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# Toxicity and taste: unequal chemical defences in a mimicry ring

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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 towards Palaemon shrimp remained relatively constant between species, toxicity levels towards brine shrimp 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.

## 1. 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 effective deterrent than distastefulness. However, distasteful compounds that are moderately toxic may also protect prey populations more effectively than highly toxic compounds [29].

To investigate the relationship between distastefulness and toxicity in mimicry systems, we investigated a putative red spot mimicry ring of nudibranch molluscs that co-occur along the east coast of Australia [30,31]. Many species of nudibranchs display vibrant warning colours to indicate that they contain defensive secondary metabolites that are sequestered, transformed from dietary sources, or synthesized *de novo* [32]. We have previously shown that one member of the putative red spot mimicry group, *Goniobranchus splendidus*, contains distasteful compounds to marine organisms and displays conspicuous colours patterns, components of which are learnt readily by reef fish predators [33]. We first examined the similarity of colour patterns in this group to a potential fish predator using spectral reflectance measurements, visual modelling, and pattern geometry analysis. Second, we conducted phylogenetic analysis to investigate shared ancestry of species. Third, we identified and quantified defensive metabolites present in each species and examined the strength of chemical defences using anti-feedant and toxicity assays with shrimp.

## 2. Methods

### (a) Study species

Nudibranch species ($n = 24$) were collected between 2012 and 2016 by hand from sites in Queensland (QLD) and New South Wales (NSW) (electronic supplementary material, table S1) either using SCUBA at depths ranging from 5–18 m, or from intertidal zones. Based on a previous paper, in which species that had red spots or patches on a (usually) white mantle were arbitrarily assigned to a 'red spot group' [30], we also identified species that exhibited a similar red spotted or red reticulate colour pattern and/or a distinctive yellow/orange mantle border, and named this the 'red spot mimicry group' (figure 1*a*, A–H). Eight species of nudibranch were assigned *a priori* to a red spot mimicry group: *Goniobranchus splendidus* (Angas, 1864) ($n = 22$), *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*b*, I–L). These 12 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) ($n = 3$), *H. 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 (electronic supplementary material, 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.), genetic sequencing of cytochrome *c* oxidase I (COI) and 16S rDNA, and comparison with sequences deposited in the GenBank database. All nudibranch specimens were then frozen and stored at −20°C until chemical extraction of chemical defences. Nudibranchs were collected under Queensland General Fisheries Permits no. 161624 and no. 183990, and NSW Scientific Collection Permits F86/2163-7.0 and P16/0052-1.0.

### (b) 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 *et al.* [34]. Details of all species used in the phylogenetic analysis are available in electronic supplementary material, table S2. All available COI and 16S data for the Chromodorididae was downloaded from GenBank (137 species) and added to newly generated data from this study (eight species; electronic supplementary material, table S2). Only species that were represented by both genes from the same individual were used. This resulted in a dataset with 145 species, representing an estimated 40% taxon completeness for the family (www.marinespecies.org). Data were aligned using the MAFFT v.7.222 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 bootstrap 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.

