
LACK OF DEFENSIVE ALKALOIDS IN THE INTEGUMENTARY TISSUE OF FOUR BRILLIANTLY COLORED AFRICAN REED FROG SPECIES (HYPEROLIIDAE: *HYPEROLIUS*)

DANIEL M. PORTIK^{1,4}, LAUREN A. SCHEINBERG², DAVID C. BLACKBURN², AND RALPH A. SAPORITO³

¹Museum of Vertebrate Zoology and Department of Integrative Biology, University of California, 3101 Valley Life Sciences Building, Berkeley, California 94720, USA

²Department of Vertebrate Zoology and Anthropology, California Academy of Sciences, San Francisco, California 94118, USA

³Department of Biology, John Carroll University, University Heights, Ohio 44118, USA

⁴Corresponding author: daniel.portik@berkeley.edu

Abstract.—The sequestration of alkaloids from prey items for the purpose of chemical defense has evolved independently in five frog families, and is documented for approximately 150 taxa. The number of currently recognized cases of defensive toxic sequestration is a conservative estimate, and there is a need for continued exploration of the occurrence of this trait across species. Here, we sampled four species of brightly colored and sexually dichromatic African Reed Frogs (*Hyperolius bolifambae*, *H. concolor*, *H. fusciventris*, and *H. ocellatus*) to determine if their integumentary tissues possess lipophilic alkaloids, the most well studied amphibian defensive compounds. The presence of lipophilic alkaloids in these species would indicate both high toxicity and the ability to sequester such defensive compounds from their diet. We did not detect these compounds, suggesting if Reed Frog coloration is aposematic or related to toxicity, that lipophilic alkaloids are not contributing to this putative toxicity. Additional work is required to characterize other potentially defensive compounds that may be present in the granular glands of reed frogs, including amines, bufadienolides, peptides, and proteins, to further test the hypothesis that their coloration may be related to toxicity.

Key Words.—Africa; alkaloids; aposematism; chemical defense; dichromatism

INTRODUCTION

Amphibians secrete a variety of defensive chemicals from granular glands in the skin, including amines, alkaloids, bufadienolides, peptides, and proteins (Daly 1995). Frogs are capable of synthesizing several of these compounds, yet others are acquired from environmental sources, such as diet. The overall number of vertebrate taxa possessing sequestered defensive compounds is extremely low, with < 1% of terrestrial vertebrate species exhibiting this trait (Saporito et al. 2012; Savitzky et al. 2012), yet there are approximately 150 species of frogs distributed across five families that sequester defensive compounds (Saporito et al. 2012). The independent evolution of the ability to sequester an alkaloid-based chemical defense from prey items has occurred in the family Bufonidae (26 species, genus *Melanophryniscus*), Dendrobatidae (90 species, *Epipedobates*, *Ameerega*, and Dendrobatinae), Eleutherodactylidae (two species, *Eleutherodactylus*), Mantellidae (17 species, *Mantella*), and Myobatrachidae (13 species, *Pseudophryne*; reviewed in Saporito et al. 2012; Hantak et al. 2013). Savitzky et al. (2012) outline several aspects of taxa that exhibit defensive toxic sequestration, namely that they consume a diet consisting of compounds considered toxic to most other animals (for amphibians, this includes a diet largely of

mites and ants), and they often have passive defense, in the form of immobility, aposematism, or mimicry. Considering the recent discovery of a fifth independent origin of this trait in the family Eleutherodactylidae (Rodríguez et al. 2011; Rodríguez et al. 2013), the number of currently recognized cases of defensive toxic sequestration in frogs is likely underestimated in anurans. There is a need for continued skin sampling from additional frog species, particularly those that satisfy the proposed generalized criteria for defensive toxic sequestration.

Hyperoliidae is the largest endemic family of frogs in Africa, and is composed of well more than 220 species across 18 genera (AmphibiaWeb. 2015. Information on amphibian biology and conservation. Available from <http://amphibiaweb.org> [Accessed June 2015]). The most diverse of these genera is *Hyperolius* (Reed Frogs), which contains more than 140 species. Members of this genus exhibit morphology consistent with arboreality (expanded toe discs, elongated limbs, enlarged eyes) and are nocturnal (Schlötter 1999; Amiet 2012). Although the diets of Reed Frogs are poorly known, the bright colors and patterns exhibited by many of these species are consistent with aposematism, which might serve as an advertisement of toxicity (Fig. 1).

