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You are what your ancestors ate: retained bufadienolide resistance in the piscivorous water cobra *Naja annulata* (Serpentes: Elapidae)

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Predators exploiting chemically defended prey are generally resistant to prey toxins. However, toxin resistance usually incurs a fitness cost and is therefore often lost when no longer needed. Bufonid toads are a frequently abundant food resource, but chemically defended by a group of cardiotonic steroids, bufadienolides. Bufophagous predators have evolved a specific and near-universal mechanism of resistance to these toxins, consisting of two amino acid substitutions in the Na⁺/K⁺-ATPase H1–H2 extracellular domain. The dynamics of loss or retention of this adaptation in secondarily non-bufophagous lineages remain inadequately understood. Here we explore this topic by showing that the piscivorous banded water cobra *Naja annulata* retains the bufadienolide-resistant genotype of the otherwise toad-eating cobra clade. This confirms a trend for secondarily non-toad-eating snakes to retain bufadienolide resistance.

Keywords: Antipredator adaptation, cardiotonic steroid, cardiac glycoside, evolution, piscivory

Cardiotonic steroids, also known as cardiac glycosides, are a common chemical defence among many plant and animal taxa. These toxins bind to and disable the ubiquitous and vital animal enzyme Na⁺/K⁺-ATPase, causing potentially lethal poisoning. However, many animals have evolved resistance to cardiotonic steroids. The main mechanism of resistance, which has evolved multiple times across the animal kingdom, consists of replacements of two of the amino acids comprising the Na⁺/K⁺-ATPase H1–H2 extracellular domain (Ujvari et al., 2015). This single, near-universal mechanism has made it possible to assess the likely susceptibility of a wide variety of animals to cardiac glycosides by simply sequencing the short segment of the Na⁺/K⁺-ATPase gene containing the H1–H2 extracellular loop (Ujvari et al., 2013; 2014; Mohammadi et al., 2016; Marshall et al., 2018).

Bufonid toads are abundant components of many freshwater and terrestrial ecosystems, and hence a potentially valuable food source for predators. However, most species are chemically defended by the group of

cardiac glycosides known as bufadienolides, which make them lethal to unadapted predators (Shine, 2010). However, amphibian predators likely to encounter toads, including multiple snake lineages (Ujvari et al., 2015; Mohammadi et al., 2016), have evolved resistance to these toxins, allowing them to exploit this food source.

Since most antitoxin defences carry a cost, there may be selection against their retention when the drivers of their initial evolution are no longer present, as may have happened in Australo-Papuan varanid lizards (Ujvari et al., 2013). On the other hand, in snakes, Mohammadi et al. (2016) found that many species that have secondarily switched away from preying on bufonid toads nevertheless retain their ancestral resistance to bufadienolides. This suggests that these mutations either carry a very limited cost, or provide other selective advantages, or that reversal would be costly, perhaps due to epistatic intramolecular effects or pleiotropic interactions with other, possibly co-evolved, traits.

Cobras (*Naja* and relatives - Kazandjian et al., 2021) are common predators of toads (Luiselli et al., 2002; Shine et al., 2007), and all members of the clade tested to date display the bufadienolide-resistant genotype of the alpha 3 isoform of the Na⁺/K⁺-ATPase H1–H2 domain (Mohammadi et al., 2016). The main exception to the generalisation of toad-eating in cobras appear to be the water cobras: these consist of a clade of three species of the subgenus *Boulengerina*, *Naja annulata*, *N. christyi* and *N. nana*, and are phylogenetically nested deep within the genus *Naja* (Wüster et al., 2007; Wallach et al., 2009; Kazandjian et al., 2021). Water cobras are generally regarded as exclusively piscivorous (Chirio & LeBreton, 2007; Pauwels & Vande weghe, 2008; Collet & Trape, 2020; Spawls & Branch, 2020), although systematic studies of their diet are lacking (Grundler, 2020). Captive specimens of *N. annulata* have been reported to consume a *Rana temporaria* and a *Lycodonomorphus bicolor* (Kratzer, 1965; Madsen & Osterkamp, 1982), and there has been speculation that the species may eat amphibians, but there are no confirmed records of this occurring in the wild (Spawls et al., 2002). In keeping with much of the available literature, we therefore treat *N. annulata* as more or less exclusively piscivorous in nature.

