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Stabilising selection on individual pattern elements of aposematic signals

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Abstract

Warning signal variation is ubiquitous but paradoxical: low variability should aid recognition and learning by predators. However, spatial variability in the direction and strength of selection for individual elements of the warning signal may allow phenotypic variation for some components, but not others. Variation in selection may occur if predators only learn particular colour pattern components rather than the entire signal. Here, we used a nudibranch mollusc, *Goniobranchus splendidus*, which exhibits a conspicuous red spot/white body/yellow rim colour pattern, to test this hypothesis. We first demonstrated that secondary metabolites stored within the nudibranch were unpalatable to a marine organism. Using pattern analysis, we demonstrated that the yellow rim remained invariable within and between populations; however, red spots varied significantly in both colour and pattern. In behavioural experiments, a potential fish predator, *Rhinecanthus aculeatus*, used the presence of the yellow rims to recognise and avoid warning signals. Yellow rims remained stable in the presence of high genetic divergence among populations. We therefore suggest that how predators learn warning signals may cause stabilizing selection on individual colour pattern elements, and will thus have important implications on the evolution of warning signals.

Introduction

Aposematic visual signals are used by prey to indicate unprofitability and/or toxicity to potential predators. Consistency in warning signals is considered beneficial to both predator and prey, as predators will be less likely to make errors when recognizing defended prey. Despite this, warning signals are often variable both between and within populations of aposematic prey [1]. Such variation might be facilitated through non-adaptive processes such as genetic drift and restricted gene flow [2, 3]. However, differences in warning signals can also relate to the variation in selective pressures, such as spatial differences in predator communities [4, 5], the abundance of other suitable prey [6], visual contrast between the habitat substrate and the warning signal [1], the availability of dietary metabolites used as chemical defences at a given location [1, 7], and geographic differences in mimetic communities [8].

An alternative hypothesis is that predators may only learn avoidance of warning signals based on individual signal elements (colour, pattern, or shape) of the aposematic signal, and therefore only these elements are under stabilizing selection. Relaxed selection may exist for other elements that are not learned or paid attention to by the predator, allowing phenotypic variation of colour patterns to persist [9]. Previous studies have shown animals often use elemental processing (signal-elemental approaches) when learning visual signals and attend to one component over others, rather than learn the stimulus in its entirety (configural-cue approaches) [10]. For example, chicks, *Gallus gallus domesticus* used colour over pattern when learning to avoid unpalatable food items [11]. Similarly, blue tits learned the colour of rewarded stimuli at a higher rate than pattern or shape, and when presented with mimetic variants of unrewarded stimuli, the birds continued to avoid stimuli based on colour rather than pattern or shape [12]. We also recently show a marine fish used colour, rather than pattern or luminance contrast, to learn an appetitive discrimination task [13].

In this study, we investigated warning signal variation of individual pattern elements in populations of aposematic prey and examined whether potential predators used a signal-elemental

approach when learning avoidance of warning signals. In addition, we used population genetics to assess whether colour pattern differences are indicative of genetic structure among populations. Our study species was a nudibranch mollusc, *Gonibranchus splendidus*, which is a common species throughout most of its range. Nudibranchs are a diverse group of marine molluscs that can deter attackers with potent chemical defences, which in most cases are sequestered and accumulated from specialized diets of sponge, ascidian, and cnidarian food sources [14]. Many nudibranchs display vibrant colour patterns thought to act as warning signals e.g. [15]. In SE Australia, *G. splendidus* is characterized by a white mantle with a red-spotted colour pattern, encircled by a conspicuous yellow rim (Figure S1a). This pattern is highly variable, with spots ranging in size from large blotches to small spots [16], and colour from bright red to maroon [17]. At the edge of its distribution, the species is rare and the rim is red [18]. This species is known to harbor a plethora of secondary metabolites [19, 20], which are accumulated in specialized glands located along the mantle rim and are thought to provide defense [21].

We first tested whether this conspicuous colour pattern was aposematic by measuring anti-feedant properties of secondary metabolites found within the nudibranch. Second, we quantified warning signal variation within and between locations where *G. splendidus* is most abundant (Southern Queensland to New South Wales, Australia) using spectral reflectance measurements, visual modeling, and pattern adjacency analysis [22]. Third, we used behavioural experiments with a potential predatory marine fish, Picasso triggerfish *Rhinecanthus aculeatus,* to examine whether fish learned individual components of the visual signal, or learned the signal in its entirety. Finally, we investigated whether colour pattern variation was indicative of genetic structure among locations. A fast evolving mitochondrial gene, Cytochrome Oxidase I (COI), was sequenced to construct a haplotype network and infer divergence among locations. A more conserved nuclear protein coding gene, Adenine Nucleotide Translocase (ANT), was also sequenced to independently test the pattern from the mitochondrial genome [23, 24]. Examining population structure can help determine if signal divergence is occurring in the presence of high genetic differentiation, or

whether genetic isolation may be driving the fixation of phenotypic variation.

Methods

Nudibranch collection

We collected individuals of *Goniobranchus splendidus* by hand on SCUBA from five sites along the south east coast of Australia: Gneerings Reef, Mooloolaba (-26° 64' S, 153° 15' E), n = 31 in March and April 2013; Shag Rock, North Stradbroke Island (-27° 41' S, 153° 52' E), n = 14 in September 2012, November 2013 and December 2014; Split Solitary Island, Coffs Harbour (-30° 31' S, 153° 15' E), n = 24 in October 2014; Seahorse Gardens, Nelson Bay (-32° 71' S, 152° 15' E), n = 20 in November 2013; and Oak Park, Sydney (-34° 06' S, 151° 15' E), n = 23 in November 2013. Specimens were transferred into larger buckets with aerated seawater and transported to a laboratory for processing. Size of individuals ranged from 10-70mm, and was significantly different between sites ($F_{4, 92} = 11.44$, $p < 0.001$), with individuals from Mooloolaba smaller than other sites (mean $\pm$ s.e. (mm): Mooloolaba = 21 $\pm$ 7, Nelson Bay 38 $\pm$ 13; Sydney 39 $\pm$ 12, Coffs Harbour 37 $\pm$ 12; North Stradbroke 37 $\pm$ 8). Nudibranchs were collected under the following permits: Queensland General Fisheries Permit (#161624); Moreton Bay Marine Park Permit (QS2012/MAN183); NSW Industry & Investment Scientific Collection Permit (F86/2163-7.0).