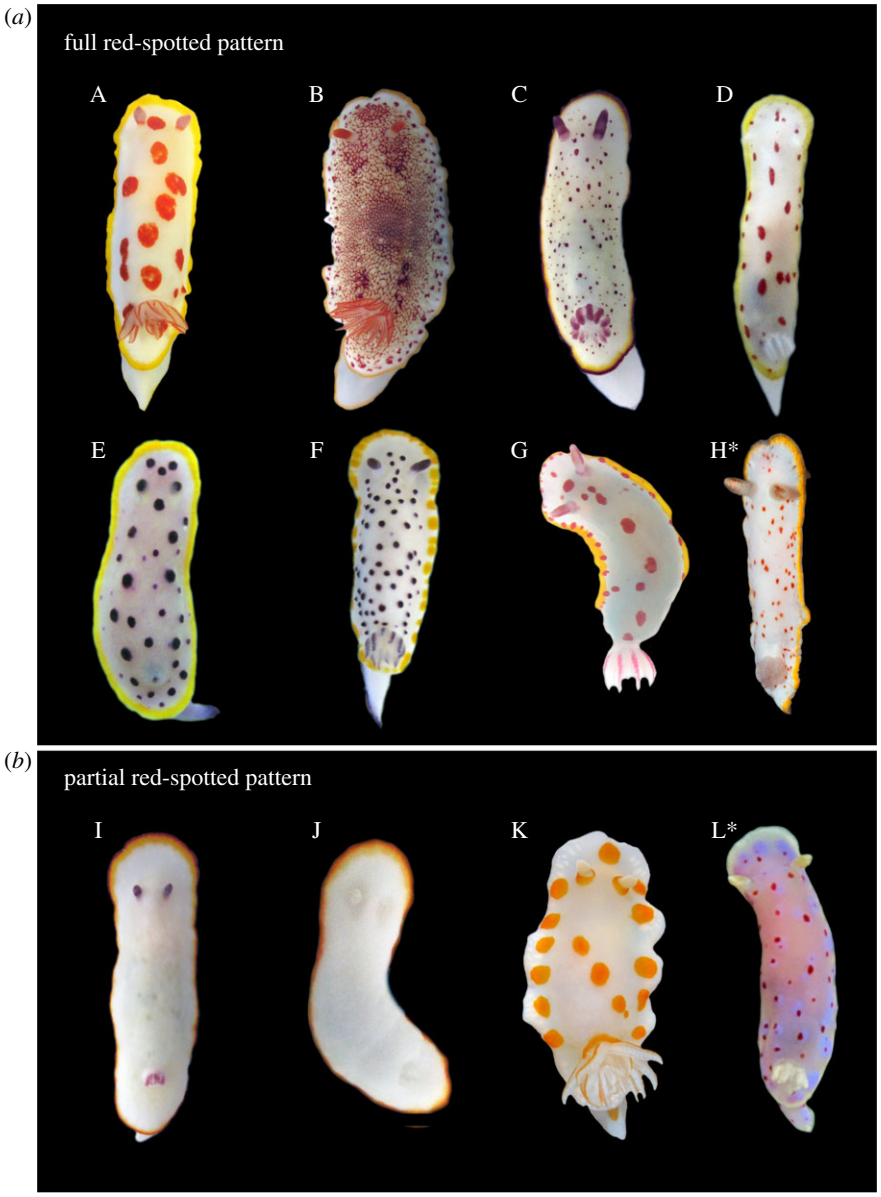


**Figure 1.** Representative photographs of the putative mimicry species investigated in this study. (*a*) Full pattern including yellow–orange mantle border, white mantle, and spots. (*b*) Partial pattern missing either spots or border. From upper left: *Goniobranchus splendidus* (A), *Goniobranchus tinctorius* (B), *Goniobranchus daphne* (C), *Goniobranchus hunterae* (D), *Mexichromis mariei* (E), *Mexichromis festiva* (F), *Hypselodoris bennetti* (G), *Verconia haliclona* (H), *Goniobranchus verrieri* (I), *Goniobranchus albonares* (J), *Goniobranchus tasmaniensis* (K), Chromodorididae *thompsoni* (L). *indicates species that were not included in the colour pattern analysis.

## (c) Spectral reflectance measurements

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

## (d) Colour and pattern analysis

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

Photon capture ($q$) generated by each given colour pattern element for each photoreceptor ($i$) 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 30 cm). 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 using the combined photon capture of the double cones, 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 nudibranchs 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 were calculated: (i) aspect ratio, (ii) colour diversity, and (iii) pattern complexity [48]. 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 evenly colours are spatially represented in the pattern. High values indicate that the relative areas of each colour class are closer to being equal; diversity was calculated by the inverse Simpson index, which yields the number of equally common (area) colours. Pattern complexity was calculated as the density of colour transitions; patterns with a greater number of pixels adjacent to a different colour class will have a higher complexity score.

## (e) Non-metric multidimensional scaling analysis

Species were differentiated in two-dimensional space using 14 characters of colour and pattern analysis by performing a non-metric multidimensional scaling (NMDS) analysis based on a Euclidean distance matrix with the metaMDS function in the vegan package [49] of R v. 3.2.2 [50]. Characters were overall pattern (plain = 1, reticulate = 2, spotted = 3 or striped = 4); chromatically contrasting rim (absent = 0, present = 1); hue, chroma, luminance of internal pattern, background colour and rim; and our three pattern geometry statistics (aspect ratio, colour diversity, pattern complexity). If there was more than one pattern present on the species, then the dominant pattern as defined by three authors was used and is stated in electronic supplementary material, table S3. If internal patterns or rims were not present on a particular species, then values calculated for background colour were used.

