Chapter 21
A Review of Chemical Defense in Poison Frogs (Dendrobatidae): Ecology, Pharmacokinetics, and Autoresistance

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21.1 Introduction

Chemical defense has evolved multiple times in nearly every major group of life, from snakes and insects to bacteria and plants (Mebs 2002). However, among land vertebrates, chemical defenses are restricted to a few monophyletic groups (i.e., clades). Most of these are amphibians and snakes, but a few rare origins (e.g., *Pitohui* birds) have stimulated research on acquired chemical defenses (Dumbacher et al. 1992). Selective pressures that lead to defense are usually associated with an organism's limited ability to escape predation or conspicuous behaviors and phenotypes that increase detectability by predators (e.g., diurnality or mating calls) (Speed and Ruxton 2005). Defended organisms frequently evolve warning signals to advertise their defense, a phenomenon known as aposematism (Mappes et al. 2005). Warning signals such as conspicuous coloration unambiguously inform predators that there will be a substantial cost if they proceed with attack or consumption of the defended prey (Mappes et al. 2005). However, aposematism is likely more complex than the simple pairing of signal and defense, encompassing a series of traits (i.e., the aposematic syndrome) that alter morphology, physiology, and behavior (Mappes and

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Alatalo 1997; Hagman and Forsman 2003; Darst et al. 2005; Despland and Simpson 2005; Ozel and Stynoski 2011; Santos and Cannatella 2011; Zhen et al. 2012).

Several lineages within five families of anurans have chemical defense and are known as poison frogs (i.e., Bufonidae, Dendrobatidae, Eleutherodactylidae, Mantellidae, and Myobatrachidae); see references for accounts in non-dendrobatid groups (e.g., Daly et al. 2005; Rodriguez et al. 2011). However, the most studied of these frogs are those within Dendrobatidae; our use of the term "poison frog" in this review will refer only to this group. For some time, defensive compounds in dendrobatids were thought to be products of biosynthetic pathways that evolved once in this family (Myers et al. 1991). However, ecological and phylogenetic studies revealed that poison frogs sequester alkaloids from their diet and that this capacity has evolved at least four times across Dendrobatidae (Fig. 21.1). This evolutionary complexity makes dendrobatids an unparalleled model clade for the study of acquired defenses among vertebrates. Although much research effort has focused on natural product discovery and characterization of chemicals found in the skin of poisonous anurans, other aspects of poison frog ecology remain poorly studied. In this review, we present an integrative summary of dendrobatid chemical ecology with an evolutionary perspective, highlighting some of these poorly studied aspects and exploring potential avenues of future research.

21.2 Evolutionary Ecology of Dendrobatid Poison Frogs

Dendrobatidae is a monophyletic clade containing more than 300 species. All members of this lineage are Neotropical endemics and common leaf litter inhabitants across tropical and mountainous forests in Central and South America (Santos et al. 2009). One species (*Dendrobates auratus*), however, occurs outside of this range in O'ahu and Maui (Hawaii, USA) since its introduction as a pest control in 1932 (McKeown 1996; Kraus and Duvall 2004). Most dendrobatid species have lowland distributions at altitudes of less than 2000 m and they tend to be diurnal, terrestrial, and locally abundant near streams. Many are popular pet trade animals, and many color morphs exist in the pet trade that do not exist in the wild (Lötters et al. 2007).

Approximately two-thirds of dendrobatids are considered to be nontoxic and cryptically colored. The remaining ~100 species are regarded as aposematic because they have both visual warning signals and defensive compounds (Summers and Clough 2001; Santos et al. 2003; Vences et al. 2003). Of the total 12 dendrobatid genera, six contain aposematic species: *Ameerega* (31 species), *Colostethus* (1 sp.), *Epipedobates* (6 spp.), *Dendrobates* (50 spp.), *Hyloxalus* (2 spp.), and *Phyllobates* (5 spp.). The genus *Dendrobates* is the most studied and the focus of frequent taxonomic reviews (Myers 1987; Bauer 1994; Grant et al. 2006; Brown et al. 2011). Seven lineages within *Dendrobates* have been proposed as new genera (Fig. 21.1), which we consider as subgenera until further taxonomic work is provided. Other chemically defended but non-aposematic dendrobatids include members of *Aromobates*, *Epipedobates*, and *Colostethus* (Myers et al. 1991; Daly et al. 1994b;

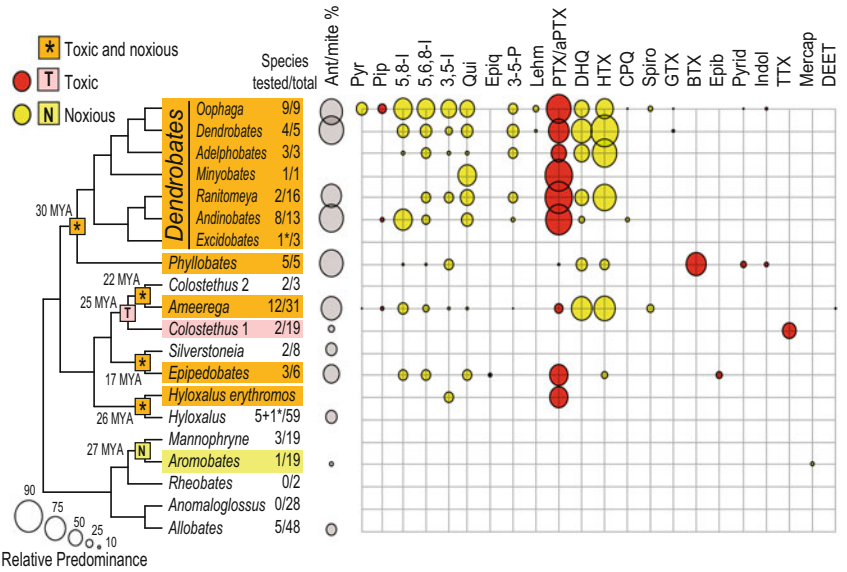

Fig. 21.1 Relative predominance (quantity and diversity) of different compound classes found in skin extracts of poison frogs of the family Dendrobatidae. Toxic and unpalatable genera are indicated by approximate age in millions of years (MYA) since diversification from their last common ancestor. *Colostethus* is split in two clades (i.e., *Colostethus* 1 and *Colostethus* 2) because it is paraphyletic with respect to *Ameerega* (i.e., *Colostethus* 2 is the sister taxa to *Ameerega*). The number of species in each genus/subgenus that has been tested for alkaloid presence is indicated over the total number of species in that genus. Diet is indicated by the percentage of the most representative prey items that are known sources of alkaloids: ant and mites. The relative predominance of each alkaloid class was determined by the sum of all the different compounds in that class multiplied by its quantity (i.e., trace, minor, major), then divided by the total number of species in the genus and finally adjusted to 100 %. Some uncertainties exist for chemical defense characterization. For example, *Dendrobates* (*Excidobates*) *captivus* and *Hyloxalus azureiventris* have tested positively for presence or ability to sequester alkaloids but the identity of alkaloids sequestered in wild populations of these species is unknown (indicated by *). The abbreviation of alkaloid classes is as follows: *Pyr* Pyrrolidines, *Pip* Piperidines, *5,8-I* 5,8-disubstituted indolizidines, *5,6,8-I* 5,6,8-trisubstituted indolizidines, *3,5-I* 3,5-disubstituted indolizidines, *Qui* Quinolizidines, *Epiq* Epiquinamide, *3,5-P* 3,5-disubstituted pyrrolizidine, *Lehm Lehmizidines*, *PTX/aPTX* Pumiliotoxins/Allopumiliotoxins, *DHQ* Decahydroquinolines, *HTX* Histrionicotoxins, *CPQ* Cyclopentaquinolizidines, *Spiro* Spiropyrrolizidines, *GTX* Gephyrotoxins, *BTX* Batrachotoxins, *Epib* Epibatidines, *Pyrid* Pyridinic alkaloids, *Indol* Indolic alkaloids, *TTX* Tetrodotoxin, *Mercap* Mercaptan-odor, *DEET* N,N-diethyltoluamide

