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Toxicity and taste: unequal chemical defences in a mimicry ring

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Abstract

Mimicry of warning signals is common, and can be mutualistic when mimetic species harbour equal levels of defence (Müllerian), or parasitic when mimics are undefended but still gain protection from their resemblance to the model (Batesian). However, whether chemically defended mimics should be similar in terms of toxicity (i.e. causing damage to the consumer) and/or unpalatability (i.e. distasteful to consumer) is unclear and in many studies remains undifferentiated. In this study, we investigated the evolution of visual signals and chemical defences in a putative mimicry ring of nudibranch molluscs. First, we demonstrated that the appearance of a group of red spotted nudibranchs molluscs was similar from the perspective of potential fish predators using visual modelling and pattern analysis. Second, using phylogenetic reconstruction, we demonstrated that this colour pattern has evolved multiple times in distantly related individuals. Third, we showed that these nudibranchs contained different chemical profiles used for defensive purposes. Finally, we demonstrated that although levels of distastefulness remained relatively constant between species, toxicity levels varied significantly. We highlight the need to disentangle toxicity and taste when considering chemical defences in aposematic and mimetic species, and discuss the implications for aposematic and mimicry signal evolution.

Key words: mimicry rings, chemical defences, aposematism, marine invertebrates, nudibranch

Introduction

Many animals use visual displays to advertise they are chemically or otherwise defended (aposematism) [1]. The efficacy of aposematic signals in deterring predation is thought to be frequency-dependent, as the warning signal must be encountered multiple times for predators to learn and remember the association between the signal and level of unpalatability [2-5]. Müllerian mimics are defended species that have a mutualistic relationship with co-mimics to increase encounters with predators and spread the burden of predator learning [6-8], whereas Batesian mimics are undefended species that parasitize the warning signal of their defended sympatric model [9]. However, mimicry systems are thought to lie on a spectrum of chemical defence strength, with well-protected Müllerian mimics at one end, unprotected Batesian mimics on the other, and a range of intermediate protection in between (quasi-Batesian mimics) [5, 10-14].

When investigating the relative strength of chemical defences for species in proposed mimicry rings, studies tend to consider the unpalatability of species (i.e. distastefulness to consumer) [15-18] and/or toxicity (i.e. harm to consumer) [19-21]. However, the relationship between distastefulness and toxicity in chemically defended prey is rarely investigated and surprisingly, in many studies remains undifferentiated (but see [22]). Indeed, distastefulness and toxicity are often used synonymously in the literature [5, 19, 23], with perhaps the assumption that they are correlated. Prey species that are distasteful but not toxic, or vice versa, may be common [5, 24], and therefore the relationship between distastefulness and toxic defence needs further consideration [25]. Distasteful compounds that are non-toxic could initially deter predators [26], but may eventually be accepted by predators [26, 27]. This could be dependent on predator satiation, how unpleasant the compound is and the abundance of other palatable prey items [28]. Therefore, toxicity could be considered a more

63 effective deterrent than distastefulness. However, distasteful compounds that are moderately
64 toxic may also protect prey populations more effectively than highly toxic compounds [29].
65 To investigate the relationship between distastefulness and toxicity in mimicry
66 systems, we investigated a putative red spot mimicry ring of nudibranch molluscs that co-
67 occur along the east coast of Australia [30, 31]. Many species of nudibranchs display vibrant
68 warning colours to indicate that they contain defensive secondary metabolites that are
69 sequestered, transformed from dietary sources, or synthesized *de novo* [32]. We have
70 previously shown that one member of the putative red spot mimicry group, *Goniobranchus*
71 *splendidus,* contains distasteful compounds to marine organisms and displays conspicuous
72 colours patterns, components of which are learnt readily by reef fish predators [33]. We first
73 examined the similarity of colour patterns in this group to a potential fish predator using
74 spectral reflectance measurements, visual modelling and pattern geometry analysis. Second,
75 we conducted phylogenetic analysis to investigate shared ancestry of species. Third, we
76 identified and quantified defensive metabolites present in each species and examined the
77 strength of chemical defences using anti-feedant and toxicity assays with shrimp.
78
79 **Methods**
80 *Study species*
81 Nudibranch species (n = 24) were collected between 2012 and 2016 by hand from
82 sites in Queensland (QLD) and New South Wales (NSW) (Table S1) either on SCUBA at
83 depths ranging from 5-18m, or from intertidal zones. Based on previous groupings [30], we
84 identified species that exhibited a similar red spotted or red reticulate colour pattern and/or a
85 distinctive yellow/orange mantle border (Figure 1, A-H). Eight species of nudibranch were
86 assigned a prioiri to a red spot mimicry group: *Goniobranchus splendidus* (Angas, 1864) (n =
87 22), *G. tinctorius* (Rüppell & Leuckart, 1830) (n = 4), *G. daphne* (Angas, 1864) (n = 8), *G.*

hunterae (Rudman, 1983) (n = 1), *Mexichromis mariei* (Crosse, 1872) (n = 4), *Mexichromis festiva* (Angas, 1864) (n = 32), *Hypselodoris bennetti* (Angas, 1864) (n = 26), and *Verconia haliclona* (Burn, 1957) (n = 1). We assigned a further four species to a partial red spot pattern group: *G. verrieri* (Crosse, 1875) (n = 2), *G. albonares* (n =5), *G. tasmaniensis* (Bergh, 1805) (n = 5), and Chromodorididae *thompsoni* (generic placement unassigned, Johnson & Gosliner 2012) (n = 3). These species exhibit part of the red spot mimicry pattern, either with spots or a coloured mantle border missing, or spots of a different colour (Figure 1, I-L). These twelve species co-occur in the study area, and seven of these species are endemic [30].