## (f) Chemical extraction and identification

To investigate the identity and strength of chemical defences for each species, the whole body tissue of specimens was extracted as per [51]. All extracts were dissolved in deuterated chloroform for $^1$H NMR analysis on a Bruker AV-500 spectrometer at 500 MHz. If necessary for identification of nudibranch metabolites, a small portion of the extract was analysed using low-resolution electrospray ionization mass spectrometry (LRESIMS) on a 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$^{-1}$) of extract for use in toxicity and palatability assays.

## (g) 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 12 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 [52,53]. 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 microfibre 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 onto 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 20 actively swimming instar I nauplii (less than 12 h after hatching) were collected with a glass pipette and added to each Petri dish with 5 ml filtered seawater. Lids were placed on top of the Petri dishes and kept under constant illumination for 24 h. Surviving nauplii (instar II/III) were then counted; nauplii were considered dead if no movement was detected after several seconds of observation. Natural mortality was controlled for using control treatments in which 0.5 ml of DCM was added to the filter paper. In all cases control deaths occurred, therefore the data were corrected using Abbott's formula % deaths = (test − control)/(100 − control) for analysis [54]. We then calculated the $LD_{50}$ of the crude extract for each nudibranch species by interpolating a line or standard curve, chosen based on $R^2$ values. $LD_{50}$ values were calculated for species with extracts that induced a response to at least 50% of the brine shrimp. $LD_{50}$ values are interpolated $x$ values (ml stock solution), where 1 ml of extract = 1 ml of tissue, and therefore reflect natural volumetric concentrations. Absolute concentrations of compounds tested are shown in electronic supplementary material, figures S2 and S3.

## (h) Anti-feedant assay

To assess the relative distastefulness, and thus feeding deterrence of nudibranch extracts, antifeedant assays were performed using the generalist rock-pool prawn (*Palaemon serenus*) between November 2013 and September 2015 as per [51,55,56]. This species has a clear carapace and digestive tract, which makes it ideal for feeding observations and preliminary studies have shown that compounds distasteful to marine fish *Tetractenos hamiltoni* and *Rhinecanthus aculeatus* are also distasteful to rock-pool shrimp [53]. Individuals were collected intertidally in SE Queensland on foot using hand nets and housed in aquaria with ample food (Ocean Nutrition, Formula 2) until used in assays. Artificial food pellets were created to approximate the nutritional content of a nudibranch with roughly 90% water, 7% squid + alginate, and 3% sand following the protocol outlined in [51,57]. Crude extracts were added in several concentrations up to that which they were found occurring naturally for each species by adding the crude stock solution or DCM without extract (control 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 min under a Nederman arm, and then the mixture was reconstituted in distilled water to make a final pellet volume of