Additional properties of hyperoliid body coloration also strengthen the need to investigate members of this

genus for the presence of chemical defenses. At sexual maturation sex steroids can trigger a change in coloration that creates bright sexual monochromatism (both sexes become brightly colored) or sexual dichromatism, in which females undergo color change and males retain juvenile coloration (Hayes and Menendez 1999). Sexual dichromatism of this kind is rare in anurans and is known from only 97 of the more than 6,500 known frog species (Bell and Zamudio 2012). Approximately 40 species of hyperoliids display ontogenetic sexual dichromatism (Fig. 1), which may be driven by factors related to sexual selection and natural selection. One possibility is sexual niche partitioning, in which males and females use different habitats or resources. The complex relationship between diet, chemical defense, coloration, and natural predation permits the possibility of sex-related differences in the chemical defenses of hyperoliid species, a condition found in different species of poison frogs, family Dendrobatidae (Daly et al. 2008; Saporito et al. 2010; Jeckel et al. 2015).

Here, we tested for the presence of alkaloids in the integumentary tissues of males and females of four species in the genus *Hyperolius*. We sampled four species of sexually dichromatic Reed Frogs from Cameroon, including *Hyperolius bolifambae*, *H. concolor*, *H. fusciventris*, and *H. ocellatus* (Fig. 1), for which females are the more brilliantly colored sex. We hypothesized that the overall coloration of these species is related to toxicity, and that they possess alkaloids. If detected, we predicted that there would be sex-related differences in alkaloid levels, with females exhibiting higher quantities of alkaloids.

MATERIALS AND METHODS

Specimen sampling.—We (DMP, LAS, and DCB) collected all live frog specimens in Manjo, Cameroon, 24–28 September 2014. We located frogs during visual surveys conducted at night. We encountered several hyperoliid species of the genus *Hyperolius*, including *H. camerunensis*, *H. bolifambae*, *H. concolor*, *H. dintelmanni*, *H. fusciventris*, and *H. ocellatus*. We collected a series of 62 specimens for *H. bolifambae*, *H. concolor*, *H. fusciventris*, and *H. ocellatus* as part of a larger study, and among other tissue types, we specifically sampled the dorsal and ventral skin. Each of these four species exhibit ontogenetic sexual dichromatism, with females typically more brilliantly colored than the males (Fig. 1). We euthanized frogs using MS-222 (tricaine), and removed the skin below the head and included all dorsal and ventral areas, but not skin of the fore or hind limbs. We divided the whole dorsal and ventral skin in two equal sections by longitudinal cuts and preserved one section in RNA Later (Ambion, Inc., Austin, Texas, USA) for analysis of

gene expression, and the other section in 100% methanol in 4 mL amber glass vials (vial: #IVA70911300; top: IVA71509316; IVA-Analysentechnik, Meerbusch, Düsseldorf, Germany). For this first screening of species presented here, we chose a representative male and female skin sample from each of the four hyperoliid species to determine alkaloid presence or absence, for a total of eight examined skin samples (Table 1). As the sequestration of defensive compounds among amphibians exhibits strong phylogenetic signal (Saporito et al. 2012), this sampling strategy is appropriate for establishing presence/absence data for lipophilic alkaloids across these species.

Experimental procedure.—We prepared individual frog skins using an acid-base alkaloid fractionation as outlined in Saporito et al. (2010), Stynoski et al. (2014), Jeckel et al. (2015), and Mina et al. (2015). As an internal standard, we added 10 µg of nicotine ((-)-nicotine ≥ 99%, Sigma-Aldrich, St. Louis, Missouri, USA) in a methanol solution to each sample prior to the fractionation process. We performed gas chromatography-mass spectrometry (GC-MS) for each individual frog extract on a Varian Saturn 2100T ion trap MS instrument (Agilent, Santa Clara, California, USA), which was coupled to a Varian 3900 GC with a 30 m × 0.25 mm i.d. Varian Factor Four VF-5ms fused silica column. The GC separation of compounds was attained using a temperature program from 100 to 280° C at a rate of 10° C per minute with helium as the carrier gas (1 mL/min). We analyzed each fraction with both electron impact MS and chemical ionization (CI) MS with methanol as the CI reagent.

