Correspondence

Pigment composition of the bright skin in the poison toad
Melanophryniscus rubriventris (Anura: Bufonidae) from Argentina

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Animals have a vast diversity of colours, which play an important role in their life history and evolution. Coloration has historically called the attention of several evolutionary biologists (e.g., Bates 1861, Cott 1940). Colour traits are involved in many inter- and intraspecific interactions, including species and sexual recognition, mimicry, as warning signals, and camouflage (Rudh & Qvarnström 2013). Previous studies assessed differences in colour patterns of individuals that differ in sex, condition, parasite load, or between individuals and their visual backgrounds (e.g., Mougeot et al. 2007, Bergman & Beehmer 2008, Cummings et al. 2008, Hutter et al. 2012, Molnár et al. 2012). Furthermore, different colour patterns have been associated with mate choice or defence (Venesky & Anthony 2007, Richards-Zawacki & Cummings 2012). Quantifying colour traits and their variation is of great relevance to understand the evolution and maintenance of phenotypic polymorphism in animals (Hoffman & Blouin 2000, Alexander & Breden 2004, Endler et al. 2005). Red, orange, and yellow colours in many fish, amphibians, reptiles, and birds are generated through the deposition of pigments in the integument (Grether et al. 2001, Hill et al. 2002, McGraw et al. 2006, Fitze et al. 2009).

Animals cannot synthesize most integumentary pigments such as carotenoids (Grether et al. 2001) and genetic factors or environmental conditions affect pigment composition (Jacot et al. 2010, San-Jose et al. 2013). Identifying light-absorbing pigments that contribute to integumentary colours might be crucial to understanding the determinants and consequences of colour variation in animals (Evans & Sheldon 2013). The majority of studies on pigment-based coloration have explored ornamental colour signals because of their potential as honest signals of phenotypic quality and have consequently become a target in the study of resource allocation trade-offs (Svensson & Wong 2011). However, pigments are also widely involved in a prey–predator context (e.g., Bezzerides et al. 2007, Sandre et al. 2007, Lindstedt et al. 2010). Several studies have shown that prey species use bright colours as a mechanism to deter predators by advertising the presence of antipredatory defences (reviewed in Stevens & Ruxton 2012). Aposematic animals advertise their defences by means of high conspicuousness, because this enhances recognition and avoidance learning by predators, leading to uniformity in signals. Long-wavelength colour patches (e.g., red, orange or yellow) are known to be effective components of many visual warning signals particularly when paired with black coloration (Exnerová et al. 2006, Maan & Cummings 2012, Stevens & Ruxton 2012). However, aposematic species with variable colours are common. For example, the combination of moderately conspicuous warning signals and colour polymorphism is commonly noticed in toxic amphibians (Mochida 2009, Noonan & Comeault 2009, Richards-Zawacki & Cummings 2012; Rojas & Endler 2013, Willink et al. 2013). Empirical evidence and theoretical approaches both support alternative scenarios in which the two components of aposematism, toxicity, and conspicuousness, could compensate in opposite ways to exert predator deterrence (Darst et al. 2006, Speed & Ruxton 2007).

In spite of many recent studies addressing the adaptive significance or ecological implications of coloration in poison frogs, most attention has been paid to its function and little is known about the relationship between pigment

composition and skin colour expression (e.g., CROTHERS & CUMMINGS 2013, RICHARDS-ZAWACKI et al. 2013, WILLINK et al. 2013). It has been stressed that a variety of pigment types could contribute to integument colour in animals to appear remarkably similar or different (MCGRAW 2004, MCGRAW et al. 2004, LINDSTEDT et al. 2010). Thus, the biochemical characterizations of skin pigments can shed light on the mechanisms responsible for the variation in colour expression, providing insights into the evolution of warning signalling in poison frogs and toads.

Melanophryniscus rubriventris (VELLARD, 1947) is an aposematic toad whose colour pattern consists of reddish orange patches combined with black ones (VAIRA 2002). The species also has many different alkaloids on the skin (DALY et al. 2007, GARRAFFO et al. 2012). Bright colours are mostly confined to specific dorsal and ventral body regions, the suprascapulae region behind the head, the gular and abdominal region, and the pelvic patch. Recent studies of such warning signals showed that the expanse and conspicuousness of bright coloration were subject to substantial variation among individuals and populations, including pinkish to reddish morphs, but also morphs with a rather cryptic black or drab colorations (BONANSEA & VAIRA 2012). However, the pigments responsible for the bright skin coloration in different morphs and populations remain unknown. Therefore, given that differences in the expression of visual warning signals could be modulated by pigment composition, identifying which pigments contribute to skin colour variability among populations of this species is an important step towards understanding their evolutionary and ecological significance.

