Evolution of Dietary Specialization and Chemical Defense in Poison Frogs (Dendrobatidae): A Comparative Analysis

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ABSTRACT: Defensive mechanisms, including noxious or toxic substances, are favored by predation-driven natural selection. The acquisition of noxious/toxic substances can be either endogenous, in which the substances are produced by the organism, or exogenous, in which the substances are produced by another organism and are sequestered. Evidence indicates that the defensive skin alkaloids of Neotropical poison frogs (Dendrobatidae) have an exogenous source: a diet of ants and other small alkaloid-containing arthropods, which we term the diet-toxicity hypothesis. A critical prediction of the diet-toxicity hypothesis is that independent origins of dietary specialization will be found to be correlicad with independent origins of skin alkaloids. We tested this prediction in an integricad framework using comparative methods with new and published data on feeding ecology and chemical defense for 15 species of dendrobatids in five genera. We found a significant correlation between alkaloid profiles and degree of dietary specialization. This reveals a recurring association of dietary specialization and alkaloid sequestration in dendrobatids, which suggests parallel evolutionary trends in the origins of defensive mechanisms.

Keywords: alkaloid sequestration, chemical defense, comparative method, Dendrobatidae, dietary specialization, noxiousness.

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Predation imposes strong selective pressures on prey, resulting in the evolution of a great diversity of defensive mechanisms, including repellent defense, such as venoms and toxins (Edmunds 1974). Toxic and/or noxious substances can come from either endogenous synthesis (the organism's metabolic machinery produces the substances) or exogenous sources (via uptake, sequestration, and/or storage of toxic substances produced by other organisms; Eisner 1970; Edmunds 1974; Mebs 2001). Toxic or otherwise unpalatable individuals are often brightly colored; this association is called aposematism (Poulton 1890). Presumably, warning coloration would be selected for after the acquisition of chemical defense, because any easily recognizable color or character that identifies the unpalatable individual to predators would be favored (Cott 1940; Edmunds 1974, 1987).

The acquisition of defensive compounds from the environment raises the possibility of integrating ecology and the evolutionary origin of chemical defense. Although feeding ecology is often regarded as a proximate factor (the current interactions between an organism and the available resources), the historical, and therefore ultimate role of diet in influencing factors of diversification may be important, particularly in organisms with diet-acquired defense. One approach to the evolutionary role of feeding ecology is to search for convergent, and therefore probably adaptive, patterns of resource use across taxa (Strong 1979; Futuyma 1983; Orians and Paine 1983). The role of diet in macroevolutionary processes has been most thoroughly studied in insects (Ehrlich and Raven 1964; Berenbaum 1983; Jeffries and Lawton 1984; Feeny 1987; Denno et al. 1990; Farrell and Mitter 1990; Becerra 1997; Becerra and Venable 1999; Dobler 2001) but has not been extensively addressed in other taxa. The relationship between diet and the sequestration of defensive compounds in the context of the evolutionary history of Neotropical poison frogs (Dendrobatidae) is the focus of this study.

The family Dendrobatidae is composed of 221 small, mostly diurnal species in at least nine genera (updated from Frost 2004). Species of *Colostethus* (plus *Mannophryne* and *Nephelobates*, which were removed from *Co-*

lostethus by La Marca [1992], [1994]) are generally non-toxic, cryptically colored dendrobatids, whereas species of *Epipedobates*, *Phyllobates*, and *Dendrobates* are those with the most noxious and/or toxic skin and brilliant, aposematic coloration (Myers et al. 1978; Daly et al. 1980, 1987, 1994*a*; Myers 1987). The skin of unpalatable dendrobatids houses a diverse range of lipophilic alkaloids (Daly et al. 1999 and references therein). These alkaloids are noxious and/or toxic to predators and therefore serve a defensive role, although the evidence for this is somewhat anecdotal (Daly and Myers 1967; Lülling 1971; Fritz et al. 1981; Szelistowski 1985). Even though the precise sister group of Dendrobatidae is not clear (Darst and Cannatella 2004), none of the apparent close relatives derives defense from alkaloids. Therefore, we assume that lack of skin alkaloids is ancestral for dendrobatids (as also suggested by Caldwell [1996]).

The first extensive molecular phylogenetic analysis of 27 dendrobatid species reconstructed a single origin of aposematism (Clough and Summers 2000). *Colostethus* was the assumed outgroup, exhibiting the cryptic, non-toxic ancestral state, followed by the transitional, unpalatable, brightly colored *Epipedobates*, then culminating in the most brightly colored, more toxic taxa *Phyllobates* and *Dendrobates*. However, with more extensive taxon sampling, especially in *Colostethus*, several independent origins of aposematism were recovered (Santos et al. 2003; Vences et al. 2003). *Colostethus* was found to be paraphyletic with respect to the more brightly colored *Allobates*, *Cryptophyllobates*, the clade *Phyllobates* + *Dendrobates*, and *Epipedobates*, the last being a polyphyletic assemblage. The multiple origins of aposematism occur at different timescales; aposematism had an ancient origin in *Phyllobates* + *Dendrobates* (a large group of toxic species), whereas other origins are recent, with little genetic divergence between brightly colored species and their cryptic sister taxa.

Evidence is mounting that alkaloids in the skin of poison frogs are accumulated from dietary sources: small, leaf-litter arthropods, particularly ants (Daly et al. 1994*a*, 1999, 2000, 2002, 2003; Spande et al. 1999; Saporito et al. 2003, 2004). It has been shown that the few species of aposematic dendrobatids examined have specialized diets with a larger percentage of ants, larger number of prey per individual, and smaller niche breadths (Toft 1980, 1995; Donnelly 1991; Simon and Toft 1991; Caldwell 1996; Parmelee 1999); however, these conclusions were made in the context of a single origin of aposematism. Thus, dietary specialization and the evolution of an uptake system for alkaloids were postulated to be key innovations leading to the development of chemical defense and permitting the evolution of aposematism and diversification in dendrobatids (Caldwell 1996).