Cipriani and Rivera 2009). The remaining dendrobatids in the genera *Allobates*, *Anomaloglossus*, *Hyloxalus*, *Mannophryne*, *Rheobates*, and *Silverstoneia* are largely considered to lack chemical defenses (Grant et al. 2006). However, at least one species of *Hyloxalus* (Santos et al. 2014) has defensive alkaloids (i.e., *H. erythromos*) and another (*H. azureiventris*) might be able to sequester them (Saporito et al. 2009; Santos and Cannatella 2011). In both instances, each species is closely related to non-defended *Hyloxalus* species, suggesting that there might be two independent origins of chemical defense in this clade, in addition to the three other

well-studied origins in *Ameerega*, *Dendrobates* + *Phyllobates*, and *Epipedobates*. More biochemical work is necessary to determine the extent of chemical defenses present in other purportedly non-aposematic species.

Both aposematic and non-aposematic dendrobatids breed nearly continuously throughout the year, but especially during rainy seasons. Almost all species use audiovisual signals during courtship, and their advertisement calls make them easy to locate (Zimmermann and Zimmermann 1988; Hödl and Amezquita 2001; Santos et al. 2014). Reproduction is largely terrestrial with egg masses deposited in leaf litter or phytotelmata (pools of water in leaf axils or bromeliads). Parental care is nearly ubiquitous in the group. Paternal care is the most common mode of parenting, but maternal and biparental types of care have also been reported (Summers et al. 2006). Nursing frogs usually guard and hydrate eggs, transport tadpoles, and in some species, provide unfertilized eggs as food to offspring reared in phytotelmata (Weygoldt 1987; Summers and McKeon 2004). Only recently it was discovered that these unfertilized eggs contain trace alkaloids, suggesting that some dendrobatid mothers may provide both food and chemical defense through parental care (Stynoski et al. 2014a, b).

Natural selection via predation may have driven the evolution of chemical defense in poison frogs. There are few reports of predation on aposematic dendrobatids, suggesting that their chemical defense is highly effective (Poulin et al. 2001; Santos and Cannatella 2011). Nevertheless, experimental evidence using clay model frogs suggests that avian predators are relatively important (Summers and Clough 2001; Saporito et al. 2007b; Noonan and Comeault 2009; Rojas et al. 2014) and visual perception models support that birds are likely to recognize brightly colored poison frog species as conspicuous (Maan and Cummings 2012). However, natural history anecdotes suggest a diversity of predators including birds (e.g., *Baryphthengus martii*; see Alvardo et al. 2013) and snakes (e.g., *Rhadinaea decorate*; see Lenger et al. 2014). In fact, most accounts refer to snake predation (69 % or 25/36 events), followed by spiders (17 % or 6/36), then birds (6 % or 2/35), and a few others (9 % including ants, fish, and crabs). Consequently, poison frogs might be under selection by multiple predators with diverse sensory biases and varied tolerances to dendrobatid defenses (Santos and Cannatella 2011). Alternatively, it is possible that aposematic species do not have specialized predators and that most predation events come from inexperienced individuals sampling aposematic frogs for the first time. Such learned avoidance is a critical prediction of the evolution and maintenance of aposematism (Speed and Ruxton 2005).

Dendrobatid defensive compounds appear to deter diverse organisms (Tables 21.1, 21.2 and 21.3). However, the toxic and repellent effects of these substances on predators and their possible accumulation at higher trophic levels are relatively unstudied. Dendrobatid species with significant quantities of toxic alkaloids, such as members of *Phyllobates*, *Dendrobates* sensu lato, *Ameerega*, and *Epipedobates* should have a large impact on their ecological communities. Organisms that consume these poison frogs may become toxic to their own predators, cascading the effects of alkaloid accumulation up the trophic chain. Likewise, poison frogs may impact lower trophic levels by altering the diversity and abundance of their arthropod prey because most

Table 21.1 Diversity of dendrobatid poison frog compounds, environmental source, and their defensive/antipredator properties

	Compounds[a]		Environmental source[a]		Number of species by compound predominance[b]			Defensive/antipredator properties[c]			
	N	*N* Unique[d]	Demonstrated	Proposed	Major	Minor	Trace	Tox	Unp	ATB	AF
Pyrrolidines	10	7	Ants|mites	–	4	0	1	–	R	A	A
Piperidines	26	23	Ants	–	1	5	3	T	R	A	A
5,8-Disubstituted indolizidines	63	27	Ants|mites	–	10	8	13	t	B*,R	A	–
5,6,8-Trisubstituted indolizidines	47	24	Mites	Ants	4	12	13	–	B*	–	–
3,5-Disubstituted indolizidines	15	7	Ants|mites	–	6	2	10	t	B*	–	–
1,4-Disubstituted quinolizidines	11	3	Mites	–	2	13	11	–	B*	–	–
4,6-Disubstituted quinolizidines	2	1	Ants|mites	–				–	B*	–	–
Epiquinamide	1	1	–	–	0	0	1	No	No	–	–
3,5-Disubstituted pyrrolizidines	13	4	Ants	–	1	6	9	–	B*	–	–
Lehmizidines	9	9	–	Ants	0	0	7	–	B*	–	–
Pumiliotoxins	31	7	Ants|mites	–	25	7	7	T	R	A	A
Allopumiliotoxins	18	12	Ants|mites	–				T	R	–	–
Deoxypumiliotoxins	5	2	Mites	Ants				T	–	–	–
Desmethylpumiliotoxins	1	1	–	Ants|mites				T	–	–	–
Homopumiliotoxins	4	1	Mites	Ants				T	–	–	–
Desmethylhomopumiliotoxins	1	0	–	Ants|mites				T	–	–	–
Deoxyhomopumiliotoxins	3	2	–	Ants|mites				T	–	–	–
Decahydroquinolines	40	28	Ants	–	14	12	7	t	B	A	–
Histrionicotoxins	16	16	Ants	–	20	10	2	t	B,R	A	–
Cyclopentaquinolizidines	9	9	–	–	1	0	1	–	B*	–	–
Spiropyrrolizidines	5	1	Mites|millipedes	–	3	2	1	–	R	–	–
Gephyrotoxins	2	2	–	Ants	0	0	1	t	–	–	–
Batrachotoxins	6	6	–	Beetles	3	0	2	T	B,R	–	–

(continued)

Table 21.1 (continued)

	Compounds[a]		Environmental source[a]		Number of species by compound predominance[b]			Defensive/antipredator properties[c]			
Epibatidines	4	4	–	Plant to arthropod	0	0	2	T	B	–	–
Pyridinic alkaloids[e]	3	2	–	Plant to arthropod	0	0	4	T	B,R	–	–
Indolic alkaloids[f]	2	2	–	Plant to arthropod	0	0	4	t	R	–	–
Tetrodotoxin	1	0	–	Bacteria|endogenous	1	0	0	T	–	–	–
Mercaptan-odor	1	–	–	Endogenous	0	0	1	–	R	–	–
N,N-diethyltoluamide	1	–	–	Moths	0	0	1	t	R	–	–
Other tricyclics	31	21	Mites|beetles	–	1	2	3	–	–	–	–
Unclassified alkaloids	144	115	–	–	–	–	–	–	–	–	–
Total	525	337									