Anti-feedant assays

Individuals from each location were combined to yield a total tissue volume of at least 2ml (Mooloolaba n=21, Stradbroke Island n=7, Coffs Harbour n=16, Nelson Bay n=13, Sydney n=6). Specimens were then chopped, extracted with acetone and sonicated for 2 minutes. The extract was then concentrated under vacuum and partitioned with diethyl ether (Et_2O) and water. The organic layer was dried with anhydrous Na_2SO_4, before concentration under nitrogen. The dry weight of each crude extract was recorded to the nearest 0.01mg using an electronic balance (ER-182A; A&D Mercury Pty. Ltd.) as per [19, 20, 25, 26].

To assess whether *G. splendidus* secondary metabolites were used as chemical defence, and whether strength of defence varied between sites, we conducted anti-feedant assays using rock-pool shrimp (*Palaemon serenus*). Although these species are not considered nudibranch predators, these crustaceans are commonly used to assay nudibranch chemical defences [27, 28]. Assays were performed using general protocols outlined in [25]. Briefly, artificial food pellets were created using a mixture of freeze-dried squid mantle, alginic acid, purified sea sand and red food dye. Crude extracts from each nudibranch population were added to pellets at four concentrations, and control pellets were made without extract. Ten shrimp were used for each treatment and control group (total n = 50 for each population). Pellets were given to shrimp and after 60 min the presence of a red spot in the transparent gastric mill of the shrimp indicated acceptance, and the absence of a spot indicated rejection. The concentration at which 50% of shrimp rejected the pellets (ED_{50}, effective-dose response) was calculated by interpolating a sigmoidal curve.

Pattern geometry

We quantified variation in size and distribution of red colour patches for individuals from each population. Individuals were submerged in seawater within a petri dish in the laboratory and photographed with a size standard in an extended crawling position. The nudibranch outline was manually traced using a magnetic lasso tool and extracted from the background using Adobe Photoshop CS5. The nudibranch image was then stylized for analysis by placing a transparent layer over the original image and using the pencil tool to define the red spot pattern [22]. The yellow border, rhinophores, and gills were removed for two reasons: rhinophores and gills are often withdrawn when nudibranchs are disturbed, making it unlikely they are used as a signal when under threat of attack, and the yellow rim was difficult to conduct pattern analysis on as it is often folded towards the foot, and thus not fully captured within the image. However, to assess yellow rim variation, we measured rim width and body length for each individual using the line and measure tools in Image J. We then calculated a rim-width: body-length ratio. Images were then normalized

for size by rescaling the images to a standard body area of 5000 pixels, converted into CIE colour space, and intermediate pixels were grouped into two clusters (red or white) using the *kmeans* cluster analysis function in the MATLAB statistical toolbox. Pattern measurements were taken from at least 13 individuals per population (Mooloolaba n=26, Stradbroke Island n=13, Coffs Harbour n=22, Nelson Bay n=20, Sydney n=23).

Pattern properties were quantified using the adjacency analysis method [22]. We used the fraction of transitions (FOT) statistic for our analysis, which is a relative measure of the total number of transitions between red and white pixels within the pattern. This provides a good estimation of variation in spot size and frequency. Animals with fewer transitions tend to have larger, less frequent spots, while animals with more transitions have more frequent spots (Figure S1b).

Spectral reflectance

We assessed differences in colour patches among locations by measuring spectral reflectance of white mantle, red spots and yellow rim with an Ocean Optics USB2000 spectrometer (Dunedin, FL, USA) and Ocean Optics OOIBASE32 software. Individuals were submerged in seawater within a petri dish in the laboratory and we used a 200μm bifurcated optic UV/visible fibre held at a 45° angle connected to a PX-2 pulse xenon light (Ocean Optics). A Spectralon 99% white reflectance standard was used to calibrate the percentage of light reflected at each wavelength from 300-700 nm (LabSphere, NH, USA). At least 10 measurements were taken per colour patch, and three different areas of each colour patch were measured and averaged per individual. Colour measurements were taken from multiple individuals per population: Mooloolaba n = 19, Stradbroke Island n = 10, Coffs Harbour n = 22, Nelson Bay n = 15, Sydney n = 5 (due to equipment failure).

To estimate colour variation of individual nudibranch pattern elements, we used spectral contrast measurements from the perspective of our model fish predator, Picasso triggerfish *Rhinecanthus aculeatus,* using the receptor noise limited vision model [29]. The model calculates

distance (ΔS) between colours in a trichromatic visual space. Colours that appear similar to a specific visual system result in low ΔS values, while those that are chromatically contrasting have high values. We used the spectral sensitivities of Picasso triggerfish λ_{max} = 413 nm, 480 nm, 528 nm [30] because this species: 1) was used in our behavioural experiment (below), 2) is found throughout the range where *G. splendidus* is abundant, from southern Queensland to Sydney, New South Wales [31]), 3) is omnivorous with a diet including molluscs [32], and 4) is representative of a common trichromatic visual system found in many reef fish [33]. This species has relatively low visual acuity at 1.75 cycles per degree [34] which is similar to other reef fish**.**

As per previous studies [15, 35], we assumed a 1:2:2 ratio for the weber fraction *(ω)*, LWS noise threshold was set at 0.05, and, colours were modelled using illumination measurements at a water depth of 5m (as per [35]). To assess colour pattern variation within and between sites, we calculated the colour contrast (ΔS) between the spectral reflectance of each individual colour patch and the average for that site (within-site variation) or the average for all sites combined (between-site variation).

