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Front cover: secretary bird attacking a model of a snake. Drawing by Sven Bellanger from an original photo by Jason Shallcross, with permission.

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Chapter 1. Introduction and Thesis Outline

In this chapter, we provide a brief overview of the issues to be addressed in this thesis, followed by an outline of the chapters.

A toxin is any substance of biological origin that can cause serious injury or death, if it enters the body of an organism, by disrupting normal physiological processes (Casewell, Wüster, Vonk *et al.*, 2013; Fry, Roelants, Champagne *et al.*, 2009). Venoms are cocktails of toxins that an animal injects into another species through a hollow fang or sting. Toxins and venoms may be used by animals to overcome their prey, or to protect themselves from attackers. Some amphibians secrete them on their skin to deter predators.

A few species exposed to toxins have molecular adaptations in the receptors targeted by toxins. These adaptations lead to changes in the receptor protein that prevent one or more toxins from binding to it. The animals that possess these changes are said to show some degree of 'resistance' to the toxin. Examples include the Asian mongoose which is resistant to the venom of the cobra (Barchan, Kachalsky, Neumann et al., 1992) and the North American garter snake, which is resistant to the tetrodotoxin of its salamander prey (Brodie, Ridenhour, Brodie et al., 2002; Geffeney, Fujimoto, Brodie et al., 2005; McGlothlin, Kobiela, Feldman et al., 2016).

The toxic animal may in turn evolve counter-measures to overcome that resistance. For example, snakes typically have dozens of toxins in their venom, possibly to target a range of different receptors and mechanisms. In any case, the give-and-take adaptation in predators and prey has been likened to a co-evolutionary 'arms race' (discussed by (Khan, Dashevsky, Kerkkamp *et al.*, 2020) .

Toxin resistance is not only of great intrinsic interest as a model of evolution, it also has important implications for wildlife conservation. For example, the Cane Toad, an introduced species in Australia, has decimated native fauna that are not resistant to the toad's cardiac glycoside toxins (Shine, 2010). Studying venom resistance genes may also have other important

applications. Many tens of thousands of people per year — including agricultural workers and other people in rural areas who cannot access antivenoms quickly, or cannot afford them — are killed or seriously injured in tropical countries from snake-bite (envenomation) (Chippaux, 1998; Kasturiratne, Wickremasinghe, de Silva *et al.*, 2008). When the money-earner in a family is killed or injured by a snakebite, the entire family may suffer severely; a single snakebite can therefore have a huge societal impact.

In this thesis, we have focussed on the nicotinic acetyl choline receptor (nAChR), the receptor targeted by snake α-neurotoxins. We have carried out fieldwork in Pakistan and Australia to harvest DNA from numerous vertebrate species, in order to look for resistance-associated molecular adaptations in the nAChR.

In Chapter 2, we have made a comprehensive review of the evolutionary processes and molecular mechanisms underlying the evolutionary arms race of toxin resistance in animals. We use a multidisciplinary approach to understanding toxin resistance by examining adaptations in the molecular targets of toxins; how these adaptations have evolved in different lineages; and the trophic interactions, among toxic and resistant species, that drive the evolution of resistance adaptations. This chapter considers resistance to snake venom α-neurotoxins, cardiac glycosides, guanidinium toxins and many others. The result is a unique database of α-neurotoxin receptor sequences in vertebrates.

Chapter 3 addresses the issue of whether lizards are resistant to cobra α-neurotoxin, in view of the fact that they are an important part of the diet of many venomous snakes. We made two principle studies: (i) a search form resistance-associated adaptations to α-neurotoxin in a wide range of lizard families including: Helodermatidae, Anguidae, Varanidae, Agamidae and Iguanidae; (ii) a functional toxicity assay using embryos of the central bearded dragon (*Pogona vitticeps*) the chicken (*Gallus gallus*) the three-spined stickleback (*Gasterosteus aculeatus*) and the zebrafish (*Danio rerio*). We chose these species in part because we and other had previously found

that the bearded dragon (Khan *et al.*, 2020) and stickleback (Gunasekaran, Sridhar, Suryanarayanan *et al.*, 2017) have a modified ligand-binding domain subunit of nAChR consistent with α-neurotoxin resistance. Our assay results show relatively high susceptibility to the toxic effects of cobra toxin in chicken and zebrafish. We discuss the possible putative adaptive role of this resistance in the species examined.

In **Chapter 4**, we have studied the ligand-binding domain of the α-subunit of the nAChR in a range of birds of prey because snake-eating (ophiophagy) is common among these birds. I did extensive fieldwork in Pakistan funded by a special grant from the Leiden University Funds. This work was in my home town, the city of Multan, as well as the Rohi desert Bahawalpur, Ali Pur, and Lahore. We found no evidence from sequencing the samples collected on these trips, of any modification in the α-subunit of the nAChR. My results lead me to suggest that birds of prey that eat snakes may rely on other types of defences against snakebites. Interestingly, the nAChR from two crocodile species were sequenced, and found to have resistance-related changes like those seen in some snake-eating mammals (Khan *et al.*, 2020).

In **Chapter 5**, we studied molecular adaptation of α-neurotoxin resistance in the ligand-binding domain of the α-subunit nAChR in 76 species of different snakes (Khan *et al.*, 2020). Of these sequences, 66 were generated *de novo* by us from DNA and tissue from collaborating institutions in the Netherlands, Australia, and the National Institute of Health, Islamabad, Pakistan. Previously, resistance had only been shown in the Egyptian Cobra (*Naja haje*) (Takacs, Wilhelmsen & Sorota, 2001) and the dice snake (*Natrix tessellata*) (Neumann, Barchan, Horowitz *et al.*, 1989). In this chapter, however, we find widespread convergent evolution of the N-glycosylation form of resistance in several snake subfamilies (Khan *et al.*, 2020). We hypothesise that these snakes have evolved the adaptation as a protection against predation by cobras, whereas in the cobra itself, it is thought to be a form of auto resistance. Signals-of-selection analyses showed several sites, within the ligand-binding domain, under significant positive selection.

Chapter 6 provides a summary of the thesis, and a discussion of its main findings and conclusions and a Dutch translation of the same summary and conclusions.

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Chapter 2: A Biological Arms Race: Animal Venoms, Resistance and Evolution

This chapter is primarily the work of the candidate. It is submitted for publication as: Jory van Thiel, Muzaffar A. Khan, Roel M. Wouters, Richard J. Harris, Nicholas R. Casewell, Bryan G. Fry, R. Manjunatha Kini, Stephen P. Mackessy, Freek J. Vonk, Wolfgang Wüster, and Michael K. Richardson (2021) *Biological Reviews* (under review). The candidate is joint first author (equal contribution) on that submission.

Abstract

Toxins are poisons of biological origin that cause disruption of physiological processes leading to incapacitation or death. Venoms are mixtures of peptide and protein produced in a venom gland and injected into the tissues of other animals via specialised structures such as fangs or stings. Some of the animals targeted by toxins and venoms have the ability to withstand their effects, a phenomenon known as resistance. At least three types of resistance are seen: (i) predator resistance, exemplified by in North American garter snakes that are resistant to tetrodotoxin in their prey, the rough-skinned newt; (ii) prey resistance, as in the mongooses, which are resistant to snake α-neurotoxins; and (iii) autoresistance, where a venomous animal is resistant to its own toxins, as exemplified by the Egyptian cobra. Venomous animals may, in parallel, evolve adaptations to overcome the resistance in their target species (more potent toxins, for instance). This reciprocal adaptation (co-evolution) in the toxic animal and its target species has been characterized as a co-evolutionary 'arms race'. Our main focus in this chapter are the molecular mechanisms of toxin resistance. We review studies on resistance to a wide range of toxin classes. Resistance strategies that we discuss include: modified transmembrane toxin-receptors, ion channels and serum factors (inhibitors). We also briefly consider non-molecular strategies (behavioural, cognitive, anatomical, etc.) for avoiding envenomation in the first place. We conclude that there is a great deal of work to be done on resistance, given the diverse and numerous animal toxins that are known.

Introduction

Biological toxins are poisonous molecules of biological origin, and are produced by animals, plants and many species of microorganisms (Fry, Roelants, Champagne *et al.*, 2009b). They include small molecules, peptides and proteins. Toxins have very high pharmacological potency and typically bind with high affinity to a particular molecular target. And so, when they enter the body of an animal, even in relatively small doses, they activate or disrupt normal physiological processes leading to incapacitation or death.

The physiological effects produced by a toxin depend on its molecular target but may include cell death, neurotoxicity, cardiotoxicity or other effects (Casewell, Wüster, Vonk *et al.*, 2013; Fry, Roelants, Champagne *et al.*, 2009a). Animals may use toxins offensively (to overpower their prey), or defensively (to deter predators or other attackers) (Casewell *et al.*, 2013; Schendel, Rash, Jenner *et al.*, 2019). In some cases, toxins are introduced into the tissue of the target animal by means of hollow teeth (fangs) or stings. In these cases, the injected toxin or toxin mixture is called a 'venom'.

Toxin resistance is the increased ability of an animal to survive the exposure to one or more toxins without being functionally affected. As a result, toxin resistance has evolved in at least three distinct ecological contexts (Figure 1) namely: *predator resistance*, where a predator is resistant to the toxins of its prey (Figure 1A-C); *prey resistance*, where the prey is resistant to the toxins of a predator (Figure 1D); or *autoresistance*, where an animal is resistant to its own toxins (Figure 1E).

In the case of most venomous snakes it is assumed that toxins are used primarily for prey capture. This is suggested by a study on a snake that evolved a habit of living largely on a diet of fish eggs; this species shows evolutionary degeneration of its venom delivery apparatus (venom gland and fangs) (Gopalakrishnakone & Kochva, 1990; Li, Fry & Kini, 2005; McCarthy, 1987). This suggests that the snake had previously used its venom exclusively for capturing living prey and not for defense. By contrast, spitting

cobras are clearly able to use their venom defensively, squirting it into the eyes of an attacker (Kazandjian, Petras, Robinson *et al.*, 2021).

Predator toxins have evolved to bind highly conserved protein targets in the prey (Takacs, Wilhelmsen & Sorota, 2001). The high potency of toxins is presumably due to strong positive selection acting over millions of years (Sunagar & Moran, 2015). One possible driver of this selection could be a need to incapacitate the target animal as quickly and effectively as possible (Casewell *et al.*, 2013; Fry *et al.*, 2009a). This scenario assumes that rapid incapacitation can mean the difference between life and death for the animal using the toxin. Rapid incapacitation of the prey might theoretically be advantageous for two reasons: (i) to prevent the prey from having time to attack the snake (ii) to prevent the prey from escaping. Although these are plausible scenarios, there are objections to them (R. M. Kini, *personal communication*). Often when snakes bite, they only inject a fraction of the total volume of venom available in the gland. Furthermore, their venom sometimes seems to have pharmacological potency far exceeding the apparent need (for example the venom of the Inland taipan (*Oxyuranus microlepidotus*) can kill 250,000 mice) (Broad, Sutherland & Coulter, 1979). This suggests that snakes already possess 'overkill', and so why would there be positive selection for further enhancement of toxin pharmacology? (Barlow, Pook, Harrison *et al.*, 2009; Sasa, 1999). A possible explanation is that a constant enhancement of toxin potency is needed to overcome continuously-evolving prey resistance, as in the 'arms-race' scenario (Duda & Palumbi, 1999a).

Toxins

Animal toxins are typically small molecules found, for example, on the skin surface of some amphibians as secretions. The origin of these toxins is not always known, but in at least some cases, are not synthesised by the toxic animal itself, but by some organism in its diet (such as a plant or microorganism). Several species of amphibians and fishes produce the small

molecule neurotoxins tetrodotoxin (TTX) or bufagenin (Brodie, 1990; Mackessy & Castoe; Ujvari, Casewell, Sunagar *et al.*, 2015). The presence of these toxins serves for protection against predators. Some snakes preying on toxic amphibians have evolved resistance to these toxins (Figure 1 (Brodie, 1990; Geffeney, Fujimoto, Brodie *et al.*, 2005; McGlothlin, Kobiela, Feldman *et al.*, 2016).

Prey and predator interaction against TTX is one of the examples often cited of an evolutionary arms race (Brodie, 1990; Geffeney *et al.*, 2005; McGlothlin *et al.*, 2016). Interestingly, in this example, both prey (*Taricha granulosa*) and predator (*Thamnophis sirtalis*) have developed resistance to TTX (Brodie, 1990; Geffeney *et al.*, 2005; McGlothlin *et al.*, 2016; Toledo, Hanifin, Geffeney *et al.*, 2016; Ujvari *et al.*, 2015; Venkatesh, Lu, Dandona *et al.*, 2005). The physiological action of TTX is to block the function of the sodium channel in neurons (Brodie, Hensel & Johnson, 1974; Kaneko, Matsumoto & Hanyu, 1997b). Bufagenin inhibits the activity of cardiac muscle Na^+/K^+-ATPase (Ujvari *et al.*, 2015). Resistance to bufagenin in some snakes, lizards and mammals is attributed to a mutation in the ATP1a3 gene coding for Na^+/K^+-ATPase (Mohammadi, Savitzky, Lohr *et al.*, 2017c; Ujvari *et al.*, 2015).

Venoms

Venom toxins not only incapacitate or kill the prey, they may also serve the important function of initiating the digestion of its tissues (Berthe, Westhoff & Bleckmann, 2013; Chippaux, Williams & White, 1991; Greene, 1983). The composition of snake venoms shows remarkable species variation (Casewell *et al.*, 2013). One very interesting finding has been that many rattlesnakes, and indeed also the spider *Cupiennius salei*, appear to be able to inject a volume of venom according to the size of the prey (HAYES, 1995; Malli, Kuhn-Nentwig, Imboden *et al.*, 1999; McCue, 2006).

Resistance

The term 'resistance' is used to describe the capacity of animals to endure the venomous effects of a toxin or venom without suffering serious harm (Edmunds, 1974). Resistance among reptiles and mammals, to different snake venoms, has been examined for many years (Allyn, 1937; Calmette, 1895). Toxin resistance is common in those animals who are liable to be frequently exposed to venomous or toxic animals, for example, when there is a predator prey-relationship and their territories overlap geographically (Biardi, Chien & Coss, 2006; Brodie Jr, Ridenhour, Brodie III *et al.*, 2002; Drabeck, Dean & Jansa, 2015; Geffeney *et al.*, 2005). The toxin-producing animal may, in turn, develop countermeasures to overcome prey resistance through adaptive mutation and toxin gene duplication (Benkman, Parchman, Favis *et al.*, 2003; Casewell *et al.*, 2013; Dawkins & Krebs, 1979; Duda & Palumbi, 1999b; Fry, Wüster, Kini *et al.*, 2003).

Animals use a variety of strategies to avoid being adversely effected by venoms or toxins (Khan, Dashevsky, Kerkkamp *et al.*, 2020). For examples of strategies of resistance, see Table 2; and for examples of selected molecular modifications relevant to this review, see Figure 2). These strategies include not only the molecular strategies that are the subject of my thesis, but might also include less obvious things such as the scaly skin on the legs of birds that might provide a physical barrier to envenomation, and the behavioural agility of mammals and birds (Figure 3) that allows them to avoid being bitten in the first place (Khan *et al.*, 2020).

The molecular mechanisms of resistance in the vertebrates against toxins offer a significant insight into the understanding of the evolutionary arms race (Geffeney *et al.*, 2005; Takacs *et al.*, 2001; Toledo *et al.*, 2016; Ujvari *et al.*, 2015; Venkatesh *et al.*, 2005). Moreover, inter-specific competition and a long-time presence of predator and prey in the same geographic area are factors that help drive the arms race (Williams, 2013). Among vertebrates, there are a small number of examples of such an arms race (Barchan, Kachalsky, Neumann *et al.*, 1992a; Barchan, Ovadia, Kochva *et al.*, 1995; Drabeck *et al.*, 2015; McGlothlin *et al.*, 2016; Voss & Jansa, 2012). Our aim

here is to review the literature relevant to toxin resistance in general, and the evolutionary arms race in particular, in the vertebrates.

Serum factors resistance against snake venom toxins

One cause of resistance to snake toxins is the presence of neutralising factors in the serum (Ovadia & Kochva, 1977). Thus, it has been reported that, in many families of the snakes, namely, Viperidae, Crotalidae, Elapidae and Colubridae as well as the hamster (*Mesocricetus aerates*) have humoral factors that neutralize *Vipera palaestinae* venom activity. *V. palaestinae* serum can neutralize its own venom neurotoxic and haemorrhagic activity (Ovadia *et al.*, 1977). Likewise, the serum of rattlesnakes (*Crotalus* sp.) and the Eastern king snake (*Lampropeltis getula*) are able to counteract the anti-haemorrhagic activity of *Crotalus* sp. venom (Moussatché & Perales, 1989).

Mammals such as the Californian beechey ground squirrel (*Spermophilus beecheyi*), and the Douglas ground squirrel (*Spermophilus beecheyi douglasii*) have a plasma protein called snake venom metalloprotease inhibitor (SVMPI) Table 1.

Table 1: Serum resistance factors in mammals.

Serum factor	**Species**	**References**
venom inhibitors (SVMPI)	ground squirrels (resistance to venom metalloprotease of the pacific and black diamond rattlesnake)	(Biardi, Coss & Smith, 2000)
venom inhibitors	Viriginia opossum (resistance to the venom of Brazilian pit vipers, eastern diamondback rattlesnake, timber rattlesnake, cottonmouth, Russell's viper, and monocled cobra)	(Catanese & Kress, 1993; Kilmon Sr, 1976; Moussatché *et al.*, 1989; Werner & Vick, 1977)

This neutralizes the venom metalloprotease activity of the pacific rattlesnake (*Crotalus viridis oreganus*) and the black diamond rattlesnake (*Crotalus oreganus helleri*) (Biardi, Ho, Marcinczyk *et al.*, 2011; Biardi *et al.*,

2006; Biardi *et al.*, 2000). The serum of the rock squirrel (*Spermophilus variegates*) acts specifically against the metalloprotease and haemolytic activity of venoms of *Crotalus* sp. (Biardi & Coss, 2011).

The plasma resistance factors against rattlesnake toxin in squirrels have evolved due to the presence of rattlesnakes in their home ranges. In contrast, squirrels which never encounter rattle snakes in their home ranges have no resistance factors against rattlesnake venom (Biardi *et al.*, 2006). The Virginia opossum (*Didelphis virginiana*) is extremely resistant to the venoms of the monocled cobra (*Naja kaouthia*) and a wide range of *Crotalus* spp. (Catanese *et al.*, 1993; Kilmon Sr, 1976; Moussatché *et al.*, 1989; Werner *et al.*, 1977). This resistance is due to a plasma proteins known as opossum serum α1-proteinase inhibitor (α1-PI) (Catanese *et al.*, 1993; Kilmon Sr, 1976; Moussatché *et al.*, 1989; Werner *et al.*, 1977). In the presence of α1-PI, the plasma protein serpin, a protease inhibitor, remains active and eventually inactivates venom metalloproteinase (Catanese *et al.*, 1993).

Lizards and Birds

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(Jones, Harris & Fry, 2021; Khan *et al.*, 2020). 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 adaptations (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 envenoming is comparable to what we propose for snake-eating birds (Figure 3). To explain this apparent paradox, we propose that a set of

morphological and behavioural traits in snake-eating birds prevent envenoming in the first place (Figure 3). This could also explain why these birds did not evolve any molecular adaptions, whereas other snake-eating lineages did (e.g., mongoose, honey badger; (Drabeck *et al.*, 2015; Khan *et al.*, 2020).Many birds prey on venomous snakes, including snake specialists such as the Secretary Bird (*Sagittarius serpentarius*), Snake Eagles (*Circaetus* spp.), and Seriemas (family Cariamidea; (Mori, Vyas & Upadhyay, 2017; Portugal, Murn, Sparkes *et al.*, 2016; Redford & Peters, 1986). Birds do not show any known resistance-related modifications associated with α-neurotoxins (Khan *et al.*, 2020). I will discuss lizards in more detail in Chapter 3, and birds in more detail in Chapter 4.

Resistance to snake α-neurotoxins

Snake α-neurotoxins target the highly conserved α-subunit of the nicotinic acetyl choline receptor (nAChR) of the neuromuscular junction (Asher, Lupu-Meiri, Jensen *et al.*, 1998b; Barchan *et al.*, 1995; Fry, Casewell, Wüster *et al.*, 2012; Kularatne & Senanayake, 2014; Takacs *et al.*, 2001). When the toxin binds, it causes paralysis of skeletal muscles (Barchan *et al.*, 1995). Elapid snakes that produce α-neurotoxins are resistant to their own toxins, and are therefore often cited as examples of autoresistance (Takacs *et al.*, 2001; Toledo *et al.*, 2016). Several mammals that attack and eat snakes have also evolved some kind of resistance to cobra venom Table 3 (Drabeck *et al.*, 2015). The resistant animals (cobras and mammals) show convergent evolution of molecular modifications in the α-subunit nAChR (Figure 2; (Drabeck *et al.*, 2015; Neumann, Barchan, Horowitz *et al.*, 1989; Ovadia *et al.*, 1977).

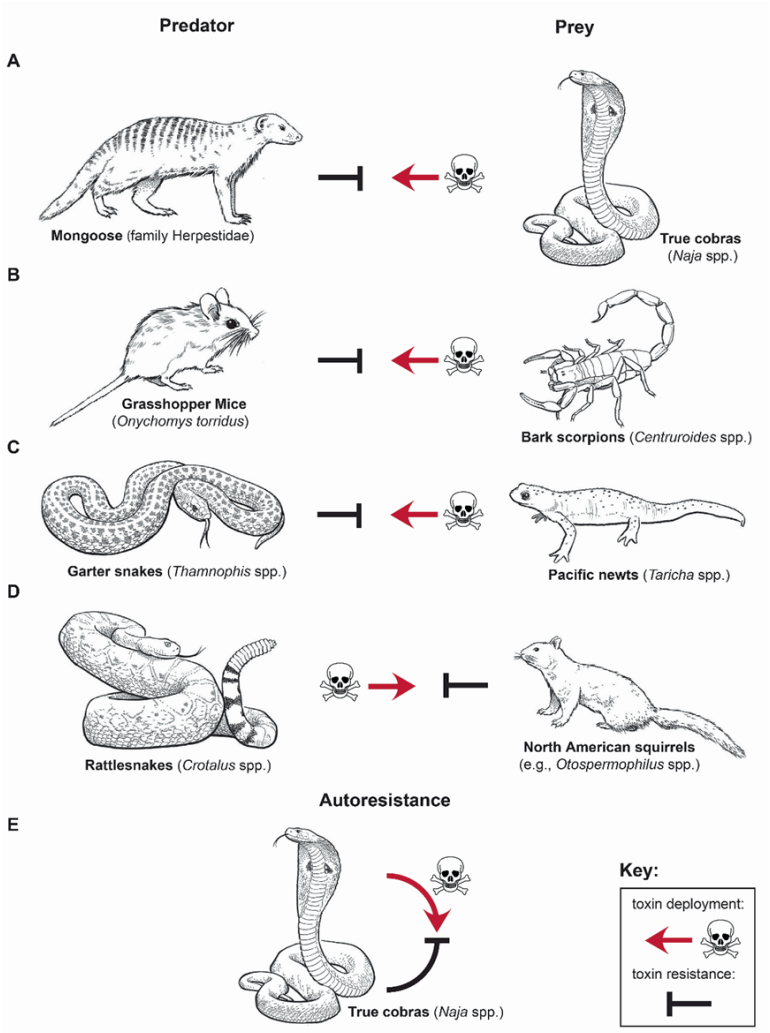

Figure 1. Classic examples of ecological contexts underpinning toxin resistance. A-C. predator resistance, where a predator is resistant to the toxins of its prey. A) the mongoose is known to predate on true cobras. B) The grasshopper mouse preys on bark scorpions. C) Garter snakes prey on toxic newts. 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, where an animal is resistant to its own toxins. The example shown here is of true cobras that show resistance to cobra α-neurotoxins. Drawings by Sven Ballinger, based on an original idea by Muzaffar Khan, Michael Richardson and Jory van Thiel.