[a]Alkaloid/compound diversity, quantity, and environmental sources for dendrobatid poison frogs derived from literature (Jones and Jacobson 1968; Daly et al. 1987, 1994a, b, 1999, 2005, 2009; Myers et al. 1991; Ujvary 1999; Saporito et al. 2003, 2007a, 2012; Dumbacher et al. 2004; Fitch et al. 2009, 2010; Mebs et al. 2010, 2014; Chau et al. 2011; Jones et al. 2012)

[b]The designation of major, minor, and trace indicates the amount of each type of substance found in frog skin (Daly et al. 1987). Major represents >50 μg, minor >5 μg, and trace <5 μg per 100 mg frog skin (Daly et al. 2009). We used this information to assign dendrobatid species to each predominance category

[c]Antipredator properties are mostly based on tests using mouse/rat models. We used these experiments to estimate toxicity (Tox): more toxic "T" or less toxic "t". Defensive properties are indicated by unpalatable (Unp): demonstrated as bitter "B", presumed bitter "B*", or repellent "R". Antibiotic (ATB) or antifungal (AF) properties are indicated by "A". All unknown states are indicated by "–"

[d]Number of unique alkaloids found in dendrobatid poison frogs is based on (Saporito et al. 2012)

[e]Nicotine, noranabasamine, pyridylnicotine

[f]Chimonanthine and calycanthine

Table 21.2 Effects of dendrobatid compounds on mammals and actions on muscular and neuronal receptors and channels

		Mouse model			Receptor/ion channels				
	Toxicity	LD50 ug/ mouse	Compound Tested	Effect	Compound Tested	nACh	Na	K	References
Piperidines	High	9.375	253 J (solenopsin)	Depressed cardiorespiratory function, seizures, and death	253 J 241D	Block[a]	–	–	Howell et al. (2005)
Pyrrolidines	–	–	–	–	197B	Block[a]	–	–	Daly et al. (1999)
3,5-Disubstituted indolizidines	Low	>200	239CD	Locomotor difficulties, piloerection, prostration with recovery > 4 h	223AB 239AB 239CD	Block[a]	–	–	Daly and Spande (1986), Daly et al. (1999)
Other indolizidines	–	–	–	–	205A, 207A 209B 235B′, 235B″	Block[a]	–	–	Daly et al. (1999), Tsuneki et al. (2004)
Spiropyrrolizidine	–	–	–	–	222, 236 238	Block[a]	–	–	Daly et al. (1999)
Coccinelline-like	–	–	–	–	205B[b]	Block[a]	–	–	Daly et al. (1999)
Epiquinamide	None	–	196	Inactive	196	None	None	None	Fitch et al. (2009)

(continued)

Table 21.2 (continued)

	Toxicity	Mouse model			Receptor/ion channels				
		LD50 ug/ mouse	Compound Tested	Effect	Compound Tested	nACh	Na	K	References
Pumiliotoxins	Medium to high	200	251D[c]	Pain; hyperactivity; convulsions; cardiac depressant; death < 6 min	251D	–	Block	Block Alter deactivation	Daly and Spande (1986), Daly et al. (1999)
		20–50	307A (PTX A) 323A (PTX B)	Locomotor difficulties; paralysis; convulsions; death < 20 min; Potentiates/prolongs muscle contraction; repetitive neuronal firing	307A 323A	–	Prevent inactivation	–	Daly et al. (1990), Vandendriessche et al. (2008)
Allopumiliotoxins	High	50[d]	323B 339A 339B	Potentiate and prolong muscle contraction; cardiotonic	323B 339A 339B	–	Prevent inactivation	–	Daly and Spande (1986), Daly et al. (1999)
		40	267A	Pain; hyperactivity; locomotor difficulties; death in < 6 min[e]		–	–	–	
Decahydroquinolines	Low	>400	219A	Locomotor difficulties at >125 mg/kg; convulsions and death in 10 min	195A 219A	Block[a]	Block	Block	Daly and Spande (1986), Daly et al. (1999)

Histrionicotoxins	Low	>1000	283A 285A	Locomotor difficulties; prostration	283A 285C 285B 287A 287B 291A	Block[a]	Block	Block	Daly and Spande (1986), Daly et al. (1999)
Gephyrotoxins	Low	>500	287C 289B	Weak muscarinic antagonist (e.g., cognitive impairment)	287C 289B	Block[a,f]	Block[g]	Block (at high levels)	Daly and Spande (1986), Daly et al. (1999)
Batrachotoxins	Very high	0.1	BTX h-BTX BTX A	Arrhythmia; cardiac arrest; anesthesia; death; depolarization of muscle and neuronal membranes	BTX h-BTX BTX A	–	Prevent inactivation, alter Activation	Prevent inactivation[g]	Daly and Spande (1986)
Pyridine alkaloids	High to very high	66.8	162 (nicotine)	Cardiac depression; paralysis/ convulsion; death	162 222/224	Agonist Agonist	–	–	Lazutka et al. (1969), Daly et al. (1999), Fitch et al. (2010)
		0.4	208/210 (epibatidine)	Analgesia; locomotor depression; reduced body temperature; changes in blood pressure; bradycardia; death	208/210	Agonist	–	–	
Indole alkaloids	Low	880	346C (calycanthine)	Cardiac depression, paralysis, and convulsion	346C[h]	–	–	–	Chebib et al. (2003)

(continued)

Table 21.2 (continued)

	Toxicity	Mouse model			Receptor/ion channels				
		LD50 ug/ mouse	Compound Tested	Effect	Compound Tested	nACh	Na	K	References
Tetrodotoxin	Very high	0.1–0.214	TTX	Respiratory failure; death	TTX	–	Block	–	Daly and Spande (1986), Bane et al. (2014)
N,N-diethyltoluamide	Low	>2000	DEET	Irritant; neurological damage	DEET[i]	–	–	–	Sudakin and Trevathan (2003)

[a]Non-competitive blocker
[b]Results are from a test of a synthetic enantiomer
[c]PTX 251D is more toxic for insects and less toxic for mammals than PTX A or PTX B
[d]Tests were done with pig atria, so lethal dose is extrapolated from other studies of comparably toxic compounds (i.e., PTX A)—see Daly and Spande (1986)
[e]Daly et al. (2003) state that aPTX 267A is toxic to mice while Daly and Spande (1986) state that it is not
[f]May also block glutamatergic NMDA receptors
[g]Effect speculated by cited authors
[h]Blocks calcium channels, GABA receptors
[i]Inhibits cholinesterases

