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 cardioxins (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 (Löters et al., 2011). Many species are brightly colored on all or part of their bodies (Fig. 3c; Löters et al., 2011), and these colors may act as aposematic signals to warn potential predators of their toxicity (Röller et al., 2019). Harlequin toads are smooth skinned and lack the large parotoid glands commonly observed in other bufonids. Instead, Atelopus granular glands are small and evenly distributed across their bodies (Mcdiarmid, 1971). Concentrated within the granular glands and skin epithelium (Mebi et al., 2018a) are two classes of toxic chemicals: bufadienolides and guanidinium alkaloids (Daly et al., 1997). With the possible exception of Clinotarsus curtipes (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). 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 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

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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 for a complete list). Becker et al. (2011) claims to have detected zetekitoxins in *Atelopus seteki* via HPLC, a method which would require a zetekitoxin AB standard for positive identification (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. seteki*. 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 (including Brown, 1972) 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/documentatio/n/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, 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 (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; Mêb et al., 1995) and *A. ignescens* (collected in Colombia and Ecuador; Brown, 1972; Daly et al., 1994; Plier et al., 1980) is ambiguous based on the collection locations. We designate these populations as *A. spumarius sensu lato* and *A. ignescens*, following Lotters et al. (2002) and Quillido et al. (2005), respectively. Similarly, the population of toads designated *A. oxyrhynchus* by Mêb and Schmidt (1989) and Yotsu-Yamashita et al. (1992) has since been identified as *A. carboneriensis*, which is likely extinct (see Table S2 for a more complete discussion of taxonomy; Lotters 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. glyptus*, *A. limosus*, *A. seteki*, *A. chiriquiensis*, *A. varius*, *A. senex*) have been tested for toxins and six ( *A. senex* excluded) have had their toxins chemically analyzed (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 (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; Rosegini et al., 1976, 1987, 1989). 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-epitetrodotoxin, 4,9-anhydro-tetrodotoxin, chiriquitoxin, and zetekitoxin AB. We also review toxins which have been detected but whose properties and structures are relatively unknown.

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-epitetrodotoxin, 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 Na1.5, Na1.8 and Na1.9 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 NaV 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 (Regyi et al., 2012, 2013).

### 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, 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, 2017), wild-caught Atelopus and newts retain (Mebs et al., 1995, 2018a; 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 (Cardall et al., 2004; Mailho-Fontana et al., 2019). However, we propose that similar patterns of TTX uptake 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 inculcate captive 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; Itô 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 and 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).

4.1.3. Chiriquitoxin

Chiriquitoxin (CHXT) 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). CHXT binds with particularly low affinity to human Na\textsubscript{A}1.7, 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, CHXT can also interfere with the function of potassium voltage gated ion channels (Yang and Rao, 1992). Nevertheless, CHXT 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-epiTTX, is not an aqueous equilibrium product of TTX. It has been proposed that CHXT 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, Adelobates galactonotus, and A. castaneotcus) metabolize an ingested pumiliotoxin, PTX (+)-251D, stereoeselectively 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 body tissues but remained in their original forms (Kudo et al., 2017). In contrast, parotid-gland-associated bacteria are known to biotransform bufadie-nolides in the toad Rhinella marina (Kamalakannan et al., 2017). Nevertheless, no bacteria have been found that can produce CHXT or modify TTX into any analog (Yotsu-Yamashita et al., 2013).

4.1.4. Zetekitoxin AB

Zetekitoxin AB (ZTX AB) is unique among Atelopus guanidinium alkaloids; it is an analog of the paralytic shellfish toxin saxitoxin and contains two guanidinium moieties (Fig. 1). Furthermore, ZTX AB is the only natural chemical known to possess an 1,2-oxazolidine ring-fused lactam moiety (Yotsu-Yamashita et al., 2004). Despite structural differences, ZTX AB is remarkably similar to TTX in potency, with an LD50 of 11 μg/kg as compared to 10 μg/kg for TTX (Brown et al., 1977; Fuhrman et al., 1976). Symptomatically, ZTX AB poisoning is virtually indistinguishable from TTX poisoning, except that it more commonly induces cardiac arrhythmia (Brown et al., 1977; Fuhrman et al., 1976). Unlike many other saxitoxin analogs but like TTX, ZTX AB causes hypotension (Brown et al., 1977; Durán-Riveroll and Cembella, 2017). Unfortunately, only limited amounts of ZTX AB have been available for pharmacological and biophysical study. As a result, little is known about its binding specificity.

