Chemical defences indicate distinct colour patterns with reduced variability in aposematic nudibranchs

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Abstract

The selective factors that shape phenotypic diversity in prey communities with aposematic animals are diverse and coincide with similar diversity in the strength of underlying secondary defences. However, quantitative assessments of colour pattern variation and the strength of chemical defences in assemblages of aposematic species are lacking. We quantified colour pattern diversity using Quantitative Colour Pattern Analysis (QCPA) in 13 Dorid nudibranch species (Infraorder: Doridoidei) that varied in the strength of their chemical defences. We accounted for the physiological properties of a potential predator's visual system (a triggerfish, *Rhinecanthus aculeatus*) and modelled the appearance of nudibranchs from multiple viewing distances (2cm and 10cm). We identified distinct colour pattern properties associated with the presence and strength of chemical defences. Colour patterns were also less variable among species with chemical defences when compared to undefended species. This confirms correlations between secondary defences and diverse, bold colouration while showing that chemical defences coincide with decreased colour pattern variability among species. Our study suggests that complex spatiochromatic properties of colour patterns perceived by potential predators can be used to make inferences on the presence and strength of chemical defences.

Keywords: Aposematism, crypsis, predator psychology, purifying selection, defensive animal colouration, signal honesty, escape and radiate, visual signalling

1. Introduction

Many animals use aposematic colour patterns to warn potential predators of underlying defences [1], with aposematic species in prey communities exhibiting a remarkable diversity of primary (i.e., colour patterns) and secondary defences (i.e., secondary metabolites) [2–4]. However, mechanisms shaping diversity within and among aposematic species in prey communities are complex, and it is poorly understood how the presence and strength of secondary defences correlate with phenotypic diversity in a natural prey community (see [5,6] for discussion). Factors shaping within-species diversity tend to coincide with factors affecting among-species variation in aposematic species (e.g. [7]). This complex mixture of selective

mechanisms in natural systems makes it challenging to understand the relationships between primary and secondary defences in prey communities.

Stabilising selection is a crucial driver underlying the distinct appearance of a given aposematic species in a species community. Once aposematism has evolved, stabilising selection is expected to constrain colour pattern diversity within species and Mullerian mimicry rings as predators learn to associate a visual signal with unprofitability [8–13]. Specifically, an invariant appearance across aposematic individuals may facilitate and strengthen predator learning and memorisation. In contrast, variation in signal design may cause predators to make errors when attacking prey and decrease rates of predator learning and increase rates of forgetting [10,14–16]. However, colour pattern diversity within and among aposematic species is ubiquitous. It is thought to be driven by countervailing evolutionary and ecological factors such as genetic drift, gene flow, variation in resource abundance, variation in predator species, and environmental biotic and abiotic variability at different spatial and temporal scales [5,10,17,18]. Aposematism in a spatially homogeneous and temporally stable environment coincides with selection towards reduced colour pattern variability within a population (e.g. [19,20]). In contrast, variability of biotic (e.g. predators) and abiotic factors (e.g. temperature) at spatial and temporal scales can favour selection on phenotypic diversity within aposematic species (e.g. [21–24]) as well as among them (e.g. [25,26]).

Investing in chemical defences is costly (see [6,27] for review) and, as a result, can favour the evolution of various forms of mimicry among prey species (e.g. [28]). Mimicry leads to specific, general or partial (e.g. [29–33]) resemblance among species, reducing phenotypic diversity among chemically defended species and undefended mimics. However, key innovations such as chemical defences are thought to enable niche expansions and, as a result, facilitate speciation [25,34–36]. Adapting to diverse ecological niches, in turn, may lead to phenotypic diversity among aposematic species, especially if such niche specialisations underly changes in the signalling environment, such as the distinctiveness from background habitats or signalling in differing light environments. Indeed, a distinct appearance not only from the background, but also from conspecifics, may aid predator learning [37] and can provide a mechanism to defend against the parasitic effects of certain types of mimicry, such as Batesian and quasi-Batesian mimicry [38–41]. However, long-standing predictions of the benefit of distinctiveness among aposematic species (e.g. [42,43]) are mainly theoretical, with no known studies investigating correlations between distinctiveness and secondary defences among aposematic species in nature.

Attacking well-defended prey is also costly; therefore, predators may generalise more broadly between the colour patterns of previously attacked prey and the prey they subsequently encounter, likely confounded by the cost of making an error (e.g. [44–46]). However, how predator generalisation between and within aposematic species and their mimics influences correlations between secondary defences and colour pattern diversity is complex, highly debated and likely varies among taxa (see [5,6] for discussion). Furthermore, selection for or against colour pattern variability within and among species can act on individual colour pattern elements or perceptual properties rather than the entire animal, depending on which elements of the signal predators learn or pay attention to (e.g. [47]). Therefore, animal colour patterns should be considered complex multicomponent phenotypes [48] under multiple selective pressures (e.g. [48,49]).

When interpreting the ecological relevance of phenotypic variation, it is essential to consider how the appearance of an organism's colours and patterns change as a function of observer acuity and viewing distance [50]. For example, colour patterns may be cryptic when viewed from a distance but may become aposematic as a predator approaches [50,51]. Animals detect objects and decide their identity and quality based on varying combinations of spatiochromatic features [52–56]. Consequently, predator learning of associations between primary and secondary prey defences, or the subsequent retrieval of formed associations from memory, might happen at a specific range of viewing distances concerning specific spatiochromatic properties of prey appearance. However, the scarce empirical evidence on the ecological significance of colour pattern variability in aposematic animals remains restricted to investigations of colour alone and do not account for the visual acuity of ecologically relevant observers and viewing distance (e.g. [57,58]).

Here, we examined how highly defended aposematic nudibranch species differ from less well-defended species in appearance to a potential predator and if, among species, variation in perceived colour patterning varies with the presence and strength of chemical defences. Specifically, we hypothesised that chemical defences would correlate with increases or decreases in colour pattern distinctiveness between species as perceived by a potential predator. We further hypothesised that colour patterns in chemically defended species were less variable than in species without chemical defences as perceived by a potential predator. To do this, we modelled the visual appearance of 13 sympatric Dorid nudibranch species across multiple viewing distances corresponding to the later stages of an escalating predation sequence [14,59]. We quantified the perception of within-species colour pattern variability using the Quantitative

Colour Pattern Analysis (QCPA) [60], allowing for the consideration of colour, luminance and spatial vision of triggerfish (*Rhinecanthus aculeatus*). Using exploratory factor analysis, we identified latent variables to compare the colour pattern appearance of individuals belonging to three levels of chemical defence. Chemical defences were defined using previously published assay data [61,62]. We then investigated differences in the perceived appearance and variability of colour patterns for species belonging to each level of chemical defences.

2. Materials and Methods

(a) Study species

We used digital photographs of 311 Dorid nudibranchs using a calibrated Olympus EPL-5 with a 60mm macro lens (see the Supplement for details on camera calibration). These individuals belonged to 13 species: *Aphelodoris varia* (N=31), *Chromodoris elisabethina* (N=31), *Chromodoris kuiteri* (N=49), *Chromodoris lochi* (N=8), *Chromodoris magnifica* (N=14); *Dendrodoris krusensterni* (N=7); *Discodoris* sp. (N=10); *Doriprismatica atromarginata* (N=35); *Glossodoris vespa* (N=32); *Hypselodoris bennetti* (N=13); *Phyllidia ocellata* (N=32), *Phyllidia varicosa* (N= 9), *Phyllidiella pustulosa* (N=40) (Fig. 1) from five locations on the east coast of Australia: Mackay (QLD), Sunshine Coast (SE Queensland, QLD), Gold Coast (SE QLD), Cook Island (New South Wales, NSW) and Nelson Bay (NSW) between March 2016 and February 2021. Two out of 13 species (*Doriprismatica atromarginata, Goniobranchus splendidus*) were sampled across sites in QLD and NSW in high numbers, whereas the other species were only sampled in either NSW or QLD or with highly uneven numbers between sites (Table S1). Two individuals of *Chromodoris magnifica* were provided by an aquarium supplier (Cairns Marine, Pty Ltd, Cairns, QLD). These species were selected as they were relatively abundant at our study sites and covered a broad range of visual appearances and strengths of chemical defences. Furthermore, we have previously provided data on the strength and identity of chemical defences in these species sampled from the same locations as individuals from this study [61,62].