RESULTS

The GC-MS analysis of skin extracts of male and female *H. ocellatus*, *H. fusciventris*, *H. concolor*, and *H. bolifambae* yielded no detectable dietary derived alkaloids (Fig. 2). However, the alkaloid nicotine (internal standard) was detected in all samples, as well as tricaine (MS-222), which was used to euthanize the frogs (Fig. 2). A small number of fatty acid methyl esters (FAMES) and phthalates were also identified in the skin samples, but they were minor peaks in comparison to that of nicotine and tricaine. No contaminants (plasticizers, mold release agents, rubbers, etc.) were detected in any of the samples.

DISCUSSION

We did not detect lipophilic alkaloids in the skin of males or females of four brilliantly colored species of *Hyperolius*. In this species-rich genus, bright coloration has often been regarded as aposematic (Schjötz 1999). Although the color of these species may indeed serve as

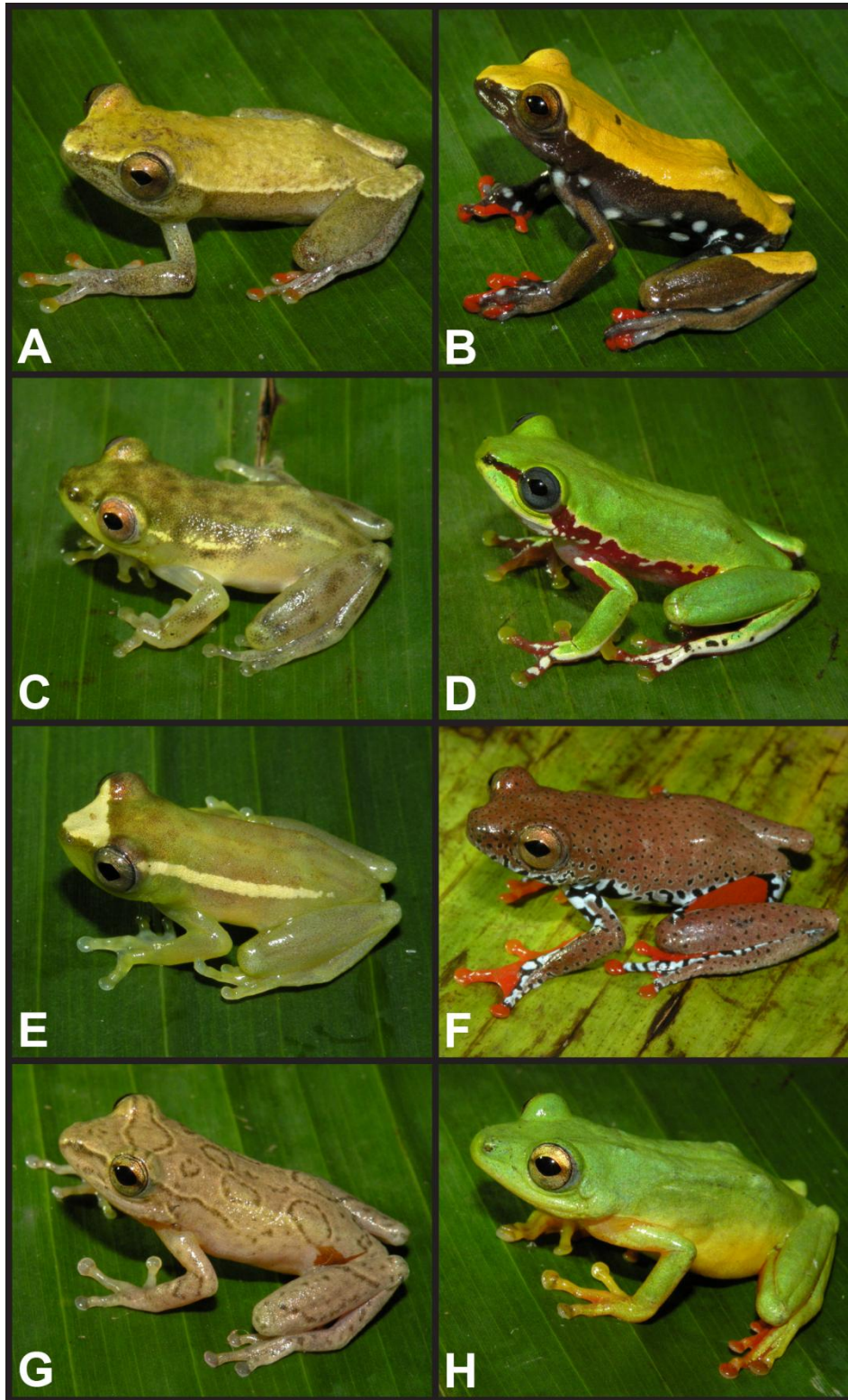


FIGURE 1. Representative males and females of four sexually dichromatic species of hyperoliid frogs from the present study in Cameroon: *Hyperolius bolifambae* (A: male, B: female), *H. fusciventris* (C: male, D: female), *H. ocellatus* (E: male, F: female), and *H. concolor* (G: male, H: female). (Photographed by Daniel M. Portik).

