to detect on coral reefs; therefore, our sampling may have been biased towards species with highly contrasting patterns. However, we also specifically targeted cryptic species using knowledge of their ecology to locate individuals.

Our prediction that an increase in body size and/or in pattern element size within the visual display strengthens the avoidance response of warning colouration by predators was based on behaviour responses of avian predators, but to our knowledge, this prediction has not been explicitly tested in fish. The light environment of marine habitats and differences in



the visual systems of fish (Marshall *et al.*, 2006) may impact the way in which fish predators respond to visual stimuli and may explain why we did not find any evidence to support our prediction. Marine fish have been shown to avoid unpalatable prey based on visual cues (colour and pattern) alone (Miller & Pawlik, 2013), and fish exhibit response bias towards particular colours (Cheney *et al.*, 2013); however, more behavioural experiments are needed with marine predators.

Factors other than an increase in body size and/or in pattern element size within the visual display may impact the avoidance response of warning colouration by predators. Although most nudibranchs are thought to contain some level of chemical defence, little is known about the abundance, relative strength and chemical profiles of these toxins and how they influence warning signal form and function (but see Cortesi & Cheney, 2010). However, highly conspicuous nudibranchs, which may have the strongest chemical defences (Cortesi & Cheney, 2010), should maximize the deviation of their visual signals from the natural spatial frequency of the surrounding visual environment to elicit a strong avoidance response by predators (Zylinski *et al.*, 2011; Stevens & Ruxton, 2012). Olfactory cues may also increase avoidance response by predators (Ritson-Williams & Paul, 2007). This may be enhanced by the secretion of pungent substances (Behrens, 2005; personal observations) in some species, for example *Phyllidiella pustulosa*, that may be detected before an attack.

The ability of nudibranchs to produce certain colours and patterns may also be restricted by diet, habitat and physiological mechanisms, as found in many colourful animals (Fox & Vevers, 1960). However, the variety of colours produced by the relatively closely related bivalve marine molluscs, such as the giant clam family (Tridacnidae), suggests that the palette available to this phylum, using both pigmentary and structural mechanisms, is large. Additional selective pressures that have confounded results from terrestrial model systems (e.g. insects, frogs) such as thermoregulation and intra-specific signalling (e.g. individual recognition and sexual signalling) are not applicable to our system. Nudibranch eyes are simple structures; their visual abilities are limited and only used for simple behaviours such as phototaxis (Barth, 1964).

As expected, both measures of pattern (intensity variance and spatial frequency power spectra) were highly correlated; however, we found no relationship between pattern statistics and spectral contrast, a measurement that is frequently used in studies investigating the function and evolution of animal visual signals. Therefore, nudibranchs that exhibited the most highly contrasting patterns compared with their background, did not necessarily exhibit the most contrasting colours. The relative importance of each signal component (e.g. colour, pattern, luminance) and how they interact is often unclear. In terrestrial systems, chicks appear to use

colours, rather than pattern, when learning and memorizing a signal (Osorio *et al.*, 1999; Aronsson & Gambralle-Stille, 2008), indicating that specific colours may transmit information, but pattern may attract attention to the signal and increase learning of a specific signal (Osorio *et al.*, 1999). Pigeons appear to use only one visual cue (shape or colour) when discriminating between visual stimuli (Reynolds, 1961; Johnson & Cumming, 1968). However, Spottiswoode & Stevens (2010) found that host birds use both colour and pattern to discriminate and reject parasitic eggs from nests. Furthermore, honeybees use more than one cue when processing information about signals (shape, colour and scent), and are able to store information about each one (Gould, 1984). To our knowledge, there has been little work done on the importance of colour and pattern in aquatic signalling systems.

Often studies use spectral contrast as a sole measure of conspicuousness, which may be misleading as colour distance may not be a linear measure of conspicuousness: once colour distance goes beyond the threshold of just noticeable differences, we do not know how spectral information is processed. Also, two spectra that are separated by equal distances in different directions in the colour space may not be equally distinguishable. Whether an increase in colour distance is directly related to an increase in conspicuousness or detectability of a colour signal should be tested empirically with behavioural experiments. Measures of conspicuousness are largely based on estimates of retinal input but do not consider neural processing of visual information. Until more is known on the relative roles and perception of visual cues component, multiple measures of conspicuousness should be used when addressing the function and evolution of visual signals.

In conclusion, we show that in nudibranch molluscs crypsis is not limited to small individuals, and we highlight the need to consider pattern in addition to spectral contrast when investigating the function and evolution of animal visual signals. We would also encourage more studies into the relative importance of colour, pattern and luminance in visual cues. We believe that that nudibranchs will be a fruitful model system in which to test a number of evolutionary hypotheses regarding the evolution of visual signals, but further information is needed on the relative strength of their chemical defences.

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Supporting information

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

Figure S1 Spatial frequency analysis of six simulated and real example images.

Figure S2 Density plots of 1000 trees obtained from MrBayes analysis to highlight phylogenetic uncertainty.

Figure S3 Relative measurements of traits plotted on phylogenetic tree.

Figure S4 Scatterplot of spectral contrast (colour distance) as perceived by a dichromatic reef fish (*Chaetodon kleinii*; λ max = 496, 530) against average body size of each nudibranch species, (a) against the background habitat and (b) within animal colour pattern.

Table S1 Species and data used in each analysis and GenBank Accession Numbers.

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