Behavioural experiment

We conducted behavioural experiments to investigate whether Picasso triggerfish learned individual pattern elements (e.g. red spots or yellow rim), or learned the colour pattern of *G. splendidus* in its entirety [10]. Picasso triggerfish are easy to keep in aquaria, and highly trainable [30, 34]. Thirty Picasso triggerfish were collected on snorkel using hand-nets in the lagoon near Lizard Island, Great Barrier Reef, Australia (14^{o}40'S, 145^{o} 28'E) from depths of 1-3m and shipped to the University of Queensland or tested at the research station. Fish standard length ranged from 4-15cm. Experiments were conducted between June-September 2014, and February-March 2017. Fish were kept in individual tanks ranging from 50-100L (W: 30-50cm; L: 40-100cm; H: 30-40cm) depending on body size, and were allowed to acclimatize for at least one week before testing. Fish were collected under the Queensland General Fisheries Permit #161624 and Great Barrier Reef

Marine Parks Authority Permit # G12/35688.

Thirty Picasso triggerfish were trained with one 'non-aposematic' and one 'aposematic' circular stimulus (2.5cm diameter; Figure 1i), printed using a HP Officejet H470 inkjet printer on matte photo-quality paper, laminated and attached in the centre of a white feeding board 10cm apart. The feeding board was placed vertically at one end of the tank and fish were trained to peck stimuli to receive a food reward. The non-aposematic stimulus was a plain white circle. In experiment 1a, the aposematic stimulus for fish in Group A (n = 8) was a yellow rim and red spot, while for fish in Group B (n = 7), the aposematic stimulus featured a red spot with no coloured rim (Figure 1i). In experiment 1b, Group C (n = 8) were presented with just a yellow rim and Group D (n = 7) were again given a yellow rim and red spot. Colours of aposematic stimuli exhibited spectral reflectance similar to *G. splendidus* (Figure S7). If fish pecked the non-aposematic stimulus, they were rewarded with palatable food held by forceps from above; if they pecked the aposematic stimulus, they were given unpalatable food. This method of food delivery ensured fish did not use olfactory cues during experiments. Palatable food was prepared by combining 6g frozen squid mantle, 3g gelatine and 10ml water; while unpalatable food consisted of 6g sodium alginate and 10ml water. Both food types had a semi-solid consistency and were similar in colour and texture. Fish given a small piece of unpalatable food immediately spat it out (> 95% of trials), while palatable food was readily consumed (> 95% of trials).

Trials commenced with the insertion of an opaque partition across the centre of the tank to keep the fish away from the feeding board featuring the pair of stimuli. Once the partition was removed, fish were permitted to peck a stimulus and obtain the associated food. Four trials were conducted per session and fish completed 15-20 sessions in total, with one or two sessions per day (total 60-80 trials per fish). The position (left or right) was pseudo-randomised so it did not remain the same for more than 2 successive sessions. Fish were considered to have learned the task of avoiding the aposematic stimulus once they achieved 80% avoidance over 5 consecutive sessions with a maximum of 1 incorrect peck allowed per session.

Once fish met the avoidance criteria in Group A (N = 8), they then proceeded to a generalisation experiment (experiment 2) in which they were tested using a paired-choice paradigm with 3 novel stimuli (yellow border/ no spot, yellow border/ 5 red spots, and no border/ 5 red spots, Figure 1ii) presented in a pseudorandomised order and position. Fish were permitted to peck twice on either stimulus but did not receive food during these sessions to avoid confounding the learned avoidance acquired during experiment 1. Fish were tested on one pair of test stimuli per session, with 1-2 sessions per day and fish encountered any given stimulus pair between 1-6 times. To ensure fish maintained avoidance of the original unpalatable stimulus, reinforcement training was conducted 1-2 hours before each generalisation session following the method of experiment 1. Fish took approximately 3 weeks to complete learning experiments and a further 2 weeks for the generalisation experiment.

Population-level genetic analysis

Tissue samples were taken from at least 12 *G. splendidus* per population (Mooloolaba n= 31, Stradbroke Island n=12, Coffs Harbour n=20, Nelson Bay n=19, Sydney n=23). The genomic DNA from individuals was extracted and purified with a DNeasy blood and tissue kit (Qiagen). These extracts were used in PCR reactions amplifying two fragments of DNA. This included the mitochondrial gene Cytochrome Oxidase I (COI) and the nuclear protein coding Adenine Nucleotide Translocase (ANT). Primers and cycling conditions are given in (Table S1). These products were purified and sequenced at the Australian Genome Research Facility on an ABI PRISM 3730. Bidirectional reads were assembled and edited in Geneious v7, aligned with MAFFT v7.017 [36]. Protein coding genes were translated to check for stop codons. Haplotypic diversity was displayed using a haplotype network constructed in PopArt using the statistical parsimony TCS algorithm [37]. F_{ST} indices for locations were calculated in Arlequin v.3.5.1.2 [38] and visualized using the heatmap.2 function in R Studio v0.98 [39] in gplots [40].

247 ***Statistical Analysis***

248 All statistical analyses were conducted in R v.3.1.3 [39]. For colour analysis, we used a
249 linear mixed-effects model (LMM) to examine whether variation in colour contrast (ΔS) differed
250 between colours within the pattern and among locations. Individual ID was included as a random
251 factor. For pattern and rim analyses, we used a one-way ANOVA to examine whether FOT and rim
252 differed among locations, with a posteriori Tukey-Kramer HSD post-hoc test to interpret significant
253 interactions between collection sites. In the models, ΔS and rim-width: body-length ratio were log
254 transformed to meet the assumptions of normality.

