Amphibia-Reptilia 33 (2012): 11-24

Geographic and intrapopulational variation in colour and patterns of an aposematic toad, *Melanophryniscus rubriventris* (Amphibia, Anura, Bufonidae)

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Abstract. The aposematic toad genus *Melanophryniscus* is a polymorphic group with noticeable variation in colour and pattern. Here, we objectively evaluate variation in spectral reflectance and quantify variation in patterning within and among populations of the species *M. rubriventris* in NW Argentina. We conducted spectrophotometric analysis on 69 individuals and recorded dorsal and ventral pattern morphs of 727 individuals from six populations. We found high divergence in the reflectance spectra, the extent of brightly coloured areas, and the skin alkaloid profiles with no correlation among coloration varieties, alkaloid profiles, and the geographic distance between populations. Our analyses imply subdivision of sampled populations groupings based mostly on different dorsal colorations. Our results also reveal that populations with very similar patterns may differ markedly in colour and vice versa. It is striking that these aposematic toads show a pronounced variation in colour and patterning among and within populations showing individuals with a conspicuous bright dorsal colouration but also morphs with a rather cryptic black or drab colouration. However, the known presence of several alkaloids classes in all populations suggests that all morphs might be equally unpalatable.

Keywords: alkaloids, poison toad, polymorphism, spectral reflectance.

Introduction

Amphibians display an extensive array of colour and patterning with widespread polymorphisms for which a variety of mechanisms have been proposed (Hoffman and Blouin, 2000). Knowledge of variation is of prime importance to understand the maintenance of polymorphism (Bond, 2007). Changes in colour are likely controlled by different evolutionary and developmental pathways than those which produce a pattern (Frost-Mason, Morrison and Mason, 1994; Hoffman and Blouin, 2000). The effects of different types of selection acting on different components of individual phenotypes may result in the composite of colour and patterns that are ultimately displayed (Grether, Kolluru and Merissian, 2004; Wollenberg et al., 2008).

While several studies have assessed the variation of colour and patterns across species and populations of anurans, these were mostly focused on the colourful Neotropical poison frog family Dendrobatidae (e.g. Summers et al., 1997; Summers and Clough, 2001; Summers, Cronin and Kennedy, 2003; Roberts et al., 2007; Wang and Shaffer, 2008; Wollenberg et al., 2008). Intraspecific aposematic polymorphisms are frequently observed in such poison frogs (Summers et al., 2003; Wang and Shaffer, 2008; Wollenberg et al., 2008) and increasing evidence are showing frequent shifts in colouration with multiple losses of bright dorsal colouration (Wang and Shaffer, 2008; Wang and Summers, 2010).

The importance of bright colour in signaling unpalatability has been shown in several studies (Rowe and Guilford, 2000; Exnerová et al., 2006; Aronsson and Gamberale-Stille, 2008). In contrast, dull colourations of many anurans are commonly viewed as being for concealment (Duellman and Trueb, 1994; Toledo and Haddad, 2009). However, it was predicted that highly defended prey should evolve less

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 DOI:10.1163/156853811X619754

conspicuous colouration because their chances of surviving attacks are enhanced, and therefore, costs involved with conspicuous signaling can be reduced (Speed and Ruxton, 2007). Experienced avian predators learn to avoid red-black aposematically coloured insects but also demonstrate a similar degree of avoidance learning over pale forms that also possess functional stink glands (Gamberale-Stille, Johansen and Tullberg, 2010).

The South American aposematic toad genus *Melanophryniscus* comprises a highly polymorphic group with colour and pattern variation apparent both among and within species (Kwet et al., 2005). Despite an increasing knowledge in the diversity of these polymorphic poison toads, there have been a few descriptions that quantify variation in patterns and colour either within or among populations (Vaira, 2002; Cairo and Di Tada, 2005; Kwet et al., 2005). Noticeable colour variation has been described within and among populations of one species from NW Argentina, *Melanophryniscus rubriventris*, where also skin analysis has revealed an array of 46 different lipophilic alkaloids with considerable variation in population-level profiles (Laurent, 1973; Vaira, 2002; Daly et al., 2007). Although the species was first formally described as a toad with a black background and bright orange colouration covering the scapular region and partially the head, dorsum and flanks, with a uniform red ventral colouration (Vellard, 1947), different morphs of this species, diagnosed as subspecies, have appeared subsequently in the literature where some populations have toads that predominately showed a more cryptic olive to black dorsal pattern (Laurent, 1973). However, it was shown that populations showing "cryptic" colourations contained higher numbers of potentially toxic skin alkaloids than the present in populations with a rather "brighter" colouration (Daly et al., 2007). Similar results, challenging the tenet that greater conspicuousness coevolves with increased toxicity were recently presented by two species of poison frogs (Darst, Cummings and Cannatella, 2006; Wang, 2011).

The ability to detect and quantify subtle colour differences is critical for studies involving colour evolution, and this is especially true of questions regarding the evolution and maintenance of aposematic signals (Speed and Ruxton, 2007). Spectrophotometric methods have become increasingly important in evolutionary and ecological studies because they are not subject to the biases of visual systems (Bennett, Cuthill and Norris, 1994; Isaksson et al., 2008). The quantitative detail provided by spectral data may afford insights into the evolution of colour polymorphism by allowing the investigation of small colour differences among species or populations (e.g. Summers et al., 2003; Wollenberg et al., 2008).

Here, we quantify variation in colour and quantitatively describe patterning variation in six different populations of *Melanophryniscus rubriventris* across the range of the species in NW Argentina. The aim of this paper is threefold. First, we assess the magnitude of geographic divergence of dorsal and ventral bright colouration and pattern morph among six populations. Secondly, we account for relationships among the extent of bright colouration with the skin alkaloid profiles previously known in four populations, and thirdly, we compare the skin alkaloids compositions among these populations accounting for the number of shared alkaloids between populations. This will enable us to examine the influence of population origin and/or geographic distances among populations on the variation of colour, pattern morph, and shared alkaloids.

Materials and methods

We sampled 6 different localities across NW Argentina (fig. 1): El Nogalar de Los Toldos (S 22°16′912″; W 64°43′ 6.45″; 1635 m asl); Canto del Monte (S 22°22′3.9″; W 64°43′16.03″; 1659 m asl); Cedral de Baritú (S 22°27′ 35.76″; W 64°44′32.94″; 1689 m asl); Tablada (S 23°05′ 917″; W 64°51′43.20″; 1725 m asl); Abra Colorada (S 23° 40′52.36″; W 64°54′493″; 1722 m asl), and Angosto de