Autoresistance to α-neurotoxin is seen in the Egyptian cobra (*Naja haje*) and is associated with the presence of a glycosylated asparagine (N) at position 189 (the position is numbered according to the human peptide; (Figure 2; (Asher, Lupu-Meiri, Jensen *et al.*, 1998a; Drabeck *et al.*, 2015). In the Egyptian mongoose the same change is seen at position 187 (Drabeck *et al.*, 2015). The European hedgehog the honey badger and the domestic pig all show a change of an aromatic residue to arginine (R) at position 187 (Asher *et al.*, 1998a; Drabeck *et al.*, 2015); this change was not present in a wide range of other mammals examined.

These findings are potentially interesting because of the popular (anecdotal) reports that the mongoose and cobra frequently fight each other; furthermore, the honey badger is reported to eat poisonous snakes (Begg, Begg, Du Toit *et al.*, 2003). Physiological assays have shown that the hedgehog is highly resistant to α-bungarotoxin (α-BTX, which is an α-neurotoxin), and that this is not due to the serum factors (Barchan *et al.*, 1995). It also has the same genetic modification as the honey badger (Barchan *et al.*, 1995; Drabeck *et al.*, 2015). The domestic pig probably also has an additional form or resistance: its tough skin (Table 2; (Drabeck *et al.*, 2015). In one study, α-BTX binding was examined using a site-specific antibody (Kachalsky, Aladjem, Barchan *et al.*, 1993; Mochly-Rosen & Fuchs, 1981). It was found that α-BTX binds to the α-subunit of the mouse nAChR, but does not bind to the mongoose α-subunit (Kachalsky *et al.*, 1993). This was confirmed in further studies which showed that α-BTX did not bind to the α-subunit of the cobra and mongoose nAChR; as mentioned above, these species have evolved a modification in the amino acids at positions 187 and 189 (Table 3; (Asher *et al.*, 1998a; Dellisanti, Yao, Stroud *et al.*, 2007; Kachalsky *et al.*, 1993).

Table 2. Details of strategies in vertebrates for avoiding the adverse effects of venoms or toxin. The strategies include resistance of various types, and various means of avoiding envenomation.

Ecological context	**Strategy**	**Examples**	**References**
autoresistance	target-site modification leading to reduced sensitivity	cobra exposure to its own α-neurotoxin	(Takacs *et al.*, 2001)
	target-site modification leading to reduced sensitivity	newt resistance to tetrodotoxin (TTX)	(Brodie, 1990; Geffeney, Brodie & Ruben, 2002; Kaneko, Matsumoto & Hanyu, 1997a).
	target-site modification leading to reduced sensitivity	puffer fish to bacterial TTX	(Soong & Venkatesh, 2006; Venkatesh *et al.*, 2005)
	target-site modification leading to reduced sensitivity	soft shell clam to bacterial TTX	(Bricelj, Connell, Konoki *et al.*, 2005; Soong *et al.*, 2006; Wiese, D'Agostino, Mihali *et al.*, 2010)
predator resistance	target-site modification leading to reduced sensitivity; also, physical avoidance (thick skin)	domestic pig, resistance to cobra venom	(Drabeck *et al.*, 2015)
	target-site modification leading to reduced sensitivity	garter snake, resistance to newt TTX	(Geffeney *et al.*, 2002)
	target-site modification leading to reduced sensitivity	mongoose, resistance to cobra α-neurotoxin	(Barchan *et al.*, 1992a)

	target-site modification leading to reduced sensitivity	honey badger, resistance to cobra α-neurotoxin	(Drabeck *et al.*, 2015)
	target-site modification leading to reduced sensitivity	African and Asian varanid lizards, resistance to cane toad bufagenins	(Ujvari *et al.*, 2015)
	target-site modification leading to reduced sensitivity	cobra, forest cobra, rhinoceros viper, resistance to cane toad bufagenins	(Ujvari *et al.*, 2015)
	target-site modification leading to reduced sensitivity	European hedgehog and muroid rodents, resistance to cane toad bufagenins	(Ujvari *et al.*, 2015)
	off-target repurposing	grasshopper mice, resistance to bark scorpion venom	(Rowe, Xiao, Rowe *et al.*, 2013b)
	unknown	pallid bat, resistance to bark scorpion venom	(Hopp, Arvidson, Adams *et al.*, 2017)
prey resistance	unknown	African plated lizard, eastern glass lizard and rainbow lizard, resistance to cobra α-neurotoxin and α-bungarotoxin	(Burden, Hartzell & Yoshikami, 1975)
	unknown	*Egernia cunninghami*, *E. striolata* and *E. whitii* and *Ctenotus robustus,* resistance to venom of Australian tiger snake, the eastern brown snake and the death adder	(Minton Jr & Minton, 1981)
behavioural avoidance	physical and behavioural avoidance (strategies to avoid envenomation: scaly or feathered skin; superior intelligence and agility)	snake-eating (ophiophagous) birds , protection against snake envenomation	(Khan *et al.*, 2020)
	aversive behaviour	*Pseudechis porphyriacus* toward cane toad bufagenins	(Phillips & Shine, 2006)
	aversive behaviour	*Heloderma horridum* toward venomous and non-venomous snakes	(Balderas-Valdivia & Ramírez-Bautista, 2005)
Other	Batrachotoxin resistance	Pitohui resistance to batrachotoxins of choresine, monarch butterflies and various plants	(Dumbacher, Menon & Daly, 2009; Dumbacher, Wako, Derrickson *et al.*, 2004).

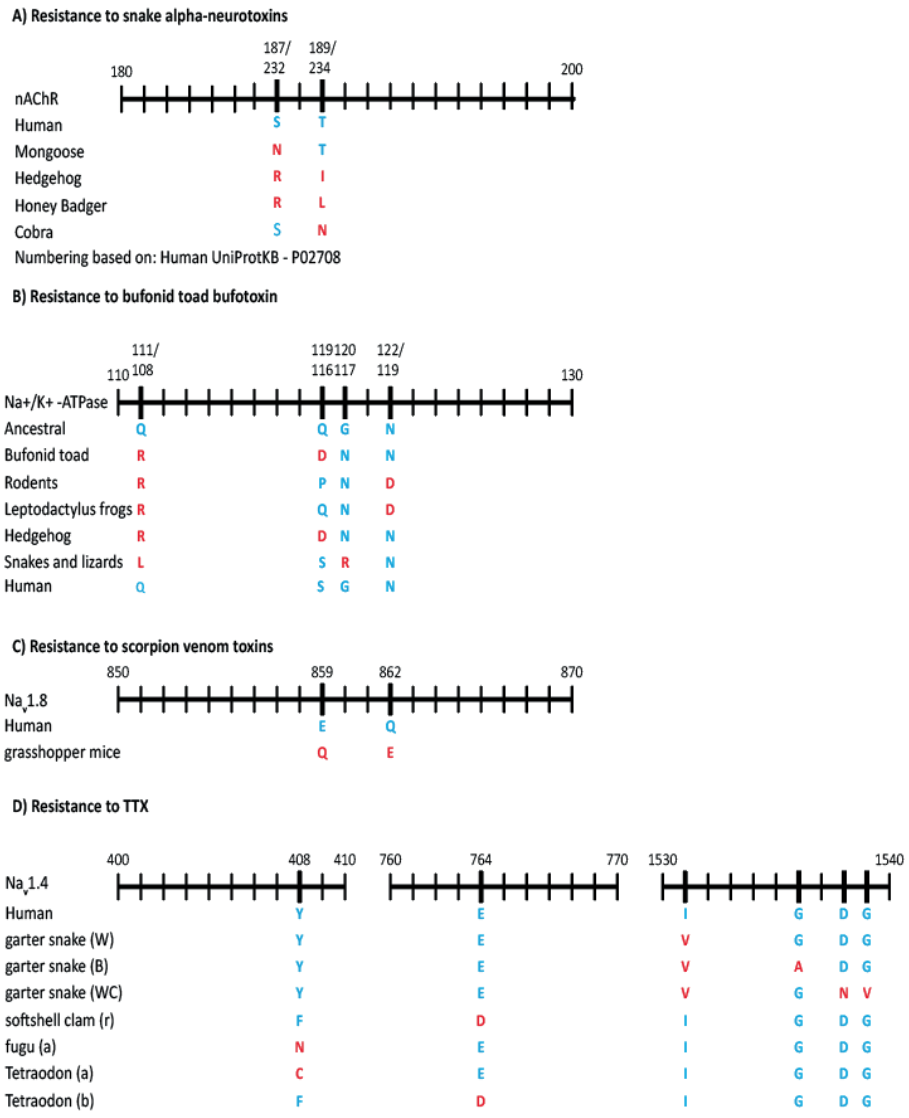

Figure 2. Examples of changes in amino acid sequence related to venom or toxin resistance. Figure by Muzaffar Khan and Harald Kerkkamp; style of presenting sequences is based on (Arbuckle, Rodríguez de la Vega & Casewell, 2017; Drabeck et al., 2015). A) Shows the molecular adaptations leading to resistance to snake alpha neurotoxin. Numbering is based on human acetylcholine receptor subunit alpha (UniProtKB - P02708). B) Shows the molecular adaptations leading to resistance to bufotoxin.

Numbering is based on human Na+/K+ ATPase subunit alpha-3 (UniProt accession number: KB - P13637). C) Shows the molecular adaptations leading to resistance to scorpion venoms. Numbering is based on human sodium channel protein type 10 subunit alpha (UniProtKB - Q9Y5Y9). D) Shows the molecular adaptations leading to resistance to tetrodotoxin. Different *Thamnophis sirtalis* populations are indicated in brackets (W) is Warrenton, (B) is Benton and (WC) is Willow Creek. indicated the numbering is based on human Sodium channel protein type 4 subunit alpha (UniProt accession number: KB - P35499). Key: Blue, amino acid not linked to resistance; Red, amino acid linked to resistance to the toxin. Amino acid coding: F (Phenylalanine), N (Asparagine), W (Tryptophan), R (Arginine), I (Isoleucine), V (Valine), C (cysteine), E (Glutamic acid), D (Aspartic acid), Q (Glutamine), L (Leucine).

Resistance to the venom of the bark scorpion

The bark scorpion (*Centruroides* sp.) is a major prey item for grasshopper mice (*Onychomys torridus*) (Rowe *et al.*, 2013b). The grasshopper mice have evolved analgesic effects against the extremely painful sting of the bark scorpion. Domain II of the grasshopper mouse Na^+ channel (Nav1.8) Figure 3) has either glutamine at position 859 or glutamic acid at position 862, while in the house mouse (*Mus musculus*) and human (*Homo sapiens*) the positions are switched in that they have glutamic acid at position 859 and glutamine at position 862. Therefore, It has been suggested in grasshopper mice that the presence in particular of the negatively-charged glutamic acid at position 862 may underlie the insensitivity to pain (Rowe *et al.*, 2013b).

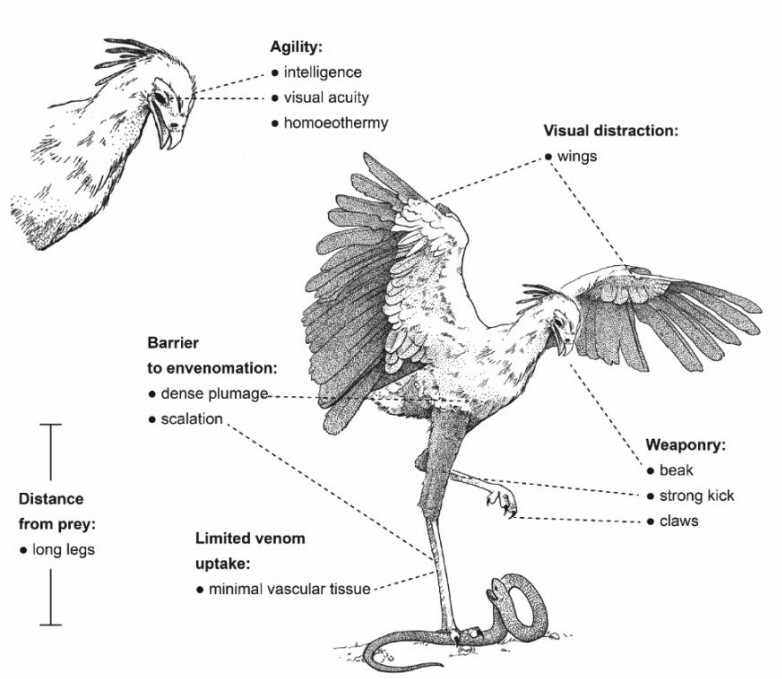

Figure 4. Morphological and behavioural traits proposed to negate selection pressures for evolving molecular resistance in snake-eating birds, such as the Secretary Bird (*Sagittarius serpentarius*). Plumage and leg scales may provide a physical barrier against snakebite. Additionally, bird legs are mainly transmit tendons and lack highly vascular tissue such as skeletal muscle; this may limit the uptake of venom if the bird is bitten. The secretary bird attacks snakes aggressively, directing kicks to the head and neck (Portugal *et al.*, 2016). Its elongated tibiotarsus and tarsometarsus 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 which may minimise the risk of snakebite (Potier, Lieuvin, Pfaff *et al.*, 2020). The red-legged seriema (*Cariama cristata*) uses its beak to grab the prey behind the neck and then shakes the prey violently so as to fracture its spine (Silva, Nunes, Estrela *et al.*, 2016). Drawings by Sven Ballinger, based on an original idea by Muzaffar Khan, Michael Richardson and Jory van Thiel.

Table 3: Autoresistance sites of α-subunit nAChR in snakes and mammals.

Species	Toxin	Toxin Target (TT)	Species	Amino acid substitutions at TT*
Egyptian cobra (*Naja haje*)	α–neurotoxin	α-subunit (nAChR)	Egyptian cobra (*Naja haje*); Krait (*Bungarus multicinctus*)	F189N
"	α–neurotoxin	α-subunit (nAChR)	Egyptian mongoose (*Herpestes ichneumon*)	W187N

*Key to amino acid substitutions at the target toxin site. F (Phenylalanine) → N (Asparagine), W (Tryptophan) → R (Arginine).

The pallid bat (*Antrozous pallidus*) preys on the bark scorpion (Hopp *et al.*, 2017). Interestingly, during the attack, the bat may be stung a number of times (Hopp *et al.*, 2017). The bat has been observed to attack again with no change of behaviour and without apparent ill-effects from being stung (Hopp *et al.*, 2017). It was shown that the pallid bat does not have the modification of its Na^+ channel seen in the grasshopper mouse Table 4. Therefore, more work is required to identify the mechanism of resistance in this bat (Hopp *et al.*, 2017).

Table 4: Grasshopper and pallid bat pain resistance sites vs. Arizona bark scorpion venom.

Species	Toxin	Toxin Target (TT)	Species	Amino acid substitutions at TT*	References
Arizona bark scorpion (*Centruroides.* Spp)	csev1 (neurotoxin 1)	voltage-gated Na^+ channel Nav1.8 Domain II (DII)	grasshopper mouse (*Onychomys torridus*)	E859Q Q862E	(Rowe *et al.*, 2013b)
"	"	voltage-gated Na^+ channel Nav1.8 Domain II (DII)	pallid bat (*Antrozous pallidus*)	unknown	(Hopp *et al.*, 2017)

*Key to amino acid substitutions at the target toxin site. E (Glutamic acid) → Q (Glutamine), Q (Glutamine) → E (Glutamic acid). The sequence data are show graphically in Figure 5

Resistance to tetrodotoxin (TTX)

Tetrodotoxin (TTX) is a neurotoxic small molecule (Brodie, 1990) that can cause death due to respiratory failure (Brodie, 1968) by binding to Na^+ channels. It is a guanidinium alkaloid. In general, the α-subunit of Na^+ channels is formed from four parallel domains (I-IV) each of which further holds six transmembrane segments designated S1-S6 (Marban, Yamagishi & Tomaselli, 1998). The resistance to toxins is due to amino acid substitutions in one or more domains of the Na^+ channel (Venkatesh *et al.*, 2005). Here, we will discuss resistance of the North American garter snake (*Thamnophis sirtalis*) to the tetrodotoxin of its prey, the rough-skinned newt (*Taricha granulosa*). We will also discuss the resistance of the pufferfish (*Tetraodon nigroviridis*) to its own (food-web derived) TTX (Figure 5).

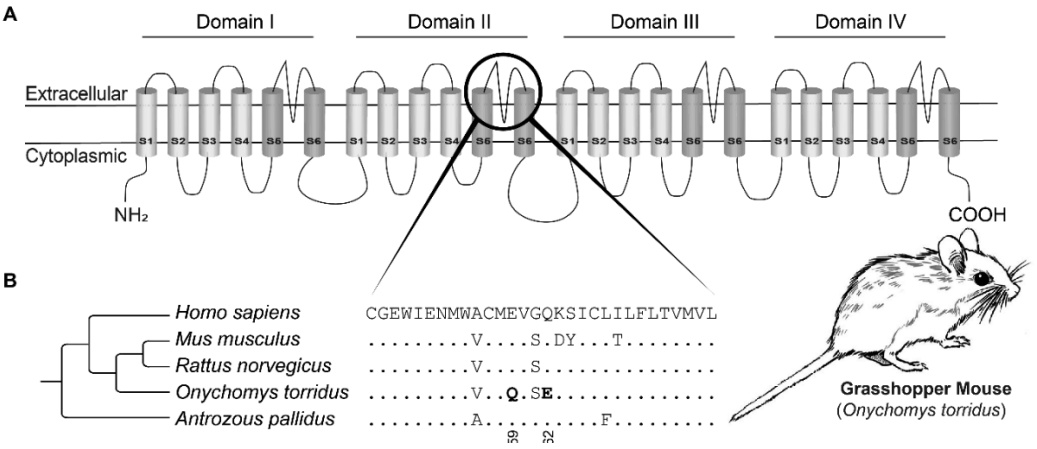

Figure 5. Resistance against pain-inducing scorpion venom in Grasshopper Mouse (*Onychomys torridus*). A. Unfolded protein structure of voltage-gated Na+ channel (Nav 1.8). Black circle indicates the outer pore associated with scorpion-venom binding in the Nav 1.8 channel. Structure was based on (Shen, Zhou, Pan et al., 2017). B. Partial sequence alignment of the outer pore of the α-subunit of domain II of Nav 1.8 channel. The displayed 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, Stecher, Suleski et al., 2017). Drawings by Sven Ballinger, based on an original idea by Jory van Thiel and Muzaffar Khan.

Garter snake

The garter snake has evolved a modification of the domain-IV segments S5-S6 of the $Na_v1.4$ in its skeletal muscles. This modification consists of the replacement of isoleucine by valine in at position 1,561 Table 5; (Brodie, 1990; Feldman, Durso, Hanifin *et al.*, 2015; Geffeney *et al.*, 2002; Geffeney *et al.*, 2005; Venkatesh *et al.*, 2005). Isoleucine is present at this position in the majority of the vertebrates that have been studied (Geffeney *et al.*, 2005). The molecular adaptation that confers resistance in garter snakes to

the newt TTX has presumably evolved because the two species share a common geographical distribution and the newt is a major part of the diet of the garter snake (McGlothlin *et al.*, 2016; Williams, Brodie & Brodie, 2004). The source of the TTX in the newt is a matter of discussion (Hanifin, 2010), but may be bacterial as it is in the pufferfish (see Table 5 (Bane, Lehane, Dikshit *et al.*, 2014; Cardall, Brodie, Brodie *et al.*, 2004). Interestingly, it has been shown that the newt, in captivity, has the capability to produce TTX in its granular skin glands and secrete it onto its dorsal skin surface (Cardall *et al.*, 2004). Physiological assay has shown that the newt resistance to TTX is not humoral-based.

Teleosts

Several marine teleosts have evolved autoresistance to TTX (Venkatesh *et al.*, 2005). Resistance in the fugu (pufferfish; *Fugu pardalis*) is due to the presence of an asparagine in place of phenylalanine at position 401 in domain-I of the $Na_v1.4a$ Table 5 (Venkatesh *et al.*, 2005). The fugu has a cysteine in domain-I, position 401 in place of phenylalanine, and further, in $Na_v1.4b$ due to aspartic acid in place of glutamic acid (domain-II, position 758; (Kaneko *et al.*, 1997a; Soong *et al.*, 2006; Venkatesh *et al.*, 2005; Yotsu-Yamashita, Nishimori, Nitanai *et al.*, 2000).

Saxitoxin

A few species of pufferfish including *Tetraodon fangi* and *T. cutcutia*, have evolved resistance to the chemically-related neurotoxin saxitoxin (Landsberg, Hall, Johannessen *et al.*, 2006; Sato, Kodama, Ogata *et al.*, 1997; Venkatesh *et al.*, 2005). Saxitoxin (STX) is a potent neurotoxin (Schantz, Ghazarossian, Schnoes *et al.*, 1975; Wiese *et al.*, 2010) that is accumulated by several teleosts from eukaryotic dinoflagelates and prokaryotic cyanobacteria in their diet (Bricelj *et al.*, 2005; Wiese *et al.*, 2010; Yotsu-Yamashita, Kim, Dudley *et al.*, 2004b). In addition Zetekitoxin AB, an analog of STX (Yotsu-Yamashita, Kim, Dudley *et al.*, 2004a), has been found in the Panamanian golden frog (*Atelopus zeteki*; (Wiese *et al.*, 2010; Yotsu-

Yamashita *et al.*, 2004a; Yotsu-Yamashita *et al.*, 2004b); its source is unknown. Autoresistance to STX is found in *Tetraodon fangi, T. cutcutia*. It is due to the presence of an asparagine in domain-II of the $Na_v1.4b$ channel (Venkatesh *et al.*, 2005). The soft-shell clam (*Mya arenaria*) contains STX. The clam has evolved resistance to STX due to the presence of aspartic acid at position 758 in place of glutamic acid in domain-II of its neuronal Nav1.4 channel (Soong *et al.*, 2006).

Steroids Toxins

Steroid toxins include several plant toxins such as cardenolides, found in the round-leafed navel-wort (*Cotyledon orbiculata*), kalanchoe pinnata (*Bryophyllum pinnatum*), butterfly weed (*Asclepias tuberosa)*, the oleander (*Nerium oleander*) and the foxglove (*Digitalis purpure*a; (Agrawal, Petschenka, Bingham *et al.*, 2012; Anderson, Schultz, Kellerman *et al.*, 1985; Krenn & Kopp, 1998; Supratman, Fujita, Akiyama *et al.*, 2000).

Bufagenins

Steroid toxins include several plant toxins such as cardenolides, found in the round-leafed navel-wort (*Cotyledon orbiculata*), kalanchoe pinnata (*Bryophyllum pinnatum*), butterfly weed (*Asclepias tuberosa)*, the oleander (*Nerium oleander*) and the foxglove (*Digitalis purpure*a; (Agrawal *et al.*, 2012; Anderson *et al.*, 1985; Krenn *et al.*, 1998; Supratman *et al.*, 2000). Bufagenins are toxic cardiac glycosides chemically related to the cardenolides of plants mentioned above. The cane toad (*Rhinella marina* [*Bufo marinus*]) produces bufagenins in its parotid glands (Phillips *et al.*, 2006). Bufagenins are also found in insects of the families Chrysomelidae and Lampyridae (Van Oycke, Braekman, Daloze *et al.*, 1987). In susceptible predators bufagenins disrupt the activity of Na^+/K^+-ATPase and eventually cause cardiotoxicity (Kamalakkannan, Salim & Capon, 2017; Ujvari *et al.*, 2015). In the cane toad they act as anti-predator defenses (Kamalakkannan *et al.*, 2017; Ujvari *et al.*, 2015).