Source. ZTX AB has only ever been detected in Atelopus zeteki and A. varius, and its source remains uninvestigated. Cyanobacteria and dinoflagellates, however, are well established as the source of saxitoxin (Durán-Riveroll and Cembella, 2017), and saxitoxin-producing cyanobacteria are found in freshwater systems (Smith et al., 2011). Given that Atelopus are riparian and possess skin-associated cyanobacteria (Becker et al., 2014), it seems plausible that ZTX AB has a cyanobacterial origin. Unlike TTX, the genetic basis of saxitoxin synthesis is known (Hackett et al., 2009), so metagenomic techniques 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 (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; Duran-Riveroll and Cembella, 2017; Murtha, 1966). Unfortunately, insufficient quantities of ZTX C were purified for structural analysis (Brown et al., 1977).

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 Ca²⁺ 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; Rodriguez 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; Lemaerts 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; Rodriguez et al., 2017) in the skin and brain of B. viridis (Lichtenstein 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 (Rodriguez 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; Kama-lakkannan 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

![Fig. 2. Bufadienolides detected in Atelopus.](image-url)
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 (Filier 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. seteki and has not been further investigated (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. seteki (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 (Mebes 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. chiriquisensis 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 (Matsumoto 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 protein. Previous studies (before 1977) used distilled water for the initial extraction, and thus may have reported lower toxin levels than were present in the toads tested. With a few exceptions (Daly et al., 1994, 1997; Mebs et al., 1995), subsequent studies on Atelopus toxins followed Pavelka et al. (1977) and performed acid extractions.

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

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

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 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 11 μg of TTX; Brown et al., 1977). Alternatively, the spotted TLC plate can be sprayed with an alkaline solution and heated (Mebes and Schmidt, 1989). This converts guanidinium alkaloids into 2-aminoquinazolino 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. (1989). This liquid chromatography-fluorescence detection system (LC-FLD) takes advantage of the fluorescence of 2-aminoquinazolino 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-epiTTX and 4,9-anhydroTTX in Atelopus extracts (Yotsu-Yamashita et al., 1992; Yotsu et al.,
Detection and quantification methods utilized in studies of Atelopus toxins.

