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Phylogenetic comparative analysis supports aposematic colouration–body size association in millipede assassins (*Hemiptera: Reduviidae: Ectrichodiinae*)

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Abstract

The diversity of colour patterns and its importance in interactions with the environment make colouration in animals an intriguing research focus. Aposematic colouration is positively correlated with body size in certain groups of animals, suggesting that warning colours are more effective or that crypsis is harder to achieve in larger animals. Surprisingly, this relationship has not been recovered in studies investigating insects, which may have been confounded by a focus on aposematic taxa that are also gregarious. Millipede assassin bugs (Hemiptera: Reduviidae: Ectrichodiinae) comprise species with cryptic and aposematic colour patterns across a range of body sizes, are typically solitary as adults and are thus an excellent model for investigating a possible association between colouration and body size. Here, we use a comprehensive phylogeny for Ectrichodiinae, ancestral state reconstruction of colouration, and phylogenetic comparative methods to test for a colouration–body size association. The ancestor of Ectrichodiinae is reconstructed as cryptically coloured, with multiple subsequent transitions between aposematic and cryptic colouration. Aposematic colouration is positively associated with male body length and supports the hypothesis that selection on Ectrichodiinae body size may influence evolutionary transitions between aposematic and cryptic colouration or alternatively that selection for aposematic colouration influences body size evolution.

Introduction

Scientists have long worked towards understanding the evolution and various functions of the astounding diversity of colour patterns in animals, which are involved in environmental (e.g. thermoregulation), intraspecific (e.g. sexual selection) and interspecific interactions (e.g. predator evasion; Areekul & Quicke, 2006; Protas & Patel, 2008; Endler & Mappes, 2017). Cryptic colouration allows individuals to blend into the environmental background. In contrast, some structurally or chemically defended species have evolved conspicuous colours that function as warning, or aposematic, signals to deter potential predators (Poulton, 1890; Edmunds, 1987) and to enhance predator learning and memorization (see Ruxton *et al.*, 2004; Exnerová *et al.*, 2006). Such colour patterns may also afford protection against predators in defenseless species because they are perceived as aposematic due to Batesian mimicry or innate or learned predator aversion (Bates, 1862; Smith, 1975; Guilford, 1988; Nilsson & Forsman, 2003). Traditionally, aposematic colouration has been thought of as a combination of red, orange, yellow or white with black (Cott, 1940; Fabricant *et al.*, 2014), but some studies suggest that metallic or iridescent patterns may also function as aposematic signals alone or by enhancing 'traditional' aposematic colours (e.g. Arrow, 1951; Schultz, 2001; Fabricant *et al.*, 2014; Pegram & Rutowski, 2014).

Empirical studies have shown that large aposematic signals are more effective than smaller signals, with the size increase being the result of larger body size (e.g. Gamberale & Tullberg, 1996a; Rudh, 2013), larger

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pattern elements (e.g. Forsman & Merilaita, 1999; Lindström *et al.*, 1999) or gregariousness (e.g. Gamberale & Tullberg, 1996b, 1998; Finkbeiner *et al.*, 2012). This suggests that aposematically coloured species may be subject to selection for any of these three features (Forsman & Merilaita, 1999). Most studies have investigated this hypothesis in a microevolutionary context (Härlin & Härlin, 2003; Stevens, 2015). However, it has been suggested that aposematic colouration may evolve more easily in species with large body size (Hagman & Forsman, 2003), predicting an association of cryptic to aposematic colour transitions with body size increases at macroevolutionary scales. This hypothesis can be tested using phylogenetic comparative approaches, but surprisingly few studies focusing on the association of colouration and body size or gregariousness have taken advantage of these approaches.

Among these, studies on poison dart frogs (Anura: Dendrobatidae) (Hagman & Forsman, 2003) and butterflies and moths (Insecta: Lepidoptera) (Tullberg & Hunter, 1996) have found positive relationships between aposematic colouration and body size or gregariousness, respectively. In contrast, a study on marine nudibranchs found that the prevalence of aposematic colouration decreases as body size increases, possibly driven by the marine habitat, habitat homogeneity or diet (Cheney *et al.*, 2014). A comparative study of 578 moth species also failed to detect an association between larval body size and colouration, leaving the authors to posit that either lifestyle (solitary vs. gregarious) or conflicting selection on larvae and adults may have confounded this association (Nilsson & Forsman, 2003). Given the mixed evidence for a positive colouration–body size association in phylogenetic comparative studies, the critical need to test this hypothesis using additional and possibly less complex model systems is evident. Speciose clades of predominantly solitary and hemimetabolous insects may provide such models, but have been underutilized for the investigation of colouration–body size associations. We propose that the millipede assassin bugs (Heteroptera: Reduviidae: Ectrichodiinae) may be such a clade.

The predominantly circumtropical, leaf-litter inhabiting millipede assassin bugs are likely the largest group of specialized millipede predators with 886 species in 137 genera (see Maldonado, 1990, 1996; Weirauch, 2010; Rédei *et al.*, 2012; Ishikawa *et al.*, 2015; Davranoglou, 2016; Forthman *et al.*, 2016; Forthman & Weirauch, 2017; Weirauch *et al.*, 2017). While certain species are cryptically coloured, many display striking red, yellow, orange, white, black and/or metallic patterns (Fig. 1). Although not experimentally verified in millipede assassin bugs, these conspicuous colours (which we refer to as aposematic colouration in the following) may function as aposematic signals given that ectrichodiines possess piercing mouthparts used for defensive behaviours in assassin bugs (Walker *et al.*, 2016) and two sets of defense glands (Weirauch, 2008). Ectrichodiinae also feed on chemically defended millipedes (Forthman & Weirauch, 2012), and we speculate that millipede assassin bugs might sequester compounds from prey for their own defense, a hypothesis that remains to be tested.

