Title: A review of chemical defense in harlequin toads (Bufonidae; *Atelopus)*

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Abstract

Toads of the genus *Atelopus* are chemically defended by a unique combination of endogenously synthesized cardiotoxins (bufadienolides) and neurotoxins which may be sequestered (guanidinium alkaloids). Investigation into *Atelopus* small-molecule chemical defenses has been primarily concerned with identifying and characterizing various forms of these toxins while largely overlooking their ecological roles and evolutionary implications. In addition to describing the extent of knowledge about *Atelopus* toxin structures, pharmacology, and biological sources, we review the detection, identification, and quantification methods used in studies of *Atelopus* toxins to date and conclude that many known toxin profiles are unlikely to be comprehensive because of methodological and sampling limitations. Patterns in existing data suggest that both environmental (toxin availability) and genetic (capacity to synthesize or sequester toxins) factors influence toxin profiles. From an ecological and evolutionary perspective, we summarize the possible selective pressures acting on *Atelopus* toxicity and toxin profiles, including predation, intraspecies communication, disease, and reproductive status. Ultimately, we intend to provide a basis for future ecological, evolutionary, and biochemical research on *Atelopus*.

1. Introduction

Harlequin toads (Anura: Bufonidae: *Atelopus*) are small, diurnal, and poisonous amphibians native to South and Central America (Lötters et al., 2011). Many species are brightly colored on all or part of their bodies (Fig. 3c; Lötters et al., 2011), and these colors may act as aposematic signals to warn potential predators of their toxicity (Rößler et al., 2019). Despite being members of the family Bufonidae, Harlequin toads are smooth skinned and lack the large parotoid glands commonly observed in other toads. Instead, *Atelopus* granular glands are small and evenly distributed across their bodies (Mcdiarmid, 1971). Concentrated within the granular glands and skin epithelium (Mebs et al., 2018a) are two classes of toxic chemicals: bufadienolides and guanidinium alkaloids (Daly et al., 1997). With the

possible exception of *Clinotarsus curtripes* (see Section 4.1.1; Gosavi et al., 2014), the cooccurrence of these toxins is unique to *Atelopus*, and extensive research has focused on describing the chemicals found in *Atelopus* skin – uncovering several toxins found nowhere else in the natural world (Yotsu-Yamashita et al., 2004; Yotsu et al., 1990b). However, toxin assessment of *Atelopus* species has been geographically and taxonomically biased, and most species have not been evaluated. Furthermore, the ecology and evolution of *Atelopus* chemical defenses have received little investigation.

Amphibians have experienced severe and widespread declines in recent decades (Stuart et al., 2004). *Atelopus* have suffered a particularly drastic decline; a major survey in 2005 found that, of species with sufficient population trend data (52 of 113 known species), 81% were in decline and 56% were possibly extinct. Chytridiomycosis, a disease caused by the fungal pathogen *Batrachochytrium dendrobatidis,* is implicated in many of the declines (La Marca et al., 2005; Lampo et al., 2017), and habitat loss and degradation are likely also important drivers (Gómez-Hoyos et al., 2020; Jorge et al., 2020b; Santa-Cruz et al., 2017)**.** Recently, several *Atelopus* species thought to be extinct or locally extirpated have been rediscovered (Barrio Amorós et al., 2020; Enciso-Calle et al., 2017; Escobedo-Galván et al., 2013; Tapia et al., 2017); however, these rediscovered populations are still at risk of extinction due to habitat loss, invasive species, low genetic diversity, and chytridiomycosis (Byrne et al., 2020; González-Maya et al., 2018; Kardos et al., 2021). *Atelopus* extinctions not only risk the loss of irreplaceable biodiversity but also threaten the persistence of toxins that are unique to the genus.

Here we review the available data on *Atelopus* small-molecule (i.e., non-peptide) chemical defenses and to identify geographic and taxonomic gaps in *Atelopus* toxin sampling. We describe known *Atelopus* toxin diversity, as well as the chemical features, pharmacology, and sources of individual toxins. Then we collate the methods used to assess *Atelopus* toxins and detail their capabilities and pitfalls. Finally, while taking into account these methodological biases and gaps in sampling, we review the available data from an ecological and evolutionary perspective. We aim to provide a foundation for

future research programs on the chemical defenses of this highly threatened genus of Neotropical toads.

2. Methods

2.1 Literature Review

We examined peer-reviewed literature published prior to November 2021 describing the composition and toxicity of *Atelopus* chemical defenses as well as auxiliary literature that describes the pharmacology of relevant toxins, toxin detection and quantification methods, and *Atelopus* ecology, morphology, taxonomy, and evolution. Articles were found using keyword searches through the UC Berkeley Library, Google Scholar, and Google Search with phrases such as "*Atelopus* toxic," "*Atelopus* peptides," "atelopidtoxin," "chiriquitoxin," "zetekitoxin," "*Atelopus* bufadienolides," etc. An exhaustive search was performed specifically for literature detailing the detection, quantification, and identification of *Atelopus* small-molecule toxins; in total, seventeen peer-reviewed papers were identified that met one or more of these criteria (see supplementary Table S1 sources for a complete list). Becker et al. (2011) claims to have detected zetekitoxins in *Atelopus zeteki* via HPLC, a method which would require a zetekitoxin AB standard for positive identification (see Table 1). Given the extraordinary rarity of purified zetekitoxin AB (Yotsu-Yamashita et al., 2004), it seems highly unlikely Becker et al. (2011) had access to sufficient quantities for use as an HPLC standard. Thus, we exclude this paper from our analyses. Additionally, we reviewed a PhD thesis (Brown, 1972) describing toxicity assessments of several *Atelopus* species, as well as the isolation and detection of guanidinium alkaloids in *A. zeteki.* Some of the data presented therein appears to have been published elsewhere (Brown et al., 1977; Fuhrman et al., 1969), but we include the unpublished data from Brown (1972) in our analyses. Two additional papers were identified that detailed the presence or absence of skin peptides produced by *Atelopus* that may or may not be used in defense (Ellison et al., 2014; Woodhams et al., 2006). Owing to a lack of information

on *Atelopus* skin peptide diversity and function, we focus our review on guanidinium alkaloids and cardiac glycosides.

2.2 Geographic and phylogenetic mapping of Atelopus toxin profiles

Sixteen of the eighteen *Atelopus* toxin assessment papers compiled during literature review described sampling location. In a few papers, only country-level sampling locations were provided or the species identification was dubious, so we excluded some of these samples from our combined assessments (see Table S2 for details). When GPS coordinates were not provided, we obtained coordinates using the geocoding service provided by Google Maps (https://developers-dot-devsite-v2-prod.appspot.com/maps/documentation/utils/geocoder). If the specific location name provided in a paper was not available, coordinates were determined by inputting larger geographic regions known to contain the locations of interest. See Supplementary Table S2 for a complete inventory of sampling locations, location names, and coordinates. Maps were generated through the ArcGIS Online application, Map Viewer Classic (Esri, Redlands, CA, USA), and edited using Adobe Illustrator (Adobe Inc., 2021).

To visualize the phylogenetic distribution of *Atelopus* toxins (Fig. 3a), we obtained a chronogram of *Atelopus* species from Ramírez et al. (2020) and pruned it to include a single tip per species in R v3.6.1 (R Core Team, 2019) using packages phytools v0.7.70 (Revell, 2012) and ape v5.5 (Paradis and Schliep, 2019).

3. Taxonomic and Geographic Gaps in *Atelopus* Toxin Assessments

The literature review yielded toxicity and small-molecule toxin composition data for sixteen *Atelopus* species (Fig. 3a, Supp. Table S1), approximately 15% of the recognized diversity of the genus (AmphibiaWeb, 2021). The amount of research dedicated to each of the sixteen species screened for toxins or toxicity varies: nine have been investigated in a single study, and four species have been investigated in four or more studies (Suppl. Table S1). Some species identifications in older papers make interpretation of the data difficult. In one case, Brown (1972) measured the toxicities of two *Atelopus* populations identified as *A. varius ambulatorius* and *A. cruciger.* Based on reported collection location, and the known distribution of these species, these individuals were likely misidentified and may represent other species. Furthermore, the identification of populations classified as *A. spumarius* (collected in Amapá, Brazil; Daly et al., 1994; Mebs et al., 1995) and *A. ignescens* (collected in Colombia and Ecuador; Brown, 1972; Daly et al., 1994; Flier et al., 1980) is ambiguous based on the collection locations. We designate these populations as *A. spumarius* sensu lato and *A. "ignescens*," following Lötters et al. (2002) and Quilindo et al. (2005), respectively. Similarly, the population of toads designated *A. oxyrhynchus* by Mebs and Schmidt (1989) and Yotsu-Yamashita et al. (1992) has since been identified as *A. carbonerensis,* which is likely extinct (see Supp. Table S2 for a more complete discussion of taxonomy; Lötters et al., 2019).

The extent of toxin research on *Atelopus* is geographically biased, with Central American *Atelopus* receiving the most focus. Of the nine described Central American harlequin toads (Ramírez et al., 2020; Veselý and Batista, 2021), seven (*A. certus, A. glyphus, A. limosus, A. zeteki, A. chiriquiensis, A. varius, A. senex*) have been tested for toxins and six (*A. senex* excluded) have had their toxins chemically analyzed (Supp. Table S1). However, the majority of *Atelopus* species are found outside of Central America and therefore large geographic and taxonomic gaps in sampling exist (Fig. 3b). Amazonian and Central Andean species have received particularly little investigation. Although Ecuador is a center of

Atelopus diversity (25 described species, of which 17 are endemic; Tapia et al., 2017), populations from only two *Atelopus* species (*A. planispina* and *A. "ignescens*") in Ecuador have been assessed (Supp. Table S2). We note that the inconsistent toxin sampling of *Atelopus* limits the generalizability of conclusions drawn in this review.

4. *Atelopus* Toxins – Chemical structures, Pharmacology, and Sources

Two chemically and pharmacologically distinct toxin classes have been detected in *Atelopus* tissues: guanidinium alkaloids, which are neurotoxins that may be sequestered from exogenous sources (i.e. symbiotic bacteria; Magarlamov et al., 2017), and bufadienolides, which are cardiac glycosides that are endogenously synthesized (Chiadao and Osuch, 1969; Garraffo and Gros, 1986; Porto and Gros, 1971). *Atelopus* do not appear to possess lipophilic alkaloids (Daly et al., 1984), and have not been assessed for indole alkaloids, a class of compounds commonly detected in amphibian skin and found in particularly large quantities in other bufonids (Rodríguez et al., 2017; Roseghini et al., 1989, 1988, 1976). In this section we review the modes of action, relative strengths, and possible sources of guanidinium and bufadienolide toxins detected in *Atelopus,* paying special attention to the five guanidinium alkaloids with described structures: tetrodotoxin, 4-*epi*tetrodotoxin, 4,9-anhydrotetrodotoxin, chiriquitoxin, and zetekitoxin AB. We also review toxins which have been detected but whose properties and structures are relatively unknown.