A critical prediction of this diet-toxicity hypothesis is that independent origins of dietary specialization will be correlated with independent origins of chemical defense. Given the recent discovery of multiple origins of aposematism, one would predict multiple origins of diet specialization. Santos et al. (2003, their fig. 2) inferred at least two and possibly three origins of ant specialization, but this inference was not based on an explicit analysis. To test the prediction of the diet-toxicity hypothesis in an integrated framework, we used the molecular phylogeny of Santos et al. (2003) and a complex of ecologically relevant traits compiled from 15 species (new data from nine species and published data for eight species) in five genera. We explore the evolutionary and behavioral ecology of alkaloid sequestration through quantification of diet contents and dietary niche and through an indirect assessment of predator-defense mechanism using an assay for skin alkaloids. This is one of the first hypothesis-driven tests of an association between diet and defense related to warning coloration in a vertebrate system.

Material and Methods

Specimen Collection

Fieldwork was conducted in the Amazonian lowland rain forest, Western Andean slopes, and Pacific lowlands of Ecuador. The four collection sites were Estación Científica Yasuní, Francisco de Orellana Province (*Allobates femoralis*, *Colostethus* sp. D ["bocagei" complex: Santos et al. 2003], *Colostethus insperatus*, *Colostethus sauli*, *Epipedobates bilinguis*, and *Epipedobates hahneli*); Estación Biológica Jatun Sacha, Napo Province (*Allobates zaparo*, *E. bilinguis*, *E. hahneli*, and *Epipedobates parvulus*); Zarayunga, the Pasaje, Cuenca road at 540 m, El Oro Province (*Epipedobates anthonyi*); and 6 km (airline) WNW of Pedro Vicente Maldonado and 9 km W of Santo Domingo de los Colorados (on the road to Chone), Pichincha Province (*Epipedobates boulengeri*). The taxonomy follows Frost (2004).

Frogs were collected by hand (when possible, in a plastic cup so as not to contaminate the skin by handling) and euthanized by pithing or freezing to avoid contamination by chemical agents. The skin and digestive tract were removed and preserved as soon as possible after collection, usually within 1–2 h, in order to prevent further digestion of consumed prey and to complement Caldwell (1996). Skins were fixed in 100% methanol; digestive organs were stored in 90% ethanol. Specimens were fixed in 10% formalin and transferred to 70% ethanol. Voucher specimens and stomach contents are deposited at Museo de Zoología, Pontificia Universidad Católica del Ecuador (QCAZ).

Diet Analyses

Gastrointestinal tract contents were sorted using a dissecting microscope, and prey items were identified to the lowest taxonomic category possible, usually order, although some items (particularly Hymenoptera, Homoptera, and Coleoptera) were identified to family; specifically, all ants were identified to Formicidae, and, therefore, all subsequent mention of Hymenoptera excludes ants. The length and width of each intact prey item was measured to 0.01 mm (with digital calipers) and volume was calculated using the formula for a prolate spheroid (Dunham 1983):

$$V = \frac{4\pi}{3} \left(\frac{\text{length}}{2} \right) \times \left(\frac{\text{width}}{2} \right)^2.$$

Length measurements excluded antennae and ovipositors. Width was recorded at the midpoint of the prey item, excluding appendages. For each species, a histogram of percent of total prey by volume in the 15 most frequent prey categories was used to represent the distribution of prey items in the diet (fig. 1).

Diet was quantified using six variables (see table A2 in the online edition of the *American Naturalist*). Three explored general aspects of the diet, and three focused on the importance of ants: the proportion of individuals in each species with ants in the gastrointestinal tract (%INDANTS), the percentage of ants by number in the total prey (%ANTSNUM), the percentage of ants by volume in the total prey (%ANTSVOL), the number of prey per individual frog (NUMPREY), and the niche breadth of each species calculated for prey number (NBNUM) and prey volume (NBVOL). Caldwell (1996) computed these same variables for 212 frogs in eight species, using the protocol described above. If diet data for a species were available from both our gut-content analyses and Caldwell (1996), the data were combined for use in comparative analyses. Niche breadth was calculated using the inverse of Simpson's (1949) formula,

$$B = \frac{1}{\sum_{i=1}^{n} p_i^2},$$

where i is the resource category, p is the proportion of resource category i, and n is the total number of prey categories (Pianka 1986). Niche breadth values vary from 1.0 (exclusive use of a single prey category) to n (all prey categories used equally).

It has been argued that electivities, which measure the proportion of prey eaten as compared to the prey availability (the proportions of prey in the leaf litter), are more realistic quantifications of dietary specialization than niche breadth (Ivlev 1961; Jacobs 1974; Toft 1995). We did not calculate electivities because Toft (1980) found that distribution and categorization of dendrobatids along a specialist-generalist continuum were the same no matter whether niche breadth or electivity values were used.

Skin Chemistry Analyses

As a proxy for direct measures of unpalatability to predators, we assessed alkaloid profiles from 161 dendrobatid skins (135 of which belonged to the animals used in diet analyses) using thin layer chromatography (TLC; Myers and Daly 1976, their app. 1). Individual skins were placed into polyethylene NUNC vials with 1–1.5 mL 100% methanol for at least 3 weeks to extract the skin alkaloids. The skins were then removed and stored in fresh methanol and deposited in the collections of QCAZ. A sample of 10 μL of each original methanol extract was applied as a small spot to an aluminum-backed silica gel TLC plate (60F$_{265}$; EM Science), followed by development of the plate with a 1 : 10 mixture of methanol and chloroform. After drying, the plate was placed in a chamber containing iodine crystals and was heated to vaporize the iodine; this allows visualization of alkaloids as orange brown spots on a light background. Digital photographs of all TLC plates were taken because the iodine vapor produces only a semipermanent record. Each lane on the TLC plate was scored as positive, negative, or trace for alkaloids. A positive result was recorded when a substantial orange-brown streak, usually containing two to six dark spots, appeared on exposure to the iodine vapor; a negative result was recorded when nothing appeared; and a trace result was recorded only when a very faint, singular spot appeared. Individual extracts of various species were compared side by side.