255 For behavioural experiment 1 (learning experiment), data were analysed with a survival
256 model, using the function survdiff in survival package [41] to examine the differences in the
257 number of sessions fish from different groups took to achieve the learning criteria. For behavioural
258 experiment 2 (generalisation experiment), data were analysed using the GenDavidson formula, part
259 of the Davidson model in the Bradley–Terry 2 package [42]. This model does not allow random
260 factors to be incorporated; therefore, to account for differences between individual fish, the data
261 was also analysed without tied data, using the original Bradley-Terry 2 model (glmmPQL:
262 Generalised mixed model using Penalized Quasi-Likelihood) in which FishID was included as a
263 random factor.

264

265 **Results**

266 ***Anti-feedant assays***

267 Crude extracts were obtained in the following concentrations for each population:
268 Mooloolaba (25.7 mg/ml), Stradbroke Island (24.6 mg/ml), Coffs Harbour (32.4 mg/ml), Nelson
269 Bay (10.3 mg/ml), Sydney (20.8 mg/ml). There were numerous compounds in extracts from each
270 site, but most were identified to be spongian diterpenes, rearranged spongian diterpenes, and
271 spongian norditerpenes as per [19, 20, 26].

All extracts exhibited a dose response to the rock-pool shrimp *Palaemon serenus*; however, the response of extracts from Nelson Bay was relatively weak (Figure 2). Extracts from Mooloolaba, Stradbroke, Coffs Harbour, and Sydney were unpalatable at less than half the concentration naturally occurring within the nudibranchs, while the extract from Nelson Bay was only unpalatable at roughly natural concentration.

Pattern geometry

We found strong variation in red spot colour pattern between sites (Figure 3). There were significant differences in FOT among sites (one-way ANOVA, $F_{4,99} = 43.07$, $p < 0.001$; Figure 3). Individuals from Northern locations (Mooloolaba, Stradbroke Island, Coffs Harbour) had larger, less frequent spots (lower FOT), while individuals from southern locations (Nelson Bay, Sydney) had smaller, more frequent spots (higher FOT) (mean FOT± standard error: Mooloolaba 0.26 ± 0.02, Stradbroke Island 0.31 ± 0.02, Coffs Harbour 0.36 ± 0.02, Nelson Bay 0.48 ± 0.02, Sydney 0.53 ± 0.02). Individuals from neighbouring sites did not differ except in the case of Coffs Harbour and Nelson Bay.

The yellow rim pattern component encircling the mantle was present in all individuals. Variation of the yellow rim was minimal with a mean width of 0.65 mm ± 0.03 standard error across all sites. There was no difference in rim-width: body-length ratio (mean ± standard error) between individuals from Mooloolaba, Stradebroke, Nelson Bay or Sydney; however, this measurement was slightly smaller for individuals from Coffs Harbour (0.017 ± 0.001) compared to Mooloolaba (0.023 ± 0.001; $p < 0.001$) and Nelson Bay (0.022 ± 0.001; $p = 0.008$) (ANOVA $F_{4,92} = 6.03$, $p < 0.001$). However, because differences in individuals from Coffs Harbour are very small, we believe they would not be functionally significant based on the visual acuity of the fish [34].

Spectral reflectance

297 For the colour contrast (ΔS) between spectral reflectance of each individual and the average
298 for all sites combined (between-site variation) there was a significant interaction between colour
299 patch and collection site ($X^2_{4,12}$ = 35.05, p < 0.001). We found similar results for within-site
300 variation ($X^2_{4,12}$ = 14.73, p = 0.005). The main effect of colour patch indicates higher ΔS (more
301 variation) for red spots than yellow rims for all sites except Sydney (which did not significantly
302 differ) (Figure 4; Figure S2), though the magnitude varies across collection sites. Results for ΔS of
303 white mantles compared red spots are reported and visualized in Figure S3.
304 Individuals from Mooloolaba were collected in March-April, while samples from other sites
305 were collected in October-December. Therefore, we collected and measured an additional n = 12
306 individuals from Mooloolaba in October 2016. There were slight differences in spectral reflectance
307 curves between seasons (Figure S4a); however, we still found higher variation for red spots than
308 yellow rims in both seasons (Figure S4b).
309
310 ***Behavioural experiment***
311 In experiment 1a (learning experiment), fish learned to avoid unprofitable aposematic
312 signals more quickly when a yellow border was present than when only a red spot was present (χ^2 =
313 9.5, df = 1, p = 0.002; Figure 1i). Surprisingly, all fish from Group B (n = 7) failed to learn the task
314 over the given time frame when only a red spot was present. In experiment 1b, there was no
315 difference in the time taken to avoid unprofitable stimuli comprised of only a yellow border (Group
316 C) and a yellow border and red spot (Group D) (χ^2 = 0.4, df = 1, p = 0.53). There was also no
317 difference between the two groups trained to avoid the yellow border/red spot signal in experiment
318 1a and 1b (Group A and D) (χ^2 = 0.5, df = 1, p = 0.50) and so the data for these two groups was
319 combined for analysis.
320
321 In experiment 2 (generalisation experiment), fish were much more likely to peck the no
322 border/ 5 red spots stimulus (Z = 3.65, df = 95, p < 0.0001) compared with chance (Figure 1ii) but

continued to avoid both stimuli featuring a yellow border (yellow border/ no spot; $Z = -2.66$, $df = 95$, $p < 0.008$ and yellow border/ 5 red spots; $Z = -0.53$, $df = 95$, $p = 0.05$).