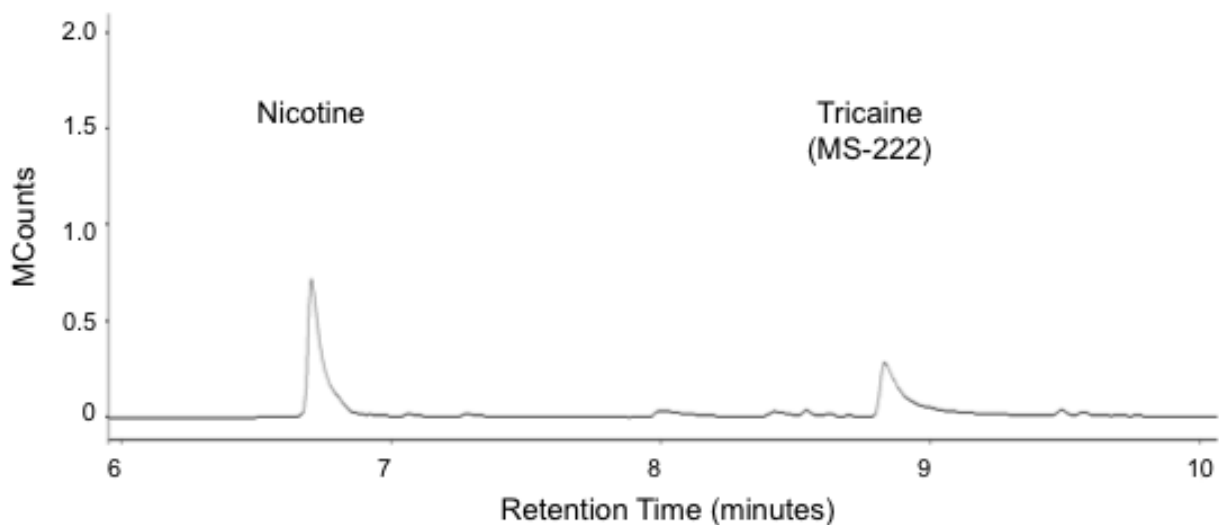
TABLE 1. Specimens included in this study, all of which were collected from Manjo, Littoral Region, Cameroon. Museum No. CAS refers to the California Academy of Sciences.

Species	Museum No.	Sex	Latitude	Longitude
<i>Hyperolius bolifambae</i>	CAS 256907	F	4°50'58.85"N	9°46'18.73"E
<i>Hyperolius bolifambae</i>	CAS 256908	M	4°50'58.85"N	9°46'18.73"E
<i>Hyperolius concolor</i>	CAS 256899	M	4°50'43.23"N	9°46'36.12"E
<i>Hyperolius concolor</i>	CAS 256900	F	4°50'43.23"N	9°46'36.12"E
<i>Hyperolius fusciventris</i>	CAS 256720	F	4°50'58.85"N	9°46'18.73"E
<i>Hyperolius fusciventris</i>	CAS 256721	M	4°50'58.85"N	9°46'18.73"E
<i>Hyperolius ocellatus</i>	CAS 256701	F	4°48'53.11"N	9°46'8.92"E
<i>Hyperolius ocellatus</i>	CAS 256702	M	4°48'53.11"N	9°46'8.92"E

an advertisement of toxicity, our results rule out alkaloids as contributing to the putative toxicity of the skin. There are other chemical compounds produced in the granular glands of amphibians, including amines, bufadienolides, peptides, proteins, and tetrodotoxin (TTX), that have yet to be surveyed across Reed Frog species. These chemicals may be involved in hyperoliid defensive skin secretions; however, additional chemical work will be required to identify specific compounds.

