ANNUAL REVIEWS

Annual Review of Ecology, Evolution, and Systematics

The Diverse Mechanisms that Animals Use to Resist Toxins

Rebecca D. Tarvin, Kannon C. Pearson, Tyler E. Douglas, Valeria Ramírez-Castañeda, and María José Navarrete

Museum of Vertebrate Zoology and Department of Integrative Biology, University of California, Berkeley, California, USA; email: rdtarvin@berkeley.edu, kannonpearson@berkeley.edu

Annu. Rev. Ecol. Evol. Syst. 2023. 54:283–306

First published as a Review in Advance on August 8, 2023

The *Annual Review of Ecology, Evolution, and Systematics* is online at ecolsys.annualreviews.org

https://doi.org/10.1146/annurev-ecolsys-102320-102117

Keywords

toxin resistance, tetrodotoxin, batrachotoxin, cardiotonic steroids, pyrrolizidine alkaloids, chemical defense

Abstract

Biological toxins are entrenched within ecosystems. Thus, animals are often exposed to such toxins, and how they adapt can be a key determinant of their evolutionary trajectories. In this review, we provide an overview of the diversity of toxin resistance mechanisms, with a focus on animals that sequester toxins from their diet and their natural predators and parasites. We propose a structured framework in which to study toxin resistance by recategorizing and reorganizing known mechanisms into avoidance, metabolism, and target categories. Then, using this framework, we review evidence regarding how animals resist four widely studied compounds: tetrodotoxin, batrachotoxin, cardiotonic steroids, and pyrrolizidine alkaloids. Based on the available data, we conclude that toxin resistance and sequestration are interrelated from both ecological and evolutionary perspectives. To conclude, we highlight open questions regarding toxin resistance and review its importance as a field.

En los ecosistemas las toxinas de origen biológico son componentes intrínsecos. Por esta razón, los animales se ven expuestos frecuenciamente a dichas toxinas y la forma en que se adaptan puede ser un factor que determina su trayectoria evolutiva. Esta revisión ofrece una visión general de la diversidad de mecanismos de resistencia a toxinas, centrándose en animales que secuestran toxinas de su dieta y en sus depredadores y parásitos naturales. En este texto se propone un marco estructural para estudiar la resistencia a toxinas mediante la recategorización y reorganización de mecanismos conocidos

en categorías de: evación, metabolismo y moléculas diana. A continuación, utilizando este marco, revisamos la literatura científica en busca de evidencia sobre cómo los animales resisten a cuatro compuestos ampliamente estudiados: tetrodotoxina, batracotoxina, esteroides cardiotónicos y alcaloides de pirrolizidina. A partir de los datos disponibles, llegamos a la conclusión de que la resistencia y la retención de toxinas están interrelacionadas tanto desde el punto de vista ecológico como evolutivo. Por último, destacamos algunas preguntas abiertas en torno a la resistencia a las toxinas y resaltamos su importancia como campo de estudio en el futuro.

INTRODUCTION

From the depths of the ocean, where bacteria lace the sediments with neurotoxins, to the skies, where migrating butterflies transport cardiotoxins between continents, natural toxins are universal features of ecosystems. All animals must navigate this world of biological toxins, and toxin resistance is a pivotal trait that can propel animals down one of two evolutionary trajectories. In the first, animals may evolve toxin avoidance, a resistance mechanism that prevents or limits toxin exposure (Després et al. 2007). In the second, animals may evolve to survive toxin exposure through resistance mechanisms that prevent or lessen a toxin's adverse effects. For animals in this second trajectory, subsequent toxin exposure may select for additional adaptations over time, including toxin sequestration or attraction to toxins. Whether organisms evolve to avoid or cope with toxins in turn shapes interspecific relationships and results in emergent, community-level changes (Agrawal et al. 2012, Boppré & Schneider 1989). Based on an appreciation of the multidimensional impacts of small quantities of neurotoxins, Zimmer & Ferrer (2007) proposed that toxins produced by organisms can act as keystone molecules.

Biological toxins have also influenced the development of evolutionary theory. Early natural historians sought to understand why animals had warning colors, undertaking extensive tests that showed brightly colored animals were often unpalatable (Carpenter 1942, Poulton 1887, Windecker 1939). Toxins played a central role in the development of coevolution as a field, with early studies by Berenbaum (1983) and Ehrlich & Raven (1964) demonstrating that toxins were a centerpiece in the escape-and-radiate dynamics between plants and herbivores. Simultaneously, the recurrent origins of pesticide resistance prompted researchers to identify the genetic and metabolic basis of resistance to toxin-derived pesticides (Georghiou 1972, Milani 1963). Toxins have also provided insight into the drivers of major life history trade-offs in plants and animals (Agrawal 2011, Hague et al. 2018, Singer 2008). In addition, preserving human and ecosystem health has depended on understanding how animals are affected by toxin exposure (Ames 1966). The relationship between animals and toxins continues to generate powerful questions and biological insight, especially with new tools in molecular biology [e.g., high-throughput electrophysiology, CRISPR-Cas9 gene editing, and spatial mass spectrometry (Dreisbach et al. 2023, Karageorgi et al. 2019, Zdenek et al. 2019)]. Yet, much remains to be described. Given the pivotal roles of toxin resistance and sequestration in constructing ecological relationships, it becomes evident that toxins influence the evolutionary trajectory of complete communities. Therefore, gaining a better understanding of toxin resistance will provide new insight into how key molecules generate biodiversity. Furthermore, understanding how animals deal with toxins helps us predict and examine the impact of introduced molecules resulting from environmental pollution, species range shifts, or habitat alteration.

In this review, we organize and outline known toxin resistance mechanisms in animals. We then review the resistance data available for animals that interact with tetrodotoxin (TTX), batrachotoxin (BTX), cardiotonic steroids (CTs), and pyrrolizidine alkaloids (PAs), demonstrating

toxin- and taxa-specific patterns in resistance mechanisms. With this data set, we evaluate hypotheses about the role of toxin resistance in toxin sequestration. Lastly, we curate a selection of open research questions in the field of toxin resistance. Although we focus on animals that are exposed to biologically produced, small-molecule toxins through their diet or by endogenous synthesis, our framework could be applied more broadly (e.g., to venom peptides or heavy metal toxins). We note that the terms tolerance and resistance are sometimes used interchangeably to describe strategies employed by animals to withstand toxin exposure. However, in plants the terms imply different strategies: Tolerance describes plants that withstand the cost of herbivory, while resistance refers to plants that deter herbivores (Mitchell et al. 2016). We favor the use of resistance rather than tolerance to describe strategies employed by animals to avoid intoxication.

