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Myrmecophagy and alkaloid sequestration in amphibians: a study on *Ameerega picta* (Dendrobatidae) and *Elachistocleis* sp. (Microhylidae) frogs

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Abstract. Frogs of the family Dendrobatidae and Microhylidae are myrmecophagous, i.e., they feed on ants. It is well accepted that toxic alkaloids in the skin secretion of dendrobatid frogs are of dietary origin and may derive from ants. Alcohol extracts from specimens of *Ameerega picta* (Dendrobatidae) and of *Elachistocleis* sp. (Microhylidae) from two locations in Bolivia were analyzed by gas chromatography-mass spectrometry. Whereas the extracts of *Ameerega picta* specimens exhibited a typical profile of toxic alkaloids, those from *Elachistocleis* sp. were alkaloid-free. Fecal samples from both frogs consisted of ant remnants. These results indicate that myrmecophagy provides dendrobatids with alkaloids, which they sequester and store in their skin, but an alkaloid sequestering system is absent in the microhylid *Elachistocleis* sp.

Key words. Amphibia, Anura, Dendrobatidae, Microhylidae, myrmecophagy, skin alkaloids.

Introduction

Amphibian skin secretions are a rich source of biologically active compounds including a wide variety of chemical substances such as amines, terpenes, steroids, alkaloids, peptides and proteins. Whereas a number of these compounds are synthesized by the amphibians itself, such as steroids by salamanders (*Salamandra* spp.) and toads (*Bufo* spp.), peptides by frogs (*Phyllomedusa* spp. etc.) and orange-speckled toads (*Bombina* spp.), others like tetrodotoxin or alkaloids are obviously of exogenous origin, either produced by symbiotic bacteria as suggested for tetrodotoxin in the case of newts (*Taricha, Notophthalmus* spp.) and toads (*Atelopus* spp.), or originate from the diet (e.g., Mebs 2002). More than 800 alkaloids have been identified in skin extracts of frogs of the families Dendrobatidae, Mantellidae and Myobatrachidae, but also from toads of the genus *Melanophryniscus* (Daly et al. 1984, 1993, 2005, 2008, Garraffo et al. 1993, Smith et al. 2002, Mebs et al. 2005, 2007). Most of these compounds are toxic and have been found to be of dietary origin. They are predominantly produced by ants, mites and other arthropods (Daly et al. 2000, 2002). When breeding dendrobatid frogs in captivity and feeding the froglets with crickets or *Drosophila*, which are free of alkaloids, the adult frogs are entirely non-poisonous, i.e., lack even traces of alkaloids (Daly et al. 1997). This also stresses the importance of a special food source for becoming poisonous. Ants, termites and mites are the major food constituents of Dendrobatidae and Mantellidae frogs (Toft 1980a,b, 1981, 1995, Lieberman, 1986, Bonilla & La Marca 1986, Donnelly 1991, Caldwell 1996, Biavati et al. 2004). Alkaloids like the pumiliotoxins have been detected in formicine ants of the genera *Brachymyrmex* and *Paratrechina* (Saporito et al. 2004) as well as in mites of the family Oribatidae (Takada et al. 2005, Saporito et al. 2007). Dietary specialization on ants, i.e., myrmecophagy, and alkaloid sequestration appear to be important factors in the evolution of the Dendrobatidae (Vences & Kniel 1998, Vences et al. 1997/98, Santos et al. 2003).

Besides dendrobatids many Microhylidae frogs are ant feeders, such as *Phrynomantis* species, which are reported to have noxious skin secretions (Passmore & Carruthers 1995). However, they lack skin alkaloids, but rather secrete a complex mixture of peptides (Mebs, unpublished results). Moreover, the microhylid frogs *Elachistocleis bicolor, E. erythrogaster* and *E. ovalis* from the Auracaria forest in Brazil were found to feed exclusively on ants and termites (Solé et al. 2002, 2005). The diet of *E. bicolor* specimens from Northern Uruguay was composed mainly of *Pheidole* and *Solenopsis* ants and termites (Berazategui et al. 2007).