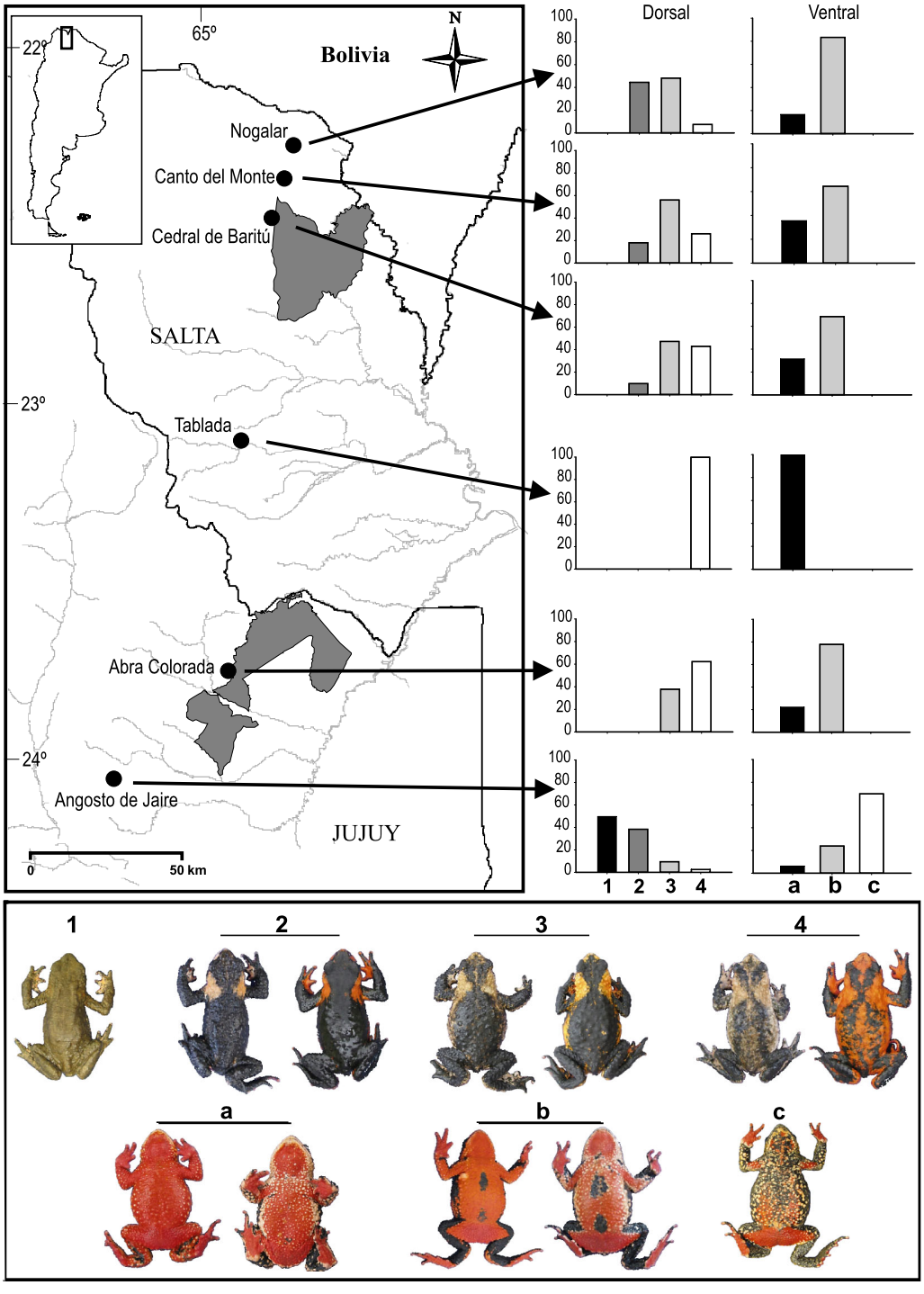

Figure 1. Geographic distribution of studied populations of *Melanophryniscus rubriventris* in NW Argentina (left corner) and representative individuals from dorsal and ventral pattern morphs (bottom). Bar charts in right corner show percent occurrence of each pattern in the studied populations. Number 1-4 and letters "a"-"c" in bar charts and photographs indicates dorsal and ventral pattern scores respectively (see text for description of each pattern scores). This figure is published in colour in the online version.

Jaire (S 24°01′23.19″; W 65°23′24.49″; 1665 m asl). A total of 727 toads were captured by hand during two rainy seasons (November to January, 2007-2008 and 2008-2009). For each individual we took dorsal and ventral images with a digital camera under natural lighting conditions for subsequent analysis of colour pattern. Toads were returned to their point of capture within 24 h with the exception of 69 individuals that were transferred to the laboratory to do spectrophotometric measurements. For these we measured reflectance spectra from five non-black body regions of adult toads to obtain a reasonably comprehensive characterization of the bright toad colouration: from the dorsum – right and left scapular area and for the ventral region – throat, belly, and pelvic patch. Those patches exist for individuals from every population and were of sufficient size to allow at least one reflectance measure. The measurements from each body region were averaged separately by dorsum and venter for subsequent statistical analysis (see below).

We measured the spectral reflectance characteristics (at ±3.5 nm resolution) of body regions with an Ocean Optics USB-2000 spectrophotometer (Ocean Optics, Inc., Dunedin, Florida) with illumination provided by a PX-2 pulsed xenon light source (effective range of emission from 220 to 750 nm) fitted to a 400 mm fibre-optic cable and calibrated against a barium sulphate standard following Bleiweiss (2007). Before measuring each specimen, a new calibration was done to correct for possible shifts in the performance of the spectrophotometer. Skin was illuminated and reflected light collected at 45° to the body surface. A bifurcated fibre optic cable was used, which allowed for illumination and detection to occur through the same probe. The probe was mounted in a prismatic probe holder to standardize measuring distance and to exclude ambient light, and was held (not pressed), over the selected region of the study skin. The diameter of the measured area was approximately 6 mm, and the distance between the probe and the skin was 25 mm.

Colorimetric analyses were made over the 320-700 nm spectral range. We excluded reflectance values below 320 nm because of considerable noise at these wavelengths. Variables considered in this study were average reflectance values for 5 nm bins for each individual. The colour variables derived for each spectrum were calculated following Bleiweiss (2007); Pryke, Andersson and Lawes (2001) and Pryke, Lawes, and Andersson (2001). We used spectra to derive traditional measures of brightness or luminance of the light spectrum (spectral intensity), hue (spectral location), and chroma (saturation or spectral purity). 'Brightness' (hereafter $R_{320\text{-}700}$) was calculated as the sum of reflectance from 320 to 700 nm; 'Hue' (λR50), as the wavelength at which reflectance is halfway between its minimum (λR_{min}) and its maximum (λR_{max}); and 'Chroma' ($R_{contrast}$), which depends on several reflectance shape aspects such as both slope height and steepness. To avoid a fixed arbitrary spectral segment, we used λR_{min} as the segment divider, and computed $R_{contrast}$ using the formula $(R_{\lambda R\,min\,\text{-}700} - R_{320\text{-}\lambda R\,min})/R_{320\text{-}700}$. Pryke et al. (2001a) observed that some reflectance components explain significant variation in human colour perceptions of hue, and chroma as estimated by the CIE (International Commission on Illumination) system of human colour space. Therefore, we used these components of reflectance as an objective way to infer at least human-perceived colour differences.

Avian predators are believed to be the predominant predators of many aposematic species, including dendrobatid frogs. In several field-based experiments, frog models were considered frequently attacked by birds (Saporito et al., 2007; Noonan and Comeault, 2009). Because birds perceive colours differently from us and many species are sensitive to ultraviolet wavelengths (Chen, Collins and Goldsmith, 1984; Eaton and Lanyon, 2003) we also measured wavelengths of peak reflectance in the ultraviolet part between 320-400 nm (λR_{UVpeak}), and half-maximal values for the UV peak (λR_{UV50}).