Population-level genetic analysis

Mitochondrial COI sequences produced a network with strong geographic structuring and many private haplotypes, and subsequently showed little haplotype sharing among locations (Figure 5). Indeed, only two haplotypes were shared, one between Coffs Harbour and Mooloolaba and another between Nelson Bay and Sydney. All individuals from Stradbroke Island possessed a unique haplotype.

The nuclear ANT sequences produced a more conserved network of two haplotypes (Figure 5) that did not contradict the mitochondrial signal. The first haplotype was shared among the three northernmost locations (Mooloolaba, Stradbroke Island, Coffs Harbour), while the second haplotype is shared among the four southernmost locations (Stradbroke Island, Coffs Harbour, Nelson Bay, Sydney). There was no haplotype sharing between Mooloolaba and Nelson Bay or Sydney.

The high F_{ST} values seen here indicate a significant lack of gene flow among populations (Figure S5). For COI, Coffs Harbour and Mooloolaba were the least diverged (FST 0.349), while Stradbroke Island and Nelson Bay were the most divergent (FST 0.941). For the more conserved ANT, the neighbouring populations of Coffs Harbour and Stradbroke Island, as well as Sydney and Nelson Bay showed no detectable differentiation, while zero gene flow could be inferred between the most northerly population of Mooloolaba and the most southerly populations of Sydney and Nelson Bay.

Discussion

We investigated the hypothesis that warning signal variation can be explained by variation in selection on individual pattern elements. First, using anti-feedant assays, we confirmed our

model species, the conspicuous nudibranch *Goniobranchus splendidus*, possessed unpalatable chemical defences. Second, using quantitative pattern analysis, we show individual pattern elements exhibited different degrees of divergence within and between populations. The red-spotted element of this colour pattern was highly variable in both colour and pattern, in comparison to the yellow rim element, which was relatively constrained. Third, in behavioural experiments, a potential fish predator used the yellow rim to avoid the colour pattern and did not use alternative pattern elements (i.e. red spots) when deciding whether to attack the stimulus. The red spot element did not further enhance avoidance learning, and there is little evidence suggesting it is part of the warning display. We therefore demonstrate visually hunting predators pay attention to certain pattern components when learning to avoid complex colour patterns. Finally, there was little gene flow between northern and southern populations, and spot pattern was correlated with genetic structure among populations. We propose that while limited gene flow can permit variation in colour patterns, the mechanisms behind predator learning may allow for stabilising selection on more salient pattern elements.

When learning visual signals, some animals only learn one element of a stimulus (stimulus-element learning) [10, 43, 44], or base behavioural decisions on the most noticeable element, which overshadows others [12]. In our behavioural experiments, fish learned avoidance of the signal when both yellow border and red spot pattern was present, but surprisingly, failed to learn the task when only a red spot was present. Furthermore, once fish learned avoidance of the yellow rim/red spot pattern, fish avoided novel stimuli when the yellow rim was present. This indicates they did not learn the pattern as a whole, but instead learned the yellow rim as an individual element. If fish had a fully configural mechanism, they would have exhibited no preference for any novel stimuli as all differed substantially in at least one aspect from the original learned stimuli [10, 45]. We therefore propose that preferential learning of the yellow rim by fish predators selects for reduced variability of this element while no such selection exists for the red-spotted pattern allowing it to vary within and between populations. In terrestrial systems, red is frequently used in warning signals; however,

375 in the marine environment, longer wavelengths of light are attenuated first and therefore would
376 have reduced signal efficacy. Furthermore, the visual systems of marine organisms including fish
377 have reduced sensitivity to long wavelengths [38].

378 However, it is possible red spots help camouflage individuals when viewed against a
379 heterogeneous reef background from a distance. Indeed, the idea that colour patterns may act as
380 camouflage from a distance and warning signals in close proximity has been suggested for other
381 species, e.g. [46]. Predator communities may vary between geographic locations [6], and these may
382 select for differences in pattern among populations, depending on predator spectral sensitivities or
383 visual acuity. In addition, geographical locations may have different habitat backgrounds against
384 which *G. splendidus* is viewed [1], requiring a shift in pattern design among populations. However,
385 underwater images of individuals from a northern site (Mooloolaba) and a southern site (Nelson
386 Bay) suggest differences in habitat backgrounds are not pronounced (Figure S6), but this cannot be
387 discounted without further pattern analysis of background pattern characteristics. Increased spot
388 frequency in southern populations may also match the warning signal of a putative red-spotted
389 mimicry ring, which includes nudibranchs from *Goniobranchus, Hypselodoris, Mexichromis and*
390 *Noumea* genera, and is more prevalent in New South Wales [47].

391 However, variation in colour patterns can also be facilitated through non-adaptive processes
392 such as genetic drift and restricted gene flow [2, 3]. We suggest restricted gene flow among
393 populations of *G. splendidus* would allow variation in spot pattern, since the red spots do not appear
394 to contribute to the warning signal. We found a gradual change in spot pattern from northern to
395 southern populations with the greatest differences in FOT values among populations with the least
396 gene flow. Therefore, it is likely there is a genetic component to the distribution of colour patches
397 in this species. In other molluscs variation in shell patterns have been attributed to Mendelian
398 inheritance [48]. The red spot pattern may also be driven by a reaction-diffusion mechanism
399 proposed for pattern formation in the external shells of molluscs [49]; however, how colour patterns
400 form in shell-less nudibranchs is unclear. Genetic drift and restricted gene flow may also contribute

401 to the slight differences in the width of the yellow rim for individuals from Coffs Harbour; but we
402 suggest this very small difference is unlikely to be perceived by fish based on their visual acuity
403 [34].

404 In contrast with the spatial distribution of pattern elements, differences in red colouration
405 among populations was not related to gene flow or geographic distance among populations. Colour
406 pigments in *G. splendidus* warning signals may be acquired from dietary sources as has been
407 described in other nudibranch species [50, 51], such as yellow and pink aplysillid sponges upon
408 which they are found feeding [52].