Table 21.3 Effects of dendrobatid compounds on insects, bacteria, and fungi

	Arthropods				Bacteria/fungi			
	Compound Tested	mM	Organisms	Effect	Compound Tested	μg	Effect	References
Pyrrolidines	–	–	Ants Termites	Repellent	Synthetic	10–30	Antibiotic antifungal	Jones et al. (1989), Daly et al. (1999), Macfoy et al. (2005)
Piperidines	253 J (solenopsin)	11–33	*Solenopsis invicta*	Reduced ambulation; deterrent	253 J Synthetic	10–50	Antibiotic antifungal	Daly et al. (2005), Macfoy et al. (2005), Weldon et al. (2013)
5,8-Disubstituted indolizidines	205A 235B	33–100	*S. invicta*	Reduced ambulation	235B′	100	Antibiotic	Macfoy et al. (2005), Weldon et al. (2013)
3,5-Disubstituted indolizidines	223AB	100	*S. invicta*	None	239AB	50	Inactive	Macfoy et al. (2005), Weldon et al. (2013)
Pumiliotoxins	267C 323A 251D 307A	0.001–33	*S. invicta* *Heliothis virescens* *Aedes aegypti*	Convulsions; reduced ambulation	251D 307A Synthetic	50 50 30–50	None or Antibiotic antifungal	Bargar et al. (1995), Macfoy et al. (2005), Weldon et al. (2006, 2013)
	237A	100	*S. invicta*	None	–	–	–	Weldon et al. (2013)
Allopumiliotoxins	267A	0.01–0.04	*S. invicta*	Reduced ambulation	–	–	–	Weldon et al. (2013)
Decahydroquinolines	cis-223 F	100	*S. invicta*	None	trans-243A	100	Antibiotic	Macfoy et al. (2005), Weldon et al. (2013)
Histrionicotoxins	291A 259A 285A	0.33–100	*S. invicta*	Convulsions; reduced ambulation	Synthetic	100	Antibiotic	Macfoy et al. (2005), Weldon et al. (2013)
Spiropyrrolizidines	236	100	*S. invicta*	None	236	200	None	Macfoy et al. (2005), Weldon et al. (2013)

(continued)

Table 21.3 (continued)

	Arthropods				Bacteria/fungi			
	Compound Tested	mM	Organisms	Effect	Compound Tested	µg	Effect	References
	151B (Polyzonamine)	0.1–10	*Formica exsectoides Periplaneta americana Phormia regina*	Repellent; irritant	–	–	–	Smolanoff et al. (1975), Ujvary (1999)
Gephyrotoxins	287C	100	*S. invicta*	None	–	–	–	Weldon et al. (2013)
Batrachotoxins	BTX	33–100	*S. invicta*	Convulsions; reduced ambulation	BTX	20	None	Macfoy et al. (2005), Weldon et al. (2013)
	BTX-A	100	*S. invicta*	None	BTX-A	50	None	Macfoy et al. (2005), Weldon et al. (2013)
	hBTX	–	Chewing lice (Phthiraptera)	Reduced life span	–	–	–	Dumbacher (1999)
Pyridinic alkaloids	162 (Nicotine)	0.33–1	*S. invicta*	Reduced ambulation; distasteful	Synthetic	50–200	None	Macfoy et al. (2005), Weldon et al. (2013)
Tetrodotoxin	TTX	–	*Musca domestica*	High affinity for Na+ channels; likely insecticide	–	–	–	Pauron et al. (1985), How et al. (2003), Gordon et al. (2007)
N,N-diethyltoluamide	191C (DEET)	0.48–10	*A. aegypti*	Repellent	–	–	–	Jones and Jacobson (1968), Bohbot and Dickens (2010)

defensive substances sequestered by these frogs come from specialized diets (Daly et al. 1994a; Caldwell 1996). More ecological studies will reveal the impact of toxic dendrobatids on the ecological dynamics of their communities.

21.2.1 Diet and Defense in Dendrobatidae

There is a tight association between diet and defense within Dendrobatidae (Daly 1998). In the wild, dendrobatids feed continuously during the day and actively defend territories that are likely associated with food and chemical defense resources (Caldwell 1996; Pröhl 2005). Diet specialization on alkaloid-bearing arthropods such as ants and mites is phylogenetically correlated with origins of chemical defense (Darst et al. 2005); both have evolved in parallel in at least three clades of aposematic dendrobatids (Fig. 21.1; *Epipedobates*, *Ameerega*, and *Dendrobates*). These lineages include diet specialists that have morphological and biomechanical adaptations that allow them to consume large quantities of diminutive prey (i.e., microphagy), including changes in tongue shape and use (elongated, narrow, fast and shooting), reduction of teeth, and compaction of cranial shape (Toft and Duellman 1979; Toft 1980, 1981, 1995; Emerson 1985; Lieberman 1986; Donnelly 1991; Simon and Toft 1991; Vences et al. 1998).

Most dendrobatid alkaloids appear to have a dietary origin. Identical alkaloids have been found in leaf litter arthropods (e.g., ants, mites, and millipedes) and dendrobatids; moreover, wild-caught frogs kept in captivity show a marked reduction in alkaloid diversity and quantity (Daly et al. 1992, 2000, 2002; Saporito et al. 2003, 2004; Jones et al. 2012). However, a few chemicals found in dendrobatids may have another source (Tables 21.1, 21.2 and 21.3). For example, one cryptic dendrobatid *Colostethus panamansis* has tetrodotoxin (TTX) (Daly et al. 1994b). The mechanisms of acquisition, transport, and storage of TTX by this frog are unknown, but in other TTX-defended systems this substance is usually produced by endosymbiont bacteria or sequestered following the consumption of other TTX-defended prey (Daly et al. 1997; Chau et al. 2011; Wood et al. 2012; Bane et al. 2014). Another interesting case is *Aromobates nocturnus*, a nocturnal species that releases a pungent (mercaptan) defensive odor when handled (Myers et al. 1991). Unfortunately, individuals of this species have not been found for at least 20 years (Barrio-Amoros et al. 2011). However, *Aromobates* relatives are common at lower elevations (~2000 m) in geographic regions close to the known distribution of *A. nocturnus* in the Merida Andes in Venezuela. Interestingly, some of these sympatric *Aromobates* (e.g., *A. saltuensis*) release a similar mercaptan odor when manipulated (Barrio-Amoros and Santos 2012; JCS *pers. obs.*).

The ultimate origin of dendrobatid alkaloids is a topic of continuous research, although many are found in arthropods, including ants (formicine and myrmicine), coccinellid beetles, siphonotid millipedes, and oribatid mites (Table 21.1; see also Saporito et al. 2009). Some alkaloids might be produced by plants, which are consumed by arthropods and then taken up by poison frogs (Daly et al. 1999; Saporito et al. 2012). For example, the chimonanthine and calycanthine alkaloids found in

Phyllobates terribilis (Daly et al. 1999) might originate from syntopic *Psychotria* (Rubiaceae) plants (Verotta et al. 1998). Several genera of ants are attracted to and consume *Psychotria* including *Solenopsis* (*Diplorhoptrum*) sp., which is an ant genus found in stomachs of several poison frog species (Born et al. 2010; Arce-Dominguez and Rengifo-Mosquera 2013; Bieber et al. 2013). Alternatively, evidence also supports that ants (and to a lesser extent, mites) are in fact producing alkaloids, rather than acting as intermediaries (Jones et al. 2012; Saporito et al. 2011). Recent observations (LAO unpublished data) of the diet of *D.* (*Oophaga*) *sylvaticus* show a prevalence of several species of fungus-growing ants, which are known to be in close association with diverse bacteria and fungi symbionts (Mueller et al. 1998; Currie et al. 1999). Hence, some dendrobatid alkaloids may actually originate in plants and microbes, but so far they have only been traced to their arthropod diet.

