



# Convergent evolution of toxin resistance in animals

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## ABSTRACT

Convergence is the phenomenon whereby similar phenotypes evolve independently in different lineages. One example is resistance to toxins in animals. Toxins have evolved many times throughout the tree of life. They disrupt molecular and physiological pathways in target species, thereby incapacitating prey or deterring a predator. In response, molecular resistance has evolved in many species exposed to toxins to counteract their harmful effects. Here, we review current knowledge on the convergence of toxin resistance using examples from a wide range of toxin families. We explore the evolutionary processes and molecular adaptations driving toxin resistance. However, resistance adaptations may carry a fitness cost if they disrupt the normal physiology of the resistant animal. Therefore, there is a trade-off between maintaining a functional molecular target and reducing toxin susceptibility. There are relatively few solutions that satisfy this trade-off. As a result, we see a small set of molecular adaptations appearing repeatedly in diverse animal lineages, a phenomenon that is consistent with models of deterministic evolution. Convergence may also explain what has been called ‘autoresistance’. This is often thought to have evolved for self-protection, but we argue instead that it may be a consequence of poisonous animals feeding on toxic prey. Toxin resistance provides a unique and compelling model system for studying the interplay between trophic interactions, selection pressures and the molecular mechanisms underlying evolutionary novelties.

*Key words:* convergent evolution, toxin resistance, molecular adaptation, functional constraint, deterministic evolution, co-evolutionary arms races.

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## I INTRODUCTION

Convergent evolution is the independent emergence of similar traits across different lineages (Storz, 2016). Toxins are key innovations that have evolved throughout the tree of life (Yamaguchi, Park & Inouye, 2011; Casewell *et al.*, 2013). They act on specific molecular targets (e.g. receptors, ion-channels, enzymes, and plasma membranes), causing a range of pathophysiological disruptions throughout the cardiovascular, circulatory and nervous systems (Fry *et al.*, 2009; Casewell *et al.*, 2013). Venom is a mixture of proteinaceous toxins exploited for a variety of functions, including prey capture and defence, ultimately resulting in severe pain, incapacitation or death. By definition, venoms are typically injected into a target system *via* a mechanical wound caused for example by fangs (e.g. snakes, centipedes and spiders), spines (e.g. fish), nematocysts (e.g. jellyfish and sea anemones) or stingers (bees, wasps and other arthropods). Poisons on the other hand tend to consist of small-molecule toxins (e.g. low-molecular-weight alkaloid or steroidal-based compounds) that are almost exclusively utilised to deter predators – often by causing rapid pain or paralysis upon contact or ingestion (Duran-Riveroll & Cembella, 2017; Botelho *et al.*, 2019).

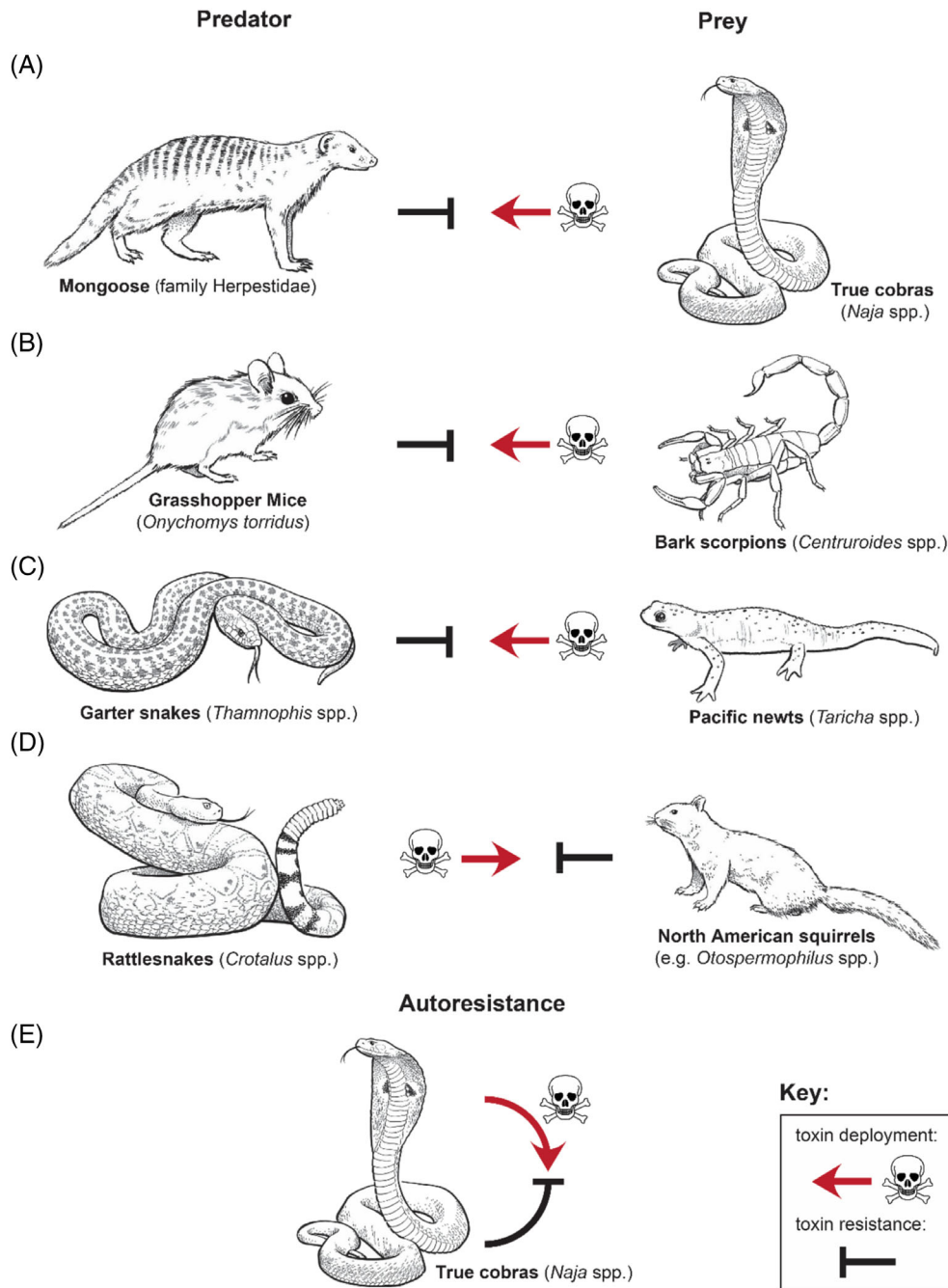
Therefore, toxins can be considered ecologically functional traits that mediate antagonistic interactions between predator and prey, driven by natural selection. To counteract the deleterious effects of these toxins, some animals have evolved resistance. Toxin resistance is the increased ability of an animal to survive exposure to one or more toxins without being functionally affected. As a result, toxin resistance has evolved in at least three distinct ecological contexts (Fig. 1): *predator resistance*, where a predator is resistant to the toxins of its prey (Fig. 1A–C); *prey resistance*, where the prey is resistant to the toxins of a predator (Fig. 1D); or *autoresistance*, where an animal is resistant to its own toxins (Fig. 1E).

Resistance particularly evolves in molecular targets involving key regulatory processes that are under strong selection

pressures, i.e. that are crucial for the survival of an organism. There are several molecular mechanisms underlying toxin resistance [reviewed in Holding *et al.*, 2016b and Arbuckle, Rodriguez de la Vega & Casewell, 2017]. First, *target modification* is a change in the gene sequences that encode receptors or circulating proteins to which specific toxins bind, resulting in a reduction of binding affinity of the toxin towards the target (Barchan *et al.*, 1992; Geffeny *et al.*, 2005; Jansa & Voss, 2011; Tarvin *et al.*, 2017; Karageorgi *et al.*, 2019). Second, *off-target repurposing* is the molecular modification of a previously non-target site that increases its toxin-binding affinity so that the physiological effect induced by the toxin is altered, and the desired effect has thereby been repurposed (Rowe *et al.*, 2013). Finally, *toxin scavenging* involves serum-based components that patrol the circulatory system and inhibit the activity of enzymatic toxins (Perez, Pichyangkul & Garcia, 1979; Biardi *et al.*, 2011; Gibbs *et al.*, 2020).

Venom systems have been proposed as models for studying processes underlying evolutionary adaptations (Zancolli & Casewell, 2020), including convergent evolution (Casewell *et al.*, 2019; Kazandjian *et al.*, 2021; Walker *et al.*, 2021). The well-defined function (e.g. ecological interactions) and the discrete genotype–phenotype association (e.g. molecular adaptations reducing the binding affinity of a certain toxin) involving toxin resistance provide a compelling model system for studying evolutionary adaptations and drivers of fundamental processes in biology. Similar toxins are found in many different animals (Casewell *et al.*, 2013; Schendel *et al.*, 2019), and therefore they are involved in many different trophic interactions (i.e. ecological interplay between species related to diet) across many animal lineages. This provides a fascinating opportunity to study the extent of convergent evolution across unrelated taxa by incorporating the evolutionary drivers stimulating molecular adaptations leading to toxin resistance.

Here, we review current knowledge on the convergent evolution of toxin resistance, with a particular emphasis on its molecular basis and evolutionary drivers. We discuss the molecular



**Fig. 1.** Well-known examples of ecological contexts underpinning toxin resistance. (A–C) predator resistance, where a predator is resistant to the toxins of its prey. (A) The mongoose is known to predate on true cobras. (B) The grasshopper mouse preys on bark scorpions. (C) Garter snakes prey on toxic newts. (D) Prey resistance is resistance of a prey species to the toxins of a predator and is exemplified here by rattlesnakes preying on North American ground squirrels. (E) Autoresistance is where an animal is resistant to its own toxins. The example shown here is of true cobras that show resistance to cobra  $\alpha$ -neurotoxins.

mechanisms underpinning toxin resistance across a wide range of toxin families, with diverse evolutionary drivers. We then discuss several intriguing evolutionary aspects of toxin resistance, namely: (i) the evolutionary framework underlying the appearance of toxin resistance; (ii) the role of functional constraints on molecular targets leading to convergence; and (iii) a re-examination of the evolutionary drivers of autoresistance in poisonous animals.

## II CONVERGENT MECHANISMS OF RESISTANCE

### (1) Snake venom metalloproteinases

Snake venom metalloproteinases (SVMPs) are zinc-dependent proteinases capable of exerting coagulopathic

and haemorrhagic effects (Ferraz *et al.*, 2019), and they are particularly abundant in viper (family Viperidae) venoms (Tasoulis & Isbister, 2017). Many animals have evolved an innate immune response mediated by SVMP inhibitors (SVMPIs). SVMPIs are acidic glycoproteins present in the circulatory system that neutralise the activity of SVMPs using a toxin-scavenging mechanism. These ‘scavenging’ inhibitors display a tight-binding reaction mechanism, mediated by the formation of non-covalent interactions and ultimately preventing the pathophysiological effects of SVMPs (Valente *et al.*, 2001). Although sharing similar functionality, SVMPIs are related to three different supergene families: (i) ficolin/opsonin p35 (Omori-Satoh, Yamakawa & Mebs, 2000); (ii) immunoglobulin (Hood, Kronenberg & Hunkapiller, 1985); or (iii) cystatin (Rawlings & Barrett, 1990). SVMPIs evolved independently across many distinct mammal (class Mammalia) and snake (suborder Serpentes) lineages.

