

Warning signal properties covary with toxicity but not testosterone or aggregate carotenoids in a poison frog

Laura Crothers¹ · Ralph A. Saporito² · Justin Yeager³ · Kathleen Lynch⁴ · Caitlin Friesen⁵ · Corinne L. Richards-Zawacki^{6,7} · Kevin McGraw⁸ · Molly Cummings⁵

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Abstract Aposematic (warning) coloration is a highly conspicuous trait that is found throughout the animal kingdom. In several aposematic species, warning signals have been co-opted for use in conspecific communication systems; for example, in the toxic and bright orange Solarte population of the strawberry poison frog (*Oophaga [Dendrobates] pumilio*), the brightness of male warning coloration serves as a sexual signal by both attracting females and repelling rivals. Here, we investigate correlations between bright male warning coloration and several physiological characteristics (e.g., circulating testosterone and carotenoids and noxious alkaloids in the skin), to gain insights into the mechanisms underlying the signal variation in this population and to inform hypotheses regarding the evolutionary stability of this trait. We find that although measures of male brightness (viewer-dependent or viewer-independent) do not correlate with two classic

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✉ Laura Crothers
crothers@utexas.edu

¹ University of California, Davis, Davis, CA 95616, USA

² Department of Biology, John Carroll University, University Heights, OH 44118, USA

³ Department of Ecology and Evolutionary Biology, Tulane University, 400 Lindy Boggs Bldg., New Orleans, LA 70118, USA

⁴ Department of Biology, Hofstra University, Gittleson 324, Hempstead, NY 11549, USA

⁵ Department of Integrative Biology, University of Texas at Austin, 1 University Station C0990, Austin, TX 78712, USA

⁶ Department of Biological Sciences, University of Pittsburgh, 4249 Fifth Avenue, Pittsburgh, PA 15260, USA

⁷ Smithsonian Tropical Research Institute, Apartado Postal 0843-03092, Panamá, Republic of Panama

⁸ School of Life Sciences, Arizona State University, Life Sciences C-wing Room 522, Tempe, AZ 85287, USA

correlates of sexually selected traits (circulating testosterone and aggregate carotenoids in the skin), male reflectance does show a positive correlation with concentrations of two xanthophyll carotenoids. Total reflectance (a viewer-independent measure of male brightness) also shows a negative relationship with aggregate pumiliotoxin in the skin, which is considered one of the major classes of defensive alkaloids in this species. Because the alkaloids used in this species' chemical defense are acquired from dietary sources, the magnitude of the reflectance intensity of a male's warning signal can potentially provide viewers with reliable information regarding territory quality, health, and/or current condition.

Keywords Aposematism · Chemical defense · Conspicuousness · *Oophaga [Dendrobates] pumilio* · Sexual selection · Signal reliability

Introduction

Colorful sexual ornaments can function as reliable signals of the health, body condition, and territorial status of an animal (Andersson 1994; Maynard Smith and Harper 2003; Whiting et al. 2003). Signal conspicuousness, for example, has been shown to correlate with an individual's health, condition, and/or nutritional state across a broad range of taxonomic groups (e.g., Hamilton and Zuk 1982; Folstad and Karter 1992; Ryan and Keddy-Hector 1992; Andersson 1994). The reliability of such “indicator” signals is often governed by physiological costs, such as diet-limited pigment molecules (e.g., carotenoids; Olson and Owens 1998). However, while many studies have focused on the physiological correlates of sexual signals, they have largely ignored another class of conspicuous animal signals. Aposematic “warning” signals, instead of evolving through sexual selection, evolve through natural selection to advertise to predators that a chemically defended prey item is unprofitable to attack (Wallace 1867).

Several lines of evidence indicate that physiological costs may also be associated with aposematic signals. The sequestration, modification, and storage of noxious compounds by chemically defended organisms are thought to be oxidatively stressful processes (Ahmad 1992; Blount et al. 2009; Santos and Cannatella 2011). Furthermore, many aposematic signals comprise red, orange, and yellow coloring, which in non-aposematic organisms is often controlled by pigments acquired from the diet that are involved in homeostatic redox reactions (Hill and Johnson 2012; Kodric-Brown 1989; McGraw 2005; Olson and Owens 1998). Interestingly, whereas predators were historically thought to be the agents shaping the evolution of aposematic signals (Müller 1879), recent research suggests that aposematic signals can function in the context of conspecific communication and that sexual selection may influence the direction of aposematic trait evolution (Jiggins et al. 2001; Maan and Cummings 2009; Nokelainen et al. 2011). Because aposematic species are highly conspicuous and can simultaneously signal to both predators and conspecifics with the same trait (Jiggins et al. 2001; Maan and Cummings 2008; Nokelainen et al. 2011; Saporito et al. 2007a, b), investigating the physiological correlates common to these traits can help clarify the underlying constraints governing the evolution of signals.

A staggering intraspecific and interspecific diversity in signal expression is observed in aposematic signals, causing some to speculate that they function as “magic traits” (sensu Gavrilets 2004), so-called “speciation” traits that simultaneously drive non-random mating

and are under divergent natural selection. The coloration of the phenotypically variable strawberry poison frog (*Oophaga* [*Dendrobates*] *pumilio*), whose populations feature 15–30 unique color patterns throughout western Panama, is regarded as a magic trait (Servedio et al. 2011). Both viewer-independent and viewer-dependent measures of signal brightness function as honest indicators of toxicity among *O. pumilio* populations (Maan and Cummings 2012). Females of this species show evidence of positive assortative mating by male color pattern (Summers et al. 1999) and a widespread preference for brighter males (Maan and Cummings 2009). However, populations do vary in these preferences; females of some populations do not prefer the local male visual signal (Dreher and Pröhl 2014; Maan and Cummings 2008) and female preferences for acoustic signals override visual signal preference in some populations (Dreher and Pröhl 2014). Bright warning coloration also appears to have been co-opted as an agonistic indicator signal within the bright orange and highly toxic Solarte population (Crothers et al. 2011; Crothers and Cummings 2015). Furthermore, males of this population also exhibit substantial variation in brightness (Crothers and Cummings 2013) and brighter males appear to be more aggressive (Crothers and Cummings 2015).

There are several biomolecules that commonly correlate with conspicuous sexual signals and are thus candidates for investigating as correlates of warning signals. First, the development or expression of sexually selected bright signals is often testosterone dependent (Folstad and Karter 1992; Johnstone and Norris 1993; Sinervo et al. 2000; reviewed in Whiting et al. 2003). Second, carotenoid pigments predominate in orange/red animal colors (Kodric-Brown 1989; McGraw 2005), and such pigment-based traits often serve as honest signals because the carotenoids used in coloration can be limited dietarily and have important health roles (Andersson 1994; Bezzerides et al. 2007; Blount et al. 2009; Hill and Johnson 2012; McGraw and Ardia 2003; Olson and Owens 1998). Finally, color or brightness expression may correlate with skin toxins within a population (as predicted by theoretical studies, e.g., Blount et al. 2009; Holen and Svennungsen 2012; Lee et al. 2011), because they have been shown to correlate across populations of this and other aposematic species (Bezzarides et al. 2007; Maan and Cummings 2012; Saporito et al. 2007a, b).