MECHANISMS OF TOXIN RESISTANCE

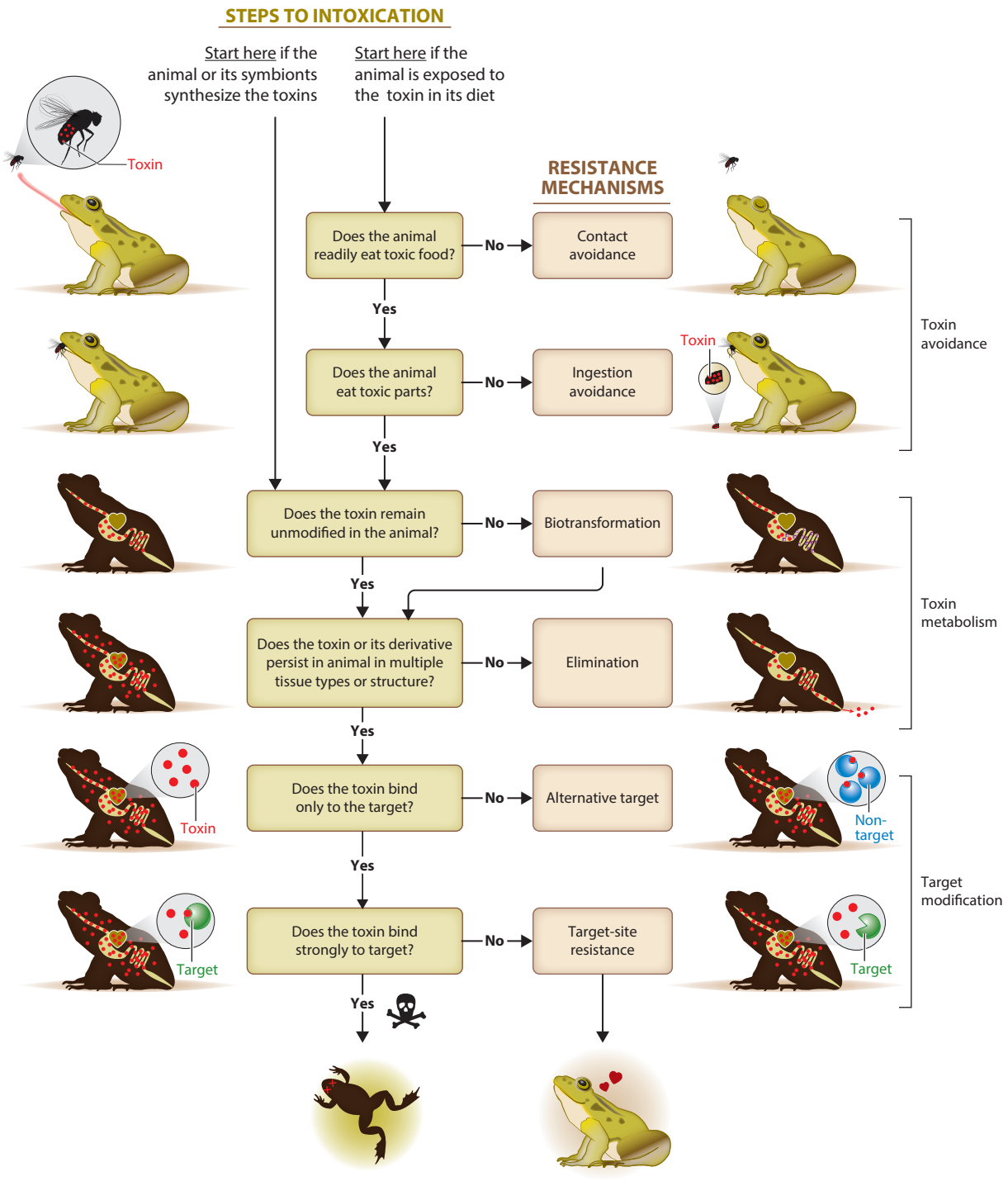
Framework

The molecular basis of toxin resistance, such as resistance-conferring substitutions in proteins targeted by toxins, is remarkably convergent across taxa and toxins. This convergence may be the result of trade-offs between molecular function and toxin-binding affinity (van Thiel et al. 2022). However, convergence in toxin resistance is also evident at the level of the organism: Animals consistently eschew, metabolize, and inhibit toxins. At this level, convergence may arise from the relatively standard path that toxins take to reach their targets, first entering the body, then moving through the body toward sensitive tissues, before finally reaching and interacting with the targets. Resistance-conferring mechanisms can occur at each point in this path. We present a structured framework (**Figure 1**) based on this convergent physiological trajectory to help shape future research on toxin resistance and sequestration.

To standardize the terms used to describe toxin resistance, we organize known mechanisms into three categories that reflect the convergence of resistance phenotypes: toxin avoidance, toxin metabolism, and target modification (**Table 1**, **Figure 1**). Within each category, we organize toxin resistance mechanisms into two types, where Type A generally results in lower risk of intoxication than Type B. The proposed types and categories are not mutually exclusive, and some animals may have both absence and presence data for each. Throughout the article we use abbreviated names for each type as described in **Figure 1** and **Table 1** (alternative target, elimination, etc.). However, we recognize that not all mechanisms intuitively match their abbreviated name, and future research may lead to the discovery of new mechanisms that better fit the Type A or Type B labels rather than the terminology we use here.

Avoidance

The first line of defense against intoxication is behavioral avoidance of toxin exposure (**Figure 1**). Avoidance can be innate, reactive, or learned (Lunceford & Kubanek 2015) and can also be energy-state dependent (Barnett et al. 2007). This category includes contact avoidance (Type A), the innate or learned aversion of a toxic food source, and ingestion avoidance (Type B), the selective consumption of nontoxic components of a toxic food source. Contact avoidance allows animals to completely avoid toxin exposure if they can recognize its source and may thus select for changes in sensory systems (Lunceford & Kubanek 2015). In contrast, ingestion avoidance may expose animals to small amounts of toxin and selects for changes in food handling behavior (Hurlbert 1970). Avoidance behavior (or lack thereof) does not necessarily depend on the true lethal risk of the compound to an animal (Glendinning 1994); some toxins, such as many of those found



(Caption appears on following page)

Figure 1 (*Figure appears on preceding page*)

A framework for studying toxin resistance in animals based on the general steps toward intoxication: toxin entering the body, toxin moving toward sensitive tissues, and toxin interacting with target proteins or processes. For toxins that are consumed, animals may first prevent toxins from entering their body through contact or ingestion avoidance (toxin avoidance). If animals cannot prevent toxins from entering the body, they may then modify the toxin to be less toxic or eliminate the toxins quickly to limit intoxication (toxin metabolism). If animals cannot avoid or metabolize the toxin, they may limit intoxication by evolving a novel target or modifying the existing target to prevent the toxin from adversely affecting organismal function (target modification). If animals cannot avoid the toxin, metabolize the toxin, or modify the target of the toxin, they may die from intoxication. Note that animals can have more than one mechanism of resistance. See **Table 1** for definitions.

in poison frogs, are bitter or repellant rather than lethal (Santos et al. 2016). Evidence for this category often is derived from food choice experiments and observations of food handling and consumption (**Table 1**).

Metabolism

Once exposed, an animal can limit the negative effects of a toxin through metabolism (**Figure 1**). Toxin metabolism in animals involves three phases, generally described as transformation, conjugation, and excretion. We refer to changes in transformation and conjugation as biotransformation (Type A); changes in excretion are referred to as elimination (Type B). Biotransformation often involves overexpression, neo- or subfunctionalization, and/or copy number variation of genes encoding detoxification enzymes (Heidel-Fischer & Vogel 2015, Li et al. 2007). Other examples of biotransformation include hosting microbial symbionts that break down toxins or upregulating the genes encoding proteases to break down venoms [acquired immunity (Holding et al. 2016)]. While we focus on biotransformation as a resistance mechanism, it is worth noting that species may biotransform toxins for additional reasons [e.g., pheromones, increasing toxicity of defensive toxins (Boppré & Schneider 1989)]. In contrast, elimination involves changes in toxin diffusion and excretion by altering transcellular or paracellular transport (Li et al. 2007). Altering existing elimination mechanisms may also result in tissue-specific toxin accumulation [i.e., sequestration (Strauss et al. 2013)]. Evidence for metabolism mechanisms often comes from experiments comparing the effect of toxin consumption or injection on gene expression and measuring toxin quantities in different tissues, including excrement (**Table 1**).

Target

If a toxin persists in an animal, intoxication can be avoided by altering the toxin's ability to bind its target (**Figure 1**). Alternative target (Target Type A) describes toxin-binding proteins that reduce the amount of free toxin in the organism and thus limit toxin–target interactions (Yotsu-Yamashita et al. 2001). While elimination (metabolism Type B) mechanisms may also involve proteins that bind toxins, we differentiate the two in that elimination directionally transports toxins across cell membranes, but alternative targets do not. In one relatively unique alternative target mechanism found in the grasshopper mouse, referred to as off-target repurposing, a novel toxin-binding site competes with the primary target for the toxin, limiting the amount of toxin that binds its primary target. Once bound to the novel site, the toxin provides a pain-killing effect by repurposing the toxin's ability to block nerve signals (Rowe et al. 2013). Target-site resistance (TSR; Target Type B) includes amino acid substitutions in the toxin target that decrease toxin-binding affinity. Type B target mechanisms are also commonly referred to as target-site insensitivity, though they do not always provide complete insensitivity. Evidence for target mechanisms often comes from ligand-binding assays, electrophysiology experiments, and sequencing (**Table 1**).