A further 12 species were assigned to a non-mimic group: *Ceratosoma amoenum* (Cheeseman, 1886) (n = 4), *Chromodoris kuiteri* Rudman, 1982 (n = 4), *C. lochi* Rudman, 1982 (n = 3), *C. elisabethina* (Bergh, 1877) (n=6), *Doriprismatica atromarginata* (Cuvier, 1804) (n = 4), *Goniobranchus decorus* (Pease, 1860) (n = 2), *G. geometricus* (Risbec, 1928) (n = 2), *Hypselodoris jacksoni* Wilson and Willan 2007 (n = 2), *H. obscura* (Stimpson, 1855) (n = 6), *H. tryoni* (Garrett, 1873) (n = 3), *H. whitei* (Adams and Reeve, 1850) (n = 3), *Risbecia godeffroyana* (Bergh, 1877) (n = 2). These species do not appear to closely resemble the red spot mimicry group in terms of colour combinations or pattern (Figure S1).

All specimens were placed in buckets with aerated seawater, transported to the laboratory and placed in a petri dish of seawater for processing. The extended crawling length (cm) of each individual was measured, individuals were photographed, the spectral reflectance of each distinct colour pattern element was measured in the water with a spectrophotometer, and a small portion of tissue from the tail was placed in ethanol for phylogenetic analysis. Species identifications were confirmed through expert examination (N.G.W) and genetic sequencing of Cytochrome *c* Oxidase I (COI) and 16S rDNA and comparison with sequences deposited on the database GenBank. All nudibranch specimens were then frozen and stored at -20°C until chemical extraction of chemical defences.

Phylogenetic relatedness

Representative individuals of newly-collected species selected for the phylogeny were extracted with a DNeasy blood and tissue kit (Qiagen). These were used in PCR reactions to amplify two mitochondrial genes, COI and 16S, using the primers and methods of Wilson, Maschek [34]. Details of all species used in the phylogenetic analysis are available in Table S2. All available COI and 16S data for the Chromodorididae was downloaded from GenBank and added to newly generated data from this study (COI GenBank XXXXX; 16S GenBank XXXXX). Only individuals that were represented by both genes from the same individual were used. This resulted in a data set with 146 species, representing an estimated 40% taxon completeness for the family (www.marinespecies.org). Data were aligned using the MAFFT v7.222 algorithm implemented in Geneious v 9.0.5, trimmed of primer regions, and checked for translation (COI). Data for each gene fragment were analysed separately in a maximum-likelihood (ML) framework for error checking and then concatenated but partitioned, applying the optimal models of evolution simultaneously estimated and selected with the Bayesian Information Criterion in ModelFinder [35] executed in IQ-TREE [36]. To estimate support at each node we used the ultrafast boostrap function, implementing 1000 replicates using a maximum of 1000 iterations and a minimum correlation coefficient of 0.99 as a stopping rule [37]. Outgroups from the putative sister group Actinocyclidae were added, as well as other members of the Doridiidae, allowing for outgroup uncertainty recently highlighted [38]. The tree was rooted with *Doris kerguelenensis***.** We mapped ancestral traits of red spot mimic colour signals (0, no red spot pattern; 1, partial red spot pattern; 2, full red spot pattern) using stochastic character mapping (SCM) [39] in Mesquite v 3.2 [40]. We selected 'MK1' as the evolutionary model, which assumes an equal probability for a particular character change.

138

139

140 *Spectral reflectance measurements*

141 Spectral reflectance measurements of each nudibranch colour pattern element were
142 obtained by placing individuals in a dish immersed in seawater and measurements were taken
143 with an Ocean Optics USB2000 spectrophotometer (Dunedin, FL, USA) and Ocean Optics
144 OOIBASE32 software. We used a 200 μm bifurcated optic UV/visible fibre held underwater
145 at 45° angle connected to a PX-2 pulse xenon light (Ocean Optics). The percentage of light
146 reflected at each wavelength from 300-700 nm was calibrated using a Spectralon 99% white
147 reflectance standard (LabSphere, NH, USA) placed in the petri dish of seawater with the
148 nudibranch. At least 10 measurements were taken of each colour pattern element and
149 averaged per individual. Spectral reflectance data were not obtained for specimens of
150 *Verconia haliclona* or Chromodorididae *thompsoni* due to equipment failure and therefore
151 these species were not included in the colour pattern analysis.

152

153 *Colour and pattern analysis*

154 We first quantified colour pattern elements from the perspective of a potential
155 trichromatic fish predator, the triggerfish *Rhinecanthus aculeatus* (photoreceptor λ_{max} of 413
156 nm, 480 nm, 528 nm and transmission measurements through cornea, vitreus and lens, all as
157 per [41]). We used this species to model the visual characteristics of nudibranchs as it is an
158 omnivorous fish known to prey on molluscs, found throughout the range of the proposed red
159 spot mimicry group (OZCAM.com.au) and is also representative of a common trichromatic
160 visual system found in many marine fish species [42].

161 Photon capture generated by each given colour pattern element (i) for each
162 photoreceptor q_i was calculated as per equation 1 in [43]. Irradiance measurements, $I(\lambda)$, were

taken at a depth of 5 m (as per [44]). Photon loss by transmittance in function of distance was ignored due to the relative clarity of the water in shallow reefs and the small distance assumed between object and viewer (max 30cm). In order to incorporate colour constancy, cone capture quanta were transformed using the von Kries correction as per equation 2 in [43].

Each colour pattern element was defined as an internal pattern (spots, stripes, reticulate), overall body (background) colour and, if present, a contrasting rim. Colour pattern elements were plotted in a trichromatic visual space (Maxwell's triangle) and we measured hue (the angle of the colour coordinate relative to the achromatic point), chroma (or saturation, defined as its distance from the achromatic point) and luminance (measured used the combined photon capture of the double cone, which process luminance in reef fish [45]) from each colour pattern element. Methods were modified from [46, 47].