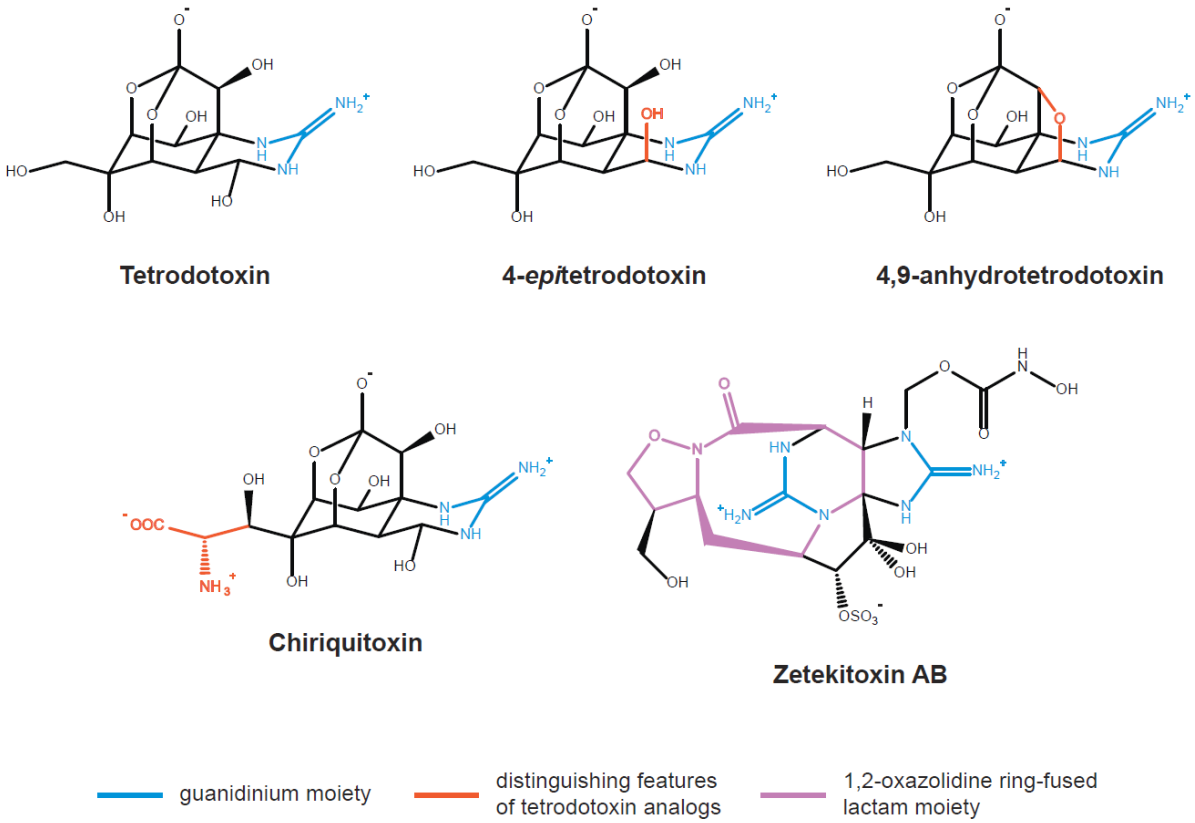


Figure 1: Guanidinium alkaloids detected in *Atelopus.* Purified quantities of Zetekitoxin C have been insufficient to estimate chemical structure (Brown et al., 1977).

4.1 Guanidinium alkaloids

Guanidinium alkaloids are low molecular weight neurotoxins that target voltage-gated sodium channels (VGSCs). The eponymous positively charged guanidinium moiety (Fig. 1) interacts with the extracellular facing end of the sodium ion channel, while the rest of the molecule effectively seals off the pore (Narahashi, 2008). With the flow of sodium ions occluded, nerves lose the ability to produce action potentials and thus can no longer send signals (Narahashi et al., 1964). Guanidinium alkaloid

poisoning is characterized by tingling, ataxia, paralysis, and death by respiratory failure or bradycardia (Durán-Riveroll and Cembella, 2017; How et al., 2003).

Although guanidinium alkaloids have been detected in many marine animals (Chau et al., 2011), their occurrence in terrestrial taxa is limited to five amphibian families: Salamandridae, Dendrobatidae (*Colostethus),* Brachycephalidae, Rhacophoridae (*Polypedates)*, and Bufonidae (*Atelopus*; Daly et al., 1994; Kim et al., 1975; Lüddecke et al., 2018; Pires et al., 2005; Tanu et al., 2001). Tetrodotoxin has also been reported in a single species of the salamander family Ambystomatidae (Yotsu et al., 1990a), however that finding has since been called into question (Hanifin, 2010). Lastly, tetrodotoxin has been suggested to cooccur with bufadienolides in *Clinotarsus cultripes* (Gosavi et al., 2014), a ranid, and chiriquitoxin has been suggested to occur in *Hypsiboas crepitans,* a hylid (Lamadrid-Feris et al., 2015); however, these findings are based on preliminary data that have not been verified by more sensitive techniques. Five guanidinium alkaloids have been detected and structurally identified in *Atelopus*: tetrodotoxin, 4,9-anhydrotetrodotoxin, 4-*epi*tetrodotoxin, chiriquitoxin, and zetekitoxin AB (Fig. 1). While not structurally identified, zetekitoxin C has been detected in *Atelopus zeteki* and is likely also a guanidinium alkaloid (Brown et al., 1977).

4.1.1 Tetrodotoxin

Tetrodotoxin (TTX) has a complex structure, of which the most functionally important portion is its single guanidinium group (Woodward, 1964). The strength of TTX binding is dependent on the characteristics of a given voltage gated sodium channel. In mammals, for instance, VGSC subtypes $Na_V1.5$, $Na_V1.8$ and $Na_V1.9$ are considered TTX-resistant (Thottumkara et al., 2014; Tsukamoto et al., 2017). Multiple vertebrate taxa (including some pufferfish, newts, and snakes) have evolved TTX resistance in Na_V proteins 1.4 and/or 1.7, which is thought to minimize or prevent TTX poisoning (Feldman et al., 2012; Hanifin and Gilly, 2015; McGlothlin et al., 2016; Venkatesh et al., 2005).

Tetrodotoxin-sensitive calcium channels have been identified in canine heart tissue (Hegyi et al., 2013, 2012).

Source. While a bacterial origin of TTX is well-supported for marine taxa (Campbell et al., 2009; Chau et al., 2011; Li et al., 2020; Magarlamov et al., 2017; Wu et al., 2005), the source of TTX in amphibians remains unresolved (see Hanifin, 2010; Stokes et al., 2014; Lukowski and Narayan, 2019). Although a complete review of all evidence regarding the source of TTX defenses in amphibians is outside the scope of this text, we evaluate existing data from *Atelopus* considering recent research in *Taricha* newts. Specifically, we propose that the absence of TTX in captive-born *A. varius* and newts (Daly et al., 1997, Kudo et al., 2015; Kudo et al., 2017) and the detection of TTX-producing bacteria in newts (Vaelli et al., 2020) are suggestive of an exogenous, bacterial origin of TTX defenses in amphibians.

Although adult, captive-born *Atelopus* and newts lack TTX (Daly et al., 1997; Mebs and Yotsu-Yamashita, 2021; Kudo et al., 2015; Kudo et al., 2017), wild-caught *Atelopus* and newts retain (Mebs et al., 2018a, 1995; Yotsu-Yamashita et al., 1992) or accumulate TTX in captivity (but see Yotsu-Yamashita et al., 2012; Hanifin et al., 2002). Together these data suggest that captive conditions do not necessarily prevent individuals from being toxic, yet without exposure to a natural environment, individuals appear incapable of initiating toxicity. Along these lines, wild-caught newts forced to secrete gland contents are able to replenish their TTX defenses over time (Cardall et al., 2004). The maintenance and regeneration of TTX in captive-held, wild amphibians has been interpreted as evidence for endogenous production of TTX by the newts themselves (Cardall et al., 2004; Mailho-Fontana et al., 2019). However, we propose that similar patterns of TTX upkeep and accumulation might be expected in a system where symbiotic bacteria obtained from a natural environment produce toxins for the captive amphibians.

The detection of TTX-producing bacteria is complicated by the unknown genetic basis of TTX synthesis (Lukowski and Narayan, 2019). While one early study was unable to detect bacterial DNA in TTX-rich tissues of the salamandrid *Taricha granulosa* (Lehman et al., 2004), multiple strains of TTX-producing bacteria have recently been cultured from the skin of the same species (Vaelli et al., 2020). These findings bolster the possibility that *Atelopus* similarly hosts bacteria capable of guanidinium alkaloid biosynthesis. Future research could assess whether wild-caught *Atelopus* individuals possess TTX-producing bacteria or attempt to inoculate non-toxic newts or toads with isolated strains of TTX-producing bacteria.

It is worth noting that, while a dietary origin for TTX defense is unsupported in newts (Cardall et al., 2004; Gall et al., 2012; Hanifin et al., 2002), we cannot rule it out in *Atelopus* given the current evidence. Pufferfish, for instance, are chemically defended by TTX and while TTX-producing bacteria have been cultured from their tissues (Campbell et al., 2009; Li et al., 2020; Wu et al., 2005; Yu et al., 2011), pufferfish are also capable of sequestering TTX from their diet (Honda et al., 2005; Itoi et al., 2018; Zhang et al., 2020). TTX has been detected in a terrestrial invertebrate (Stokes et al., 2014), which could serve as a source of TTX in terrestrial food chains.

In summary, the origin of TTX in amphibians generally, and *Atelopus* specifically, remains unclear, as conclusive evidence for bacterial or endogenous production is lacking. With this caveat in mind and given the overwhelming evidence for a bacterial origin in marine systems along with ambiguous evidence for endogenous production of TTX by any vertebrate, we consider the bacterial origin more likely and discuss its implications in the remainder of this review (see sections 6, 7 and 8).

4.1.2 4,9-anhydrotetrodotoxin and 4-epitetrodotoxin

4,9-anhydrotetrodotoxin (4,9-anhydroTTX) is a tetrodotoxin analog wherein the two hydroxyl substituents at positions C4 and C9 have been replaced with an ester linkage connecting the carbons

(Fig. 1; Deguchi, 1967). 4,9-anhydroTTX is generally a weaker VGSC ligand than TTX, with 40 to 231 times as much 4,9-anhydroTTX needed to achieve the same inhibition as a given amount of TTX on a human VGSC (Rosker et al., 2007). As a result 4,9-anhydroTTX is the least toxic TTX analog found in *Atelopus*: the LD50 (mouse, intravenous injection) is more than a hundred times that of TTX (Deguchi, 1967). Interestingly, 4,9-anhydroTTX is also more selective in its binding targets, strongly inhibiting the human $Na_V1.6$ (Rosker et al., 2007; Teramoto et al., 2012) and $Na_V1.1$ proteins (Denomme et al., 2020). Despite differences in targeting and strength between the toxins, the symptoms of 4,9-anhydroTTX poisoning are similar to those of TTX poisoning (Deguchi, 1967).

4-*epi*tetrodotoxin (4-*epi*TTX) is a simple epimer of TTX, meaning it has the same chemical formula and differs only by the arrangement of substituents at the C4 position (Fig. 1). This change results in a sevenfold reduction in toxicity (Nakamura and Yasumoto, 1985). There seems to have been less investigation into the pharmacological nature of 4-*epi*TTX as compared to other TTX analogs.