Table 1 Overview of toxin resistance mechanisms, some potential costs and modulators, relevant methods, and synonymous terms

Category	Mechanisms	Example phenomena	Potential cost	Ecological and evolutionary hypotheses	Example study methods	Synonymous terms
Avoidance	**Type A: contact avoidance** behavioral avoidance or learned aversion upon recognition of a toxin source	Cattle (*Bos taurus*) learned to avoid feed pellets when associated with larkspur extract (Olsen & Ralphs 1986). The secretary bird (*Sagittarius serpentarius*) uses attack strategies that limit exposure to snake venom (van Thiel et al. 2022). The silver-sided sector spider (*Zygiella x-notata*) demonstrates learned avoidance of toxic aphid prey (Malcolm 1986, 1989).	Increased time spent foraging	Modulated by ability to detect the toxin or its source May select for niche partitioning or changes in sensory systems	Behavioral trials presenting organism with toxic prey or another food infused with toxin Behavioral trials after blocking sensory receptors Observations in the wild	Contact avoidance (Després et al. 2007) Behavioral resistance (FAO 2012)
	Type B: ingestion avoidance selective consumption of nontoxic components and/or physical manipulation of a toxin source to avoid exposure	The turtle *Chrysemys picta* tends to consume only the belly and viscera of the tetrodotoxin-defended newt *Notophthalmus viridescens*, avoiding the toxic dorsal skin (Hurlbert 1970). The snake *Oligodon fasciolatus* disembowels *Duttaphrynus melanostictus* toads and consumes the gut, avoiding bufadienolide-containing toxin glands on the dorsum (Bringsøe & Holden 2021). Monarch butterfly caterpillars (*Danaus plexippus*) cut leaf veins to divert cardenolide-rich latex away from where they are feeding (Helmus & Dussourd 2005). Floodplain death adders (*Acanthophis praelongus*) envenomate peptide-defended frogs and delay consumption, allowing the peptides to degrade first (Phillips & Shine 2007).	Fewer resources obtained per prey item	Modulated by prior experience consuming the toxic resource May select for changes in food-handling behavior	Behavioral trials presenting organism with toxic prey Observations in the wild	Ingestion avoidance (Després et al. 2007) Behavioral resistance (FAO 2012)

(*Continued*)

Table 1 (*Continued*)

Category	Mechanisms	Example phenomena	Potential cost	Ecological and evolutionary hypotheses	Example study methods	Synonymous terms
Metabolism	**Type A: biotransformation** changes in Phase I and II metabolic processes resulting in enhanced, decreased (in the case of protoxins), or novel xenobiotic enzyme activity from the animal or its microbiota that alters toxin transformation and/or conjugation	Duplication of *Cyp28d1* and *Cyp28d2* genes encoding xenobiotic enzymes increase nicotine resistance in *Drosophila melanogaster* (Chakraborty et al. 2019). The leaf beetle *Oreina cacaliae* prevents reduction (activation) of senecionine by suppressing gut enzyme activity (Hartmann et al. 1999). The coffee berry borer (*Hypothenemus hampei*) harbors *Pseudomonas* bacteria that degrade caffeine and prevent the beetle from experiencing intoxication (Ceja-Navarro et al. 2015). The large milkweed bug (*Oncopeltus fasciatus*) biotransforms the polar cardenolide ouabain in the Malpighian tubules and the nonpolar cardenolide digitoxin in both the Malpighian tubules and midgut (Scudder et al. 1986).	Energetic cost in producing enzymes or harboring specific bacteria	Modulated by the existing metabolic detoxification strategies of an organism relevant to the specific toxin May select for more frequent ingestion of toxic prey, and/or additional changes in metabolic and target resistance mechanisms	Feeding assays paired with differential gene expression and toxin quantification Tracer experiments with isotopes or radioactive atoms to determine biotransformation into specific compounds Incubation of tissue or gut extracts with toxins followed by identification of metabolites	Degradation of the toxin (Després et al. 2007) Detoxification by ubiquitous enzymes, horizontal gene transfer (of enzymes) (Heidel-Fischer & Vogel 2015) Acquired immunity (Holding et al. 2016) Metabolic detoxification (Feyereisen 1995, FAO 2012) Tolerance (Wilson 2001) Metabolic resistance (FAO 2012, Labbé et al. 2011)
	Type B: elimination changes in Phase III metabolic processes resulting in the alteration of toxin transport that prevents the toxin from entering an at-risk tissue, uptake of the toxin into a low-risk tissue, or rapid excretion of the toxin	The nervous tissues of two species of hawkmoth (*Daphnis nerii* and *Manduca sexta*) are impermeable to the polar cardenolide ouabain (Petschenka et al. 2013). A diffusion barrier in the gut of mantids (Mantodea) prevents intoxication when tetrodotoxin is ingested (Mebs et al. 2016). Pyrrolizidine alkaloids are quickly taken up by midgut cells in *Oreina* spp. beetles, avoiding activation by gut enzymes (Narberhaus et al. 2004). *Poekilocerus pictus* grasshoppers excrete large quantities of cardenolides from the salivary gland while actively consuming cardenolide-rich plant material (Pugalenthi & Livingstone 1995).	Elimination changes that cause uptake can result in toxin accumulation, increasing the risk of lethal intoxication	Modulated by the chemical properties of the toxin (e.g., polarity), the diffusion properties of the tissue, and the relative protection from further intoxication if toxin is bioaccumulated May select for additional mechanisms of resistance; if the result is bioaccumulation, the animal may be subject to further selection from parasites and predators, potentially leading to active sequestration	Estimates of tissue-specific toxin concentration Studies of toxin diffusion and elimination kinetics Visualization of toxins using fluorescent labels Comparison of effect on animal when feeding versus injecting toxin to identify presence of gut diffusion barrier	Toxin barriers (Heidel-Fischer & Vogel 2015) Sequestration (Heidel-Fischer & Vogel 2015) Excretion (Després et al. 2007) Penetration (FAO 2012, Labbé et al. 2011, Wilson 2001)

(*Continued*)

Table 1 (*Continued*)

Category	Mechanisms	Example phenomena	Potential cost	Ecological and evolutionary hypotheses	Example study methods	Synonymous terms
Target	**Type A: alternative target** increased expression and/or generation of an alternative target that, when bound by the toxin, produces less-adverse effects than the original target	The Southern grasshopper mouse (*Onychomys torridus*) evolved a novel binding site for bark scorpion (*Centruroides sculpturatus*) venom, which functions as an analgesic (Rowe et al. 2013). Proteins in the blood of California ground squirrels (*Otospermophilus beecheyi*) bind to Northern Pacific rattlesnake (*Crotalus oreganus*) venom proteins with high affinity and broad specificity (Gibbs et al. 2020). Saxiphilin binds saxitoxin in frogs, protecting voltage-gated sodium channels (Abderemane-Ali et al. 2021, Morabito 1994). Pufferfish (*Fugu pardalis*) have tetrodotoxin-binding proteins (Yotsu-Yamashita et al. 2001).	Energetic costs in producing binding proteins	Modulated by changes in gene expression, gene duplication, and subfunctionalization May select for more frequent ingestion of toxic prey	Profiling of proteins extracted from tissues and mixed with toxin to determine differences in thermal stability Mixing of fluorophore- or biotin-labeled toxins with plasma or homogenized tissue Mixing of protein extract with toxin followed by injection into another animal (bioassay) Recombinant protein expression and toxin binding assays Search for genes encoding known binding proteins in the genome Computational modeling and molecular docking assays	Altered targets, repurposed toxins (Holding et al. 2016) Off-target repurposing, toxin scavenging (Arbuckle et al. 2017, van Thiel et al. 2022) Venom inhibitors (Holding et al. 2016) Sequestration (Feyereisen 1995, FAO 2012, Labbé et al. 2011)
	Type B: target-site resistance substitutions, duplications, or conformational change in the target of the toxin	Poison frogs (Anura: Dendrobatidae) have amino acid substitutions in a nicotinic acetylcholine receptor that provide resistance to the neurotoxin epibatidine (Tarvin et al. 2017). Newts (Caudata: Salamandridae) have amino acid substitutions in voltage-gated sodium channels that provide resistance to tetrodotoxin (Hanifin & Gilly 2015). The large milkweed bug (*Oncopeltus fasciatus*) has three copies of an Na^+/K^+-ATPase gene, one of which is highly sensitive to cardenolides and expressed in nervous tissue and the others of which are expressed in gut tissue and are involved in resistance (Lohr et al. 2017). Monarch butterflies (*Danaus plexippus*) possess three Na^+/K^+-ATPase substitutions, each of which provides resistance to sequestered cardenolides and one of which epistatically reduces the fitness costs of the others (Karageorgi et al. 2019).	Change in protein function or efficiency	Modulated by compensatory substitutions, changes in gene expression, or gene duplication and subfunctionalization May select for further ingestion of toxic prey and bioaccumulation for antipredatory or antiparasitic purposes	Sequencing of target proteins Electrophysiology comparing the target function in the presence and absence of toxin Computational models predicting the effect of mutating the target on the ability of the toxin to bind	Target-site mutation (Després et al. 2007) Target-site insensitivity (Arbuckle et al. 2017, Feyereisen 1995) Target-site resistance (Heidel-Fischer & Vogel 2015, Wilson 2001) Target modification (van Thiel et al. 2022) Target-site modification (Labbé et al. 2011) Reduced sensitivity at the target site (FAO 2012)