There is extensive biotransformation of bufagenins in the cane toad by Gram-positive bacteria (*Bacillus* sp.; (Kamalakkannan *et al.*, 2017). The eggs and tadpoles of the cane toad contain bufagenins making them toxic to predators (Shine, 2018). Bufagenins are also present in the adult parotid gland and in the secretion of skin glands (Chen & Kovaříková, 1967). In 1935, the cane toad was released into the sugar cane fields of Australia in the belief that it would control pests (Haynes, 2015; Sabath, Boughton & Easteal, 1981; Shine, 2018). It did not do so, and instead has since become a very troublesome, invasive species. The bufagenins of the cane toad have become a serious threat to Australian wildlife, because they result in the poisoning of many the many Australian native animals that prey on cane toads, and have not evolved any resistance. These predators include snakes, monitor lizards and crocodiles (Phillips, Brown, Greenlees *et al.*, 2007; Shine, 2010). It has been shown that African varanid lizards (*Varanus niloticus, V. albigularis, V. exanthematicus*), Asian varanid lizards (*V. dumerilii, V. bengalensis, V. rudicollis, V.salvator*), the European hedgehog (*Erinaceus europaeus*), and murid rodents (Muridae), that feed on cane toads, have evolved resistance to bufagenin. Further, two species of elapid and viper show resistance (Ujvari *et al.*, 2015).

The resistance in all of these animals is associated with the presence of leucine and arginine in the H1–H2 extracellular domain of the Na^+/K^+-ATPase at positions 111 and 120, respectively (Brodie, 1977; Ujvari *et al.*, 2015). By contrast, the Australian varanid lizard (*Varanus varius*) which is not resistant to bufagenin has a glutamine (Q) at position 111 and glycine (G) at position 120 (Losos & Greene, 1988; Ujvari *et al.*, 2015; Ujvari, Mun, Conigrave *et al.*, 2013) Table 6. In Japan, the Japanese tiger keelback snake (*Rhabdophis tigrinus*) preys on cane toads (Kojima & Mori, 2015). It has a specialised nuchal gland in which bufagenins from the ingested toads are sequestered and then re-used for antipredator defense (Figure 6). In this snake there may also be an endocrine adaptation to the toad toxins. Thus, when the snake ingests a cane toad, its plasma concentration of the stress hormone corticosterone decreases, and that of the mineralocorticoid aldosterone

increases (Mohammadi, French, Neuman-Lee *et al.*, 2017a; Mohammadi, French, Neuman-Lee *et al.*, 2017b). This physiological response is not seen in non-resistant snake species (Mohammadi *et al.*, 2017a).

Table 5. Newt, Fugu, pufferfish and soft shell clams Na+ channel sites of tetrodotoxin resistance.

Species	Toxin	Toxin Target (TT)	Species	Amino acid substitutions at TT*	References
rough-skinned newt (*Taricha granulosa*)	tetrodotoxin (TTX)	skeletal muscle voltage-gated Na+ channel	garter snake (*Thamnophis sirtalis*)	I1561V	(Feldman *et al.*, 2015; Geffeney *et al.*, 2005)
marine bacteria (*Vibrio* sp., *Pseudomonas* sp.) marine actinomycete (*Nocardiopsis dassonvillei*), starfish, gastropods and shrimps.	"	skeletal muscle voltage-gated Na+ channel	fugu (*Fugu pardalis*)	C401N	(Soong *et al.*, 2006; Venkatesh *et al.*, 2005)
"	"	skeletal muscle voltage-gated Na^+ channel	pufferfish (*Tetraodon nigroviridis*)	E758D	(Soong *et al.*, 2006; Venkatesh *et al.*, 2005)
ocean water dinoflagellates and fresh water cyanobacteria	saxitoxin (STX)	Na+ channel (Nav1.4b) domain II	soft-shell clams (*Mya arenaria*)	E945D	(Bricelj *et al.*, 2005; Soong *et al.*, 2006; Wiese *et al.*, 2010)

*Key to amino acid substitutions at the target toxin site. I (Isoleucine) → V (Valine),C (cysteine) → N (Asparagine), E (Glutamic acid) → D (Aspartic acid).

In Australia, the black snake (*Pseudechis porphyriacus*) appears to have evolved both physiological resistance to bufagenin, presumably due to the presence of the cane toad in its geographical range. This resistance is accompanied by behavioural avoidance of the cane toad as potential prey (Phillips *et al.*, 2006). Interestingly, these changes have evolved rapidly in the snake, i.e. in around 23 generations (Phillips *et al.*, 2006). Toxicity testing in Australia shows that the saltwater crocodile (*Crocodylus porosus*) is less susceptible to bufagenin than is the freshwater crocodile *Crocodylus johnstoni* (Smith & Phillips, 2006). Whether the saltwater crocodile has evolved some mechanism of resistance is not known. However, we notice here that the saltwater crocodile has an amino acid leucine (L) at position 111 H1–H2 extracellular domain of the Na^+/K^+-ATPase as do species resistant to bufagenins.

Table 6. Different vertebrates bufotoxins resistance sites.

Species	**Toxin**	**Toxin Target (TT)**	**Species**	**Amino acid substitutions at TT***
bufonids toads (Bufonidae)	bufotoxins	H1–H2 extracellular domain of the Na+/K+ -ATPase	African and Asian varanid lizards, Indian Cobra, forest cobra, puff adder, rhinoceros vipers, European vipers, muroid rodents	Q111L G120R

*Key to amino acid substitutions at the target toxin site Q (Glutamine) → L (Leucine), Q (Glutamine) → R (Arginine).

Batrachotoxins

In 1963, first time the venom was extracted from the skin of the Colombian black-legged poison dart frog (*Phyllobates bicolor*) (Maerki & Witkop, 1963). The name batrachotoxin was given to the major active toxin of this species (Daly, Witkop, Bommer *et al.*, 1965). Batrachotoxins are neurotoxic, lipophilic alkaloids which bind to vertebrate Na^+ channels in nerves and muscles. They have been classified in three highly toxic alkaloids: (i) batrachotoxin; (ii) homobatrachotoxin; and (iii) batrachotoxin A (reviewed in (Daly, 1995; Daly, Brown, Mensah-Dwumah *et al.*, 1978)). It is thought that the poison dart frogs acquire the batrachotoxins from items in their diet, possibly from Melyrid beetles (*Choresine*) (Dumbacher *et al.*, 2004) Figure 6). Batrachotoxins have also been identified on the feathers of certain passerine birds of New Guinea which belong to genus *Pitohui* (Dumbacher, Beehler, Spande *et al.*, 1992). The toxins come from the uropygial glands of these birds and is transferred onto the feathers during preening. The batrachotoxins may help the birds to protect against infections, ectoparasites and potential predators including snakes and birds of prey (Dumbacher *et al.*, 1992; Jacob, 1978; Poulsen, 1994). The batrachotoxins originate from items in the diet of the pitohui birds including beetles of the genus *Choresine*, monarch butterflies (*Danaus plexippus*) and various plants (Figure 6) (Dumbacher *et al.*, 2009; Dumbacher *et al.*, 2004).

Interestingly, as a result of eating these insects *Pitohui* species have developed resistance to homobatrachotoxin (Dumbacher *et al.*, 1992; Dumbacher, Deiner, Thompson *et al.*, 2008). Despite the high concentrations found in these passerine birds, there are no resistance-related modifications in the Na_v channels ($Na_v1.4$ and Na_v 1.5, respectively) which could suggest a comparable strategy as proposed in poison dart frogs (Abderemane-Ali, Rossen, Kobiela *et al.*, 2021).

Resistance and the so-called 'co-evolutionary arms race'

In any predator-prey relationship involving a poisonous or venomous participant, it seems likely that there will be selection for resistance. Given sufficient reciprocal selection in the predator, one can envisage a co-evolutionary arms race, where the prey evolves continuously evolves more effective resistance, and the predator evolves more effective toxins (discussed by (Khan *et al.*, 2020). By 'effective' I mean more potent, and faster acting. 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 (Khan *et al.*, 2020; Ujvari *et al.*, 2015). This suggests that their might be some fitness cost to maintaining resistance.

Evolutionary 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, Brodie, Brodie *et al.*, 2012; Ujvari *et al.*, 2015); mammalian mesopredators feeding on venomous snakes (Drabeck *et al.*, 2015; Drabeck, Rucavado, Hingst-Zaher *et al.*, 2020) and grasshopper mice eating bark scorpions (Rowe, Xiao, Rowe *et al.*, 2013a). 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 preying on moray eels (Heatwole & Poran, 1995) and rattlesnakes preying on North American squirrels and other rodents (de Wit, 1982; Gibbs, Sanz, Perez *et al.*, 2020; Holding, Biardi & Gibbs, 2016). 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.*, 2016; Margres, Wray, Hassinger *et al.*, 2017).

Evolutionary theory predicts that resistance is unlikely to evolve when selection pressure is low, for example: i) when predation by a venomous predator is a relatively unimportant selective force for the prey because of scarcity of encounters, ii) a short temporal window of exposure (Marques, Martins, Develey *et al.*, 2012), or iii) when behavioural avoidance of toxic prey is more advantageous than evolving resistance (Brodie Iii, 1993; Portugal *et al.*, 2016; Smith, 1977); see also Figure 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). 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.

Competing selection pressures and convergent evolution

Evolutionary trade-offs usually come with some kind of fitness disadvantage (Blanchard & Moreau, 2017; Brodie Iii & Brodie Jr, 1999; Hague, Toledo, Geffeney *et al.*, 2018). It is important that resistance modifications do not disrupt the physiology of the resistant animal. For example, resistance modifications of the neuromuscular junction, that reduce the binding of snake α–neurotoxins, should not interfere with the physiological binding of the animal's own neurotransmitter (acetyl choline) (Fuchs, Barchan, Kachalsky *et al.*, 1993). Indeed this appears to be the case: multiple substitutions have convergently evolved to reduce snake α-neurotoxin binding, but without compromising the amino acid residues vital for acetylcholine binding (Barchan, Kachalsky, Neumann *et al.*, 1992b; Khan *et al.*, 2020). These observations support the concept of a trade-off between a functional target (e.g., binding site of the endogenous ligand) and the modifications that enhance toxin-resistance.

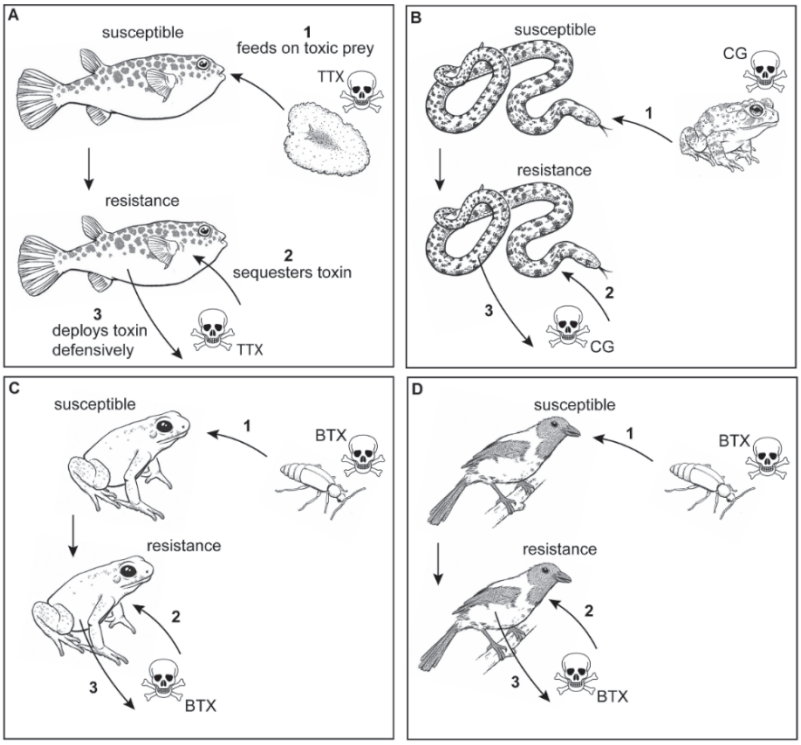

Figure 6. Hypothesised evolutionary arms scenarios for autoresistance in poisonous animals. It is generally assumed that autoresistance is a phenomenon of self-protection. Here, we propose a three-step evolution scenario for the origins of autoresistance: 1) predator resistance is followed by 2) sequestration of the toxin by the predator, and 3) exploitation of the toxin for defence. As this figure indicates, a similar three-step process has been seen in very diverse lineages, suggesting evolutionary convergence. The displayed examples include, A) pufferfish (family Tetraodontidae) feeding on TTX-bearing flatworms, gastropods, and echinoderms, B) keelback snakes (Rhabdophis spp.) feeding on cane toads. C) poison dart frogs (family Dendrobatidae) feeding on toxic arthropods, D) pitohui birds (Pitohui spp.) feeding among others on BTX-bearing melyrid beetles, Key: TTX (tetrodotoxin), CG (cardiac glycosides), BTX (batrachotoxin). Drawings by Sven Ballinger, based on an original idea by Jory van Thiel, Muzaffar Khan and Michael Richardson.

Similar trade-offs may exist in the case of the garter snakes, and their resistance to the tetrodotoxin of newts. Convergent adaptations have been found not only in garter snakes but in multiple distinct colubrid snakes, and

the adaptations are associated with tetrodotoxin resistance. These adaptations have been shown to be mediated by a functional trade-off between ion channel function and tetrodotoxin-insensitivity (Feldman *et al.*, 2012; Lee, Jones, Ahern *et al.*, 2011). A further example of the possible trade-offs involved in toxin resistance is seen in the case of the evolution of cardiac glycoside resistance. This has evolved several times, by means of two or three substitutions (respectively at positions 111, 119, 120 or 122) in the Na^+/K^+-ATPase (Dobler, Dalla, Wagschal *et al.*, 2012; Karageorgi, Groen, Sumbul *et al.*, 2019; Ujvari *et al.*, 2015). In summary, there may be a limited number of amino acid changes that can reduce the binding affinity of toxins, without disrupting the normal physiology of the animal. These same amino acid changes are seen repeatedly in different species under similar selection pressures. We believe that this is a persuasive example of convergent evolution: the arrival at the same solution in independent lineages of animals, in response to similar selection pressures. Only in this way can the animals develop resistance, while maintaining their normal physiology

Origins of autoresistance in poisonous animals

Some animals are resistant to their own toxins, a phenomenon referred to as autoresistance. However, here we argue that this is a much more complicated evolutionary scenario in the case of toxins (e.g., tetrodotoxin, cardiac glycosides, batrachotoxin). The complexity of the issue has already been partially touched upon in previous literature (Santos, Tarvin & O'Connell, 2016; Saporito, Donnelly, Spande *et al.*, 2012). We propose a scenario in which there was a three-step evolution of resistance across phylogenetically distinct poisonous animals: first, (i) predator resistance, followed by (ii) sequestration of the toxin by the predator and finally, (iii) exploitation of the toxin for defence.

Over the course of evolution, predation on a toxic species leads to frequent exposure to a specific toxin or toxins through generalized 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 capitalize on abundant, often underutilized prey species. This then provided an evolutionary selection pressure favouring resistance.

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, Arakawa & Takatani, 2006; Saporito, Donnelly, Jain *et al.*, 2007; Yotsu-Yamashita, Gilhen, Russell *et al.*, 2012). Some toxins (e.g., alkaloid or steroidal-based toxins) are not destroyed in the gut, and can thus accumulate in the body. Ultimately this enabled the exploitation of the accumulated toxins for defensive purposes in poisonous animals (as reviewed in (Savitzky, Mori, Hutchinson *et al.*, 2012). Therefore, we hypothesise that autoresistance primarily evolved as predator resistance rather than in its own right.

Conclusions

Toxin resistance provides a fascinating model system for the understanding of convergent evolution. We hope that our review will lead to novel insights into complex evolutionary processes provided by integrating molecular biology, evolution and ecology. Functional constraints on molecular targets explain the convergence of resistant traits that are seen across the animal kingdom. Toxin resistance is an evolved response seen at many trophic levels, underscoring how relatively simple adaptations can bring solutions to complicated problems.

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Chapter 3: Molecular and Functional Study of Cobra α-Neurotoxin Resistance in Lizards, Fish and the Chicken

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Abstract

Snakes of the family Elapidae (cobras, kraits and others) contain peptide toxins of the α-neurotoxin group in their venom. This toxin contributes to the ability of cobra venom to incapacitate the prey or attacker of the cobra. Some of the prey or attacker species have evolved a degree of resistance to cobra venom in general and to α-neurotoxin(s) in particular. The resistance is due to alterations in the ligand-binding domain of the nAChR. Examples are substitution of a glycosylated asparagine for the ancestral aromatic residue at position 187 and 189. These resistance-related substations are well documented in some mammals. However It is not known whether other species such as lizards and fish have evolved similar molecular adaptations. To address this issue, we have analyzed the sequences of the α-neurotoxin ligand-binding domain of nAChR in seventeen species of lizards and five species of teleost fish. We did *de novo* sequencing of DNA and tissue samples from collaborators around the world and retrieved other sequences from scientific databases. We also performed developmental LC_{50} and LD_{50} assays of cobra venom toxicity in four species. Of the lizards examined, Central bearded dragon has asparginine at position 187 and proline replacement to leucine (P→L) at position 194. By contrast the lizards Australian water dragon, Gilbert's dragon, transvolcanic alligator lizard have the amino acid replacement proline to leucine (P →L) at position 194. No other lizards showed resistance-related mutations. Among the seven teleosts examined, the Three-spined stickleback and Reedfish had asparginine at position 189. Our functional assays shows Central bearded dragon tolerated cobra venom approximately around five times that of chicken (LD_{50}: 1.870 vs. 0.340 mg/mL) and Three-spined stickleback ten times more venom than Zebrafish (LC_{50}: 0.673 vs. 0.062 mg/mL). These findings suggest that some fishes and lizards have evolved changes in the nAChR consistent with a potential role in providing resistances. We discuss the possible putative adaptive role of this resistance in the species examined.

Introduction

Animals have developed a range of morphological, behavioral and physiological mechanisms and strategies to protect themselves against predators (Biardi, Chien & Coss, 2006; Biardi & Coss, 2011; Biardi, Coss & Smith, 2000; Broeckhoven, Diedericks & Mouton, 2015; Glinski & Buczek, 1999). For example, the armadillo girdled lizard (*Ouroborus cataphractus*) has evolved body armour against its potential terrestrial mammalian predators, namely: (i) the meerkat (*Suricata suricatta*); (ii) the Egyptian mongoose (*Herpestes ichneumon*); (iii) the Cape gray mongoose (*Galerella pulverulenta*) and (iv) the yellow mongoose (*Cynictis penicillata*) (Broeckhoven *et al.*, 2015). The yellow-spotted goanna (*Varanus panoptes*) and the lace monitors (*Varanus varius*) in Queensland, which have co-existed with the toxic cane toad for more than 70 years, are still sensitive to cane toad bufagenin toxins but show behavioral modifications such that they avoid feeding on the toads (Pinch, Madsen & Ujvari, 2017). In a study of molecular resistance to the cane toad toxins, it was found that another Australian reptile, the bearded dragon (*P. vitticeps*), has no molecular adaption against cane toad bufagenins (Ujvari, Casewell, Sunagar *et al.*, 2015).

In the south-eastern United States, the green anole (*Anolis carolinensis*), the Eastern fence lizard (*Sceloporus undulates*) and the broad-head skink (*Eumeces laticeps*) rarely ingest toxic fireflies (*Photinus* sp.) — again, due to avoidance behavior. Those fireflies contain toxic steroidal pyrone lucibufagins that are structurally similar to cane toad bufagenins and plant cardenolides. In one case study it was noted that two bearded dragons (*Pogona vittceps*) might have died from the ingestion of *Photinus* sp. in Australia (Knight, Glor, Smedley *et al.*, 1999). However, the bearded dragon shows no correlated behavioral avoidance towards *Photinus* sp. Possible explanations for these observations are that, in the past, the bearded dragon did not encounter *Photinus* in Australia, or that Australian fireflies might secondarily lack lucibufagins (Knight *et al.*, 1999).

The ringneck snake (*Diadophis punctatus*) preys on the red-backed salamander (*Plethodon cinereus*), which is able to shed its own tail (self-amputation) as a defense mechanism, thereby, it is thought, increasing its chances of survival (Lancaster & Wise, 1996). One remarkable study found that newly-hatched white-throated savannah monitors (*Exanthematicus albigularis*) show behavioral differences towards venomous and non-venomous snakes (Phillips & Alberts, 1992). The newly-hatched monitors had a 100% attack rate on non-venomous prey, such as land snails, corn crickets (*Acanthoplus discoidalis*), grasshoppers (Pamphagidae) and sand snakes (*Psammophis leightoni*) (Phillips *et al.*, 1992). However, in the presence of venomous snake carcass, they exhibited behavior such as high-pitched hissing and tail-slapping. The authors suggest that neonatal monitors may be able to distinguish venomous from non-venomous animals using chemoreception (Phillips *et al.*, 1992).

Harvester ants (*Pogonomyrmex* sp.) use powerful stings for the delivery of venom to their vertebrate predators. Texas horned lizards (*Phrynosoma cornutum*) are the main vertebrate predators of these ants (Pianka & Parker, 1975) and have evolved a neutralizing blood plasma factor against the venom of *Pogonomyrmex maricopa.* Moreover, experiments show that the Texas horned lizard is more resistant than the blue-spotted spiny Lizard (*Sceloporus jarrovii*), and 1,500 times more resistant than mice, to the venom of *P. maricopa* (Schmidt, Sherbrooke & Schmidt, 1989).

In summary, there is considerable evidence that toxin resistance is common in those animals who are liable to be frequently exposed to venomous or toxic animals, for example, when there is a predator prey-relationship and their territories overlap geographically(Barchan, Kachalsky, Neumann *et al.*, 1992b; Barchan, Ovadia, Kochva *et al.*, 1995; Biardi *et al.*, 2006; Burden, Hartzell & Yoshikami, 1975). The toxin-producing animal may, in turn, develop countermeasures to overcome prey resistance through adaptive mutation and toxin gene duplication (Ahmed, El-Din, Mohamed *et al.*, 1974; Gunasekaran, Sridhar, Suryanarayanan *et al.*, 2017; Hamburger & Hamilton, 1951; Liu & Xu, 1990; Minton Jr & Minton, 1981). For these and other

reasons, resistance against toxins may be a valuable model offering insight into evolutionary processes. In some cases, a similar adaptation that causes resistance to the same type of toxin has occurred in different vertebrate lineages. The binding of α-neurotoxin of snakes to nicotinic acetylcholine receptor (nAChR) causes paralysis of skeletal muscles in the prey (Barchan *et al.*, 1995). The nAChR of some snakes, lizards and mammals are insensitive to snake α-neurotoxin (Barchan, Kachalsky, Neumann *et al.*, 1992a; Burden *et al.*, 1975). It has been proposed that modifications to the nicotinic acetylcholine receptor (nAChR) evolved in Squamata in response to more primitive reptilian toxins, before the appearance of α-neurotoxins (Burden *et al.*, 1975; Liu *et al.*, 1990). Physiological assays have shown that some lizards – the African plated lizard (*Cordylus jonesi*), the Eastern glass lizard (*Ophisaurus ventralis*) and *Lacerta* sp – have skeletal muscle that is resistant to α- neurotoxin, α-atratoxin and α- bungarotoxin (Burden *et al.*, 1975). In a study of Australian skinks (*Ctenotus robustus*, *Egerina striolata* and *E. whitii*) evidence was found of high resistance to the venom of the four Australian elapids snakes (Minton Jr *et al.*, 1981). These Australian elapids have α-neurotoxin in their venom. In venomous lizards (Toxicofera), the molecular mechanism of resistance to snake neurotoxins is unclear.

Furthermore, there is little or no information available on the phylogenetic distribution of the molecular adaptation in lizards that confers resistance to snake α-neurotoxin. Our goal here is to further investigate toxin resistance in lizards (Helodermatidae, Anguidae, Varanidae, Agamidae and Iguania) to fill the evolutionary gaps in our knowledge of toxin resistance to snake α-neurotoxin. During online data mining, I found that the three-spined stickleback *(Gasterosteus aculeatus*) and Reedfish (*Erpetoichthys calabaricus*) have asparginine at position 189 in ligand bind domain of nAChR like cobra α-neurotoxin resistant animals (Khan, Dashevsky, Kerkkamp *et al.*, 2020). So, we will also discuss that species in this chapter.