For pattern analysis, we used images of nudibranch that were normalized for size by rescaling the images to a standard body area of 5000 pixels. The outline of each animal was then 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 [48]. This ensured individual colour pattern elements were correctly recognized by the MATLAB code required to run the analysis. Pattern properties of the entire nudibranch pattern were quantified using the adjacency analysis method [48]. Briefly, the method quantifies the distribution of transitions within and between colour pattern elements on an animal. Three relevant statistics [48] were calculated: 1) aspect ratio, 2) colour diversity and 3) pattern complexity. Aspect ratio was calculated by dividing the vertical patch size by the horizontal patch size (patch size was determined by calculating the average number of pixels along a vertical or horizontal transect until a zone transition). Colour diversity described how

188 spatially evenly colours are represented in the pattern. High values indicate that the relative
189 areas of each colour class are more close to being equal; diversity was calculated by the
190 inverse Simpson index which yields the equivalent number of equally common (area) colours.
191 Pattern complexity was calculated as the density of colour transitions; patterns with a greater
192 number of pixels adjacent to a different colour class will have a higher complexity score.
193
194 *Non-metric multidimensional scaling analysis (NMDS)*
195 Species were differentiated in two-dimensional space using 14 characters of colour
196 and pattern analysis by performing a non-metric multidimensional scaling analysis based on a
197 Euclidean distance matrix with the metaMDS function in the vegan package [49] of R v 3.2.2
198 [50]. Characters were overall pattern (plain = 1, reticulate = 2, spotted = 3 or striped = 4);
199 chromatically contrasting rim (absent = 0, present = 1); hue, chroma, luminance of internal
200 pattern, background colour and rim; and our three pattern geometry statistics (aspect ratio,
201 colour diversity, pattern complexity). If there was more than one pattern present on the
202 species, then the dominant pattern was used as defined by 3 authors and is stated in Table S3.
203 If internal patterns or rims were not present on a particular species, then values calculated for
204 background colour were used.
205
206 *Chemical extraction and identification*
207 To investigate the identity and strength of chemical defences for each species, the
208 whole body tissue of specimens were extracted as per [51]. All extracts were dissolved in
209 deuterated chloroform for ^{1}H NMR analysis on a Bruker AV-500 spectrometer at 500 MHz.
210 If necessary for identification of nudibranch metabolites, a small portion of the extract was
211 analyzed using low-resolution electrospray ionisation mass spectrometry (LRESIMS) on a
212 Bruker Esquire HCT mass spectrometer. The ^{1}H NMR and LRESIMS data of crude extracts

were compared with the respective literature to identify known compounds. Where necessary, a small portion of the extract was subjected to silica flash chromatography, and the various fractions produced were further separated into individual compounds by normal phase high performance liquid chromatography (NP HPLC), eluting with various ratios of hexanes/ethyl acetate. Dried extracts were placed in solution with dichloromethane (DCM) at the recorded specimen volume to provide a stock solution at the natural concentration (mg/mL) of extract for use in toxicity and palatability assays.

Toxicity Assay

In order to measure the relative toxic properties of crude extracts from each species of nudibranch, brine shrimp (*Artemia* sp.) LD_{50} (Lethal dose at 50%) assays were conducted between November 2013 and September 2015 on six of the twelve red spot species for which there was enough biological material (*G. splendidus, G. tinctorius, G. daphne, G. tasmaniensis, M. festiva, H. bennetti*). Comparative studies using extracts from marine sponges have demonstrated that brine shrimp can be a good first indicator of bioactivity, and show similar results to assays tested against fish [52, 56]. Assays were carried out as per methods in [51]. Briefly, a stock solution of the crude extract for each species was prepared by adding a volume of DCM equivalent to that of the extracted tissue. One glass microfiber filter paper (Whatman GF/C 47 mm diam.) was placed into individual glass petri dishes (55 mm diam.) then 0.005, 0.05, 0.5 mL of stock solution were transferred on to the filter papers with a glass pipette. The solvent was left to evaporate from the filter paper under a Nederman arm for 10 min. Brine shrimp eggs were hatched in artificial seawater (Tropic Marin) and twenty actively swimming instar I nauplii (< 12 h after hatching) were collected with a glass pipette and added to each petri dish with 5 mL filtered sea water. Lids were placed on top of the petri dishes and kept under constant illumination for 24 hours. Surviving nauplii (instar

238 II/III) were then counted; nauplii were considered dead if no movement was detected after
239 several seconds of observation. Natural mortality was controlled for using control treatments
240 in which 0.5 mL of DCM was added to the filter paper. In all cases control deaths occurred,
241 therefore the data was corrected using Abott's formula % deaths = (test – control)/(100 –
242 control) for analysis [53]. We then calculated the LD_{50} of the crude extract for each
243 nudibranch species by interpolating a line or standard curve, chosen based on R^2 values. LD_{50}
244 values were calculated for species with extracts that induced a response to at least 50% of the
245 brine shrimp. LD_{50} values are interpolated x values (mL stock solution), where 1 mL of
246 extract = 1 mL of tissue, and therefore reflect natural volumetric concentrations. Absolute
247 concentrations of compounds tested are shown in Figures S2 and S3.
248
249 *Anti-feedant assay*
250 To assess the relative distastefulness, and thus feeding deterrence of nudibranch
251 extracts, antifeedant assays were performed using the generalist rock-pool prawn (*Palaemon*
252 *serenus*) between November 2013 and September 2015 as per [51, 54, 55]. This species has a
253 clear carapace and digestive tract, which makes it ideal for feeding observations and
254 preliminary studies have shown that compounds distasteful to marine fish *Tetractenos*
255 *hamiltoni* and *Rhinecanthus aculeatus* are also distasteful to rock-pool shrimp [56].
256 Individuals were collected intertidally in SE Queensland on foot using hand nets and housed
257 in aquaria with ample food (Ocean Nutrition, Formula 2) until used in assays. Artificial food
258 pellets were created to approximate the nutritional content of a nudibranch with roughly 90%
259 water, 7% squid + alginate, and 3% sand following the protocol outlined in [51, 57]. Crude
260 extracts were added in several concentrations up to that which they were found occurring
261 naturally for each species by adding the crude stock solution or DCM without extract (control
262 pellets) to a dry mixture (50 mg freeze-dried squid mantle, 30 mg alginic acid, 30 mg purified

sea sand). The DCM of each treatment and control was allowed to evaporate for 30 minutes under a Nederman arm, and then the mixture was reconstituted in distilled water to make a final pellet volume of 0.5 mL. Shrimps were selected randomly and placed individually in small compartments (135mm x 98mm x 90mm) with adequate aeration and water flow. Shrimp were allowed to acclimatize for at least 3 days and fed green fish flakes (Ocean Nutrition, Formula 2) once per day. Shrimp were then starved for 2 days prior to trials. Ten shrimp were randomly selected for each extract-treated and control group. Pellets were offered to shrimp using tweezers and then observed for 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. Shrimp that rejected a pellet were then offered a control pellet and observed for a further 30 minutes. Shrimp that did not eat control pellets were removed from the analysis. Shrimp were not re-used. The ED_{50} of crude extracts was calculated as above.