We predicted that conspicuous warning signals would show similar physiological underpinnings to those of sexual signals. Here, we investigate several potential physiological correlates of warning coloration in the phenotypically variable and sexually dimorphic Solarte *O. pumilio* population, to begin to understand the underlying mechanistic basis for inter-male color variation in this poison frog population. We predicted that *O. pumilio* males with greater total reflectance or viewer-dependent (avian or *O. pumilio*) measures of conspicuousness would have (1) higher circulating testosterone levels, (2) greater quantities of skin carotenoids, and (3) greater quantities of noxious skin alkaloids.

Methods

Body measurements

Calling territorial adult males were located in the field during early daytime hours in August to September of 2011 and July of 2012 on Isla Solarte, in Bocas del Toro, Panama (N 09°20.014', W 82°13.197'). Males were captured and kept individually in plastic 475 mL deli containers moistened with ultraviolet (UV) purified water until body

measurements were taken. All males ($N = 43$) were measured for spectral reflectance and were photographed on a standard background that included a ruler, using a Canon Rebel 10.1 XS DSLR camera, as done previously (Crothers et al. 2011; Crothers and Cummings 2015). In 2011, twenty-three males were measured beneath a tent in the field within several hours of capture, and then transported to the Smithsonian Tropical Research Institute (STRI) in Bocas del Toro, Panama, for blood sampling (see below). In 2012, twenty males were first transported to the Smithsonian Tropical Research Institute (STRI) in Bocas del Toro, Panama, and then were measured there within 24 h of capture, followed by skin carotenoid and alkaloid measurements (see below). For a subset of the males caught in 2012 ($N = 10$) we recorded perch height in the male's territory prior to capture in order to characterize any physiological correlates with microhabitat use.

Spectral reflectance measurements and analysis of brightness

Spectral reflectance measurements for each individual were taken at the head and dorsum (two measurements per region in 2011; four measurements per region in 2012) using an EPP2000 UV–VIS portable spectrometer and R600-8 UV–VIS-SR reflectance probe (StellarNet Inc., Tampa, FL) and a PX2 Xenon flash lamp outfitted with a custom-made 50 Hz trigger input (Ocean Optics, Dunedin, FL). Spectralon white standard measurements were taken frequently to account for lamp drift. Males captured in 2011 were briefly housed individually at STRI until they were returned to their territories in the field.

We averaged measurements of the head and dorsum to calculate both the total reflectance $\left[\sum_{300\text{ nm}}^{700\text{ nm}} R(\lambda) \right]$, a perceptually unbiased estimate of male brightness, and long-wave chroma. Long-wave chroma (\sim redness) assesses the proportion of the total reflectance in the long-wave band: $\left[\frac{\sum_{i=600\text{ nm}}^{700\text{ nm}} R(\lambda)}{\sum_{i=300\text{ nm}}^{700\text{ nm}} R(\lambda)} \right]$.

Because the conspicuousness of a frog depends both on the unique characteristics of a viewer's visual system and on the spectral properties of the signaling background, we also evaluated viewer-specific measures of male brightness or luminance contrast (ΔL). In addition, we evaluated viewer-specific measures of male color contrast (ΔS) and overall conspicuousness (OC), which represents a bivariate measure of luminance and color contrast (as in Maan and Cummings 2012). We estimated these parameters for a frog's dorsum when viewed against two common signaling backgrounds in the Solarte population (palm leaf and *Heliconia* sp. leaf) and using an average irradiance measurement from that population (see Crothers and Cummings 2013 for details). We used a *Sturnus vulgaris*-specific visual model and an *O. pumilio*-specific visual model (as in Crothers and Cummings 2013) to estimate conspicuousness for what is considered the species' most common class of predator (birds: Maan and Cummings 2012; Dreher et al. 2015) and for a conspecific viewer, respectively. Overall conspicuousness (OC) for these two visual systems was calculated as the Euclidean distance of two detection parameters (ΔL and ΔS) in the viewer-specific color space.

Circulating testosterone

We housed the 23 calling males that we collected in 2011 in individual terraria at STRI ($\sim 37\text{ cm} \times 22\text{ cm} \times 24\text{ cm}$), containing water, leaf litter, and arthropods, for approximately 24 h prior to blood collection. Blood was collected from the orbital sinus using a

heparinized capillary tube with a tapered end produced with a micropipette puller, as done by Lynch et al. (2006). One frog died following blood collection, but the others were returned to their points of capture in the field after sampling. In July of 2013, blood was also collected from six additional adult males in a captive colony maintained in the Richards-Zawacki laboratory at Tulane University; these samples were pooled and were used to perform a serial dilution validation for the hormone analysis. These six frogs were first sacrificed by double pithing and then blood was immediately collected from the orbital sinus and from an incision in the right hind leg with a heparinized micropipette tip. All blood samples were immediately centrifuged at $\sim 10,000$ rpm for 6 min and the plasma layer collected and frozen for several days on dry ice while in transport to the University of Texas, where they were maintained at -20°C until hormone analyses were performed in February of 2014.

We measured circulating levels of total testosterone using enzyme-linked immunosorbent assay (Enzo Life Sciences, cat. # ADI-900-065). *O. pumilio* plasma samples were analyzed for parallelism with the kit's standard curve using a series of six dilutions from the pooled plasma stock (1:10, 1:20, 1:40, 1:80, 1:160, and 1:320). The dilutions ran parallel to the standard curve (homogeneity of slopes ANCOVA: $F_{1,7} = 0.019$, $P = 0.895$), validating the kit's suitability for use with this species. Individual male plasma samples were diluted at 1:64 (16 samples), 1:86 (5 samples), 1:142.5 (1 sample), and 1:213.5 (1 sample) in assay buffer and the kit protocol was strictly followed. Each sample was run in duplicate. The use of lower dilution concentrations for some samples was due to the exceptionally small available plasma quantities ($<2\mu\text{L}$) for those samples. The plates were read with a conventional plate reader at 405 nm (SpectraMax M3, Molecular Devices). To compute circulating testosterone levels, the percent bound for each of the standards was calculated and a logarithmic curve was generated. This curve was then used to compute the circulating testosterone levels for the males (in ng/mL). Circulating testosterone levels ranged from 0.67 to 2.68 ng/mL (mean = 1.34, SD = 0.65). Intra-assay variation was 17.64 %, and inter-assay variation was 7.14 %. Cross-reactivity in the testosterone kit was <0.001 % for dihydrotestosterone.

Skin carotenoids

Immediately following body measurements at STRI (size and spectral measurements), all 20 males captured in 2012 were sacrificed by double pithing. A small sample of dorsal skin was collected for skin alkaloid analysis from each male, as described below. The remaining dorsal skin was dissected and frozen in liquid nitrogen for transport to the United States, after which samples were stored at -80°C until their carotenoid content was analyzed in October 2013. Carotenoid levels in the dorsal skin tissue were quantified (in μg of carotenoid per g of tissue; tissue was weighed to the nearest 0.00001 g with a digital balance) using high performance liquid chromatography (HPLC), following a modified version of previously established protocols (McGraw et al. 2006).