Materials and Methods

Ethics statement

All animal experimental procedures were conducted in accordance with local and international regulations. DNA sampling in the Netherlands was done in accordance the *Wet op de dierproeven* (Article 9) of Dutch Law (National) and the same law administered by the Bureau of Animal Experiment Licensing, Leiden University (Local). This local regulation serves as the implementation of Guidelines on the protection of experimental animals by the Council of Europe, Directive 86/609/EEC. The samples from Australia were collected under University of Melbourne Animal Ethics approval number 03126.

Field Work

In November 2018, I went to Queensland, Australia, for the collection of field samples of different lizards under the budget of the KNAW ecology funds grant (KNAWWF/713/18015) that was awarded to me in 2018. With the support of our collaborator and fieldwork host, Associate Professor Bryan G. Fry, at the Venom Evolution Laboratory, University of Queensland, Australia, I got the opportunity to collect unique DNA samples of those species of lizards which were expected to be resistant to α-neurotoxin. The genomic DNA were shipped to the Institute of Biology Leiden University (IBL), the Netherlands, under the export permits of Professor Fry.

DNA extraction

DNA extraction was performed using a QIAGEN DNeasy kit according to prescribed procedures (Qiagen, Inc., Valencia, CA, USA). The manufacturer's instructions were followed. DNA was extracted from tissue samples preserved in 70% ethanol. The tissues were rinsed with 10% phosphate-buffered saline (PBS), then cut into small pieces and transferred to 180 µL DNA tissue lysis buffer with 20µL/mL Proteinase K (ProtK) overnight with gentle shaking at 56°C digital heat block (VWR International).

After incubation, the 200µL lysis buffer was added and mixed thoroughly by vortex, followed by incubation at 56°C digital heat block for 10 minutes. After incubation, 200 µL ethanol was added and mixed thoroughly by vortex. The mixture was then pipetted into the DNeasy mini spin column (Qiagen, Inc., Valencia, CA, USA) and placed in a 2 mL collection tube, then centrifuged for 1 minute at 8,000 rpm. After centrifugation, the collection tube was discarded. The mini spin column was replaced with a fresh collection tube. 500mL of lysis buffer was added to the mini spin column and centrifuged for 1 min at 8,000 rpm. After centrifugation the collection tube was discarded. The mini spin column was replaced with a fresh collection tube. 500 mL of lysis buffer 2 was added to the mini spin column and centrifuged for 3 minutes at 14,000 rpm). The spin column was transferred to a 1.5 mL Eppendorf tube. The DNA was eluted by adding 200 µL of elution buffer to the center of the spin column membrane. The spin column was incubated for 1 min at room temperature and centrifuged for 1 min at 8,000 rpm for DNA elution into an Eppendorf tube. The DNA concentration was determined using a NanoDrop 1000 UV/Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, United States) at an absorbance of 26 nm.

Amplification of the ligand-binding domain of the α-neurotoxin nAChR

Samples were processed and sequenced separately. Primers specific for the ligand-binding domain of the nicotinic acetylcholine receptor (nAChR; Figure 7) were designed based on the alignment of reference sequences of the following lizards species: Green anole (*Anolis carolinensis*), Central bearded dragon *(Pogona vitticeps*) and Common wall lizard (*Podarcis muralis*) Table 7. Successively, an amplicon of 400 bp of the ligand-binding domain α-neurotoxin from the gene nAChR (Figure 7) was amplified. PCR was performed in a volume of 25µL mixture according to the instructions of

manufacturer (Qiagen, Inc., California, USA). PCR reaction conditions with an annealing temperature of 65°C for 10s (-1/cycle). As a quality check, the PCR products were electrophoresed for 30 min, and visualized on gel documentation apparatus on the Red™ Imaging System from Alpha Innotech (California, United States).

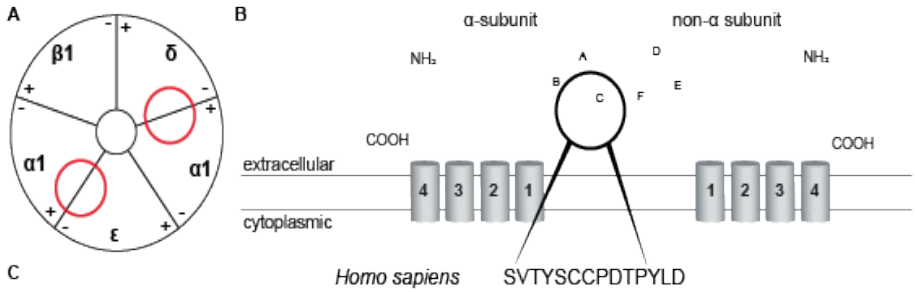

Figure 7. Schematic representation of the α-1 muscle-type nicotinic acetylcholine receptor (nAChR). Red circle indicates the position of the ligand-binding domain of α-neurotoxins in the nAChR. Figure was based on (Kini, 2019). B) Unfolded protein structure of an α-subunit and a non-α-subunit of the muscle-type nAChR. The black circle indicates the C-loop involved in α-neurotoxin binding. C) Sequence alignment of α1-nAChR ligand-binding domain. The displayed reference amino acid sequence is from humans (Homo sapiens). Based on an original idea by Muzaffar Khan and Jory van Thiel and with input from Prof. R. M. Kini.

Sequencing

The amplified PCR products of nAChR for all lizards were sequenced by Baseclear B.V., the Netherlands. The sequences were translated into protein *in silico* and aligned using the program CLC main workbench v. 7.6.4 (Qiagen, California, USA). The ligand-binding domain in the lizard nAChR was examined and compared with the orthologous region of reference sequences of snake α-neurotoxin resistant animal species from NCBI. All sequences were submitted to The National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/) and can be found under accession numbers Table 8.

Table 7. Primers used for the amplification of lizard nAChR, ligand-binding domain.

Forward (F1)	Forward TAGGTAAGTGAACGTCCAGAC
Forward (F2)	TCCAGACCTGAGTAACTACATGG
Forward (F3) (alternative)	TGAGTAACTACATGGGGAGTGG
Reverse (R1)	TGTGGGTAGATAAAACACTAATCC
Reverse (R2)	AATGAGAACAGGAGGCAAGG
	Added to the 5'end was an M13 tail as follows:
	TGTAAAACGACGGCCAGT
	CAGGAAACAGCTATGAC

Toxicity Assays Using Embryos

Functional assays of *Naja naja* (spectacled cobra) venom were performed on the following animals. *Gallus gallus* (chicken embryos, 5 d incubation); *Pogona vitticeps* (bearded dragon embryos, 5–7 d incubation); *Gasterosteus aculeatus* (three-spined stickleback larvae, 4 days post fertilization (4 dpf)); and *Danio rerio* (zebrafish larvae, 4 dpf). The bearded dragon and stickleback have a modified ligand-binding domain subunit of α1nAChR consistent with -toxin resistance, while the chicken and zebrafish do not have that change.

Table 8. Sequences included in this study, with accession numbers and species names. Species in the same order as in (Figure 9), (Figure 10) Key: 'Source' indicates the origin of the sequence or the DNA sample. In the case of the sequences determined by me de novo in this study, who sourced the DNA samples are listed in the column headed using the following abbreviations: BGF, Bryan G. Fry; FJV, Freek J. Vonk; JvT, Jory van Thiel; MAGdB, Merijn A.G. de Bakker; RMW, Roel M. Wouters. The remaining sequences were obtained from NCBI (NCBI, National Center for Biotechnology Information, Bethesda, Maryland, United States).

Accession No.	Scientific Name	Common Name	Database	Tissue sample source
NM_131445.1	*Danio rerio*	Zebrafish	GenBank, NCBI	-
VCAZ01000208.1	*Bagarius yarrelli*	Giant devil catfish	GenBank, NCBI	-
XM_020601562.1	*Monopterus albus*	Swamp eel	GenBank, NCBI	-
VDFK01000470.1	*Gasterosteus aculeatus*	Three-spined stickleback	GenBank, NCBI	-
AY295875.1	*Takifugu rubripes*	Japanese puffer	GenBank, NCBI	-
CAAE01015010.1	*Tetraodon nigroviridis*	Spotted green pufferfish	GenBank, NCBI	-
XM_028808143.1	*Erpetoichthys calabaricus*	Reedfish	GenBank, NCBI	-
XM_015426640.1	*Gekko japonicus*	Schlegel's Japanese gecko	GenBank, NCBI	-
XM_033167788.1	*Lacerta agilis*	Sand lizard	GenBank, NCBI	-
XM_028749253.1	*Podarcis muralis*	Common wall lizard	GenBank, NCBI	-

XM_003226425.3	*Anolis carolinensis*	Green anole	GenBank, NCBI	-
MT249123	*Iguana iguana*	Common green iguana	Pet trade	BGF
MT249130	*Uromastyx aegyptia*	Egyptian spiny-tailed lizard	Pet trade	BGF
MT249127	*Intellagama lesueurii,*	Eastern water dragon	Pet trade	BGF
MT249122	*Pogona vitticeps*	Bearded dragon	Reptielenhuis de Aarde, Breda, the Netherlands	MAGdB
MT249128	*Lophognathus gilberti*	Gilbert's lashtail	Pet trade	BGF
MT249129	*Varanus komodoensis*	Komodo dragon	Pet trade	BGF
MT249118	*Varanus mertensi*	Mertens' water monitor	Pet trade	BGF
MT249131	*Varanus giganteus*	Perentie	Pet trade	BGF
MT249121	*Pseudopus apodus*	Scheltopusik, Pallas's glass lizard	Terrariumspeciaalzaak Kameleon, Tilburg, the Netherlands	JvT & RMW
MT249120	*Gerrhonotus infernalis*	Texas alligator lizard	Pet trade	BGF
MT249126	*Barisia imbricata*	Transvolcanic alligator lizard	Pet trade	BGF
MT249119	*Abronia graminea*	Mexican alligator lizard	Pet trade	BGF
MN337817	*Anilios bituberculatus*	Prong-snouted blind snake	Pet trade	FJV

Preparation of venom stock solution

Naja naja (spectacled cobra) venom was used in LD_{50} and LC_{50} functional assays. The venom was supplied by Freek J. Vonk (FJV). The venom was freeze-dried (lyophilised) and stored at 20°C. For the experiments on the chicken and bearded dragon embryos, 7.7 mg/mL venom stock solution in sterile HBSS (Hanks' balanced salt solution; Sigma Aldrich, H9269) was prepared. For *Gasterosteus aculeatus* and *Danio rerio*, the stock solution was also 7.7 mg/mL, but was prepared in egg water and tap water, respectively (that is, the swimming water for those two species). This yielded stock solutions with a venom concentration of 7.7 mg/mL. The stock solution was divided into 30 tubes in an amount of 100 µL per tube. These were stored at 80°C

Embryo set-up

This LD_{50} assay was performed using embryos of *Gallus gallus* and *Pogona vitticeps*. The embryos were stored in a humidified incubator on stationary at 38°C. *Pogona vitticeps* eggs were supplied from Reptielenhuis De Aarde, Breda, and Terrariumspeciaalzaak Kameleon, Tilburg, the Netherlands. *Pogona vitticeps* eggs were incubated in a humidified incubator at 28°C. *Gasterosteus aculeatus* larvae were kindly provided Dr. Jörn Scharsack, Institute for Evolution and Biodiversity, Universität Münster, Germany, and were incubated at 17°C. *Danio rerio* were obtained from the zebrafish facility of the Institute of Biology, Leiden University, and were incubated at 28°C.

LD_{50} Assay in *Gallus gallus* (Domestic Chicken) Embryos

Gallus gallus embryos of 5 day incubation were injected with 10 µL of venom solution. This solution was dropped onto the punctured vitelline membrane of the embryo, as described in (Ahmed, El-Din, Mohamed *et al.*, 1974). A hole was made in the vitelline membrane with a tungsten needle. Four different venom concentrations were used: 1X (stock), 16X, 32X, and 64X, plus a control consisting of 10 µL of Hanks' salt solution. The embryos were staged as described in (Hamburger *et al.*, 1951). The embryos were at stage

24 (Hamburger *et al.*, 1951). Then, 10 µL venom was dripped onto the embryo with a Gilson P20 pipette through the previously-made hole. The egg was sealed afterwards with adhesive tape and returned to the incubator at 38°C. The embryos were inspected 24 h after injection to see whether they were alive or dead.

LD_{50} Assay in *Pogona vitticeps* (Inland Bearded Dragon) Embryos

There is no method described in the literature for LD_{50} assay on lizard embryos. Lizard eggs have a leathery, non-calcified shell and no air sac, and are thus extremely difficult to open without damage using the standard chicken embryo approach of 'windowing'. This is because the egg contents are very liable to herniate through the opened hole. We thus developed a new technique, which we describe here. The lizard embryos were staged as closely as possible to the Hamburger–Hamilton series. The position of the embryo was determined by candling, and a hole was made in the shell and shell membrane, just beyond the position of the embryo, using a sterilized syringe needle of gauge 26, L 1/2 inch.

We then removed 30 to 50 µL of egg albumen using a sterile hypodermic 1 mL syringe. Then, 10 µL of venom solution was injected through the hole and under the shell membrane near the embryo using a Gilson P20 pipette. We did not, as we had in the chicken, puncture the vitelline membrane with a tungsten needle because of the danger of herniation of egg contents or damage to the embryo. However, it is at least possible that, in some cases, the vitelline membrane may have been ruptured by the hypodermic needle. This could not be determined, however, because of the lack of an air sac for windowing. The egg was sealed with an instant adhesive (Loctite 406; Henkel Adhesives, Düsseldorf, Germany) and incubated at 28°C. The embryos were inspected 24 h after injection to determine whether the embryos were alive or dead.

LC_{50} Assay on *Gasterosteus aculeatus* (Three-Spined Stickleback) and *Danio rerio* (Zebrafish) Developmental Stages

A geometric series was used, namely, 1X (stock), 2X, 4X, 8X, 16X, 32X, 64X, 128X, and 256X, plus a control consisting of 10 µL of vehicle (embryo medium). The diluted venom (60 µL) was introduced to each well of a 24-well tissue culture plate (VWR, 734-2325, VWR International, Radnor, Pennsylvania, USA) in which the single larvae were cultured, giving a total volume per well of 600 µL. One column of wells in the plate counted as controls. These control wells contained only 600 µL of embryo medium and a single larva. The mortality of the developing G. aculeatus) and D. rerio was recorded after 24 h. The following three criteria needed to be met for embryo to be scored as 'dead': tissue opaque (milky-white) in appearance instead of transparent; heart not beating; and fish motionless (no locomotor activity). The LC_{50} values of *N. naja* venom were determined based on mortality scoring using Regression Probit analysis. This was achieved using the dose–response curve (drc) package in RStudio© (version 1.1.456; https://rstudio.com/).

Results and Discussion

Bioinformatics

Our study examines resistance to cobra α-neurotoxin cobra in lizards and fish. We investigated the sequences of the ligand-binding domain of α subunit of nAChR in a seven teleost and lizard families including Helodermatidae, Anguidae, Varanidae, Agamidae and Iguania. We sequenced 12 lizard species and derived five further sequences from the NCBI database. We compared these sequences with the ligand-binding domain of the α-subunit of nAChR of snakes and mammals that have known modifications to their receptor (Barchan et al., 1995a) see (Figure 8). Amino acid alignments of the ligand-binding domain of the α-subunit of nAChR showing in a range of reference species. The numbers 187 and 189 indicate the amino acid positions relative to the human sequence and * indicates an

amino acid linked to neurotoxin resistance.. We found that, *Pogona vitticeps* (inland bearded dragon) both shared the 187–189NVT motif, which has been described in *H. ichneumon* and *Naja naja* (Barchan et al., 1995a, Takacs et al., 2001(Khan *et al.*, 2020)). However, in our sequence analysis, we found that several species possess proline replacements at positions 194 and 197, identical to those that have been previously associated with resistance (Kachalsky, Jensen, Barchan *et al.*, 1995).

The 194L mutation is particularly widespread, and was found in the following: *Suricata suricatta* (meerkat); all three of the Australian agamids studied (*Intellagama lesueurii* (water dragon), *Lophognathus gilberti* (Gilbert's dragon), and *P. vitticeps*); the anguimorph lizard *Barisia imbricata* (transvolcanic alligator lizard); a 194T mutation in the anguimorph lizard *Gerrhonotus infernalis* (Texas alligator lizard)(Khan *et al.*, 2020). The exact impact of these mutations is difficult to predict because the study that identified them suggested that there are complex patterns of interaction between mutations at positions 194 and 197, as well as between these mutations and those associated with steric hindrance resistance at positions 187 and 189 (Kachalsky *et al.*, 1995). Thus, the results of species in this study identified as having replacements of prolines at positions 194 or 197 must be interpreted with caution. As mentioned above, even those specific substitutions that have been demonstrated to confer resistance in one taxon cannot confidently be stated to do so in others, especially mutations to amino acids that have never specifically been associated with resistance.

For instance, α-neurotoxins have been found to bind the ligand-binding domain sequences of both the radiated ratsnake (*Coelognathus radiates*, which contains the 194L mutation) and Schlegel's Japanese gecko (*Gekko japonicus*, which contains the 194T mutations) with higher affinity than they do to other species tested (Harris, Zdenek, Debono *et al.*, 2020; Harris, Zdenek, Harrich *et al.*, 2020; Zdenek, Harris, Kuruppu *et al.*, 2019). These findings underscore the fact that not all substitutions at these sites confer resistance (e.g., (Dellisanti, Yao, Stroud *et al.*, 2007)) and that complex interactions, involving multiple amino acids, may be involved in conferring

resistance. Thus, with the exception of the well-validated resistance conferred by N-glycosylation present at positions 187 or 189, other mutations cannot be attributed as conferring resistance until validated as such through functional testing.

To support our conjecture that the N-glycosylated asparagine confers resistance in additional species, we demonstrated decreased mortality following exposure to α neurotoxins in two species with these mutations, compared with two species without these mutations Table 9. In this series of developmental toxicity assays, we used embryos of *P. vitticeps* and *G. aculeatus,* which possess mutations 187–189NVT and 189–191NYS, respectively (Khan *et al.*, 2020). For comparison, we used the embryos of *Gallus gallus* (domestic chicken) and *Danio rerio* (zebrafish), both of which lack relevant mutations. The embryos were exposed to *Naja naja* venom in a concentration series to calculate the lethal dose or lethal concentration for 50% of embryos/larvae (LD_{50} and LC_{50}, respectively). *G. aculeatus* tolerated approximately ten times more venom than D. rerio (LD_{50}: 0.673 vs. 0.062 mg/mL), *and P. vitticeps* around five times that of *G. gallus* (LD_{50}: 1.870 vs. 0.340 mg/mL). In this chapter we performed DNA sequencing and analysis of the ligand-binding domain of the α-subunit of nAChR in 16 lizards species. We also examined seven teleost fish sequences because of the surprising find, during the earlier days of my Ph.D. studies, that the three-spined stickleback and reedfish have glycosylation at position 189 (Gunasekaran *et al.*, 2017; Khan *et al.*, 2020; Figure 7).

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Furthermore, the lack of any sites in the ligand binding domain of the nAChR under positive selection (Chapter 4), and the relatively strong negative selection across the ligand-binding domain (Chapter 4), it is likely that these modifications in the two teleosts are the result of an evolutionary process unrelated to our hypothesis about resistance. Nonetheless, we show experimentally that the 189–191NYS motif in *G. aculeatus* does indeed reduce susceptibility to Indian cobra venom Table 9. As discussed below, it appears that there is a fitness disadvantage to the N-glycosylation, with it being secondarily lost in lineages (e.g., *Vipera berus*) that have radiated into areas outside the range of neurotoxic elapid snakes. Thus, the presence of the modification in two unrelated lineages of fish is intriguing and a fascinating area for future research. As our functional testing showed that *G. aculeatus* is indeed resistant to neurotoxins Table 9, and putatively *E. calabaricus* as suggested by its N-glycosylation site, a hypothesis to test would be if this modification confers resistance to anatoxin-α (also known as very fast death factor), a powerfully neurotoxic bicyclic amine alkaloidal cyanotoxin secreted by freshwater cyanobacteria that potently binds to nicotinic acetylcholine receptors (Aráoz, Molgó & Tandeau de Marsac, 2010).

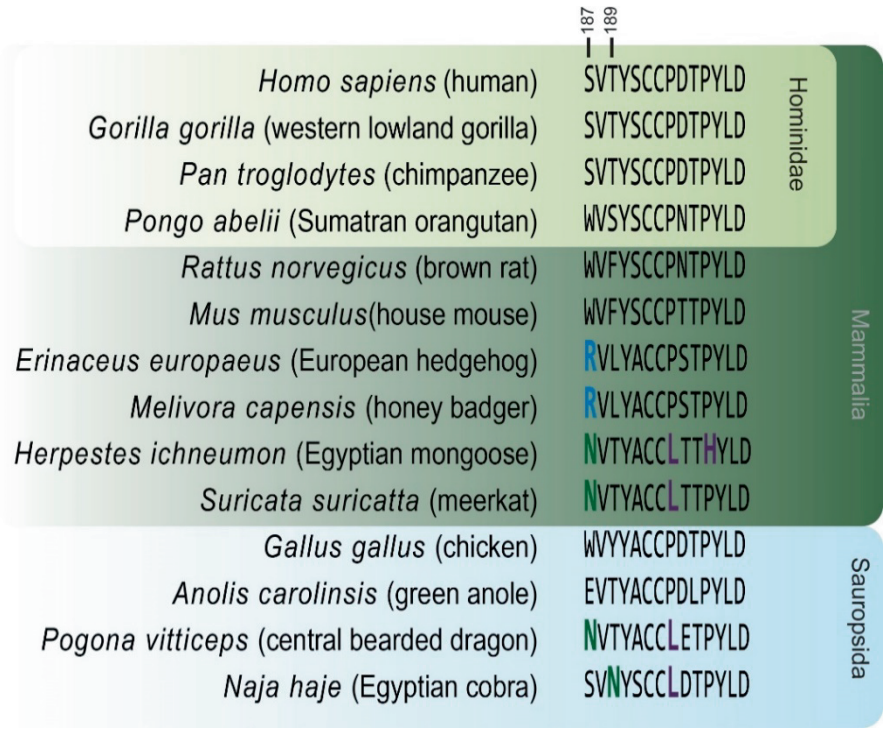

Figure 8. Amino acid alignments of the ligand-binding domain of the α-subunit of nAChR showing in a range of reference species. The numbers 187 and 189 indicate the amino acid positions relative to the human sequence and * indicates an amino acid linked to neurotoxin resistance.

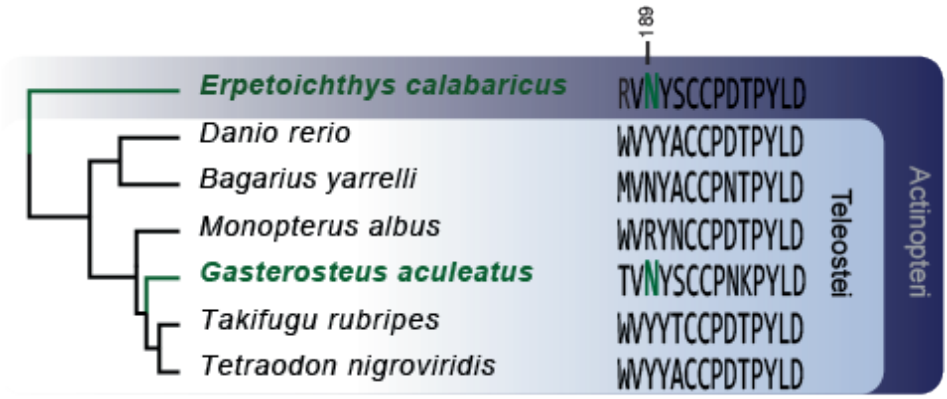

Figure 9. Tree showing phylogeny of the Fish species studied in this chapter and the nACHR ligands binding domain amino acid sequence. Key: Green highlight corresponds to the three-spined stickleback (Gasterosteus aculeatus) and Reedfish (Erpetoichthys calabaricus) which has an asparagine at position 187. This sequence modification is also found in some snake-eating mammals (Barchan et al., 1995; Kachalsky et al., 1995; Khan et al., 2020; Takacs, Wilhelmsen & Sorota, 2004).