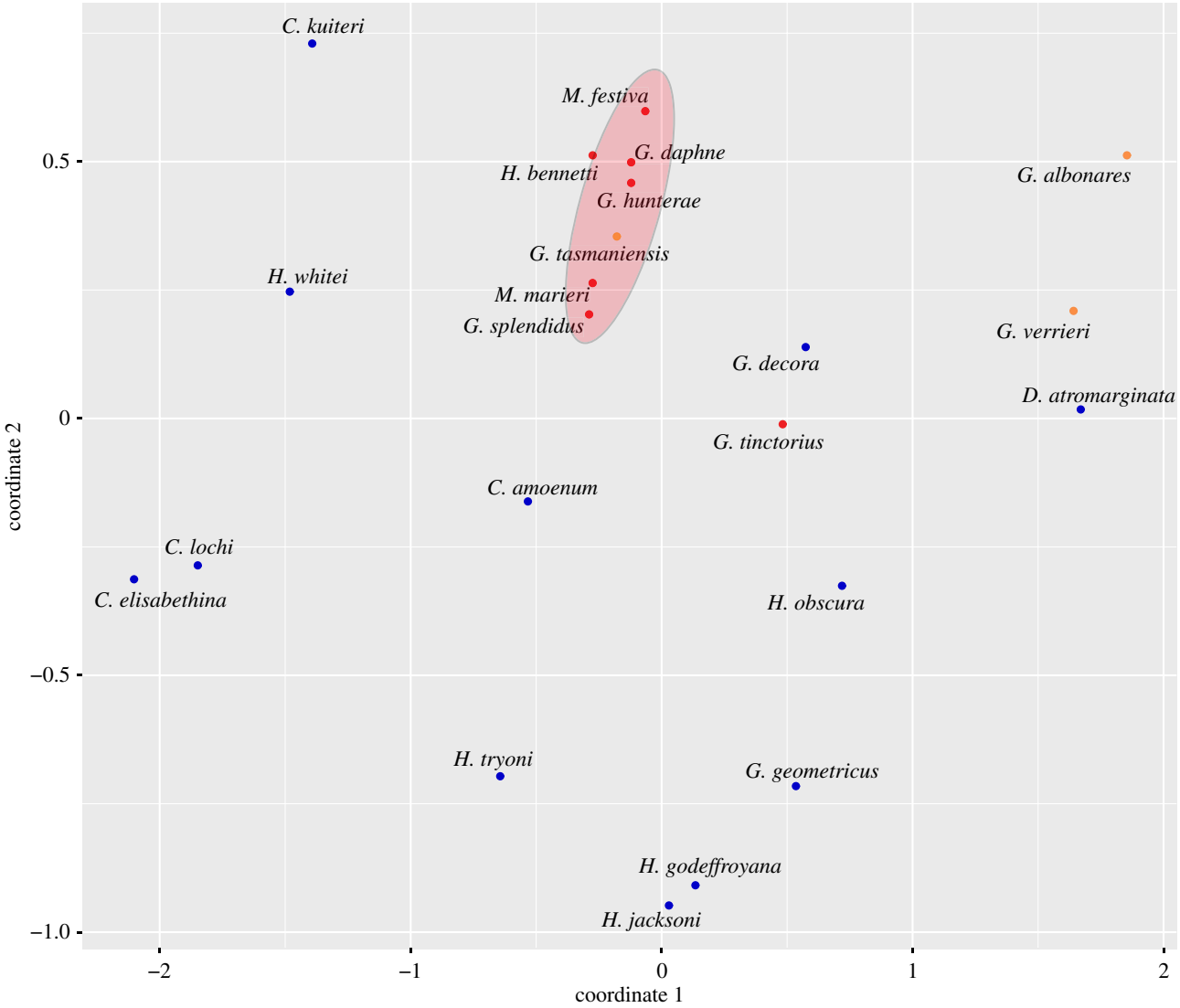


**Figure 2.** Nudibranch colour patterns differentiated in ordinal space (NMDS) based on 14 metrics of the hue, chroma and luminance of colour pattern element and overall nudibranch pattern geometry. The *a priori* predicted red spotted group is shown in red. Partial red spotted pattern species are shown in orange and non-red spot group are shown in blue. The red ellipse shows the clustering of many red spotted species.

0.5 ml. Shrimps were selected randomly and placed individually in small compartments (135 × 98 × 90 mm) 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 min. 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 v. 3.2.2 [50].

## 3. Results

### (a) Colour and pattern analysis

Data for colour and pattern parameters are reported in electronic supplementary material, 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.