To consider whether a correlation existed between distastefulness and toxicity while considering phylogenetic relatedness between species, we used a Generalized Least Squares (GLS) regression model. We first pruned the tree to leave only the six species on which we had conducted assays and then created a chronogram using the chronos function in the ape package v 5.0 [58]. We used the Brownian model [59] as this had the lowest AIC values using corBrownian, in comparison to models run with corGrafen and corMartin. Phylogenetic regression analysis was conducted in R version 3.2.2 [50].

Results

Colour and pattern analysis

Data for colour and pattern parameters are reported in Table S3 and were visualized in ordinal spacing using NMDS. The red spotted species *Goniobranchus splendidus, G. daphne*, *G. hunterae*, *Mexichromis mariei, M. festiva* and *Hypselodoris bennetti* formed a

close cluster of similar colour pattern characteristics (Figure 2) from the perspective of a potential predator. *Goniobranchus tasmaniensis* also clustered closely with this group, even though it does not have a yellow rim and spots are orange to human eyes. *Goniobranchus tinctorius* did not cluster close to the main species, presumably due to the presence of a reticulate pattern rather than well-defined spots. Partial red spotted species that did not cluster with the main group were *G. verrieri* and *G. albonares* but neither of these possessed a spotted pattern. Species that were placed in the non-mimic group were widely distributed in the plot. Therefore, our *a priori* groupings based on human vision appeared to be validated, with the exception of the exclusion of *G. tinctorius* and the inclusion of *G. tasmaniensis,* which may reflect differences between human and triggerfish vision.

Phylogenetic relatedness

The phylogeny generated and stochastic ancestral state reconstruction demonstrates that the red spot group occurs in six parts of the phylogenetic tree (Figure 3) with these included taxa. However, incomplete taxon sampling may affect the reconstruction for some groups, and more conservative estimates might be warranted. However, although the results indicates that shared ancestry may account for similarities in colour pattern for species within the genus *Goniobranchus* and between those in the genus *Mexichromis*, it would not do so between these genera or the other red spot species *Verconia haliclona*, or *Hypselodoris bennetti*. Thus, the red spot pattern has been independently acquired within the family Chromodorididae.

Chemical identification

Nudibranch species from the red spot mimicry group contained different compounds (Table 1). Species from the genus *Goniobranchus* possessed spongian diterpenes, rearranged

313 diterpenes, and norditerpenes as per [60], and there were significant differences in chemical
314 profiles between species. Species from *Hypselodoris* and *Mexichromis* species possessed
315 furanosesquiterpenes (Table 1), and the extracts of *M. festiva* from Nelson Bay and the Gold
316 Coast possessed the same compounds. Compound names and structures are listed in Table S4.
317
318 *Toxicity and palatability assays*
319 Red spot species differed both in terms of toxicity and distastefulness (Figure 4).
320 Species with extracts that were toxic to brine shrimp included *G. tasmaniensis*, *H. bennetti*,
321 and *M. festiva* (Figure 4A). A dose response was also observed for the extract of *G. daphne*,
322 but this response did not reach above 50% mortality, and no dose response was observed for
323 *G. tinctorius* or *G. splendidus*. All extracts produced a dose response to the shrimp *Palaemon*
324 *serenus*, though this response did not reach above 50% for the extract of *M. festiva* (Figure
325 4B). Importantly, using the phylogenetic generalised least square (GLS) regression model,
326 we did not find an association between toxicity and distastefulness ($t_6 = 0.89$, $p = 0.42$;
327 Figure S3).
328
329 **Discussion**
330 This study presents quantitative evidence of visual similarities between species in a
331 putative mimicry group using colour and pattern analysis, and demonstrates that shared
332 pattern elements of these co-occurring species are distinct from other, closely related species.
333 Phylogenetic analysis indicates that this red spot pattern evolved at multiple times, suggesting
334 this pattern has resulted from convergent evolution rather than shared ancestry. Members of
335 the mimicry group possess different chemical profiles used for defensive purposes, and these
336 suites of compounds provide unequal levels of defence in terms of a toxic response. However,
337 the level of distastefulness of these compounds appears to be relatively similar to a marine

338 shrimp. These data therefore do not support the assumption that distasteful compounds
339 honestly signal levels of toxicity, at least in this mimicry system, and in many systems,
340 toxicity may not be related to distastefulness [25]. However, cumulative ingestion could be
341 toxic over time and cause incremental damage or illness. This study should encourage
342 researchers to disentangle terms such as toxicity and distastefulness as modes of chemical
343 defences when investigating aposematic and mimicry systems.

344 Many theoretical models of mimicry rings with unequal defences exist [e.g. 13, 56-
345 63]. Weakly defended co-mimics may degrade the warning signal of the model [15, 64]; for
346 instance, in an experiment using birds, an increase in abundance of a moderately defended
347 artificial prey increased per capita predation on both the mimic and the highly defended
348 model prey when population densities were low [15]. However, the relationship between
349 species with comparably weak defences and that of their co-mimics remains unclear. In
350 some studies, unequal defences still appear to be mutualistic [14, 65]. For example, highly
351 defended models coupled with moderately defended mimics can have a decrease in per capita
352 mortality when population densities are high [14].

353 However, the mode of chemical defence is often not defined in such models and
354 unequal defences in mimicry systems are sometimes only discussed in terms of quantity (but
355 see [29]). Prey that store distasteful, but otherwise non-toxic compounds that would not
356 damage or incur costs on the host, may repel predators due to their unpleasant nature.
357 Predators may quickly learn they are not harmed after consuming such prey and may still
358 consume distasteful prey when other food is scarce and predators are hungry [61, 62]. If
359 compounds are equally distasteful, we propose that predators may be unable to discriminate
360 levels of toxicity between species. Therefore, non-toxic species may benefit from resembling
361 their toxic counterparts, but not incur costs involved in harbouring toxins. It is also possible
362 that species may mimic the taste of toxic compounds with those that are non-toxic.