409 All populations of *G. splendidus* were unpalatable to palaemon shrimp, although palatability
410 varied among geographic locations. The extract from the Nelson Bay population was only weakly
411 unpalatable in comparison with other geographic locations. *Goniobranchus* nudibranchs are
412 assumed to sequester defensive chemicals from their diet [53]. Thus, the strength of chemical
413 defences from each population likely reflects the availability of different dietary sponges. Indeed,
414 chemical variation in other nudibranch species has been shown to depend on the dietary origin of
415 the metabolites [54]. Though how nudibranch chemical differences influence avoidance learning
416 and selection by predators requires further study.

417 Our results demonstrate the importance of measuring individual elements of colour patterns
418 to help us better understand how predator learning can influence the design of aposematic warning
419 signals. We demonstrate that elements within the pattern of an aposematic nudibranch differ in
420 salience potentially driving stabilising selection on the yellow rim and indicating red spots may not
421 contribute to the warning signal. Geographic variance in the red-spotted pattern may vary across
422 populations due to interactions between restricted gene flow and differences in selection among
423 populations, while differences in colour are likely related to availability of sponge food sources and
424 may be linked to differences in chemical defences. These results have important implications for
425 the selective pressures acting on aposematic warning signals in the marine environment.
426

427 Ethics

428 Experiments were conducted in accordance with the University of Queensland's Animal
429 Ethics Committee (SBS/111/14/ARC).

430

431 Data accessibility

432 Data will be made available through Dryad prior to publication.

433

434 Competing interests

435 We have no competing interests.

436

437 Author's contributions

438 AEW participated in fieldwork, lab-work, data analyses, design of the study, and drafted the
439 manuscript; NFG participated in fieldwork, lab-work, data analyses, and drafting the manuscript;
440 NGW participated the conception of the study, fieldwork, lab-work, data analyses, and drafting the
441 manuscript, MJH participated in data analyses and drafting the manuscript, MJG advised on lab-
442 work and participated in drafting the manuscript, NJM advised on data analyses, KLC conceived of,
443 coordinated, and designed the study, participated in fieldwork, lab-work, data analyses, and drafting
444 the manuscript. All authors gave final approval for publication.

445

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578

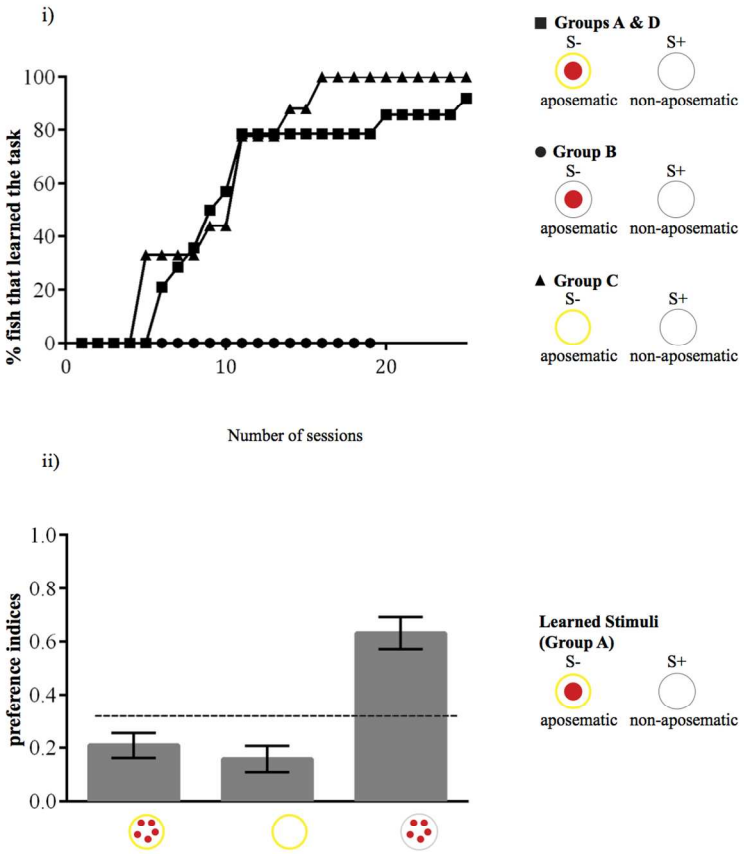
Figure 1. i) The % of fish that learned avoidance of an aposematic stimulus (S-) over a non-aposematic stimulus (S+) after a given number of sessions. In Group A and D the aposematic stimulus contained a yellow rim and red spots (represented by squares). In Group B the aposematic stimulus contained red spots but no rim (represented by circles), and in Group C was only a yellow rim (represented by triangles). ii) The learning experiment was followed by a generalization experiment on fish from Group A. Fish were presented with novel stimuli displayed on the x-axis. Preference indices (mean ± standard error) indicate the likelihood of each stimuli being chosen (pecked). The expected preference index if choices were random is indicated with a dashed line.

Figure 2. Rejection of pellets by the shrimp *Palaemon serenus* (ED_{50}) for crude extracts from each population of *G. splendidus*. The y-axis is reversed so extracts with higher activity (low volume of extract needed to induce unpalatability) are at the top. Where interpolated x values fall within the range of the standard curve, values are graphed along with 95% confidence intervals. Where interpolated x values were extrapolated beyond the reported range, 95% confidence intervals were not calculated.

Figure 3. Representative zone maps for each population of *G. splendidus* are pictured beside a map of collection locations. Differences in our measure of pattern, fraction of transition values (FOT), are displayed in a bar graph with mean ± standard error. Most sites were significantly different. However, sites that did not differ ($p > 0.05$) are indicated with ~.