Source. In aqueous solutions, TTX readily undergoes epimerization and subsequent dehydration to form 4-*epi*TTX and 4,9-anhydroTTX, respectively (Watanabe et al., 2019). These two analogs have been found in almost all terrestrial taxa that possess TTX (Hanifin, 2010). 4,9-anhydroTTX is the most stable of the three under basic conditions (Goto et al., 1965). Given that frog skin is slightly basic (Civan and Peterson-Yantorno, 1986), it might be expected for all TTX to be ultimately converted to the less toxic 4,9-anhydroTTX in *Atelopus*. However, this is inconsistent with observations of TTX analog ratios in *Atelopus*, where TTX is present in larger amounts than 4-*epi*TTX and 4,9-anhydroTTX (Daly et al., 1994; Mebs et al., 2018a, 1995; Yotsu-Yamashita et al., 1992). Similar data are observed in wild-caught pufferfish, which maintain a relatively constant ratio of the three chemicals across their tissues (Nakamura and Yasumoto, 1985). In lab-raised pufferfish, the fate of TTX is dependent on the route of administration: intramuscularly injected TTX is mostly converted to 4,9-anhydroTTX while dietarily administered TTX remains unmodified as the major component (Kono et al., 2008). Thus, it is important

to use biologically relevant administration methods when conducting toxin experiments. Future research could investigate whether TTX-binding proteins, which are known from pufferfish and gastropods (Hwang et al., 2007; Matsui et al., 2000; Matsumoto et al., 2010; Yotsu-Yamashita et al., 2001), can prevent the interconversion of TTX and analogs.

4.1.3 Chiriquitoxin

Chiriquitoxin (CHTX) is a tetrodotoxin analog found exclusively in *Atelopus* toads. It differs from tetrodotoxin by the replacement of a hydroxyl substituent with a glycine residue at the C11 position (Fig. 1; Yotsu et al., 1990b). CHTX binds with particularly low affinity to human $Na_V1.7$, which may be attributable to the loss of a ligand/channel hydrogen bond which involves the C11 hydroxyl group in TTX (Tsukamoto et al., 2017). Unlike TTX, CHTX can also interfere with the function of potassium voltage gated ion channels (Yang and Kao, 1992). Nevertheless, CHTX is only slightly less toxic than TTX upon injection in mice, and produces similar symptoms (Fuhrman et al., 1976).

Source. Chiriquitoxin is the most structurally complex tetrodotoxin analog found in *Atelopus*, and, unlike 4,9-anhydroTTX and 4-*epi*TTX, is not an aqueous equilibrium product of TTX. It has been proposed that CHTX is generated by a reaction between glycine and either tetrodotoxin or an oxidized derivative thereof (Yotsu et al., 1990b). Whether this conversion is performed by the toads themselves or by microorganisms living on their skin is unknown, but there is precedence for amphibians modifying sequestered toxins. Four species of dendrobatid poison frogs (*Dendrobates auratus, D. tinctorius, Adelophobates galactonotus*, and *A. castaneoticus*) metabolize an ingested pumiliotoxin, PTX (+)-251D, stereoselectively hydroxylating it to form a more potent derivative, aPTX (+)-267A (Alvarez-Buylla et al., 2020; Daly et al., 2003). However, preliminary investigations have not shown animals to be capable of interconverting TTX analogs (Yotsu-Yamashita et al., 2013). A study of a TTX-bearing newt (*Cynops pyrrhogaster*) demonstrated that ingested TTX and putative biosynthetic precursors accumulated in

283 body tissues but remained in their original forms (Kudo et al., 2017). In contrast, parotoid-gland-
284 associated bacteria are known to biotransform bufadienolides in the toad *Rhinella marina*
285 (Kamalakkannan et al., 2017). Nevertheless, no bacteria have been found that can produce CHTX or
286 modify TTX into any analog (Yotsu-Yamashita et al., 2013).
287
288 4.1.4 Zetekitoxin AB
289 Zetekitoxin AB (ZTX AB) is unique among *Atelopus* guanidinium alkaloids; it is an analog of the
290 paralytic shellfish toxin saxitoxin and contains two guanidinium moieties (Fig. 1). Furthermore, ZTX AB is
291 the only natural chemical known to possess an 1,2-oxazolidine ring-fused lactam moiety (Yotsu-
292 Yamashita et al., 2004). Despite structural differences, ZTX AB is remarkably similar to TTX in potency,
293 with an LD50 (mouse, intraperitoneal) of 11 ug/kg as compared to 10 ug/kg for TTX (Brown et al., 1977;
294 Fuhrman et al., 1976). Symptomatically, ZTX AB poisoning is virtually indistinguishable from TTX
295 poisoning, except that it more commonly induces cardiac arrhythmia (Brown et al., 1977; Fuhrman et
296 al., 1976). Unlike many other saxitoxin analogs but like TTX, ZTX AB causes hypotension (Brown et al.,
297 1977; Durán-Riveroll and Cembella, 2017). Unfortunately, only limited amounts of ZTX AB have been
298 available for pharmacological and biophysical study. As a result, little is known about its binding
299 specificity.

300 *Source.* ZTX AB has only ever been detected in *Atelopus zeteki* and *A. varius,* and its source
301 remains uninvestigated. Cyanobacteria and dinoflagellates, however, are well established as the source
302 of saxitoxin (Durán-Riveroll and Cembella, 2017), and saxitoxin-producing cyanobacteria are found in
303 freshwater systems (Smith et al., 2011). Given that *Atelopus* are riparian and possess skin-associated
304 cyanobacteria (Becker et al., 2014), it seems plausible that ZTX AB has a cyanobacterial origin. Unlike
305 TTX, the genetic basis of saxitoxin synthesis is known (Hackett et al., 2013), so metagenomic techniques
306 could be applied to the *Atelopus* microbiome to test for the presence of bacteria with gene clusters

similar to the saxitoxin gene cluster (Lukowski and Narayan, 2019). While *A. zeteki* from El Valle de Antón, Panama are the most studied sources of ZTX AB (Suppl. Table S1), the use of metagenomic analyses on the microbiome of this species is complicated by the possible extinction of *A. zeteki* in the wild, and uncertainty regarding whether captive *A. zeteki* retain ZTX AB (Lukowski and Narayan, 2019). However, populations of *A. varius* persist in El Copé, Coclé, Panama as of 2016 (Byrne et al., 2020) and ZTX AB was detected in *A. varius* collected near El Copé in 1971 (Yotsu-Yamashita et al., 2004). These *A. varius* populations could be promising subjects for metagenomic research in search of ZTX AB-producing bacteria.

4.1.5 Zetekitoxin C

What was once referred to as atelopidtoxin (Fuhrman et al., 1969; Shindelman et al., 1969) is now known to be a mixture of ZTX AB and zetekitoxin C (ZTX C). ZTX C appears to only have been isolated once, as a minor component of *Atelopus zeteki* skin alkaloids. It is much less toxic than ZTX AB. Chemically, ZTX C has features in common with guanidinium alkaloids, including solubility in water and basicity (Brown, 1972; Brown et al., 1977). The symptoms produced by its injection in dogs – hypotension, ventricular fibrillation, and death – are also consistent with inhibition of voltage-gated sodium channels (Brown et al., 1977; Durán-Riveroll and Cembella, 2017; Murtha, 1960). Unfortunately, insufficient quantities of ZTX C were purified for structural analysis (Brown et al., 1977).

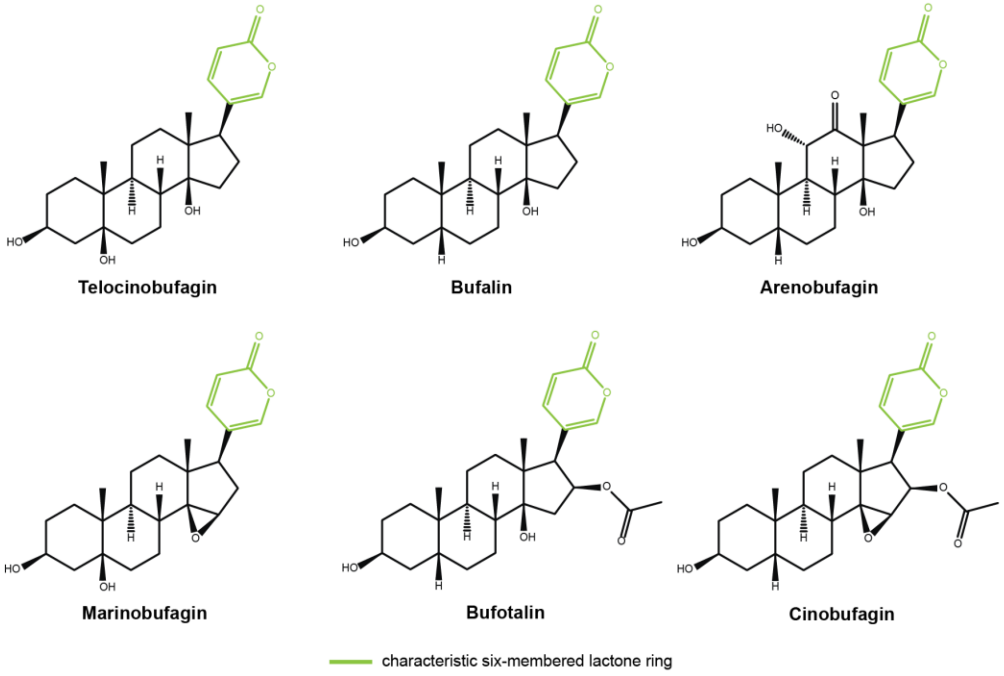


Figure 2: Bufadienolides detected in *Atelopus.*

4.2 Bufadienolides

Bufadienolides are cardiac glycosides (CGs), steroidal toxins that bind to and inhibit Na^+/K^+-ATPases (Fig. 2; Botelho et al., 2019). Na^+/K^+-ATPase inhibition ultimately causes a buildup of Ca^{2+} ions within nerve and muscle cells, which increases the contractility of muscle tissues (Blaustein et al., 2009). CG poisoning manifests as hypertension, gastrointestinal distress, abnormal heart rate, and – in high enough doses – death (Roberts et al., 2016). CG inhibition of Na^+/K^+-ATPase also alters some signaling pathways and is the topic of intense research for potential anticancer therapies (Reddy et al., 2020). Whereas other CGs have a five membered lactone ring attached to the central steroid structure, bufadienolides are characterized by a six membered lactone ring (Fig. 2; Rodríguez et al., 2017).

Source. All four *Atelopus* species that have been tested for bufadienolides were found to possess this class of toxins (Daly et al., 1997; Flier et al., 1980). Bufadienolides are endogenously

synthesized by toads, likely from cholesterol (Chiadao and Osuch, 1969; Garraffo and Gros, 1986; Porto and Gros, 1971). Interestingly, bufadienolides and other CGs are present at low levels in mammal and amphibian tissues, and likely have a highly conserved role as endogenous hormones (Dmitrieva et al., 2000; Flier et al., 1980; Lenaerts et al., 2018; Schoner and Scheiner-Bobis, 2005). Bufadienolides may also be used for sodium and water regulation in toads. For example, exposure to saline solutions altered the concentration of digitalis-like compounds (likely bufadienolides, see Dufresnes et al., 2019; Rodríguez et al., 2017) in the skin and brain of *Bufotes viridis* (Lichtstein et al., 1991). Thus, a possible evolutionary pathway for bufadienolide defense in toads is via natural selection on the regulation of endogenous CGs (Flier et al., 1980) coupled with the development of Na^+/K^+-ATPase target site insensitivity, whereby amino acid substitutions result in a weaker affinity of Na^+/K^+-ATPase for CGs. Target site insensitivity to CGs has been demonstrated in the α3 Na^+/K^+ ATPase subunit of bufonid toads – including *Atelopus spumarius* – and toad-feeding reptiles (Moore et al., 2009; Ujvari et al., 2015) and in a tandem duplicate of the α1 Na^+/K^+ ATPase in toad-feeding frogs (*Leptodactylus;* Mohammadi et al., 2021). More than one hundred different bufadienolides have been detected in the skins, eggs, or granular gland secretions of bufonid toads (Rodríguez et al., 2017). The mechanisms underlying the diversity of bufadienolides in toads has been largely uninvestigated, though microbial biotransformation may play a role (Hayes et al., 2009b; Kamalakkannan et al., 2017).