To assess the relative surface areas that were black (or drab) versus brightly coloured we used the standardized high-resolution colour digital photographs of the dorsum and ventral parts imported into the public domain image processing program ImageJ 1.41o (National Institutes of Health, USA, available at http://rsb.info.nih.gov/ij). Images were converted to grayscale of 8 bits, and outlines of the entire body area and the black area were drawn. The extent of both brightly coloured and black areas were then calculated from outline areas and determined as percentages of the total area of the body.

Because colour measurements obtained by spectrophotometric analyses may represent some degree of redundancy, we conducted a principal components analysis (PCA) to reduce the number of variables. The principal components axes were Varimax-rotated to maximize their correlation (loading) with original variables. To analyze possible geographic divergences among colouration traits, we ran a discriminant function analysis (DFA) on the colouration variables obtained from the reflectance spectra and on the extent of the area of bright colour. DFA were performed using colour readings and bright area from dorsal and ventral values as the dependent variable and populations as the grouping variable. We examined how well individuals could be classified into their original populations (based on Mahalanobis distances to each population centroid), and identify the discriminant function that best characterized the differences among populations. The degree of divergence was directly estimated from the standardized coefficients in the discriminant functions. The higher the absolute value of the coefficient the larger the among-populations divergence of the corresponding variable.

Endler and Mielke (2005) advise that multivariate techniques should not be used to tests for differences among colours obtained from radiance spectra because some fundamental assumptions seem to be violated when applied to spectrophotometric data. However, they considered multivariate techniques appropriate for exploratory data analysis. So we used PCA only to explore and describe patterns of variation in the data, and DFA only to analyze which variables best discriminate and contribute the most variation among prespecified groups (populations).

The pattern morphs were described by one of us (MIB) scoring every individual based on a subjective categorization scheme for dorsal and ventral patterns separately. Dorsal patterns of toads were scored from 1 (almost completely

black or drab) to 4 (an equal proportion of black and bright colouration) (see fig. 1). Ventral patterns were binned into three categories from 1 (brightly uniform colouration) to 3 (completely speckled) (fig. 1). We tested the null hypothesis of no effect of population origin on the variations of pattern morphs scores. Because the variance of frequency of dorsal and ventral pattern morph was not similar across populations, we analyzed the effect of population origin on the pattern variation score using non-parametric Jonckheere-Terpstra tests (Pirie, 1983).

To assess whether there was an association between geographic distances and colouration, average pattern morph, and shared alkaloids distances among populations, we used a Mantel (1967) non-parametric matrix permutation approach. We first calculated all pairwise population distances (Mahalanobis generalized distances) from colouration variables and average pattern morphs. A Bray-Curtis dissimilarity index of alkaloid content was calculated between all populations pairwise. Also, pairwise straight-line geographic distances were calculated between all populations. We tested for association between each pair of the resulting dissimilarity matrices via permutation techniques using PASSaGE version 2 (Rosenberg and Anderson, 2011) with 10 000 permutations. All other tests were performed with SPSS for Windows 15.0 (SPSS Inc., Chicago, IL, U.S.A.).

Results

We found marked variation in colouration across populations with no discrete differences in spectral measurements. Rather, spectral values appeared to vary continuously with considerable overlap between populations in many cases ranging from 520 to 595 nm (table 1). All dorsal and ventral spectra have a major reflectance band extended over longer (500-700 nm) visible wavelengths that accounted for more of the total reflectance and a band of low reflectance in the near-UV with a minimum between 320 and 430 nm depending on the body region and population considered (fig. 2). However, among populations spectra of dorsal colour did differ in a secondary smaller reflectance band that extended from short visible (450 nm) through near-UV (320-400 nm) wavelengths. The reflectance of incident UV wavelengths varied markedly across the populations sampled. Toads from Cedral de Baritú, Tablada and Angosto de Jaire reflected less than 5% of the UV wavelengths. By contrast, toads from Abra Colorada and Nogalar de Los Toldos have a UV reflectance greater than 5% and 10% respectively, and individuals from Canto del Monte reflect in excess of 15% of incident UV wavelengths (fig. 2).

Geographic variation, mostly on the dorsal area, was evident not only in colouration but also in the extent of brightly coloured areas (fig. 3). The colouration variables and median pattern morphs values for each locality are listed in table 1. Our PCA of dorsal colouration reduced to two principal components that explained 73% of total variance with large positive loadings on the three colour measures (brightness, hue and chroma). PCA for ventral colouration also reduced to two principal components explaining 68% of the original variance with large loadings on two colour measures (hue and chroma) and reflectance characteristics of UV wavelengths (table 2). The first two functions of the discriminant function analysis summarized over 96% of the colour variation. DF1 alone explained 71% of variation among populations and revealed the largest differences to be in dorsal colouration (see table 3). Our analyses imply subdivision of sampled populations groupings based on different dorsal colourations. Populations of Nogalar de Los Toldos, Canto del Monte, and Angosto de Jaire show dull yellow or pinkish colouration with similar extents of bright colouration. Toads from Cedral de Baritú, Tablada, and Abra Colorada show more intense colourations ranging from orange to vivid red, with the extent of bright colouration also considerably higher than in the preceding three populations (fig. 3).

Even with geographic distance between locales spanning 10 to 200 kilometers (fig. 1), we found no significant matrix correlations between geographic distance and colouration (fig. 4). We also found no such correlation between both dorsal and ventral colour Mahalanobis distances ($r_{\text{dorsal}} = 0.20$, $P_{\text{dorsal}} = 0.49$; $r_{\text{ventral}} = 0.24$, $P_{\text{ventral}} = 0.43$).

Populations of *Melanophryniscus rubriventris* also differed significantly in colouration patterns (figs 1 and 3). The species is polymorphic throughout much of its geographic range

Table 1. Population differences in spectral variables in each body region (dorsal and ventral) for six populations of *Melanophryniscus rubriventris* in NW Argentina.