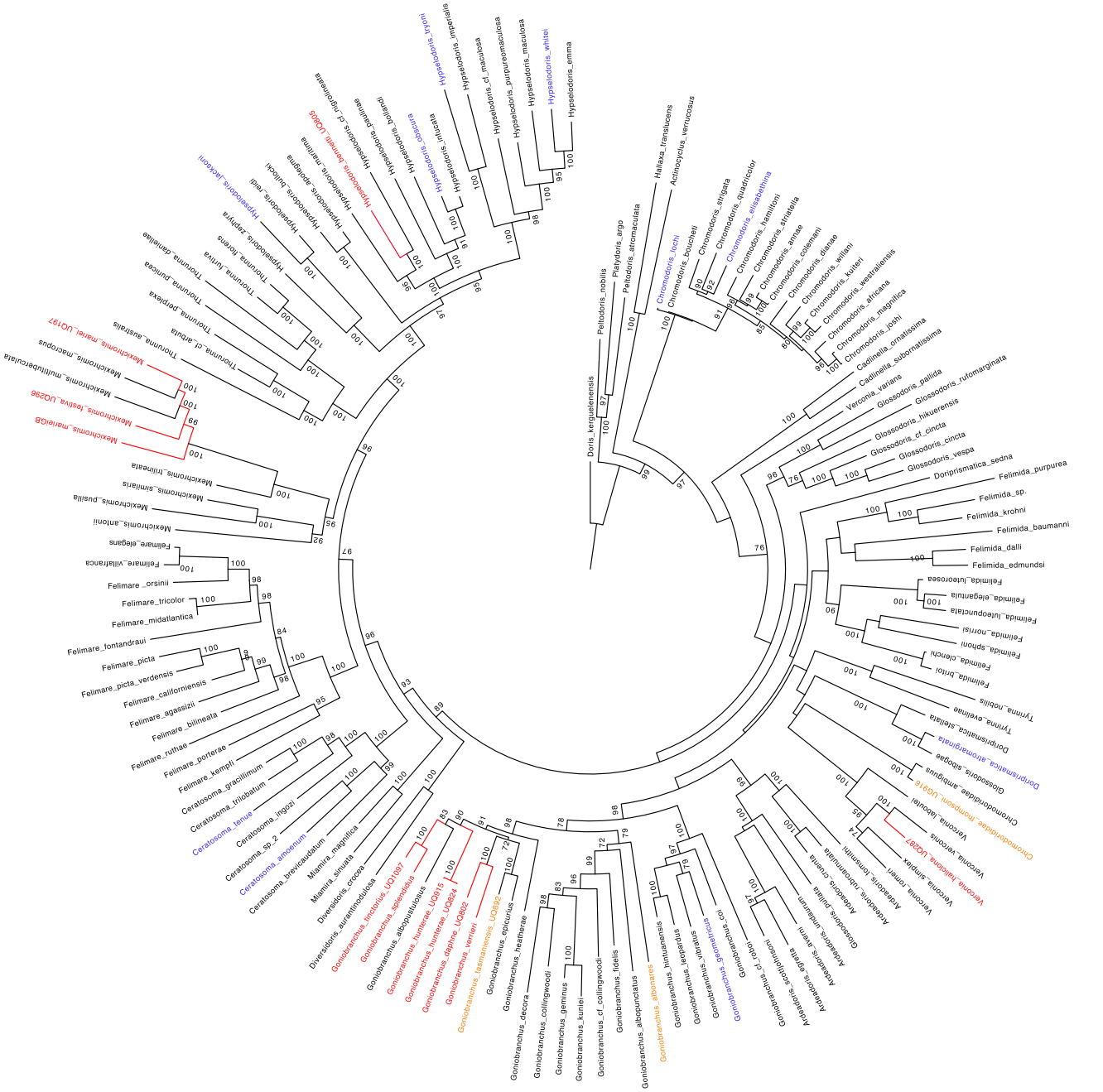


**Figure 3.** Maximum-likelihood topology of Chromodorididae taxa. Species that were assigned to a red spotted group are shown in red, the partial red spot group in orange, and those not assigned to the non-red spot group in blue. Bootstrap values are shown for clades with over 70% support. Ancestral state reconstruction of the red spotted colour pattern was performed using ML analysis and marginal probability reconstruction with model Mk1 (rate 0.24 log likelihood, −54.77).

## (b) 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 indicate 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.

## (c) Chemical identification

Nudibranch species from the red spot mimicry group contained different compounds (table 1). Species from the genus *Goniobranchus* possessed spongian diterpenes, rearranged diterpenes, and norditerpenes as per [60], and there were significant differences in chemical profiles between species. *Hypselodoris* and *Mexichromis* species possessed furanosesquiterpenes (table 1), and the extracts of *M. festiva* from Nelson Bay and the Gold Coast possessed the same compounds. Compound names and structures are listed in electronic supplementary material, table S4.