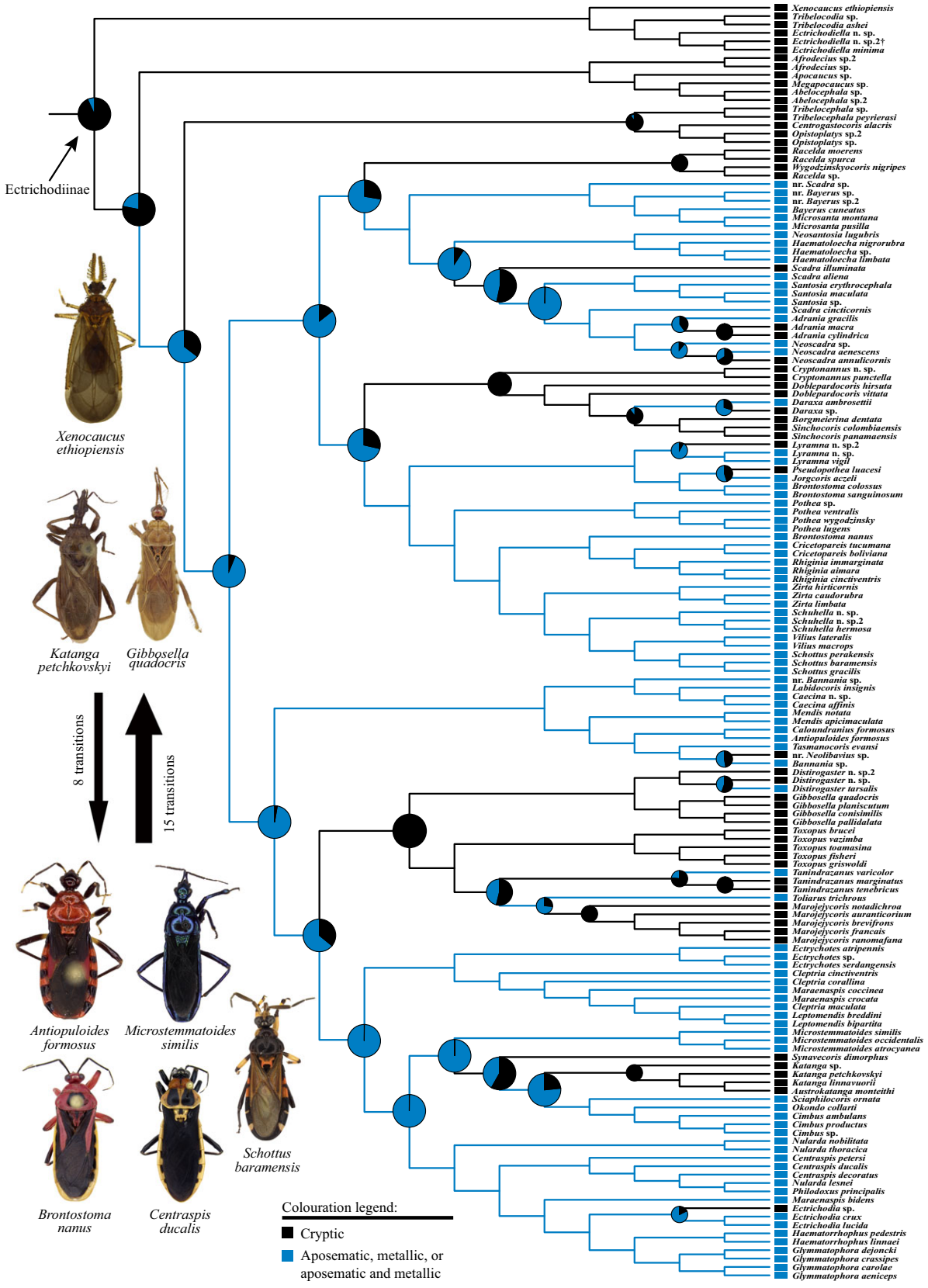
Some of the smallest and largest Ectrichodiinae species are either cryptically [e.g. *Ectrichodiella* Fracker & Bruner, 1924 (3–5 mm); *Xenorhyncocoris* Miller, 1938 (32–37 mm)] or aposematically coloured [e.g. *Schuhella* Dougherty, 1995 (6 mm); *Centraspis* Schaum, 1862 (25–40 mm)], suggesting that colouration and body size may not be correlated in this clade. Although communal predation has been documented for immatures in some species, millipede assassin bugs are typically solitary (see Forthman & Weirauch, 2012; pers. obs.). Furthermore, colouration in immatures and adults is similar, making this group a model system less prone to confounding effects from gregariousness or differing selection on immatures and adults. Given the size of the group, diversity of colour forms across a range of body sizes, and an available phylogenetic framework, millipede assassin bugs present an opportunity to explore, for the first time, the evolution of colouration and its association with body length in a group of non-gregarious insects. We use a recently published, comprehensive phylogeny of the Ectrichodiinae to investigate the evolution of colouration with ancestral state reconstruction (ASR) methods. A phylogenetic comparative analysis is subsequently conducted to test for a relationship between aposematic colouration and body length.