21.2.2 Toxicity and Unpalatability

Poison frog alkaloids can be toxic (i.e., causing damage to the consumer), unpalatable (i.e., distasteful or repellent to predators), or both. Among the first to notice the toxicity of dendrobatids were the Native Americans of the Chocoan Emberá and Noanamá tribes in Western Colombia (Myers et al. 1978). Several anthropologists, ethnologists, and zoologists have described in detail how these indigenous people use skin extracts of *Phyllobates* frogs to poison blowgun darts to hunt large game (Cochrane 1825; Posada-Arango 1883; Wassen 1935; Marki and Witkop 1963). These observations attracted the attention of biochemists, who were interested in understanding the basis of poison frog toxicity. Early work focused on the physiological effects of dendrobatid alkaloids in model organisms such as mice, rats, and frogs (Daly and Myers 1967; Daly and Spande 1986; Daly et al. 1999). Thereafter, the rapid development of gas chromatography–mass spectrometry stimulated intense work on alkaloid structure, classification, synthesis, and mechanism of action (Daly et al. 1978, 1987, 1999; Saporito et al. 2012). A summary of the toxic and unpalatable effects of these dendrobatid compounds is provided in Tables 21.1, 21.2 and 21.3.

An early method used to detect chemical defense in anurans was tasting (e.g., licking) their skin secretions (Neuwirth et al. 1979). Species rich in alkaloids (e.g., *Dendrobates* sensu lato) were usually described as bitter and causing burning and numbing sensations (Myers and Daly 1976; Neuwirth et al. 1979). These observations led researchers to propose that some dendrobatid chemical defenses may not actually be lethal (Daly et al. 2005). Certain classes of dendrobatid compounds with low toxicity instead appear to be distasteful to a broad diversity of predators, even at small quantities (Daly et al. 2005). Such repellents may allow potential predators to rapidly develop an aversion to consuming poison frogs because they will recall their unpleasant taste (Darst and Cummings 2006; Bassoli et al. 2007). Among these unpalatable compounds are histrionicotoxins (HTX) and decahydroquinolines (DHQ), which have relatively low toxicity in mammals (Daly and Spande 1986),

and several types of izidines (e.g., indolizidines, pyrrolizidines, and quinolizidines), which are considered bitter or unpalatable (Table 21.1). However, aside from their unpleasant taste, some HTXs, DHQs, and izidines are surprisingly toxic to arthropods that might parasitize poison frogs (Table 21.3). Interestingly, these substances also appear to have antibiotic and antifungal properties although this should be further investigated (Macfoy et al. 2005).

The evolutionary significance of toxicity versus unpalatability in dendrobatids is a topic of continuous research. To frame this hypothesis phylogenetically, we determined relative measurements of both properties based on how predominant each compound class is among the genera of poison frogs (Fig. 21.1). However, we emphasize that many of the species that potentially have defensive compounds have not been analyzed and some surveys show significant variation among species and even individuals (Daly et al. 1978, 1992; Saporito et al. 2007a). Given these caveats, we summarize the following observations. Most species within *Dendrobates* sensu lato are both toxic and unpalatable as many of their members have PTX/aPTXs (toxic) and DHQ, HTX, and izidines (unpalatable) as predominant alkaloid classes (i.e., >50 μg/100 mg of frog skin). Most notable in this group is the subgenus *Oophaga*, >90 % species of which have PTX/aPTX as its major alkaloid type. *Phyllobates* and *Epipedobates*, on the other hand, are toxic with predominance of BTX, PTX 251D and epibatidine alkaloids respectively, but they are relatively poor in unpalatable compounds such as DHQs, HTXs, and izidines. *Ameerega* is mostly unpalatable and less toxic, with a predominance of DHQ and HTX alkaloids. Insect repellent compounds have also been isolated from *Ameerega* species, including DEET (N,N-diethyltoluamide) and polyzonamine, which might function against a broad spectrum of ectoparasites.

21.3 Acquiring Chemical Defenses: A Pharmacokinetics Perspective

Although very little is known about the physiological mechanisms of toxin sequestration and chemical modification in poison frogs, recent technological advances have opened the possible exploration of these unique biological mechanisms like never before. The question of how poisonous amphibians have evolved physiological mechanisms to sequester dietary chemical defenses is essentially a study in pharmacokinetics. Here we discuss current and future directions in advancing our understanding of the mechanistic basis of toxin acquisition and storage in amphibians. Approaching this topic of toxin physiology will require pursuing questions within an integrative framework and combining methods in genomics, proteomics, and pharmacology.

A dendrobatid's ability to sequester alkaloids is genetic, as captive-raised dendrobatids fed with alkaloid-dusted fruit flies are able to uptake, modify, and accumulate most alkaloids (Daly et al. 1994a, 2003; Saporito et al. 2009). Dermal granular glands are the main alkaloid storage organs and are responsible for their

release, but some reports also suggest that small traces of alkaloids are present in other tissues including liver, muscle, and oocytes (Neuwirth et al. 1979; Delfino et al. 2010; Saporito et al. 2010b, 2012; Prates et al. 2012; Stynoski et al. 2014a). The number and size of granular skin glands increases allometrically with body size, allowing larger and older frogs to accumulate more alkaloids than juveniles (Saporito et al. 2010b). However, the physiological mechanisms of alkaloid sequestration are topics of ongoing research.

As poison frogs acquire chemical defenses through their diet (Saporito et al. 2012), the first candidate tissue to uptake small molecule lipophilic alkaloids is the intestine. The gut has long been known to play a major role in the oral bioavailability of compounds in the context of orally prescribed drug absorption in humans (Zhang and Benet 2001). Lipophilic compounds, like alkaloid toxins found on many poisonous amphibians, undergo passive absorption in the gut, but that does not imply that lipophilic compounds pass through the gut into the blood unhindered. In many organisms, the first line of defense against ingested toxic substances is a series of membranes between the gut epithelium and the blood. Within these barriers are highly expressed protein families that interact with dietary compounds, including the ABC (ATP-binding cassette) transporters that promiscuously bind compounds for transport either back into the lumen for excretion or into the blood for circulation, and the cytochrome p450 family of enzymes that metabolize compounds (Chan et al. 2004). Some ABC efflux proteins are known to transport alkaloid compounds that are used in cancer treatments (Chan et al. 2004), and it is likely they transport other lipophilic alkaloids as well, making them good candidates for sequestration of alkaloid toxins in amphibians. It is currently unknown if dietary alkaloids that successfully surpass these filtering mechanisms are sequestered in the skin (rather than being excreted) or if poisonous amphibians have altered expression patterns of these protein families in the gut to prevent loss and favor uptake of dietary alkaloids.

Much of the circulating blood is filtered through the liver, an environment rich in enzymes that metabolize and neutralize potentially toxic substances. The cytochrome p450 superfamily of enzymes is highly abundant in the intestines and especially in the liver, and is best known for metabolism of drugs through oxidation (Danielson 2002). Although many of the frog alkaloid toxins are sequestered unchanged through the diet, there is one example of a compound that is chemically modified by frogs. Some species of South American frogs can stereoselectively hydroxylize PTX (+)-251D to aPTX (+)-267A, which is roughly fivefold more toxic than its precursor (Daly et al. 2003). Although only a few species in the Dendrobatidae clade were tested, only frogs in the *Dendrobates* genus were able to metabolize pumiliotoxin into allopumiliotoxin, whereas this chemical modification was not observed in species in the genus *Phyllobates* or in the more distant *Epipedobates*. As the conversion from PTX (+)-251D to aPTX (+)-267A involves a 7-hydroxylation, obvious candidate enzymes for this metabolism are members of the cytochrome p450 family. The question of how dendrobatids can accomplish this conversion, but *Phyllobates* or *Epipedobates* cannot, remains to be determined. With next-generation sequencing technologies becoming common for nontraditional model species, a molecular evolution study comparing the cytochrome p450 family would be a step forward towards understanding these differences in toxin metabolism.