Here, we analyse the composition of pigments associated with the reddish orange coloration of the skin of *Melanophryniscus rubriventris* and examine their variation between populations. Our results could provide insights into the mechanisms involved in the expression of colours in aposematic species.

We analysed 30 adult male specimens of *M. rubriventris* from 7 different populations in NW Argentina whose bright dorsal and ventral colorations vary substantially (see BONANSEA & VAIRA 2012 for details on geographic distribution and colour patterns). Individuals of each population were captured by hand during the same day (from November of 2008 to February of 2009) and euthanised with a topic overdose of ethyl-p-aminobenzoate (benzocaine) so that skin extractions could be performed immediately. We obtained five specimens from El Nogalar de Los Toldos (22°16'912" S, 64°43'6.45" W, 1,635 m a.s.l.); five from Canto del Monte (22°22'3.9" S, 64°43'16.03" W, 1,659 m a.s.l.); four from Cedral de Baritú; (22°27'35.76" S, 64°44'32.94" W, 1,689 m a.s.l.); four from Tablada (23°05'917" S, 64°51'43.20" W, 1,725 m a.s.l.); six from Abra Colorada (23°40'32.36" S, 64°53'52.35" W, 1,722 m a.s.l.); three from Angosto de Jaire (24°01'23.19" S, 65°23'24.49" W, 1,665 m a.s.l.), and three from La Almona (24°16'29.21" S, 65°24'30.62" W, 1,729 m a.s.l.). Sampled specimens were deposited in the herpetological collection at Universidad Nacional de Jujuy, Argentina (collector numbers MV470–474, MV 451–455, MV 481–484, MV 686–689, MV 573–575, MV 407–409, MV 440–445).

Skin samples were excised from three different parts of the body: (i) a triangular piece from the suprascapulae region behind the head; (ii) the central portion of the abdomen; and (iii) the entire section of skin covering the pelvic patch. Each skin sample was rinsed with water to rid it of spilled blood that might contaminate them and weighed with an analytic scale (Ohaus® ± 0.0001 g). Skins were placed in vials with 2 ml of acetone for 24 h in the dark to separate carotenoids from other possible pigments such as pteridines (insoluble in acetone).

Pigment separation and comparison with reference standards were first conducted by thin-layer chromatography (TLC) (aluminium sheets 10 × 10 cm, 0.2 mm thickness) with silica gel 60 F_{254} support (Merck® KGaA), using hexane and acetone, 70:30 v/v as a solvent. In addition, extracts were analysed with high-performance liquid chromatography (HPLC). The chromatographic system consisted of a Bds Hypersil® C18, 5 µm (250 × 4.6 mm) reverse-phase column, with isocratic elution system of methanol-acetonitrile (90:10 v/v) at a flow rate of 2 ml/min, with a runtime of 45 min, an injection volume of 20 µl, with a detection of absorbance at 450 nm. Absorption spectra were recorded from 400 to 600 nm. Compound identification was carried out by comparison with authentic standards of β-carotene (Sigma-Aldrich Co. LLC., Type I 95% UV); astaxanthin and canthaxanthin (DSM Nutritional Products Ltd., purity > 95%); as well as lycopene from tomato extracted in the laboratory following MARTÍ & DÍAZ SÁNCHEZ (2008). Major pigment fractions in extracts were identified using the HPLC system software by comparing their retention time (Rt), visible absorption spectra, and spectral fine structure. The former was defined as the ratio of the peak heights between absorption bands (%III/II), taking the minimum of the two bands as a baseline compared with those of pigment standards and literature data.

We performed a permutational multivariate analysis of variance (PERMANOVA) based on a Jaccard similarity matrix to test for differences in pigment composition between the species' populations based on the presence/absence of different pigments in the skin samples. This method compares the variance between groups with the variance within groups in a dissimilarity matrix by means of the sum of squared distances in an ANOVA design (ANDERSON 2001). To run PERMANOVA, we used the 'adonis' function of the vegan package in the R 3.0.1 software (R Core Team 2013).