Several taxonomic problems exist in the genus *Elachistocleis* (De la Riva et al. 2000, Lavilla et al. 2003). Regarding *E. ovalis*, *E. bicolor* and *E. surinamensis*, Lavilla et al. (2003) suggested the following key identification of *Elachistocleis* species: species of this genus with immaculate, yellow, ventral coloration, which inhabit the northern part of their original range of distribution, should be considered as *E. ovalis.* Species with immaculate venter inhabiting the southern part of their original range should be considered as *E. bicolor.* Reichle (2006) mentioned that in

Bolivia there are at least two species with a mottled venter: *Elachistocleis* cf. *skotogaster* from the Andean Dry Valleys and the Tucuman Bolivian Forest, and one species from the lowlands (*Elachistocleis* sp.). However, even more species may exist. The *Elachistocleis* specimens in this study are from the eastern lowlands of Bolivia and have a mottled venter, and are, therefore, named *Elachistocleis* sp.

In the present study, the dendrobatid *Ameerega picta* and the microhylid *Elachistocleis* sp. that live sympatrically in the same habitat, a savannah region of Bolivia, were investigated for the alkaloid content of their skin secretions, since both are myrmecophagous species. In skin extracts of *A. flavopicta* from Brazil, several characteristic alkaloids had been identified such as pumiliotoxin **251D**, histrionicotoxin **285D** and the two decahydroquinolines **219A** and **243A** (Mortari et al. 2004). So far no data exist about constituents of skin secretions of *Elachistocleis* species.

Material and methods

Elachistocleis sp. (Fig. 1) and *Ameerega picta* (Fig. 2) were collected in the area of the Hacienda San Sebastián, Department of Santa Cruz, and at Selva Blue, Department of Beni, Bolivia (for exact locations see Table 1). Three specimens of *Ameerega* and nine of *Elachistocleis* were individually placed in 70% ethanol for museum purposes. The content of fecal samples of the frogs was analyzed microscopically.

The first ethanol extracts containing alcohol-soluble compounds were analyzed for the presence of alkaloids, which were identified by gas chromatography combined with mass spectrometry (GC/MS). Extracts were evaporated to dryness with a stream of air at 25 °C and dissolved in 100 or 200 µl chloroform. One µl was subjected to GC/MS, performed on an Agilent Technologies (Waldbronn, Germany) HP6890 GC equipped with an autosampler HP6890 ALS and interfaced to a HP5973 MSD. A Factor Four MS capillary column (CP 8912, 30 m × 0.25 mm I.D., 0.25 µm film thickness) from Varian (Darmstadt, Germany), which was protected by a guard column, deactivated (diphenyltetramethyldisiloxane) glass capillary (1.5 m × 0.25 mm I.D.) from BGB Analytik AG (Anwil, Switzerland), was used with 1.0 mL/min helium as carrier gas. Splitless injection was performed at 230 °C injection port temperature and a temperature program from 80 °C, which was held for 2 min. and increased by 12 °C/min. to 310 °C, held for 6.5 min., was applied. The MS transfer line was maintained at 280 °C, the ion source at 250 °C, and operated with 70 eV ionization energy. Mass spectra were recorded in full scan mode from *m/z* 43 to *m/z* 550. Data analysis was performed using the HP ChemStation software (Rev. B.01.00). Synthetic pumiliotoxin **251D** (Sudau et al. 2002), kindly provided by U. Nubbemeyer, University of Mainz, was used as standard compound. Identification of the major alkaloids was achieved by comparison with published data (Daly & Spande 1986, Daly et al. 1993) and with MS-spectra provided by the software NIST/EPA/NIH Mass Spectral Database (Rev C.00.00). Code names were assigned to the alkaloids in the manner of Daly et al. (1987, 2005). Bold-faced numbers indicate the nominal mass, the bold-faced letter is used for identification of the alkaloids exhibiting the same nominal mass according to the recent tabulation of anuran alkaloids.

