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

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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.

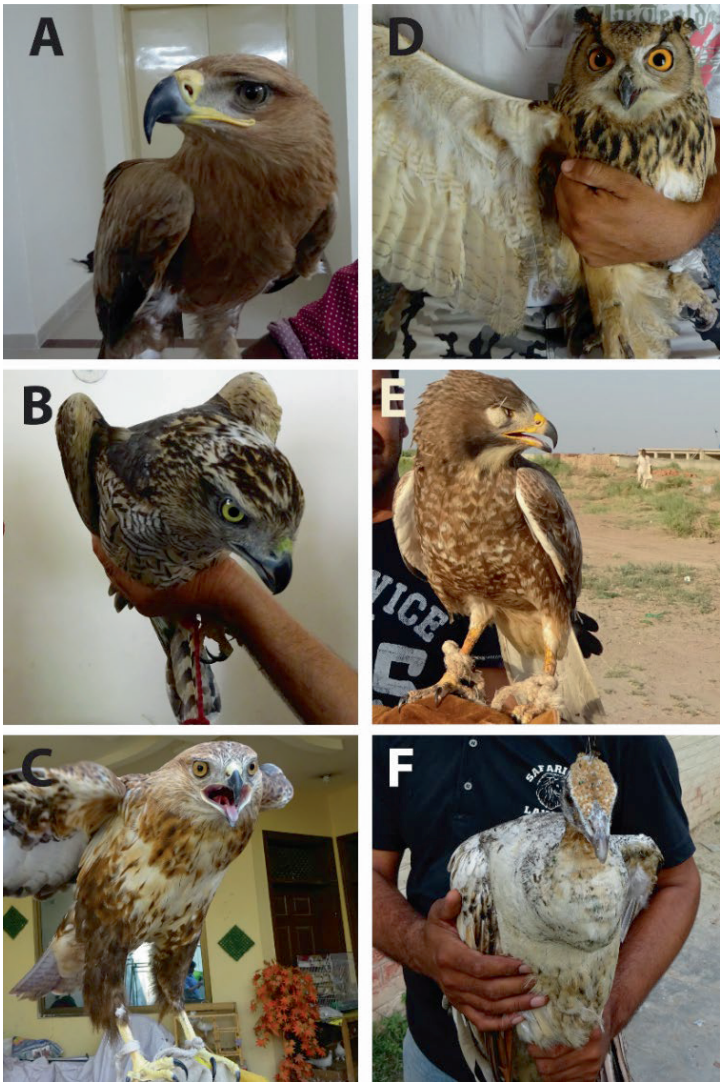

Figure 11. Representative field photos of sampled birds. A, *Aquila rapax* (Tawny eagle); B, *Accipiter genitis* (Goshawk); C *Buteo buteo* (Common buzzard); D, *Bubo bubo* (Eurasian eagle-owl); E *Butastur liventer* (Rufous-winged buzzard); F, *Pavo cristatus* (Peacock).

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.

