

Comment on Amézquita *et al.* (2017) “Conspicuousness, color resemblance, and toxicity in geographically diverging mimicry: The pan-Amazonian frog *Allobates femoralis*”

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Comment on Amézquita *et al.* (2017) “Conspicuousness, color resemblance, and toxicity in geographically diverging mimicry: The pan-Amazonian frog *Allobates femoralis*”

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RAS and TG contributed equally to the writing of this manuscript.

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Abstract. Amézquita *et al.* (2017) recently concluded that species of the *Allobates femoralis* group are toxic to mice at levels equivalent to syntopic alkaloid-containing poison frogs, which they attributed to the presence of alkaloids in skin secretions. However, the chemical composition of skin secretions was not analyzed, and here we present additional data supporting the absence of alkaloids in skin secretions of the *Allobates femoralis* group. Instead, we suggest the observed toxicity was caused by the anesthetic benzocaine, which was applied to the buccal cavity to euthanize frogs prior to skin removal. We show that orally administered benzocaine is rapidly incorporated into the skin of species that sequester and do not sequester alkaloids, which casts doubt on the conclusion that *Allobates femoralis* group skin secretions are toxic and makes the results of experiments with alkaloid-containing species of *Adelphobates* and *Ameerega* uninterpretable. To prevent experimental errors and misinterpretations in studies of amphibian chemical defense, we encourage researchers to test the chemical composition of samples prior to experimentation, include all necessary controls to detect false positives, conduct small pilot studies for new methods, and consider the limitations of particular methods and their ability to address the intended research questions.

Keywords. Alkaloid, Anura, benzocaine, chemical defense, poison frogs, toxicity.

Main text.

Brightly colored poison frogs of the family Dendrobatidae secrete alkaloid-based chemical defenses that are sequestered from dietary arthropods and provide protection from predators and pathogens (Saporito *et al.* 2012; Mina *et al.* 2015). Sequestration as a defensive strategy appears to have evolved independently in four lineages of dendrobatids, with 97 dendrobatid species in 11 genera known or believed (based on coloration and phylogeny) to employ alkaloid-based defenses (Grant *et al.* 2006, 2017; Saporito *et al.* 2012; Santos *et al.* 2014). No species of the sister group of Dendrobatidae, Aromobatidae, is known to possess alkaloid-based chemical defenses (Grant *et al.* 2006, 2017), despite the astonishing resemblance in coloration and skin texture of *Allobates femoralis* group species to several alkaloid-containing species of the dendrobatid genus *Ameerega*. Daly *et al.* (1987: 1078) reported a trace amount of the 5,8-disubstituted indolizidine **181B** from *Allobates femoralis* in Napo, Ecuador but detected no alkaloids in frogs from five other localities (Lago Agrio, Ecuador; Putumayo, Colombia; Pebas, Peru; Rio Llullapichis, Peru; and Cataracts, Surinam), and J.W. Daly clarified that the Napo sample actually contained a “trace, trace” amount (pers. comm. to TG and RAS) that should not be treated “as evidence for significant ability for accumulation of alkaloids in this species” (*in litt.* to TG, 2 February 2000). We further confirm the absence of lipophilic alkaloids in samples of *Allobates femoralis* from Reserva Extrativista Riozinho Liberdade, Brazil (Fig. 1), although samples from other populations and/or feeding experiments could lead to different results. Similarly, Darst *et al.* (2005: 59) characterized *Allobates zaparo*, also part of the *Allobates femoralis* group, as possessing “trace (T), possibly very small amount of alkaloids,” but their assessment of skin secretions used thin layer chromatography and the identity of the supposed alkaloids was not determined. Furthermore, injected skin secretions of both *Allobates femoralis* and *Allobates zaparo* have been found to be non-toxic to the house mouse, *Mus musculus* (Darst and Cummings 2006; Darst *et al.* 2006). Consequently, species of the *Allobates femoralis* group are believed to lack skin alkaloids and be Batesian mimics of sympatric species of alkaloid-containing species of the genus *Ameerega* (Darst and Cummings 2006; Darst *et al.* 2006).

Contrary to those previous findings, Amézquita *et al.* (2017) recently concluded that *Allobates femoralis* group skin secretions are toxic, using behavioral assays with mice, at levels equivalent to syntopic alkaloid-containing dendrobatids in the genera *Adelphobates* and *Ameerega*, suggesting they are Müllerian, not Batesian, mimics. Moreover, they attributed the toxicity of *Allobates femoralis* group species to the presence of alkaloids in the frog skin

secretions. However, critical oversights in their experimental procedures call those conclusions into question.