As alkaloid toxins are sequestered in various tissues, they must eventually be bound and transported in the blood, passing through various tissues to eventually accumulate in the skin; however the identity of such alkaloid transporters in poison frogs is unknown. There is little known about proteins that bind neurotoxins in general, with the exception of saxitoxin, a shellfish alkaloid neurotoxin that targets sodium channels in a manner similar to tetrodotoxin (Terlau et al. 1991). In plasma, saxitoxin is bound by saxiphilin, a unique member of the transferrin family of Fe^{3+}-binding proteins that has evolved to bind alkaloid toxins (Morabito and Moczydlowski 1994). Saxiphilin was initially discovered in the plasma of the North American bullfrog (*Rana catesbeiana*) (Mahar et al. 1991), but it was then determined that this protein is highly conserved throughout animals as a mechanism to bioaccumulate shellfish toxins encountered in the environment away from the animal's nervous system (Llewellyn et al. 1997). It is possible that a similar mechanism has evolved in poison frogs, with neofunctionalization (e.g., a paralog gene that takes on a totally new function) of transport carrier proteins to bind and transport alkaloids in plasma en route to the skin granular glands where toxins are stored until secretion (Neuwirth et al. 1979). Once more genetic information becomes available for poison frogs, molecular evolution methods could be employed to determine candidate genes that may play a similar role as saxiphilin in the binding and transport of alkaloid toxins compared to nontoxic amphibians.

Well-studied examples of toxin sequestration and storage mechanisms and their fitness consequences are insect herbivores that specialize on alkaloid-producing plants. In many of these cases, the host-plant derived alkaloids are pyrrolizidines coopted for defense by some moths, beetles, and other insects (see Opitz and Muller 2009 for detailed review). Some plants synthesize these pyrrolizidines in a non-toxic N-oxide form, which is unstable and easily reduced to a toxic form in the insect gut by cytochrome p450s (Hartmann et al. 1997, 1999; Narberhaus et al. 2005). Moths adapted to specialize on pyrrolizidine-producing plants have developed a monooxygenase that reoxidizes the toxic alkaloid into the N-oxide form where it is stored in tissues inaccessible to cytochrome p450s (Lindigkeit et al. 1997; Naumann et al. 2002). Interestingly, controlled feeding experiments with labeled alkaloids have shown beetles that specialize on pyrrolizidine-producing plants have a specific membrane carrier for the pyrrolizidine alkaloid senecionine (Narberhaus et al. 2004), although the exact protein has yet to be identified. In some arctiid insects, many of these alkaloids sequestered from the diet are stored and repurposed for critical roles in reproductive behavior (Conner et al. 1981, 2000). Females store alkaloids in eggs (Dussourd et al. 1988), which has recently been shown to occur in poison frogs (Stynoski et al. 2014a). Similarly, male arctiid insects incorporate alkaloids into spermatophores (Dussourd et al. 1988) and/or convert them into pheromones (Hartmann and Witte 1995; Hartmann et al. 2003). Females are highly attracted to these alkaloid-derived pheromones and prefer males with higher alkaloid content (Bogner and Boppre 1989), potentially selecting for more efficient sequestration mechanisms (Eisner and Meinwald 1995; Opitz and Muller 2009). These studies in insects can inform new directions for researchers interested in poison frog sequestration mechanisms, such as specific membrane

transporters for alkaloids or how sequestration of alkaloids may play a role in poison frog reproduction and mate choice.

An example of the importance of considering pharmacokinetics in the study of frog chemical ecology is the strawberry dart frog [*Dendrobates* (*Oophaga*) *pumilio*]. In this species, females have a greater quantity and diversity of alkaloids present in the skin than males (Saporito et al. 2010a). Although such differences tend to be explained by ecologists as purely environmental (i.e., males and females may have different foraging preferences, although diet was not examined in the above study), there is evidence in humans that there are many gender effects in pharmacokinetics of orally administered compounds, especially involving drug binding and metabolism (Harris et al. 1995). It is possible then that there are also sex differences in absorption and/or metabolism of small lipophilic alkaloids in anurans and the relative contributions of diet and physiology need to be further dissected.

Although great and insightful work in chemical ecology of poisonous amphibians has mostly focused on descriptive ecological contributions (Saporito et al. 2012), the emergence of new high-throughput technologies has opened the doors to understanding the chemical ecology of poisonous amphibians in a mechanistic way. For example, RNA sequencing could be applied to dietary studies in poisonous frogs to determine how gene expression changes across various tissues with a toxic or non-toxic diet. Whole transcriptome sequencing for many species is now possible and creates many opportunities to examine sequence variation in protein families that may be involved in toxin sequestration and metabolism. Moreover, the ease of sequencing a transcriptome now makes high-throughout proteomics possible, enabling the identification of proteins that bind alkaloids, which would have been extremely difficult five to ten years ago. We predict that harnessing these technologies will lead to rapid and informative advances in the field of amphibian chemical ecology, moving the field past correlative ecology and into a deeper mechanistic understanding of how amphibians have evolved these unique traits. It is even more remarkable that toxin sequestration in anurans has independently evolved several times (Santos et al. 2003), and in similar ways (i.e., to sequester the same families of small molecule compounds from the diet). Once more is known about the physiological mechanisms of toxin sequestration in a model poison frog family, one can begin to ask if the convergent evolution of toxin sequestration among amphibians involves a convergence in underlying mechanism or entirely different molecular pathways.

The physiological adaptation of sequestering a large variety of small molecule alkaloids in poison frogs is distinctive among complex biological systems and represents a unique opportunity to investigate binding properties of protein systems. Poison frogs have presumably evolved a set of proteins that bind small molecule alkaloids with some selectivity and yet have promiscuous enough binding properties to bind a range of alkaloids. The evolution and kinetics of this yet-to-be-discovered protein family promises not only rich insights into the evolution of toxicity in amphibians but also general insights into protein systems that bind toxins targeting the nervous system. Such studies focusing on basic mechanisms could lead to more translational research of therapeutics for human pathologies and chemical defense strategies.

21.4 Autoresistance as a Component of Chemical Defense

Chemical defense is a complex phenotype that involves a suite of ecological, morphological, physiological, and genetic changes over time (Härlin and Härlin 2003; Santos and Cannatella 2011). One often-overlooked component is autoresistance—how do chemically defended organisms resist self-intoxication, and how does resistance evolve? Long-term exposure to low levels of toxins may select for some degree of toxin resistance in organisms without any chemical defense (Hua et al. 2013). In species with diet-derived defense, both chemical sequestration and diet specialization increase toxin exposure, which in turn would select for greater resistance (Dobler et al. 2011). Hence, autoresistance in aposematic organisms may evolve as a combination of pre-existing resistance and resistance that evolves in parallel with chemical defense. In this section, we describe the evolution of toxin resistance in a number of taxa and explore the potential complexity of this phenotype in dendrobatid poison frogs. These amphibians are an ideal study system for the evolution of autoresistance because within this clade chemical defense has evolved independently at least four times at various evolutionary timescales (Fig. 21.1).