A well-studied example of SVMP resistance can be seen in several North American squirrels (e.g. *Otospermophilus* spp., *Ictidomys* sp. and *Sciurus* sp.) that are sympatric (i.e. occurring in the same geographical area) with rattlesnakes (*Crotalus* spp.; Martinez *et al.*, 1999; Biardi *et al.*, 2011; Biardi & Coss, 2011; Pomento *et al.*, 2016). This is in contrast to squirrels that are allopatric (i.e. occurring in distinct, non-overlapping geographical areas) with rattlesnakes, which show less resistance (Poran, Coss & Benjamini, 1987; Holding, Biardi & Gibbs, 2016a; Pomento *et al.*, 2016; Gibbs *et al.*, 2020). Holding *et al.* (2016a) showed that local rattlesnake populations demonstrate higher SVMP activity in their venom; this higher activity was linked to the increased SVMPI activity in sympatric squirrels. The latter was not observed in allopatric populations. This suggests that rattlesnake venom has become adapted to maintain its selective advantage in overcoming squirrel resistance (Holding *et al.*, 2016a). This highlights the evolutionary interplay between predator and prey, resulting in convergent, geographically restricted adaptations within and across different squirrel species.

Another example is the opossum family (Didelphidae), which is sympatric with pitvipers (subfamily Crotalinae). Interestingly, they have reciprocal trophic relationships: opossums predate upon pitvipers (Oliveira & Santori, 1999; Almeida-Santos *et al.*, 2000) and pitvipers prey on opossums (Voss, 2013). Many opossum species show resistance to injected pitviper venoms (Werner & Vick, 1977; Perales *et al.*, 1994), a resistance mediated by serum SVMPIs. Evolutionary drivers underpinning resistance are likely to be species dependent; more data on trophic interactions will help address this issue (Voss, 2013).

Various other mammals, including several North American rodents (*Sigmodon* sp., *Microtus* sp. and *Neotoma* spp.), have evolved resistance to the SVMPs of sympatric pitviper species (Pichyangkul & Perez, 1981; de Wit, 1982; Garcia & Perez, 1984). Furthermore, some animals that prey on snakes, including the Indian grey mongoose (*Herpestes edwardsii*) and the European hedgehog (*Erinaceus europaeus*), also have serum SVMPIs. Most mammalian SVMPIs are related to the immunoglobulin family; however, erinacin, isolated from *E. europaeus*, is

related to the ficolin/opsonin p35 family (Mebs *et al.*, 1996; Omori-Satoh *et al.*, 2000). An overview of mammalian SVMPIs derived from the literature is provided as online supporting information in Table S1.

In addition to mammals, several snakes have also evolved serum SVMPIs that may confer resistance. In pitvipers this is likely an example of autoresistance, but in the eastern indigo snake (*Drymarchon couperi*) and Ryukyu odd-tooth snake (*Lycodon semicarinatus*) it may rather be predator resistance because these species are known to prey on sympatric pitvipers (Tomihara *et al.*, 1988; Goetz *et al.*, 2019). Another snake with serum SVMPIs is the Burmese python (*Python bivittatus*; Duan *et al.*, 2017), which likely evolved resistance in response to predation by venomous, snake-eating snakes (Jones *et al.*, 2020; Smith *et al.*, 2021). While most research has focused on serum SVMPIs, some snakes also have SVMPIs in their venom and venom glands (Munekio & Mackessy, 2005; Yee *et al.*, 2016; Valente *et al.*, 2018). This could be an example of autoresistance, whereby the SVMPIs protect the secretory epithelium of the venom gland and venom components from harmful effects of endogenous SVMPs (Mackessy & Baxter, 2006; Valente *et al.*, 2018). An overview of snake SVMPIs derived from the literature is provided in Table S2.

## (2) Snake venom phospholipases A<sub>2</sub>

Phospholipases A<sub>2</sub> (PLA<sub>2</sub>s) are esterolytic enzymes that can cause a variety of pathological effects including myotoxicity, neurotoxicity and haemotoxicity (Manjunatha Kini, 2003; Ferraz *et al.*, 2019). They are major venom components across different snake lineages (Tasoulis & Isbister, 2017). Several animals have evolved an innate immune response mediated by PLA<sub>2</sub> inhibitors (PLA<sub>2</sub>Is) that neutralise the activity of PLA<sub>2</sub>s using a toxin-scavenging mechanism (Lizano, Domont & Perales, 2003). PLA<sub>2</sub>Is form stable complexes, and by mimicking PLA<sub>2</sub>-acceptors prevent binding to the cell membrane, ultimately resulting in the inhibition of the pathological effects of PLA<sub>2</sub>s (Perales *et al.*, 1995). PLA<sub>2</sub>Is are assigned to three structural classes (reviewed in Lizano *et al.*, 2003): PLA<sub>2</sub>I- $\alpha$  (C-type lectin domain); PLA<sub>2</sub>I- $\beta$  (leucine-rich repeats domain); and PLA<sub>2</sub>I- $\gamma$  (three-finger domain). These PLA<sub>2</sub>Is evolved convergently in snakes and opossums.

Serum-derived PLA<sub>2</sub>Is are predominantly found in venomous snakes, including species with an abundance of PLA<sub>2</sub>s in their venom (Tasoulis & Isbister, 2017). Interestingly, all elapid inhibitors are classified as PLA<sub>2</sub>I- $\gamma$  class, whereas viperid inhibitors are also in the PLA<sub>2</sub>I- $\alpha$  and PLA<sub>2</sub>I- $\beta$  classes (see Table S3 for an overview of PLA<sub>2</sub>Is in snakes). PLA<sub>2</sub>I- $\gamma$  of some non-venomous snakes may represent prey resistance that evolved in response to predation from venomous snakes (Thwin *et al.*, 2000; Zhong & Huang, 2016; Fortes-Dias *et al.*, 2020; Rodrigues *et al.*, 2020, 2021). PLA<sub>2</sub>Is have also been characterised in the venom and venom gland itself, possibly representing a form of autoresistance (Mackessy & Baxter, 2006; Valente *et al.*, 2018).

The only PLA<sub>2</sub>Is known from mammals are those of opossums. A PLA<sub>2</sub>I has been isolated from the white-eared

opossum (*Didelphis albiventris*; Soares *et al.*, 1997). PLA<sub>2</sub>Is are also known in the big-eared opossum (*D. aurita*). Interestingly, they show homology with the immunoglobulin supergene family. This is significant because SVMPIs isolated from other didelphids share high sequence similarities with their PLA<sub>2</sub>I-counterpart, suggesting that similar inhibitors act on distinct snake venom toxins (Rocha *et al.*, 2002).

### (3) Snake venom C-type lectins

C-type lectins (CTLs) are members of the lectin family and are predominantly found in the venom of vipers (Tasoulis & Isbister, 2017). CTLs bind to glycoprotein 1b and von Willebrand factor (vWF), thereby promoting abnormal platelet aggregation. This resistance convergently evolved within the opossum lineage, which are known to have trophic interactions with pitvipers (Oliveira & Santori, 1999; Almeida-Santos *et al.*, 2000; Voss, 2013). Several opossum species (*Didelphis* spp., *Philander* spp. and *Lutreolina* sp.) show modifications of the CTL-binding site of the vWF protein (A1 domain) under positive selection (Jansa & Voss, 2011). The modified vWF protein has substitutions that change its hydrophobicity and net charge, which is hypothesised to inhibit the binding of CTLs (Jansa & Voss, 2011). This hypothesis was supported by functional *in vitro* experiments revealing significant decreases in platelet aggregation in opossum plasma exposed to CTLs (Drabeck *et al.*, 2020). This is the first documented example of resistance-associated adaptations in a non-receptor protein targeted by venom.