First, Amézquita *et al.* (2017) did not analyze the chemical composition of the skin secretions they studied, so their conclusion that the inferred toxicity of samples was due to alkaloids is unfounded. Second, although not specified in Materials and Methods, Amézquita *et al.* (2017) euthanized frogs by buccal application of an undetermined quantity of 20% benzocaine in gel form (Benzotop, DFL Indústria e Comércio, Rio de Janeiro, RJ, Brazil; A.P. Lima, *in litt.* to TG, 23 March 2017) prior to skin removal. Benzocaine (10–20% solution) is a synthetic anesthetic commonly used for euthanizing frogs (McDiarmid, 1994), typically by direct application to the skin where it is quickly absorbed; however, this poses a significant problem when studying the physiological and/or behavioral effects of skin secretions on other organisms. At the molecular level, frog alkaloids and benzocaine share similar modes of action: frog alkaloids interfere with various subtypes of nicotinic receptors and a diversity of ion channels (e.g., Na⁺, K⁺, Ca²⁺ channels; Daly *et al.* 1999, 2005; Santos *et al.* 2016), and benzocaine interferes with Na⁺ channels (Schneider and Dubois 1986; Wang and Wang 1994; Quan *et al.* 1996). Also, benzocaine is known to inhibit locomotor activity in mice (Reith *et al.* 1985) and cause methemoglobinemia (Davis *et al.* 1993), the symptoms of which vary in humans from cyanosis, dizziness, lethargy, and syncope to seizures, arrhythmias, shock, and even death, depending on methemoglobin concentration (Rodriguez *et al.* 1994). In non-human animals, even a low concentration of methemoglobin (10%) “results in a direct decrement in O₂-carrying capacity of erythrocytes, which can affect performance studies” (Davis *et al.* 1993: 1325). In an attempt to circumvent this problem, Amézquita *et al.* (2017) applied benzocaine to the buccal cavity of frogs, assuming this would prevent benzocaine from contaminating the skin secretions.

Unfortunately, orally administered benzocaine accumulates rapidly in the highly vascularized skin of frogs and is present in skin extracts following euthanasia. On the basis of new data presented below, oral administration of lethal doses of benzocaine results in the accumulation of large amounts of benzocaine in euthanized individuals of both alkaloid-sequestering and non-alkaloid-sequestering frogs (Fig. 2; for chemical profiles of individuals of these poison frog species euthanized by other means, see Daly *et al.* 1987, Jeckel *et al.* 2015a,b). In the bufonid poison frog *Melanophryniscus moreirae*, the amount of benzocaine in the skin following oral administration of 10 µL of liquid Orajel ® containing 20% benzocaine (Church & Dwight Co., Inc., Ewing, New Jersey, USA) was equivalent to almost 50% of the total amount of the naturally occurring chemical defenses (Fig. 2a). The large quantity of benzocaine was

greater than the amount of bufotenine and almost as great as the amount of pumiliotoxin **267C**, the most abundant skin alkaloid in this species (Jeckel *et al.* 2015a). In identical experiments with a captive bred individual of the dendrobatid poison frog *Dendrobates auratus* and the non-alkaloid sequestering ranid frog *Lithobates clamitans*, benzocaine also rapidly accumulated in the skin in large amounts (Fig. 2b & 2c). As such, following the procedure of Amézquita *et al.* (2017), skin samples that originally contained and did not contain lipophilic alkaloids would all contain large amounts of benzocaine. Although a detailed understanding of the pharmacokinetics of benzocaine in amphibians is lacking, in fish benzocaine is rapidly distributed to highly perfused tissues prior to elimination (Meinertz *et al.* 1999), and we suspect the same occurs in amphibians. When used as an agent for euthanasia, benzocaine elimination does not proceed, which might explain its presence in such large amounts in the skins of euthanized frogs.