Most nudibranchs were photographed underwater against their natural habitat (n = 182) with the camera in an Olympus PT-EP10 underwater housing and using white LED illumination from a combination of VK6r and PV62 Scubalamp video lights. The remaining nudibranchs (n = 129) were collected for separate studies on their chemical defences, taken back to the laboratory, submerged in water in a petri dish and photographed against a white background with the same camera. In the laboratory, nudibranchs were illuminated with 400nm-700nm full-

spectrum white LED lights. The Supplementary Information (Table S1) details collection sites and dates, and camera and illumination spectra are provided in [60]. A sub-sample of these images was previously used to investigate distance-dependent signalling regarding colour pattern detectability and boldness [63]. Nudibranchs were collected under the Queensland General Fisheries Permit 183990, 205961 and NSW Scientific Collection Permit P16/0052-1.0.

(b) Image analysis

We used ImageJ [64] and the MICA toolbox [65] to manually segment the images into regions of interest (ROI). This was done by outlining and selecting the animal from its background and defining a size standard. All nudibranchs were aligned head up in the image before analysis with QCPA [60], with the rotation angle determined by the rotation, causing most of each animal to be aligned vertically. To analyse the nudibranch colour patterns, we used the visual system parameters of a trichromatic triggerfish, *Rhinecanthus aculatus* [66–71], a common shallow reef inhabitant found throughout the Indo-Pacific, which feeds on invertebrates, algae, and detritus [72].

We analysed colour patterns for viewing distances of 2cm and 10cm, using the estimated spatial acuity of the triggerfish of three cycles per degree [66,70]. A viewing distance of 2cm represents the spatiochromatic information available to a triggerfish upon immediate contact with a nudibranch. A viewing distance of 10cm more likely represents visual information available to a triggerfish at a short distance where a subjugation attempt has not yet been made. Following acuity modelling, the images were processed with a Receptor Noise Limited (RNL) ranked filter (falloff: 3, radius: 5, repetition: 5) and clustered using RNL clustering with a colour threshold of 2 ΔS [71,73] and a luminance contrast threshold of 4 ΔS [74] for all analyses except the local edge intensity analysis (LEIA) which does not require RNL clustering but is recommended to be subjected to RNL ranked filtering [60]. We calculated receptor-specific Weber fractions based on a relative photoreceptor abundance of 1:2:2:2 (sw:mw:lw:dbl) and photoreceptor noise of 0.05, resulting in 0.07:0.05:0.05:0.05.

QCPA analysis was achieved using a custom batch script [75] running on high-performance computing (HPC) infrastructure. We analysed each animal colour pattern using: 1) colour adjacency analysis (CAA), which describes pattern geometry in a segmented image; 2) visual contrast analysis (VCA), which describes pattern boldness based on chromatic and spatial pattern element properties in a clustered image; 3) boundary strength analysis (BSA), which describes the colour and luminance contrast of boundaries between pattern elements at

the scale of an animal in an unclustered image; and 4) local edge intensity analysis (LEIA) which describes the strength of colour and luminance contrast at the scale of an edge-detecting receptive field in an unclustered image. This resulted in a highly descriptive array of 157 colour pattern statistics for each animal. A detailed description of each pattern statistic can be found in [60]. Here, we use CAA, VCA, BSA, and LEIA as prefixes for each type of analysis.

All pattern analyses, except LEIA, used a segmented image and measured transitions between pixels along vertical (along body axis) and horizontal (perpendicular to body axis) sampling transects in a transition matrix. Statistics ending with 'vrt' or 'hrz' are the vertical (i.e., up-down in image) and horizontal version (analysing the respective transition matrix only) of their respective statistic (analysing the full transition matrix) and can represent differential directionality sensitivity in the visual system of an observer and directionality in patterns such as stripes [76–78]. LEIA does not use a transition matrix due to the lack of image segmentation but equally discriminates between horizontal and vertical edge contrast by describing the shape of a histogram drawn from edge contrast measurements in a given image or region of interest [60].

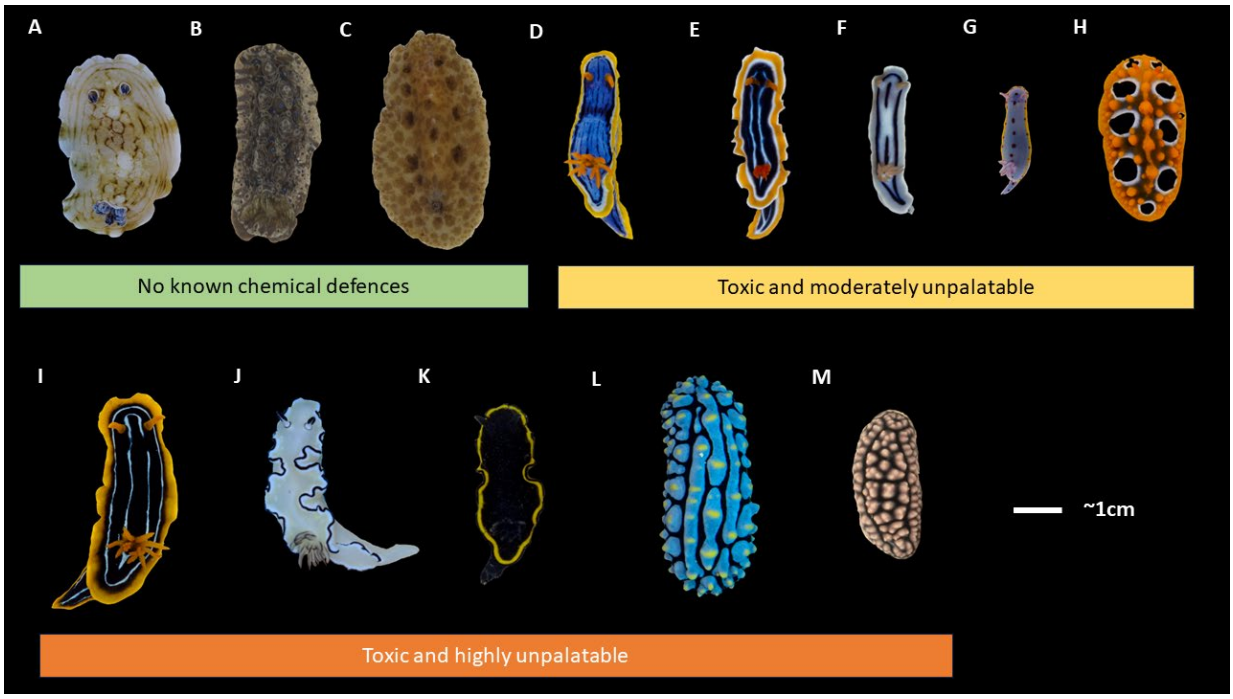

Figure 1. Representative photographs of the 13 species used in this study grouped into categories of chemical defences based on whole-body extract assays with palaemon shrimp to assess unpalatability (1-Effective Dose, ED_{50}) and brine shrimp to assess toxicity (1-Lethal Dose, LD_{50}) values as per [61,62]: A) *Aphelodoris varia;* B) *Dendrodoris krusensterni*; C) *Discodoris sp*; D) *Chromodoris elisabethina*; E) *Chromodoris magnifica;* F) *Chromodoris lochi;* G) *Hypselodoris bennetti;* H) *Phyllidia ocellata*; I) *Chromodoris kuiteri*; J) *Doriprismatica atromarginata;* K) *Glossodoris vespa*; L) *Phyllidia varicosa;* M) *Phyllidiella pustulosa.*