Our study species had very different chemical profiles: *Hypselodoris* and *Mexichromis* nudibranchs contained furanosesquiterpenes while *Goniobranchus* nudibranchs and Chromodorididae *thompsoni* contained spongian diterpenes, nor-diterpenes, and rearranged diterpenes, which appeared to be less toxic than furanosesquiterpenes. Although all chemical extracts in this study were distasteful to *Palaemon* shrimp, this effect was weak for the extract of *M. festiva* (Nelson Bay), which did not induce a response to 50% of the shrimp. *M. festiva* extracts were more concentrated, but contained fewer metabolites than that of *H. bennetti*, which showed enhanced activity in both assays. Therefore, toxicity of these extracts is instead likely to be largely influenced by differences in metabolites. did not test for an emetic response, which has been shown before in nudibranch compounds [55]. From our results, it appears that chemical defences, both in terms of palatability and of toxicity, are not equal in this mimicry ring. Ideally, toxicity and unpalatability assays would have been conducted on a potential fish predator of nudibranchs, as the response of different taxa to particular compounds may be variable. However, there are considerable ethical implications of conducting toxicity assays with vertebrates.

Our red spot mimetic species clustered together and shared very similar visual characteristics; however, there are some species that shared only some visual similarities and may be considered imperfect mimics. It is predicted that selection on quasi-Batesian mimicry rings should be similar to Batesian systems, with an evolutionary arms race in warning signal design between well-defended and weakly defended species [12, 63]. In this scenario species with greater chemical defences would be selected to differentiate their warning signal from those with weaker defences. However, this hypothesis was not supported in this system, where the colour patterns of the two species with the most potent chemical defences (*G. tasmaniensis* and *H. bennetti*) clustered well with other co-mimics. Alternatively, predators may select for imperfect mimicry in complex Müllerian systems when defended and

388 palatable prey types are discriminated based on certain components of the visual signal [64],
389 with relaxed selection on other components of the visual signal that are generalized [65].
390 Indeed, we have recently shown that when learning a red spot / yellow rim colour pattern,
391 triggerfish paid most attention to the yellow border when learning to avoid distasteful food,
392 and disregarded the internal red pattern. We also found that the yellow rim was a more
393 consistent part of the visual signal in populations of *Goniobranchus splendidus*, although
394 there was considerable variation in the red spot component [33] . Highly contrasting body
395 outlines may help nudibranchs to stand out against their background and increase
396 conspicuousness, which is an important characteristic of warning signal designs [5].
397 However, this does not explain the lack of mantle border in five species in this study.

398 We believe that this is the first study of an aposematic mimicry ring to include
399 detailed chemical profiles and to assess both the toxicity and distastefulness of contributing
400 species. We have demonstrated that there may not be a correlation between toxicity and
401 distastefulness, and therefore highlight the importance of testing multiple modes of defence
402 to inform future models of mimicry systems. It is likely that warning signal designs and
403 chemical profiles vary geographically [56]; therefore, the impact of geographical differences
404 in dietary resources and predation pressure on warning signal design, chemical profiles, and
405 anti-predator activity of co-mimics would be an interesting direction for future research.
406

407 **Data accessibility**

408 Data will be made available through Dryad prior to publication.
409

410 **Competing interests**

411 We have no competing interests.
412

Author's contributions

AEW participated in fieldwork, lab-work, data analyses, design of the study, and drafted the manuscript; NGW participated the conception of the study, fieldwork, lab-work, data analyses, and drafting the manuscript. CPvdB participated in data analyses, MJH participated in data analyses and drafting the manuscript, JAE participated in data analyses, NJM advised on data analyses, AMW conducted lab-work and identified metabolites. MJG advised on lab-work, assisted with metabolite identification and participated in drafting the manuscript. KLC conceived of, coordinated, and designed the study, participated in fieldwork, lab-work, data analyses, and drafting the manuscript. All authors provided comments on final version and gave approval for publication.

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587
588

589 **Figure Legends**

590 **Figure 1.** Representative photographs of the putative mimicry species investigated in this
591 study. **Top panel:** Full pattern including yellow-orange mantle border, white mantle, and
592 spots. **Bottom panel:** partial pattern missing either spots or border. From upper left:
593 *Goniobranchus splendidus* (A), *Goniobranchus tinctorius* (B), *Goniobranchus daphne* (C),
594 *Goniobranchus hunterae* (D), *Mexichromis mariei* (E), *Mexichromis festiva* (F),
595 *Hypselodoris bennetti* (G), *Verconia haliclona* (H), *Goniobranchus verrieri* (I),
596 *Goniobranchus albonares* (J), *Goniobranchus tasmaniensis* (K), Chromodorididae *thompsoni*
597 (L).
598
599 **Figure 2.** Nudibranch colour patterns differentiated in ordinal space (NMDS) based on 14
600 metrics of the hue, chroma and luminance of colour pattern element and overall nudibranch
601 pattern geometry. The *a priori* predicted red spotted group is shown in red. Partial red
602 spotted pattern species are shown in orange and non-red spot group are shown in black. The
603 red ellipse shows the clustering of many red spotted species.
604
605 **Figure 3.** Maximum-likelihood topology of Chromodorididae taxa. Species that were
606 assigned to a red spotted group are shown in red, the partial red spot group in orange, and
607 those not assigned to the non-red spot group in blue. Bootstrap values are shown for clades
608 with over 70% support. Ancestral state reconstruction of the red colour pattern was
609 performed using ML analysis and marginal probability reconstruction with model Mk1
610 (rate 0.24 Log likelihood, −54.77).
611
612
613 **Figure 4**. **a*) Toxicity assay:*** LD_{50} values based on mortality of Brine shrimp, *Artemia* sp. ***b)***
614 **Anti-feedant assay**. ED_{50} values based on rejection of pellets by Palaemon shrimp,
615 *Palaemon serenus.* Values are represented as proportion of natural concentration found in the
616 mantle of the nudibranchs. Circles indicate LD_{50} values calculated from the data, nr indicate
617 no response at the highest concentration tested. Absolute concentrations are shown in Figure
618 S2.
619
620
621

Figure 1.