To create a continuous, composite score for alkaloid profiles, our TLC data were combined with alkaloid data from Summers and Clough (2001), which were compiled from Daly et al. (1987). Summers and Clough (2001) called this score "total toxicity," which is perhaps a misnomer given that Daly et al. (1987) did not present toxicity data but rather only structural information and a classification of dendrobatid skin alkaloids. Further, not all dendrobatid alkaloids are toxic: many are distasteful, some are noxious, and others are highly toxic (Daly et al. 1987). We therefore refer to Summers and Clough's "total toxicity" as "composite alkaloid score" (table 1). We used the composite alkaloid score for nine species directly from Summers and Clough with the following exceptions. Because data for *E. boulengeri* were not available, Summers and Clough (2001) assigned it the same score as its closest relative, *E. espinosai*, which Daly et al. (1987) scored as "+" on the +, ++, or +++ scale for number of alkaloids detected per 100 mg

Table 1: Measures of alkaloid levels in species of Dendrobatidae

Species	No. skins	TLC	Diversity	Quantity	Lethality	Composite alkaloid score	Binary alkaloid score
Allobates femoralis	15	N	.17	.17	1	1.2	0
Allobates zaparo	20	T	…	…	…	1.7	0
Colostethus sp. D	22	N	…	…	…	0	0
Colostethus insperatus	12	N	…	…	…	0	0
Colostethus sauli	10	N	…	…	…	0	0
Colostethus talamancae	…	…	0	0	0	0	0
Dendrobates auratus	…	…	16.8	2.56	2	6.2	1
Dendrobates pumilio	…	…	16.0	2.25	2	5.9	1
Dendrobates ventrimaculatus	…	…	12.5	2.0	2	5.3	1
Epipedobates anthonyi	10	P	2.0	1	1	2.2	1
Epipedobates bilinguis	24	P	6.0	1.5	1	3.1	1
Epipedobates boulengeri	20	N	…	…	…	0	0
Epipedobates hahneli	16	P	6.0	2.0	1	3.6	1
Epipedobates parvulus	12	P	…	…	…	3.4	1
Phyllobates lugubris	…	…	1.7	.67	3	3.8	1

Note: Thin layer chromatography (TLC) results are reported as positive (P), presence of alkaloids in skin extract; as negative (N), no alkaloids; or trace (T), possibly very small amount of alkaloids. Diversity, quantity, and lethality of species are taken from Summers and Clough (2001) and our combined scores of composite alkaloid score and binary alkaloid score. Alkaloids were detected in 15 out of 16 *Epipedobates hahneli* skin extracts.

skins. In our sample of 20 skin extracts of *E. boulengeri*, no alkaloids were detected using TLC, so this species was scored as 0.