Variable	Body region	Nogalar ($n = 9$)	Canto del Monte ($n = 10$)	Cedral de Baritú ($n = 10$)	Tablada ($n = 11$)	Abra Colorada ($n = 19$)	Angosto de Jaire ($n = 10$)
λR_{50} (hue)	dorsal	537 ± 8.7	540 ± 19.4	573 ± 4.8	582 ± 4.8	572 ± 8.7	527 ± 13.8
	ventral	587 ± 2.5	586 ± 9.5	590 ± 4	593 ± 4.2	589 ± 3.5	582 ± 8.7
$R_{contrast}$ (chroma)	dorsal	0.03 ± 0.002	0.03 ± 0.01	0.04 ± 0.002	0.04 ± 0.002	0.03 ± 0.002	0.02 ± 0.004
	ventral	0.04 ± 0.004	0.04 ± 0.005	0.04 ± 0.004	0.04 ± 0.002	0.04 ± 0.002	0.03 ± 0.003
$R_{320\text{-}700}$ (brightness)	dorsal	1615 ± 429	2250 ± 528	1459 ± 174	1318 ± 261	1678 ± 316	896 ± 560
	ventral	1208 ± 348	1197 ± 468	1111 ± 219	1076 ± 123	1170 ± 202	963 ± 309
λR_{min}	dorsal	324 ± 5.5	333 ± 34.3	350 ± 42.2	363 ± 39.5	405 ± 42.9	326 ± 10
	ventral	399.7 ± 49.7	401.2 ± 50.3	432.2 ± 60.6	397.2 ± 42.1	409.2 ± 43.7	424.2 ± 6.0
λR_{UVpeak}	dorsal	390 ± 16.6	394 ± 2.1	347 ± 19.6	362 ± 28.9	350 ± 21.7	361 ± 25.5
	ventral	348.5 ± 18.2	358.7 ± 29.8	340.8 ± 15.4	346.2 ± 25.5	343.8 ± 16.2	340.7 ± 9.8
Percent of coloured area	dorsal	12 ± 4.9	10 ± 8.2	26 ± 7.8	25 ± 9.3	31 ± 10.0	9 ± 12.6
	ventral	80 ± 5.7	78 ± 7.0	83 ± 7.3	86 ± 3.8	86 ± 5.1	72 ± 8.9
Pattern morphs	dorsal	3 (2-4)	3 (2-4)	3 (2-4)	4 (4-4)	4 (3-4)	2 (1-4)
	ventral	2 (1-2)	2 (1-2)	2 (1-2)	1 (1-1)	2 (1-2)	3 (1-3)

Note: Values represent means ± SD except for Pattern morphs expressed as median and ranges.

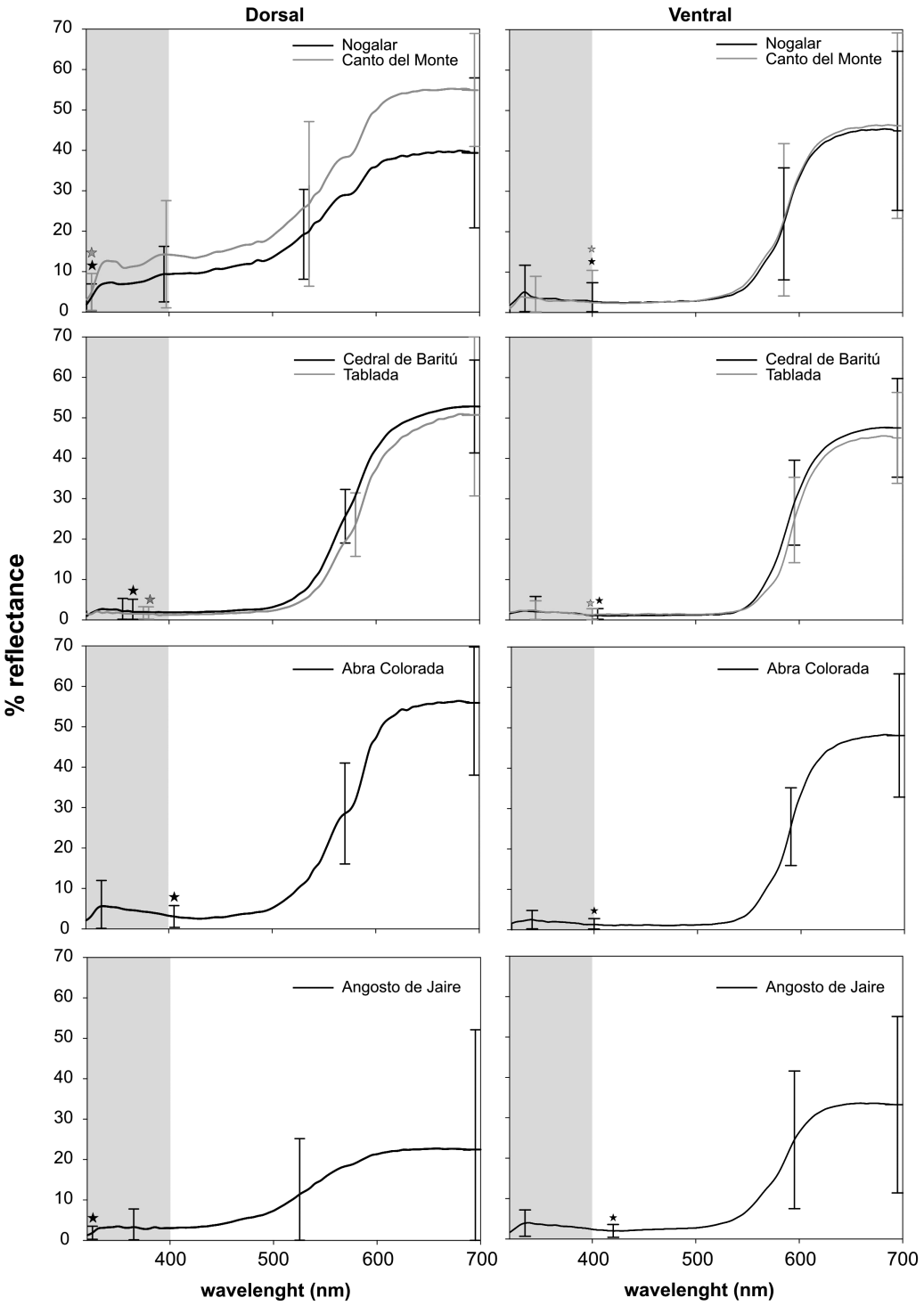

Figure 2. Results of the reflectance spectrometric analyses from dorsal and ventral colouration of six populations of *Melanophryniscus rubriventris* from NW Argentina. Each figure indicates population mean reflectance curves (dark and light line) obtained from three skin measurements per individual. Variation among toads of the same population is shown by the thin range bars for λR_{min} (indicated by a star), $R\lambda_{UVpeak}$, λR_{50}, and R_{max} values (see text for description of spectral components).