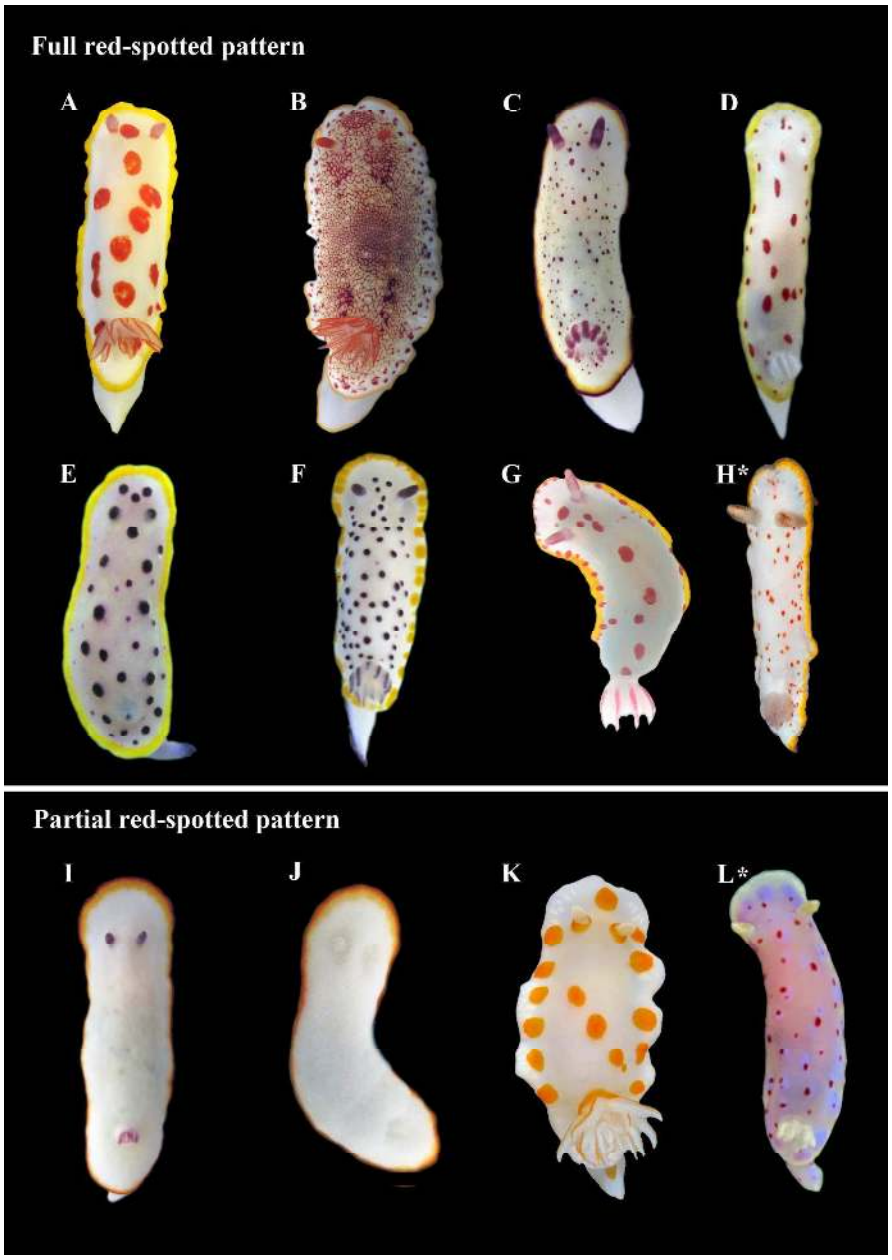
Full red-spotted pattern
A
B
C
D
E
F
G
H*
Partial red-spotted pattern
I
J
K
L*

Figure 2.

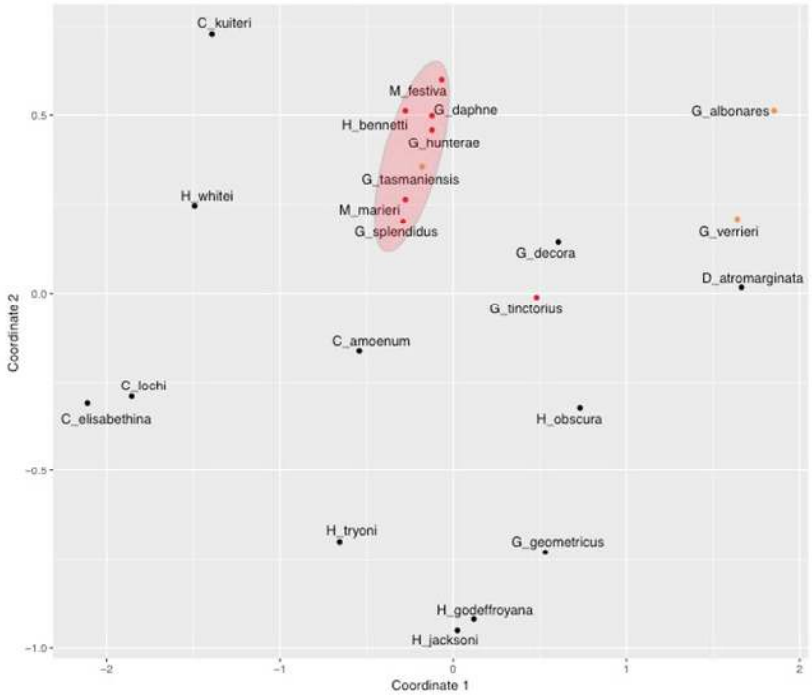
C_kuiteri
M_festiva
G_daphne
H_bennetti
G_hunterae
G_albonares
G_tasmaniensis
H_whitei
M_marieri
G_splendidus
G_verrieri
G_decora
D_atromarginata
G_tinctorius
C_amoenum
C_lochi
C_elisabethina
H_obscura
H_tryoni
G_geometricus
H_godeffroyana
H_jacksoni
Coordinate 1
Coordinate 2
0.5
0.0
−0.5
−1.0
−2
−1
0
1
2

Figure 3.