We found amino acid substitutions (W→N), at position 187 (W187N) and (P → L) at position 194 (P194L) in the bearded dragon. As a result of these substitutions, we see the motif (N-X-T/Y) at positions 187-189 and at position 194 (P194L) the bearded dragon (Figure 8) . This motif is also seen in the Egyptian mongoose (*Herpestes ichneumon*) and the meerkat (*Suricata suricatta*), which both show venom resistance (Barchan *et al.*, 1992a; Drabeck, Dean & Jansa, 2015; Kachalsky *et al.*, 1995; Khan *et al.*, 2020; Figure 8).

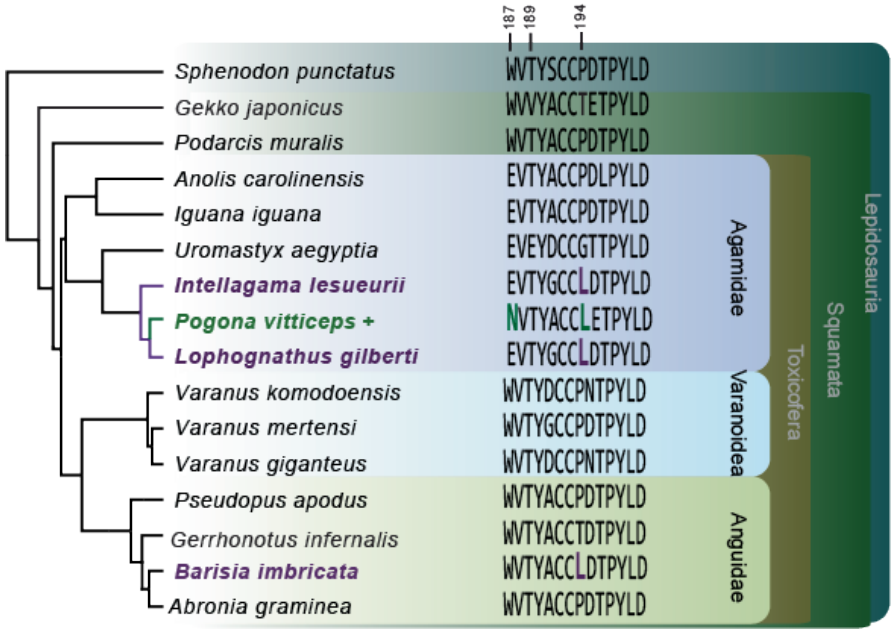

Figure 10. Tree showing phylogeny of the lizard species studied in this chapter and the nACHR ligands binding domain amino acid sequence. Key: Green highlight corresponds to the eastern beard dragon (Pogona vitticeps) which has an asparagine at position 187 and a proline replaced with leucine at position 194. This sequence modification is also found in some snake-eating mammals (Barchan et al., 1995; Kachalsky et al., 1995; Khan et al., 2020; Takacs et al., 2004). Purple highlight corresponds to lizards (Intellagama lesueurii, Lophognathus gilberti, Barisia imbricata) with a proline replaced with leucine at position 194. This replacement is identical to the substitution that is associated with α-neurotoxin resistance in mammals (Kachalsky et al., 1995).

In the Egyptian cobra (*Naja haje*) the motif (N-X-S/Y) is known to be present at positions 189-191; this species is resistant to its own toxins (Takacs, Wilhelmsen & Sorota, 2001). The same motif has been predicted to be strongly associated with glycosylation of asparagine (Barchan *et al.*, 1992a; Gunasekaran *et al.*, 2017; Khan *et al.*, 2020). The mammals resistant to α-

neurotoxins share a conserved consensus sequence pattern (N-X-T/Y) for glycosylation, as predicted by (Gavel & Heijne, 1990).

It has been suggested (Mononen & Karjalainen, 1984) that the presence of proline in positions X and Y of the consensus sequence (N-X-T/Y) should reduce the chances of glycosylation. Further, in our sequence analysis, we found that several species possess proline replacements at position 194 identical to those that have been previously associated with resistance (Kachalsky et al., 1995). The 194L mutation is particularly widespread, and was found in the following species *Suricata suricatta* (meerkat); all three of the Australian agamids studied (*Intellagama lesueurii* (water dragon), *Lophognathus gilberti* (Gilbert's dragon), and *P. vitticeps*) and the anguimorph lizard *Barisia imbricata* (transvolcanic alligator lizard); (Khan *et al.*, 2020) We have summarised some key details of resistance-related motifs in Table 11 in Chapter 5.

One model of molecular resistance to α-neurotoxins is that the proline residues block the acceptor sites for glycosylation in the consensus sequence (Gavel *et al.*, 1990; Mononen *et al.*, 1984). A previous study using physiological assays has shown that some lizards have skeletal muscle that is resistant to cobra α-neurotoxin, α-atratoxin and α-bungarotoxin (Burden *et al.*, 1975). The lizards assayed in that study were: the African plated lizard (*Gerrhosaurus validus*), the Eastern glass lizard (*Ophisaurus ventralis*) and various *Lacerta* sp. (Burden *et al.*, 1975). Another study using physiological assays showed that some Australian skinks which are striped skink , tree skink and White's rock-skink (*Plestiodon fasciatus*, *Lamprolepis smaragdina, Liopholis whitii*) show high resistance to the venom of the Australian elapids the mainland tiger snake, the eastern brown snake (*Pseudonaja textilis*) and the death adder (*Acanthophis antarcticus*), (Minton Jr *et al.*, 1981).

Table 9. Toxicity Assays of cobra venom toxicity. Probit analysis was used to calculate the LD_{50} or LC_{50}. For full details of the statistical analysis see Supplementary File 3 and Refs.(Faraggi, Izikson & Reiser, 2003; Paige, Chapman & Butler, 2011; Ritz, Baty, Streibig *et al.*, 2016).

	Concentration of *Naja naja* venom (mg/mL)										
	0.00	0.03	0.06	0.12	0.24	0.48	0.945	1.89	3.78	7.7	LD_{50} or LC_{50} mg/mL
Bearded dragon											**1.87**
alive	5	-	-	5	5	5	-	-	-	0	
dead	0	-	-	0	0	0	-	-	-	5	
Chicken											**0.340**
alive	5	-	-	5	5	0	-	-	-	0	
dead	0	-	-	0	0	5	-	-	-	5	
Stickleback											**0.673**
alive	8	8	8	8	8	8	0	0	0	0	
dead	0	0	0	0	0	0	8	8	8	8	
Zebrafish											**0.062**
alive	8	8	5	0	0	0	0	0	0	0	
dead	0	0	3	8	8	8	8	8	8	8	

Here, we have not found comparable alterations in the ligand-binding domain of nACHR of the other lizard species examined, namely: Schlegel's Japanese gecko (*Gekko japonicus*), the Common wall lizard (*Podarcis muralis*), Rio Fuerte beaded lizard (*Heloderma exasperatum*), the European glass lizard (*Pseudopus apodus*), the Texas alligator lizard (*Gerrhonotus infernalis*), the Imbricate Alligator Lizard (*Barisia imbricata*), the terrestrial arboreal alligator lizard (*Abronia graminea*), Mertens' water monitor (*Varanus mertensi*), the perentie (*Varanus giganteus*), the Komodo dragon (*Varanus komodoensis*), the Egyptian spiny–tailed lizard (*Uromastyx aegyptia*), the Australian water dragon (*Intellagama lesueurii*), Gilbert's Dragon (*Lophognathus gilberti*), the American iguana (*Iguana iguana*) and the green anole (*Anolis carolinensis*). It is possible that these lizards have evolved anti-predator defenses other than resistance to toxins. Thus, behavioral adaptations are seen in some species as I shall now discuss.

It has been shown that the Western banded gecko (*Coleonyx variegate*) can recognize skin chemicals of the lizard-eating (saurophagous) snake, the spotted leafnose (*Phyllorhynchus decurtatus*), and the non-lizard-eater, the Western shovel-nose snake (*Chionactis occipitalis*) (Dial, Weldon & Curtis, 1989). In response to the chemical signals of the lizard-eating snake, the gecko showed defensive behavior and tail display. However, the tail display was not observed when the gecko encountered skin chemicals from the non-lizard-eater (Dial *et al.*, 1989). This suggests that at least some species have evolved behavioral patterns that may constitute a form of resistance towards a venomous predator. For further examples, see Refs. (Balderas-Valdivia & Ramírez-Bautista, 2005; Cooper Jr., 1989; Cooper, 1994; Downes & Shine, 1998; Durand, Legrand, Tort *et al.*, 2012; Knight *et al.*, 1999; Phillips *et al.*, 1992). In this chapter, we used *in vivo* assays to assess the toxicity of Indian cobra venom. This assay employed the embryonated eggs of the bearded dragon and compared it with the same test using chicken embryos. In these embryo assays, we found that the LD_{50} of India cobra venom in the bearded dragon embryo was 1.870 mg/mL while in the chicken embryo it was 0.340 mg/mL . This suggests that the chicken is much more susceptible

toward cobra venom than the bearded dragon. This is consistent with my finding that the bearded dragon has W187N compared to the chicken, which has ancestral tryptophan at position 187.

An examination of sequences in the public NCBI database, showed that the three-spined stickleback and reedfish also has F189N in the acetylcholine receptor (Khan *et al.*, 2020). We then found that the change had been reported before in the stickleback (Gunasekaran *et al.*, 2017). This is the change seen in animals presumed to be resistant to cobra α-neurotoxin (see Refs. (Drabeck *et al.*, 2015; Takacs *et al.*, 2001). Interestingly, We found that the zebrafish did not have that change. My assays on the larvae of these fish found that the LC_{50} of Indian cobra venom in three-spined stickleback larvae was 0.673 mg/mL while in the zebrafish it was 0.062 mg/mL. We, therefore conclude that the zebrafish is more susceptible to Indian cobra venom than is the three-spined stickleback. At present, it is unclear what advantage, if any, this resistance provided to the three-spined stickleback.

The work in this chapter provides new insight into the pattern of resistance to cobra venom in vertebrate species. In particular, it expands the range of animals known to have modifications of the nAChR. To date, such modifications had been found in a few mammals (Drabeck *et al.*, 2015), the Egyptian Cobra (*Naja haje*) (Takacs *et al.*, 2001), the Chinese cobra (*Naja atra*) and the dice snake (*Natrix tessellata*) (Neumann, Barchan, Horowitz *et al.*, 1989). We show here that the resistance-associated modification of the nAChR is also present in the bearded dragon. Our functional assays suggest that the modification does indeed confer resistance. A similar correlation between the modification and resistance was seen in my fish assays. Further work is needed to validate the bearded dragon assay and to explore a wider range of lizards.

Acknowledgements

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Chapter 4: A Search for Evidence of Cobra α-Neurotoxin Resistance in the Nicotinic Acetylcholine Receptor of Snake-eating Birds and Crocodilians

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Abstract

As we have seen in previous chapters, a number of animals that prey on snakes show resistance to cobra α-neurotoxins. The resistance is due to amino acid changes in the α-subunit of the nicotinic acetylcholine receptor (nAChR) of the neuromuscular junction. These changes inhibit snake α-neurotoxin binding to the receptor. In this chapter I want to determine whether birds of prey, peacocks, or other snake-eating (ophiophagous) birds have acquired similar changes. I also examine the crocodilians because these are a sister group to birds. A total of 25 DNA samples from wild and captive birds, together with sequences from public databases, were analyzed. The material I harvested in the wild from Pakistan represents the first large, multispecies DNA samples collected from birds of prey for the purpose of toxin study. DNA from the Nile crocodile (*Crocodylus niloticus*) and four crocodilian sequences from public databases was analyzed. Species identifications of the bird DNA samples were validated by DNA barcoding. Surprisingly, we found no evidence of sequence changes that might correlate with resistance in any of the birds sampled, even though these birds are known to attack and eat snakes. We discuss several possible explanations for these finding.

Introduction

Thousands of people die each year from snakebites in many countries (Chippaux, 1998; Kasturiratne, Wickremasinghe, de Silva *et al.*, 2008). In Australia, the number of deaths from snakebites has remained constant over the last 30 years despite the advanced healthcare system in that country. The incidence is 2.4 per 100,000 people (Bradley, 2008; Welton, Liew & Braitberg, 2017a) and the death rate 0.13 per 100,000 people per year (Welton *et al.*, 2017a; Welton, Williams & Liew, 2017b). Interestingly, some wild animals that are thought to prey on snakes, have evolved some kind of venom resistance. Examples include the honey badger (*Mellivora capensis*), the Egyptian mongoose (*Herpestes ichneumon*), the meerkat (*Suricata suricatta*) the European hedgehog (*Erinaceus europaeus*) and the domestic pig (*Sus scrofa*). These animals are thought to include snakes in their diet, and also show modification of the α-subunit nAChR (Farquhar, 1986b; Welton *et al.*, 2017b).

The nAChR itself is composed of alpha, beta, epsilon and gamma subunits (Kreienkamp, Sine, Maeda *et al.*, 1994). Neumann *et al.* and Barchan *et al.* have sequenced the α-neurotoxin-binding domain of the nAChR of the cobra and the mongoose (Barchan, Kachalsky, Neumann *et al.*, 1992; Neumann, Barchan, Horowitz *et al.*, 1989). This revealed a replacement of aromatic residues (tryptophan and phenylalanine) of the ligand-binding domain with a non-aromatic (asparagine) residue, which provides a site for glycosylation. The addition of the glycosylic group at the binding site is thought to be the main reason for α-toxin resistance the mongoose as well, interestingly, in the Egyptian cobra itself (*Naja haje*) (Takacs, Wilhelmsen & Sorota, 2001).

In addition to the species just mentioned, there are many birds that are thought to eat snakes. A well-known example is the Indian blue peafowl (*Pavo cristatus*), an omnivorous bird that consumes insects,

worms, lizards, toads and snakes (Chopra & Kumar). This bird is often kept in captivity, not least because its alleged snake-eating (ophiophagous) habit is valued by the owner (Jackson, 2006). In Sanskrit the name of this species means 'killer of snakes' (Jackson, 2006). There are also reports of ophiophagy in birds of prey (raptors) (Fitch & Bare, 1978; Leatherman) but very little is known about how birds of prey avoid being poisoned by the venomous snakes that they prey on. In many hawk species, snakes are part of their diet (Bent, 1937; Knight & Erickson, 1976).

Buteo jamaicensis (the red-tailed hawk) relies heavily on snakes (Knight *et al.*, 1976). In one study it was recorded that their diet content contained (by mass): 16.8% *Coluber constrictor*, 30.9% *Pituophis melanoleucus*, 0.4% *Thamnophis* sp. and *Crotalus viridis* (1.1%) (Knight *et al.*, 1976). *Geranoaetus albicaudatus* (the white-tailed hawk) can also prey on venomous snakes with apparent impunity (Farquhar, 1986b; Fitch *et al.*, 1978; Leatherman).

In another study, Iguanids, tree monitors, vipers, elapids and colubrid were caught by, or found in the diet of, the tawny eagle (*Aquila rapax*) (Steyn, 1982). A range of other raptors species belonging to the families Accipitridae, Falconidae and Strigidae may also include snakes in their diet (Bent, 1937; Farquhar, 1986a; Fitch *et al.*, 1978; Gehlbach, 1995; Henry & Gehlbach, 1999; Kannan & James, 1998; Kilham, 1989; Knight *et al.*, 1976; Kochert, Bammann, Steenhof *et al.*, 1975; Leatherman; Ogden, 1974; Parker, 1999; Sherrod, 1978; Sparkman, Bronikowski, Billings *et al.*, 2013).

Other presumed ophiophagous birds include the secretary bird (*Saggitarius cristatus*) (Portugal, Murn, Sparkes *et al.*, 2016) and the red-legged seriema (*Cariama cristata*) (Ridgely, 2016). We were interested in determining whether birds of prey, peacocks and other ophiophagous birds have acquired similar types of amino acid replacement in the toxin-binding region of the α-subunit of the nAChR

as seen in mammals. Here, we amplify and sequence the toxin-binding region in a range of birds of prey, peacock breeds and the red-legged seriema. We also examine sequences from crocodilians, because these are the extant sister group of birds.

Stomach content analysis shows that the Nile crocodile (*Crocodylus niloticus*) preys on multiple snake species (B.Cott, 1961). Another study found brown water python (*Liasis fuscus*) and aru mangrove snake (*Myron richardsoni*) in the stomach contents of the saltwater crocodile (*Crocodylus porosus*) (Taylor, 1979). We compare all these sequences with those from a range of other vertebrates that are known to be resistant to the α-neurotoxin of the cobra. Blood samples were collected from a range of wild birds of prey and peacocks in Pakistan. Further, DNA was collected from the feathers of captive *Cariama cristata* specimens and from embryonic tissue of *Crocodylus niloticus*. The DNA was sequenced and the sequences compared so as to explore potential venom resistance.

Materials and Methods

Ethical statement

Samples were provided by Dr. Jawad Nazir and Muzaffar Ali Khan, who were both qualified veterinary surgeons in permanent government (university) employment in Pakistan at the time of writing. The project was approved by the Ethics Committee of the University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan. No data reported here came from birds on the International Union for Conservation of Nature (IUCN) red list of endangered species. The birds sampled were pre-existing in the trade in Pakistan. No birds were caught in the wild at our request and no money was paid to the owners.

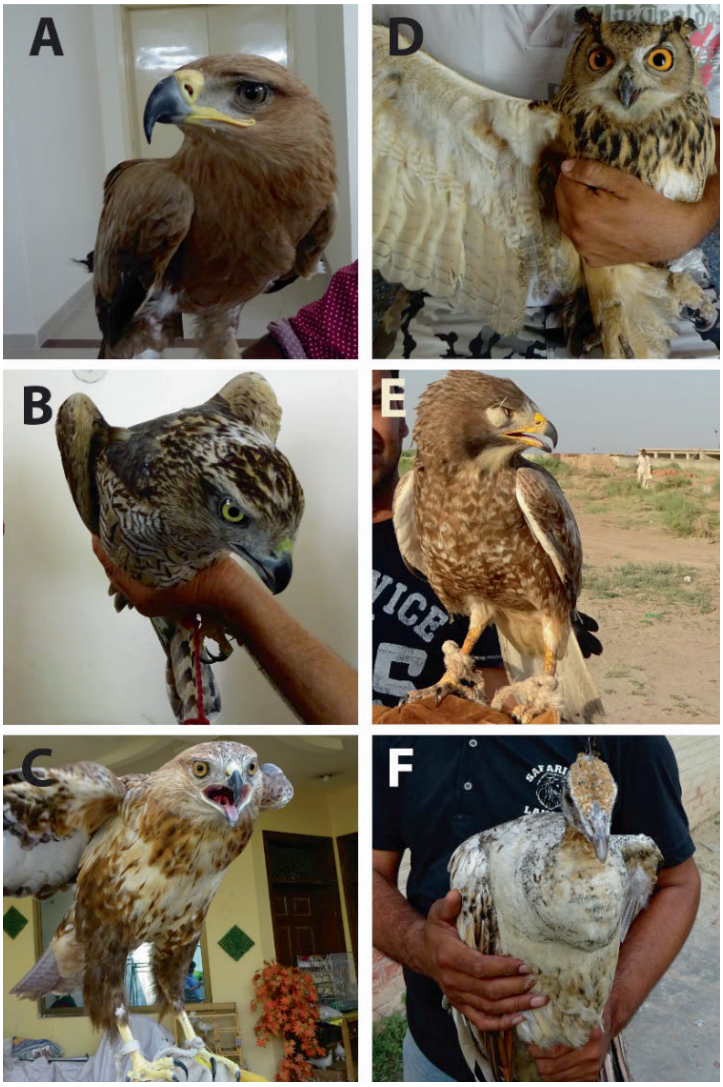

Figure 11. Representative field photos of sampled birds. A, *Aquila rapax* (Tawny eagle); B, *Accipiter genitis* (Goshawk); C *Buteo buteo* (Common buzzard); D, *Bubo bubo* (Eurasian eagle-owl); E *Butastur liventer* (Rufous-winged buzzard); F, *Pavo cristatus* (Peacock).

The animals had been captured previously, without any communication from us. Other birds sampled were captive bred in zoological gardens. No anaesthesia was given before the blood samples were taken, but every effort was made to cause minimal stress and anxiety to the birds. Indeed, this gentle handling was insisted upon very strongly by the 'owners', to whom the birds have a substantial financial value.

Fieldwork

The wetlands of Pakistan are home to many species of bird. Every year, 0.7 million to 1.2 million birds migrate to Pakistan by using a migratory route called the Indus flyway, which runs from Siberia to the Indus plains (Ali & Akhtar, 2006). According to unpublished personal observations of two of the authors (MAK and JN), people in the Multan, Alipur, Khar pur Saddat, Rohi Desert, and Bahawalpur regions see (Figure 12) often trap these birds of prey illicitly to keep as pets, to use for hunting other birds (falconry), or to sell on to the lucrative falconry market in the Gulf States.

Collection of blood samples was carried out by MAK and JN during field trips between September 2015 and January 2016, the migratory season of birds using the Indus flyway. All the fieldwork was carried out with support of local people, who were not paid for allowing us to take blood samples. The collection of blood samples from the birds of prey and wild peacocks was done in the following locations in Pakistan: Multan; Lahore Zoo Safari Park; Alipur; Khar pur Saddat; and the Rohi Desert, Bahawalpur. Only adults were sampled. The GPS locations of all samplings were recorded with a GPS device (eTrex® 20). The peacocks were the only birds sampled in the safari park, Lahore, Pakistan, with the assistance of the safari park veterinary officer.

DNA extraction from samples

A standard blood collection procedure was adopted (Arctander, 1988). A total of 24 blood samples were collected from birds of prey and peacocks. One mL of blood was withdrawn from the wing vein using a 1 mL sterile hypodermic needle and syringe. Half of the blood was transferred to an evacuated blood collection tube (Becton, Dickinson and Company, Franklin Lakes, New Jersey, United States) and the other half to 10 mL of 100% ethanol. The samples were then transported to the Department of Microbiology, UVAS Lahore, Pakistan, on ice. DNA was extracted using the QIAGEN DNeasy kit (Qiagen, Inc., Valencia, CA, and USA) in the molecular biology lab of the Department of Microbiology, UVAS Lahore, Pakistan. Finally, the DNA was transported to Leiden University by courier in compliance with their biological shipment procedures. Fertilized eggs of *Crocodylus niloticus* were obtained from La Ferme aux Crocodiles, Pierrelatte, France.

Amplification and sequencing of the nicotinic acetyl choline receptor gene (CHRNA1)

Each blood sample was processed and sequenced separately; there was no pooling of samples. Primers specific for the α-subunit of the nicotinic acetylcholine receptor (nAChR) were designed, based on the chicken sequence (NM_204816.1) in the database of the National Center for Biotechnology Information (NCBI). The ligand-binding domain in the chicken was identified by alignment with the corresponding reference protein of the honey badger (*Mellivora capensis*) (Drabeck, Dean & Jansa, 2015) to be in exon 6. This chicken sequence was aligned with the sequences of the bald eagle (*Haliaeetus leucocephalusused*, XM_010566433.1) and the peregrine falcon (*Falco peregrinus*, XM_013298866.1). M13 primer sites were added for easier sequencing.