Costs of Toxin Resistance Mechanisms May Spur Additional Adaptation

Toxin resistance mechanisms are thought to come with some cost, so animals that evolve resistance may experience selection for additional adaptations (**Table 1**). For example, avoidance mechanisms can reduce foraging efficiency and may select for changes in sensory systems that help an animal learn to quickly recognize or deal with a toxic food source; in turn, animals that lack avoidance mechanisms may lose the ability to taste aversive compounds (Imai et al. 2012). Other resistance mechanisms such as biotransformation and alternative targets require increased protein production and are predicted to incur an energetic cost (Després et al. 2007), though this has not been widely substantiated (Reid & Ahn 2020). Some elimination mechanisms result in tissue-specific accumulation, which puts the organism at further risk of poisoning if they do not possess additional mechanisms of resistance such as TSR (Petschenka & Agrawal 2015). In turn, TSR often comes at a cost in protein function, as it selects for compensatory substitutions in the protein target (Tarvin et al. 2017), gene or exon duplication (Mohammadi et al. 2021), or other physiological compensation. Importantly, the costs and benefits of evolving toxin resistance must be evaluated in the ecological context of each species (and population) because competitors, predators, and parasites can also exert strong selective pressures on toxin ingestion (Bernays & Graham 1988).

ANIMALS EMPLOY DIVERSE MECHANISMS TO RESIST TOXINS

Supplemental Material >

We conducted a literature review of resistance mechanisms using Google Scholar with each toxin and species name listed in **Supplemental Table 2** as search terms and backward chaining of citations in older literature that is less available in digital databases. Specifically, we reviewed the evidence supporting the presence or absence of resistance mechanisms in organisms reported to have ecologically relevant interactions with and/or possess resistance mechanisms to four types of toxins: TTX and BTX, which are neurotoxic alkaloids that interfere with the function of voltage-gated sodium (Na_V) channels; CTs, which inhibit the sodium–potassium pump; and PAs, which often occur as protoxins that are bioactivated by detoxifying enzymes into alkylating agents that cross-link DNA (Rosenkranz & Wink 2007, Stevens et al. 2011). In addition, we reviewed evidence of sequestration (tissue-specific accumulation), offspring provisioning, the proximate source of the toxin (e.g., synthesized or dietary), and toxin attraction. Species were included in the database if they possessed information for any reviewed category, except for species that had data only for toxin source. A description of the evidence used to categorize data is provided in **Supplemental Table 1**. **Supplemental Table 2** contains all references and descriptions of data for each species. **Supplemental Table 3** provides a numerical tabulation of the data used in generating the numbers discussed below and shown in **Supplemental Figures 1–4**.

Patterns in Available Evidence

In total, we obtained evidence of toxin resistance in 1,210 species, 56 of which had information for more than one focal toxin, 333 of which had information for more than one type of mechanism, and 44 of which had information for all three resistance categories (**Supplemental Table 3**). Only a few species are well studied for each toxin. For TTX, this included several species of *Takifugu* spp. pufferfish, some gastropods (*Tanea* spp. and *Oliva* spp.), the snake *Thamnophis sirtalis*, and two species of newts (*Taricha torosa*, *Cynops pyrrhogaster*). For BTX, *Phyllobates bicolor* was the only species with information for all categories, but important information was also available for *Pitohui dichrous*. For cardenolides, twenty species had data for each category. The most intensively studied were the milkweed bug, *Oncopeltus fasciatus*, and the monarch butterfly, *Danaus plexippus*. Less

information was available for bufadienolides (which includes the lucibufagins); three species had data in each category: the beetle *Photuris versicolor*, the toad *Rhinella marina*, and the snake *Rhabdophis tigrinus*. For PAs, only a few species had data for all categories, including several erebid moths (e.g., *Arctia caja* and *Creatonotos transiens*) and the grasshopper *Zonocerus variegatus*. Other well-studied PA-exposed species included *Estigmene acrea*, *Oreina cacaliae*, *Tyria jacobaeae*, and *Utetheisa ornatrix*.

Supplemental Material >

Across toxins, elimination, avoidance, and TSR were the most well-studied mechanisms (**Supplemental Figure 1**). The source of the toxin is more well-known for CTs and PAs than TTX and BTX. We found relatively more evidence for alternative targets and elimination in animals that interact with TTX, for TSR in animals that interact with CTs, and for avoidance for animals that interact with PAs (**Supplemental Figure 1**). Other categories had similar amounts of evidence across toxins. A large number of species were found to provision offspring with sequestered toxins: 70 for TTX (51% of TTX-sequestering species), 0 for BTX, 20 for CTs, and 26 for PAs. These patterns differed by ability to sequester; for example, contact avoidance in PA-exposed animals was most common in species without sequestration data (**Supplemental Figure 2**). To visualize the phenotypic space occupied by different phyla for each toxin (**Supplemental Figure 3**) (not phylogenetically controlled), we used nonmetric multidimensional scaling (metaMDS function with distance = "bray" and $k = 3$) and calculated convex hulls for each phylum using R version 4.1.2 (Oksanen et al. 2022, R Core Team 2021). A few patterns were apparent: Vertebrates with TTX represented the broadest set of phenotypes, closely followed by arthropods with TTX or cardenolides. The phenotypic space (i.e., the extent of space described by the nonmetric multidimensional scaling axes) occupied by species interacting with TTX versus those interacting with CTs and PAs appeared distinct, likely because of the predominance of biotransformation mechanisms in PA- and CT-exposed animals. In the following sections, we provide more detailed information regarding these patterns (for a full list of references, see **Supplemental Table 2**).

Tetrodotoxin

We identified 291 species that interact with TTX in nature and/or possess TTX resistance mechanisms (93 families in 56 orders and 9 phyla), of which 136 possess TTX in a tissue outside of the digestive tract, suggesting some amount of toxin accumulation or sequestration (**Supplemental Figure 1**). Information regarding resistance to TTX was available for at least 163 of these taxa; many have not been tested directly.

Avoidance. Only 18 species were reported to possess avoidance, while 38 species demonstrated a lack of avoidance. Among the contact avoiders (16 species) were several salmonid fish capable of tasting TTX and a few species of birds including *Falco sparverius* and *Charadrius vociferus*. In addition, juvenile *Taricha torosa* are known to detect TTX and avoid highly toxic adult newts, and *Thamnophis sirtalis* can self-assess to avoid consuming newts that are too toxic for their resistance level. Little information exists for ingestion avoidance, but the crayfish *Procambarus clarkii* avoids eating the skin of adult *Notophthalmus viridescens*, and the turtle *Chrysemys picta* consumes only the belly and viscera of *N. viridescens*, avoiding its toxic dorsal skin. Raccoons (*Procyon lotor*), who readily eat toxic newts, roll them on the ground or in water prior to consuming them. Twenty-two species were attracted to TTX, including several puffer fish (which potentially used TTX as a pheromone) and snails (which likely used TTX to find prey).