Materials and methods

Ectrichodiinae phylogenetic hypothesis

To investigate the evolution of colouration, we used the preferred phylogenetic hypothesis from Forthman & Weirauch (2017), that is the maximum likelihood (ML) best tree of the combined morphological and molecular dataset of 152 taxa of Ectrichodiinae. The taxa included in the total evidence dataset exhibit all major colour patterns across differently sized Ectrichodiinae species, making it ideal for our purposes.

Fig. 1 BayesTraits Multistate maximum likelihood (ML) ancestral state reconstruction of colour character 1 on the ML phylogram (tree converted to cladogram for visual). Terminal taxa coded as cryptic (black) or either aposematic, metallic, or aposematic and metallic (blue). Pie charts at select nodes show probability values and are used to visualize transitions between states; branches are coloured to reflect the most probable colour state at that branch.


Ectrichodiinae
Xenocaucus ethiopiensis
Katanga petchkovskyi
Gibbosella quadocris
8 transitions
15 transitions
Antiopuloides formosus
Microstemmatoides similis
Schottus baramensis
Brontostoma nanus
Centraspis ducalis
Colouration legend:
Cryptic
Aposematic, metallic, or aposematic and metallic
Xenocaucus ethiopiensis
Tribelocodia sp.
Tribelocodia ashei
Ectrichodiella n. sp.
Ectrichodiella n. sp.2†
Ectrichodiella minima
Afrodecius sp.2
Afrodecius sp.
Apocaucus sp.
Megapocaucus sp.
Abelocephala sp.
Abelocephala sp.2
Tribelocephala sp.
Tribelocephala peyrierasi
Centrogastocoris alacris
Opistoplatys sp.2
Opistoplatys sp.
Racelda moerens
Racelda spurca
Wygodzinskyocoris nigripes
Racelda sp.
nr. Scadra sp.
nr. Bayerus sp.
nr. Bayerus sp.2
Bayerus cuneatus
Microsanta montana
Microsanta pusilla
Neosantosia lugubris
Haematoloecha nigrorubra
Haematoloecha sp.
Haematoloecha limbata
Scadra illuminata
Scadra aliena
Santosia erythrocephala
Santosia maculata
Santosia sp.
Scadra cinciticornis
Adrania gracilis
Adrania macra
Adrania cylindrica
Neoscadra sp.
Neoscadra aenescens
Neoscadra annulicornis
Cryptonannus n. sp.
Cryptonannus punctella
Doblepardocoris hirsuta
Doblepardocoris vittata
Daraxa ambrosettii
Daraxa sp.
Borgmeierina dentata
Sinchocoris colombiaensis
Sinchocoris panamaensis
Lyramna n. sp.2
Lyramna n. sp.
Lyramna vigil
Pseudopothea luacesi
Jorgecoris aczeli
Brontostoma colossus
Brontostoma sanguinosum
Pothea sp.
Pothea ventralis
Pothea wygodzinsky
Pothea lugens
Brontostoma nanus
Cricetopareis tucumana
Cricetopareis boliviana
Rhiginia immarginata
Rhiginia aimara
Rhiginia cinctiventris
Zirta hirticornis
Zirta caudorubra
Zirta limbata
Schuhella n. sp.
Schuhella n. sp.2
Schuhella hermosa
Vilius lateralis
Vilius macrops
Schottus perakensis
Schottus baramensis
Schottus gracilis
nr. Bannania sp.
Labidocoris insignis
Caecina n. sp.
Caecina affinis
Mendis notata
Mendis apicimaculata
Caloundranius formosus
Antiopuloides formosus
Tasmanocoris evansi
nr. Neolibavius sp.
Bannania sp.
Distirogaster n. sp.2
Distirogaster n. sp.
Distirogaster tarsalis
Gibbosella quadocris
Gibbosella planiscutum
Gibbosella conisimilis
Gibbosella pallidalata
Toxopus brucei
Toxopus vuzimba
Toxopus toamasina
Toxopus fisheri
Toxopus griswoldi
Tanindrazanus varicolor
Tanindrazanus marginatus
Tanindrazanus tenebricus
Toliarus trichrous
Marojejycoris notadichroa
Marojejycoris auranticorium
Marojejycoris brevifrons
Marojejycoris francais
Marojejycoris ranomafana
Ectrychotes atripennis
Ectrychotes sp.
Ectrychotes serdangensis
Cleptria cinctiventris
Cleptria corallina
Maraenaspis coccinea
Maraenaspis crocata
Cleptria maculata
Leptomendis breddini
Leptomendis bipartita
Microstemmatoides similis
Microstemmatoides occidentalis
Microstemmatoides atrocyanea
Synavocoris dimorphus
Katanga sp.
Katanga petchkovskyi
Katanga linnavuorii
Austrokatanga monteithi
Sciaphilocoris ornata
Okondo collarti
Cimbus ambulans
Cimbus productus
Cimbus sp.
Nularda nobilitata
Nularda thoracica
Centraspis petersi
Centraspis ducalis
Centraspis decoratus
Nularda lesnei
Philodoxus principalis
Maraenaspis bidens
Ectrichodia sp.
Ectrichodia crux
Ectrichodia lucida
Haematorrhophus pedestris
Haematorrhophus linnaei
Glymmatophora dejoncki
Glymmatophora crassipes
Glymmatophora carolae
Glymmatophora aeniceps