The PCR was performed in 25 µL reactions using 1.0 ml of 10 mM CHRNA1F1M13 (5'-GTTTTCCCAGTCACGACCCTGA-TCTGAGTAACTTCAT GGAGAG-3') primer solution, 1.0 mL of 10 mM CHRNA1R1M13 (5'-CAGGAAA-CAGCTATGACAAGGAGAAG-AGCAGGCAGGG-3') primer solution, 0.2µL DNA polymerase, concentrations of buffer CL (recommended by Qiagen, Inc., Valencia, CA, USA), and dNTPs. Reactions were performed for 30 cycles of melting 95°C for 5 minutes, followed by annealing at 95°C for 10 seconds, and extension at 65°C for 10 s. Reactions were preceded by a 1 minute denaturation at 95°C and included a final extension at 72°C for 20 minutes. Primers of the crocodilian are in the chapter supplementary data.

nAChR sequence analysis

The amplified PCR products of nAChR for all birds and one Nile crocodile were sequenced by Baseclear B.V., the Netherlands. The sequences were translated into protein and aligned with the program Vector NTI (Thermo Fisher Scientific; Waltham, Massachusetts, United States). The ligand-binding domain in the avian nAChR was examined and compared with the orthologous region in a range of other vertebrates, using sequences from NCBI. All sequences were submitted to The National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/) and can be found under accession numbers see Table 10.

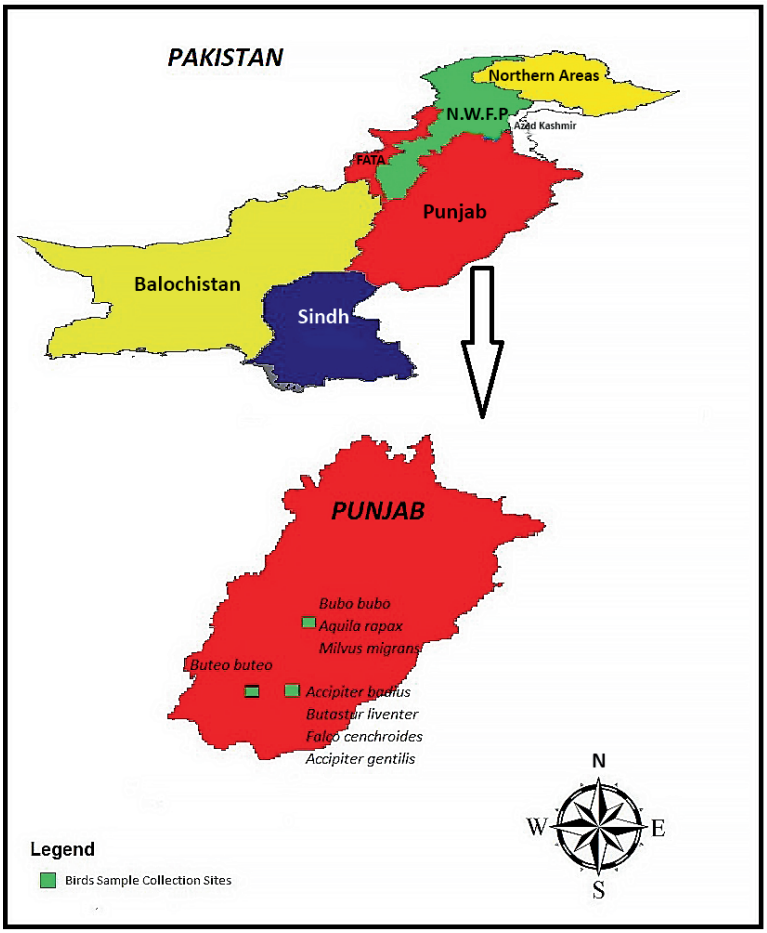

Figure 12. Bird samples collection sites. Top (in yellow, green, red and purple), the provinces of Pakistan, with red indicating Punjab. Bottom with light green boxes: cities of Der a Ghazi Khan, Multan, and Bahawalpur in the province of Punjab. Source: Geographic Information System (GIS). The sites of collection of the birds of prey are noted with their species names in italics. With acknowledgements to associate professor Dr Muhammad Jehanzeb Masud Cheema, Faculty of Agricultural Engineering & Technology, Pir Mehr Ali Shah (PMAS) Arid Agriculture University Rawalpindi, Pakistan for providing access to GIS.

Results and Discussion

DNA was collected from the feathers of the one red-legged seriema, from embryos of the Nile crocodile, and from blood samples taken from 16 individual birds of prey, seven individual peacocks and one chicken. Sequencing of these materials generated ten bird and four crocodilian sequences for the ligand-binding domain of the nAChR. We then screened these sequences for the presence or absence of resistance-related sequences changes. In Chapter 2 of this thesis, resistance-related mutations were found in lizards and some fish. Our hypothesis was that resistance-related mutations would be found in birds too, especially the birds sampled here which prey on snakes. Contrary to expectations, we did not find any such mutations in any of the birds that we studied see (Figure 13). This lack of resistance motifs in *Circaetus pectoralis* (black-chested snake eagle) and *Sagittarius serpentarius* (secretary bird) was particularly unexpected because they are snake-eating (ophiophagous) species (Figure 3) Ophiophagy (predation upon snakes) is common in birds of prey (Bent, 1937; Farquhar, 1986a; Fitch *et al.*, 1978; Knight *et al.*, 1976). Furthermore, *Pavo cristatus* (the Indian blue peafowl), and *Cariama cristata* (the red-legged seriema) also sometimes feed on snakes (Chopra & Kumar, 2014). Some birds, such as *Circaetus* sp. (snake eagles) and *Sagittarius serpentarius* (secretary birds), are snake-specialist predators (Sinclair, Hockey & Tarboton, 2012). For these reasons, we predicted that resistance to α-neurotoxins would be present in birds.

Table 10. DNA barcoding of sampled bird species. The species identification of the birds of prey was confirmed by DNA barcoding using cytochrome c oxidase I (COI). Key: no of samples, species name, Sequence identification number (ID).

No. Individuals sampled	Scientific name	Common name	Sequence ID
-	*Pelodiscus sinensis*	Chinese soft-shelled turtle	XM_006119477.3
-	*Alligator sinensis*	Chinese alligator	XM_006020803.2
-	*Alligator mississippiensis*	American alligator	XM_006267516.3
-	*Gavialis gangeticus*	Gharial	XM_019522952.1
-	*Crocodylus porosus*	Saltwater crocodile	XM_019554696.1
1	*Crocodylus niloticus*	Nile crocodile	MT249132
-	*Dromaius novaehollandiae*	Emu	XM_026092832.1
7	*Pavo cristatus*	Indian peafowl	MT231212
1	*Gallus Gallus*	Chicken	MT274612
1	*Cariama cristata*	Red-legged seriema	MT262918
1	*Bubo bubo*	Eurasian eagle-owl	MT231210
1	*Falco tinnunculus*	Common kestrel	MT231209
1	*Falco cenchroides*	Nankeen kestrel	MT231206
-	*Sagittarius serpentarius*	Secretary bird	VWYJ01026266.1

-	*Circaetus pectoralis*	Black-chested snake eagle	VZZV01000171.1
1	*Aquila rapax*	Tawny eagle	MT231205
1	*Accipiter badius*	Shikra	MT231204
1	*Accipiter genitis*	Goshawk	MT231203
1	*Milvus migrans*	Black kite	MT231207
8	*Butastur liventer*	Rufous-winged buzzard	MT231209
1	*Buteo buteo*	Common buzzard	MT231211

However, we found no resistance mutations in any of the birds studied. Furthermore, we showed in Chapter 2 using LD_{50} testing on *Gallus gallus* embyos is relatively susceptible to spectacled cobra venom. In Chapter 2 of this thesis, resistance-related mutations were found in lizards and some fish. Our hypothesis was that resistance-related mutations would be found in birds too, especially the birds sampled here which prey on snakes. Contrary to expectations, we did not find any such mutations in any of the birds that we studied (Figure 13). This lack of resistance motifs in predatory birds *Circaetus pectoralis* (black-chested snake eagle) and *Sagittarius serpentarius* (secretary bird) was particularly unexpected because they are snake-eating (ophiophagous) species (Figure 3).

Ophiophagy (predation upon snakes) is common in birds of prey (Bent, 1937; Farquhar, 1986a; Fitch *et al.*, 1978; Knight *et al.*, 1976). Furthermore, *Pavo cristatus* (the Indian blue peafowl), and *Cariama cristata* (the red-legged seriema) also sometimes feed on snakes (Chopra & Kumar, 2014). Some birds, such as *Circaetus* sp. (snake eagles) and *Sagittarius serpentarius* (secretary birds), are snake-specialist predators (Sinclair, Hockey & Tarboton, 2012). For these

reasons, we predicted that resistance to α-neurotoxins would be present in birds. However, we found no resistance mutations in any of the birds studied. Furthermore, we showed in Chapter 2 using LD_{50} testing on *Gallus gallus* embyos is relatively susceptible to spectacled cobra venom. The observations of this chapter and the previous chapter, suggest that many birds lack resistance to snake α-neurotoxins. This lack of resistance might help explain why the invasion of *Boiga irregularis* (brown tree snake) on the island of Guam led to the eradication of so many local bird populations (Pawlak, Mackessy, Fry *et al.*, 2006). *B. irregularis* venom is primarily composed of α-neurotoxins including the dimeric irditoxin, which binds especially well to the receptors of diapsids.

One possible explanation for the lack of resistance in predatory birds is that they already possess traits that potentially help them avoid envenomation. These include behavioural resistance traits such as agility, high visual acuity, intelligence; and physical resistance traits such as thick, protective scalation on the legs, and feathers on the body (Figure 3 and see also (Ellemberg, Lewis, Liu *et al.*, 1999; Potier, Lieuvin, Pfaff *et al.*, 2020). Furthermore, birds typically rely on size of the prey and an ambush predation strategy which likely reduces the risk of experiencing a defensive bite (Hedenström & Rosén, 2001). Thus, the absence of resistance motifs within predatory birds that feed regularly on venomous snakes is suggestive of a fitness disadvantage for evolving neurotoxin resistance, whereby the advantage gained must outweigh the corresponding disadvantage. This suggestion is supported by secondary loss of resistance in viperid snakes that have radiated outside the range of neurotoxic predatory snakes. We suggest that predatory birds are not vulnerable to snakebite thanks to their behavioural and mechanical forms of defense and avoidance. Therefore, they are not under selection pressure to evolve resistance. Any random mutation conferring resistance would be under negative

purifying selection if it imparted a fitness disadvantage not offset by a greater fitness advantage. Interestingly, for the first time, we have shown in this chapter that the Nile crocodile (*Crocodylus niloticus*) and the saltwater crocodile (*Crocodylus porosus*) have an aromatic residue (arginine) at position 189 of the nACHR ligand-binding domain. This modification has already been found at position 187 in snake α-neurotoxin-resistant mammals, namely, the European hedgehog (*Erinaceus europaeus*) the honey badger (*Mellivora capensis*) and the domestic pig (*Sus scrofa*) (see refs (Asher, Lupu-Meiri, Jensen *et al.*, 1998; Drabeck *et al.*, 2015)). The resistance in *Erinaceus* species, *Mellivora capensis*, and *Sus scrofa* by an arginine at position 187 is due to particular site-specific interaction, thus an arginine mutation at 189 does not automatically imply resistance. Thus, the mutation 189R revealed in the Crocodylus species *C. niloticus* and *C. porosus* cannot be attributed as conferring resistance in the absence of functional testing.

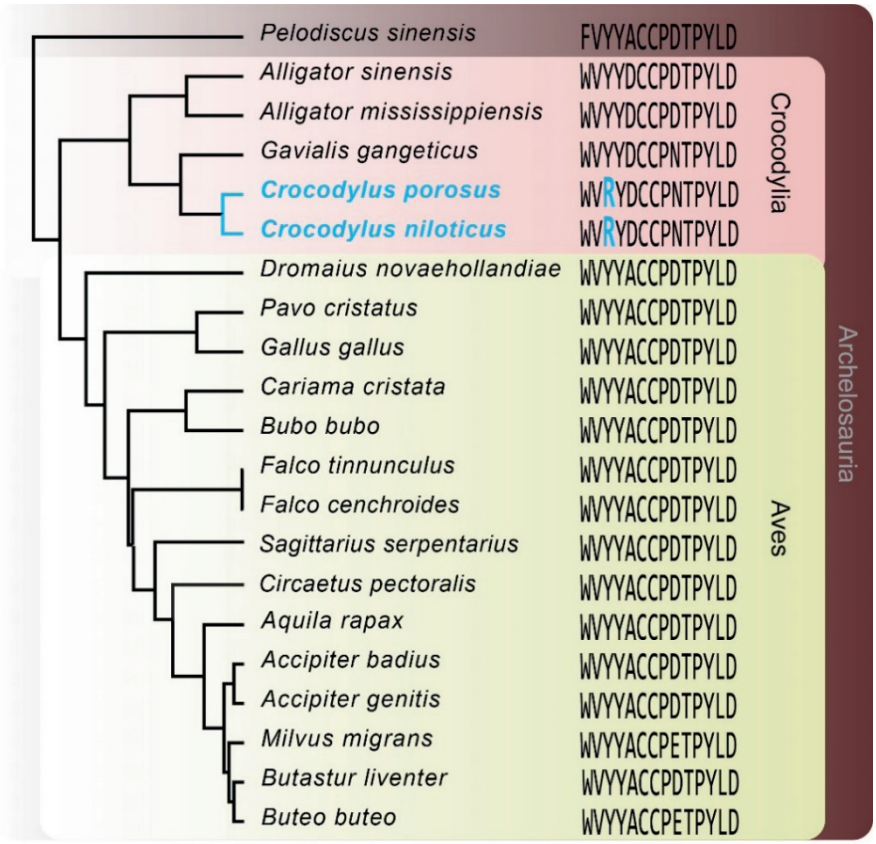

Figure 13. Archosaur (bird + crocodilian) phylogeny constructed from refs (Green, Braun, Armstrong et al., 2014; Jiang, Chen, Wang et al., 2015; Oaks, 2011; Oatley, Simmons & Fuchs, 2015; Prum, Berv, Dornburg et al., 2015; Stein, Brown & Mooers, 2015. Key: blue terminal branches correspond to those sequences having an arginine (R) at position 189 i.e. the sequence modification found in some snake-eating mammals (Drabeck et al., 2015). On the right of the figure is the amino acid alignment of the ligand-binding domain in the archosaur nAChR. The alignment of translated proteins of birds shows that there are no amino acid changes in the cys-loops (highly conserved amino acid region of the nAChRs) corresponding to those that are thought to confer resistance in the mongoose, honey badger, hedgehog or cobra. However the alignments of the

sequence from the saltwater crocodile and Nile crocodile show a positively-charged arginine (R) at position 189 in the highly conserved amino acid region of the nAChRs, (Khan, Dashevsky, Kerkkamp et al., 2020) corresponding to those that are thought to confer resistance in the honey badger, hedgehog and domestic pig (Sus scrofa). Figure made by Muzaffar Khan and Merijn de Bakker.

The presence of 189R only in *Crocodylus*, but not in other crocodilian sequences, may be explained by the biogeographical history of the clade. Crocodylus diversified 13.6–8.3 million years ago (MYA) in Australasia after the split from all other Crocodylia 50 MYA. The Crocodylia in turn diversified 70 MYA from Alligatoridae (Oaks, 2011) [69]. The diversification of the Elapidae began around 35 MYA in Asia(Lee, Sanders, King *et al.*, 2016). This suggests that the speciation of the genus Crocodylus occurred in an environment occupied by Elapidae, while all other Crocodylia (alligators, gharials, and caiman) had diversified prior to that(Oaks, 2011) (Lee *et al.*, 2016) . What remains unclear is the extent to which crocodiles interact with elapids when they share an environment. However, crocodiles are generalist predators, and given the common association between elapids and water bodies, it is plausible to posit that younger individuals may opportunistically predate upon elapids.

It must be emphasized that there is no evidence that 189R confers resistance, and thus it cannot be inferred that Crocodylus species are resistant to snake neurotoxins. However, this is a rich area for future testing, as would sequencing of South American caimans that occur sympatrically with the aquatic elapid *Micrurus surinamensis* (aquatic coral snake).

Acknowledgements

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Chapter 5: Evolution of Molecular Resistance to Snake Venom α-Neurotoxins in Snakes, and the Broader Implications of this Thesis Research

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Abstract

Venomous snakes are important subjects of study in evolution, ecology, and biomedicine. Many venomous snakes have alpha-neurotoxins (α-neurotoxins) in their venom. These toxins bind the alpha-1 nicotinic acetylcholine receptor (nAChR) at the neuromuscular junction, causing paralysis and asphyxia. Several venomous snakes and their predators have evolved resistance to α-neurotoxins. The resistance is conferred by steric hindrance from N-glycosylated asparagines at amino acids 187 or 189, by an arginine at position 187 that has been hypothesized to either electrostatically repulse positively charged neurotoxins or sterically interfere with α-neurotoxin binding, or proline replacements at positions 194 or 197 of the nAChR ligand-binding domain to inhibit α-neurotoxin binding through structural changes in the receptor. In this chapter, we analyzed this domain in 76 snakes species, and assessed its amino acid sequences for resistance-associated mutations. We also looked for signals of selection in the sequences. Of the snake sequences analysed, 66 were sequenced *de novo*. This represents a major new data set studying toxin resistance in reptiles. We find widespread convergent evolution of the N-glycosylation form of resistance in several snake subfamilies, namely: Viperinae (5/6 species), Natricinae (3/3 species), Colubrinae (4/13 species), and Dipsadinae (1/5 species). We discuss our data in the context of the arms race model of co-ecvolution. We also make a sysntesis of the data in the context of the previous two experimental chapters. Looking at the broader implications of this thesis research, we show important venom resistance – or the lack of it - can be in the context of invasive snakes species. The work in this thesis underscores the inter-connectedness of the biosphere and the ripple effects that one adaption can have across global ecosystems.

Introduction

Venoms have evolved independently in multiple animal lineages (Casewell, Wüster, Vonk *et al.*, 2013; Fry, Roelants, Champagne *et al.*, 2009). When a venomous animal injects venom into a target animal (an event called 'envenomation'), venom toxins disrupt physiological processes, causing pain, incapacitation or death. The fitness costs associated with envenomation can spur a co-evolutionary "arms race" between predator and prey (Brodie & Brodie, 1999; Cott, 1940; Dawkins & Krebs, 1979; Thompson, 1999). The snake α-neurotoxins are members of the three-finger toxin (3FTx) family (Barber, Isbister & Hodgson, 2013; Chang, 1999; Dutertre, Nicke & Tsetlin, 2017; Utkin, Sunagar, Jackson *et al.*, 2015; Vonk, Casewell, Henkel *et al.*, 2013) and are major components of venoms from the families Elapidae and Colubridae (Bourne, Talley, Hansen *et al.*, 2005; Chang & Lee, 1963; Fry, Lumsden, Wuster *et al.*, 2003; Fry, Wuster, Ryan Ramjan *et al.*, 2003; Pawlak, Mackessy, Sixberry *et al.*, 2009; Suryamohan, Krishnankutty, Guillory *et al.*, 2020). Venomous snakes in these families are of considerable scientific interest, not least because they are responsible for numerous human fatalities (Chauhan & Thakur, 2016), and because they can cause ecological destruction as invasive species (Savidge, 1987).

In species susceptible to α-neurotoxins, the toxins bind to the nicotinic acetylcholine receptor (nAChR) α1-subunit (Figure 14A). A number of species that are frequently envenomated by elapids, including predators and prey, or the snakes when they accidently bite themselves, have evolved resistance to these toxins (Arbuckle, Rodriguez de la Vega & Casewell, 2017; Edmunds, 1974; Geffeney, Fujimoto, Brodie *et al.*, 2005; Takacs, Wilhelmsen & Sorota, 2001;

Toledo, Hanifin, Geffeney *et al.*, 2016; Ujvari, Casewell, Sunagar *et al.*, 2015; Venkatesh, Lu, Dandona *et al.*, 2005).

The mechanism of resistance in these cases is modification of the ligand-binding domain of the nAChR. For example, several studies demonstrate that the binding of α-neurotoxins is disrupted by glycosylation of asparagine residues. The NXS/T motif (where X = any amino acid except proline), is an indicator of N-glycosylation (Gavel & Heijne, 1990; Mellquist, Kasturi, Spitalnik *et al.*, 1998; Ohtsubo & Marth, 2006). Previous research has shown this motif to have evolved convergently in the Egyptian cobra (*Naja haje*) and its predator, the Egyptian mongoose (*Herpestes ichneumon*) but at different sites within the ligand-binding domain of the α-1 subunit. N-glycosylation at positions 187 (mongoose) and 189 (cobra) impedes binding via steric hindrance due to the long carbohydrate chain preventing docking by the α-neurotoxins, rendering both species resistant (Asher, Lupu-Meiri, Jensen *et al.*, 1998; Takacs *et al.*, 2001; Takacs, Wilhelmsen & Sorota, 2004). Additionally, mutations to the proline subsite of the ligand-binding domain of the mongoose nAChR (194L and 197H), and testing of an artificial variant of the mouse sequence with a 194S mutation, result in decreased α-neurotoxin affinity (Kachalsky, Jensen, Barchan *et al.*, 1995). This is presumably due to changes in the conformation of the binding pocket.

Other resistant animals, including the honey badger (*Mellivora capensis*), hedgehogs (*Erinaceus concolor* and *E. europaeus*), and pig (*Sus scrofa*), have independently evolved amino acid substitutions from an aromatic residue to the positively charged arginine at position 187, which greatly reduces the affinity of α-bungarotoxin (Asher *et al.*, 1998; Barchan, Kachalsky, Neumann *et al.*, 1992; Drabeck, Dean & Jansa, 2015; Fuchs, Barchan, Kachalsky *et al.*, 1993), possibly due to electrostatic repulsion of the positively charged neurotoxins.

However, recent modelling has suggested that this mutation may impart resistance due to steric hindrance instead (Rahman, Teng, Worrell *et al.*, 2020).

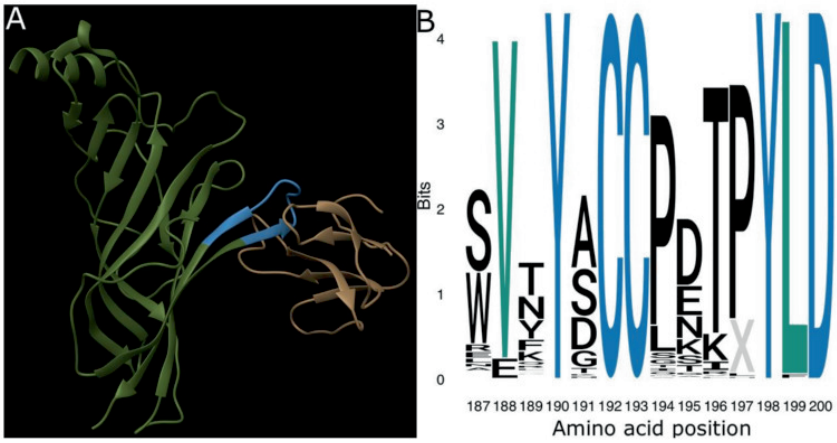


Figure 14. The ligand-binding domain of the nAChR. **A.** Ribbon model of α-bungarotoxin (brown) forming a complex with the ligand-binding domain (blue) on the extracellular domain of a single human α1-nAChR subunit (green). This structure is publicly available from the RCSB PDB under the ID 2QC1 (Dellisanti, Yao, Stroud et al., 2007a). B. Sequence logo showing the information value and amino acid content of the ligand-binding domain sequences in our dataset. Note the complete conservation of positions 190, 192, 193, 198, and 200 (blue) and strong conservation of positions 188 and 199 (teal) from (Khan, Dashevsky, Kerkkamp et al., 2020).

Table 11. Key sites and mutations that confer α-neurotoxin resistance.