The fact that large amounts of benzocaine accumulate in the skin following oral administration of a lethal dose of that anesthetic casts doubt on Amézquita *et al.*'s (2017) conclusions about frog toxicity. Injection of *Allobates femoralis* group skin extracts lacking naturally occurring skin alkaloids but containing large amounts of benzocaine are expected to elicit similar responses to those reported by Amézquita *et al.* (2017), with variation in the extent of responses caused by differences in the unquantified amounts of benzocaine used to euthanize frogs. Further, the occurrence of large and variable amounts of benzocaine in skins of alkaloid-containing species of *Adelphobates* and *Ameerega* makes the results of experiments with those species uninterpretable, since responses would vary as a function of both the well-documented individual variation in naturally occurring skin alkaloids (Saporito *et al.* 2012) and the different amounts of benzocaine used to euthanize frogs.

Despite our criticisms, studies like that of Amézquita *et al.* (2017) aiming to investigate the relationship between geographic variation in coloration and chemical defenses are essential to understand the ecology and evolution of chemical defense in anurans, and it is not our aim to discourage efforts in this area. To the contrary, we hope this critique will help future studies to take the necessary steps to ensure the integrity of their results. To that end, we offer the following general guidelines. First, it is imperative that the chemical composition of samples be tested prior to any subsequent experimentation. We have shown that oral administration of lethal doses of benzocaine result in the accumulation of large amounts of the anesthetic in the skin of euthanized frogs, and other anesthetics commonly used to euthanize amphibians, including tricaine methanesulfonate (MS-222) and lidocaine, are likely to behave similarly. In

contrast, euthanasia by immersion in ethanol would result in the extraction of alkaloids from skin, which would also alter the results of subsequent experimentation. Chemical analysis of skins would have allowed Amézquita *et al.* (2017) to detect benzocaine in their samples and either choose a different method of euthanasia (e.g., Shine *et al.*, 2015; Lillywhite *et al.* 2017) or include benzocaine control injections in their procedures. Second, studies must include all necessary controls to detect false positives. Amézquita *et al.* (2017) appropriately injected saline solution into mice as a negative control, but the inclusion of a species confirmed to lack alkaloids (e.g., a non-poison frog) would have enabled them to determine if some other component of their experimental treatment was confounding their results. Third, we strongly recommend conducting small pilot studies, especially when experiments require individuals to be euthanized. A small pilot study that tested chemical compositions and included all necessary controls would have allowed Amézquita *et al.* (2017) to correct their procedures before euthanizing the 95 frogs included in their study.

Finally, the limitations of particular research methods must be considered. For example, lipophilic alkaloids are not the only sources of chemical defenses in anurans, so it is possible for skin secretions of species that lack lipophilic alkaloids to elicit positive toxicity responses. All amphibians possess granular glands that presumably secrete defensive chemicals (Toledo and Jared, 1995), yet fewer than 200 species are known or believed to secrete alkaloids. Even among dendrobatids, two species that lack lipophilic alkaloids possess water-soluble toxins (Daly *et al.* 1994; Grant, 2007), and amphibians generally are known to secrete biogenic amines, bufadienolides, peptides and proteins, and several classes of alkaloids (Erspamer, 1994). Similarly, chemical defenses evolved in relation to naturally occurring pathogens and predators, not the mouse. Mice are the standard test subjects in many toxicity studies because they are believed to be a good proxy for humans, but, given that mice are not considered a potential natural predator or closely related to any potential predator of poison frogs and defensive secretions are ingested, not injected, the ecological or evolutionary relevance of the effects of these injections on mice is unclear (for further discussion, see Bolton *et al.* 2017; Weldon, 2017). That is, injection of skin secretions might be highly toxic to mice while ingestion might have little or no effect on potential avian, snake, or arthropod predators, or they might have no effect on mice, but be extremely effective at inhibiting pathogens across a broad spectrum. The reliance on particular and perhaps inappropriate species for bioassays of toxicity can be avoided by instead focusing on the chemical composition of secretions, but care in interpretation is required here as well, as such studies provide no evidence regarding the

functional effects of secretions on potential pathogens and predators. Consequently, studies on the alkaloid composition of poison frogs cannot say whether a frog with one set of alkaloids is more or less defended ("toxic") than another frog with a different set of alkaloids, even if the total number or quantity of alkaloids differs greatly.

Chemical defense is a fundamental aspect of the biology of amphibians that has shaped their evolution and governs their ecology, and interdisciplinary studies that focus on chemical defense are essential to construct a complete understanding of this clade. Nevertheless, it is imperative that researchers understand both the biology and the chemistry in order to avoid mistakes that undermine or invalidate their conclusions.