Ancestral state reconstructions

The use of either ultrametric trees or phylograms in ASRs has been controversial (e.g. Litsios & Salamin, 2012; Cusimano & Renner, 2014). The assumption behind ultrametric trees is that phenotypic change is related to the amount of time along a branch and that the rate of mutation is the same across all lineages (Bromham *et al.*, 2002; Litsios & Salamin, 2012; Cusimano & Renner, 2014). Correlations between molecular and phenotypic rates of evolution have been demonstrated in some studies (e.g. Omland, 1997; Smith & Donoghue, 2008), questioning the accuracy of ASRs on ultrametric trees. However, there is currently no consensus regarding the greater accuracy of ultrametric trees versus phylograms for ASRs (Litsios & Salamin, 2012; Cusimano & Renner, 2014). To investigate whether tree choice matters for ASR of colour characters in Ectrichodiinae, we estimated ancestral states on the ML phylogram and three ultrametric trees that were generated by Forthman & Weirauch (2017) using penalized likelihood (Sanderson, 2002, 2003) and different penalties corresponding to parametric estimation, penalty on rate change, and a global molecular clock (see Forthman & Weirauch, 2017; for details), following Litsios & Salamin (2012). While eight branches with very short, nonzero branch lengths were collapsed in the ultrametric trees, we used these trees as most collapsed branches were near the terminals and within clades in which terminals had the same state.

Colouration was treated as a categorical character, and states were assigned based on observations by five independent observers: cryptic, traditional aposematic (i.e. metallic appearances excluded), uniform metallic and aposematic and metallic. For each of the 152 species, each observer was presented with at least one insect specimen (same specimens used for each observer) and, when available, images of individuals in natural habitat (16 species) (Table S1, See Dryad). In four cases where a majority opinion was not achieved, ties were broken by the first author's assignments made prior to surveying observers. In our first ASR dataset, colouration was coded as (0) cryptic or (1) aposematic, uniform metallic, or aposematic and metallic (colouration character 1). As some studies suggest uniform metallic appearances may serve a cryptic function in other taxa (e.g. Parker, 1998; Thomas *et al.*, 2007), we alternatively coded a second colouration dataset (colouration character 2) as (0) cryptic or uniform metallic or (1) aposematic or aposematic and metallic. Colouration character data for both data sets are provided in Appendix S1 (See Dryad).

We performed parsimony ASR on the phylogram in Mesquite v3.04 (Maddison & Maddison, 2015) and ML and Markov Chain Monte Carlo (MCMC) reconstructions on all trees with BayesTraits v2 MultiState (Pagel *et al.*, 2004). For the ML approach, 1000 iterations were conducted under free or equal rate parameters and compared with a likelihood ratio test (LTR); we report ASRs based on restricted rates ($P > 0.09$, d.f. = 1). Under the MCMC framework, we used a hyperprior on an exponential distribution drawn from a uniform distribution (interval between 0 and 10). Twenty million generations were conducted, with every 2000 generations sampled under a free rates and equal rates model. The first 25% of the generations were discarded as burn-in, and acceptance rates were between 0.2–0.4. Tracer v1.6 (Rambaut *et al.*, 2014) was used to obtain the mean state value for each node, with ESS values > 200 for all nodes reconstructed. Bayes factors (BF) were calculated to compare the free and equal rate models; we report ASRs based on restricted rates (BF < 2).

Phylogenetic comparative analyses

To examine the relationship between a binary dependent trait [i.e. colouration (each colouration dataset analysed separately)] and a continuous independent trait (i.e. body length) in a phylogenetic context, phylogenetic logistic regression (PLR) with Firth correction was implemented using PLogReg.m (Ives & Garland, 2010) in MATLAB R2015b. This approach uses a two-state Markov process to model the evolution of the dependent trait along a phylogeny and to estimate the strength of phylogenetic signal (Ives & Garland, 2010). Subsequently, the independent trait influences the dependent trait, with the evolutionary rate no longer dependent on phylogenetic signal but on the independent trait's regression coefficient (Ives & Garland, 2010). While the PLR approach standardizes tip-to-tip distances for noncontemporaneous trees, the standardization procedure may result in one tip being nearest to another, less closely related tip (Ives & Garland, 2010). Thus, we only used the ultrametric trees for PLR.

The ultrametric trees were pruned to exclude 20 terminals lacking male body length data and converted to phylogenetic variance–covariance matrices using the PDAP package (Midford *et al.*, 2009) in Mesquite. We used male body length because males are smaller than females and, thus, provide conservative estimates. Where possible, we obtained average male body length by measuring from the clypeal apex to the posterior abdominal margin for up to five specimens per species. When male specimens were not available, male body length was determined from taxonomic descriptions; if a range was provided, the average of the minimum and maximum lengths was used. Male body lengths are provided in Appendix S1 (See Dryad). Prior to analysis, log-transformed male body length was standardized to have a mean equal to zero and standard deviation equal to one following the recommendation of Ives & Garland (2010). A bootstrap procedure was used for 2500 replicates to generate confidence intervals and test

for statistical significance of the regressions (Ives & Garland, 2010). In all but one case, some replicates failed to converge. Removal of nonconverged replicates resulted in slightly different parameter and confidence interval values, which are reported with estimates based on converged and nonconverged replicates together.