Site	Mutation	Mechanism	Reference
187	NXS/T	Steric	(Barchan *et al.*, 1992)
	R	Steric	(Barchan, Ovadia, Kochva *et al.*, 1995)
189	NXS/T	Steric	(Takacs *et al.*, 2001)
194	L	Proline	(Kachalsky *et al.*, 1995)
	S	Proline	(Kachalsky *et al.*, 1995)
197	H	Proline	(Kachalsky *et al.*, 1995)

Given the convergence of these mutations across a diversity of resistant taxa, and in light of the trophic importance of snake venoms, we posit that α-neurotoxin resistance may be present in more species than is currently documented. In this study, therefore, we assess α1-nAChR sequences from 76 snake species for evidence of resistance mutations within the ligand-binding domain, with a particular focus upon the *N*-glycosylated asparagine form of steric hindrance. This is a far larger species sample, with a greater taxonomic range, than has previously been analysed. This allows us to look for multiple independent instances of evolutionary change and gain new insight patterns of resistance evolution. We examine amino acid sites associated with α-neurotoxin resistance (Table 11).

Material Methods

Ethics statement

All animal experimental procedures were conducted in accordance with local and international regulations. The milking of snakes for venom is not considered an animal experiment in accordance with the Experiments on Animals Act (Wet op de dierproeven, 2014), the applicable legislation in the Netherlands, and its implementation the European guidelines (EU directive no. 2010/63/EU). The milking was executed in a licenced establishment for the breeding and use of experimental animals, and subject to internal regulations and guidelines; advice was taken from the Leiden University Ethics Committee to minimise suffering. In the case of snake embryos used for DNA extractions, no license is required by Council of Europe (1986), Directive 86/609/EEC. DNA samples from the NIH in Pakistan were harvested under local regulations of the National Institute of Health Islamabad, Pakistan. No live animals in Australia were used; all samples studied were from existing tissue libraries (collected originally under University of Melbourne Animal Ethics approval number 03126).

Tissue samples

For the species examined, and the origins of the tissues samples see Table 12.

Table 12 Sequences included in this study, with accession numbers and species names. Key: 'Source' indicates the origin of the sequence or the DNA sample. The sequences determined by me de novo in this study and who sourced the DNA samples are listed in the column headed using the following abbreviations: BGF, Bryan G. Fry; FJV, Freek J. Vonk; HMIK, Harald M.I. Kerkkamp; JvT, Jory van Thiel; MAGdB, Merijn A.G. de Bakker; MAK, RMW, Roel M. Wouters, Muzaffar A. Khan and National Institute of

Health (NIH) Islamabad, Pakistan. The remaining sequences were obtained from NCBI (NCBI, National Center for Biotechnology Information, Bethesda, Maryland, United States).

Accession No.	Scientific Name	Common Name	Database	Tissue sample source
XM_018572442.1	*Nanorana parkeri*	High Himalaya frog	GenBank, NCBI	-
XM_002934601.5	*Xenopus tropicalis*	African clawed frog	GenBank, NCBI	-
XM_029605825.1	*Rhinatrema bivittatum*	Two-lined caecilian	GenBank, NCBI	-
XM_033946945.1	*Geotrypetes seraphini*	Gaboon caecilian	GenBank, NCBI	-
XM_030209957.1	*Microcaecilia unicolor*	Tiny Cayenne caecilian	GenBank, NCBI	-
XM_001514832.4	*Ornithorhynchus anatinus*	Platypus	GenBank, NCBI	-
XM_003763981.2	*Sarcophilus harrisii*	Tasmanian devil	GenBank, NCBI	-
XM_001376625.4	*Monodelphis domestica*	Gray short-tailed opossum	GenBank, NCBI	-
XM_007940110.1	*Orycteropus afer*	Aardvark	GenBank, NCBI	-
XM_023542827.1	*Loxodonta africana*	African savanna elephant	GenBank, NCBI	-
XM_004476894.2	*Dasypus novemcinctus*	Nine-banded armadillo	GenBank, NCBI	-

XM_006151116.1	*Tupaia chinensis*	Chinese tree shrew	GenBank, NCBI	-
XM_011991880.1	*Mandrillus leucophaeus*	Drill	GenBank, NCBI	-
NM_001039523.3	*Homo sapiens*	Human	GenBank, NCBI	-
XM_003478585.3	*Cavia porcellus*	Domestic guinea pig	GenBank, NCBI	-
XM_013028276.1	*Dipodomys ordii*	Ord's kangaroo rat	GenBank, NCBI	-
XM_004660327.1	*Jaculus jaculus*	Lesser Egyptian jerboa	GenBank, NCBI	-
XM_021649964.1	*Meriones unguiculatus*	Mongolian gerbil	GenBank, NCBI	-
U17016.1	*Erinaceus concolor*	Southern white-breasted hedgehog	GenBank, NCBI	-
XM_008138537.2	*Eptesicus fuscus*	Big brown bat	GenBank, NCBI	-
XM_006921218.1	*Pteropus alecto*	Black flying fox	GenBank, NCBI	-
XM_021075437.1	*Sus scrofa*	Pig	GenBank, NCBI	-
KR477832.1	*Mellivora capensis*	Honey badger	GenBank, NCBI	-
XM_003990883.5	*Felis catus*	Domestic cat	GenBank, NCBI	-
XM_007074557.2	*Panthera tigris*	Tiger	GenBank, NCBI	-

M93639.1	*Herpestes ichneumon*	Egyptian mongoose	GenBank, NCBI	-
XM_029932975.1	*Suricata suricatta*	Meerkat	GenBank, NCBI	-
MN337817	*Anilios bituberculatus*	Prong-snouted blind snake	Pet trade	FJV
MT274611	*Indotyphlops braminus*	Brahminy blind snake	Pet trade	BGF
MN337822	*Boa constrictor*	Common boa	Pet trade	BGF
MN337841	*Corallus hortulanus*	Garden tree boa	Pet trade	BGF
MN337819	*Aspidites melanocephalus*	Black-headed python	Pet trade	BGF
MN337856	*Malayopython reticuatus*	Reticulated python	Pet trade	BGF
XM_007444717	*Python bivittatus*	Burmese python	GenBank, NCBI	
MN337828	*Liasis mackloti*	Macklot's water python	Pet trade	BGF
MN337853	*Morelia spilota*	Carpet python	Pet trade	BGF
MN337818	*Acrochordus granulatus*	Banded file snake	Pet trade	BGF
MN337801	*Causus rhombeatus*	Rhombic night adder	Pet trade	FJV
MN337797	*Daboia russelii*	Russell's viper	NIH, Islamabad, Pakistan	MAK
GCA_000800605.1	*Vipera berus*	European adder	GenBank, NCBI	-

MN337798	*Echis carinatus*	Saw-scaled viper	NIH, Islamabad, Pakistan	MAK
MN337800	*Bitis gabonica*	Gaboon viper	Pet trade	BGF
MN337851	*Tropidolaemus subannulatus*	North Philippine temple pitviper	Pet trade	BGF
MN337844	*Deinagkistrodon acutus*	Chinese moccasin	Gifttierhause Eimsheim, Germany.	JvT & RMW
MN337836	*Trimeresurus albolabris*	White-lipped tree viper	Pet trade	BGF
MN337837	*Trimeresurus hageni*	Indonesian pit viper	Pet trade	FJV
XM_015815894.1	*Protobothrops mucrosquamatus*	Brown-spotted pit viper	GenBank, NCBI	
MN337854	*Bothrops asper*	Fer-de-lance	Gifttierhause Eimsheim, Germany.	JvT & RMW
MT262920	*Bothrops alternatus*	Urutu	Gifttierhause Eimsheim, Germany.	JvT & RMW
MN337838	*Agkistrodon bilineatus*	Cantil viper	Gifttierhause Eimsheim, Germany.	JvT & RMW
JPMF01213521.1	*Crotalus pyrrhus*	Speckled rattlesnake	GenBank, NCBI	-
LVCR01006207.1	*Crotalus horridus*	Timber rattlesnake	GenBank, NCBI	-

MN337852	*Crotalus vegrandis*	Uracoan Rattlesnake	Pet trade	JvT & RMW
MN337824	*Erpeton tentaculatum*	Tentacle snake	Pet trade	BGF
MN337825	*Homalopsis buccata*	Puff-faced water snake	Pet trade	BGF
MN337848	*Pseudoxenodon bambusicola*	Bamboo false cobra	Pet trade	BGF
MN337792	*Erythrolamprus poecilogyrus*	Yellow-bellied water snake	Pet trade	BGF
MN337846	*Philodryas baroni*	Baron's green racer	Pet trade	BGF
MN337832	*Oxyrhopus rhombifer*	Diamondback flame snake	Pet trade	BGF
MN337813	*Helicops leopardinus*	Leopard keelback	Pet trade	BGF
MN337842	*Ahaetulla prasina*	Asian vine snake	Pet trade	BGF
MN337847	*Platyceps florulentus*	Egyptian whip snake	Pet trade	BGF
MN337814	*Thrasops jacksonii*	Black tree snake	Pet trade	BGF
MN337815	*Dispholidus typus*	Boomslang	Pet trade	BGF
MN337811	*Thelotornis capensis*	Savanna vine snake	Pet trade	BGF
MN337850	*Trimorphodon biscutatus*	Western lyre snake	Pet trade	BGF
MN337810	*Oligodon cyclurus*	Cantor's kukri snake	Pet trade	BGF

MT262919	*Coelognathus radiatus*	Radiated ratsnake	Pet trade	BGF
JTLQ01052499	*Pantherophis guttatus*	Corn snake	GenBank, NCBI	
MN337833	*Pantherophis spiloides*	Grey rat snake	Pet trade	FJV
MN337849	*Stegonotus cucullatus*	Slaty grey snakes	Pet trade	BGF
MN337823	*Dasypeltis scabra*	Common egg-eating snake	Pet trade	BGF
MN337793	*Boiga irregularis*	Brown tree snake	Pet trade	BGF
MN337843	*Boiga dendrophila*	Mangrove snake	Pet trade	BGF
XM_032237666.1	*Thamnophis elegans*	Western terrestrial garter snake	GenBank, NCBI	-
MN337812	*Natrix natrix*	European grass snake	Pet trade	FJV
M26389.1	*Natrix tessellata*	Checkered water snake	GenBank, NCBI	
MN337835	*Pseudaspis cana*	Mole snake	Pet trade	BGF
MN337831	*Malpolon monspessulanus*	Montpellier snake	Pet trade	BGF
MN337834	*Psammophis mossambicus*	Olive grass Snake	Pet trade	BGF
MN337829	*Macrelaps microlepidotus*	Natal black snake	Pet trade	BGF
MN337840	*Atractaspis bibronii*	Bibron's stiletto snake	Pet trade	BGF

MN337839	*Atractaspis fallax*	False mole viper	Pet trade	BGF
MN337821	*Atractaspis microlepidota*	Small-scaled burrowing asp	Pet trade	BGF
MN337826	*Boaedon fuliginosus*	African house snake	Pet trade	FJV
MN337827	*Leioheterodon madagascariensis*	Malagasy giant hognose snake	Pet trade	BGF
MN337830	*Madagascarophis ocellatus*	Ocellated cat snake	Pet trade	BGF
MN337805	*Calliophis bivirgatus*	Blue Malaysian coral snake	Pet trade	BGF
MN337802	*Aspidelaps lubricus*	Cape coral cobra	Pet trade	FJV
AF077763.1	*Naja haje*	Egyptian cobra	GenBank, NCBI	
MN337806	*Naja kaouthia*	Monocled cobra	Pet trade	FJV
MN337807	*Naja naja*	Indian cobra	NIH, Islamabad, Pakistan	MAK
ETE71672.1	*Ophiophagus hannah*	King cobra	GenBank, NCBI	-
MN337804	*Bungarus caeruleus*	Common krait	NIH, Islamabad, Pakistan	MAK
MN337816	*Acanthophis rugosus*	Rough-scaled death adder	Gifttierhause Eimsheim, Germany.	JvT & RMW

XM_026696730.1	*Pseudonaja textilis*	Eastern brown snake	GenBank, NCBI	-
MN337809	*Oxyuranus microlepidotus*	Inland taipan	Pet trade	BGF
XM_026677744.1	*Notechis scutatus*	Mainland tiger snake	GenBank, NCBI	-
MN337808	*Hydrophis curtus*	Shaw's sea snake	Pet trade	BGF
MN337803	*Aipysurus mosaicus*	Mosaic sea snake	Weipa, Queensland, Australia	BGF
MN337831	*Malpolon monspessulanus*	Montpellier snake	Pet trade	BGF
MN337834	*Psammophis mossambicus*	Olive grass Snake	Pet trade	BGF
MN337829	*Macrelaps microlepidotus*	Natal black snake	Pet trade	BGF
MN337840	*Atractaspis bibronii*	Bibron's stiletto snake	Pet trade	BGF
MN337839	*Atractaspis fallax*	False mole viper	Pet trade	BGF

DNA was extracted from tissue samples preserved in 70% ethanol. The tissues were rinsed with 10% phosphate-buffered saline (PBS), then cut into small pieces and transferred to DNA lysis buffer containing 10% sodium dodecyl sulfate (SDS) and 10 µL /mL Proteinase K (ProtK) overnight with gentle shaking at 55 °C digital heat block (VWR International). After the incubation the buffer samples were centrifuged at high speed (20,238 rpm) for 15 min. The supernatant was mixed with 0.6 µL isopropanol to precipitate the DNA and then centrifuged at high speed. The resultant pellet was treated with 70%

ethanol, air dried and dissolved in RNA/DNA free water at 65 °C for 45-60 min.

Amplification and sequencing of the ligand binding domain of α-neurotoxin nAChR

Primers specific for the ligand-binding domain of the nicotinic acetylcholine receptor (nAChR) were designed based on the alignment of reference sequences of the following snake species: Egyptian cobra (*Naja haje*), Burmese python (*Python bivittatus*) and king cobra (*Ophiophagus hannah*). Primer sequences are shown in Supplementary File 4 and the amplicon sequences in Supplementary File 5. Successively, an amplicon of 400 bp of the ligand binding domain α-neurotoxin from the gene nAChR was amplified. PCR was performed in a volume of 25 µL mixture according to the instructions of manufacturer (Qiagen, Inc., California, USA). PCR reaction conditions included an annealing temperature of 65 °C for 10 s (-1/cycle). As a quality check, the PCR products were electrophoresed for 30 min, and visualised on gel documentation apparatus (Westburg, the Netherlands). The amplified PCR products of nAChR for all snake species were Sanger-sequenced in both directions by BaseClear B.V., the Netherlands. All sequences were submitted to The National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/) and can be found under accession numbers Table 12.

Analysis of site-specific selection

Nucleotide sequences of the ligand-binding domain from other species were downloaded from NCBI. The relevant accession numbers are given in Table 12. The nucleotide sequences were translated into amino acids, manually aligned, and trimmed down to the 14 codons of the ligand-binding domain using AliView 1.18 (Larsson, 2014). A

phylogeny of all the species included in our dataset was compiled from a consensus generated by TimeTree.org and reconciled with taxon-specific phylogenies (Alencar, Quental, Grazziotin *et al.*, 2016; Betancur-R, Wiley, Arratia *et al.*, 2017; dos Reis, Inoue, Hasegawa *et al.*, 2012; Kumar, Stecher, Suleski *et al.*, 2017; Lerner & Mindell, 2005; Portillo, Stanley, Branch *et al.*, 2019; Prum, Berv, Dornburg *et al.*, 2015; Šmíd & Tolley, 2019; Zaher, Murphy, Arredondo *et al.*, 2019). The data set was separated into five major clades: Actinopterygii, Mammalia, Archelosauria, toxicoferan lizards, and Serpentes. The tree data are given in Supplementary File S4 . These were analysed using the FUBAR (Fast Unconstrained Bayesian Approximation) and MEME (Mixed Effects Model of Evolution) programs implemented in HyPhy (Hypothesis Testing Using Phylogenies) 2.220150316beta (Murrell, Moola, Mabona *et al.*, 2013; Murrell, Wertheim, Moola *et al.*, 2012; Pond, Frost & Muse, 2005).

Results and Discussion

We sequenced *de novo* the nAChR ligand binding domain of 66 snake species and obtained sequences for a further 11 species from The National Center for Biotechnology Information Table 12. A preliminary search for sites under positive selection was made independently using a smaller subset of the main sequence collection (Methods, Supplementary File S5). Positively selected sites inferred under posterior probability PP>0.95 were found (172, 177, 181, 187, 194 and 206). These positively-selected sites include sites 187 and 194, modifications of which are associated with toxin resistance.

In our analysis of the full dataset, we included a wide range of vertebrates for comparison with the snakes (Figure 15). This was in order to help us understand the significance of any signals of selection.

We identified a number of highly-conserved sites, which is interesting given that our dataset covers a broad taxonomic scope and contains an over-representation of resistant species (Figure 14B).

Conserved sites includes the tyrosine residues at 190 and 198 which are known to interact directly with ligands; and the cysteine doublet at 192-193 which is crucial to the structure of the ligand-binding domain (Kini & Evans, 1996). The conservation of these residues across such a diverse sampling of vertebrates suggests that they may be important to the physiological function of the nAChR. By contrast, sites 187 and 189, sites of known α-neurotoxin resistance mutations, are far more variable than 194 and 197. Even though most of the observed variation at these sites comes from mutations different from those that are known to produce resistance, we demonstrate that these α-neurotoxin resistance mutations are widespread among vertebrates. However, as we noted in Chapter 3, they are not found in any of the birds that we studied not even the snake specialists in the genus *Circaetus* (snake eagles) and *Sagittarius serpentarius* (secretary bird; Figure 13).

A number of species possess substitutions equivalent to those previously identified as offering α-neurotoxin resistance, via steric hindrance imparted by *N*-glycosylation of an asparagine as they possess the well-documented signature NXS/T motif Additionally, we find the 189-191NYS motif in all elapid snakes we examined, in addition to the *Naja* species in which it was originally characterised (Takacs *et al.*, 2001). Variants of the NXS/T motif were found in other snakes that we sampled, occurring within subfamilies Viperinae (5/6 species), Natricinae (3/3 species), Colubrinae (4/13 species), and Dipsadinae (1/5 species).

In our sequence analysis we also found that several species possess proline replacements at positions 194 and 197 identical to those that have been previously associated with resistance (Kachalsky *et al.*, 1995). The 194L mutation is particularly widespread, and was found in the yellow-bellied water snake (*Erythrolamprus poecilogyrus*), the dice snake (*Natrix tessellata*), the radiated ratsnake (*Coelognathus radiatus*), and most of the non-hydrophine elapids (6/7 species).

We also found the 194S mutation in the reticulated python (*Malayopython reticuatus*) and basal crotalines (3/12 species). No other snake species in our dataset possessed the 197H mutation found in the mongoose. The exact impact of these mutations is difficult to predict since the study that identified them suggested that there are complex patterns of interaction between mutations at these two sites, as well as between these mutations and those associated with steric hindrance resistance (Kachalsky *et al.*, 1995). Thus these results must be interpreted with caution. As mentioned above, even those specific substitutions which have been demonstrated to confer resistance in one taxon cannot confidently be stated to do so in others, especially mutations to amino acids which have never specifically been associated with resistance. For instance, α-neurotoxins have been found to bind the ligand-binding domain sequences of both the radiated ratsnake (Coelognathus radiates, which contains the 194L mutation) and Schlegel's Japanese gecko (*Gekko japonicus*, which contains the 194T mutations) with higher affinity than they do to other species tested (Harris, Zdenek, Debono *et al.*, 2020; Harris, Zdenek, Harrich *et al.*, 2020; Zdenek, Harris, Kuruppu *et al.*, 2019). These findings underscore the fact that not all substitutions at these sites confer resistance (e.g. (Dellisanti, Yao, Stroud *et al.*, 2007b), and that complex interactions, involving multiple amino acids, may be involved in conferring resistance.

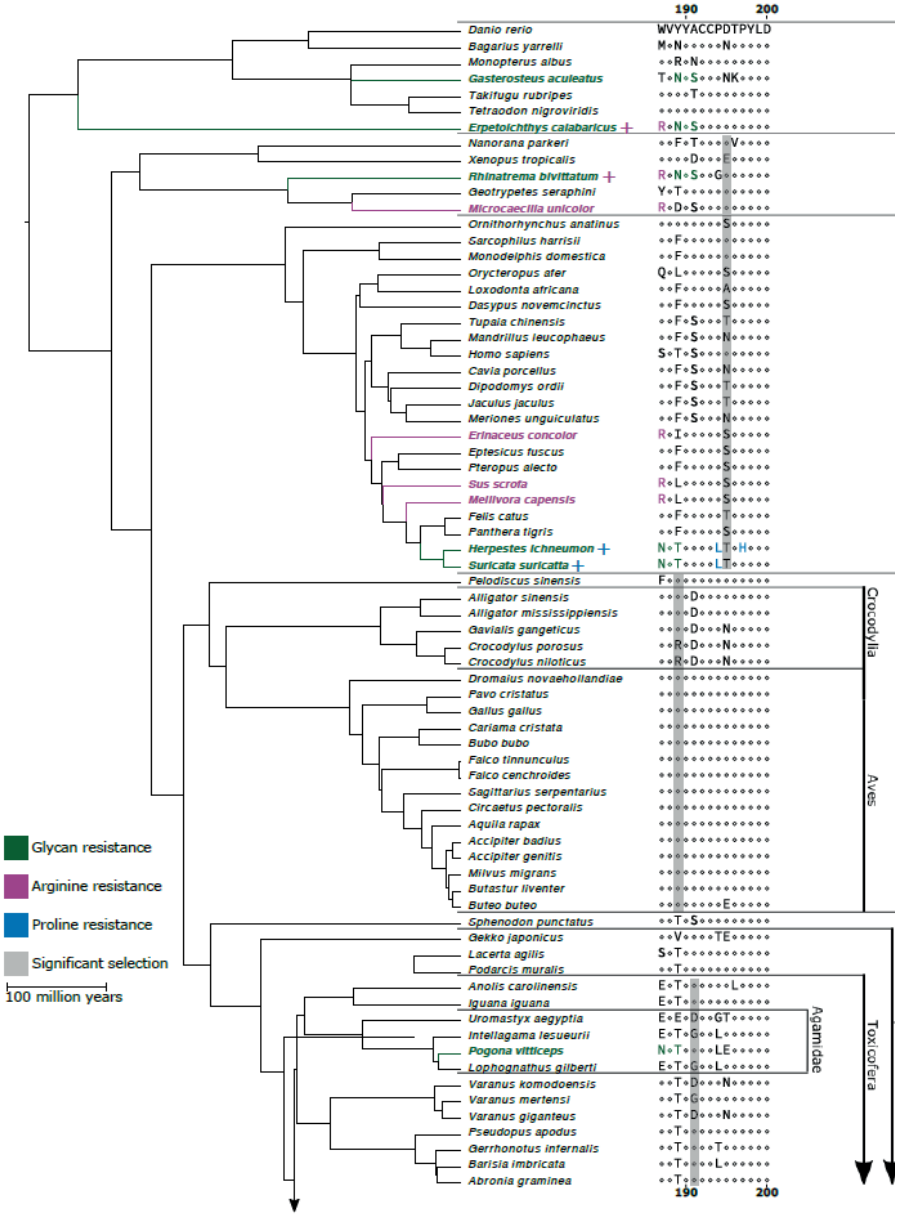
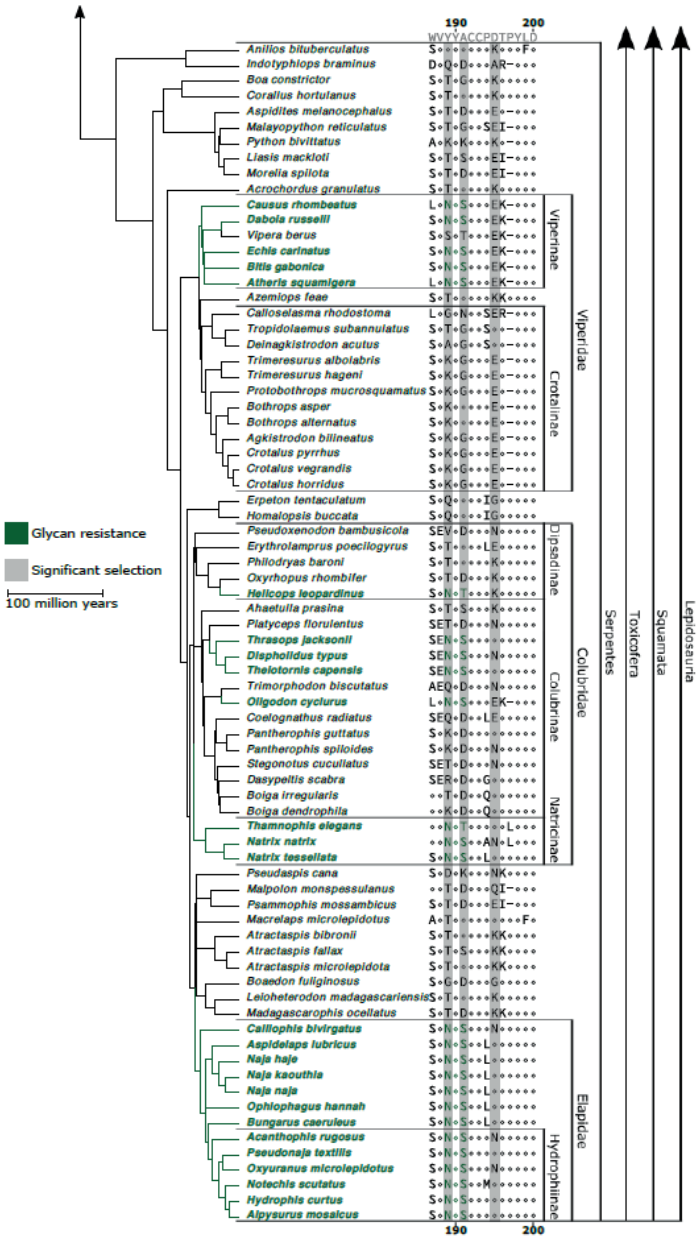