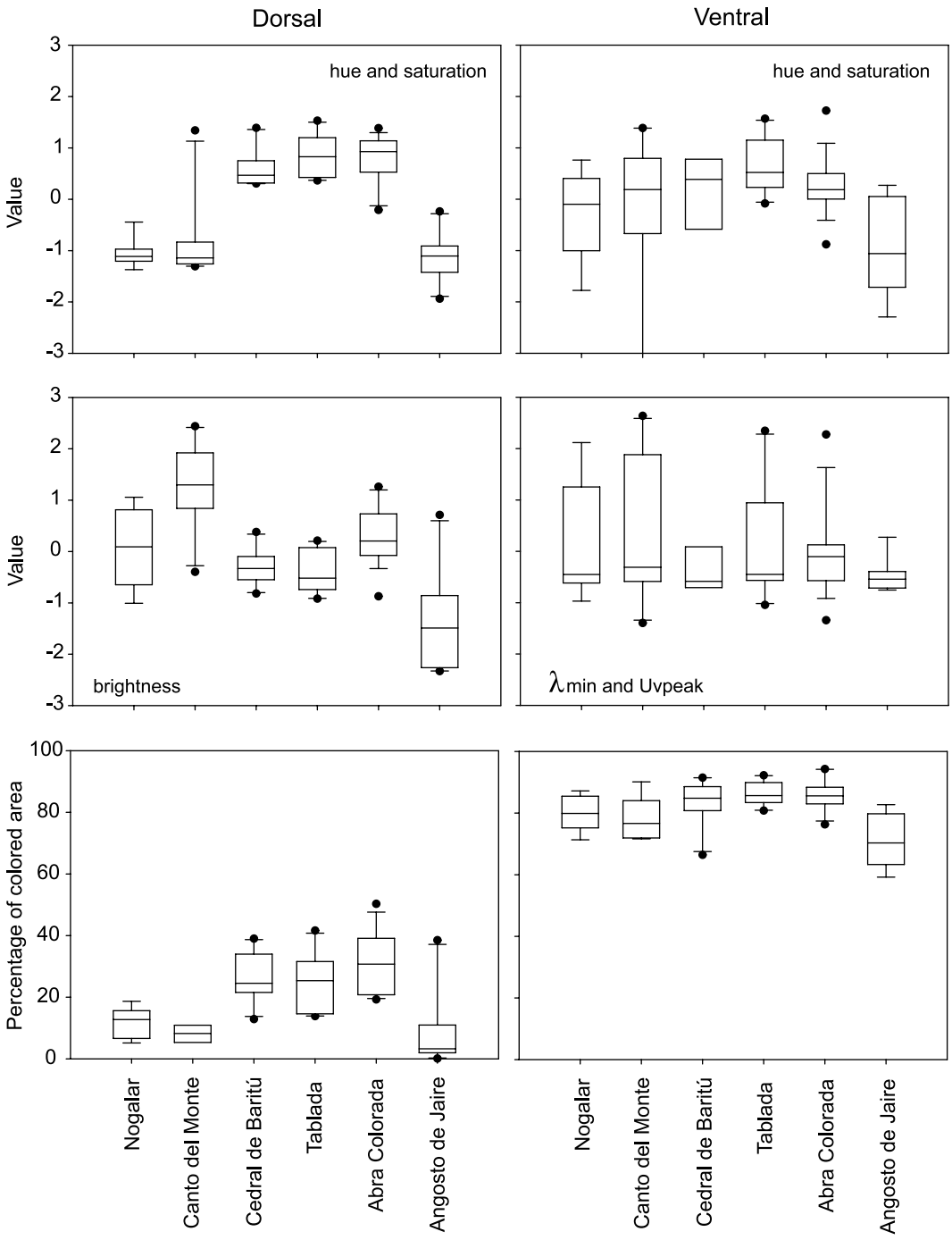

Figure 3. Geographic variation in dorsal and ventral colouration traits among six populations of *Melanophryniscus rubriventris* from NW Argentina. Values correspond to principal component scores (see table 2) and the percent of coloured area correspond to the amount of bright skin with respect with total body area. Bottom and top of the box is the 25th and 75th percentile. Horizontal line represents the media. Whiskers are the lowest and highest still within 1.5 IQR of the upper and lower quartile. Outliers are represented by a dot.

Table 2. Principal component analyses summarising variation in dorsal and ventral colouration of *Melanophryniscus rubriventris* from six populations from NW Argentina.

	PC1	PC2
Dorsal		
λR50 (hue)	**0.941**	−0.006
$R_{contrast}$ (chroma)	**0.853**	−0.176
$R_{320\text{-}700}$ (brightness)	−0.057	**0.982**
λR_{min}	0.742	0.108
λR_{UVpeak}	−0.645	0.264
Eigenvalue	2.64	1.02
% of variance explained	52.8	20.3
Ventral		
λR50 (hue)	**0.910**	−0.039
$R_{contrast}$ (chroma)	**0.893**	0.114
$R_{320\text{-}700}$ (brightness)	−0.584	0.055
λR_{min}	−0.104	**−0.846**
λR_{UVpeak}	−0.112	**0.843**
Eigenvalue	1.99	1.44
% of variance explained	39.8	28.8

Note: Numbers represent loadings of original variables on principal components. The highest loadings are outlined in bold, when their value was higher than 0.8.

Table 3. Standardised coefficients (and percentage of explained variation) of the first two canonical discriminant functions that predict population membership of *Melanophyniscus rubriventris* according to colouration.

	DF1 (70.9%)	DF2 (25.4%)
PC1 dorsal	**0.811**	0.103
PC2 dorsal	−0.058	**1.016**
Area of colour dorsal	0.544	−0.256
PC1 ventral	0.604	−0.188
PC2 ventral	−0.259	0.451
Area of colour ventral	0.019	0.270

Note: Variables are principal components that summarise measured variables of colouration (see table 2) and the extent of the area of bright colour. The highest coefficients in each discriminant function are outlined in bold.

with two to four different dorsal patterns and two and three ventral patterns within and among populations. We found a significant effect of population origin in frequency of dorsal and ventral patterns (Jonckheere-Terpstra test statistic for dorsal patterns $= -5.97$, $P < 0.001$; J-T test for ventral patterns $= 10.03$, $P < 0.01$). Individuals from Tablada had a mostly uniform dorsal and ventral pattern with only slight variation among individuals. Toads from Nogalar de

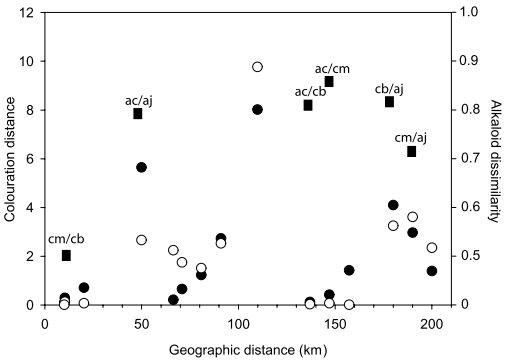

Figure 4. Pairwise relationships between geographic distances, colouration distances and shared alkaloids among populations of *Melanophryniscus rubriventris* from NW Argentina. Each data point represents the contrasts between two populations (black circles: dorsal colouration; white circles: ventral colouration; black squares: alkaloids). Letters above the black square represent population pairwise. (**cm**: Canto del Monte; **cb**: Cedral de Baritú; **ac**: Abra Colorada; **aj**: Angosto de Jaire.) Colouration distance are the Mahalanobis' generalized distances calculated from the scores of two principal components that summarize original variables (see table 2) and the average pattern morphs. Alkaloid dissimilarities are expressed as Bray-Curtis dissimilarity index values.

Los Toldos, Canto del Monte, Cedral de Baritú, and Abra Colorada show similar variable dorsal and ventral patterns within and among populations. Strikingly, the southern population of Angosto de Jaire shows a pronounced intrapopulational variation with strongly variable dorsal and ventral patterns with a continuum from a complete absence of bright colouration to grades of dull yellow and/or reddish patches (dorsum) and dots (belly) (see fig. 1 and table 1).

Previous population-level analyses indicate that lipophilic alkaloids were present in the skin of toads from four populations analyzed in this study (Daly et al., 2007). Forty-six different alkaloids were detected in populations of Canto del Monte (23 alkaloids), Cedral de Baritú (12 alkaloids), Abra Colorada (10 alkaloids), and Angosto de Jaire (listed as Tiraxi in Daly et al., 2007), 19 alkaloids. However, only 13 alkaloids were shared by at least two populations and just two were common to the four populations (table 4; see also table 4 in Daly et al., 2007). The less-conspicuous and most distant

Table 4. Population differences in skin alkaloid profiles for four populations of *Melanophryniscus rubriventris* in NW Argentina. Listed structural classes of alkaloids and number per class (in parentheses) were obtained from Daly et al. (2007).