211 (c) Chemical defences

212 To categorise the level of chemical defences for each species, we used previously
213 published data on the deterrent properties from feeding rejection assays with rockpool shrimp
214 (*Palaemon serenus*), which demonstrate similar results to assays performed with triggerfish
215 and toadfish [61] and toxicity assays with brine shrimp [61,62]. Assays were conducted by
216 adding extracted nudibranch compounds to food pellets made from squid mantle at increasing
217 concentrations. Effective dose (ED_{50}) and lethal dose (LD_{50}) values in [61,62] were calculated
218 based on the concentration that elicited a rejection response in, or mortality of, at least 50% of
219 the shrimp. For this study, we averaged ED_{50} and LD_{50} values from [61] when multiple extracts
220 from the same species were reported. We considered only whole-body extracts (rather than
221 mantle-only values) to make assay values comparable between species. We then subtracted
222 these values from 1 so that values close to 0 were the most palatable/non-toxic, and values close
223 to 1 were the least palatable/ toxic (Table S2). Although *C. magnifica* was not included in [61],
224 [79] demonstrated that this species also stores latrunculin A as the sole defensive compound in
225 the mantle rim, and this is at concentrations between those found in *C. elisabethina* and *C*
226 *kuiteri* [80]. We, therefore, set unpalatable ED_{50} values as the average from these two sister
227 species for *C. magnifica*. Lastly, assay data for *G. vespa* is presented in [62].

228 Like Winters et al. [61], we binned the species into categories indicating chemical
229 defence strength to account for our dataset's highly uneven spread in toxicity and palatability
230 values and the difference in sampling levels between colour pattern data and chemistry data.
231 Our categorisation differed from that of Winters et al. [61] in that we based our categories on
232 the assumption of a sigmoidal dose-effect response similar to a psychometric curve. Species
233 were allocated in the following classes (Fig. 1), where we treated NR values from [61] as 0:

234 1.) Not defended ($1 - ED_{50} / LD_{50} = 0$)
235 2.) Toxic and moderately unpalatable ($0.25 < 1 - ED_{50} > 0.74$ and $LD_{50} > 0$),
236 3.) Toxic and highly unpalatable ($0.74 < 1 - ED_{50}$ and $LD_{50} > 0$).

237 The threshold to distinguish between medium and high levels of unpalatability was 0.74 ,
238 representing the median $1 – ED_{50}$ value of chemically defended species while also being very
239 close to the point-of-inflexion in a sigmoidal response curve. Only 3 out of 10 species with
240 chemical defences had $1 – LD_{50}$ values below 0.5, yet 6 out of 10 had values above 0.80.
241 Therefore, we did not distinguish between different toxicity levels in our dataset. Treating
242 toxicity as present/absent and distinguishing between medium and high levels of unpalatability

243 ensured at least three species in each category, allowing the investigation of differences in
244 animal colouration between variable levels of chemical defences.

245 (d) Statistical analysis

246 Our study considers many of the more commonly found Dorid nudibranchs in the study
247 sites (e.g. [22–24]). To analyse the large dataset derived from the QCPA analysis, we only kept
248 images that did not produce any missing value for any pattern metrics. VCA, CAA, and BSA
249 metrics can produce NaN or infinite values if a colour pattern has less than two colour pattern
250 elements following RNL clustering [60]. LEIA metrics do not suffer from this limitation. Nine
251 available images from *Discodoris sp* were rejected from analysis due to this constraint, resulting
252 in the reported sample size.

253 We then applied a Latent variable Exploratory Factor Analysis (EFA) with the R package
254 *psych* using the factoring method of Ordinary Least Squares 'ols', and the orthogonal rotation
255 'varimax'. To prepare the dataset for the EFA, we first filtered the number of highly correlated
256 QCPA metrics by keeping only those that were less correlated than 0.6 (Pearson correlation),
257 which reduced their number from 157 to 15. We then run the factor analysis based on three
258 factors. The number of factors was selected by comparing the eigenvalues calculated from the
259 original dataset to the median eigenvalues extracted from 10,000 randomly generated datasets
260 with the same number of rows and columns of the original data. We selected factors with
261 eigenvalues greater than the median of the eigenvalues from the simulated data. We also
262 computed bootstrapped confidence intervals of the loadings by iterating the factor analysis 1000
263 times.

264 Looking at the loadings of each factor, we can identify what latent variable they
265 describe. While it would be possible to discuss each factor extensively, we keep their
266 description to loadings of +/- 0.4 (out of 0 -1) to capture their main properties. Due to data
267 filtering for metrics less correlated than 0.6, the QCPA parameter listed for a given loading is
268 likely synonymous with various other parameters in our 157-dimension colour pattern space
269 (Table S5). Therefore, the precise wording to describe each factor can vary depending on which
270 colour pattern metrics are put into focus—for example, *BSA.BMSL.Vrt* is positively associated
271 with factor 1 (Fig. 2) but is simply a placeholder for *BSA.BMSL* (both considering horizontal
272 and cumulative transitions) as it is 92-96% correlated with these metrics and 97% correlated
273 with *BSA.BML* (Table S2). Unlike *BSA.BMSL* (which describes boundary contrast using the
274 mean RNL luminance contrast between colour pattern elements relative to the fraction of the

respective pattern border), *VCA.BML* captures boundary contrast calculated by the Weber contrast of cone catches in the luminance channel between colour pattern elements relative to the fraction of a given boundary type. Thus, it would be more appropriate to say that animals with high values of factor 1 are associated with stronger achromatic colour pattern boundary contrast rather than explicitly referring to the randomly retained value only. A complete list of all colour pattern parameters with more than 0.6 Pearson correlation with parameters associated with factors 1-3 shown in Fig. 2 can be found in the Supplement (Table S2).

The scores of the factors extracted from the EFA were then used to implement three phylogenetic, distributional linear mixed models to compare the colour patterns of nudibranchs with different levels of chemical defences. Models were run in R v 4.1.2 (https://www.r-project.org/) using the brms package [81], which fits Bayesian models using Stan (https://mc-stan.org/). To account for the phylogenetic dependency of closely related species, all models included the phylogenetic tree of the 13 species of nudibranchs (Fig. S1), with the tree from [82] pruned and missing species added according to their taxonomic classification in the World Register of Marine Species [83]. The phylogenetic model was implemented following the guidelines of the online *brms* vignette (https://cran.rproject.org/web/packages/brms/vignettes/brms_phylogenetics.html) based on de Villemeruil & Nakagawa [84].

The first model investigated differences in scores for latent *factor 1* between nudibranchs with different levels of chemical defences (see chemical defences section) using a Student distribution. The model estimated the effect of the main categorial predictors level of *chemical defence* (undefended; toxic and moderately unpalatable; toxic and highly unpalatable) and *observer distance* (2 cm and 10 cm) and their interaction on both the mean and the residual standard deviation of the response distribution. To account for repeated measurements of each species, we also included *species ID* as a random intercept to the model. We further included random slopes over distance because their relationship with the value of the response *factor 1* varied among species. The second and third models were identical to the first but used *factor 2* and *factor 3* as response variables.

All models were fitted using weakly informative prior distributions (normal with mean = 0 and sd = 5 for intercept and coefficients, exponential (1) for standard deviations). Their performance was evaluated using posterior predictive model checking, which compares model predictions with observed data to assess overall model fit. We ran four Markov-Chain-Monte-

Carlo (MCMC) chains for each model and obtained coefficient estimates from 16,000 post-warm-up samples. All model parameters reached reliable conversion indicators [85]: A Monte Carlo standard error smaller than 5% of the posterior standard deviation, an effective posterior sample size greater than 10% of the total sample size, and a $\hat{R}$ statistic value smaller than 1.01.