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The apparent loss of bufophagy in *N. annulata* raises the question whether this species has retained the resistance to bufadienolides that arose during the initial divergence of the cobra clade (Mohammadi et al., 2016), or whether, in the absence of selection for cardiac glycoside resistance, this species has reverted to the sensitive genotype. To add to our understanding of the dynamics of gain and loss of this adaptation in cobras and their relatives, we here report on additional sequences of the Na⁺/K⁺-ATPase H1–H2 domain in elapid snakes, with particular focus on *N. annulata*.

Our methods largely follow Mohammadi et al. (2016) and Marshall et al. (2018). We extracted DNA from tissue samples collected from pet trade specimens or by colleagues at Liverpool School of Tropical Medicine in the course of other work (Kazandjian et al., 2021), using Qiagen Dneasy Blood and Tissue Kits following the manufacturer's instructions. The alpha 3 isoforms of the Na⁺/K⁺-ATPase H1–H2 domain were amplified using the primers ATP1a3Fwd (CGAGATGGCCCCAATGCTCTCA) and ATP1a3Rvs (TGGTAGTAGGAGAAGCAGCCGGT) (Mohammadi et al., 2016). For details of PCR conditions and the cleaning of PCR products, see Marshall et al. (2018). PCR products were sent to Macrogen (Seoul, South Korea) for sequencing using both forward and reverse primers.

The sequence trace files were checked for errors and aligned in MEGA 11 (Tamura et al., 2021). We then aligned them with the relevant sequences from Mohammadi et al. (2016), and used Genome BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to obtain corresponding

sequences from the published genomes of *Bungarus multicinctus*, *Laticauda colubrina*, *L. laticaudata*, *Naja naja* and *Ophiophagus hannah* (Vonk et al., 2013; Kishida et al., 2019; Suryamohan et al., 2020; Zhang et al., 2022). The coding sequences for the Na⁺/K⁺-ATPase H1–H2 domain were translated into amino acid sequences in MEGA 11 and checked for the key Q111L and G120R substitutions that confer resistance to cardiac glycosides (Ujvari et al., 2015; Mohammadi et al., 2016).

We obtained new sequences of *Aspidelaps scutatus*, *Hemachatus haemachatus*, *N. annulata*, *N. kaouthia*, *N. naja*, *N. nigricollis*, *N. nivea* and *N. subfulva*. With existing sequences, we aligned a total of 19 sequences of 16 species (Table 1). The translated amino acid sequences show that all members of the cobra clade share the resistance-conferring Q111L and G120R substitutions. This includes the piscivorous *N. annulata* as well as the most basal lineage of the group, *Aspidelaps*. All other elapids analysed lack these resistance-conferring mutations, except that *B. multicinctus* has Q111L but not G120R. The amino-acid sequences of the H1–H2 domain mapped onto the phylogeny of the Elapidae are shown in Figure 1. All new sequences were deposited in GenBank (Accession numbers OQ338149–OQ338156 – Table 1)

Our results shed new light on the dynamics of the evolution of cardiac glycoside resistance in elapid snakes. With increasing phylogenetic breadth and depth (i.e. representation of *Aspidelaps* and the subgenus *Uraeus* through inclusion of *N. nivea*) and additional species of the other subgenera of *Naja* (Wallach et al., 2009), our results strongly support the inference that

Table 1. Taxa included in the analysis, origin of new material, GenBank accession numbers and original reference