In brief, carotenoids were extracted using a micronizer in the presence of solvent (1.4 mL hexane:tert butyl methyl ether, 1:1, v/v), using 0.1 g of skin. Tissue and solvent were centrifuged, and the supernatant was recovered and dried down for carotenoid analysis. HPLC analyses follow those in McGraw et al. (2006), using a Waters 2695 instrument (Waters, Milford, MA). Because of the presence of ketocarotenoids in the samples, the analytical method was slightly modified. First, the HPLC column (Waters YMC Carotenoid column, 5 mm, 4.6 mm #250 mm) was pretreated with 1 % orthophosphoric acid in methanol for 30 min at 1 mL/min. Secondly, solvent composition

Table 1 Skin carotenoids found in Solarte males

Carotenoid	Median (µg/g)	Mean (µg/g)	Range (µg/g)	SD (µg/g)	% Indiv.	Correlation coefficient
Apocarotenoid	5.74	5.67	0–13.75	4.88	68	
Canary Xanthophyll	17.05	21.29	1.06–64.07	17.26	100	0.26
Canthaxanthin	6.05	11.93	0–73.74	16.73	79	0.22
Xanthophyll	3.77	6.47	0–24.78	6.73	84	<i>0.48</i>
cis-Ketocarotenoid	6.34	8.30	0–40.62	9.99	74	0.32
Echinenone	0	4.76	0–51.99	14.43	11	
3-Hydroxy-echinenone	34.41	47.31	0–179.22	43.29	95	0.27
Lutein Ester (1)	23.79	34.76	2.2–83.87	27.05	100	0.03
cis-Xanthophyll	18.84	22.26	0–71.22	20.03	84	0.12
Canary Xanthophyll Ester (1)	47.94	56.55	4.01–214.11	55.92	100	0.03
B-Carotene	152.63	195.08	37.96–482.83	134.50	100	0.11
Canary Xanthophyll Ester (2)	60.04	76.67	5.98–226.29	63.44	100	0.23
Ketocarotenoid Ester (2)	0	2.63	0–35.21	8.45	16	
Canary Xanthophyll Ester (3)	21.81	30.86	0–90.07	26.58	95	<i>0.47</i>
Canthaxanthin Ester	46.99	50.64	1.66–135.09	32.31	100	0.16
Ketocarotenoid Ester	18.82	24.77	0–95.38	22.81	84	0.25
Xanthophyll Ester	8.67	10.37	0–29.14	8.96	79	0.128
Total Carotenoids	459.73	610.32	86.04–1421.51	407.45	–	0.20

The penultimate column presents the percentage of individuals in the dataset ($N = 19$ males) that had the carotenoid present in their skin. The final column contains correlation coefficients for that carotenoid and male brightness (total reflectance) for those carotenoids found in 70 % or more of males. Coefficients are italicized for positive linear regressions in which $P < 0.05$ (not corrected for multiple hypothesis testing)

and flow rate were altered to optimize separation of different ketocarotenoids. At a constant flow rate of 1.2 mL/min, an isocratic elution with 42:42:16 (v/v/v) methanol:acetonitrile:dichloromethane was first used for 11 min followed by a linear gradient up to 42:23:35 (v/v/v) methanol:acetonitrile:dichloromethane through 21 min, holding those conditions until minute 25, and finishing with a return to the original isocratic conditions from 25 to 29.5 min. Carotenoid types were identified by comparison to authentic standards from CaroteNature (Ostermundigen, Switzerland). External standard curves were used to quantify concentrations of each carotenoid type. One sample was lost during HPLC analysis, resulting in a final sample size of 19 males for this dataset. Total carotenoid quantities ranged from 86.04 to 1421.51 µg/g. We identified 17 unique carotenoids within the analyzed samples (presented in Table 1).

Skin alkaloids

A sample of mid-dorsal skin tissue, referred to herein as a “disc,” was removed from the 20 recently sacrificed males captured in 2012 (described above) using a 4 mm-diameter circular biopsy punch. Biopsies were consistently performed on the same side and approximate location on the dorsal skin surface. Skin samples were stored individually at room temperature in methanol-filled glass vials for subsequent alkaloid analysis. Individual alkaloid fractions were prepared in April 2013 from methanol extracts of each skin and characterized using gas chromatography in combination with mass spectrometry (GC–MS)

using the methods detailed in Saporito et al. (2010), with final dilution volumes modified to accommodate the smaller tissue sizes. GC–MS was performed on a Varian 3900 GC with a 30 m × 0.25 mm i.d. Varian Factor Four VF-5 ms fused silica column coupled to a Varian Saturn 2100T ion trap MS instrument. Alkaloids were separated using a GC temperature program from 100 to 280 °C at a rate of 10 °C per minute with helium as the carrier gas (1 mL/min). Each alkaloid fraction was analyzed using electron impact MS and chemical ionization MS with methanol as the ionizing reagent.

Comparisons of mass spectrometry properties and GC retention times with those described in previous studies allowed us to identify individual alkaloids for each skin sample (Daly et al. 2005; Saporito et al. 2007a, b, 2010). All alkaloids within a fraction were quantified (in µg/disc) by comparison of the alkaloid's peak area to the peak area of a nicotine internal standard using a Varian MS Workstation v.6.9 SPI. There was a broad range of both alkaloid quantity (1.59–153.41 µg, median = 5.64, SD = 33.60) and alkaloid diversity (8–48 unique alkaloids, median = 13.5, SD = 9.38) within the twenty 4 mm-diameter samples of dorsal skin (Table 2).

Statistical analysis

All statistical tests were performed in R 2.15.1 (R Development Core Team 2012). We first assessed the relationship between circulating testosterone (in ng/mL) and male warning coloration by fitting linear regressions (LM) with the viewer-dependent (ΔL , ΔS , OC) and viewer-independent (total reflectance and long-wave chroma) measures of conspicuousness as predictor variables. Two samples were off the standard curve and were not included in these analyses, resulting in a final sample size of 21 males for the hormone analyses.

We then tested the relationship between total skin carotenoids (µg/g) and male coloration by fitting linear regressions with the conspicuousness measures described in the paragraph above as predictor variables. For prevalent carotenoids (found in >70 % of individuals), we performed individual linear regressions of those carotenoids on male total reflectance (Table 1).

Because the alkaloid dataset contained two exceptionally toxic males (>6x that of the median alkaloid quantity for the dataset), we converted alkaloid quantity in the skin (in µg), and total alkaloid diversity (number of unique alkaloids) to rank data. Small sample size (N = 20) precluded the use of ordinal logistic regression, thus we modeled the relationship between male color measurements (brightness, long-wave chroma, ΔL , ΔS , OC) and these alkaloid measures using Kendall's rank correlations (Kendall 1955). We also modeled the relationship between viewer-independent estimates of conspicuousness and alkaloids/carotenoids with Wilcoxon rank sum tests using a dichotomous total reflectance and long-wave chroma measure ("brighter" or redder than the median versus "duller" or less red than the median total reflectance or long-wave chroma for the dataset, respectively; as in Crothers and Cummings 2013). We performed two additional analyses, using Kendall's rank correlations, to assess the relationships between male warning signals and common alkaloids. First, we assessed whether conspicuousness measures co-varied with aggregate quantity of pumiliotoxins, considered a major class of toxic alkaloids found in the skin of poison frogs of the *Dendrobates/Oophaga* genera (Daly and Myers 1967; Daly et al. 1999). Second, we assessed the relationship between male total reflectance and the five specific alkaloids found in >70 % of male skins (results presented in Table 2).