We present the medians of latent factors values and their 95% credible intervals of the posterior distributions of fitted values for the population average, obtained from the joint posterior distributions of the model parameters for the combination of chemical defences and distance [85,86] (Fig. 2). The same posterior distribution of fitted values was used to compute pairwise differences and their 95% credible intervals between all the combinations of the same two categorical predictors using the 'emmeans' R package [87]. To compare variances of responses between all predictor groups, we also computed the posterior distribution of all pairwise differences of the residual standard deviation on the original scale (back-transformed from the log scale). The effect size of pairwise differences increases with increasing deviation of such differences from zero, and the robustness of the result increases with decreasing degree of overlap of the 95% Credible Intervals (CIs) with zero.

3. Results

We identified three latent factors describing overall differences in colour pattern appearance to a triggerfish (*R. aculeatus*). We describe each factor at 2cm and 10cm, respectively.

While not intended to identify a maximal amount of variability in colour pattern variation in our dataset, the three factors still explain 38% of the total variation (factor 1: 14%; factor 2: 13%; factor 3: 11%) (Fig 2).

<u>(a) Factor 1:</u> *Colour patterns with high achromatic contrast have low colour contrast*

Contrasts [difference (+- 95% CI)] between groups of chemical defences indicate that toxic species with high levels of unpalatability differed in appearance from toxic species with moderate levels of unpalatability (Fig. 2b, Table S3). However, undefended species did not differ from chemically defended species for factor 1. At a 2cm viewing distance, undefended species are not different in appearance from toxic and highly unpalatable species (0.99 (-2.31 / 0.31)). In contrast, toxic and moderately defended species have a lower score (-1.23 (-1.74 / -0.70)) for factor 1 compared to highly unpalatable toxic species (Fig. 2b). This is true at immediate contact between the triggerfish and prey at 2cm, as well as at 10cm (undefended vs. toxic and highly unpalatable: -0.60 (-2.00 / 0.81); toxic and medium unpalatable vs. toxic and

338 highly unpalatable: -1.01 (-1.67 / -0.33)). Toxic animals with medium levels of unpalatability
339 did not differ from undefended species regarding factor 1 at either 2cm (0.21 (-1.10 / 1.56) or
340 10cm (0.40 (-1.09 / 1.82). We found no indication of differences in colour pattern variability
341 in species of different groups as captured by factor 1 (Table S4).

342 Factor 1 describes 14% of colour pattern variability in our dataset. It is associated with
343 high loadings of luminance contrast between colour patches as a function of their patch size,
344 which VCA describes. We can see high loadings for mean and standard deviation variation
345 measures of pattern contrast measured as cone catches of the luminance channel (e.g. *VCA.CVL*)
346 and using the RNL model (e.g. *VCA.MSL*). We also find high luminance pattern contrast
347 captured by factor 1 as an expression of the boundary contrast (BSA), which refers to contrast
348 scaled by the length of boundaries between colour patches rather than their size. Given that
349 larger patches tend to have longer boundaries, it is not surprising that we find similar loadings
350 for measures relative to either. The negative loadings for chromatic colour pattern contrast (e.g.
351 *VCA.MDmax*) indicate that patterns with strong and variable achromatic contrast tend to have
352 a reduced level of average chromaticity contrast. High factor values would indicate the presence
353 of black and white, pale hues or long wavelength colours that appear of low chromaticity to the
354 visual system of a triggerfish. Therefore, our results indicate higher levels of achromatic
355 contrast and lower levels of chromatic contrast present in the colour patterns of highly
356 unpalatable toxic species compared to the other groups, with the increase in achromatic contrast
357 coinciding with more prominent relatively achromatic colour pattern elements.

358 <u>(b) Factor 2:</u> *Highly contrasting colour patterns are more regular and vertically elongated*

359 Contrasts [difference (+- 95% CI)] between the different groups of chemical defences indicate
360 that chemically defended species do not have higher scores for factor 2 than undefended species
361 (Fig. 2d, Table S5). There was also no difference in factor values between toxic and medium
362 unpalatable animals and toxic and highly unpalatable animals at either 2cm (0.05 (-0.84 / 0.94)
363 or 10cm (0.06 (-0.91 / 0.92)). However, at 2cm viewing distance, undefended species have
364 more variable colour patterns than toxic and moderately unpalatable species (0.40 (0.14 / 0.74)
365 as well as toxic and highly unpalatable species (0.31 (0.06 / 0.67)) (Table S6).

366 Factor 2 explains 13% of colour pattern variability in our dataset. It describes the
367 relationship between decreases in the aspect ratio of colour patterns (*CAA. Asp*) coinciding with
368 decreases in average patch size (*CAA.Pt*) as well as decreases in the average luminance contrast
369 (e.g. *VCA.ML*) and its variability (e.g., *VCA.sL*) between patches in the horizontal axis and

increases in various measures of chromatic and achromatic colour pattern contrast variability relative to the mean contrast in a given colour pattern (e.g. *VCA.CVSL, VCA.CVS*) as well as increases in colour pattern transition regularity (e.g., *CAA.Qt*).

(c) Factor 3: *Colour patterns with variable edge contrast have reduced spatial evenness*

Contrasts [estimate (+- 95% CI)] calculated between the different groups of chemical defences indicate no overall differences between groups (Fig. 2, Table S7). This is the case for both 2cm (undefended vs. toxic and medium unpalatable: 0.05 (-1.16 / 1.14); undefended vs. toxic and highly unpalatable: -0.10 (-1.29 / 1.03); toxic and medium unpalatable vs. toxic and highly unpalatable: -0.16 (-0.77 / 0.46)). We found no indication of differences in colour pattern variability in species of different groups captured by factor 3 (Table S8).

Factor 3 explains 11% of colour pattern variability in our dataset. It describes positive changes in colour (e.g. *Col.kurtosis*) and luminance (e.g. *Lum.kurtosis*) contrast variability relative to the average contrast in an animal coinciding with reduced colour pattern evenness (e.g. $CAA.Q_C$) as well as decreased average luminance contrast of boundaries between colour pattern elements (e.g. *Lum.mean*) and decreased overall colour pattern complexity (*CAA.C*).