<table>
<thead>
<tr>
<th>Method</th>
<th>Capabilities</th>
<th>Limitations</th>
<th>Relevant Atelopus Studies</th>
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<tr>
<td><strong>BIOASSAYS</strong></td>
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<td></td>
<td>(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, 2005; Yotsu-Yamashita and Tateki, 2010; Yotsu et al., 1990b)</td>
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<tr>
<td>Mouse Bioassay (MBA)</td>
<td>- quantifies in vivo toxicity of extracts or of purified toxins</td>
<td>- variance in standardization between studies</td>
<td>(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, 2005; Yotsu-Yamashita and Tateki, 2010; Yotsu et al., 1990b)</td>
</tr>
<tr>
<td></td>
<td>- provides a preliminary determination of toxin identity</td>
<td>- not specific; toxins with similar biological effects cannot be distinguished</td>
<td>(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, 2005; Yotsu-Yamashita and Tateki, 2010; Yotsu et al., 1990b)</td>
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<td></td>
<td></td>
<td>- requires use of live animals</td>
<td>(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; Shindelman et al., 1969; Yotsu-Yamashita et al., 1992, 2005; Yotsu-Yamashita and Tateki, 2010; Yotsu et al., 1990b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- only provides estimate of toxicity to mammals</td>
<td>(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; Shindelman et al., 1969; Yotsu-Yamashita et al., 1992, 2005; Yotsu-Yamashita and Tateki, 2010; Yotsu et al., 1990b)</td>
</tr>
<tr>
<td>Binding Inhibition Assays</td>
<td>- detect and quantify compounds that interact with guanidinium alkaloid or Bufadienolide binding sites on VGSCs and Na+/K+ ATPase, respectively</td>
<td>- not specific, measures all compounds with the same binding behavior</td>
<td>(Daly et al., 1994, 1997; Flier et al., 1980)</td>
</tr>
<tr>
<td>Immunohistochemistry (IH)</td>
<td>- detects TTX and visualizes distribution within tissues</td>
<td>- capable of application to other guanidinium alkaloids (Smolowitz and Doucette, 1995)</td>
<td>(Mebs et al., 2018a)</td>
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<tr>
<td>PHYSICOCHEMICAL</td>
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<tr>
<td>Nuclear Magnetic Resonance (NMR)</td>
<td>- detects and quantifies TTX, 4-epiTTX, 4,9-anhydroTTX (Nakamura and Yamanoto, 1985), CHTX, ZTX AB</td>
<td>- matrix components can reduce spectra quality (Bane et al., 2014)</td>
<td>(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)</td>
</tr>
<tr>
<td>Thin Layer Chromatography (TLC)</td>
<td>- determines purity and preliminary identity of guanidinium alkaloids</td>
<td>- cannot provide a quantitative estimate of toxin amounts</td>
<td>(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)</td>
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<tr>
<td>High Pressure Liquid Chromatography (HPLC)</td>
<td>- detects and quantifies TTX, 4,9-anhydroTTX, CHTX</td>
<td>- requires standards of each compound being identified</td>
<td>Flier et al. (1980)</td>
</tr>
<tr>
<td>Liquid Chromatography with Fluorescence Detection (LC-FLD)</td>
<td>- detects and quantifies TTX, 4-epiTTX, 4,9-anhydroTTX, CHTX</td>
<td>- can’t detect ZTX AB or low quantities of CHTX</td>
<td>(Daly et al., 1994; Mebs et al., 1995; Yotsu-Yamashita et al., 1992; Yotsu-Yamashita and Tateki, 2010)</td>
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<tr>
<td>Gas Chromatography with Mass Spectrometry (GC-MS)</td>
<td>- detects and quantifies TTX and its analogs, and some individual lipophilic alkaloids</td>
<td>- Antelopus guanidinium alkaloids are not volatile, chemical derivation required (Guznaga and Kotoku, 1980)</td>
<td>(Daly et al., 1984; Mebs and Schmidt, 1989)</td>
</tr>
<tr>
<td>Electrospray Ionization with Mass Spectrometry (ESI-MS)</td>
<td>- detects TTX, 4,9-anhydroTTX (Wang et al., 2010), CHTX, ZTX AB</td>
<td>- not specific, TTX and analogs cannot be distinguished (Magalhamov et al., 2017)</td>
<td>(Mebs et al., 1995; Yotsu-Yamashita and Tateki, 2010)</td>
</tr>
<tr>
<td>High Resolution Hydrophilic Interaction Liquid Chromatography/Mass Spectrometry (HR-HILIC-MS)</td>
<td>- detects and quantifies TTX, 4-epiTTX, 4,9-anhydroTTX (Nakagawa et al., 2006), CHTX, ZTX AB</td>
<td>- matrices components can reduce spectra quality (Bane et al., 2014)</td>
<td>(Mebs et al., 2018a)</td>
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</table>