4.3 Unidentified Toxins

Toxin diversity in *Atelopus* is incompletely characterized, and toxins whose identities are unknown have been detected in multiple species. For various reasons, including small quantities and methodological limitations, investigation into these chemicals has been insufficient to clarify their structures, pharmacology, and/or chemical characteristics (see Section 5.2 for a discussion of the methods used to identify *Atelopus* toxins).

Several unidentified toxins that mirror guanidinium alkaloids in effect or chemistry have been detected in *Atelopus*. The only toxin found in *A. certus* is water soluble and likely positively charged, both of which are features of guanidinium alkaloids. While this unknown chemical was determined to not be TTX, too little was purified for further analysis (Yotsu-Yamashita and Tateki, 2010). In competitive binding assays, *A. spurrelli* skin extracts inhibit saxitoxin binding, a characteristic of guanidinium alkaloids. Given that TTX is a minor component of *A. spurrelli* skin extracts, one or more unidentified TTX-like toxins are believed responsible for *A. spurrelli* toxicity (Daly et al., 1994). Similarly, TTX is a trace component in *A. "ignescens*," and the tetrodotoxin-like chemicals which account for the remaining toxicity of *A. "ignescens"* skin extracts to mice are uncharacterized (Daly et al., 1994). Finally, aqueous *A. senex* skin extracts injected into mice caused the same symptoms as known guanidinium alkaloids (Brown, 1972). While *A. senex* skin extracts likely contain guanidinium alkaloids, the individual identities of these toxins have not been determined.

There are unidentified *Atelopus* toxins which either differ substantially from guanidinium alkaloids or whose properties are almost completely unknown. Aqueous skin extracts of *A. planispina* injected in mice cause symptoms that differ from those of guanidinium alkaloid poisoning, specifically cessation of respiration before cardiac arrest (Fuhrman et al., 1969). The unidentified toxin is unlikely a bufadienolide because bufadienolides are weakly soluble in water (Flier et al., 1980) and do not cause the symptoms observed with *A. planispina* toxins (Roberts et al., 2016). Thus, *A. planispina* represents a likely source of novel *Atelopus* toxins, which warrants further research. Secondly, an unidentified major toxin has been detected in a single specimen of *A. zeteki and* has received no further investigation (Yotsu-Yamashita and Tateki, 2010). However, the method used on that specimen was incapable of detecting ZTX AB, the most common major toxin found in *A. zeteki* (Supp. Table S2; Yotsu-Yamashita and Tateki, 2010), so it is plausible that the chemical was ZTX AB.

5. *Atelopus* Toxin Extraction, Quantification, and Identification Methods

In this section we give a general overview of the methods used to isolate, quantify, and identify toxins in *Atelopus*. We do not attempt to describe every step, but rather focus on those which impact the accuracy and completeness of the toxin assessment. Furthermore, we describe how these methods have changed over time, and the consequences of those changes. We also note methods which may prove useful in future *Atelopus* toxin studies.

5.1 Extraction and purification

In most studies, extractions are performed on isolated skin or eggs, though whole-body extractions are also possible (Mebs et al., 2018a; Yotsu-Yamashita et al., 1992). Usually, tissues are broken into small pieces and suspended in a solvent with properties most amenable to the toxin type of interest. If the tissues are homogenized, subsequent dialysis is performed to separate soluble chemicals from the slurry (Fuhrman et al., 1969; Pavelka et al., 1977; Shindelman et al., 1969). A variety of extract cleaning methods can be used, many of which involve some form of filtration via chromatography (Daly et al., 1994; Mebs and Schmidt, 1989; Shindelman et al., 1969). Final toxin separation and purification may be performed through chromatography or free-flowing electrophoresis (Brown et al., 1977; Yotsu-Yamashita et al., 2004).

A study published in 1977 found that higher levels of guanidinium alkaloids were extracted from *A. chiriquiensis* eggs and skin when 3% acetic acid was used as opposed to water, with the effect most pronounced in egg extractions (Pavelka et al. 1977). Following acid extraction, the toxins exhibited enhanced solubility in water. The authors suggest guanidinium alkaloids in *Atelopus* may exist to some extent in an insoluble bound form, from which the toxins are released following hydrolysis with acid (Pavelka et al., 1977). Several species of TTX-possessing pufferfish (Matsui et al., 2000; Matsumoto et al., 2010; Yotsu-Yamashita et al., 2001) and gastropods (Hwang et al., 2007) are known to possess TTX-binding proteins, thus another possibility is the acidic denaturation of a guanidinium alkaloid-binding

411 protein. Previous studies (before 1977) used distilled water for the initial extraction, and thus may have
412 reported lower toxin levels than were present in the toads tested. With a few exceptions (Daly et al.,
413 1997, 1994; Mebs et al., 1995), subsequent studies on *Atelopus* toxins followed Pavelka et al. (1977) and
414 performed acidic extractions.

415 While bufadienolides have a variety of structures and physical properties (Rodríguez et al.,
416 2017), they tend to be poorly soluble in water (Flier et al., 1980; Li et al., 2009; Zhang et al., 2008).
417 Furthermore, bufadienolides degrade over 24-hour timescales in highly acidic or basic solutions (Li et al.,
418 2015). Thus, aqueous and/or acidic extractions of *Atelopus* tissues may largely exclude bufadienolides
419 and mouse bioassays of such extractions likely do not account of the contribution of bufadienolides to
420 *Atelopus* toxicity. Bufadienolide-specific toad extractions commonly employ methanol as a solvent
421 (Barnhart et al., 2017; Daly et al., 1997; Flier et al., 1980; Inoue et al., 2020; Petroselli et al., 2018).

422 Considering the severity of *Atelopus* declines (La Marca et al., 2005), nonfatal extraction
423 methods may be critical for future research on toxins in wild *Atelopus* populations. One method involves
424 collecting small skin punches from animals in the field, and has been utilized to measure TTX levels in
425 salamanders but has not been benchmarked yet for accuracy against whole-body extractions (Bucciarelli
426 et al., 2014; Hanifin et al., 2002). Completely noninvasive methods involve the collection of granular
427 gland secretion via manual or electrical stimulation of amphibian skin (Conceição et al., 2007; Rozek et
428 al., 1998). Although these methods have not been thoroughly tested on amphibians that possess
429 guanidinium alkaloids, we suspect that they would be fruitful. The sampling of museum specimens for
430 toxins represents another avenue for *Atelopus* research and could enable the assessment of species that
431 have gone extinct. In a couple of studies, analyses were performed on the storage alcohol of *Atelopus*
432 museum specimens (Mebs et al., 2018a, 1995). However, toxins in museum specimens may degrade
433 over time and specimens stored in formalin are not suitable for toxin analyses (Mebs et al., 1995).

Method	Capabilities	Limitations	Relevant *Atelopus* Studies
BIOASSAYS			
Mouse Bioassay (MBA)	- quantifies *in vivo* toxicity of extracts or of purified toxins - provides a preliminary determination of toxin identity	- variance in standardization between studies - not specific; toxins with similar biological effects cannot be distinguished - requires use of live animals - only provides estimate of toxicity to mammals	(Brown, 1972; Brown et al., 1977; Daly et al., 1994; Fuhrman et al., 1969, 1976; Kim et al., 1975; Mebs et al., 1995; Mebs and Schmidt, 1989; Pavelka et al., 1977; Shindelman et al., 1969; Yotsu-Yamashita et al., 1992, 2004; Yotsu-Yamashita and Tateki, 2010; Yotsu et al., 1990b)
Binding Inhibition Assays	- detect and quantify compounds that interact with guanidinium alkaloid or bufadienolide binding sites on VGSCs and Na+/K+ ATPase, respectively	- not specific, measures all compounds with the same binding behavior	(Daly et al., 1997, 1994; Flier et al., 1980)
IMMUNOLOGICAL			
Immunohistochemistry (IH)	- detects TTX - visualizes TTX distribution within tissues - capable of application to other guanidinium alkaloids (Smolowitz and Doucette, 1995)		(Mebs et al 2018a)
PHYSICOCHEMICAL			
Nuclear Magnetic Resonance (NMR)	- detects and quantifies TTX, 4-*epi*TTX, 4,9-anhydroTTX (Nakamura and Yasumoto, 1985), CHTX, ZTX AB	- matrix components can reduce spectra quality (Bane et al., 2014)	(Fuhrman et al., 1976; Kim et al., 1975; Pavelka et al., 1977; Shindelman et al., 1969; Yotsu-Yamashita et al., 2004; Yotsu-Yamashita and Tateki, 2010; Yotsu et al., 1990b)
Thin Layer Chromatography (TLC)	- determines purity and preliminary identity of guanidinium alkaloids - paired with Weber Reagent or UV fluorescence tests to verify guanidinium alkaloid presence	- cannot provide a quantitative estimate of toxin amounts	(Brown, 1972; Brown et al., 1977; Daly et al., 1994; Flier et al., 1980; Kim et al., 1975; Mebs and Schmidt, 1989; Shindelman et al., 1969; Yotsu-Yamashita et al., 2004; Yotsu et al., 1990b)
High Pressure Liquid Chromatography (HPLC)	- isolates and preliminarily identifies individual bufadienolides - paired with UV absorption measurements to detect presence of bufadienolide α-pyrone ring	- requires standards of individual compounds being identified	(Flier et al., 1980)
Liquid Chromatography with Fluorescence Detection (LC-FLD)	- detects and quantifies TTX, 4-*epi*TTX, 4,9-anhydroTTX, CHTX	- can't detect ZTX AB or low quantities of CHTX - differences in fluorescent intensities of analogs complicates analysis (Shoji et al., 2001) - standards of each analog required for quantification (Yotsu et al., 1989), but are difficult to obtain (Bane et al., 2014)	(Daly et al., 1994; Mebs et al., 1995; Yotsu-Yamashita et al., 1992; Yotsu-Yamashita and Tateki, 2010)
Gas Chromatography with Mass Spectrometry (GC-MS)	- detects and quantifies TTX and its analogs, and some individual lipophilic alkaloids	- *Atelopus* guanidinium alkaloids are not volatile, chemical derivation required (Suenaga and Kotoku, 1980) - not specific, TTX and analogs cannot be distinguished (Magarlamov et al., 2017)	(Daly et al., 1984; Mebs and Schmidt, 1989)
Electrospray Ionization with Mass Spectrometry (ESI-MS)	- detects TTX, 4,9-anhydroTTX (Wang et al., 2010), CHTX, ZTX AB		(Mebs et al., 1995; Yotsu-Yamashita and Tateki, 2010)

High Resolution Hydrophilic Interaction Liquid Chromatography/Mass Spectrometry (HR-HILIC-LC/MS)	- detects and quantifies TTX, 4-*epi*TTX, 4,9-anhydroTTX (Nakagawa et al. 2006), CHTX, ZTX AB		(Mebs et al., 2018a)

Table 1: Detection and quantification methods utilized in studies of *Atelopus* toxins

5.2 Toxin Identification

After extraction and purification, a variety of methods can be applied to determine guanidinium alkaloid identity (Table 1). Thin layer chromatography (TLC) and nuclear magnetic resonance (NMR) were among the earliest and most used of these in *Atelopus* studies. TLC separates chemicals and allows for assessment of purity. Spots on a TLC plate can be subsequently sprayed with the Weber reagent, an aqueous solution of sodium nitroprusside, potassium ferricyanide, and sodium hydroxide (Weber, 1928) that turns red in the presence of fifty or more mouse units of guanidinium alkaloids (approximately equivalent to 11ug of TTX; Brown et al., 1977). Alternatively, the spotted TLC plate can be sprayed with an alkaline solution and heated (Mebs and Schmidt, 1989). This converts guanidinium alkaloids into 2-aminoquinazoline derivatives that fluoresce under UV light (Nakamura and Yasumoto, 1985). 1H NMR and C-13 NMR spectra give information on the electronic environments, neighboring atoms, and quantities of carbon and hydrogen atoms in a molecule, respectively (Klein, 2017). In addition to serving as a method of detection, NMR has been critical in determining the chemical structures of *Atelopus* guanidinium alkaloids (Yotsu-Yamashita et al., 2004; Yotsu et al., 1990b).