Metabolism. In our data set, more species lacked biotransformation or elimination mechanisms (26 and 101, respectively) than possessed them (5 and 16 species, respectively). The only evidence

of biotransformation was modification of TTX or altered gene expression in *Takifugu* spp. pufferfish. Animals that possessed elimination mechanisms demonstrated gut impermeability (e.g., mantids and crayfish) and rapid clearance or selective excretion of TTX (e.g., some pufferfish and mantids). Species that lacked biotransformation mechanisms demonstrated a lack of toxin degradation through long-term retention of unmodified TTX. Similarly, animals lacking elimination demonstrated the presence of TTX throughout the body or slowed clearance (*Limnephilus* spp. caddisflies and *Takifugu rubripes* pufferfish). In a few cases, TTX has been tracked from digestion to the tissues where it is stored (some pufferfish and the ribbon worm *Cephalothrix* cf. *simula*).

Target. Twenty-six species possessed alternative target mechanisms, and 49 possessed TSR. Convincing evidence for the presence of TTX-binding proteins existed in *Arothron* spp. pufferfish, many gastropods, and *Hemigrapsus* spp. shore crabs, with more tenuous evidence for *Taricha* spp. newts and *Carcinoscorpius* spp. horseshoe crabs. Evidence for TSR was observed for 49 taxa, including newts, snakes, pufferfish, gastropods, and ribbon worms. Evidence for a lack of alternative targets was sparse. Lack of TSR was identified in at least three taxa, including *Heterodon* spp. snakes and shore crabs.

Additional comments. Much of the data for marine species come from public health monitoring. Although our focus was on TTX, many of the same species are also exposed to saxitoxin and in some cases are better adapted to resist and/or sequester saxitoxin than TTX (e.g., *Pao suvattii*). We also note that a potential error in the literature has been propagated: Chaetognatha arrow worms may not be TTX defended as previously suggested (Mahon 1999); we exclude these from our analyses.

Batrachotoxin

BTX was found in 34 taxa across 13 families in 6 orders and 2 phyla. Information regarding toxin resistance mechanisms or lack thereof was available for only 15 of these species. Currently, 11 taxa are reported as possessing BTX in a tissue outside of the digestive tract (sequestration) (**Supplemental Table 1**).

Supplemental Material >

Avoidance. We found data on toxin avoidance mechanisms for seven species. For contact avoidance, the snake *Erythrolamprus epinephelus* rejects large *Phyllobates terribilis* frog prey, and the snake *Rhadinaea taeniata* rejects *Phyllobates* spp. of any size, though *R. taeniata* is not a natural predator of *Phyllobates* species. In addition, a few species of chewing lice avoid BTX-containing feathers. The only ingestion avoidance mechanism known for BTX was employed by humans, who rarely eat toxic *Ifrita* spp. and *Pitohui* spp. birds but skin them first if they do. Only two species have been shown to lack avoidance: The snake *Erythrolamprus epinephelus* consumes juvenile *Phyllobates* spp. (the same species avoids large *P. terribilis*), and *Phyllobates bicolor* consumes BTX-laced food.

Metabolism. No mechanism of metabolic resistance is known for BTX. However, several species lacked biotransformation, characterized by long-term retention of the (untransformed) toxin (e.g., in *Phyllobates* spp. frogs). Other species such as some *Pitohui* spp. birds maintain BTX in several tissues, suggesting a lack of diffusion barrier, and poison frogs such as *Phyllobates terribilis* can retain BTX for more than 6 years in captivity, indicating a lack of elimination.

Target. Although no evidence was available regarding alternative target mechanisms for BTX, it has been hypothesized that a BTX-binding protein exists in *Phyllobates* spp. frogs and *Pitohui* spp. birds. For TSR, the evidence is mixed with different studies using different methods supporting or negating TSR in *Phyllobates* spp. and *Pitohui* spp.

Additional comments. BTX remains a poorly studied compound, at least in ecologically relevant scenarios. The colubrid snake *Erythrolamprus epinephelus* was the only known natural predator of BTX-bearing organisms and is thought to possess TSR, although this has not been verified experimentally. Both invertebrates and vertebrates appear to be able to sense and avoid BTX, suggesting that either early symptoms or potentially the taste of BTX (which is bitter to humans) can result in avoidance. Future studies could benefit from further investigating the natural predators of BTX-defended animals as well as its putative beetle source.

Cardiotonic Steroids

Supplemental Material >

We identified a total of 482 species that interact with CTs in nature and/or possess CT resistance mechanisms (from 101 families in 40 orders and 3 phyla), of which 140 are known to sequester or synthesize CTs and 364 have some amount of information on resistance mechanisms (**Supplemental Figure 1**). Importantly, this class of chemicals varies in polarity, and thus, animals often had resistance mechanisms that interact differently with nonpolar and polar compounds. The two main categories of CT are cardenolides and bufadienolides, which possess five- and six-membered lactone rings, respectively. While cardenolides are synthesized by plants and insects, bufadienolides are synthesized by plants, insects, and toads. Perhaps as a consequence of these different sources, most cardenolide data came from arthropods (170 of 241 species), and most bufadienolide data came from vertebrates (210 of 249 species). Synthesizing species store CTs in defensive glands, and we coded these taxa as sequestering for purposes of our analyses.

Avoidance. We found CT avoidance data for 179 species, of which 44 exhibit contact avoidance and 34 exhibit ingestion avoidance. Contact avoidance was taxonomically widespread, found in birds, mammals, reptiles, amphibians, insects, and spiders. Species with ingestion avoidance included at least nine insects that have vein-cutting behavior (e.g., *Labidomera clivicollis*) or other techniques to limit exposure to CT-containing latex from host plants. Other insects (e.g., *Dacus siliqualactis*) selectively lay eggs on host plant tissues that are particularly low in cardenolides. Predators of toads (Anura: Bufonidae) often avoid ingestion of skin and parotoid glands, which are enriched in bufadienolides, by eviscerating them or exclusively consuming their tongues (e.g., *Milvus migrans*). A few predators of sequestering insects similarly tear apart their prey, such as the mantis *Hierodula membranacea*, which avoids consuming the cardenolide-containing midgut. Evidence supporting the absence of contact avoidance was found in 118 species, including butterflies, lygaeid bugs, birds, and toads. For example, *Pheucticus melanocephalus* grosbeaks readily predate cardenolide-sequestering *Danaus* spp. butterflies. Forty-two species lacked ingestion avoidance, of which at least 22 were frogs, snakes, lizards, and crocodiles that swallow prey whole. Nine species were attracted to CTs or CT-containing food sources, including four lygaeid bugs, three colubrid snakes, one grasshopper (*Phymateus leprosus*), and one toad (*Rhinella marina*). All but two of these species (*Macropisthodon rudis* and *Rhinella marina*) sequester CTs from dietary sources. *Rhinella marina* tadpoles synthesize and are attracted to bufadienolides and may use bufadienolides as cues to find and cannibalize conspecific eggs. Interestingly, the stronger the Na^+/K^+-ATPase inhibitory effect of the bufadienolides, the greater their attractiveness to tadpoles, suggesting that Na^+/K^+-ATPase activity and attraction may be linked (Crossland et al. 2021).