Figure 4: Mean colour contrast (ΔS) between the spectral reflectance of *G. splendidus* yellow and red colour patches are displayed for each population with mean ± standard error. For each population, mean (ΔS) was calculated between i) the average for all sites combined (between-site variation) or ii) the average for that site (within-site variation).

Figure 5. COI and ANT haplotype networks for *G. splendidus* populations. Circles represent haplotypes, size represents number of individuals that possess each haplotype, and colours represent the collection site for individuals. Black circles represent an inferred missing haplotype (not found in individuals sampled) and bars represent mutational steps between haplotypes.



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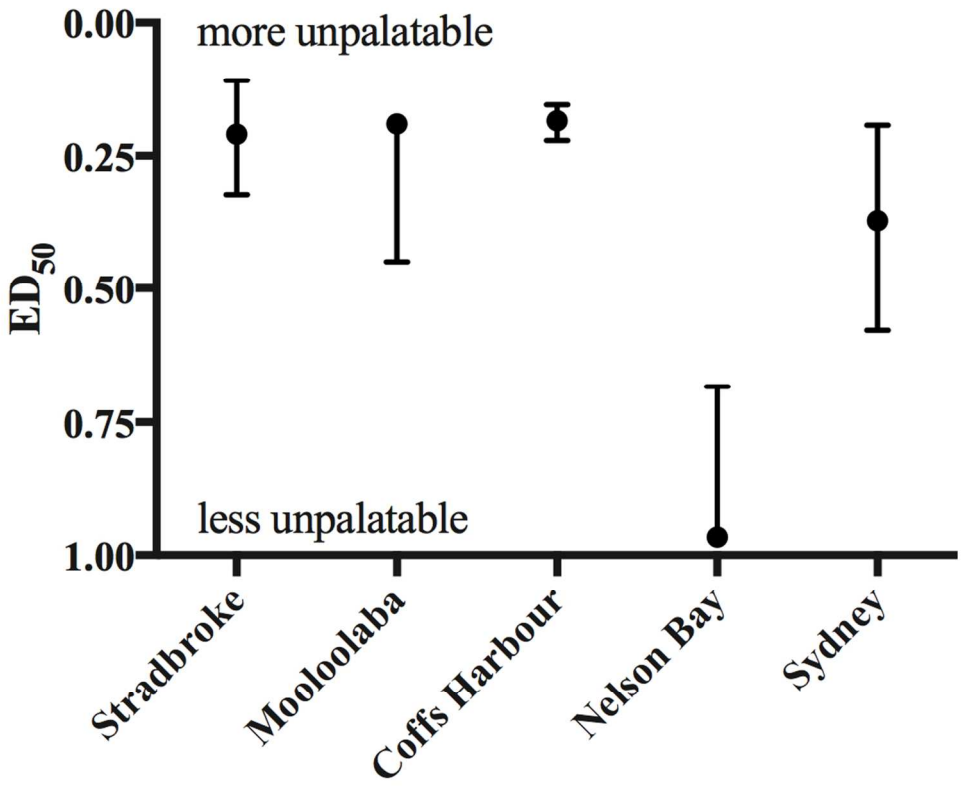

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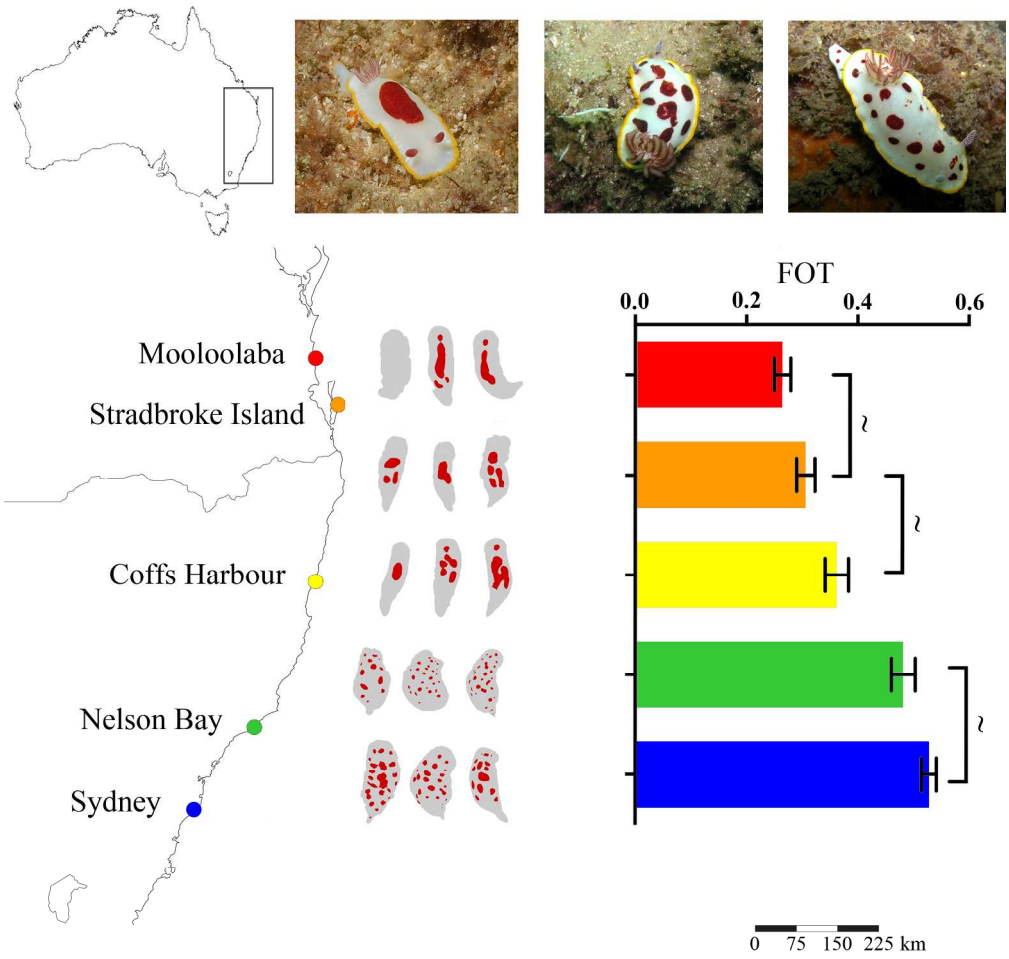