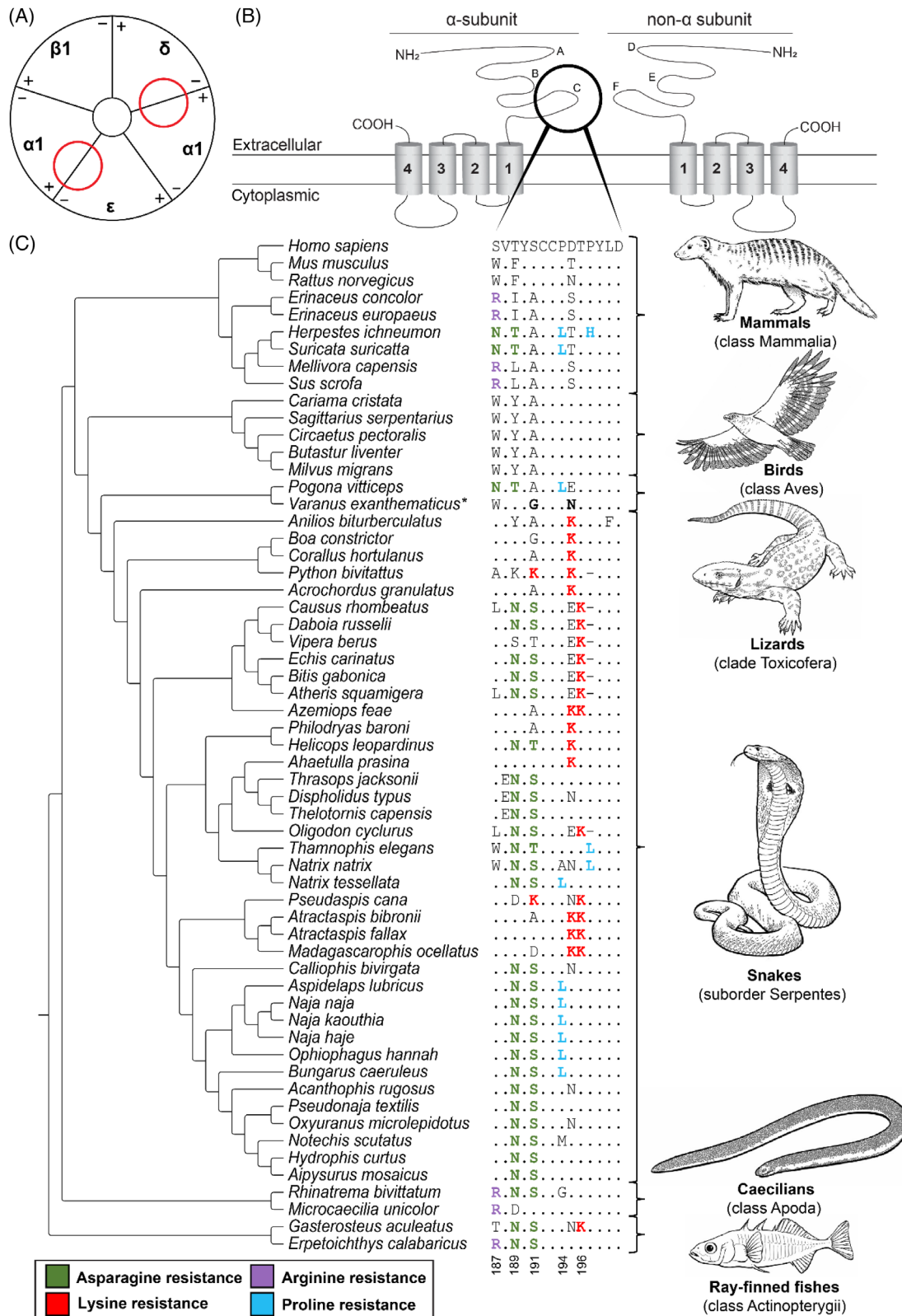
### (4) Snake venom three-finger toxins

Three-finger toxins (3FTX) are one of the most abundant non-enzymatic toxin families in elapid (family Elapidae) and some colubrid (family Colubridae) venoms (Tasoulis & Isbister, 2017; Modahl & Mackessy, 2019), and their principal effects are cytotoxicity and neurotoxicity. The basal activity is post-synaptic neurotoxicity through antagonistic binding to the muscle-type nicotinic acetylcholine receptor (nAChR), causing muscular paralysis and respiratory failure (Barchan *et al.*, 1995; Takacs, Wilhelmsen & Sorota, 2004). These neurotoxic effects are caused by  $\alpha$ -neurotoxins, which primarily bind to Loop C of the ligand-binding domain ( $\alpha$ 1-subunit) of the nAChR. Furthermore, they also show interaction with the Cys Loop, Loop F, and neighbouring delta and gamma subunits (Rahman *et al.*, 2020). Resistance to  $\alpha$ -neurotoxins is underpinned by different molecular modifications of the ligand-binding domain of the nAChR, causing a significant reduction in their binding affinity (Barchan *et al.*, 1995; Kachalsky *et al.*, 1995; Asher *et al.*, 1998; Takacs, Wilhelmsen & Sorota, 2001; Takacs *et al.*, 2004; Dellisanti *et al.*, 2007; Rahman *et al.*, 2020; Harris & Fry, 2021; Jones *et al.*, 2021). These resistance adaptations can be generally categorised as one of four different amino acid substitutions. First, asparagine resistance is characterised by a change to asparagine, resulting in a glycosylation motif (187–189NVT or 189–191NXS/T). As a result, the *N*-

glycosylation of asparagine sterically hinders the binding of  $\alpha$ -neurotoxins (Asher *et al.*, 1998; Takacs *et al.*, 2001, 2004; Rahman *et al.*, 2020). Secondly, arginine resistance is characterised by a replacement to arginine (187R). This change sterically hinders the binding of  $\alpha$ -neurotoxins (Rahman *et al.*, 2020). However, since arginine is a positively charged amino acid, it has also been suggested to affect toxin binding by means of electrostatic repulsion of the positively charged  $\alpha$ -neurotoxins (Dellisanti *et al.*, 2007; Drabeck, Dean & Jansa, 2015). Lysine resistance is characterised by a replacement to lysine (191K, 195K and/or 196K). This change to the positively charged amino acid affects the binding by means of electrostatic repulsion of  $\alpha$ -neurotoxins (Harris & Fry, 2021). Finally, proline resistance is characterised by a replacement from proline to either a leucine or histidine (194L, 197L/H). These replacements change the structural conformation of the ligand-binding domain, and therefore affect the binding of  $\alpha$ -neurotoxins (Kachalsky *et al.*, 1995). An overview of the key sites and mutations that confer  $\alpha$ -neurotoxin resistance is provided in Table S4.  $\alpha$ -Neurotoxin resistance has evolved convergently across a broad diversity of vertebrates (Fig. 2).

A classic example of  $\alpha$ -neurotoxin resistance is seen in mongooses (family Herpestidae). For example, the Egyptian mongoose (*Herpestes ichneumon*) and the meerkat (*Suricata suricatta*) both possess a combination of asparagine resistance (187–189NVT) and proline resistance (194L; Fig. 2; Barchan *et al.*, 1992; Kachalsky *et al.*, 1995; Asher *et al.*, 1998; Khan *et al.*, 2020). Furthermore, the Egyptian mongoose also shows an additional proline replacement (197H; Fig. 2). These adaptations presumably evolved in response to predation on venomous snakes including true cobras (*Naja* spp.; Stuart, 1983; Struhsaker & McKey, 1975). However, more studies are needed to characterise the extent of trophic interactions between these mammals and snakes in the wild. Other resistant mammals include the honey badger (*Mellivora capensis*), hedgehogs (*Erinaceus europaeus* and *E. concolor*), and the wild boar (*Sus scrofa*), all of which show arginine resistance (187R; Fig. 2; Barchan *et al.*, 1995; Drabeck *et al.*, 2015; Harris & Fry, 2021). The honey badger predates upon venomous snakes, including the Cape cobra (*Naja nivea*), which has  $\alpha$ -neurotoxin in its venom (Begg *et al.*, 2003). Wild boars and hedgehogs are also known occasionally to prey on snakes (Reeve, 1994; Tanaka & Mori, 2000; Jolley *et al.*, 2010; Wilcox, 2015). Interestingly, a recent study of primates highlighted that some groups that are sympatric with  $\alpha$ -neurotoxic snakes had a reduced susceptibility towards  $\alpha$ -neurotoxins of true cobras (*Naja* spp.). Members of the subfamily Homininae (*Homo*, *Pan* and *Gorilla* spp.) showed the lowest degree of susceptibility compared to other primates (Harris, Nekaris & Fry, 2021). In principle, this pattern could be explained in terms of the long history of interactions between primates and venomous snakes (Isbell, 2009; Kazandjian *et al.*, 2021).

Many birds prey on venomous snakes, including snake specialists such as the secretary bird (*Sagittarius serpentarius*), snake eagles (*Circaetus* spp.), and seriemas (family Cariamidea;



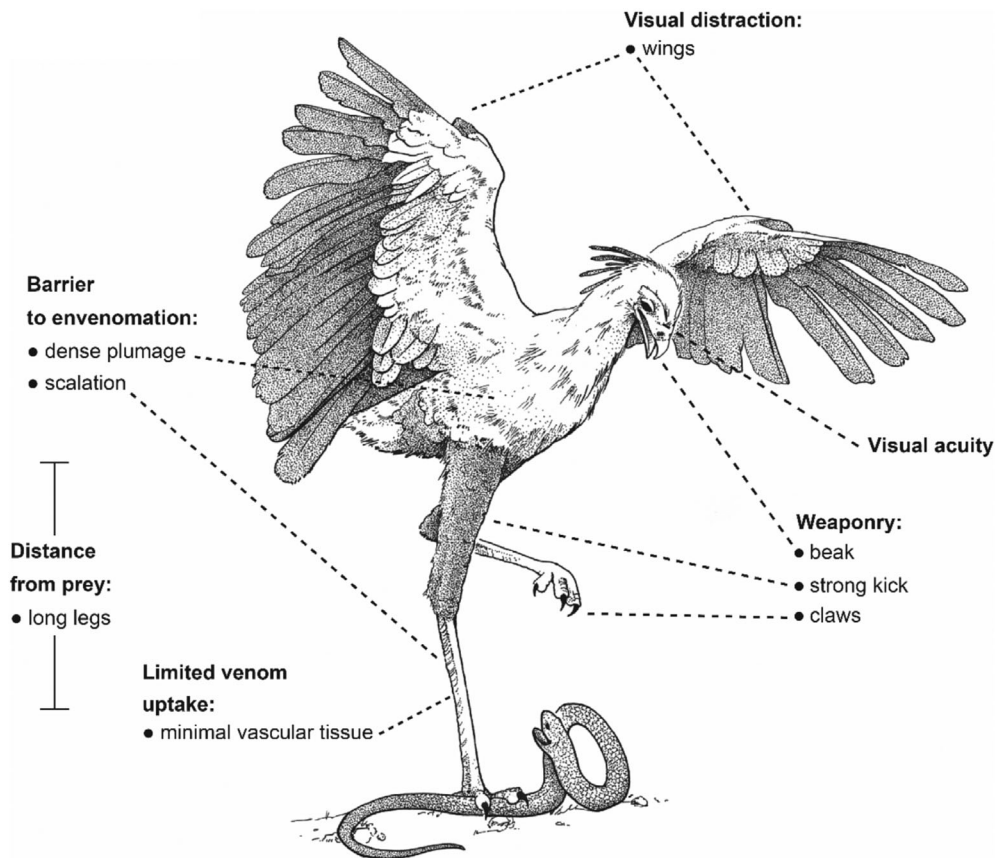
**Fig. 2.** Convergent evolution of  $\alpha$ -neurotoxin resistance in animals. (A) Schematic representation (based on Kini, 2019) of the  $\alpha$ -1 muscle-type nicotinic acetylcholine receptor (nAChR). Red circles indicate the position of the ligand-binding domain of  $\alpha$ -neurotoxins in the nAChR. (B) Protein topology of an  $\alpha$ -subunit and a non- $\alpha$ -subunit of the muscle-type nAChR. A-F indicate the loop structures at the extracellular domain in the respective subunits (Rahman et al., 2020). The black circle indicates the C-loop involved in  $\alpha$ -neurotoxin binding. (C) Sequence alignment of the  $\alpha$ 1-nAChR ligand-binding domain. The reference amino acid sequence is from humans (*Homo sapiens*) and differences from this sequence are displayed for all other species. Substitutions associated with resistance are highlighted in coloured font. The asterisk (\*) in *Varanus exanthematicus* indicates that the two substitutions shown are associated with reduced binding affinity (Jones, Harris & Fry, 2021). Tree topology based on Khan et al. (2020). Sequence accession numbers are provided in Table S5.

Redford & Peters, 1986; Portugal *et al.*, 2016; Mori, Vyas & Upadhyay, 2017). Birds do not show any known resistance-related modifications associated with  $\alpha$ -neurotoxins (Fig. 2); Khan *et al.*, 2020). To explain this apparent paradox, we propose that a set of morphological exaptations and behavioural traits in snake-eating birds prevent envenomation in the first place (Fig. 3). Some examples of morphological exaptations are plumage and leg scales that may provide a physical barrier against snakebite envenomation (Lucas & Stettenheim, 1972). Additionally, bird legs mainly contain tendons and lack highly vascular tissue such as skeletal muscle; this may limit the uptake of venom if the bird is bitten. In particular, the secretary bird attacks snakes aggressively, directing kicks to the head and neck (Portugal *et al.*, 2016). Its elongated tibiotarsus and tarsometatarsus may facilitate a powerful kick (Portugal *et al.*, 2016). Birds of prey, many of which are snake-eaters, have high visual acuity and ambush hunting strategies that may minimise the risk of snakebite (Potier *et al.*, 2020). The red-legged seriema (*Cariama cristata*) uses its beak to grasp prey behind the neck and then shakes the prey violently so as to fracture its spine (Silva *et al.*, 2016). In sum, these bird-specific morphological and behavioural traits might explain why molecular adaptations conferring resistance have not

evolved among them, in contrast to other snake-eating lineages [e.g. mongoose, honey badger (Drabeck *et al.*, 2015; Khan *et al.*, 2020)].