Metabolism. We found 28 species with evidence relevant to biotransformation, of which 9 had data suggesting its presence. Among species interacting with cardenolides, the milkweed bug *Oncopeltus fasciatus* biotransforms ouabain and digitoxin, the aphid *Aphis nerii* upregulates genes that encode detoxification enzymes and transporters [including cytochrome P450 family members (*CYP450*s), hydrolases, and ATPase-binding cassette transporters (*ABC*s)], and *Danaus* spp.

butterflies may biotransform specific ingested cardenolides. Two toads (*Bufo gargarizans* and *Rhinella marina*) exhibit lineage-specific expansions of *CYP450*s, the sequestering firefly *Photuris versicolor* biotransforms lucibufagin to compounds with higher polarity, and the snake *Rhabdophis tigrinus* alters the structure of bufadienolides sequestered from toad prey. Notably, the skin-associated bacteria of *Rhinella marina* may biotransform bufadienolides of its host. Some species (18) lacked biotransformation mechanisms, including lygaeid bugs, aphids, and *Danaus* spp. butterflies that maintain sequestered toxins for long periods, rodents that do not metabolize ouabain, and a mantid that excretes consumed cardenolides unchanged (*Hierodula membranacea*). Several cardenolide-exposed species (24) possessed elimination, including selective excretion of cardenolides in some hemipterans, diffusion barriers in the nymphalid *Euploea core* and the hawkmoth *Daphnis nerii*, and tissue-specific uptake of toxins and effective excretion in at least two orthopteran species and rodents. No evidence supporting the presence of elimination was found for bufadienolide-exposed species. Many species (39) lacked elimination, as evidenced by the presence of CTs in multiple tissues, the evolution of specific storage glands such as nuchal glands in keelback snakes (*Rhabdophis* spp.), and selective sequestration of cardenolides in five lygaeid bugs, four aphids, two *Danaus* spp. butterflies, and the oleander moth *Syntomeida epilais*.

Target. We found few examples of alternative target mechanisms in animals that interact with CTs; only the milkweed bug *Oncopeltus fasciatus* and some mammals have alternative target mechanisms, which in both cases involve circulating toxin-binding proteins (Baggot & Davis 1973). However, the *Oncopeltus fasciatus* protein may actually be a transport protein encoded by *ABC*s (elimination), as seen in other cardenolide-adapted animals such as the beetle *Chrysochus auratus*. No species were identified to lack alternative target mechanisms. By contrast, at least 237 species possessed TSR to CTs including many beetles, hemipterans, varanid lizards, snakes, frogs, and lepidopterans. CT TSR is highly convergent across these and other taxa, with substitutions in the H1–H2 extracellular loop of the Na^+/K^+-ATPase appearing frequently. The accumulation of Na^+/K^+-ATPase sequence data seems to have outpaced the collection of ecological records, as a large number of species (114) are known to have TSR-relevant substitutions but are not known to interact with CTs in nature. Some species also have duplications of the sodium–potassium pump gene, e.g., many lygaeid bugs, leptodactylid frogs, and at least one species of weevil that does not sequester (*Rhyssomatus lineaticollis*). Many species (55) were found to lack TSR, including a number of danaine butterflies and newly exposed predators of the invasive *Rhinella marina* in Australia.

Additional comments. Bufonid toads, some fireflies, and some chrysomelid leaf beetles endogenously synthesize CTs; thus, CTs are the only toxins included in this review that are broadly synthesized by animals (a few ant species are thought to synthesize PAs). The resistance mechanisms of CT-synthesizing animals are largely uninvestigated. Toxin synthesis necessarily involves biotransformation of chemicals; however, it is unknown whether biotransformation also plays a role in resistance. Not included in our general review were several parasitic mistletoes (Plantae: Solanales) that sequester cardenolides from oleander.

Pyrrolizidine Alkaloids

We identified 458 species that interact with PAs in nature and/or possess PA resistance mechanisms (from 93 families in 35 orders and 4 phyla), of which 153 likely have some level of toxin accumulation or sequestration (**Supplemental Figure 1**). Information regarding PA resistance mechanisms was available for at least 355 species.

Supplemental Material >

Avoidance. We found evidence of the presence or absence of avoidance mechanisms in 304 species. Many of the species with contact avoidance present were birds (41% of 139 species), given several large-scale studies on the topic. Other species included spiders (e.g., *Nephila maculata*), chrysomelid beetles, frogs, primates, and lizards. The two species with ingestion avoidance present included the ant *Monomorium floricola*, which avoids consuming the cuticle of the toxic moth *Eucereon maia*, and the oriole *Icterus cayanensis*, which eats the thorax and abdomen of the moth *Histioea cepheus* but leaves its head and genitalia untouched. Species that lacked contact avoidance (191 species) predominantly included lepidopterans (98 species in 5 families) that were also often attracted to PAs. Other species lacking contact avoidance include humans, who consume toxic *Zonocerus* grasshoppers and use PA-containing plants as medicine; some birds such as chickens (*Gallus gallus*); a few species of mantids; and a number of chloropid flies and chrysomelid beetles that were attracted to PAs. Species lacking ingestion avoidance (43 in total) were almost exclusively species known to sequester PAs that clearly seek out and consume PAs, such as the grasshopper *Zonocerus variegatus*, which actually avoids eating nontoxic plant tissues. In total, 202 species were attracted to PAs, which reflects the predominance of their use as a pheromone or feeding cue.

Metabolism. We found evidence for biotransformation and elimination mechanisms in a relatively large proportion of species (137 of 355, or 39%). PAs normally occur in a protoxic form that becomes toxic only when metabolized (bioactivated) into a tertiary or free base. Thus, the metabolic response to PA exposure in most species involves biotransformation, so it is not surprising that 125 species possessed this mechanism, including chrysomelid beetles, erebid moths, nymphalid butterflies, grasshoppers, humans, and livestock. A subset of these species possessed additional biotransformation mechanisms such as some *Longitarsus* spp. beetles and many erebid moths that *N*-oxidize the toxic tertiary PA into a nontoxic PA that cannot be transformed into its toxic state again. In some cases, this novel metabolic function has been traced to a monooxygenase gene duplication [e.g., in *T. jacobaeae*, *Apantesis incorrupta* (previously *Grammia geneura*)]. In addition, many species of erebid moths transform ingested PAs into nontoxic pheromones. Given the protoxic nature of most PAs, lack of biotransformation can also lead to resistance. We identified evidence supporting the absence of biotransformation mechanisms in 16 species. For example, guinea pigs (*Cavia porcellus*) downregulate the *CYP450* genes normally responsible for bioactivation of ingested PAs. The chrysomelid beetle *Oreina cacaliae* takes up ingested PAs into secretion glands so quickly that it avoids bioactivation.

We found evidence supporting the presence of elimination mechanisms in 34 species, some of which were characterized by rapid toxin excretion (e.g., the beetle *Chrysolina coerulans*, the noctuid moth *Spodoptera littoralis*, humans). In other cases, elimination was present in PA-sequestering species, for example, rapid transport from hemolymph into storage glands (e.g., *Platyphora* spp. beetles) or selective elimination of specific PAs (*Utetheisa ornatrix*). Evidence for the absence of elimination was much more prevalent, with data for 90 species. Most of these were PA-sequestering nymphalid butterflies, erebid moths, chrysomelid beetles, and pyrgomorphid grasshoppers that avoid excretion of some subset of ingested PAs. In some species, such as *Creatonotos transiens*, a portion of the PAs consumed by larvae are retained through metamorphosis into adulthood.

Target. We found evidence supporting the presence of alternative target mechanisms in eight erebid moths and one grasshopper (*Zonocerus variegatus*), which each possess novel monooxygenase enzymes that can *N*-oxidize tertiary PAs. No species was identified to lack alternative targets, and no information was found regarding the potential presence or absence of TSR for PAs. One

plausible TSR mechanism (though not encoded in **Supplemental Table 1**) is a reduction in the function of *CYP450s* that normally bioactivate PA, which is known only in the beetle *Oreina cacaliae* and the guinea pig *Cavia porcellus*.

Supplemental Material >

Additional comments. In nearly every case, the ultimate source of PAs was synthesis by plants. The only exceptions were a fungus that synthesizes a PA, which was subsequently stolen by a parasitic plant (Stermitz & Harris 1987) (not included in our analyses), and a few *Megalomyrmex* spp. ants that appear to synthesize PAs as major components of their venom (Jones et al. 1991). At least 24 dendrobatid frogs and 8 mantellid frogs obtain PAs from their diet (ultimate source unknown), but resistance to PAs is unknown. At least one dendrobatid frog consumes PAs and provisions them to offspring (*Oophaga sylvatica*).