The next technology that became widely used in *Atelopus* toxin studies was developed by Yotsu et al. in 1989. This liquid chromatography-fluorescence detection system (LC-FLD) takes advantage of the fluorescence of 2-aminoquinazoline derivatives (generated by heating guanidinium alkaloids in alkaline solutions) to identify guanidinium alkaloids that have been separated by liquid chromatography, and was the first method used to detect 4-*epi*TTX and 4,9-anhydroTTX in *Atelopus* extracts (Yotsu-Yamashita et al., 1992; Yotsu et al., 1989). LC-FLD utilizes reverse phase chromatography, which is

456 incapable of separating all TTX analogs (Bane et al., 2014). Additionally, because TTX analogs exhibit a
457 wide range of fluorescent intensities, analogs may not all be detectable under the same set of
458 conditions (Shoji et al., 2001). For instance, detection of 11-deoxytetrodotoxin, a common analog in
459 newts and brachycephalid toads (Hanifin, 2010), requires higher post column reaction temperatures
460 than does the detection of TTX (Yotsu et al., 1989). Studies of *Atelopus* toxins which utilized LC-FLD
461 either employed post-column reaction temperatures below those needed for 11-deoxytetrodotoxin
462 detection (Yotsu-Yamashita et al., 1992) or neglect to specify the post-column reaction temperature
463 (Daly et al., 1994; Mebs et al., 1995; Yotsu-Yamashita and Tateki, 2010). All *Atelopus* LC-FLD studies
464 utilized a C18 chromatography column but varied in mobile phase composition (heptafluorobutyric acid
465 or trifluoroacetic acid) and concentration of NaOH in the post-column reaction (ranging from 2.8N to
466 4N; Daly et al., 1994; Mebs et al., 1995; Yotsu-Yamashita et al., 1992, Yotsu-Yamashita and Tateki, 2010).
467 LC-FLD cannot detect low levels of CHTX, and it is incapable of detecting ZTX AB (Yotsu-Yamashita et al.,
468 1992; Yotsu-Yamashita and Tateki, 2010). Four *Atelopus* species have had their guanidinium alkaloids
469 studied exclusively through LC-FLD: *A. "ignescens*," *A. spumarius* sensu lato*, A. spurrelli,* and *A.*
470 *subornatus* (Daly et al., 1994; Mebs et al., 1995). Thus, these species could have undetected CHTX, ZTX
471 AB, or 11-deoxytetrodotoxin.

472 Most recently, methods that incorporate electrospray ionization−mass spectrometry (ESI-MS)
473 have emerged as promising guanidinium alkaloid assays in *Atelopus* studies (Mebs et al., 1995; Mebs et
474 al., 2018a; Yotsu-Yamashita and Tateki, 2010). Whereas quantification by LC-FLD requires calibration
475 with standards of each TTX analog being measured, quantification of TTX analogs can be performed by
476 ESI-MS with the exclusive use of a TTX standard (Chen et al., 2011; Nakagawa et al., 2016; Shoji et al.,
477 2001). The simplest use of ESI-MS is to directly screen toad extracts for ions indicative of toxin presence,
478 such as the MH+ ion of TTX (320 *m/*z; Mebs et al., 1995). However, the presence of other chemicals in
479 the extract/matrix can reduce sensitivity (Bane et al., 2014), thus chromatography can be used prior to

ESI-MS for chemical separation (Yotsu-Yamashita and Tateki, 2010). Mebs et al. (2018a) is the only study of *Atelopus* toxins to employ high resolution hydrophilic interaction liquid chromatography (HR-HILIC), a highly sensitive method which achieves better separation of analogs than reverse phase chromatography (EFSA CONTAM Panel et al., 2017; Nakagawa et al., 2016). Subsequent analysis by ESI-MS detected only TTX and 4-*epi*TTX in an *A. hoogmoedi* extract (Mebs et al., 2018a). As HR-HILIC-LC/MS can detect nearly every major analog of TTX found in nature (Yotsu-Yamashita et al., 2013), the exclusive detection of TTX and 4-*epi*TTX suggests that *A. hoogmoedi* lacks other TTX analogs including 6-*epi*tetrodotoxin and 11-deoxytetrodotoxin, which are frequently detected in newts (Hanifin, 2010). This is consistent with the apparent absence of 6-*epi*tetrodotoxin and tetrodonic acid in *A. carbonerensis* (Mebs et al., 1995; Yotsu-Yamashita et al., 1992). Application of HR-HILIC-LC/MS to other *Atelopus* species could determine whether 6-*epi*tetrodotoxin, 11-deoxytetrodotoxin, and tetrodonic acid are universally absent in the genus.

HR-HILIC-LC/MS forms the basis of a mass spectrometry-guided screening method developed by Kudo et al. (2014, 2020): it enables the detection of unknown compounds for subsequent structural analysis. The pattern of unique TTX analogs and other chemicals detected in newts via this method has led to the determination of a putative TTX biosynthetic pathway (Kudo et al., 2014; Kudo et al., 2016; Kudo et al., 2020; Kudo et al., 2021; Kudo and Yotsu-Yamashita et al., 2019). Use of this screening method on *Atelopus* could help determine the extent of convergence in the biosynthetic pathway(s) of amphibian associated TTX. Furthermore, the search for genes in amphibians and bacteria which encode for inferred biosynthetic enzymes could give powerful insight into the ultimate source of TTX (Kudo et al., 2014).

The identification of individual *Atelopus* bufadienolides has only been attempted once (Flier et al., 1980). After verification of bufadienolide presence, Flier et al. (1980) performed HPLC on *Atelopus "ignescens"* skin extractions. By comparing the elution order with those of bufadienolide standards, the

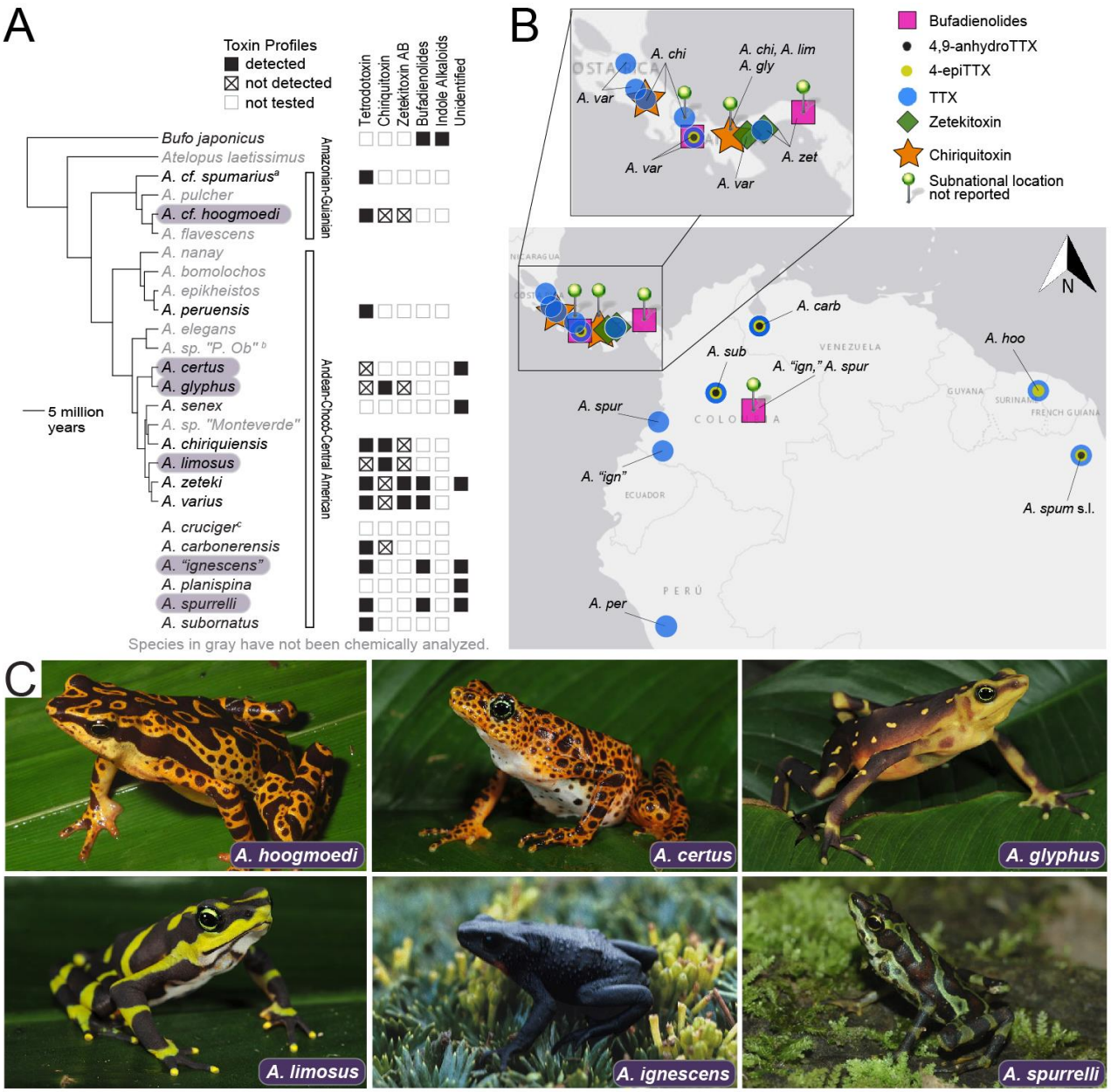
preliminary identities of six *Atelopus* bufadienolides were determined. A variety of methods are now available which make it possible to identify many bufadienolides precisely and sensitively (Zhan et al., 2020).

5.3 Quantification of Toxicity and Pharmacological Activity

Most of the methods described in Section 5.2 can quantify individual chemicals, subject to their detection and identification limitations (Table 1). In contrast, the methods described below can be used to determine the combined toxicity or pharmacological activity of multiple toxins.

The mouse bioassay (MBA) is the most frequently used method of toxicity quantification in *Atelopus* toxin studies. MBAs may also be used throughout the toxin extraction and purification process to identify toxic fractions (Shindelman et al., 1969; Yotsu-Yamashita and Tateki, 2010; Yotsu et al., 1990b). MBAs involve injecting varying doses of toxin intraperitoneally into mice and measuring survival times (Brown et al., 1977). The symptoms observed during MBAs may suggest the presence or absence of different toxins (Fuhrman et al., 1969). Toxin quantities determined by MBAs are reported in mouse units (MUs). The standard definition of one MU is that it is enough toxin to kill a 20g male mouse in 30 minutes by intraperitoneal injection and is equivalent to 0.22 ug of tetrodotoxin (Kawabata, 1978; Yasumoto, 1991). However, *Atelopus* toxin studies published prior to 1988 use conflicting definitions of MUs based on post-injection survival times of 20 minutes (Brown et al., 1977; Pavelka et al., 1977), 30 minutes (Mebs and Schmidt, 1989) and one hour (Kim et al., 1975; Shindelman et al., 1969). Other studies do not specify the survival time used in their MU definition (Fuhrman et al., 1969, 1976). Lastly, female mice and mice of different strains were sometimes used for MBAs (Pavelka et al., 1977). Post 1989, all *Atelopus* studies that use MBAs for toxin quantification apply the standard MU definition or use the standard MU to TTX equivalent conversion outlined in Yasumoto (1991). The variability in MU definition between *Atelopus* studies may complicate the comparability of the toxicities they report.