Results

Two or three major alkaloids were detected in the ethanol extracts of *Ameerega picta*, e.g., 2.5 disubstituted decahydroquinoline, histrionicotoxins and a pumiliotoxin. The extract of the specimen from the Beni province exhibited a different profile with histrionicotoxins of higher masses (Table 1). Although this is not an exhaustive analysis and minor alkaloids present in the extracts have not been further characterized, the data clearly demonstrate that *A. picta* is able to sequester and accumulate alkaloids in its body, particularly in the skin.

On the other hand, all of the alcohol extracts of nine *Elachistocleis* sp. were entirely free of alkaloids and contained not even traces of these compounds. Microscopic examination of fecal samples from *A. picta* (one sample from Hacienda San Sebastián) and two from *Elachistocleis* sp. revealed the presence of ants of similar species (Fig.

Figure 1. *Elachistocleis* sp. from San Sebastián, Department of Santa Cruz, Bolivia.

Figure 2. *Ameerega picta* from Selva Blue, Department of Beni, Bolivia.

Table 1. Major alkaloids in ethanol extracts from three *Ameerega picta* specimens. Abbreviations: DHQ – 2.5 disubstituted decahydroquinoline, HTX- histrionicotoxin, PTX- pumiliotoxin (for chemical structures see Mebs et al. 2008); SMF – Senckenberg Museum Frankfurt.

Sample	Location	Alkaloids
SMF 88379	Hacienda San Sebastián, Santa Cruz (S 16°21.749′, W 62°01.317′, 537m)	DHQ **195B**, HTX **235**[a]
SMF 88377	Same	DHQ **219A**, HTX **235A**, PTX **267A**
SMF 88378	Selva Blue, Beni (S 12°46.147′, W 65°48.920′, 153m)	HTX **285A, 287A, 291A**

3). These results indicate that despite using the same food source, mainly ants, only the dendrobatid frog *A. picta* is able to store toxic alkaloids and thus becomes poisonous, whereas *Elachistocleis* sp. lacks this ability.

Discussion

Myrmecophagy is a common phenomenon in frogs and toads. Ants appear to be an important source of alkaloids to be sequestered by members of the Dendrobatidae family. In Myrmicinae ants, several pyrrolidine, pyrrolizidine, piperidine, indolizidine, quinolizidine and decahydroquinoline alkaloids have been identified (Daly et al. 1994a, 2000, Jones et al. 1999, Clark et al. 2005). Moreover, pumiliotoxins have been found in formicine ants of the genera *Brachymyrmex* and *Paratrechina* (Saporito et al. 2004).

The dietary origin of skin alkaloids in dendrobatid frogs is well accepted and the high variability in the alkaloid profiles of individual frogs is considered to originate from temporal and spatial variations as well as from the availability of alkaloid-containing prey arthropods (Saporito et al. 2006, Mebs et al. 2008). This phenomenon is demonstrated in the third specimen of *A. picta* from the Beni region by its exhibiting a profile of the major alkaloid constituents that is different from that of the two specimens from the Santa Cruz region. The presence of pumiliotoxin PTX **267A** in one of the *A. picta* samples is of particular interest, because is it suggested that it is a derivative of PTX **251D** enzymatically hydroxylated by the frog as observed in other dendrobatid species by Daly et al. (2003). PTX **267A** is more toxic than the parent compound PTX **251D**.

It is also interesting to note that the alkaloid epibatidine, which has been found in skin extracts from the Ecuadorian *Epipedobates tricolor* besides the major alkaloid PTX **251D** (Spande et al. 1992), was neither detected in the extracts of the present study nor had it been identified in the Brazilian *Ameerega flavopicta* (Mortari et al. 2004).

Myrmecophagy in anurans is not necessarily leading to toxicity, which has been clearly shown in the present study.