Our PLR analysis assumes that colouration evolves subsequent to body size, but it could be argued that aposematically coloured taxa might subsequently be under selection for larger body size. As such, we conducted phylogenetic ANOVA on our datasets – where body size is the dependent variable – and pruned ultrametric trees using phylANOVA (Garland *et al.*, 1993; Harmon *et al.*, 2008; Revell, 2012) in the R 3.4.1 (R Core Team, 2017) phytools package (Revell, 2012), with nsim = 500 000 for significance testing, post hoc pairwise tests performed, and *P*-values adjusted using the Holm correction (Holm, 1979) to correct for multiple comparisons.

Results

When analysing colour character 1, the ancestor of Ectrichodiinae was reconstructed as cryptically coloured, regardless of analytical approach or tree used (Fig. 1; Figs S1 and Table S2, See Dryad). Our results also recovered an early evolution of aposematic colouration in the phylogeny, except deeper nodes were reconstructed as cryptically coloured or ambiguous in the parsimony reconstruction (Fig. S1, See Dryad). Based on the phylogram, at least 15 reversals to cryptic colouration were recovered in the ML ASR, as well as seven additional gains of aposematic colouration. In general, parsimony reconstructions (Fig. S1, See Dryad) were congruent with the highest probability ML reconstructions on the phylogram near the tips but were more ambiguous at deeper nodes. Our ML reconstructions on ultrametric trees (Table S2, See Dryad) were similar to those based on the phylogram with several exceptions. At three nodes, the most probable state was opposite of that in the ML phylogram reconstruction. The *Toliarus* + *Marojejycoris* node was reconstructed as cryptically coloured (aposematic in phylogram reconstruction), which may be the result of the subtending, cryptically coloured polytomous *Marojejycoris* node in our ultrametric tree and the cryptically coloured ancestral node (i.e. *Tanindrazanus* + *Toliarus* + *Marojejycoris*). This particular reconstruction also required one transition to aposematic colouration along the terminal *Toliarus* branch, whereas our phylogram reconstruction required one gain of aposematic colouration and a subsequent reversal to cryptic colouration. The second node, which unites the three terminal *Adrania* species, was also reconstructed as cryptically coloured when analyses were performed on ultrametric trees; regardless of how this node was reconstructed, one additional transition to either cryptic or aposematic colouration was required beyond this node. Lastly, our ultrametric reconstruction recovered an aposematically coloured *Adrania* + *Neoscadra* + *Santosia* + *Scadra* node, which produced a more parsimonious set of transitions similar to those seen for the *Adrania* node. Five additional nodes, all of which join two terminal branches, had ambiguous reconstructions in our ultrametric trees. Thus, 11–16 reversals to cryptic colouration and 5–10 transitions to aposematic colouration are possible from our ultrametric tree reconstructions. These ranges overlapped with the number of transitions observed in our phylogram reconstructions. The MCMC reconstructions were highly similar to ML reconstructions (Table S2, See Dryad). Results of our PLRs on ultrametric trees indicate that aposematic colouration was associated with larger male body length ($P < 0.05$; Table 1).

Table 1 Phylogenetic logistic regression parameter estimates for the effects of log-transformed male body length on colouration (colour character 1) in 132 Ectrichodiinae species. Parameters are phylogenetic signal (a), intercept (b_0) and the regression coefficient (b_1).

Tree/data set	Par	Estimate	SE	*P*	BS mean (C and NC)†	BS confidence interval (C and NC)†	BS *P* (C and NC)†	BS mean (C only)	BS confidence interval (C only)
Ultra0	*a*	−0.6208			−0.0933	−3.4576, −0.4597	**0.0180**	−0.9365	−3.4482, −0.4129
	*b*0	−0.2458	0.5559	0.6592	−0.2911	−1.5741, 1.0498	0.6160	−0.2907	−1.5610, 1.0496
	*b*1	0.7983	0.2325	**0.0008**	0.8945	−0.3999, 1.6021	**0.0008**	0.8944	−0.4014, 1.6000
Ultra10	*a*	−0.0238			−0.5099	−3.9681, 1.0646	**0.0240**	−0.5153	−3.8989, 1.0510
	*b*0	−0.1197	0.6771	0.8598	−0.1841	−1.5967, 1.3802	0.7960	−0.1840	−1.5876, 1.3802
	*b*1	0.7557	0.2309	**0.0014**	0.8776	0.3657, 1.5349	**<0.0001**	0.8774	0.3666, 1.5349
Ultra10000	*a*	−0.0233			−0.4881	−2.9396, 0.9724	**0.0168**	−0.4949	−2.9080, 0.9357
	*b*0	−0.1195	0.6772	0.8598	−0.1738	−1.5637, 1.3268	0.7976	−0.1741	−1.5596, 1.3268
	*b*1	0.7557	0.2308	**0.0014**	0.8779	0.3791, 1.5382	**0.0008**	0.8786	0.3798, 1.5382

Ultra0, ultrametric tree generated with penalty = 0; Ultra10, ultrametric tree generated with penalty = 10; Ultra10000, ultrametric tree generated with penalty = 10 000; Par, parameter; SE, standard error; BS, bootstrap; C, converged; NC, nonconverged.

†Parametric bootstrapping was performed to obtain means and confidence intervals, as well as test the null hypotheses that there is no phylogenetic signal in the residuals (H_0: $a = -4$, 1-tailed test) and that the regression coefficient equals 0 (H_0: $b_1 = 0$, 2-tailed test).

$P < 0.05$ are indicated in bold.

Results from our phylANOVA analysis recovered a statistically significant difference in body size among differently coloured taxa ($F = 34.66$, $P < 0.003$ across all ultrametric trees). Results of ASRs and PLRs when treating cryptic colouration and uniform metallic colouration as one character (i.e. colour character 2) recovered similar ASR and PLR trends observed for colour character 1 (Table S3–S4, Figs. S2–S3, See Dryad); similar phylANOVA trends were also recovered ($F = 22.97$, $P < 0.02$ across all ultrametric trees).