## (d) Toxicity and palatability assays

Red spot species differed both in terms of toxicity and distastefulness (figure 4). Species with extracts that were toxic to brine shrimp included *G. tasmaniensis*, *H. bennetti*, and *M. festiva* (figure 4*a*). A dose response was also observed for the extract of *G. daphne*, but this response did not reach above 50% mortality, and no dose response was observed for *G. tinctorius* or *G. splendidus*. All extracts produced a

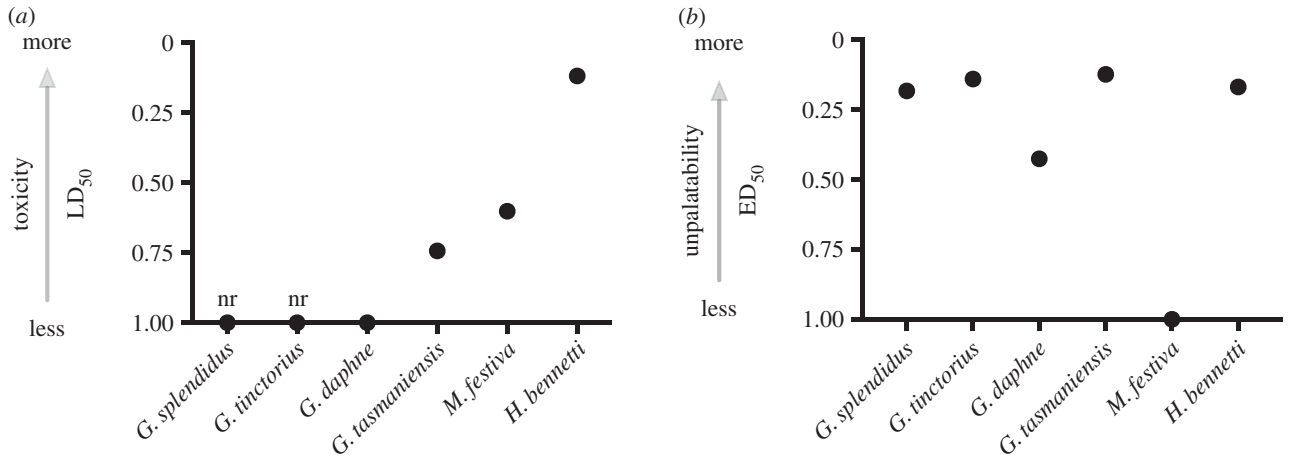


**Figure 4.** (*a*) Toxicity assay: $LD_{50}$ values based on mortality of brine shrimp, *Artemia* sp. (*b*) Anti-feedant assay. $ED_{50}$ values based on rejection of pellets by Palaemon shrimp, *Palaemon serenus*. Values are represented as proportion of natural concentration found in the mantle of the nudibranchs. Circles indicate $LD_{50}$ values calculated from the data, nr indicates no response at the highest concentration tested. Absolute concentrations are shown in electronic supplementary material, figure S2.

**Table 1.** Summary table of secondary metabolites indicating the chemical type (A, spongian diterpenes; B, rearranged diterpenes; C, norditerpenes; D, furanone diterpenes; E, furanosesquiterpenes) and concentration of crude chemical extracts (mg $ml^{-1}$) for each species. gcbs refers to the extract from Gold Coast individuals used in the Brine shrimp assay; nbps refers to the extract from Nelson Bay individuals used in the Palaemon shrimp assay.

| | species | type | crude mg $ml^{-1}$ |
|---|---|---|---|
| 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* | n.a. | n.a. |
| partial red spot species | *Goniobranchus verrieri* | B, C | 19.3 |
| | *Goniobranchus albonares* | n.a. | n.a. |
| | *Goniobranchus tasmaniensis* | A, B | 37.6 |
| | Chromodorididae *thompsoni* | B | 19.1 |

dose response to the shrimp *Palaemon serenus*, though this response did not reach above 50% for the extract of *M. festiva* (figure 4*b*). Importantly, using the phylogenetic generalized least square (GLS) regression model, we did not find an association between toxicity and distastefulness ($t_6 = 0.89$, $p = 0.42$; electronic supplementary material, figure S3).

## 4. Discussion

This study presents quantitative evidence of visual similarities between species in a putative mimicry group using colour and pattern analysis, and demonstrates that shared pattern elements of these co-occurring species are distinct from other, closely related species. Phylogenetic analysis indicates that this red spot pattern evolved at multiple times, suggesting this pattern has resulted from convergent evolution rather than shared ancestry. Members of the mimicry group possess different chemical profiles used for defensive purposes, and these suites of compounds provide unequal levels of defence in terms of a toxic response. However, the level of distastefulness of these compounds appears to be relatively similar to a marine shrimp. These data therefore do not support the assumption that distasteful compounds honestly signal levels of toxicity, at least in this mimicry system, and in many systems, toxicity may not be related to distastefulness [25]. This study should encourage researchers to disentangle terms such as toxicity and distastefulness as modes of chemical defences when investigating aposematic and mimicry systems.

