Evolution of molecular resistance to snake venom α-neurotoxins in vertebrates

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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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