Resistance-related mutations have been documented in lizards (clade Toxicofera) that are potentially vulnerable to predation by sympatric, neurotoxic snakes, such as the central bearded dragon (*Pogona vitticeps*; 187–189NYT, 194L) and the savannah monitor [*Varanus exanthematicus*; 191G and 195N; Fig. 2 (Khan *et al.*, 2020; Jones *et al.*, 2021)]. However, resistance has not been documented in monitor lizards (*Varanus* spp.) that have been suggested to prey on neurotoxic snakes (Jones *et al.*, 2021). Several studies hypothesised that morphological exaptations (thick, osteodermic scales) and prey-handling behaviour negated selection pressure for molecular resistance in these lizards (Jones *et al.*, 2021; Youngman, Llinas & Fry, 2021). The evolution of such strategies to avoid envenomation is comparable to what we propose for snake-eating birds (Fig. 3).

$\alpha$ -neurotoxin resistance is particularly widespread in snakes, which have convergently evolved asparagine resistance (189–191NXS/T), lysine resistance (191K, 195K and/or 196K) and/or, proline resistance (194L, 197L) to elapid  $\alpha$ -neurotoxins (Fig. 2; Khan *et al.*, 2020; Harris & Fry,



**Fig. 3.** Morphological exaptations and behavioural traits proposed to negate selection pressures for evolving molecular resistance in snake-eating birds such as the secretary bird (*Sagittarius serpentarius*). This figure represents multiple examples of traits unique to birds, and particularly to snake-eating birds, that might contribute to the prevention of snakebite envenomation. Drawing from an original photograph by Jason Shallcross, with permission.

2021). Resistance to these toxins in snakes is particularly interesting because – uniquely among vertebrates – snakes show all three ecological functions of resistance, namely, predator resistance, prey resistance and autoresistance. The driver of this trait is either due to selection pressure from sympatric venomous snakes or autoresistance. Elapids, including true cobras (*Naja* spp.) and the king cobra (*Ophiophagus hannah*), prey on other snakes, and this includes cannibalism (Shine *et al.*, 2007; Layloo, Smith & Maritz, 2017; Maritz, Alexander & Maritz, 2019; Jones *et al.*, 2020), and numerous species are specialised snake predators, which may have been the ancestral condition of the clade (Shine, 1991; Kgaditse, 2016). It has been suggested that predation from snake-eating elapids may have contributed to the evolution of resistance in multiple non-elapid snake lineages, an assertion that is supported by ecological observations of predation events and diet studies (Alexander & Maritz, 2010; Maritz *et al.*, 2019; Jones *et al.*, 2020). Notably, the European viper (*Vipera berus*) shows a reversal of the asparagine-resistance genotype (Fig. 2), secondary to vipers radiating into geographic areas lacking sympatric neurotoxic snakes; this may indicate that resistance mutations carry a fitness cost in species that no longer encounter  $\alpha$ -neurotoxins (Khan *et al.*, 2020). On the other hand, this resistance may also prevent self-venomation (e.g. accidental occasions when a venomous snake bites itself) in snakes with  $\alpha$ -neurotoxins. All elapid snakes sequenced to date share the *N*-glycosylation form of resistance to  $\alpha$ -neurotoxins found in their own venom (Fig. 2; Khan *et al.*, 2020). Resistance to  $\alpha$ -neurotoxins is less common in non-elapid neurotoxic snakes – but is ubiquitous within the elapids (Khan *et al.*, 2020; Harris & Fry, 2021), perhaps suggesting a strong selection pressure for the evolution of autoresistance.

Less well-documented examples have been observed in both caecilians (clade Apoda) and ray-finned fishes (class Actinopterygii). Two caecilian species have convergently evolved resistance elements: the tiny Cayenne caecilian (*Microcaecilia unicolor*; 187R) and the two-lined caecilian (*Rhinatrema bivittatum*; 189–191NYS and 187R; Fig. 2; Khan *et al.*, 2020). Both species are sympatric with caecilian-eating coral snakes (*Micrurus* spp.), which could explain this resistance (Martins & Ermelinda Oliveira, 1998). Additionally, asparagine resistance (189–191NYS) has evolved in the three-spined stickleback (*Gasterosteus aculeatus*) and the reedfish (*Erethoichthys calabaricus*; Fig. 2; Khan *et al.*, 2020). The three-spined stickleback additionally evolved lysine resistance (196K) whereas the reedfish additionally shows arginine resistance (187R; Fig. 2). The ecological role of these modifications, if any, is unknown. A highly speculative possibility is that it could have evolved against anatoxin-a (which is an  $\alpha$ -neurotoxin) secreted by freshwater cyanobacteria (Ar oz, Molg  & Tandeau de Marsac, 2010).

### (5) Pain-inducing scorpion toxins

The venom of bark scorpions (*Centruroides* spp.) is potentially lethal, and their venom rapidly induces intense pain,

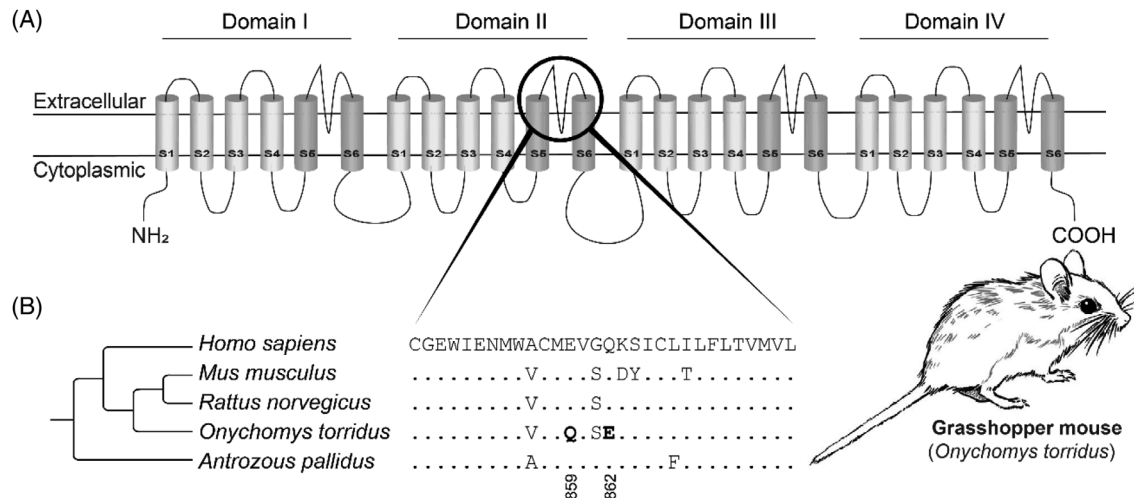
presumably to deter attackers. The pain is caused by the activation of voltage-gated  $\text{Na}^+$  channels ( $\text{Na}_v$  1.7), which are responsible for transmitting pain signals (nociceptive action potentials) to the central nervous system (Rowe *et al.*, 2011, 2013). The grasshopper mouse (*Onychomys torridus*) preys on arthropods including bark scorpions. It shows toxin resistance towards bark scorpion venom characterised by a diminished pain response (Rowe & Rowe, 2008; Rowe *et al.*, 2013). This resistance is underpinned by two substitutions: glutamine (859Q) and glutamic acid (862E) in the  $\text{Na}_v$  1.8 channel (Fig. 4; Rowe *et al.*, 2013). Interestingly, these substitutions do not occur in the original targeted  $\text{Na}^+$  channel ( $\text{Na}_v$  1.7) but in the previously non-target  $\text{Na}^+$  channel ( $\text{Na}_v$  1.8), an example of off-target repurposing (Rowe *et al.*, 2013). The negatively charged glutamic acid facilitates binding of the toxin to  $\text{Na}_v$  1.8 channels, inhibiting the transmission of pain signals by the neuron, inducing an analgesic effect (Rowe *et al.*, 2013). The pallid bat (*Antrozous pallidus*) also preys on bark scorpions (Bell, 1982; Johnston & Fenton, 2001; Lenhart, Mata-Silva & Johnson, 2010) and shows resistance to the venom of the Arizona bark scorpion (*Centruroides sculpturatus*; Hopp *et al.*, 2017). However, the unknown mechanism of resistance appears to be different; the pallid bat does not possess the resistant genotype observed in the grasshopper mouse (Fig. 4; Hopp *et al.*, 2017). This highlights that similar selection pressures do not always stimulate convergence at the molecular level.

### (6) Guanidinium toxins

Guanidinium toxins, including tetrodotoxin (TTX) and saxitoxin (STX), are alkaloids that bind to the outer pores of voltage-gated  $\text{Na}^+$  channels ( $\text{Na}_v$ ) on excitable tissues, causing muscle paralysis and even death (Duran-Riveroll & Cembella, 2017). Animals that deploy these toxins for defence presumably sequester them from their diet or produce them by means of symbiotic microorganisms (Hwang *et al.*, 1989; Duran-Riveroll & Cembella, 2017). Guanidinium toxins are synthesised by several species of bacteria and then enter food webs, where they are assimilated by a wide variety of organisms (Miyazawa & Noguchi, 2001). Resistance against TTX and STX is attributed to amino acid substitutions in the  $\alpha$ -subunit of skeletal muscle-type ( $\text{Na}_v$  1.4) and neuronal-type ( $\text{Na}_v$  1.6 and  $\text{Na}_v$  1.7) voltage-gated  $\text{Na}^+$  channels (Soong & Venkatesh, 2006). These substitutions alter TTX binding by changing the outer pore structures and/or by altering the electrostatic interaction between TTX and the pore residues (Geffeney *et al.*, 2005; Feldman *et al.*, 2012). Guanidinium toxin resistance has evolved independently in phylogenetically distinct animal lineages (Fig. 5).