Alkaloid sequestration, modification, and storage are believed to be oxidatively stressful for chemically defended organisms (Ahmad 1992; Blount et al. 2009; Santos and Canatella 2011). We thus used two methods to assess whether the most abundant carotenoids

Table 2 Skin alkaloids in Solarte males

Alkaloid	Median (µg/disc)	Mean (µg/disc)	Range (µg/disc)	SD (µg/disc)	% Indiv.	Correlation coefficient
3,5-I 195B	0.00	0.087	0–0.877	0.216	20	–0.03
3,5-I 251K	0.00	0.010	0–0.202	0.045	5	
3,5-P 209K	0.00	0.002	0–0.049	0.011	5	
3,5-P 223H	0.00	0.479	0–8.509	1.896	25	
3,5-P 223H (<i>iso</i>)	0.00	0.009	0–0.166	0.037	10	
5,6,8-I 277E	0.00	0.009	0–0.172	0.038	5	
5,6,8-I 277E (<i>iso</i>)	0.00	0.001	0–0.022	0.005	5	
5,6,8-I 221U	0.00	0.001	0–0.019	0.004	5	
5,6,8-I 223A	0.00	0.036	0–0.439	0.104	20	
5,6,8-I 223X	0.00	0.001	0–0.015	0.003	5	
5,6,8-I 231B	0.00	0.004	0–0.024	0.007	25	
5,6,8-I 237C	0.00	0.109	0–1.424	0.320	40	
5,6,8-I 249C	0.00	0.002	0–0.034	0.008	5	
5,6,8-I 253H	0.00	0.021	0–0.235	0.056	25	
5,6,8-I 263A	0.00	0.007	0–0.122	0.027	10	
5,8-I (<i>unidentified</i>)	0.00	0.004	0–0.071	0.016	5	
5,8-I 195A	0.00	0.046	0–0.920	0.206	5	
5,8-I 195I	0.00	0.011	0–0.220	0.049	5	
5,8-I 205A	0.00	0.018	0–0.183	0.048	15	
5,8-I 207A	0.00	0.065	0–0.755	0.171	30	
5,8-I 221A	0.00	0.001	0–0.030	0.007	5	
5,8-I 223D	0.00	0.032	0–0.235	0.076	20	
5,8-I 233D	0.00	0.352	0–5.880	1.327	10	–0.12
5,8-I 233D (<i>iso</i>)	0.00	0.001	0–0.016	0.004	5	
5,8-I 233D (<i>iso</i>)	0.00	0.007	0–0.075	0.018	30	
5,8-I 233D (<i>iso</i>)	0.04	0.251	0–2.337	0.543	65	
5,8-I 233D (<i>iso</i>)	0.00	0.023	0–0.336	0.078	10	
5,8-I 235B	0.07	3.757	0–59.84	13.285	70	
5,8-I 243B	0.00	0.014	0–0.192	0.046	15	
5,8-I 245B	0.00	0.014	0–0.289	0.065	5	
5,8-I 251B	0.00	0.059	0–0.879	0.196	30	
5,8-I 251B (<i>iso</i>)	0.00	0.002	0–0.036	0.008	5	
5,8-I 253B	0.00	0.003	0–0.052	0.012	5	–0.12
5,8-I 259B	0.00	0.003	0–0.067	0.015	5	
aPTX 305A	0.00	0.052	0–0.798	0.178	20	
aPTX 305A (<i>iso</i>)	0.00	0.001	0–0.027	0.006	5	
aPTX 323B	0.00	0.014	0–0.273	0.061	5	
aPTX 325A	0.00	0.291	0–4.892	1.095	25	
aPTX 325B	0.00	0.017	0–0.346	0.077	5	
d-5,8-I 221V	0.00	0.003	0–0.054	0.012	5	
d-5,8-I 201A	0.00	0.003	0–0.039	0.010	10	
DHQ 195A	0.10	2.852	0–37.37	8.314	75	

Table 2 continued

Alkaloid	Median ($\mu\text{g}/\text{disc}$)	Mean ($\mu\text{g}/\text{disc}$)	Range ($\mu\text{g}/\text{disc}$)	SD ($\mu\text{g}/\text{disc}$)	% Indiv.	Correlation coefficient
DHQ 195A (<i>iso</i>)	0.00	0.005	0–0.106	0.024	5	
DHQ 195A (<i>iso</i>)	0.00	0.011	0–0.205	0.046	15	
DHQ 211A	0.00	0.123	0–1.352	0.317	25	
Izidine 209D	0.00	0.002	0–0.037	0.008	5	
Izidine 211C	0.00	0.002	0–0.015	0.005	20	
Lehm 275A	0.00	0.003	0–0.058	0.013	5	
Pip 211J	0.00	0.004	0–0.076	0.017	5	
PTX 251D	0.00	0.026	0–0.513	0.115	5	
PTX 277B	0.00	0.018	0–0.238	0.058	10	
PTX 307A	0.66	2.356	0–21.02	5.107	75	–0.34
PTX 307B	0.00	0.090	0–1.176	0.265	35	
PTX 307F'	0.00	0.007	0–0.109	0.024	15	
PTX 307F''	0.00	0.022	0–0.222	0.067	10	
PTX 307G	0.00	0.013	0–0.250	0.056	5	
PTX 309A	0.00	0.027	0–0.196	0.060	20	
PTX 321A	0.00	0.236	0–4.420	0.986	15	
PTX 323A	0.04	1.495	0–10.60	3.196	50	
PTX 323A (<i>iso</i>)	0.00	0.001	0–0.015	0.003	5	
PTX 323A (<i>iso</i>)	0.00	0.046	0–0.455	0.112	20	
PTX 323A (<i>iso</i>)	0.00	0.801	0–4.549	1.239	45	
PTX 323A (<i>iso</i>)	0.00	0.006	0–0.082	0.020	10	
PTX 325B	0.00	0.027	0–0.330	0.084	10	
Spiro 236	0.00	0.011	0–0.219	0.049	5	
Spiro 252A	0.00	0.019	0–0.258	0.057	30	
Tri (<i>unidentified</i>)	0.00	0.003	0–0.061	0.014	5	
Tri 191B	0.57	0.555	0–1.214	0.297	90	0.04
Tri 191B (<i>iso</i>)	0.00	0.005	0–0.029	0.010	25	
Tri 203B	0.01	0.092	0–0.367	0.140	55	
Tri 203B (<i>iso</i>)	0.00	0.039	0–0.517	0.126	10	
Tri 205B	0.25	0.256	0.166–0.53	0.076	100	0.02
Tri 205B (<i>iso</i>)	0.03	0.200	0–0.952	0.313	55	
Tri 205H	0.00	0.001	0–0.019	0.004	5	
<i>Unclassified 279I</i>	0.00	0.072	0–0.812	0.197	20	
<i>Unidentified</i>	0.00	0.011	0–0.127	0.034	10	
<i>Unidentified</i>	0.00	0.004	0–0.084	0.019	5	

Isomers are indicated by the designation (*iso*). The penultimate column contains the percentage of individuals in the dataset ($N = 20$ males) that had the alkaloid present in their skin. The final column contains the Kendall's tau rank correlation coefficient of that alkaloid and male brightness (total reflectance), for the few alkaloids found in 70 % or more of males

(3,5-I: 3,5-disubstituted indolizidine; 3,5-P: 3,5-disubstituted pyrrolizidine; 5,6,8-I: 5,6,8-trisubstituted indolizidine; 5,8-I: 5,8-disubstituted indolizidine; aPTX: allopumiliotoxin; d-5,8-I: dehydro-5,8-disubstituted indolizidine; DHQ: 2,5-disubstituted decahydroquinoline; Lehm: lehmizidine; Pip: piperidine; PTX: pumiliotoxin; Spiro: spiropyrrolizidine; Tri: tricyclic)

co-varied with the most abundant alkaloids within samples; rank correlations were used to assess the relationships between beta-carotene/xanthophyll (the most abundant carotenoids) and aggregate quantity of tricyclics/pumiliotoxins (the most abundant alkaloid classes), and linear regression was used to determine whether the proportions of these alkaloids co-varied with the proportions of these carotenoids.