Discussion

Here, we have used a group of millipede-feeding, typically solitary assassin bugs, the Ectrichodiinae, to investigate the hypothesis that aposematic colouration co-evolves with body size. Our comparative phylogenetic results support a positive association between body size and aposematic colouration, which evolved multiple times within the clade. This constitutes the first well-documented case of a positive aposematic colouration–body size association in nongregarious insects, but similar associations have previously been documented in some vertebrate taxa [e.g. poison dart frogs (Hagman & Forsman, 2003; Rudh, 2013)]. In gregarious insects, a phylogenetic comparative study on moths found gregariousness – another means of achieving large signal size – to be correlated with aposematic colouration (Tullberg & Hunter, 1996). Based on these and other experimental studies, species that have recently acquired aposematic colouration are hypothesized to be under selection for a larger body size (Forsman & Merilaita, 1999; Hagman & Forsman, 2003). Alternatively, large body size may impair the effectiveness of cryptic colouration, and, thus, aposematic colouration may evolve more easily in already large species (Hagman & Forsman, 2003). The colouration–body size association was supported in our study regardless of which trait was treated as the dependent variable, indicating that the two traits are tightly correlated and hindering our ability to assess cause and effect between the traits. Thus, the positive relationship between colouration and body size in our study is congruent with both hypotheses.

In addition to signal size, aposematic colouration may also be positively correlated with other traits as shown in phylogenetic comparative studies on poison dart frogs. For example, Santos *et al.* (2003) detected an apparent association with diet specialization (ants, termites and mites) despite substantial missing dietary data in their analysis. A positive relationship with toxicity has also been reported (Summers & Clough, 2001), which has been found in some microevolutionary studies (e.g. Maan & Cummings, 2012). Similar associations between aposematic colouration and prey specialization or toxicity are conceivable in millipede assassin bugs. Forthman & Weirauch (2012), as well as additional unpublished data, documented feeding behaviors of 20 species of Ectrichodiinae – the majority of which are aposematically coloured – on chemically defended millipedes. Although millipede prey specialization has been inferred for the most recent common ancestor of all Ectrichodiini, the most speciose clade within the Ectrichodiinae (Hwang & Weirauch, 2012; Forthman & Weirauch, 2017), feeding observations for almost all cryptic and many aposematically coloured species are unavailable. Thus, we cannot dismiss the possibility of colour transitions being linked to shifts in prey range. Similarly, our unpublished observations on *Centraspis ducalis* Distant, 1902, and a study on a species of *Ectrichodia* (Peschke *et al.*, 2002) confirm that these two aposematically coloured species emit substances from the metathoracic glands that cause skin and eye irritations in vertebrates that are unknown to that degree from other assassin bugs, including cryptic Ectrichodiinae. Our study has provided the first step towards teasing apart the relative potential roles of size, diet and toxicity in shaping the evolution of this charismatic group of insects.

Our study demonstrates the value of phylogenetic comparative methods in investigating the evolution of aposematic colouration in animals. It presents the first case of positive aposematic colouration–body size association in nongregarious insects, a phenomenon predicted in the evolutionary literature but rarely tested and recovered. During the early evolutionary history of millipede assassin bugs, aposematic colouration evolved from cryptic colour patterns, followed by multiple transitions and reversals between cryptic and aposematic patterns. Other than the association with body length, we speculate that prey specialization and/or toxicity could also be correlated with colour transitions in these insects. Although requiring substantial new ecological and behavioral data across Ectrichodiinae, we posit that millipede assassin bugs are a promising system to investigate the potential roles of size, diet and toxicity in shaping the evolution of aposematism in animals.

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References

Areekul, B. & Quicke, D.L.J. 2006. The use of colour characters in phylogenetic reconstruction. *Biol. J. Linn. Soc.* **88**: 193–202.

Arrow, G.J. 1951. *Horned Beetles: A Study of the Fantastic in Nature*. Dr W. Junk Publishers, The Hague.

Bates, H.W. 1862. XXXII. Contributions to an insect fauna of the Amazon Valley. Lepidoptera: Heliconidae. *Trans. Linn. Soc. Lond.* **23**: 495–566.

Bromham, L., Woolfit, M., Lee, M.S.Y. & Rambaut, A. 2002. Testing the relationship between morphological and molecular rates of change along phylogenies. *Evolution* **56**: 1921–1930.

Cheney, K.L., Cortesi, F., How, M.J., Wilson, N.G., Blomberg, S.P., Winters, A.E. *et al.* 2014. Conspicuous visual signals do not coevolve with increased body size in marine sea slugs. *J. Evol. Biol.* **27**: 676–687.

Cott, H.B. 1940. *Adaptive Coloration in Animals*. Methuen & Co., Ltd, London, UK.

Cusimano, N. & Renner, S.S. 2014. Ultrametric trees or phylograms for ancestral state reconstruction: does it matter? *Taxon* **63**: 721–726.

Davranoglou, L.R. 2016. *Redeicephala taylori*, a new genus and species of Reduviidae from New Guinea, with notes on a few morphological features of the Tribelocephalinae (Hemiptera: Heteroptera). *Acta Entomol. Mus Natl. Pragae* **56**: 39–50.