528 Binding assays can be used to determine the quantity of chemicals with specific pharmacological
529 activities. While other variants of toxin binding assays exist (Stokes et al., 2012), those applied in studies
530 of *Atelopus* toxins thus far measure the binding inhibition of radioactively labeled reference chemicals in
531 homogenized brain tissue or red blood cells by toad skin extracts. Binding inhibition assays for
532 guanidinium alkaloids and bufadienolides use [^{3}H]Saxitoxin and [^{3}H]Ouabain as reference chemicals,
533 respectively (Daly et al., 1997, 1994; Flier et al., 1980). Modern binding assays can be an order of
534 magnitude more sensitive than the mouse bioassay in detecting guanidinium alkaloids (Kawabata, 1978;
535 Stokes et al., 2012), and have fewer ethical considerations (Stern et al., 2018; Taylor et al., 2011; Wilder-
536 Kofie et al., 2011).


A
Toxin Profiles
detected
not detected
not tested
Tetrodotoxin
Chiriquitoxin
Zetekitoxin AB
Bufadienolides
Indole Alkaloids
Unidentified
Bufo japonicus
Atelopus laetissimus
A. cf. spumarius[a]
A. pulcher
A. cf. hoogmoedi
A. flavescens
A. nanay
A. bomolochos
A. epikheistos
A. peruensis
A. elegans
A. sp. "P. Ob"[b]
A. certus
A. glyphus
A. senex
A. sp. "Monteverde"
A. chiriquiensis
A. limosus
A. zeteki
A. varius
A. cruciger[c]
A. carbonerensis
A. "ignescens"
A. planispina
A. spurrelli
A. subornatus
Amazonian-Guianan
Andean-Choco-Central American
5 million years
Species in gray have not been chemically analyzed.
B
A. chi
A. chi, A. lim
A. gly
A. var
A. var
A. var
A. zet
Bufadienolides
4,9-anhydroTTX
4-epiTTX
TTX
Zetekitoxin
Chiriquitoxin
Subnational location not reported
N
A. carb
A. hoo
A. sub
A. "ign," A. spur
A. spur
A. "ign"
A. spum s.l.
A. per
VENEZUELA
GUYANA
SURINAME
FRENCH GUIANA
ECUADOR
PERÚ
C
A. hoogmoedi
A. certus
A. glyphus
A. limosus
A. ignescens
A. spurrelli

538 **Figure 3. A)** The phylogenetic distribution of toxic non-proteinaceous chemicals in skin, granular gland, and egg extracts of *Atelopus*. Bars to
539 the right of the chronogram correspond to clades described by Lötters et al. (2011) and supported by Ramírez et al. (2020). Species listed
540 below the chronogram were not included in the original phylogenetic analysis (Ramírez et al., 2020), and have been placed in the Andean-
541 Chocó-Central American clade based on Lötters et al. (2011) and/or geographic range (Amphibiaweb, 2021). Species names highlighted in
542 purple have corresponding images in Fig. 3C. **B)** Geographic distribution of *Atelopus* toxins. Samples for which subnational data weren't
543 reported (green pin) are mapped only when they are the sole sample containing a particular toxin collected from a given species in that
544 country. The locations of these points were selected for ease of visualization. See supplementary Table S2 for coordinate data. **C)** Selected
545 images of *Atelopus* species which have been subjected to chemical analysis. Photo credits: *A. hoogmoedi* by Pedro L. V. Peloso via
546 calphotos.berkeley.edu (© 2010, with permission)*; A. certus, A. glyphus,* and *A. limosus* by Brian Freiermuth via calphotos.berkeley.edu
547 (© 2013, with permission); *A. ignescens* by Luis A. Coloma via bioweb.bio (CC BY-NC-ND 4.0)*, A. spurrelli* by RD Tarvin (2014, Termales,
548 Chocó, Colombia).
549
550 [a] Whereas *A. cf. spumarius* samples from Ecuador were used in the estimation of the chronogram (Ramírez et al., 2020), the associated toxin
551 profile data is derived from *A. spumarius* sensu lato collected in Colombia (Supp. Table S2; Daly et al., 1994).
552 [b] "P. Ob" is an abbreviation of "Puerto Obaldia-Capurgana."
553 [c] Per Mebs and Schmidt (1989)*, A. cruciger* are nontoxic.
554
555 References: *Atelopus:* See Table S1. *Bufo japonicus*: (Erspamer et al., 1964; Inoue et al., 2020)

6. Geographic and Phylogenetic Distribution of *Atelopus* Toxins

Atelopus are distributed throughout much of the Andes from Bolivia to Venezuela, continuing into Central America, with the most northern species found in Costa Rica. A disjunct group of species occupies the eastern Amazonian Basin and the Guiana Shield (Lötters et al., 2011). *Atelopus* are found at elevations ranging from 0 to 4800m (La Marca et al., 2005) and occupy a variety of habitats, including Chocó-Darién moist forests (Veselý and Batista, 2021), treeless high-altitude páramo (Rueda Solano et al., 2016), and lowland Amazonian rainforest (Jorge et al., 2020a). Harlequin toads live in riparian areas, with males often staying close to streams and females ranging further into the surrounding areas (Mcdiarmid, 1971).

Atelopus toxins similarly exhibit geographic and phylogenetic patterns, with tetrodotoxin found in the majority of species and toxin diversity concentrated in Central American toads (Fig. 3a). It is important to note that the distribution patterns derived from existing research may not reflect the complete distributions of those toxins due to sampling biases (Section 3) and methodological limitations (Section 5.2). For instance, four South American *Atelopus* species have been analyzed exclusively using a method that cannot detect low levels of CHTX or any amount of ZTX AB (Daly et al., 1994; Mebs et al., 2018a, 1995; Yotsu-Yamashita et al., 2013; Yotsu-Yamashita and Tateki, 2010). In contrast to Central American *Atelopus,* only a minority of South American species have been chemically analyzed. Thus, there is a possibility that some South American *Atelopus* could possess CHTX or ZTX AB, or other chemicals that are yet to be discovered. In this section we describe the geographic and phylogenetic distribution of each *Atelopus* toxin, given the available data, and note any patterns possibly indicative of genetic or environmental factors influencing toxin composition.

6.1 Guanidinium alkaloids

Sequestered toxin profiles are constrained by the availability of toxins (an environmental factor; Mebs et al., 2018b; Yoshida et al., 2020) and the capacity of the organism to sequester those chemicals

(a genetic factor; Davison et al., 2021). *Atelopus* may sequester guanidinium alkaloids from bacteria, and, if so, it is unclear whether such bacteria are horizontally or vertically transmitted. In the case of horizontal transmission, *Atelopus* toxin profiles would be constrained by the geographic distributions of guanidinium-alkaloid synthesizing bacteria, whereas vertical transmission would ensure the availability of particular guanidinium alkaloids, regardless of any biogeographic patterns in microbial diversity. In both scenarios, *Atelopus* guanidinium alkaloid profiles would also be shaped by the genetic capacity to sequester specific toxins, establish symbioses with toxin-producing bacteria, or, in the possible case of CHTX, to modify sequestered chemicals (see Section 4.1.3). If, instead, *Atelopus* guanidinium alkaloids are endogenously synthesized, toxin profiles should reflect genetically controlled synthetic abilities and, possibly, plasticity in defensive responses to environmental cues (e.g., predation attempts, reproductive status, etc). Thus it is likely that both genetic and environmental factors shape *Atelopus* guanidinium toxin profiles.

TTX. Tetrodotoxin is the most widespread *Atelopus* toxin, having been detected in ten of the sixteen chemically assessed species (Fig. 3a). It is usually a major toxin component and has been found in *Atelopus* toads from across the entire geographic range of the genus (Fig. 3b) and within both major clades (Fig. 3a). *Atelopus* is the only taxon in Bufonidae known to possess guanidinium alkaloids (Rodríguez et al., 2017); toxin assessments of species within the sister taxon of *Atelopus* (*Oreophrynella;* Kok et al., 2018) and other "atelopodid" bufonids (*Melanophryniscus* and *Dendrophryniscus*; Graybeal, 1997) have failed to detect guanidinium alkaloids (Daly et al., 1994; Mebs et al., 1995). Thus, the phylogenetic distribution of TTX within Bufonidae suggests a single origin of TTX defense in the common ancestor of *Atelopus* with possible secondary losses in some species, i.e., *A. glyphus, A. limosus, and A. cruciger* (Fig. 3a). If TTX is sequestered from bacteria in *Atelopus,* the absence of TTX could be reflective of differences in microbiome composition rather than the loss of sequestration ability. However, better

603 sampling of the Amazonian-Guianan *Atelopus* clade and of Bufonidae more generally is needed before a
604 definitive model of the origin and evolution of TTX defense in *Atelopus* can be proposed.
605 4,9-anhydroTTX and 4-*epi*TTX frequently cooccur with TTX, which is expected given the aqueous
606 equilibrium between the three chemicals. Interestingly, 4-*epi*TTX has once been detected in the absence
607 of 4,9-anhydroTTX in a single male specimen of *A. hoogmoedi* from Suriname (Fig. 3b; Mebs et al.,
608 2018a).

609 *CHTX.* Chiriquitoxin was discovered in the now-extinct *Atelopus chiriquiensis* in 1975 and was
610 thought to be unique to that species until its detection in *A. limosus* and *A. glyphus* more than three
611 decades later (Kim et al., 1975; Yotsu-Yamashita and Tateki, 2010). CHTX is a major component in all
612 three species (Suppl. Table S1), and appears to be restricted to Central America, having only been found
613 in Costa Rican and Panamanian *Atelopus* (Fig. 3b). The three species with CHTX form a polyphyletic
614 group. *A. certus* and *A. senex* possess guanidinium alkaloid-like toxins (Brown, 1972; Fuhrman et al.,
615 1969), and their phylogenetic placement (Fig. 3a) and Central American ranges (Kahn et al., 2005; Veselý
616 and Batista, 2021) suggest they would be promising targets for CHTX testing. However, *A. senex* is
617 extinct (IUCN, 2021). TTX was also a major component in *A. chiriquiensis*, consistently detected
618 alongside CHTX (Suppl. Table S1). Although TTX is the likely metabolic precursor of CHTX (Yotsu et al.,
619 1990b), TTX is not known to be present in *A. glyphus* or *A. limosus* (Yotsu-Yamashita and Tateki, 2010),
620 indicating that TTX, if present in these species, could be completely converted to CHTX.