In their review of noxious compounds in the skin of amphibians, Daly et al. (1987) summarized information on the presence of alkaloids for a number of amphibian families and genera. Within Hyperoliidae, the genus *Hyperolius* is listed as having tested negative for the

presence of lipophilic alkaloids. However, this observation is based on a single species without information on the species identification, limiting the utility of this finding. Daly et al. (1987) reported the hyperoliid genus *Kassina* lacked lipophilic alkaloids, also based on a single exemplar. Patterns of alkaloid-based chemical defense occurring in anurans indicate this trait can be restricted to a single genus in diverse families (e.g., Bufonidae, Mantellidae, Myobatrachidae) or even to a small portion of species occurring within a single large genus (e.g., *Eleutherodactylus*). Therefore, although our results indicate a lack of lipophilic alkaloids in the skin of *Hyperolius bolifambae*, *H. concolor*, *H. fusciventris*, and *H. ocellatus*, and Daly et

**FIGURE 2.** Representative gas chromatogram illustrating presence of nicotine (internal standard) and tricaine, MS-222 (ethanizing agent; *Hyperolius fusciventris*; female; specimen no. CAS 256720). The quantity of nicotine is equivalent to 40 $\mu\text{g}/\text{skin}$.

al. (1987) report the same for the genus *Kassina*, we caution against extrapolating these results to the family level. There are 16 other genera occurring in the family that currently lack data for lipophilic alkaloids, which can be sampled for future work.

In addition to summarizing the occurrence of lipophilic alkaloids in amphibians, Daly et al. (1987) also provided information about the presence of amines, bufadienolides, peptides, and proteins occurring in amphibian skin. For the single exemplar in the genus *Hyperolius*, low levels of bufadienolides or related compounds were also detected, but no information was available for amines, peptides, or proteins. As there are over 140 named species in the genus *Hyperolius*, detailed studies of different classes of compounds across multiple species of *Hyperolius* will be required to thoroughly investigate this genus. Daly et al. (1987) reported the genus *Kassina* contained tryptamine (biogenic amine), caeruleins and physalaemins (peptides), and low levels of bufadienolides or related compounds. These findings have led to research and discovery of novel skin peptides with various properties in the genus *Kassina* (Mattute et al. 2000), some of which may provide specific biological defense strategies (Chen et al. 2005; Conlon et al. 2005; Wang et al. 2009a, b, c). These innovative discoveries highlight the need for continued research on skin compounds for this genus and for other genera occurring in the family Hyperoliidae.

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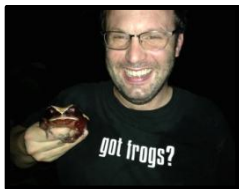
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DANIEL M. PORTIK is a Ph.D. candidate at the Museum of Vertebrate Zoology in the Integrative Biology Department at the University of California, Berkeley, USA. He received a B.Sc. from John Carroll University (2007) and an M.Sc. from Villanova University (2009). His dissertation is focused on the molecular systematics and morphological evolution of hyperoliid frogs in tropical Africa, but he is broadly interested in biogeography, phylogeography, trait evolution, and the natural history of a variety of African reptiles and amphibians. (Photographed by Lauren A. Scheinberg).



LAUREN A. SCHEINBERG is a Curatorial Assistant in the Department of Herpetology at the California Academy of Sciences, USA. She received her B.S. in Biology from the University of California Santa Cruz (2006) and is currently finishing her M.S. in Ecology, Evolution, and Conservation Biology at San Francisco State University. (Photographed by Daniel M. Portik).



DAVID C. BLACKBURN is the Associate Curator of Herpetology at the California Academy of Sciences, USA. He received an A.B. from the University of Chicago (2001) and a Ph.D. from Harvard University (2008). One goal of his research program is to generate an understanding of African frog diversity and evolution since the Cretaceous. He is known to rock a microphone. (Photographed by Daniel M. Portik).



RALPH A. SAPORITO is an Associate Professor of Biology at John Carroll University, University Heights, Ohio, USA. He received his Ph.D. from Florida International University (2007). His research is focused on the ecology and evolution of antipredator adaptations in animals, particularly chemical defenses and aposematism. Most of his research surrounds the study of conspicuously colored poison frogs and the arthropod prey from which they sequester alkaloid-based chemical defenses. (Photographed by Vincent Farallo).