Resistance against TTX has evolved convergently in at least six colubrid snake lineages (Feldman *et al.*, 2012). TTX-resistance is underpinned by numerous substitutions that decrease the binding affinity of TTX with the  $\text{Na}_v$  1.4 channel (Fig. 5). Among these colubrid snakes, North American garter snakes (*Thamnophis* spp.) are one of the best-



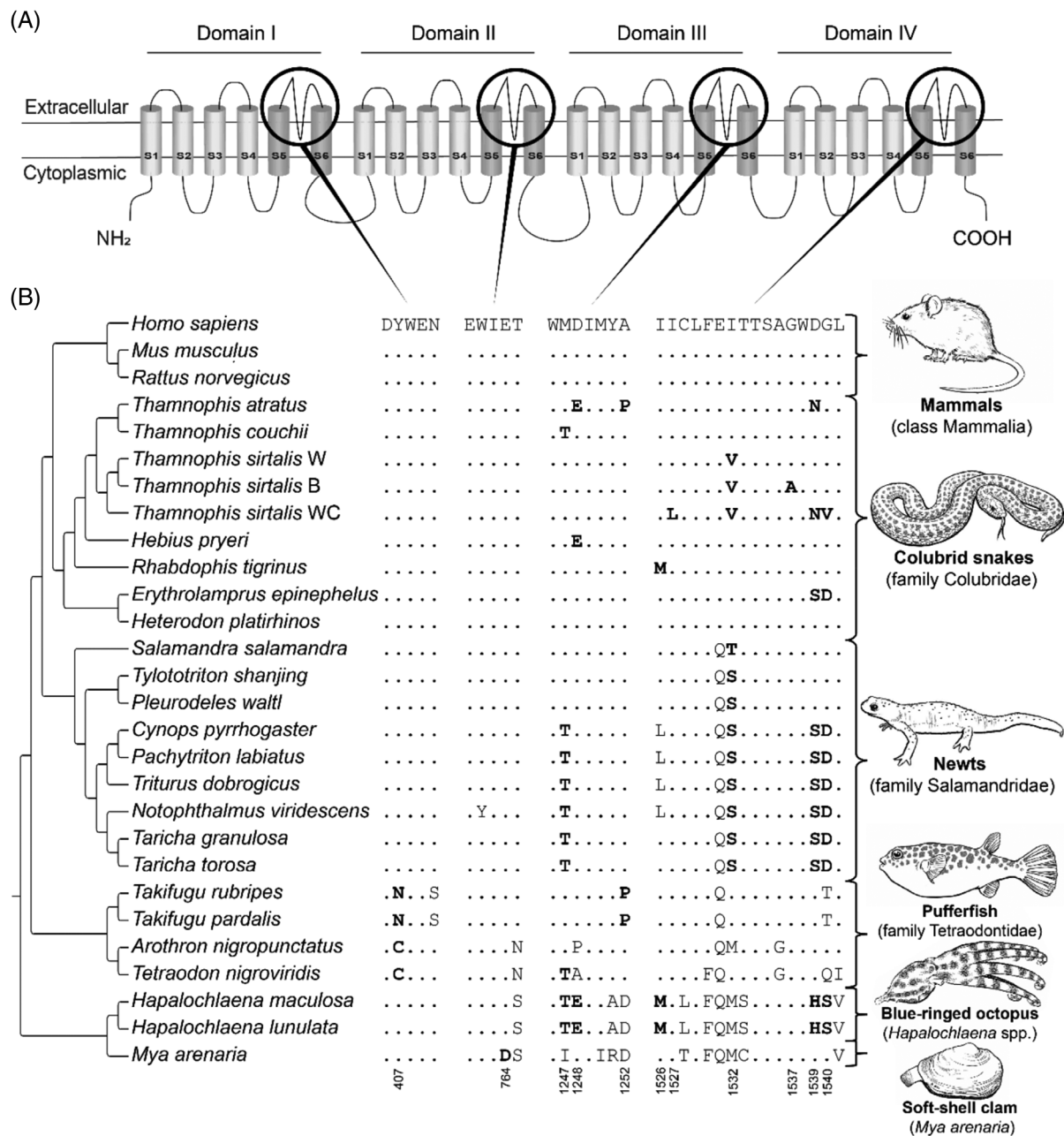


**Fig. 4.** Resistance against pain-inducing scorpion venom in grasshopper mouse (*Onychomys torridus*). (A) Protein topology of voltage-gated Na<sup>+</sup> channel (Na<sub>v</sub> 1.8). The black circle indicates the outer pore associated with scorpion-venom binding in the Na<sub>v</sub> 1.8 channel. Structure based on Shen et al. (2017). (B) Partial sequence alignment of the outer pore of the α-subunit of domain II of the Na<sub>v</sub> 1.8 channel. The reference amino acid sequence is from humans (*Homo sapiens*) and differences from this sequence are displayed for all other species. Substitutions associated with resistance are highlighted in bold. Tree topology based on TimeTree.org (Kumar et al., 2017). For sequence accession numbers, see Table S6.

studied examples of TTX resistance (Brodie, 1990; Geffeney, Brodie & Ruben, 2002; Brodie III et al., 2005; Geffeney et al., 2005; Feldman et al., 2009, 2010). Interestingly, resistance has evolved independently among species as well as within one species, as observed in distinct populations of the common garter snake (*T. sirtalis*; Fig. 5; Geffeney et al., 2002, 2005), highlighting the ecological importance of this resistance trait. Common garter snakes also evolved TTX resistance in two additional Na<sub>v</sub> paralogs: Na<sub>v</sub> 1.6 and Na<sub>v</sub> 1.7 (McGlothlin et al., 2014). These paralogs are exclusively expressed in the peripheral nervous system, which is frequently exposed to TTX. By contrast, the Na<sub>v</sub> paralogs in the central nervous system (Na<sub>v</sub> 1.1–1.3), which are not exposed to TTX, lack any resistance modifications (McGlothlin et al., 2014). The resistant Na<sub>v</sub> paralogs (Na<sub>v</sub> 1.6 and Na<sub>v</sub> 1.7) were present in the common ancestor of all snakes – which initially allowed predation on toxic prey. Subsequently, this facilitated the evolution of resistance at the skeletal muscle Na<sub>v</sub> 1.4 channel (McGlothlin et al., 2016). This resistance in garter snakes is tightly linked to predation on Pacific newts (*Taricha* spp.) that deploy TTX on their skin for defence against predators (Brodie III & Brodie Jr., 1999; Williams, Brodie & Brodie, 2004; Hanifin & Gilly, 2015). Additionally, several geographically widespread colubrids (e.g. *Hebius* sp., *Rhabdophis* sp., and *Erythrolamprus* sp.) have also evolved substitutions conferring TTX-resistance (Feldman et al., 2012), facilitating predation on distinct TTX-bearing amphibians (Fig. 5; Feldman et al., 2012). However, the eastern hognose snake (*Heterodon platirhinos*) displays high levels of TTX resistance, but lacks these Na<sub>v</sub> channel substitutions, suggesting a different mechanism of adaptation that remains to be elucidated (Fig. 5; Feldman et al., 2016).

Another example of TTX resistance is provided by newts (family Salamandridae). One or more substitutions associated with TTX resistance evolved among these amphibians, providing them with the ability to accumulate TTX from dietary sources or (symbiotic) microorganisms and to exploit it for defensive purposes (Hanifin & Gilly, 2015; Vaelli et al., 2020). Interestingly, all species show a single substitution (1532T/S) associated with TTX resistance in the Na<sub>v</sub> 1.4 channel (Fig. 5; Hanifin & Gilly, 2015). However, some newts show three additional substitutions (1247T, 1539S, and 1540D), conferring an increased level of TTX resistance and additionally deploying higher concentrations of TTX, and they are therefore more toxic to adversaries (Fig. 5; Hanifin & Gilly, 2015). Interestingly, a recent study revealed that TTX resistance in newts is not exclusively restricted to the Na<sub>v</sub> 1.4 channel but extends across the entire Na<sub>v</sub> gene family, which is driven by positive selection, relaxed constraints and gene conversion events (Gendreau et al., 2021).

The pufferfishes (family Tetraodontidae) are another group of animals that show TTX resistance. Different substitutions (407N/C, 1247T, and 1252P) evolved in Na<sub>v</sub> 1.4 channels conferring resistance to TTX (Fig. 5; Yotsu-Yamashita et al., 2000; Venkatesh et al., 2005; Jost et al., 2008). Pufferfish exploit their resistant genotype for different ecological functions, enabling them to prey on TTX-bearing species such as gastropods and echinoderms (Noguchi, Arakawa & Takatani, 2006b). Pufferfish thus accumulate high concentrations of dietary TTX in their organs, including liver, skin and ovaries (Noguchi et al., 2006b). Notably, captive pufferfish kept on a TTX-free diet are not toxic, but when fed a TTX-containing diet, they started to accumulate the toxins (Noguchi, Arakawa & Takatani, 2006a; Noguchi et al., 2006b). These accumulated TTXs are primarily