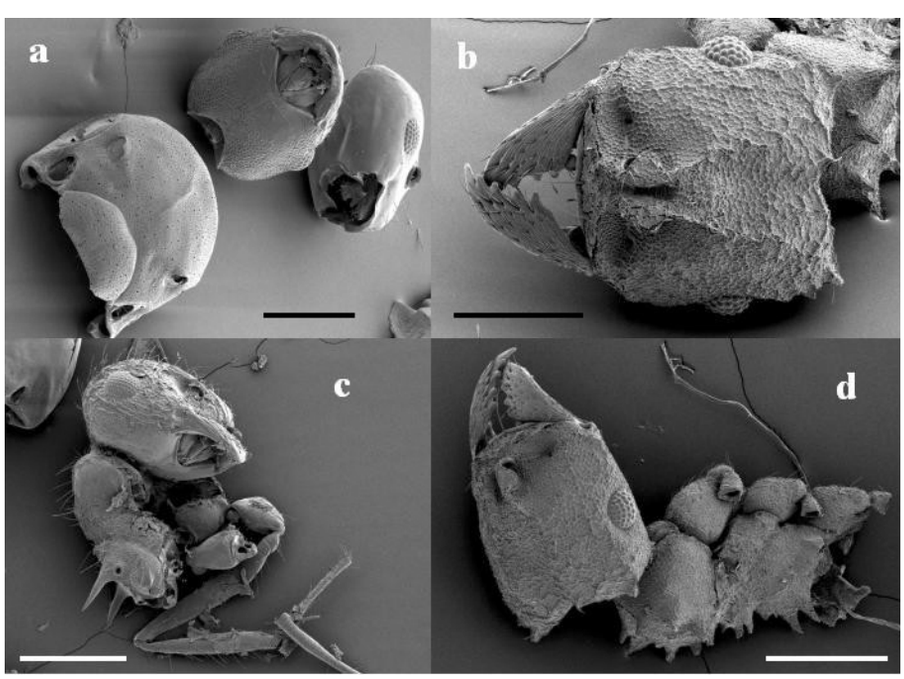

Figure 3. Feces from *Elachistocleis* sp. containing various remnants of ants (scanning electron micrographs): a – Formicinae or Dolichoderinae ants (bar: 400 µm); b and d – *Mycocepurus* spp., Myrmicinae, tribus Atti (bar: 300 and 400 µm); c – Myrmicinae (*Crematogaster*?) (bar: 600 µm). The analysis of a fecal sample from *Ameerega picta* revealed a similar composition.

Alcohol extracts from the microhylid frog *Elachistocleis* sp. from the same area and habitat as *A. picta*, the Chiquitano Dry-Forest of the Hacienda San Sebastián, Santa Cruz, Bolivia, were entirely free of alkaloids. Fecal samples from both frogs consisted of similar remnants of ants. It is well established that *Elachistocleis* species feed exclusively on ants and termites (Solé et al. 2002, 2005; Berazategui et al. 2007).

In a similar study on a specimen of *Silverstoneia flotator* from the Golfito region of southern Costa Rica, it was shown that the alcohol extract was alkaloid-free (Mebs, unpublished results). Many Colostethinae and Hyloxalinae species within the Dendrobatidae family do not contain alkaloids in their skin (Daly et al. 1994). In feeding experiments with a mixture of several alkaloids, Daly et al. (1994b) demonstrated that these anurans are not able to accumulate these compounds.

Sequestration of toxic alkaloids from the diet such as arthropods requires a special uptake and transport system from the gut to the granular skin glands where these compounds are stored and secreted. Dendrobatid and mantelline frogs, but most probably all other alkaloid-containing anurans developed a highly efficient and specific sequestering system (Daly et al. 1994b, 1997), which appears to be well-conserved and merely overexpressed in these animals (Daly 1998). Such a system is obviously absent in *Elachistocleis* sp., a obligatory myrmecophagous frog.

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