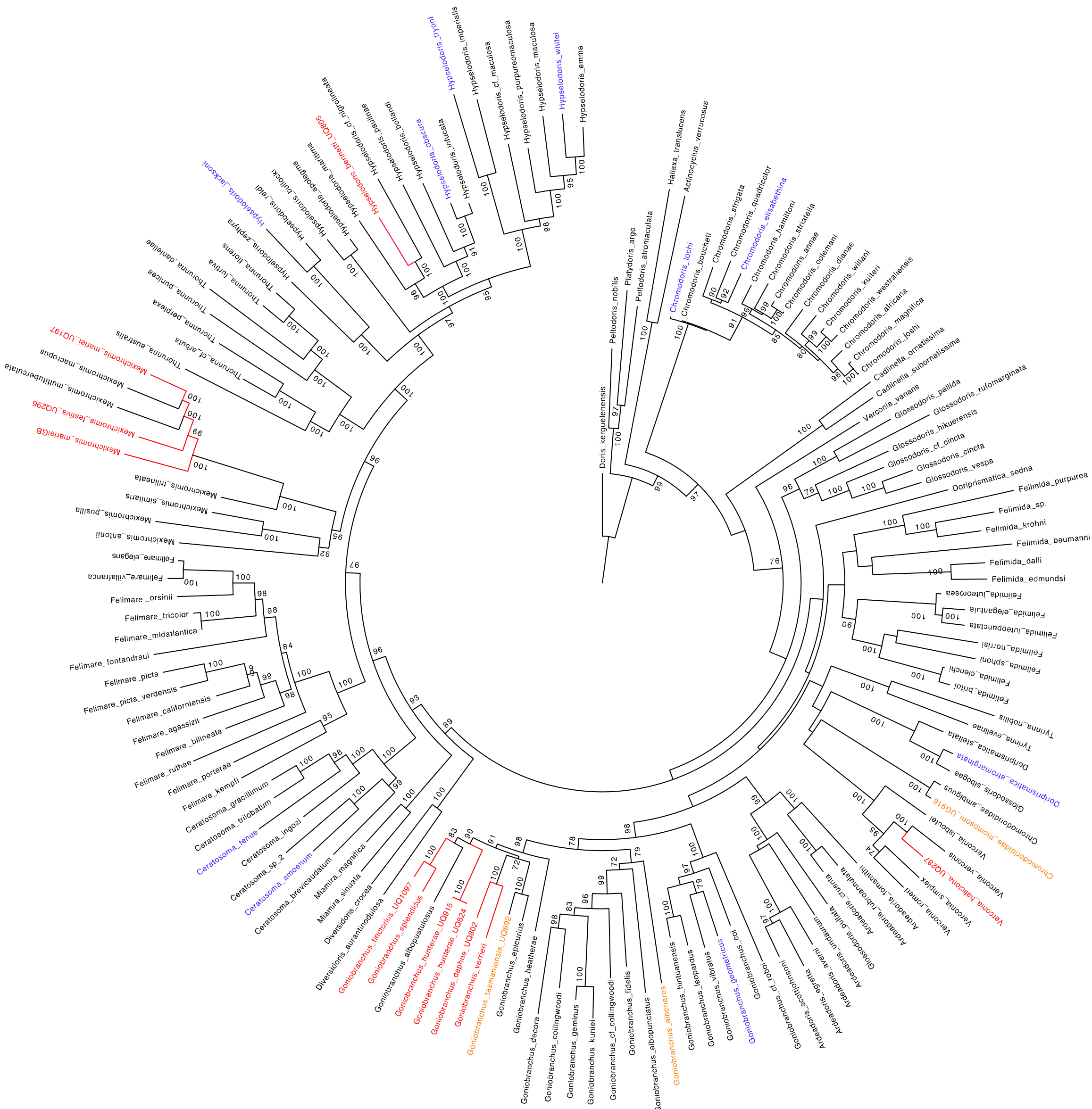
Hypselodoris_tryoni
Hypselodoris_imperialis
Hypselodoris_cf_maculosa
Hypselodoris_purpureomaculosa
Hypselodoris_maculosa
Hypselodoris_whitei
Hypselodoris_emma
Hypselodoris_infucata
Hypselodoris_obscura
Hypselodoris_bollandi
Hypselodoris_paulinae
Hypselodoris_bennetti_UQ805
Hypselodoris_maritima
Hypselodoris_apolegma
Hypselodoris_bullockii
Hypselodoris_reidi
Hypselodoris_jacksoni
Hypselodoris_zephyra
Thorunna_florens
Thorunna_furtiva
Thorunna_daniellae
Thorunna_punicea
Thorunna_perplexa
Thorunna_cf_arbuta
Thorunna_australis
Mexichromis_mariei_UQ197
Mexichromis_macropus
Mexichromis_multituberculata
Mexichromis_festiva_UQ296
Mexichromis_mariei_GB
Mexichromis_trilineata
Mexichromis_similaris
Mexichromis_pusilla
Mexichromis_antonii
Felimare_elegans
Felimare_villafranca
Felimare_orsinii
Felimare_tricolor
Felimare_midatlantica
Felimare_fontandraui
Felimare_picta
Felimare_picta_verdensis
Felimare_californiensis
Felimare_agassizii
Felimare_bilineata
Felimare_ruthae
Felimare_porterae
Felimare_kempfi
Ceratosoma_gracillimum
Ceratosoma_trilobatum
Ceratosoma_tenue
Ceratosoma_ingozi
Ceratosoma_sp_2
Ceratosoma_amoenum
Ceratosoma_brevicaudatum
Miamira_magnifica
Miamira_sinuata
Diversidoris_crocea
Diversidoris_aurantionodulosa
Goniobranchus_tinctorius_UQ1097
Goniobranchus_splendidus
Goniobranchus_albopustulosus
Goniobranchus_hunterae_UQ915
Goniobranchus_hunterae_UQ824
Goniobranchus_daphne_UQ802
Goniobranchus_verrieri
Goniobranchus_tasmaniensis_UQ892
Goniobranchus_epicurius
Goniobranchus_heatherae
Goniobranchus_decora
Goniobranchus_collingwoodi
Goniobranchus_geminus
Goniobranchus_kuniei
Goniobranchus_cf_collingwoodi
Goniobranchus_fidelis
Goniobranchus_albopunctatus
Goniobranchus_albonares
Goniobranchus_hintuanensis
Goniobranchus_leopardus
Goniobranchus_vibratus
Goniobranchus_geometricus
Ardeadoris_egretta
Ardeadoris_scottjohnsoni
Ardeadoris_averni
Glossodoris_undaurum
Glossodoris_pullata
Ardeadoris_cruenta
Ardeadoris_rubroannulata
Ardeadoris_tomsmithi
Verconia_romeri
Verconia_simplex
Verconia_haliclona_UQ287
Verconia_verconis
Verconia_laboutei
Glossodoris_sibogae
Doriprismatica_atromarginata
Doriprismatica_stellata
Tyrinna_evelinae
Tyrinna_nobilis
Felimida_britoi
Felimida_clenchi
Felimida_sphoni
Felimida_norrisi
Felimida_luteopunctata
Felimida_elegantula
Felimida_luteorosea
Felimida_edmundsi
Felimida_dalli
Felimida_baumanni
Felimida_krohni
Felimida_sp.
Felimida_purpurea
Doriprismatica_sedna
Glossodoris_vespa
Glossodoris_cincta
Glossodoris_cf_cincta
Glossodoris_hikuerensis
Glossodoris_rufomarginata
Glossodoris_pallida
Verconia_varians
Cadlinella_subornatissima
Cadlinella_ornatissima
Chromodoris_joshi
Chromodoris_magnifica
Chromodoris_africana
Chromodoris_westraliensis
Chromodoris_kuiteri
Chromodoris_willani
Chromodoris_dianae
Chromodoris_colemani
Chromodoris_annae
Chromodoris_striatella
Chromodoris_hamiltoni
Chromodoris_elisabethina
Chromodoris_quadricolor
Chromodoris_strigata
Chromodoris_boucheti
Chromodoris_lochi
Actinocyclus_verrucosus
Hallaxa_translucens
Peltodoris_atromaculata
Platydoris_argo
Peltodoris_nobilis
Doris_kerguelenensis