Also, Summers and Clough (2001) used alkaloid data reported by Daly et al. (1987) for *Epipedobates tricolor* and *E. anthonyi*. The populations reported by Daly et al. (1987) differ quite markedly in distribution and abundance of alkaloids, but they are all referable to *E. anthonyi* (not *E. tricolor*) based on the localities (Graham et al. 2004). The type locality of *E. anthonyi* is in southwestern Ecuador (Salvia, El Oro Province). The type locality of *E. tricolor* is in the western Andean slopes of central Ecuador (El Porvenir, Bolívar Province), and the alkaloid profile of this species has not been assessed. Using TLC, we found skins of *E. anthonyi* (road between Pasaje and Cuenca, El Oro Province) to contain alkaloids, in accordance with Daly et al. (1987). We used the composite alkaloid score for *E. anthonyi* from Pasaje, El Oro, Ecuador as reported by Summers and Clough (2001) because of concordant locality information and because it is the score least likely to reject the null hypothesis.
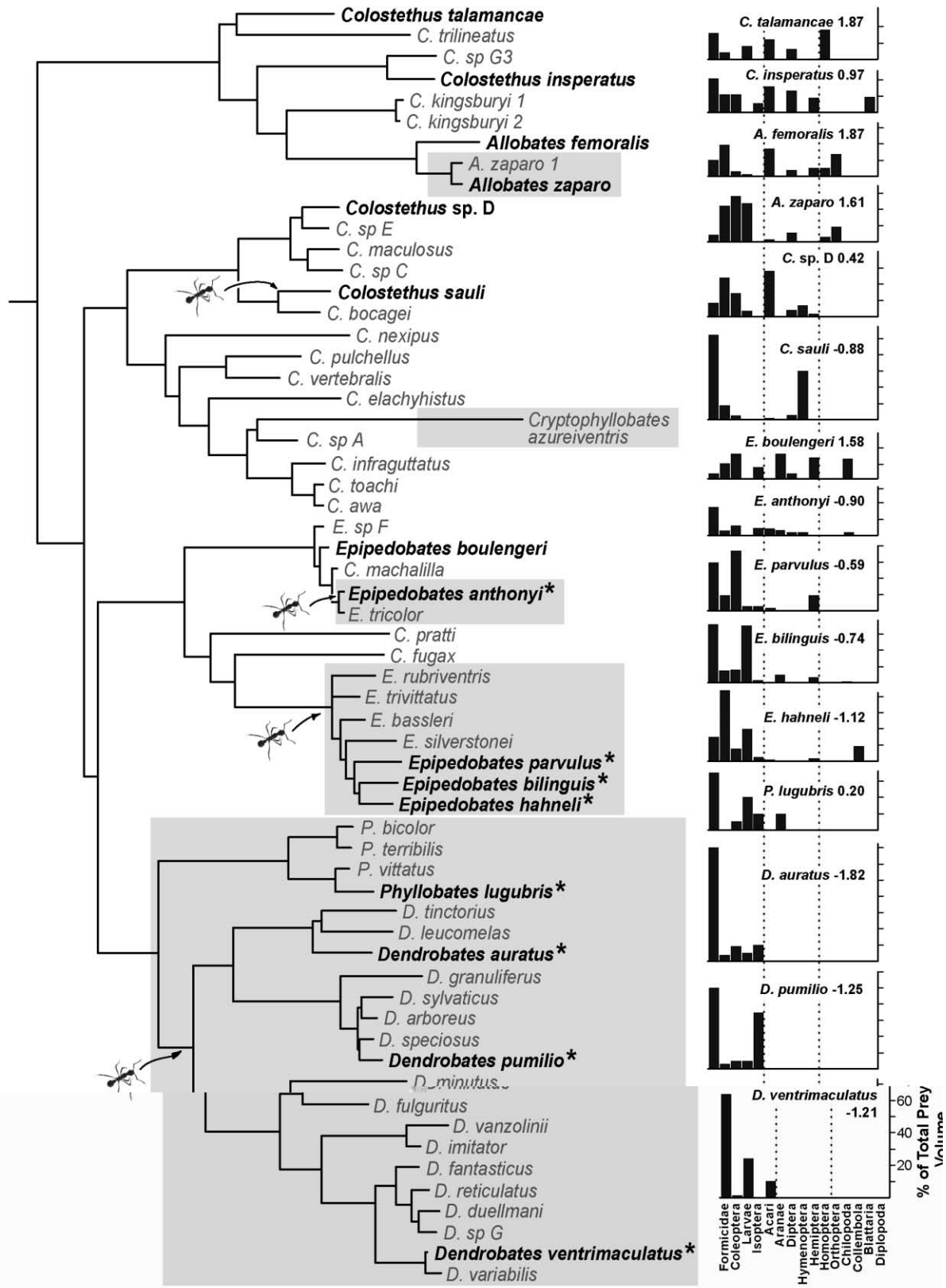
Although we detected no alkaloids in *A. femoralis*, we used the published score of 1.2 for consistency. *Allobates zaparo* displayed at most trace amounts of alkaloids, and side-by-side comparisons of TLC data for that species with *A. femoralis* (1.2 composite alkaloid score), and *E. anthonyi* (2.2 composite alkaloid score) indicated a score for *A. zaparo* intermediate between the other two; we therefore assigned it a composite alkaloid score of 1.7.

We also assigned presence/absence alkaloid scores (bi-

nary alkaloid score; table 1) to all 15 species. Some researchers (e.g., Daly et al. 2002) have questioned the realism in the total toxicity scores of Summers and Clough (2001), and therefore a more conservative measure might be desirable. Using the binary alkaloid score, the statistical tests for association between alkaloid presence and diet will be less likely to reject the null hypothesis. Also, there is little doubt that species can be divided into two groups: containing alkaloids or not. Species were assigned the number 1 based on either presence of alkaloid bands on the TLC plates or data in Daly et al. (1987). Species for which no alkaloids were detected using TLC, or that were reported by Daly and colleagues to lack alkaloids, were assigned the number 0. Three species warrant explanation. *Allobates femoralis* was reported to have one trace alkaloid in one individual out of six populations (Daly et al. 1987); our TLC analysis detected no alkaloids from 15 skins from one locality; this species was scored as 0. Because all *A. zaparo* examined displayed only a trace alkaloid band and because our observations suggest *A. zaparo* are not unpalatable to predators (C. R. Darst, personal observation), we scored it as 0.

Comparative Method Analyses Using Generalized Least Squares

Statistical analyses employed JMP (SAS Institute 2000). Niche breadth variables (NBNUM and NBVOL) were log-transformed; %ANTSNUM, %ANTSVOL, and %INDANTS were arcsine-transformed as appropriate for per-

Phylogenetic tree with taxa:

Colostethus talamancae
C. trilineatus
C. sp G3
Colostethus insperatus
C. kingsburyi 1
C. kingsburyi 2
Allobates femoralis
A. zaparo 1
Allobates zaparo
Colostethus sp. D
C. sp E
C. maculosus
C. sp C
Colostethus sauli
C. bocagei
C. nexipus
C. pulchellus
C. vertebralis
C. elachyhistus
Cryptophyllobates azureiventris
C. sp A
C. infraguttatus
C. toachi
C. awa
E. sp F
Epipedobates boulengeri
C. machalilla
Epipedobates anthonyi *
E. tricolor
C. pratti
C. fugax
E. rubriventris
E. trivittatus
E. bassleri
E. silverstonei
Epipedobates parvulus *
Epipedobates bilinguis *
Epipedobates hahneli *
P. bicolor
P. terribilis
P. vittatus
Phyllobates lugubris *
D. tinctorius
D. leucomelas
Dendrobates auratus *
D. granuliferus
D. sylvaticus
D. arboreus
D. speciosus
Dendrobates pumilio *
D. minutus
D. fulguritus
D. vanzolinii
D. imitator
D. fantasticus
D. reticulatus
D. duellmani
D. sp G
Dendrobates ventrimaculatus *
D. variabilis

0.05 substitutions/site

Diet histograms:
C. talamancae 1.87
C. insperatus 0.97
A. femoralis 1.87
A. zaparo 1.61
C. sp. D 0.42
C. sauli -0.88
E. boulengeri 1.58
E. anthonyi -0.90
E. parvulus -0.59
E. bilinguis -0.74
E. hahneli -1.12
P. lugubris 0.20
D. auratus -1.82
D. pumilio -1.25
D. ventrimaculatus -1.21

% of Total Prey Volume
60
40
20

Prey categories:
Formicidae
Coleoptera
Larvae
Isoptera
Acari
Aranae
Diptera
Hymenoptera
Hemiptera
Homoptera
Orthoptera
Collembola
Chilopoda
Blattaria
Diplopoda

centages (Sokal and Rohlf 1981). All transformations improved the distributional properties of the data. All subsequent references to these variables refer to transformed data.