Finally, for the ten males on which we collected perch data, we used Kendall's rank correlation to assess whether measures of male conspicuousness may correlate with differences in microhabitat by correlating male conspicuousness measures with a male's perch height (in m) in his territory.

Results

Circulating testosterone

There was no relationship between circulating testosterone concentration and male total reflectance (Fig. 1a; LM: $N = 21$, $t = 0.545$, $P = 0.592$, $r = 0.12$), long-wave chroma (LM: $t = -1.011$, $P = 0.325$, $r = -0.23$), luminance contrast (ΔL , all $P > 0.49$; all $r < 0.16$), color contrast (ΔS , all $P > 0.27$; all $r < 0.25$), or overall conspicuousness (all $P > 0.75$; all $r < 0.07$) for the bird or frog visual models (see table S1).

Skin Carotenoids

There was no relationship between male total reflectance or long-wave chroma and total quantity of dorsal skin carotenoids, both when assessed as a linear relationship (see Fig. 1b for total reflectance plot; LM: $N = 19$, both $t < 0.94$, both $P > 0.360$, both $r \leq 0.2$) and when these color characteristics were coded as dichotomous variables (Wilcoxon rank sum test: *total reflectance*: $W = 39$, $P = 0.661$; *long-wave chroma*: $W = 46$, $P = 0.968$). Analyses using the viewer-specific estimates of ΔL , ΔS , and overall conspicuousness (OC) were also non-significant (LM: all $P > 0.55$, all $r < 0.15$; see table S1). However, the quantity of two specific carotenoids exhibited a positive relationship with total reflectance (xanthophyll: $P = 0.04$, and a canary xanthophyll ester: $P = 0.04$; Table 1; Fig. 1c, d), though these relationships were no longer significant after correction for multiple hypothesis testing (not shown). Xanthophyll and canary xanthophyll did not correlate with viewer-dependent conspicuousness measures (Table S2).

Skin alkaloids

Alkaloid quantity and alkaloid diversity were positively correlated (Kendall's rank correlation: $N = 20$, $z = 3.375$, $P = 0.0007$, tau coefficient = 0.56). There was no correlation between total reflectance and alkaloid quantity (Fig. 2a; Kendall's rank correlation: $N = 20$, $z = -1.3627$, $P = 0.173$, tau coefficient = 0.22) or alkaloid diversity (Fig. 2a; $z = -1.212$, $P = 0.225$, tau coefficient = 0.20), or between long-wave chroma and these measures (Kendall's rank correlations: both $P > 0.299$, both tau coefficients < 0.17); results were similarly non-significant when assessing alkaloid quantity and diversity and ΔL , ΔS , and overall conspicuousness (OC) for the bird and conspecific visual models (all $P > 0.14$; all tau coefficients < 0.24 ; see table S1). However, when males were categorized into dichotomous total reflectance categories, "brighter"-than-average males had

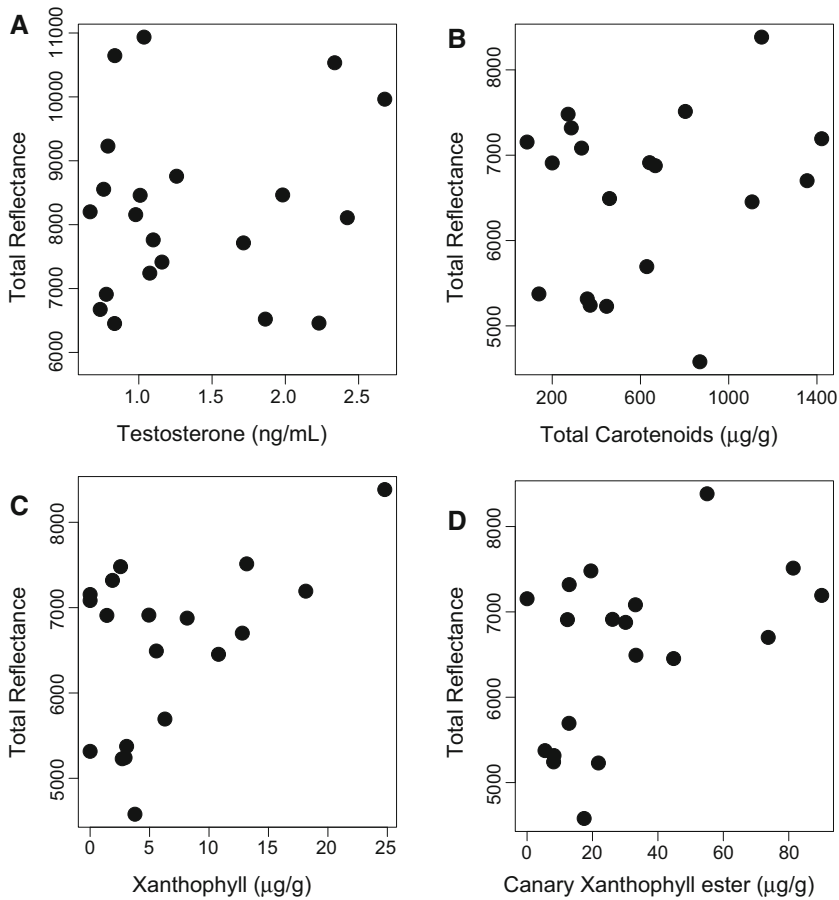


Fig. 1 Plots of male total reflectance and **a** circulating testosterone, **b** total carotenoid concentration, **c** xanthophyll concentration, and **d** canary xanthophyll ester concentration in the dorsal skin. For **c** and **d**, the significant positive relationship detected between these carotenoids and total reflectance, and presented in Table 1, has not been corrected for multiple hypothesis testing

significantly less alkaloid diversity than “duller”-than-average males (Fig. 2a; Wilcoxon rank sum test: $W = 23$, $P = 0.043$), but did not differ significantly from duller males in terms of alkaloid quantity (Fig. 2a; Wilcoxon rank sum test: $W = 25$, $P = 0.063$). There was a significant negative relationship between male total reflectance and aggregate quantity of pumiliotoxins (Fig. 2b; Kendall’s rank correlation: $N = 20$, $z = -2.160$, $P = 0.031$, tau coefficient = -0.36), but no relationship between long-wave chroma and aggregate pumiliotoxin (Kendall’s rank correlation: $z = 0.589$, $P = 0.556$, tau coefficient = 0.10) or between aggregate pumiliotoxin and ΔL , ΔS , and overall conspicuousness (OC) to the bird or frog visual models (all $P > 0.15$, all tau coefficients < 0.24 ; see table S1). Finally, there was a significant negative relationship between the quantity of one of the pumiliotoxins (PTX 307A; $P = 0.04$) and total reflectance, but not the other common alkaloids (Table 2). PTX 307A did not correlate with viewer-dependent measures of conspicuousness (Table S2).