	Canto del Monte	Cedral de Baritú	Abra Colorada	Angosto de Jaire	Present in two pops	Present in all pops
Pumilitoxins (14)	9	6	3	7	8	1
Deoxyhomopumiliotoxins (1)	1	–	–	–	–	–
Allopumiliotoxins (1)	–	–	–	1	–	–
5,8-Disubstituted indolizidines (3)	–	–	1	2	–	–
3,5-Disubstituted indolizidines (1)	1	–	–	–	–	–
5,6,8-Trisubstituted indolizidines (3)	3	1	1	2	1	1
Izidines (8)	1	2	4	2	1	–
Tricyclics (10)	4	3	1	3	2	–
Unclass (5)	4	–	–	2	1	–
Total number of alkaloids	23	12	10	19	13	2
Non shared alkaloids	10	3	7	11		

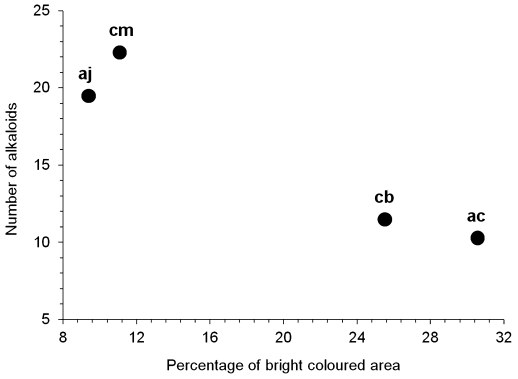

Figure 5. Pairwise relationships between the percent of extent of bright colouration and number of alkaloids among populations of *Melanophryniscus rubriventris* from NW Argentina. Letters above the black square represent populations. (**cm**: Canto del Monte; **cb**: Cedral de Baritú; **ac**: Abra Colorada; **aj**: Angosto de Jaire.)

populations analyzed (Canto del Monte and Angosto de Jaire), showed low dissimilarity index and the highest number of alkaloids detected. We found no significant matrix correlations between geographic distance and number of shared alkaloids between populations (figs 4 and 5).

Discussion

Melanophryniscus rubriventris is a highly polymorphic species with significant divergence in colouration as revealed by spectrophotometric analysis and in the extent of bright colouration. These differences are uncorrelated with between-population geographic distance. Moreover, closest populations are characterized by the highest colour and pattern polymorphism expressed mainly as differences in spectral location and purity (hue and chroma), and pattern morph of dorsal body regions. Populations of Nogalar de Los Toldos, Canto del Monte, and Angosto de Jaire (northernmost and southernmost populations) predominately showed a more cryptic olive to black dorsal pattern with dull yellow to pinkish dorsum. Meanwhile populations of Cedral de Baritú (a northern population), Tablada and Abra Colorada (central populations) have individuals with bright uniform dorsum, differing mainly in the extent of black patches. Concomitantly, individuals from northern and central populations have a mostly uniform pinkish to red belly whereas the southern population of Angosto de Jaire has toads with well-demarcated yellow, red and black speckled bellies.

Pronounced differences in warning dorsal colour and pattern, including the loss of bright colouration, among adjacent geographic populations with rather stable polymorphisms within populations is known in other aposematic frogs (Wang and Shaffer, 2008; Wollenberg et al.,

2008; Comeault and Noonan, 2011). Such variation in the degree of the aposematic trait may have no evolutionary consequences if colour variants perform equally well regarding detection and recognition by receivers. It is possible that drab individuals from northern and southern populations are not by implication cryptic forms and they merely have relatively inconspicuous dorsal warning displays but equally effective with the local predator ensemble. Recent studies showed that predators could perceive frogs aposematic signals differently across small spatial scales (Noonan and Comeault, 2009; Comeault and Noonan, 2011).

Potential predators may also simultaneously evaluate more than a colour or pattern property; instead of this a combination of several traits may determine the warning signal recognition. In a study of colouration and pattern of a dendrobatoid frog, the bright dorsal colour-pattern component were considered a multicomponent signal clearly visible from a bird, mammal or reptile perspective (named the "displayed" component) and appeared to be an adaptive trait. Meanwhile, the ventral colour-pattern (named the "concealed" component) was considered a non-adaptive trait (Wollenberg et al., 2008). However, ventral pattern and colouration in *Melanophryniscus rubriventris* could also turn visible when broadcast via postural adjustments. Typically, this toad compresses the body and elevates the head, arms, and foot during a stereotyped defensive display considered as an "Unken Reflex" to expose part of the ventral colouration (Laurent, 1973). A geographical variation in such aposematic behaviour paralleling differences in ventral aposematic colouration was found in a poison salamander (Mochida, 2009). Notably, the ventral colouration and patterning of *Melanoprhyniscus rubriventris* were less variable than the dorsum along the studied populations.

Our spectrophotometric results revealed that UV reflectance is a significant component of dorsal colouration in some populations of *M. rubriventris*. Birds perceive colours differently from us and many species are sensitive to ultraviolet wavelengths (Endler and Mielke, 2005). Consequently, a significant portion of the colour pattern of the toads might be available only to birds that are physiologically capable of seeing UV wavelengths (Chen et al., 1984). Even when some colour morphs of *M. rubriventris* have relatively little reflectance in the ultraviolet part of the spectrum, research has shown that even small UV differences (less than 5%) can influence birds' behaviours (Hunt et al., 2001). It would be interesting to test the ability of potential avian predators of *M. rubriventris* to perceive these subtle UV differences with broad implications for future studies.

Carotenoids are largely responsible of red, orange, and yellow colours in amphibians (Hoffman and Blouin, 2000). The reflectance spectra of a carotenoid-based colouration show a pronounced reflectance peak in the ultraviolet (UV) wavelength (between 300 and 400 nm), low reflectance in short to middle wavelengths (around 450 nm) because of the light-absorbing properties of carotenoid pigments, and a high plateaulike reflectance curve in the long waveband between 550 and 700 nm (Bleiweiss, 2007). The spectral reflection curves obtained in this study suggest that red, pink, and yellow colourations in *Melanophryniscus rubriventris* may be due to a carotenoid origin. It is possible that a different mixture of carotenoid pigments in the skin could be responsible for the bright colouration differences observed in the different populations. Additional biochemical evidence is required to support this assumption.

Our analyses also reveal populations with similar patterns that differ markedly in colour and vice versa, suggesting that colour and pattern can vary independently. It is possible that different developmental mechanisms might be responsible of patterning and colour evolution. Colour patterning in *Melanophryniscus rubriventris* was defined mainly by the extension and shape of dorsal and ventral melanic black patches. Melanin-based pigmentation in vertebrates are widely studied and many differ-

ent molecular and developmental changes were demonstrated to affect the type, density and distribution of melanin synthesized endogenously by the animal (Hubbard et al., 2010). Several studies have linked remarkable variations in the melanin-based pigmentation with environmental heterogeneity (Hoekstra, 2006). On the other hand, if we can assume that bright colouration in *M. rubriventris* appears to be originate from carotenoids, we would expect less genetic control on this trait that are tightly linked to the availability of such pigments through diet and being condition-dependent (Hubbard et al., 2010). Consequently, proximate mechanism responsible for colour and patterning variation could be substantially different driving to different combinations in each population.