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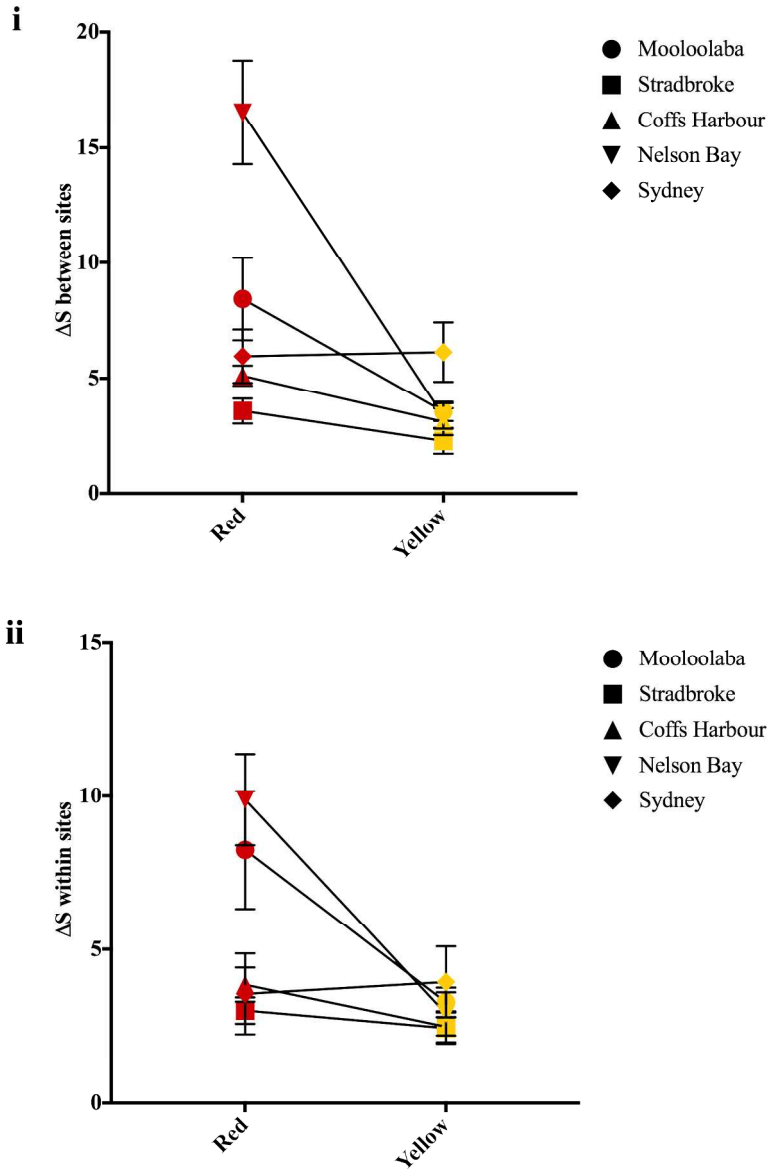

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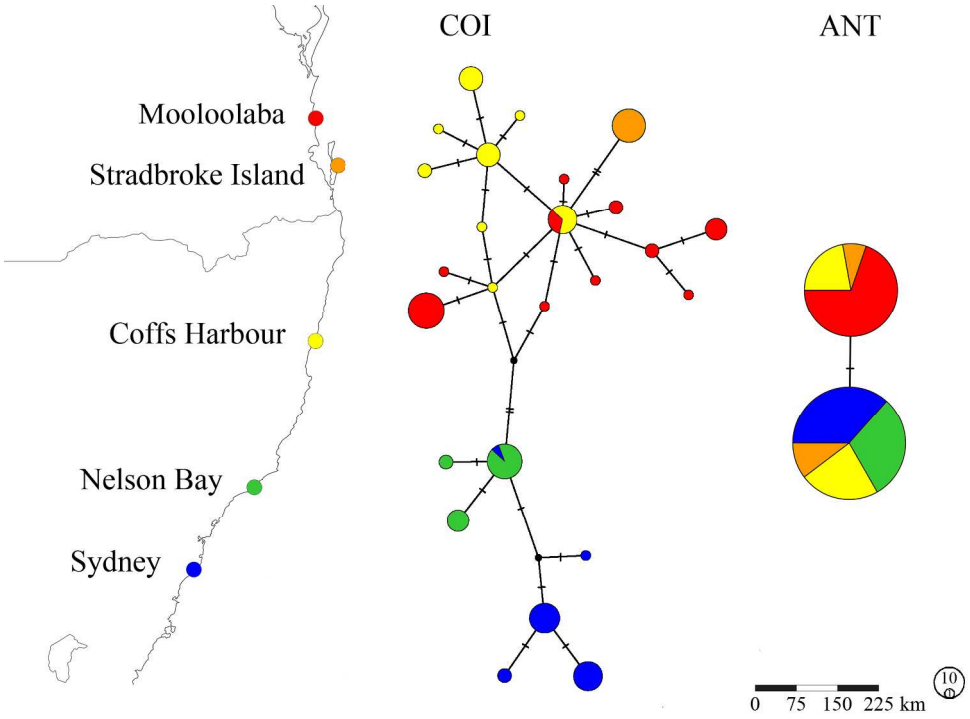

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