Our statistical analyses were designed to answer two questions: Do measures of diet specificity co-vary with phylogenetic divergence (as measured by DNA sequences)? and, Are diet variables correlated with alkaloid profiles? We used two general methods of analysis to examine the relationship between diet and skin alkaloids while accounting for similarity due to common descent: generalized least squares (GLS) analysis of transformed variables (Martins and Hansen 1997; Pagel 1997) and conversion of variables to independent contrasts (Felsenstein 1985; Garland et al. 1992). Both methods require a model phylogeny with branch lengths, and this was taken from Santos et al. (2003). Santos et al. (2003) treated *E. anthonyi* under the name *E. tricolor*. However, *E. tricolor* 1 (Santos et al. 2003) is referable to *E. anthonyi* (Graham et al. 2004) and was therefore used for *E. anthonyi* branch length data. Our *A. zaparo* is *A. zaparo* 2 (Santos et al. 2003). At the level of analysis used here, this phylogeny is unambiguous and well supported with respect to the placement of the focal species.

Sillén-Tullberg (1993) argued that the intensity of taxon sampling, and therefore the relative abundance of character states, can bias the results of comparative methods. This was discussed in the context of Maddison's (1990) concentrated changed test; however, the argument applies to all comparative methods. Although our sample of taxa is smaller than perhaps is desirable, this experimental design only makes the tests more conservative and less likely to reject the null hypothesis.

Principal component analyses (from correlation matrices) of the transformed variables were used to generate new linear combinations of variables and thus to reduce dimensionality of the dataset. Both NBNUM and NBVOL were thus analyzed, creating PCNB, the score of each species on the first principal component. Similarly, %ANTSNUM and %ANTSVOL were summarized using the first principal component (PC%ANTS). Also, the first principal component of all six measures of diet speciali-

zation was calculated to summarize all diet variables (PCALL).

The GLS approach can be used to examine the degree to which trait variation is related to phylogeny as well as the degree to which two or more traits co-vary in a phylogenetic context (Freckleton et al. 2002). Both questions are special cases of a general method that integrates information about phylogeny and phenotypic variation and summarizes this as an "evolutionary regression coefficient" (Pagel 1993).

The matrix form of the GLS model is

$$y = \beta X + e.$$

A species trait is treated as the y value to be predicted from a regression of that trait on some predictor variable X where β is the regression coefficient. The maximum likelihood estimate of β is found by

$$\beta = (X'V^{-1}X)^{-1}(X'V^{-1}Y),$$

where β is the vector of regression coefficients, X is a matrix of predictor variables (see below), Y is the matrix of response variables, and V is the species-by-species variance-covariance matrix of the shared branch lengths of the tree.

To address the degree to which trait variation is related to phylogeny, the predictor X is a matrix of the sum of branch lengths from the root for each species. In this case, β estimates the amount of change in y value per unit change of evolutionary divergence (e.g., as measured by branch lengths indicating genetic divergence or time). In this type of analysis, the variance in the observed trait y is explained by the regression equation, and thus one can assess whether y co-varies with the amount of evolutionary divergence. In other words, are the observed values of y independent of the phylogeny, as would be expected if strong selection had obliterated phylogenetic information in the trait, or in contrast, do the values of y co-vary with the phylogeny, as expected if change in y follows a Brownian motion process?

The GLS model can also be used to assess the covariance between two traits. The matrix X consists of one or more

Figure 1: Maximum likelihood phylogeny of Dendrobatidae from Santos et al. (2003); the gray boxes represent conspicuous species. The species names in bold are those examined in this study. Asterisks after species names denote presence of alkaloids. We divided the species in this study evenly into two groups by PCNB (the first principal component of niche breadth measured by prey number and prey volume), eight specialists (negative component score) and seven generalists (positive component score), using a cutoff between *Epipedobates parvulus* (−0.59) and *Phyllobates lugubris* (0.20). The ant icons indicate evolutionary origins of specialized diets based on this categorization. It should be noted, however, that the specialist-generalist spectrum is a continuum; therefore, a characterization as generalist versus specialist based on a continuous trait is arbitrary and should be used for heuristic purposes rather than as an invariant character of a species. Histograms depict percentage of total prey volume for the 15 most abundant prey categories; values of PCNB are given for comparison on the histograms immediately following each species name.

species traits that are used to predict a matrix of species traits **Y**. Thus, one can estimate the evolutionary correlation between **Y** and one or more predictor variables, **X**. In this case, β estimates the change in **Y** per unit change in **X** along branches in the tree. This type of analysis can produce a result essentially identical to that using independent contrasts under certain assumptions.

We used GLS as implemented in Continuous (Pagel 1997, 1999; see also Martins and Hansen 1997). To assess the degree to which a trait co-varies with phylogeny, Continuous provides a maximum likelihood estimate, termed λ, of the regression coefficient. The parameter λ measures the correlation between a trait and total divergence as 0 if the trait is completely independent of the phylogeny (no "phylogenetic effect") and 1 if the trait follows a Brownian motion process (complete covariance with the phylogeny). Continuous also provides a likelihood ratio test of the estimate of λ. For the variables used here, we tested the null hypothesis that $\lambda = 0$ (no phylogenetic effect) under a general assumption of Brownian motion evolution of the trait.