Edmunds, M. 1987. Color in opisthobranchs. *Am. Malacol. Bull.* **5**: 185–196.

Endler, J.A. & Mappes, J. 2017. The current and future state of animal coloration research. *Philos. Trans. R. Soc. B. Biol. Sci.* **372**: 20160352.

Exnerová, A., Svádová, K., Štys, P., Barcalová, S., Landová, E., Prokopová, M. *et al.* 2006. Importance of colour in the reaction of passerine predators to aposematic prey: experiments with mutants of *Pyrrhocoris apterus* (Heteroptera). *Biol. J. Linn. Soc.* **88**: 143–153.

Fabricant, S.A., Exnerová, A., Ježová, D. & Štys, P. 2014. Scared by shiny? The value of iridescence in aposematic signalling of the hibiscus harlequin bug. *Anim. Behav.* **90**: 315–325.

Finkbeiner, S.D., Briscoe, A.D. & Reed, R.D. 2012. The benefit of being a social butterfly: communal roosting deters predation. *Proc. Biol. Sci.* **279**: 2769–2776.

Forsman, A. & Merilaita, S. 1999. Fearful symmetry: pattern size and asymmetry affects aposematic signal efficacy. *Evol. Ecol.* **13**: 131–140.

Forthman, M. & Weirauch, C. 2012. Toxic associations: a review of the predatory behaviors of millipede assassin bugs (Hemiptera: Reduviidae: Ectrichodiinae). *Eur. J. Entomol.* **109**: 147–153.

Forthman, M. & Weirauch, C. 2017. Millipede assassins and allies (Heteroptera: Reduviidae: Ectrichodiinae, Tribelocephalinae): evolution of sexual dimorphism based on combined phylogenetic hypotheses and a revised subfamily and tribal classification. *Syst. Entomol.* **42**: 575–595.

Forthman, M., Chłond, D. & Weirauch, C. 2016. Taxonomic monograph of the endemic millipede assassin bug fauna of Madagascar (Hemiptera: Reduviidae: Ectrichodiinae). *Bull. Am. Mus. Nat. Hist.* **400**: 1–152.

Gamberale, G. & Tullberg, B.S. 1996a. Evidence for a peak-shift in predator generalization among aposematic prey. *Proc. Biol. Sci.* **263**: 1329–1334.

Gamberale, G. & Tullberg, B.S. 1996b. Evidence for a more effective signal in aggregated aposematic prey. *Anim. Behav.* **52**: 597–601.

Gamberale, G. & Tullberg, B.S. 1998. Aposematism and gregariousness: the combined effect of group size and coloration on signal repellence. *Proc. Biol. Sci.* **265**: 889–894.

Garland, T. Jr, Dickerman, A.W., Janis, C.M. & Jones, J.A. 1993. Phylogenetic analysis of covariance by computer simulation. *Syst. Biol.* **24**: 265–292.

Guilford, T. 1988. The evolution of conspicuous coloration. *Am. Nat.* **131**: S7–S21.

Hagman, M. & Forsman, A. 2003. Correlated evolution of conspicuous coloration and body size in poison frogs (Dendrobatidae). *Evolution* **57**: 2904–2910.

Härlin, C. & Härlin, M. 2003. Towards a historization of aposematism. *Evol. Ecol.* **17**: 197–212.

Harmon, L.J., Weir, J.T., Brock, C.D., Glor, R.E. & Challenger, W. 2008. GEIGER: investigating evolutionary radiations. *Bioinformatics* **24**: 129–131.

Holm, S. 1979. A simple sequentially rejective multiple test procedure. *Scand. J. Stat.* **6**: 65–70.

Hwang, W.S. & Weirauch, C. 2012. Evolutionary history of assassin bugs (Insecta: Hemiptera: Reduviidae): insights from divergence dating and ancestral state reconstruction. *PLoS One* **7**: e45523.

Ishikawa, T., Cai, W. & Tomokuni, M. 2015. The assassin bug subfamily Tribelocephalinae (Hemiptera: Heteroptera: Reduviidae) from Japan, with descriptions of eight new species in the genera *Opistoplatys* and *Abelocephala*. *Zootaxa* **3936**: 151–180.

Ives, A.R. & Garland, T. Jr 2010. Phylogenetic logistic regression for binary dependent variables. *Syst. Biol.* **59**: 9–26.

Lindström, L., Alatalo, R.V., Mappes, J., Riipi, M. & Vertainen, L. 1999. Can aposematic signals evolve by gradual change? *Nature* **397**: 249–251.

Litsios, G. & Salamin, N. 2012. Effects of phylogenetic signal on ancestral state reconstruction. *Syst. Biol.* **61**: 533–538.

Maan, M.E. & Cummings, M.E. 2012. Poison frog colors are honest signals of toxicity, particularly for bird predators. *Am. Nat.* **179**: E1–E14.

Maddison, W.P. & Maddison, D.R. 2015. Mesquite: a modular system for evolutionary analysis. Version 3.02. http://mesquiteproject.org

Maldonado, J. 1990. *Systematic Catalogue of the Reduviidae of the World (Insecta: Heteroptera)*. University of Puerto Rico, San Juan, Puerto Rico. *Caribb. J. Sci.* Special Edition, pp. 1–694.

Maldonado, J. 1996. New taxa and key to the tribes and genera in Tribelocephalinae Stål 1866 (Heteroptera: Reduviidae). *Proc. Entomol. Soc. Wash.* **98**: 138–144.