The reddish orange colour of the skin patches disappeared within one hour after immersion in acetone, taking on a greyish colour that discards the presence of other yellow-orange pigments, such as pteridines. TLC and HPLC analyses identified four carotenoids whose profiles were not consistent in all populations. β-carotene (λmax = 452, retention time = 24 min) and canthaxanthin (λmax = 475, retention time = 3.6 min) were identified in 97 and 53% of the sampled toads, respectively, followed by lycopene (λmax = 471, retention time = 18.8 min) that was present

Table 1. Populational differences in carotenoid profiles from the bright skin of seven populations of *Melanophryniscus rubriventris*. The values indicate the percentages of individuals of the total sample per population (in parentheses) where each carotenoid type was detected.

Populations	Carotenes		Xanthophylls	
	β-Carotene	Lycopene	Astaxanthin	Canthaxanthin
Nogalar de los Toldos (n=5)	80%	40%	20%	60%
Canto del Monte (n=5)	100%	40%	40%	100%
Cedral de Baritú (n=4)	100%	25%	-	50%
Tablada (n=4)	100%	50%	-	50%
Abra Colorada (n=6)	100%	-	-	16.7%
Angosto de Jaire (n=3)	100%	33.3%	33.3%	33.3%
La Almona (n=3)	100%	-	-	66.7%

in five populations (27% of sampled toads), and astaxanthin (λmax = 476, retention time = 2 min) in three populations (13% of the sampled toads). None of the pigments were exclusive to a single population (Table 1). There were no differences in pigment composition between the seven populations ($F_{6,23}$ = 1.39, P = 0.181). There were also four unidentified peaks in the chromatograms of 12 specimens that could not be identified because they did not match any of the standards utilized (Fig. 1). Three of them have a UV–vis spectrum with λmax of 458, 461, and 469 nm and retention times of 6.9, 9.6, and 40 min, respectively, in HPLC analyses. A fourth pigment has a UV–vis spectrum with λmax between 446 and 473 nm and a retention time of 32.3 min.

We found that the reddish orange skin pigments of the aposematic toad, *Melanophryniscus rubriventris*, stem from carotenoids, as is evidenced by the extraction of pigments with acetone, which leads to the loss of bright coloration in skin patches. We provide evidence that at least four different primary pigments consist of a suite of orange-red carotenes and yellow xanthophylls and so contribute to the bright skin colour in different populations of the species. Similar pigment compositions were observed in the integuments of other aposematic amphibian species such as the

newts *Cynops pyrrhogaster* and *Notophthalmus viridescens*, and the fire-bellied toad, *Bombina orientalis* (FORBES et al. 1973, FROST & ROBINSON 1984, MATSUI et al. 2002). Although pteridines were also reported to be present in amphibian skins (OBIKA & BAGNARA 1964), we did not find this pigment in the bright patches of *M. rubriventris*.

While our results showed no significant differences in pigment composition between populations, a former study showed that toads from different populations of the species consume different prey types (QUIROGA et al. 2011), suggesting the potential role of individual foraging on the expression of bright colours. Most of the arthropods that constitute the diet of *M. rubriventris* (see BONANSEA & VAIRA 2007) were reported as potential sources of β-carotene, canthaxanthin, lycopene, and astaxanthin (GOODWIN 1954, KAYSER 1982). Thus, assuming that coloration might reflect the type of pigments incorporated in the integument (but see below), populational differences in the expression of bright colour might be a consequence of different availabilities of pigments from the diet or different foraging patterns of these toads. Spatial variation in the availability of dietary pigments could be important in this species, whose diet consists of small arthropods that might contain small
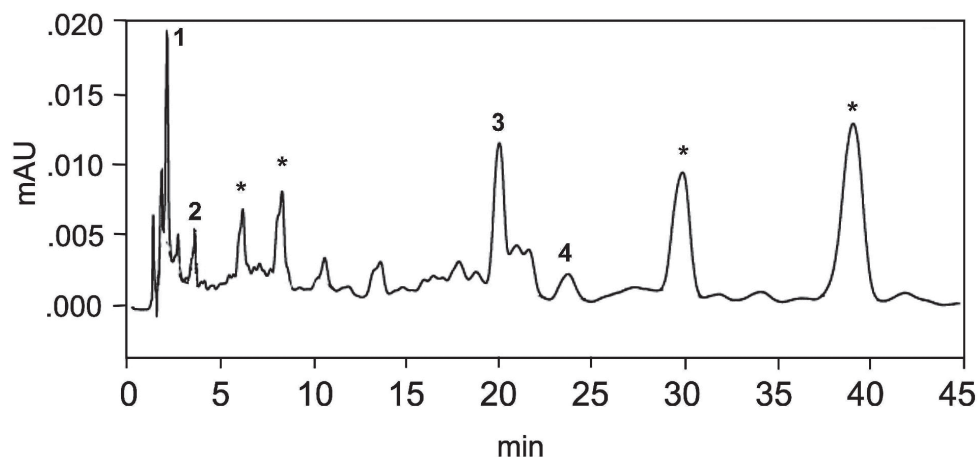

