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

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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.

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