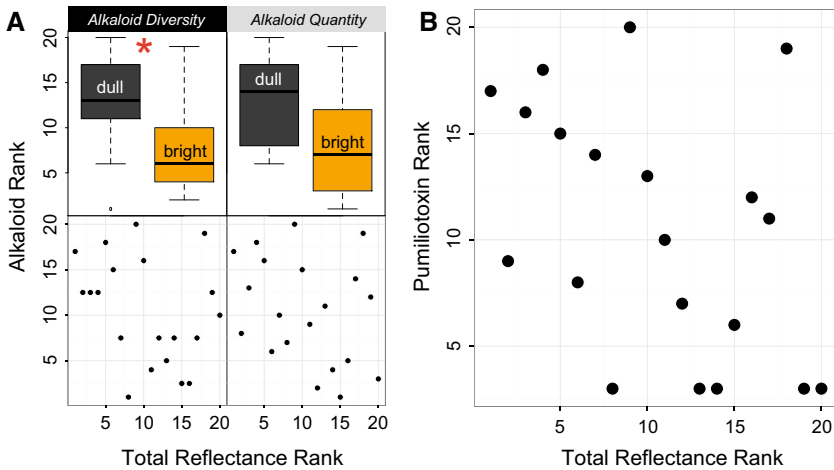


Fig. 2 Relationships between brightness (total reflectance) and skin alkaloids. **a** Total reflectance (*top* as a dichotomous measure, *bottom* as a rank measure) and rankings of alkaloid diversity (*left* the number of unique alkaloids present in the skin) and alkaloid quantity (*right* total μg of alkaloids present in the standardized skin samples) in the dataset of 20 males. Higher rankings indicate larger values. **b** Relationship between ranked total reflectance and ranked quantity of aggregate pumiliotoxins (Kendall's rank correlation: $N = 20$, $z = -2.160$, $P = 0.031$)

Relationships between alkaloids and carotenoids

There was no correlation between total carotenoid quantity and total alkaloid quantity (Kendall's rank correlation: $N = 19$, $z = 0.105$, $P = 0.916$, tau coefficient = 0.02). Examining the proportional relationships between skin alkaloids and skin carotenoids, we found that the proportion of tricyclic alkaloid quantity was positively correlated with the proportion of beta-carotene (Fig. 3a; LM: $N = 19$, $t = 2.426$, $P = 0.027$, $r = 0.51$) and negatively correlated with the proportion of xanthophyll (Fig. 3b; LM: $N = 19$, $t = -2.548$, $P = 0.021$, $r = -0.53$). The proportion of pumiliotoxin alkaloids was negatively correlated with the proportion of beta-carotene (Fig. 3c; LM: $N = 19$, $t = -2.609$, $P = 0.018$, $r = -0.53$), but not correlated with the proportion of xanthophyll (Fig. 3d; LM: $N = 19$, $t = 1.639$, $P = 0.119$, $r = 0.37$). Rank correlations of these measures were all non-significant (all $P > 0.22$, all tau coefficients < 0.21).

Lastly, for the males with perch data, we found a significant positive relationship between male dorsal total reflectance and perch height (Fig. 4a; $N = 10$, Kendall's rank correlation: $T = 36$, $P = 0.017$, tau coefficient = 0.6). However, a significant relationship for the viewer-dependent conspicuousness measures was only found for overall conspicuousness (OC) to an avian viewer (but not *O. pumilio* viewer) and perch height against a palm background ($P = 0.02$, tau coefficient = 0.6; Fig. 4b; see table S1 for other results).

Discussion

We sought to address the relationships between measures of male warning signal brightness and several common condition- or quality-related correlates of conspicuous sexual and aposematic signals. We found that, though *O. pumilio* male dorsal total reflectance (an

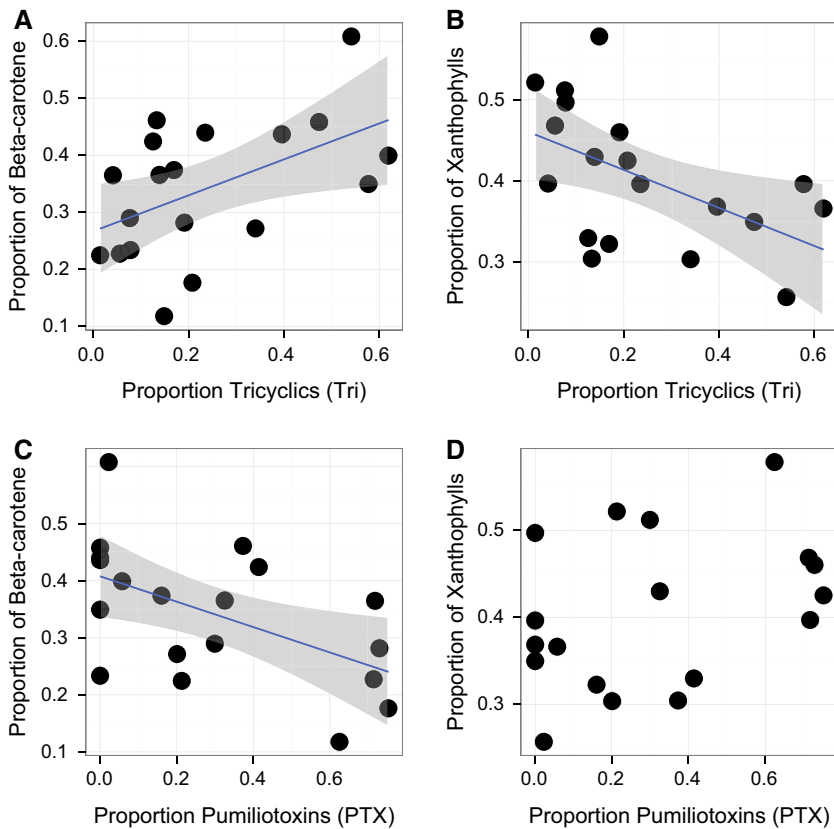


Fig. 3 Relationships of the most common alkaloids and carotenoids found in Solarte male skin samples. *Top* Proportional tricyclic alkaloids (x-axis) and **a** beta-carotene and **b** xanthophylls (y-axis). *Bottom* Proportional pumiliotoxins (x-axis) and **c** beta-carotene and **d** xanthophylls. *Lines* and *shaded areas* flanking the lines represent the predicted line and smoothed 95 % confidence intervals of the statistically significant linear regressions, respectively

inherent and viewer-independent measure of brightness) does not correlate with total skin carotenoids or circulating testosterone levels, it was positively linked to concentrations of two specific xanthophyll carotenoids and negatively linked to quantities of a key class of alkaloid toxins. Viewer-dependent estimates of conspicuousness did not correlate with these measures.