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Figure Legends

Figure 1. Gas chromatography (GC) trace of two individual *Allobates femoralis* from Reserva Extrativista Riozinho Liberdade, Brazil. No alkaloids were detected in either individual. Note: the unlabeled peaks represent fatty acids. MCounts (Mega Counts) is a measure of the total ion count (TIC) for each compound present in the GC trace. Retention time represents the time interval between sample injection and the maximum peak height for each compound in the GC trace. See *Supplementary Information* for experimental methods.

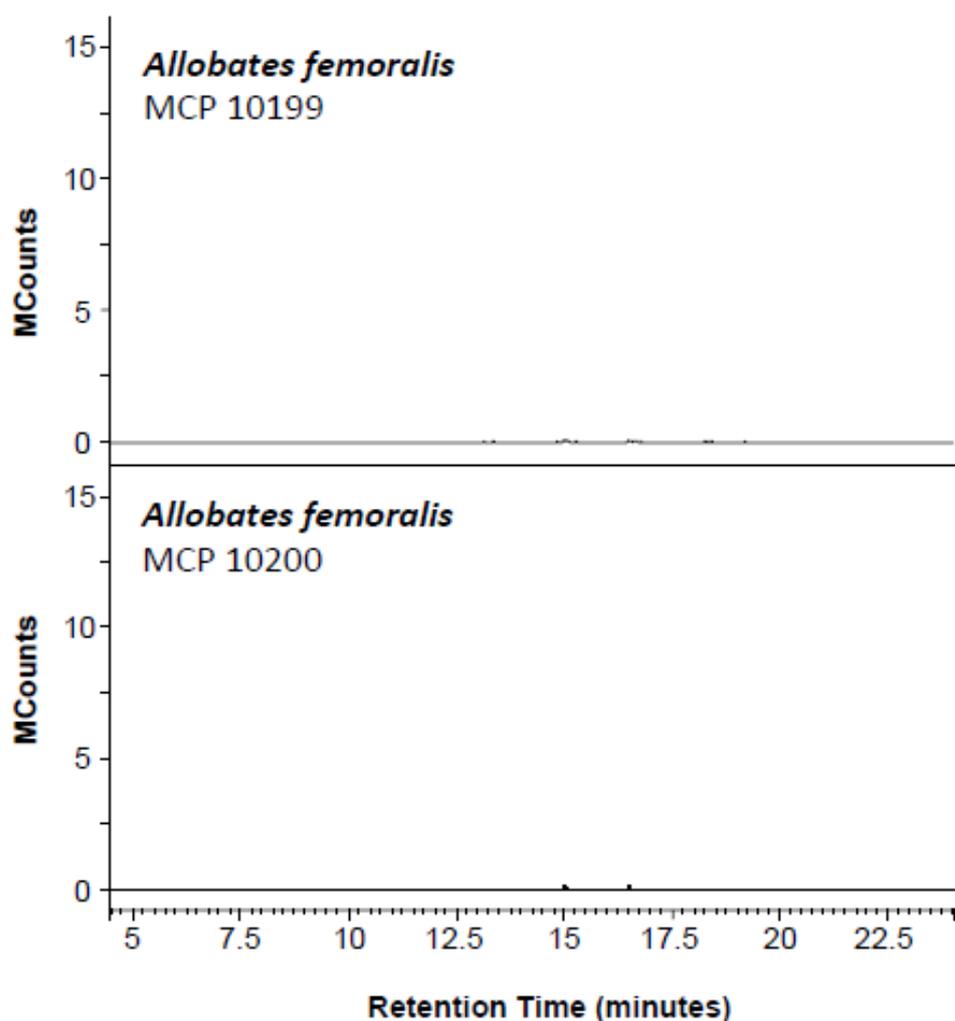


Figure 2. (a) Gas chromatography (GC) trace of individual *Melanophryniscus moreirae* skin/methanol extract. Alkaloid classes: tricyclic (Tri), pumiliotoxin (PTX), and allopumiliotoxin (aPTX). The unlabeled peaks represent fatty acids and fatty acid methyl esters (FAMES); (b) GC trace of individual *Dendrobates auratus* skin/methanol extract; (c) GC trace of individual *Lithobates clamitans* skin/methanol extract. The unlabeled peaks represent fatty acids. MCounts (Mega Counts) is a measure of the total ion count (TIC) for each compound present in the GC trace. Retention time represents the time interval between sample injection and the maximum peak height for each compound in the GC trace. See *Supplementary Information* for experimental methods.