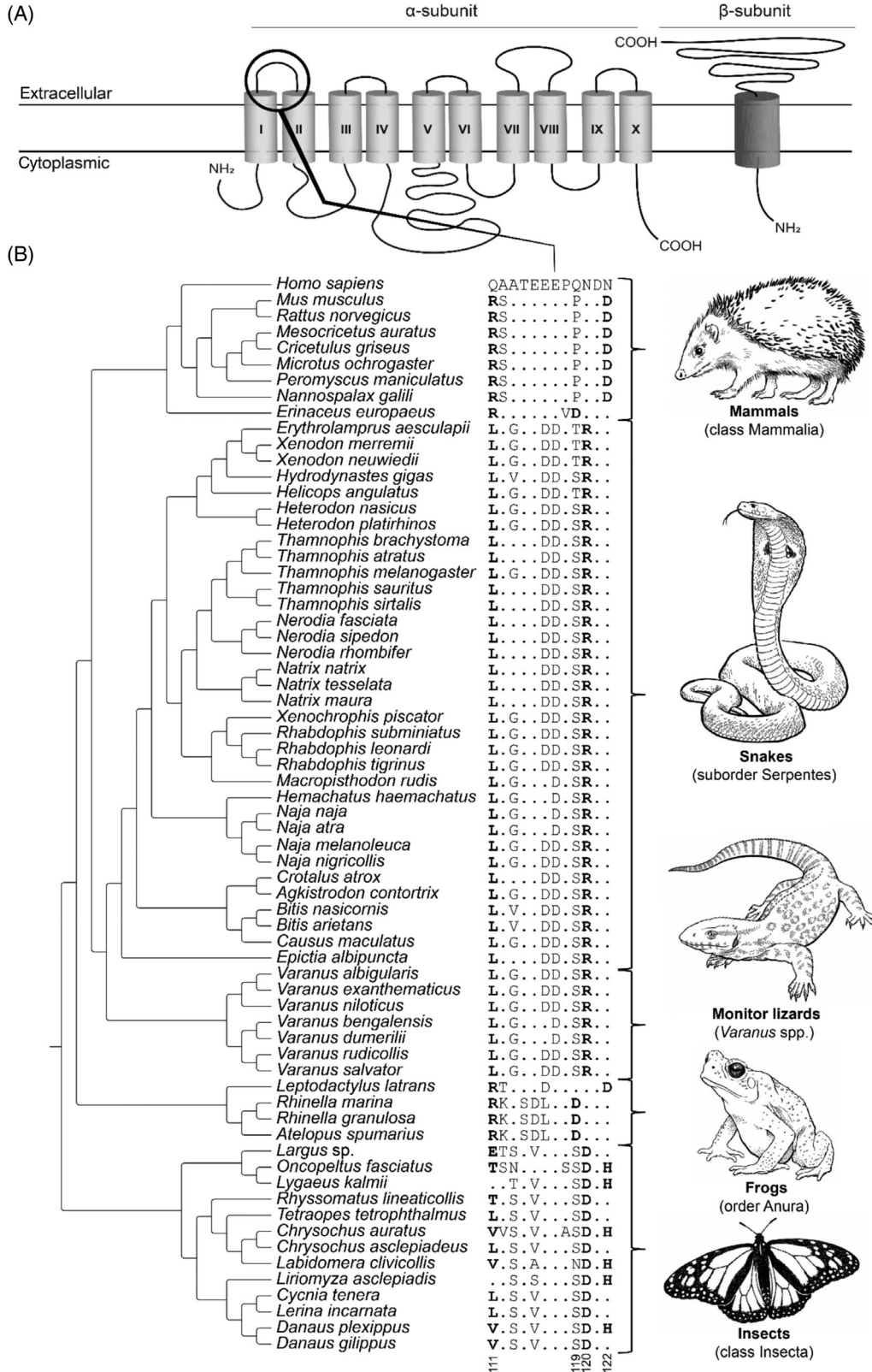


**Fig. 5.** Convergent evolution of guanidinium toxin resistance in animals. (A) Protein topology of the voltage-gated  $\text{Na}^+$  channel,  $\text{Na}_v 1.4$ . The black circles indicate the outer pores involved in guanidinium toxin binding. Structure based on Shen *et al.* (2017). (B) Partial sequence alignments of the outer pores of the voltage-gated  $\text{Na}^+$  channel  $\text{Na}_v 1.4$ . The reference amino acid sequence is from humans (*Homo sapiens*) and differences from this sequence are displayed for all other species. Substitutions associated with resistance are highlighted in bold, and their respective amino-acid positions are numbered based on  $\text{Na}_v 1.4$  from *Homo sapiens*. Tree topology based on TimeTree.org (Kumar *et al.*, 2017) and taxon-specific phylogenies (Geffeney *et al.*, 2005; Feldman *et al.*, 2012; Hanifin & Gilly, 2015). Key for the *Thamnophis sirtalis* populations: B, Benton County; W, Warrenton; WC, Willow Creek. For sequence accession numbers, see Table S7.

exploited to deter predators of this otherwise innocuous fish. When threatened, pufferfish inflate their body, erecting spines and releasing TTX from the skin, thereby deterring predators (Kodama, Ogata & Sato, 1985).

TTX resistance has also evolved in some invertebrates. The greater blue-ringed octopus (*Hapalochlaena lunulata*) and

southern blue-ringed octopus (*H. maculosa*) show five amino acid substitutions (1247T, 1248E, 1526M, 1539H, 1540S) associated with TTX resistance (Fig. 5; Geffeney *et al.*, 2019; Whitelaw *et al.*, 2020). Produced by symbiotic bacteria, this toxin is sequestered by these octopuses in their posterior salivary glands (Sheumack *et al.*, 1978), and in their skin and



**Fig. 6.** Convergent evolution of cardiac glycoside resistance in animals. (A) Protein topology of the sodium–potassium pump ( $\text{Na}^+/\text{K}^+$ -ATPase). The black circle indicates the H1–H2 extracellular loop that is involved in cardiac glycoside binding. Structure based on Bagrov, Shapiro & Fedorova (2009). (B) Sequence alignment of the H1–H2 extracellular domain of  $\text{Na}^+/\text{K}^+$ -ATPase. The reference amino acid sequence is from humans (*Homo sapiens*) and differences from this sequence are displayed for all other species. Substitutions associated with resistance are highlighted in bold. Tree topology based on TimeTree.org (Kumar *et al.*, 2017) and taxon-specific phylogenies (Dobler *et al.*, 2012; Ujvari *et al.*, 2015; Mohammadi *et al.*, 2016). For sequence accession numbers, see Table S8.

other organs, so TTX is assumed to play a role in both prey capture as well as predator deterrence (Yotsu-Yamashita, Mebs & Flachsenberger, 2007; Williams & Caldwell, 2009). Another example is the soft-shell clam (*Mya arenaria*), which has evolved resistance towards STX, a toxin produced by algal blooms of dinoflagellates (*Alexandrium* spp.; Bricelj *et al.*, 2005; Phillips *et al.*, 2018). Softshell clam populations frequently exposed to blooms have evolved resistance underpinned by an aspartic acid substitution (764D; Fig. 5; Bricelj *et al.*, 2005; Phillips *et al.*, 2018). This resistance increases their capacity to accumulate these toxins, thereby enhancing the risk of paralytic shellfish poisoning after human consumption (Bricelj *et al.*, 2005).

Another relatively underexplored strategy associated with guanidinium toxin resistance involves toxin-binding proteins. The best-studied example is saxiphilin, which is a soluble, well-characterised STX-binding protein (Yen *et al.*, 2019). Saxiphilin has particularly been studied in frogs (Mahar *et al.*, 1991; Yen *et al.*, 2019), but STX-binding activity has also been detected in other amphibians, reptiles, fish and some arthropods (Llewellyn, Bell & Moczydlowski, 1997). By contrast, STX-binding activity has not been detected in any mammal or bird (Llewellyn *et al.*, 1997). Other soluble guanidinium toxin-binding proteins have been identified in pufferfish (Yotsu-Yamashita *et al.*, 2000, 2010), crabs (Lin *et al.*, 2015) and gastropods (Hwang *et al.*, 2007; Takati *et al.*, 2007). Toxin-binding proteins have been identified in plasma, haemolymph, and a diverse range of tissues [e.g., liver, stomach, kidney and heart (Mahar *et al.*, 1991; Llewellyn *et al.*, 1997; Yotsu-Yamashita *et al.*, 2010)]. Remarkably, such toxin-binding proteins not only interact with STX and/or TTX but also with some other small-molecule neurotoxins [e.g. batrachotoxin and decahydroquinoline (Mahar *et al.*, 1991; Llewellyn *et al.*, 1997; Abderemane-Ali *et al.*, 2021; O'Connell *et al.*, 2021)]. Ultimately, toxin-binding proteins such as saxiphilin can provide resistance against guanidinium toxins and are proposed to play a role in sequestration mechanisms that may facilitate autoresistance.

### (7) Batrachotoxins

Batrachotoxin (BTX) is a steroidal alkaloid that targets voltage-gated Na<sup>+</sup> channels (Na<sub>v</sub>), causing irreversible depolarisation of muscles and nerves leading to paralysis, cardiac arrest, and other harmful effects (Albuquerque, Daly & Witkop, 1971). Resistance against BTX has evolved in poison dart frogs (family Dendrobatidae) and a few passerine bird species (e.g. *Pitohui* spp.).

Poison dart frogs are known for sequestering a variety of toxins including BTX (Daly, 1995). These frogs selectively sequester such toxins from their diet (Daly *et al.*, 1994; Clark *et al.*, 2005; Saporito *et al.*, 2007b), and these are then used for chemical defence that is generally accompanied by vivid aposematic warning patterns (Summers & Clough, 2001). The golden poison dart frog (*Phyllobates terribilis*), which contains high BTX concentrations in its tissues, shows BTX resistance

(Daly *et al.*, 1980). Partial sequencing of the skeletal muscle Na<sub>v</sub> 1.4 channel suggested convergence of several substitutions conferring BTX resistance in poison frogs (Tarvin *et al.*, 2016). Wang & Wang (2017) showed that one of these substitutions reduces BTX sensitivity and suggested that a single amino acid replacement confers BTX resistance. However, a more recent study failed to support the idea that Na<sub>v</sub> 1.4 channel mutations confer resistance in poison dart frogs (Abderemane-Ali *et al.*, 2021). It has been hypothesised that these frogs may have autoresistance based on toxin-binding proteins (Abderemane-Ali *et al.*, 2021).

Certain passerine birds native to New Guinea (*Pitohui* spp. and *Ifrita kowaldi*) are known to sequester dietary BTXs (Dumbacher *et al.*, 1992, 2004; Dumbacher, Spande & Daly, 2000). BTXs have been identified in significant concentrations across several organs (e.g. heart, skeletal muscle and liver), but the highest abundance is present in their skin and feathers (Dumbacher, Menon & Daly, 2009). Therefore, BTX is likely utilised for chemical defence against ectoparasites and/or predators (Dumbacher, 1999; Dumbacher *et al.*, 2000). Despite the high concentrations found in these passerine birds, there are no resistance-related modifications in the Na<sub>v</sub> channels (Na<sub>v</sub> 1.4 and Na<sub>v</sub> 1.5, respectively), which could suggest a comparable strategy using toxin-binding proteins as proposed in poison dart frogs (Abderemane-Ali *et al.*, 2021).

### (8) Cardiac glycosides

Cardiac glycosides (e.g. cardenolides and bufadienolides) are steroidal compounds that cause cardiotoxicity by inhibiting the sodium–potassium pump (Na<sup>+</sup>/K<sup>+</sup>-ATPase; Schoner, 2002). A variety of animals and plants exploit cardiac glycosides as defensive poisons to deter potential predators (Botelho *et al.*, 2019). As a response, many animals have evolved resistance underpinned by substitutions in the H1–H2 extracellular domain of the Na<sup>+</sup>/K<sup>+</sup>-ATPase (Ujvari *et al.*, 2015; Karageorgi *et al.*, 2019). These substitutions are predominantly characterised by the replacement of neutral amino acids with charged amino acids, causing a reduction in binding affinity of cardiac glycosides (Ujvari *et al.*, 2015). Cardiac glycoside resistance has evolved across the animal kingdom (Fig. 6).

Cardiac glycoside resistance evolved at least twice in mammals (Fig. 6). The European hedgehog (*Erinaceus europaeus*) shows two substitutions (111R and 119D), facilitating predation on cardiac glycoside-containing toads, and could also be associated with the hedgehog's habit of anointing itself with the toad toxins (Brodie, 1977; Ewert & Traud, 1979; Ujvari *et al.*, 2015). Also, some rodents (Muroidea) show resistance characterised by two amino acid substitutions (111R and 122D) – most likely associated with feeding on bufonid toads, insects or plants that contain cardiac glycosides (Ujvari *et al.*, 2015).

Reptiles of the order Squamata have convergently evolved resistance mediated by identical substitutions in their Na<sup>+</sup>/K<sup>+</sup>-ATPase (111L and 120R; Fig. 6; Ujvari *et al.*, 2015; Mohammadi *et al.*, 2016, 2018). This resistant genotype is widespread

across the snake phylogeny, and likely contributes to their ability to prey on toads (Ujvari *et al.*, 2015; Mohammadi *et al.*, 2016). Interestingly, keelback snakes (*Rhabdophis* spp.) not only consume toxin-bearing amphibians, but they also sequester these toxins in their nuchal glands for antipredator defence (Hutchinson *et al.*, 2007; Mori *et al.*, 2012). Additionally, varanid lizards (*Varanus* spp.) native to Africa and Asia that occasionally feed on toxin-bearing toads also have evolved this resistant genotype (Ujvari *et al.*, 2013). However, their Australian congeners, which originally did not share their habitat with bufonid toads, were found to have a reversal of this genotype (Ujvari *et al.*, 2013, 2015). This lack of resistance in Australian varanid lizards resulted in major population declines after the introduction of the invasive cane toad (*Rhinella marina*), on which they occasionally predate (Madsen & Ujvari, 2009; Jolly, Shine & Greenlees, 2015).