1989). LC-FLD utilizes reverse phase chromatography, which is incapable of separating all TTX analogs (Bane et al., 2014). Additionally, because TTX analogs exhibit a wide range of fluorescent intensities, analogs may not all be detectable under the same set of conditions (Shoji et al., 2001). For instance, detection of 11-deoxytetrodotoxin, a common analog in newts and brachycephalid toads (Hamlin, 2010), requires higher post-column reaction temperatures than does the detection of TTX (Yotsu et al., 1989). Studies of Atelopus toxins which utilized LC-FLD either employed post-column reaction temperatures below those needed for 11-deoxytetrodotoxin detection (Yotsu-Yamashita et al., 1992) or neglect to specify the post-column reaction temperature (Daly et al., 1994; Mebs et al., 1995; Yotsu-Yamashita and Tateki, 2010). All Atelopus LC-FLD studies utilized a C18 chromatography column but varied in mobile phase composition (heptfluorobutyric acid or trifluoroacetic acid) and concentration of NaOH in the post-column reaction (ranging from 2.8N to 4N; Daly et al., 1994; Mebs et al., 1995; Yotsu-Yamashita et al., 1992, 2010). LC-FLD cannot detect low levels of CHTX, and it is incapable of detecting ZTX AB (Yotsu-Yamashita et al., 1992; Yotsu-Yamashita and Tateki, 2010).
alkaloid assays in Atelopus studies (Mebs et al., 1995, 2018a; Yotsu-Yamashita and Tateki, 2010). Whereas quantification by LC-FLD requires calibration with standards of each TTX analog being measured, quantification of TTX analogs can be performed by ESI-MS with the exclusive use of a TTX standard (Chen et al., 2011; Nakagawa et al., 2006; Shoji et al., 2001). The simplest use of ESI-MS is to directly screen toad extracts for ions indicative of toxin presence, such as the MH+ ion of TTX (320 m/z; Mebs et al., 1995). However, the presence of other chemicals in 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 (Knutsen et al., 2017). Subsequent analysis by ESI-MS detected only TTX and 4-epi-TTX in an A. hoogmoedii 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. hoogmoedii lacks other TTX analogs including 6-epitetrodotoxin and 11-deoxytetrodotoxin, which are frequently detected in newts (Hanifin, 2010). This is consistent with the apparent absence of 6-epitetrodotoxin 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-epitetrodotoxin, 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, 2016, 2020, 2021; Kudo and Yotsu-Yamashita, 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 A. “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., 1999b). 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 min by intraperitoneal injection and is equivalent to 0.22 µg 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 min (Brown et al., 1977; Pavelka et al., 1977), 30 min (Mebs and Schmidt, 1989) and 1 h (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.

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

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 (Löters 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, 2001), 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 (Mediarmid, 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., 1995, 2018a; 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 gua
nidiun 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 Bufo
nidae known to possess guanidinium alkaloids (Rodríguez et al., 2017);
toxin assessments of species within the sister taxon of Atelopus (Oreo-
ophrynella; Kok et al., 2018) and other “ateloid” bufonids (Melano-
phryniscus 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. glyphs, 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 sampling of the Amazonian-Guianan clade and of Bufo
nidae more generally is needed before a definitive model of the origin
and evolution of TTX defense in Atelopus can be proposed.

4,9-anhydroTTX and 4-epiTTX frequently cooccur with TTX, which is
expected given the aqueous equilibrium between the three chemicals.
Interestingly, 4-epiTTX has once been detected in the absence of 4,9-
anhydroTTX in a single male specimen of A. hoogmoedii from Suriname
(Fig. 3b; Mebs et al., 2018a).