621 *ZTX AB.* Zetekitoxin AB (ZTX AB) has only been found in two of the seven assessed Central
622 American *Atelopus*: *A. varius* and *A. zeteki* (Fig. 3a). These sister species are closely related (Fig. 3a;
623 Lötters et al., 2011; Ramírez et al., 2020) and a recent whole-genome analysis does not support the
624 species boundary between them (Byrne et al., 2020), suggesting that *A. varius* and *A. zeteki* are the
625 same species. *A. varius* and *A. zeteki* exhibit intraspecific variation in the presence and absence of TTX
626 and ZTX AB. The exclusive presence of ZTX AB in *A. varius* and *A. zeteki* suggests that ZTX AB

sequestration or synthesis is under some degree of genetic control. This is corroborated by the occurrence of *Colostethus panamensis,* a dendrobatid poison frog, in El Valle de Antón. *C. panamensis* occupies the same habitat as *A. zeteki* but possesses only TTX, not ZTX AB (Daly et al., 1994). Nevertheless, the paucity of data makes it difficult to draw any conclusion on the broader phylogenetic or geographic distribution of this toxin.

6.2 Bufadienolides

Bufadienolides have been detected in all *Atelopus* which have been tested with methods that are sensitive to these substances: *A. "ignescens*," *A. spurrelli, A. varius,* and *A. zeteki* (Fig. 3a; Daly et al., 1997; Flier et al., 1980). Thus, while cardiac glycosides appear geographically restricted to Andean and Central American harlequin toads (Fig. 3b), this is likely an artifact of incomplete sampling. Identification of *Atelopus* bufadienolides has been attempted only once, revealing the likely presence of major components telocinobufagin and bufotalin, and minor components including marinobufagin, cinobufagin, bufalin, and arenobufagin, as well as two unidentified bufadienolides (Fig. 2; Flier et al., 1980). In other bufonids, bufadienolide profiles are highly variable between populations, species, and life history stages. Factors implicated in this variation include population structuring, environmental factors like climate and habitat quality, and microbial toxin biotransformation (Bókony et al., 2019, 2016; Cao et al., 2019; Hayes et al., 2009b, 2009a; Inoue et al., 2020; Kamalakkannan et al., 2017). Consequently, there is likely undiscovered bufadienolide diversity and variation within *Atelopus.*

7. *Atelopus* Chemical Defense Characteristics: Ecological and Evolutionary Perspectives

The ecological roles of *Atelopus* toxins have not been investigated. However, the localization of toxins within granular glands that can be emptied in response to threatening stimuli and the possibly aposematic colorations of many *Atelopus* species (see Figure 3c and Rößler et al., 2019) suggest that *Atelopus* toxins may serve as an antipredator defense (Mebs et al., 2018a). There are few known

Atelopus predators. *Erythrolamprus epinephalus* is a colubrid snake that has been observed eating *A. varius* and *A. zeteki* in the wild (Greene, 1997; Lindquist et al., 2007) and has consumed *A. elegans* and *A. zeteki* while in captivity to no ill effect (Myers et al., 1978). However, Myers et al., (1978) does not specify whether these toads were wild-caught or captive-raised. The genetic basis of guanidinium alkaloid resistance in *Erythrolamprus* snakes may include amino acid substitutions in the skeletal muscle VGSC, $Na_V1.4$ (Feldman et al., 2012; Ramírez-Castañeda, 2017). Interestingly, *E. epinephalus* is also resistant to the effects of dendrobatid lipophilic alkaloids (Myers et al., 1978). In 2019, a fish (*Hoplerythrinus unitaeniatus*) and aquatic insect (*Abedus spp.)* were observed preying on *A. hoogmoedi* and *A. varius*, respectively (González-Maya et al., 2019; Lima et al., 2019). The predators appeared to suffer no ill effects, which suggests multiple predator species may possess resistance to *Atelopus* toxins. It remains to be seen whether *Atelopus* toxins prevent attacks or consumption by predators that lack resistance to bufadienolides and/or guanidinium alkaloids.

Atelopus toxins may have functions that extend beyond antipredator defense, as seen in other amphibian systems. Toxins can serve as intraspecific cues: *Taricha* larvae can detect tetrodotoxin and use this cue to avoid cannibalism by toxic adults (Zimmer et al., 2006). *Rhinella marina* tadpoles cannibalize eggs and are attracted to the bufadienolides found within them (Crossland et al., 2012). In another toad species, *Bufo bufo,* tadpoles produce greater concentrations of bufadienolides when living in ponds with high tadpole densities, suggesting bufadienolides could also act as a control mechanism for competition (Bókony et al., 2016). Defensive bufadienolides may also act as regulators of sodium and water levels in toads and could have evolved from endogenous chemicals (see Section 4.2). Lastly, bufadienolides and guanidinium alkaloids may also play a role in the immune system or as antimicrobial defenses. Two bufadienolides found in *A. "ignescens*," marinobufagin and telocinobufagin, have antimicrobial activities (Cunha-Filho et al., 2005; Flier et al., 1980). Along with arenobufagin, another known *Atelopus* bufadienolide (Flier et al., 1980), telocinobufagin inhibits in vitro growth of the

pathogen, *Batrachochytrium dendrobatidis* (Bd; Barnhart et al., 2017). In *Taricha,* TTX levels are negatively associated with total parasite richness and likelihood of Bd infection, but not with nematode infection load (Johnson et al., 2018). Similarly, although TTX exposure reduces survivorship of trematodes in the lab (Calhoun et al., 2017), there is no relationship between TTX levels and infection by nematode, trematode, and cestode endoparasites in *Notophthalmus viridescens* (Mebs et al., 2020). The dynamic between *Atelopus* toxins and Bd is important considering the role of Bd in *Atelopus* declines (La Marca et al., 2005), though a direct mechanism is not immediately obvious given the absence of Na+/K+-ATPases and VGSCs in fungi (Johnson et al., 2018; Rodríguez-Navarro and Benito, 2010). In summary, complex selective pressures relating to predation, communication, physiology, and immune function may be acting on *Atelopus* chemical defenses, underscoring the necessity of further research into *Atelopus* chemical ecology. In this section, we propose explanations for patterns in *Atelopus* toxin profiles and overall toxicity and suggestions for further research.

7.1 Atelopus Toxin Profiles

While many individual *Atelopus* toxins have been detected and characterized both chemically and pharmacologically, the adaptive importance of the specific compositions of *Atelopus* toxin cocktails remains uninvestigated. From an antipredator perspective, for instance, it is unclear whether possessing ZTX AB or CHTX rather than TTX as a major toxin component would affect harlequin toad fitness. It is possible that the unique binding patterns of different guanidinium alkaloids (see Section 4.1) result in functional differences relevant to warding off species-specific predators. Alternatively, considering the similar toxicities of TTX, CHTX, and ZTX to mice, the identity of the major alkaloid component in each *Atelopus* species may be of no adaptive significance. Future studies that involve exposing potential *Atelopus* predators to different guanidinium alkaloids could determine whether major toxin component identity influences the effectiveness of *Atelopus* chemical defenses.

699 The implications of simultaneously maintaining guanidinium alkaloids and cardiac glycosides,
700 two chemically and pharmacologically distinct toxin classes, are worth consideration. Having diverse
701 toxin types can enable organisms to be defended against multiple natural enemies, as demonstrated in
702 chemically defended plants (Lindroth and Hwang, 1996). Furthermore, toxins can synergize to magnify
703 each other's effects (Nelson and Kursar, 1999; Raaymakers et al., 2017). The respective targets of
704 bufadienolides and guanidinium alkaloids, VGSC and Na^+/K^+-ATPase proteins, both influence sodium ion
705 concentrations and may thus interact physiologically. In astrocytes, a type of glial cell, inhibition of
706 VGSCs results in lower Na^+/K^+-ATPase activity. VGSCs may maintain Na^+ ion concentrations at levels
707 necessary for Na^+/K^+-ATPase function (Sontheimer et al., 1994). The interaction between these
708 membrane proteins could have consequences for the function of *Atelopus* toxin cocktails. Interestingly,
709 tetrodotoxin reduces the toxicity of cardiac glycosides (CGs) when injected directly into the brain of cats
710 but potentiates CG toxicity when given intravenously (Peres-Gomes and Ribeiro, 1979). The difference in
711 effect between TTX administered to the brain and TTX administered intravenously is probably a
712 consequence of the impermeability of the blood-brain barrier to TTX (Zimmer, 2010). Tissue-specific
713 expression of uncharacterized receptors for toxin-binding proteins could also result in delivery-
714 dependent differences in poisoning symptoms. In dogs, intravenous TTX increases survival times and
715 reduces cardiac arrhythmia following cardiac glycoside poisoning (Bernstein, 1969). The prevalence of
716 system- and delivery-dependent results highlight the need to investigate the interactions of
717 bufadienolide and guanidinium alkaloids in predator systems that are biologically relevant to *Atelopus*.

718 If *Atelopus* sequester guanidinium alkaloids, bufadienolide biosynthesis could provide some
719 level of chemical defense when said sequestration is disrupted. Captive-raised *Atelopus* that lack
720 guanidinium alkaloids retain bufadienolides (Daly et al., 1997). *Melanophryniscus* is another genus of
721 bufonid toads where autogenous toxin production may compensate for variability in toxin sequestration
722 (Cei et al., 1968; Hantak et al., 2013). Although *Melanophryniscus* that are fed an alkaloid-free diet

gradually lose lipophilic alkaloids from their skins (Mebs et al., 2018b) *Melanophryniscus* may be able to upregulate the synthesis of indole alkaloids when lipophilic alkaloids are low (Jeckel et al., 2015). Similarly, the myobatrachid, *Pseudophryne semimarmorata,* synthesizes more indole alkaloids when raised in captivity without access to dietary lipophilic alkaloids (Smith et al., 2002). In contrast to *Melanophryniscus* and *Pseudophryne*, bufadienolide quantities are similar in *A. varius* with and without guanidinium alkaloids, suggesting that bufadienolide production is not upregulated in response to low TTX levels (Daly et al., 1997). More investigation is needed to clarify the functional role and regulation of autogenous toxins in *Atelopus.*