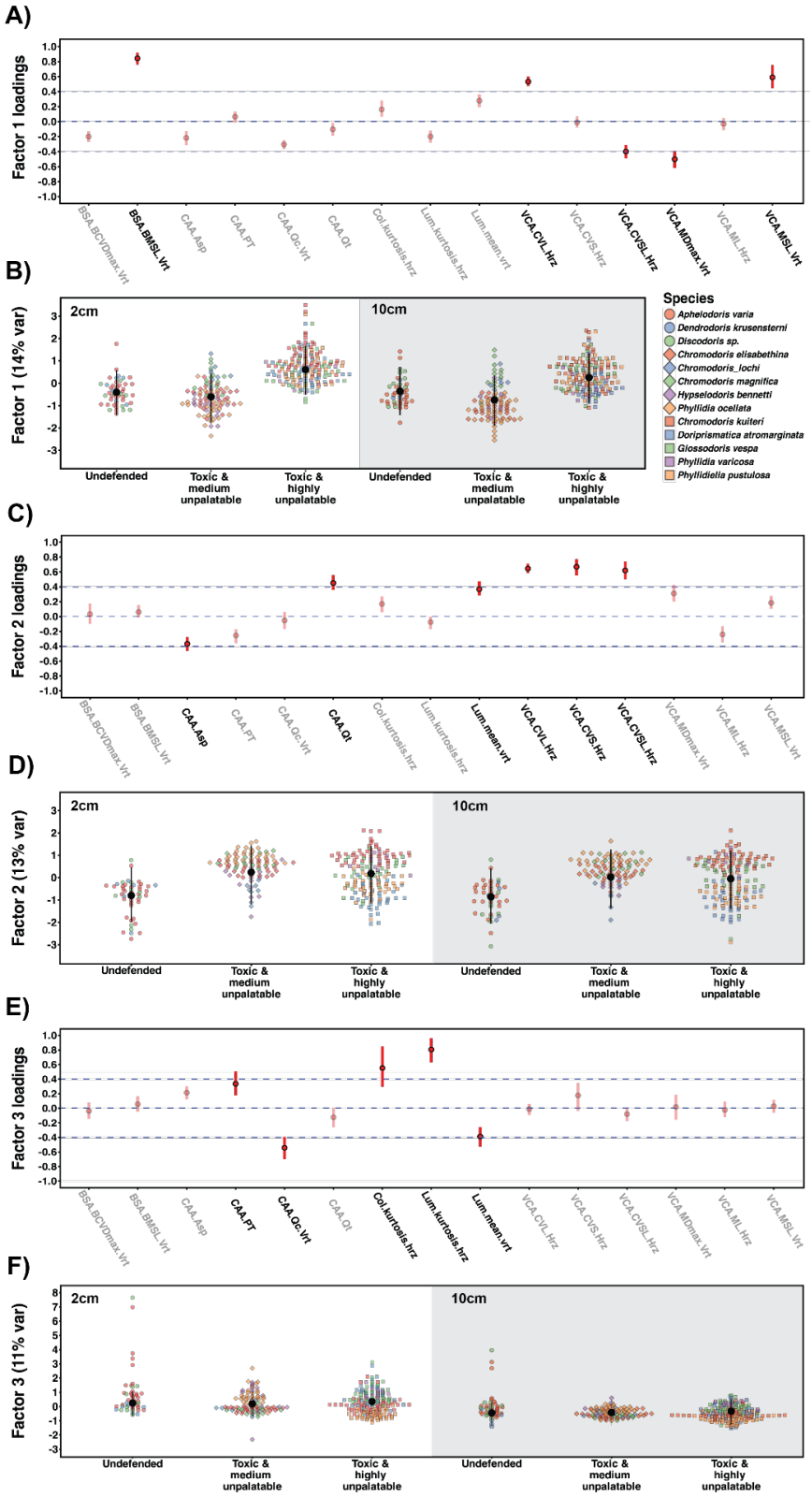

Figure 2: Detailed visual representation of the loadings of factor 1 (A), factor 2 (C) and factor 3 (E). Greyed-out factor loadings indicate colour pattern descriptors with minor contributions to each factor. Factor values for each group with different strength of chemical defences are given for factor 1 (B), factor 2 (D) and factor 3 (F). Estimates are given for 2cm viewing distance (left panel half, white) and 10cm (right panel half, grey). Coloured points represent repeated observations for each species (N = 13). Black vertical bars represent group predicted medians and 95% compatibility intervals (CIs) derived from the joint posterior distributions of the model

385

386 **4. Discussion**

387 We identified three latent variables that captured differences in appearance between
388 distinct differences in colour patterns between our three levels of chemically defended groups
389 of nudibranch molluscs (Fig. 2). Our analysis captures a significant proportion of variability in
390 the dataset (38%) and indicates substantial colour pattern variation among sampled species
391 across multiple viewing distances as perceived by a potential predator (Fig. 2). We found
392 differences in appearance both between chemically defended and undefended species and also
393 between toxic/moderately unpalatable species and toxic/highly unpalatable species. These
394 differences in colour patterns between species belonging to different levels of chemical
395 defences are likely visible to a potential predator at close contact (2cm) and from further away
396 (10cm) and might be used by predators to infer the presence and strength of underlying chemical
397 defences based on the general appearance of prey animals.

398 The colour patterns of chemically defended species were less variable than those of
399 undefended species (Fig. 2d, Table S3). Specifically, the variability of colour and luminance
400 contrast and the spatial arrangement of colour pattern elements was reduced in species with
401 chemical defences compared to those without. Furthermore, the colour patterns of toxic species
402 with high levels of unpalatability were different in appearance from toxic species with moderate
403 levels of unpalatability (Fig 2b, Table S3). Specifically, species with high levels of
404 unpalatability showed increased levels of achromatic contrast between colour pattern elements
405 when compared to more palatable toxic species. This increase in achromatic contrast in highly
406 unpalatable species coincides with a decrease in the mean level of chromatic contrast relative
407 to toxic species with lower levels of unpalatability. Overall, the differences in the visual
408 appearance to a potential predator between species of nudibranchs with different levels of
409 chemical defences describe general colour pattern properties (such as pattern regularity and
410 spectral contrast) associated with aposematic signalling (Fig. 2). Therefore, in agreement with
411 existing literature (e.g. [2,88]), we find that Dorid nudibranch colour patterns are highly diverse
412 and that the presence of chemical defences correlates with the presence of boldly contrasting
413 colour patterns.

414 The observed differences in animal colouration between groups of species with varying
415 levels of chemical defences generally agree with and can be interpreted as indicating selective
416 factors driving between-species pattern diversity in conjunction with the presence of secondary
417 defences. Such drivers of phenotypic diversity can favour distinctiveness among chemically

defended species, either as a means to defend against Batesian mimicry (e.g. [38]), as well as the potential need to optimise signalling efficacy across a complex, spatially and temporally variable biotic and abiotic environment (e.g. [5,17,21,22,25,89,90]). Thus, our results agree with predictions made by assuming facilitated niche expansion and subsequent speciation and adaptation to visually diverse habitats [25,34–36] as potential drivers of phenotypic diversity in chemically defended species.

Our results further suggest the general presence of secondary defences to coincide with reduced colour pattern variability among species when viewed up close by a potential predator (Fig. 2e, Table S3). Reduced variability among chemically defended species may suggest the presence of broadly generalisable, qualitative signalling properties underlying aposematic signalling in the species considered in this study. However, the presence of distinct colour pattern appearance at a quantitative scale (i.e., comparing species with different levels of chemical defences) would align with chemical defences, favouring visual distinctiveness from co-occurring Batesian or quasi-Batesian mimics (e.g. [38]). In other words, considering colour patterns as complex, multicomponent signals, it is possible to think of certain colour pattern properties indicating the qualitative presence of secondary defences ('is the animal defended or not'). In contrast, others indicate the quantitative presence of secondary defences ('how potent are the defences'), thus allowing different parts of simultaneously perceived visual information elicited by animal colouration to be under seemingly opposing selection pressures towards and away from general resemblance. In addition to these perceptual modalities being realised simultaneously, trade-offs between selective pressures for and against multiple, seemingly contractionary signalling properties of colour patterns can be mediated by distance-dependent signalling (e.g. [63,94]). Our results suggest both to be possible, with colour pattern variability only differing between species with and without chemical defences at 2cm viewing distance but not 10cm. In contrast, toxic and highly unpalatable species differ in their appearance from toxic and moderately defended species as well as undefended ones at 2cm and 10cm.

Phenotypic diversity within (e.g. polymorphism and polyphenism) and among chemically defended species is generally described as a detriment to predator learning, with selection towards resemblance underlying purifying selection at the species level (e.g. [10,14–16]) and Mullerian mimicry at the community level (e.g. [7,32,91,92]). However, phenotypic diversity among chemically defended species might, contrary to general assumptions, benefit predator learning as it can lead to more stable, generalisable associations [93] and, thus, provide

mutual benefits among chemically defended species considered in the context of qualitative and quantitative signal honesty and mimicry. Experimental investigations into the importance of signal variability for avoidance learning in non-human animals would be of great interest for future research as it, in turn, would inform our assumptions on the mechanisms underlying the evolution and maintenance of colour pattern diversity within and among chemically defended species.