Species	GenBank Accession number	Origin of new material	Source
<i>Laticauda colubrina</i>	BLBF01000071		Kishida et al. (2019)
<i>Laticauda laticaudata</i>	BHFT01056349		Kishida et al. (2019)
<i>Bungarus multicinctus</i>	CM042835		Zhang et al. (2022)
<i>Ophiophagus hannah</i>	AZIM01007862		Vonk et al. (2013)
<i>Dendroaspis angusticeps</i>	KU738076		Mohammadi et al. (2016)
<i>Dendroaspis polylepis</i>	KU738077		Mohammadi et al. (2016)
<i>Aspidelaps scutatus</i>	OQ338149	UK pet trade specimen, locality of origin unknown	This study
<i>Hemachatus haemachatus</i>	KU738087		Mohammadi et al. (2016)
<i>Hemachatus haemachatus</i>	OQ338153	LSTM Hem Hae ZAF003, South Africa	This study
<i>Naja (Uraeus)nivea</i>	OQ338155	LSTM Naj Niv ZAF001, South Africa	This study
<i>Naja (Naja) naja</i>	OQ338151	UK pet trade specimen, Sri Lanka	This study
<i>Naja (Naja) naja</i>	CM019160		Suryamohan et al. (2020)
<i>Naja (Naja) atra</i>	KU738098		Mohammadi et al. (2016)
<i>Naja (Naja) kaouthia</i>	OQ338152	UK pet trade specimen, Malaysia	This study
<i>Naja (Afronaja) nigricollis</i>	OQ338154	LSTM Naj Mos TZA004, Tanzania	This study
<i>Naja (Afronaja) nigricollis</i>	KU738100		Mohammadi et al. (2016)
<i>Naja (Boulengerina) subfulva</i>	OQ338156	LSTM Naj Mel CMR002, Cameroon	This study
<i>Naja (Boulengerina) melanoleuca</i>	KU738099		Mohammadi et al. (2016)
<i>Naja (Boulengerina) annulata</i>	OQ338156	UK pet trade specimen, locality of origin unknown	This study