190
200
WVYYACCPDTPYLD
Danio rerio
Bagarius yarrelli
Monopterus albus
Gasterosteus aculeatus
Takifugu rubripes
Tetraodon nigroviridis
Erpetoichthys calabaricus
Nanorana parkeri
Xenopus tropicalis
Rhinatrema bivittatum
Geotrypetes seraphini
Microcaecilia unicolor
Ornithorhynchus anatinus
Sarcophilus harrisii
Monodelphis domestica
Orycteropus afer
Loxodonta africana
Dasypus novemcinctus
Tupaia chinensis
Mandrillus leucophaeus
Homo sapiens
Cavia porcellus
Dipodomys ordii
Jaculus jaculus
Meriones unguiculatus
Erinaceus concolor
Eptesicus fuscus
Pteropus alecto
Sus scrofa
Mellivora capensis
Felis catus
Panthera tigris
Herpestes ichneumon
Suricata suricatta
Pelodiscus sinensis
Alligator sinensis
Alligator mississippiensis
Gavialis gangeticus
Crocodylus porosus
Crocodylus niloticus
Dromaius novaehollandiae
Pavo cristatus
Gallus gallus
Cariama cristata
Bubo bubo
Falco tinnunculus
Falco cenchroides
Sagittarius serpentarius
Circaetus pectoralis
Aquila rapax
Accipiter badius
Accipiter genitis
Milvus migrans
Butastur liventer
Buteo buteo
Sphenodon punctatus
Gekko japonicus
Lacerta agilis
Podarcis muralis
Anolis carolinensis
Iguana iguana
Uromastyx aegyptia
Intellagama lesueurii
Pogona vitticeps
Lophognathus gilberti
Varanus komodoensis
Varanus mertensi
Varanus giganteus
Pseudopus apodus
Gerrhonotus infernalis
Barisia imbricata
Abronia graminea
Crocodylia
Aves
Agamidae
Toxicofera
Glycan resistance
Arginine resistance
Proline resistance
Significant selection
100 million years

Figure 15. Sites of positive selection in α1-nAChR ligand-binding domain in a wide range of vertebrate taxa. Topology constructed from the consensus of TimeTree.org and taxon-specific phylogenies (Alencar et al., 2016; Betancur-R et al., 2017; dos Reis et al., 2012; Kumar et al., 2017; Lerner et al., 2005; Portillo et al., 2019; Prum et al., 2015; Šmíd et al., 2019; Zaher et al., 2019). The most common amino acid sequence of the α1-nAChR ligand-binding is displayed for one species (Danio rerio) and differences from this sequence are displayed for all other species. Sites showing significant positive selection are highlighted in grey for the relevant clade. Green taxa and amino acids indicate resistance conferred through the glycosylated NXS/T motif, purple signify the 187R mutation, and blue indicates resistance granted by proline subsite mutations. Scale bar indicates 100 million years of branch length. Continued in next figure.



Continuation of **Figure 15**.

A number of mutations apparent in our dataset have not previously been discussed in the context of α-neurotoxin resistance. For instance, since the steric hindrance from *N*-glycosylation inhibits the binding of α-neurotoxins at positions 187 and 189, one might suspect that the steric hindrance created by arginine might similarly confer resistance to those species with a 189R mutation. These species the egg-eating snake (*Dasypeltis scabra*; Colubridae).

However, we find this unlikely since steric hindrance from the 187R mutation is imposed due to very specific interactions between the toxin and the ligand-binding domain and positions 187 and 189 interact with different parts of the toxin (Dellisanti *et al.*, 2007b; Rahman *et al.*, 2020). This is in contrast to the steric hindrance by N-glycosylation which, due to the large glycan emerging from the asparagine, presents a much larger obstacle to binding which can hinder the process from a wider variety of positions within the binding pocket of the nicotinic acetylcholine receptor.

We used signals-of-selection analyses to calculate the ratio of nonsynonymous (β) to synonymous (α) substitutions within the α1-nAChR nucleotide sequence. Non-synonymous mutations affect the biochemistry of the final gene product; synonymous mutations do not. A scarcity of non-synonymous mutations suggests that deviations from the ancestral state may be deleterious, and therefore selected against. Conversely, an overabundance of non-synonymous compared to synonymous mutations implies an adaptive process selecting for change or diversity, and this is a hallmark of evolutionary arms races. We therefore used this ratio to infer negative selection ($\alpha > \beta$), neutral

evolution ($\alpha = \beta$), and positive selection ($\alpha < \beta$) of sites within the nAChR sequence.

The analyses found several sites under significant positive selection (at the threshold of $p < 0.1$ for these conservative algorithms) within the ligand-binding domain (Figure 15). In the main analysis described here (Figure 15), we used Mixed Effects Model of Evolution (MEME) and Fast Unconstrained Bayesian Approximation (FUBAR) to analyse the following clades: Actinopterygii, Mammalia, Archelosauria, Toxicoferan lizards, and Serpentes. MEME is designed to detect sites that have undergone episodic diversification, whereas FUBAR is built to detect sites with more pervasive positive selection throughout their evolutionary history. Due to these differences, we would expect MEME to determine a greater number of sites as significant than would FUBAR. While there was no significant positive selection within Actinopterygii, Amphibia (position 195: MEME $p = 0.03$, FUBAR $p = 0.06$), Mammalia (position 195: MEME $p = 0.04$, FUBAR $p = 0.06$), Archelosauria (position 189, MEME $p = 0.01$, FUBAR $p = 0.18$), and Toxicoferan lizards (position 191, MEME $p = 0.06$, FUBAR $p = 0.07$) all have one site under positive selection. In Serpentes, three positions: 189 (MEME $p = 0.13$, FUBAR $p = 0.01$), 191 (MEME $p = 0.09$, FUBAR $p = 0.004$) , and 195 (MEME $p = 0.001$, FUBAR $p = 0.001$)

were found to be significant by at least one of the analyses. Both algorithms indicate that most remaining sites are subject to negative

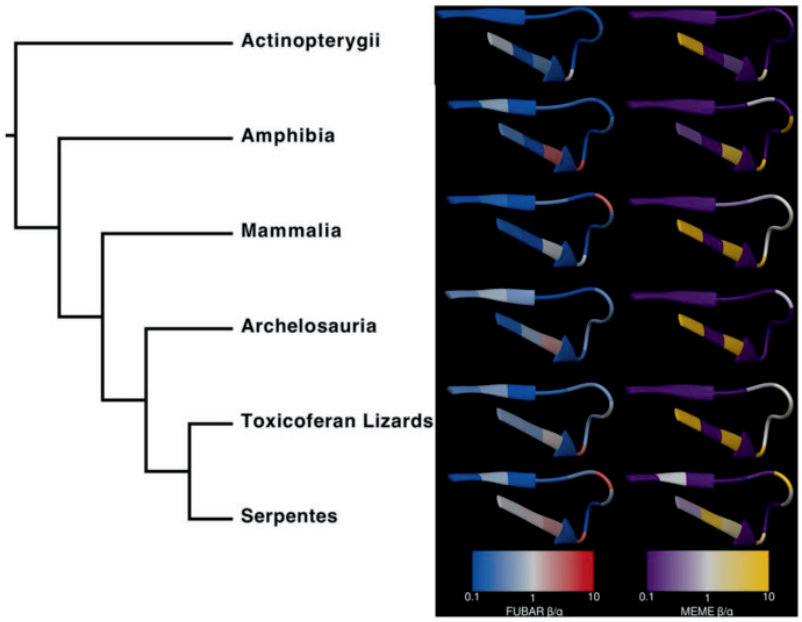


Figure 16. Amino acids in the α1-nAChR ligand-binding domains of snakes are subject to stronger and more pervasive positive selection than other taxa. Predicted surface of the ligand-binding domain (blue residues in Figure 13) coloured according to FUBAR β/α and MEME weighted β/α where red and yellow denote positive selection while blue and purple represent negative selection. This structure is publicly available from the RCSB PDB under the ID 2BG9; see also (Zouridakis, Giastas, Zarkadas et al., 2014).

selection (α > β); Sites that appear be nearly neutral (α ≈ β) in some clades include those at positions 192, 193, 198, 199, and 200, which are strongly conserved across our species. This is an artefact arising from extraordinarily strong negative selection, which eliminates all or

almost all mutations, including those that are non-synonymous, as discussed in (Dashevsky & Fry, 2018).

We shall now discuss those taxa not mentioned in previous chapters. Neither of the frog species included in our analyses possessed a resistance mutation, but two of the three caecilian species did. The resistant species are both South American which could be because of coral snakes (*Micrurus*) which are known to prey on caecilians specifically including the tiny Cayenne caecilian (Martins and Oliveira 1998). From our phylogeny it is impossible to be certain whether the 187R mutation is ancestral to all caecilians and further mutated to 187Y in the lineage leading to the Gaboon caecilian or whether it is a convergent mutation in the tiny Cayenne caecilian and two-lined caecilian. Since these lineages predate the evolution of elapids, our hypothesis predicts the latter scenario. Further research into these enigmatic amphibians will be necessary to confirm or deny this prediction. The significant positive selection at site 195 across the amphibians could allude to a possible association with resistance, though how mutations at this site might affect the binding of α-neurotoxins remains unclear.

We identified an additional species (the meerkat) that was previously not known to possess resistance. The meerkat is closely related to the mongoose and has a similar foraging strategy. This strongly suggests that the identical 187-189NVT sequence and the 194L mutations shared by these two taxa is a homologous trait that was present in their most recent common ancestor, which likely also preyed on venomous snakes. As with the amphibians, the consistent positive selection of site 195 across all mammals tested could be related to α-neurotoxin resistance, but this remains hypothetical until an actual effect or mechanism can be demonstrated.

Considering all the previously described resistance mutations, and the experimental evidence of resistance described in previous studies (*Eryx*, *Laticauda*, *Naja*, and *Natrix*) (Takacs *et al.*, 2001), our results suggest that the *N*-glycosylated asparagine form of α-neurotoxin resistance has evolved convergently at least six times within the snakes alone. The phylogenetic pattern provides evidence to suggest that these are independent origins of resistance rather than multiple losses. This is an extraordinary level of convergence of this very effective form of resistance.

We found mutations *N*-glycosylated asparagine form of α-neurotoxin resistance were particularly widespread in two of the major venomous snake families, Elapidae and Viperidae but also occurred within Colubridae. Within the elapids, the 189-191NYS mutation is present in all 13 species examined, but is not in other closely related snake families. This suggests that it evolved once in the common ancestor of the elapids, paralleling the explosive diversification of α-neurotoxins within this family (Fry, Wuster, Kini *et al.*, 2003). Within the viperids, only the Viperinae subfamily contains the *N*-glycosylated asparagine form of steric hindrance resistance (189-191NYS), which suggests that the selection pressure for resistance may postdate the divergence between these subfamilies. This leads us to posit that predation from ophiophagous elapids may have contributed to the evolution of this mutation given that the origin of elapids is thought to postdate the split between Viperinae and Crotalinae (Alencar *et al.*, 2016; Lee, Sanders, King *et al.*, 2016; Zaher *et al.*, 2019; Zheng & Wiens, 2016). Interestingly, the European adder (*Vipera berus*), a viperine, is the only species examined here with a reversal of the *N*-glycosylated form of steric resistance (NXS/T mutation). This adder has a very broad distribution across northern Eurasia, however it is found at relatively

high latitudes and is not sympatric with any elapid (Wallach, Williams & Boundy, 2017).

This reversal may therefore indicate that resistance mutations carry a fitness cost in a species that is no longer encountering α-neurotoxins. Such a scenario has been shown in several other cases of resistance to toxins (Carlo, Leblanc, Brodie Jr *et al.*, 2016; Ujvari *et al.*, 2015). Members of Colubridae are known to produce abundant α-neurotoxins within their venom which could lead to the evolution of autoresistance (Dashevsky *et al.*, 2018; Fry, Scheib, van der Weerd *et al.*, 2008; Pawlak, Mackessy, Fry *et al.*, 2006; Pla, Sanz, Whiteley *et al.*, 2017). Some of the taxa that possess resistance mutations are also sympatric with ophiophagous elapids, but in other cases such as Natricinae it is less clear whether there was sufficient overlap between ancestral populations to lead to predator-prey coevolution.

While additional mechanisms of resistance may have evolved, such as mutations to the proline subsite, this will require future functional testing to validate. All of the non-hydrophine elapids, except for the Malaysian blue coral snake (*Calliophis bivirgatus*), share the 194L mutation. This suggests that it may have evolved subsequent to the divergence of the Asian coral snakes, and reverted in the lineage that colonized Australia. Within the viperids, the Crotalinae subfamily has the proline replacement (194S).

It should be noted that the evidence linking this type mutation to α-neurotoxin resistance comes from a structure-function study of mammalian receptors which demonstrated these changes resulted in significant resistance (Kachalsky *et al.*, 1995). However, as the proline replacement mutations involve complex interplays between amino acids in the binding pocket, as opposed to the simple steric hindrance imposed by *N*-glycosylation of an asparagine, this mutation might not

confer resistance in the context of the other differences between the mammalian and crotaline sequences.

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Chapter 6. Thesis Summary and General Discussion

We have examined sequences from the ligand-binding domain of the nicotinic acetyl choline receptor (nAChR) in 148 vertebrate species. We are in interested in this receptor because the α-neurotoxins of many venomous snakes binds to this receptor in its location at the neuromuscular junction in all vertebrates. Furthermore, some animals have evolved resistance to snake venoms and show modifications in the ligand binding domain of the nAChR which inhibit the binding of snake α-neurotoxins. Our analysis has shown that numerous vertebrate species, most of which were not previously known to possess α-neurotoxin resistance, do actually contain resistance-related modifications. These modifications are present in most of the taxa in our dataset, with the unexpected exclusion of the birds. It was particularly surprising to us that the snake-specialist predatory birds *Circaetus pectoralis* (black-chested snake eagle) and *Sagittarius serpentarius* (secretary bird) did not possess resistance modifications. There were also relatively few resistance-related mutations within the mammals. By contrast, there were multiple convergent evolutions of the well-characterised N-glycosylation motif within the squamate reptiles—particularly the snakes. We also identified a number of sites under positive selection, such as mutations to the proline subsite. Future functional testing will be needed to validate that these modifications do indeed confer resistance. To provide functional confirmation that resistance-related modifications do indeed reduce susceptibility to toxins, we used developmental bioassays. These assays showed that two species possessing resistance-related modifications of the nAChR (stickleback and bearded dragon) were less susceptible to the toxic effects of cobra venom than two species that lacked such modifications (zebrafish and chicken). In summary, we demonstrate that the range of mechanisms along with the

phylogenetic distribution of resistance to snake α-neurotoxin appears to be more extensive than was previously appreciated. It also shows strong evidence of the convergent evolution of the same resistance mutations in independent linages. Our findings also support the notion that the mutations we have identified in this thesis may represent adaptive change in response to selective pressures exerted by α-neurotoxic snake venoms in an evolutionary arms race. Thus, we conclude that the evolutionary arms race between predator and prey appears to be a pervasive feature of the trophic interactions surrounding venomous snakes, which is shaping the molecular evolution of the nAChR in the vertebrates.

Samenvatting proefschrift en algemene discussie

Wij onderzochten sequenties van het ligandbindende domein van de nicotinerge acetylcholinereceptor (nAChR) in 148 gewervelde soorten. We zijn geïnteresseerd in deze receptor, omdat de α-neurotoxinen van veel giftige slangen zich binden aan deze receptor op zijn locatie op de neuromusculaire verbinding in alle gewervelde dieren. Bovendien hebben sommige dieren resistentie ontwikkeld tegen slangengif en vertonen modificaties in het ligandbindende domein van de nAChR die de binding van α-neurotoxinen van slangen onderdrukken. Onze analyse heeft aangetoond dat een groot aantal gewervelde soorten, waarvan bij de meeste niet eerder α-neurotoxineresistentie bekend was, wel degelijk resistentie-gerelateerde modificaties hebben. Deze modificaties zijn in de meeste taxa in onze dataset aanwezig, met onverwachte uitsluiting van de vogels. Het was voor ons bijzonder verbazingwekkend dat de in slangen gespecialiseerde roofvogels *Circaetus pectoralis* (zwartborstslangenarend) en *Sagittarius serpentarius* (secretarisvogel) geen resistentiemodificaties hadden.
Ook waren er relatief weinig resistentie-gerelateerde mutaties bij de zoogdieren. Daarentegen waren er meerdere convergente evoluties van het goed gedocumenteerde N-glycosyleringsmotief bij de schubreptielen, in het bijzonder de slangen. We hebben ook een aantal plaatsen geïdentificeerd die onder positieve selectie vallen en niet eerder met resistentie in verband gebracht werden. Als functionele bevestiging dat resistentie-gerelateerde modificaties inderdaad de gevoeligheid voor toxines verminderen, hebben we ontwikkelingsbioassays gebruikt. Deze tests toonden aan dat twee soorten met resistentie-gerelateerde modificaties van de nAChR (stekelbaars en baardagaam) minder vatbaar waren voor de toxische

effecten van cobragif dan twee soorten die dergelijke modificaties niet bezaten (zebravis en kip). Samengevat tonen wij aan dat de reeks mechanismen samen met de fylogenetische distributie van resistentie tegen slangen-α-neurotoxine uitgebreider lijkt te zijn dan tot nu toe werd aangenomen. Ons onderzoek levert ook duidelijk bewijs van de convergente evolutie van dezelfde resistentiemutaties in onafhankelijke afstammingslijnen. Daarnaast ondersteunen onze bevindingen de gedachte dat de mutaties die wij in dit proefschrift hebben geïdentificeerd, een adaptieve verandering kunnen zijn als reactie op de selectiedruk uitgeoefend door α-neurotoxisch slangengif in een evolutionaire wapenwedloop. Derhalve concluderen wij dat de evolutionaire wapenwedloop tussen roofdier en prooi een algemeen kenmerk lijkt te zijn van de trofische interacties rond gifslangen, hetgeen de moleculaire evolutie van de nAChR in gewervelde dieren vormgeeft.

Curriculum Vitae

Muzaffar Ali Khan was born on June 19, 1978, in the city Khanewal, province of Punjab, Pakistan. His native language is Punjabi. In 2001, Mr Khan graduated as a Doctor of Veterinary Medicine (DVM) from the college of Veterinary sciences (CVS), Lahore. The CVS has now become the University of Veterinary and Animal Sciences Lahore, Pakistan. After his graduation in 2003, Mr Khan completed M.Sc. (Hons) in microbiology at the University of Veterinary and Animal Sciences Lahore, Pakistan. His master research thesis title was Isolation and Characterization of Canine Parvovirus. In June 2004, he became a permanent lecturer at the Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan, Pakistan. He married on November 16, 2008, and has two daughters and one son. His long-term goal was to do a Ph.D. from a prestigious international university. So, in March, 2015, he got an opportunity to do a Ph.D. as a self-funded student at the Institute of Biology Leiden (IBL) at Leiden University. He started the journey of his Ph.D. under the supervision of Prof. Michael Richardson (IBL). Mr Khan won two grants and one scholarship. One was for €13,500 from the Leids Universiteit Fonds (LUF; Elise Mathilde Fonds) in June, 2016, to conduct fieldwork in Pakistan. A second grant was the Academy Ecology Fund of the Royal Netherlands Academy Of Arts And Sciences for €5000 in October, 2018 to conduct field work in Queensland, Australia. Mr. Khan also worked as a visiting research student, the University of Queensland, Australia, from November 16, 2018, to December 17, 2018. A third grant was Leiden University Fund/Swaantje Mondt Fonds for €435 in November 2018 to study stay at University of Queensland, Australia. He was awarded a scholarship of US$12,000 on September 17, 2018, under the programme Partial Support for Ph.D. Studies Abroad from the Higher Education Commission (HEC), Islamabad, Pakistan, for the final year of

his Ph.D. studies. . He has assisted the IBL teaching program as student assistant for the Human Evolution (minor) Skeleton Practical, Chicken Embryo Practicum and Rat Dissection practical courses. In May 2017, he travelled to the Alistair Reid Venom Research Unit, Liverpool School of Tropical Medicine, United Kingdom, to learn experimental procedures relating to the study of the snake venom gland.

Publications

1. **Muzaffar A. Khan**, Daniel Dashevsky, Harald Kerkkamp, Dušan Kordiš, Merijn A. G. de Bakker, Roel Wouters, Jory van Thiel, Bianca op den Brouw, Freek J. Vonk, R. Manjunatha Kini, Jawad Nazir, Bryan G. Fry and Michael K. Richardson. Widespread Evolution of Molecular Resistance to Snake Venom α-Neurotoxins in Vertebrates. Toxins 2020, 12, 1-20.
2. **Muzaffar Ali Khan**, Harald M.I. Kerkkamp, Merijn de Bakker, Bryan G. Fry, Michael K. Richardson. Molecular adaptation and resistance, to the A-neurotoxin of elapid snakes, in Squamata, aves and fishes. April 2020 Toxicon 177 Suppl 1: S37-S3 (Conference Abstract) .
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4. Razzaq, F., Khosa, T., Ahmad, S., Hussain, M., Saeed, Z., **Khan, M.A.**, Shaikh, R.S., Ali, M. and Iqbal, F (2015). Prevalence of *Anaplasma phagocytophilum* in horses from Southern Punjab (Pakistan). Tropical Biomedicine, 32(2), 233-239.
5. K. Zahra, **M. A. Khan**, F. Iqbal (2014). Oral supplementation of *Ocimum basilicum* has the potential to improve the locomotory, exploratory, anxiolytic behavior and learning in adult male albino mice. Neurologic Sciences, 36, 73–78.

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7. **M. A. Khan**, M. Rabbani, K. Muhammad, N. Murtaza and J. Nazir, (2006) Isolation and characterization *canine Parvovirus*. International Journal of Agriculture and Biology, 8 (4): 898-900
8. Naveed murtaza, Abdul Qayyum and **Muzaffar Ali Khan** (2005). Comparative Study of the Soluble Storage Proteins in *Gossypium hirsutum L. Germplasm* through Electrophoresis. International Journal of Agriculture and Biology. 7, 253-256.
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Rear cover image: a true cobra (*Naja* sp.). Drawing by Sven Ballinge