Our methodology is tailored to reflect the fact that colour pattern elements and signalling properties do not exist in isolation, thus warranting an 'agnostic' approach to deduce correlations between predictor and dependent variables in the context of a complex trait described by a high-dimensional dataset (i.e., colour pattern space) [55,60,95]. Therefore, even if specific colour pattern features might be under purifying selection among certain species (e.g., as a result of mimicry), this was not captured by latent variables capturing overarching differences between individuals and species in the data set. Our results indicate that aposematic species' overall colour pattern phenotype might indeed be selected for less variability when compared to that of undefended species. However, our methodology does not address the possibility that specific colour pattern elements and signalling properties among aposematic species and putative mimics could be under purifying selection. Examples of this have been documented both within and between species of nudibranchs [3,47] and could apply to our dataset with representatives of a putative yellow-rim mimicry ring [96] (Fig. 1). This consideration is of broad relevance across all studies using methodology describing the cumulative colour pattern appearance of an animal, rather than specific colour pattern elements or body areas.

Data accessibility

The data can be accessed on UQ's e-space: https://doi.org/10.48610/a596710

Authors' contributions

CPvdB: Conceptualisation, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, software, validation, visualisation, writing – original draft, writing – review & editing. MS: Data analysis, writing - review and editing. JAE:

project funding acquisition, writing - review and editing. BRD, CS, NW: investigation. KLC: Funding acquisition, project administration, resources, validation, writing - review & editing.

Competing interests

We declare we have no competing interests.

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References

1. Poulton EB. 1890 *The Colours of Animals*. Kegan Paul, Trench & Trubner.
2. Cortesi F, Cheney KL. 2010 Conspicuousness is correlated with toxicity in marine opisthobranchs. *J. Evol. Biol.* **23**, 1509–18. (doi:10.1111/j.1420-9101.2010.02018.x)
3. Winters AE, Wilson NG, van den Berg CP, How MJ, Endler JA, Marshall NJ, White AM, Garson MJ, Cheney KL. 2018 Toxicity and taste: unequal chemical defences in a mimicry ring. *Proc. R. Soc. B Biol. Sci.* **285**, 20180457. (doi:10.1098/rspb.2018.0457)
4. Arias M, Mappes J, Théry M, Llaurens V. 2016 Inter-species variation in unpalatability does not explain polymorphism in a mimetic species. *Evol. Ecol.* **30**, 419–433.

(doi:10.1007/s10682-015-9815-2)

5. Briolat ES, Burdfield-Steel ER, Paul SC, Katja HR, Seymoure BM, Stankowich T, Stuckert AMM. 2019 Diversity in warning coloration: selective paradox or the norm? *Biol. Rev.* **94**, 388–414. (doi:10.1111/brv.12460)

6. Ruxton GD, Allen WL, Sherratt TN, Speed MP. 2018 *Avoiding Attack*. New York: Oxford University Press. (doi:10.1093/oso/9780199688678.001.0001)

7. Franks DW, Ruxton GD, Sherratt TN. 2009 Warning signals evolve to disengage batesian mimics. *Evolution (N. Y).* **63**, 256–267. (doi:10.1111/j.1558-5646.2008.00509.x)

8. Borer M, Van Noort T, Rahier M, Naisbit RE. 2010 Positive frequency-dependent selection on warning color in alpine leaf beetles. *Evolution (N. Y).* **64**, 3629–3633. (doi:10.1111/j.1558-5646.2010.01137.x)

9. Endler JA, Greenwood JJD. 1988 Frequency-dependent predation, crypsis and aposematic coloration. *Philos. Trans. R. Soc. London. Ser. B Biol. Sci.* **319**, 505–523. (doi:10.1098/rstb.1988.0062)

10. Endler JA, Mappes J. 2004 Predator Mixes and the Conspicuousness of Aposematic Signals. *Am. Nat.* **163**, 532–547. (doi:10.1086/382662)

11. Mallet JLB, Barton NH. 1989 Strong Natural Selection in a Warning-Color Hybrid Zone. *Evolution (N. Y).* **43**, 421. (doi:10.2307/2409217)

12. Sherratt TN, Speed MP, Ruxton GD. 2004 Natural selection on unpalatable species imposed by state-dependent foraging behaviour. *J. Theor. Biol.* **228**, 217–226. (doi:10.1016/j.jtbi.2003.12.009)

13. Brower LP, Ryerson WN, Coppinger LL, Glazier SC. 1968 Ecological Chemistry and the Palatability Spectrum. *Science.* **161**, 1349–1350. (doi:10.1126/science.161.3848.1349)

14. Endler JA. 1991 Interactions between predators and prey. In *Behavioural Ecology* (eds JR Krebs, NB Davies), pp. 169–196. Oxford: Blackwell Scientific.

15. Karpestam E, Merilaita S, Forsman A. 2014 Natural levels of colour polymorphism reduce performance of visual predators searching for camouflaged prey. *Biol. J. Linn. Soc.* **112**, 546–555. (doi:10.1111/bij.12276)

16. Mappes J, Marples N, Endler JA. 2005 The complex business of survival by aposematism. *Trends Ecol. Evol.* **20**, 598–603. (doi:10.1016/j.tree.2005.07.011)

17. Speed MP, Turner JRG. 1999 Learning and memory in mimicry: II. Do we understand the mimicry spectrum? *Biol. J. Linn. Soc.* **67**, 281–312. (doi:10.1006/bijl.1998.0310)

18. Turner JRG. 1981 Adaptation and Evolution in Heliconius: A Defense of NeoDarwinism. *Annu. Rev. Ecol. Syst.* **12**, 99–121. (doi:10.1146/annurev.es.12.110181.000531)

19. Sherratt TN. 2002 The coevolution of warning signals. *Proc. R. Soc. B Biol. Sci.* **269**, 741–746. (doi:10.1098/rspb.2001.1944)
20. Summers K, Speed MP, Blount JD, Stuckert AMM. 2015 Are aposematic signals honest? A review. *J. Evol. Biol.* **28**, 1583–1599. (doi:10.1111/jeb.12676)
21. Cadena V, Rankin K, Smith KR, Endler JA, Stuart-Fox D. 2018 Temperature-induced colour change varies seasonally in bearded dragon lizards. *Biol. J. Linn. Soc.* **123**, 422–430. (doi:10.1093/biolinnean/blx152)
22. Larkin MF, Smith SDA, Willan RC, Davis TR. 2018 Diel and seasonal variation in heterobranch sea slug assemblages within an embayment in temperate eastern Australia. *Mar. Biodivers.* **48**, 1541–1550. (doi:10.1007/s12526-017-0700-9)
23. Schubert J, Smith SDA. 2020 Sea slugs—"rare in space and time"—but not always. *Diversity* **12**, 1–14. (doi:10.3390/d12110423)
24. Smith SDA, Davis TR. 2019 Slugging it out for science: volunteers provide valuable data on the diversity and distribution of heterobranch sea slugs. *Molluscan Res.* **39**, 214–223. (doi:10.1080/13235818.2019.1594600)
25. Ehrlich PR, Raven PH. 1964 Butterflies and Plants: A Study in Coevolution. *Evolution (N. Y).* **18**, 586. (doi:10.2307/2406212)
26. Suchan T, Alvarez N. 2015 Fifty years after Ehrlich and Raven, is there support for plant-insect coevolution as a major driver of species diversification? *Entomol. Exp. Appl.* **157**, 98–112. (doi:10.1111/eea.12348)
27. Broom M, Higginson AD, Ruxton GD. 2010 Optimal investment across different aspects of anti-predator defences. *J. Theor. Biol.* **263**, 579–586. (doi:10.1016/j.jtbi.2010.01.002)
28. Balogh ACV, Gamberale-Stille G, Leimar O. 2008 Learning and the mimicry spectrum: from quasi-Bates to super-Müller. *Anim. Behav.* **76**, 1591–1599. (doi:10.1016/j.anbehav.2008.07.017)
29. Arias M, Davey JW, Martin S, Jiggins C, Nadeau N, Joron M, Llaurens V. 2020 How do predators generalise warning signals in simple and complex prey communities? Insights from a videogame. *Proc. R. Soc. B Biol. Sci.* **287**. (doi:10.1098/rspb.2020.0014)
30. Howse PE, Allen JA. 1994 Satyric mimicry: the evolution of apparent imperfection. *Proc. R. Soc. London. Ser. B Biol. Sci.* **257**, 111–114. (doi:10.1098/rspb.1994.0102)
31. Stevens M. 2007 Predator perception and the interrelation between different forms of protective coloration. *Proc. R. Soc. B Biol. Sci.* **274**, 1457–64. (doi:10.1098/rspb.2007.0220)
32. Dalziell AH, Welbergen JA. 2016 Mimicry for all modalities. *Ecol. Lett.* **19**, 609–619.