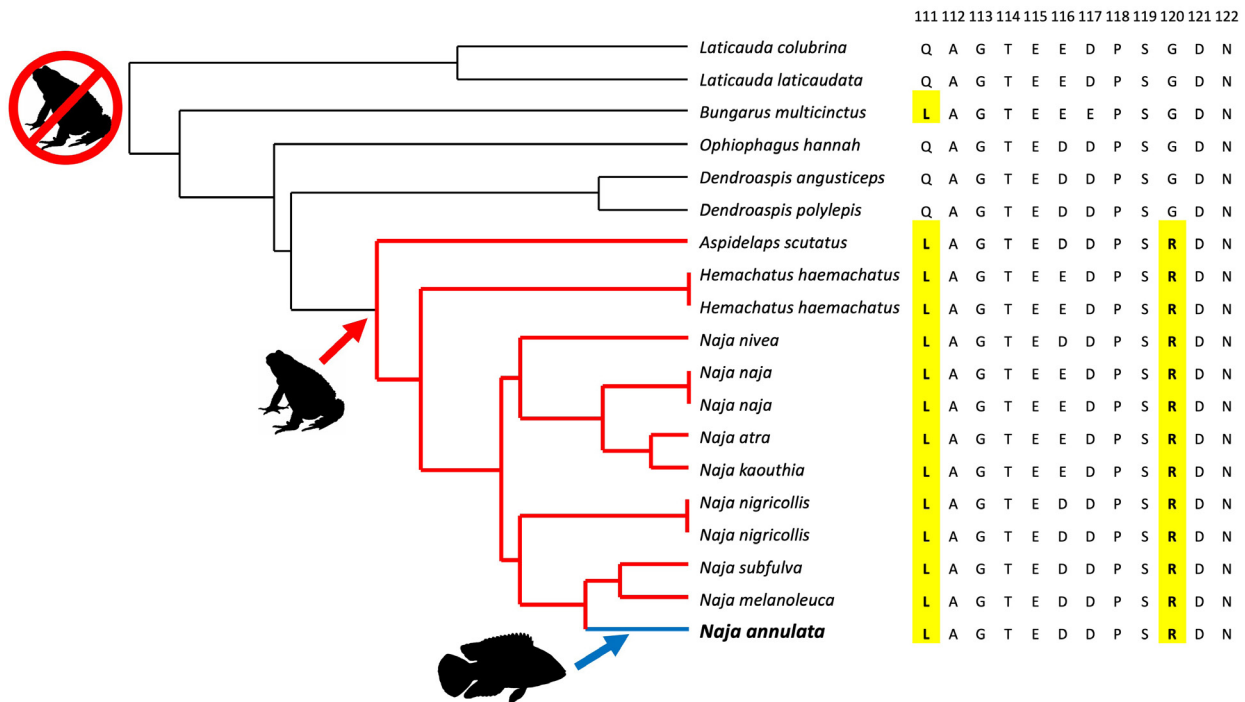


Figure 1. Amino acid sequences of the H1–H2 extracellular loop of all species analysed in this study mapped onto the phylogeny of the cobras and their relatives. Phylogeny redrawn from Kazandjian et al. (2021); the timing of the divergence between *Laticauda colubrina* and *L. laticaudata* is taken from Buehler (2020). Bold type on a yellow background indicates amino-acids likely to confer bufadienolide resistance, bold red branches indicate inferred bufophagous lineages, and the bold blue branch indicates the origin of piscivory in water cobras.

bufadienolide resistance most likely evolved once at the base of the cobra radiation (Mohammadi et al., 2016). Virtually all members of this clade are occasional or frequent toad predators (Luiselli et al., 2002; Shine et al., 2007; Grundler, 2020), including its most basal lineage, *Aspidelaps* (Broadley & Baldwin, 2006). For the most part, bufadienolide resistance is thus restricted to toad-feeding elapid snakes.

The notable exception to this pattern is *N. annulata*, which appears to be a highly specialised fish predator, but is nested deep within the bufophagous cobra clade and retains the resistant genotype. This species therefore appears to follow the pattern identified by Mohammadi et al. (2016), that while bufadienolide resistance evolves readily in toad-eating lineages, it is often retained in descendant lineages that no longer feed on bufonids. This provides additional impetus to the study of the costs and benefits of bufadienolide resistance in snakes.

Another point of interest in our results is that the many-banded krait *B. multicinctus*, a member of a largely snake-eating clade, displays one, but not both, of the two substitutions associated with cardiac glycoside resistance. It is questionable whether substitution Q111L alone confers significant resistance against cardiac glycosides, so the functional consequences of this mutation in *B. multicinctus* remain unknown (Mohammadi et al., 2016). The topic of bufadienolide resistance in kraits may repay further investigation, as there are several records of one species, *B. caeruleus*, eating bufonids (Slowinski, 1994). In addition, it is worth noting that

ophiophagous snakes are potentially at risk of secondary cardiac glycoside poisoning through the consumption of recently-fed bufophagous snakes, especially if the latter sequester bufadienolides (Hutchinson et al., 2007). However, there are virtually no documented observations of such encounters, except that Hesed (2006) observed a non-resistant king cobra *Ophiophagus hannah* feeding on a *Rhabdophis nigrocinctus*, a species known to sequester bufadienolides in specialised nuchal glands (Takeuchi et al., 2018). Unfortunately, the king cobra was not followed after the event, so any ill-effects would have remained undocumented. In any case, the risk of secondary bufadienolide poisoning may provide a selective explanation for the retention of resistance in some of the non-bufophagous snake-eating species, such as *Clelia clelia*, *Erythrolamprus aesculapii* and *E. bizonus*, noted by Mohammadi et al. (2016). It should also be noted that only the alpha 3 isoform of the Na⁺/K⁺-ATPase H1–H2 domain has been sequenced for most snakes: resistance-conferring mutations in other isoforms are found in other clades (Ujvari et al., 2015), and could potentially also occur in squamates, providing additional pathways to resistance.

In conclusion, our results have shed new light on the dynamics of the evolution of bufadienolide resistance in the family Elapidae: they confirm the single origin of this adaptation at the base of the clade of cobra-like elapids, and the tendency for resistance to be retained in snakes descended from toad-eating ancestors, in this instance the water cobra *N. annulata*, and highlight the potential

interest of the genus *Bungarus* for further research on this phenomenon. Taxonomically fine-grained studies of clades with variable diets, as here, are particularly helpful to better understand the selective advantages and costs of bufadienolide resistance in snakes, and whether retention of this adaptation is better explained by a low cost of resistance, or a high cost of reversal due to factors such as intramolecular epistasis (Mohammadi et al., 2021) or potential pleiotropic links to co-evolved physiological adaptations.

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