Bright colouration in *Melanophryniscus*, as in other poison frogs and toads, was considered mainly to serve in interspecific communication, signaling their distastefulness to potential predators as a consequence of the presence of lipophilic alkaloids in the skin (Daly, 1995). A study of alkaloids contents in the skin of four populations of *M. rubriventris* considered in the present work showed strong variation in the profiles among these populations (Daly et al., 2007). Population-level analyses indicate that lipophilic alkaloids were present in the skin of toads from every population most of them unshared but differences were unrelated with geographic distance between populations. Although Canto del Monte and Angosto de Jaire populations showed the less-conspicuous dorsal colourations, the highest numbers of alkaloids were detected in their skins. However, given the small and unbalanced sample sizes of toads that were analyzed those results should be cautiously considered (see Daly et al., 2007).

Recent work in poison frogs revealed that less conspicuous color morphs might be even more toxic than the brightest morphs (Darst et al., 2006; Wang, 2011). The level of toxicity has not been measured in the studied populations of *M. rubriventris*, but the presence of an important set of shared and unshared alkaloids in all populations despite the colour or pattern suggest that all morphs might be at least unpalatable to any potential predator attempting to attack any toad. Although the evolution of aposematism requires some degree of toxicity, there need not be a positive correlation between level of toxicity and level of aposematic colouration (Speed and Ruxton, 2007). However, we do not know whether or not the different morphs of *M. rubriventris* differ in unpalatability just because their alkaloids profiles are different, and if the degree of unpalatability could affect the avoidance learning of predators of the species.

Our present data do not allow us to identify the mechanisms underlying the striking colour variation within and between populations of *M. rubriventris.* We argue that the patterns of colour variation within and among populations of *M. rubriventris* must be considered with reference to both the suite of predators that may be present and the physical environment that may influence detection probabilities. Colouration may also have functions not related exclusively to avoid predation. The lowest reflectance band in the spectra of bright morphs of *M. rubriventris* were predominantly in the middle wavelength region (400-550 nm), which corresponds to the spectral sensitivities reported from the rod and cone photoreceptors in a diurnal poison frog, *Dendrobates pumilio* (Siddiqi et al., 2004). Females of this species were noted to discriminate among potential mates using colours as a visual cue displaying a significant preference for their own morph (Summers et al., 1999). It is possible, then than female choice can be an important factor in the colour divergence of *M. rubriventris* populations.

As a final consideration, thermoregulation has also been invoked as an important consideration in the evolution of anuran colour (Hoffman and Blouin, 2000; Vences et al., 2002). For example, black colouration may be important for heat absorbance and may influence individual capacity to regulate temperature (Forsman, 2000). Polymorphic populations with different capabilities and tolerances may therefore

arise in response to varying climate (Forsman and Hagman, 2009). Darkly pigmented morphs could arise as a result of selection for the ability to absorb heat more readily, a clear advantage in cooler climates. The fact that the relative abundance of dorsal colour patterns varies across populations may suggests that the association between colour and thermoregulation could be important in *Melanoprhyniscus rubriventris*. This species occurs typically at altitudes between 1000 to 2000 m asl in subtropical montane forests with cool climates at the highest portions (Vaira, 2002).

The study of the role of predation avoidance, sexual selection, and temperature as well as others factors (e.g. diet; health) affecting the expression of colouration might allow better understanding of the evolution of aposematic colouration in *Melanophryniscus rubriventris*.

Acknowledgements. This research was supported by a Secter-UNJu grant # D-073 and a PIP Conicet grant # 228. Permits for sample collection of the species were provided by Delegación Técnica de Parques Nacionales, Regional Noroeste and Dirección Provincial de Políticas Ambientales y Recursos Naturales, Jujuy. We are especially grateful to O. Peinado Reviglione and J. Nuñez for the help provided during fieldwork. We are particularly indebted to S. Lougheed for providing us the spectrophotometer and valuable comments on the manuscript. Early versions of the manuscript were greatly improved by the comments of two anonymous referees.

References

Aronsson, M., Gamberale-Stille, G. (2008): Domestic chicks primarily attend to colour, not pattern, when learning an aposematic coloration. Anim. Behav. **75**: 417-423.

Bennett, A.T., Cuthill, I.C., Norris, K.J. (1994): Sexual selection and the mismeasure of colour. Am. Nat. **144**: 848-860.

Bleiweiss, R. (2007): On the ecological basis of interspecific homoplasy in carotenoid-bearing signals. Evolution **61**: 2861-2878.

Bohlin, T., Tullberg, B.S., Merilaita, S. (2008): The effect of signal appearance and distance on detection risk in an aposematic butterfly larva (*Parnassius apollo*). Anim. Behav. **76**: 577-584.

Bond, A.B. (2007): The evolution of color polymorphism: crypticity, searching images, and apostatic selection. Annu. Rev. Ecol. Evol. Syst. **38**: 489-514.

Cairo, S.L., Di Tada, I. (2005): Patrones de coloración de *Melanophryniscus* sp. (Anura: Bufonidae) en Sierra de la Ventana (Buenos Aires, Argentina). Bol. Asoc. Herpetol. Esp. **16**: 44-49.

Chen, D., Collins, J.S., Goldsmith, T.H. (1984): The ultraviolet receptor of bird retinas. Science **225**: 337-340.

Comeault, A.A., Noonan, B.P. (2011): Spatial variation in the fitness of divergent aposematic phenotypes of the poison frog, *Dendrobates tinctorius*. J. Evol. Biol. **24**: 1374-1379.

Daly, J.W. (1995): The chemistry of poisons in amphibian skin. Proc. Natl. Acad. Sci. USA **92**: 9-13.

Daly, J.W., Wilham, W.M., Spande, T.F., Garraffo, H.M., Gil, R.R., Silva, G.L., Vaira, M. (2007): Alkaloids in bufonid toads (*Melanophryniscus*): temporal and geographic determinants for two Argentinian species. J. Chem. Ecol. **33**: 871-887.

Darst, C.R., Cummings, M.E., Cannatella, D.C. (2006): A mechanism for diversity in warning signals: conspicuousness versus toxicity in poison frogs. Proc. Natl. Acad. Sci. USA **103**: 5852-5857.

Duellman, W.E., Trueb, L. (1994): Biology of Amphibians. The Johns Hopkins University Press, Baltimore.

Eaton, M.D., Lanyon, S.M. (2003): The ubiquity of avian ultraviolet plumage reflectance. Proc. R. Soc. B **270**: 1721-1726.

Endler, J.A., Mielke Jr., P.W. (2005): Comparing entire colour patterns as birds see them. Biol. J. Linn. Soc. **86**: 405-431.

Exnerová, A., Svádová, K., Stys, P., Barcalová, S., Landová, E., Prokopová, M., Fuchs, R., Socha, R. (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.