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

ZTX AB. Zetekitoxin AB (ZTX AB) has only been found in two of the
seven assessed Central American Atelopus: A. varius and A. zeteki
(Fig. 3a). These sister species are closely related (Fig. 3a; Lötters et al.,
2011; Ramirez et al., 2020) and a recent whole-genome analysis does
not support the species boundary between them (Bryne et al., 2020),
suggesting that A. varius and A. zeteki are the same species. A. varius
and A. zeteki exhibit intraspecific variation in the presence and absence
of TTX 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. Identifi-
cation of Atelopus bufadienolides has been attempted only once,
revealing the likely presence of major components telocinobufagin and
bufotalin, and minor components including marinobufagin, cinobufa-
gin, bufalin, and arenobufagin, as well as two unidentified bufadieno-
lides (Fig. 2; Flier et al., 1980). In other bufonids, bufadienolide profiles
are highly variable between populations, species, and life stages. Factors
implicated in this variation include population structuring, environ-
mental factors like climate and habitat quality, and microbial
toxin biotransformation (Bokony et al., 2016, 2019; Cao et al., 2019;
Hayes et al., 2009a, 2009b; Inoue et al., 2020; Kamalakkannan et al.,
2017). Consequently, there is likely undiscovered bufadienolide diver-
sity 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 Fig. 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. Erythrolampremus
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) do not specify
whether these toads were wild-caught or captive-raised. The genetic
basis of guanidinium alkaloid resistance in Erythrolampremus snakes may
include amino acid substitutions in the skeletal muscle VGSC, Naq.1.4
(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 (Hoploerythrus unia-
tenius) and aquatic insect (Abedus spp.) were observed preying on
A. hoogmoedii 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 intra-
specific cues: Taricha larvae can detect tetrodotoxin and use this cue to
avoid cannibalism by toxic adults (Zimmer et al., 2006). Rhinella marina
tadpoles can 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 (Bokony 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 telocinobu-
faqin, 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 sur-
vivourship 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 Notopthalmus 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, under scoring 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 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.

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

If Atelopus sequester guanidinium alkaloids, bufadienolide biosynthesis could provide some level of chemical defense when said sequestration is disrupted. Captive-raised Atelopus that lack guanidinium alkaloids retain bufadienolides (Daly et al., 1997). Melanophryniscus is another genus of bufoinid toads where autogenous toxin production may compensate for variability in toxin sequestration (Cei et al., 1968; Hantak et al., 2013). Although Melanophryniscus that are fed an alkaid-free diet gradually lose lipophilic alkaloids from their skins (Mebs et al., 2018a) Melanophryniscus may be able to upregulate the synthesis of indole alkaloids when lipophilic alkaloids are low (Jeeckel et al., 2015). Similarly, the myobatrachid, Pseudophryne seminirmorata, 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.

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).

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

Atelopus toxicity may be influenced by selection pressure from predators. In Taricha, a genus of newts that possess as many as 60,000 MU of TTX per individual, high toxin levels are thought to be driven by a coevolutionary relationship with TTX-resistant predatory garter snakes (Thamnophis) (Hague et al., 2020; Hanifin et al., 2002; Williams et al., 2003). The intensity of reciprocal selection between these species varies geographically, resulting in populations with drastically different toxicities and toxin resistances (Hague et al., 2020). A similar situation is possible between Atelopus and one or more predator species (such as Erythrolamprus epinephalus). Future studies could investigate covariation in Atelopus predator toxin resistance and Atelopus toxicity across the sympatric ranges of both taxa to see if a coevolutionary arms race is taking place.

Some of the observed intrapopulation variation in Atelopus toxicity (Daly et al., 1994; Kim et al., 1975; Mebs et al., 1995; Pavelka et al., 1977) might be attributable to the experiences of sampled individuals. While also found in the skin epithelium and the liver, Atelopus toxins are primarily localized in the granular glands, which are distributed across the body and can eject their contents when a toad feels threatened (Mebs et al., 2018a; Toledo and Jared, 1995). A toad that was recently attacked may have temporarily diminished its skin-associated stores of alkaloids and steroids. Over longer time periods, encounters with predators could lead to higher toxicity in Atelopus individuals. Predator cue exposure and
simulated predator attacks induce increased toxicity in some amphibian species that possess guanidinium alkaloids or bufadienolides (Benard and Fordyce, 2003; Bucciarelli et al., 2017), but not in others (Brossman et al., 2014; Üveges et al., 2017). The plasticity of Atelopus chemical defenses needs investigation and could provide insight into the ecological significance of their toxins.

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

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

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., 2014, 2019), 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 (Furhman 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 (Ramirez et al., 2020), has the highest diversity of guanidinium alkaloids. If not a result of sampling biases, it is unclear what shapes the interplay between VGSCs and Na\textsuperscript{+}\textsuperscript{+}. Feeding experiments 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. Probiosomes to this article can be found online at https://doi.org/10.1016/j.toxcx.2022.100092.

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