21.4.1 Tetrodotoxin as an Example of the Evolution of Toxin Resistance

One of the best-studied naturally occurring toxins is tetrodotoxin (TTX). This neurotoxic alkaloid is found in many organisms including pufferfish, frogs, newts, molluscs, crabs and the blue-ringed octopus, among other animals (Noguchi et al. 1984, 1986; Tsuruda et al. 2002; Soong and Venkatesh 2006; Hwang et al. 2007). In anurans, TTX is found in *Brachycephalus*, *Polypedates*, *Atelopus*, and the dendrobatid *Colostethus panamansis* (Daly et al. 1994b; Tanu et al. 2001; Pires et al. 2005). The toxicity of TTX is well characterized: it binds to and blocks voltage-gated sodium channels (VGSCs), cell membrane proteins encoded by the Nav1 gene family that mediate neural communication and muscle contraction (Wang and Wang 1998; Cestele and Catterall 2000). The Nav1 genes are functionally important, evident by the maintenance of gene duplications and their subfunctionalization in a number of lineages (six genes in amphibians, eight in teleosts, and ten in mammals) and the extremely high conservation of amino acid (AA) sequences in these genes across animals (Zakon 2012). By disrupting VGSCs, TTX causes paralysis or respiratory failure; at low doses it slows muscle reaction and diminishes sensory input (Soong and Venkatesh 2006). However, AA substitutions in VGSCs at the TTX binding site prevent TTX from binding; hence, TTX resistance can be traced to specific AA substitutions in Nav1 genes. Alternatively, TTX resistance could potentially be conferred by compartmentalization or metabolic inactivation of TTX (Saporito et al. 2012). However, there is overwhelming support that high levels of TTX resistance is conferred by nonsynonymous (i.e., AA changing) substitutions in Nav1 genes.

VGSCs have tissue-specific expression and TTX can cross cell membranes, so whole-body resistance to TTX likely requires AA substitutions in all Nav1 paralogs. In pufferfish, TTX resistance has independently evolved in four species (*Takifugu rubripes*, *Tetraodon nigroviridis*, *Canthigaster solandri*, *Arothron nigropunctatus*) via convergent AA substitutions in all eight Nav1 paralogs (Jost et al. 2008). However, in *Thamnophis sirtalis*, a snake that consumes TTX-defended newts (*Taricha*), Nav1 genes expressed in the peripheral nervous system have TTX-resistant AA substitutions, but those expressed in the central nervous system do not, suggesting that selection for whole-body TTX resistance is less in garter snakes than in pufferfish (McGlothlin et al. 2014). TTX-defended newts (*Cynops pyrrhogaster*, *Taricha granulosa*) and other species of snakes (*T. couchii*, *T. sierrae*, *T. atratus*, *Liophis epinephelus*, *Amphiesma pryeri*, *Rhabdophis tigrinus*) that consume TTX-defended amphibians (*Taricha*, *Cynops*, *Polypedates*, *Atelopus*) have convergent AA substitutions in the skeletal muscle VGSC Nav1.4 (Kaneko et al. 1997; Hanifin et al. 1999; Feldman et al. 2009, 2012). However, other Nav1 paralogs in these species have not yet been sequenced, so patterns of whole-body resistance cannot be assessed across taxa.

Other defensive alkaloids and steroids affect ion channels and transporters in a similar way as TTX and resistance can also be traced via nonsynonymous AA substitutions in target proteins. Many organisms are exposed to low levels of these compounds, so pre-existing resistance may sometimes play a role in the evolution of chemical defense. For example, in milkweed butterflies (Nymphalidae, Danaini) and leaf beetles (Chrysomelidae, *Chrysochus*), some resistance to cardenolides via one AA substitution in the sodium-potassium pump evolved prior to diet specialization on and sequestration from cardenolide-rich plants (Dobler 2001; Dobler et al. 2011; Aardema et al. 2012). In diet specialized and toxic lineages, three additional substitutions conferring greater levels of resistance are found (Petschenka et al. 2013a). Similarly, in salamanders, one TTX-resistance-conferring AA substitution in the Nav1.4 pore evolved prior to the origin of chemical defense in this clade, followed by the accumulation of four other AA substitutions that provide higher levels of resistance in the toxic lineages (Hanifin and Gilly 2015). Pre-existing resistance may have also contributed to the multiple recurrent origins of TTX-defended newt consumption by garter snakes and of bufadienolide-defended toads by natricine snakes (Savitzky et al. 2012; McGlothlin et al. 2014). Resistance to plant secondary compounds in insects other than monarchs and beetles has arisen many times, but whether or not some of this resistance evolved prior to chemical defense remains largely uninvestigated (Dobler et al. 2011).

21.4.2 Evolution of Alkaloid Resistance in Dendrobatidae

Chemically defended poison frogs sequester alkaloids from arthropod prey, deposit them in granular dermal glands, and release them as defense (Neuwirth et al. 1979; Daly et al. 1994c; Saporito et al. 2009). These substances are generally unpalatable; some are toxic and highly effective deterrents against diverse predators and

parasites (Tables 21.1, 21.2 and 21.3). Like TTX, dendrobatid alkaloids affect the function of ion channels and transporters causing neuromuscular disruption in sensitive organisms (Karalliedde 1995; Daly et al. 2003; Vandendriessche et al. 2008; Petschenka et al. 2013a). Although resistance could be conferred by metabolic inactivation (see Petschenka et al. 2013b), most alkaloids are taken up and released without any sort of modification (Daly et al. 2003), suggesting the absence of such a detoxification process. Hence, poison frog autoresistance should be conferred by key AA substitutions in target proteins like in other chemically defended systems (Wang et al. 2006; Jost et al. 2008; Dobler et al. 2011; Feldman et al. 2012; Zakon 2012; Petschenka et al. 2013a). We propose that this is the major mechanism of autoresistance in dendrobatids.

Dendrobatids have an outstanding diversity of alkaloids (Table 21.1; ~520 types), with substantial variation across species, populations, and individuals (Clark et al. 2005; Daly et al. 2005; Darst and Cummings 2006; Saporito et al. 2007a). The sheer variation of alkaloid profiles among species is perhaps the most predictable aspect of poison frog defense. This makes dendrobatid clades difficult to categorize by their alkaloid profiles (Fig. 21.1). The only two types of alkaloids that appear to have a synapomorphic (unique to a single clade) distribution are batrachotoxin (BTX) in *Phyllobates* and epibatidines in *Epipedobates* (*Saporito* et al. 2009). However, the large diversity of alkaloid types within a single species of dendrobatids presents a unique problem for the evolution of autoresistance. This particularity of dendrobatid chemical ecology clearly contrasts with other chemical defense systems that predominately involve one toxin that targets only one type of ion channel.

We propose that because most, if not all, dendrobatids consume some amount of alkaloid-rich prey (Table 21.1: ants and mites) there must exist some common alkaloid resistance that evolved prior to and facilitated the four origins of chemical defense in the group (Fig. 21.1). The significant correlation between diet specialization on alkaloid-bearing arthropods and the evolution of chemical defense in dendrobatids highlights the evolutionary ties between alkaloid exposure, sequestration, and presumed resistance (Darst et al. 2005). Increasing exposure to alkaloids via diet specialization likely resulted in selection for increased resistance, closely timed with the evolution of chemical sequestration and defense (Saporito et al. 2012; Savitzky et al. 2012). From these patterns, two major questions arise: (1) how have dendrobatids responded to selection for diverse alkaloid resistance; and (2) what role has alkaloid resistance played in the evolution of dendrobatids?

21.4.3 How Have Dendrobatids Responded to Selection for Diverse Alkaloid Resistance?

Despite numerous studies of TTX resistance in multiple organisms, there is almost no information regarding autoresistance in poison frogs, perhaps because of its inherent complexity. Dendrobatid alkaloids target at least five families of ion channel genes (>30 genes) and bind to different regions of the channels, diversely affecting their function (Table 21.2). Moreover, many ion channel proteins are composed of

multiple subunits, so one ion channel complex may be encoded by more than four distinct genes or isoforms (Hille 2001). Most dendrobatid alkaloids are lipophilic, meaning that they cross cell membranes and may permeate the blood–brain barrier. Hence, all expressed channels targeted by poison frog alkaloids should be subject to selection for resistance, even if their expression is limited to specific tissues. However, there may be variation in the level of selection on genes with tissue-specific expression correlated with alkaloid concentrations of each tissue, as is seen in garter snakes (McGlothlin et al. 2014). Moreover, the role of ion channel isoforms in autoresistance across alkaloid-defended taxa is virtually unstudied. Such attributes make in vitro studies of ion channel/receptor proteins challenging and imply that the genetic basis of autoresistance in Dendrobatidae is rather complex.