Figure 4.

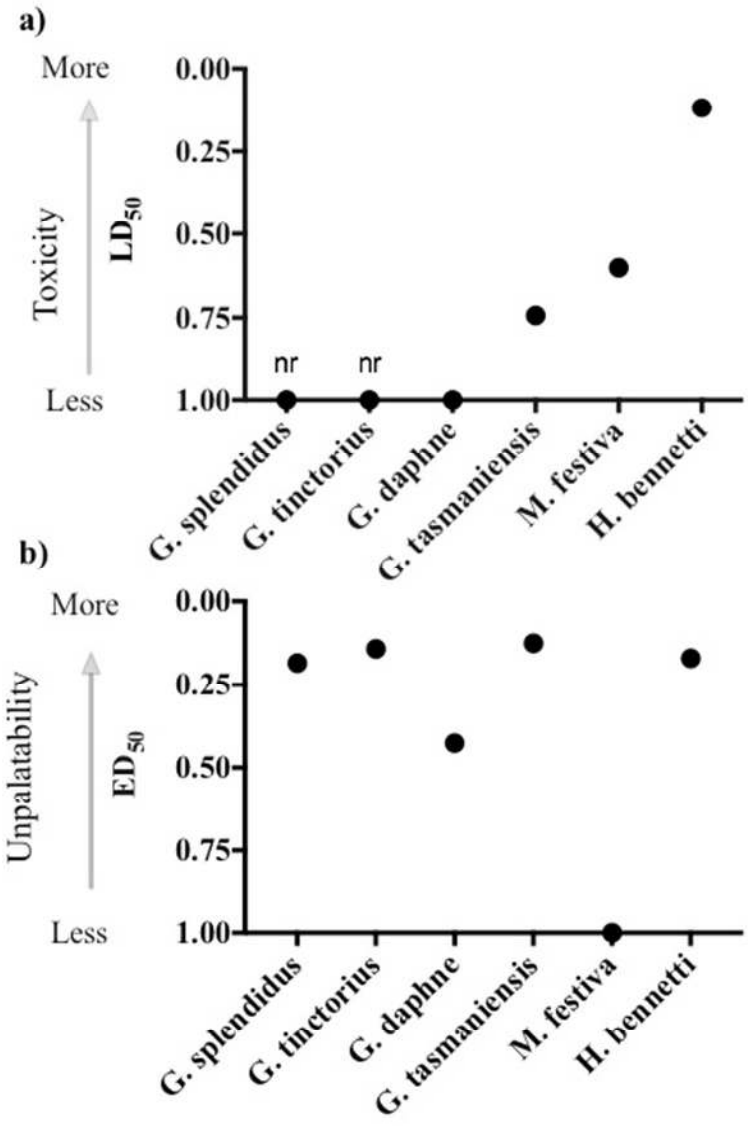
a)
More
Toxicity
Less
LD50
0.00
0.25
0.50
0.75
1.00
nr
nr
G. splendidus
G. tinctorius
G. daphne
G. tasmaniensis
M. festiva
H. bennetti
b)
More
Unpalatability
Less
ED50
0.00
0.25
0.50
0.75
1.00
G. splendidus
G. tinctorius
G. daphne
G. tasmaniensis
M. festiva
H. bennetti

Table 1.

	Species	Type	Crude mg/ml
Red-spot mimicry species	*Goniobranchus splendidus*	A, B, C, D	32.4
	Goniobranchus tinctorius	A, B	19.9
	Goniobranchus daphne	B, C	12.3
	Goniobranchus hunterae	B	35.0
	Mexichromis mariei	E	15.3
	Mexichromis festiva	E	17.8 (gcbs) 29.2 (nbps)
	Hypselodoris bennetti	E	15.2
	Veronica haliclona	NA	NA
Partial red-spot species	*Goniobranchus verrieri*	B, C	19.3
	Goniobranchus albonares	NA	NA
	Goniobranchus tasmaniensis	A, B	37.6
	Chromodorididae *thompsoni*	B	19.1