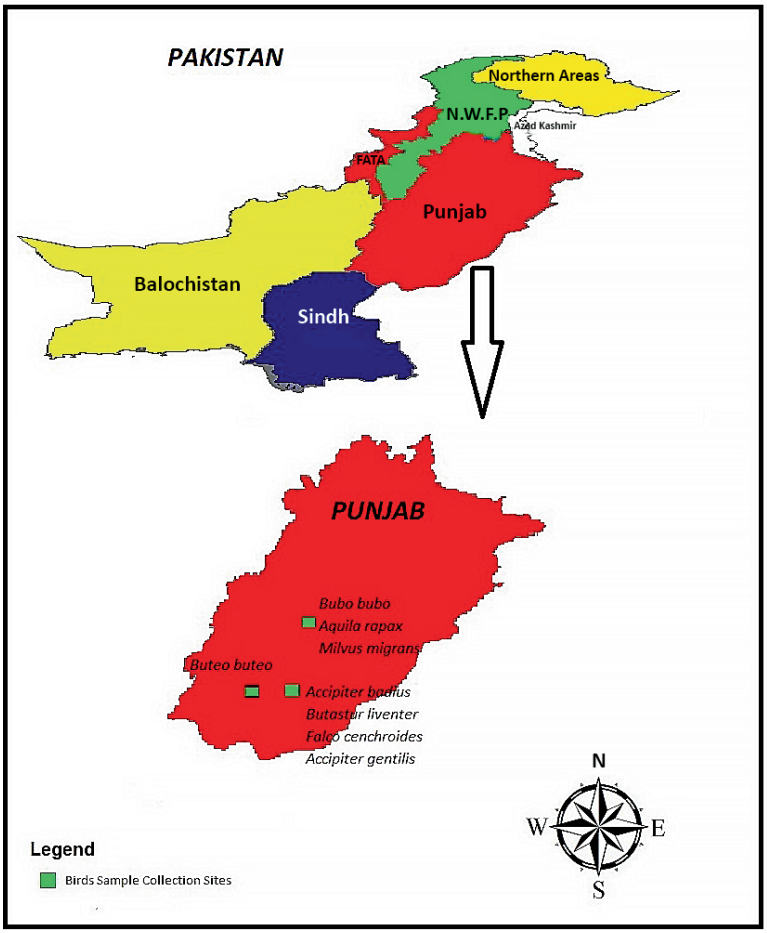

Figure 12. Bird samples collection sites. Top (in yellow, green, red and purple), the provinces of Pakistan, with red indicating Punjab. Bottom with light green boxes: cities of Der a Ghazi Khan, Multan, and Bahawalpur in the province of Punjab. Source: Geographic Information System (GIS). The sites 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.

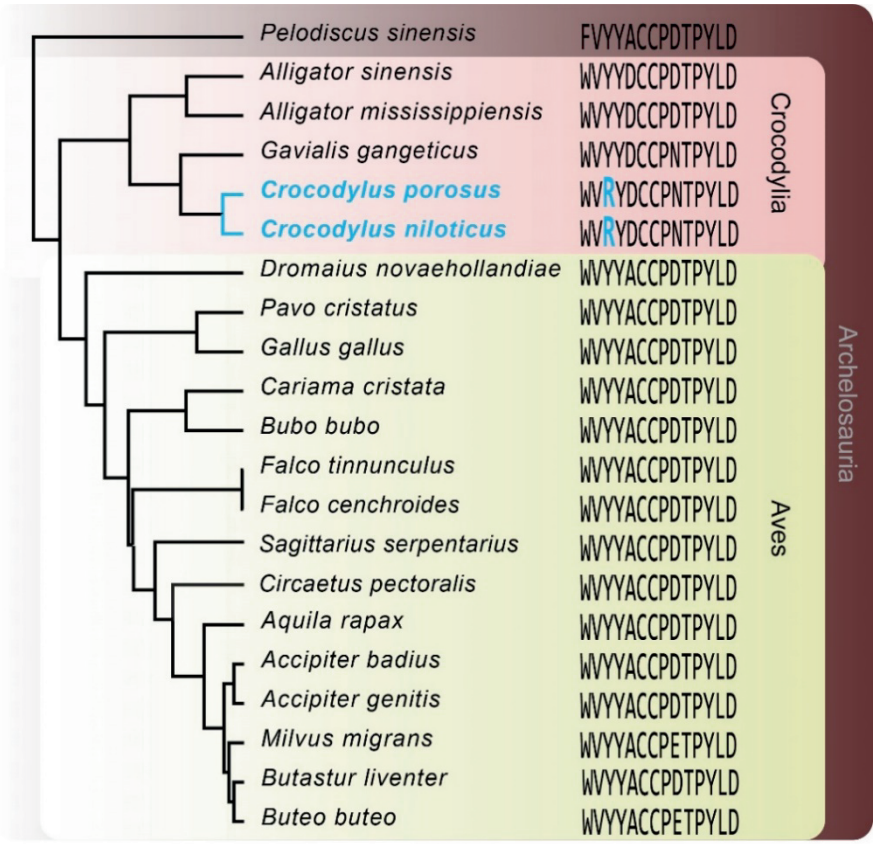

Figure 13. Archosaur (bird + crocodilian) phylogeny constructed from refs (Green, Braun, Armstrong et al., 2014; Jiang, Chen, Wang et al., 2015; Oaks, 2011; Oatley, Simmons & Fuchs, 2015; Prum, Berv, Dornburg et al., 2015; Stein, Brown & Mooers, 2015. Key: blue terminal branches correspond to those sequences 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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