Amphibians of the order Anura have convergently evolved resistance towards cardiac glycosides on two occasions (Ujvari *et al.*, 2015). Bufonid toads (family Bufonidae) evolved resistance under strong positive selection based on two substitutions in Na<sup>+</sup>/K<sup>+</sup>-ATPase (111R and 119D; Fig. 6) – presumably enabling the synthesis of their cardiac glycosides which they exploit to deter aggressors (Moore *et al.*, 2009; Ujvari *et al.*, 2015). By contrast, the South American spotted grassfrog (*Leptodactylus latrans*) evolved two substitutions in Na<sup>+</sup>/K<sup>+</sup>-ATPase (111R and 122D; Fig. 6), allowing them to feed upon otherwise chemically protected bufonid prey (Ujvari *et al.*, 2015; Mohammadi *et al.*, 2021).

Another example of resistance in invertebrates is provided by insects (class Insecta). At least 21 lineages of insects have independently evolved the ability to feed on plants that contain cardiac glycosides and to sequester those toxins (Karageorgi *et al.*, 2019). These feeding habits are facilitated by toxin resistance underpinned by combinations of two or three substitutions at residues 111, 120 and/or 122 in Na<sup>+</sup>/K<sup>+</sup>-ATPase (111E, 111T, 111L, 111V, 120D, and/or 122H; Fig. 6; Holzinger, Frick & Wink, 1992; Dobler *et al.*, 2012; Zhen *et al.*, 2012; Ujvari *et al.*, 2015; Karageorgi *et al.*, 2019).

### (9) Epibatidine

Epibatidine is an alkaloid toxin that targets neural-type nicotinic acetylcholine receptors (nAChRs), causing muscle paralysis (Tarvin *et al.*, 2017). This rarely observed form of resistance has evolved independently in distinct poison dart frog lineages. Poison dart frogs are known to exploit multiple toxins, including epibatidine, for chemical defence (Daly, 1995). Three distinct dendrobatid frog clades (*Oophaga* spp., *Ameerega* spp., and *Epipedobates* spp.) have convergently evolved resistance mediated by a single amino acid replacement to a cysteine in the  $\beta$ 2 subunit of neural-type nAChR (108C; Fig. 7; Tarvin *et al.*, 2017). The cysteine residue has a sulphur-containing side chain that is bulkier compared to the naïve serine residue. This substitution occurs at a key position for epibatidine binding and is therefore hypothesised to alter the epibatidine–receptor interaction (Tarvin *et al.*, 2017). Additionally, another amino acid replacement to a valine (118V) in the *Ameerega* lineage has also been shown to

reduce epibatidine binding (Fig. 7; Tarvin *et al.*, 2017). The resistant phenotype might allow storage of epibatidine in the granular skin glands, which subsequently can be used for deterring adversaries.

## III EVOLUTIONARY IMPLICATIONS OF TOXIN RESISTANCE

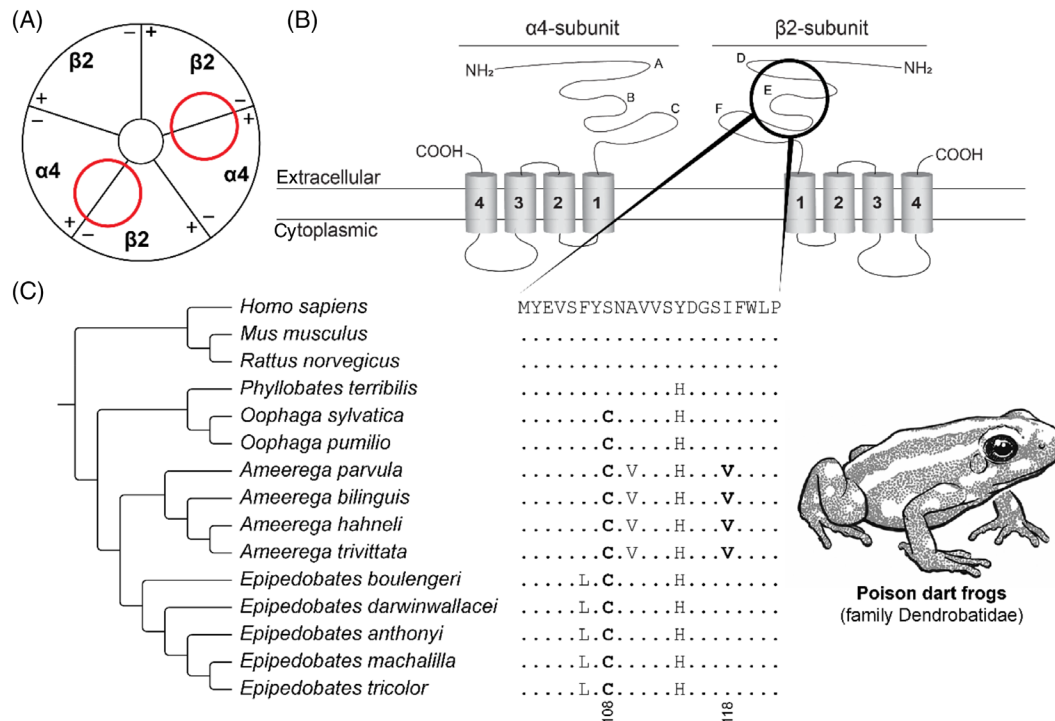
### (1) When does toxin resistance evolve?

Prey and predator inevitably exert reciprocal selection pressures on each other. A prey species is under selection to avoid capture, whereas a predator is under selection to acquire the energy resources contained in the prey. In any predator–prey relationship involving a poisonous or venomous participant, this will translate into selection to evade these toxic armaments. Given sufficient reciprocal selection, this can in turn trigger an evolutionary response in the toxic participant to maintain the effectiveness of its weaponry, potentially leading to an evolutionary arms race.

The intensity and symmetry of selective forces between prey and predator are highly variable, depending on the importance of the prey species as a resource to the predator, and the importance of the predator as a cause of loss in fitness to the prey. For example, as we discussed above, some animals show a reversal of their resistant genotype in the absence of their toxic counterparts (Ujvari *et al.*, 2015; Khan *et al.*, 2020).

Life-history theory predicts that toxin resistance is most likely to evolve when the poisonous or venomous opponent exerts strong selection, whether as prey or as predator. In predators of toxic prey, resistance is most likely to evolve when the predator is under strong selection to exploit an abundant but toxic food source. Examples include many reptiles that prey on toxic amphibians (Feldman *et al.*, 2012; Ujvari *et al.*, 2015), mammalian mesopredators feeding on venomous snakes (Drabeck *et al.*, 2015, 2020) and grasshopper mice eating bark scorpions (Rowe *et al.*, 2013). In prey species subject to predation by a venomous predator, prey resistance will most likely evolve if the predator is an important overall cause of mortality, e.g. sea kraits (*Laticauda* spp.), a lineage unrelated to other sea snakes, preying on moray eels (*Gymnothorax* spp. Heatwole & Poran, 1995) and rattlesnakes preying on North American ground squirrels and other rodents (de Wit, 1982; Holding *et al.*, 2016a; Gibbs *et al.*, 2020). In the latter example, reciprocal adaptation has been demonstrated, as rattlesnakes match their venom phenotype to the resistance profile of local prey to retain a selective advantage (Holding *et al.*, 2016a; Margres *et al.*, 2017), contrary to the predictions of the ‘Life-Dinner Principle’ (Dawkins *et al.*, 1979). That principle implies asymmetric selection, such that the prey is under greater selection pressure to escape capture, compared to the selection pressure on the predator to secure a meal.

By contrast, life-history theory predicts that resistance is unlikely to evolve when selection pressure is low, for example:



**Fig. 7.** Convergent evolution of epibatidine resistance in poison dart frogs. (A) Schematic representation (based on Kini, 2019) of the neural-type nicotinic acetylcholine receptor [nAChR;  $(\alpha 4)_2(\beta 2)_3$ ]. Red circles indicate the ligand-binding domain of epibatidine in the nAChR. (B) Protein topology of the  $\alpha 4$ -subunit and the  $\beta 2$ -subunit of the neural-type nAChR. A–F indicate the loop structures at the extracellular domain in the respective subunits (Rahman *et al.*, 2020). The black circle indicates the E-loop involving the ligand-binding domain of epibatidine. (C) Sequence alignment of the  $\beta 2$ -nAChR ligand-binding domain. The reference amino acid sequence is from humans (*Homo sapiens*) and differences from this sequence are displayed for all other species. Substitutions associated with resistance are highlighted in bold. Tree topology based on Tarvin *et al.* (2017). For sequence accession numbers, see Table S9.