Many theoretical models of mimicry rings with unequal defences exist (e.g. [13,53,57–63]). Weakly defended co-mimics may degrade the warning signal of the model [15,64]; for instance, in an experiment using birds, an increase

in abundance of a moderately defended artificial prey increased *per capita* predation on both the mimic and the highly defended model prey when population densities were low [15]. However, the relationship between species with comparably weak defences and that of their co-mimics remains unclear. In some studies, unequal defences still appear to be mutualistic [14,65]. For example, highly defended models coupled with moderately defended mimics can have a decrease in *per capita* mortality when population densities are high [14].

However, the mode of chemical defence is often not defined in such models, and unequal defences in mimicry systems are sometimes only discussed in terms of quantity (but see [29]). Prey that store distasteful, but otherwise non-toxic compounds that would not damage or incur costs on the host, may repel predators due to their unpleasant nature. Predators may quickly learn they are not harmed after consuming such prey and may still consume distasteful prey when other food is scarce and predators are hungry [61,62]. If compounds are equally distasteful, we propose that predators may be unable to discriminate levels of toxicity between species. Therefore, non-toxic but distasteful species may benefit from resembling their toxic counterparts, but not incur costs involved in harbouring toxins. It is also possible that species may mimic the taste of toxic compounds with those that are non-toxic [63].

Our study species had very different chemical profiles: *Hypselodoris* and *Mexichromis* nudibranchs contained furanosesquiterpenes while *Goniobranchus* nudibranchs and Chromodorididae *thompsoni* contained spongian diterpenes, norditerpenes, 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. We also did not test for an emetic response, which has been shown before using nudibranch compounds [56]. 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. For example, extracts from two-spot ladybirds (*Adalia bipunctata*) were toxic to *Daphnia pulex* [64], but nestling blue tits (*Parus caeruleus*) that were fed two-spot ladybirds over a few days did not appear to suffer any adverse effects, such as increased mortality, decreased weight gain or liver damage [65]. However, there are considerable ethical implications of conducting toxicity assays with vertebrates [66].

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 [12,67]. 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 palatable prey types are discriminated based on certain components of the visual signal [68], with relaxed selection on other components of the visual signal that are generalized [69]. Indeed, we have recently shown that when learning a red spot/yellow rim colour pattern, triggerfish paid most attention to the yellow border when learning to avoid distasteful food, and disregarded the internal red pattern. We also found that the yellow rim was a more consistent part of the visual signal in populations of *Goniobranchus splendidus*, although there was considerable variation in the red spot component [33]. Highly contrasting body outlines may help nudibranchs to stand out against their background and increase conspicuousness, which is an important characteristic of warning signal designs [5]. However, this does not explain the lack of mantle border in five species in this study.

We believe that this is the first study of an aposematic mimicry ring to include detailed chemical profiles and to assess both the toxicity and distastefulness of contributing species. We have demonstrated that there may not be a correlation between toxicity and distastefulness, and therefore highlight the importance of testing multiple modes of defence to inform future models of mimicry systems. It is likely that warning signal designs and chemical profiles vary geographically [53]; therefore, the impact of geographical differences in dietary resources and predation pressure on warning signal design, chemical profiles, and anti-predator activity of co-mimics would be an interesting direction for future research.

Ethics. Research on the animals used in this study does not require ethical approval.

Data accessibility. Additional data are provided in the electronic supplementary material.

Authors' contributions. A.E.W. participated in fieldwork, laboratory work, data analyses, design of the study, and drafted the manuscript; N.G.W. participated the conception of the study, fieldwork, laboratory work, data analyses, and drafting the manuscript. C.P.v.d.B. participated in data analyses, M.J.H. participated in data analyses and drafting the manuscript, J.A.E. participated in data analyses, N.J.M. advised on data analyses, A.M.W. conducted laboratory work and identified metabolites. M.J.G. advised on laboratory work, assisted with metabolite identification and participated in drafting the manuscript. K.L.C. conceived of, coordinated, and designed the study, participated in fieldwork, laboratory work, data analyses, and drafting the manuscript. All authors provided comments on final version and gave approval for publication.

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