Although exaggerated color expression is due to high levels of testosterone in many species of bird (Folstad and Karter 1992; Kimball and Ligon 1999; Whiting et al. 2006) and lizard (reviewed in Cooper and Greenberg 1992), little is known of how testosterone controls bright coloration in amphibians (Richards 1982). While brighter males of this population are more aggressive (Crothers et al. 2011; Crothers and Cummings 2015), we find here that males with higher total reflectance (and higher associated values of conspicuousness to bird and frog viewers) did not have higher testosterone levels. Several mechanisms may explain the lack of relationship between variation in male signal qualities and circulating testosterone. Though testosterone can correlate positively with amphibian calling behavior (Emerson 2001; Marler and Ryan 1996) and induce changes in color

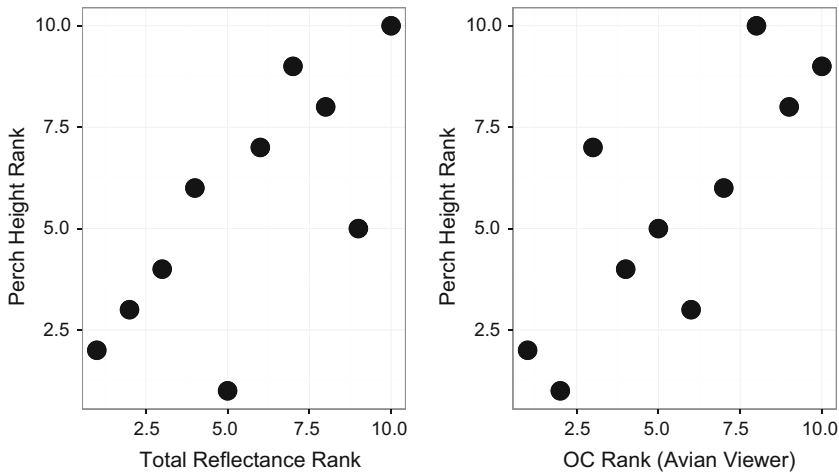


Fig. 4 Ranks of **a** male total reflectance and **b** overall conspicuousness (OC) to an avian viewer against a palm leaf background and males' perch height in their territories in the field at the time of capture. Higher rankings indicate larger values for those variables

pattern (Richards 1982), effects of testosterone on amphibian aggression have not been well investigated (Wilczynski et al. 2005). It is possible that differences in circulating testosterone levels among males may only be detected when the hormone is rapidly modulated during short periods of social instability, as it is in other taxa (Goyman et al. 2007; Wingfield et al. 1990). However, we were unable to test this “challenge hypothesis” (Wingfield et al. 1990) with our dataset. Our experimental methodology may also have precluded us from observing subtle differences in testosterone among males because of stress-induced changes in their hormone profiles (e.g., Mosconi et al. 2007). Furthermore, though the warning signal in this population has evidently been co-opted as a sexual signal, the base color and pattern of various *O. pumilio* morphs are present prior to sexual maturation in both sexes (L. R. Crothers, *pers. obs.*) and thus may not rely on gonadal steroid input.

Contrary to common perception, the maintenance of conspicuous signals is not always testosterone-dependent (Owens and Short 1995) and other hormones have been implicated in the control of both conspicuous male signals and male behavior. Corticosterone (Cote et al. 2010; Moore and Jessop 2003) and melanocortins (Ducrest et al. 2008) both exhibit complex relationships with aggression and male signal expression in other taxa, but their impact on amphibian aggression and coloration are less understood (Wilczynski et al. 2005). Exogenous supplementation of arginine vasotocin has recently been shown to increase aggressive calling in the Neotropical frog *Eleutherodactylus coqui* (Ten Eyck and ul Haq 2012), suggesting that vasotocin may be an especially promising candidate hormone for control of aggression in *O. pumilio*.

Although the skins of *O. pumilio* contain a strikingly complex mixture of carotenoids (Table 1), skin color characteristics such as total reflectance, long-wave chroma, and avian and frog ΔL , ΔC and OC did not correlate with total concentration of skin carotenoids. Though these results were unexpected, a lack of correlation between plumage coloration and aggregate carotenoid content in the integument has been observed in some birds (Saks et al. 2003), and pigments other than carotenoids can often contribute to orange and red

coloration in animals (e.g., pteridines; Bagnara 2003; McGraw 2005; Weiss et al. 2012). However, we did find statistical support for a positive relationship between total reflectance (but not other conspicuousness measures) and two carotenoids that are associated with yellow coloration: xanthophyll and a canary xanthophyll ester (Saks et al. 2003; presented in Table 1; Fig. 1c, d). While these two xanthophylls were not the most abundant carotenoids present in the skin samples, they may play an important function in regulating total reflectance in this population. Little work has been conducted on pigmentary control of amphibian coloration, though pteridines, carotenoids, and melanin can all contribute to color (Bagnara 1976; Obika and Bagnara 1964; reviewed in Rudh and Qvarnström 2013). It should also be noted that although Solarte frogs are generally a uniform orange–red on their dorsal and ventral surfaces, some individuals do also have small, pinprick-sized dark spots on their dorsum. To our knowledge the impact of this spotting on conspicuousness and the pigment molecules controlling this pattern have not yet been explored in this population or in other populations of this species where patterning is more pronounced. Ongoing investigations may elucidate whether other pigment molecules (e.g., pteridines, *L. Freeborn, pers. comm*) or these two xanthophyll carotenoids control aspects of brightness in this population of *O. pumilio*.

We found a significant negative relationship between one conspicuousness measure, dorsal total reflectance, and the quantity of skin defensive chemicals (alkaloids) males of this population. “Brighter”-than-average males had a lower diversity of alkaloids and exhibited a marginal trend for lower alkaloid quantities in their skin (Fig. 2a), but only when total reflectance was treated as a dichotomous measure and not when analyzed as a rank measure. Further testing is necessary to determine the magnitude of this relationship and whether it is linear. Males with a higher total reflectance also had less aggregate pumiliotoxin, which is considered one of the major classes of toxic alkaloids in the genus (Daly et al. 2005). PTX 307A, a pumiliotoxin that we found negatively correlates with total reflectance but not other measures of conspicuousness (Table 2), is one of the few noxious alkaloids found in poison frogs that have been assessed for toxicity. Lethal dosage (LD50) values for this alkaloid are 50 µg/mouse, indicating high toxicity (Daly et al. 2005).

Interestingly, we also detected correlations between the proportions of the most common alkaloids (pumiliotoxins and tricyclics) and the most common skin carotenoids (beta-carotene and xanthophylls) found within these males. Correlations among chemical defenses and pigments have also been observed in ladybirds, where elytral carotenoids correlate negatively with some alkaloids and positively with others, sometimes in a sex-dependent manner (Blount et al. 2012). There has been some speculation that the alkaloid sequestration and chemical modification performed by chemically defended species is oxidatively costly (Ahmad 1992; Blount et al. 2009). For example, there may be a molecular tradeoff where carotenoids can be allocated to buffer the damaging effects of alkaloid sequestration/modification or allocated to the integument as a visual signal. The negative correlations we observe between particular carotenoids and alkaloids (Fig. 3b, c) provide indirect support for this hypothesis, even though carotenoid quantity and alkaloid quantity did not correlate.