THE POTENTIAL IMPACT OF TOXIN RESISTANCE MECHANISMS ON TOXIN SEQUESTRATION

Avoidance Mechanisms

Effective contact or ingestion avoidance should preclude toxin sequestration; thus, the loss of avoidance mechanisms may be required for sequestration to evolve. In total we identified 112 species with the presence of toxin sequestration and absence of contact avoidance, while only 12 had both present, supporting the idea that avoidance may be incompatible with sequestration (the latter 12 species included animals that can eat toxins but prefer not to if given the choice). The evolution of toxin sequestration may in turn select for toxin attraction, which could ensure a steady supply of toxin. In our study, at least 138 species showed attraction to PAs, 14 to TTX, and 9 to CTs. Toxin attraction is often associated with pheromonal use of toxins and reproduction (Trigo & Motta 1990). For example, some male erebid moths are attracted to PA-producing plants, which they consume and modify into nontoxic pheromones (del Campo et al. 2007). In some species of pufferfish, a nontoxic analog of TTX acts as pheromone (Noguchi et al. 2022). Such dual-trait chemicals possess both ecological and reproductive functions, similar to insect cuticular hydrocarbons that mediate mate selection and desiccation resistance (Chung & Carroll 2015). Predators on species that use toxins as defenses and pheromones may thus indirectly affect reproduction and drive speciation. Further investigation into the ecological roles of toxins outside of predation and herbivory may give a more complete understanding of how toxins influence biodiversity. Comprehensive assessment of avoidance and attraction toward toxins is necessary to understand how toxic defenses evolve, yet careful biologically relevant studies are known for very few species (e.g., Lawrence et al. 2023).

Metabolism Mechanisms

Xenobiotic metabolism is a baseline strategy that nonsequestering animals commonly employ to survive toxin exposure (Groen et al. 2017). Metabolic processes often convert nonpolar into polar molecules to facilitate their excretion, yet toxin sequestration requires the retention of molecules. Thus, the initial polarity of an ingested compound and whether or not it is metabolically altered could influence sequestration mechanisms.

We hypothesized that nonpolar toxins would be more commonly sequestered than polar toxins because nonpolar compounds can passively diffuse across cell membranes and do not necessarily require specific transport systems (Agrawal et al. 2012, Groen et al. 2017). In support of this hypothesis, more insect taxa are known to preferentially sequester nonpolar cardenolides (eight species) than polar cardenolides (two species). However, our data also indicate that TTX (polar) is sequestered by at least 136 species spanning 6 phyla, while sequestration of CTs (nonpolar

and polar) and BTX (nonpolar) is known for far fewer species (69 and 6, respectively). Further, monarch butterflies preferentially sequester polar cardenolides (Frick & Wink 1995). For PAs, animals can sequester ingested compounds in either their polar or nonpolar state (Hartmann et al. 1999). For example, the beetle *Oreina cacaliae* suppresses PA bioactivation, actively taking up polar *N*-oxides from the gut into the hemolymph and secretion glands. The erebid moth *T. jacobaeae* bioactivates ingested PAs, which then passively diffuse in their nonpolar form to the hemolymph, where they are *N*-oxidized prior to active uptake into the integument. In addition, several *Platyphora* spp. beetles bioactivate PAs and take up and store the nonpolar form in secretion glands (Hartmann et al. 2001). Although these patterns are influenced by the geographic and taxonomic distribution of each toxin and the available evidence, it does not appear that polarity is indicative of the propensity for a compound to be sequestered.

Perhaps unintuitively, toxin metabolism may provide the initial substrate for toxin sequestration. Biotransformation permits *Drosophila melanogaster* to consume enough nicotine to increase survival against an endoparasite (Douglas et al. 2022). Poison frogs known to sequester dietary compounds also demonstrate ongoing toxin breakdown and modification (Jeckel et al. 2022). Monarch butterflies convert consumed toxins into less-toxic compounds, which are then sequestered (Agrawal et al. 2021). The upregulation of genes encoding Phase I detoxification enzymes (*CYP450*s) can also increase feeding rates (Snyder & Glendinning 1996). Even with elimination mechanisms, animals can still obtain a defensive benefit from consuming toxins. For example, xanthid crabs retain TTX within the digestive gland, potentially to protect other tissues (Tsai et al. 1997), but digestive gland contents can also be expelled as a form of defense (Freitas et al. 1995). Thus, despite toxin modification, metabolism mechanisms often allow animals to increase their overall food and toxin intake, which may then select for additional toxin resistance or sequestration.

Target Mechanisms

Given the extent to which toxin-sequestering animals are exposed to toxins, it has been predicted that TSR is required for sequestration to evolve (Després et al. 2007, Petschenka & Agrawal 2015). With TSR, toxin exposure at the target site has little impact on organismal function, and thus, animals may become completely unaffected by extreme toxin exposure and may even begin to passively accumulate toxins (Karageorgi et al. 2019). Using a Pearson's Chi-squared Test (in R) we find that presence of TSR and sequestration cooccur more often than by chance ($X^2 = 45.9$, $df = 9$, $p = 0.00000063$; $N = 89$ species with data on both types of mechanisms). When controlling for phylogeny ($N = 65$), this pattern is not significant [Pagel's 1994 test, *fitPagel* in *phytools* (Revell 2012); X^2 (4) $= 3.65$, $p = 0.455$] (**Supplemental Figure 4**). Thus, our data are suggestive but not conclusive that a relationship between these traits exists. In some systems, TSR exists, but encounter rates with toxic prey seem to be extremely low (Durso et al. 2021), and it is also possible to sequester without evolving TSR (Abderemane-Ali et al. 2021, Reimche et al. 2022).

Supplemental Material >

OPEN RESEARCH TOPICS AND QUESTIONS

How Does Target-Site Resistance Evolve?

A great deal of research in the last 20 years has focused on the evolution of TSR, providing exemplary cases of convergent evolution (Dobler et al. 2012) and coevolution at both macro- and microevolutionary scales (Holding et al. 2016). TSR for TTX and CTs is highly predictable, likely driven by functional constraint, and has been reviewed elsewhere (Mohammadi et al. 2022, van Thiel et al. 2022). Because amino acid substitutions providing resistance can be costly, animals often evolve additional compensatory substitutions (Mohammadi et al. 2021, Tarvin et al. 2017).

A recent study (Karageorgi et al. 2019) used CRISPR-Cas9 in *D. melanogaster* to recapitulate in vivo the evolutionary trajectory leading to TSR in monarch butterflies, including its costly intermediate steps. Interestingly, no definitive evidence is available for TSR in any BTX- or PA-bearing organism.

To date, most species with TSR appear fixed for resistant alleles. However, sampling more populations may reveal greater diversity. For example, the snakes *Thamnophis sirtalis* and *Erythrolamprus* spp. and the clam *Mya arenaria* each possess TTX-sensitive and TTX-resistant alleles for a Na_V channel gene (Geffeney et al. 2005, Phillips et al. 2018, Ramírez-Castañeda 2017). Duplication of target genes followed by subfunctionalization can also offset the cost of TSR (Lohr et al. 2017, Mohammadi et al. 2021). Comparing these three types of systems (one gene, one allele; one gene, two alleles; two genes, two alleles) could shed light on the mechanisms facilitating or preventing the evolution of TSR. Niche partitioning, the context-specific cost of resistant and sensitive alleles, the strength of selection for resistance, the presence or absence of other resistance mechanisms, and neutral processes may all shape the avenues to TSR. The recent focus on TSR without necessarily investigating other mechanisms of resistance (**Table 1**) may produce an incomplete and possibly misleading picture of resistance, at least for these toxins (Abderemane-Ali et al. 2021, Reimche et al. 2022).

How Do Toxins Regulate Physiology in Toxin-Resistant Species?