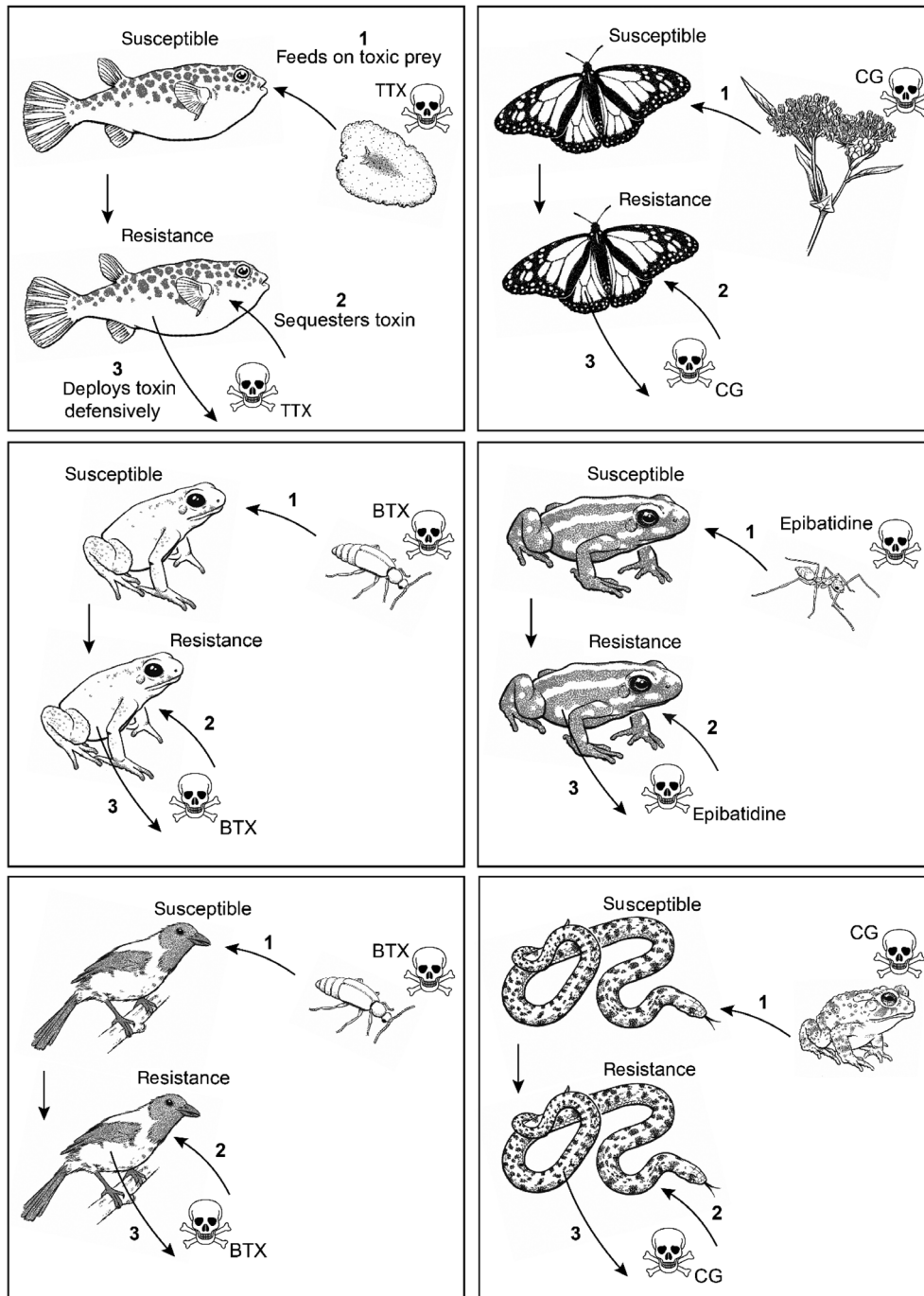
(i) when predation by a venomous predator is a relatively unimportant selective force for the prey because of the scarcity of encounters; (ii) a short temporal window of exposure exists (Marques *et al.*, 2012); or (iii) when behavioural avoidance of toxic prey is more advantageous than evolving resistance (Smith, 1977; Brodie III, 1993; Portugal *et al.*, 2016); see also Fig. 3).

Finally, it is also possible that resistance is most likely to evolve in situations where incremental increases in resistance confer an increasing selective advantage. Relatively low-level resistance could be adaptive where prey toxicity varies geographically (Feldman *et al.*, 2012). It could also be adaptive where different life stages differ in their toxin content, as appears to be the case in cane toads (*Rhinella marina*), for example (Hayes *et al.*, 2009). Finally, low-level resistance could be adaptive where partial failure of predatory envenomation is common, as seen in venomous snakes (Whitford *et al.*, 2019). In summary, resistance is seen in many diverse ecological contexts and can be interpreted under a range of evolutionary scenarios. Despite the complex routes towards resistance, a few outcomes are repeatedly seen in unrelated lineages.

## (2) Competing selection pressures and convergent evolution

Evolutionary trade-offs usually come with a fitness disadvantage (Brodie III & Brodie Jr, 1999; Blanchard & Moreau, 2017; Hague *et al.*, 2018). It is important that resistance modifications do not disrupt the physiology of the resistant animal. Therefore, there is a trade-off between a functional target (e.g. binding site of the endogenous ligand) and the modifications enhancing toxin resistance. The emergence of similar adaptations is likely mediated by constraints on a functional target when subjected to similar selection pressures. Some examples of this can be seen across the animal kingdom.

Poison dart frog clades convergently evolved an identical substitution conferring epibatidine-resistance, causing a decrease in acetylcholine sensitivity. As a result, this was then compensated by additional substitutions to maintain the receptor function (Tarvin *et al.*, 2017). A similar phenomenon can be observed in  $\alpha$ -neurotoxin resistance. Multiple substitutions convergently evolved to reduce  $\alpha$ -neurotoxin binding but without compromising the amino acid residues vital for acetylcholine binding (Barchan *et al.*, 1992; Khan *et al.*, 2020). Similar convergent adaptations are found in multiple



**Fig. 8.** Hypothesised convergent evolutionary scenarios for autoresistance in poisonous animals. It is generally assumed that autoresistance is a self-protection phenomenon. Here, we propose a three-step evolution scenario for the origins of autoresistance: (1) predator resistance, followed by (2) sequestration of the toxin by the predator, and (3) exploitation of the toxin for defence. As this figure indicates, a similar three-step process can be seen in diverse lineages, suggesting evolutionary convergence. The displayed examples include (A) pufferfish (family Tetraodontidae) feeding on TTX-bearing flatworms, gastropods and echinoderms, (B) herbivorous insects feeding on CG-containing plants, (C, D) poison dart frogs (family Dendrobatidae) feeding on toxic arthropods, (E) pitohui birds (*Pitohui* spp.) feeding on (among others) BTX-bearing melyrid beetles, and (F) keelback snakes (*Rhabdophis* spp.) feeding on CG-bearing anuran amphibians. BTX, batrachotoxin; CG, cardiac glycosides; TTX, tetrodotoxin.

distinct colubrid snakes showing tetrodotoxin resistance. This trait is mediated by a functional trade-off between ion channel function and tetrodotoxin insensitivity (Lee *et al.*, 2011; Feldman *et al.*, 2012). Cardiac glycoside resistance consistently evolved many times by two or three substitutions (respectively positioned at 111, 119, 120 or 122) – suggesting that these widespread genotypes also are constrained (Dobler *et al.*, 2012; Ujvari *et al.*, 2015; Karageorgi *et al.*, 2019).

In summary, toxin resistance shows fascinating examples of non-random and deterministic evolution mediated by constraints on sequence plasticity, while retaining receptor functionality. Thus, there may be a limited number of functional amino acid substitutions that reduce the binding affinity of toxins, even when different species are under similar selection pressures. This limited number of functional solutions available for adaptive evolution results in repeated funnelling of the same molecular pathway, leading to convergence.

### (3) Origins of autoresistance in poisonous animals

Some animals are resistant to their own toxins, referred to as autoresistance. Venomous animals likely evolved resistance both for the prevention of self-venomation (autoresistance) and for defence against other venomous animals (see Section II.(4)). However, we suggest that this is a much more complicated evolutionary scenario in the case of poisons (e.g. tetrodotoxin, cardiac glycosides, batrachotoxin, epibatidine) – which has already been partially touched upon in previous literature (Saporito *et al.*, 2012; Santos, Tarvin & O'Connell, 2016). We propose a scenario in which there was a three-step evolution of resistance across phylogenetically distinct poisonous animals: (i) predator resistance, followed by (ii) sequestration of the toxin by the predator, and finally (iii) exploitation of the toxin for defence (Fig. 8).

Over the course of evolution, predation on a toxic species leads to frequent exposure to one or more specific toxins through generalised trophic interactions. In most cases, naïve predators feeding on highly toxic prey (such as TTX-containing newts) are rapidly eliminated, with negative selection on the wild type thus favouring toxic prey avoidance. However, if variants that are capable of tolerating potent toxins exist in the population, then positive selection should favour the resistant phenotype, as this allows the predator to capitalise on abundant, often underutilised prey species. This then provides an evolutionary selection pressure on evolving, and maybe in some cases even maintaining, a less-susceptible genotype. Interestingly, several animals (e.g. poison dart frogs and pufferfish) have been shown to be toxic only after the ingestion of a toxic diet, indicating that the toxins originated exogenously (Noguchi *et al.*, 2006a; Saporito *et al.*, 2007a; Yotsu-Yamashita *et al.*, 2012). Subsequently, the resistant phenotype increases the accumulation capacity of the toxin compared to non-resistant animals (for example, as observed in clams; Bricelj *et al.*, 2005). This phenomenon is not likely to occur in predators of venomous animals due to the proteinaceous nature of venom toxins that are easily metabolised after ingestion. By contrast, poisons

(e.g. alkaloid or steroidal-based toxins) are less easily metabolised and thus accumulate in the body. Ultimately, this enabled the exploitation of the accumulated toxins for defensive purposes in poisonous animals (reviewed in Savitzky *et al.*, 2012). Therefore, we hypothesise that autoresistance primarily evolved as predator resistance rather than as an evolutionary driver itself, suggesting that multiple widespread taxa convergently evolved this three-step evolution of resistance (Fig. 8).

## IV CONCLUSIONS

(1) Toxin resistance is an adaptive response seen at many trophic levels, underscoring how relatively simple adaptations can lead to solutions to complicated problems. This review has shown that molecular adaptations conferring toxin resistance have evolved repeatedly in diverse animal lineages, highlighting how different selection pressures can result in convergence at the molecular level.

(2) Convergent evolution involving toxin resistance can be explained by functional constraints. These constraints are mediated by a trade-off between maintaining a functional molecular target and reducing toxin susceptibility. This trade-off limits the functional solutions available for adaptive evolution.

(3) We propose a novel scenario for the evolution of 'autoresistance' in poisonous animals. We suggest that autoresistance did not evolve primarily as a form of self-protection, but as a consequence of those animals feeding on toxic prey. This would imply that multiple diverse taxa convergently evolved this scenario.

(4) Similar selection pressures do not always lead to convergent molecular adaptations (as shown in certain bird, mammal, and reptile species). Molecular adaptations are only one of the ways in which organisms deal with toxins. We propose that some animals have elaborated or exploited existing behavioural or morphological traits, which may be exaptations, as alternative strategies to prevent intoxication in the first place.

(5) Toxin resistance is a phenomenon existing at the crossroads between molecular evolution, selection pressures and ecological interactions. The emergence of new or improved research technologies (e.g. -omics, functional assays and genetic modification techniques), combined with more robust ecological models, will provide opportunities to study novel and unexplored forms of resistance, as well as fundamental knowledge on how animals cope with direct or indirect exposure to toxic molecules in their environment. Toxin resistance is a compelling and multidisciplinary model system for studying evolutionary novelties with relevance in many branches of biology.

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## VII. Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Snake venom metalloproteinase inhibitors (SVMPIs) from mammalian serum, plasma or muscle tissue.

**Table S2.** Snake venom metalloproteinase inhibitors (SVMPIs) derived from snake serum, plasma or liver tissue.

**Table S3.** Phospholipase A<sub>2</sub> inhibitors (PLA<sub>2</sub>Is) from snake serum, plasma or liver.

**Table S4.** Overview of the key sites on the muscle-type nicotinic acetylcholine receptor (nAChR) and mutations that confer α-neurotoxin resistance.

**Table S5.** Accession numbers of sequences included in Fig. 2.

**Table S6.** Accession numbers of sequences included in Fig. 4.

**Table S7.** Accession numbers of sequences included in Fig. 5.

**Table S8.** Accession numbers of sequences included in Fig. 6.

**Table S9.** Accession numbers of sequences included in Fig. 7.

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