(doi:10.1111/ele.12602)

33. Mallet J, Gilbert LE. 1995 Why are there so many mimicry rings? Correlations between habitat, behaviour and mimicry in Heliconius butterflies. *Biol. J. Linn. Soc.* **55**, 159–180. (doi:10.1111/j.1095-8312.1995.tb01057.x)

34. Arbuckle K, Speed MP. 2015 Antipredator defenses predict diversification rates. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 13597–13602. (doi:10.1073/pnas.1509811112)

35. Arbuckle K, Brockhurst M, Speed MP. 2013 Does chemical defence increase niche space? A phylogenetic comparative analysis of the Musteloidea. *Evol. Ecol.* **27**, 863–881. (doi:10.1007/s10682-013-9629-z)

36. Arbuckle K, Harris RJ. 2021 Radiating pain: venom has contributed to the diversification of the largest radiations of vertebrate and invertebrate animals. *BMC Ecol. Evol.* **21**, 1–12. (doi:10.1186/s12862-021-01880-z)

37. Zahavi A. 1993 The fallacy of conventional signalling. *Philos. Trans. - R. Soc. London, B* **340**, 227–230. (doi:10.1098/rstb.1993.0061)

38. Merilaita S, Ruxton GD. 2007 Aposematic signals and the relationship between conspicuousness and distinctiveness. *J. Theor. Biol.* **245**, 268–277. (doi:10.1016/j.jtbi.2006.10.022)

39. Polnaszek TJ, Rubi TL, Stephens DW. 2017 When it's good to signal badness: using objective measures of discriminability to test the value of being distinctive. *Anim. Behav.* **129**, 113–125. (doi:10.1016/j.anbehav.2017.05.009)

40. Sherratt TN. 2002 The evolution of imperfect mimicry. *Behav. Ecol.* **13**, 821–826. (doi:10.1093/beheco/13.6.821)

41. Sherratt TN, Beatty CD. 2003 The evolution of warning signals as reliable indicators of prey defense. *Am. Nat.* **162**, 377–389. (doi:10.1086/378047)

42. Turner JRG. 1977 Butterfly Mimicry: The Genetical Evolution of an Adaptation. In *Evolutionary Biology*, pp. 163–206. Boston, MA: Springer US. (doi:10.1007/978-1-4615-6953-4_3)

43. Fisher RA. 1958 *The genetical theory of natural selection*. 2nd rev. e. New York: Dover Publications.

44. Banich MT, Caccamise D. 2010 *Generalisation of knowledge : multidisciplinary perspectives*. New York: Psychology Press.

45. Gamberale G, Tullberg BS. 1996 Evidence for a peak-shift in predator generalisation among aposematic prey. *Proc. Biol. Sci.* **263**, 1329–1334. (doi:10.1098/rspb.1996.0195)

46. Rowe C. 1999 Receiver psychology and the evolution of multicomponent signals. *Anim.*

Behav. **58**, 921–931. (doi:10.1006/anbe.1999.1242)

47. Winters AE, Green NF, Wilson NG, How MJ, Garson MJ, Marshall NJ, Cheney KL. 2017 Stabilising selection on individual pattern elements of aposematic signals. *Proc. R. Soc. B Sci.* **284**.

48. Hebets EA, Papaj DR. 2005 Complex signal function: Developing a framework of testable hypotheses. *Behav. Ecol. Sociobiol.* **57**, 197–214. (doi:10.1007/s00265-004-0865-7)

49. Postema EG, Lippey MK, Armstrong-Ingram T. 2022 Color under pressure: how multiple factors shape defensive coloration. *Behav. Ecol.* , 1–13. (doi:10.1093/beheco/arac056)

50. Endler JA. 1978 A predator's view of animal color patterns. *Evol. Biol.* **11**, 320–364. (doi:10.1007/978-1-4615-6956-5_5)

51. Barnett JB, Scott-Samuel NE, Cuthill IC. 2016 Aposematism: balancing salience and camouflage. *Biol. Lett.* **12**, 20160335. (doi:10.1098/rsbl.2016.0335)

52. Endler JA, Houde AE. 1995 Geographic variation in female preferences for male traits in *Poecilia reticulata*. *Evolution (N. Y).* **49**, 456. (doi:10.2307/2410270)

53. Rosselli FB, Alemi A, Ansuini A, Zoccolan D. 2015 Object similarity affects the perceptual strategy underlying invariant visual object recognition in rats. *Front. Neural Circuits* **9**, 1–22. (doi:10.3389/fncir.2015.00010)

54. Sibeaux A, Cole GL, Endler JA. 2019 The relative importance of local and global visual contrast in mate choice. *Anim. Behav.* **154**, 143–159. (doi:10.1016/j.anbehav.2019.06.020)

55. van den Berg CP, Endler JA, Papinczak DEJ, Cheney KL. 2022 Using colour pattern edge contrast statistics to predict detection speed and success in triggerfish (*Rhinecanthus aculeatus*). *J. Exp. Biol.* (doi:10.1242/jeb.244677)

56. Troscianko J, Osorio D. 2022 A model of colour appearance based on efficient coding of natural images. , 1–16. (doi:10.1371/journal.pcbi.1011117)

57. Rönkä K, De Pasqual C, Mappes J, Gordon S, Rojas B. 2018 Colour alone matters: no predator generalisation among morphs of an aposematic moth. *Anim. Behav.* **135**, 153–163. (doi:10.1016/j.anbehav.2017.11.015)

58. Nokelainen O, Galarza JA, Kirvesoja J, Suisto K, Mappes J. 2022 Genetic colour variation visible for predators and conspecifics is concealed from humans in a polymorphic moth. *J. Evol. Biol.* **35**, 467–478. (doi:10.1111/jeb.13994)

59. Endler JA. 1986 Defense Against Predators. In *Predator-prey relationships, Perspectives and Approaches from the Study of Lower Vertebrates*, pp. 109–134. Chicago: University of Chicago Press.