Most studies of dendrobatid alkaloids were performed in the 1970–1980s using mice or frog nerve and muscle preps (Daly and Spande 1986; Daly et al. 1999). These assays elucidate physiological effects of alkaloids but do not always reveal specific target channels and binding sites; for these, in vitro expression assays are required (e.g., Vandendriessche et al. 2008). Experiments of alkaloid resistance using dendrobatid tissues are limited to *Dendrobates* (*Oophaga*) *histrionicus*, *Phyllobates terribilis*, and *P. aurotaenia* (Albuquerque et al. 1973, 1974; Daly et al. 1980). These assays demonstrated that *Phyllobates* frogs are physiologically resistant to batrachotoxin (BTX) while *Rana pipiens* (not defended) and *D. histrionicus* (alkaloid defended but not by BTX) were not. For HTX toxicity, *D. histrionicus* were more resistant than *R. pipiens*, although at high concentrations, *D. histrionicus* were not completely resistant; *Phyllobates* were not tested. These authors also showed through a breeding experiment that the basis for alkaloid resistance in *P. terribilis* is genetic. We now know that BTX interacts with the inner pore of voltage-gated sodium channels and that BTX resistance is conferred by amino acid substitutions in these regions (Wang and Wang 1998, 2003; Wang et al. 2006). However, no published studies have investigated the genetics of alkaloid resistance in dendrobatids, although data suggest that various species of *Phyllobates*, *Dendrobates*, *Epipedobates*, and *Ameerega* have multiple AA substitutions in the inner pore of Nav1.4, of which at least two appear to be unique to *Phyllobates* and may provide BTX resistance (Frezza et al. 2010; Marquez and Amezquita 2014; Tarvin et al. 2014). No other information of sensitivity to BTX, PTX, or other compounds is available. Much more is to be learned from studying these channels across dendrobatids.

21.4.4 What Role Has Alkaloid Resistance Played in the Evolution of Dendrobatids?

Evolutionary patterns of poison frog alkaloid resistance likely influenced the evolutionary trajectories of alkaloid defense in dendrobatids. If some resistance is ancestral to Dendrobatidae (Darst et al. 2005; Savitzky et al. 2012), this exaptation (pre-adaptation) may have facilitated early stages of alkaloid sequestration and dietary specialization by diminishing the negative effects of increased alkaloid exposure. Therefore, phylogenetic patterns of autoresistance in ion channel gene

families across the poison frog phylogeny should correlate with alkaloid profiles and toxicity of each lineage, reflecting their co-evolutionary history.

Alkaloid profiles are dependent on available arthropods, by the ability to sequester alkaloids, and variation in sequestration rates of the different compounds across species (Daly et al. 1994c; Clark et al. 2005; Saporito et al. 2007a, 2009). Similar alkaloid profiles in other defended anuran groups (i.e., Mantellidae: *Mantella*, Myobatrachidae: *Pseudophryne*, Bufonidae: *Melanophryniscus*, Eleutherodactylidae: *Eleutherodactylus*) suggest a common metabolic pattern of chemical sequestration (Daly et al. 1987; Smith et al. 2002; Clark et al. 2005; Rodriguez et al. 2011). Sequestration of the same classes of alkaloids is likely also evidence of either their broad dietary availability and/or a selective advantage in sequestering these particular compounds. For example, pumiliotoxins (PTX) are found in all five groups (Daly et al. 1999). One of these toxins, PTX 251D, modulates both voltage-gated sodium and potassium channels and is known to deter mosquitoes and ants (Table 21.2). Perhaps this compound is particularly effective in defense; however, resistance to PTX 251D would require genetic changes in more genes than is required for resistance to BTX or TTX. Alternatively, resistance to PTX 251D may require AA substitutions in less-conserved regions of ion channels that are subject to lower levels of purifying selection.

If alkaloid resistance affects alkaloid profiles, selection for resistance to compounds like PTX 251D with diverse physiological effects may promote diversification in alkaloid defense. The most toxic alkaloid found in dendrobatids, BTX, occurs in *Phyllobates* at the level of oldest origin of chemical defense (Dendrobatinae: *Phyllobates* + *Dendrobates*; Fig. 21.1). *Dendrobates* species presented with BTX-dusted fruit flies reject them as prey and are physiologically sensitive to BTX, suggesting that *Dendrobates* may either be incapable of sequestering BTX or not resistant enough to do so (Daly et al. 1980, 1999). It may be possible that the accumulation of AA substitutions providing resistance to various alkaloids in the *Dendrobates* sensu lato clade facilitated the evolution of resistance to and sequestration of BTX in *Phyllobates*. However, more rigorous experiments of alkaloid resistance, availability, and sequestration are needed to clarify what determines the alkaloid profile of a species and why certain alkaloids are more common than others. Further analyses of alkaloid diversity may reveal unexpected patterns in alkaloid resistance and vice versa.

21.5 Future Directions

Many questions remain regarding the chemical ecology of poison frogs. How are alkaloids transported from the digestive system to the skin? What physiological changes during metamorphosis facilitate the sequestration of alkaloids from diet? What are the relative roles of resistance and dietary specialization in the evolution of alkaloid sequestration and defense? Do defended diet generalists have different patterns of resistance than defended diet specialists, owing to their exposure to a broader array of alkaloids? How does dietary conservatism of predators (Thomas

et al. 2003; Marples et al. 2005; Darst and Cummings 2006; Lee et al. 2010) affect these patterns? If AA substitutions are not found in all alkaloid-affected proteins, how is the expression of both sensitive and resistant forms modulated and what effect does this have on sequestration, defense, and resistance? Moreover, how does the evolution of autoresistance and sequestration in poison frogs compare to that of frogs that synthesize their own toxins (e.g., *Pseudophryne*; see Smith et al. 2002)? New next-generation sequencing methods will facilitate the genetic aspects of these studies (Li et al. 2013).

The large volume of pharmacological research on poison frog alkaloids highlights its significance. Some of these compounds have extremely promising applications in development of novel anesthetics (e.g., epibatidine) and basic research on neuromuscular functions (e.g., pumiliotoxins, batrachotoxins, and izidines) (Daly 2005). The work of John Daly and colleagues (Savitzky and Saporito 2012) has resulted in a prolific field of artificial synthesis of dendrobatid alkaloids and design of therapeutic agents based on their structures (Toyooka and Nemoto 2002). However, detailed in vitro assays of their function are scarce except for a few compounds (BTX, PTX 251D, epibatidine) and progress in studying these compounds in general has slowed under stricter legal regulations in response to concerns regarding bioprospecting for commercial drugs (Angerer 2011). Nevertheless, it is surprising that the synthesis of these compounds has not ameliorated some of these problems. New emphasis should be given to collaborative efforts between native and international researchers to further the exploration and causal bases of dendrobatid chemical defenses. Our labs are working towards uncovering the basis of resistance and sequestration across Dendrobatidae; in conjunction with a more complete survey of alkaloid profiles across dendrobatids and more in-depth physiological studies of these alkaloids, we should soon be able to answer many questions regarding dendrobatid evolution.

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