Figure 1. Representative HPLC chromatogram for carotenoid pigments found in the skin of an individual of *Melanophryniscus rubriventris* from the Cedral de Baritú population. Main pigments include: astaxanthin (1), canthaxanthin (2), lycopene (3), and β-carotene (4). Peaks marked with asterisks are unidentified pigments that did not conform to any of the standards utilized.

quantities of carotenoids (Bonansea & Vaira 2007). It was shown that a change in dietary pigments across environments is one proximate factor that may lead to populational differences in the feather coloration in birds (McGraw & Toomey 2010). Also, the availability of dietary carotenoids can directly influence skin coloration in captive frogs (Ogilvy et al. 2012). It remains to be demonstrated whether toads prefer prey with specific pigment contents and this affects bright colours. To our knowledge there are no studies evaluating if prey sources bearing different carotenoid types change across habitats or the existence of selectivity of such prey types in amphibians.

However, our study provides indirect evidence that differences in the coloration of individuals of *M. rubriventris* cannot be merely ascribed to differences in their skin pigment profiles (i.e., pigment types) as a consequence of differential pigment availability. Also, the idea of differential foraging of individuals related to carotenoid limitation in the environment was contrasted by Hudon (1994), who proposed that differences in coloration in animals do not necessarily relate to conditions limiting pigment availability, but most probably reflect their physiological condition. Nutrition, health status, and reproduction can obligate to divert carotenoids to maintenance-related processes against the deposition of carotenoids in the integument, and then this may also contribute to differences in coloration. Several experiments conducted mainly in birds have shown that the expression of colours is correlated with the individual's nutritional state (McGraw 2005), immune system activation (McGraw & Ardia 2003) or parasitic infections (Hill et al. 2009, but see Pröhl et al. 2013 who found that the effect of parasitic infections on the skin colour of a poison frog was minor). Most studies on these topics have focused on fishes, reptiles, and birds, and very little attention has as yet been devoted to aposematic amphibians.

Also, differences in the way toads from different populations process ingested carotenoids are worth investigating. Several studies show that some birds can grow red plumage by oxidizing yellow dietary precursor pigments (reviewed in Hill & Johnson 2012). In *M. rubriventris*, interpopulational differences may occur as a result of differential abilities to process or modify dietary carotenes and/or xanthophylls from the arthropods they consume. The mechanisms related to the uptake, absorption, and tissue accumulation of carotenoids and their influence on skin coloration are still unknown in this species. It is clear that more experimental studies manipulating carotenoid access are needed to better understand how and why coloration evolved.

On the other hand, skin colour differences might be related to genetically determined mechanisms unrelated to external conditions such as pigment types availability or resource limitation. Previous studies described how bright skin colorations in animals stemmed from a combination of pigments and structural components in three contiguous cell layers (Grether et al. 2004). In most cases, complex interactions between these components contributed

to the perceived variation in chromatic changes of individuals or populations (Frost & Robinson 1984, Brown et al. 2013, San-Jose et al. 2013). In the outermost layer, carotenoids absorb light between 400–500 nm. Two background components interact with them, iridophores in the middle layer that contain crystalline platelets reflecting light whereas melanins in the basal layer modulate iridophore-based reflectance (Grether et al. 2004). Thus, a more robust understanding of the extant variation in the skin colour pattern is needed to incorporate information regarding background structures and pigment types acting simultaneously on the observed differences of the reddish orange colours of *M. rubriventris*. While we did not examine the effects of underlying background components of the skin in this study, it is possible that similar carotenoid profiles in different interactions with iridophores and melanophores could determine populational differences in skin coloration.

To our knowledge this is the first study to explore the factors involved in the expression of skin colours in a member of the aposematic genus *Melanophryniscus* whose species exhibit major differences in their colour patterns (Kwet et al. 2005). Our results set the stage for future research on the complex interactions of different factors affecting the expression of carotenoid-based skin colours in aposematic species. The multiple functions of carotenoids may create trade-offs between investing in warning signalling or self-maintenance (Romero-Diaz et al. 2013), and determine the differences in coloration between species.

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