Midford, P.E., Garland, T. Jr. & Maddison, W.P. 2009. PDAP package of Mesquite. Version 1.16. http://mesquiteproject.org/pdap_mesquite/

Nilsson, M. & Forsman, A. 2003. Evolution of conspicuous colouration, body size and gregariousness: a comparative analysis of lepidopteran larvae. *Evol. Ecol.* **17**: 51–66.

Omland, K.E. 1997. Correlated rates of molecular and morphological evolution. *Evolution* **51**: 1381–1393.

Pagel, M., Meade, A. & Barker, D. 2004. Bayesian estimation of ancestral character states on phylogenies. *Syst. Biol.* **53**: 673–684.

Parker, A. 1998. The diversity and implications of animal structural colours. *J. Exp. Biol.* **201**: 2343–2347.

Pegram, K.V. & Rutowski, R.L. 2014. Relative effectiveness of blue and orange warning colours in the contexts of innate avoidance, learning and generalization. *Anim. Behav.* **92**: 1–8.

Peschke, K., Gack, C. & Mahsberg, D. 2002. *o*-Nitrobenzaldehyde sprayed as a sneezing-powder by an assassin bug (*Ectrichodia* spec.) [abstract]. 19th Annual Meeting of the International Society of Chemical Ecology, University of Hamburg, Germany.

Poulton, E.B. 1890. *The Colors of Animals*. Trübner & Co., Ltd, London, UK.

Protas, M.E. & Patel, N.H. 2008. Evolution of coloration patterns. *Annu. Rev. Cell Dev. Biol.* **24**: 425–446.

R Core Team. 2017. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Rambaut, A., Suchard, M.A., Xie, W. & Drummond, A.J. 2014. Tracer. Version 1.61. http://tree.bio.ed.ac.uk/software/tracer/

Rédei, D., Ren, S. & Bu, W. 2012. A new synonymy in the genus *Rhysostethus* (Hemiptera: Heteroptera: Reduviidae). *Acta Entomol. Mus. Natl. Pragae* **52**: 341–348.

Revell, L.J. 2012. Phytools: an R package for phylogenetic comparative biology (and other things). *Methods Ecol. Evol.* **3**: 217–223.

Rudh, A. 2013. Loss of conspicuous coloration has co-evolved with decreased body size in populations of poison dart frogs. *Evol. Ecol.* **27**: 755–767.

Ruxton, G.D., Sherratt, T.N. & Speed, M.P. 2004. *Avoiding Attack: The Evolutionary Ecology of Crypsis, Warning Signals, and Mimicry*. Oxford University Press, Oxford, UK.

Sanderson, M.J. 2002. Estimating absolute rates of molecular evolution and divergence times: a penalized likelihood approach. *Mol. Biol. Evol.* **19**: 101–109.

Sanderson, M.J. 2003. r8s: inferring absolute rates of molecular evolution and divergence times in the absence of a molecular clock. *Bioinformatics* **19**: 301–302.

Santos, J.C., Coloma, L.A. & Cannatella, D.C. 2003. Multiple, recurring origins of aposematism and diet specialization in poison frogs. *Proc. Natl. Acad. Sci. USA* **100**: 12792–12797.

Schultz, T.D. 2001. Tiger beetle defenses revisited: alternative defense strategies and colorations of two Neotropical tiger beetles, *Odontocheila nicaraguensis* Bates and *Pseudoxycheila tarsalis* Bates (Carabidae: Cincindelinae). *Coleopt. Bull.* **55**: 153–163.

Smith, S.M. 1975. Innate recognition of coral snake pattern by a possible avian predator. *Science* **187**: 759–760.

Smith, S.A. & Donoghue, M.J. 2008. Rates of molecular evolution are linked to life history in flowering plants. *Science* **322**: 86–89.

Stevens, M. 2015. Anti-predator coloration and behaviour: a longstanding topic with many outstanding questions. *Curr. Zool.* **61**: 702–707.

Summers, K. & Clough, M.E. 2001. The evolution of coloration and toxicity in the poison frog family (Dendrobatidae). *Proc. Natl. Acad. Sci. USA* **98**: 6227–6232.

Thomas, D., Seago, A. & Robacker, D. 2007. Reflections on golden scarabs. *Am. Entomol.* **53**: 224–230.

Tullberg, B.S. & Hunter, A.F. 1996. Evolution of larval gregariousness in relation to repellent defences and warning coloration in tree-feeding Macrolepidoptera: a phylogenetic analysis based on independent contrasts. *Biol. J. Linn. Soc.* **57**: 253–276.

Walker, A.A., Weirauch, C., Fry, B.G. & King, G.F. 2016. Venoms of heteropteran insects: a treasure trove of diverse pharmacological toolkits. *Toxins (Basel)* **8**: 43.

Weirauch, C. 2008. Cladistic analysis of Reduviidae (Heteroptera: Cimicomorpha) based on morphological characters. *Syst. Entomol.* **33**: 229–274.

Weirauch, C. 2010. *Tribelocodia ashei*, new genus and new species of Reduviidae (Insecta: Hemiptera), has implications on character evolution in Ectrichodiinae and Tribelocephalinae. *Insect Syst. Evol.* **41**: 103–122.

Weirauch, C., Forthman, M., Grebennikov, V. & Baňař, P. 2017. From Eastern Arc Mountains to extreme sexual dimorphism: systematics of the enigmatic assassin bug tribe Xenocaucini (Reduviidae: Tribelocephalinae). *Org. Divers. Evol.* **17**: 421–445.

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