Using Continuous, we also estimated the correlation coefficient (taking phylogeny into account) between the two measures of alkaloid profiles (composite and binary) and the diet variables. Continuous provides a likelihood ratio test of the significance of the correlation coefficients. In this test, λ was estimated (rather than set to be 0 or 1) under both the null and alternative hypotheses, and the covariance between the two traits under the null hypothesis was constrained to be 0.

Comparative Method Analyses Using Independent Contrasts

Using Comparative Analysis by Independent Contrasts (CAIC; Purvis and Rambaut 1994), we computed independent contrasts for composite alkaloid scores, the six measures of diet, and the principal component scores. We plotted the absolute value of each set of standardized contrasts versus the standard deviation for that variable to check that each contrast had been adequately standardized (Garland et al. 1991, 1992). Hereafter, all mentions of contrasts refer to standardized contrasts. Comparative Analysis by Independent Contrasts was also used to calculate nodal values for selected variables.

To assess the relationship between diet and toxicity, we used two approaches. First, correlation coefficients (forced through the origin) between composite alkaloid score and each diet variable were calculated from the contrasts. Second, the contrasts of NUMPREY, %INDANTS, PC%ANTS, and PCNB were used as variables in a stepwise multiple regression (through the origin) to determine which measure of dietary specialization was the best pre-

dictor of alkaloid profile. In the stepwise regression, we used the contrasts from the principal components (PC%ANTS and PCNB) rather than contrasts from the %ANTSNUM, %ANTSVOL, NBNUM, and NBVOL because %ANTSNUM and %ANTSVOL are highly correlated, as are NBNUM and NBVOL. In this way we avoided potential problems with collinearity in predictor variables in the multiple regression analysis (Neter et al. 1996). All analyses using contrasts were repeated with and without the data for *E. anthonyi* because the contrast computed between *E. anthonyi* and *E. boulengeri* was an extreme outlier due to the large differences in diet and alkaloid profiles between the two species.

Results

Diet Analyses

A total of 3,390 prey items in 46 prey categories were identified from 135 specimens of nine species of dendrobatid frogs, summarized in figure 1, table A1, and table A2. The first principal component derived from NBVOL and NBNUM (PCNB) summarized 83% of the variance in those two variables; PC%ANTS summarized 70% of the variance in %ANTSNUM and %ANTSVOL; and PCALL (for all six measures of diet specialization) accounted for 68% of the total variance.

To visualize interspecific variation in diet specificity, histograms of percent of total prey volume for the 15 most abundant prey categories are presented with the phylogeny (fig. 1). The value for PCNB follows the species name (fig. 1). Species with negative values of PCNB are interpreted as having a narrow diet. Ants (Formicidae) are frequently the most abundant prey by volume in the species with negative PCNB values; however, PCNB is a measure of general diet specialization, not just specialization on ants. Correspondingly, the histograms of specialists have a few tall bars (e.g., *Dendrobates auratus*); in contrast, species with positive PCNB values are interpreted as having a broad diet with more shorter bars in the histograms (e.g., *Epipedobates boulengeri*).

Skin Chemistry Analyses

Of the ten species examined using thin layer chromatography, no alkaloids were detected in five species (*Allobates femoralis*, *E. boulengeri*, and all three *Colostethus*; table 1). Alkaloids were detected in all *Epipedobates anthonyi*, *Epipedobates bilinguis* and *Epipedobates parvulus* individuals assayed, and in 15 of 16 individuals of *Epipedobates hahneli*. All individuals of *Allobates zaparo* displayed a score of "trace" for alkaloids: a very faint, single spot as opposed to dark, multiple spotting or streaking.

Relationship between Traits and Phylogeny

The λs for all diet variables, except NUMPREY and NBNUM, were found to be indistinguishable from 0. The null hypothesis of λ = 0 was rejected for both binary alkaloid score and composite alkaloid score (table 2).

Relationship between Diet and Skin Alkaloids

Using the GLS model and the composite alkaloid score from all species, all dietary specialization measures were significantly correlated ($P < .05$) with the composite alkaloid score except %ANTSNUM (table 2). Using the binary alkaloid score from all species, all dietary specialization measures were significantly correlated ($P < .05$) with the binary alkaloid score except NUMPREY (table 2).

Based on the correlation coefficients calculated from contrasts, all diet-specialization measures were significantly correlated ($P < .05$) with composite alkaloid score except NUMPREY (see fig. B1 in the online edition of the *American Naturalist*). In the analyses that excluded *E. anthonyi*, all diet variables except %ANTSVOL were significantly correlated ($P < .05$) with composite alkaloid score (fig. B1). Using a stepwise multiple regression of diet variable contrasts and composite alkaloid score contrasts (both with and without *E. anthonyi*), PCNB was found to be the best predictor of the composite alkaloid score.

Discussion

Diet and Phylogeny

How many times has a specialized diet evolved in dendrobatids? We divided the species evenly into two groups by PCNB (other variables could be used), eight specialists

(negative component score) and seven generalists (positive component score), using a cutoff between *Epipedobates parvulus* (−0.59) and *Phyllobates lugubris* (0.20). Mapping these two categories onto the phylogeny (fig. 1) indicates that a specialized diet evolved at least four times: in *Colostethus sauli*, in *Epipedobates anthonyi*, in the clade of three *Epipedobates* (*hahneli*, *bilinguis*, and *parvulus*), and in the large clade of *Dendrobates*. However, a specialist-generalist spectrum is a continuum; therefore, a characterization as generalist versus specialist based on a continuous trait is arbitrary. Categorization should be used for heuristic purposes rather than as an invariant character of a species, particularly given that the species on either side of the cutoff may be different depending on which diet variable is used.