60. van den Berg CP, Troscianko J, Endler JA, Marshall NJ, Cheney KL. 2020 Quantitative

Colour Pattern Analysis (QCPA): A comprehensive framework for the analysis of colour patterns in nature. *Methods Ecol. Evol.* **11**, 316–332. (doi:10.1111/2041-210X.13328)

61. Winters AE, Chan W, White AM, Berg CP, Garson MJ, Cheney KL. 2022 Weapons or deterrents? Nudibranch molluscs use distinct ecological modes of chemical defence against predators. *J. Anim. Ecol.* **91**, 831–844. (doi:10.1111/1365-2656.13643)
62. Winters AE, White AM, Dewi AS, Mudianta IW, Wilson NG, Forster LC, Garson MJ, Cheney KL. 2018 Distribution of Defensive Metabolites in Nudibranch Molluscs. *J. Chem. Ecol.* **44**, 384–396. (doi:10.1007/s10886-018-0941-5)
63. van den Berg CP, Endler JA, Cheney KL. 2023 Signal detectability and boldness are not the same: the function of defensive coloration in nudibranchs is distance-dependent. *Proc. R. Soc. B Biol. Sci.* **290**, 2022.12.20.521213. (doi:10.1098/rspb.2023.1160)
64. Schneider CA, Rasband WS, Eliceiri KW. 2012 NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9**, 671–675. (doi:10.1038/nmeth.2089)
65. Troscianko J, Stevens M. 2015 Image calibration and analysis toolbox – a free software suite for objectively measuring reflectance, colour and pattern. *Methods Ecol. Evol.* **6**, 1320–1331. (doi:10.1111/2041-210X.12439)
66. Champ CM, Wallis G, Vorobyev M, Siebeck U, Marshall J. 2014 Visual acuity in a species of coral reef fish: *Rhinecanthus aculeatus*. *Brain. Behav. Evol.* **83**, 31–42. (doi:10.1159/000356977)
67. Champ CM, Vorobyev M, Marshall NJ. 2016 Colour thresholds in a coral reef fish. *R. Soc. Open Sci.* **3**, 160399. (doi:10.1098/rsos.160399)
68. Cheney KL, Newport C, McClure EC, Marshall NJ. 2013 Colour vision and response bias in a coral reef fish. *J. Exp. Biol.* **216**, 2967–2973. (doi:10.1242/jeb.087932)
69. Pignatelli V, Champ CM, Marshall J, Vorobyev M. 2010 Double cones are used for colour discrimination in the reef fish, *Rhinecanthus aculeatus*. *Biol. Lett.* **6**, 537–539. (doi:10.1098/rsbl.2009.1010)
70. Cheney KL, Hudson J, de Busserolles F, Luehrmann M, Shaughnessy A, van den Berg C, Green NF, Marshall NJ, Cortesi F. 2022 Seeing Picasso: an investigation into the visual system of the triggerfish *Rhinecanthus aculeatus*. *J. Exp. Biol.* **225**, jeb243907. (doi:10.1242/jeb.243907)
71. Green NF, Guevara E, Osorio DC, Endler JA, Marshall NJ, Vorobyev M, Cheney KL. 2022 Colour discrimination thresholds vary throughout colour space in a reef fish (*Rhinecanthus aculeatus*). *J. Exp. Biol.* **225**, jeb243533. (doi:10.1242/jeb.243533)
72. Randall JE, Allen GR, Steene RC. 1997 *Fishes of the Great Barrier Reef and Coral Sea*.

Crawford House Publishing.

73. Cheney KL, Green NF, Vibert AP, Vorobyev M, Marshall NJ, Osorio DC, Endler JA. 2019 An Ishihara-style test of animal colour vision. *J. Exp. Biol.* **222**, jeb189787. (doi:10.1242/jeb.189787)

74. van den Berg CP, Hollenkamp M, Mitchell LJ, Watson EJ, Green NF, Marshall NJ, Cheney KL. 2020 More than noise: Context-dependant luminance contrast discrimination in a coral reef fish (*Rhinecanthus aculeatus*). *J. Exp. Biol.* **223**, jeb.232090. (doi:10.1242/jeb.232090)

75. van den Berg CP, Condon ND, Conradsen CI, White TE, Cheney KL. 2023 Automated workflows using Quantitative Colour Pattern Analysis (QCPA): A guide to batch processing and downstream data analysis. *bioRxiv* (doi:https://doi.org/10.1101/2023.02.02.526788)

76. Endler JA. 2012 A framework for analysing colour pattern geometry: Adjacent colours. *Biol. J. Linn. Soc.* **107**, 233–253. (doi:10.1111/j.1095-8312.2012.01937.x)

77. Endler JA, Cole GL, Kranz AM. 2018 Boundary strength analysis: Combining colour pattern geometry and coloured patch visual properties for use in predicting behaviour and fitness. *Methods Ecol. Evol.* **9**, 2334–2348. (doi:10.1111/2041-210X.13073)

78. Hubel DH, Wiesel TN. 1962 Receptive fields, binocular interaction and functional architecture in the cat's visual cortex. *J. Physiol.* **160**, 106–154. (doi:10.1113/jphysiol.1962.sp006837)

79. Cheney KL, White A, Mudianta IW, Winters AE, Quezada M, Capon RJ, Mollo E, Garson MJ. 2016 Choose your weaponry: Selective storage of a single toxic compound, latrunculin a, by closely related nudibranch molluscs. *PLoS One* **11**, 1–16. (doi:10.1371/journal.pone.0145134)

80. Chan W. 2022 The Chemical Ecology of Nudibranchs.

81. Bürkner P-C. 2018 Advanced Bayesian Multilevel Modeling with the R Package brms. *R J.* **10**, 395. (doi:10.32614/RJ-2018-017)

82. Cheney KL, Cortesi F, How MJ, Wilson NG, Blomberg SP, Winters AE, Umanzör S, Marshall NJ. 2014 Conspicuous visual signals do not coevolve with increased body size in marine sea slugs. *J. Evol. Biol.* **27**, 676–87. (doi:10.1111/jeb.12348)

83. Appeltans W *et al.* 2012 World Register of Marine Species. See http://www.marinespecies.org.

84. de Villemereuil P, Nakagawa S. 2014 General Quantitative Genetic Methods for Comparative Biology. In *Modern Phylogenetic Comparative Methods and Their*

Application in Evolutionary Biology: Concepts and Practice (ed LZ Garamszegi), pp. 287–303. Berlin, Heidelberg: Springer Berlin Heidelberg. (doi:10.1007/978-3-662-43550-2_11)

85. Korner-Nievergelt F, Roth T, von Felten S, Guélat J, Almasi B, Korner-Nievergelt P. 2015 *Bayesian Data Analysis in Ecology Using Linear Models with R, BUGS, and STAN*. Elsevier. (doi:10.1016/C2013-0-23227-X)
86. Santon M, Korner-Nievergelt F, Michiels NK, Anthes N. 2023 A versatile workflow for linear modelling in R. *Front. Ecol. Evol.* **11**, 1–15. (doi:10.3389/fevo.2023.1065273)
87. Lenth R V. 2023 emmeans: Estimated Marginal Means, aka Least-Squares Means.
88. Rudman WB. 1991 Purpose in pattern - the evolution of color in chromodorid Nudibranchs. *J. Molluskan Stud.* **57**, 5–21.
89. Speed M. 2000 Warning signals, receiver psychology and predator memory. *Anim. Behav.* **60**, 269–278. (doi:10.1006/anbe.2000.1430)
90. Franks DW, Noble J. 2004 Warning signals and predator–prey coevolution. *Proc. R. Soc. London. Ser. B Biol. Sci.* **271**, 1859–1865. (doi:10.1098/rspb.2004.2795)
91. Skelhorn J, Halpin CG, Rowe C. 2016 Learning about aposematic prey. *Behav. Ecol.* **27**, 955–964. (doi:10.1093/beheco/arw009)
92. Kikuchi DW, Sherratt TN. 2015 Costs of Learning and the Evolution of Mimetic Signals. *Am. Nat.* **186**, 321–332. (doi:10.1086/682371)
93. Raviv L, Lupyan G, Green SC. 2022 How variability shapes learning and generalisation. *Trends Cogn. Sci.* **26**, 462–483. (doi:10.1016/j.tics.2022.03.007)
94. Barnett JB, Cuthill IC. 2015 Distance-dependent defensive coloration. *Curr. Biol.* **24**, R1157–R1158. (doi:10.1016/j.cub.2014.11.015)
95. White TE, Umbers KDL. 2021 Meta-analytic evidence for quantitative honesty in aposematic signals. *Proc. R. Soc. B Biol. Sci.* **288**. (doi:10.1098/rspb.2021.0679)
96. Green NF, Urquhart HH, van den Berg CP, Marshall NJ, Cheney KL. 2018 Pattern edges improve predator learning of aposematic signals. *Behav. Ecol.* **29**, 1481–1486. (doi:10.1093/beheco/ary089)