There has been much disagreement over whether warning signals should be quantitatively honest, with a relatively tight correlation between conspicuousness and toxicity, or qualitatively honest, where the presence of a signal, regardless of its magnitude, sufficiently advertises secondary defense (Blount et al. 2009; Lee et al. 2011; Speed and Ruxton 2007; Speed et al. 2010). Theoretical investigations have predicted both negative and positive relationships between toxicity and conspicuousness (Blount et al. 2009; Holen and Sæviak 2012; Speed and Ruxton 2007). The few empirical studies that have

investigated the relationship between toxicity and conspicuousness have yielded seemingly conflicting results, both interspecifically (e.g., Cortesi and Cheney 2010; Darst et al. 2006; Summers and Clough 2001; reviewed in Summers et al. 2015) and intraspecifically (Bezzarides et al. 2007; Daly and Myers 1967; Maan and Cummings 2012; reviewed in Speed et al. 2012). Furthermore, it appears that the relationship between toxicity and color can be relatively stochastic across time (as suggested by Daly et al. 2002; Saporito et al. 2006, 2007a, b).

Here we found that while there is a positive relationship between measures of brightness/conspicuousness and toxicity *across* phenotypically distinct *O. pumilio* populations (Maan and Cummings 2012), there is evidently a weak but negative relationship between these traits *within* the Solarte population. Our results may be understood in the context of Holen and Svennungsen's resource allocation tradeoff hypothesis (2012), which explains that aposematic signals can function as handicaps, where a limited resource (e.g., pigments) is used both to buffer the costs of chemical defense and as a component of the visual display. A key prediction of this hypothesis is that there will not necessarily be a perfect correspondence between physiological state and signal magnitude as long as there is enough information for predators to respond appropriately. The across- and within-population relationships between toxicity and coloration in *O. pumilio* fit with these predictions; *across* the archipelago, population-level conspicuousness positively correlates with measures of toxicity, while *within* the exceptionally conspicuous and exceptionally toxic Solarte population a tight correspondence between conspicuousness and chemical defense may not be strategically beneficial. However, it is important to note a caveat when interpreting our results: our inter- and intra-population assessments of 'toxicity' differ significantly. For the inter-population study (Maan and Cummings 2012), the researchers used a measure of noxiousness or irritability (assays using skin extracts injected into sleeping mice), whereas in this intra-population study we assess toxicity based on alkaloid identification and quantification and assume that having greater aggregate pumiliotoxin imparts greater noxiousness/toxicity. Additionally, although pumiliotoxins are commonly considered the important toxic class of alkaloids in this genus, it is not known which particular alkaloids may be most important in chemical defense in *O. pumilio*, and whether that differs depending upon the predator type.

There are several possible explanations for our finding that males with higher total reflectance have less alkaloid diversity and aggregate pumiliotoxin in their skin. Brighter males may be sampled more frequently by predators, and thus expel their toxins more frequently, because they differ behaviorally or visually from duller males (Crothers et al. 2011; Crothers and Cummings 2013). Furthermore, there may be a metabolic tradeoff between brightness and toxicity (e.g., Blount et al. 2009), and/or a correlation between brightness, toxicity, and age. Finally, a strategic trade-off between conspicuousness and toxicity, whereby individuals of a population can gain protection from predators through investing in either toxicity or conspicuousness, is a pattern that has been observed among poison frog species (Darst et al. 2006) but has not yet been identified within a species. Because male warning signal reflectance exhibits a negative relationship with diet-dependent skin alkaloids, it may function as a reliable indicator of territory quality or current condition. Past findings that females prefer brighter males (Maan and Cummings 2009) are thus somewhat surprising in this context, since females may prefer a more conspicuous but physiologically compromised male phenotype within this population. However, further investigations will need to be performed to see whether brighter males do indeed have higher mating success in this population. It should also be noted that the patterns we find in the highly conspicuous and toxic Solarte population may not be observed in other

populations of this species. Sexual preferences and sexual signaling differ across the populations of the archipelago (e.g., Dreher and Pröhl 2014; Maan and Cummings 2008), with some of these differences appearing to be explained by a population's average level of chemical defense and/or conspicuousness (Rudh et al. 2011, 2013).

Cummings and Crothers (2013) argued that predators may impose a selective regime in this species whereby, (1) populations above a toxicity–brightness threshold are at liberty to diversify via sexual selection and below which populations are constrained to maintain a stricter resemblance to a more cryptic population mean, and (2) synergistic/additive effects of inter- and intrasexual selection could drive the evolution of brighter males within populations above the toxicity threshold. Thus we would expect in populations of low average toxicity that individuals deviating from the average aposematic signal of the population would be more vulnerable to detection by predators. However, in populations that are strongly defended, such as Solarte, predators may largely be tolerant to signal variation due to predator generalization (Darst et al. 2006) or perceptual limitations (Crothers and Cummings 2013). Predator permissiveness to signal variation in Solarte, whether driven by predator generalization or the inability of predators to detect the variation, would result in the pattern we observe here—namely, the lack of relationship between conspicuousness and chemical defense.

Intriguingly, brighter males were caught calling at higher locations within their territories (Fig. 4). Kime et al. (2000) found that frog call recordings played at higher locations in the forest experience less degradation than calls played at ground level. The bright males perched at higher locations may therefore be able to project their advertisement calls across larger distances and communicate to a greater number of potential mates. Our within-population findings also fit the across-population findings of Rudh et al. (2011) and Rudh et al. (2013), where males of more conspicuous *O. pumilio* populations were found to inhabit more exposed calling sites and to be more aggressive and exhibit more explorative behavior. However, using higher perches likely comes with trade-offs. Higher males may have less dietary access to alkaloid-containing arthropods found in the leaf litter or may be sampled more frequently by avian predators—which are believed to be the major predator of this species (Dreher et al. 2015; Hegna et al. 2011; Maan and Cummings 2012; Saporito et al. 2007a, b)—and thus expel their accumulated toxins more frequently. A recent clay-model predation study by Dreher et al. (2015) found that avian predation rates are especially high in this population compared to others of the archipelago. This could mean that brighter males' reduced toxicity may indeed be based on more frequent predator sampling because they are more exposed to birds by virtue of their higher perch heights. Alternatively, our past theoretical investigations with a model avian visual system revealed that birds are unlikely to discriminate much of the brightness variation found within the Solarte population, implying that there may be little fitness tradeoff for brighter, less toxic males in terms of predation (Crothers and Cummings 2013).

A benefit of investigating signal evolution in *O. pumilio* is that it is evident that the processes of selection are all impacting the same quantifiable trait: aposematic coloration. In the highly conspicuous and toxic Solarte population, warning signal brightness has seemingly been co-opted as an agonistic indicator trait (Crothers and Cummings 2015). Many status-signaling models require that the signals be strategically costly in order to be evolutionarily stable (Berglund et al. 1996; Maynard Smith and Harper 2003). Sexually selected brightness in this population has previously been shown to correlate with body temperature and a call characteristic that contributes to mating success (Crothers et al. 2011), and there also appears to be a tradeoff between long-wave chroma (~redness) and measures of brightness in some orange/red taxa (e.g., Grether 2000), including this

population (Crothers and Cummings 2013; Maan and Cummings 2009). Brightness, however, does not appear to correlate with several classic measures of body condition, such as length-mass residuals (Crothers et al. 2011), circulating testosterone or aggregate amounts of pigments acquired from the diet. Instead, the costs of color expression may be imparted through a physiological measure unique to chemically defended species: alkaloids derived from the diet and sequestered in the skin. Future investigations may elucidate the physiological or strategic trade-offs driving the relationships we describe here.

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