Santos et al. (2003) postulated another origin of diet specialization in the brightly colored *Allobates zaparo* based on data from Almendáriz (1987; 74% ants by number in the diet), the only available diet data for *A. zaparo* at the time. We found *A. zaparo* to have a much more generalized diet than reported by Almendáriz (1987). The *A. zaparo* diet data used in our comparative analyses is from April 2003, northern Jatun Sacha, Ecuador (26% ants by number). Data from the Río Arajuno, southern Jatun Sacha, Ecuador, from February 2004 also suggests *A. zaparo* is a generalist (37% ants by number), as does data from Morona-Santiago in southern Ecuador collected in March 2004 (11% ants in the diet). Dietary variation in *A. zaparo* does exist between sites, as would be expected in an opportunistic generalist, and it is possible that the high percentage of ants found by Almendáriz (1987) reflects this. The discrepancy may also be due to the length of time between when a frog was captured and when it was preserved and/or perhaps to the use of older museum specimens in Almendáriz (1987). Frogs, particularly dendro-

Table 2: Correlations of diet measures with phylogeny (λ) and correlations of diet measures with composite and binary alkaloid scores

Dietary specialization measure	λ	Probability (H₀: λ = 0)	Composite alkaloid score		Binary alkaloid score	
			Correlation coefficient	Probability (H₀: r = 0)	Correlation coefficient	Probability (H₀: r = 0)
NUMPREY	1.000	.009	.793	.025	.467	.130
%INDANTS	.068	.857	.790	<.001	.869	<.001
NBNUM	.553	.030	−.538	.016	−.706	<.001
NBVOL	.493	.204	−.761	.002	−.641	.005
%ANTSNUM	.000	1.000	.393	.085	.643	.005
%ANTSVOL	.725	.099	.677	.030	.504	.042
PCNB	.530	.072	−.717	.002	−.743	.004
PC%ANTS	.019	.963	.632	.016	.673	.002
PCALL	.434	.206	.811	<.001	.798	<.001

Note: λs and correlation coefficients were calculated using the GLS model. Composite alkaloid score: λ = 0.893; P (H₀: λ = 0) = 0.005; binary alkaloid score: λ = 0.616; P (H₀: λ = 0) = 0.037.

batids, are capable of digesting their prey rapidly, particularly less-chitinous prey (Caldwell 1996); there is a skew toward more chitinous prey in all dendrobatid diet data in Almendáriz (1987) as compared to our data, to Caldwell (1996), and to Toft (1980, 1981, 1995). Misidentification is another possibility, given that the color pattern of *A. zaparo* is very similar to *E. parvulus* and *E. bilinguis*, both of which are more diet specialized than *A. zaparo*.

Additional data from the literature are consistent with our interpretation of four evolutionary origins of dietary specialization. Toft (1995) found the mean dietary niche breadth of three species of *Phyllobates* to be larger than that of the mean values for *Dendrobates* and most species of *Epipedobates*. She also reported the niche breadth of *Colostethus pratti* (included in her mean value for *Colostethus*) to be larger than that of *Phyllobates*, *Epipedobates*, and *Dendrobates*. The position of *C. pratti* in our tree is consistent with a plesiomorphic generalist diet in the clade containing *Epipedobates boulengeri*, a generalist species, as well as *E. parvulus*, *E. hahneli*, and *E. bilinguis*, species with smaller dietary niches.

The homoplasy that is evident in mapping PCNB onto the tree is also evident in the degree to which diet variables co-vary with the phylogeny. All diet variables except NUMPREY and NBNUM were found to have λs that cannot be distinguished from 0, indicating no phylogenetic effect (table 2). The lack of covariance with phylogeny in the majority of diet variables is consistent with natural selection promoting an evolutionary shift from more generalized to more specialized diet in dendrobatids.

Correlation of Diet and Skin Alkaloids

Our results indicate that divergences from the ancestral, nontoxic state are significantly correlated with divergences in degree of dietary specialization in dendrobatids. In other words, we have verified the critical prediction of the diet-toxicity hypothesis in the context of multiple origins of aposematism: Independent origins of dietary specialization are correlated with independent origins of skin alkaloids. However, this correlation does not entail a one-to-one correspondence between aposematism and dietary specialization. For example, *A. zaparo* is brightly colored and a generalist but displays only a trace score for skin alkaloids. Also, *A. zaparo* was observed to be palatable to potential predators, suggesting possible Batesian mimicry with *E. bilinguis* and *E. parvulus* (C. R. Darst, personal observation). *Colostethus sauli* is cryptic and has no skin toxins but was found to eat mainly ants (fig. 1). *Phyllobates lugubris* is brightly colored, and some populations are known to contain the highly toxic *Phyllobates*-specific alkaloid batrachotoxin (Daly et al. 1999; but see Dumbacher et al. 1992, 2000); however, *P. lugubris* does not exhibit a

particularly specialized diet. Toft (1995) found the mean dietary niche breadth of three species of *Phyllobates* (*P. lugubris*, *Phyllobates aurotaenius*, and *Phyllobates vittatus*, the latter two of which have been consistently found to contain batrachotoxin; Daly et al. 1999) to be larger than that of the mean values for *Dendrobates* and most species of *Epipedobates*. The dietary source for batrachotoxin remains unknown; Daly et al. (1999) propose it may be a prey item larger than ants.