Forsman, A. (2000): Some like it hot: intra-population variation in behavioral thermoregulation in color-polymorphic pygmy grasshoppers. Evol. Ecol. **14**: 25-38.

Forsman, A., Hagman, M. (2009): Association of coloration mode with population declines and endangerment in Australian frogs. Conserv. Biol. **23**: 1535-1543.

Frost-Mason, S., Morrison, R., Mason, K. (1994): Pigmentation. In: Amphibian Biology, Vol. 1. The Integument, p. 64-97. Heatwole, H., Barthalmus, G.T., Eds, Surrey Beatty & Sons, Chipping Norton, NSW.

Gamberale-Stille, G., Johansen, A.I., Tullberg, B.S. (2010): Change in protective coloration in the striated shield-bug *Graphosoma lineatum* (Heteroptera: Pentatomidae): predator avoidance and generalization among different life stages. Evol. Ecol. **24**: 423-432.

Grether, G.F., Kolluru, G.R., Nersissian, K. (2004): Individual colour patches as multicomponent signals. Biol. Rev. **79**: 583-610.

Hoffman, E.A., Blouin, M. (2000): A review of colour and pattern polymorphisms in anurans. Biol. J. Linn. Soc. **70**: 633-665.

Hoekstra, H.H. (2006): Genetics, development and evolution of adaptive pigmentation in vertebrates. Heredity **97**: 222-234.

Hubbard, J.K., Uy, J.A.C., Hauber, M.E., Hoekstra, H.H., Safran, R.J. (2010): Vertebrate pigmentation: from underlying genes to adaptive function. Trends Genet. **25**: 231-239.

Hunt, S., Cuthill, I.C., Bennett, A.T.D., Church, S.C., Partridge, J.C. (2001): Is the ultraviolet waveband a special communication channel in avian mate choice? J. Exp. Biol. **204**: 2499-2507.

Isaksson, C., Ornborg, J., Prager, M., Andersson, S. (2008): Sex and age differences in reflectance and biochemistry of carotenoid-based colour variation in the great tit *Parus major*. Biol. J. Linn. Soc. **95**: 758-765.

Kwet, A., Maneyro, R., Zillikens, A., Mebs, D. (2005): Advertisement calls of *Melanophryniscus dorsalis* (Mertens, 1933) and *M. montevidensis* (Philippi, 1902), two parapatric species from southern Brazil and Uruguay, with comments on morphological variation in the *Melanophryniscus stelzneri* group (Anura: Bufonidae). Salamandra **41**: 3-20.

Laurent, R.F. (1973): Variación geográfica en *Melanophryniscus rubriventris* (Vellard). Acta zool. lilloana **26**: 317-335.

Mantel, N. (1967): The detection of disease clustering and a generalized regression approach. Cancer Res. **27**: 209-220.

Noonan, B.P., Comeault, A.A. (2009): The role of predator selection on polymorphic aposematic poison frogs. Biol. Lett. **5**: 51-54.

Pirie, W. (1983): Jonckheere tests for ordered alternatives. In: Encyclopedia of Statistical Sciences, p. 315-318. Kotz, S., Johnson, N.L., Read, C.B., Eds, Wiley, New York.

Pryke, S.R., Lawes, M.J., Andersson, S. (2001a): Agonistic carotenoid signalling in male red-collared widowbirds: aggression related to the colour signal of both the territory owner and model intruder. Anim. Behav. **62**: 695-704.

Pryke, S.R., Andersson, S., Lawes, M.J. (2001b): Sexual selection of multiple handicaps in the red-collared widowbird: female choice of tail length but not carotenoid display. Evolution **55**: 1452-1463.

Roberts, J.L., Brown, J.L., Schulte, R., Arizabal, W., Summers, K. (2007): Rapid diversification of colouration among populations of a poison frog isolated on sky peninsulas in the central cordilleras of Peru. J. Biogeogr. **34**: 417-426.

Rosenberg, M.S., Anderson, C.D. (2011): PASSaGE: Pattern Analysis, Spatial Statistics and Geographic Exegesis. Version 2. Meth. Ecol. Evol. **2**: 229-232. Available from passagesoftware.net/index.php [accessed 20 June 2011].

Rowe, C., Guilford, T. (2000): Aposematism: to be red or dead. Trends Ecol. Evol. **15**: 261-262.

Saporito, R.A., Zuercher, R., Roberts, M., Gerow, K.G., Donnelly, M.A. (2007): Experimental evidence for aposematism in the dendrobatid poison frog *Oophaga pumilio*. Copeia **2007**: 1006-1011.

Siddiqi, A., Cronin, T.W., Loew, E.R., Vorobyev, M., Summers, K. (2004): Interspecific and intraspecific views of color signals in the strawberry poison frog *Dendrobates pumilio*. J. Exp. Biol. **207**: 2471-2485.

Speed, M.P., Ruxton, G.D. (2007): How bright and how nasty: explaining diversity in warning signal strength. Evolution **61**: 623-635.

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.

Summers, K., Bermingham, E., Weigt, L., McCafferty, S., Dahlstrom, L. (1997): Phenotypic and genetic divergence in three species of dart-poison frogs with contrasting parental behavior. J. Hered. **88**: 8-13.

Summers, K., Symula, R., Clough, M.E., Cronin, T.W. (1999): Visual mate choice in poison frogs. Proc. R. Soc. Lond. B **266**: 2141-2145.

Summers, K., Cronin, T.W., Kennedy, T. (2003): Variation in spectral reflectance among populations of *Dendrobates pumilio*, the strawberry poison frog, in the Bocas del Toro Archipielago, Panamá. J. Biogeogr. **30**: 35-53.

Toledo, L.F., Haddad, C.F.B. (2009): Colors and some morphological traits as defensive mechanisms in Anurans. Int. J. Zool. **2009**: 1-12.

Vaira, M. (2002): Variación de la coloración en poblaciones argentinas de *Melanophryniscus rubriventris* (Vellard, 1947). Cuad. herpetol. **16**: 151-163.

Vellard, J. (1947): Un nuevo batracio del Norte Argentino. Acta zool. lilloana **4**: 115-119.

Vences, M., Galán, P., Vieites, D.R., Puente, M., Oetter, K., Wanke, S. (2002): Field body temperatures and heating rates in a montane frog population: the importance of black dorsal pattern for thermoregulation. Ann. Zool. Fennici **39**: 209-220.

Wang, I.J. (2011): Inversely related aposematic traits: reduced conspicuousness evolves with increased toxicity in a polymorphic poison-dart frog. Evolution **65**: 1637-1649.

Wang, I.J., Shaffer, H.B. (2008): Rapid color evolution in an aposematic species: a phylogenetic analysis of color variation in the strikingly polymorphic strawberry poison-dart frog. Evolution **62**: 2742-2759.

Wang, I.J., Summers, K. (2010): Genetic structure is correlated with phenotypic divergence rather than geographic isolation in the highly polymorphic strawberry poison-dart frog. Mol. Ecol. **19**: 447-458.

Wollenberg, K.C., Lötters, S., Mora-Ferrer, C., Veith, M. (2008): Disentangling composite colour patterns in a poison frog species. Biol. J. Linn. Soc. **93**: 433-444.

Received: August 4, 2011. Accepted: November 14, 2011.