Toxins often mimic endogenous processes, which begs the question of how toxins affect animals that are mostly resistant. For example, PAs can be morphogenic: In the moth *Creatonotos transiens*, PA amount is associated with the growth and size of the scent organ (Egelhaaf et al. 1992). In some pufferfish, TTX alters immune and stress responses (Amano et al. 2019, Lee et al. 2007) and penetrates the blood–brain barrier, accumulating to levels as high as in the skin (Amano et al. 2022). Endogenously synthesized CTs are particularly interesting, as CTs are implicated as physiological regulators in many taxa; mammals, birds, fish, and nonbufonid frogs may all possess miniscule, nontoxic quantities of CTs (Flier et al. 1980, Kajimura et al. 2004, Manunta et al. 2009, Wei et al. 1996). In small amounts, CTs stimulate Na^+/K^+-ATPase activity; endogenous CTs in mammals are also known to regulate blood pressure and activate multiple signaling pathways and are sometimes classified as hormones (Manunta et al. 2009). Despite occurring at high levels as defensive chemicals, bufadienolides in toads seem to retain their function as physiological regulators of water balance (Lichtstein et al. 1991). Toads possess a highly resistant *ATP1a1* isoform, but toad brain ATPases are sensitive to CTs (Morris et al. 1997). Thus, the evolution of CT defensive synthesis may have involved increased production of an endogenous regulator paired with TSR in some but not all target proteins. These systems could provide insight into the evolution of novelty in highly constrained phenotypes.

How Do Species Resist Multiple Toxins?

Ecosystems may often contain several keystone molecules either because a single organism secretes a cocktail of toxins and/or because multiple organisms possess different toxins. In fact, several reviewed species are adapted to multiple toxins (e.g., some *Oreina* spp. beetles, *Danaus* spp. butterflies, and erebid moths sequester CTs and PAs) (**Supplemental Table 2**). Thus, some species possess a single resistance mechanism that is effective against multiple toxins. Mantids, for example, have a gut epithelium that prevents TTX and CTs from diffusing out of the gut (Mebs et al. 2016, 2017). Other species possess resistance mechanisms against multiple toxins that are toxin-specific. For example, the garter snake *Thamnophis sirtalis* feeds on prey defended by bufadienolides (toads) and TTX (*Taricha* spp. newts) and has TSR in Na^+/K^+-ATPases and

Supplemental Material >

Na_V channels (McGlothlin et al. 2016, Mohammadi et al. 2016). Studying multiple mechanisms and toxins could expand our understanding of evolutionary predictability beyond single toxin resistance adaptations to more complex (and realistic) systems.

How Do Species Adapt to Introduced Toxins?

Human activities drastically alter the distributions of species, which in turn change the global distributions of keystone molecules. For instance, predators in Australia lacked exposure to toad bufadienolides until the introduction of cane toads (*Rhinella marina*). Consequently, many Australian species are highly susceptible to intoxication by toads and die off in massive numbers along the invasion front (Smith & Phillips 2006). Others appear to be successfully taking advantage of toads as a new food source [i.e., murid rodents and birds (Cabrera-Guzmán et al. 2015)]. Thus, this system is a powerful case study for the adaptation of communities to new toxins. Avoidance is particularly common in Australian predators of cane toads (47%), potentially the first line of defense against novel toxins. By contrast, none of these predators are known to possess metabolic or target resistance, with the exception of *Crocodylus porosus*, which has TSR, and Australian murid rodents, which may possess the TSR substitutions common in their family. Intriguingly, populations of the Australian blue tongue skink *Tiliqua scincoides* that have had historical exposure to invasive, bufadienolide-laden *Bryophyllum* spp. plants are less sensitive to injections of toad secretions (Price-Rees et al. 2012). The introduction of keystone molecules through invasive species has profound effects on an ecosystem. Studying these invasions may reveal the temporal sequence of toxin resistance evolution and provide predictive insight into how species adapt to pollution and other novel molecules.

What Are the Molecular Mechanisms of Toxin Sequestration?

As we have shown above, some toxin resistance mechanisms can lead to toxin sequestration. Thus, studies of toxin resistance can also shed light on sequestration. Feeding assays are one of the best ways to study toxin resistance and sequestration, yet few such experiments have been done for BTX, TTX, or CTs; in contrast, many exist for PAs (Hartmann et al. 1999). Documenting toxin diffusion kinetics and tissue-specific concentrations (Jeckel et al. 2020, Melnikova & Magarlamov 2022) can help generate new hypotheses regarding toxin sequestration (Malykin et al. 2021, Zhang et al. 2020). New tools are also advancing our understanding of sequestration, for example, through the identification of a new binding protein in poison frogs using photo-labeled toxins (Alvarez-Buylla et al. 2022) and the generation of a pufferfish model with a knocked-out toxin-binding protein (Kato-Unoki et al. 2018). The role of xenosensors, which are toxin-sensing transcription factors that activate the expression of metabolic genes (Nakata et al. 2006), is largely unexamined in toxin-sequestering taxa, yet they may play an important role in the origin of sequestration. Some toxin storage and synthesis mechanisms described in plants, such as the roles of *CYP450*s in transgenic *Arabidopsis thaliana* (Kristensen et al. 2005) and of vacuoles in *Senecio* spp. (Ehmke et al. 1988), could also provide inspiration for research avenues in toxin-sequestering animals.

ABCs can provide toxin resistance through diffusion barriers (Groen et al. 2017, Petschenka et al. 2013), yet they are also emerging candidate genes for toxin sequestration. In the beetle *Chrysochus auratus*, three different ABCs differentially bind cardenolides in a tissue-specific manner, preventing their diffusion into nervous tissue and facilitating their accumulation in defensive glands (Kowalski et al. 2020). An ABC also facilitates glucoside accumulation in the defensive glands of *Chrysomela populi* (Strauss et al. 2013). Similar trends exist in other species, but the potential role for ABCs has not been investigated; examples include the active, selective uptake of cardenolides across the midgut in *Oncopeltus fasciatus* and the active, selective uptake of digoxin in

midgut cells of *Syntomeida epilais* (for references, see **Supplemental Table 2**). There is no research on ABCs in bufadienolide-sequestering species, although bufadienolides can inhibit ABC activity in cancer cells (Zeino et al. 2015). ABCs may also be involved in toxin sequestration in puffer fish (Zhang et al. 2022) and poison frogs (O'Connell et al. 2021). We foresee future mechanistic studies delving into the dual role of ABC transporters in both toxin resistance and sequestration.

Supplemental Material >

CONCLUSION

As keystone molecules, toxins are integrated into multiple facets of organismal ecology and physiology. Toxin resistance research can thus help us understand the great diversity of insect herbivores, the evolutionary scenarios that gave rise to charismatic and poisonous vertebrates, and mechanisms that pests use to evade insecticides. Such areas of interest have led to a few well-developed and phylogenetically distinct systems (e.g., pufferfish, lygaeid beetles, erebid moths, *Rhabdophis* spp. snakes), yet research into toxin resistance has been far from systematic. Herein, we propose a framework in which to ask questions about the ecology, evolution, and mechanisms shaping toxin resistance, which in turn will provide insight into the origins of toxin sequestration. We encourage researchers to investigate multiple mechanisms of resistance to provide a more holistic understanding of how animals deal with toxic compounds. Understanding toxin resistance can help us predict how animals may adapt to environmental pollution. Revealing the diversity of molecular mechanisms by which animals modulate toxin metabolism, transport, and storage will also aid in better drug design and improved drug efficacy. Thus, understanding adaptations that underlie toxin resistance and sequestration will contribute foundational data for human medicine and unique insight into organismal ecology and evolution. Toxin resistance is a complex adaptation that interconnects many aspects of biology, providing a naturally integrative lens on living organisms, the challenges they face, and the solutions they employ.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

We thank Stuart Macdonald, Shabnam Mohammadi, members of the Tarvin Lab, and an anonymous reviewer for helpful comments on earlier versions of the manuscript. We thank Madison Pearson and Avi Berger for assistance with data transfer. We acknowledge that relevant studies may have been unintentionally overlooked during the process of writing this review. R.D.T. was supported by start-up funds from the University of California, Berkeley. T.E.D. was supported by the Hellman Fellow Faculty Fund awarded to R.D.T.

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