The persistence of accumulated skin alkaloids in dendrobatids (Daly et al. 1994b) suggests that the coupling of diet and defense can be relatively loose; a broader range of prey can be consumed as long as the frog eats enough

The association of dietary specialization and sequestration of defensive compounds is not novel to frogs. Lepidoptera (Brower 1958; Brower and Brower 1964; Alpin et al. 1968), Coleoptera (Eisner et al. 1962; Rowell-Rahier 1984), Orthoptera (von Euw et al. 1967), Hemiptera (Scudder and Duffey 1972; Braekman et al. 1982; Aliabadi et al. 2002); Hymenoptera (Schaffner et al. 1994), nudibranch mollusks (Thompson 1960; Edmunds 1966), birds (Dumbacher et al. 1992, 2000), and possibly reptiles (Mori and Burghardt 2000) sequester defensive chemicals from their diet. However, the repeated association of these traits in the context of an explicit phylogeny has rarely been demonstrated.

Amphibian skin has two kinds of glands: mucous glands and serous (or granular) glands, which are the "poison glands" of amphibians (Duellman and Trueb 1986). Neuwirth et al. (1979) suggested that serous glands are plesiomorphic among amphibians and that their original function was probably other than poison synthesis, and, therefore, the glands were co-opted for toxin production and/or storage of sequestered compounds. The actual process of sequestration in poison frogs is unknown. In Lepidoptera, substances to be sequestered are reabsorbed through the gut membrane (but not broken down), transported into the hemolymph, and deposited in particular sites of the body (Duffey 1980; Nishida 2002). Many sequestered chemicals are distributed nonrandomly, concentrated in the peripheral integument and wings. In poison frogs, alkaloids sequestered in the skin are not found in the muscle or internal organs (Daly et al. 1994*b*). Also, bitter taste is common in many naturally occurring compounds, including the skin alkaloids of dendrobatids (Daly and Myers 1967; Nishida 2002). Peripheral distribution and bitter taste of alkaloids may have been important in facilitating the evolution of aposematism because predators could directly sample frog tissues without inflicting lethal injuries.

Sequestration has evolved independently in at least four lineages of frogs: Dendrobatidae, *Mantella* of the Madagascan Mantellidae, the Australian myobatrachid *Pseudophryne* (although this frog may endogenously synthesize toxins as well; Smith et al. 2002), and possibly the South American bufonid *Melanophryniscus* (Garraffo et al. 1993*a*, 1993*b*; Daly et al. 1996, 1997). Six classes of frog alkaloids are known to come from ants, one from beetles, and one from a millipede (Daly et al. 1997, 1999, 2002; Jones et al. 1999; Saporito et al. 2003, 2004). However, as far as is known, dendrobatid frogs are unique among vertebrates in their recurring evolutionary association of chemical defense and dietary specialization. The recurrence of correlated instances of diet and skin alkaloids in Dendrobatidae suggests either the presence of an ancestral sequestration mechanism or that the evolution of a se-

questration mechanism may not be particularly complex or difficult to attain (Caldwell 1996; Summers and Clough 2001; Santos et al. 2003; Summers 2003). But, if all dendrobatids have the capacity to sequester toxins, why don't all species do it? Daly (1998) suggested that an uptake system is primitive and merely over expressed as sequestration in frogs that accumulate alkaloids. Daly et al. (1994*b*) found that *Colostethus talamancae* and *C. inguinalis* did not accumulate alkaloids into their skin after being fed with alkaloid-dusted fruit flies for 5 weeks; an identical feeding regimen did result in accumulation of alkaloids in *Dendrobates auratus* and *Phyllobates bicolor*. Interestingly, no adverse effects were reported in *Colostethus* that did not accumulate the alkaloids, suggesting that ingestion of these noxious and/or toxic alkaloids without injurious effects may be plesiomorphic in Dendrobatidae; this ability may or may not be related to the "primitive uptake system" proposed by Daly (1998). In contrast, Daly et al. (1980) reported that the tissues of *Dendrobates histrionicus* were sensitive to *Phyllobates*-specific batrachotoxin (to which *Phyllobates* tissues are insensitive, whether captive raised or wild caught) and that in feeding experiments *Dendrobates* rejected fruit flies dusted with batrachotoxin (Daly et al. 1999), suggesting the ability to ingest batrachotoxin, the most toxic dendrobatid alkaloid found only in *Phyllobates*, is more derived than the ability to ingest other noxious alkaloids. For example, *C. sauli* does not have skin alkaloids but has a small niche breadth, and ants are significant prey (table A2; fig. 1). In fact, most if not all dendrobatids eat at least some ants and appear unaffected by a diet of this generally undesirable prey. These data support the possibility that ingestion of noxious alkaloids with no adverse effects may be ancestral in Dendrobatidae. It may be that sequestration of alkaloids in toxic dendrobatids is a modification of a system that allows all dendrobatid frogs to consume noxious/toxic prey without harm.

Diurnality and Terrestriality as Possible Innovations

Myers et al. (1991) postulated that diurnal and terrestrial habits evolved early in dendrobatid phylogeny based on the nocturnal and aquatic habits of *Aromobates nocturnus*, which was hypothesized to be the sister species of all other dendrobatids. Given this, diurnality and the transition from aquatic (*A. nocturnus*) to riparian (various species of *Colostethus*) and finally to terrestrial (all other dendrobatids, although a few species have become arboreal) habits have preceded the origins of dietary specialization. The evolution of diurnality and/or the shift to terrestriality may be the innovations that contributed to the parallel evolution of toxicity mediated by predation (Toft 1981; Vences et al. 1997/1998). This shift may have exposed frogs to

new food sources, assuming the diurnal leaf-litter arthropod community is different than the nocturnal arthropod community and/or that new prey species were encountered in the move to terrestriality. If a small degree of unpalatability (possibly acquired as a modification of a system that allows ingestion of noxious prey with no adverse effects) conferred at least some protection from predation, then natural selection exerted by predators would continually favor defended individuals, thereby promoting greater dietary specialization and a more efficient sequestration mechanism. For example, at least three species of *Dendrobates* have evolved a specific enzyme that converts a pumiliotoxin to a much more toxic allopumiliotoxin

Acknowledgments

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