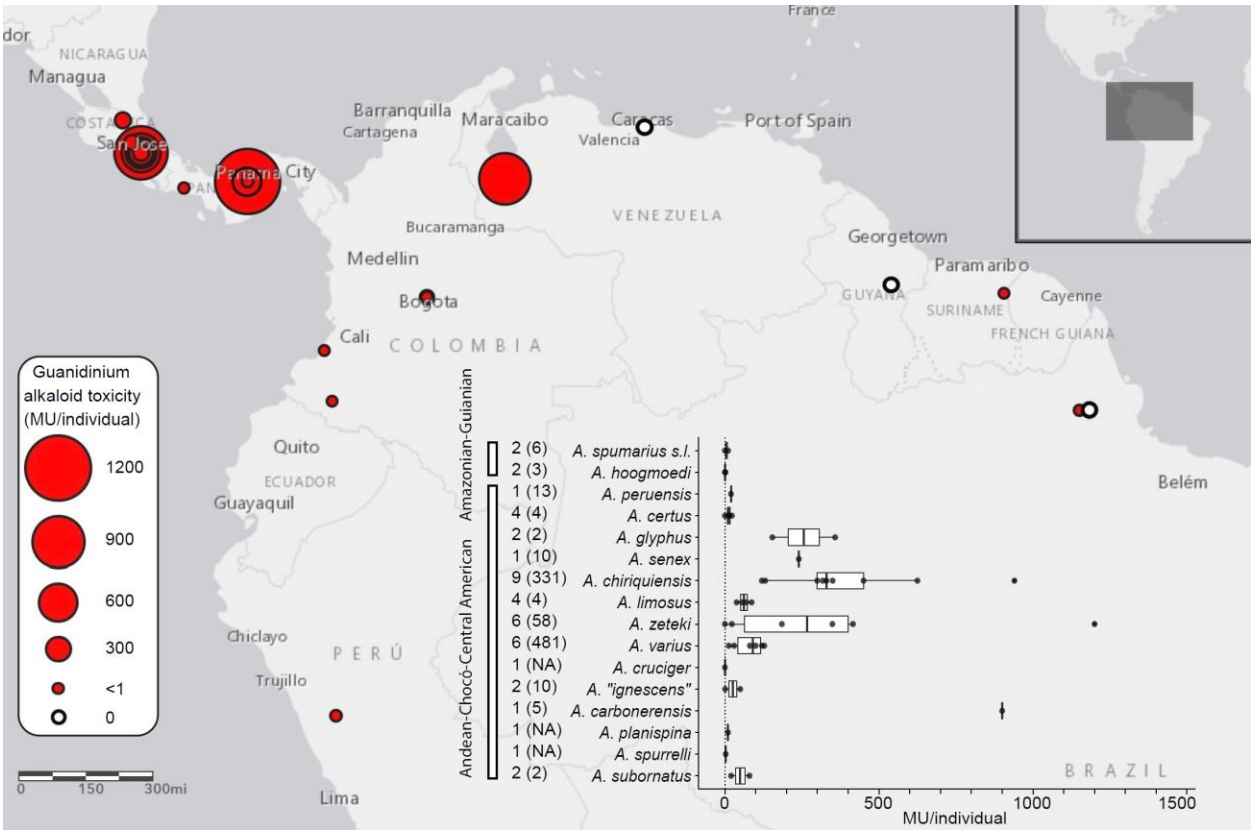


Figure 4: Geographic (N = 892 individuals) and species-level (inset) variation in adult *Atelopus* toxicity. Toxicity values primarily reflect quantity of guanidinium alkaloids (see section 5.1). Numbers left of species names detail the total number of toxicity assessments and number of specimens assessed (in parentheses) for each species. One mouse unit (MU) is sufficient to kill a

single average-weight mouse in thirty minutes upon injection (Yasumoto, 1991). When toxicity values were given in TTX equivalents or when TTX quantity alone was given, conversion to MUs used the conversion factor 1 MU = 0.22 ug TTX (Yasumoto, 1991). See supplementary Table S2 for coordinates, toxicity values, species names, sources, and details on unreported sample size data.

Toxicity data summarized and graphed using ggplot2 v3.3.3 (Wickham, 2016), dplyr v1.0.6 (Wickham et al., 2021), and cowplot v1.1.1 (Wilke, 2020).

7.2 Variation in Toxicity Between and Within Species

A common characteristic of chemically defended organisms is variation in toxicity, from the individual to the species level and in response to temporal, environmental, and physiological changes (Speed et al., 2012). *Atelopus* is no different. Individual harlequin toads range from completely nontoxic to toxic enough to kill thousands of mice (Fig. 4 inset). The causes of this variation are unknown and presumably depend on the source of a toxin and the ability of the toad to bioaccumulate the toxin or host its producers; however, some patterns do emerge which parallel those observed in better-studied systems. It is important to note that the toxicity values reported for *Atelopus* are primarily reflective of the guanidinium alkaloids present in their skin because the acidic aqueous extraction methods commonly used prior to toxin quantification likely exclude some or all bufadienolides from the resulting toxic fractions (see Section 5.1). When methods sensitive to bufadienolides were used, bufadienolide quantities in *Atelopus* were found to be large enough to contribute to the overall toxicity of the toads (Daly et al., 1997; Flier et al., 1980). Studies published prior to 1989 employed alternative and conflicting mouse unit definitions, which impede the comparability of reported *Atelopus* toxicities (see Section 5.3 for an expanded discussion).

759 The most toxic harlequin toads (presently known) are found in Central America (e.g. up to 1200
760 MU/toad for *A. zeteki* and 948 MU/toad for *A. chiriquiensis;* Kim et al., 1975; Pavelka et al., 1977) and
761 the montane cloud forests of the state of Mérida, Venezuela (*A. carbonerensis,* up to 1000 MU/toad;
762 Dole and Durant, 1974; Yotsu-Yamashita et al., 1992). *Atelopus* from the Guiana Shield and the Andes
763 south of Venezuela generally have low toxin levels (Fig. 4). However, while a minimum of 889 Central
764 American harlequin toads have been assessed for toxicity, at least 39 from all other geographic regions
765 have been tested (Fig 4 inset, see Supp. Table S2 for details on reported sample sizes). The lack of
766 standardization in older *Atelopus* toxicity studies also prevents confirmation of these patterns. Future
767 research on *Atelopus* toxicity could prioritize the sampling of toads from the Andes and Guiana Shield.

768 *Atelopus* toxicity may be influenced by selection pressure from predators. In *Taricha,* a genus of
769 newts that possesses as many as 60,000 MU of TTX per individual, high toxin levels are thought to be
770 driven by a coevolutionary relationship with TTX-resistant predatory garter snakes (*Thamnophis*) (Hague
771 et al., 2020; Hanifin, 2010; Williams et al., 2003). The intensity of reciprocal selection between these
772 species varies geographically, resulting in populations with drastically different toxicities and toxin
773 resistances (Hague et al., 2020). A similar situation is possible between *Atelopus* and one or more
774 predator species (such as *Erythrolamprus epinephalus*). Future studies could investigate covariance in
775 *Atelopus* predator toxin resistance and *Atelopus* toxicity across the sympatric ranges of both taxa to see
776 if a coevolutionary arms race is taking place.

777 Some of the observed intrapopulation variation in *Atelopus* toxicity (Daly et al., 1994; Kim et al.,
778 1975; Mebs et al., 1995; Pavelka et al., 1977) might be attributable to the experiences of sampled
779 individuals. While also found in the skin epithelium and the liver, *Atelopus* toxins are primarily localized
780 in the granular glands, which are distributed across the body and can eject their contents when a toad
781 feels threatened (Mebs et al., 2018a; Toledo and Jared, 1995). A toad that was recently attacked may
782 have temporarily diminished its skin-associated stores of alkaloids and steroids. Over longer time

783 periods, encounters with predators could lead to higher toxicity in *Atelopus* individuals. Predator cue
784 exposure and simulated predator attacks induce increased toxicity in some amphibian species that
785 possess guanidinium alkaloids or bufadienolides (Benard and Fordyce, 2003; Bucciarelli et al., 2017), but
786 not in others (Brossman et al., 2014; Üveges et al., 2017). The plasticity of *Atelopus* chemical defenses
787 needs investigation and could provide insight into the ecological significance of their toxins.

788 Reproductive cycles and development may also play a role in toxicity variation. Gravid *Atelopus*
789 *chiriquiensis* females have lower skin-associated toxin levels than males but are comparably poisonous
790 when the toxicities of their eggs are accounted for (Pavelka et al., 1977). Thus, *Atelopus* may provision
791 their eggs with toxins, possibly as a defensive measure, and this process likely involves the diversion of
792 skin toxins into the reproductive system (Pavelka et al., 1977; Yotsu-Yamashita and Tateki, 2010). More
793 generally, toxicity may vary with *Atelopus* age and/or metamorphic stage, as seen in other
794 bufadienolide-defended toads (Hayes et al., 2009a; Üveges et al., 2017) and tetrodotoxin-defended
795 newts (Gall et al., 2011; Tsuruda et al., 2002) and octopi (Williams, 2008; Williams et al., 2011). In other
796 toxin-sequestering frog species, body size has been positively correlated with granular gland capacity
797 (Saporito et al., 2010) and overall toxin quantity (Jeckel et al., 2015). *Atelopus* toxin provisioning and
798 ontogenetic changes in toxicity could be investigated for mechanisms involved in toxin transport and
799 accumulation.

800 Most speculatively, unidentified environmental factors may influence the success of microbe-
801 toad symbioses, which are possible sources of guanidinium alkaloids in *Atelopus.* Some *Atelopus* species
802 have extremely high site fidelity and individuals may thus be exposed to relatively constant
803 microenvironments throughout their lives (Crump, 1986; Tarvin et al., 2014). *Atelopus* occupy a variety
804 of habitats, and the possibility of covariance between toxicity and abiotic factors such as temperature
805 and precipitation remains an area of interest.
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8. Concluding Remarks

A half-century of research into *Atelopus* chemical defenses has resulted in the discovery of individual chemicals and toxin profiles found in no other biological system. Yet, only a fraction of *Atelopus* species have been assessed for toxins, and the most characterized species are geographically and phylogenetically clustered. Furthermore, varying standards and detection abilities reduce what conclusions can be drawn from existing data. There is likely undiscovered toxin diversity in the genus, representing chemicals with possible medical or scientific value. Of the known *Atelopus* toxins, several appear restricted to a few species or populations of harlequin toads. For instance, the only known extant source of ZTX AB is *A. varius* (Yotsu-Yamashita et al., 2004). ZTX AB cannot be synthesized at this time (Adachi et al., 2019, 2014), and is consequently at risk of disappearing. If efforts by host countries or by collaborations supported by host countries are not made to chemically analyze declining *Atelopus* species, novel chemicals could be lost before being identified and characterized. Such a situation may have already occurred with the unidentified *A. planispina* toxin which induced unique poisoning symptoms (Fuhrman et al., 1969). *A. planispina* was last observed in 1985 and may be extinct (IUCN, 2021).

Many questions remain regarding the evolution of *Atelopus* chemical defenses. While it is commonly assumed that *Atelopus* toxins provide protection against predators, the potential ecological and physiological roles of toxins in *Atelopus* remain unstudied. Few *Atelopus* predators are known, and investigation into *Atelopus* predator-prey relationships could bring a clearer understanding of what causes toxicity variation within and between species as well as the adaptive significance of *Atelopus* toxin cocktails. We suggest that toxins could have additional functions unrelated to antipredator defense, including communication, defense against pathogens, and physiological regulation.

The clade of endemic Central American *Atelopus,* which diverged from South American *Atelopus* more than three million years ago (Ramírez et al., 2020), has the highest diversity of guanidinium

alkaloids. If not a result of sampling biases, it is unclear what shapes the chemical defense characteristics that are potentially unique to this subclade. Have Central American toads evolved unique sequestration or synthesis mechanisms? Are they forming symbiotic relationships with different guanidinium alkaloid-producing or modifying cyanobacteria? The genetic underpinnings of putative toxin sequestration and bacterial symbioses in *Atelopus* have not been studied. Furthermore, the case for bacteria as the source of *Atelopus* guanidinium alkaloids is speculative and requires further investigation. Feeding experiments and bacterial inoculation studies are likely to be fruitful areas of research in the future.

Krogh's principle holds that the answers to biological questions can be most efficiently pursued through the study of organisms with features relevant to those questions (Krebs, 1975; Krogh, 1929). Thus, loss of organismal diversity necessarily impedes research in the life sciences. Harlequin toad chemical defenses represent a promising study system for multiple broad evolutionary and ecological questions – including the interplay between VGSCs and Na^+/K^+-ATPase in regulating vertebrate physiology, the evolution of toxin sequestration and synthesis, and the regulation of bacteria-amphibian symbioses – however, *Atelopus* have experienced precipitous declines in recent decades (La Marca et al., 2005). A few species are stable in the wild (Lampo et al., 2017; Lips, 2008), and several conservation efforts (e.g., Centro Jambatu: http://www.anfibiosecuador.ec/, Atelopus Survival Initiative: https://www.atelopus.org/, Amphibian Rescue & Conservation Project: http://amphibianrescue.org/) are ensuring the ex-situ survival of species at risk of extinction. There is much to be discovered by studying *Atelopus* and their toxins, highlighting the importance of continued investment in conservation.

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